LIPID NANOPARTICLES COMPRISING CODING RNA MOLECULES FOR USE IN GENE EDITING AND AS VACCINES AND THERAPEUTIC AGENTS

TECHNICAL FIELD

[0001]The present disclosure generally relates to the field of nucleic acid lipid nanoparticle (LNP) compositions and delivery thereof for use as vaccines and/or therapeutics for the treatment of disease. The disclosure further relates to compositions comprising LNPs formulated with coding RNAs, including linear and/or circular mRNAs, for the delivery of encoded vaccine antigens and/or therapeutic proteins for the vaccination against infectious agents and/or treatment of disease, including infectious disease and cancer.

BACKGROUND

[0002]There are many challenges associated with the delivery of nucleic acids to affect a desired response in a biological system, such as an immune response or the production of a therapeutically beneficial protein to treat a disease. Nucleic acid-based therapeutics and vaccines have enormous potential but there remains a need for more effective delivery of nucleic acids to appropriate sites within a cell or organism in order to realize this potential.

[0003]Nucleic acid-based therapeutics and vaccines are generally composed of DNA or RNA. DNA is known to be relatively stable and easy to handle, however, the use of DNA bears the risk of undesired insertion into a cell's genome which potentially may produce mutagenic events. As a further concern, the delivery of DNA is associated with unwanted immunogenicity and the production of anti-DNA antibodies. Yet another concern in the use of DNA is the limited expression level of the encoded peptide or protein that is achievable due to the requirement that the administered DNA must first enter the nucleus to undergo transcription prior to translation into a desired protein product (e.g., antigen or therapeutic protein).

[0004]In contrast to DNA, the use of RNA is substantially safer because RNA does not involve the risk of being integrated into the genome of a transfected cell, thus eliminating the concern that the introduced genetic material will disrupt the normal functioning of an essential gene or cause a mutation. In addition, RNA-based agents do not require extraneous promoter sequences for effective expression of an encoded protein and are also less immunogenic than DNA-based agents, in part because RNA has a relatively short half-life unlike DNA. In addition, while DNA must enter the nuclease to perform its function, RNA performs its function outside of the nucleus and is therefore more efficient.

[0005]Despite the advantages of using RNA-based therapeutics and vaccines, the stability of RNA (e.g., mRNA) is far lower than DNA, especially when it reaches the cytoplasm of a cell and is exposed to RNA-degrading enzymes. In addition, the presence of a hydroxyl group on the second carbon of the sugar moiety in RNA causes steric hindrance that prevents the RNA from forming a more stable double helix structure like in the case of DNA, thus making RNA more prone to hydrolytic degradation than DNA.

[0006]To circumvent these challenges, delivery of RNA vaccines (e.g., mRNA vaccines) and therapeutics has recently focused on the use of lipid nanoparticles (LNPs). Indeed, LNPs have emerged as the most promising nonviral delivery vehicle for exogenous mRNA (see e.g., Guan et al., "Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems," *Gene Ther*, 24 (2017), pp. 133-143). The LNP is a complex nanostructured body that provides protection to payload RNA molecules encapsulated within from the harshly degrading nuclease environment in vivo while facilitating intracellular delivery. LNPs are formed through self-assembly by combining the RNA payload with several lipid components, including an ionizable lipid that plays a central role in delivery efficacy (e.g., Miao et al., "Delivery of mRNA vaccines with heterocyclic lipids increases anti-tumor efficacy by STING-mediated immune cell activation," *Nat. Biotechnol.*, 27 (2019), pp. 1174-1185). Entrapment of RNA is achieved by mixing RNA with lipids at an acidic pH at which the ionizable lipid is positively charged, thus ensuring a charge-driven interaction with the negatively charged RNA molecules (e.g., Mindy et al., "Mechanism of macromolecular structure evolution in self-assembled lipid nanoparticles for siRNA delivery," *Langmuir*, 20 (2014), pp. 4613-4622). The pH is then adjusted to above the pKa of the ionizable lipid, which results in a near-neutral surface charge desirable for clinical administration (see Id.). In addition, the incorporation of a pegylated lipid into the mixture achieves a sterically stabilized core shell nanoparticle useful for clinical applications as vaccines and/or therapeutics.

[0007]In spite of the development of LNPs, the delivery of RNA payloads to cells *in vivo* in a targeted manner that also allows for sufficient levels of protein production (e.g., production of vaccine antigens or therapeutic proteins) remains an important and significant challenge.

[0008]Genome editing tools encompass a diverse set of technologies that can make many types of genomic alterations in various contexts. These technologies have evolved over the last couple of decades to provide a range of user-programmable editing tools that include ZFN (zinc finger) nuclease editing systems, meganuclease editing systems, and TALENS (transcription activator-like effector nucleases). The past decade has seen an explosive growth in a new generation of genome editing systems based on components from bacterial immune pathways, including CRISPR (clustered regularly interspaced short palindromic repeats) and the associated CRISPR-associated proteins (e.g., CRISPR-Cas9) (Jinek *et al.*, "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity," *Science*, Vol. 337 (6096), pp. 816-821), meganuclease editors (Boissel et al., "megaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering," *Nucleic Acids Research* 42: pp. 2591-2601) and bacterial retron systems (Schubert *et al.*, "High-throughput functional variant screens via in vivo production of single-stranded DNA," *PNAS*, April 27, 2021, Vol. 118(18), pp. 1-10). In particular, CRISPR-Cas9 has been derivatized in numerous ways to expand upon its guide RNA-based programmable double-strand cutting activity to form systems ranging from finding alternative CRISPR Cas nuclease enzymes having different PAM requirements and cutting properties (e.g., engineered Cas9 proteins and other naturally-occurring Cas9 homologs,

including, but not limited to, Cas12a, Cas12f, Cas13a, and Cas13b, and their engineered variants) to base editing (Komor *et al.*, "Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage," *Nature*, May 19, 2016, 533 (7603); pp. 420-424 [cytosine base editors or CBEs] and Gaudelli *et al.*, "Programmable base editing of A-T to G-C in genomic DNA without DNA cleavage," *Nature*, Vol. 551, pp. 464-471 [adenine base editors or ABEs]) to prime editing (Anzalone *et al.*, "Search-and-replace genome editing without double-strand breaks or donor DNA," *Nature*, Dec 2019, 576 (7789): pp. 149-157) to twin prime editing (Anzalone *et al.*, "Programmable deletion, replacement, integration and inversion of large DNA sequences with twin prime editing," *Nature Biotechnology*, Dec 9, 2021, vol. 40, pp. 731-740) to epigenetic editing (Kungulovski and Jeltsch, "Epigenome Editing: State of the Art, Concepts, and Perspective," *Trends in Genetics*, Vol.32, 206, pp. 101-113) to CRISPR-directed integrase editing (Yarnell *et al.*, "Drag-and-drop genome insertion of large sequences without double-stranded DNA cleavage using CRISPR-directed integrases," *Nature Biotechnology*, Nov 24, 2022, ("PASTE")).

[0009]While the expansion of genome editing tools has exploded, the development of safe and effective gene editing tool delivery systems has lagged behind. There remain numerous challenges associated with the delivery of gene editing tools—including, but not limited to, CRISPR-Cas9 and alternative Cas nuclease editors, retron editors, base editors, prime editors, twin prime editors, epigenetic editors, and integrase editors—to achieve safe and effective therapeutic application of such tools in cells and patients for treating disease and/or otherwise modifying the nucleotide sequence of a target nucleic acid molecule (e.g., a gene or genome) particularly as it relates to delivery *in vivo*. That said, the use of lipid nanoparticles (LNPs) has emerged as a leading delivery option for the safe, effective, and targeted delivery of gene editing tools to target tissues and cells. However, there remains a need for improved LNPs, including better performing ionizable lipids, that will enhance the targeted delivery of LNP-based gene editing tools. Preferably, such improved LNPs would protect payloads from degradation and clearance while achieving targeted delivery, be suitable for systemic or local delivery, and provide delivery of RNA cargo, including those relating to a wide variety of gene editing tools, such as those mentioned above. In addition, such improved LNP-based therapeutics should exhibit low toxicity and provide an adequate therapeutic index, such that patient treatment at an effective dose of the LNP minimizes risk to the patient while maximizing therapeutic benefit.

[0010]Thus, improved LNPs that enhance the delivery of LNP-based RNA vaccines and therapeutics to cells, tissues, and bodily sites and which are more protective of RNA payloads would advance the art. Preferably, such improved LNPs would protect RNA payloads from degradation and clearance while achieving delivery, be suitable for *ex vivo* or *in vivo* delivery , and provide delivery of any target, including RNA in linear and/or circular and/or modified form.. In addition, such improved LNP-based RNA vaccines and therapeutics should exhibit low toxicity and provide an adequate therapeutic index, such that patient treatment at an effective dose of the LNP minimizes risk to the

patient while maximizes therapeutic benefit. The present disclosure provides these and related advantages.

SUMMARY

[0011]Described herein are compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA medicines (e.g., vaccines and gene-editing therapeutics). In particular, described herein are compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA medicines (e.g., vaccines and gene editing therapeutics) for the delivery of one or more coding and/or non-coding RNA molecules. In various embodiments, the non-coding RNAs may comprise one or more guide RNAs relating to a gene editing system, such as one based on CRISPR-Cas9 or CRISPR-Cas12a, each of which require complexing with a guide RNA that facilitates the localizing the protein-RNA complex to a target sequence having an enzyme-specific PAM site (protospacer adjacent motif – recognized by the CRISPR enzyme) and a target nucleotide sequence (i.e., the protospacer) that is complementary to a portion of the guide RNA (i.e., to the spacer region). In other embodiments, the coding RNA may encode any protein component of LNP-based RNA medicine, such as, but limited to a virus antigen (e.g., a viral envelope spike protein), a therapeutic protein (e.g., a functional version of a defective protein), or one or more gene editing components (e.g., a programmable nuclease or other effector protein, such as a deaminase or reverse transcriptase). Further described herein are compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA medicines (e.g., vaccines and/or gene editing therapeutics) for the delivery of one or more RNA molecules, e.g., a coding RNA that codes for one or more therapeutic proteins for the prophylactic and/or therapeutic treatment of one or more diseases or a symptom thereof, or a non-coding RNA, such as, but not limited to a guide RNA for a gene editing system. In various embodiments, the RNA molecule delivered by the herein disclosed LNPs can be a linear mRNA. In other embodiments, the RNA molecule delivered by the herein disclosed LNPs can be a circular mRNA. In still other embodiments, the RNA molecule delivered by the herein disclosed LNPs can include both linear and circular forms of mRNA. In further embodiments, the RNA may comprise one or more modifications, including chemical modifications (e.g., ribonucleotide analogs, alternative phosphate chain linkers), sequence modification (e.g., relative to a wild type sequence), and/or structural modification (e.g., secondary-folded structures, such as, but not limited to, stem-loops, hairpins, and G-quadruplexes, and tertiary structural elements, such as, but not limited to, helical duplexes and triple-stranded structures). In various other embodiments, the disclosure provides novel lipid components of the herein disclosed LNPs, including, but not limited to, novel ionizable lipids.

[0012]The present disclosure describes improved LNP-based RNA medicines (e.g., vaccines and therapeutics) for use in treating and/or immunization against disease. In particular, the disclosure describes improved LNPs, including better performing ionizable lipids, that enhance the targeted

delivery of LNP-based RNA vaccines and therapeutics based on linear and/or circular mRNAs. The improved LNPs protect linear and/or circular mRNA cargos (i.e., the circular and/or linear mRNA molecules encapsulated by the LNPs) from degradation and clearance while achieving targeted systemic or local delivery for use as enhanced vaccines and/or therapeutic agents.

[0013]In an aspect of the disclosure, provided herein is a compound having a structure of Formula (TL) or a pharmaceutically acceptable salt thereof, or any lipid in Table (I) or a salt, solvate, stereoisomer, or enantiomer thereof, *see* below, collectively referred to as "Lipids of the Disclosure" and each individually referred to as a "Lipid of the Disclosure."

[0014]In an aspect of the disclosure, provided herein is a pharmaceutical composition comprising a compound as disclosed herein, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

[0015]In an aspect, provided herein is a pharmaceutical composition comprising: a) at least one lipid nanoparticle comprising at least one compound having a structure of any of Formula (TL) or a pharmaceutically acceptable salt thereof, or any lipid in Table (I) or a salt, solvate, stereoisomer, or enantiomer thereof; and b) at least one nucleobase editing system.

[0016]In an aspect, provided herein is a method of delivering a nucleobase editing system to a subject in need thereof, the method comprising administering to the subject the pharmaceutical composition disclosed herein.

[0017]In an aspect of the disclosure, provided herein is a lipid nanoparticle (LNP) comprising a compound having a structure of any of Formula (TL) or a pharmaceutically acceptable salt thereof, or any lipid in Table (I) or a salt, solvate, stereoisomer, or enantiomer thereof.

[0018]In another aspect of the disclosure, provided herein is a method for delivering a nucleic acid to a cell comprising contacting the cell with a LNP disclosed herein or a pharmaceutical composition disclosed herein.

[0019]In another aspect of the disclosure, provided herein is a method for treating a disease characterized by a deficiency of a functional protein, the method comprising administering to a subject having the disease, a LNP formulation comprising a LNP disclosed herein, wherein the mRNA encodes the functional protein or a protein having the same biological activity as the functional protein.

[0020]In another aspect of the disclosure, provided herein is a method for treating a disease characterized by overexpression of a polypeptide, comprising administering to a subject having the disease a LNP formulation comprising a LNP disclosed herein and a siRNA, wherein the siRNA targets expression of the overexpressed polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021]FIG. 1 is a diagram illustrating the LNP-based RNA vaccines and therapeutics disclosed herein which are encapsulated with RNA payloads (e.g., linear and/or circular mRNAs).

[0022]FIG. 2 is a diagram illustrating an originator polynucleotide construct of the present disclosure which may be linear or circular.

DETAILED DESCRIPTION

I. Introduction

[0023]The instant specification describes compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA medicines (e.g., vaccines, gene therapies, or gene-editing therapeutics). In various embodiments, the LNP-based RNA medicines comprise an LNP delivery system (as described in detail herein) and an encapsulated cargo/payload (e.g., RNA in the case of RNA medicines).

[0024]In various embodiments and as described further herein, the LNP delivery vehicle is a complex nanostructured body that provides protection to an encapsulated RNA payload (i.e., one or more RNA molecules) environmental damage (e.g., an intracellular environment). LNPs are formed through self-assembly of multiple lipid components, including (i) an ionizable lipid (e.g., ALC-0315 as in COMIRNATY® (Pfizer-BioNTech), SM-102 as in SPIKEVAX® (Moderna), or MC3 as in ONPATTRO® (Alnylam), or those ionizable lipids described herein), (ii) a helper lipid (such as, but not limited to, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC)), (iii) a sterol (e.g., cholesterol), and (iv) a PEG-lipid (e.g., PEG-DSPE).

[0025]In various embodiments and as described further herein, the RNA payload in the herein described LNP-based medicines may comprise coding and/or non-coding RNA, and/or mixtures thereof. The particular RNA payload constituents will generally reflect the medicine. For example, an LNP-based vaccine or therapeutic may comprise only coding RNA for expressing a vaccine antigen or a therapeutic protein, respectively. However, an LNP-based gene editing medicine may comprise a combination of coding RNA (e.g., encoding a CRISPR nuclease) and non-coding RNAs (e.g., guide RNAs). In various embodiments, the RNA molecule delivered by the herein disclosed LNPs can be a linear mRNA. In other embodiments, the RNA molecule delivered by the herein disclosed LNPs can be a circular mRNA. In still other embodiments, the RNA molecule delivered by the herein disclosed LNPs can include both linear and circular forms of mRNA. In further embodiments, the RNA may comprise one or more modifications, including chemical modifications (e.g., ribonucleotide analogs, alternative phosphate chain linkers), sequence modification (e.g., relative to a wild type sequence), and/or structural modification (e.g., secondary-folded structures, such as, but not limited to, stem-loops, hairpins, and G-quadruplexes, and tertiary structural elements, such as, but not limited to, helical duplexes and triple-stranded structures).

A. LNP-Based RNA Vaccines

[0026]Described herein in certain aspects are improved LNP-based RNA vaccines for use in immunization against disease. In various aspects, the disclosure describes improved LNPs, including better performing ionizable lipids, that enhance the targeted delivery of LNP-based RNA vaccines and

therapeutics based on linear and/or circular mRNAs. The improved LNPs protect linear and/or circular mRNA cargos (i.e., the circular and/or linear mRNA molecules encapsulated by the LNPs) from degradation and clearance while achieving targeted systemic or local delivery for use as enhanced vaccines.

[0027]The instant specification describes compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA vaccines. In particular, as described herein are compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA vaccines for the delivery of an RNA molecule that codes for one or more immunogenic viral antigens for use as vaccine and/or immunogenic compositions. In various embodiments, the RNA molecules delivered by the herein disclosed LNPs can be linear mRNA. In other embodiments, the RNA molecules delivered by the herein disclosed LNPs can be circular mRNA. In still other embodiments, the RNA molecule delivered by the herein disclosed LNPs can include both linear and circular forms of mRNA. In further embodiments, the RNA may comprise one or more modifications, including chemical modifications (e.g., ribonucleotide analogs, alternative phosphate chain linkers), sequence modification (e.g., relative to a wild type sequence), and/or structural modification (e.g., secondary-folded structures, such as, but not limited to, stem-loops, hairpins, and G-quadruplexes, and tertiary structural elements, such as, but not limited to, helical duplexes and triple-stranded structures). In various other embodiments, the disclosure provides novel lipid components of the herein disclosed LNPs, including, but not limited to, novel ionizable lipids.

B.LNP-Based RNA Therapeutics

[0028]Described herein in certain aspects are improved LNP-based RNA therapeutics for use in treating disease or a symptom thereof. In various aspects, the disclosure describes improved LNPs, including better performing ionizable lipids, that enhance the targeted delivery of LNP-based RNA therapeutics based on linear and/or circular mRNAs. The improved LNPs protect linear and/or circular mRNA cargos (i.e., the circular and/or linear mRNA molecules encapsulated by the LNPs) from degradation and clearance while achieving targeted systemic or local delivery for use as enhanced therapeutic agents.

[0029]The instant specification describes compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA therapeutics. In particular, described herein are compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA therapeutics for the delivery of an RNA molecule that codes for one or more therapeutic proteins for use treating a disease or a symptom thereof. Further described herein are compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA therapeutics for the administration of an RNA molecule that codes for one or more therapeutic proteins for the prophylactic and/or therapeutic treatment of one or more diseases or a symptom

thereof. In various embodiments, the RNA molecules delivered by the herein disclosed LNPs can be linear mRNA. In other embodiments, the RNA molecules delivered by the herein disclosed LNPs can be circular mRNA. In still other embodiments, the RNA molecules delivered by the herein disclosed LNPs can include both linear and circular forms of mRNA. In further embodiments, the RNA may comprise one or more modifications, including chemical modifications (e.g., ribonucleotide analogs, alternative phosphate chain linkers), sequence modification (e.g., relative to a wild type sequence), and/or structural modification (e.g., secondary-folded structures, such as, but not limited to, stem-loops, hairpins, and G-quadruplexes, and tertiary structural elements, such as, but not limited to, helical duplexes and triple-stranded structures). In various other embodiments, the disclosure provides novel lipid components of the herein disclosed LNPs, including, but not limited to, novel ionizable lipids.

C.LNP-Based Gene Editing Therapeutics

[0030]Also described herein are LNP compositions comprising gene editing systems for use in treating disease and/or otherwise modifying the sequence and/or expression of target nucleotide sequences. The disclosure provides LNPs capable of delivering a gene editing system to a target organ, tissue, and/or cell. The gene editing systems may be delivered to cells under *in vitro* or *ex vivo* conditions and to organs, tissues, or cells under *in vivo* conditions (e.g., administered to a subject in an effective amount).

[0031]The disclosure also provides in various aspects therapeutic or pharmaceutical compositions comprising LNPs comprising gene editing systems or one or more components thereof. The gene editing systems may comprise DNA components, RNA components, protein components, nucleoprotein components, polysaccharide components, or combinations thereof. In other aspects, the disclosure provides nucleic acid molecules (e.g., RNA or DNA) that encode and/or constitute various componentry of the deliverable gene editing systems contemplated herein. In addition, other aspects of the disclosure provide nucleic acid molecules as components of the herein contemplated gene editing systems, such as, but not limited to plasmids or vectors encoding one or more components of a gene editing system, RNAs encoding one or more components of a gene editing system (e.g., mRNAs coding for a nuclease domain of a gene editing system), and non-coding RNAs (e.g., guide RNAs capable of complexing with and targeting a nucleic acid-programmable DNA binding domain to a specific target nucleotide sequence or a retron ncRNAs).

[0032]In further embodiments, the nucleic acid components (e.g., RNA) may comprise one or more modifications, including chemical modifications (e.g., ribonucleotide analogs, alternative phosphate chain linkers), sequence modification (e.g., relative to a wild type sequence), and/or structural modification (e.g., secondary-folded structures, such as, but not limited to, stem-loops, hairpins, and G-quadruplexes, and tertiary structural elements, such as, but not limited to, helical duplexes and triple-stranded structures).

[0033]The disclosure, in other aspects, describes various protein components (which may be encoded by the nucleic acid components described herein) of the various gene editing systems contemplated herein, including, but not limited to, user-programmable DNA binding proteins and various effector proteins, such as nucleases, polymerases, reverse transcriptases, recombinases, integrases, endonucleases, exonucleases, transposases, and deaminases.

[0034]The disclosure also describes nucleoprotein components of the gene editing systems contemplated herein, such as, but not limited to nuclease-guide RNA complexes. The disclosure also provides methods of modifying the sequence and/or expression level of a target nucleic acid molecule through the delivery and/or administration of an LNP described herein that comprises a gene editing system or components thereof. Still further, the disclosure provides methods of treating a disease by administering a therapeutically effective amount of an LNP-based gene editing system that results in the modification in the sequence and/or expression level of a target nucleic acid molecule (e.g., a disease-associated gene or regulatory sequence, such as a promoter, transcription factor binding site, or gene enhancer site).

[0035]The gene editing systems deliverable by the herein disclosed LNPs can be any type of gene editing system. Without limitation, the gene editing systems contemplated herein can include (A) nucleobase gene editing systems which result in one or more the changes to the *sequence* of a target nucleic acid molecule (e.g., a gene or gene regulatory sequence) (sequence modifications may include, but are not limited to, an insertion of one or more base pairs, a deletion of one of more base pairs, a substitution or one or more base pairs, a conversion of a base pair to another base pair (e.g., a G:C pair converted to an A:T pair), an inversion, or a translocation), (B) an epigenetic editing system which results in one or more modifications to the *epigenome* to bring about an effect on gene expression without altering the sequence of a nucleic acid molecule, and (C) gene editing systems that combine the features of nucleobase editing systems and epigenetic editing systems (e.g., combining components from both types of systems to change the sequence and an epigenomic component with one system).

[0036]Nucleobase editing systems include a wide array of configurations with various combinations of protein functionalities and/or nucleic acid molecule components, all of which are contemplated herein. In general, nucleobase editing systems comprise at least a (i) DNA binding domain that is user-programmable to target a specific sequence in a nucleic acid molecule and optionally (ii) one or more effector domains that facilitate the modification of the sequence of the nucleic acid molecule. User-programmability may comprise amino acid sequence-programmable DNA binding domains (e.g., TALENS, zinc finger-binding domains, meganucleases (or homing endonucleases)) or nucleic acid sequence-programmable DNA binding domains or proteins ("naspDBP") (e.g., CRISPR-Cas9, CRISPR-Cas12a, CRISPR-Cas12f, CRISPR-Cas13a, CRISPR-Cas13b, or TnpB).

[0037]Similarly, epigenetic editing systems comprise at least a (i) DNA binding domain that targets a specific sequence in a nucleic acid molecule and (ii) one or more effector domains that facilitates the modification of one or more epigenomic features of the nucleic acid molecule.

[0038]Gene editing systems may comprise one or more effector domains that provide various functionalities that facilitate changes in nucleotide sequence and/or gene expression, such as, but not limited to, single-strand DNA binding proteins, nucleases, endonucleases, exonucleases, deaminases (e.g., cytidine deaminases or adenosine deaminases), polymerases (e.g., reverse transcriptases), integrases, recombinases, etc., and fusion proteins comprising one or more functional domains linked together.

[0039]In addition, gene editing systems that utilize a nucleic acid sequence-programmable DNA binding domain or protein (naspDBP) may also comprise one or more non-coding nucleic acids, such as, one or more guide RNAs which complex with the nucleic acid programmable DNA binding protein (naspDBP) and target the complex to a specific nucleotide sequence. In the case of prime editing, the guide RNA may be a prime editing guide RNA ("pegRNA") which comprises a specialized RNA template molecule that provides a template or coding sequence for a reverse transcriptase of the prime editing system. In some embodiments, the RNA template molecule may be coupled to a guide RNA as an extension arm at the 5' or 3' end of the guide RNA. In other embodiments, the RNA template molecule may be provided *in trans* as a separate molecule in a manner such that the RNA template molecule may itself become localized and associated with the target sequence and/or the gene editing system at the site of editing. In some embodiments, co-localization of an *in trans* RNA template molecule may be achieved with an aptamer or other RNA structure which binds to a binding partner that is coupled to, integrated with, or otherwise associated with the editing complex.

[0040]In the case of editing systems comprising a nucleic acid sequence-programmable DNA binding protein (naspDBP), such as a CRISPR-Cas9 or CRISPR-Cas12a nuclease, appropriate guides may be designed and synthesized using methods, software, and commercial sources which are well known to those having ordinary skill in the art such that guide RNAs for any given naspDBP may be obtained without undue experimentation.

[0041]Reference may be made to the following references providing information and tools for the design, synthesis, modification, and structural configuration of guide RNAs: (1) Mohr SE, Hu Y, Ewen-Campen B, Housden BE, Viswanatha R, Perrimon N. CRISPR guide RNA design for research applications. FEBS J. 2016 Sep;283(17):3232-8. doi: 10.1111/febs.13777. Epub 2016 Jun 22. PMID: 27276584; PMCID: PMC5014588; (2) Hoberecht L, Perampalam P, Lun A, Fortin JP. A comprehensive Bioconductor ecosystem for the design of CRISPR guide RNAs across nucleases and technologies. Nat Commun. 2022 Nov 2;13(1):6568. doi: 10.1038/s41467-022-34320-7. PMID: 36323688; PMCID: PMC9630310; (3) Cram D, Kulkarni M, Buchwaldt M, Rajagopalan N, Bhowmik P, Rozwadowski K, Parkin IAP, Sharpe AG, Kagale S. WheatCRISPR: a web-based guide

RNA design tool for CRISPR/Cas9-mediated genome editing in wheat. BMC Plant Biol. 2019 Nov 6;19(1):474. doi: 10.1186/s12870-019-2097-z. PMID: 31694550; PMCID: PMC6836449; (4) Pliatsika V, Rigoutsos I. "Off-Spotter": very fast and exhaustive enumeration of genomic lookalikes for designing CRISPR/Cas guide RNAs. Biol Direct. 2015 Jan 29;10:4. doi: 10.1186/s13062-015-0035-z. PMID: 25630343; PMCID: PMC4326336; (5) Hoof JB, Nødvig CS, Mortensen UH. Genome Editing: CRISPR-Cas9. Methods Mol Biol. 2018;1775:119-132. doi: 10.1007/978-1-4939-7804-5_11. PMID: 29876814; (6) Labun K, Krause M, Torres Cleuren Y, Valen E. CRISPR Genome Editing Made Easy Through the CHOPCHOP Website. Curr Protoc. 2021 Apr;1(4):e46. doi: 10.1002/cpz1.46. PMID: 33905612; (7) Lee CM, Davis TH, Bao G. Examination of CRISPR/Cas9 design tools and the effect of target site accessibility on Cas9 activity. Exp Physiol. 2018 Apr 1;103(4):456-460. doi: 10.1113/EP086043. Epub 2017 Apr 12. PMID: 28303677; PMCID: PMC7266697; (8) Ma S, Lv J, Feng Z, Rong Z, Lin Y. Get ready for the CRISPR/Cas system: A beginner's guide to the engineering and design of guide RNAs. J Gene Med. 2021 Nov;23(11):e3377. doi: 10.1002/jgm.3377. Epub 2021 Jul 28. PMID: 34270141; (9) Hiranniramol K, Chen Y, Wang X. CRISPR/Cas9 Guide RNA Design Rules for Predicting Activity. Methods Mol Biol. 2020;2115:351-364. doi: 10.1007/978-1-0716-0290-4_19. PMID: 32006410; (10) Wiles MV, Qin W, Cheng AW, Wang H. CRISPR-Cas9-mediated genome editing and guide RNA design. Mamm Genome. 2015 Oct;26(9-10):501-10. doi: 10.1007/s00335-015-9565-z. Epub 2015 May 20. PMID: 25991564; PMCID: PMC4602062; (11) Creutzburg SCA, Wu WY, Mohanraju P, Swartjes T, Alkan F, Gorodkin J, Staals RHJ, van der Oost J. Good guide, bad guide: spacer sequence-dependent cleavage efficiency of Cas12a. Nucleic Acids Res. 2020 Apr 6;48(6):3228-3243. doi: 10.1093/nar/gkz1240. PMID: 31989168; PMCID: PMC7102956; (12) Heigwer F, Boutros M. Cloud-Based Design of Short Guide RNA (sgRNA) Libraries for CRISPR Experiments. Methods Mol Biol. 2021;2162:3-22. doi: 10.1007/978-1-0716-0687-2_1. PMID: 32926374; (13) Dronina J, Samukaite-Bubniene U, Ramanavicius A. Towards application of CRISPR-Cas12a in the design of modern viral DNA detection tools (Review). J Nanobiotechnology. 2022 Jan 21;20(1):41. doi: 10.1186/s12951-022-01246-7. PMID: 35062978; PMCID: PMC8777428; (14) Krysler AR, Cromwell CR, Tu T, Jovel J, Hubbard BP. Guide RNAs containing universal bases enable Cas9/Cas12a recognition of polymorphic sequences. Nat Commun. 2022 Mar 25;13(1):1617. doi: 10.1038/s41467-022-29202-x. PMID: 35338140; PMCID: PMC8956631; (15) Shin HR, Kweon J, Kim Y. Gene Manipulation Using Fusion Guide RNAs for Cas9 and Cas12a. Methods Mol Biol. 2021;2162:185-193. doi: 10.1007/978-1-0716-0687-2_10. PMID: 32926383; (16) Schubert MS, Thommandru B, Woodley J, Turk R, Yan S, Kurgan G, McNeill MS, Rettig GR. Optimized design parameters for CRISPR Cas9 and Cas12a homology-directed repair. Sci Rep. 2021 Sep 30;11(1):19482. doi: 10.1038/s41598-021-98965-y. PMID: 34593942; PMCID: PMC8484621; (17) Crone MA, MacDonald JT, Freemont PS, Siciliano V. gDesigner: computational design of synthetic gRNAs for Cas12a-based transcriptional repression in mammalian cells. NPJ Syst Biol Appl. 2022 Sep 16;8(1):34. doi: 10.1038/s41540-022-00241-w.

PMID: 36114193; PMCID: PMC9481559; (18) Konstantakos V, Nentidis A, Krithara A, Paliouras G. CRISPR-Cas9 gRNA efficiency prediction: an overview of predictive tools and the role of deep learning. Nucleic Acids Res. 2022 Apr 22;50(7):3616-3637. doi: 10.1093/nar/gkac192. PMID: 35349718; PMCID: PMC9023298; (19) Wang J, Zhang X, Cheng L, Luo Y. An overview and metanalysis of machine and deep learning-based CRISPR gRNA design tools. RNA Biol. 2020 Jan;17(1):13-22. doi: 10.1080/15476286.2019.1669406. Epub 2019 Sep 27. PMID: 31533522; PMCID: PMC6948960; and (20) Cram D, Kulkarni M, Buchwaldt M, Rajagopalan N, Bhowmik P, Rozwadowski K, Parkin IAP, Sharpe AG, Kagale S. WheatCRISPR: a web-based guide RNA design tool for CRISPR/Cas9-mediated genome editing in wheat. BMC Plant Biol. 2019 Nov 6;19(1):474. doi: 10.1186/s12870-019-2097-z. PMID: 31694550; PMCID: PMC6836449; each of which are incorporated herein by reference in their entireties.

[0042]In the case of prime editing, in particular, further reference may be made to the following references providing information and tools for the design, synthesis, modification, and structural configuration of pegRNAs: (1) Hsu JY, Grünewald J, Szalay R, Shih J, Anzalone AV, Lam KC, Shen MW, Petri K, Liu DR, Joung JK, Pinello L. PrimeDesign software for rapid and simplified design of prime editing guide RNAs. Nat Commun. 2021 Feb 15;12(1):1034. doi: 10.1038/s41467-021-21337-7. PMID: 33589617; PMCID: PMC7884779; (2) Li Y, Chen J, Tsai SQ, Cheng Y. Easy-Prime: a machine learning-based prime editor design tool. Genome Biol. 2021 Aug 19;22(1):235. doi: 10.1186/s13059-021-02458-0. PMID: 34412673; PMCID: PMC8377858; (3) Zhang W, Petri K, Ma J, Lee H, Tsai CL, Joung JK, Yeh JJ. Enhancing CRISPR prime editing by reducing misfolded pegRNA interactions. bioRxiv [Preprint]. 2023 Aug 15:2023.08.14.553324. doi: 10.1101/2023.08.14.553324. PMID: 37645936; PMCID: PMC10462064; (4) Jin S, Lin Q, Gao Q, Gao C. Optimized prime editing in monocot plants using PlantPegDesigner and engineered plant prime editors (ePPEs). Nat Protoc. 2023 Mar;18(3):831-853. doi: 10.1038/s41596-022-00773-9. Epub 2022 Nov 25. PMID: 36434096; (5) Lin Q, Jin S, Zong Y, Yu H, Zhu Z, Liu G, Kou L, Wang Y, Qiu JL, Li J, Gao C. High-efficiency prime editing with optimized, paired pegRNAs in plants. Nat Biotechnol. 2021 Aug;39(8):923-927. doi: 10.1038/s41587-021-00868-w. Epub 2021 Mar 25. PMID: 33767395; (6) Standage-Beier K, Tekel SJ, Brafman DA, Wang X. Prime Editing Guide RNA Design Automation Using PINE-CONE. ACS Synth Biol. 2021 Feb 19;10(2):422-427. doi: 10.1021/acssynbio.0c00445. Epub 2021 Jan 19. PMID: 33464043; PMCID: PMC7901017; (7) Zhang W, Petri K, Ma J, Lee H, Tsai CL, Joung JK, Yeh JJ. Enhancing CRISPR prime editing by reducing misfolded pegRNA interactions. bioRxiv [Preprint]. 2023 Aug 15:2023.08.14.553324. doi: 10.1101/2023.08.14.553324. PMID: 37645936; PMCID: PMC10462064; (8) Chow RD, Chen JS, Shen J, Chen S. A web tool for the design of prime-editing guide RNAs. Nat Biomed Eng. 2021 Feb;5(2):190-194. doi: 10.1038/s41551-020-00622-8. Epub 2020 Sep 28. PMID: 32989284; PMCID: PMC7882013; each of which are incorporated herein by reference in their entireties.

[0043]Reference may also be made to the following commercial vendors which sell guide RNAs for CRISPR editing applications (including base editing and prime editing) and provide various tools and instruction for the ordering, design, synthesis, modification, and structural configuration of guide RNAs: GENSCRIPT, SYNTHEGO, TAKARA BIO, INTEGRATED DNA TECHNOLOGIES, LC SCIENCES, HORIZON DISCOVERY; SIGMA-ALDRICH; ORIGENE, and TWIST BIOSCIENCES, among others.

[0044]In addition, guide RNA may be modified with chemical modifications and/or structural modifications for enhancing various properties thereof, including specificity, stability, and limiting off-target activity. One of ordinary skill in the art will be able to modify a guide RNA with any known modification without undue experimentation. Guide modifications are discussed in the following references: (1) Ke Y, Ghalandari B, Huang S, Li S, Huang C, Zhi X, Cui D, Ding X. 2'-*O*-Methyl modified guide RNA promotes the single nucleotide polymorphism (SNP) discrimination ability of CRISPR-Cas12a systems. Chem Sci. 2022 Feb 1;13(7):2050-2061. doi: 10.1039/d1sc06832f. PMID: 35308857; PMCID: PMC8848812; (2) Allen D, Rosenberg M, Hendel A. Using Synthetically Engineered Guide RNAs to Enhance CRISPR Genome Editing Systems in Mammalian Cells. Front Genome Ed. 2021 Jan 28;2:617910. doi: 10.3389/fgeed.2020.617910. PMID: 34713240; PMCID: PMC8525374; (3) Basila M, Kelley ML, Smith AVB. Minimal 2'-O-methyl phosphorothioate linkage modification pattern of synthetic guide RNAs for increased stability and efficient CRISPR-Cas9 gene editing avoiding cellular toxicity. PLoS One. 2017 Nov 27;12(11):e0188593. doi: 10.1371/journal.pone.0188593. PMID: 29176845; PMCID: PMC5703482; (4) Sakovina L, Vokhtantsev I, Vorobyeva M, Vorobyev P, Novopashina D. Improving Stability and Specificity of CRISPR/Cas9 System by Selective Modification of Guide RNAs with 2'-fluoro and Locked Nucleic Acid Nucleotides. Int J Mol Sci. 2022 Nov 3;23(21):13460. doi: 10.3390/ijms232113460. PMID: 36362256; PMCID: PMC9655745; (5) Shapiro J, Tovin A, Iancu O, Allen D, Hendel A. Chemical Modification of Guide RNAs for Improved CRISPR Activity in CD34+ Human Hematopoietic Stem and Progenitor Cells. Methods Mol Biol. 2021;2162:37-48. doi: 10.1007/978-1-0716-0687-2_3. PMID: 32926376; (6) Filippova J, Matveeva A, Zhuravlev E, Stepanov G. Guide RNA modification as a way to improve CRISPR/Cas9-based genome-editing systems. Biochimie. 2019 Dec;167:49-60. doi: 10.1016/j.biochi.2019.09.003. Epub 2019 Sep 4. PMID: 31493470; (7) Hendel A, Bak RO, Clark JT, Kennedy AB, Ryan DE, Roy S, Steinfeld I, Lunstad BD, Kaiser RJ, Wilkens AB, Bacchetta R, Tsalenko A, Dellinger D, Bruhn L, Porteus MH. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat Biotechnol. 2015 Sep;33(9):985-989. doi: 10.1038/nbt.3290. Epub 2015 Jun 29. PMID: 26121415; PMCID: PMC4729442; (8)_ Ryan DE, Taussig D, Steinfeld I, Phadnis SM, Lunstad BD, Singh M, Vuong X, Okochi KD, McCaffrey R, Olesiak M, Roy S, Yung CW, Curry B, Sampson JR, Bruhn L, Dellinger DJ. Improving CRISPR-Cas specificity with chemical modifications in single-guide RNAs. Nucleic Acids Res. 2018 Jan 25;46(2):792-803. doi: 10.1093/nar/gkx1199. Erratum in: Nucleic Acids

Res. 2022 Mar 21;50(5):2986. PMID: 29216382; PMCID: PMC5778453; (9) Palumbo CM, Gutierrez-Bujari JM, O'Geen H, Segal DJ, Beal PA. Versatile 3' Functionalization of CRISPR Single Guide RNA. Chembiochem. 2020 Jun 2;21(11):1633-1640. doi: 10.1002/cbic.201900736. Epub 2020 Mar 5. PMID: 31943634; PMCID: PMC7323579; (10) Mullally G, van Aelst K, Naqvi MM, Diffin FM, Karvelis T, Gasiunas G, Siksnys V, Szczelkun MD. 5' modifications to CRISPR-Cas9 gRNA can change the dynamics and size of R-loops and inhibit DNA cleavage. Nucleic Acids Res. 2020 Jul 9;48(12):6811-6823. doi: 10.1093/nar/gkaa477. PMID: 32496535; PMCID: PMC7337959; (12) Lu S, Zhang Y, Yin H. Chimeric DNA-RNA Guide RNA Designs. Methods Mol Biol. 2021;2162:79-85. doi: 10.1007/978-1-0716-0687-2_6. PMID: 32926379; each of which are incorporated by reference herein in their entireties.

[0045]In the specific case of prime editing, pegRNAs may be modified with chemical modifications and/or structural modifications for enhancing various properties thereof, including specificity, stability, and limiting off-target activity. One of ordinary skill in the art will be able to modify a pegRNA for prime editing with any known modification without undue experimentation. pegRNA modifications are discussed in the following references: (1) Nelson JW, Randolph PB, Shen SP, Everette KA, Chen PJ, Anzalone AV, An M, Newby GA, Chen JC, Hsu A, Liu DR. Engineered pegRNAs improve prime editing efficiency. Nat Biotechnol. 2022 Mar;40(3):402-410. doi: 10.1038/s41587-021-01039-7. Epub 2021 Oct 4. Erratum in: Nat Biotechnol. 2021 Dec 8;: PMID: 34608327; PMCID: PMC8930418; (2) Liu B, Dong X, Cheng H, Zheng C, Chen Z, Rodríguez TC, Liang SQ, Xue W, Sontheimer EJ. A split prime editor with untethered reverse transcriptase and circular RNA template. Nat Biotechnol. 2022 Sep;40(9):1388-1393. doi: 10.1038/s41587-022-01255-9. Epub 2022 Apr 4. PMID: 35379962; each of which are incorporated by reference herein in their entireties.

[0046]Other specialized guide RNAs may be included depending upon the requirements and/or nature of the gene editing system and the cognate nucleic acid programmable proteins. For example, TnpB enzymes require a specialized guide RNA referred to as reRNA. Also, guide RNAs have different characteristics (e.g., PAM preferences, the spacer length, and the scaffold portion that binds to the nuclease protein) depending upon the programmable nuclease requirements.

[0047]The gene editing systems contemplated here may introduce a wide variety of changes, including (A) a change in the sequence of the target nucleic acid molecule, such as, but not limited to, (i) a nucleobase substitution (e.g., a purine to a pyrimidine), (ii) a deletion of one or more nucleobases, (iii) an insertion of one or more nucleobases, (iv) a combination of a deletion and insertion of one or more nucleobases, (v) an inversion of a nucleobase sequence, a (vi) translocation of a nucleobase sequence, and (vii) a combination or two or more such modifications, and (B) one or more modifications to the *epigenome* to bring about an effect on gene expression without altering the sequence of a nucleic acid molecule wherein said epigenetic change results in altered gene expression through altered chromatin structure or accessibility.

[0048]The LNP compositions and/or gene editing systems described herein may include a variety of coding RNA molecules that code for the various components of gene editors. In various aspects, the coding RNA may be linear mRNA. In other embodiments, the coding RNA may be circular mRNA. In various aspects, the improved LNPs protect linear and/or circular mRNA cargos from degradation and clearance while achieving targeted systemic or local delivery for use as enhanced gene editing platforms and/or therapeutic agents.

[0049]In various other aspects, the LNP compositions and/or gene editing systems described herein may also include a repair template, e.g., an homology-directed repair (HDR)-dependent repair template (or HDR template). Such HDR templates are well-known in the art and can include single-strand or double-stranded DNA (e.g., oligos) or RNA. Further information regarding HDR and HDR templates for use in editing systems for various applications, such as gene knock-in, may be found in Fu YW, Dai XY, Wang WT, Yang ZX, Zhao JJ, Zhang JP, Wen W, Zhang F, Oberg KC, Zhang L, Cheng T, Zhang XB. Dynamics and competition of CRISPR-Cas9 ribonucleoproteins and AAV donor-mediated NHEJ, MMEJ and HDR editing. Nucleic Acids Res. 2021 Jan 25;49(2):969-985. doi: 10.1093/nar/gkaa1251. PMID: 33398341; PMCID: PMC7826255; Iyer S, Mir A, Vega-Badillo J, Roscoe BP, Ibraheim R, Zhu LJ, Lee J, Liu P, Luk K, Mintzer E, Guo D, Soares de Brito J, Emerson CP Jr, Zamore PD, Sontheimer EJ, Wolfe SA. Efficient Homology-Directed Repair with Circular Single-Stranded DNA Donors. CRISPR J. 2022 Oct;5(5):685-701. doi: 10.1089/crispr.2022.0058. Epub 2022 Sep 7. PMID: 36070530; PMCID: PMC9595650; and Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotechnol. 2016 Mar;34(3):339-44. doi: 10.1038/nbt.3481. Epub 2016 Jan 20. PMID: 26789497, each of which are incorporated herein by reference in their entireties.

[0050]Accordingly, the instant specification describes compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based gene editing systems as therapeutic compositions. Further described herein are compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based gene editing therapeutics for the prophylactic and/or therapeutic treatment of one or more diseases or a symptom thereof. The components capable of being encapsulated by or otherwise incorporated by the LNPs described herein may be referred to as LNP "payloads" and may include all of the biological materials described above, including DNA molecules, RNA molecules (coding and/or non-coding), proteins, and nucleoproteins (e.g., Cas/guide RNA complexes).

II.LNP delivery systems

[0051]The RNA payloads (e.g., linear and circular mRNAs) described herein may be encapsulated and delivered by lipid nanoparticles (LNPs) and compositions and/or formulations comprising RNA-encapsulated LNPs.

[0052]Below describes LNPs that may be used as the RNA payload delivery vehicles contemplated herein, as well as the various ionizable lipids, structural lipids, PEGylated lipids, and phospholipids that may be used to make the herein LNPs for delivery RNA payloads to cells. In addition, below describes additional LNP components that are contemplated, such as targeting moieties and other lipid components.

A. Lipid Nanoparticle Compositions

[0053]In one aspect, the present disclosure further provides delivery systems for delivery of a therapeutic payload (e.g., the RNA payloads described herein which may encode a polypeptid of interest, e.g., an antigen or a therapeutic protein) disclosed herein. In some embodiments, a delivery system suitable for delivery of the therapeutic payload disclosed herein comprises a lipid nanoparticle (LNP) formulation.

[0054]In some embodiments, an LNP of the present disclosure comprises an ionizable lipid, a structural lipid, a PEGylated lipid (aka PEG lipid), and a phospholipid. In alternative embodiments, an LNP comprises an ionizable lipid, a structural lipid, a PEGylated lipid (aka PEG lipid), and a zwitterionic amino acid lipid. In some embodiments, an LNP further comprises a 5th lipid, besides any of the aforementioned lipid components. In some embodiments, the LNP encapsulates one or more elements of the active agent of the present disclosure. In some embodiments, an LNP further comprises a targeting moiety covalently or non-covalently bound to the outer surface of the LNP. In some embodiments, the targeting moiety is a targeting moiety that binds to, or otherwise facilitates uptake by, cells of a particular organ system.

[0055]In some embodiments, an LNP has a diameter of at least about 20nm, 30 nm, 40nm, 50nm, 60nm, 70nm, 80nm, or 90nm. In some embodiments, an LNP has a diameter of less than about 100nm, 110nm, 120nm, 130nm, 140nm, 150nm, or 160nm. In some embodiments, an LNP has a diameter of less than about 120 nm. In some embodiments, an LNP has a diameter of less than about 100nm. In some embodiments, an LNP has a diameter of less than about 90nm. In some embodiments, an LNP has a diameter of less than about 80nm. In some embodiments, an LNP has a diameter of about 60-100nm. In some embodiments, an LNP has a diameter of about 50-120nm. In some embodiments, an LNP has a diameter of about 75-80nm.

[0056]In some embodiments, the lipid nanoparticle compositions of the present disclosure are described according to the respective molar ratios of the component lipids in the formulation. As a non-limiting example, the mol-% of the ionizable lipid may be from about 10 mol-% to about 80 mol-%. As a non-limiting example, the mol-% of the ionizable lipid may be from about 20 mol-% to about 70 mol-%. As a non-limiting example, the mol-% of the ionizable lipid may be from about 30 mol-% to about 60 mol-%. As a non-limiting example, the mol-% of the ionizable lipid may be from about 35 mol-% to about 55 mol-%. As a non-limiting example, the mol-% of the ionizable lipid may be from about 40 mol-% to about 50 mol-%. As a non-limiting example, the mol-% of the ionizable lipid may be from about 30 mol-% to about 40 mol-%. As a non-limiting example, the mol-% of the ionizable

lipid may be from about 25 mol-% to about 35 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 10 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 15 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 20 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 25 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 30 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 33 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 35 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 40 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 45 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 55 mol-%. In some embodiments, the mol-% of the ionizable lipid is about 60 mol-%.

[0057]In some embodiments, the mol-% of the phospholipid may be from about 1 mol-% to about 50 mol-%. In some embodiments, the mol-% of the phospholipid may be from about 2 mol-% to about 45 mol-%. In some embodiments, the mol-% of the phospholipid may be from about 3 mol-% to about 40 mol-%. In some embodiments, the mol-% of the phospholipid may be from about 4 mol-% to about 35 mol-%. In some embodiments, the mol-% of the phospholipid may be from about 5 mol-% to about 30 mol-%. In some embodiments, the mol-% of the phospholipid may be from about 10 mol-% to about 20 mol-%. In some embodiments, the mol-% of the phospholipid may be from about 5 mol-% to about 20 mol-%. In some embodiments, the mol-% of the phospholipid is from about 30 mol-% to about 60 mol-%. In some embodiments, the mol-% of the phospholipid is from about 35 mol-% to about 55 mol-%. In some embodiments, the mol-% of the phospholipid is from about 35 mol-% to about 45 mol-%. In some embodiments, the mol-% of the phospholipid is about 10 mol-%. In some embodiments, the mol-% of the phospholipid is about 15 mol-%. In some embodiments, the mol-% of the phospholipid is about 20 mol-%. In some embodiments, the mol-% of the phospholipid is about 25 mol-%. In some embodiments, the mol-% of the phospholipid is about 30 mol-%. In some embodiments, the mol-% of the phospholipid is about 35 mol-%. In some embodiments, the mol-% of the phospholipid is about 40 mol-%. In some embodiments, the mol-% of the phospholipid is about 45 mol-%. In some embodiments, the mol-% of the phospholipid is about 55 mol-%. In some embodiments, the mol-% of the phospholipid is about 60 mol-%.

[0058]In some embodiments, the mol-% of the phospholipid as described above comprises two or more phospholipids at an individual mol-% that totals to an aforementioned amount. In certain embodiments, the mol-% of the phospholipid is about 20 mol-% each of two phospholipids. In certain embodiments, the mol-% of the phospholipid is about 15 mol-% each of two phospholipids. In certain embodiments, the mol-% of the phospholipid is about 25 mol-% each of two phospholipids. In certain embodiments, the mol-% of the phospholipid is about 30 mol-% each of two phospholipids. In certain embodiments, the mol-% of the phospholipid is about 15 mol-% of a first phospholipid and about 20 mol-% of a second phospholipid. In certain embodiments, the mol-% of the phospholipid is about 30 mol-% of a first phospholipid and about 10 mol-% of a second phospholipid. In certain embodiments, the mol-% of the phospholipid is about 25 mol-% of a first phospholipid and about 10

mol-% of a second phospholipid. In certain embodiments, the mol-% of the phospholipid is about 25 mol-% of a first phospholipid and about 20 mol-% of a second phospholipid. In certain embodiments, the mol-% of the phospholipid is about 15 mol-% of a first phospholipid and about 20 mol-% of a second phospholipid.

[0059]In some embodiments, the mol-% of the structural lipid may be from about 10 mol-% to about 80 mol-%. In some embodiments, the mol-% of the structural lipid may be from about 20 mol-% to about 70 mol-%. In some embodiments, the mol-% of the structural lipid may be from about 30 mol-% to about 60 mol-%. In some embodiments, the mol-% of the structural lipid may be from about 35 mol-% to about 55 mol-%. In some embodiments, the mol-% of the structural lipid may be from about 40 mol-% to about 50 mol-%.

[0060]In some embodiments, the mol-% of the PEG lipid may be from about 0.1 mol-% to about 10 mol-%. In some embodiments, the mol-% of the PEG lipid may be from about 0.2 mol-% to about 5 mol-%. In some embodiments, the mol-% of the PEG lipid may be from about 0.5 mol-% to about 3 mol-%. In some embodiments, the mol-% of the PEG lipid may be from about 1 mol-% to about 2 mol-%. In some embodiments, the mol-% of the PEG lipid may be about 1.5 mol-%. In some embodiments, the mol-% of the PEG lipid may be about 2.5 mol-%.

[0061]Where reference is made above to "mol-%" or "mol %", the amount of the noted LNP component is intended to be the mol% of the specific component as compared to the total lipid component content of the lipid nanoparticle.

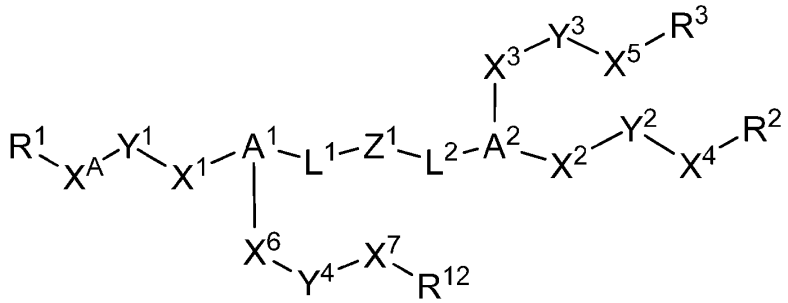
i. Ionizable lipids

[0062]In some embodiments, an LNP disclosed herein comprises an ionizable lipid. In some embodiments, an LNP comprises two or more ionizable lipids.

[0063]Described below are a number of exemplary ionizable Lipids of the Disclosure.

Formula (TL'')

[0064]The present disclosure, in some embodiments, provides compounds of Formula (TL'')



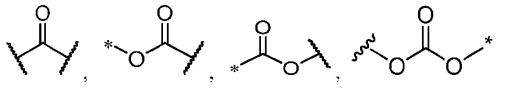
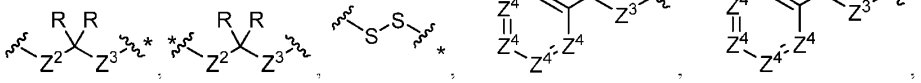
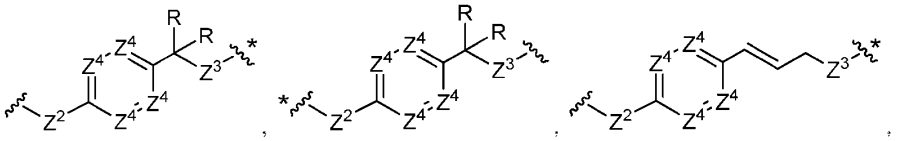
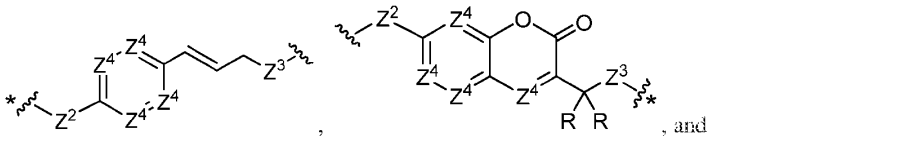
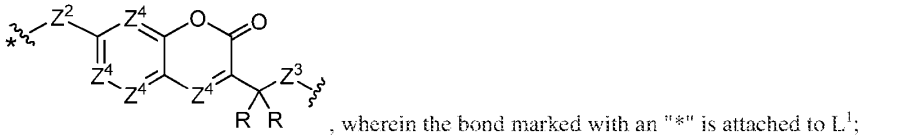
(TL''),

or a pharmaceutically acceptable salt thereof, wherein:

A^1 is selected from CH and N;

A^2 is selected from CH and N;

L^1 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

Z^1 is selected from the group consisting of: , , , , and , wherein the bond marked with an "*" is attached to L^1;

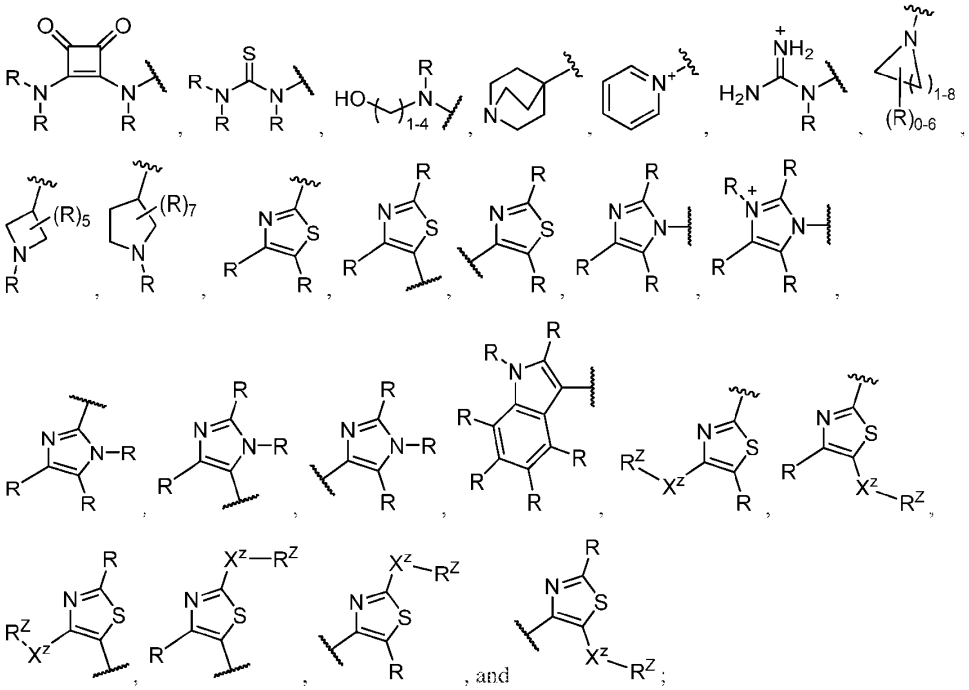
Z^2 is selected from the group consisting of -O-, -NR-, and -S-;

Z^3 is selected from the group consisting of a bond, -O-, -NR-, -S-, -OC(O)-, -C(O)O-, -NRC(O)-, -C(O)NR-,-NRC(O)O-, or -OC(O)NR-;

each Z^4 is independently selected from =CR- and =N-;

L^2 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^1 is selected from the group consisting of -OH, -OAc, -NR_2,



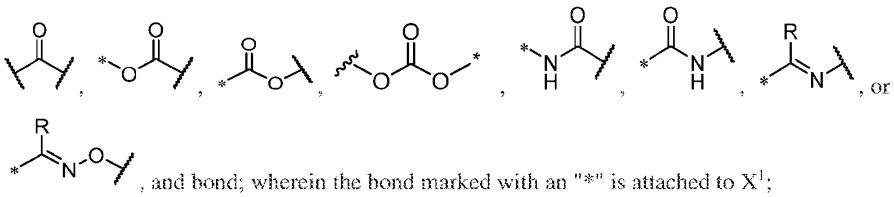
each R is independently -H or C_1-C_6 aliphatic;

X^Z is a bond or optionally substituted C_1-C_6 aliphatic;

R^Z is NR_2 or OH;

X^1 and X^A are each independently a bond or optionally substituted C_1-C_6 aliphatic;

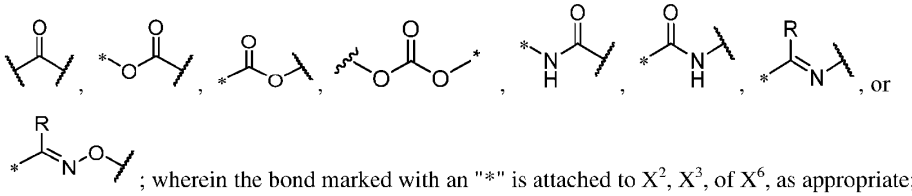
Y^1 is selected from the group consisting of



, and bond; wherein the bond marked with an "*" is attached to X^1;

each of X^2, X^3, and X^6 is independently a bond or optionally substituted C_1-C_{12} aliphatic;

each of Y^2, Y^3, and Y^4 is independently selected from the group consisting of



; wherein the bond marked with an "*" is attached to X^2, X^3, of X^6, as appropriate;

each of X^4, X^5, and X^7 is independently optionally substituted C_1-C_6 aliphatic;

R^2 is -CH(OR^6)(OR^7), -CH(SR^6)(SR^7), -CH(R^6)(R^7), -R^{10}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{10}, wherein one or more methylene linkages of R^2 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^3 is -CH(OR^8)(OR^9), -CH(SR^8)(SR^9), -CH(R^8)(R^9), -R^{11}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{11}, wherein one or more methylene linkages of R^3 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^{12} is -CH(OR^{13})(OR^{14}), -CH(SR^{13})(SR^{14}), -CH(R^{13})(R^{14}), -R^{15}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{15}, wherein one or more methylene linkages of R^{12} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^6 and R^7 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{10}, or optionally substituted -C_1-C_{14} aliphatic-R^{10}; wherein one or more methylene linkages of R^6 and R^7 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

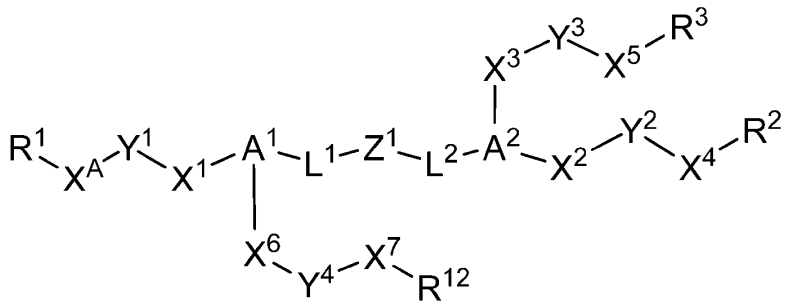
R^8 and R^9 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{11}, or optionally substituted -C_1-C_{14} aliphatic-R^{11}; wherein one or more methylene linkages of R^8 and R^9 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^{13} and R^{14} are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{15}, or optionally substituted -C_1-C_{14} aliphatic-R^{15}; wherein one or more methylene linkages of R^{13} and R^{14} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

each of R^{10}, R^{11}, and R^{15} is independently an optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic C_4-C_{14} cycloalkyl or optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic 4-14 membered heterocyclyl, or two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic C_4-C_{14} cycloalkyl or optionally substituted bridged bicyclic or multicyclic 4-14 membered heterocyclyl.

Formula (TL')

[0065]The present disclosure, in some embodiments, provides compounds of Formula (TL')



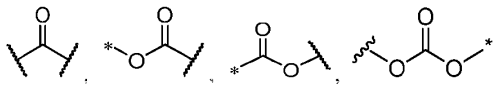
(TL'),

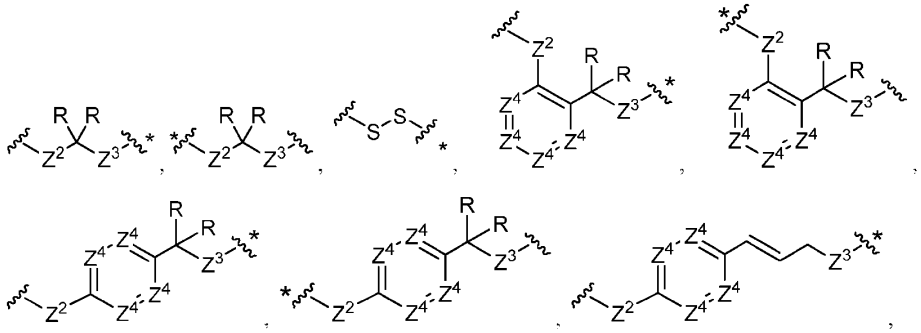
or a pharmaceutically acceptable salt thereof, wherein:

A^1 is selected from CH and N;

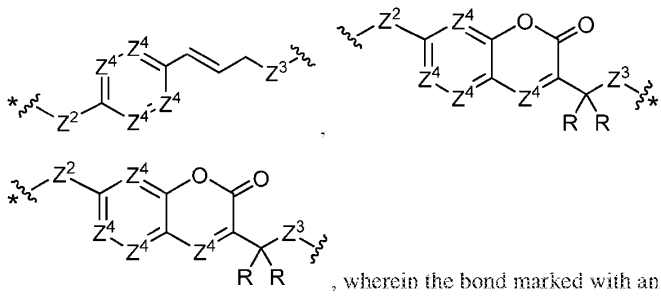
A^2 is selected from CH and N;

L^1 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

Z^1 is selected from the group consisting of: 



, and



, wherein the bond marked with an "*" is attached to L^1;

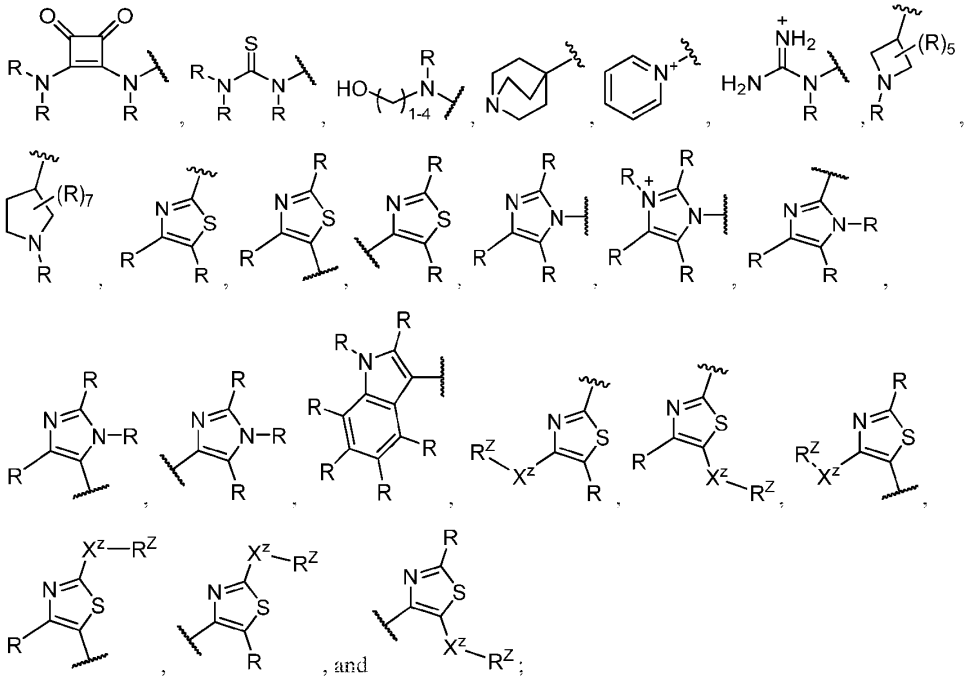
Z^2 is selected from the group consisting of -O-, -NR-, and -S-;

Z^3 is selected from the group consisting of a bond, -O-, -NR-, -S-, -OC(O)-, -C(O)O-, -NRC(O)-, -C(O)NR-,-NRC(O)O-, or -OC(O)NR-;

each Z^4 is independently selected from =CR- and =N-;

L^2 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^1 is selected from the group consisting of -OH, -OAc, -NR_2,



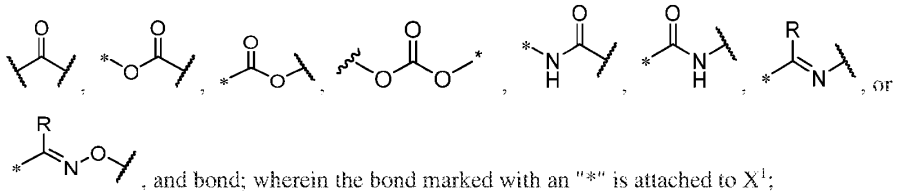
each R is independently -H or C_1-C_6 aliphatic;

X^Z is a bond or optionally substituted C_1-C_6 aliphatic;

R^Z is NR_2 or OH;

X^1 and X^A are each independently a bond or optionally substituted C_1-C_6 aliphatic;

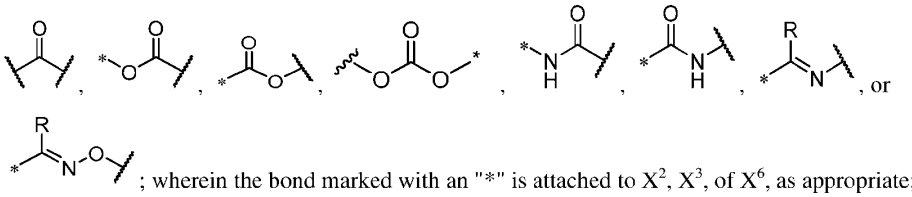
Y^1 is selected from the group consisting of



, and bond; wherein the bond marked with an "*" is attached to X^1;

each of X^2, X^3, and X^6 is independently a bond or optionally substituted C_1-C_{12} aliphatic;

each of Y^2, Y^3, and Y^4 is independently selected from the group consisting of



; wherein the bond marked with an "*" is attached to X^2, X^3, of X^6, as appropriate;

each of X^4, X^5, and X^7 is independently optionally substituted C_1-C_6 aliphatic;

R^2 is -CH(OR^6)(OR^7), -CH(SR^6)(SR^7), -CH(R^6)(R^7), -R^{10}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{10}, wherein one or more methylene linkages of R^2 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^3 is -CH(OR^8)(OR^9), -CH(SR^8)(SR^9), -CH(R^8)(R^9), -R^{11}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{11}, wherein one or more methylene linkages of R^3 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^{12} is -CH(OR^{13})(OR^{14}), -CH(SR^{13})(SR^{14}), -CH(R^{13})(R^{14}), -R^{15}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{15}, wherein one or more methylene linkages of R^{12} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^6 and R^7 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{10}, or optionally substituted -C_1-C_{14} aliphatic-R^{10}; wherein one or more methylene linkages of R^6 and R^7 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

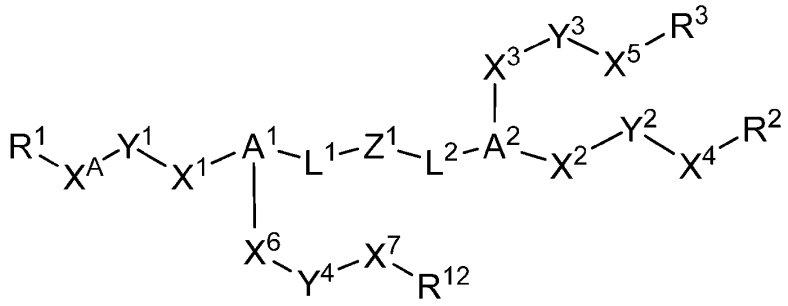
R^8 and R^9 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{11}, or optionally substituted -C_1-C_{14} aliphatic-R^{11}; wherein one or more methylene linkages of R^8 and R^9 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^{13} and R^{14} are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{15}, or optionally substituted -C_1-C_{14} aliphatic-R^{15}; wherein one or more methylene linkages of R^{13} and R^{14} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

each of R^{10}, R^{11}, and R^{15} is independently an optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic C_4-C_{14} cycloalkyl or optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic 4-14 membered heterocyclyl, or two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic C_4-C_{14} cycloalkyl or optionally substituted bridged bicyclic or multicyclic 4-14 membered heterocyclyl.

Formula (TL)

[0066]The present disclosure, in some embodiments, provides compounds of Formula (TL)



(TL),

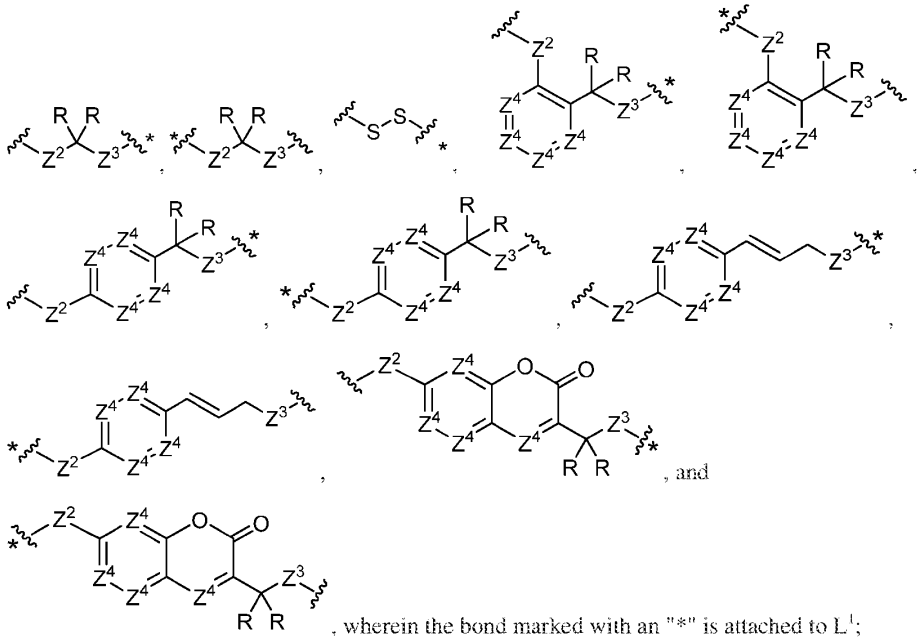
or a pharmaceutically acceptable salt thereof, wherein:

A^1 is selected from CH and N;

A^2 is selected from CH and N;

L^1 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

Z^1 is selected from the group consisting of



, wherein the bond marked with an "*" is attached to L^1;

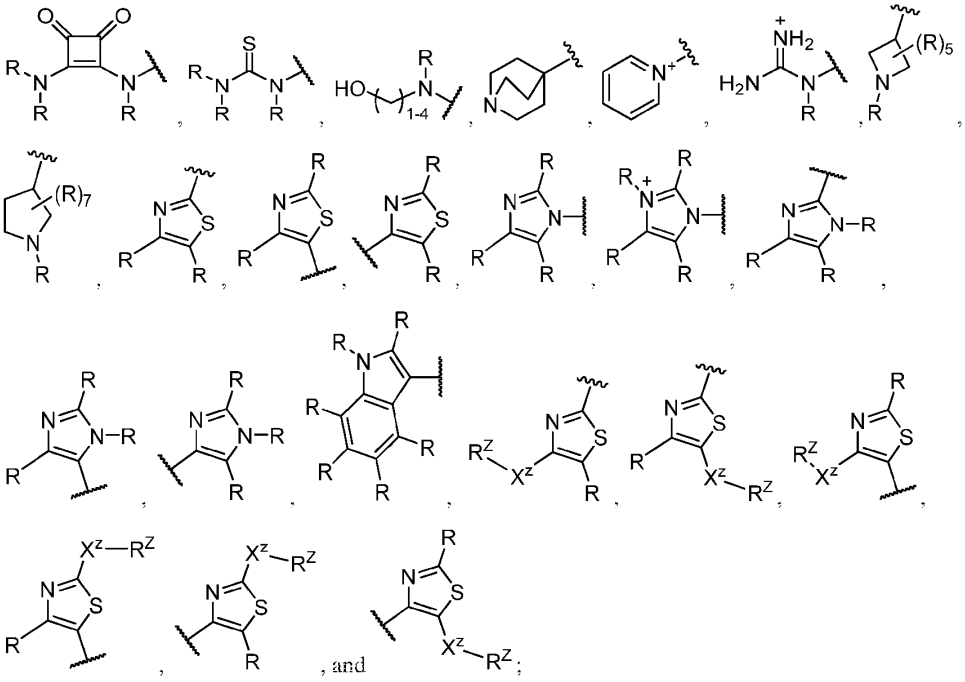
Z^2 is selected from the group consisting of -O-, -NR-, and -S-;

Z^3 is selected from the group consisting of a bond, -O-, -NR-, -S-, -OC(O)-, -C(O)O-, -NRC(O)-, -C(O)NR-,-NRC(O)O-, or -OC(O)NR-;

each Z^4 is independently selected from =CR- and =N-;

L^2 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^1 is selected from the group consisting of -OH, -OAc, -NR_2,



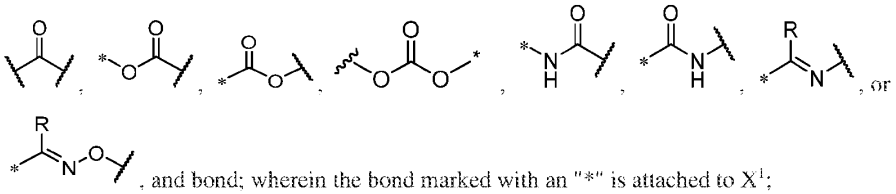
each R is independently -H or C_1-C_6 aliphatic;

X^Z is a bond or optionally substituted C_1-C_6 aliphatic;

R^Z is NR_2 or OH;

X^1 and X^A are each independently a bond or optionally substituted C_1-C_6 aliphatic;

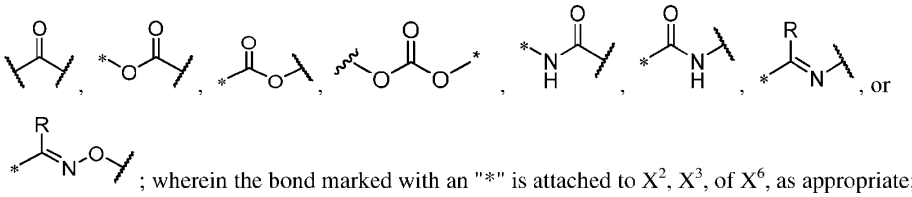
Y^1 is selected from the group consisting of



, and bond; wherein the bond marked with an "*" is attached to X^1;

each of X^2, X^3, and X^6 is independently a bond or optionally substituted C_1-C_{12} aliphatic;

each of Y^2, Y^3, and Y^4 is independently selected from the group consisting of



; wherein the bond marked with an "*" is attached to X^2, X^3, of X^6, as appropriate;

each of X^4, X^5, and X^7 is independently optionally substituted C_1-C_6 aliphatic;

R^2 is -CH(OR^6)(OR^7), -CH(SR^6)(SR^7), -CH(R^6)(R^7), -R^{10}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{10}, wherein one or more methylene linkages of R^2 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^3 is -CH(OR^8)(OR^9), -CH(SR^8)(SR^9), -CH(R^8)(R^9), -R^{11}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{11}, wherein one or more methylene linkages of R^3 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^{12} is -CH(OR^{13})(OR^{14}), -CH(SR^{13})(SR^{14}), -CH(R^{13})(R^{14}), -R^{15}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{15}, wherein one or more methylene linkages of R^{12} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^6 and R^7 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{10}, or optionally substituted -C_1-C_{14} aliphatic-R^{10}; wherein one or more methylene linkages of R^6 and R^7 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

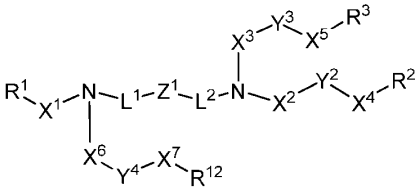
R^8 and R^9 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{11}, or optionally substituted -C_1-C_{14} aliphatic-R^{11}; wherein one or more methylene linkages of R^8 and R^9 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^{13} and R^{14} are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{15}, or optionally substituted -C_1-C_{14} aliphatic-R^{15}; wherein one or more methylene linkages of R^{13} and R^{14} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

each of R^{10}, R^{11}, and R^{15} is independently an optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic C_4-C_{14} cycloalkyl or optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic 4-14 membered heterocyclyl, or two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic C_4-C_{14} cycloalkyl or optionally substituted bridged bicyclic or multicyclic 4-14 membered heterocyclyl.

Formula (TL-A)

[0067]The present disclosure, in some embodiments, provides compounds of Formula (TL-A)

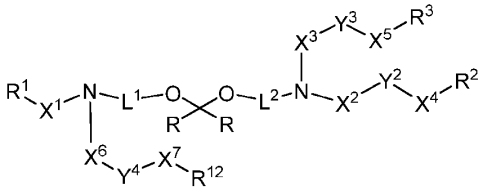


(TL-A),

or a pharmaceutically acceptable salt thereof, wherein R^1, R, X^1, L^1, L^2, Z^1, X^2, X^3, X^4, X^5, Y^2, Y^3, Y^4, R^2, R^3, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formula (TL-B)

[0068]The present disclosure, in some embodiments, provides compounds of Formula (TL-B)

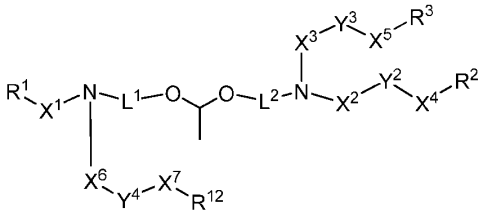


(TL-B),

or a pharmaceutically acceptable salt thereof, wherein R^1, R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, Y^4, R^2, R^3, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formula (TL-B')

[0069]The present disclosure, in some embodiments, provides compounds of Formula (TL-B')

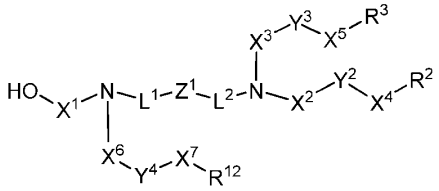


(TL-B'),

or a pharmaceutically acceptable salt thereof, wherein R^1, R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, Y^4, R^2, R^3, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formula (TL-C)

[0070]The present disclosure, in some embodiments, provides compounds of Formula (TL-C)

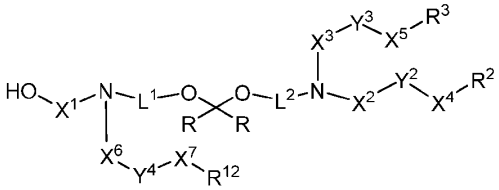


(TL-C),

or a pharmaceutically acceptable salt thereof, wherein Z^1, R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, Y^4, R^2, R^3, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formula (TL-D)

[0071]The present disclosure, in some embodiments, provides compounds of Formula (TL-D)

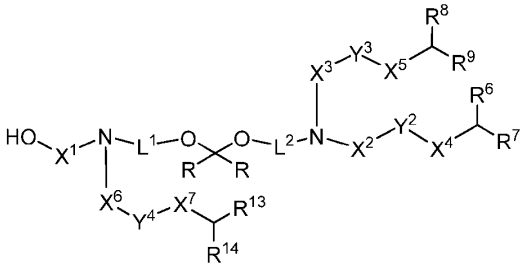


(TL-D),

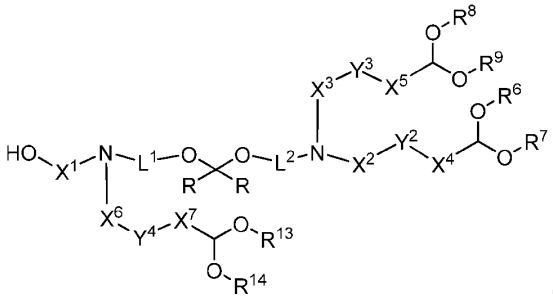
or a pharmaceutically acceptable salt thereof, wherein R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, Y^4, R^2, R^3, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{12}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formulae (TL-E), (TL-E'), and (TL-E'')

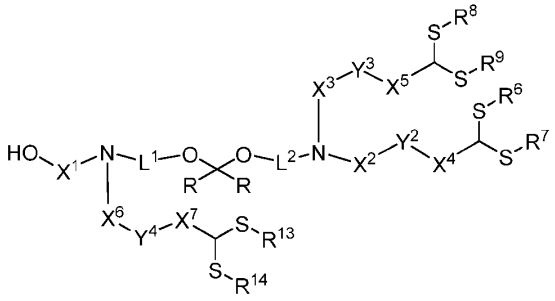
[0072]The present disclosure, in some embodiments, provides compounds of Formula (TL-E), (TL-E'), and (TL-E'')



(TL-E),



(TL-E'),

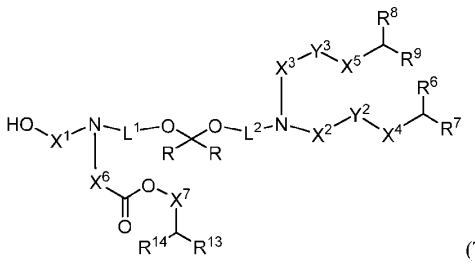


(TL-E''),

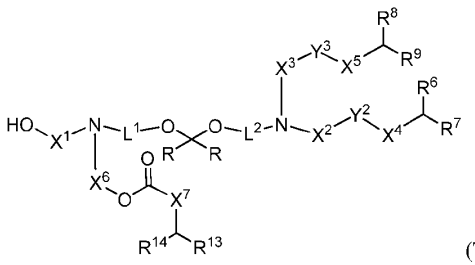
or a pharmaceutically acceptable salt thereof, wherein R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, Y^4, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formulae (TL-E-1), (TL-E-2), (TL-E'-1), (TL-E'-2), (TL-E''-1), and (TL-E''-2)

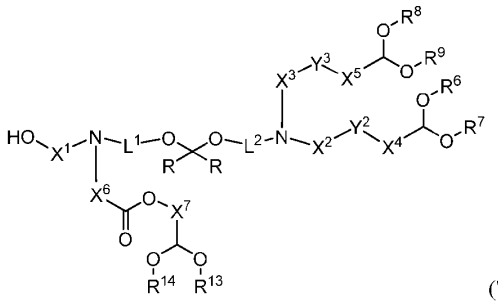
[0073]The present disclosure, in some embodiments, provides compounds of Formula (TL-E-1), (TL-E-2), (TL-E'-1), (TL-E'-2), (TL-E''-1), and (TL-E''-2)



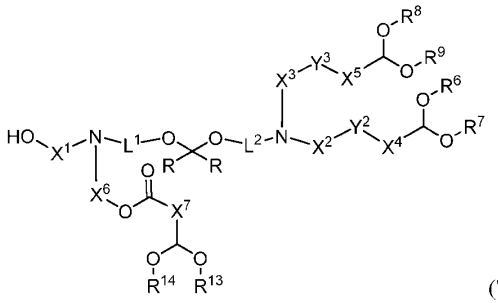
(TL-E-1),



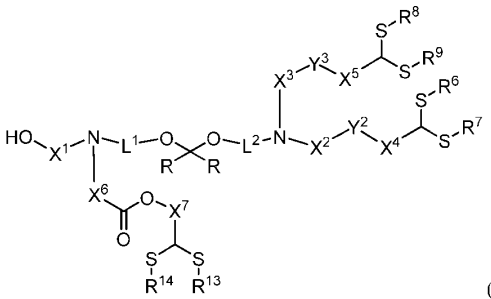
(TL-E-2),



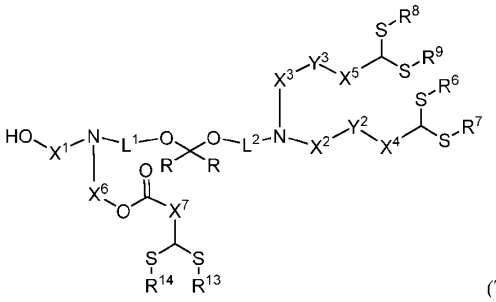
(TL-E'-1),



(TL-E'-2),



(TL-E''-1),

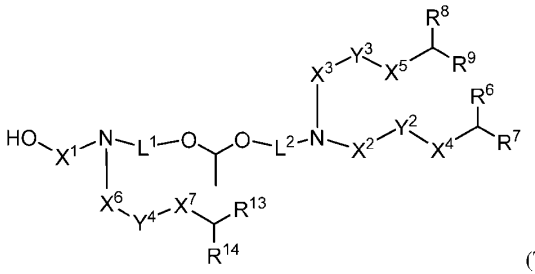


(TL-E''-2),

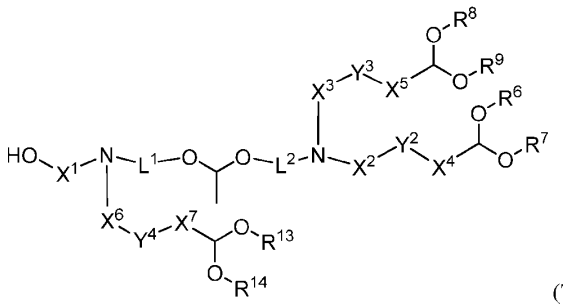
or a pharmaceutically acceptable salt thereof, wherein R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formulae (TL-F), (TL-F'), and (TL-F'')

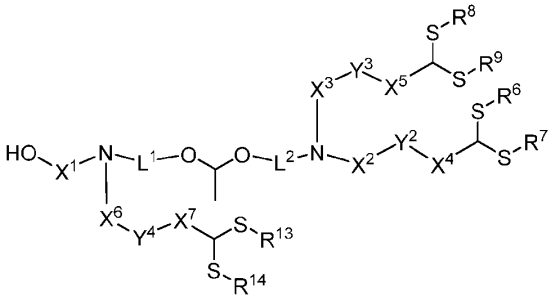
[0074]The present disclosure, in some embodiments, provides compounds of Formula (TL-F), (TL-F'), and (TL-F'')



(TL-F),



(TL-F'),

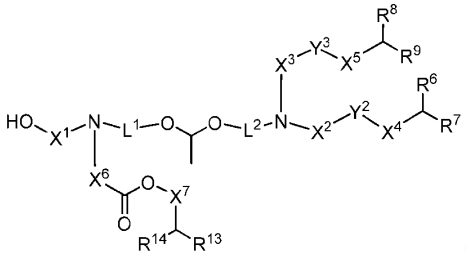


(TL-F''),

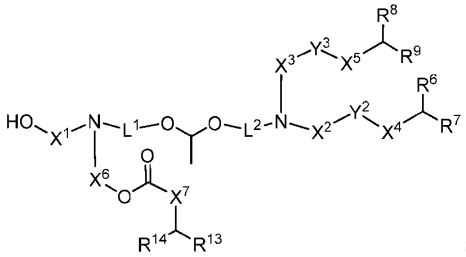
or a pharmaceutically acceptable salt thereof, wherein R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, Y^4, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formulae (TL-F-1), (TL-F-2), (TL-F'-1), (TL-F'-2), (TL-F''-1), and (TL-F''-2)

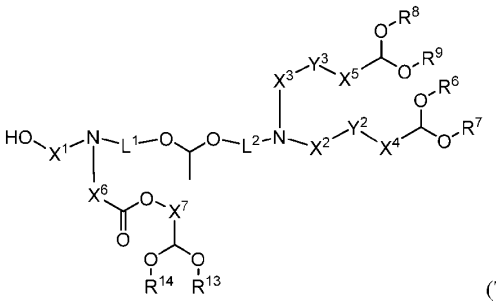
[0075]The present disclosure, in some embodiments, provides compounds of Formula (TL-F-1), (TL-F-2), (TL-F'-1), (TL-F'-2), (TL-F''-1), and (TL-F''-2)



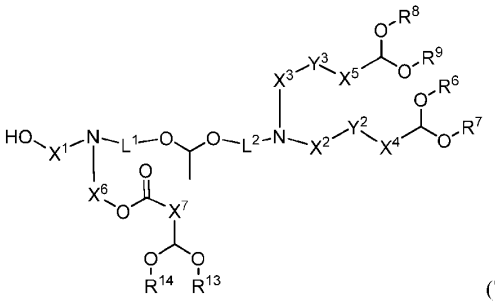
(TL-F-1),



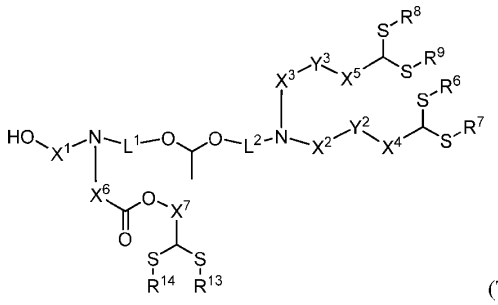
(TL-F-2),



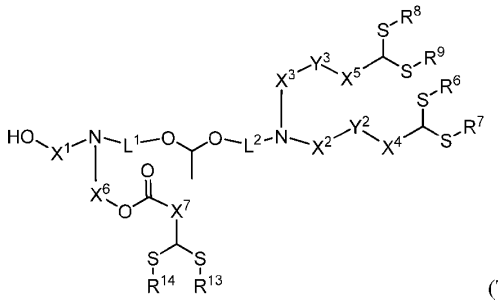
(TL-F'-1),



(TL-F'-2),



(TL-F''-1),

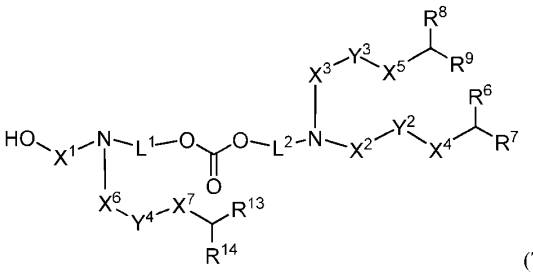


(TL-F''-2),

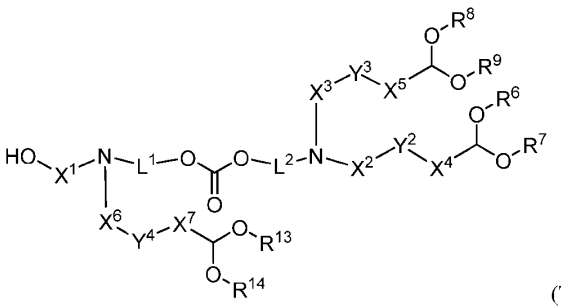
or a pharmaceutically acceptable salt thereof, wherein R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formulae (TL-G), (TL-G'), and (TL-G'')

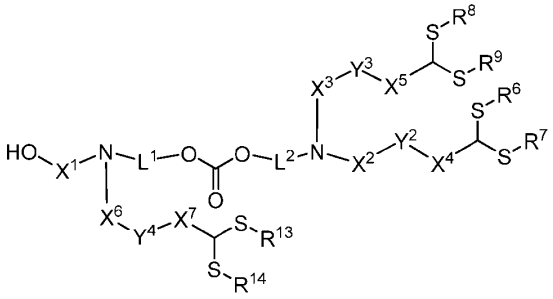
[0076]The present disclosure, in some embodiments, provides compounds of Formula (TL-G), (TL-G'), and (TL-G'')



(TL-G),



(TL-G'),

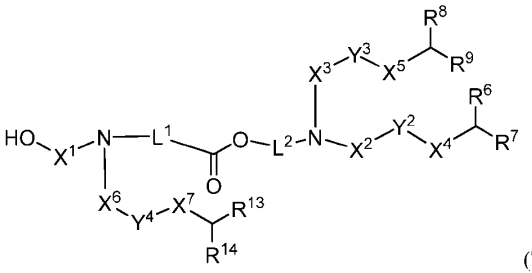


(TL-G''),

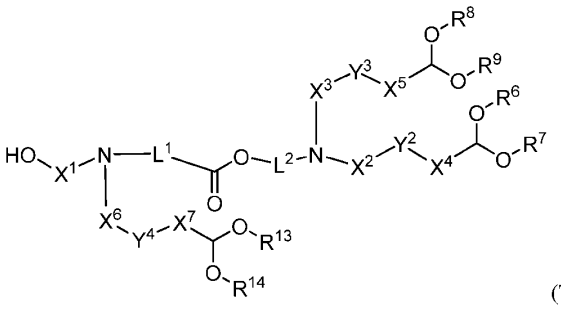
or a pharmaceutically acceptable salt thereof, wherein R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, Y^4, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

Formulae (TL-H), (TL-H'), and (TL-H'')

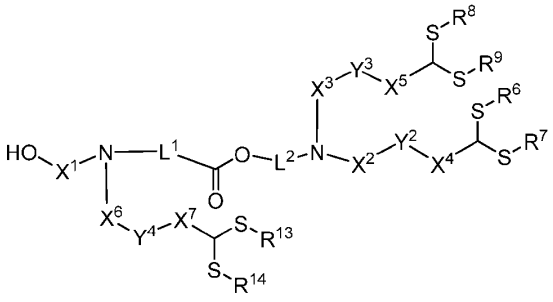
[0077]The present disclosure, in some embodiments, provides compounds of Formula (TL-H), (TL-H'), and (TL-H'')



(TL-H),

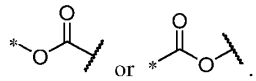


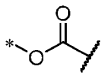
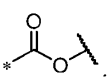
(TL-H'),



(TL-H''),

or a pharmaceutically acceptable salt thereof, wherein R, X^1, L^1, L^2, X^2, X^3, X^4, X^5, Y^2, Y^3, Y^4, R^6, R^7, R^8, R^9, R^{10}, R^{11}, R^{13}, R^{14}, and R^{15} are as described in Formula (TL) or as otherwise described in any embodiments below.

[0078]In certain embodiments, the compound is of Formula (TL), (TL-A), (TL-B), (TL-B'), (TL-C), (TL-D), (TL-E), (TL-E'), (TL-E''), (TL-F), (TL-F'), or (TL-F'') wherein Y^2, Y^3, and Y^4 are each  or .

[0079]In certain embodiments, the compound is of Formula (TL), (TL-A), (TL-B), (TL-B'), (TL-C), (TL-D), (TL-E), (TL-E'), (TL-E''), (TL-F), (TL-F'), (TL-F''), (TL-G), (TL-G'), (TL-G''), (TL-H), (TL-H'), and (TL-H'') wherein Y^2, Y^3, and Y^4 are each  or .

A^1 and A^2

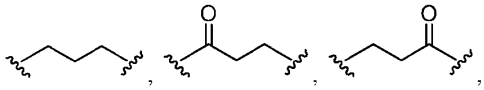
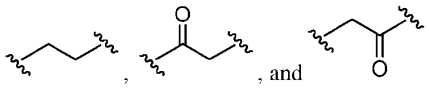
[0080]As disclosed in Formula (TL), in certain embodiments, A^1 is selected from CH and N, and A^2 is selected from CH and N.

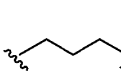
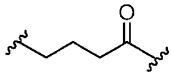
[0081]In certain embodiments, A^1 is CH. In certain embodiments, A^1 is N. In certain embodiments, A^2 is CH. In certain embodiments, A^2 is N. In certain embodiments, A^1 and A^2 are both CH. In certain embodiments, A^1 and A^2 are both N. In certain embodiments, A^1 is CH and A^2 is N. In certain embodiments, A^1 is N and A^2 is CH.

L^1

[0082]As disclosed in Formula (TL), in certain embodiments, L^1 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^1 is unsubstituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^1 is unsubstituted C_1-C_8 aliphatic, wherein one methylene linkage of L^1 is replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^1 is unsubstituted C_1-C_8 aliphatic, wherein one methylene linkage of L^1 is replaced with -C(O)-. In certain embodiments, L^1 is unsubstituted C_1-C_8 aliphatic. In certain embodiments, L^1 is unsubstituted C_1-C_6 aliphatic. In certain embodiments, L^1 is optionally substituted C_1-C_8 alkylene. In certain embodiments, L^1 is optionally substituted C_1-C_6 alkylene. In certain embodiments, L^1 is unsubstituted C_1-C_8 alkylene. In certain embodiments, L^1 is unsubstituted C_1-C_6 alkylene. In certain embodiments, L^1 is optionally substituted C_1-C_8 alkylene, wherein one methylene linkage of L^1 is replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^1 is optionally substituted C_1-C_8 alkylene, wherein one methylene linkage of L^1 is replaced with -C(O)-. In certain embodiments, L^1 is optionally substituted C_1-C_6 alkylene, wherein one methylene linkage of L^1 is replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -

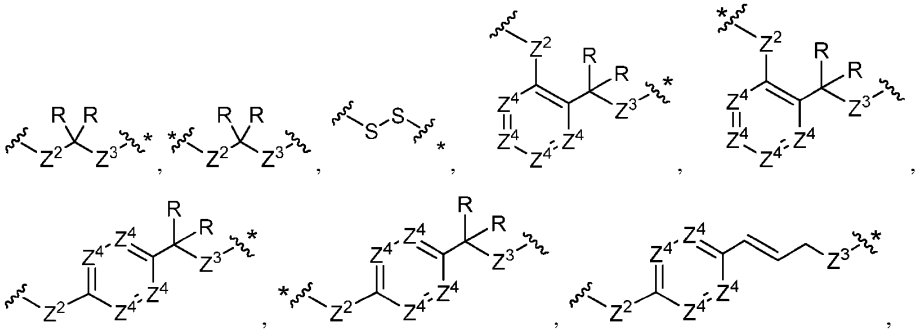
OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^1 is optionally substituted C_1-C_6 alkylene, wherein one methylene linkage of L^1 is replaced with -C(O)-. In certain embodiments, L^1 is optionally substituted C_1-C_4 alkylene. In certain embodiments, L^1 is unsubstituted C_1-C_4 alkylene. In certain embodiments, L^1 is optionally substituted C_1-C_4 alkylene, wherein one methylene linkage of L^1 is replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^1 is optionally substituted C_1-C_4 alkylene, wherein one methylene linkage of L^1 is replaced with -C(O)-. In certain embodiments, L^1 is optionally substituted C_1-C_2 alkylene. In certain embodiments, L^1 is unsubstituted C_1-C_2 alkylene. In certain embodiments, L^1 is unsubstituted C_2-C_6 alkylene. In certain embodiments, L^1 is optionally substituted methylene. In certain embodiments, L^1 is optionally substituted C_2 alkylene. In certain embodiments, L^1 is optionally substituted C_3 alkylene. In certain embodiments, L^1 is optionally substituted C_4 alkylene. In certain embodiments, L^1 is optionally substituted C_5 alkylene. In certain embodiments, L^1 is optionally substituted C_6 alkylene. In certain embodiments, L^1 is $-(CH_2)-$. In certain embodiments, L^1 is $-(CH_2)_2-$. In certain embodiments, L^1 is $-(CH_2)_3-$. In certain embodiments, L^1 is $-(CH_2)_4-$. In certain embodiments, L^1 is $-(CH_2)_5-$. In certain embodiments, L^1 is $-(CH_2)_6-$.

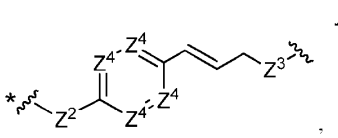
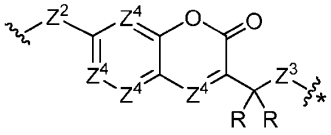
[0083]In certain embodiments, L^1 is selected from , , and .

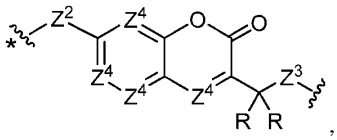
[0084]In some embodiments, L^1 , or .

Z^1

[0085]As disclosed in Formula (TL), in certain embodiments, Z^1 is selected from the group consisting of



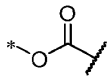
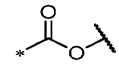
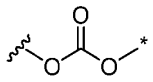


, and

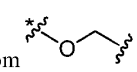
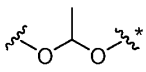
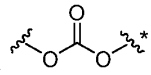

, wherein the bond marked with an "*" is attached to L^1;

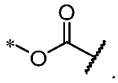
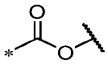
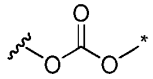
Z^2 is selected from the group consisting of -O-, -NR-, and -S-;

Z^3 is selected from the group consisting of a bond, -O-, -NR-, -S-, -OC(O)-, -C(O)O-, -NRC(O)-, -C(O)NR-, -NRC(O)O-, or -OC(O)NR-;

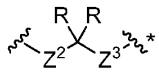
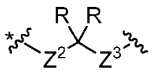
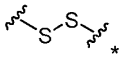
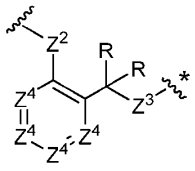
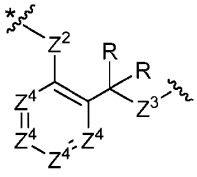
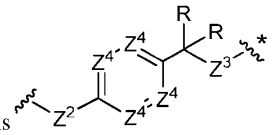
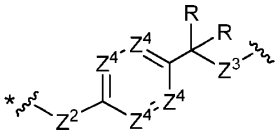
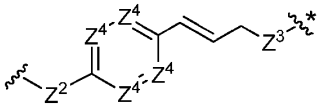
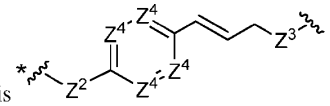
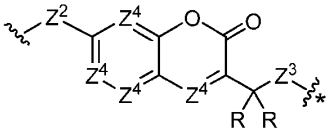
each Z^4 is independently selected from =CR- and =N-;

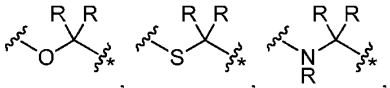
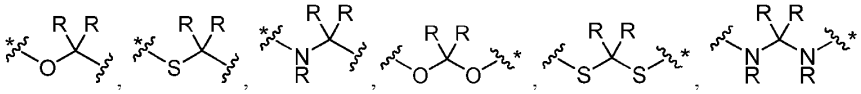
[0086]In certain embodiments Z^1 is , , , or  wherein the bond marked with an "*" is attached to L^1.

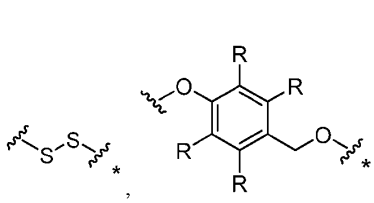
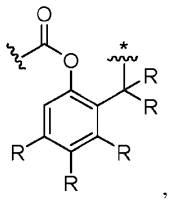
[0087] In some embodiments, Z^1 is selected from , , and , wherein the bond marked with an "*" is attached to L^1.

[0088]In certain embodiments Z^1 is . In certain embodiments Z^1 is . In certain embodiments Z^1 is . In certain embodiments Z^1 is .

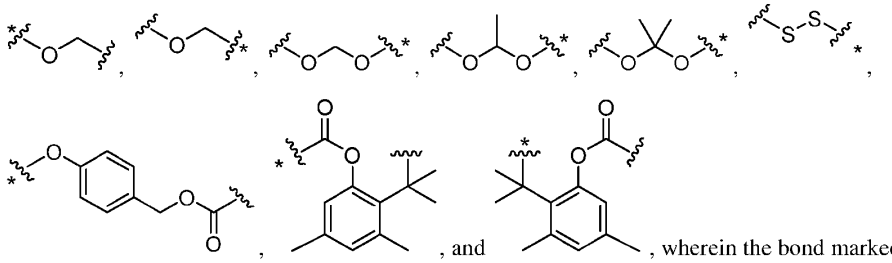
[0089]In some embodiments, Z^2 is -O-. In some embodiments, Z^2 is -NR-. In some embodiments, Z^2 is -S-. In some embodiments, Z^3 is a bond. In some embodiments, Z^3 is -O-. In some embodiments, Z^3 is -NR-. In some embodiments, Z^3 is -S-. In some embodiments, Z^3 is -OC(O)-. In some embodiments, Z^3 is -C(O)O-. In some embodiments, Z^3 is -NRC(O)-. In some embodiments, Z^3 is -C(O)NR-. In some embodiments, Z^3 is -NRC(O)O-. In some embodiments, Z^3 is -OC(O)NR-. In some embodiments, each Z^4 is =CR-. In some embodiments, one Z^4 is =N- and the remaining Z^4 are =CR-.

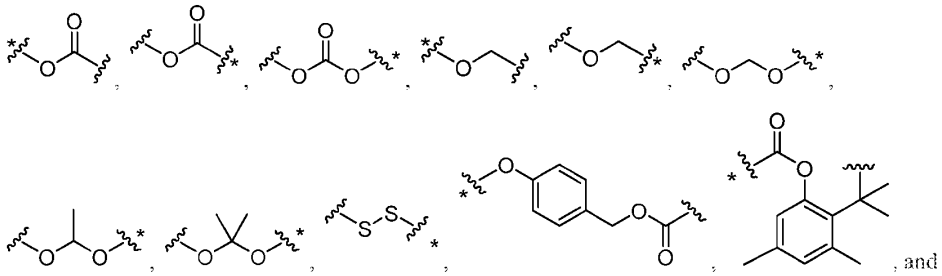
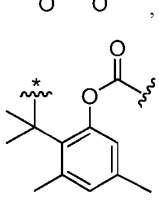
[0090]In some embodiments, Z^1 is . In some embodiments, Z^1 is . In some embodiments, Z^1 is . In some embodiments, Z^1 is . In some embodiments, Z^1 is . In some embodiments, Z^1 is . In some embodiments, Z^1 is . In some embodiments, Z^1 is . In some embodiments, Z^1 is . In some embodiments, Z^1 is .

[0091]In some embodiments, Z^1 is selected from , .



, wherein the bond marked with an "*" is attached to L^1.

[0092]In some embodiments, Z^1 is selected from

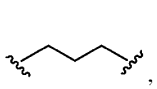
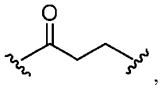
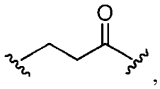
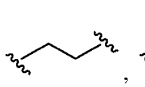
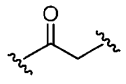
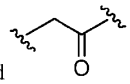
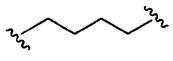

, wherein the bond marked with an "*" is attached to L^1. In some embodiments, Z^1 is selected from



, wherein the bond marked with an "*" is attached to L^1.

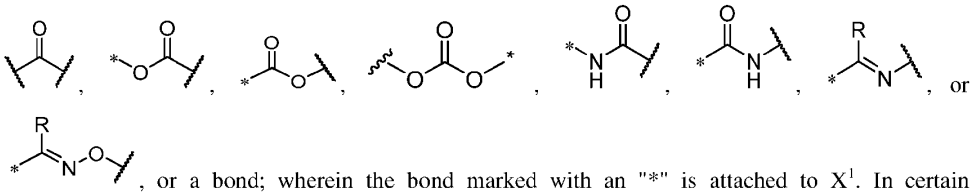
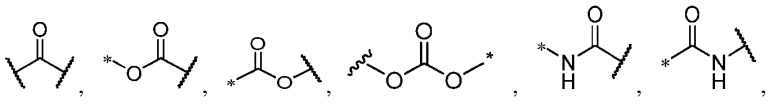
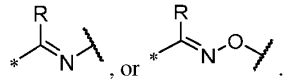
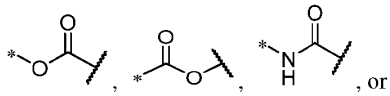
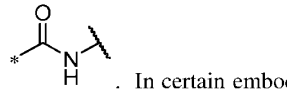
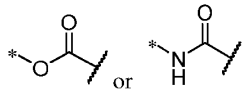
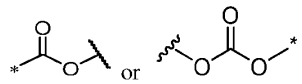
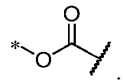
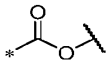
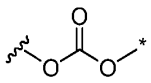
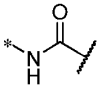
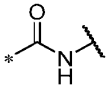
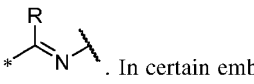
L^2

[0093]As disclosed in Formula (TL), in certain embodiments, L^2 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^2 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^2 is unsubstituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^2 are

each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^2 is unsubstituted C_1-C_8 aliphatic, wherein one methylene linkage of L^2 is replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^2 is unsubstituted C_1-C_8 aliphatic, wherein one methylene linkage of L^2 is replaced with -C(O)-. In certain embodiments, L^2 is unsubstituted C_1-C_8 aliphatic. In certain embodiments, L^2 is unsubstituted C_1-C_6 aliphatic. In certain embodiments, L^2 is optionally substituted C_1-C_8 alkylene. In certain embodiments, L^2 is optionally substituted C_1-C_6 alkylene. In certain embodiments, L^2 is unsubstituted C_1-C_8 alkylene. In certain embodiments, L^2 is unsubstituted C_1-C_6 alkylene. In certain embodiments, L^2 is optionally substituted C_1-C_8 alkylene, wherein one methylene linkage of L^2 is replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^2 is optionally substituted C_1-C_8 alkylene, wherein one methylene linkage of L^2 is replaced with -C(O)-. In certain embodiments, L^2 is optionally substituted C_1-C_6 alkylene, wherein one methylene linkage of L^2 is replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^2 is optionally substituted C_1-C_6 alkylene, wherein one methylene linkage of L^2 is replaced with -C(O)-. In certain embodiments, L^2 is optionally substituted C_1-C_4 alkylene. In certain embodiments, L^2 is unsubstituted C_1-C_4 alkylene. In certain embodiments, L^2 is optionally substituted C_1-C_4 alkylene, wherein one methylene linkage of L^2 is replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-. In certain embodiments, L^2 is optionally substituted C_1-C_4 alkylene, wherein one methylene linkage of L^2 is replaced with -C(O)-. In certain embodiments, L^2 is optionally substituted C_1-C_2 alkylene. In certain embodiments, L^2 is unsubstituted C_1-C_2 alkylene. In certain embodiments, L^2 is unsubstituted C_2-C_6 alkylene. In certain embodiments, L^2 is optionally substituted methylene. In certain embodiments, L^2 is optionally substituted C_2 alkylene. In certain embodiments, L^2 is optionally substituted C_3 alkylene. In certain embodiments, L^2 is optionally substituted C_4 alkylene. In certain embodiments, L^2 is optionally substituted C_5 alkylene. In certain embodiments, L^2 is optionally substituted C_6 alkylene. In certain embodiments, L^2 is $-(CH_2)-$. In certain embodiments, L^2 is $-(CH_2)_2-$. In certain embodiments, L^2 is $-(CH_2)_3-$. In certain embodiments, L^2 is $-(CH_2)_4-$. In certain embodiments, L^2 is $-(CH_2)_5-$. In certain embodiments, L^2 is $-(CH_2)_6-$.

[0094]In certain embodiments, L^2 is selected from , , , , , and . In certain embodiments, L^2 is selected from ,

[0095]As disclosed in Formula (TL), in certain embodiments, Y^1 is

, or a bond; wherein the bond marked with an "*" is attached to X^1. In certain embodiments, Y^1 is , or . In certain embodiments, Y^1 is , or . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is  or . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is . In certain embodiments, Y^1 is a bond.

X^1

[0096]As disclosed in Formula (TL), in certain embodiments, X^1 is a bond or optionally substituted C_1-C_6 aliphatic. In certain embodiments, X^1 is a bond. In certain embodiments, X^1 is a bond or unsubstituted C_1-C_6 aliphatic. In certain embodiments, X^1 is unsubstituted C_1-C_6 aliphatic. In certain embodiments, X^1 is a bond or optionally substituted C_1-C_6 alkylene. In certain embodiments, X^1 is optionally substituted C_1-C_6 alkylene. In certain embodiments, X^1 is a bond or unsubstituted C_1-C_6 alkylene. In certain embodiments, X^1 is unsubstituted C_1-C_6 alkylene. In certain embodiments, X^1 is a bond or unsubstituted C_1-C_4 alkylene. In certain embodiments, X^1 is unsubstituted C_1-C_4 alkylene. In certain embodiments, X^1 is a bond or unsubstituted C_1-C_2 alkylene. In certain embodiments, X^1 is unsubstituted C_1-C_2 alkylene. In certain embodiments, X^1 is unsubstituted C_2-C_6 alkylene. In certain embodiments, X^1 is optionally substituted methylene. In certain embodiments, R^2 is optionally substituted C_2 alkylene. In certain embodiments, X^1 is optionally substituted C_3 alkylene. In certain

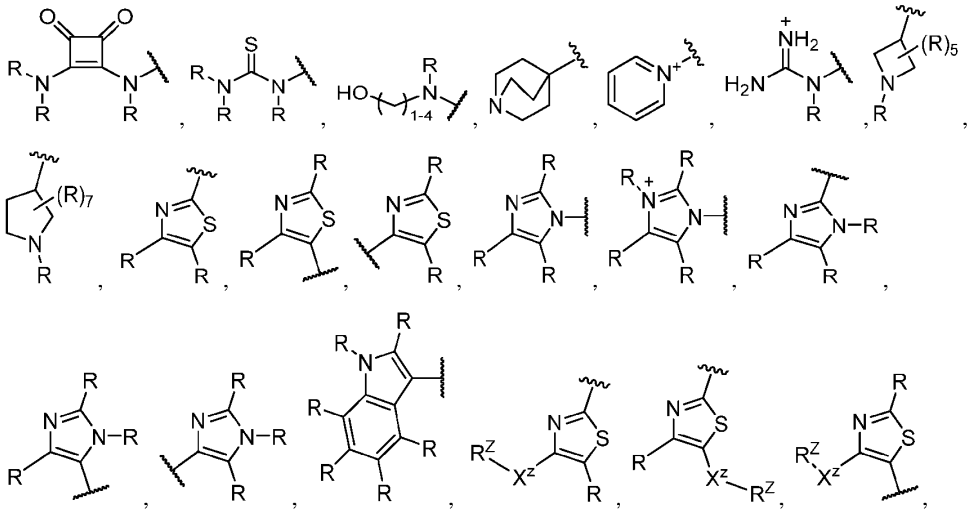
embodiments, X^1 is optionally substituted C_4 alkylene. In certain embodiments, X^1 is optionally substituted C_5 alkylene. In certain embodiments, X^1 is optionally substituted C_6 alkylene. In certain embodiments, X^1 is $-(CH_2)-$. In certain embodiments, X^1 is $-(CH_2)_2-$. In certain embodiments, X^1 is $-(CH_2)_3-$. In certain embodiments, X^1 is $-(CH_2)_4-$. In certain embodiments, X^1 is $-(CH_2)_5-$. In certain embodiments, X^1 is $-(CH_2)_6-$.

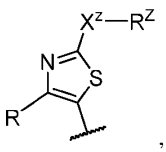
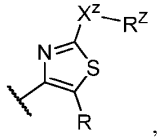
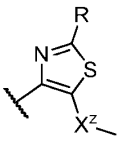
X^A

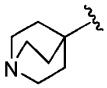
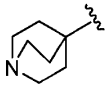
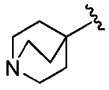
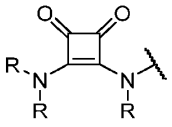
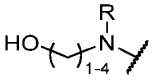
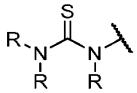
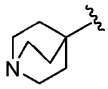
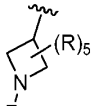
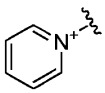
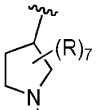
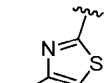
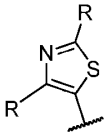
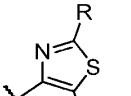
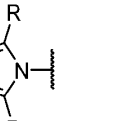
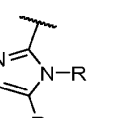
[0097]As disclosed in Formula (TL), in certain embodiments X^A is a bond or optionally substituted C_1-C_6 aliphatic. In certain embodiments, X^A is a bond. In certain embodiments, X^A is a bond or unsubstituted C_1-C_6 aliphatic. In certain embodiments, X^A is unsubstituted C_1-C_6 aliphatic. In certain embodiments, X^A is a bond or optionally substituted C_1-C_6 alkylene. In certain embodiments, X^A is optionally substituted C_1-C_6 alkylene. In certain embodiments, X^A is a bond or unsubstituted C_1-C_6 alkylene. In certain embodiments, X^A is unsubstituted C_1-C_6 alkylene. In certain embodiments, X^A is a bond or unsubstituted C_1-C_4 alkylene. In certain embodiments, X^A is unsubstituted C_1-C_4 alkylene. In certain embodiments, X^A is a bond or unsubstituted C_1-C_2 alkylene. In certain embodiments, X^A is unsubstituted C_1-C_2 alkylene. In certain embodiments, X^A is unsubstituted C_2-C_6 alkylene. In certain embodiments, X^A is optionally substituted methylene. In certain embodiments, R^2 is optionally substituted C_2 alkylene. In certain embodiments, X^A is optionally substituted C_3 alkylene. In certain embodiments, X^A is optionally substituted C_4 alkylene. In certain embodiments, X^A is optionally substituted C_5 alkylene. In certain embodiments, X^A is optionally substituted C_6 alkylene. In certain embodiments, X^A is $-(CH_2)-$. In certain embodiments, X^A is $-(CH_2)_2-$. In certain embodiments, X^A is $-(CH_2)_3-$. In certain embodiments, X^A is $-(CH_2)_4-$. In certain embodiments, X^A is $-(CH_2)_5-$. In certain embodiments, X^A is $-(CH_2)_6-$.

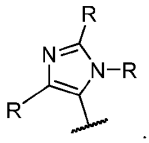
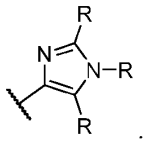
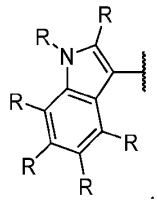
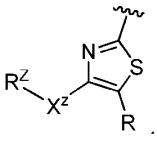
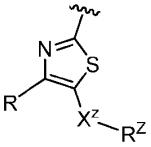
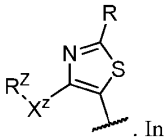
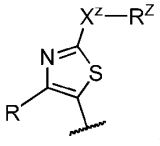
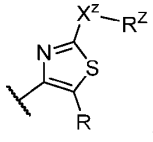
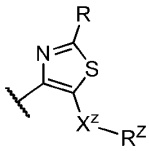
R^1

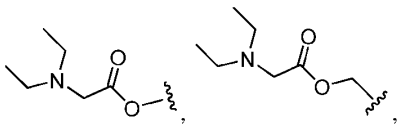
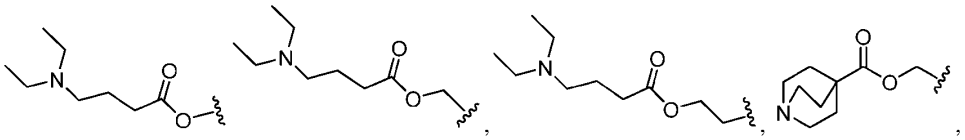
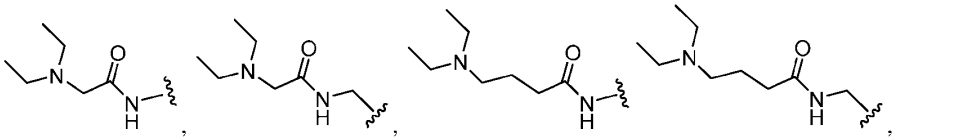
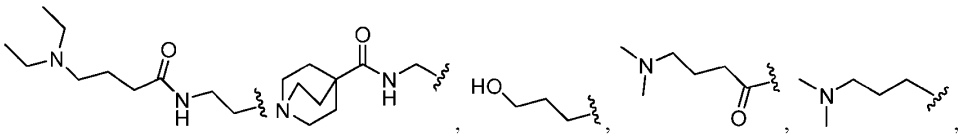
[0098]As disclosed in Formula (TL), in certain embodiments R^1 is selected from -OH, -OAc, $-NR_2$,

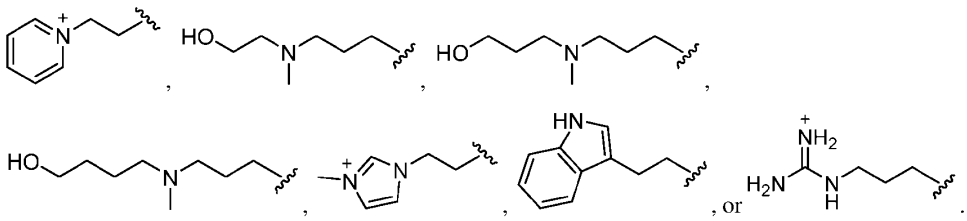





; wherein X^Z is a bond or optionally substituted C_1-C_6 aliphatic; R^Z is NR_2 or OH;

[0099]In certain embodiments, R^1 is OH, $-NR_2$, or . In certain embodiments, R^1 is $-NR_2$ or . In certain embodiments, R^1 is $-NMe_2$, $-NEt_2$, or . In certain embodiments, R^1 is -OH. In certain embodiments, R^1 is -OAc. In certain embodiments, R^1 is $-NR_2$. In certain embodiments, R^1 is $-NH_2$. In certain embodiments, R^1 is $-NMe_2$. In certain embodiments, R^1 is $-NEt_2$. In certain embodiments, R^1 is . n certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is .

embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is . In certain embodiments, R^1 is .

[00100]In certain embodiments, $-X^1-Y^1-X^A-R^1$ is , , , , .



R

[00101]As disclosed in Formula (TL), in certain embodiments each R is independently -H or C_1-C_6 aliphatic. In certain embodiments, R is -H. In certain embodiments, R is C_1-C_6 aliphatic. In certain embodiments, R is or C_1-C_6 alkyl. In certain embodiments, R is C_1-C_4 alkyl. In certain embodiments, R is C_1-C_2 alkyl. In certain embodiments, R is unsubstituted C_1-C_6 alkyl. In certain embodiments, R is unsubstituted C_1-C_4 alkyl. In certain embodiments, R is unsubstituted C_1-C_2 alkyl. In certain embodiments, R is methyl or ethyl. In certain embodiments, R is methyl. In certain embodiments, R is ethyl.

X^2, X^3 and X^6

[00102]As disclosed in Formula (TL), in certain embodiments, X^2, X^3 and X^6 are each independently a bond or optionally substituted C_1-C_{12} aliphatic. In certain embodiments, X^2 and X^3 are the same. In certain embodiments, X^2 and X^3 are different. In certain embodiments, X^2, X^3 and X^6 are the same. In certain embodiments, X^2, X^3 and X^6 are each different from each other. In certain embodiments, X^2 and X^3 are the same, and X^6 is different from the other two.

[00103]In certain embodiments, X^2 is a bond. In certain embodiments, X^2 is an optionally substituted C_1-C_{12} alkylene. In certain embodiments, X^2 is an optionally substituted C_1-C_{12} alkenylene. In certain embodiments, X^2 is an optionally substituted C_1-C_{10} aliphatic. In certain embodiments, X^2 is an optionally substituted C_1-C_{10} alkylene. In certain embodiments, X^2 is an optionally substituted C_1-C_{10} alkenylene. In certain embodiments, X^2 is an optionally substituted C_1-C_8 aliphatic. In certain embodiments, X^2 is an optionally substituted C_1-C_8 alkylene. In certain embodiments, X^2 is an optionally substituted C_1-C_8 alkenylene. In certain embodiments, X^2 is an optionally substituted C_1-C_6 aliphatic. In certain embodiments, X^2 is an optionally substituted C_1-C_6 alkylene. In certain embodiments, X^2 is an optionally substituted C_1-C_6 alkenylene. In certain embodiments, X^2 is an optionally substituted C_2-C_{12} aliphatic. In certain embodiments, X^2 is an optionally substituted C_2-C_{12} alkylene. In certain embodiments, X^2 is an optionally substituted C_2-C_{12} alkenylene. In certain embodiments, X^2 is an optionally substituted C_4-C_{12} aliphatic. In certain embodiments, X^2 is an optionally substituted C_4-C_{12} alkylene. In certain embodiments, X^2 is an optionally substituted C_4-C_{12} alkenylene. In certain embodiments, X^2 is an optionally substituted C_4-C_{10} aliphatic. In certain embodiments, X^2 is an optionally substituted C_4-C_{10} alkylene. In certain embodiments, X^2 is an optionally substituted C_4-C_{10} alkenylene. In certain embodiments, X^2 is an optionally substituted C_6-C_8 aliphatic. In certain embodiments, X^2 is an optionally substituted C_6-C_8 alkylene. In certain

embodiments, X^2 is an optionally substituted C_6-C_8 alkenylene. In certain embodiments, X^2 is an optionally substituted C_7-C_9 aliphatic. In certain embodiments, X^2 is an optionally substituted C_7-C_9 alkylene. In certain embodiments, X^2 is an optionally substituted C_7-C_9 alkenylene. In certain embodiments, X^2 is a bond or an optionally substituted C_1-C_4 aliphatic. In certain embodiments, X^2 an optionally substituted C_1-C_4 alkylene. In certain embodiments, X^2 is an unsubstituted C_1-C_4 aliphatic. In certain embodiments, X^2 is an unsubstituted C_1-C_4 alkylene. In certain embodiments, X^2 is an optionally substituted C_1-C_2 aliphatic. In certain embodiments, X^2 is an optionally substituted C_1-C_2 alkylene. In certain embodiments, X^2 is an unsubstituted C_1-C_2 aliphatic. In certain embodiments, X^2 is an unsubstituted C_1-C_2 alkylene. In certain embodiments, X^2 is $-(CH_2)-$. In certain embodiments, X^2 is $-(CH_2)_2-$. In certain embodiments, X^2 is $-(CH_2)_3-$. In certain embodiments, X^2 is $-(CH_2)_4-$. In certain embodiments, X^2 is $-(CH_2)_5-$. In certain embodiments, X^2 is $-(CH_2)_6-$. In certain embodiments, X^2 is $-(CH_2)_7-$. In certain embodiments, X^2 is $-(CH_2)_8-$. In certain embodiments, X^2 is $-(CH_2)_9-$. In certain embodiments, X^2 is $-(CH_2)_{10}-$. In certain embodiments, X^3 is $-(CH_2)_{11}-$. In certain embodiments, X^3 is $-(CH_2)_{12}-$.

[00104] In certain embodiments, X^3 is a bond. In certain embodiments, X^3 is an optionally substituted C_1-C_{12} alkylene. In certain embodiments, X^3 is an optionally substituted C_1-C_{12} alkenylene. In certain embodiments, X^3 is an optionally substituted C_1-C_{10} aliphatic. In certain embodiments, X^3 is an optionally substituted C_1-C_{10} alkylene. In certain embodiments, X^3 is an optionally substituted C_1-C_{10} alkenylene. In certain embodiments, X^3 is an optionally substituted C_1-C_8 aliphatic. In certain embodiments, X^3 is an optionally substituted C_1-C_8 alkylene. In certain embodiments, X^3 is an optionally substituted C_1-C_8 alkenylene. In certain embodiments, X^3 is an optionally substituted C_1-C_6 aliphatic. In certain embodiments, X^3 is an optionally substituted C_1-C_6 alkylene. In certain embodiments, X^3 is an optionally substituted C_1-C_6 alkenylene. In certain embodiments, X^3 is an optionally substituted C_2-C_{12} aliphatic. In certain embodiments, X^3 is an optionally substituted C_2-C_{12} alkylene. In certain embodiments, X^3 is an optionally substituted C_2-C_{12} alkenylene. In certain embodiments, X^3 is an optionally substituted C_4-C_{12} aliphatic. In certain embodiments, X^3 is an optionally substituted C_4-C_{12} alkylene. In certain embodiments, X^3 is an optionally substituted C_4-C_{12} alkenylene. In certain embodiments, X^3 is an optionally substituted C_4-C_{10} aliphatic. In certain embodiments, X^3 is an optionally substituted C_4-C_{10} alkylene. In certain embodiments, X^3 is an optionally substituted C_4-C_{10} alkenylene. In certain embodiments, X^3 is an optionally substituted C_6-C_8 aliphatic. In certain embodiments, X^3 is an optionally substituted C_6-C_8 alkylene. In certain embodiments, X^3 is an optionally substituted C_6-C_8 alkenylene. In certain embodiments, X^3 is an optionally substituted C_7-C_9 aliphatic. In certain embodiments, X^3 is an optionally substituted C_7-C_9 alkylene. In certain embodiments, X^3 is an optionally substituted C_7-C_9 alkenylene. In certain embodiments, X^3 is an optionally substituted C_1-C_4 aliphatic. In certain embodiments, X^3 is an optionally substituted C_1-C_4 alkylene. In certain embodiments, X^3 is an unsubstituted C_1-C_4 aliphatic. In certain embodiments, X^3 is an unsubstituted C_1-C_4 alkylene. In certain embodiments, X^3 is an

optionally substituted C_1-C_2 aliphatic. In certain embodiments, X^3 is an optionally substituted C_1-C_2 alkylene. In certain embodiments, X^3 is an unsubstituted C_1-C_2 aliphatic. In certain embodiments, X^3 is an unsubstituted C_1-C_2 alkylene. In certain embodiments, X^3 is $-(CH_2)-$. In certain embodiments, X^3 is $-(CH_2)_2-$. In certain embodiments, X^3 is $-(CH_2)_3-$. In certain embodiments, X^3 is $-(CH_2)_4-$. In certain embodiments, X^3 is $-(CH_2)_5-$. In certain embodiments, X^3 is $-(CH_2)_6-$. In certain embodiments, X^3 is $-(CH_2)_7-$. In certain embodiments, X^3 is $-(CH_2)_8-$. In certain embodiments, X^3 is $-(CH_2)_9-$. In certain embodiments, X^3 is $-(CH_2)_{10}-$. In certain embodiments, X^3 is $-(CH_2)_{11}-$. In certain embodiments, X^3 is $-(CH_2)_{12}-$.

[00105]In certain embodiments, X^6 is a bond. In certain embodiments, X^6 is an optionally substituted C_1-C_{12} alkylene. In certain embodiments, X^6 is an optionally substituted C_1-C_{12} alkenylene. In certain embodiments, X^6 is an optionally substituted C_1-C_{10} aliphatic. In certain embodiments, X^6 is an optionally substituted C_1-C_{10} alkylene. In certain embodiments, X^6 is an optionally substituted C_1-C_{10} alkenylene. In certain embodiments, X^6 is an optionally substituted C_1-C_8 aliphatic. In certain embodiments, X^6 is an optionally substituted C_1-C_8 alkylene. In certain embodiments, X^6 is an optionally substituted C_1-C_8 alkenylene. In certain embodiments, X^6 is an optionally substituted C_1-C_6 aliphatic. In certain embodiments, X^6 is an optionally substituted C_1-C_6 alkylene. In certain embodiments, X^6 is an optionally substituted C_1-C_6 alkenylene. In certain embodiments, X^6 is an optionally substituted C_2-C_{12} aliphatic. In certain embodiments, X^6 is an optionally substituted C_2-C_{12} alkylene. In certain embodiments, X^6 is an optionally substituted C_2-C_{12} alkenylene. In certain embodiments, X^6 is an optionally substituted C_4-C_{12} aliphatic. In certain embodiments, X^6 is an optionally substituted C_4-C_{12} alkylene. In certain embodiments, X^6 is an optionally substituted C_4-C_{12} alkenylene. In certain embodiments, X^6 is an optionally substituted C_4-C_{10} aliphatic. In certain embodiments, X^6 is an optionally substituted C_4-C_{10} alkylene. In certain embodiments, X^6 is an optionally substituted C_4-C_{10} alkenylene. In certain embodiments, X^6 is an optionally substituted C_6-C_8 aliphatic. In certain embodiments, X^6 is an optionally substituted C_6-C_8 alkylene. In certain embodiments, X^6 is an optionally substituted C_6-C_8 alkenylene. In certain embodiments, X^6 is an optionally substituted C_7-C_9 aliphatic. In certain embodiments, X^6 is an optionally substituted C_7-C_9 alkylene. In certain embodiments, X^6 is an optionally substituted C_7-C_9 alkenylene. In certain embodiments, X^6 is an optionally substituted C_1-C_4 aliphatic. In certain embodiments, X^6 is an optionally substituted C_1-C_4 alkylene. In certain embodiments, X^6 is an unsubstituted C_1-C_4 aliphatic. In certain embodiments, X^6 is an unsubstituted C_1-C_4 alkylene. In certain embodiments, X^6 is an optionally substituted C_1-C_2 aliphatic. In certain embodiments, X^6 is an optionally substituted C_1-C_2 alkylene. In certain embodiments, X^6 is an unsubstituted C_1-C_2 aliphatic. In certain embodiments, X^6 is an unsubstituted C_1-C_2 alkylene. In certain embodiments, X^6 is $-(CH_2)-$. In certain embodiments, X^6 is $-(CH_2)_2-$. In certain embodiments, X^6 is $-(CH_2)_3-$. In certain embodiments, X^6 is $-(CH_2)_4-$. In certain embodiments, X^6 is $-(CH_2)_5-$. In certain embodiments, X^6 is $-(CH_2)_6-$. In certain embodiments, X^6 is $-(CH_2)_7-$. In certain embodiments, X^6 is $-(CH_2)_8-$. In certain embodiments, X^6 is $-(CH_2)_9-$. In

certain embodiments, X^6 is $-(CH_2)_{10}-$. In certain embodiments, X^6 is $-(CH_2)_{11}-$. In certain embodiments, X^6 is $-(CH_2)_{12}-$.

[00106]In certain embodiments, X^2 and X^3 are each independently a bond or an optionally substituted C_6-C_{10} aliphatic. In certain embodiments, X^2 and X^3 are each independently a bond or an optionally substituted C_6-C_{10} alkylene. In certain embodiments, X^2 and X^3 are each independently a bond or an unsubstituted C_6-C_{10} aliphatic. In certain embodiments, X^2 and X^3 are each independently a bond or an unsubstituted C_6-C_{10} alkylene. In certain embodiments, X^2 and X^3 are each independently a bond or an optionally substituted C_7-C_9 aliphatic. In certain embodiments, X^2 and X^3 are each independently a bond or an optionally substituted C_7-C_9 alkylene. In certain embodiments, X^2 and X^3 are each independently a bond or an unsubstituted C_7-C_9 aliphatic. In certain embodiments, X^2 and X^3 are each independently a bond or an unsubstituted C_7-C_9 alkylene.

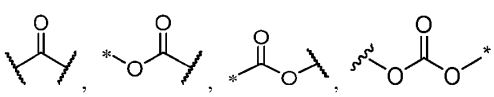
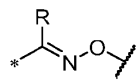
[00107]In certain embodiments, X^2, X^3 and X^6 are each independently a bond or an optionally substituted C_6-C_{10} aliphatic. In certain embodiments, X^2, X^3 and X^6 are each independently a bond or an optionally substituted C_6-C_{10} alkylene. In certain embodiments, X^2, X^3 and X^6 are each independently a bond or an unsubstituted C_6-C_{10} aliphatic. In certain embodiments, X^2, X^3 and X^6 are each independently a bond or an unsubstituted C_6-C_{10} alkylene. In certain embodiments, X^2, X^3 and X^6 are each independently a bond or an optionally substituted C_7-C_9 aliphatic. In certain embodiments, X^2, X^3 and X^6 are each independently a bond or an optionally substituted C_7-C_9 alkylene. In certain embodiments, X^2, X^3 and X^6 are each independently a bond or an unsubstituted C_7-C_9 aliphatic. In certain embodiments, X^2, X^3 and X^6 are each independently a bond or an unsubstituted C_7-C_9 alkylene.

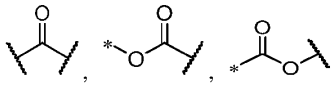
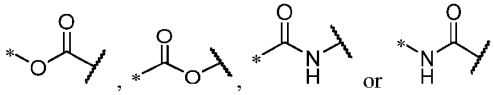
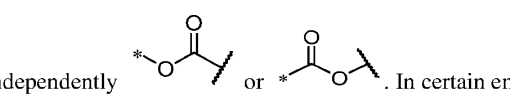
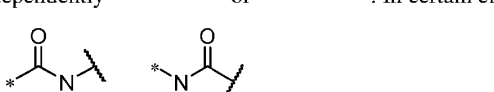
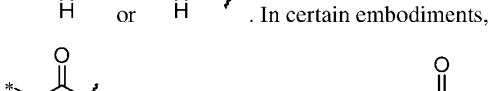
[00108]In certain embodiments, X^2 and X^3 are both a bond or an optionally substituted C_6-C_{10} aliphatic. In certain embodiments, X^2 and X^3 are both a bond or an optionally substituted C_6-C_{10} alkylene. In certain embodiments, X^2 and X^3 are both a bond or an unsubstituted C_6-C_{10} aliphatic. In certain embodiments, X^2 and X^3 are both a bond or an unsubstituted C_6-C_{10} alkylene. In certain embodiments, X^2 and X^3 are both a bond or an optionally substituted C_7-C_9 aliphatic. In certain embodiments, X^2 and X^3 are both a bond or an optionally substituted C_7-C_9 alkylene. In certain embodiments, X^2 and X^3 are both a bond or an unsubstituted C_7-C_9 aliphatic. In certain embodiments, X^2 and X^3 are both a bond or an unsubstituted C_7-C_9 alkylene.

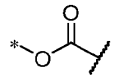
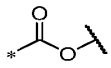
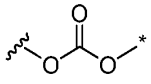
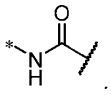
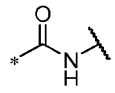
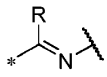
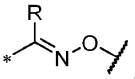
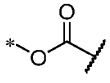
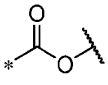
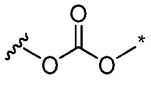
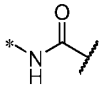
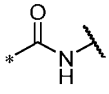
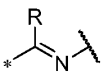
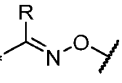
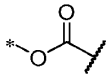
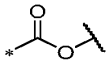
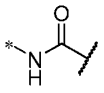
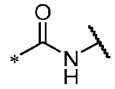
[00109]In certain embodiments, X^2, X^3 and X^6 are each a bond or an optionally substituted C_6-C_{10} aliphatic. In certain embodiments, X^2, X^3 and X^6 are each a bond or an optionally substituted C_6-C_{10} alkylene. In certain embodiments, X^2, X^3 and X^6 are each a bond or an unsubstituted C_6-C_{10} aliphatic. In certain embodiments, X^2, X^3 and X^6 are each a bond or an unsubstituted C_6-C_{10} alkylene. In certain embodiments, X^2, X^3 and X^6 are each a bond or an optionally substituted C_7-C_9 aliphatic. In certain embodiments, X^2, X^3 and X^6 are each a bond or an optionally substituted C_7-C_9 alkylene. In certain embodiments, X^2, X^3 and X^6 are each a bond or an unsubstituted C_7-C_9 aliphatic. In certain embodiments, X^2, X^3 and X^6 are each a bond or an unsubstituted C_7-C_9 alkylene.

[00110]In certain embodiments, X^2 and X^3 are both bonds. In certain embodiments, X^2 and X^3 are both $-(CH_2)_6-$. In certain embodiments, X^2 and X^3 are both $-(CH_2)_7-$. In certain embodiments, X^2 and X^3 are both $-(CH_2)_8-$. In certain embodiments, X^2 and X^3 are both $-(CH_2)_9-$. In certain embodiments, X^2 and X^3 are both $-(CH_2)_{10}-$. In certain embodiments, X^2, X^3 and X^6 are each bonds. In certain embodiments, X^2, X^3 and X^6 are each $-(CH_2)_6-$. In certain embodiments, X^2, X^3 and X^6 are each $-(CH_2)_7-$. In certain embodiments, X^2, X^3 and X^6 are each $-(CH_2)_8-$. In certain embodiments, X^2, X^3 and X^6 are each $-(CH_2)_9-$. In certain embodiments, X^2, X^3 and X^6 are each $-(CH_2)_{10}-$.

Y^2, Y^3 and Y^4

[00111]As disclosed in Formula (TL), in certain embodiments, Y^2, $Y^3$and Y^4 are each independently , , wherein the bond marked with an "*" is attached to X^2 for Y^2, or X^3 for Y^3, or X^6 for Y^4. In certain embodiments, Y^2 and Y^3 are the same. In certain embodiments, Y^2 and Y^3 are different. In certain embodiments, Y^2, $Y^3$and Y^4 are all the same. In certain embodiments, Y^2, $Y^3$and Y^4 are all different. In certain embodiments, two of Y^2, $Y^3$and Y^4 are the same and the third is different. In certain embodiments, Y^2 and Y^3 are the same, and Y^4 is different from the other two.

[00112]In certain embodiments, Y^2 and Y^3 are each independently . In certain embodiments, Y^2 and Y^3 are each independently . In certain embodiments, Y^2 and Y^3 are each independently . In certain embodiments, Y^2 and Y^3 are each independently . In certain embodiments, Y^2 and Y^3 are each independently . In certain embodiments, Y^2 is . In certain embodiments, Y^2 is

. In certain embodiments, Y^2 is . In certain embodiments, Y^2 is . In certain embodiments, Y^2 is . In certain embodiments, Y^2 is . In certain embodiments, Y^2 is . In certain embodiments, Y^2 is . In certain embodiments, Y^3 is . In certain embodiments, Y^3 is . In certain embodiments, Y^3 is . In certain embodiments, Y^3 is . In certain embodiments, Y^3 is . In certain embodiments, Y^3 is . In certain embodiments, Y^3 is . In certain embodiments, Y^3 is . In certain embodiments, Y^3 and Y^2 are both . In certain embodiments, Y^3 and Y^2 are both . In certain embodiments, Y^3 and Y^2 are both . In certain embodiments, Y^3 and Y^2 are both .

X^4, X^5 and X^7

[00113]As disclosed in Formula (TL), in certain embodiments, X^4, X^5 and X^7 are each independently a bond or optionally substituted C_1-C_6 aliphatic. In certain embodiments, X^4 and X^5 are the same. In certain embodiments, X^4 and X^5 are different. In certain embodiments, X^4, X^5 and X^7 are the same. In certain embodiments, X^4, X^5 and X^7 are each different from each other. In certain embodiments, X^4 and X^5 are the same, and X^7 is different from the other two.

[00114]In certain embodiments, X^4 is a bond. In certain embodiments, X^4 is an optionally substituted C_1-C_6 aliphatic. In certain embodiments, X^4 is an optionally substituted C_1-C_6 alkylene. In certain embodiments, X^4 is an optionally substituted C_1-C_6 alkenylene. In certain embodiments, X^4 is an optionally substituted C_2-C_5 aliphatic. In certain embodiments, X^4 is an optionally substituted C_2-C_5

alkylene. In certain embodiments, X^4 is an optionally substituted C_2-C_5 alkenylene. In certain embodiments, X^4 is an optionally substituted C_3-C_4 aliphatic. In certain embodiments, X^4 is an optionally substituted C_3-C_4 alkylene. In certain embodiments, X^4 is an optionally substituted C_3-C_4 alkenylene. In certain embodiments, X^4 is $-(CH_2)-$. In certain embodiments, X^4 is $-(CH_2)_2-$. In certain embodiments, X^4 is $-(CH_2)_3-$. In certain embodiments, X^4 is $-(CH_2)_4-$. In certain embodiments, X^4 is $-(CH_2)_5-$. In certain embodiments, X^4 is $-(CH_2)_6-$.

[00115] In certain embodiments, X^5 is a bond. In certain embodiments, X^5 is an optionally substituted C_1-C_6 aliphatic. In certain embodiments, X^5 is an optionally substituted C_1-C_6 alkylene. In certain embodiments, X^5 is an optionally substituted C_1-C_6 alkenylene. In certain embodiments, X^5 is an optionally substituted C_2-C_5 aliphatic. In certain embodiments, X^5 is an optionally substituted C_2-C_5 alkylene. In certain embodiments, X^5 is an optionally substituted C_2-C_5 alkenylene. In certain embodiments, X^5 is an optionally substituted C_3-C_4 aliphatic. In certain embodiments, X^5 is an optionally substituted C_3-C_4 alkylene. In certain embodiments, X^5 is an optionally substituted C_3-C_4 alkenylene. In certain embodiments, X^5 is $-(CH_2)-$. In certain embodiments, X^5 is $-(CH_2)_2-$. In certain embodiments, X^5 is $-(CH_2)_3-$. In certain embodiments, X^5 is $-(CH_2)_4-$. In certain embodiments, X^5 is $-(CH_2)_5-$. In certain embodiments, X^5 is $-(CH_2)_6-$.

[00116]In certain embodiments, X^7 is a bond. In certain embodiments, X^7 is an optionally substituted C_1-C_6 aliphatic. In certain embodiments, X^7 is an optionally substituted C_1-C_6 alkylene. In certain embodiments, X^7 is an optionally substituted C_1-C_6 alkenylene. In certain embodiments, X^7 is an optionally substituted C_2-C_5 aliphatic. In certain embodiments, X^7 is an optionally substituted C_2-C_5 alkylene. In certain embodiments, X^7 is an optionally substituted C_2-C_5 alkenylene. In certain embodiments, X^7 is an optionally substituted C_3-C_4 aliphatic. In certain embodiments, X^7 is an optionally substituted C_3-C_4 alkylene. In certain embodiments, X^7 is an optionally substituted C_3-C_4 alkenylene. In certain embodiments, X^7 is $-(CH_2)-$. In certain embodiments, X^7 is $-(CH_2)_2-$. In certain embodiments, X^7 is $-(CH_2)_3-$. In certain embodiments, X^7 is $-(CH_2)_4-$. In certain embodiments, X^7 is $-(CH_2)_5-$. In certain embodiments, X^7 is $-(CH_2)_6-$.

[00117]In certain embodiments, X^4 and X^5 are each independently a bond or an optionally substituted C_2-C_5 aliphatic. In certain embodiments, X^4 and X^5 are each independently a bond or an optionally substituted C_2-C_5 alkylene. In certain embodiments, X^4 and X^5 are each independently a bond or an unsubstituted C_2-C_5 aliphatic. In certain embodiments, X^4 and X^5 are each independently a bond or an unsubstituted C_2-C_5 alkylene. In certain embodiments, X^4 and X^5 are each independently a bond or an optionally substituted C_3-C_4 aliphatic. In certain embodiments, X^4 and X^5 are each independently a bond or an optionally substituted C_3-C_4 alkylene. In certain embodiments, X^4 and X^5 are each independently a bond or an unsubstituted C_3-C_4 aliphatic. In certain embodiments, X^4 and X^5 are each independently a bond or an unsubstituted C_3-C_4 alkylene.

[00118]In certain embodiments, X^4, X^5 and X^7 are each independently a bond or an optionally substituted C_2-C_5 aliphatic. In certain embodiments, X^4, X^5 and X^7 are each independently a bond or

an optionally substituted C_2-C_5 alkylene. In certain embodiments, X^4, X^5 and X^7 are each independently a bond or an unsubstituted C_2-C_5 aliphatic. In certain embodiments, X^4, X^5 and X^7 are each independently a bond or an unsubstituted C_2-C_5 alkylene. In certain embodiments, X^4, X^5 and X^7 are each independently a bond or an optionally substituted C_3-C_4 aliphatic. In certain embodiments, X^4, X^5 and X^7 are each independently a bond or an optionally substituted C_3-C_4 alkylene. In certain embodiments, X^4, X^5 and X^7 are each independently a bond or an unsubstituted C_3-C_4 aliphatic. In certain embodiments, X^4, X^5 and X^7 are each independently a bond or an unsubstituted C_3-C_4 alkylene.

[00119]In certain embodiments, X^4 and X^5 are both a bond or an optionally substituted C_2-C_5 aliphatic. In certain embodiments, X^4 and X^5 are both a bond or an optionally substituted C_2-C_5 alkylene. In certain embodiments, X^4 and X^5 are both a bond or an unsubstituted C_2-C_5 aliphatic. In certain embodiments, X^4 and X^5 are both a bond or an unsubstituted C_2-C_5 alkylene. In certain embodiments, X^4 and X^5 are both a bond or an optionally substituted C_3-C_4 aliphatic. In certain embodiments, X^4 and X^5 are both a bond or an optionally substituted C_3-C_4 alkylene. In certain embodiments, X^4 and X^5 are both a bond or an unsubstituted C_3-C_4 aliphatic. In certain embodiments, X^4 and X^5 are both a bond or an unsubstituted C_3-C_4 alkylene.

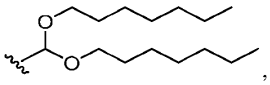
[00120]In certain embodiments, X^4, X^5 and X^7 are each a bond or an optionally substituted C_2-C_5 aliphatic. In certain embodiments, X^4, X^5 and X^7 are each a bond or an optionally substituted C_2-C_5 alkylene. In certain embodiments, X^4, X^5 and X^7 are each a bond or an unsubstituted C_2-C_5 aliphatic. In certain embodiments, X^4, X^5 and X^7 are each a bond or an unsubstituted C_2-C_5 alkylene. In certain embodiments, X^4, X^5 and X^7 are each a bond or an optionally substituted C_3-C_4 aliphatic. In certain embodiments, X^4, X^5 and X^7 are each a bond or an optionally substituted C_3-C_4 alkylene. In certain embodiments, X^4, X^5 and X^7 are each a bond or an unsubstituted C_3-C_4 aliphatic. In certain embodiments, X^4, X^5 and X^7 are each a bond or an unsubstituted C_3-C_4 alkylene.

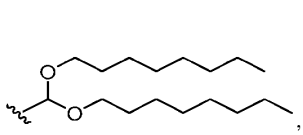
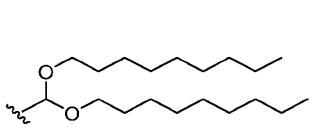
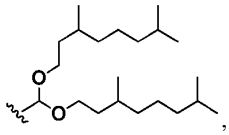
[00121]In certain embodiments, X^4 and X^5 are both bonds. In certain embodiments, X^4 and X^5 are both $-(CH_2)_6$-. In certain embodiments, X^4 and X^5 are both $-(CH_2)_5$-. In certain embodiments, X^4 and X^5 are both $-(CH_2)_4$-. In certain embodiments, X^4 and X^5 are both $-(CH_2)_3$-. In certain embodiments, X^4 and X^5 are both $-(CH_2)_2$-. In certain embodiments, X^4 and X^5 are both $-(CH_2)$-. In certain embodiments, X^4, X^5 and X^7 are each bonds. In certain embodiments, X^4, X^5 and X^7 are each $-(CH_2)_6$-. In certain embodiments, X^4, X^5 and X^7 are each $-(CH_2)_5$-. In certain embodiments, X^4, X^5 and X^7 are each $-(CH_2)_4$-. In certain embodiments, X^4, X^5 and X^7 are each $-(CH_2)_3$-. In certain embodiments, X^4, X^5 and X^7 are each $-(CH_2)_2$-. In certain embodiments, X^4, X^5 and X^7 are each $-(CH_2)$-.

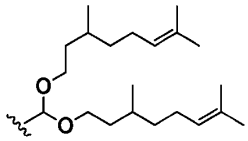
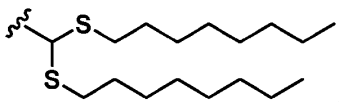
R^2

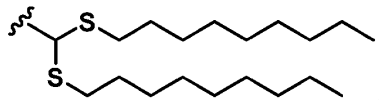
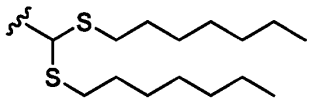
[00122]As disclosed in Formula (TL), in certain embodiments, R^2 is $-CH(OR^6)(OR^7)$, $-CH(SR^6)(SR^7)$, $-CH(R^6)(R^7)$, $-R^{10}$, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{10}, wherein one or more methylene linkages of R^2 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -

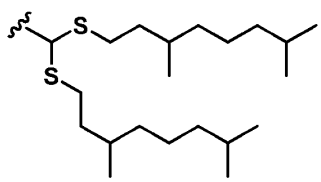
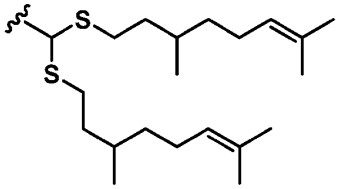
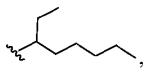
OC(O)-, -NHC(O)- or -C(O)O-. In certain embodiments, R^2 is -CH(OR^6)(OR^7). In certain embodiments, R^2 is -CH(R^6)(R^7). In certain embodiments, R^2 is -CH(SR^6)(SR^7). In certain embodiments, R^2 is -R^{10}. In certain embodiments, R^2 is optionally substituted C_5-C_{18} aliphatic. In certain embodiments, R^2 is optionally substituted C_1-C_{14} aliphatic-R^{10}.

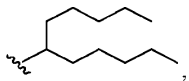
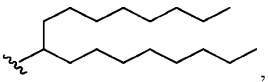
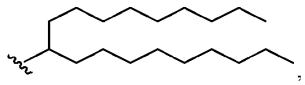
[00123]In certain embodiments, R^2 is selected from ,

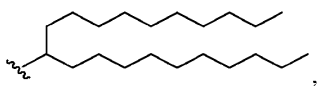
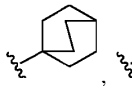
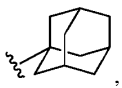
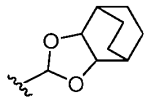
, , ,

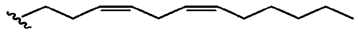
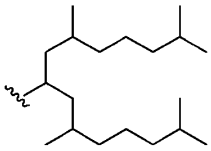
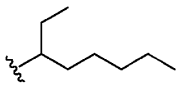
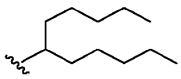
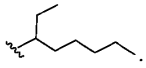
, ,

, ,

, , ,

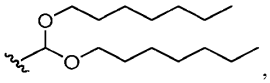
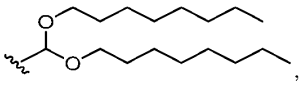
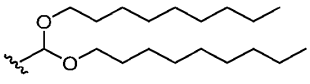
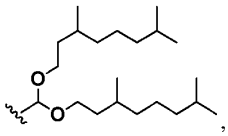
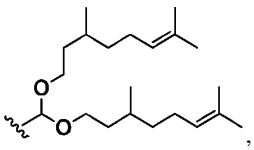
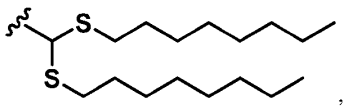
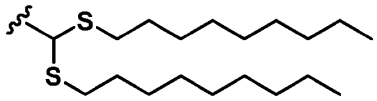
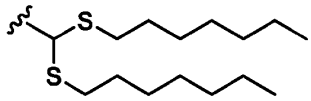
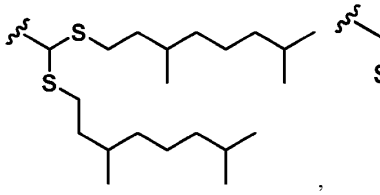
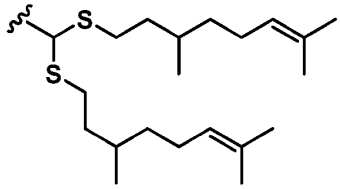
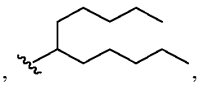
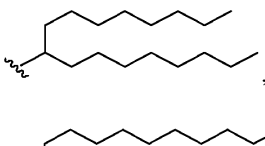
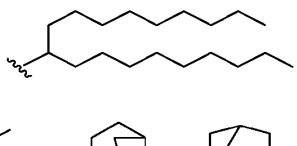
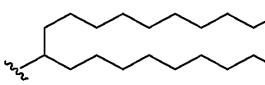
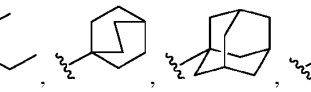
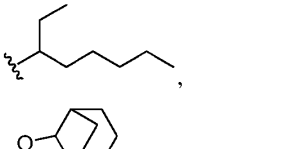
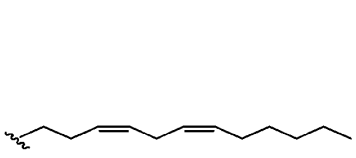
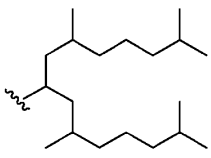
, , ,

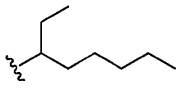
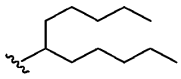
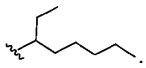
, , , , and

. In certain embodiments, R^2 is . In certain embodiments, R^2 is . In some embodiments, R^2 is  or .

R^3

[00124]As disclosed in Formula (TL), in certain embodiments, R^3 is $-CH(OR^8)(OR^9)$, $-CH(SR^8)(SR^9)$, $-CH(R^8)(R^9)$, $-R^{11}$, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{11}, wherein one or more methylene linkages of R^3 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)- or -C(O)O-. In certain embodiments, R^3 is $-CH(OR^8)(OR^9)$. In certain embodiments, R^3 is $-CH(R^8)(R^9)$. In certain embodiments, R^3 is $-CH(SR^8)(SR^9)$. In certain embodiments, R^3 is $-R^{11}$. In certain embodiments, R^3 is optionally substituted C_5-C_{18} aliphatic. In certain embodiments, R^3 is optionally substituted C_1-C_{14} aliphatic-R^{11}.

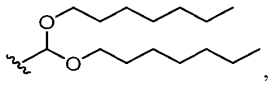
[00125]In certain embodiments, R^3 is selected from , , , , , , , , , , , , , , , , and . In certain embodiments, R^3 is . In

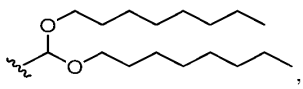
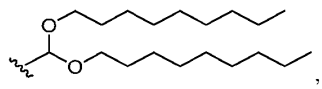
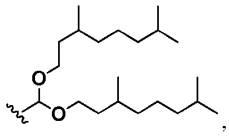
certain embodiments, R^3 is . In some embodiments, R^3 is  or .

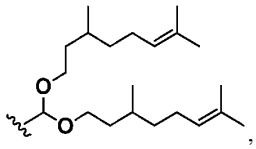
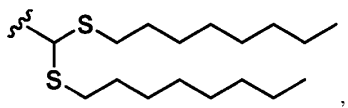
[00126]In certain embodiments, R^2 and R^3 are the same. In certain embodiments, R^2 and R^3 are different.

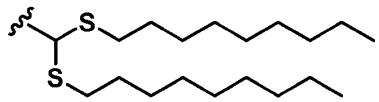
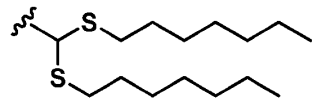
R^{12}

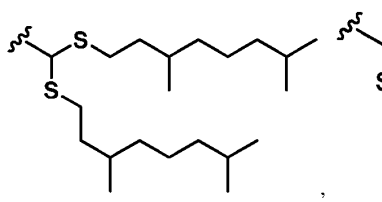
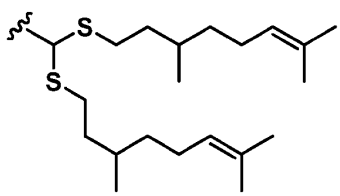
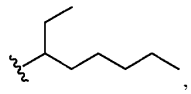
[00127]As disclosed in Formula (TL), in certain embodiments, R^{12} is $-CH(OR^{13})(OR^{14})$, $-CH(SR^{13})(SR^{14})$, $-CH(R^{13})(R^{14})$, $-R^{15}$, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{15}, wherein one or more methylene linkages of R^{12} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)- or -C(O)O-. In certain embodiments, R^{12} is $-CH(OR^{13})(OR^{14})$. In certain embodiments, R^{12} is $-CH(R^{13})(R^{14})$. In certain embodiments, R^{12} is $-CH(SR^{13})(SR^{14})$. In certain embodiments, R^{12} is $-R^{15}$. In certain embodiments, R^{12} is optionally substituted C_5-C_{18} aliphatic. In certain embodiments, R^{12} is optionally substituted C_1-C_{14} aliphatic-R^{15}.

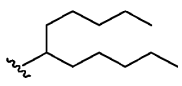
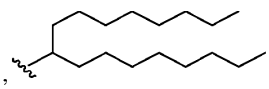
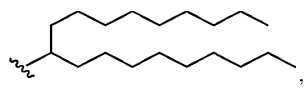
[00128]In certain embodiments, R^{12} is selected from ,

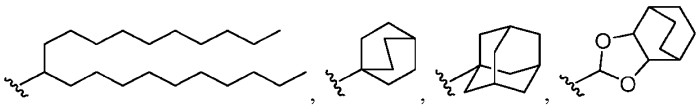
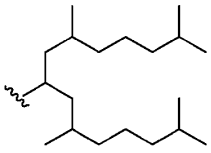
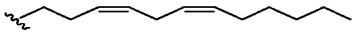
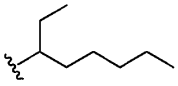
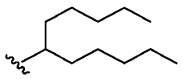
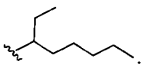
, , ,

, ,

, ,

, , ,

, , ,


, and 

. In certain embodiments, R^{12} is
. In certain embodiments, R^{12} is 
. In some embodiments, R^{12} is 
or 
.

[00129]In certain embodiments, R^2, R^3 and R^{12} are the same. In certain embodiments, R^2, R^3 and R^{12} are different. In certain embodiments, R^2 and R^3 are the same, and R^{12} is different from the other two.

R^6 and R^7

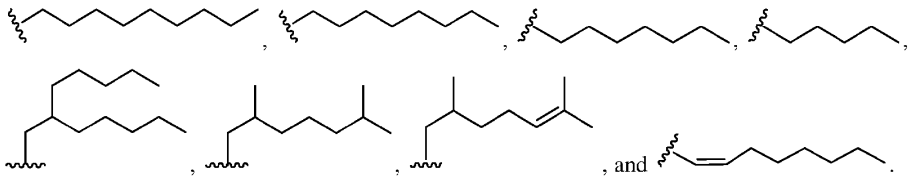
[00130]As disclosed in Formula (TL), in certain embodiments, R^6 and R^7 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{10}, or optionally substituted -C_1-C_{14} aliphatic-R^{10}; wherein one or more methylene linkages of R^6 and R^7 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)- or -C(O)O-.

[00131]In certain embodiments, R^6 and R^7 are the same. In certain embodiments, R^6 and R^7 are different.

[00132]In certain embodiments, R^6 is optionally substituted C_1-C_{14} aliphatic. In certain embodiments, R^6 is optionally substituted C_1-C_{14} alkyl. In certain embodiments, R^6 is optionally substituted C_1-C_{14} branched alkyl. In certain embodiments, R^6 is optionally substituted C_1-C_{14} straight chain alkyl. In certain embodiments, R^6 is optionally substituted C_1-C_{14} alkenyl. In certain embodiments, R^6 is optionally substituted C_1-C_{14} branched alkenyl. In certain embodiments, R^6 is optionally substituted C_1-C_{14} straight chain alkenyl. In certain embodiments, R^6 is optionally substituted C_4-C_{10} alkyl. In certain embodiments, R^6 is optionally substituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^6 is unsubstituted C_6-C_{10} alkyl. In certain embodiments, R^6 is unsubstituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^6 is optionally substituted C_6-C_{10} alkyl. In certain embodiments, R^6 is optionally substituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^6 is unsubstituted C_4-C_{10} alkyl. In certain embodiments, R^6 is unsubstituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^6 is optionally substituted $-(CH_2)_5CH_3$. In certain embodiments, R^6 is optionally substituted $-(CH_2)_6CH_3$. In certain embodiments, R^6 is optionally substituted $-(CH_2)_7CH_3$. In certain embodiments, R^6 is optionally substituted $-(CH_2)_8CH_3$. In certain embodiments, R^6 is optionally substituted $-(CH_2)_9CH_3$.

[00133]In certain embodiments, R^7 is optionally substituted C_1-C_{14} aliphatic. In certain embodiments, R^7 is optionally substituted C_1-C_{14} alkyl. In certain embodiments, R^7 is optionally substituted C_1-C_{14} branched alkyl. In certain embodiments, R^7 is optionally substituted C_1-C_{14} straight chain alkyl. In certain embodiments, R^7 is optionally substituted C_1-C_{14} alkenyl. In certain embodiments, R^7 is optionally substituted C_1-C_{14} branched alkenyl. In certain embodiments, R^7 is optionally substituted C_1-C_{14} straight chain alkenyl. In certain embodiments, R^7 is optionally substituted C_4-C_{10} alkyl. In certain embodiments, R^7 is optionally substituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^7 is unsubstituted C_6-C_{10} alkyl. In certain embodiments, R^7 is unsubstituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^7 is optionally substituted C_6-C_{10} alkyl. In certain embodiments, R^7 is optionally substituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^7 is unsubstituted C_4-C_{10} alkyl. In certain embodiments, R^7 is unsubstituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^7 is optionally substituted $-(CH_2)_5CH_3$. In certain embodiments, R^7 is optionally substituted $-(CH_2)_6CH_3$. In certain embodiments, R^7 is optionally substituted $-(CH_2)_7CH_3$. In certain embodiments, R^7 is optionally substituted $-(CH_2)_8CH_3$. In certain embodiments, R^7 is optionally substituted $-(CH_2)_9CH_3$.

[00134]In certain embodiments, each R^6 and R^7 are each independently selected from



R^8 and R^9

[00135]As disclosed in Formula (TL), in certain embodiments, R^8 and R^9 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{11}, or optionally substituted -C_1-C_{14} aliphatic-R^{11}; wherein one or more methylene linkages of R^8 and R^9 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)- or -C(O)O-.

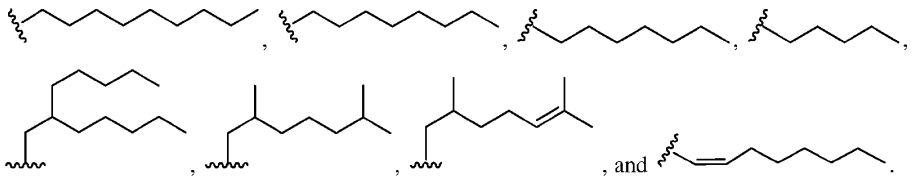
[00136]In certain embodiments, R^8 and R^9 are the same. In certain embodiments, R^8 and R^9 are different.

[00137]In certain embodiments, R^8 is optionally substituted C_1-C_{14} aliphatic. In certain embodiments, R^8 is optionally substituted C_1-C_{14} alkyl. In certain embodiments, R^8 is optionally substituted C_1-C_{14} branched alkyl. In certain embodiments, R^8 is optionally substituted C_1-C_{14} straight chain alkyl. In certain embodiments, R^8 is optionally substituted C_1-C_{14} alkenyl. In certain embodiments, R^8 is optionally substituted C_1-C_{14} branched alkenyl. In certain embodiments, R^8 is optionally substituted C_1-C_{14} straight chain alkenyl. In certain embodiments, R^8 is optionally substituted C_4-C_{10} alkyl. In certain embodiments, R^8 is optionally substituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^8 is unsubstituted C_6-C_{10} alkyl. In certain embodiments, R^8 is unsubstituted straight chain C_4-C_{10}

alkyl. In certain embodiments, R^8 is optionally substituted C_6-C_{10} alkyl. In certain embodiments, R^8 is optionally substituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^8 is unsubstituted C_4-C_{10} alkyl. In certain embodiments, R^8 is unsubstituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^8 is optionally substituted $-(CH_2)_5CH_3$. In certain embodiments, R^8 is optionally substituted $-(CH_2)_6CH_3$. In certain embodiments, R^8 is optionally substituted $-(CH_2)_7CH_3$. In certain embodiments, R^8 is optionally substituted $-(CH_2)_8CH_3$. In certain embodiments, R^8 is optionally substituted $-(CH_2)_9CH_3$.

[00138]In certain embodiments, R^9 is optionally substituted C_1-C_{14} aliphatic. In certain embodiments, R^9 is optionally substituted C_1-C_{14} alkyl. In certain embodiments, R^9 is optionally substituted C_1-C_{14} branched alkyl. In certain embodiments, R^9 is optionally substituted C_1-C_{14} straight chain alkyl. In certain embodiments, R^9 is optionally substituted C_1-C_{14} alkenyl. In certain embodiments, R^9 is optionally substituted C_1-C_{14} branched alkenyl. In certain embodiments, R^9 is optionally substituted C_1-C_{14} straight chain alkenyl. In certain embodiments, R^9 is optionally substituted C_4-C_{10} alkyl. In certain embodiments, R^9 is optionally substituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^9 is unsubstituted C_6-C_{10} alkyl. In certain embodiments, R^9 is unsubstituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^9 is optionally substituted C_6-C_{10} alkyl. In certain embodiments, R^9 is optionally substituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^9 is unsubstituted C_4-C_{10} alkyl. In certain embodiments, R^9 is unsubstituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^9 is optionally substituted $-(CH_2)_5CH_3$. In certain embodiments, R^9 is optionally substituted $-(CH_2)_6CH_3$. In certain embodiments, R^9 is optionally substituted $-(CH_2)_7CH_3$. In certain embodiments, R^9 is optionally substituted $-(CH_2)_8CH_3$. In certain embodiments, R^9 is optionally substituted $-(CH_2)_9CH_3$.

[00139]In certain embodiments, each R^8 and R^9 are each independently selected from



R^{13} and R^{14}

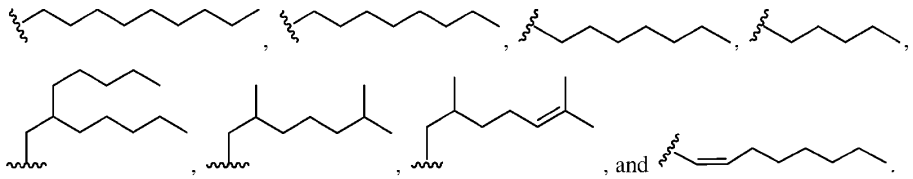
[00140]As disclosed in Formula (TL), in certain embodiments, R^{13} and R^{14} are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{15}, or optionally substituted -C_1-C_{14} aliphatic-R^{15}; wherein one or more methylene linkages of R^{13} and R^{14} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)- or -C(O)O-.

[00141]In certain embodiments, R^{13} and R^{14} are the same. In certain embodiments, R^{13} and R^{14} are different.

[00142]In certain embodiments, R^{13} is optionally substituted C_1-C_{14} aliphatic. In certain embodiments, R^{13} is optionally substituted C_1-C_{14} alkyl. In certain embodiments, R^{13} is optionally substituted C_1-C_{14} branched alkyl. In certain embodiments, R^{13} is optionally substituted C_1-C_{14} straight chain alkyl. In certain embodiments, R^{13} is optionally substituted C_1-C_{14} alkenyl. In certain embodiments, R^{13} is optionally substituted C_1-C_{14} branched alkenyl. In certain embodiments, R^{13} is optionally substituted C_1-C_{14} straight chain alkenyl. In certain embodiments, R^{13} is optionally substituted C_4-C_{10} alkyl. In certain embodiments, R^{13} is optionally substituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^{13} is unsubstituted C_6-C_{10} alkyl. In certain embodiments, R^{13} is unsubstituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^{13} is optionally substituted C_6-C_{10} alkyl. In certain embodiments, R^{13} is optionally substituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^{13} is unsubstituted C_4-C_{10} alkyl. In certain embodiments, R^{13} is unsubstituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^{13} is optionally substituted $-(CH_2)_5CH_3$. In certain embodiments, R^{13} is optionally substituted $-(CH_2)_6CH_3$. In certain embodiments, R^{13} is optionally substituted $-(CH_2)_7CH_3$. In certain embodiments, R^{13} is optionally substituted $-(CH_2)_8CH_3$. In certain embodiments, R^{13} is optionally substituted $-(CH_2)_9CH_3$.

[00143]In certain embodiments, R^{14} is optionally substituted C_1-C_{14} aliphatic. In certain embodiments, R^{14} is optionally substituted C_1-C_{14} alkyl. In certain embodiments, R^{14} is optionally substituted C_1-C_{14} branched alkyl. In certain embodiments, R^{14} is optionally substituted C_1-C_{14} straight chain alkyl. In certain embodiments, R^{14} is optionally substituted C_1-C_{14} alkenyl. In certain embodiments, R^{14} is optionally substituted C_1-C_{14} branched alkenyl. In certain embodiments, R^{14} is optionally substituted C_1-C_{14} straight chain alkenyl. In certain embodiments, R^{14} is optionally substituted C_4-C_{10} alkyl. In certain embodiments, R^{14} is optionally substituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^{14} is unsubstituted C_6-C_{10} alkyl. In certain embodiments, R^{14} is unsubstituted straight chain C_4-C_{10} alkyl. In certain embodiments, R^{14} is optionally substituted C_6-C_{10} alkyl. In certain embodiments, R^{14} is optionally substituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^{14} is unsubstituted C_4-C_{10} alkyl. In certain embodiments, R^{14} is unsubstituted straight chain C_6-C_{10} alkyl. In certain embodiments, R^{14} is optionally substituted $-(CH_2)_5CH_3$. In certain embodiments, R^{14} is optionally substituted $-(CH_2)_6CH_3$. In certain embodiments, R^{14} is optionally substituted $-(CH_2)_7CH_3$. In certain embodiments, R^{14} is optionally substituted $-(CH_2)_8CH_3$. In certain embodiments, R^{14} is optionally substituted $-(CH_2)_9CH_3$.

[00144]In certain embodiments, each R^{13} and R^{14} are each independently selected from



R^{10}, R^{11} and R^{15}

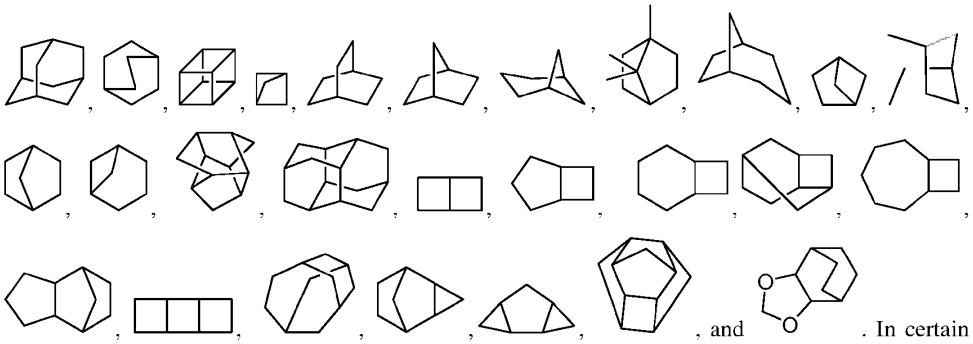
[00145]As disclosed in Formula (TL), in certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted cyclic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic C_4-C_{14} cycloalkyl or optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic 4-14 membered heterocyclyl, or two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic C_4-C_{14} cycloalkyl or optionally substituted bridged bicyclic or multicyclic 4-14 membered heterocyclyl.

[00146]In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted monocyclic C_4-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted monocyclic C_6-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted monocyclic C_6-C_8 cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bicyclic C_4-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bicyclic C_6-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bicyclic C_8-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bicyclic C_6-C_{10} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged bicyclic C_4-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged bicyclic C_6-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged bicyclic C_8-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged bicyclic C_6-C_{10} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted multicyclic C_4-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted multicyclic C_6-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted multicyclic C_8-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged multicyclic C_4-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged multicyclic C_6-C_{14} cycloalkyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged multicyclic C_8-C_{14} cycloalkyl. In certain embodiments, two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic C_4-C_{14} cycloalkyl. In certain embodiments, two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic C_6-C_{14} cycloalkyl. In certain embodiments, two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic C_8-C_{14} cycloalkyl.

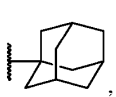
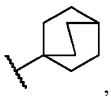
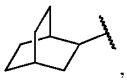
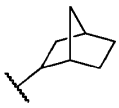
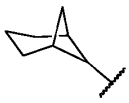
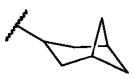
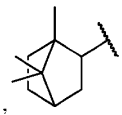
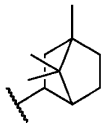
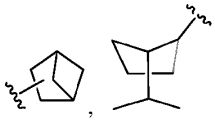
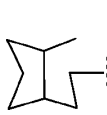
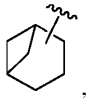
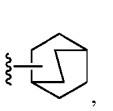
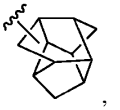
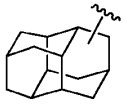
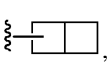
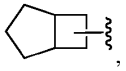
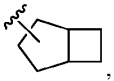
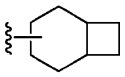
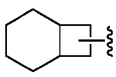
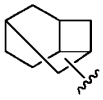
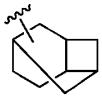
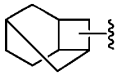
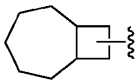
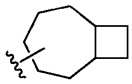
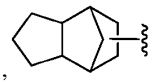
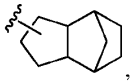
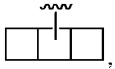
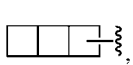
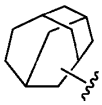
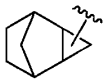
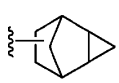
[00147]In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted monocyclic 4-14 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted monocyclic 6-14 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted monocyclic C_6-C_8

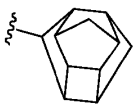
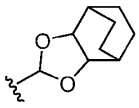
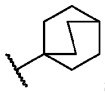
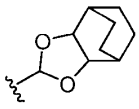
heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bicyclic 4-14 membered heterocyclyl. In certain embodiments, each R^{10} and R^{11} are independently an optionally substituted bicyclic 6-14 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bicyclic C_8-C_{14} heterocyclyl. In certain embodiments, each R^{10} and R^{11} are independently an optionally substituted bicyclic 6-12 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged bicyclic 4-14 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged bicyclic 6-14 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged bicyclic C_8-C_{14} heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged bicyclic 6-12 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted multicyclic 4-14 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted multicyclic 6-14 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted multicyclic C_8-C_{14} heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged multicyclic 4-14 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged multicyclic 6-14 membered heterocyclyl. In certain embodiments, each R^{10}, R^{11} and R^{15} are independently an optionally substituted bridged multicyclic C_8-C_{14} heterocyclyl. In certain embodiments, two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic 4-14 membered heterocyclyl. In certain embodiments, two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic 6-14 membered heterocyclyl. In certain embodiments, two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic 8-14 membered heterocyclyl.

[00148]In certain embodiments, each R^{10}, R^{11} and R^{15} is independently an optionally substituted monovalent cyclic group selected from



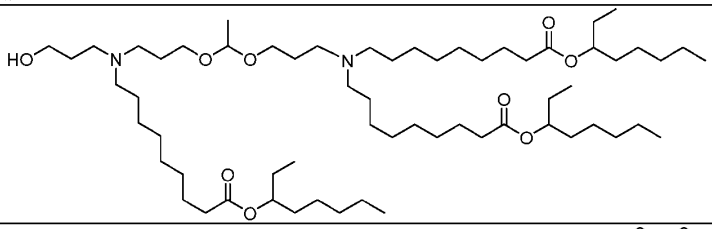
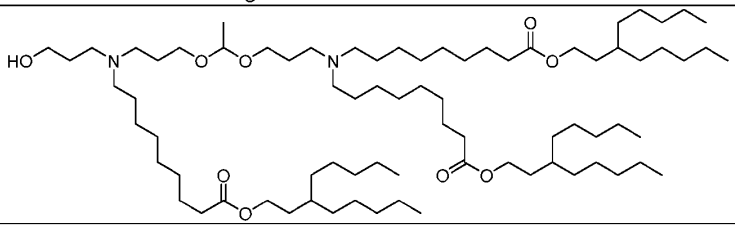
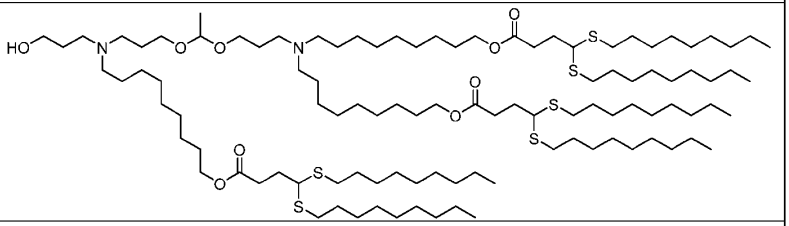
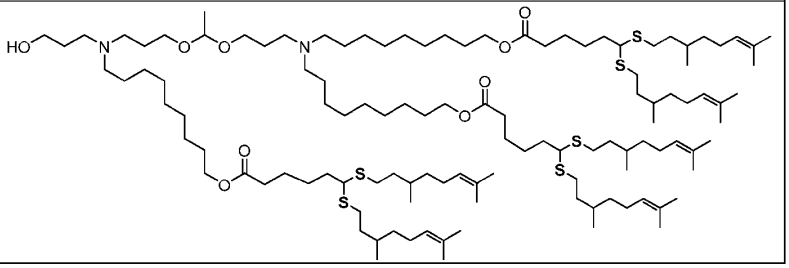
. In certain

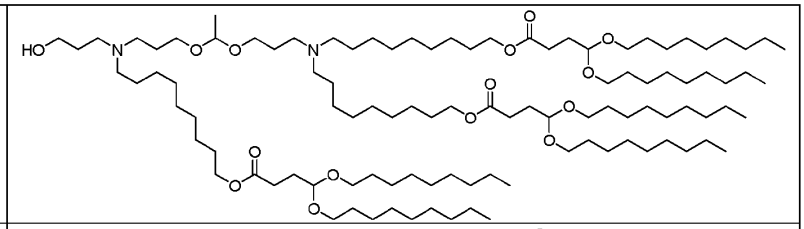
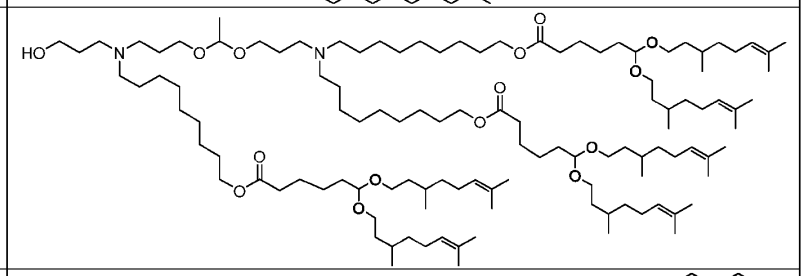
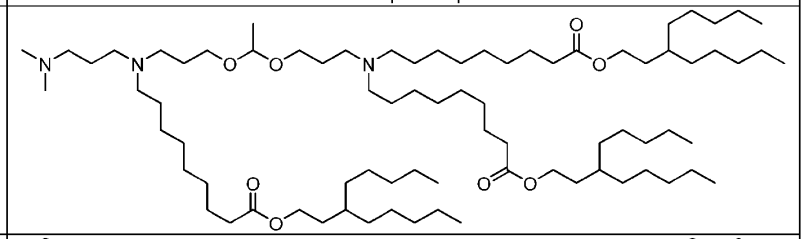
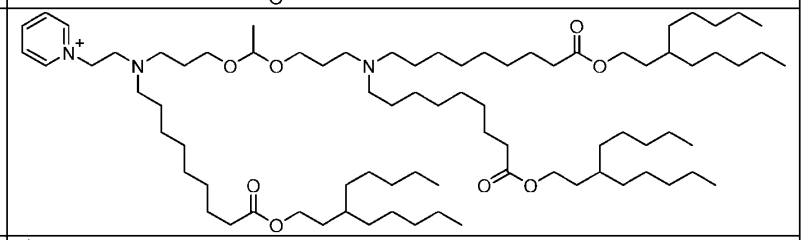
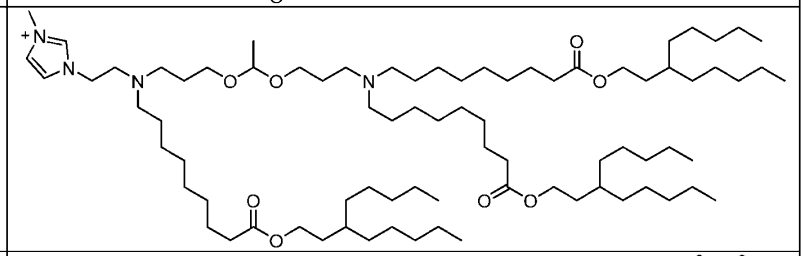
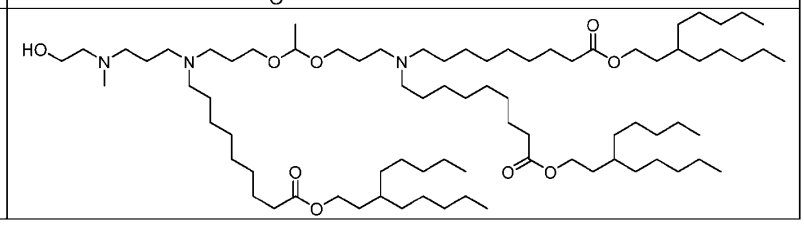
embodiments, each R^{10}, R^{11} and R^{15} is independently a structure selected from , , , , , , , , , , , and . In certain embodiments, each R^{10}, R^{11} and R^{15} is independently a structure selected from , , , , , , , , , , , , , , , , , , , , , , , , , , , , , , , and . In certain embodiments, each R^{10}, R^{11} and R^{15} is independently a structure selected from , , , , , , , , , , , , , , , , , , ,

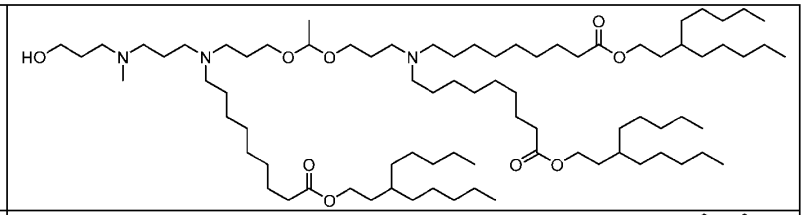
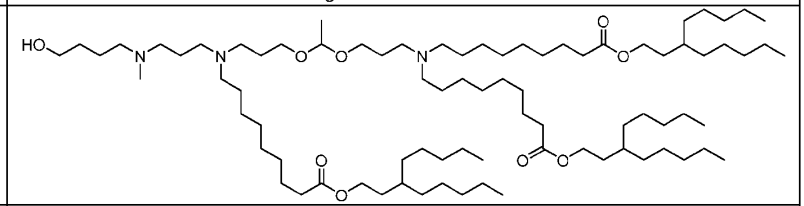
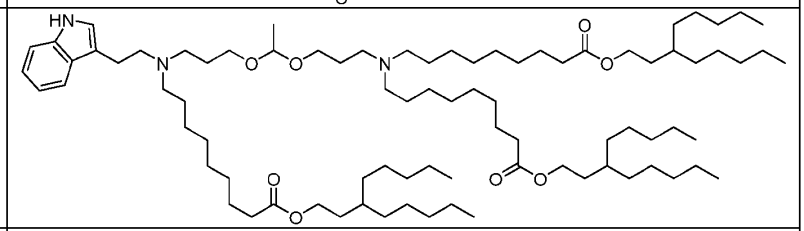
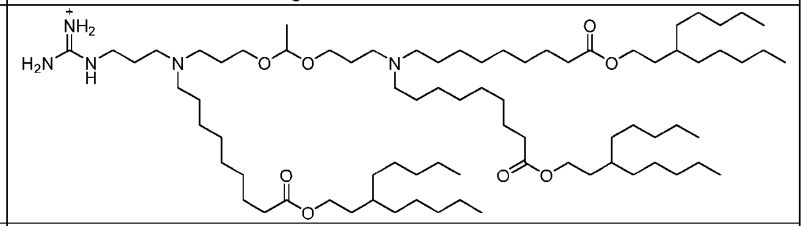
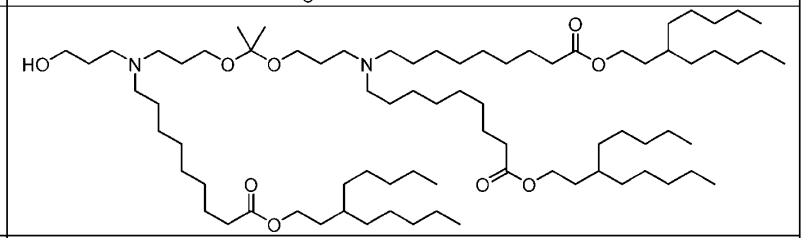
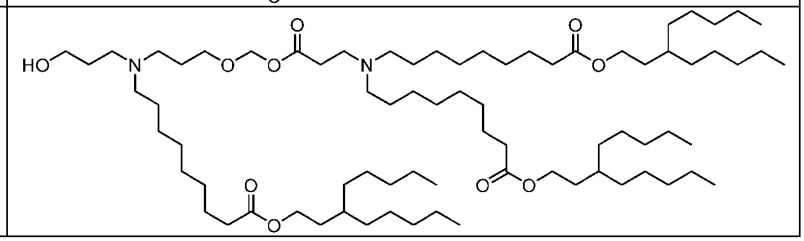
, and . In certain embodiments, each R^{10}, R^{11} and R^{15} is independently a structure selected from  and .

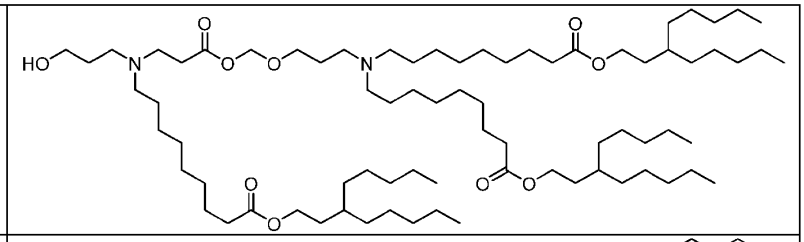
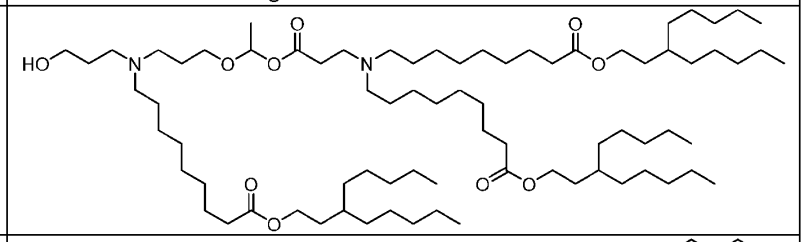
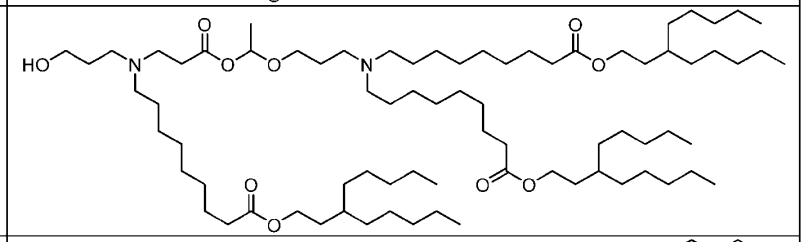
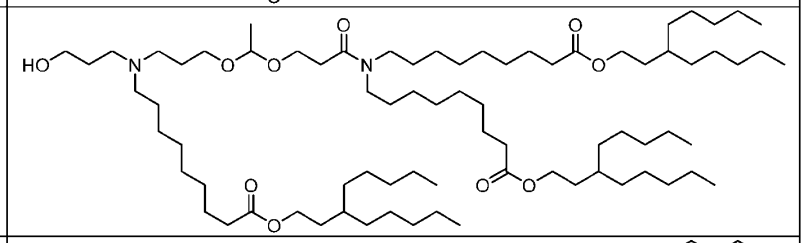
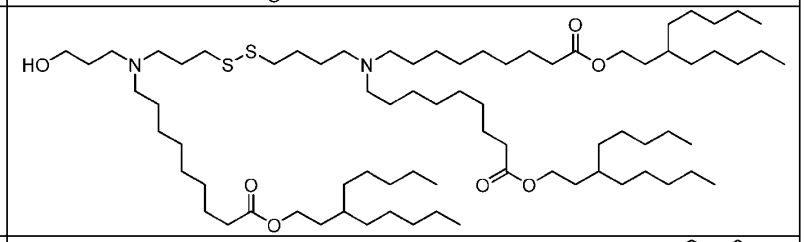
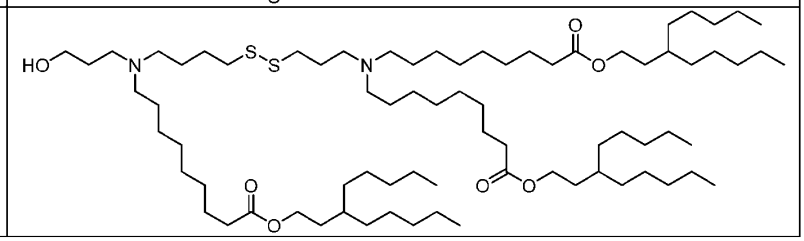
[00149]In some embodiments, Lipids of the Disclosure are selected from any lipid in Table (I) below or a pharmaceutically acceptable salt, solvate, stereoisomer, or enantiomer thereof:

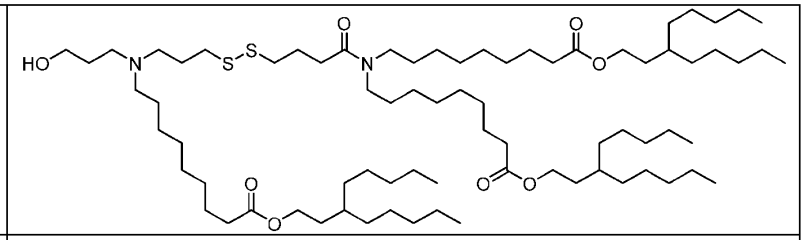
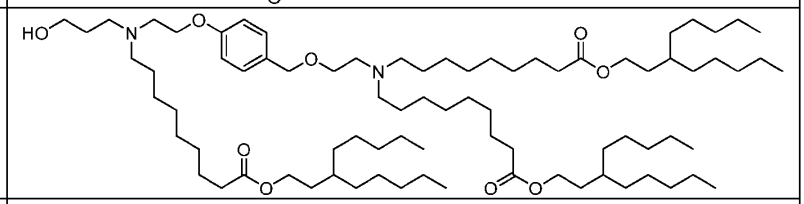
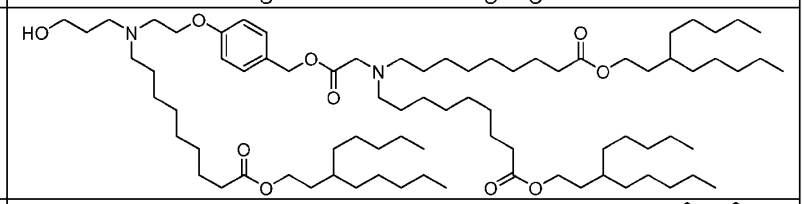
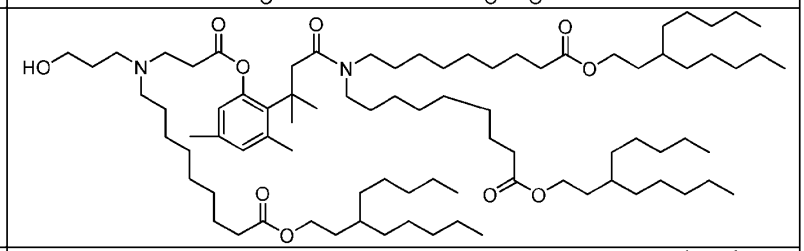
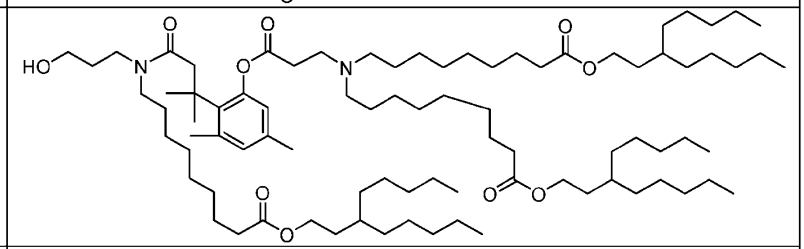
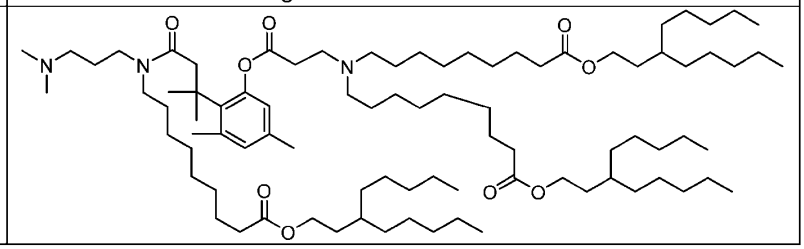
Table (I). Non-Limiting Examples of Ionizable Lipids of the Disclosure

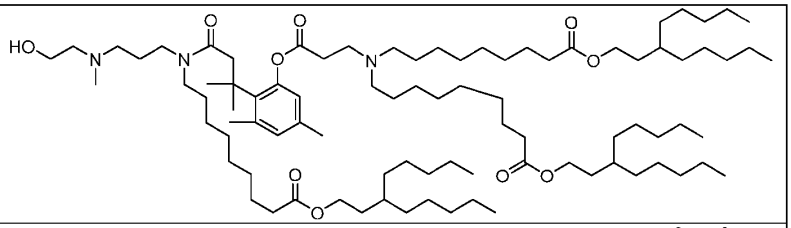
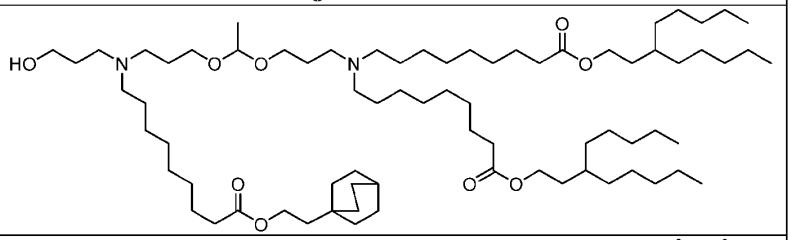
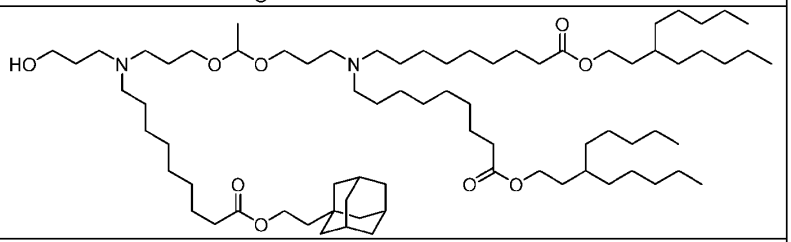
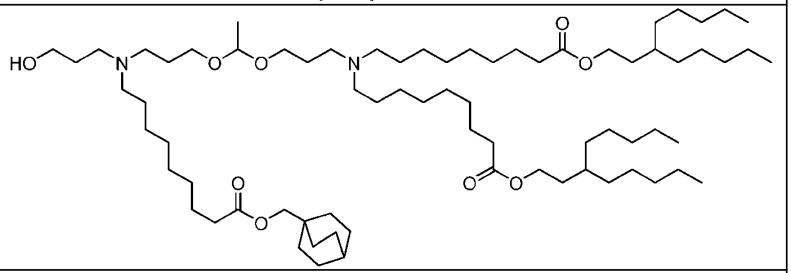
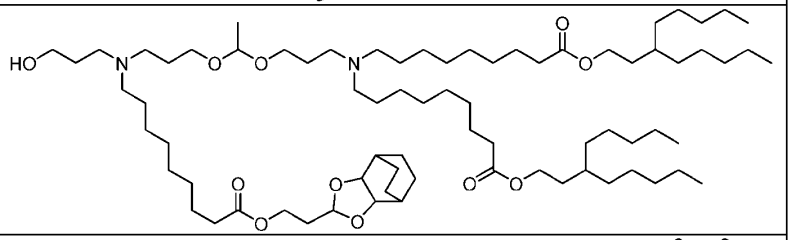
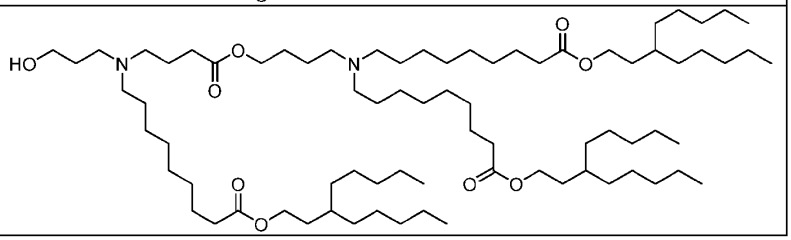
Cmpd	Structure
TL-1	
TL-2	
TL-3	
TL-4	

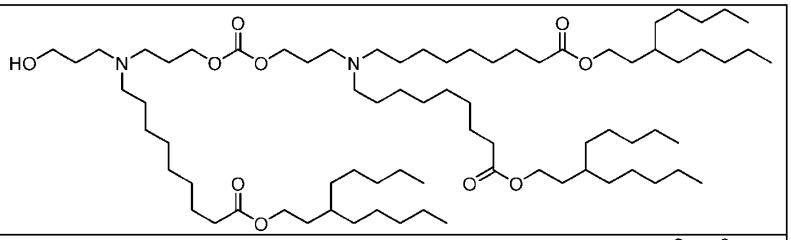
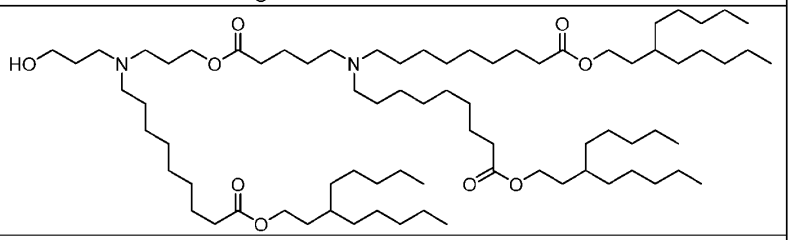
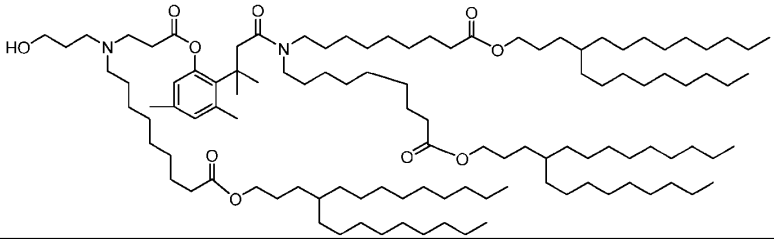
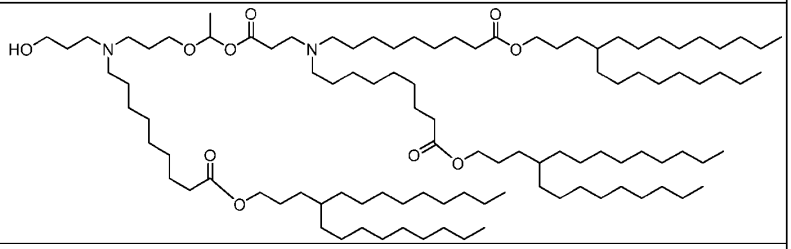
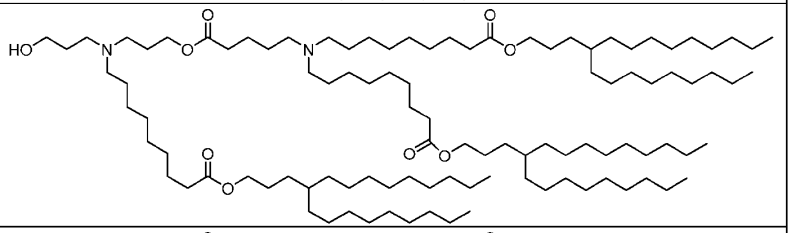
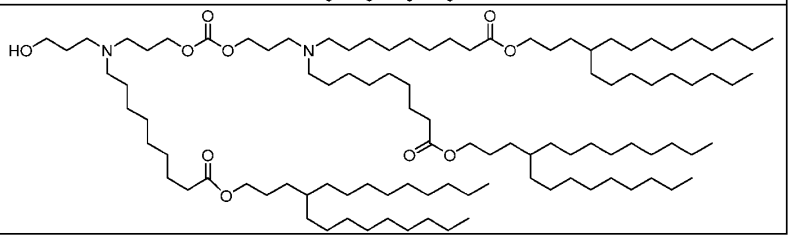
TL-5	
TL-6	
TL-7	
TL-8	
TL-9	
TL-10	

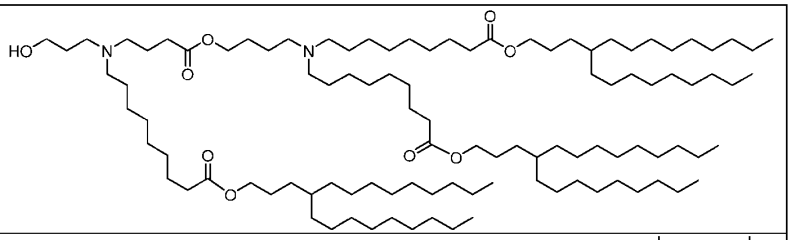
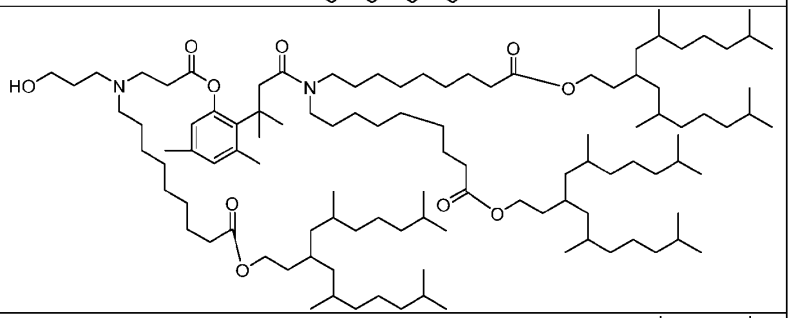
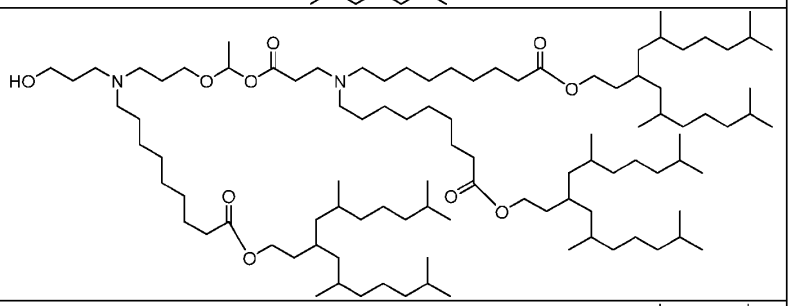
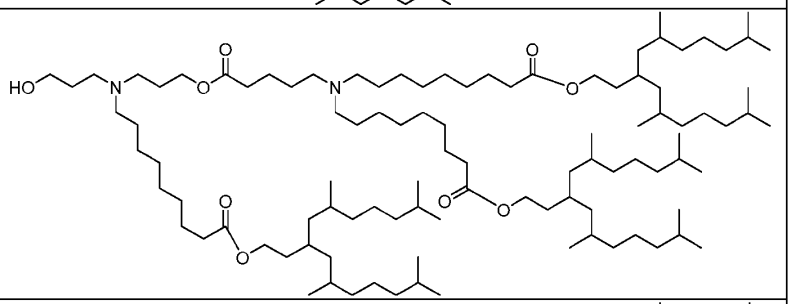
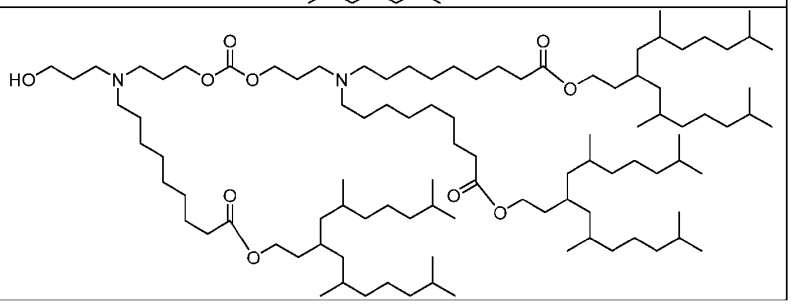
TL-11	
TL-12	
TL-13	
TL-14	
TL-15	
TL-16	

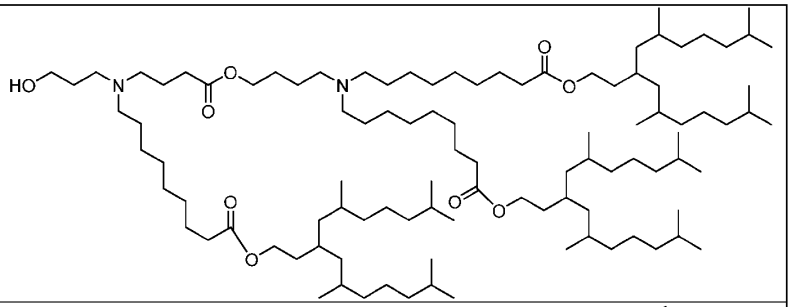
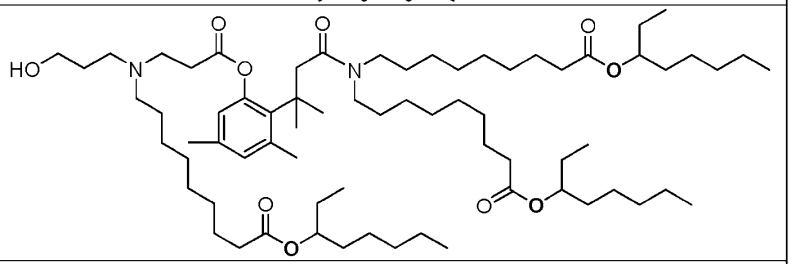
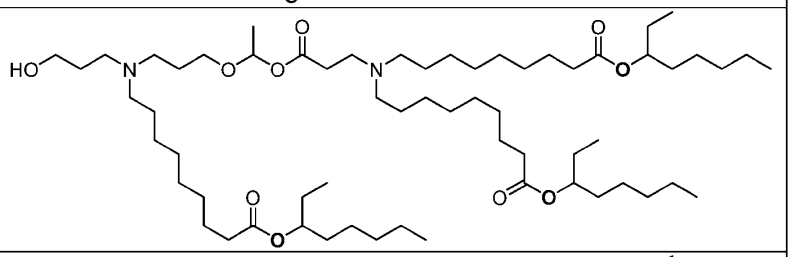
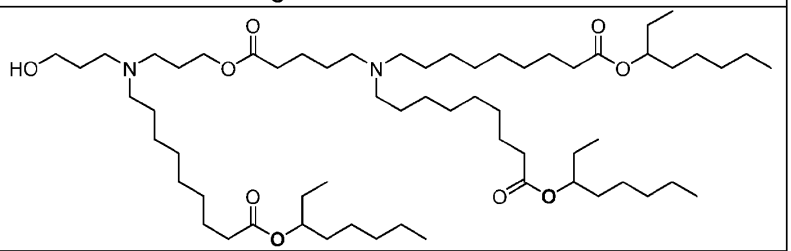
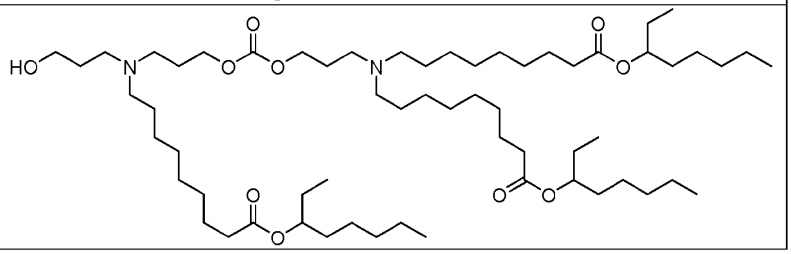
TL-17	
TL-18	
TL-19	
TL-20	
TL-21	
TL-22	

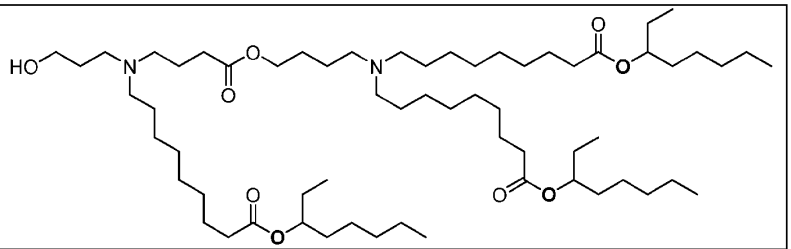
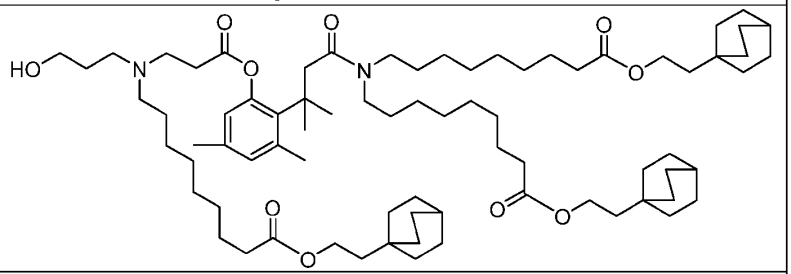
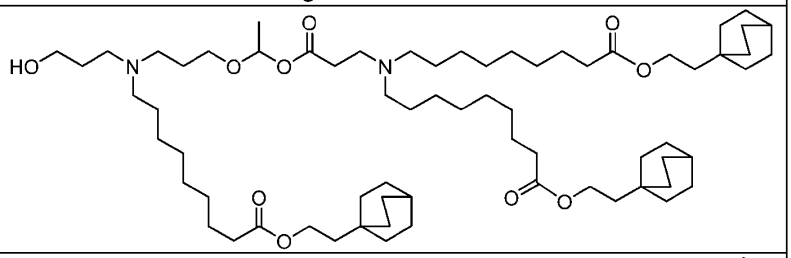
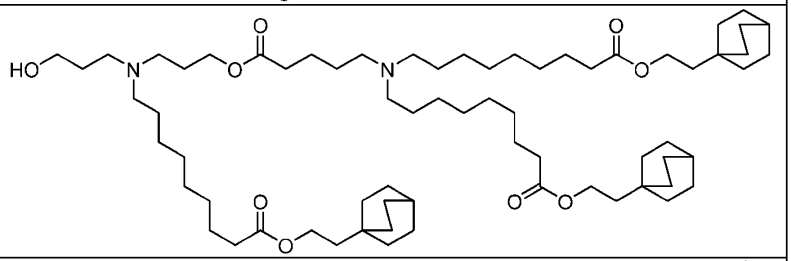
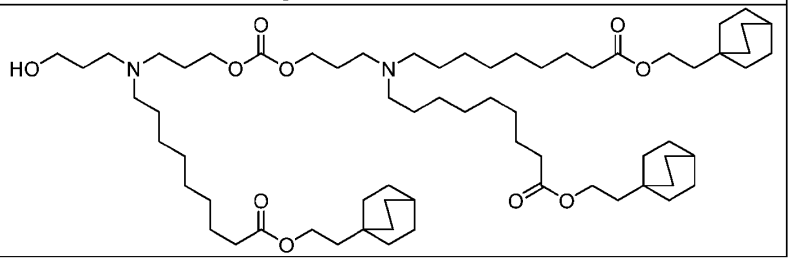
TL-23	
TL-24	
TL-25	
TL-26	
TL-27	
TL-28	

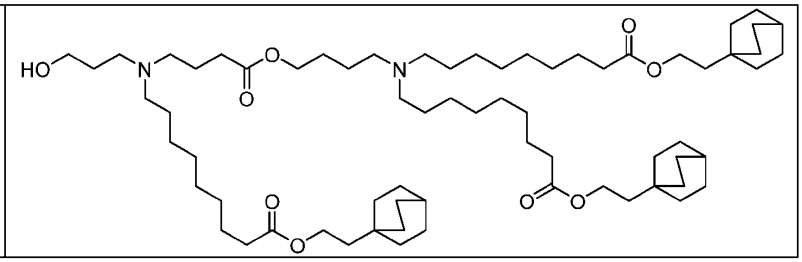
TL-29	
TL-30	
TL-31	
TL-32	
TL-33	
TL-34	

TL-35	
TL-36	
TL-37	
TL-38	
TL-39	
TL-40	

TL-41	
TL-42	
TL-43	
TL-44	
TL-45	

TL-46	
TL-47	
TL-48	
TL-49	
TL-50	

TL-51	
TL-52	
TL-53	
TL-54	
TL-55	

TL-56	

[00150]In some embodiments, Lipids of the Disclosure are selected from any lipid in Table (I) above, an enantiomer thereof, or any mixture of enantiomers thereof, or a pharmaceutically acceptable salt of any of the aforementioned.

ii.Structural lipids

[00151]In some embodiments, an LNP comprises a structural lipid. In some embodiments, an LNP comprises two or more structural lipids. Structural lipids can be selected from the group consisting of, but are not limited to, cholesterol, fecosterol, fucosterol, beta sitosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, cholic acid, sitostanol, litocholic acid, tomatine, ursolic acid, alpha-tocopherol, Vitamin D3, Vitamin D2, Calcipotriol, botulin, lupeol, oleanolic acid, beta-sitosterol-acetate and mixtures thereof. In some embodiments, the structural lipid is cholesterol. In some embodiments, the structural lipid is a cholesterol analogue disclosed by Patel, et al., Nat Commun., 11, 983 (2020), which is incorporated herein by reference in its entirety. In some embodiments, the structural lipid includes cholesterol and a corticosteroid (such as prednisolone, dexamethasone, prednisone, and hydrocortisone), or any combinations thereof. In some embodiments, a structural lipid is described in international patent application WO2019152557A1, which is incorporated herein by reference in its entirety.

[00152]In some embodiments, a structural lipid is a cholesterol analog. Using a cholesterol analog may enhance endosomal escape as described in Patel et al., Naturally-occurring cholesterol analogues in lipid nanoparticles induce polymorphic shape and enhance intracellular delivery of mRNA, Nature Communications (2020), which is incorporated herein by reference.

[00153]In some embodiments, a structural lipid is a phytosterol. Using a phytosterol may enhance endosomal escape as described in Herrera et al., Illuminating endosomal escape of polymorphic lipid nanoparticles that boost mRNA delivery, Biomaterials Science (2020), which is incorporated herein by reference.

[00154]In some embodiments, a structural lipid contains plant sterol mimetics for enhanced endosomal release.

[00155]In some embodiments, the structural lipid is cholesteryl hemisuccinate (CHEMS). In some embodiments, the structural lipid is 3-(4-((2-(4-morpholinyl)ethyl)amino)-4-oxobutanoate) (Mochol).

iii. PEGylated lipids

[00156]A PEGylated lipid is a lipid modified with polyethylene glycol. The term "PEGylated lipid" is used interchangeably herein with the shortened term "PEG lipid".

[00157]In some embodiments, an LNP comprises one, two or more PEGylated lipid or PEG-modified lipid. A PEGylated lipid may be selected from the non-limiting group consisting of PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols, and mixtures thereof. For example, a PEG lipid may be PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPC, or a PEG-DSPE lipid.

[00158]In some embodiments, the PEGylated lipid is selected from (R)-2,3-bis(octadecyloxy)propyl-1-(methoxypoly(ethyleneglycol)2000)propylcarbamate, PEG-S-DSG, PEG-S-DMG, PEG-PE, PEG-PAA, PEG-OH DSPE C18, PEG-DSPE, PEG-DSG, PEG-DPG, PEG-DOMG, PEG-DMPE Na, PEG-DMPE, PEG-DMG2000, PEG-DMG C14, PEG-DMG, PEG-DMA, PEG-Ceramide C16, PEG-C-DOMG, PEG-c-DMOG, PEG-c-DMA, PEG-cDMA, PEGA, PEG750-C-DMA, PEG400, PEG2k-DMG, PEG2k-C11, PEG2000-PE, PEG2000P, PEG2000-DSPE, PEG2000-DOMG, PEG2000-DMG, PEG2000-C-DMA, PEG2000, PEG200, PEG(2k)-DMG, PEG DSPE C18, PEG DMPE C14, PEG DLPE C12, PEG Click DMG C14, PEG Click C12, PEG Click C10, N(Carbonyl-methoxypolyethylenglycol-2000)-l,2-distearoyl-sn-glycero3-phosphoethanolamine, Myrj52, mPEG-PLA, MPEG-DSPE, mPEG3000-DMPE, MPEG-2000-DSPE, MPEG2000-DSPE, mPEG2000-DPPE, mPEG2000-DMPE, mPEG2000-DMG, mDPPE-PEG2000, l,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG2000, HPEG-2K-LIPD, Folate PEG-DSPE, DSPE-PEGMA 500, DSPE-PEGMA, DSPE-PEG6000, DSPE-PEG5000, DSPE-PEG2K-NAG, DSPE-PEG2k, DSPE-PEG2000maleimide, DSPE-PEG2000, DSPE-PEG, DSG-PEGMA, DSG-PEG5000, DPPE-PEG-2K, DPPE-PEG, DPPE-mPEG2000, DPPE-mPEG, DPG-PEGMA, DOPE-PEG2000, DMPE-PEGMA, DMPE-PEG2000, DMPE-Peg, DMPE-mPEG2000, DMG-PEGMA, DMG-PEG2000, DMG-PEG, distearoyl-glycerol-polyethyleneglycol, C18PEG750, C18PEG5000, C18PEG3000, C18PEG2000, C16PEG2000, C14PEG2000, C18-PEG5000, C18PEG, C16PEG, C16 mPEG (polyethylene glycol) 2000 Ceramide, C14-PEG-DSPE200, C14-PEG2000, C14PEG2000, C14-PEG 2000, C14-PEG, C14PEG, 14:0-PEG2KPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG2000, (R)-2,3-bis(octadecyloxy)propyl-1-(methoxypoly(ethyleneglycol)2000)propylcarbamate, (PEG)-C-DOMG, PEG-C-DMA, and DSPE-PEG-X. In certain embodiments, the PEGylated lipid is PEG-DPPE. In certain embodiments, the PEGylated lipid is PEG2k-DPPE.

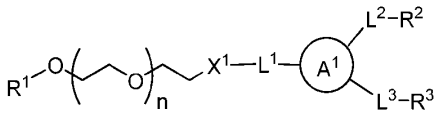
[00159]In some embodiments, the LNP comprises a PEGylated lipid disclosed in one of US 2019/0240354; US 2010/0130588; US 2021/0087135; WO 2021/204179; US 2021/0128488; US 2020/0121809; US 2017/0119904; US 2013/0108685; US 2013/0195920; US 2015/0005363; US 2014/0308304; US 2013/0053572; WO 2019/232095A1; WO 2021/077067; WO 2019/152557; US 2015/0203446; US 2017/0210697; US 2014/0200257; or WO 2019/089828A1, each of which is

incorporated by reference herein in their entirety. In some embodiments, the LNP comprises a PEGylated lipid described or disclosed in PCT/US2023/072878 filed August 25, 2023, which is incorporated by reference herein in its entirety.

[00160]In some embodiments, the LNP comprises a PEGylated lipid substitute in place of the PEGylated lipid. All embodiments disclosed herein that contemplate a PEGylated lipid should be understood to also apply to PEGylated lipid substitutes. In some embodiments, the LNP comprises a polysarcosine-lipid conjugate, such as those disclosed in US 2022/0001025 A1, which is incorporated by reference herein in its entirety. In some embodiments the LNP comprises a polyoxazoline-lipid conjugate, such as those disclosed in US 2022/0249695 A1, which is incorporated by reference herein in its entirety.

[00161]In some embodiments, the LNP comprises a PEGylated lipid disclosed and described in PCT Application PCT/US2023/072878, which is incorporated by reference herein, in its entirety. In certain embodiments, the PEGylated lipid is a lipid of any one of formulas PL-I', PL-I'', PL-I, PL-Ia, PL-Ib, PL-Iaa, PL-Iab, PL-Iac, PL-Iad, PL-Iae, PL-Iaf, PL-Iag, PL-Iah, PL-Iba, PL-Ibb, PL-Ibc, PL-Ibd, PL-Ibe, PL-Ibf, PL-Ibg, PL-Ibh, PL-Ica, PL-Icb, PL-Icc, PL-Icd, PL-Id PL-Ie, PL-If, PL-Ig, PL-Ih, PL-Ii, PL-Iha, PL-Ihb, PL-Ihc, PL-Ihd, PL-Iia, PL-Iib, PL-Iic, PL-Iid, PL-Ij, PL-Ik, L-Il, PL-Im, PL-In, PL-Io, PL-Ip, PL-Iq, PL-Ioa, PL-Iob, PL-Ioc, PL-Iod, PL-Ioe, PL-Iof, PL-Iog, PL-Ioh, PL-Ipa, PL-Ipb, PL-Ipc, PL-Ipd, PL-Ipe, PL-Ipf, PL-Ipg, PL-Iph, PL-Iqa, PL-Iqb, PL-Iqc, PL-Iqd, PL-Ir, PL-Is, PL-It, PL-Iu, PL-Iv, PL-Iw, PL-Iva, PL-Ivb, PL-Ivc, PL-Ivd, PL-Iwa, PL-Iwb, PL-Iwc, PL-Iwd, PL-Ix, PL-Ixx, PL-Iy, PL-Iyy, PL-Iyyy, PL-Iz, PL-Izz, PL-Izzz, PL-II', PL-II'', PL-II, PL-IIc, PL-IId, PL-IIe, PL-IIf, PL-IIg, PL-IIh, PL-IIa, PL-IIb, PL-IIk, PL-IIm or PL-IIn.

[00162]In some embodiments, the PEGylated lipid is a compound of formula **PL-I'**:



PL-I'

or a pharmaceutically acceptable salt thereof, wherein:

A^1 is a saturated 5-6 membered carbocyclic ring or a saturated 5-6 membered heterocyclic ring containing 1 or 2 heteroatoms independently selected from nitrogen, oxygen, and sulfur, wherein the carbocyclic ring and heterocyclic ring are substituted with t occurrences of R^4;

X^1 is -N(H)-, -N(C_{1-6} alkyl)-, -C_{1-6} aliphatic-N(H)-, -C_{1-6} aliphatic-N(C_{1-6} alkyl)-, -O- or -C_{1-6} aliphatic-O-;

L^1 is -C(O)(C_{1-6} aliphatic)C(O)-N(R)-, -C(O)(C_{1-6} aliphatic)-N(R)C(O)-, -C(O)(C_{1-6} aliphatic)C(O)O-, -C(O)(C_{1-6} aliphatic)C(O)-, -C(O)(C_{1-6} aliphatic)C(O)OCH_2-, -C(O)(C_{1-6} aliphatic)-, -C(O)(C_{1-6} aliphatic)-N(R)-, or -C(O)-;

L^2 and L^3 are independently a covalent bond or C_{1-6} alkylene wherein one methylene unit of the C_{1-6} alkylene is optionally replaced with -O-, -NR-, -S-, -S-S-, -S(O)-, -$S(O)_2$-, -C(O)-, -C(O)O-, -OC(O)-, -OC(O)O-, -OC(O)N(R)-, -N(R)C(O)O-, -C(O)N(R)-, -N(R)C(O)-, -N(R)C(O)N(R)-, -$C(R^5)$=N-, or -$C(R^5)$=N-O-;

R^1 is H, C_{1-6} alkyl, -(C_{1-6} alkyl)-N_3, -(C_{1-6} alkyl)-SH, or C_{3-8} alkynyl;

R^2 and R^3 are independently a straight or branched C_{6-30} alkyl, straight or branched C_{6-30} alkenyl, or straight or branched C_{6-30} alkynyl; wherein 1, 2, or 3 methylene units are independently and optionally replaced by a saturated or partially unsaturated C_{3-6} carbocyclic ring or phenylene; wherein the alkyl, alkenyl, and alkynyl and any carbocyclic ring or phenylene is substituted with m instances of R^x;

R^4 is C_{1-4} alkyl;

R^5 is C_{1-6} alkyl or C_{2-14} alkenyl;

each R is independently hydrogen or an optionally substituted group selected from C_{1-6} aliphatic, a 3-8 membered saturated or partially unsaturated monocyclic carbocyclic ring, phenyl, an 8-10 membered bicyclic aromatic carbocyclic ring, a 4-8 membered saturated or partially unsaturated monocyclic heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, and sulfur, a 5-6 membered monocyclic heteroaromatic ring having 1-4 heteroatoms independently selected from nitrogen, oxygen, and sulfur, or an 8-10 membered bicyclic heteroaromatic ring having 1-5 heteroatoms independently selected from nitrogen, oxygen, or sulfur;

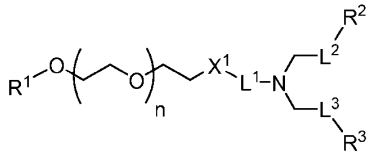
each R^x is independently halogen, -CN, -OR, -SR, -C(O)R, -C(O)OR, or -OC(O)OR;

n is an integer from 10-75, inclusive;

m is 0, 1, 2, 3, or 4; and

t is 0, 1, or 2.

[00163]In some embodiments, the PEGylated lipid is a compound of formula **PL-II'**:



PL-II'

or a pharmaceutically acceptable salt thereof, wherein:

X^1 is -N(H)-, -N(C_{1-6} alkyl)-, -C_{1-6} aliphatic-N(H)-, -C_{1-6} aliphatic-N(C_{1-6} alkyl)-, -O- or -C_{1-6} aliphatic-O-;

L^1 is -C(O)(C_{1-6} aliphatic)C(O)-, -C(O)(C_{1-6} aliphatic)-, or -C(O)-;

L^2 and L^3 are a covalent bond or C_{1-6} alkylene wherein one methylene unit of the C_{1-6} alkylene is optionally replaced with -O-, -NR-, -S-, -S-S-, -S(O)-, -$S(O)_2$-, -C(O)-, -C(O)O-, -OC(O)-, -OC(O)O-, -OC(O)N(R)-, -N(R)C(O)O-, -C(O)N(R)-, -N(R)C(O)-, -N(R)C(O)N(R)-, -$C(R^6)$=N-, or -$C(R^6)$=N-O-;

R^1 is H, C_{1-6} alkyl, -(C_{1-6} alkyl)-N_3, -(C_{1-6} alkyl)-SH, or C_{3-8} alkynyl;

R^2 and R^3 are independently straight or branched C_{6-30} alkyl, straight or branched C_{6-30} alkenyl, or straight or branched C_{6-30} alkynyl; wherein 1, 2, or 3 methylene units are independently and optionally replaced by a saturated or partially unsaturated C_{3-6} carbocyclic ring or phenylene; wherein the alkyl, alkenyl, and alkynyl and any carbocyclic ring or phenylene is substituted with m instances of R^x;

R^6 is C_{1-6} alkyl or C_{2-14} alkenyl;

each R is independently hydrogen or an optionally substituted group selected from C_{1-6} aliphatic, a 3-8 membered saturated or partially unsaturated monocyclic carbocyclic ring, phenyl, an 8-10 membered bicyclic aromatic carbocyclic ring, a 4-8 membered saturated or partially unsaturated monocyclic heterocyclic ring having 1-2 heteroatoms independently selected from nitrogen, oxygen, and sulfur, a 5-6 membered monocyclic heteroaromatic ring having 1-4 heteroatoms independently selected from nitrogen, oxygen, and sulfur, or an 8-10 membered bicyclic heteroaromatic ring having 1-5 heteroatoms independently selected from nitrogen, oxygen, or sulfur;

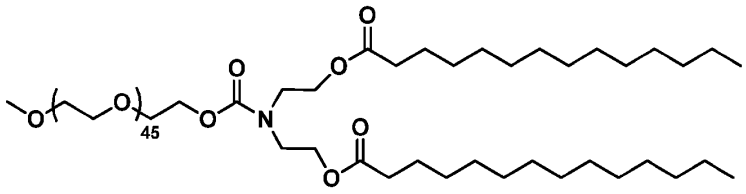
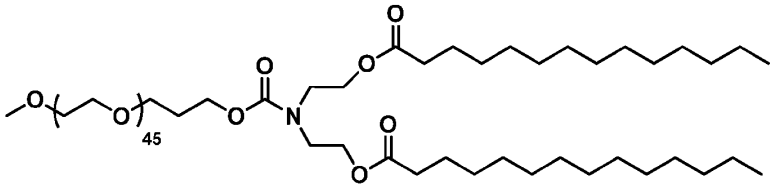
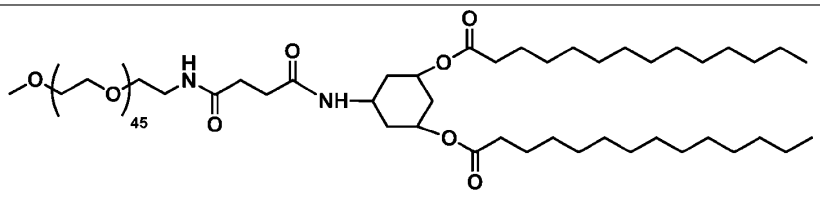
each R^x is independently halogen, -CN, -OR, -SR, -C(O)R, -C(O)OR, or OC(O)OR;

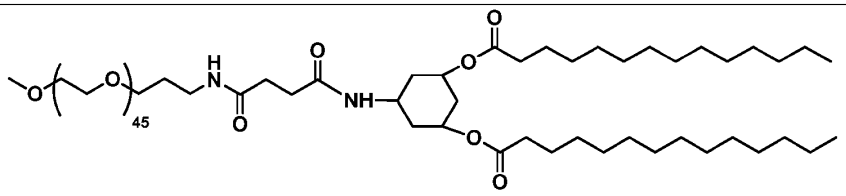
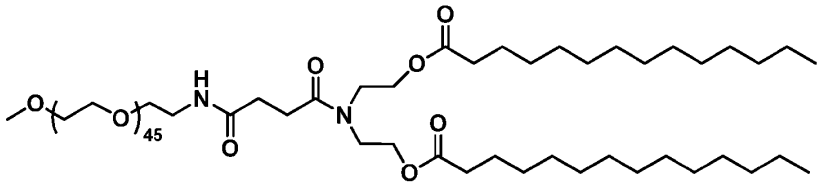
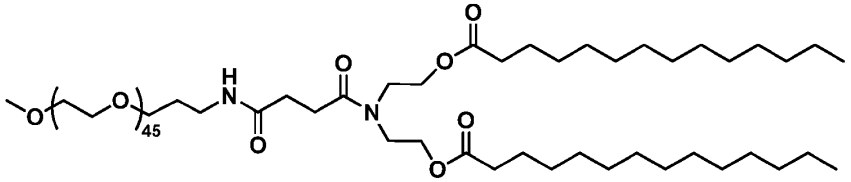
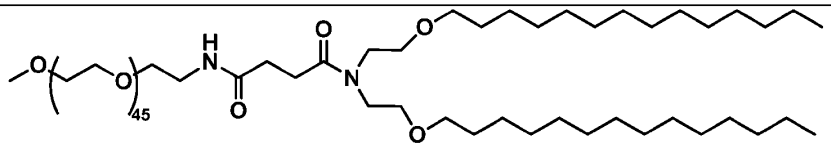
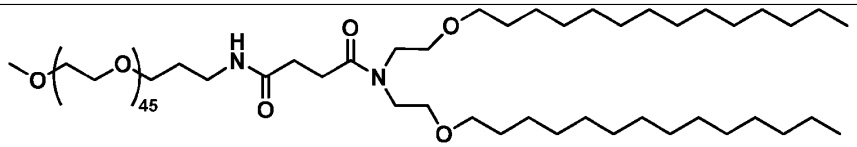
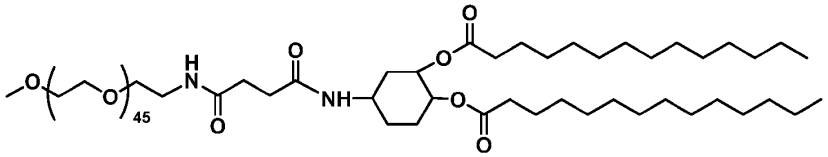
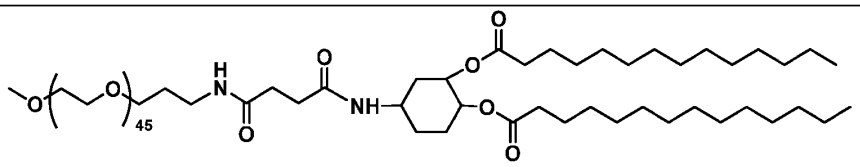
n is an integer from 10-75, inclusive; and

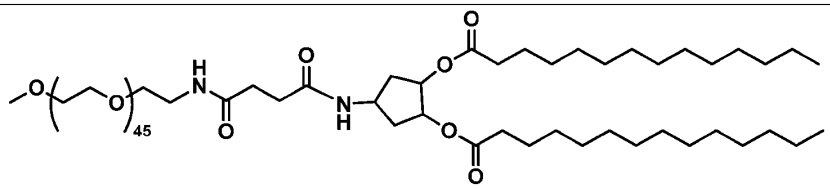
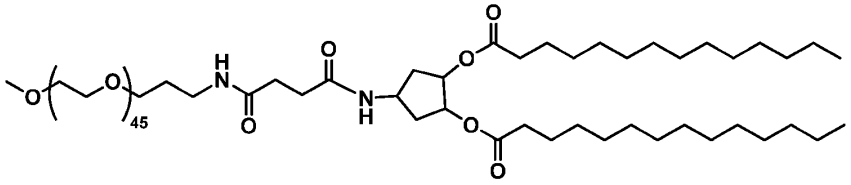
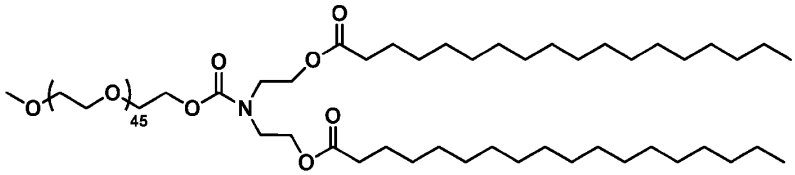
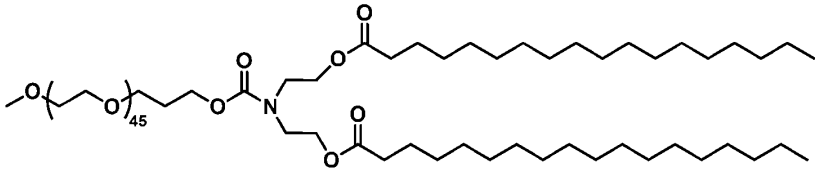
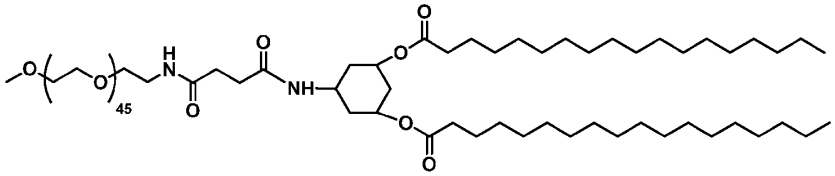
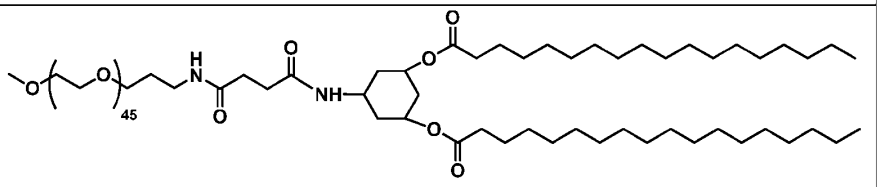
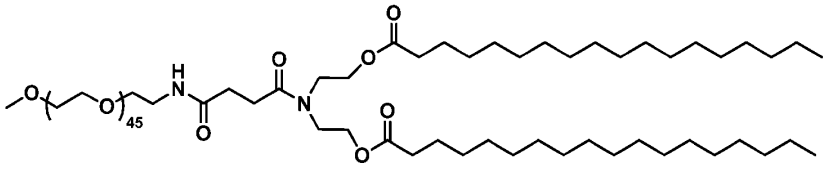
m is 0, 1, 2, 3, or 4.

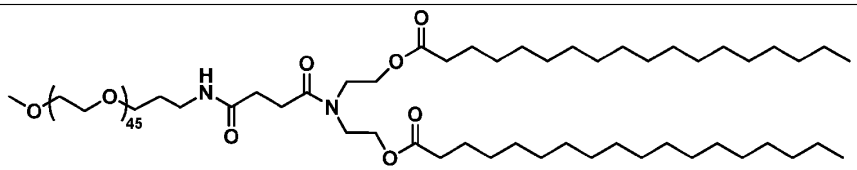
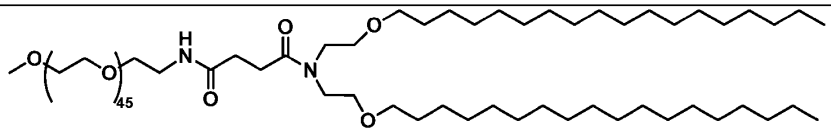
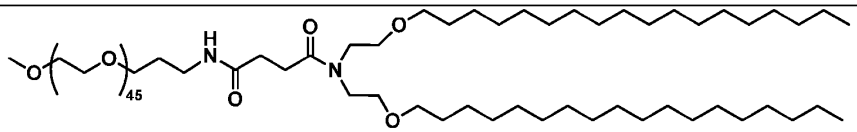
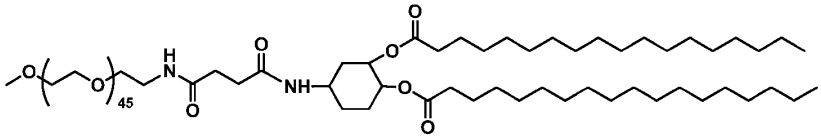
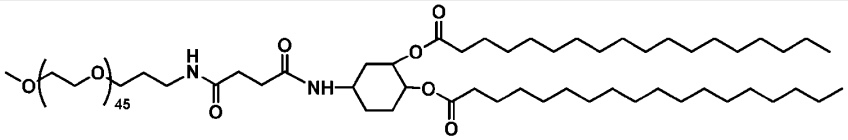
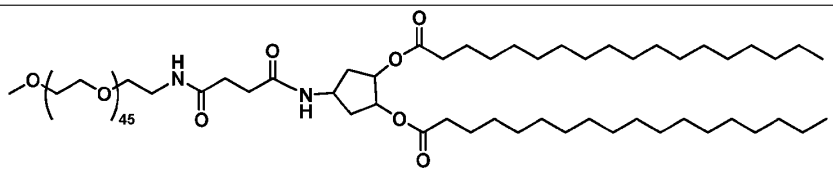
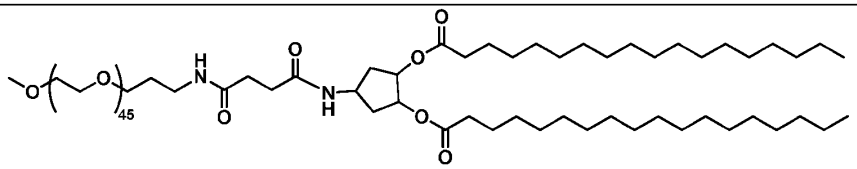
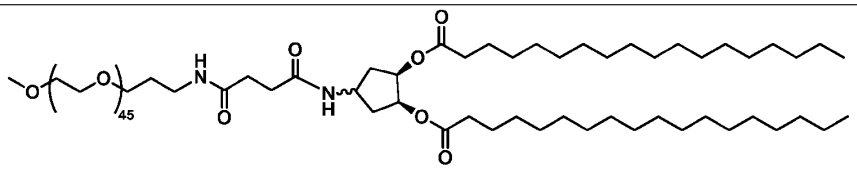
[00164]In some embodiments, the PEGylated lipid compound is one of those shown in Table (I-X), or a pharmaceutically acceptable salt thereof.

Table (I-X). Exemplary PEGylated Compounds

Compound No.	Structure
PL-1	
PL-1A	
PL-2	

Compound No.	Structure
PL-2A	
PL-3	
PL-3A	
PL-4	
PL-4A	
PL-5	
PL-5A	

Compound No.	Structure
PL-6	
PL-6A	
PL-7	
PL-7A	
PL-8	
PL-8A	
PL-9	

Compound No.	Structure
PL-9A	
PL-10	
PL-10A	
PL-11	
PL-11A	
PL-12	
PL-12A	
PL-12B	

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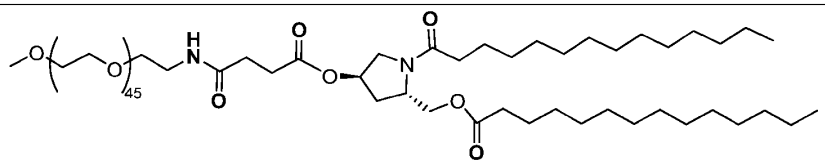
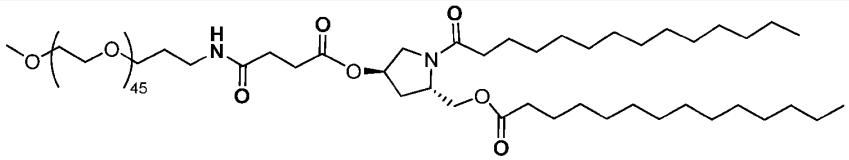
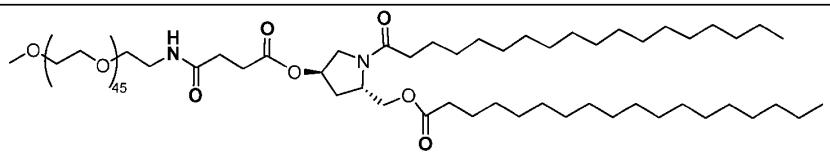
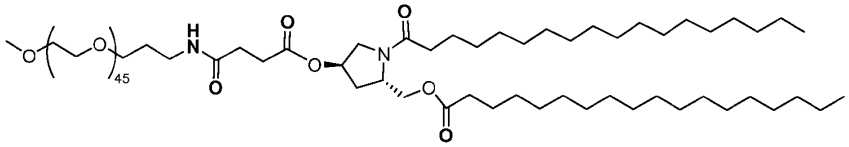
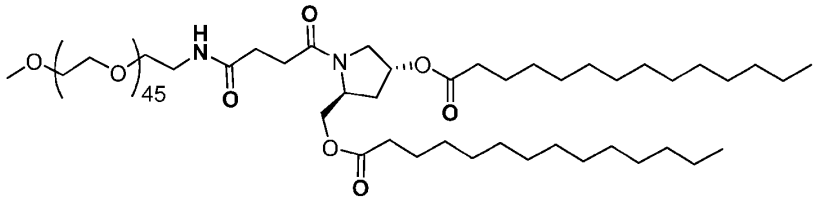
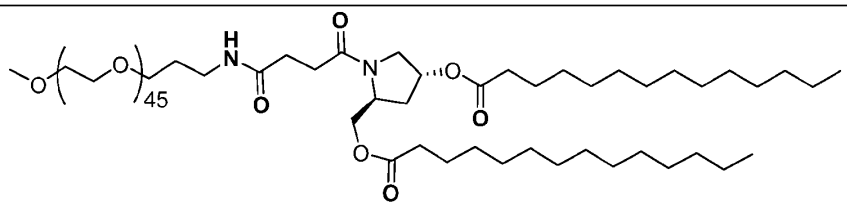
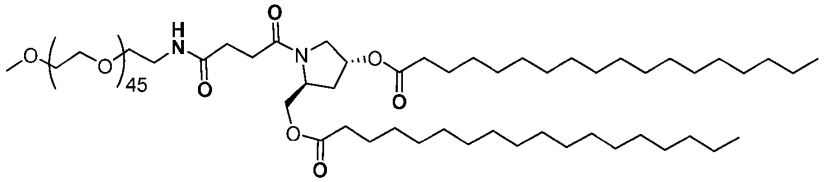
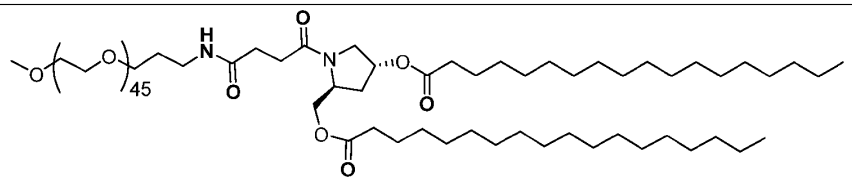
PL-13	
PL-13A	
PL-14	
PL-14A	
PL-15	
PL-15A	
PL-16	

Compound No.	Structure
PL-16A	

iv. Phospholipids

[00165]In some embodiments, an LNP of the present disclosure comprises a phospholipid. In some embodiments, an LNP of the present disclosure comprises two or more phospholipids. AsPhospholipids useful in the compositions and methods may be selected from the non-limiting group consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1.2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocho line (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sodium (S)-2-ammonio-3-((((R)-2-(oleoyloxy)-3-(stearoyloxy)propoxy)oxidophosphoryl)oxy)propanoate (L-α-phosphatidylserine; Brain PS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE), 1,2-Dielaidoyl-sn-phosphatidylethanolamine (DEPE), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), 1,2-dioleoyl-sn-glycero-3-phosphate (18:1 PA; DOPA), ammonium bis((S)-2-hydroxy-3-(oleoyloxy)propyl) phosphate (18:1 DMP; LBPA), 1,2-dioleoyl-sn-glycero-3-

phospho-(1'-myo-inositol) (DOPI; 18:1 PI), 1,2-distearoyl-sn-glycero-3-phospho-L-serine (18:0 PS), 1,2-dilinoleoyl-sn-glycero-3-phospho-L-serine (18:2 PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (16:0-18:1 PS; POPS), 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (18:0-18:1 PS), 1-stearoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (18:0-18:2 PS), 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:1 Lyso PS), 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:0 Lyso PS), and sphingomyelin. In some embodiments, an LNP includes DSPC. In certain embodiments, an LNP includes DOPE. In some embodiments, an LNP includes both DSPC and DOPE.

[00166]In some embodiments, the LNP comprises a phospholipid selected from 1-pentadecanoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine, 1-myristoyl-2-stearoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-myristoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-myristoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-palmitoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine, 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine, 1-oleoyl-2-myristoyl-sn-glycero-3-phosphocholine, 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine, 1-oleoyl-2-stearoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-acetyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',4'-bisphosphate), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',5'-bisphosphate), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',4',5'-trisphosphate), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3'-phosphate), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4'-phosphate), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-5'-phosphate), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine, and 1-(8Z-octadecenoyl)-2-palmitoyl-sn-glycero-3-phosphocholine.

[00167]In some embodiments, the LNP comprises a phospholipid selected from DSPS (Distearoylphosphatidylserine), DSPG (1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol)), DSPA (1,2-Distearoyl-sn-glycero-3-phosphate), diPhyPC (1,2-diphytanoyl-sn-glycero-3-phosphocholine), diPhy-diether-PC (1,2-di-O-phytanyl-sn-glycero-3-phosphocholine), diPhyPE (1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine), diPhy-diether-PE (1,2-di-O-phytanyl-sn-glycero-3-phosphoethanolamine), diPhyPS (1,2-diphytanoyl-sn-glycero-3-phospho-L-serine), diPhyPG (1,2-diphytanoyl-sn-glycero-3-phospho-(1'-rac-glycerol)), diPhyPA (1,2-diphytanoyl-sn-glycero-3-phosphate), Egg PA (L-α-phosphatidic acid), and Soy PA (L-α-phosphatidic acid).

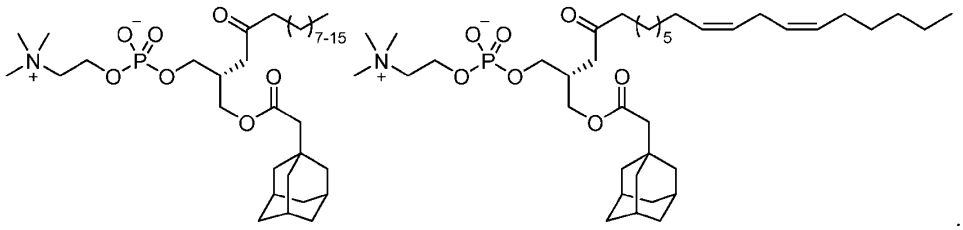
[00168]In some embodiments, the LNP comprises a phospholipid selected from 18:1 (Δ9-Cis) PE (DOPE), 18:0-18:1 PE (SOPE), C16-18:1 PE, 16:0-18:1 PE (POPE), 18:1 BMP (S,R), 18:0-18:1 PC (SOPC), 16:0-18:1 PC (POPC), 4ME 16:0 Diether PE (4Me), 18:1 (Δ9-Trans) PE (DEPE), 16:1 PE

(DPPE), and CL. In certain embodiments, the LNP comprises a phospholipid described or disclosed in Alvarez-Benedicto, et al. (Biomater. Sci., 2022, 10, 549) and Li, et al. (Asian Journal of Pharmaceutical Sciences, 2015, 10, 81-98).

[00169]In certain embodiments, the phospholipid is a sphingoid lipid or sphingolipid, such as, but not limited to sphingomyelin. As used herein, the terms "sphingoid lipid" and "sphingolipid" are meant to refer to a class of lipids containing a backbone comprising a sphingoid base. An exemplary sphingoid base is sphingosine. In certain embodiments, the LNP comprises a sphingolipid selected from Egg Sphingomyelin (Egg SM / ESM / (2S,3R,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl (2-(trimethylammonio)ethyl) phosphate), Brain or Porcine Sphingomyelin (Brain SM / (2S,3R,E)-3-hydroxy-2-stearamidooctadec-4-en-1-yl (2-(trimethylammonio)ethyl) phosphate), Milk or Bovine Sphingomyelin (Milk SM / (2S,3R,E)-3-hydroxy-2-tricosanamidooctadec-4-en-1-yl (2-(trimethylammonio)ethyl) phosphate), 28:0 SM (N-octacosanoyl-D-erythro-sphingosylphosphorylcholine), 14:0 SM (N-myristoyl-D-erythro-sphingosylphosphorylcholine), 16:1 SM (N-palmitoleoyl-D-erythro-sphingosylphosphorylcholine), 12:0 Dihydro SM (N-lauroyl-D-erythro-sphinganylphosphorylcholine), Lyso SM (Sphingosylphosphorylcholine), Lyso SM (Sphingosylphosphorylcholine), Lyso SM (dihydro) (Sphinganine Phosphorylcholine), 24:1 SM (N-nervonoyl-D-erythro-sphingosylphosphorylcholine), 24:0 SM (N-lignoceroyl-D-erythro-sphingosylphosphorylcholine), 18:1 SM (N-oleoyl-D-erythro-sphingosylphosphorylcholine), 18:0 SM (N-stearoyl-D-erythro-sphingosylphosphorylcholine), 17:0 SM (N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine), 16:0 SM (N-palmitoyl-D-erythro-sphingosylphosphorylcholine), 12:0 SM (N-lauroyl-D-erythro-sphingosylphosphorylcholine), 06:0 SM (N-hexanoyl-D-erythro-sphingosylphosphorylcholine), 02:0 SM (N-acetyl-D-erythro-sphingosylphosphorylcholine), 3-O-methyl Lyso SM (3-O-methyl-spingosylphosphorylcholine), 3-O-methyl-N-methyl Lyso SM (3-O-methyl-N-methyl-spingosylphosphorylcholine), and 3-N-methyl Lyso SM (3-N-methyl-spingosylphosphorylcholine).

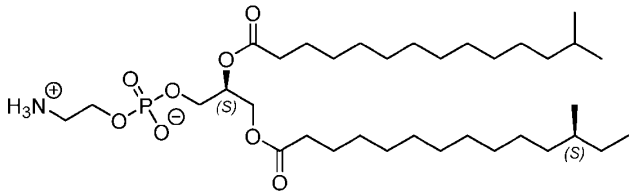
[00170]In some embodiments, the LNP comprises at least two phospholipids. In certain embodiments, at least a portion of the overall phospholipid content comprises a non-phosphatidylcholine phospholipid, wherein a "non-phosphatidylcholine phospholipid" is a phospholipid that does not comprise a phosphatidylcholine moiety. Exemplary non-phosphatidylcholine phospholipids include DOPE, DSPS, and DSPG. In certain embodiments, the LNP comprises at least 5 mol% of a non-phosphatidylcholine phospholipid. In certain embodiments, the LNP comprises at least 6 mol% of a non-phosphatidylcholine phospholipid. In certain embodiments, the LNP comprises at least 10 mol% of a non-phosphatidylcholine phospholipid.

[00171]In some embodiments, the LNP comprises a phospholipid comprising at least one constrained tail, such as those described by Gan, et al. (Bioeng Transl Med. 2020 Sep; 5(3): e10161.). In certain embodiments, the phospholipid is one selected from:



[00172]In some embodiments, the LNP comprises a phospholipid comprising a ceramide analogue having a triazole linkage, such as those described by Kim et al., Bioorg. Med. Chem. Lett., 17(16), 2007, 4584-4587.

[00173]In some embodiments, the LNP comprises a phospholipid disclosed in WO 2023/141470, which is incorporated by reference herein, in its entirety. In certain embodiments, the phospholipid is



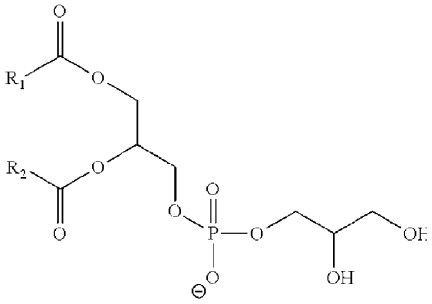
(a15:0-i15:0 PE).

[00174]In some embodiments, the LNP comprises a phospholipid disclosed in WO 2022/040641, which is incorporated by reference herein, in its entirety.

[00175]In some embodiments, a phospholipid tail may be modified in order to promote endosomal escape as described in U.S. 2021/0121411, which is incorporated herein by reference.

[00176]In some embodiments, the LNP comprises a phospholipid disclosed in one of US 2019/0240354; US 2010/0130588; US 2021/0087135; WO 2021/204179; US 2021/0128488; US 2020/0121809; US 2017/0119904; US 2013/0108685; US 2013/0195920; US 2015/0005363; US 2014/0308304; US 2013/0053572; WO 2019/232095A1; WO 2021/077067; WO 2019/152557; US 2017/0210697; or WO 2019/089828A1, each of which is incorporated by reference herein in their entirety.

[00177]In some embodiments, phospholipids disclosed in US 2020/0121809 have the following structure:



wherein R1 and R2 are each independently a branched or straight, saturated or unsaturated carbon chain (e.g., alkyl, alkenyl, alkynyl).

v.Targeting moieties

[00178]In some embodiments, the lipid nanoparticle further comprises a targeting moiety. The targeting moiety may be an antibody or a fragment thereof. The targeting moiety may be capable of binding to a target antigen. In certain embodiments, the lipid nanoparticle comprises more than one targeting moiety. In certain embodiments, the lipid nanoparticle comprises more than one targeting moiety, wherein the targeting moieties target at least two different receptors, and in some embodiments, the at least two different receptors are prevalent on different types of cells or tissues.

[00179]In some embodiments, the pharmaceutical composition comprises a targeting moiety that is operably connected to a lipid nanoparticle. In some embodiments, the targeting moiety is capable of binding to a target antigen. In some embodiments, the target antigen is expressed in a target organ. In some embodiments, the target antigen is expressed more in the target organ than it is in the liver.

[00180]In some embodiments, the targeting moiety is an antibody as described in WO2016189532A1, which is incorporated herein by reference. For example, in some embodiments, the targeted particles are conjugated to a specific anti-CD38 monoclonal antibody (mAb), which allows specific delivery of the siRNAs encapsulated within the particles at a greater percentage to B-cell lymphocytes malignancies (such as MCL) than to other subtypes of leukocytes.

[00181]In some embodiments, the targeting moiety targets a receptor selected from CD20, CCR7, CD3, CD4, CD5, CD8, CD16, CD19, CD20, CD21, CD22, CD25, CD28, CD35, CD40, CD45RA, CD45RO, CD52, CD62L, CD80, CD95, CD127, and CD137. In some embodiments, the targeting moiety targets a receptor selected from CD1, CD2, CD3, CD5, CD7, CD8, CD16, CD25, CD26, CD27, CD28, CD30, CD38, CD39, CD40L, CD44, CD45, CD62L, CD69, CD73, CD80, CD83, CD86, CD95, CD103, CD119, CD126, CD150, CD153, CD154, CD161, CD183, CD223, CD254, CD275, CD45RA, CXCR3, CXCR5, FasL, IL18R1, CTLA-4, 0X40, GITR, LAG3, ICOS, PD-1, leu-12, TCR, TLR1, TLR2, TLR3, TLR4, TLR6, NKG2D, CCR, CCR1, CCR2, CCR4, CCR6, and CCR7. In some embodiments, the targeting moiety targets a receptor selected from CD2, CD3, CD5 and CD7. In some embodiments, the targeting moiety targets a receptor selected from CD2, CD3, CD5, CD7, CD8, CD4, beta 7 integrin, beta 2 integrin, and C1q. In some embodiments, the targeting moiety targets CD117. In some embodiments, the targeting moiety targets CD90. In some embodiments, the targeting moiety targets a receptor selected from a mannose receptor, CD206 and C1q. In some embodiments, the targeting moiety is selected from T-cell receptor motif antibodies, T-cell α chain antibodies, T-cell β chain antibodies, T-cell γ chain antibodies, T-cell δ chain antibodies, CCR7 antibodies, CD3 antibodies, CD4 antibodies, CD5 antibodies, CD7 antibodies, CD8 antibodies, CD11b antibodies, CD11c antibodies, CD16 antibodies, CD19 antibodies, CD20 antibodies, CD21 antibodies, CD22 antibodies, CD25 antibodies, CD28 antibodies, CD34 antibodies, CD35 antibodies, CD40 antibodies, CD45RA antibodies, CD45RO antibodies, CD52 antibodies, CD56 antibodies,

CD62L antibodies, CD68 antibodies, CD80 antibodies, CD90 antibodies, CD95 antibodies, CD117 antibodies, CD127 antibodies, CD133 antibodies, CD137 (4-1BB) antibodies, CD163 antibodies, F4/80 antibodies, IL-4Rα antibodies, Sca-1 antibodies, CTLA-4 antibodies, GITR antibodies GARP antibodies, LAP antibodies, granzyme B antibodies, LFA-1 antibodies, transferrin receptor antibodies, and fragments thereof. In certain embodiments, the targeting moiety is any one described or contemplated in US20230312713A1, US20230203538A1, US20230320995A1, US20160145348, and US20110038941, each of which is incorporated by reference herein in its entirety.

[00182]In some embodiments, the lipid nanoparticles may be targeted when conjugated/attached/associated with a targeting moiety such as an antibody, or a fragment thereof.

vi. Zwitterionic amino lipids

[00183]In some embodiments, an LNP comprises a zwitterionic lipid. In some embodiments, an LNP comprising a zwitterionic lipid does not comprise a phospholipid. In some embodiments, an LNP comprising a zwitterionic lipid further comprises a phospholipid.

[00184]Zwitterionic amino lipids have been shown to be able to self-assemble into LNPs without phospholipids to load, stabilize, and release mRNAs intracellularly as described in U.S. Patent Application 20210121411, which is incorporated herein by reference in its entirety. Zwitterionic, ionizable cationic and permanently cationic helper lipids enable tissue-selective mRNA delivery and CRISPR-Cas9 gene editing in spleen, liver and lungs as described in Liu et al., Membrane-destablizing ionizable phospholipids for organ-selective mRNA delivery and CRISPR-Cas gene editing, Nat Mater. (2021), which is incorporated herein by reference in its entirety.

[00185]The zwitterionic lipids may have head groups containing a cationic amine and an anionic carboxylate as described in Walsh et al., Synthesis, Characterization and Evaluation of Ionizable Lysine-Based Lipids for siRNA Delivery, Bioconjug Chem. (2013), which is incorporated herein by reference in its entirety. Ionizable lysine-based lipids containing a lysine head group linked to a long-chain dialkylamine through an amide linkage at the lysine α-amine may reduce immunogenicity as described in Walsh et al., Synthesis, Characterization and Evaluation of Ionizable Lysine-Based Lipids for siRNA Delivery, Bioconjug Chem. (2013).

vii. Additional lipid components

[00186]In some embodiments, the LNP compositions of the present disclosure further comprise one or more additional lipid components capable of influencing the tropism of the LNP. In some embodiments, the LNP further comprises at least one lipid selected from DDAB, EPC, 14PA, 18BMP, DODAP, DOTAP, and C12-200 (see Cheng, et al. Nat Nanotechnol. 2020 April; 15(4): 313–320.; Dillard, et al. PNAS 2021 Vol. 118 No. 52.).

[00187]In some embodiments, an LNP of the present disclosure further comprises one or more additional ionizable lipids, such as, but not limited to those disclosed in one of US 2023/0053437; US 2019/0240354; US 2010/0130588; US 2021/0087135; WO 2021/204179; US 2021/0128488; US 2020/0121809; US 2017/0119904; US 2013/0108685; US 2013/0195920; US 2015/0005363; US

2014/0308304; US 2013/0053572; WO 2019/232095A1; WO 2021/077067; WO 2019/152557; US 2017/0210697; or WO 2019/089828A1, each of which is incorporated by reference herein in their entirety. In certain embodiments, an LNP of the present disclosure further comprises one or more additional ionizable lipids selected from those disclosed in WO2023044343A1 or WO2023044333A1, both of which are incorporated by reference herein in their entirety.

[00188]In some embodiments, the LNP compositions of the present disclosure comprise, or further comprise one or more lipids selected from 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine (18:3 PC), Acylcarnosine (AC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), N-oleoyl-sphingomyelin (SPM) (C18:l), N-lignoceryl SPM (C24:0), N-nervonoylshphingomyelin (C24:l), Cardiolipin (CL), l,2-bis(tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine (DC8-9PC), dicetyl phosphate (DCP), dihexadecyl phosphate (DCP1), 1,2-Dipalmitoylglycerol-3-hemisuccinate (DGSucc), short-chain bis-n-heptadecanoyl phosphatidylcholine (DHPC), dihexadecoyl-phosphoethanolamine (DHPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), l,2-dilauroyl-sn-glycero-3-PE (DLPE), dimyristoyl glycerol hemisuccinate (DMGS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleyloxybenzylalcohol (DOBA), 1,2-dioleoylglyceryl-3-hemisuccinate (DOGHEMS), N-[2-(2-{2-[2-(2,3-Bis-octadec-9-enyloxy-propoxy)-ethoxy]-ethoxy}-ethoxy)-ethyl]-3-(3,4,5-dihydroxy-6-hydroxymethyl-1etrahydro-pyran-2-ylsulfanyl)-propionamide (DOGP4αMan), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), histaminedistearoylglycerol (HDSG), 1,2-Dipalmitoylglycerol-hemisuccinate-Nα-Histidinyl-Hemisuccinate (HistSuccDG), N-(5'-hydroxy-3'-oxypentyl)-10-12-pentacosadiynamide (h-Pegi-PCDA), 2-[l-hexyloxyethyl]-2-devinylpyropheophorbide-a (HPPH), hydrogenatedsoybeanphosphatidylcholine (HSPC), 1,2-Dipalmitoylglycerol-O-α-histidinyl-Nα-hemisuccinate (IsohistsuccDG), mannosialized dipalmitoylphosphatidylethanolamine (ManDOG), l,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (MCC-PE), 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16:0 PE), 1-myristoyl-2-hydroxy-sn-glycero-phosphocholine (MHPC), a thiol-reactive maleimide headgroup lipid e.g.1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)but-yramid (MPB-PE), Nervonic Acid (NA), sodium cholate (NaChol), l,2-dioleoyl-sn-glycero-3-[phosphoethanolamine-N-dodecanoyl (NC12-DOPE), 1-oleoyl-2-cholesteryl hemisuccinoyl-sn-glycero-3-phosphocholine

(OChemsPC), phosphatidylethanolamine lipid (PE), PE lipid conjugated with polyethylene glycol(PEG) (e.g., polyethylene glycol-distearoylphosphatidylethanolamine lipid (PEG-PE)), phosphatidylglycerol (PG), partially hydrogenated soy phosphatidylchloline (PHSPC), phosphatidylinositol lipid (PI), phosphotidylinositol-4-phosphate (PIP), palmitoyloleoylphosphatidylcholine (POPC), phosphatidylethanolamine (POPE), palmitoyloleyolphosphatidylglycerol (POPG), phosphatidylserine (PS), lissamine rhodamineB-phosphatidylethanolamine lipid (Rh-PE), purifiedsoy-derivedmixtureofphospholipids (SIOO), phosphatidylcholine (SM), 18-1-trans-PE,1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), soybean phosphatidylcholine (SPC), sphingomyelins (SPM), alpha,alpha-trehalose-6,6'-dibehenate (TDB), l,2-dielaidoyl-sn-glycero-3-phophoethanolamine (transDOPE), ((23S,5R)-3-(bis(hexadecyloxy)methoxy)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methylmethylphosphate, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleyl-sn-glycero-3-phosphoethanolamine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 16-O-monomethyl PE, 16-O-dimethyl PE, and dioleylphosphatidylethanolamine.

B.Exemplary LNP Compositions

[00189]In some embodiments, provided herein are LNPs comprising (a): at least one Lipid of the Disclosure; (b) at least one PEG lipid; (c) at least one structural lipid; and (d) at least one non-ionizable lipid and/or a zwitterionic lipid. In some embodiments, the LNPs further comprise an additional ionizable lipid, besides a compound of Formula (AX).

[00190]In some embodiments, the PEG-lipid is selected from the group consisting of PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPC, and PEG-DSPE.

[00191]In some embodiments, the structural lipid is selected from the group consisting of cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, ursolic acid, and alpha-tocopherol.

[00192]In some embodiments, the non-ionizable lipid is a phospholipid selected from the group consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1.2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocho line (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-

didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sodium (S)-2-ammonio-3-((((R)-2-(oleoyloxy)-3-(stearoyloxy)propoxy)oxidophosphoryl)oxy)propanoate (L-α-phosphatidylserine; Brain PS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), 1,2-dioleoyl-sn-glycero-3-phosphate (18:1 PA; DOPA), ammonium bis((S)-2-hydroxy-3-(oleoyloxy)propyl) phosphate (18:1 DMP; LBPA), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol) (DOPI; 18:1 PI), 1,2-distearoyl-sn-glycero-3-phospho-L-serine (18:0 PS), 1,2-dilinoleoyl-sn-glycero-3-phospho-L-serine (18:2 PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (16:0-18:1 PS; POPS), 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (18:0-18:1 PS), 1-stearoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (18:0-18:2 PS), 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:1 Lyso PS), 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:0 Lyso PS), and sphingomyelin.

[00193]In some embodiments, the non-ionizable lipid is a phospholipid selected from the group consisting of Egg Sphingomyelin (Egg SM / ESM / (2S,3R,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-yl (2-(trimethylammonio)ethyl) phosphate), Brain or Porcine Sphingomyelin (Brain SM / (2S,3R,E)-3-hydroxy-2-stearamidooctadec-4-en-1-yl (2-(trimethylammonio)ethyl) phosphate), Milk or Bovine Sphingomyelin (Milk SM / (2S,3R,E)-3-hydroxy-2-tricosanamidooctadec-4-en-1-yl (2-(trimethylammonio)ethyl) phosphate), 28:0 SM (N-octacosanoyl-D-erythro-sphingosylphosphorylcholine), 14:0 SM (N-myristoyl-D-erythro-sphingosylphosphorylcholine), 16:1 SM (N-palmitoleoyl-D-erythro-sphingosylphosphorylcholine), 12:0 Dihydro SM (N-lauroyl-D-erythro-sphinganylphosphorylcholine), Lyso SM (Sphingosylphosphorylcholine), Lyso SM (Sphingosylphosphorylcholine), Lyso SM (dihydro) (Sphinganine Phosphorylcholine), 24:1 SM (N-nervonoyl-D-erythro-sphingosylphosphorylcholine), 24:0 SM (N-lignoceroyl-D-erythro-sphingosylphosphorylcholine), 18:1 SM (N-oleoyl-D-erythro-sphingosylphosphorylcholine), 18:0 SM (N-stearoyl-D-erythro-sphingosylphosphorylcholine), 17:0 SM (N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine), 16:0 SM (N-palmitoyl-D-erythro-sphingosylphosphorylcholine), 12:0 SM (N-lauroyl-D-erythro-sphingosylphosphorylcholine), 06:0 SM (N-hexanoyl-D-erythro-

sphingosylphosphorylcholine), 02:0 SM (N-acetyl-D-erythro-sphingosylphosphorylcholine), 3-O-methyl Lyso SM (3-O-methyl-spingosylphosphorylcholine), 3-O-methyl-N-methyl Lyso SM (3-O-methyl-N-methyl-spingosylphosphorylcholine), and 3-N-methyl Lyso SM (3-N-methyl-spingosylphosphorylcholine).

[00194]In some embodiments, (a) the PEG lipid is PEG2k-DMG or PEG2k-DSPE or a mixture thereof; (b) the structural lipid is cholesterol; and (c) the phospholipid, non-ionizable lipid or zwitterionic lipid is a sphingolipid or DSPC or a mixture thereof.

[00195]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 0 mol% to about 10 mol% of PEG lipid; (b) about 0 mol% to about 30 mol% structural lipid; (c) about 20 mol% to about 45 mol% phospholipid, non-ionizable lipid or zwitterionic lipid; and (d) about 30 mol% to about 60 mol% of a Lipid of the Disclosure.

[00196]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 1 mol% to about 2 mol% of PEG lipid; (b) about 25 mol% to about 40 mol% structural lipid; (c) about 20 mol% to about 45 mol% phospholipid, non-ionizable lipid or zwitterionic lipid; and (d) about 30 mol% to about 60 mol% of a Lipid of the Disclosure.

[00197]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 2 mol% of PEG lipid; (b) about 25 mol% structural lipid; (c) about 40 mol% phospholipid, non-ionizable lipid or zwitterionic lipid; and (d) about 33 mol% of a Lipid of the Disclosure.

[00198]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 0.5 mol% to about 10 mol% of PEG lipid; (b) about 0.5 mol% to about 50 mol% structural lipid; (c) about 5 mol% to about 45 mol% non-ionizable lipid (e.g., phospholipid), non-ionizable lipid or zwitterionic lipid; and (d) about 30 mol% to about 60 mol% of a Lipid of the Disclosure.

[00199]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 0.5 mol% to about 5 mol% of PEG lipid; (b) about 20 mol% to about 45 mol% structural lipid; (c) about 35 mol% to about 45 mol% non-ionizable lipid (e.g., phospholipid), non-ionizable lipid or zwitterionic lipid; and (d) about 30 mol% to about 40 mol% of a Lipid of the Disclosure.

[00200]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 1.5 mol% to about 3.5 mol% of PEG lipid; (b) about 20 mol% to about 45 mol% structural lipid; (c) about 35 mol% to about 45 mol% non-ionizable lipid (e.g., phospholipid), non-ionizable lipid or zwitterionic lipid; and (d) about 30 mol% to about 40 mol% of a Lipid of the Disclosure.

[00201]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 0.5 mol% to about 5 mol% of PEG lipid; (b) about 20 mol% to about 45 mol% structural lipid; (c) about 5 mol% to about 15 mol% non-ionizable lipid (e.g., phospholipid), non-ionizable lipid or zwitterionic lipid; and (d) about 45 mol% to about 55 mol% of a Lipid of the Disclosure.

[00202]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 1.5 mol% to about 3.5 mol% of PEG lipid; (b) about 20 mol% to about 45 mol% structural lipid; (c) about 5

mol% to about 15 mol% non-ionizable lipid (e.g., phospholipid), non-ionizable lipid or zwitterionic lipid; and (d) about 45 mol% to about 55 mol% of a Lipid of the Disclosure.

[00203]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 2.5 mol% of PEG lipid; (b) about 39 mol% structural lipid; (c) about 10 mol% phospholipid, non-ionizable lipid or zwitterionic lipid; and (d) about 48.5 mol% of a Lipid of the Disclosure.

[00204]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 1.5 mol% of PEG lipid; (b) about 40 mol% structural lipid; (c) about 10 mol% phospholipid, non-ionizable lipid or zwitterionic lipid; and (d) about 48.5 mol% of a Lipid of the Disclosure.

[00205]In certain embodiments, the lipid component of the nanoparticle composition comprises about 30 mol % to about 60 mol % ionizable lipid, about 0 mol % to about 30 mol % phospholipid, about 18.5 mol % to about 48.5 mol % structural lipid, and about 0 mol% to about 10 mol% of PEG lipid, provided that the total mol % does not exceed 100%. In certain embodiments, the lipid component of the nanoparticle composition comprises about 20 mol % to about 45 mol % ionizable lipid, about 30 mol % to about 60 mol % phospholipid, about 10 mol % to about 30 mol % structural lipid, and about 0 mol% to about 10 mol% of PEG lipid, provided that the total mol % does not exceed 100%. In some embodiments, the lipid component of the nanoparticle composition comprises about 35 mol % to about 55 mol % ionizable lipid, about 5 mol % to about 25 mol % phospholipid, about 30 mol % to about 40 mol % structural lipid, and about 0 mol % to about 10 mol % of PEG lipid, provided that the total mol % does not exceed 100%. In some embodiments, the lipid component of the nanoparticle composition comprises about 30 mol % to about 40 mol % ionizable lipid, about 35 mol % to about 45 mol % phospholipid, about 20 mol % to about 30 mol % structural lipid, and about 0.5 mol % to about 5 mol % of PEG lipid, provided that the total mol % does not exceed 100%. In certain embodiments, the lipid component of the nanoparticle composition comprises about 25 mol % to about 45 mol % ionizable lipid, about 35 mol % to about 50 mol % phospholipid, about 10 mol % to about 25 mol % structural lipid, and about 1 mol% to about 5 mol% of PEG lipid, provided that the total mol % does not exceed 100%. In a particular embodiment, the lipid component comprises about 50 mol % ionizable lipid, about 10 mol % phospholipid, about 38.5 mol % structural lipid, and about 1.5 mol% of PEG lipid. In another particular embodiment, the lipid component comprises about 40 mol % ionizable lipid, about 20 mol % phospholipid, about 38.5 mol % structural lipid, and about 1.5 mol % of PEG lipid. In another particular embodiment, the lipid component comprises about 48.5 mol % ionizable lipid, about 10 mol % phospholipid, about 40 mol % structural lipid, and about 1.5 mol % of PEG lipid. In another particular embodiment, the lipid component comprises about 48.5 mol % ionizable lipid, about 10 mol % phospholipid, about 39 mol % structural lipid, and about 2.5 mol % of PEG lipid. In another particular embodiment, the lipid component comprises about 33 mol % ionizable lipid, about 40 mol % phospholipid, about 25 mol % structural lipid, and about 2 mol % of PEG lipid. In some embodiments, the phospholipid is DOPE or DSPC. In some embodiments, the phospholipid is DSPC. In some embodiments, the phospholipid is a sphingolipid. In some

embodiments, the phospholipid is sphingomyelin. In some embodiments, the phospholipid is egg sphingomyelin (ESM). In other embodiments, the PEG lipid is PEG-DMG (eg. PEG2K-DMG). In other embodiments, the PEG lipid is PEG-DSPE (eg. PEG2K-DSPE). In other embodiments, the PEG lipid is PEG-DMPE (eg. PEG2K-DMPE). In other embodiments, the structural lipid is cholesterol. In other embodiments, the PEG lipid is PEG-DMG and/or the structural lipid is cholesterol. In some embodiments, the PEG lipids is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is DSPC. In some embodiments, the PEG lipids is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is sphingomyelin. In some embodiments, the PEG lipids is PEG-DMG, the structural lipid is cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In certain embodiments, the LNP comprises about 33mol% ionizable lipid (eg. at least one ionizable lipid of a Formula described herein), about 40mol% of a sphingolipid, about 25mol% cholesterol and about 2mol% PEG2K-DMG. In some embodiments, the PEG lipids is PEG2K-DSPE, the structural lipid is cholesterol, and the phospholipid is DSPC. In some embodiments, the PEG lipids is PEG2K-DSPE, the structural lipid is cholesterol, and the phospholipid is sphingomyelin. In some embodiments, the PEG lipids is PEG-DSPE, the structural lipid is cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In some embodiments, the PEG lipids is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is DOPE. In some embodiments, the PEG lipids is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is DOPC. In some embodiments, the PEG lipids is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is DLPC. In some embodiments, the PEG lipids is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is DOPS. In some embodiments, the PEG lipids is PEG-DMG, the structural lipid is cholesterol, and the phospholipid is a mixture of a phosphatidylcholine lipid and a sphingolipid. In some embodiments, the PEG lipids is PEG-DMG, the structural lipid is cholesterol, and the phospholipid is a mixture of a phosphatidylcholine lipid and phosphatidylserine lipid. In some embodiments, the PEG lipids is PEG-DMG, the structural lipid is cholesterol, and the phospholipid is a mixture of a phosphatidylcholine lipid and a phosphoethanolamine lipid. In some embodiments, the PEG lipids is PEG-DMG, the structural lipid is cholesterol, and the phospholipid is a mixture of a sphingolipid and phosphatidylserine lipid. In some embodiments, the PEG lipids is PEG-DMG, the structural lipid is cholesterol, and the phospholipid is a mixture of a sphingolipid and a phosphoethanolamine lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 20mol% of a sphingolipid, about 20mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 10mol% of a sphingolipid, about 30mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 30mol% of a sphingolipid, about 10mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable

lipid, about 20mol% sphingomyelin, about 20mol% of a DSPC, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 10mol% sphingomyelin, about 30mol% of a DSPC, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 30mol% sphingomyelin, about 10mol% of a DSPC, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 25mol% cholesterol, about 2mol% of a PEGylated lipid, and about 40% of a mixture of phosphatidylcholine, phosphatidylserine, phosphoethanolamine, and sphingoid lipids. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 25mol% cholesterol, about 2mol% of a PEGylated lipid, and about 40% of a mixture of phosphatidylcholine, phosphatidylserine, phosphoethanolamine, and sphingoid lipids, wherein each of the phosphatidylcholine, phosphatidylserine, phosphoethanolamine, and sphingoid lipids is present in an amount less than 30 mol% of the total lipid component of the LNP. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 25mol% cholesterol, about 2mol% of a PEGylated lipid, and about 40% of a mixture of phosphatidylcholine, phosphatidylserine, phosphoethanolamine, and sphingoid lipids, wherein each of the phosphatidylcholine, phosphatidylserine, phosphoethanolamine, and sphingoid lipids is present in an amount less than 25 mol% of the total lipid component of the LNP. In certain embodiments, LNP is any one of the aforementioned in this paragraph wherein the PEG lipid is PEG2k-DMG. In certain embodiments, LNP is any one of the aforementioned in this paragraph wherein the PEG lipid is PEG2k-DSPE.

[00206]In another particular embodiment, LNP comprises about 33 mol % ionizable lipid, about 40 mol % DSPC, about 25 mol % cholesterol, and about 2 mol % of PEG lipid. In another particular embodiment, LNP comprises about 33 mol % ionizable lipid, about 40 mol % sphingomyelin, about 25 mol % cholesterol, and about 2 mol % of PEG lipid. In another particular embodiment, LNP comprises about 33 mol % ionizable lipid, about 40 mol % DOPE, about 25 mol % cholesterol, and about 2 mol % of PEG lipid. In another particular embodiment, LNP comprises about 33 mol % ionizable lipid, about 40 mol % DOPC, about 25 mol % cholesterol, and about 2 mol % of PEG lipid. In another particular embodiment, LNP comprises about 33 mol % ionizable lipid, about 40 mol % DLPC, about 25 mol % cholesterol, and about 2 mol % of PEG lipid. In another particular embodiment, LNP comprises about 33 mol % ionizable lipid, about 40 mol % DOPS, about 25 mol % cholesterol, and about 2 mol % of PEG lipid. In another particular embodiment, LNP comprises about 33 mol % ionizable lipid, about 40 mol % phospholipid, about 25 mol % cholesterol, and about 2 mol % of PEG lipid. In another particular embodiment, LNP comprises about 33 mol % ionizable lipid, about 20 mol % sphingomyelin, about 20 mol% DSPC, about 25 mol % cholesterol, and about 2 mol % of PEG lipid. In certain embodiments, LNP is any one of the aforementioned in this paragraph wherein the PEG lipid is PEG2k-DMG. In certain embodiments, LNP is any one of the aforementioned in this paragraph wherein the PEG lipid is PEG2k-DSPE.

[00207]In certain embodiments, the LNP comprises about 43mol% ionizable lipid, about 15mol% of a sphingolipid, about 15mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 25mol% of a sphingolipid, about 15mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 15mol% of a sphingolipid, about 25mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In some embodiments, the PEG lipid is PEG2K-DSPE, the structural lipid is cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In some embodiments, the PEG lipid is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 40mol% cholesterol and about 1.5mol% PEG2K-DSPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 40mol% cholesterol and about 1.5mol% PEG2K-DMG. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 39mol% cholesterol and about 2.5mol% PEG2K-DSPE.

[00208]In another particular embodiment, the lipid component includes about 48.5 mol % ionizable lipid, about 10 mol % phospholipid, about 38.5 mol % structural lipid, and about 3 mol % of PEG lipid. In another particular embodiment, the lipid component includes about 48.5 mol % ionizable lipid, about 10 mol % phospholipid, about 38 mol % structural lipid, and about 3.5 mol % of PEG lipid. In some embodiments, the PEG lipid is PEG2K-DPPE, the structural lipid is cholesterol, and the phospholipid is a DSPC or a mixture of DSPC and sphingomyelin. In some embodiments, the PEG lipid is PEG2K-DPPE, the structural lipid is cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 40mol% cholesterol and about 1.5mol% PEG2K-DPPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 39.5 mol% cholesterol and about 2 mol% PEG2K-DPPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 39mol% cholesterol and about 2.5mol% PEG2K-DPPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 38.5 mol% cholesterol and about 3 mol% PEG2K-DPPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 38 mol% cholesterol and about 3.5mol% PEG2K-DPPE. In some embodiments, the PEG lipid is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is a DSPC or a mixture of DSPC and sphingomyelin. In some embodiments, the PEG lipid is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about

10mol% of a phospholipid (such as DSPC), about 40mol% cholesterol and about 1.5mol% PEG2K-DMG. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 39.5 mol% cholesterol and about 2 mol% PEG2K-DMG. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 39mol% cholesterol and about 2.5mol% PEG2K-DMG. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 38.5 mol% cholesterol and about 3 mol% PEG2K-DMG. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 38 mol% cholesterol and about 3.5mol% PEG2K-DMG. In some embodiments, the PEG lipid is PEG2K-DSPE, the structural lipid is cholesterol, and the phospholipid is a DSPC or a mixture of DSPC and sphingomyelin. In some embodiments, the PEG lipid is PEG2K-DSPE, the structural lipid is cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 40mol% cholesterol and about 1.5mol% PEG2K-DSPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 39.5 mol% cholesterol and about 2 mol% PEG2K-DSPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 39mol% cholesterol and about 2.5mol% PEG2K-DSPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 38.5 mol% cholesterol and about 3 mol% PEG2K-DSPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 38 mol% cholesterol and about 3.5mol% PEG2K-DSPE.

[00209]In some embodiments, the LNP further comprises a targeting moiety. In some embodiments, the targeting moiety is an antibody or a fragment thereof.

[00210]In some embodiments, the LNP further comprises an active agent. In some embodiments, the active agent is a nucleic acid. In some embodiments, the nucleic acid is a ribonucleic acid. In some embodiments, the ribonucleic acid is at least one ribonucleic acid selected from the group consisting of a small interfering RNA (siRNA), an asymmetrical interfering RNA (aiRNA), a microRNA (miRNA), a Dicer-substrate RNA (dsRNA), a small hairpin RNA (shRNA), a messenger RNA (mRNA), and a long non-coding RNA (lncRNA). In some embodiments, the nucleic acid is a messenger RNA (mRNA) or a circular RNA. In some embodiments, the mRNA comprises an open reading frame encoding a cancer antigen. In some embodiments, the mRNA comprises an open reading frame encoding an immune checkpoint modulator. In some embodiments, the mRNA comprises at least one motif selected from the group consisting of a stem loop, a chain terminating nucleoside, a polyA sequence, a polyadenylation signal, and a 5' cap structure. In some embodiments, the nucleic acid is suitable for a genome editing technique. In some embodiments, the genome editing technique is clustered regularly interspaced short palindromic repeats (CRISPR) or transcription

activator-like effector nuclease (TALEN). In some embodiments, the nucleic acid is at least one nucleic acid suitable for a genome editing technique selected from the group consisting of a CRISPR RNA (crRNA), a trans-activating crRNA (tracrRNA), a single guide RNA (sgRNA), and a DNA repair template. In some embodiments, the mRNA is at least 30 nucleotides in length. In some embodiments, the mRNA is at least 300 nucleotides in length. In some embodiments, the nucleic acid encodes a therapeutic protein. In some embodiments, the therapeutic protein is a CAR or TCR complex protein. In some embodiments, the CAR or TCR complex protein comprises an antigen binding domain specific for an antigen selected from the group: CD 19, CD123, CD22, CD30, CD171, CS-1, C-type lectin-like molecule- 1, CD33, epidermal growth factor receptor variant III (EGFRvIII), disialoganglioside GD2, disaloganglioside GD3, TNF receptor family member, B cell maturation antigen (BCMA), Tn antigen ((Tn Ag) or (GalNAca-Ser/Thr)), prostate- specific membrane antigen (PSMA), Receptor tyrosine kinase-like orphan receptor 1 (ROR1), Fms-Like Tyrosine Kinase 3 (FLT3), Tumor-associated glycoprotein 72 (TAG72), CD38, CD44v6, Carcinoembryonic antigen (CEA), Epithelial cell adhesion molecule (EPCAM), B7H3 (CD276), KIT (CD 117), Interleukin- 13 receptor subunit alpha-2, mesothelin, Interleukin 11 receptor alpha (IL-l lRa), prostate stem cell antigen (PSCA), Protease Serine 21, vascular endothelial growth factor receptor 2 (VEGFR2), Lewis(Y) antigen, CD24, Platelet-derived growth factor receptor beta (PDGFR-beta), Stage-specific embryonic antigen-4 (SSEA-4), CD20, Folate receptor alpha, HER2, HER3, Mucin 1, cell surface associated (MUC1), epidermal growth factor receptor (EGFR), neural cell adhesion molecule (NCAM), Prostase, prostatic acid phosphatase (PAP), elongation factor 2 mutated (ELF2M), Ephrin B2, fibroblast activation protein alpha (FAP), insulin-like growth factor 1 receptor (IGF-I receptor), carbonic anhydrase IX (CAIX), Proteasome (Prosome, Macropain) Subunit, Beta Type, 9 (LMP2), glycoprotein 100 (gplOO), oncogene fusion protein consisting of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl), tyrosinase, ephrin type- A receptor 2 (EphA2), Fucosyl GM1, sialyl Lewis adhesion molecule (sLe), ganglioside GM3, transglutaminase 5 (TGS5), high molecular weight-melanoma-associated antigen (HMWMAA), o-acetyl-GD2 ganglioside (0AcGD2), Folate receptor beta, tumor endothelial marker 1 (TEM1/CD248), tumor endothelial marker 7 -related (TEM7R), claudin 6 (CLDN6), claudin 18.2 (CLDN18.2), thyroid stimulating hormone receptor (TSHR), G protein-coupled receptor class C group 5, member D (GPRC5D), chromosome X open reading frame 61 (CXORF61), CD97, and CD179a.

III. LNP payload

[00211]The instant specification describes compositions, methods, processes, kits and devices for the selection, design, preparation, manufacture, formulation, and/or use of LNP-based RNA medicines (e.g., vaccines, gene therapies, or gene-editing therapeutics). In various embodiments, the LNP-based RNA medicines comprise an LNP delivery system (as described in detail herein) and an encapsulated cargo/payload (e.g., RNA in the case of RNA medicines).

[00212]In the case of RNA medicines, the payload can be one or more RNA molecules, including coding RNA (e.g., linear or circular mRNA) or non-coding RNA (e.g., guide RNA, pegRNA, or retron ncRNA).

[00213]In various other embodiments, the payloads can include any type of nucleic acid molecule, including coding RNA molecules (e.g., mRNA), guide RNAs for editing systems (e.g., Cas9 guides, Cas12a guides, base editor guides, and prime editor guides), other non-coding RNAs relating to editing systems (e.g., retron ncRNAs), small RNAs (sRNAs)—which refer to a wide variety of polymeric RNA molecules that are generally less than 200 nucleotides in length with various functionalities, such as RNA interference, and include small-interfering RNA (siRNA), microRNAs (miRNA), piwi-interacting RNA (piRNA), repeat associated small interfering RNA (rasiRNA), small nuclear RNA (snRNA or U-RNA), small nucleolar RNA (snoRNA), small rDNA-derived RNA (srRNA), rRNA fragment (tRF), and Y RNA-derived small RNA, tRNA, rRNA, and self-amplifying RNA (saRNA)—and DNA molecules, such as DNA vectors, DNA plasmids, HDR donors, oligonucleotides, primers, etc., and chimeric molecules comprising both DNA and RNA. The cargo nucleic acid molecules may be single-stranded or double-stranded. Such nucleic acid cargo may comprise exactly one molecule. Such nucleic acid cargo may comprise exactly two molecules. Such nucleic acid cargo may comprise exactly three molecules. Such nucleic acid cargo may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 distinct molecules. Such nucleic acid cargo may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 distinct molecules. Such nucleic acid cargo may comprise between 1-25, or 5-30, or 10-35, or 20-40, or up to 100, or more distinct molecules.

[00214]In various other aspects, the LNPs described herein may be used to deliver any payload of interest to a biological target, e.g., to a cell or a bodily tissue. The term "payload" refers to an active substance (i.e., not limited to RNA or DNA), such as a small molecule, polypeptide, peptide, carbohydrate, or nucleic acid molecule, and includes, without limitation, mRNA molecules (including linear and circular mRNA) or non-coding RNA molecules (e.g., guide RNAs, pegRNAs, retron ncRNAs) which are encapsulated within the LNPs described herein. In some embodiments, the LNP cargo may comprise an RNP or ribonucleoprotein, such as a gene editing nuclease protein complexed with a cognate guide RNA.

[00215]In various embodiments, the payload is an RNA molecule, which may be linear or circular and may comprise one or more functional nucleotide sequences of interest, which may include, but are not limited to coding and non-coding nucleotide sequences. In various embodiments, the non-coding nucleotide sequences may comprise regulatory elements that influence RNA post-transcriptional processing, nuclear translation control sequences, and sequences which encode one or more biological products of interest, e.g., a therapeutic protein or antigen, among other sequence elements that may impact the functioning of the RNA or its encoded products. As used herein, the term "coding region of interest" or "product coding region" or the like may be used to refer to the

encoded one or more biological products of interest. Equivalently, a product coding region may be referred to as a "product expression sequence."

[00216]In various embodiments herein, the specification refers to "originator constructs" (or "originator polynucleotide constructs") and "benchmark constructs" (or "benchmark polynucleotide constructs"), which are embodiments of payloads comprising nucleic acid molecules, i.e., embodiments of linear and/or circular mRNA payloads, and which may comprise a product coding region that encodes a polypeptide, such as, but not limited to an antigen or a therapeutic protein or to components of a gene editing system (e.g., a programmable nuclease).

[00217]FIG. 2 shows an example of an originator construct **100**, which may be a linear or circular mRNA molecule. The originator construct **100** may include at least one product coding region **10** which is or encodes a polypeptide of interest, such as, but not limited to a vaccine antigen or a therapeutic protein. The originator construct **100** may contain 1 or 2 flanking regions 20. The flanking regions 2**0** may be located 5' to the product coding region **10** and/or 3' to the product coding region **10**. In some instances the originator construct **100** does not contain a flanking region 2**0**. The flanking region **20** of the originator construct **100** may include at least one regulatory region **30**. At least one flanking region **20** of the originator polynucleotide construct **100** may include at least one identifier region **40**. The identifier region **40** may be, but is not limited to, a barcode, label, signal and/or tag. Additionally, the identifier region **40** may be located within the product coding region **10** or may be located in the product coding region **10** and at least one flanking region **20**.

[00218]In some embodiments, the originator construct comprises from about 5 to about 10,000 nucleotides in length. As a non-limiting examples, the length of the originator construct may be from 5 to 30, from 5 to 50, from 5 to 100, from 5 to 250, from 5 to 500, from 5 to 1,000, from 5 to 1,500, from 5 to 3,000 from 5 to 5,000, from 5 to 7,000, from 5 to 10,000 from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 3,000 to 5,000, from 3,000 to 7,000, from 3,000 to 10,000, from 5,000 to 7,000, from 5,000 to 10,000, and from 7,000 to 10,000 nucleotides in length.

[00219]In some embodiments, the length of the product coding region is greater than about 5 nucleotides in length such as, but not limited to, at least or greater than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000,

1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000 or more than 10,000 nucleotides in length.

[00220]In some embodiments, the flanking region may range independently from 0 to 10,000 nucleotides in length such as, but not limited to, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, and 10,000 nucleotides in length.

[00221]In some embodiments, the regulatory region may range independently from 0 to 3,000 nucleotides in length such as, but not limited to, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000 nucleotides in length.

[00222]In some embodiments, the originator construct may be circularized. In other embodiments, the originator construct may be concatemerized.

[00223]Originator constructs which include at least one identifier 40 or "identifier region" 40 (e.g., barcodes, labels, signals and/or tags) may also be referred to as "benchmark constructs" or "benchmark polynucleotide constructs." The benchmark construct may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more identifiers which may be the same or different throughout the benchmark polynucleotide construct.

[00224]In some embodiments, the identifier region may range independently from 1 to 3,000 nucleotides in length such as, but not limited to, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000. As a non-limiting example the identifier region may be 1-5 residues, 2-5 residues, 3-5 residues, 2-7 residues, 3-7 residues, 1-10 residues, 2-10 residues, 3-10 residues, 5-10 residues, 7-10 residues, 1-15 residues, 2-15 residues, 3-15 residues, 5-15 residues, 7-15 residues, 10-15 residues, 12-15 residues, 1-20 residues, 2-20 residues, 3-20 residues, 5-20 residues, 7-20 residues, 10-20 residues, 12-20 residues, 15-20 residues, 17-20 residues, 1-25 residues, 2-25 residues, 3-25 residues, 5-25 residues, 7-25 residues, 10-25 residues, 12-25 residues, 15-25 residues, 17-25 residues, 20-25 residues, 1-30 residues, 2-30 residues, 3-30 residues, 5-30 residues, 7-30 residues, 10-30 residues, 12-30 residues, 15-30 residues, 17-30 residues, 20-30 residues, 25-30 residues, 1-35 residues, 2-35 residues, 3-35 residues, 5-35 residues, 7-35 residues, 10-35 residues, 12-35 residues, 15-35 residues, 17-35 residues, 20-35 residues, 25-35 residues, 30-35 residues, 1-35 residues, 2-35 residues, 3-35 residues, 5-35 residues, 7-35 residues, 10-35 residues, 12-35 residues, 15-35 residues, 17-35 residues, 20-35 residues, 25-35 residues, 30-35 residues, 1-40 residues, 2-40 residues, 3-40 residues, 5-40 residues, 7-40 residues, 10-

40 residues, 12-40 residues, 15-40 residues, 17-40 residues, 20-40 residues, 25-40 residues, 30-40 residues, 35-40 residues, 1-45 residues, 2-45 residues, 3-45 residues, 5-45 residues, 7-45 residues, 10-45 residues, 12-45 residues, 15-45 residues, 17-45 residues, 20-45 residues, 25-45 residues, 30-45 residues, 35-45 residues, 40-45 residues, 1-50 residues, 2-50 residues, 3-50 residues, 5-50 residues, 7-50 residues, 10-50 residues, 12-50 residues, 15-50 residues, 17-50 residues, 20-50 residues, 25-50 residues, 30-50 residues, 35-50 residues, 40-50 residues, or 45-50 nucleotides in length.

[00225]In some embodiments, the identifier region in the benchmark construct overlaps with the product coding region. As used herein, "overlap" means that at least one nucleotide of the identifier region extends into the product coding region. In some aspects the identifier region overlaps with the product coding region by 1 nucleotide, 2 nucleotides, 3 nucleotides, 4 nucleotides, 5 nucleotides, 6 nucleotides, 7 nucleotides, 8 nucleotides, 9 nucleotides, 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23 nucleotides, 24 nucleotides, 25 nucleotides, 26 nucleotides, 27 nucleotides, 28 nucleotides, 29 nucleotides, 30 nucleotides, 31 nucleotides, 32 nucleotides, 33 nucleotides, 34 nucleotides, 35 nucleotides, 36 nucleotides, 37 nucleotides, 38 nucleotides, 39 nucleotides, 40 nucleotides 41 nucleotides, 42 nucleotides, 43 nucleotides, 44 nucleotides, 45 nucleotides, 46 nucleotides, 47 nucleotides, 48 nucleotides, 49 nucleotides, 50 nucleotides or more than 50 nucleotides. In some aspects the identifier region overlaps with the product coding region by 1-5 nucleotides, 2-5 nucleotides, 3-5 nucleotides, 2-7 nucleotides, 3-7 nucleotides, 1-10 nucleotides, 2-10 nucleotides, 3-10 nucleotides, 5-10 nucleotides, 7-10 nucleotides, 1-15 nucleotides, 2-15 nucleotides, 3-15 nucleotides, 5-15 nucleotides, 7-15 nucleotides, 10-15 nucleotides, 12-15 nucleotides, 1-20 nucleotides, 2-20 nucleotides, 3-20 nucleotides, 5-20 nucleotides, 7-20 nucleotides, 10-20 nucleotides, 12-20 nucleotides, 15-20 nucleotides, 17-20 nucleotides, 1-25 nucleotides, 2-25 nucleotides, 3-25 nucleotides, 5-25 nucleotides, 7-25 nucleotides, 10-25 nucleotides, 12-25 nucleotides, 15-25 nucleotides, 17-25 nucleotides, 20-25 nucleotides, 1-30 nucleotides, 2-30 nucleotides, 3-30 nucleotides, 5-30 nucleotides, 7-30 nucleotides, 10-30 nucleotides, 12-30 nucleotides, 15-30 nucleotides, 17-30 nucleotides, 20-30 nucleotides, 25-30 nucleotides, 1-35 nucleotides, 2-35 nucleotides, 3-35 nucleotides, 5-35 nucleotides, 7-35 nucleotides, 10-35 nucleotides, 12-35 nucleotides, 15-35 nucleotides, 17-35 nucleotides, 20-35 nucleotides, 25-35 nucleotides, 30-35 nucleotides, 1-35 nucleotides, 2-35 nucleotides, 3-35 nucleotides, 5-35 nucleotides, 7-35 nucleotides, 10-35 nucleotides, 12-35 nucleotides, 15-35 nucleotides, 17-35 nucleotides, 20-35 nucleotides, 25-35 nucleotides, 30-35 nucleotides, 1-40 nucleotides, 2-40 nucleotides, 3-40 nucleotides, 5-40 nucleotides, 7-40 nucleotides, 10-40 nucleotides, 12-40 nucleotides, 15-40 nucleotides, 17-40 nucleotides, 20-40 nucleotides, 25-40 nucleotides, 30-40 nucleotides, 35-40 nucleotides, 1-45 nucleotides, 2-45 nucleotides, 3-45 nucleotides, 5-45 nucleotides, 7-45 nucleotides, 10-45 nucleotides, 12-45 nucleotides, 15-45 nucleotides, 17-45 nucleotides, 20-45 nucleotides, 25-45 nucleotides, 30-45 nucleotides, 35-45 nucleotides, 40-45 nucleotides, 1-50 nucleotides, 2-50

nucleotides, 3-50 nucleotides, 5-50 nucleotides, 7-50 nucleotides, 10-50 nucleotides, 12-50 nucleotides, 15-50 nucleotides, 17-50 nucleotides, 20-50 nucleotides, 25-50 nucleotides, 30-50 nucleotides, 35-50 nucleotides, 40-50 nucleotides, or 45-50 nucleotides.

[00226]In some embodiments, the benchmark polynucleotide construct comprises a product coding region and an identifier region. The identifier region may be located 5' to the product coding region, 3' to the product coding region, or the identifier region may overlap with the 5' end or the 3'end of the product coding region.

[00227]In some embodiments, the benchmark polynucleotide construct comprises a product coding region and two identifier regions. Each identifier region may independently be located 5' to the product coding region, 3' to the product coding region, or the identifier region may overlap with the 5' end or the 3'end of the product coding region.

[00228]As a non-limiting example, the first identifier region is located 5' to the product coding region and the second identifier region is located 3' to the product coding region. As a non-limiting example, the first and second identifier regions are located 5' to the product coding region. As a non-limiting example, the first and second identifier regions are located 3' to the product coding region.

[00229]As a non-limiting example, the first identifier region is inverted and is located 5' to the product coding region and the second identifier region is located 3' to the product coding region. As a non-limiting example, the first identifier region is inverted and is located 5' to the product coding region and the second identifier region is inverted and is located 3' to the product coding region. As a non-limiting example, the first identifier region is located 5' to the product coding region and the second identifier region is inverted and is located 3' to the product coding region. As a non-limiting example, the first and second identifier regions are both inverted and are located 5' to the product coding region. As a non-limiting example, the first and second identifier regions are located 5' to the product coding region and the first identifier region is inverted. As a non-limiting example, the first and second identifier regions are located 5' to the product coding region and the second identifier region is inverted. As a non-limiting example, the first and second identifier region are both inverted and located 3' to the product coding region. As a non-limiting example, the first and second identifier regions are located 3' to the product coding region and the first identifier region is inverted. As a non-limiting example, the first and second identifier regions are located 3' to the product coding region and the second identifier region is inverted.

[00230]As a non-limiting example, the first identifier region is located 5' to the product coding region and overlaps with the product coding region and the second identifier region is located 3' to the product coding region. As a non-limiting example, the first identifier region is located 5' to the product coding region and the second identifier region is located 3' to the product coding region and overlaps with the product coding region.

[00231]As a non-limiting example, the first and second identifier regions are located 5' to the product coding region and the second identifier region overlaps with the product coding region. As a non-

limiting example, the first and second identifier regions are located 3' to the product coding region and the first identifier region overlaps with the product coding region.

[00232]As a non-limiting example, the first identifier region is inverted, is located 5' to the product coding region and overlaps with the product coding region, and the second identifier region is located 3' to the product coding region. As a non-limiting example, the first identifier region is inverted and is located 5' to the product coding region and the second identifier region is located 3' to the product coding region and overlaps with the product coding region. As a non-limiting example, the first identifier region is inverted, is located 5' to the product coding region, the second identifier region is located 3' to the product coding region, and both of the first and second identifier regions overlap with the product coding region.

[00233]As a non-limiting example, the first identifier region is inverted, is located 5' to the product coding region and overlaps with the product coding region, and the second identifier region is inverted and is located 3' to the product coding region. As a non-limiting example, the first identifier region is inverted and is located 5' to the product coding region and the second identifier region is inverted, is located 3' to the product coding region and overlaps with the product coding region. As a non-limiting example, the first identifier region is inverted and is located 5' to the product coding region, and the second identifier region is inverted and is located 3' to the product coding region, and both of the first and second identifier regions overlap with the product coding region.

[00234]As a non-limiting example, the first identifier region is located 5' to the product coding region and overlaps with the product coding region, and the second identifier region is inverted and is located 3' to the product coding region. As a non-limiting example, the first identifier region is located 5' to the product coding region and the second identifier region is inverted, is located 3' to the product coding region and overlaps with the product coding region. As a non-limiting example, the first identifier region is located 5' to the product coding region and the second identifier region is inverted and is located 3' to the product coding region, and both of the first and second identifier regions overlap with the product coding region.

[00235]As a non-limiting example, the first and second identifier regions are both inverted and are located 5' to the product coding region, and the second identifier region overlaps with the product coding region. As a non-limiting example, the first and second identifier regions are located 5' to the product coding region and the first identifier region is inverted, and the second identifier region overlaps with the product coding region. As a non-limiting example, the first and second identifier regions are located 5' to the product coding region and the second identifier region is inverted and overlaps with the product coding region. As a non-limiting example, the first and second identifier region are both inverted and located 3' to the product coding region, and the first identifier region overlap with the product coding region. As a non-limiting example, the first and second identifier regions are located 3' to the product coding region and the first identifier region is inverted and overlaps with the product coding region. As a non-limiting example, the first and second identifier

regions are located 3' to the product coding region and the second identifier region is inverted, and the first product coding region overlap with the product coding region.

[00236]In some embodiments, at least one identifier moiety may be associated with the benchmark polynucleotide construct. The benchmark polynucleotide construct may have 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more identifier moieties associated with the benchmark polynucleotide construct which may be the same moiety or different moieties associated with the benchmark polynucleotide construct. Each identifier moiety may independently be located on the flanking region 5' to the product coding region, on the flanking region 3' to the product coding region, or the location of the identifier moiety may span the 5' end or the 3'end of the product coding region and a flanking region. In some aspects the location of the identifier moiety may include one or more nucleotides of the product coding region such as, but not limited to, 1 nucleotide, 2 nucleotides, 3 nucleotides, 4 nucleotides, 5 nucleotides, 6 nucleotides, 7 nucleotides, 8 nucleotides, 9 nucleotides, 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23 nucleotides, 24 nucleotides, 25 nucleotides, 26 nucleotides, 27 nucleotides, 28 nucleotides, 29 nucleotides, 30 nucleotides, 31 nucleotides, 32 nucleotides, 33 nucleotides, 34 nucleotides, 35 nucleotides, 36 nucleotides, 37 nucleotides, 38 nucleotides, 39 nucleotides, 40 nucleotides 41 nucleotides, 42 nucleotides, 43 nucleotides, 44 nucleotides, 45 nucleotides, 46 nucleotides, 47 nucleotides, 48 nucleotides, 49 nucleotides, 50 nucleotides or more than 50 nucleotides. In some aspects the location of the identifier moiety may include one or more nucleotides of the product coding region such as, but not limited to, 1-5 nucleotides, 2-5 nucleotides, 3-5 nucleotides, 2-7 nucleotides, 3-7 nucleotides, 1-10 nucleotides, 2-10 nucleotides, 3-10 nucleotides, 5-10 nucleotides, 7-10 nucleotides, 1-15 nucleotides, 2-15 nucleotides, 3-15 nucleotides, 5-15 nucleotides, 7-15 nucleotides, 10-15 nucleotides, 12-15 nucleotides, 1-20 nucleotides, 2-20 nucleotides, 3-20 nucleotides, 5-20 nucleotides, 7-20 nucleotides, 10-20 nucleotides, 12-20 nucleotides, 15-20 nucleotides, 17-20 nucleotides, 1-25 nucleotides, 2-25 nucleotides, 3-25 nucleotides, 5-25 nucleotides, 7-25 nucleotides, 10-25 nucleotides, 12-25 nucleotides, 15-25 nucleotides, 17-25 nucleotides, 20-25 nucleotides, 1-30 nucleotides, 2-30 nucleotides, 3-30 nucleotides, 5-30 nucleotides, 7-30 nucleotides, 10-30 nucleotides, 12-30 nucleotides, 15-30 nucleotides, 17-30 nucleotides, 20-30 nucleotides, 25-30 nucleotides, 1-35 nucleotides, 2-35 nucleotides, 3-35 nucleotides, 5-35 nucleotides, 7-35 nucleotides, 10-35 nucleotides, 12-35 nucleotides, 15-35 nucleotides, 17-35 nucleotides, 20-35 nucleotides, 25-35 nucleotides, 30-35 nucleotides, 1-35 nucleotides, 2-35 nucleotides, 3-35 nucleotides, 5-35 nucleotides, 7-35 nucleotides, 10-35 nucleotides, 12-35 nucleotides, 15-35 nucleotides, 17-35 nucleotides, 20-35 nucleotides, 25-35 nucleotides, 30-35 nucleotides, 1-40 nucleotides, 2-40 nucleotides, 3-40 nucleotides, 5-40 nucleotides, 7-40 nucleotides, 10-40 nucleotides, 12-40 nucleotides, 15-40 nucleotides, 17-40 nucleotides, 20-40 nucleotides, 25-40 nucleotides, 30-40 nucleotides, 35-40 nucleotides, 1-45 nucleotides, 2-45 nucleotides, 3-45 nucleotides, 5-45 nucleotides, 7-45 nucleotides, 10-45 nucleotides, 12-45

nucleotides, 15-45 nucleotides, 17-45 nucleotides, 20-45 nucleotides, 25-45 nucleotides, 30-45 nucleotides, 35-45 nucleotides, 40-45 nucleotides, 1-50 nucleotides, 2-50 nucleotides, 3-50 nucleotides, 5-50 nucleotides, 7-50 nucleotides, 10-50 nucleotides, 12-50 nucleotides, 15-50 nucleotides, 17-50 nucleotides, 20-50 nucleotides, 25-50 nucleotides, 30-50 nucleotides, 35-50 nucleotides, 40-50 nucleotides, or 45-50 nucleotides.

[00237]In some embodiments, one identifier moiety may be associated with the benchmark polynucleotide construct. As a non-limiting example, the identifier moiety may be associated with the benchmark polynucleotide construct on the 5' end of the benchmark polynucleotide construct. As a non-limiting example, the identifier moiety may be associated with the benchmark polynucleotide construct on the 5' flanking region. As a non-limiting example, the identifier moiety may be associated with the benchmark polynucleotide construct on the 3' flanking region. As a non-limiting example, the identifier moiety may be associated with the benchmark polynucleotide construct on the 3' end of the benchmark polynucleotide construct. As a non-limiting example, the identifier moiety may be associated with the benchmark polynucleotide construct on the product coding region. As a non-limiting example, the benchmark polynucleotide construct comprises an identifier moiety and the location of the identifier moiety spans the 5' end of the product coding region and the 5' flanking region. As a non-limiting example, the benchmark polynucleotide construct comprises an identifier moiety and the location of the identifier moiety spans the 3' end of the product coding region and the 3' flanking region.

[00238]In some embodiments, two identifier moieties are associated with the benchmark polynucleotide construct. As a non-limiting example, the first identifier moiety and the second identifier moiety are located on the 5' flanking region. As a non-limiting example, the first identifier moiety and the second identifier moiety are located on the product coding region. As a non-limiting example, the first identifier moiety and the second identifier moiety are located on the 3' flanking region. As a non-limiting example, the first identifier moiety and the second identifier moiety are located on the 5' end of the benchmark polynucleotide construct. As a non-limiting example, the first identifier moiety and the second identifier moiety are located on the 3' end of the benchmark polynucleotide construct.

[00239]As a non-limiting example, the first identifier moiety is located on the 5' end of the benchmark polynucleotide construct and the second identifier moiety is located on the 5' flanking region. As a non-limiting example, the first identifier moiety is located on the 5' end of the benchmark polynucleotide construct and the second identifier moiety is located on the product coding region. As a non-limiting example, the first identifier moiety is located on the 5' end of the benchmark polynucleotide construct and the second identifier moiety is located on the 3' flanking region. As a non-limiting example, the first identifier moiety is located on the 5' end of the benchmark polynucleotide construct and the location of the second identifier moiety spans the 5' flanking region and the product coding region. As a non-limiting example, the first identifier moiety is located on the

5' end of the benchmark polynucleotide construct and the location of the second identifier moiety spans the 3' flanking region and the product coding region. As a non-limiting example, the first identifier moiety is located on the 5' end of the benchmark polynucleotide construct and the second identifier moiety is located on the 3' end of the benchmark polynucleotide construct.

[00240]As a non-limiting example, the first identifier moiety is located on the 5' flanking region and the second identifier moiety is located on the product coding region. As a non-limiting example, the first identifier moiety is located on the 5' flanking region and the second identifier moiety is located on the 3' flanking region. As a non-limiting example, the first identifier moiety is located on the 5' flanking region and the location of the second identifier moiety spans the 5' flanking region and the product coding region. As a non-limiting example, the first identifier moiety is located on the 5' flanking region and the location of the second identifier moiety spans the 3' flanking region and the product coding region. As a non-limiting example, the first identifier moiety is located on the 5' flanking region and the second identifier moiety is located on the 5' end of the benchmark polynucleotide construct. As a non-limiting example, the first identifier moiety is located on the 5' flanking region and the second identifier moiety is located on the 3' end of the benchmark polynucleotide construct.

[00241]As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the second identifier moiety is located on the 5' end of the benchmark polynucleotide construct. As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the second identifier moiety is located on the 5' flanking region. As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the second identifier moiety is located on the product coding region. As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the location of the second identifier moiety spans the 3' flanking region and the product coding region. As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the second identifier moiety is located on the 3' flanking region. As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the second identifier moiety is located on the 3' end of the benchmark polynucleotide construct.

[00242]As a non-limiting example, the first identifier moiety is located on the product coding region and the second identifier moiety is located on the 5' end of the benchmark polynucleotide construct. As a non-limiting example, the first identifier moiety is located on the product coding region and the second identifier moiety is located on the 5' flanking region. As a non-limiting example, the first identifier moiety is located on the product coding region and the location of the second identifier moiety spans the 5' flanking region and the product coding region. As a non-limiting example, the first identifier moiety is located on the product coding region and the location of the second identifier moiety spans the 3' flanking region and the product coding region. As a non-limiting example, the

first identifier moiety is located on the product coding region and the second identifier moiety is located on the 3' flanking region. As a non-limiting example, the first identifier moiety is located on the product coding region and the second identifier moiety is located on the 3' end of the benchmark polynucleotide construct.

[00243]As a non-limiting example, the location of the first identifier moiety spans the 3' flanking region and the product coding region and the second identifier moiety is located on the 5' end of the benchmark polynucleotide construct. As a non-limiting example, the location of the first identifier moiety spans the 3' flanking region and the product coding region and the second identifier moiety is located on the 5' flanking region. As a non-limiting example, the location of the first identifier moiety spans the 3' flanking region and the product coding region and the location of the second identifier moiety spans the 5' flanking region and the product coding region. As a non-limiting example, the location of the first identifier moiety spans the 3' flanking region and the product coding region and the second identifier moiety is located on the product coding region. As a non-limiting example, the location of the first identifier moiety spans the 3' flanking region and the product coding region and the second identifier moiety is located on the 3' flanking region. As a non-limiting example, the location of the first identifier moiety spans the 3' flanking region and the product coding region and the second identifier moiety is located on the 3'end of the benchmark polynucleotide construct.

[00244]As a non-limiting example, the location of the first identifier moiety spans the 3' flanking region and the product coding region and the second identifier moiety is located on the 5' flanking region. As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the second identifier moiety is located on the product coding region. As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the location of the second identifier moiety spans the 3' flanking region and the product coding region. As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the second identifier moiety is located on the 3' flanking region. As a non-limiting example, the location of the first identifier moiety spans the 5' flanking region and the product coding region and the second identifier moiety is located on the 3' end of the benchmark polynucleotide construct.

[00245]As a non-limiting example, the first identifier moiety is located on the 3' flanking region and the second identifier moiety is located on the 5' end of the benchmark polynucleotide construct. As a non-limiting example, the first identifier moiety is located on the 3' flanking region and the second identifier moiety is located on the 5' flanking region. As a non-limiting example, the first identifier moiety is located on the 3' flanking region and the location of the second identifier moiety spans the 5' flanking region and the product coding region. As a non-limiting example, the first identifier moiety is located on the 3' flanking region and the second identifier moiety is located on the product coding region. As a non-limiting example, the first identifier moiety is located on the 3' flanking region and the location of the second identifier moiety spans the 3' flanking region and the product coding region.

As a non-limiting example, the first identifier moiety is located on the 3' flanking region and the second identifier moiety is located on the 3' end of the benchmark polynucleotide construct.

[00246]As a non-limiting example, the first identifier moiety is located on the 3' end of the benchmark polynucleotide construct and the second identifier moiety is located on the 5' end of the benchmark polynucleotide construct. As a non-limiting example, the first identifier moiety is located on the 3' end of the benchmark polynucleotide construct and the second identifier moiety is located on the 5' flanking region. As a non-limiting example, the first identifier moiety is located on the 5' end of the benchmark polynucleotide construct and the location of the second identifier moiety spans the 5' flanking region and the product coding region. As a non-limiting example, the first identifier moiety is located on the 3' end of the benchmark polynucleotide construct and the second identifier moiety is located on the product coding region. As a non-limiting example, the first identifier moiety is located on the 5' end of the benchmark polynucleotide construct and the location of the second identifier moiety spans the 3' flanking region and the product coding region. As a non-limiting example, the first identifier moiety is located on the 3' end of the benchmark polynucleotide construct and the second identifier moiety is located on the 3' flanking region.

[00247]In some embodiments, three identifier moieties are associated with the benchmark polynucleotide construct. In some embodiments, four identifier moieties are associated with the benchmark polynucleotide construct. In some embodiments, five identifier moieties are associated with the benchmark polynucleotide construct. In some embodiments, six identifier moieties are associated with the benchmark polynucleotide construct. In some embodiments, seven identifier moieties are associated with the benchmark polynucleotide construct. In some embodiments, eight identifier moieties are associated with the benchmark polynucleotide construct. In some embodiments, nine identifier moieties are associated with the benchmark polynucleotide construct. In some embodiments, ten identifier moieties are associated with the benchmark polynucleotide construct.

[00248]In some embodiments, the product coding region encodes a biologically active molecule such as, but not limited to a therapeutic protein or an antigen. As used herein, the term "biologically active" refers to a characteristic of any agent that has activity in a biological system, and particularly in an organism. For instance, an agent that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active. In some embodiments, the CROI encodes one or more prophylactically- or therapeutically-active proteins, polypeptides, or other factors. As a non-limiting example, the CROI may encode an agent that enhances tumor killing activity such as, but not limited to, TRAIL or tumor necrosis factor (TNF), in a cancer. As another non-limiting example, the CROI may encode an agent suitable for the treatment of conditions such as muscular dystrophy (e.g., CROI encodes Dystrophin), cardiovascular disease (e.g., CROI encodes SERCA2a, GATA4, Tbx5, Mef2C, Hand2, Myocd, etc.), neurodegenerative disease (e.g., CROI encodes NGF, BDNF, GDNF, NT-3, etc.), chronic pain (e.g., CROI encodes GlyRal), an enkephalin, or a glutamate decarboxylase (e.g., CROI encodes GAD65, GAD67, or another isoform), lung disease (e.g., CROI encodes CFTR),

hemophilia (e.g., CROI encodes Factor VIII or Factor IX), neoplasia (e.g., CROI encodes PTEN, ATM, ATR, EGFR, ERBB2, ERBB3, ERBB4, Notchl, Notch2, Notch3, Notch4, AKT, AKT2, AKT3, HIF, HI Fla, HIF3a, Met, HRG, Bcl2, PPARalpha, PPAR gamma, WT1 (Wilms Tumor), FGF Receptor Family members (5 members: 1, 2, 3, 4, 5), CDKN2a, APC, RB (retinoblastoma), MEN1, VHL, BRCA1, BRCA2, AR (Androgen Receptor), TSG101, IGF, IGF Receptor, Igfl (4 variants), Igf2 (3 variants), Igfl Receptor, Igf2 Receptor, Bax, Bcl2, caspases family (9 members: 1, 2, 3, 4, 6, 7, 8, 9, 12), Kras, Ape), age-related macular degeneration (e.g., CROI encodes Aber, Ccl2, Cc2, cp (ceruloplasmin), Timp3, cathepsin D, Vldlr), schizophrenia (e.g. Neuregulin (Nrgl), Erb4 (receptor for Neuregulin), Complexin-l (Cplxl), Tphl Tryptophan hydroxylase, Tph2 Tryptophan hydroxylase 2, Neurexin 1, GSK3, GSK3a, GSK3b, 5-HIT (Slc6a4), COMT, DRD (Drdla), SLC6A3, DAOA, DTNBPI, Dao (Daol)), trinucleotide repeat disorders (e.g., HTT (Huntington's Dx), SBMA/SMAXI/AR (Kennedy's Dx), FXN/X25 (Friedrich's Ataxia), ATX3 (Machado-Joseph's Dx), ATXNI and ATXN2 (spinocerebellar ataxias), DMPK (myotonic dystrophy), Atrophin-1 and Atnl(DRPLA Dx), CBP (Creb-BP-global instability), VLDLR (Alzheimer's), Atxn7, Atxn10), fragile X syndrome (e.g., CROI encodes FMR2, FXRI, FXR2, mGLUR5), secretase related disorders (e.g., CROI encodes APH-1 (alpha and beta), Presenilin (Psenl), nicastrin (Ncstn), PEN-2), ALS (e.g., CROI encodes SOD1, ALS2, STEX, FUS, TARD BP, VEGF (VEGF-a, VEGF-b, VEGF-c)), autism (e.g., CROI encodes Mecp2, BZRAP1, MDGA2, Sema5A, Neurexin 1), Alzheimer's disease (e.g., CROI encodes El, CHIP, UCH, UBB, Tau, LRP, PICALM, Clusterin, PS1, SORL1, CR1, Vldlr, Ubal, Uba3, CHIP28 (Aqpl, Aquaporin 1), Uchll, Uchl3, APP), inflammation (e.g., CROI encodes IL-10, IL-1 (IL-Ia, IL-Ib), IL-13, IL-17 (IL-17a (CTLA8), IL-17b, IL-17c, IL-17d, IL-171), 11-23, Cx3crl, ptpn22, TNFa, NOD2/CARD15 for IBD, IL-6, IL-12 (IL-12a, IL-12b), CTLA4, Cx3cll), Parkinson's Disease (e.g., x-Synuclein, DJ-1, LRRK2, Parkin, PINK1), blood and coagulation disorders, such as, e.g., anemia, bare lymphocyte syndrome, bleeding disorders, hemophagocytic lymphohistiocytosis disorders, hemophilia A, hemophilia B, hemorrhagic disorders, leukocyte deficiencies and disorders, sickle cell anemia, and thalassemia (e.g., CROI encodes CRAN1, CDA1, RPS19, DBA, PKLR, PK1, NT5C3, UMPH1, PSNI, RHAG, RH50A, NRAMP2, SPTB, ALAS2, ANH1, ASB, ABCB7, ABC7, ASAT, TAPBP, TPSN, TAP2, ABCB3, PSF2, RING11, MHC2TA, C2TA, RFX5, RFXAP, RFX5, TBXA2R, P2RX1, P2X1, HF1, CFH, HUS, MCFD2, FANCA, FAC A, FA1, FA, FA A, FAAP95, FAAP90, FLJ34064, FANCB, FANCC, FACC, BRCA2, FANCDI, FANCD2, FANCD, FACD, FAD, FANCE, FACE, FANCF, XRCC9, FANCG, BR1PI, BACH1, FANCJ, PHF9, FANCL, FANCM, KIAA1596, PRF1, HPLH2, UNC13D, MUNC13-4, HPLH3, HLH3, FHL3, F8, FSC, PI, ATT, F5, ITGB2, CD18, LCAMB, LAD, EIF2B1, EIF2BA, EIF2B2, EIF2B3, EIF2B5, LVWM, CACH, CLE, EIF2B4, HBB, HBA2, HBB, HBD, LCRB, HBA1), B-cell non-Hodgkin lymphoma or leukemia (e.g., CROI encodes BCL7A, BCL7, ALI, TCL5, SCL, TAL2, FLT3, NBS1, NBS, ZNFN1AI, 1KI, LYF1, HOXD4, HOX4B, BCR, CML, PHL, ALL, ARNT, KRAS2, RASK2, GMPS, AFIO, ARHGEF12, LARG, KIAA0382, CALM, CLTH, CEBPA, CEBP,

CHIC2, BTL, FLT3, KIT, PBT, LPP, NPMI, NUP214, D9S46E, CAN, CAIN, RUNXI, CBFA2, AML1, WIISC1LI, NSD3, FLT3, AF1Q, NPMI, NUMA1, ZNF145, PLZF, PML, MYL, STAT5B, AF1Q, CALM, CLTH, ARL11, ARLTS1, P2RX7, P2X7, BCR, CML, PHL, ALL, GRAF, NF1, VRNF, WSS, NFNS, PTPNII, PTP2C, SHP2, NS1, BCL2, CCND1, PRAD1, BCL1, TCRA, GATA1, GF1, ERYF1, NFE1, ABLI, NQO1, DIA4, NMOR1, NUP214, D9S46E, CAN, CAIN), inflammation and immune related diseases and disorders (e.g., CROI encodes KIR3DL1, NKAT3, NKB1, AMB11, K1R3DS1, IFNG, CXCL12, TNFRSF6, APT1, FAS, CD95, ALPS1A, IL2RG, SCIDX1, SCIDX, IMD4, CCL5, SCYA5, D17S136E, TCP228, IL10, CSIF, CMKBR2, CCR2, CMKBR5, CCCKR5 (CCR5), CD3E, CD3G, AICDA, AID, HIGM2, TNFRSF5, CD40, UNG, DGU, HIGM4, TNFSFS, CD40LG, HIGM1, IGM, FOXP3, IPEX, AIID, XPID, PIDX, TNFRSF14B, TACI), inflammation (e.g., CROI encodes IL-10, IL-1 (IL-1A, IL-IB), IL-13, IL-17 (IL-17a (CTLA8), IL-17b, IL-17c, IL-17d, IL-171), 11-23, Cx3crl, ptpn22, TNFa, NOD2/CARD15 for IBD, IL-6, IL-12 (IL-12a, IL-12b), CTLA4, Cx3cII), JAK3, JAKL, DCLREIC, ARTEMIS, SCIDA, RAG1, RAG2, ADA, PTPRC, CD45, LCA, IL7R, CD3D, T3D, IL2RG, SCIDXI, SCIDX, IMD4), metabolic, liver, kidney and protein diseases and disorders (e.g., CROI encodes TTR, PALB, APOA1, APP, AAA, CVAP, ADI, GSN, FGA, LYZ, TTR, PALB, KRT18, KRT8, CIRH1A, NAIC, TEX292, KIAA1988, CFTR, ABCC7, CF, MRP7, SLC2A2, GLUT2, G6PC, G6PT, G6PT1, GAA, LAMP2, LAMPB, AGL, GDE, GBE1, GYS2, PYGL, PFKM, TCF1, HNF1A, MODY3, SCOD1, SCOl, CTNNB1, PDGFRL, PDGRL, PRLTS, AX1NI, AXIN, CTNNB1, TP53, P53, LFS1, IGF2R, MPRI, MET, CASP8, MCH5, UMOD, HNFJ, FJHN, MCKD2, ADMCKD2, PAH, PKU1, QDPR, DHPR, PTS, FCYT, PKHD1, ARPKD, PKD1, PKD2, PKD4, PKDTS, PRKCSH, G19P1, PCLD, SEC63), muscular/skeletal diseases and disorders (e.g., CROI encodes DMD, BMD, MYF6, LMNA, LMN1, EMD2, FPLD, CMDIA, HGPS, LGMDIB, LMNA, LMNI, EMD2, FPLD, CMDIA, FSHMD1A, FSHD1A, FKRP, MDC1C, LGMD2I, LAMA2, LAMM, LARGE, KIAA0609, MDC1D, FCMD, TTID, MYOT, CAPN3, CANP3, DYSF, LGMD2B, SGCG, LGMD2C, DMDA1, SCG3, SGCA, ADL, DAG2, LGMD2D, DMDA2, SGCB, LGMD2E, SGCD, SGD, LGMD2F, CMD1L, TCAP, LGMD2G, CMD1N, TRIM32, HT2A, LGMD2H, FKRP, MDCIC, LGMD21, TTN, CMD1G, TMD, LGMD2J, POMT1, CAV3, LGMD1C, SEPN1, SELN, RSMD1, PLEC1, PLTN, EBS1, LRP5, BMNDl, LRP7, LR3, OPPG, VBCH2, CLCN7, CLC7, OPTA2, OSTMI, GL, TCIRG1, TIRC7, OC116, OPTB1, VAPB, VAPC, ALS8, SMN1, SMA1, SMA2, SMA3, SMA4, BSCL2, SPG17, GARS, SMAD1, CMT2D, HEXB, IGHMBP2, SMUBP2, CATF1, SMARD1), neurological and neuronal diseases and disorders (e.g., CROI encodes SOD1, ALS2, STEX, FUS, TARDBP, VEGF (VEGF-a, VEGF-b, VEGF-c), APP, AAA, CVAP, ADI, APOE, AD2, PSEN2, AD4, STM2, APBB2, FE65LI, NOS3, PLAU, URK, ACE, DCPI, ACEI, MPO, PAC1PI, PAXIPIL, PTIP, A2M, BLMH, BMH, PSEN1, AD3, Mecp2, BZRAP1, MDGA2, Sema5A, Neurexin 1, GLOl, MECP2, RTT, PPMX, MRX16, MRX79, NLGN3, NLGN4, KIAA1260, AUTSX2, FMR2, FXR1, FXR2, mGLUR5, HD, IT15, PRNP, PRIP, JPH3, JP3, HDL2, TBP, SCA17, NR4A2, NURR1, NOT, TINUR, SNCAIP, TBP,

SCA17, SNCA, NACP, PARK1, PARK4, DJI, PARK7, LRRK2, PARK8, PINK1, PARK6, UCHL1, PARK5, SNCA, NACP, PARKl, PARK4, PRKN, PARK2, PDJ, DBII, NDUFV2, MECP2, RTT, PPMX, MRX16, MRX79, CDKL5, STK9, MECP2, RTT, PPMX, MRX16,MRX79, x-Synuclein, DJ-1, Neuregulin-l (Nrgl), Erb4 (receptor for Neuregulin), Complexin-l (Cplxl), Tphl Tryptophan hydroxylase, Tph2, Tryptophan hydroxylase 2, Neurexin 1, GSK3, GSK3a, GSK3b, 5-HTT (Slc6a4), CONT, DRD (Drdla), SLC6A , DAOA, DTNBP1, Dao (Daol), APH-l(alpha and beta), Presenilin (Psenl), Nicastrin, (Ncstn), PEN-2, Nosl, Parpl, Natl, Nat2, HTT, SBMA/SMAX1/AR, FXN/X25, ATX3, TXN, ATXN2, DMPK, Atrophin-1, Atnl, CBP, VLDLR, Atxn7, and AtxnlO), and ocular diseases and disorders (e.g., Aber, Ccl2, Cc2, cp (ceruloplasmin), Timp3, cathepsin-D, Vldlr, Ccr2, CRYAA, CRYA1, CRYBB2, CRYB2, PITX3, BFSP2, CP49, CP47, CRYAA, CRYAI, PAX6, AN2, MGDA, CRYBA1, CRYB1, CRYGC, CRYG3, CCL, LIM2, MP19, CRYGD, CRYG4, BFSP2, CP49, CP47, HSF4, CTM, HSF4, CTM, MIP, AQPO, CRYAB, CRYA2, CTPP2, CRYBB1, CRYGD, CRYG4, CRYBB2, CRYB2, CRYGC, CRYG3, CCL, CRYAA, CRYAI, GJA8, CX50, CAE1, GJA3, CX46, CZP3, CAE3, CCM1, CAM, KRIT1, APOA1, TGFBI, CSD2, CDGG1, CSD, BIGH3, CDG2, TACSTD2, TROP2, M1SI, VSX1, RINX, PPCD, PPD, KTCN, COL8A2, FECD, PPCD2, PIP5K3, CFD, KERA, CNA2, MYOC, TIGR, GLCIA, JO AG, GPOA, OPTN, GLC1E, FIP2, HYPL, NRP, CYP1BI, GLC3A, OPA1, NTG, NPG, CYP1BI, GLC3A, CRB1, RP12, CRX, CORD2, CRD, RPGRIPI, LCA6, CORD9, RPE65, RP20, AIPL1, LCA4, GUCY2D, GUC2D, LCA1, CORD6, RDH12, LCA3, ELOVL4, ADMD, STGD2, STGD3, RDS, RP7, PRPH2, PRPH, AVMD, AOFMD, and VMD2).

[00249]In some embodiments, the product coding region of the RNA payloads described herein encodes a factor that can affect the differentiation of a cell. As a non-limiting example, the expression of one or more of Oct4, Klf4, Sox2, c-Myc, L-Myc, dominant-negative p53, Nanog, Glisl, Lin28, TFIID, mir-302/367, or other miRNAs can cause the cell to become an induced pluripotent stem (iPS) cell.

[00250]In some embodiments, the product coding region of the RNA payloads described herein encodes a factor for transdifferentiating cells. Non-limiting examples of factors include: one or more of GATA4, Tbx5, Mef2C, Myocd, Hand2, SRF, Mespl, SMARCD3 for cardiomyocytes; Ascii, Nurrl, LmxlA, Bm2, Mytll, NeuroDl, FoxA2 for neural cells; and Hnf4a, Foxal, Foxa2 or Foxa3 for hepatic cells.

[00251]Additional product coding regions of the RNA payloads described herein are described elsewhere.

A.Nucleic acid payloads

[00252]In various embodiments, the LNP compositions described herein can be used to deliver a nucleic acid or polynucleotide payload, e.g., a DNA HDR donor, a linear or circular mRNA, or a chimeric DNA/RNA guide.

[00253]In some embodiments, a LNP is capable of delivering a polynucleotide to a target cell, tissue, or organ. A polynucleotide, in its broadest sense of the term, includes any compound and/or substance that is or can be incorporated into an oligonucleotide chain. Exemplary polynucleotides for use in accordance with the present disclosure include, but are not limited to, one or more of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) including messenger mRNA (mRNA), hybrids thereof, RNAi-inducing agents, RNAi agents, siRNAs, shRNAs, miRNAs, antisense RNAs, ribozymes, catalytic DNA, RNAs that induce triple helix formation, aptamers, vectors, etc. RNAs useful in the compositions and methods described herein can be selected from the group consisting of but are not limited to, shortimers, antagomirs, antisense, ribozymes, short interfering RNA (siRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), Dicer substrate RNA (dsRNA), short hairpin RNA (shRNA), transfer RNA (tRNA), messenger RNA (mRNA), and mixtures thereof. In some embodiments, a polynucleotide is mRNA. In some embodiments, a polynucleotide is circular RNA. In some embodiments, a polynucleotide encodes a protein, e.g., a vaccine antigen, a therapeutic protein, or a nucleobase editing enzyme. A polynucleotide may encode any polypeptide of interest, including any naturally or non-naturally occurring or otherwise modified polypeptide. A polypeptide may be of any size and may have any secondary structure or activity. In some embodiments, a polypeptide encoded by an mRNA may have a therapeutic effect when expressed in a cell.

[00254]In other embodiments, a polynucleotide is an siRNA. An siRNA may be capable of selectively knocking down or down regulating expression of a gene of interest. For example, an siRNA could be selected to silence a gene associated with a particular disease, disorder, or condition upon administration to a subject in need thereof of a nanoparticle composition including the siRNA. An siRNA may comprise a sequence that is complementary to an mRNA sequence that encodes a gene or protein of interest. In some embodiments, the siRNA may be an immunomodulatory siRNA.

[00255]In some embodiments, a polynucleotide is an shRNA or a vector or plasmid encoding the same. An shRNA may be produced inside a target cell upon delivery of an appropriate construct to the nucleus. Constructs and mechanisms relating to shRNA are well known in the relevant arts.

[00256]A polynucleotide may include a first region of linked nucleosides encoding a polypeptide of interest (e.g., a coding region), a first flanking region located at the 5'-terminus of the first region (e.g., a 5'-UTR), a second flanking region located at the 3'-terminus of the first region (e.g., a 3'-UTR), at least one 5'-cap region, and a 3'-stabilizing region. In some embodiments, a polynucleotide further includes a poly-A region or a Kozak sequence (e.g., in the 5'-UTR). In some cases, polynucleotides may contain one or more intronic nucleotide sequences capable of being excised from the polynucleotide. In some embodiments, a polynucleotide (e.g., an mRNA) may include a 5'cap structure, a chain terminating nucleotide, a stem loop, a polyA sequence, and/or a polyadenylation signal.

[00257]In various embodiments, the nucleic acid payloads may contain one or more modifications. Such modifications include various chemical and/or structural modifications. For example, in the

case of RNA, the RNA may comprise one or more modifications, including chemical modifications (e.g., ribonucleotide analogs, alternative phosphate chain linkers), sequence modification (e.g., relative to a wild type sequence), and/or structural modification (e.g., secondary-folded structures, such as, but not limited to, stem-loops, hairpins, and G-quadruplexes, and tertiary structural elements, such as, but not limited to, helical duplexes and triple-stranded structures). To date, hundreds of different RNA modifications have been characterized. Among them, several RNA modifications, including N^6-methyladenosine (m^6A), N^6,2'-O-dimethyladenosine (m^6Am), 8-oxo-7,8-dihydroguanosine (8-oxoG), pseudouridine (Ψ), 5-methylcytidine (m^5C), and N^4-acetylcytidine (ac^4C), have been shown to regulate mRNA stability, consequently affecting diverse cellular and biological processes. Any known modification to RNA or DNA is contemplated herein.

[00258]In some embodiments, a nucleic acid may include one or more alternative components (e.g., an alternative nucleoside). For example, the 3'-stabilizing region may contain an alternative nucleoside such as an L-nucleoside, an inverted thymidine, or a 2'-O-methyl nucleoside and/or the coding region, 5'-UTR, 3'-UTR, or cap region may include an alternative nucleoside such as a 5-substituted uridine (e.g., 5-methoxyu ridine), a 1-substituted pseudouridine (e.g., 1-methyl pseudouridine or 1-ethyl-pseudouridine), and/or a 5-substituted cytidine (e.g., 5-methyl-cytidine). In some embodiments, a polynucleotide contains only naturally occurring nucleosides. Nucleic acid modifications are well known in the art and are further discussed in the following references: (1) Crooke ST, Witztum JL, Bennett CF, Baker BF. RNA-Targeted Therapeutics. Cell Metab. 2018 Apr 3;27(4):714-739. doi: 10.1016/j.cmet.2018.03.004. Erratum in: Cell Metab. 2019 Feb 5;29(2):501. PMID: 29617640; (2) JP. Wen W, Zhang F, Oberg KC, Zhang L, Cheng T, Zhang XB. Dynamics and competition of CRISPR-Cas9 ribonucleoproteins and AAV donor-mediated NHEJ, MMEJ and HDR editing. Nucleic Acids Res. 2021 Jan 25;49(2):969-985. doi: 10.1093/nar/gkaa1251. PMID: 33398341; PMCID: PMC7826255; (3) Pradeep SP, Malik S, Slack FJ, Bahal R. Unlocking the potential of chemically modified peptide nucleic acids for RNA-based therapeutics. RNA. 2023 Apr;29(4):434-445. doi: 10.1261/rna.079498.122. Epub 2023 Jan 18. PMID: 36653113; PMCID: PMC10019372; (4) Haruehanroengra P, Zheng YY, Zhou Y, Huang Y, Sheng J. RNA modifications and cancer. RNA Biol. 2020 Nov;17(11):1560-1575. doi: 10.1080/15476286.2020.1722449. Epub 2020 Feb 7. PMID: 31994439; PMCID: PMC7567502; (5) Heidenreich O, Pieken W, Eckstein F. Chemically modified RNA: approaches and applications. FASEB J. 1993 Jan;7(1):90-6. doi: 10.1096/fasebj.7.1.7678566. PMID: 7678566; (6) Zhang HY, Du Q, Wahlestedt C, Liang Z. RNA Interference with chemically modified siRNA. Curr Top Med Chem. 2006;6(9):893-900. doi: 10.2174/156802606777303676. PMID: 16787282; (7) Jin G, Xu M, Zou M, Duan S. The Processing, Gene Regulation, Biological Functions, and Clinical Relevance of N4-Acetylcytidine on RNA: A Systematic Review. Mol Ther Nucleic Acids. 2020 Jun 5;20:13-24. doi: 10.1016/j.omtn.2020.01.037. Epub 2020 Feb 8. PMID: 32171170; PMCID: PMC7068197; (8) Gao M, Zhang Q, Feng XH, Liu J. Synthetic modified messenger RNA for therapeutic applications. Acta Biomater. 2021 Sep 1;131:1-

15. doi: 10.1016/j.actbio.2021.06.020. Epub 2021 Jun 13. PMID: 34133982; PMCID: PMC8198544; (9) Filippova JA, Semenov DV, Juravlev ES, Komissarov AB, Richter VA, Stepanov GA. Modern Approaches for Identification of Modified Nucleotides in RNA. Biochemistry (Mosc). 2017 Nov;82(11):1217-1233. doi: 10.1134/S0006297917110013. PMID: 29223150; (10) Röthlisberger P, Berk C, Hall J. RNA Chemistry for RNA Biology. Chimia (Aarau). 2019 May 29;73(6):368-373. doi: 10.2533/chimia.2019.368. PMID: 31118118; and (11) Elkhalifa D, Rayan M, Negmeldin AT, Elhissi A, Khalil A. Chemically modified mRNA beyond COVID-19: Potential preventive and therapeutic applications for targeting chronic diseases. Biomed Pharmacother. 2022 Jan;145:112385. doi: 10.1016/j.biopha.2021.112385. Epub 2021 Oct 28. PMID: 34915673; PMCID: PMC8552589; (12) Boo SH, Kim YK. The emerging role of RNA modifications in the regulation of mRNA stability. Exp Mol Med. 2020 Mar;52(3):400-408. doi: 10.1038/s12276-020-0407-z. Epub 2020 Mar 24. PMID: 32210357; PMCID: PMC7156397; (13) Varshney D, Spiegel J, Zyner K, Tannahill D, Balasubramanian S. The regulation and functions of DNA and RNA G-quadruplexes. Nat Rev Mol Cell Biol. 2020 Aug;21(8):459-474. doi: 10.1038/s41580-020-0236-x. Epub 2020 Apr 20. PMID: 32313204; PMCID: PMC7115845; each of which are incorporated herein by reference in their entireties.

[00259]In some cases, a polynucleotide is greater than 30 nucleotides in length. In another embodiment, the poly nucleotide molecule is greater than 35 nucleotides in length. In another embodiment, the length is at least 40 nucleotides. In another embodiment, the length is at least 45 nucleotides. In another embodiment, the length is at least 55 nucleotides. In another embodiment, the length is at least 50 nucleotides. In another embodiment, the length is at least 60 nucleotides. In another embodiment, the length is at least 80 nucleotides. In another embodiment, the length is at least 90 nucleotides. In another embodiment, the length is at least 100 nucleotides. In another embodiment, the length is at least 120 nucleotides. In another embodiment, the length is at least 140 nucleotides. In another embodiment, the length is at least 160 nucleotides. In another embodiment, the length is at least 180 nucleotides. In another embodiment, the length is at least 200 nucleotides. In another embodiment, the length is at least 250 nucleotides. In another embodiment, the length is at least 300 nucleotides. In another embodiment, the length is at least 350 nucleotides. In another embodiment, the length is at least 400 nucleotides. In another embodiment, the length is at least 450 nucleotides. In another embodiment, the length is at least 500 nucleotides. In another embodiment, the length is at least 600 nucleotides. In another embodiment, the length is at least 700 nucleotides. In another embodiment, the length is at least 800 nucleotides. In another embodiment, the length is at least 900 nucleotides. In another embodiment, the length is at least 1000 nucleotides. In another embodiment, the length is at least 1100 nucleotides. In another embodiment, the length is at least 1200 nucleotides. In another embodiment, the length is at least 1300 nucleotides. In another embodiment, the length is at least 1400 nucleotides. In another embodiment, the length is at least 1500 nucleotides. In another embodiment, the length is at least 1600 nucleotides. In another embodiment, the length is at least 1800

nucleotides. In another embodiment, the length is at least 2000 nucleotides. In another embodiment, the length is at least 2500 nucleotides. In another embodiment, the length is at least 3000 nucleotides. In another embodiment, the length is at least 4000 nucleotides. In another embodiment, the length is at least 5000 nucleotides, or greater than 5000 nucleotides.

[00260]In some embodiments, a polynucleotide molecule, formula, composition or method associated therewith comprises one or more polynucleotides comprising features as described in WO2002/098443, WO2003/051401, WO2008/052770, WO2009/127230, WO2006/122828, WO2008/083949, WO2010/088927, WO2010/037539, WO2004/004743, WO2005/016376, WO2006/024518, WO2007/095,976, WO2008/014979, WO2008/077592, WO2009/030481, WO2009/095226, WO2011/069586, WO2011/026641, WO2011/144358, WO2012/019780, WO2012/013326, WO2012/089338, WO2012/113513, WO2012/116811, WO2012/116810, WO2013/113502, WO2013/113501, WO2013/113736, WO2013/143698, WO2013/143699, WO2013/143700, WO2013/120626, WO2013/120627, WO2013/120628, WO2013/120629, WO2013/174409, WO2014/127917, WO2015/024669, WO2015/024668, WO2015/024667, WO2015/024665, WO2015/024666, WO2015/024664, WO2015/101415, WO2015/101414, WO2015/024667, WO2015/062738, WO2015/101416, all of which are incorporated by reference herein.

[00261]In some embodiments, a polynucleotide comprises one or more microRNA binding sites. In some embodiments, a microRNA binding site is recognized by a microRNA in a non-target organ. In some embodiments, a microRNA binding site is recognized by a microRNA in the liver. In some embodiments, a microRNA binding site is recognized by a microRNA in hepatic cells.

B.Linear mRNA payloads

[00262]In various embodiments, the LNP-based RNA vaccines, RNA therapeutics and pharmaceutical compositions thereof described herein can be used to deliver an RNA payload that is a linear mRNA molecule.

[00263]In various embodiments, the LNP-based pharmaceutical compositions described herein, e.g., LNP-based gene editing systems, may include one or more linear mRNA molecules or linear mRNA payloads. In various embodiments, the mRNA payloads may encode one or more components of the herein described gene editing systems. For example, an mRNA payload may encode an amino acid sequence-programmable DNA binding domain (e.g., TALENS and zinc finger-binding domains) or a nucleic acid sequence-programmable DNA binding domain (e.g., CRISPR Cas9, CRISPR Cas12a, CRISPR Cas12f, CRISPR Cas13a, CRISPR Cas13b, or TnpB).

[00264]mRNA payloads may also encode, depending upon the nature of the gene editing system, one or more effector domains that provide various functionalities that facilitate changes in nucleotide sequence and/or gene expression, such as, but not limited to, single-strand DNA binding proteins, nucleases, endonucleases, exonucleases, deaminases (e.g., cytidine deaminases or adenosine

deaminases), polymerases (e.g., reverse transcriptases), integrases, recombinases, etc., and fusion proteins comprising one or more functional domains linked together.

[00265]Ribonucleic acid (RNA) is a molecule that is made up of nucleotides, which are ribose sugars attached to nitrogenous bases and phosphate groups. The nitrogenous bases include adenine (A), guanine (G), uracil (U), and cytosine (C). Generally, RNA mostly exists in the single-stranded form but can also exists double-stranded in certain circumstances. The length, form and structure of RNA is diverse depending on the purpose of the RNA. For example, the length of an RNA can vary from a short sequence (e.g., siRNA) to a long sequences (e.g., lncRNA), can be linear (e.g., mRNA) or circular (e.g., oRNA), and can either be a coding (e.g., mRNA) or a non-coding (e.g., lncRNA) sequence.

[00266]In various embodiments, the LNP-based RNA vaccines, RNA therapeutics, gene editing systems and pharmaceutical compositions thereof described herein can be used to deliver a mRNA payload that is a linear mRNA molecule. In embodiments, the mRNA payload may comprise one or more nucleotide sequences that encode a product of interest, such as, but not limited to a vaccine antigen, a component of a gene editing system (e.g., an endonuclease, a prime editor, etc.) and/or a therapeutic protein.

[00267]In some embodiments, the RNA payload may be a linear mRNA. As used herein, the term "messenger RNA" (mRNA) refers to any polynucleotide which encodes a protein of interest and which is capable of being translated to produce the encoded protein of interest *in vitro*, *in vivo*, *in situ* or *ex vivo*.

[00268]Generally, a mRNA molecule comprises at least a coding region, a 5' untranslated region (UTR), a 3' UTR, a 5' cap and a poly-A tail. In some aspects, one or more structural and/or chemical modifications or alterations may be included in the RNA which can reduce the innate immune response of a cell in which the mRNA is introduced. As used herein, a "structural" feature or modification is one in which two or more linked nucleotides are inserted, deleted, duplicated, inverted or randomized in a nucleic acid without significant chemical modification to the nucleotides themselves. Because chemical bonds will necessarily be broken and reformed to affect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide "ATCG" may be chemically modified to "AT-5meC-G".

[00269]Generally, a coding region of interest in an mRNA used herein may encode a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, a hexapeptide, a heptapeptide, an octapeptide, a nonapeptide, or a decapeptide. In another embodiment, the mRNA may encode a peptide of 2-30 amino acids, e.g. 5-30, 10-30, 2-25, 5-25, 10-25, or 10-20 amino acids. The mRNA may encode a peptide of at least 10, 11, 12, 13, 14, 15, 17, 20, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 amino acids, or a peptide that is no longer than 10, 11, 12, 13, 14, 15, 17, 20, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 amino acids.

[00270]Generally, the length of the region of the mRNA encoding a product of interest is greater than about 30 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000 or up to and including 100,000 nucleotides).

[00271]In some embodiments, the mRNA has a total length that spans from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1 ,000 to 25,000, from 1,000 to 50,000, from 1,000 to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1 ,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000 nucleotides).

[00272]In some embodiments, the region or regions flanking the region encoding the product of interest may range independently from 15-1,000 nucleotides in length (e.g., greater than 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, and 900 nucleotides or at least 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, and 1,000 nucleotides).

[00273]In some embodiments, the mRNA comprises a tailing sequence which can range from absent to 500 nucleotides in length (e.g., at least 60, 70, 80, 90, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, or 500 nucleotides). Where the tailing region is a polyA tail, the length may be determined in units of or as a function of polyA Binding Protein binding. In this embodiment, the polyA tail is long enough to bind at least 4 monomers of PolyA Binding Protein. PolyA Binding Protein monomers bind to stretches of approximately 38 nucleotides. As such, it has been observed that polyA tails of about 80 nucleotides and 160 nucleotides are functional.

[00274]In some embodiments, the mRNA comprises a capping sequence which comprises a single cap or a series of nucleotides forming the cap. The capping sequence may be from 1 to 10, e.g. 2-9, 3-8, 4-7, 1-5, 5-10, or at least 2, or 10 or fewer nucleotides in length. In some embodiments, the caping sequence is absent.

[00275]In some embodiments, the mRNA comprises a region comprising a start codon. The region comprising the start codon may range from 3 to 40, e.g., 5-30, 10-20, 15, or at least 4, or 30 or fewer nucleotides in length.

[00276]In some embodiments, the mRNA comprises a region comprising a stop codon. The region comprising the stop codon may range from 3 to 40, e.g., 5-30, 10-20, 15, or at least 4, or 30 or fewer nucleotides in length.

[00277]In some embodiments, the mRNA comprises a region comprising a restriction sequence. The region comprising the restriction sequence may range from 3 to 40, e.g., 5-30, 10-20, 15, or at least 4, or 30 or fewer nucleotides in length.

Untranslated Regions (UTRs)

[00278]In various embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems and pharmaceutical compositions thereof described herein, may comprise at least one untranslated region (UTR) which flanks the region encoding the product of interest and/or is incorporated within the mRNA molecule. UTRs are transcribed by not translated. The mRNA payloads can include 5' UTR sequences and 3' UTR sequences, as well as internal UTRs.

[00279]The RNA payloads of the present disclosure may comprise one or more regions or parts which act or function as an untranslated region. Where nucleic acids are designed to encode at least one polypeptide of interest, the nucleic acid may comprise one or more of these untranslated regions (UTRs). Wild-type untranslated regions of a nucleic acid are transcribed but not translated. In mRNA, the 5′ UTR starts at the transcription start site and continues to the start codon but does not include the start codon; whereas, the 3′ UTR starts immediately following the stop codon and continues until the transcriptional termination signal. There is growing body of evidence about the regulatory roles played by the UTRs in terms of stability of the nucleic acid molecule and translation. The regulatory features of a UTR can be incorporated into the RNA payload molecules (e.g., linear and circular mRNA molecules) of the present disclosure to, among other things, enhance the stability of the molecule. The specific features can also be incorporated to ensure controlled down-regulation of the transcript in case they are misdirected to undesired organs sites. A variety of 5′UTR and 3′UTR sequences are known and available in the art.

[00280]In various embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein, may comprise at least one UTR that may be selected from any UTR sequence listed in Tables 19 or 20 of U.S. Patent No. 10,709,779, which is incorporated herein by reference.

5′ UTR regions

[00281]In various embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein, may comprise at least one 5′ UTR.

[00282]In an embodiment, the 5' UTR comprises a sequence provided in Table (II) or a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a 5' UTR sequence provided in Table (II), or a variant or a fragment thereof (e.g., a fragment that lacks the first one, two, three, four, five, or six nucleotides of the 5' UTR sequence provided in Table (II)). In an embodiment, the 5' UTR comprises a sequence with at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, or SEQ ID NO: 29.

[00283]Table (II) – Exemplary nucleotide sequences of 5' UTRs

5' UTR Nucleotide Sequence	Sequence Identifier
ggaaaucgca aaauuugcuc uucgcguuag auuucuuuua guuuucucgc aacuagcaag cuuuuuguuc ucgccgccgc c	SEQ ID NO: 1
ggaaaucgca aaauuugcuc uucgcguuag auuucuuuua guuuucucgc aacuagcaag cuuuuuguuc ucgccgccgc c	SEQ ID NO: 2
ggaaaucgca aaauuuucuu uucgcguuag auuucuuuua guuuucuuuc aacuagcaag cuuuuuguuc ucgccgccgc c	SEQ ID NO: 3
ggaaaucgca aaauuuuugc ucuuuuucgc guuagauuuc uuuuaguuuu cuykcaacua gcaagcuuuu uguucucgcc rcc	SEQ ID NO: 4
ggaaaucccc acaaccgccu cauauccagg cucaagaaua gagcucagug uuuuguuguu uaaucauucc gacguguuuu gcgauauucg cgcaaagcag ccagucgcgc gcuugcuuuu aaguagaguu guuuuuccac ccguuugcca ggcaucuuua auuuaacaua uuuuuauuuu ucaggcuaac cuacgccgcc acc	SEQ ID NO: 5
ggaaauaaga gagaaaagaa gaguaagaag aaauauaaga ucucccugag cuucagggag ccccggcgcc gccacc	SEQ ID NO: 6
ggaaaccccc cacccccgua agagagaaaa gaagaguaag aagaaauaua agaucucccu gagcuucagg gagccccggc gccgccacc	SEQ ID NO: 7
ggagaacuuc cgcuuccguu ggcgcaagcg cuuucauuuu uucugcuacc gugacuaag	SEQ ID NO: 8
ggaaauaaga gagaaaagaa gaguaagaag aaauauaaga gccacc	SEQ ID NO: 9
ggaaauaaga gagaaaagaa gaguaagaag aaauauaaga ccccggcgcc gccacc	SEQ ID NO: 10

ggaaacuuua uuuaguguua cuuuauuuuc uguuuauuug uguuucuuca guggguuugu ucuaauuucc uuggccgcc	SEQ ID NO: 11
ggaaaaucug uauuagguug gcguguucuu uggucgguug uuaguauugu uguugauucg uuuguggucg guugccgcc	SEQ ID NO: 12
ggaaaauuau uaacaucuug guauucucga uaaccauucg uuggauuuua uuguauucgu aguuuggguu ccugccgcc	SEQ ID NO: 13
ggaaauuauu auuauuucua gcuacaauuu aucauuguau uauuuuagcu auucaucauu auuuacuugg ugaucaaca	SEQ ID NO: 14
ggaaauaggu uguuaaccaa guucaagccu aauaagcuug gauucuggug acuugcuuca ccguuggcgg gcaccgauc	SEQ ID NO: 15
ggaaaucgua gagagucgua cuuaguacau aucgacuauc gguggacacc aucaagauua uaaaccaggc caga	SEQ ID NO: 16
ggaaacccgc ccaagcgacc ccaacauauc agcaguugcc caaucccaac ucccaacaca aucccccaagc aacgccgcc	SEQ ID NO: 17
ggaaagcgau ugaaggcguc uuuucaacua cucgauuaag guuggguauc gucgugggac uuggaaauuu guuguuucc	SEQ ID NO: 18
ggaaacuaau cgaaauaaaa gagccccgua cucuuuuauu ucuauuaggu uaggagccuu agcauuugua ucuuaggua	SEQ ID NO: 19
ggaaauguga uuuccagcaa cuucuuuuga auauauugaa uuccuaauuc aaagcgaaca aaucuacaag ccauauacc	SEQ ID NO: 20
ggaaaucgua gagagucgua cuuacguggu cgccauugca uagcgcgcga aagcaacagg aacaagaacg cgcc	SEQ ID NO: 21
ggaaaucgua gagagucgua cuuagaauaa acagagucgg gucgacuugu cucugauacu acgacgucac aauc	SEQ ID NO: 22
ggaaaauuug ccuucggagu ugcguauccu gaacugccca gccuccugau auacaacugu uccgcuuauu cgggccgcc	SEQ ID NO: 23
ggaaaucuga gcaggaaucc uuugugcauu gaagacuuua gauuccucuc ugcgguagac gugcacuuau aaguauuug	SEQ ID NO: 24
ggaaagcgau ugaaggcguc uuuucaacua cucgauuaag guuggguauc gucgugggac uuggaaauuu guugccacc	SEQ ID NO: 25
ggaaaauuuu agccuggaac guuagauaac uguccuguug ucuuuauaua cuuggucccc aaguaguuug ucuuccaaa	SEQ ID NO: 26
ggaaauuuuu uuuugauauu auaagaguuu uuuuuugaua uuaagaaaau uuuuuuuuga uauuagaaga guaagaagaa auauaagacc ccggcgccgc cacc	SEQ ID NO: 27

ggaaauaaga gagaaaagaa gaguaagaag aaauauaaga gccaaaaaaa aaaaacc	SEQ ID NO: 28
ggaaaucucc cugagcuuca gggaguaaga gagaaaagaa gaguaagaag aaauauaaga ccccggcgcc gccacc	SEQ ID NO: 29

[00284]A 5′ UTR is region of an mRNA that is directly upstream (5′) from the start codon (the first codon of an mRNA transcript translated by a ribosome). A 5′ UTR does not encode a protein (is non-coding). Natural 5′UTRs have features that play roles in translation initiation. They harbor signatures like Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG (SEQ ID NO: 30), where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5′UTR also have been known to form secondary structures which are involved in elongation factor binding. 5' UTR sequences are also known to be important for ribosome recruitment to the mRNA and have been reported to play a role in translation (Hinnebusch A, et al., (2016) Science, 352:6292: 1413-6). In addition, 5' UTR sequences may confer increased half-life, increased expression and/or increased activity of a polypeptide encoded by the RNA payload described herein.

[00285]In various embodiments, the RNA payload constructs contemplated herein may include 5'UTRs that are found in nature and those that are not. For example, the 5'UTRs can be synthetic and/or can be altered in sequence with respect to a naturally occurring 5'UTR. Such altered 5'UTRs can include one or more modifications relative to a naturally occurring 5'UTR, such as, for example, an insertion, deletion, or an altered sequence, or the substitution of one or more nucleotide analogs in place of a naturally occurring nucleotide.

[00286]The 5' UTR starts at the transcription start site and continues to the start codon but does not include the start codon; whereas, the 3 'UTR starts immediately following the stop codon and continues until the transcriptional termination signal. While not wishing to be bound by theory, the UTRs may have a regulatory role in terms of translation and stability of the nucleic acid.

[00287]Natural 5' UTRs usually include features which have a role in translation initiation as they tend to include Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG, where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5'UTR also have been known to form secondary structures which are involved in elongation factor binding.

[00288]In some embodiments of the disclosure, a 5′ UTR is a heterologous UTR, i.e., is a UTR found in nature associated with a different mRNA. In another embodiment, a 5′ UTR is a synthetic UTR, i.e., does not occur in nature. Synthetic UTRs include UTRs that have been

mutated to improve their properties, e.g., which increase gene expression as well as those which are completely synthetic. Exemplary 5′ UTRs include Xenopus or human derived alpha-globin or beta-globin (e.g., US8,278,063 and US9,012,219), human cytochrome b-245 polypeptide, and hydroxysteroid (17b) dehydrogenase, and Tobacco etch virus. CMV immediate-early 1 (IE1) gene (see US20140206753 and WO2013/185069), the sequence GGGAUCCUACC (SEQ ID NO: 31) (WO2014144196) may also be used. In another embodiment, 5′ UTR of a TOP gene is a 5′ UTR of a TOP gene lacking the 5′ TOP motif (the oligopyrimidine tract) (e.g., WO/2015101414, WO2015101415, WO/2015/062738, WO2015024667, WO2015024667; 5′ UTR element derived from ribosomal protein Large 32 (L32) gene (WO/2015101414, WO2015101415, WO/2015/062738)), 5′ UTR element derived from the 5′UTR of an hydroxysteroid (17-β) dehydrogenase 4 gene (HSD17B4) (WO2015024667), or a 5′ UTR element derived from the 5′ UTR of ATP5A1 (WO2015024667) can be used. In one embodiment, an internal ribosome entry site (IRES) is used as a substitute for a 5′ UTR.

[00289]In some embodiments, a 5′ UTR of the present disclosure comprises a sequence selected from SEQ ID NO: 32 (GGGAAAUAAG AGAGAAAAGA AGAGUAAGAA GAAAUAUAAG AGCCACC), SEQ ID NO: 33 (GGGAAATAAG AGAGAAAAGA AGAGTAAGAA GAAATATAAG AGCCACC), SEQ ID NO: 34 (GGGAAAUAAG AGAGAAAAGA AGAGUAAGAA GAAAUAUAAG AGCCACC) and SEQ ID NO: 35 (GGGAAATAAG AGAGAAAAGA AGAGTAAGAA GAAATATAAG AGCCACC).

3′ UTR regions

[00290]In various embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein, may comprise at least one 3′ UTR. 3′ UTRs may be heterologous or synthetic.

[00291]A 3′ UTR is region of an mRNA that is directly downstream (3′) from the stop codon (the codon of an mRNA transcript that signals a termination of translation). A 3′ UTR does not encode a protein (is non-coding). Natural or wild type 3′ UTRs are known to have stretches of adenosines and uridines embedded in them. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al, 1995): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. C-Myc and MyoD contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A)(U/A) (SEQ ID NO: 36) nonamers. Molecules containing this type of AREs include GM-CSF and TNF-α. Class III ARES are less well defined. These U rich regions do not contain an AUUUA motif. c-Jun and Myogenin are two well-studied examples of this class. Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to

increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3′ UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message in vivo.

[00292]3' UTRs are known to have stretches of adenosines and uridines embedded in them. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al., 1995): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. C-Myc and MyoD contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A)(U/A) nonamers. Molecules containing this type of AREs include GM-CSF and TNF-a. Class III ARES are less well defined. These U rich regions do not contain an AUUUA motif. c-Jun and Myogenin are two well-studied examples of this class. Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3' UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message in vivo.

[00293]Introduction, removal or modification of 3' UTR AU rich elements (AREs) can be used to modulate the stability of the mRNA payloads described herein. For example, one or more copies of an ARE can be introduced to make mRNA less stable and thereby curtail translation and decrease production of the resultant protein. Alternatively, AREs can be identified and removed or mutated to increase the intracellular stability and thus increase translation and production of the resultant protein.

[00294]In some embodiments, the introduction of features often expressed in genes of target organs the stability and protein production of the mRNA can be enhanced in a specific organ and/or tissue. As a non-limiting example, the feature can be a UTR. As another example, the feature can be introns or portions of introns sequences.

[00295]Those of ordinary skill in the art will understand that 5′ UTRs that are heterologous or synthetic may be used with any desired 3′ UTR sequence. For example, a heterologous 5′ UTR may be used with a synthetic 3′ UTR with a heterologous 3′ UTR.

[00296]Non-UTR sequences may also be used as regions or subregions within an RNA payload construct. For example, introns or portions of introns sequences may be incorporated into regions of nucleic acid of the disclosure. Incorporation of intronic sequences may increase protein production as well as nucleic acid levels.

[00297]Combinations of features may be included in flanking regions and may be contained within other features. For example, the polypeptide coding region of interest in an mRNA payload may be flanked by a 5′ UTR which may contain a strong Kozak translational initiation signal and/or a 3′ UTR which may include an oligo(dT) sequence for templated addition of a poly-A tail. 5′ UTR may comprise a first polynucleotide fragment and a second polynucleotide fragment from the same and/or

different genes such as the 5′ UTRs described in US Patent Application Publication No. 20100293625 and PCT/US2014/069155, herein incorporated by reference in its entirety

[00298]It should be understood that any UTR from any gene may be incorporated into the regions of an RNA payload molecule (e.g., a linear mRNA). Furthermore, multiple wild-type UTRs of any known gene may be utilized. It is also within the scope of the present disclosure to provide artificial UTRs which are not variants of wild type regions. These UTRs or portions thereof may be placed in the same orientation as in the transcript from which they were selected or may be altered in orientation or location. Hence a 5′ or 3′ UTR may be inverted, shortened, lengthened, made with one or more other 5′ UTRs or 3′ UTRs. As used herein, the term "altered" as it relates to a UTR sequence, means that the UTR has been changed in some way in relation to a reference sequence. For example, a 3′ UTR or 5′ UTR may be altered relative to a wild-type or native UTR by the change in orientation or location as taught above or may be altered by the inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides. Any of these changes producing an "altered" UTR (whether 3′ or 5′) comprise a variant UTR.

[00299]In some embodiments, a double, triple or quadruple UTR such as a 5′ UTR or 3′ UTR may be used. As used herein, a "double" UTR is one in which two copies of the same UTR are encoded either in series or substantially in series. For example, a double beta-globin 3′ UTR may be used as described in US Patent publication 20100129877, the contents of which are incorporated herein by reference in its entirety.

[00300]It is also within the scope of the present disclosure to have patterned UTRs. As used herein "patterned UTRs" are those UTRs which reflect a repeating or alternating pattern, such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than 3 times. In these patterns, each letter, A, B, or C represent a different UTR at the nucleotide level.

[00301]In some embodiments, flanking regions are selected from a family of transcripts whose proteins share a common function, structure, feature or property. For example, polypeptides of interest may belong to a family of proteins which are expressed in a particular cell, tissue or at some time during development. The UTRs from any of these genes may be swapped for any other UTR of the same or different family of proteins to create a new polynucleotide. As used herein, a "family of proteins" is used in the broadest sense to refer to a group of two or more polypeptides of interest which share at least one function, structure, feature, localization, origin, or expression pattern.

[00302]The untranslated region may also include translation enhancer elements (TEE). As a non-limiting example, the TEE may include those described in US Application No. 20090226470, herein incorporated by reference in its entirety, and those known in the art.

5′ Capping

[00303]In various embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein, may comprise a 5' cap structure.

[00304]The 5' cap structure of an mRNA is involved in nuclear export, increasing mRNA stability and binds the mRNA Cap Binding Protein (CBP), which is responsible for mRNA stability in the cell and translation competency through the association of CBP with poly(A) binding protein to form the mature cyclic mRNA species. The cap further assists the removal of 5' proximal introns removal during mRNA splicing.

[00305]Endogenous mRNA molecules may be 5'-end capped generating a 5'-ppp-5'-triphosphate linkage between a terminal guanosine cap residue and the 5'-terminal transcribed sense nucleotide of the mRNA molecule. This 5'-guanylate cap may then be methylated to generate an N7-methyl-guanylate residue. The ribose sugars of the terminal and/or anteterminal transcribed nucleotides of the 5' end of the mRNA may optionally also be 2'-0-methylated. 5'-decapping through hydrolysis and cleavage of the guanylate cap structure may target a nucleic acid molecule, such as an mRNA molecule, for degradation.

[00306]Modifications to mRNA may generate a non-hydrolyzable cap structure preventing decapping and thus increasing mRNA half-life. Because cap structure hydrolysis requires cleavage of 5'-ppp-5' phosphorodiester linkages, modified nucleotides may be used during the capping reaction. For example, a Vaccinia Capping Enzyme from New England Biolabs (Ipswich, MA) may be used with a-thio-guanosine nucleotides according to the manufacturer's instructions to create a phosphorothioate linkage in the 5'-ppp-5' cap.

[00307]Additional modified guanosine nucleotides may be used such as a-methyl-phosphonate and seleno-phosphate nucleotides.

[00308]Additional modifications include, but are not limited to, 2'-0-methylation of the ribose sugars of 5 '-terminal and/or 5'-anteterminal nucleotides of the mRNA (as mentioned above) on the 2'-hydroxyl group of the sugar ring. Multiple distinct 5 '-cap structures can be used to generate the 5 '-cap of a nucleic acid molecule, such as an mRNA molecule.

[00309]Cap analogs, which herein are also referred to as synthetic cap analogs, chemical caps, chemical cap analogs, or structural or functional cap analogs, differ from natural (i.e. endogenous, wild-type or physiological) 5'-caps in their chemical structure, while retaining cap function. Cap analogs may be chemically (i.e. non-enzymatically) or enzymatically synthesized and/or linked to a nucleic acid molecule.

[00310]For example, the Anti-Reverse Cap Analog (ARCA) cap contains two guanines linked by a 5 '-5 '-triphosphate group, wherein one guanine contains an N7 methyl group as well as a 3'-0-methyl group (i.e., N7,3'-0-dimethyl-guanosine-5'-triphosphate-5 '-guanosine (m^7G-3'mppp-G; which may equivalently be designated 3' O-Me-m7G(5')ppp(5')G). The 3'-0 atom of the other, unmodified, guanine becomes linked to the 5'-terminal nucleotide of the capped nucleic acid molecule (e.g. an mRNA). The N7- and 3'-0-methlyated guanine provides the terminal moiety of the capped nucleic acid molecule (e.g. mRNA).

[00311]Another exemplary cap is mCAP, which is similar to ARCA but has a 2'-0-methyl group on guanosine (i.e., N7,2'-0-dimethyl-guanosine-5'-triphosphate-5'-guanosine, m^7Gm-ppp-G).

[00312]While cap analogs allow for the concomitant capping of a nucleic acid molecule in an in vitro transcription reaction, up to 20% of transcripts can remain uncapped. This, as well as the structural differences of a cap analog from an endogenous 5 '-cap structures of nucleic acids produced by the endogenous, cellular transcription machinery, may lead to reduced translational competency and reduced cellular stability.

[00313]mRNA may also be capped post-transcriptionally, using enzymes, in order to generate more authentic 5'-cap structures. As used herein, the phrase "more authentic" refers to a feature that closely mirrors or mimics, either structurally or functionally, an endogenous or wild type feature. That is, a "more authentic" feature is better representative of an endogenous, wild-type, natural or physiological cellular function and/or structure as compared to synthetic features or analogs, etc., of the prior art, or which outperforms the corresponding endogenous, wild-type, natural or physiological feature in one or more respects. Non-limiting examples of more authentic 5 'cap structures are those which, among other things, have enhanced binding of cap binding proteins, increased half-life, reduced susceptibility to 5' endonucleases and/or reduced 5'decapping, as compared to synthetic 5 'cap structures known in the art (or to a wild-type, natural or physiological 5 'cap structure). For example, recombinant Vaccinia Virus Capping Enzyme and recombinant 2'-0-methyltransferase enzyme can create a canonical 5 '-5 '-triphosphate linkage between the 5 '-terminal nucleotide of an mRNA and a guanine cap nucleotide wherein the cap guanine contains an N7 methylation and the 5 '-terminal nucleotide of the mRNA contains a 2'-0-methyl. Such a structure is termed the Capl structure. This cap results in a higher translational-competency and cellular stability and a reduced activation of cellular pro-inflammatory cytokines, as compared, e.g., to other 5 'cap analog structures known in the art. Cap structures include, but are not limited to, 7mG(5*)ppp(5*)N,pN2p (cap 0), 7mG(5*)ppp(5*)NlmpNp (cap 1), and 7mG(5*)-ppp(5')NlmpN2mp (cap 2).

[00314]In some embodiments, the 5' terminal caps may include endogenous caps or cap analogs.

[00315]In some embodiments, a 5' terminal cap may comprise a guanine analog. Useful guanine analogs include, but are not limited to, inosine, Nl-methyl-guanosine, 2'fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

IRES Sequences

[00316]In various embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein, may comprise one or more IRES sequences.

[00317]In some embodiments, the mRNA may contain an internal ribosome entry site (IRES). First identified as a feature Picorna virus RNA, IRES plays an important role in initiating protein synthesis in absence of the 5' cap structure. An IRES may act as the sole ribosome binding site, or may serve as one of multiple ribosome binding sites of an mRNA. An mRNA that contains more than one

functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes. Non-limiting examples of IRES sequences that can be used include without limitation, those from picornaviruses (e.g. FMDV), pest viruses (CFFV), polio viruses (PV), encephalomyocarditis viruses (ECMV), foot-and-mouth disease viruses (FMDV), hepatitis C viruses (HCV), classical swine fever viruses (CSFV), murine leukemia virus (MLV), simian immune deficiency viruses (SIV) or cricket paralysis viruses (CrPV).

[00318]In some embodiments, the IRES is from Taura syndrome virus, Triatoma virus, Theiler's encephalomyelitis virus, Simian Virus 40, Solenopsis invicta virus 1, Rhopalosiphum padi virus, Reticuloendotheliosis virus, Human poliovirus 1, Plautia stali intestine virus, Kashmir bee virus, Human rhinovirus 2, Homalodisca coagulata virus-1, Human Immunodeficiency Virus type 1, Homalodisca coagulata virus-1, Himetobi P virus, Hepatitis C virus, Hepatitis A virus, Hepatitis GB virus, Foot and mouth disease virus, Human enterovirus 71, Equine rhinitis virus, Ectropis obliqua picorna-like virus, Encephalomyocarditis virus, Drosophila C Virus, Human coxsackievirus B3, Crucifer tobamovirus, Cricket paralysis virus, Bovine viral diarrhea virus 1, Black Queen Cell Virus, Aphid lethal paralysis virus, Avian encephalomyelitis virus, Acute bee paralysis virus, Hibiscus chlorotic ringspot virus, Classical swine fever virus, Human FGF2, Human SFTPA1, Human AML1/RUNX1, Drosophila antennapedia, Human AQP4, Human AT1R, Human BAG-1, Human BCL2, Human BiP, Human c-IAP1, Human c-myc, Human eIF4G, Mouse NDST4L, Human LEF1, Mouse HIF1 alpha, Human n.myc, Mouse Gtx, Human p27kip1, Human PDGF2/c-sis, Human p53, Human Pim-1, Mouse Rbm3, Drosophila reaper, Canine Scamper, Drosophila Ubx, Human UNR, Mouse UtrA, Human VEGF-A, Human XIAP, Drosophila hairless, S. cerevisiae TFIID, S. cerevisiae YAP1, tobacco etch virus, turnip crinkle virus, EMCV-A, EMCV-B, EMCV-Bf, EMCV-Cf, EMCV pEC9, Picobirnavirus, HCV QC64, Human Cosavirus E/D, Human Cosavirus F, Human Cosavirus JMY, Rhinovirus NAT001, HRV14, HRV89, HRVC-02, HRV-A21, Salivirus A SH1, Salivirus FHB, Salivirus NG-J1, Human Parechovirus 1, Crohivirus B, Yc-3, Rosavirus M-7, Shanbavirus A, Pasivirus A, Pasivirus A 2, Echovirus E14, IIuman Parechovirus 5, Aichi Virus, Hepatitis A Virus HA16, Phopivirus, CVA10, Enterovirus C, Enterovirus D, Enterovirus J, Human Pegivirus 2, GBV-C GT110, GBV-C K1737, GBV-C Iowa, Pegivirus A 1220, Pasivirus A 3, Sapelovirus, Rosavirus B, Bakunsa Virus, Tremovirus A, Swine Pasivirus 1, PLV-CHN, Pasivirus A, Sicinivirus, Hepacivirus K, Hepacivirus A, BVDV1, Border Disease Virus, BVDV2, CSFV-PK15C, SF573 Dicistrovirus, Hubei Picorna-like Virus, CRPV, Salivirus A BNS, Salivirus A BN2, Salivirus A 02394, Salivirus A GUT, Salivirus A CH, Salivirus A SZ1, Salivirus FHB, CVB3, CVB1, Echovirus 7, CVBS, EVA71, CVA3, CVA12, EV24 or an aptamer to eIF4G.

Poly-A tails and 3' stabilizing region

[00319]In various embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein, may comprise a poly-A tail.

[00320]During RNA processing, a long chain of adenine nucleotides (poly-A tail) may be added to a polynucleotide such as an mRNA molecules in order to increase stability. Immediately after transcription, the 3' end of the transcript may be cleaved to free a 3' hydroxyl. Then poly-A polymerase adds a chain of adenine nucleotides to the free 3' hydroxyl end. The process, called polyadenylation, adds a poly-A tail of a certain length.

[00321]In some embodiments, the length of a poly-A tail is greater than 30 nucleotides in length. In another embodiment, the poly-A tail is greater than 35 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000 nucleotides) and no more than about 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, or 3000 nucleotides in length. In some embodiments, the mRNA includes a poly-A tail from about 30 to about 3,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 750, from 30 to 1,000, from 30 to 1,500, from 30 to 2,000, from 30 to 2,500, from 50 to 100, from 50 to 250, from 50 to 500, from 50 to 750, from 50 to 1 ,000, from 50 to 1,500, from 50 to 2,000, from 50 to 2,500, from 50 to 3,000, from 100 to 500, from 100 to 750, from 100 to 1,000, from 100 to 1,500, from 100 to 2,000, from 100 to 2,500, from 100 to 3,000, from 500 to 750, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 2,500, from 500 to 3,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 2,500, from 1,000 to 3,000, from 1,500 to 2,000, from 1,500 to 2,500, from 1,500 to 3,000, from 2,000 to 3,000, from 2,000 to 2,500, and from 2,500 to 3,000).

[00322]In some embodiments, the poly-A tail is designed relative to the length of the overall mRNA. This design may be based on the length of the region coding for a target of interest, the length of a particular feature or region (such as a flanking region), or based on the length of the ultimate product expressed from the mRNA.

[00323]In this context the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% greater in length than the mRNA or feature thereof. The poly-A tail may also be designed as a fraction of mRNA to which it belongs. In this context, the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, or 90% or more of the total length of the construct or the total length of the construct minus the poly-A tail. Further, engineered binding sites and conjugation of mRNA for poly-A binding protein may enhance expression.

[00324]Additionally, multiple distinct mRNA may be linked together to the PABP (Poly-A binding protein) through the 3'-end using modified nucleotides at the 3 '-terminus of the poly-A tail. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72 hr and day 7 post-transfection.

[00325]In some embodiments, the mRNA are designed to include a polyA-G Quartet. The G-quartet is a cyclic hydrogen bonded array of four guanine nucleotides that can be formed by G-rich sequences

in both DNA and RNA. In this embodiment, the G-quartet is incorporated at the end of the poly-A tail.

Stop Codons

[00326]In various embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein, may comprise one or more translation stop codons. Translational stop codons, UAA, UAG, and UGA, are an important component of the genetic code and signal the termination of translation of an mRNA. During protein synthesis, stop codons interact with protein release factors and this interaction can modulate ribosomal activity thus having an impact translation (Tate WP, et al., (2018) Biochem Soc Trans, 46(6):1615-162).

[00327]A stop element as used herein, refers to a nucleic acid sequence comprising a stop codon. The stop codon can be selected from TGA, TAA and TAG in the case of DNA, or from UGA, UAA and UAG in the case of RNA. In an embodiment, a stop element comprises two consecutive stop codons. In an embodiment, a stop element comprises three consecutive stop codons. In an embodiment, a stop element comprises four consecutive stop codons. In an embodiment, a stop element comprises five consecutive stop codons.

[00328]In some embodiments, the mRNA may include one stop codon. In some embodiments, the mRNA may include two stop codons. In some embodiments, the mRNA may include three stop codons. In some embodiments, the mRNA may include at least one stop codon. In some embodiments, the mRNA may include at least two stop codons. In some embodiments, the mRNA may include at least three stop codons. As non-limiting examples, the stop codon may be selected from TGA, TAA and TAG.

[00329]In other embodiments, the stop codon may be selected from one or more of the following stop elements of Table (III):

Table (III): Additional stop elements

Nucleotide sequence (5' to 3')	Sequence Identifier
UGAUAAUAG	SEQ ID NO: 37
UAAUAGUAA	SEQ ID NO: 38
UAAGUCUAA	SEQ ID NO: 39
UAAAGCUAA	SEQ ID NO: 40
UAAGUCUCC	SEQ ID NO: 41
UAAGGCUAA	SEQ ID NO: 42
UAAGCCCCUCCGGGG	SEQ ID NO: 43
UAAAGCUCCCCGGGG	SEQ ID NO: 44
UAAGCCCCU	SEQ ID NO: 45
UAAAGCUCC	SEQ ID NO: 46

UAAAGCUCC	SEQ ID NO: 47
UAGGGUUAA	SEQ ID NO: 48
UAAGCACCC	SEQ ID NO: 49
UGAUAGUAA	SEQ ID NO: 50
UAAAGCGCU	SEQ ID NO: 51

[00330]In some embodiments, the mRNA includes the stop codon TGA and one additional stop codon. In a further embodiment the addition stop codon may be TAA.

MicroRNA binding sites and other regulatory elements

[00331]In various embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein, may comprise one or more regulatory elements, including, but not limited to microRNA (miRNA) binding sites, structured mRNA sequences and/or motifs, artificial binding sites to bind to endogenous nucleic acid binding molecules, and combinations thereof.

Chemically unmodified nucleotides

[00332]In some embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein are not chemically modified and comprises the standard ribonucleotides consisting of adenosine, guanosine, cytosine and uridine. In some embodiments, nucleotides and nucleosides of the present disclosure comprise standard nucleoside residues such as those present in transcribed RNA (e.g. A, G, C, or U). In some embodiments, nucleotides and nucleosides of the present disclosure comprise standard deoxyribonucleosides such as those present in DNA (e.g. dA, dG, dC, or dT).

Chemically modified nucleotides

[00333]In some embodiments, the mRNA payloads of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions thereof described herein comprise, in some embodiments, comprises at least one chemical modification.

[00334]The terms "chemical modification" and "chemically modified" refer to modification with respect to adenosine (A), guanosine (G), uridine (U), thymidine (T) or cytidine (C) ribonucleosides or deoxyribnucleosides in at least one of their position, pattern, percent or population. Generally, these terms do not refer to the ribonucleotide modifications in naturally occurring 5′-terminal mRNA cap moieties. With respect to a polypeptide, the term "modification" refers to a modification relative to the canonical set 20 amino acids. Polypeptides, as provided herein, are also considered "modified" of they contain amino acid substitutions, insertions or a combination of substitutions and insertions.

[00335]Polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides), in some embodiments, comprise various (more than one) different modifications. In some embodiments, a particular region of a polynucleotide contains one, two or more (optionally different) nucleoside or

nucleotide modifications. In some embodiments, a modified RNA polynucleotide (e.g., a modified mRNA polynucleotide), introduced to a cell or organism, exhibits reduced degradation in the cell or organism, respectively, relative to an unmodified polynucleotide. In some embodiments, a modified RNA polynucleotide (e.g., a modified mRNA polynucleotide), introduced into a cell or organism, may exhibit reduced immunogenicity in the cell or organism, respectively (e.g., a reduced innate response).

[00336]Modifications of polynucleotides include, without limitation, those described herein. Polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) may comprise modifications that are naturally-occurring, non-naturally-occurring or the polynucleotide may comprise a combination of naturally-occurring and non-naturally-occurring modifications. Polynucleotides may include any useful modification, for example, of a sugar, a nucleobase, or an internucleoside linkage (e.g., to a linking phosphate, to a phosphodiester linkage or to the phosphodiester backbone).

[00337]Polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides), in some embodiments, comprise non-natural modified nucleotides that are introduced during synthesis or post-synthesis of the polynucleotides to achieve desired functions or properties. The modifications may be present on an internucleotide linkages, purine or pyrimidine bases, or sugars. The modification may be introduced with chemical synthesis or with a polymerase enzyme at the terminal of a chain or anywhere else in the chain. Any of the regions of a polynucleotide may be chemically modified.

[00338]The present disclosure provides for modified nucleosides and nucleotides of a polynucleotide (e.g., RNA polynucleotides, such as mRNA polynucleotides). A "nucleoside" refers to a compound containing a sugar molecule (e.g., a pentose or ribose) or a derivative thereof in combination with an organic base (e.g., a purine or pyrimidine) or a derivative thereof (also referred to herein as "nucleobase"). A "nucleotide" refers to a nucleoside, including a phosphate group. Modified nucleotides may by synthesized by any useful method, such as, for example, chemically, enzymatically, or recombinantly, to include one or more modified or non-natural nucleosides. Polynucleotides may comprise a region or regions of linked nucleosides. Such regions may have variable backbone linkages. The linkages may be standard phosphodiester linkages, in which case the polynucleotides would comprise regions of nucleotides.

[00339]Modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine or uracil. Any combination of base/sugar or linker may be incorporated into polynucleotides of the present disclosure.

[00340]In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) include a combination of at least two (e.g., 2, 3, 4 or more) of the aforementioned modified nucleobases.

[00341]In some embodiments, modified nucleobases in polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) are selected from the group consisting of pseudouridine (ψ), N1-methylpseudouridine ($m^1\psi$), N1-ethylpseudouridine, 2-thiouridine, 4′-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methoxyuridine and 2′-O-methyl uridine. In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) include a combination of at least two (e.g., 2, 3, 4 or more) of the aforementioned modified nucleobases.

[00342]In some embodiments, modified nucleobases in polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) are selected from the group consisting of 1-methyl-pseudouridine ($m^1\psi$), 5-methoxy-uridine (mo^5U), 5-methyl-cytidine (m^5C), pseudouridine (ψ), α-thio-guanosine and α-thio-adenosine. In some embodiments, polynucleotides includes a combination of at least two (e.g., 2, 3, 4 or more) of the aforementioned modified nucleobases.

[00343]In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise pseudouridine (ψ) and 5-methyl-cytidine (m^5C). In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise 1-methyl-pseudouridine ($m^1\psi$). In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise 1-methyl-pseudouridine ($m^1\psi$) and 5-methyl-cytidine (m^5C). In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise 2-thiouridine (s^2U). In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise 2-thiouridine and 5-methyl-cytidine (m^5C). In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise methoxy-uridine (mo^5U). In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise 5-methoxy-uridine (mo^5U) and 5-methyl-cytidine (m^5C). In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise 2′-O-methyl uridine. In some embodiments polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise 2′-O-methyl uridine and 5-methyl-cytidine (m^5C). In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise N6-methyl-adenosine (m^6A). In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) comprise N6-methyl-adenosine (m^6A) and 5-methyl-cytidine (mC).

[00344]In some embodiments, polynucleotides (e.g., RNA polynucleotides, such as mRNA polynucleotides) are uniformly modified (e.g., fully modified, modified throughout the entire sequence) for a particular modification. For example, a polynucleotide can be uniformly modified

with 5-methyl-cytidine (m^5C), meaning that all cytosine residues in the mRNA sequence are replaced with 5-methyl-cytidine (m^5C). Similarly, a polynucleotide can be uniformly modified for any type of nucleoside residue present in the sequence by replacement with a modified residue such as those set forth above.

[00345]Exemplary nucleobases and nucleosides having a modified cytosine include N4-acetyl-cytidine (ac4C), 5-methyl-cytidine (m5C), 5-halo-cytidine (e.g., 5-iodo-cytidine), 5-hydroxymethyl-cytidine (hm5C), 1-methyl-pseudoisocytidine, 2-thio-cytidine (s2C), and 2-thio-5-methyl-cytidine.

[00346]In some embodiments, a modified nucleobase is a modified uridine. Exemplary nucleobases and In some embodiments, a modified nucleobase is a modified cytosine. nucleosides having a modified uridine include 5-cyano uridine, and 4′-thio uridine.

[00347]The polynucleotides of the present disclosure may be partially or fully modified along the entire length of the molecule. For example, one or more or all or a given type of nucleotide (e.g., purine or pyrimidine, or any one or more or all of A, G, U, C) may be uniformly modified in a polynucleotide of the disclosure, or in a given predetermined sequence region thereof (e.g., in the mRNA including or excluding the polyA tail). In some embodiments, all nucleotides X in a polynucleotide of the present disclosure (or in a given sequence region thereof) are modified nucleotides, wherein X may any one of nucleotides A, G, U, C, or any one of the combinations A+G, A+U, A+C, G+U, G+C, U+C, A+G+U, A+G+C, G+U+C or A+G+C.

[00348]The polynucleotide may contain from about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, i.e., any one or more of A, G, U or C) or any intervening percentage (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100%). It will be understood that any remaining percentage is accounted for by the presence of unmodified A, G, U, or C.

[00349]The polynucleotides may contain at a minimum 1% and at maximum 100% modified nucleotides, or any intervening percentage, such as at least 5% modified nucleotides, at least 10% modified nucleotides, at least 25% modified nucleotides, at least 50% modified nucleotides, at least 80% modified nucleotides, or at least 90% modified nucleotides. For example, the polynucleotides may contain a modified pyrimidine such as a modified uracil or cytosine. In some embodiments, at least 5%, at least 10%, at least 25%, at least 50%, at least 80%, at least 90% or 100% of the uracil in the polynucleotide is replaced with a modified uracil (e.g., a 5-substituted uracil). The modified uracil

can be replaced by a compound having a single unique structure, or can be replaced by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures). In some embodiments, at least 5%, at least 10%, at least 25%, at least 50%, at least 80%, at least 90% or 100% of the cytosine in the polynucleotide is replaced with a modified cytosine (e.g., a 5-substituted cytosine). The modified cytosine can be replaced by a compound having a single unique structure, or can be replaced by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures).

C. Circular mRNA payloads

[00350] In various embodiments, the LNP-based RNA vaccines, RNA therapeutics and pharmaceutical compositions thereof described herein can be used to deliver an RNA payload that is a circular mRNA molecule or "oRNA." The circular mRNA molecule may encode a CROI, such as a vaccine antigen, cancer antigen, or therapeutic protein as described in this specification.

[00351] In various embodiments, the LNP-based pharmaceutical compositions described herein, e.g., LNP-based gene editing systems, may include one or more circular mRNA molecules or "oRNAs." In various embodiments, the circular mRNA payloads may encode one or more components of the herein described gene editing systems or other therapeutic protein of interest. For example, a circular mRNA payload may encode an amino acid sequence-programmable DNA binding domain (e.g., TALENS and zinc finger-binding domains) or a nucleic acid sequence-programmable DNA binding domain (e.g., CRISPR Cas9, CRISPR Cas12a, CRISPR Cas12f, CRISPR Cas13a, CRISPR Cas13b, or TnpB).

[00352] The circular mRNA payloads may also encode, depending upon the nature of the gene editing system, one or more effector domains that provide various functionalities that facilitate changes in nucleotide sequence and/or gene expression, such as, but not limited to, single-strand DNA binding proteins, nucleases, endonucleases, exonucleases, deaminases (e.g., cytidine deaminases or adenosine deaminases), polymerases (e.g., reverse transcriptases), integrases, recombinases, etc., and fusion proteins comprising one or more functional domains linked together.

[00353] In some embodiments, the RNA payload is a circular RNA (oRNA). As used herein, the terms "oRNA" or "circular RNA" are used interchangeably and can refer to a RNA that forms a circular structure through covalent or non-covalent bonds.

[00354] Circular RNA described herein are polyribonucleotides that form a continuous structure through covalent or non-covalent bonds. Due to the circular structure, oRNAs have improved stability, increased half-life, reduced immunogenicity, and/or improved functionality (e.g., of a function described herein) compared to a corresponding linear RNA.

[00355] In some embodiments, an oRNA binds a target. In some embodiments, an oRNA binds a substrate. In some embodiments, an oRNA binds a target and binds a substrate of the target. In some embodiments, an oRNA binds a target and mediates modulation of a substrate of the target. In some embodiments, an oRNA brings together a target and its substrate to mediate modification of the

substrate, e.g., post-translational modification. In some embodiments, an oRNA brings together a target and its substrate to mediate a cellular process (e.g., alters protein degradation or signal transduction) involving the substrate. In some embodiments, a target is a target protein and a substrate is a substrate protein.

[00356]In some embodiments, an oRNA comprises a conjugation moiety for binding to chemical compound. The conjugation moiety can be a modified polyribonucleotide. The chemical compound can be conjugated to the oRNA by the conjugation moiety. In some embodiments, the chemical compound binds to a target and mediates modulation of a substrate of the target. In some embodiments, an oRNA binds a substrate of a target and a chemical compound conjugated to the oRNA by the conjugation moiety binds the target to bring together the target and its substrate to mediate modification of the substrate, e.g., post-translational modification. In some embodiments, an oRNA binds a substrate of a target and a chemical compound conjugated to the oRNA by the conjugation moiety binds the target to bring together the target and its substrate to mediate modification of the substrate to mediate a cellular process (e.g., alters protein degradation or signal transduction) involving the substrate. In some embodiments, a target is a target protein and a substrate is a substrate protein.

[00357]In some embodiments, the oRNA may be non-immunogenic in a mammal (e.g., a human, non-human primate, rabbit, rat, and mouse).

[00358]In some embodiments, the oRNA may be capable of replicating or replicates in a cell from an aquaculture animal (e.g., fish, crabs, shrimp, oysters etc.), a mammalian cell, a cell from a pet or zoo animal (e.g., cats, dogs, lizards, birds, lions, tigers and bears etc.), a cell from a farm or working animal (e.g., horses, cows, pigs, chickens etc.), a human cell, cultured cells, primary cells or cell lines, stem cells, progenitor cells, differentiated cells, germ cells, cancer cells (e.g., tumorigenic, metastatic), non-tumorigenic cells (e.g., normal cells), fetal cells, embryonic cells, adult cells, mitotic cells, non-mitotic cells, or any combination thereof.

[00359]In one aspect, provided herein is a pharmaceutical composition comprising: a circular RNA comprising, in the following order, a 3' group I intron fragment, an Internal Ribosome Entry Site (IRES), an expression sequence encoding a polypeptide (e.g., a vaccine antigen, therapeutic protein, such as a chimeric antigen receptor (CAR) or T cell receptor (TCR) complex protein or a nucleobase editing system or component thereof), and a 5' group I intron fragment, and a transfer vehicle comprising at least one of (i) an ionizable lipid, (ii) a structural lipid, and (iii) a PEG-modified lipid, wherein the transfer vehicle is capable of delivering the circular RNA polynucleotide to a cell (e.g., a human cell, such as an immune cell present in a human subject), such that the polypeptide is translated in the cell.

[00360] In some embodiments, the pharmaceutical composition is formulated for intravenous administration to the human subject in need thereof. In some embodiments, the 3' group I intron fragment and 5' group I intron fragment are Anabaena group I intron fragments.

[00361] In certain embodiments, the 3' intron fragment and 5' intron fragment are defined by the L9a-5 permutation site in the intact intron. In certain embodiments, the 3' intron fragment and 5' intron fragment are defined by the L8-2 permutation site in the intact intron.

[00362]In some embodiments, the IRES is from Taura syndrome virus, Tiiatoma virus, Theiler's encephalomyelitis virus, Simian Virus 40, Solenopsis invicta virus 1, Rhopalosiphum padi virus, Reticuloendotheliosis virus, Human poliovirus 1, Plautia stall intestine virus, Kashmir bee virus, Human rhinovirus 2, Homalodisca coagulata virus- 1, Human Immunodeficiency Virus type 1, Homalodisca coagulata virus- 1, Himetobi P virus, Hepatitis C virus, Hepatitis A virus, Hepatitis GB virus , Foot and mouth disease virus, Human enterovirus 71, Equine rhinitis virus, Ectropis obliqua picoma-like virus, Encephalomyocarditis virus, Drosophila C Virus, Human coxsackievirus B3, Crucifer tobamovirus, Cricket paralysis virus, Bovine viral diarrhea virus 1, Black Queen Cell Virus, Aphid lethal paralysis virus, Avian encephalomyelitis virus, Acute bee paralysis virus, Hibiscus chlorotic ringspot virus, Classical swine fever virus, Human FGF2, Human SFTPA1, Human AML1/RUNX1, Drosophila antennapedia, Human AQP4, Human AT1R, Human BAG-1, Human BCL2, Human BiP, Human c-IAPl, Human c-myc, Human eIF4G, Mouse NDST4L, Human LEF1, Mouse HIFl alpha, Human n.myc, Mouse Gtx, Human p27kipl, Human PDGF2/c-sis, Human p53, Human Pim-1, Mouse Rbm3, Drosophila reaper, Canine Scamper, Drosophila Ubx, Human UNR, Mouse UtrA, Human VEGF-A, Human XIAP, Drosophila hairless, S. cerevisiae TFIID, S. cerevisiae YAP1, tobacco etch virus, turnip crinkle virus, EMCV-A, EMCV-B, EMCV-Bf, EMCV-Cf, EMCV pEC9, Picobirnavirus, HCV QC64, Human Cosavirus E/D, Human Cosavirus F, Human Cosavirus JMY, Rhinovirus NAT001, HRV14, HRV89, HRVC-02, HRV-A21, Salivirus A SHI, Salivirus FHB, Salivirus NG-J1, Human Parechovirus 1, Crohivirus B, Yc-3, Rosavirus M-7, Shanbavirus A, Pasivirus A, Pasivirus A 2, Echovirus E14, Human Parechovirus 5, Aichi Virus, Hepatitis A Virus HA 16, Phopivirus, CVA10, Enterovirus C, Enterovirus D, Enterovirus J, Human Pegivirus 2, GBV-C GT110, GBV-C K1737, GBV-C Iowa, Pegivirus A 1220, Pasivirus A 3, Sapelovirus, Rosavirus B, Bakunsa Virus, Tremovirus A, Swine Pasivirus 1, PLV-CIIN, Pasivirus A, Sicinivirus, Hepacivirus K, Hepacivirus A, BVDV1, Border Disease Virus, BVDV2, CSFV- PK15C, SF573 Dicistravirus, Hubei Picoma-like Virus, CRPV, Salivirus A BN5, Salivirus A BN2, Salivirus A 02394, Salivirus A GUT, Salivirus A CH, Salivirus A SZ1, Salivirus FHB, CVB3, CVB1, Echovirus 7, CVB5, EVA71, CVA3, CVA12, EV24 or an aptamer to eIF4G.

[00363]In some embodiments, the IRES comprises a CVB3 IRES or a fragment or variant thereof. In some embodiments, the pharmaceutical composition comprises a first internal spacer between the 3' group I intron fragment and the IRES, and a second internal spacer between the expression sequence and the 5' group I intron fragment. In certain embodiments, the first and second internal spacers each have a length of about 10 to about 60 nucleotides.

[00364]In some embodiments, the circular mRNA comprises a nucleotide sequence encoding a polypeptide of interest, such as a vaccine antigen, nucleobase editing system, or therapeutic protein (e.g., a CAR or TCR complex protein).

[00365]In embodiments where the therapeutic protein encoded by the herein RNA payload (e.g., circular or linear mRNA) is a CAR or TCR complex protein, the CAR or TCR complex protein comprises an antigen binding domain specific for an antigen selected from the group: CD 19, CD123, CD22, CD30, CD171, CS-1, C-type lectin-like molecule- 1, CD33, epidermal growth factor receptor variant III (EGFRvIII), disialoganglioside GD2, disaloganglioside GD3, TNF receptor family member, B cell maturation antigen (BCMA), Tn antigen ((Tn Ag) or (GalNAca-Ser/Thr)), prostate-specific membrane antigen (PSMA), Receptor tyrosine kinase-like orphan receptor 1 (ROR1), Fms-Like Tyrosine Kinase 3 (FLT3), Tumor-associated glycoprotein 72 (TAG72), CD38, CD44v6, Carcinoembryonic antigen (CEA), Epithelial cell adhesion molecule (EPCAM), B7H3 (CD276), KIT (CD 117), Interleukin- 13 receptor subunit alpha-2, mesothelin, Interleukin 11 receptor alpha (IL-l lRa), prostate stem cell antigen (PSCA), Protease Serine 21, vascular endothelial growth factor receptor 2 (VEGFR2), Lewis(Y) antigen, CD24, Platelet-derived growth factor receptor beta (PDGFR-beta), Stage-specific embryonic antigen-4 (SSEA-4), CD20, Folate receptor alpha, HER2, HER3, Mucin 1, cell surface associated (MUC1), epidermal growth factor receptor (EGFR), neural cell adhesion molecule (NCAM), Prostase, prostatic acid phosphatase (PAP), elongation factor 2 mutated (ELF2M), Ephrin B2, fibroblast activation protein alpha (FAP), insulin-like growth factor 1 receptor (IGF-I receptor), carbonic anhydrase IX (CAIX), Proteasome (Prosome, Macropain) Subunit, Beta Type, 9 (LMP2), glycoprotein 100 (gplOO), oncogene fusion protein consisting of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl), tyrosinase, ephrin type- A receptor 2 (EphA2), Fucosyl GM1, sialyl Lewis adhesion molecule (sLe), ganglioside GM3, transglutaminase 5 (TGS5), high molecular weight-melanoma-associated antigen (HMWMAA), o-acetyl-GD2 ganglioside (0AcGD2), Folate receptor beta, tumor endothelial marker 1 (TEM1/CD248), tumor endothelial marker 7 -related (TEM7R), claudin 6 (CLDN6), claudin 18.2 (CLDN18.2), thyroid stimulating hormone receptor (TSHR), G protein-coupled receptor class C group 5, member D (GPRC5D), chromosome X open reading frame 61 (CXORF61), CD97, and CD179a.

[00366]In further embodiments where the therapeutic protein encoded by the herein RNA payload (e.g., circular or linear mRNA) is a CAR or TCR complex protein, the CAR or TCR complex protein comprises a CAR comprising an antigen binding domain specific for CD19. In some embodiments, the CAR or TCR complex protein comprises a CAR comprising a costimulatory domain selected from the group CD28, 4-1BB, 0X40, CD27, CD30, ICOS, GITR, CD40, CD2, SLAM, and combinations thereof. In some embodiments, the CAR or TCR complex protein comprises a CAR comprising a CD3zeta signaling domain. In some embodiments, the CAR or TCR complex protein comprises a

CAR comprising a CH2CH3, CD28, and/or CD8 spacer domain. In some embodiments, the CAR or TCR complex protein comprises a CAR comprising a CD28 or CD8 transmembrane domain.

[00367]In some embodiments, the CAR or TCR complex protein comprises a CAR comprising: an antigen binding domain, a spacer domain, a transmembrane domain, a costimulatory domain, and an intracellular T cell signaling domain.

[00368]In some embodiments, the CAR or TCR complex protein comprises a multispecific CAR comprising antigen binding domains for at least two different antigens. In some embodiments, the CAR or TCR complex protein comprises a TCR complex protein selected from the group TCRalpha, TCRbeta, TCRgamma, and TCRdelta.

[00369]In some embodiments, the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing system, and pharmaceutical compositions described herein further comprise a targeting moiety. In certain embodiments, the targeting moiety mediates receptor-mediated endocytosis or direct fusion of the delivery vehicle (LNPs) into selected cells of a selected cell population or tissue in the absence of cell isolation or purification. In certain embodiments, the targeting moiety is capable of binding to a protein selected from the group CD3, CD4, CD8, CDS, CD7, PD-1, 4-1BB, CD28, Clq, and CD2. In certain embodiments, the targeting moiety comprises an antibody specific for a macrophage, dendritic cell, NK cell, NKT, or T cell antigen. In certain embodiments, the targeting moiety comprises a scFv, nanobody, peptide, minibody, polynucleotide aptamer, heavy chain variable region, light chain variable region or fragment thereof.

[00370] In some embodiments, the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing system, and pharmaceutical compositions described herein are administered in an amount effective to treat a disease in the human subject (e.g., wherein the disease can be cancer, muscle disorder, or CNS disorder, etc.). In some embodiments, the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing system, and pharmaceutical compositions have an enhanced safety profile when compared to a pharmaceutical composition comprising T cells or vectors comprising exogenous DNA encoding the same polypeptide, e.g., a CAR complex protein.

[00371] In some embodiments, the LNP-based RNA vaccines and pharmaceutical compositions thereof are administered in an amount effective to mount an immunogenic response in a human subject for the vaccination against an infectious agent and/or cancer. In some embodiments, the LNP-based RNA vaccines and pharmaceutical compositions have an enhanced safety profile when compared to state of the art vaccine compositions.

[00372] In some embodiments, the LNP-based nucleobase editing systems and pharmaceutical compositions thereof are administered in an amount effective to induce a desire precise edit in a genome. In some embodiments, the LNP-based nucleobase editing systems and pharmaceutical compositions have an enhanced safety profile when compared to state of the art gene editing delivery compositions.

[00373]In another aspect, the present disclosure provides a circular RNA comprising, in the following order, a 3' group I intron fragment, an Internal Ribosome Entry Site (IRES), an expression sequence encoding a polypeptide (e.g., a vaccine antigen, nucleobase editing system or component thereof, therapeutic protein, such as a chimeric antigen receptor (CAR) or T cell receptor (TCR) complex protein), and a 5' group I intron fragment.

[00374] In some embodiments, the 3' group I intron fragment and 5' group I intron fragment are Anabaena group I intron fragments. In certain embodiments, the 3' intron fragment and 5' intron fragment are defined by the L9a-5 permutation site in the intact intron. In certain embodiments, the 3' intron fragment and 5' intron fragment are defined by the L8-2 permutation site in the intact intron. In certain embodiments, the IRES comprises a CVB3 IRES or a fragment or variant thereof.

[00375]In some embodiments, the circular RNA comprises a first internal spacer between the 3' group I intron fragment and the IRES, and a second internal spacer between the expression sequence and the 5' group I intron fragment.

[00376]In certain embodiments, the first and second internal spacers each have a length of about 10 to about 60 nucleotides.

[00377]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein consists of natural nucleotides. In some embodiments, the circular RNA further comprises a second expression sequence encoding a therapeutic protein. In some embodiments, the therapeutic protein comprises a checkpoint inhibitor. In certain embodiments, the therapeutic protein comprises a cytokine.

[00378]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing system, and pharmaceutical compositions described herein consists of natural nucleotides.

[00379]In some embodiments, the circular RNA payload LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein comprises a nucleotide sequence that is codon optimized, either partially or fully. In some embodiments, the circular RNA is optimized to lack at least one microRNA binding site present in an equivalent pre-optimized polynucleotide. In some embodiments, the circular RNA is optimized to lack at least one endonuclease susceptible site present in an equivalent pre-optimized polynucleotide. In some embodiments, the circular RNA is optimized to lack at least one RNA-editing susceptible site present in an equivalent pre-optimized polynucleotide.

[00380]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing system, and pharmaceutical compositions described herein has an in vivo functional half- life in humans greater than that of an equivalent linear RNA having the same expression sequence. In some embodiments, the circular RNA has a length of about 100 nucleotides to about 10 kilobases. In some embodiments, the circular RNA has a functional half-life of at least about 20 hours. In some embodiments, the circular RNA has a duration of therapeutic effect in a

human cell of at least about 20 hours. In some embodiments, the circular RNA has a duration of therapeutic effect in a human cell greater than or equal to that of an equivalent linear RNA comprising the same expression sequence. In some embodiments, the circular RNA has a functional half-life in a human cell greater than or equal to that of an equivalent linear RNA comprising the same expression sequence.

[00381]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, nucleobase editing systems, RNA therapeutics and pharmaceutical compositions described herein has a half-life of at least that of a linear counterpart. In some embodiments, the oRNA has a half-life that is increased over that of a linear counterpart. In some embodiments, the half-life is increased by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, or greater. In some embodiments, the oRNA has a half-life or persistence in a cell for at least about 1 hour to about 30 days, or at least about 2 hours, 6 hours, 12 hours, 18 hours, 24 hours (1 day), 2 days, 3, days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 60 days, or longer or any time therebetween. In some embodiments, the oRNA has a half-life or persistence in a cell for no more than about 10 mins to about 7 days, or no more than about 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 24 hours (1 day), 36 hours (1.5 days), 48 hours (2 days),60 hours (2.5 days), 72 hours (3 days), 4 days, 5 days, 6 days, or 7 days.

[00382]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, nucleobase editing systems, RNA therapeutics and pharmaceutical compositions described herein has a half-life or persistence in a cell while the cell is dividing. In some embodiments, the oRNA has a half-life or persistence in a cell post division.

[00383]In certain embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein has a half-life or persistence in a dividing cell for greater than about 10 minutes to about 30 days, or at least about 10 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 24 hours (1 day), 2 days, 3, days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 60 days, or longer or any time therebetween.

[00384]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein modulates a cellular function, e.g., transiently or long term. In certain embodiments, the cellular function is stably altered, such as a modulation that persists for at least about 1 hour to about 30 days,

or at least about 2 hours, 6 hours, 12 hours, 18 hours, 24 hours (1 day), 2 days, 3, days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 60 days, or longer. In certain embodiments, the cellular function is transiently altered, e.g., such as a modulation that persists for no more than about 30 mins to about 7 days, or no more than about 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours (1 day), 36 hours (1.5 days), 48 hours (2 days), 60 hours (2.5 days), 72 hours(3 days), 4 days, 5 days, 6 days, or 7 days.

[00385]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein is at least about 20 nucleotides, at least about 30 nucleotides, at least about 40 nucleotides, at least about 50 nucleotides, at least about 75 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, at least about 300 nucleotides, at least about 400 nucleotides, at least about 500 nucleotides, at least about 1,000 nucleotides, at least about 2,000 nucleotides, at least about 5,000 nucleotides, at least about 6,000 nucleotides, at least about 7,000 nucleotides, at least about 8,000 nucleotides, at least about 9,000 nucleotides, at least about 10,000 nucleotides, at least about 12,000 nucleotides, at least about 14,000 nucleotides, at least about 15,000 nucleotides, at least about 16,000 nucleotides, at least about 17,000 nucleotides, at least about 18,000 nucleotides, at least about 19,000 nucleotides, or at least about 20,000 nucleotides. In some embodiments, the oRNA may be of a sufficient size to accommodate a binding site for a ribosome.

[00386]In some embodiments, the maximum size of the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein may be limited by the ability of packaging and delivering the RNA to a target. In some embodiments, the size of the oRNA is a length sufficient to encode polypeptides, and thus, lengths of at least 20,000 nucleotides, at least 15,000 nucleotides, at least 10,000 nucleotides, at least 7,500 nucleotides, or at least 5,000 nucleotides, at least 4,000 nucleotides, at least 3,000 nucleotides, at least 2,000 nucleotides, at least 1,000 nucleotides, at least 500 nucleotides, at least 400 nucleotides, at least 300 nucleotides, at least 200 nucleotides, at least 100 nucleotides may be useful.

[00387]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein comprises one or more elements described elsewhere herein. In some embodiments, the elements may be separated from one another by a spacer sequence or linker. In some embodiments, the elements may be separated from one another by 1 nucleotide, 2 nucleotides, about 5 nucleotides, about 10 nucleotides, about 15 nucleotides, about 20 nucleotides, about 30 nucleotides, about 40 nucleotides, about 50 nucleotides, about 60 nucleotides, about 80 nucleotides, about 100 nucleotides, about 150 nucleotides, about 200 nucleotides, about 250 nucleotides, about 300 nucleotides, about 400

nucleotides, about 500 nucleotides, about 600 nucleotides, about 700 nucleotides, about 800 nucleotides, about 900 nucleotides, about 1000 nucleotides, up to about 1 kb, at least about 1000 nucleotides.

[00388]In some embodiments, one or more elements are contiguous with one another, e.g., lacking a spacer element.

[00389]In some embodiments, one or more elements is conformationally flexible. In some embodiments, the conformational flexibility is due to the sequence being substantially free of a secondary structure.

[00390]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein comprises a secondary or tertiary structure that accommodates a binding site for a ribosome, translation, or rolling circle translation.

[00391]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein comprises particular sequence characteristics. For example, the oRNA may comprise a particular nucleotide composition. In some such embodiments, the oRNA may include one or more purine rich regions (adenine or guanosine). In some such embodiments, the oRNA may include one or more purine rich regions (adenine or guanosine).In some embodiments, the oRNA may include one or more AU rich regions or elements (AREs). In some embodiments, the oRNA may include one or more adenine rich regions.

[00392]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein comprises one or more modifications described elsewhere herein.

[00393]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein comprises one or more expression sequences and is configured for persistent expression in a cell of a subject in vivo. In some embodiments, the oRNA is configured such that expression of the one or more expression sequences in the cell at a later time point is equal to or higher than an earlier time point. In such embodiments, the expression of the one or more expression sequences can be either maintained at a relatively stable level or can increase over time. The expression of the expression sequences can be relatively stable for an extended period of time. For instance, in some cases, the expression of the one or more expression sequences in the cell over a time period of at least 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 23 or more days does not decrease by 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, or 5%. In some cases, in some cases, the expression of the one or more expression sequences in the cell is maintained at a level that does not vary by more than 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, or 5% for at least 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 23 or more days.

Regulatory Elements

[00394]In some embodiments, the circular RNA payload of the LNP-based RNA vaccines, RNA therapeutics, nucleobase editing systems, and pharmaceutical compositions described herein comprises one or more regulatory elements. As used herein, a "regulatory element" is a sequence that modifies expression of an expression sequence, e.g., a nucleotide sequence encoding an antigen, nucleobase editing system, or a therapeutic protein, i.e., a coding region of interest (CROI). The regulatory element may include a sequence that is located adjacent to a coding region of interest encoded on the circular RNA payload. The regulatory element may be operatively linked to a nucleotide sequence of the circular RNA that encodes a coding region of interest (e.g., an antigen, nucleobase editing system, or therapeutic polypeptide).

[00395]In some embodiments, a regulatory element may increase an amount of expression of a coding region of interest encoded on the circular RNA payload as compared to an amount expressed when no regulatory element exists.

[00396]In some embodiments, a regulatory element may comprise a sequence to selectively initiates or activates translation of a coding sequence of interest encoded on the circular RNA payload.

[00397]In some embodiments, a regulatory element may comprise a sequence to initiate degradation of the oRNA or the payload or cargo. Non-limiting examples of the sequence to initiate degradation includes, but is not limited to, riboswitch aptazyme and miRNA binding sites.

[00398]In some embodiments, a regulatory element can modulate translation of a coding region of interest encoded on the oRNA. The modulation can create an increase (enhancer) or decrease (suppressor) in the expression of the coding region of interest. The regulatory element may be located adjacent to the CROI (e.g., on one side or both sides of the CROI).

Translation Initiation Sequence

[00399]In some embodiments, a translation initiation sequence functions as a regulatory element. In some embodiments, the translation initiation sequence comprises an AUG/ATG codon. In some embodiments, a translation initiation sequence comprises any eukaryotic start codon such as, but not limited to, AUG/ATG, CUG/CTG, GUG/GTG, UUG/TTG, ACG, AUC/ATC, AUU, AAG, AUA/ATA, or AGG. In some embodiments, a translation initiation sequence comprises a Kozak sequence. In some embodiments, translation begins at an alternative translation initiation sequence, e.g., translation initiation sequence other than AUG/ATG codon, under selective conditions, e.g., stress induced conditions. As a non-limiting example, the translation of the circular polyribonucleotide may begin at alternative translation initiation sequence, such as ACG. As another non-limiting example, the circular polyribonucleotide translation may begin at alternative translation initiation sequence, CUG/CTG. As another non-limiting example, the translation may begin at alternative translation initiation sequence, GUG/GTG. As yet another non-limiting example, the translation may begin at a repeat-associated non-AUG (RAN) sequence, such as an alternative

translation initiation sequence that includes short stretches of repetitive RNA e.g. CGG, GGGGCC, CAG, or CTG.

[00400]In some embodiments, the oRNA encodes a polypeptide or peptide and may comprise a translation initiation sequence. The translation initiation sequence may comprise, but is not limited to a start codon, a non-coding start codon, a Kozak sequence or a Shine-Dalgarno sequence. The translation initiation sequence may be located adjacent to the payload or cargo (e.g., on one side or both sides of the coding region of interest).

[00401]In some embodiments, the translation initiation sequence provides conformational flexibility to the oRNA. In some embodiments, the translation initiation sequence is within a substantially single stranded region of the oRNA.

[00402]The oRNA may include more than 1 start codon such as, but not limited to, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15 or more than 15 start codons. Translation may initiate on the first start codon or may initiate downstream of the first start codon.

[00403]In some embodiments, the oRNA may initiate at a codon which is not the first start codon, e.g., AUG. Translation of the circular polyribonucleotide may initiate at an alternative translation initiation sequence, such as, but not limited to, ACG, AGG, AAG, CUG/CTG, GUG/GTG, AUA/ATA, AUU/ATT, UUG/TTG. In some embodiments, translation begins at an alternative translation initiation sequence under selective conditions, e.g., stress induced conditions. As a non-limiting example, the translation of the oRNA may begin at alternative translation initiation sequence, such as ACG. As another non-limiting example, the oRNA translation may begin at alternative translation initiation sequence, CUG/CTG. As yet another non-limiting example, the oRNA translation may begin at alternative translation initiation sequence, GTG/GUG. As yet another non-limiting example, the oRNA may begin translation at a repeat-associated non-AUG (RAN) sequence, such as an alternative translation initiation sequence that includes short stretches of repetitive RNA e.g. CGG, GGGGCC, CAG, CTG.

IRES Sequences

[00404]In some embodiments, the oRNA described herein comprises an internal ribosome entry site (IRES) element capable of engaging an eukaryotic ribosome. In some embodiments, the IRES element is at least about 5 nucleotides, at least about 8 nucleotides, at least about 9 nucleotides, at least about 10 nucleotides, at least about 15 nucleotides, at least about 20 nucleotides, at least about 25 nucleotides, at least about 30 nucleotides, at least about 40 nucleotides, at least about 50 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, at least about 250 nucleotides, at least about 350 nucleotides, or at least about 500 nucleotides. In one embodiment, the IRES element is derived from the DNA of an organism including, but not limited to, a virus, a mammal, and a Drosophila. Such viral DNA may be derived from, but is not limited to, picornavirus complementary DNA (cDNA), with encephalomyocarditis virus (EMCV) cDNA and poliovirus

cDNA. In one embodiment, Drosophila DNA from which an IRES element is derived includes, but is not limited to, an Antennapedia gene from Drosophila melanogaster.

[00405]In some embodiments, the IRES element is at least partially derived from a virus, for instance, it can be derived from a viral IRES element, such as ABPV_IGRpred, AEV, ALPV_IGRpred, BQCV_IGRpred, BVDV1_1-385, BVDV1_29-391, CrPV_5NCR, CrPV_IGR, crTMV_IREScp, crTMV_IRESmp75, crTMV_IRESmp228, crTMV_IREScp, crTMV_IREScp, CSFV, CVB3, DCV_IGR, EMCV-R, EoPV_5NTR, ERAV 245-961, ERBV 162-920, EV71_1-748, FeLV-Notch2, FMDV_type_C, GBV-A, GBV-B, GBV-C, gypsy_env, gypsyD5, gypsyD2, HAV_HM175, HCV_type_1a, HiPV_IGRpred, HIV-1, HoCV1_IGRpred, HRV-2, IAPV_IGRpred, idefix, KBV_IGRpred, LINE-1_ORF1_-101_to_-1, LINE-1_ORF1-302_to_-202, LINE-1_ORF2-138_to_-86, LINE-1_ORF1_-44to_-1, PSIV_IGR, PV_type1_Mahoney,PV_type3_Leon, REV-A, RhPV_5NCR, RhPV_IGR, SINV1_IGRpred, SV40_661-830, TMEV, TMV_UI_IRESmp228, TRV_5NTR, TrV_IGR, or TSV_IGR. In some embodiments, the IRES element is at least partially derived from a cellular IRES, such as AML1/RUNX1, Antp-D, Antp-DE, Antp-CDE, Apaf-1, Apaf-1, AQP4, AT1R_var1, AT1R_var2, AT1R_var3, AT1R_var4, BAG1_p36delta236 nt, BAG1_p36, BCL2, BiP_-222_-3, c-IAP1_285-1399, c-IAP1_1313-1462, c-jun, c-myc, Cat-1224, CCND1, DAPS, eIF4G, eIF4GI-ext, eIF4GII, eIF4GII-long, ELG1, ELH, FGF1A,FMR1, Gtx-133-141, Gtx-1-166, Gtx-1-120, Gtx-1-196, hairless, HAP4, HIF1a, hSNM1, Hsp101, hsp70, hsp70, Hsp90, IGF2_leader2, Kv1.4_1.2, L-myc, LamB1_-335_-1, LEF1, MNT_75-267, MNT_36-160, MTG8a, MYB, MYT2_997-1152, n-MYC, NDST1, NDST2, NDST3, NDST4L, NDST4S, NRF_-653_-17, NtHSF1, ODC1, p27kip1, 03_128-269, PDGF2/c-sis, Pim-1, PITSLRE_p58, Rbm3, reaper, Scamper, TFIID, TIF4631, Ubx_1-966, Ubx_373-961, UNR, Ure2, UtrA, VEGF-A-133-1, XIAP_5-464, XIAP_305-466, or YAP1.

[00406]In another embodiment, the IRES is an IRES sequence from Coxsackievirus B3 (CVB3), the protein coding region encodes Guassia luciferase (Gluc) and the spacer sequences are polyA-C.

[00407]In some embodiments, the IRES, if present, is at least about 50 nucleotides in length. In one embodiment, the vector comprises an IRES that comprises a natural sequence. In one embodiment, the vector comprises an IRES that comprises a synthetic sequence.

[00408]An IRES may act as the sole ribosome binding site, or may serve as one of multiple ribosome binding sites of an mRNA. A polynucleotide containing more than one functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes (e.g., multicistronic mRNA). When polynucleotides are provided with an IRES, further optionally provided is a second translatable region. Examples of IRES sequences that can be used according to the present disclosure include without limitation, those from picornaviruses (e.g., FMDV), pest viruses (CFFV), polio viruses (PV), encephalomyocarditis viruses (ECMV), foot-and mouth disease viruses (FMDV), hepatitis C viruses (HCV), classical Swine fever viruses (CSFV), murine leukemia virus (MLV), simian immune deficiency viruses (SIV) or cricket paralysis viruses (CrPV).

Termination Element

[00409]In some embodiments, the oRNA includes one or more coding regions of interest (i.e., also referred to as product expression sequences) which encode polypeptides of interest, including but not limited to vaccine antigens and therapeutic proteins. In various embodiments, the product expression sequences may or may not have a termination element.

[00410]In some embodiments, the oRNA includes one or more product expression sequences that lack a termination element, such that the oRNA is continuously translated.

[00411]Exclusion of a termination element may result in rolling circle translation or continuous expression of the encoded peptides or polypeptides as the ribosome will not stall or fall-off. In such an embodiment, rolling circle translation expresses continuously through the product expression sequence.

[00412]In some embodiments, one or more product expression sequences in the oRNA comprise a termination element.

[00413]In some embodiments, not all of the product expression sequences in the oRNA comprise a termination element. In such instances, the product expression sequence may fall off the ribosome when the ribosome encounters the termination element and terminates translation.

Rolling Circle Translation

[00414]In some embodiments, once translation of the oRNA is initiated, the ribosome bound to the oRNA does not disengage from the oRNA before finishing at least one round of translation of the oRNA. In some embodiments, the oRNA as described herein is competent for rolling circle translation. In some embodiments, during rolling circle translation, once translation of the oRNA is initiated, the ribosome bound to the oRNA does not disengage from the oRNA before finishing at least 2 rounds, at least 3 rounds, at least 4 rounds, at least 5 rounds, at least 6 rounds, at least 7 rounds, at least 8 rounds, at least 9 rounds, at least 10 rounds, at least 11 rounds, at least 12 rounds, at least 13 rounds, at least 14 rounds, at least 15 rounds, at least 20 rounds, at least 30 rounds, at least 40 rounds, at least 50 rounds, at least 60 rounds, at least 70 rounds, at least 80 rounds, at least 90 rounds, at least 100 rounds, at least 150 rounds, at least 200 rounds, at least 250 rounds, at least 500 rounds, at least 1,000 rounds, at least 1,500 rounds, at least 2,000 rounds, at least 5,000 rounds, at least 10,000 rounds, at least 10^5 rounds, or at least 10^6 rounds of translation of the oRNA.

[00415]In some embodiments, the rolling circle translation of the oRNA leads to generation of polypeptide that is translated from more than one round of translation of the oRNA. In some embodiments, the oRNA comprises a stagger element, and rolling circle translation of the oRNA leads to generation of polypeptide product that is generated from a single round of translation or less than a single round of translation of the oRNA.

Circularization

[00416]In one embodiment, a linear RNA may be cyclized, or concatemerized. In some embodiments, the linear RNA may be cyclized *in vitro* prior to formulation and/or delivery. In some embodiments, the linear RNA may be cyclized within a cell.

[00417]In some embodiments, the mechanism of cyclization or concatemerization may occur through at least 3 different routes: 1) chemical, 2) enzymatic, and 3) ribozyme catalyzed. The newly formed 5'-/3'-linkage may be intramolecular or intermolecular.

[00418]In the first route, the 5'-end and the 3 '-end of the nucleic acid contain chemically reactive groups that, when close together, form a new covalent linkage between the 5 '-end and the 3 '-end of the molecule. The 5 '-end may contain an NHS-ester reactive group and the 3 '-end may contain a 3'-amino-terminated nucleotide such that in an organic solvent the 3'-amino-terminated nucleotide on the 3 '-end of a synthetic mRNA molecule will undergo a nucleophilic attack on the 5 '-NHS-ester moiety forming a new 5 '-/3 '-amide bond.

[00419]In the second route, T4 RNA ligase may be used to enzymatically link a 5'-phosphorylated nucleic acid molecule to the 3'-hydroxyl group of a nucleic acid forming a new phosphorodiester linkage. In an example reaction, ^g of a nucleic acid molecule is incubated at 37°C for 1 hour with 1-10 units of T4 RNA ligase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. The ligation reaction may occur in the presence of a split oligonucleotide capable of base-pairing with both the 5'-and 3'-region in juxtaposition to assist the enzymatic ligation reaction.

[00420]In the third route, either the 5 '-or 3 '-end of the cDNA template encodes a ligase ribozyme sequence such that during in vitro transcription, the resultant nucleic acid molecule can contain an active ribozyme sequence capable of ligating the 5 '-end of a nucleic acid molecule to the 3 '-end of a nucleic acid molecule. The ligase ribozyme may be derived from the Group I Intron, Group I Intron, Hepatitis Delta Virus, Hairpin ribozyme or may be selected by SELEX (systematic evolution of ligands by exponential enrichment). The ribozyme ligase reaction may take 1 to 24 hours at temperatures between 0 and 37°C.

[00421]In some embodiments, the oRNA is made via circularization of a linear RNA.

[00422]In some embodiments, the following elements are operably connected to each other and, in some embodiments, arranged in the following sequence: a.) a 5′ homology arm, b.) a 3′ group I intron fragment containing a 3′ splice site dinucleotide, c.) a protein coding or noncoding region, d.) a 5′ group I intron fragment containing a 5′ splice site dinucleotide, and e.) a 3′ homology arm. In certain embodiments said vector allows production of a circular RNA that is translatable and/or biologically active inside eukaryotic cells. In some embodiments, the biologically active RNA is, for example, an miRNA sponge, or long noncoding RNA.

[00423]In some embodiments, the following elements are operably connected to each other and arranged in the following sequence: a.) a 5′ homology arm, b.) a 3′ group I intron fragment containing a 3′ splice site dinucleotide, c.) optionally, a 5′ spacer sequence, d.) optionally, an internal ribosome

entry site (IRES), c.) a protein coding or noncoding region, f.) optionally, a 3′ spacer sequence, g.) a 5′ group I intron fragment containing a 5′ splice site dinucleotide, and h.) a 3′ homology arm. In certain embodiments, said vector allows production of a circular RNA that is translatable and/or biologically active inside eukaryotic cells.

[00424]In some embodiments, the following elements are operably connected to each other and arranged in the following sequence: a.) a 5′ homology arm, b.) a 3′ group I intron fragment containing a 3′ splice site dinucleotide, c.) a 5′ spacer sequence, d.) an internal ribosome entry site (IRES), e.) a protein coding or noncoding region, f.) a 5′ group I intron fragment containing a 5′ splice site dinucleotide, and g.) a 3′ homology arm. In some embodiments, said vector allows production of a circular RNA that is translatable and/or biologically active inside eukaryotic cells.

[00425]In some embodiments, the following elements are operably connected to each other and arranged in the following sequence: a.) a 5′ homology arm, b.) a 3′ group I intron fragment containing a 3′ splice site dinucleotide, c.) a 5′ spacer sequence, d.) a protein coding or noncoding region, e.) a 3′ spacer sequence, f.) a 5′ group I intron fragment containing a 5′ splice site dinucleotide, and g.) a 3′ homology arm. In some embodiments, said vector allows production of a circular RNA that is translatable and/or biologically active inside eukaryotic cells.

[00426]In some embodiments, the following elements are operably connected to each other and arranged in the following sequence: a.) a 5′ homology arm, b.) a 3′ group I intron fragment containing a 3′ splice site dinucleotide, c.) an internal ribosome entry site (IRES), d.) a protein coding or noncoding region, e.) a 3′ spacer sequence, f) a 5′ group I intron fragment containing a 5′ splice site dinucleotide, and g.) a 3′ homology arm. In some embodiments, said vector allows production of a circular RNA that is translatable and/or biologically active inside eukaryotic cells.

[00427]In some embodiments, the following elements are operably connected to each other and arranged in the following sequence: a.) a 5′ homology arm, b.) a 3′ group I intron fragment containing a 3′ splice site dinucleotide, c.) a protein coding or noncoding region, d.) a 3′ spacer sequence, e.) a 5′ group I intron fragment containing a 5′ splice site dinucleotide, and f.) a 3′ homology arm. In some embodiments, said vector allows production of a circular RNA that is translatable and/or biologically active inside eukaryotic cells.

[00428]In some embodiments, the following elements are operably connected to each other and arranged in the following sequence: a.) a 5′ homology arm, b.) a 3′ group I intron fragment containing a 3′ splice site dinucleotide, c.) a 5′ spacer sequence, d.) a protein coding or noncoding region, e.) a 5′ group I intron fragment containing a 5′ splice site dinucleotide, and f.) a 3′ homology arm. In some embodiments, said vector allows production of a circular RNA that is translatable and/or biologically active inside eukaryotic cells.

[00429]In some embodiments, the following elements are operably connected to each other and arranged in the following sequence: a.) a 5′ homology arm, b.) a 3′ group I intron fragment containing a 3′ splice site dinucleotide, c.) an internal ribosome entry site (IRES), d.) a protein coding or

noncoding region, e.) a 5′ group I intron fragment containing a 5′ splice site dinucleotide, and f) a 3′ homology arm. In some embodiments, said vector allows production of a circular RNA that is translatable and/or biologically active inside eukaryotic cells.

[00430]In some embodiments, the following elements are operably connected to each other and arranged in the following sequence: a.) a 5′ homology arm, b.) a 3′ group I intron fragment containing a 3′ splice site dinucleotide, c.) a 5′ spacer sequence, d.) an internal ribosome entry site (IRES), e.) a protein coding or noncoding region, f) a 3′ spacer sequence, g.) a 5′ group I intron fragment containing a 5′ splice site dinucleotide, and h.) a 3′ homology arm. In some embodiments, said vector allowing production of a circular RNA that is translatable and/or biologically active inside eukaryotic cells.

[00431]In one embodiment, the 3′ group I intron fragment and/or the 5′ group I intron fragment is from a Cyanobacterium Anabaena sp. pre-tRNA-Leu gene or T4 phage Td gene.

[00432]In one embodiment, the 3′ group I intron fragment and/or the 5′ group I intron fragment is from a Cyanobacterium Anabaena sp. pre-tRNA-Leu gene.

[00433]In one embodiment, the protein coding region encodes a protein of eukaryotic or prokaryotic origin. In another embodiment, the protein coding region encodes human protein or non-human protein. In some embodiments, the protein coding region encodes one or more antibodies. For example, in some embodiments, the protein coding region encodes human antibodies. In one embodiment, the protein coding region encodes a protein selected from hFIX, SP-B, VEGF-A, human methylmalonyl-CoA mutase (hMUT), CFTR, cancer self-antigens, and additional gene editing enzymes like Cpf1, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). In another embodiment, the protein coding region encodes a protein for therapeutic use. In one embodiment, the human antibody encoded by the protein coding region is an anti-HIV antibody. In one embodiment, the antibody encoded by the protein coding region is a bispecific antibody. In one embodiment, the bispecific antibody is specific for CD19 and CD22. In another embodiment, the bispecific antibody is specific for CD3 and CLDN6. In one embodiment, the protein coding region encodes a protein for diagnostic use. In one embodiment, the protein coding region encodes Gaussia luciferase (Gluc), Firefly luciferase (Fluc), enhanced green fluorescent protein (eGFP), human erythropoietin (hEPO), or Cas9 endonuclease.

[00434]In one embodiment, the 5′ homology arm is about 5-50 nucleotides in length. In another embodiment, the 5′ homology arm is about 9-19 nucleotides in length. In some embodiments, the 5′ homology arm is at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 nucleotides in length. In some embodiments, the 5′ homology arm is no more than 50, 45, 40, 35, 30, 25 or 20 nucleotides in length. In some embodiments, the 5′ homology arm is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 nucleotides in length.

[00435]In one embodiment, the 3′ homology arm is about 5-50 nucleotides in length. In another embodiment, the 3′ homology arm is about 9-19 nucleotides in length. In some embodiments, the 3′

homology arm is at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 nucleotides in length. In some embodiments, the 3′ homology arm is no more than 50, 45, 40, 35, 30, 25 or 20 nucleotides in length. In some embodiments, the 3′ homology arm is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 nucleotides in length.

[00436]In one embodiment, the 5′ spacer sequence is at least 10 nucleotides in length. In another embodiment, the 5′ spacer sequence is at least 15 nucleotides in length. In a further embodiment, the 5′ spacer sequence is at least 30 nucleotides in length. In some embodiments, the 5′ spacer sequence is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or 30 nucleotides in length. In some embodiments, the 5′ spacer sequence is no more than 100, 90, 80, 70, 60, 50, 45, 40, 35 or 30 nucleotides in length. In some embodiments the 5′ spacer sequence is between 20 and 50 nucleotides in length. In certain embodiments, the 5′ spacer sequence is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides in length. In one embodiment, the 5′ spacer sequence is a polyA sequence. In another embodiment, the 5′ spacer sequence is a polyA-C sequence.

[00437]In one embodiment, the 3′ spacer sequence is at least 10 nucleotides in length. In another embodiment, the 3′ spacer sequence is at least 15 nucleotides in length. In a further embodiment, the 3′ spacer sequence is at least 30 nucleotides in length. In some embodiments, the 3′ spacer sequence is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or 30 nucleotides in length. In some embodiments, the 3′ spacer sequence is no more than 100, 90, 80, 70, 60, 50, 45, 40, 35 or 30 nucleotides in length. In some embodiments the 3′ spacer sequence is between 20 and 50 nucleotides in length. In certain embodiments, the 3′ spacer sequence is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides in length. In one embodiment, the 3′ spacer sequence is a polyA sequence. In another embodiment, the 5′ spacer sequence is a polyA-C sequence.

Extracellular Circularization

[00438]In some embodiments, the linear RNA is cyclized, or concatemerized using a chemical method to form an oRNA. In some chemical methods, the 5'-end and the 3'-end of the nucleic acid (e.g., a linear RNA) includes chemically reactive groups that, when close together, may form a new covalent linkage between the 5'-end and the 3'-end of the molecule. The 5'-end may contain an NHS-ester reactive group and the 3'-end may contain a 3'-amino-terminated nucleotide such that in an organic solvent the 3'-amino-terminated nucleotide on the 3'-end of a linear RNA will undergo a nucleophilic attack on the 5'-NHS-ester moiety forming a new 5'-/3'-amide bond.

[00439]In one embodiment, a DNA or RNA ligase may be used to enzymatically link a 5'-phosphorylated nucleic acid molecule (e.g., a linear RNA) to the 3'-hydroxyl group of a nucleic acid (e.g., a linear nucleic acid) forming a new phosphorodiester linkage. In an example reaction, a linear RNA is incubated at 37C for 1 hour with 1-10 units of T4 RNA ligase according to the manufacturer's protocol. The ligation reaction may occur in the presence of a linear nucleic acid capable of base-

pairing with both the 5'-and 3'-region in juxtaposition to assist the enzymatic ligation reaction. In one embodiment, the ligation is splint ligation where a single stranded polynucleotide (splint), like a single stranded RNA, can be designed to hybridize with both termini of a linear RNA, so that the two termini can be juxtaposed upon hybridization with the single-stranded splint. Splint ligase can thus catalyze the ligation of the juxtaposed two termini of the linear RNA, generating an oRNA.

[00440]In one embodiment, a DNA or RNA ligase may be used in the synthesis of the oRNA. As a non-limiting example, the ligase may be a circ ligase or circular ligase.

[00441]In one embodiment, either the 5'-or 3'-end of the linear RNA can encode a ligase ribozyme sequence such that during in vitro transcription, the resultant linear RNA includes an active ribozyme sequence capable of ligating the 5'-end of the linear RNA to the 3'-end of the linear RNA. The ligase ribozyme may be derived from the Group I Intron, Hepatitis Delta Virus, Hairpin ribozyme or may be selected by SELEX (systematic evolution of ligands by exponential enrichment).

[00442]In one embodiment, a linear RNA may be cyclized or concatemerized by using at least one non-nucleic acid moiety. In one aspect, the at least one non-nucleic acid moiety may react with regions or features near the 5' terminus and/or near the 3' terminus of the linear RNA in order to cyclize or concatermerize the linear RNA. In another aspect, the at least one non-nucleic acid moiety may be located in or linked to or near the 5' terminus and/or the 3' terminus of the linear RNA. The non-nucleic acid moieties contemplated may be homologous or heterologous. As a non-limiting example, the non-nucleic acid moiety may be a linkage such as a hydrophobic linkage, ionic linkage, a biodegradable linkage and/or a cleavable linkage. As another non-limiting example, the non-nucleic acid moiety is a ligation moiety. As yet another non-limiting example, the non-nucleic acid moiety may be an oligonucleotide or a peptide moiety, such as an aptamer or a non-nucleic acid linker as described herein.

[00443]In one embodiment, a linear RNA may be cyclized or concatemerized due to a non-nucleic acid moiety that causes an attraction between atoms, molecular surfaces at, near or linked to the 5' and 3' ends of the linear RNA. As a non-limiting example, one or more linear RNA may be cyclized or concatemerized by intermolecular forces or intramolecular forces. Non-limiting examples of intermolecular forces include dipole-dipole forces, dipole-induced dipole forces, induced dipole-induced dipole forces, Van der Waals forces, and London dispersion forces. Non-limiting examples of intramolecular forces include covalent bonds, metallic bonds, ionic bonds, resonant bonds, agnostic bonds, dipolar bonds, conjugation, hyperconjugation and antibonding.

[00444]In one embodiment, the linear RNA may comprise a ribozyme RNA sequence near the 5' terminus and near the 3' terminus. The ribozyme RNA sequence may covalently link to a peptide when the sequence is exposed to the remainder of the ribozyme. In one aspect, the peptides covalently linked to the ribozyme RNA sequence near the 5' terminus and the 3' terminus may associate with each other causing a linear RNA to cyclize or concatemerize. In another aspect, the peptides covalently linked to the ribozyme RNA near the 5' terminus and the 3' terminus may cause the linear

RNA to cyclize or concatemerize after being subjected to ligated using various methods known in the art such as, but not limited to, protein ligation.

[00445]In some embodiments, the linear RNA may include a 5' triphosphate of the nucleic acid converted into a 5' monophosphate, e.g., by contacting the 5' triphosphate with RNA 5' pyrophosphohydrolase (RppH) or an ATP diphosphohydrolase (apyrase). Alternately, converting the 5' triphosphate of the linear RNA into a 5' monophosphate may occur by a two-step reaction comprising: (a) contacting the 5' nucleotide of the linear RNA with a phosphatase (e.g., Antarctic Phosphatase, Shrimp Alkaline Phosphatase, or Calf Intestinal Phosphatase) to remove all three phosphates; and (b) contacting the 5' nucleotide after step (a) witha kinase (e.g., Polynucleotide Kinase) that adds a single phosphate.

[00446]In some embodiments, RNA may be circularized using the methods described in WO2017222911 and WO2016197121, the contents of each of which are herein incorporated by reference in their entirety.

[00447]In some embodiments, RNA may be circularized, for example, by back splicing of a non-mammalian exogenous intron or splint ligation of the 5' and 3 ' ends of a linear RNA. In one embodiment, the circular RNA is produced from a recombinant nucleic acid encoding the target RNA to be made circular. As a non-limiting example, the method comprises: a) producing a recombinant nucleic acid encoding the target RNA to be made circular, wherein the recombinant nucleic acid comprises in 5' to 3 ' order: i) a 3 ' portion of an exogenous intron comprising a 3' splice site, ii) a nucleic acid sequence encoding the target RNA, and iii) a 5 ' portion of an exogenous intron comprising a 5 ' splice site; b) performing transcription, whereby RNA is produced from the recombinant nucleic acid; and c) performing splicing of the RNA, whereby the RNA circularizes to produce a oRNA.

[00448]While not wishing to be bound by theory, circular RNAs generated with exogenous introns are recognized by the immune system as "non-self" and trigger an innate immune response. On the other hand, circular RNAs generated with endogenous introns are recognized by the immune system as "self" and generally do not provoke an innate immune response, even if carrying an exon comprising foreign RNA.

[00449]Accordingly, circular RNAs can be generated with either an endogenous or exogenous intron to control immunological self/non-self discrimination as desired. Numerous intron sequences from a wide variety of organisms and viruses are known and include sequences derived from genes encoding proteins, ribosomal RNA (rRNA), or transfer RNA (tRNA).

[00450]Circular RNAs can be produced from linear RNAs in a number of ways. In some embodiments, circular RNAs are produced from a linear RNA by backsplicing of a downstream 5' splice site (splice donor) to an upstream 3' splice site (splice acceptor). Circular RNAs can be generated in this manner by any nonmammalian splicing method. For example, linear RNAs containing various types of introns, including self-splicing group I introns, self-splicing group II

introns, spliceosomal introns, and tRNA introns can be circularized. In particular, group I and group II introns have the advantage that they can be readily used for production of circular RNAs in vitro as well as in vivo because of their ability to undergo self-splicing due to their autocatalytic ribozyme activity.

[00451]In some embodiments, circular RNAs can be produced in vitro from a linear RNA by chemical or enzymatic ligation of the 5' and 3' ends of the RNA. Chemical ligation can be performed, for example, using cyanogen bromide (BrCN) or ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDC) for activation of a nucleotide phosphomonoester group to allow phosphodiester bond formation. See e.g., Sokolova (1988) FEBS Lett 232: 153-155; Dolinnaya et al. (1991) Nucleic Acids Res., 19:3067-3072; Fedorova (1996) Nucleosides Nucleotides Nucleic Acids 15: 1 137-1 147; herein incorporated by reference. Alternatively, enzymatic ligation can be used to circularize RNA. Exemplary ligases that can be used include T4 DNA ligase (T4 Dnl), T4 RNA ligase 1 (T4 Rnl 1), and T4 RNA ligase 2 (T4 Rnl 2).

[00452]In some embodiments, splint ligation using an oligonucleotide splint that hybridizes with the two ends of a linear RNA can be used to bring the ends of the linear RNA together for ligation. Hybridization of the splint, which can be either a DNA or a RNA, orientates the 5 '-phosphate and 3' -OH of the RNA ends for ligation. Subsequent ligation can be performed using either chemical or enzymatic techniques, as described above. Enzymatic ligation can be performed, for example, with T4 DNA ligase (DNA splint required), T4 RNA ligase 1 (RNA splint required) or T4 RNA ligase 2 (DNA or RNA splint). Chemical ligation, such as with BrCN or EDC, in some cases is more efficient than enzymatic ligation if the structure of the hybridized splint-RNA complex interferes with enzymatic activity.

[00453]In some embodiments, the oRNA may further comprise an internal ribosome entry site (IRES) operably linked to an RNA sequence encoding a polypeptide. Inclusion of an IRES permits the translation of one or more open reading frames from a circular RNA. The IRES element attracts a eukaryotic ribosomal translation initiation complex and promotes translation initiation. See, e.g., Kaufman et al., Nuc. Acids Res. (1991) 19:4485-4490; Gurtu et al., Biochem. Biophys. Res. Comm. (1996) 229:295-298; Rees et al., BioTechniques (1996) 20: 102-110; Kobayashi et al., BioTechniques (1996) 21 :399-402; and Mosser et al., BioTechniques 1997 22 150-161).

[00454]In some embodiments, the circularization efficiency of the circularization methods provided herein is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or 100%. In some embodiments, the circularization efficiency of the circularization methods provided herein is at least about 40%.

Splicing Element

[00455]In some embodiments, the oRNA includes at least one splicing element. The splicing element can be a complete splicing element that can mediate splicing of the oRNA or the spicing element can be a residual splicing element from a completed splicing event. For instance, in some cases, a splicing element of a linear RNA can mediate a splicing event that results in circularization of the linear RNA, thereby the resultant oRNA comprises a residual splicing element from such splicing-mediated circularization event. In some cases, the residual splicing element is not able to mediate any splicing. In other cases, the residual splicing element can still mediate splicing under certain circumstances. In some embodiments, the splicing element is adjacent to at least one expression sequence. In some embodiments, the oRNA includes a splicing element adjacent each expression sequence. In some embodiments, the splicing element is on one or both sides of each expression sequence, leading to separation of the expression products, e.g., peptide(s) and or polypeptide(s).

[00456]In some embodiments, the oRNA includes an internal splicing element that when replicated the spliced ends are joined together. Some examples may include miniature introns (<100 nt) with splice site sequences and short inverted repeats (30-40 nt) such as AluSq2, AluJr, and AluSz, inverted sequences in flanking introns, Alu elements in flanking introns, and motifs found in (suptable4 enriched motifs) cis-sequence elements proximal to backsplice events such as sequences in the 200 bp preceding (upstream of) or following (downstream from) a backsplice site with flanking exons. In some embodiments, the oRNA includes at least one repetitive nucleotide sequence described elsewhere herein as an internal splicing element. In such embodiments, the repetitive nucleotide sequence may include repeated sequences from the Alu family of introns. See, e.g., US Patent No. 11,058,706.

[00457]In some embodiments, the oRNA may include canonical splice sites that flank head-to-tail junctions of the oRNA.

[00458]In some embodiments, the oRNA may include a bulge-helix-bulge motif, comprising a 4-base pair stem flanked by two 3-nucleotide bulges. Cleavage occurs at a site in the bulge region, generating characteristic fragments with terminal 5'-hydroxyl group and 2', 3'-cyclic phosphate. Circularization proceeds by nucleophilic attack of the 5'-OH group onto the 2', 3'-cyclic phosphate of the same molecule forming a 3', 5'-phosphodiester bridge.

[00459]In some embodiments, the oRNA may include a sequence that mediates self-ligation. Non-limiting examples of sequences that can mediate self-ligation include a self-circularizing intron, e.g., a 5' and 3' slice junction, or a self-circularizing catalytic intron such as a Group I, Group II or Group III Introns. Non-limiting examples of group I intron self-splicing sequences may includeself-splicing permuted intron-exon sequences derived from T4 bacteriophage gene td, and the intervening sequence (IVS) rRNA of Tetrahymena.

Other Circularization Methods

[00460]In some embodiments, linear RNA may include complementary sequences, including either repetitive or nonrepetitive nucleic acid sequences within individual introns or across flanking introns. In some embodiments, the oRNA includes a repetitive nucleic acid sequence. In some embodiments, the repetitive nucleotide sequence includes poly CA or poly UG sequences. In some embodiments, the oRNA includes at least one repetitive nucleic acid sequence that hybridizes to a complementary repetitive nucleic acid sequence in another segment of the oRNA, with the hybridized segment forming an internal double strand. In some embodiments, repetitive nucleic acid sequences and complementary repetitive nucleic acid sequences from two separate oRNA that hybridize to generate a single oRNA, with the hybridized segments forming internal double strands. In some embodiments, the complementary sequences are found at the 5' and 3' ends of the linear RNA. In some embodiments, the complementary sequences include about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more paired nucleotides.

[00461]In some embodiments, chemical methods of circularization may be used to generate the oRNA. Such methods may include, but are not limited to click chemistry (e.g., alkyne- and azide-based methods, or clickable bases), olefin metathesis, phosphoramidate ligation, hemiaminal-imine crosslinking, base modification, and any combination thereof. In some embodiments, enzymatic methods of circularization may be used to generate the oRNA. In some embodiments, a ligation enzyme, e.g., DNA or RNA ligase, may be used to generate a template of the oRNA or complement, a complementary strand of the oRNA, or the oRNA.

[00462]Any of the circular polynucleotides as taught in for example U.S. Provisional Application No. 61/873,010 filed Sep. 3, 2013 or U.S. Patent No. 10,709,779, may be used herein. The contents of these references are incorporated herein by reference in their entirety. In addition, any of the circular RNAs, methods for making circular RNAs, circular RNA compositions that are described in the following publications are contemplated herein and are incorporated by reference in their entireties are part of the instant specification: US Patents US 11,352,640, US 11,352,641, US 11,203,767, US 10,683,498, US 5,773,244, and US 5,766,903; US Application Publications US 2022/0177540, US 2021/0371494, US 2022/0090137, US 2019/0345503, and US 2015/0299702; and PCT Application Publications WO 2021/226597, WO 2019/236673, WO 2017/222911, WO2016/187583, WO2014/082644, WO 1997/007825, and WO 2023/182948.

[00463]Reference is also made to the following scientific publications that provide information and details on the production of circular RNA molecules: (1) Chen X, Lu Y. Circular RNA: Biosynthesis *in vitro*. Front Bioeng Biotechnol. 2021 Nov 30;9:787881. doi: 10.3389/fbioe.2021.787881. PMID: 34917603; PMCID: PMC8670002; (2) Lee KH, Kim S, Lee SW. Pros and Cons of In Vitro Methods for Circular RNA Preparation. Int J Mol Sci. 2022 Oct 31;23(21):13247. doi: 10.3390/ijms232113247. PMID: 36362032; PMCID: PMC9654983; (3) Liu X,

Zhang Y, Zhou S, Dain L, Mei L, Zhu G. Circular RNA: An emerging frontier in RNA therapeutic targets, RNA therapeutics, and mRNA vaccines. J Control Release. 2022 Aug;348:84-94. doi: 10.1016/j.jconrel.2022.05.043. Epub 2022 Jun 2. PMID: 35649485; PMCID: PMC9644292; (4) Müller S, Appel B. In vitro circularization of RNA. RNA Biol. 2017 Aug 3;14(8):1018-1027. doi: 10.1080/15476286.2016.1239009. Epub 2016 Sep 26. PMID: 27668458; PMCID: PMC5680678; (5) Petkovic S, Müller S. Synthesis and Engineering of Circular RNAs. Methods Mol Biol. 2018;1724:167-180. doi: 10.1007/978-1-4939-7562-4_14. PMID: 29322449; (6) Welden JR, Stamm S. Pre-mRNA structures forming circular RNAs. Biochim Biophys Acta Gene Regul Mech. 2019 Nov-Dec;1862(11-12):194410. doi: 10.1016/j.bbagrm.2019.194410. Epub 2019 Aug 14. PMID: 31421281; and (7) Prats AC, David F, Diallo LH, Roussel E, Tatin F, Garmy-Susini B, Lacazette E. Circular RNA, the Key for Translation. Int J Mol Sci. 2020 Nov 14;21(22):8591. doi: 10.3390/ijms21228591. PMID: 33202605; PMCID: PMC7697609; each of which are incorporated herein by reference in their entireties.

D.Gene editing systems

[00464]As described herein in various embodiments, the LNPs of the present disclosure may comprise a gene editing system. As used herein, the term "gene editing system" generally refers to a composition having one or more gene editing system components which are capable of independently or co-dependently editing, modifying, or altering a target polynucleotide sequence or a target transcript comprising a nucleic acid sequence and/or modifying the epigenome to effect a change in gene regulation. Gene editing systems for the present disclosure include any editing systems known in the art. Gene editing systems, nucleobase editing systems, nucleobase editing tools and similar terms throughout should be understood to refer to interchangeable concepts. As described herein, all references to LNPs comprising gene editing systems should be understood to also contemplate LNPs comprising polynucleotides encoding said gene editing systems.

[00465]For example, the LNP compositions herein may be used to deliver any gene editing system including CRISPR (clustered regularly interspaced short palindromic repeats) and the associated CRISPR-associated proteins (e.g., CRISPR-Cas9) (Jinek *et al.*, "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity," *Science*, Vol. 337 (6096), pp. 816-821), meganuclease editors (Boissel et al., "megaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering," *Nucleic Acids Research* 42: pp. 2591-2601) and bacterial retron systems (Schubert *et al.*, "High-throughput functional variant screens via in vivo production of single-stranded DNA," *PNAS*, April 27, 2021, Vol. 118(18), pp. 1-10). In particular, CRISPR-Cas9 has been derivatized in numerous ways to expand upon its guide RNA-based programmable double-strand cutting activity to form systems ranging from finding alternative CRISPR Cas nuclease enzymes having different PAM requirements and cutting properties (e.g., Cas12a, Cas12f, Cas13a, and Cas13b) to base editing (Komor *et al.*, "Programmable editing of a target base in genomic DNA

without double-stranded DNA cleavage," *Nature*, May 19, 2016, 533 (7603); pp. 420-424 [cytosine base editors or CBEs] and Gaudelli *et al.*, "Programmable base editing of A-T to G-C in genomic DNA without DNA cleavage," *Nature*, Vol. 551, pp. 464-471 [adenine base editors or ABEs]) to prime editing (Anzalone *et al.*, "Search-and-replace genome editing without double-strand breaks or donor DNA," *Nature*, Dec 2019, 576 (7789): pp. 149-157) to twin prime editing (Anzalone *et al.*, "Programmable deletion, replacement, integration and inversion of large DNA sequences with twin prime editing," *Nature Biotechnology*, Dec 9, 2021, vol. 40, pp. 731-740) to epigenetic editing (Kungulovski and Jeltsch, "Epigenome Editing: State of the Art, Concepts, and Perspective," *Trends in Genetics,* Vol.32, 206, pp. 101-113) to CRISPR-directed integrase editing (Yarnell *et al.*, "Drag-and-drop genome insertion of large sequences without double-stranded DNA cleavage using CRISPR-directed integrases," *Nature Biotechnology*, Nov 24, 2022, ("PASTE"). Each of these gene editing systems may be packaged up in the LNP compositions described herein and delivered to target organs, tissues, and cells to bring about the modification of a target sequence or the expression of a target gene.

[00466]The gene editing systems deliverable by the herein disclosed LNPs can be any gene editing system. The gene editing systems contemplated herein can include (A) nucleobase gene editing systems which result in one or more the modifications to the *sequence* of target nucleic acid molecule, (B) an epigenetic editing system which results in one or more modifications to the *epigenome* to bring about an effect on gene expression without altering the sequence of a nucleic acid molecule, and (C) gene editing systems that combine the features of nucleobase editing systems and epigenetic editing systems.

[00467]Nucleobase editing systems include a wide array of configurations with various combinations of protein functionalities and/or nucleic acid molecule components, all of which are contemplated herein. In general, nucleobase editing systems comprise at least a (i) DNA binding domain that is user-programmable to target a specific sequence in a nucleic acid molecule and optionally (ii) one or more effector domains that facilitate the modification of the sequence of the nucleic acid molecule. User-programmability may comprise amino acid sequence-programmable DNA binding domains (e.g., TALENS and zinc finger-binding domains) or nucleic acid sequence-programmable DNA binding domains (e.g., CRISPR Cas9, CRISPR Cas12a, CRISPR Cas12f, CRISPR Cas13a, CRISPR Cas13b, IscB, IsrB, or TnpB). Similarly, epigenetic editing systems comprise at least a (i) DNA binding domain that targets a specific sequence in a nucleic acid molecule and (ii) one or more effector domains that facilitates the modification of one or more epigenomic features of the nucleic acid molecule.

[00468]Gene editing systems may also comprise one or more effector domains that provide various functionalities that facilitate changes in nucleotide sequence and/or gene expression, such as, but not limited to, single-strand DNA binding proteins, nucleases, endonucleases, exonucleases, deaminases (e.g., cytidine deaminases or adenosine deaminases), polymerases (e.g., reverse transcriptases),

integrases, recombinases, etc., and fusion proteins comprising one or more functional domains linked together). In certain embodiments, the nucleobase editing systems include, but are not limited to, systems comprising a clustered regularly interspaced short palindromic repeats ("CRISPR")-associated ("Cas") protein, a zinc finger nuclease ("ZFN"), a transcription activator-like effector nuclease ("TALEN"), an adenosine deaminase acting on RNA ("ADAR") enzyme, an adenosine deaminase acting on transfer RNA ("ADAT") enzyme, an activation induced cytidine deaminase ("AID")/ apolipoprotein B editing complex ("APOBEC") enzyme, a meganuclease, IscB, IsrB, TnpB, or a restriction enzyme.

[00469]In some embodiments, the nucleobase editing system edits, modifies, or alters the target polynucleotide sequence *ex vivo*. In some embodiments, the nucleobase editing system edits, modifies, or alters the target polynucleotide sequence *in vivo*. In some embodiments, the nucleobase editing system edits, modifies, or alters the target polynucleotide sequence in a cell. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell.

[00470]In some embodiments, the target polynucleotide sequence is a gene or a regulatory sequence that controls transcription of a gene (e.g., a promoter, transcription binding site, enhancer sequence, etc.) or a sequence which controls the translation of a messenger RNA. In some embodiments, the target transcript comprising a nucleic acid sequence is a product of gene transcription. In some embodiments, the target transcript comprising a nucleic acid sequence is an RNA transcript such as a messenger RNA transcript, microRNA transcript or transfer RNA transcript.

[00471]The originator constructs and benchmark constructs of the present disclosure may comprise, encode or be conjugated to a cargo which is a nucleobase editing tool. As used herein, the term "nucleobase editing tool" is used interchangeably with "nucleobase editing system component" and generally refers to a compound or substance which is capable of independently or co-dependently editing, modifying, or altering a target polynucleotide sequence or a target transcript comprising a nucleic acid sequence. Nucleobase editing tools for the present disclosure include all nucleobase editing tools known in the art. In certain embodiments, the nucleobase editing tools include, but not limited to, effector proteins which modify DNA or RNA, guide elements which guide effector proteins to specific DNA or RNA sequence, repair elements which encode a nucleic acid sequence template, and supportive elements which activate or modulate the activity of another nucleobase editing tool, or activates or modulates host DNA repair enzymes.

[00472]In some embodiments, the cargo may comprise a nucleobase editing tool or a polynucleotide encoding a nucleobase editing tool. In some embodiments, the cargo may comprise one or more polynucleotides encoding a nucleobase editing tool. In some embodiments, the cargo may comprise a polynucleotide encoding one or more nucleobase editing tools. In some embodiments, the cargo may comprise a polynucleotide that is a component of the nucleobase editing tool. In some embodiments, the cargo may comprise a polynucleotide encoding one or more protein or peptide components in the nucleobase editing tool.

[00473]In some embodiments, the cargo may comprise an effector protein capable of modifying a target DNA or RNA sequence. In some embodiments, the cargo may comprise a polynucleotide encoding an effector protein. In certain embodiments, the effector proteins include polymerases, nucleases, mutator enzymes, reverse transcriptases, recombinases, integrases, endonucleases, exonucleases, transposases, and deaminases. As used herein, the term "polymerases," includes enzymes which catalyze the synthesis of DNA or RNA polymers. As used herein, the term "nucleases," includes enzymes which cleave nucleobases. In certain embodiments, nucleases include enzymes which create single-stranded breaks ("SSB") or double-stranded breaks ("DSB") in nucleic acid sequences. As used herein, the term "mutator enzymes," in its broadest sense, includes enzymes which mutate nucleic acid sequences. In certain embodiments, the cargo may comprise nucleases such as effector proteins include clustered regularly interspaced short palindromic repeats ("CRISPR")-associated ("Cas") proteins, zinc finger nucleases ("ZFNs"), transcription activator-like effector nucleases ("TALENs"), adenosine deaminase acting on RNA ("ADAR") enzymes, adenosine deaminase acting on transfer RNA ("ADAT") enzymes, activation induced cytidine deaminase ("AID")/ apolipoprotein B editing complex ("APOBEC") enzymes, meganucleases, IscB, IsrB, TnpB, or restriction enzymes.

[00474]In some embodiments, the cargo may comprise a guide element which guide effector proteins to target a DNA or RNA sequence. In some embodiments, the cargo may comprise a polynucleotide encoding a guide element. In certain embodiments, guide elements include guide RNAs ("gRNAs"), CRISPR RNAs ("crRNAs"), prime editing guide RNAs ("pegRNAs"), transcription activator-like effectors (TALEs), or antisense oligomers.

[00475]In some embodiments, the cargo may further comprise a repair element which encodes a sequence repair template. In some embodiments, the cargo may further comprise a polynucleotide encoding a repair element or sequence repair template.

[00476]In some embodiments, the cargo may further comprise a supportive element which activates or modulates the editing system. In some embodiments, the cargo may further comprise a supportive element which activates or modulates the effector protein. In some embodiments, the cargo may further comprise a polynucleotide encoding a supportive element. Non-limiting categories of supportive elements include trans-activating RNA ("tracrRNA").

CRISPR-Cas editors

[00477]In some embodiments, the LNPs may be used to deliver a CRISPR-Cas gene editing system comprising a CRISPR-Cas programmable nuclease, such as a CRISPR-Cas9 or CRISPR-Cas12a nuclease.

[00478]In general, nucleobase editing systems comprise at least a (i) DNA binding domain that is user-programmable to target a specific sequence in a nucleic acid molecule and optionally (ii) one or more effector domains that facilitate the modification of the sequence of the nucleic acid molecule. User-programmability may comprise amino acid sequence-programmable DNA binding domains

(e.g., TALENS and zinc finger-binding domains) or nucleic acid sequence-programmable DNA binding domains (e.g., CRISPR Cas9, CRISPR Cas12a, CRISPR Cas12f, CRISPR Cas13a, CRISPR Cas13b, or TnpB), and including a guide RNA which targets the programmable DNA binding protein to target sequence.

[00479]In some embodiments, the CRISPR-Cas system comprises a Cas or Cas-derived protein.

[00480]In other embodiments, the sequence-programmable DNA binding domains (e.g., RNA-guided nuclease) used for genome modification is a clustered regularly interspersed short palindromic repeats (CRISPR) system Cas nuclease. Any RNA-guided Cas nuclease capable of catalyzing site- directed cleavage of DNA to allow integration of donor polynucleotides by the HDR mechanism can be used in genome editing, including CRISPR system Class 1, Type I, II, or III Cas nucleases; Class 2, Type II nuclease (such as Cas9); a Class 2, Type V nuclease (such as Cpfl), or a Class 2, Type VI nuclease (such as C2c2). Examples of Cas proteins include Casl, CaslB, Cas2, Cas3, Cas4, Cas5, Cas5e (CasD), Cas6, Cas6e, Cas6f, Cas7, Cas8al, Cas8a2, Cas8b, Cas8c, Cas9 (Csnl or Csxl2), CaslO, CaslOd, CasF, CasG, CasH, Csyl, Csy2, Csy3, Csel (CasA), Cse2 (CasB), Cse3 (CasE), Cse4 (CasC), Cscl, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmrl, Cmr3, Cmr4, Cmr5, Cmr6, Csbl, Csb2, Csb3, Csxl7, Csxl4, CsxlO, Csxl6, CsaX, Csx3, Csxl, Csxl5, Csfl, Csf2, Csf3, Csf4, and Cul966, and homologs or modified versions thereof. In some embodiments, an LNP of the present disclosure comprises gene editing system is or comprises a Type V nuclease editing system described in International Application Publication WO2024020346A1, which is incorporated by reference herein in its entirety.

[00481]In some embodiments, a Class 1, type II CRISPR system Cas9 endonuclease is used. Cas9 nucleases from any species, or biologically active fragments, variants, analogs, or derivatives thereof that retain Cas9 endonuclease activity (*i.e.*, catalyze site-directed cleavage of DNA to generate double-strand breaks) may be used to perform genome modification as described herein. The Cas9 need not be physically derived from an organism but may be synthetically or recombinantly produced. Cas9 sequences from a number of bacterial species are well known in the art and listed in the National Center for Biotechnology Information (NCBI) database. See, for example, NCBI entries for Cas9 from: *Streptococcus pyogenes* (WP 002989955, WP_038434062, WP_011528583); *Campylobacter jejuni* (WP_022552435, YP 002344900), *Campylobacter coli* (WP 060786116); *Campylobacter fetus* (WP 059434633); *Corynebacterium ulcerans* (NC_015683, NC_017317); *Corynebacterium diphtheria* (NC_016782, NC_016786); *Enterococcus faecalis* (WP 033919308); *Spiroplasma syrphidicola* (NC 021284); *Prevotella intermedia* (NC 017861); *Spiroplasma taiwanense* (NC 021846); *Streptococcus iniae* (NC 021314); *Belliella baltica* (NC 018010); *Psychroflexus torquisl* (NC O 18721); *Streptococcus thermophilus* (YP 820832), *Streptococcus mutans* (WP 061046374, WP 024786433); *Listeria innocua* (NP 472073); *Listeria monocytogenes* (WP 061665472); *Legionella pneumophila* (WP 062726656); *Staphylococcus aureus* (WP_001573634); *Francisella tularensis* (WP_032729892, WP_014548420), *Enterococcus faecalis* (WP 033919308); *Lactobacillus*

rhamnosus (WP 048482595, WP_032965177); and *Neisseria meningitidis* (WP_061704949, YP_002342100); all of which sequences (as entered by the date of filing of this application) are herein incorporated by reference in their entireties. Any of these sequences or a variant thereof comprising a sequence having at least about 70-100% sequence identity thereto, including any percent identity within this range, such as 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity thereto, can be used for genome editing, as described herein. See also Fonfara *et al.* (2014) Nucleic Acids Res. 42(4):2577-90; Kapitonov *et al.* (2015) J. Bacterid. 198(5): 797-807, Shmakov *et al.* (2015) Mol. Cell. 60(3):385- 397, and Chylinski *et al.* (2014) Nucleic Acids Res. 42(10):6091-6105); for sequence comparisons and a discussion of genetic diversity and phylogenetic analysis of Cas9.

[00482]In another embodiment, the gene editing system delivered by the LNP-based RNA medicines described herein may comprise a CRISPR-Cas12a (Cpf1) nuclease. Cpf1 was first identified from *Prevotella* and *Francisella* 1 (Cpfl, or Cas12a) and published in Zetsche et al., "Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system," Cell, October 22, 2015, 163, pp. 759-711, which is incorporated herein by reference. Cpfl is another class II CRISPR/Cas system RNA-guided nuclease with similarities to Cas9 and may be used analogously. Unlike Cas9, Cpfl does not require a tracrRNA and only depends on a crRNA in its guide RNA, which provides the advantage that shorter guide RNAs can be used with Cpfl for targeting than Cas9. Cpfl is capable of cleaving either DNA or RNA. The PAM sites recognized by Cpfl have the sequences 5′-YTN-3′ (where "Y" is a pyrimidine and "N" is any nucleobase) or 5′-TTN-3′, in contrast to the G-rich PAM site recognized by Cas9. Cpfl cleavage of DNA produces double-stranded breaks with a sticky-ends having a 4 or 5 nucleotide overhang. For a discussion of Cpfl, see, *e.g.*, Ledford *et al.* (2015) Nature. 526 (7571):17-17, Zetsche *et al.* (2015) Cell. 163 (3):759-771, Murovec *et al.* (2017) Plant Biotechnol. J. 15(8):917-926, Zhang *et al.* (2017) Front. Plant Sci. 8:177, Fernandes *et al.* (2016) Postepy Biochem. 62(3):315-326; herein incorporated by reference.

[00483]The gene editing systems described herein can include any known Cas12a nuclease, or any variant thereof, such as any Cas12a ortholog described in US Patent Application No. 18/297,346, US Patent Application No. 18/481,393, or Intern International Application Publication WO2024020346A1, or an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 99%, or up to 100% sequence identity with any of the Cas12a orthologs described in said patent applications.

[00484]The gene editing systems described herein can include any known Cas12a nuclease, or any variant thereof, such as any Cas12a ortholog described in (1) Wu J, Gao P, Shi Y, Zhang C, Tong X, Fan H, Zhou X, Zhang Y, Yin H. Characterization of a thermostable Cas12a ortholog. Cell Insight. 2023 Oct 11;2(6):100126. doi: 10.1016/j.cellin.2023.100126. PMID: 38047138; PMCID: PMC10692460; (2) Swarts DC, Jinek M. Cas9 versus Cas12a/Cpf1: Structure-function comparisons and implications for genome editing. Wiley Interdiscip Rev RNA. 2018 Sep;9(5):e1481. doi: 10.1002/wrna.1481. Epub 2018 May 22. PMID: 29790280; (3) Kleinstiver BP, Sousa AA, Walton

RT, Tak YE, Hsu JY, Clement K, Welch MM, Horng JE, Malagon-Lopez J, Scarfò I, Maus MV, Pinello L, Aryee MJ, Joung JK. Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing. Nat Biotechnol. 2019 Mar;37(3):276-282. doi: 10.1038/s41587-018-0011-0. Epub 2019 Feb 11. Erratum in: Nat Biotechnol. 2020 Jul;38(7):901. PMID: 30742127; PMCID: PMC6401248; (4) Ma E, Chen K, Shi H, Stahl EC, Adler B, Trinidad M, Liu J, Zhou K, Ye J, Doudna JA. Improved genome editing by an engineered CRISPR-Cas12a. Nucleic Acids Res. 2022 Dec 9;50(22):12689-12701. doi: 10.1093/nar/gkac1192. PMID: 36537251; PMCID: PMC9825149; (5) Li T, Zhu L, Xiao B, Gong Z, Liao Q, Guo J. CRISPR-Cpf1-mediated genome editing and gene regulation in human cells. Biotechnol Adv. 2019 Jan-Feb;37(1):21-27. doi: 10.1016/j.biotechadv.2018.10.013. Epub 2018 Nov 3. PMID: 30399413 (6); Jacobsen T, Liao C, Beisel CL. The Acidaminococcus sp. Cas12a nuclease recognizes GTTV and GCTV as non-canonical PAMs. FEMS Microbiol Lett. 2019 Apr 1;366(8):fnz085. doi: 10.1093/femsle/fnz085. PMID: 31004485; PMCID: PMC6604746; (7) Huang H, Huang G, Tan Z, Hu Y, Shan L, Zhou J, Zhang X, Ma S, Lv W, Huang T, Liu Y, Wang D, Zhao X, Lin Y, Rong Z. Engineered Cas12a-Plus nuclease enables gene editing with enhanced activity and specificity. BMC Biol. 2022 Apr 25;20(1):91. doi: 10.1186/s12915-022-01296-1. PMID: 35468792; PMCID: PMC9040236; (8) Zhu D, Wang J, Yang D, Xi J, Li J. High-Throughput Profiling of Cas12a Orthologues and Engineered Variants for Enhanced Genome Editing Activity. Int J Mol Sci. 2021 Dec 10;22(24):13301. doi: 10.3390/ijms222413301. PMID: 34948095; PMCID: PMC8706968; (9) Gao L, Cox DBT, Yan WX, Manteiga JC, Schneider MW, Yamano T, Nishimasu H, Nureki O, Crosetto N, Zhang F. Engineered Cpf1 variants with altered PAM specificities. Nat Biotechnol. 2017 Aug;35(8):789-792. doi: 10.1038/nbt.3900. Epub 2017 Jun 5. PMID: 28581492; PMCID: PMC5548640; (10) Zhang L, Zuris JA, Viswanathan R, Edelstein JN, Turk R, Thommandru B, Rube HT, Glenn SE, Collingwood MA, Bode NM, Beaudoin SF, Lele S, Scott SN, Wasko KM, Sexton S, Borges CM, Schubert MS, Kurgan GL, McNeill MS, Fernandez CA, Myer VE, Morgan RA, Behlke MA, Vakulskas CA. AsCas12a ultra nuclease facilitates the rapid generation of therapeutic cell medicines. Nat Commun. 2021 Jun 23;12(1):3908. doi: 10.1038/s41467-021-24017-8. Erratum in: Nat Commun. 2021 Jul 19;12(1):4500. PMID: 34162850; PMCID: PMC8222333; (11) Ling X, Chang L, Chen H, Gao X, Yin J, Zuo Y, Huang Y, Zhang B, Hu J, Liu T. Improving the efficiency of CRISPR-Cas12a-based genome editing with site-specific covalent Cas12a-crRNA conjugates. Mol Cell. 2021 Nov 18;81(22):4747-4756.e7. doi: 10.1016/j.molcel.2021.09.021. Epub 2021 Oct 13. PMID: 34648747; and (12) Zhou J, Chen P, Wang H, Liu H, Li Y, Zhang Y, Wu Y, Paek C, Sun Z, Lei J, Yin L. Cas12a variants designed for lower genome-wide off-target effect through stringent PAM recognition. Mol Ther. 2022 Jan 5;30(1):244-255. doi: 10.1016/j.ymthe.2021.10.010. Epub 2021 Oct 20. PMID: 34687846; PMCID: PMC8753454; each of which are incorporated by reference in their entireties.

[00485]Any publicly known Cas12a/Cpf1 may be used as a component of the gene editing systems described herein, including but not limited to, the following publicly available amino acid sequences: GenBank Accession No. QOE76968.1; GenBank Accession No. WKU83685.1; GenBank Accession No. WBC51234.1; GenBank Accession No. QOL02411.1; GenBank Accession No. UVJ64960.1; GenBank Accession No. UVJ64958.1; GenBank Accession No. UVJ64957.1; GenBank Accession No. UVJ64956.1; GenBank Accession No. UVJ64954.1; GenBank Accession No. UVJ64953.1; GenBank Accession No. UVJ64952.1; GenBank Accession No. UVJ64951.1; GenBank Accession No. UVJ64949.1; GenBank Accession No. UVJ64947.1; GenBank Accession No. UVJ64946.1; GenBank Accession No. UVJ64945.1; GenBank Accession No. WP_320869194.1; GenBank Accession No. WOZ85592.1; GenBank Accession No. WOF96262.1; and any amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 99%, or up to 100% sequence identity with any of the aforementioned sequence, or any other publicly available Cas12a sequence.

[00486]C2c1 (Cas12b) is another class II CRISPR/Cas system RNA-guided nuclease that may be used. C2c1, similarly to Cas9, depends on both a crRNA and tracrRNA for guidance to target sites. See, *e.g.*, Shmakov *et al.* (2015) Mol Cell. 60(3):385-397, Zhang *et al.* (2017) Front Plant Sci. 8:177; herein incorporated by reference.

[00487]In one aspect, a nucleic acid sequence-programmable DNA binding domain can be associated with or complexed with at least one guide nucleic acid (e.g., guide RNA or a pegRNA), which localizes the DNA binding domain to a DNA sequence that comprises a DNA strand (i.e., a target strand) that is complementary to the guide nucleic acid, or a portion thereof (e.g., the spacer of a guide RNA which anneals to the protospacer of the DNA target). In other words, the guide nucleic-acid "programs" the DNA binding domain (e.g., Cas9 or equivalent) to localize and bind to complementary sequence of the protospacer in the DNA.

[00488]Any suitable nucleic acid sequence-programmable DNA binding domain may be used in the prime editors described herein. In various embodiments, the nucleic acid sequence-programmable DNA binding domain may be any Class 2 CRISPR-Cas system, including any type II, type V, or type VI CRISPR-Cas enzyme. Given the rapid development of CRISPR-Cas as a tool for genome editing, there have been constant developments in the nomenclature used to describe and/or identify CRISPR-Cas enzymes, such as Cas9 and Cas9 orthologs. CRISPR-Cas nomenclature is extensively discussed in Makarova et al., "Classification and Nomenclature of CRISPR-Cas Systems: Where from Here?," The CRISPR Journal, Vol.1. No.5, 2018, the entire contents of which are incorporated herein by reference.

[00489]Without being bound by theory, the mechanism of action of certain CRISPR Cas enzymes contemplated herein includes the step of forming an R-loop whereby the Cas protein induces the unwinding of a double-strand DNA target, thereby separating the strands in the region bound by the Cas protein. The guide RNA spacer then hybridizes to the "target strand" at a region that is complementary to the protospacer sequence of the DNA. In some embodiments, the Cas protein may

include one or more nuclease activities, which then cut the DNA leaving various types of lesions. For example, the Cas protein may comprises a nuclease activity that cuts the non-target strand at a first location, and/ or cuts the target strand at a second location. Depending on the nuclease activity, the target DNA can be cut to form a "double-stranded break" whereby both strands are cut. In other embodiments, the target DNA can be cut at only a single site, i.e., the DNA is "nicked" on one strand. Exemplary Cas proteins with different nuclease activities include "Cas9 nickase" ("nCas9") and a deactivated Cas9 having no nuclease activities ("dead Cas9" or "dCas9").

[00490]The below description of various Cas proteins which can be used in connection with the presently disclosed LNP-delivered gene editing systems is not meant to be limiting in any way. The gene editing systems may comprise the canonical SpCas9 or Cas12a, or any ortholog Cas9 protein or Cas12a protein, or any variant Cas9 or Cas12a protein—including any naturally occurring variant, mutant, or otherwise engineered version of Cas9—that is known or which can be made or evolved through a directed evolutionary or otherwise mutagenic process. In various embodiments, the Cas9 or Cas9 variants have a nickase activity, i.e., only cleave one strand of the target DNA sequence. In other embodiments, the Cas9 or Cas9 variants have inactive nucleases, i.e., are "dead" Cas9 proteins. Other variant Cas9 proteins that may be used are those having a smaller molecular weight than the canonical SpCas9 (e.g., for easier delivery) or having modified or rearranged primary amino acid structure.

[00491]The gene editing systems described herein may also comprise Cas9 equivalents, including Cas12a (Cpf1) and Cas12b1 proteins. The Cas proteins usable herein (e.g., SpCas9, Cas9 variant, or Cas9 equivalents) may also contain various modifications that alter/enhance their PAM specificities. The present disclosure contemplates any Cas9, Cas9 variant, or Cas9 equivalent which has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.9% sequence identity to a reference Cas9 sequence, such as a reference SpCas9 canonical sequence of *Streptococcus pyogenes* M1 (Accession No. Q99ZW2) (SEQ ID NO: 52).

[00492]The Cas proteins contemplated herein embrace CRISPR Cas 9 proteins, as well as Cas9 equivalents, variants (e.g., Cas9 nickase (nCas9) or nuclease inactive Cas9 (dCas9)) homologs, orthologs, or paralogs, whether naturally occurring or non-naturally occurring (e.g., engineered or recombinant), and may include a Cas9 equivalent from any Class 2 CRISPR system (e.g., type II, V, VI), including Cas12a (Cpf1), Cas12e (CasX), Cas12b1 (C2c1), Cas12b2, Cas12c (C2c3), C2c4, C2c8, C2c5, C2c10, C2c9 Cas13a (C2c2), Cas13d, Cas13c (C2c7), Cas13b (C2c6), and Cas13b. Further Cas-equivalents are described in Makarova et al., "C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector," Science 2016; 353(6299) and Makarova et al., "Classification and Nomenclature of CRISPR-Cas Systems: Where from Here?," The CRISPR Journal, Vol.1. No.5, 2018, the contents of which are incorporated herein by reference.

[00493]The terms "Cas9" or "Cas9 nuclease" or "Cas9 moiety" or "Cas9 domain" embrace any naturally occurring Cas9 from any organism, any naturally-occurring Cas9 equivalent or functional

fragment thereof, any Cas9 homolog, ortholog, or paralog from any organism, and any mutant or variant of a Cas9, naturally-occurring or engineered. The term Cas9 is not meant to be particularly limiting and may be referred to as a "Cas9 or equivalent." Exemplary Cas9 proteins are further described in the art and are incorporated herein by reference. As noted herein, Cas9 nuclease sequences and structures are well known to those of skill in the art (see, e.g., "Complete genome sequence of an M1 strain of Streptococcus pyogenes." Ferretti et al., J.J., McShan W.M., Ajdic D.J., Savic D.J., Savic G., Lyon K., Primeaux C., Sezate S., Suvorov A.N., Kenton S., Lai H.S., Lin S.P., Qian Y., Jia H.G., Najar F.Z., Ren Q., Zhu H., Song L., White J., Yuan X., Clifton S.W., Roe B.A., McLaughlin R.E., Proc. Natl. Acad. Sci. U.S.A.98:4658-4663(2001); "CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III." Deltcheva E., Chylinski K., Sharma C.M., Gonzales K., Chao Y., Pirzada Z.A., Eckert M.R., Vogel J., Charpentier E., Nature 471:602-607(2011); and "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J.A., Charpentier E.Science 337:816-821(2012), the entire contents of each of which are incorporated herein by reference).

[00494]In certain embodiments, a polynucleotide programmable nucleotide binding domain of a nucleobase editor itself comprises one or more domains. In one embodiment, a polynucleotide programmable nucleotide binding domain comprises one or more nuclease domains. In some embodiments, the nuclease domain of a polynucleotide programmable nucleotide binding domain comprises an endonuclease or an exonuclease. In some embodiments, the endonuclease cleaves a single strand of a double-stranded nucleobase. In some embodiments, the endonuclease cleaves both strands of a double-stranded nucleobase molecule. In some embodiments, the polynucleotide programmable nucleotide binding domain is a deoxyribonuclease. In some embodiments, the polynucleotide programmable nucleotide binding domain is a ribonuclease.

[00495]In some embodiments, the nuclease domain of a polynucleotide programmable nucleotide binding domain can cut zero, one, or two strands of a target polynucleotide. In some embodiments, the polynucleotide programmable nucleotide binding domain comprises a nickase domain. Herein the term "nickase" refers to a polynucleotide programmable nucleotide binding domain comprising a nuclease domain that is capable of cleaving only one strand of the two strands in a duplexed nucleobase molecule (e.g., DNA). In some embodiments, the nickase is derived from a fully catalytically active (e.g., natural) form of a polynucleotide programmable nucleotide binding domain by introducing one or more mutations into the active polynucleotide programmable nucleotide binding domain. In certain embodiments, where a polynucleotide programmable nucleotide binding domain comprises a nickase domain derived from Cas9. Cas12a and Cas9 nickases are known in the art and contemplated for use herein, for example as discussed in (1) Schubert MS, Thommandru B, Woodley J, Turk R, Yan S, Kurgan G, McNeill MS, Rettig GR. Optimized design parameters for CRISPR Cas9 and Cas12a homology-directed repair. Sci Rep. 2021 Sep 30;11(1):19482. doi: 10.1038/s41598-021-98965-y. PMID: 34593942; PMCID: PMC8484621; (2) Fu BXH, Smith JD,

Fuchs RT, Mabuchi M, Curcuru J, Robb GB, Fire AZ. Target-dependent nickase activities of the CRISPR-Cas nucleases Cpf1 and Cas9. Nat Microbiol. 2019 May;4(5):888-897. doi: 10.1038/s41564-019-0382-0. Epub 2019 Mar 4. PMID: 30833733; PMCID: PMC6512873; (3) Xu T, Tao X, Kempher ML, Zhou J. Cas9 Nickase-Based Genome Editing in Clostridium cellulolyticum. Methods Mol Biol. 2022;2479:227-243. doi: 10.1007/978-1-0716-2233-9_15. PMID: 35583742; (4) Wu WH, Ma XM, Huang JQ, Lai Q, Jiang FN, Zou CY, Chen LT, Yu L. CRISPR/Cas9 (D10A) nickase-mediated Hb CS gene editing and genetically modified fibroblast identification. Bioengineered. 2022 May;13(5):13398-13406. doi: 10.1080/21655979.2022.2069940. PMID: 36700476; PMCID: PMC9276056; each of which are incorporated herein by reference in their entireties.

[00496]In some embodiments, the Cas9-derived nickase has one or more mutations in the RuvC-1 domain. In one embodiment, the Cas9-derived nickase has a D10A mutation in the RuvC-1 domain. In some embodiments, the Cas9-derived nickase has one or more mutations in the REC Lobe domain. In one embodiment, the Cas9-derived nickase has a N497A, R661A, and/or Q695A mutation in the REC Lobe domain. In some embodiment, the Cas9-derived nickase has one or more mutations in the HNH domain. In one embodiment, the Cas9-derived nickase has H840A, N863A, and/or D839A in the HNH domain.

[00497]In certain embodiments, in the SpCas9-derived nickase, the residue H840 retains catalytic activity and can thereby cleave a single strand of the nucleobase duplex. In certain embodiments, a Cas9-derived nickase domain can comprise an H840A mutation, while the amino acid residue at position 10 remains a D. In certain embodiments, a Cas9-derived nickase domain can comprise an N863A mutation, while the amino acid residue at position 10 remains a D. In some embodiments, the nickase is derived from a fully catalytically active (e.g., natural) form of a polynucleotide programmable nucleotide binding domain by removing all or a portion of a nuclease domain that is not required for the nickase activity. In certain embodiments, where a polynucleotide programmable nucleotide binding domain comprises a nickase domain derived from Cas9, the Cas9-derived nickase domain comprises a deletion of all or a portion of the RuvC domain or the HNH domain.

[00498]Any of the above CRISPR-Cas editor embodiments or any variants, modifications, or derivatives thereof are contemplated herein to be delivered by the LNP systems disclosed in this specification for gene editing in cells, tissues, and/or organs under *in vitro*, *ex vivo*, or *in vivo* conditions. The various components described herein may be configured and delivered in any suitable manner. Any of the descriptions presented in this section are not intended to be strictly limiting.

Base editors

[00499]In other embodiments, the LNPs may be used to deliver a base editing system. Base editors are generally composed of an engineered deaminase and a catalytically impaired CRISPR–Cas9 variant and enzymatically convert one base to another base at a specific target site with the assistance of endogenous DNA repair systems in the cell. However, base editors may also comprise Cas12a

enzymes and/or other programmable nucleases. For example, Cas12a-configured base editors are described in (1) Chen F, Lian M, Ma B, Gou S, Luo X, Yang K, Shi H, Xie J, Ge W, Ouyang Z, Lai C, Li N, Zhang Q, Jin Q, Liang Y, Chen T, Wang J, Zhao X, Li L, Yu M, Ye Y, Wang K, Wu H, Lai L. Multiplexed base editing through Cas12a variant-mediated cytosine and adenine base editors. Commun Biol. 2022 Nov 2;5(1):1163. doi: 10.1038/s42003-022-04152-8. PMID: 36323848; PMCID: PMC9630288; (2) Wang X, Ding C, Yu W, Wang Y, He S, Yang B, Xiong YC, Wei J, Li J, Liang J, Lu Z, Zhu W, Wu J, Zhou Z, Huang X, Liu Z, Yang L, Chen J. Cas12a Base Editors Induce Efficient and Specific Editing with Low DNA Damage Response. Cell Rep. 2020 Jun 2;31(9):107723. doi: 10.1016/j.celrep.2020.107723. PMID: 32492431; and (3) Swartjes T, Staals RHJ, van der Oost J. Editor's cut: DNA cleavage by CRISPR RNA-guided nucleases Cas9 and Cas12a. Biochem Soc Trans. 2020 Feb 28;48(1):207-219. doi: 10.1042/BST20190563. PMID: 31872209; PMCID: PMC7054755; (4) Gaillochet C, Peña Fernández A, Goossens V, D'Halluin K, Drozdzecki A, Shafie M, Van Duyse J, Van Isterdael G, Gonzalez C, Vermeersch M, De Saeger J, Develtere W, Audenaert D, De Vleesschauwer D, Meulewaeter F, Jacobs TB. Systematic optimization of Cas12a base editors in wheat and maize using the ITER platform. Genome Biol. 2023 Jan 13;24(1):6. doi: 10.1186/s13059-022-02836-2. PMID: 36639800; PMCID: PMC9838060; each of which are incorporated herein by reference in their entireties.

[00500]Base editing was first described in Komor et al., "Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage," Nature, May 19, 2016, 533 (7603); pp. 420-424 in the form of cytosine base editors or CBEs followed by the disclosure of Gaudelli et al., "Programmable base editing of A-T to G-C in genomic DNA without DNA cleavage," Nature, Vol. 551, pp. 464-471 describing adenine base editors or ABEs. Subsequently, base editing has been described in numerous scientific publications, including, but not limited to (i) Kim JS. Precision genome engineering through adenine and cytosine base editing. Nat Plants. 2018 Mar;4(3):148-151. doi: 10.1038/s41477-018-0115-z. Epub 2018 Feb 26. PMID: 29483683.; (ii) Wei Y, Zhang XH, Li DL. The "new favorite" of gene editing technology-single base editors. Yi Chuan. 2017 Dec 20;39(12):1115-1121. doi: 10.16288/j.yczz.17-389. PMID: 29258982; (iii) Tang J, Lee T, Sun T. Single-nucleotide editing: From principle, optimization to application. Hum Mutat. 2019 Dec;40(12):2171-2183. doi: 10.1002/humu.23819. Epub 2019 Sep 15. PMID: 31131955; PMCID: PMC6874907; (iv) Grünewald J, Zhou R, Lareau CA, Garcia SP, Iyer S, Miller BR, Langner LM, Hsu JY, Aryee MJ, Joung JK. A dual-deaminase CRISPR base editor enables concurrent adenine and cytosine editing. Nat Biotechnol. 2020 Jul;38(7):861-864. doi: 10.1038/s41587-020-0535-y. Epub 2020 Jun 1. PMID: 32483364; PMCID: PMC7723518; (v) Sakata RC, Ishiguro S, Mori H, Tanaka M, Tatsuno K, Ueda H, Yamamoto S, Seki M, Masuyama N, Nishida K, Nishimasu H, Arakawa K, Kondo A, Nureki O, Tomita M, Aburatani H, Yachie N. Base editors for simultaneous introduction of C-to-T and A-to-G mutations. Nat Biotechnol. 2020 Jul;38(7):865-869. doi: 10.1038/s41587-020-0509-0. Epub 2020 Jun 2. Erratum in: Nat Biotechnol. 2020 Jun 5;: PMID: 32483365; (vi) Fan J,

Ding Y, Ren C, Song Z, Yuan J, Chen Q, Du C, Li C, Wang X, Shu W. Cytosine and adenine deaminase base-editors induce broad and nonspecific changes in gene expression and splicing. Commun Biol. 2021 Jul 16;4(1):882. doi: 10.1038/s42003-021-02406-5. PMID: 34272468; PMCID: PMC8285404; (vii) Zhang S, Yuan B, Cao J, Song L, Chen J, Qiu J, Qiu Z, Zhao XM, Chen J, Cheng TL. TadA orthologs enable both cytosine and adenine editing of base editors. Nat Commun. 2023 Jan 26;14(1):414. doi: 10.1038/s41467-023-36003-3. PMID: 36702837; PMCID: PMC988000; and (viii) Zhang S, Song L, Yuan B, Zhang C, Cao J, Chen J, Qiu J, Tai Y, Chen J, Qiu Z, Zhao XM, Cheng TL. TadA reprogramming to generate potent miniature base editors with high precision. Nat Commun. 2023 Jan 26;14(1):413. doi: 10.1038/s41467-023-36004-2. PMID: 36702845; PMCID: PMC987999, each of which are incorporated herein by reference in their entireties.

[00501]Amino acid and nucleotide sequences of base editors, including adenosine base editors, cytidine base editors, and others are readily available in the art. For example, exemplary base editors that may be delivered using the LNP compositions described herein can be found in the following published patent applications, each of their contents (including any and all biological sequences) are incorporated herein by reference:

US 2023/0021641 A1	CAS9 VARIANTS HAVING NON-CANONICAL PAM SPECIFICITIES AND USES THEREOF
US 11542496 B2	Cytosine to guanine base editor
US 11542509 B2	Incorporation of unnatural amino acids into proteins using base editing
US 2022/0315906 A1	BASE EDITORS WITH DIVERSIFIED TARGETING SCOPE
US 2022/0282275 A1	G-TO-T BASE EDITORS AND USES THEREOF
US 2022/0249697 A1	AAV DELIVERY OF NUCLEOBASE EDITORS

[00502]In some embodiments, the LNP cargo comprises a base editing system or a polynucleotide encoding a CRISPR-Cas base editing system. In some embodiments, the cargo comprises a component of a base editing system or a polynucleotide encoding a component of a base editing system.

[00503]Base editing does not require double-stranded DNA breaks or a DNA donor template. In some embodiments, base editing comprises creating an SSB in a target double-stranded DNA sequence and then converting a nucleobase. In some embodiments, the nucleobase conversion is an adenosine to a guanine. In some embodiments, the nucleobase conversion is a thymine to a cytosine. In some embodiments, the nucleobase conversion is a cytosine to a thymine. In some embodiments, the nucleobase conversion is a guanine to an adenosine. In some embodiments, the nucleobase conversion is an adenosine to inosine. In some embodiments, the nucleobase conversion is a cytosine to uracil.

[00504]A base editing system comprises a base editor which can convert a nucleobase. The base editor ("BE") comprises a partially inactive Cas protein which is connected to a deaminase that precisely and permanently edits a target nucleobase in a polynucleotide sequence. A base editor comprises a polynucleotide programmable nucleotide binding domain and a nucleobase editing domain (e.g., adenosine deaminase or cytosine deaminase). In some embodiments, the partially inactive Cas protein is a Cas nickase. In some embodiments, the partially inactive Cas protein is a Cas9 nickase (also referred to as "nCas9").

[00505]A polynucleotide programmable nucleotide binding domain, when in conjunction with a bound guide polynucleotide (e.g., gRNA), can specifically bind to a target polynucleotide sequence (i.e., via complementary base pairing between bases of the bound guide nucleobase and bases of the target polynucleotide sequence) and thereby localize the nucleobase editor to the target polynucleotide sequence desired to be edited. In some embodiments, the target polynucleotide sequence comprises single-stranded DNA or double-stranded DNA. In some embodiments, the target polynucleotide sequence comprises RNA. In some embodiments, the target polynucleotide sequence comprises a DNA-RNA hybrid.

[00506]In certain embodiments, polynucleotide programmable nucleotide binding domains also include nucleobase programmable proteins that bind RNA. In certain embodiments, the polynucleotide programmable nucleotide binding domain can be associated with a nucleobase that guides the polynucleotide programmable nucleotide binding domain to an RNA.

[00507]In some embodiments, the LNP-deliverable base editors may comprise a deaminase domain that is a cytidine deaminase domain. A cytidine deaminase domain may also be referred to interchangeably as a cytosine deaminase domain. In some embodiments, the cytidine deaminase catalyzes the hydrolytic deamination of cytidine (C) or deoxycytidine (dC) to uridine (U) or deoxyuridine (dU), respectively. In some embodiments, the cytidine deaminase domain catalyzes the hydrolytic deamination of cytosine (C) to uracil (U). In some embodiments, the cytidine deaminase catalyzes the hydrolytic deamination of cytidine or cytosine in deoxyribonucleic acid (DNA). Without wishing to be bound by any particular theory, fusion proteins comprising a cytidine deaminase are useful inter alia for targeted editing, referred to herein as "base editing," of nucleic acid sequences in vitro and in vivo.

[00508]One exemplary suitable type of cytidine deaminase is a cytidine deaminase, for example, of the APOBEC family. The apolipoprotein B mRNA-editing complex (APOBEC) family of cytidine deaminase enzymes encompasses eleven proteins that serve to initiate mutagenesis in a controlled and beneficial manner (see, e.g., Conticello S G. The AID/APOBEC family of nucleic acid mutators. Genome Biol. 2008; 9(6):229). One family member, activation-induced cytidine deaminase (AID), is responsible for the maturation of antibodies by converting cytosines in ssDNA to uracils in a transcription-dependent, strand-biased fashion (see, e.g., Reynaud C A, et al. What role for AID: mutator, or assembler of the immunoglobulin mutasome, Nat Immunol. 2003; 4(7):631-638). The

apolipoprotein B editing complex 3 (APOBEC3) enzyme provides protection to human cells against a certain HIV-1 strain via the deamination of cytosines in reverse-transcribed viral ssDNA (see, e.g., Bhagwat A S. DNA-cytosine deaminases: from antibody maturation to antiviral defense. DNA Repair (Amst). 2004; 3(1):85-89).

[00509]Some aspects of this disclosure relate to the recognition that the activity of cytidine deaminase enzymes such as APOBEC enzymes can be directed to a specific site in genomic DNA. Without wishing to be bound by any particular theory, advantages of using a nucleic acid programmable binding protein (e.g., a Cas9 domain) as a recognition agent include (1) the sequence specificity of nucleic acid programmable binding protein (e.g., a Cas9 domain) can be easily altered by simply changing the sgRNA sequence; and (2) the nucleic acid programmable binding protein (e.g., a Cas9 domain) may bind to its target sequence by denaturing the dsDNA, resulting in a stretch of DNA that is single-stranded and therefore a viable substrate for the deaminase. It should be understood that other catalytic domains of napDNAbps, or catalytic domains from other nucleic acid editing proteins, can also be used to generate fusion proteins with Cas9, and that the disclosure is not limited in this regard.

[00510]In some embodiments, the cytidine deaminase is an apolipoprotein B mRNA-editing complex (APOBEC) family deaminase. In some embodiments, the cytidine deaminase is an APOBEC1 deaminase. In some embodiments, the cytidine deaminase is an APOBEC2 deaminase. In some embodiments, the cytidine deaminase is an APOBEC3 deaminase. In some embodiments, the cytidine deaminase is an APOBEC3A deaminase. In some embodiments, the cytidine deaminase is an APOBEC3B deaminase. In some embodiments, the cytidine deaminase is an APOBEC3C deaminase. In some embodiments, the cytidine deaminase is an APOBEC3D deaminase. In some embodiments, the cytidine deaminase is an APOBEC3E deaminase. In some embodiments, the cytidine deaminase is an APOBEC3F deaminase. In some embodiments, the cytidine deaminase is an APOBEC3G deaminase. In some embodiments, the cytidine deaminase is an APOBEC3H deaminase. In some embodiments, the cytidine deaminase is an APOBEC4 deaminase. In some embodiments, the cytidine deaminase is an activation-induced deaminase (AID). In some embodiments, the cytidine deaminase is a vertebrate cytidine deaminase. In some embodiments, the cytidine deaminase is an invertebrate cytidine deaminase. In some embodiments, the cytidine deaminase is a human, chimpanzee, gorilla, monkey, cow, dog, rat, or mouse deaminase. In some embodiments, the cytidine deaminase is a human cytidine deaminase. In some embodiments, the cytidine deaminase is a rat cytidine deaminase, e.g., rAPOBEC1.

[00511]In some embodiments, the nucleic acid editing domain is at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to any of the cytidine deaminase domain examples above.

[00512]In other embodiments, the LNP-deliverable base editors may comprise a deaminase domain that is an adenosine deaminase domain.

[00513] The disclosure provides fusion proteins that comprise one or more adenosine deaminases. In some aspects, such fusion proteins are capable of deaminating adenosine in a nucleic acid sequence (e.g., DNA or RNA). As one example, any of the fusion proteins provided herein may be base editors, (e.g., adenine base editors). Without wishing to be bound by any particular theory, dimerization of adenosine deaminases (e.g., in cis or in trans) may improve the ability (e.g., efficiency) of the fusion protein to modify a nucleic acid base, for example to deaminate adenine. In some embodiments, any of the fusion proteins may comprise 2, 3, 4 or 5 adenosine deaminases. In some embodiments, any of the fusion proteins provided herein comprise two adenosine deaminases. Exemplary, non-limiting, embodiments of adenosine deaminases are provided herein. It should be appreciated that the mutations provided herein (e.g., mutations in ecTadA) may be applied to adenosine deaminases in other adenosine base editors, for example those provided in U.S. Patent Publication No. 2018/0073012, published Mar. 15, 2018, which issued as U.S. Pat. No. 10,113,163, on Oct. 30, 2018; U.S. Patent Publication No. 2017/0121693, published May 4, 2017, which issued as U.S. Pat. No. 10,167,457 on Jan. 1, 2019; International Publication No. WO 2017/070633, published Apr. 27, 2017; U.S. Patent Publication No. 2015/0166980, published Jun. 18, 2015; U.S. Pat. No. 9,840,699, issued Dec. 12, 2017; and U.S. Pat. No. 10,077,453, issued Sep. 18, 2018, all of which are incorporated herein by reference in their entireties.

[00514]In some embodiments, any of the adenosine deaminases provided herein is capable of deaminating adenine. In some embodiments, the adenosine deaminases provided herein are capable of deaminating adenine in a deoxyadenosine residue of DNA. The adenosine deaminase may be derived from any suitable organism (e.g., E. coli). In some embodiments, the adenosine deaminase is a naturally-occurring adenosine deaminase that includes one or more mutations corresponding to any of the mutations provided herein (e.g., mutations in ecTadA). One of skill in the art will be able to identify the corresponding residue in any homologous protein and in the respective encoding nucleic acid by methods well known in the art, e.g., by sequence alignment and determination of homologous residues. Accordingly, one of skill in the art would be able to generate mutations in any naturally-occurring adenosine deaminase (e.g., having homology to ecTadA) that corresponds to any of the mutations described herein, e.g., any of the mutations identified in ecTadA. In some embodiments, the adenosine deaminase is from a prokaryote. In some embodiments, the adenosine deaminase is from a bacterium. In some embodiments, the adenosine deaminase is from Escherichia coli, Staphylococcus aureus, Salmonella typhi, Shewanella putrefaciens, Haemophilus influenzae, Caulobacter crescentus, or Bacillus subtilis. In some embodiments, the adenosine deaminase is from E. coli.

[00515]Any two or more of the adenosine deaminases described herein may be connected to one another (e.g. by a linker) within an adenosine deaminase domain of the fusion proteins provided herein. For instance, the fusion proteins provided herein may contain only two adenosine deaminases. In some embodiments, the adenosine deaminases are the same. In some embodiments, the adenosine deaminases are any of the adenosine deaminases provided herein. In some embodiments, the

adenosine deaminases are different. In some embodiments, the first adenosine deaminase is any of the adenosine deaminases provided herein, and the second adenosine is any of the adenosine deaminases provided herein, but is not identical to the first adenosine deaminase. In some embodiments, the fusion protein comprises two adenosine deaminases (e.g., a first adenosine deaminase and a second adenosine deaminase). In some embodiments, the fusion protein comprises a first adenosine deaminase and a second adenosine deaminase. In some embodiments, the first adenosine deaminase is N-terminal to the second adenosine deaminase in the fusion protein. In some embodiments, the first adenosine deaminase is C-terminal to the second adenosine deaminase in the fusion protein. In some embodiments, the first adenosine deaminase and the second deaminase are fused directly or via a linker.

[00516]In some embodiments, the base editor comprises a deaminase enzyme. In some embodiments, the base editor comprises a cytidine deaminase. In some embodiments, the base editor comprises a Cas9 protein fused to a cytidine deaminase enzyme. In some embodiments, the base editor comprises an adenosine deaminase. In some embodiments, the base editor comprises a Cas9 protein fused to an adenosine deaminase enzyme.

[00517]In some embodiments, the base editing system comprises an uracil glycosylase inhibitor. In some embodiments, the base editing system comprises a Cas9 protein fused to an uracil glycosylase inhibitor. In some embodiments, the cargo comprises an uracil glycosylase inhibitor or a polynucleotide encoding an uracil glycosylase inhibitor. In some embodiments, the cargo comprises a Cas9 protein fused to an uracil glycosylase inhibitor or a polynucleotide encoding a Cas9 protein fused to an uracil glycosylase inhibitor.

[00518]A variety of nucleobase modifying enzymes are suitable for use in the nucleobase systems disclosed herein. In some embodiments, the nucleobase modifying enzyme is a RNA base editor. In some embodiments, the RNA base editor can be a cytidine deaminase, which converts cytidine into uridine. Non-limiting examples of cytidine deaminases include cytidine deaminase 1 (CDA1), cytidine deaminase 2 (CDA2), activation-induced cytidine deaminase (AICDA), apolipoprotein B mRNA-editing complex (APOBEC) family cytidine deaminase (e.g., APOBEC1, APOBEC2, APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D/E, APOBEC3F, APOBEC3G, APOBEC3H, APOBEC4), APOBEC1 complementation factor/APOBEC1 stimulating factor (ACF1/ASF) cytidine deaminase, cytosine deaminase acting on RNA (CDAR), bacterial long isoform cytidine deaminase (CDDL), and cytosine deaminase acting on tRNA (CDAT). In other embodiments, the RNA base editor can be an adenosine deaminase, which converts adenosine into inosine, which is read by polymerase enzymes as guanosine. In certain embodiments, adenosine deaminases include tRNA adenine deaminase, adenosine deaminase, adenosine deaminase acting on RNA (ADAR), and adenosine deaminase acting on tRNA (ADAT).

[00519]In some embodiments, in the nucleobase editing systems disclosed herein, the Cas effector may associate with one or more functional domains (e.g., via fusion protein or suitable linkers). In

some embodiments, the effector domain comprises one or more cytidine or nucleotide deaminases that mediate editing of via hydrolytic deamination. In certain embodiments, the effector domain comprises the adenosine deaminase acting on RNA (ADAR) family of enzymes. In certain embodiments, the adenosine deaminase protein or catalytic domain thereof capable of deaminating adenosine or cytidine in RNA or is an RNA specific adenosine deaminase and/or is a bacterial, human, cephalopod, or Drosophila adenosine deaminase protein or catalytic domain thereof, preferably TadA, more preferably ADAR, optionally huADAR, optionally (hu)ADAR1 or (hu)ADAR2, preferably huADAR2 or catalytic domain thereof.

[00520]In some embodiments, the cytidine deaminase is a human, rat or lamprey cytidine deaminase. In some embodiments, the cytidine deaminase is an apolipoprotein B mRNA-editing complex (APOBEC) family deaminase, an activation-induced deaminase (AID), or a cytidine deaminase 1 (CDA1).

[00521]In certain embodiments, the adenosine deaminase is adenosine deaminase acting on RNA (ADAR). In certain embodiments, the ADAR is ADAR (ADAR1), ADARB1 (ADAR2) or ADARB2 (ADAR3) (*see, e.g.*, Savva et al. Genon. Biol. 2012, 13(12):252).

[00522]In some embodiments, the gene editing system comprises AID/APOBEC (apolipoprotein B editing complex) family of enzymes deaminates cytidine to uridine, leading to mutations in RNA and DNA.

[00523]In some embodiments, the nucleobase editing system comprises ADAR and an antisense oligonucleotide. In certain embodiments, the antisense oligonucleotide is chemically optimized antisense oligonucleotide. In certain embodiments, the antisense oligonucleotide is administered for the nucleobase editing, wherein the antisense oligonucleotide activates human endogenous ADAR for nucleobase editing. Such ADAR and antisense oligonucleotide editing system provides a safer site-directed RNA editing with low off-target effect. *See, e.g.*, Merkle *et al.*, Nature Biotechnology, 2019, 37, 133-138.

[00524]Any of the above base editor embodiments or variants, modifications, or derivatives thereof are contemplated herein to be delivered by the LNP systems disclosed in this specification for gene editing in cells, tissues, and/or organs under *in vitro*, *ex vivo*, or *in vivo* conditions. The various components described herein may be configured and delivered in any suitable manner. Any of the descriptions presented in this section are not intended to be strictly limiting.

Prime editors

[00525]In various embodiments, the herein disclosed LNPs may contain a prime editing system or components thereof and which may be used to conduct prime editing of target nucleic acid sequences in cells, tissues, and organs in an *ex vivo* or *in vivo* manner.

[00526]Prime editing technology is a gene editing technology that can make targeted insertions, deletions, and all transversion and transition point mutations in a target genome. Without wishing to be bound by any particular theory, the prime editing process may search and replace endogenous

sequences in a target polynucleotide. The spacer sequence of a prime editing guide RNA ("PEgRNA" or "pegRNA") recognizes and anneals with a search target sequence in a target strand of a double stranded target polynucleotide, e.g., a double stranded target DNA. A prime editing complex may generate a nick in the target DNA on the edit strand which is the complementary strand of the target strand. The prime editing complex may then use a free 3' end formed at the nick site of the edit strand to initiate DNA synthesis, where a "primer binding site sequence" (PBS) of the PEgRNA complexes with the free 3' end, and a single stranded DNA is synthesized (by reverse transcriptase) using an editing template of the PEgRNA as a template. As used herein, a "primer binding site" is a single-stranded portion of the PEgRNA that comprises a region of complementarity to the PAM strand (i.e., the non-target strand or the edit strand). The PBS is complementary or substantially complementary to a sequence on the PAM strand of the double stranded target DNA that is immediately upstream of the nick site.

[00527]The term "prime editor (PE)" refers to the polypeptide or polypeptide components involved in prime editing, or any polynucleotide(s) encoding the polypeptide or polypeptide components. In various embodiments, a prime editor includes a polypeptide domain having DNA binding activity and a polypeptide domain having DNA polymerase activity. In some embodiments, the prime editor further comprises a polypeptide domain having nuclease activity. In some embodiments, the polypeptide domain having DNA binding activity comprises a nuclease domain or nuclease activity. In some embodiments, the polypeptide domain having nuclease activity comprises a nickase, or a fully active nuclease. As used herein, the term "nickase" refers to a nuclease capable of cleaving only one strand of a double-stranded DNA target. In some embodiments, the prime editor comprises a polypeptide domain that is an inactive nuclease. In some embodiments, the polypeptide domain having programmable DNA binding activity comprises a nucleic acid guided DNA binding domain, for example, a CRISPR-Cas protein, for example, a Cas9 nickase, a Cpf1 nickase, or another CRISPR-Cas nuclease. In some embodiments, the polypeptide domain having DNA polymerase activity comprises a template-dependent DNA polymerase, for example, a DNA-dependent DNA polymerase or an RNA-dependent DNA polymerase. In some embodiments, the DNA polymerase is a reverse transcriptase. In some embodiments, the prime editor comprises additional polypeptides involved in prime editing, for example, a polypeptide domain having 5' endonuclease activity, e.g., a 5' endogenous DNA flap endonucleases (e.g., FEN1), for helping to drive the prime editing process towards the edited product formation. In some embodiments, the prime editor further comprises an RNA-protein recruitment polypeptide, for example, a MS2 coat protein.

[00528]A prime editor may be engineered. In some embodiments, the polypeptide components of a prime editor do not naturally occur in the same organism or cellular environment. In some embodiments, the polypeptide components of a prime editor may be of different origins or from different organisms. In some embodiments, a prime editor comprises a DNA binding domain and a DNA polymerase domain that are derived from different species. In some embodiments, a prime

editor comprises a Cas polypeptide (DNA binding domain) and a reverse transcriptase polypeptide (DNA polymerase) that are derived from different species. For example, a prime editor may comprise a S. pyogenes Cas9 polypeptide and a Moloney murine leukemia virus (M-MLV) reverse transcriptase polypeptide.

[00529]In some embodiments, polypeptide domains of a prime editor may be fused or linked by a peptide linker to form a fusion protein. In other embodiments, a prime editor comprises one or more polypeptide domains provided in trans as separate proteins, which are capable of being associated to each other through non-peptide linkages or through aptamers or recruitment sequences. For example, a prime editor may comprise a DNA binding domain and a reverse transcriptase domain associated with each other by an RNA-protein recruitment aptamer, e.g., a MS2 aptamer, which may be linked to a PEgRNA. Prime editor polypeptide components may be encoded by one or more polynucleotides in whole or in part. In some embodiments, a single polynucleotide, construct, or vector encodes the prime editor fusion protein. In some embodiments, multiple polynucleotides, constructs, or vectors each encode a polypeptide domain or portion of a domain of a prime editor, or a portion of a prime editor fusion protein. For example, a prime editor fusion protein may comprise an N-terminal portion fused to an intein-N and a C-terminal portion fused to an intein-C, each of which is individually encoded by an AAV vector.

[00530]The editing template may comprise one or more intended nucleotide edits compared to the endogenous double stranded target DNA sequence. Accordingly, the newly synthesized single stranded DNA also comprises the nucleotide edit(s) encoded by the editing template. Through removal of the editing target sequence on the edit strand of the double stranded target DNA and DNA repair mechanism, the newly synthesized single stranded DNA replaces the editing target sequence, and the desired nucleotide edit(s) are incorporated into the double stranded target DNA.

[00531]Prime editing was first described in Anzalone et al., "Search-and-replace genome editing without double-strand breaks or donor DNA," Nature, Dec 2019, 576 (7789): pp. 149-157, which is incorporated herein in its entirety. Prime editing has subsequently been described and detailed in numerous follow-on publications, including, for example, (i) Liu et al., "Prime editing: a search and replace tool with versatile base changes," Yi Chuan, Nov. 20, 2022, 44(11): 993-1008; (ii) Lu C et al., "Prime Editing: An All-Rounder for Genome Editing. Int J Mol Sci. 2022 Aug 30;23(17):9862; (iii) Velimirovic M, Zanetti LC, Shen MW, Fife JD, Lin L, Cha M, Akinci E, Barnum D, Yu T, Sherwood RI. Peptide fusion improves prime editing efficiency. Nat Commun. 2022 Jun 18;13(1):3512. doi: 10.1038/s41467-022-31270-y. PMID: 35717416; PMCID: PMC9206660; (iv) Velimirovic M, Zanetti LC, Shen MW, Fife JD, Lin L, Cha M, Akinci E, Barnum D, Yu T, Sherwood RI. Peptide fusion improves prime editing efficiency. Nat Commun. 2022 Jun 18;13(1):3512. doi: 10.1038/s41467-022-31270-y. PMID: 35717416; PMCID: PMC9206660; (v) Habib O, Habib G, Hwang GH, Bae S. Comprehensive analysis of prime editing outcomes in human embryonic stem cells. Nucleic Acids Res. 2022 Jan 25;50(2):1187-1197. doi: 10.1093/nar/gkab1295. PMID: 35018468; PMCID:

PMC8789035; (vi) Marzec M, Brąszewska-Zalewska A, Hensel G. Prime Editing: A New Way for Genome Editing. Trends Cell Biol. 2020 Apr;30(4):257-259. doi: 10.1016/j.tcb.2020.01.004. Epub 2020 Jan 27. PMID: 32001098; (vii) Tao R, Wang Y, Jiao Y, Hu Y, Li L, Jiang L, Zhou L, Qu J, Chen Q, Yao S. Bi-PE: bi-directional priming improves CRISPR/Cas9 prime editing in mammalian cells. Nucleic Acids Res. 2022 Jun 24;50(11):6423-6434. doi: 10.1093/nar/gkac506. PMID: 35687127; PMCID: PMC9226529; (viii) Nelson JW, Randolph PB, Shen SP, Everette KA, Chen PJ, Anzalone AV, An M, Newby GA, Chen JC, Hsu A, Liu DR. Engineered pegRNAs improve prime editing efficiency. Nat Biotechnol. 2022 Mar;40(3):402-410. doi: 10.1038/s41587-021-01039-7. Epub 2021 Oct 4. Erratum in: Nat Biotechnol. 2021 Dec 8; PMID: 34608327; PMCID: PMC8930418; (ix) Doman JL, Sousa AA, Randolph PB, Chen PJ, Liu DR. Designing and executing prime editing experiments in mammalian cells. Nat Protoc. 2022 Nov;17(11):2431-2468. doi: 10.1038/s41596-022-00724-4. Epub 2022 Aug 8. PMID: 35941224; PMCID: PMC9799714; (x) Jiao Y, Zhou L, Tao R, Wang Y, Hu Y, Jiang L, Li L, Yao S. Random-PE: an efficient integration of random sequences into mammalian genome by prime editing. Mol Biomed. 2021 Nov 18;2(1):36. doi: 10.1186/s43556-021-00057-w. PMID: 35006470; PMCID: PMC8607425; and (xi) Awan MJA, Ali Z, Amin I, Mansoor S. Twin prime editor: seamless repair without damage. Trends Biotechnol. 2022 Apr;40(4):374-376. doi: 10.1016/j.tibtech.2022.01.013. Epub 2022 Feb 10. PMID: 35153078, all of which are incorporated herein by reference.

[00532]In addition, prime editing has been described and disclosed in numerous published patent applications, each of which their entire contents, amino acid sequences, nucleotide sequences, and all disclosures therein are incorporated herein by reference in their entireties:

Publication No.	**Publication Date**	**Title**
WO 2023/015309 A2	2/9/2023	IMPROVED PRIME EDITORS AND METHODS OF USE
WO 2023/004439 A2	1/26/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF CHRONIC GRANULOMATOUS DISEASE
WO 2023/288332 A2	1/19/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF WILSON'S DISEASE
WO 2023/283092 A1	1/12/2023	COMPOSITIONS AND METHODS FOR EFFICIENT GENOME EDITING
WO 2023/283246 A1	1/12/2023	MODULAR PRIME EDITOR SYSTEMS FOR GENOME ENGINEERING

Publication No.	Publication Date	Title
WO 2022/256714 A3	1/12/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF WILSON'S DISEASE
EP 4107273 A1	12/28/2022	PRIME EDITING TECHNOLOGY FOR PLANT GENOME ENGINEERING
WO 2022/256714 A2	12/8/2022	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF WILSON'S DISEASE
WO 2022/234051 A1	11/10/2022	SPLIT PRIME EDITING ENZYME
US 2022/0356469 A1	11/10/2022	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2022/206352 A1	10/6/2022	PRIME EDITING TOOL, FUSION RNA, AND USE THEREOF
WO 2022/212926 A1	10/6/2022	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2022/204476 A1	9/29/2022	NUCLEOTIDE EDITING TO REFRAME DMD TRANSCRIPTS BY BASE EDITING AND PRIME EDITING
WO 2022/203905 A1	9/29/2022	PRIME EDITING-BASED SIMULTANEOUS GENOMIC DELETION AND INSERTION
US 11447770 B1	9/20/2022	Methods and compositions for prime editing nucleotide sequences
WO 2022/174829 A1	8/25/2022	EDITING OF DOUBLE-STRANDED DNA WITH RELAXED PAM REQUIREMENT FIELD OF THE DISCLOSURE
WO 2022/170058 A1	8/11/2022	PRIME EDITOR SYSTEM FOR IN VIVO GENOME EDITING
WO 2022/169235 A1	8/11/2022	PRIME EDITING COMPOSITION WITH IMPROVED EDITING EFFICIENCY

Publication No.	Publication Date	Title
WO 2022/150790 A3	8/11/2022	PRIME EDITOR VARIANTS, CONSTRUCTS, AND METHODS FOR ENHANCING PRIME EDITING EFFICIENCY AND PRECISION
WO 2022/149166 A1	7/14/2022	A COCKTAIL FORMULATION FOR SELECTIVE ENRICHMENT OF GENE-MODIFIED CELLS
WO 2022/150790 A2	7/14/2022	PRIME EDITOR VARIANTS, CONSTRUCTS, AND METHODS FOR ENHANCING PRIME EDITING EFFICIENCY AND PRECISION
US 11384353 B2	7/12/2022	Inhibition of unintended mutations in gene editing
WO 2022/067130 A3	6/23/2022	PRIME EDITING GUIDE RNAS, COMPOSITIONS THEREOF, AND METHODS OF USING THE SAME
WO 2022/114815 A1	6/2/2022	COMPOSITION FOR PRIME EDITING COMPRISING TRANS-SPLICING ADENO-ASSOCIATED VIRUS VECTOR
WO 2022/100662 A1	5/19/2022	GENOMIC EDITING OF IMPROVED EFFICIENCY AND ACCURACY
WO 2022/098765 A1	5/12/2022	SPLIT PRIME EDITING PLATFORMS
WO 2022/098885 A1	5/12/2022	PRECISE GENOME DELETION AND REPLACEMENT METHOD BASED ON PRIME EDITING
WO 2022/071745 A1	4/7/2022	PRIME EDITING USING HIV REVERSE TRANSCRIPTASE AND CAS9 OR VARIANT THEREOF
WO 2022/067130 A2	3/31/2022	PRIME EDITING GUIDE RNAS, COMPOSITIONS THEREOF, AND METHODS OF USING THE SAME
WO 2022/065689 A1	3/31/2022	PRIME EDITING-BASED GENE EDITING COMPOSITION WITH ENHANCED EDITING EFFICIENCY AND USE THEREOF
US 2022/0064626 A1	3/3/2022	INHIBITION OF UNINTENDED MUTATIONS IN GENE EDITING

Publication No.	Publication Date	Title
WO 2022/032085 A1	2/10/2022	TARGETED SEQUENCE INSERTION COMPOSITIONS AND METHODS
WO 2022/025623 A1	2/3/2022	SYSTEM AND METHOD FOR PRIME EDITING EFFICIENCY PREDICTION USING DEEP LEARNING
WO 2021/226558 A8	1/13/2022	METHODS AND COMPOSITIONS FOR SIMULTANEOUS EDITING OF BOTH STRANDS OF A TARGET DOUBLE-STRANDED NUCLEOTIDE SEQUENCE
WO 2021/243289 A1	12/2/2021	SYSTEMS AND METHODS FOR STABLE AND HERITABLE ALTERATION BY PRECISION EDITING (SHAPE)
WO 2021/226558 A1	11/11/2021	METHODS AND COMPOSITIONS FOR SIMULTANEOUS EDITING OF BOTH STRANDS OF A TARGET DOUBLE-STRANDED NUCLEOTIDE SEQUENCE
WO 2021/215897 A1	10/28/2021	GENOME EDITION USING CAS9 OR CAS9 VARIANT
WO 2021/215827 A1	10/28/2021	GENOME EDITING USING CAS9 OR CAS9 VARIANT
WO 2020/191248 A8	10/21/2021	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191234 A8	10/21/2021	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2021/165508 A1	8/26/2021	PRIME EDITING TECHNOLOGY FOR PLANT GENOME ENGINEERING
WO 2021/138469 A1	7/8/2021	GENOME EDITING USING REVERSE TRANSCRIPTASE ENABLED AND FULLY ACTIVE CRISPR COMPLEXES
WO 2021/092204 A1	5/14/2021	METHODS AND COMPOSITIONS FOR NUCLEIC ACID-GUIDED NUCLEASE CELL TARGETING SCREEN
WO 2021/076876 A1	4/22/2021	GENOTYPING EDITED MICROBIAL STRAINS

Publication No.	Publication Date	Title
WO 2021/072328 A1	4/15/2021	METHODS AND COMPOSITIONS FOR PRIME EDITING RNA
WO 2020/191153 A8	12/30/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191153 A3	12/10/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191153 A9	11/12/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191171 A9	10/29/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191248 A1	9/24/2020	METHOD AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191239 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191153 A2	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191246 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191249 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191233 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191243 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191234 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191245 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191242 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES

Publication No.	Publication Date	Title
WO 2020/191171 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/191241 A1	9/24/2020	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2020/156575 A1	8/6/2020	INHIBITION OF UNINTENDED MUTATIONS IN GENE EDITING
US 10189831 B2	1/29/2019	Non-nucleoside reverse transcriptase inhibitors
WO 2019/014564 A1	1/17/2019	SYSTEMS AND METHODS FOR TARGETED INTEGRATION AND GENOME EDITING AND DETECTION THEREOF USING INTEGRATED PRIMING SITES
US 10150955 B2	12/11/2018	Stabilized reverse transcriptase fusion proteins
WO 2018/049168 A1	3/15/2018	HIGH-THROUGHPUT PRECISION GENOME EDITING
US 9783791 B2	10/10/2017	Mutant reverse transcriptase and methods of use
US 9458484 B2	10/4/2016	Reverse transcriptase mixtures with improved storage stability
WO 2023/070110 A2	4/27/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF RETINITIS PIGMENTOSA
US 2023/0059368 A1	2/23/2023	POLYNUCLEOTIDE EDITORS AND METHODS OF USING THE SAME
WO 2023/086558 A1	5/19/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF FRAGILE X SYNDROME
WO 2023/288332 A3	2/23/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF WILSON'S DISEASE
WO 2023/192655 A2	10/5/2023	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES

Publication No.	Publication Date	Title
WO 2022/256714 A2	12/8/2022	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF WILSON'S DISEASE
WO 2023/283092 A1	1/12/2023	COMPOSITIONS AND METHODS FOR EFFICIENT GENOME EDITING
WO 2023/070062 A3	5/25/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF USHER SYNDROME TYPE 3
WO 2023/081787 A3	9/28/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF FANCONI ANEMIA
WO 2023/086389 A1	5/19/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS
WO 2023/096847 A2	6/1/2023	METHODS AND COMPOSITIONS FOR INIIIBITING MISMATCII REPAIR
WO 2023/081426 A1	5/11/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF FRIEDREICH'S ATAXIA
WO 2023/015318 A2	2/9/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF CYSTIC FIBROSIS
WO 2022/212926 A1	10/6/2022	METHODS AND COMPOSITIONS FOR EDITING NUCLEOTIDE SEQUENCES
WO 2023/028180 A3	8/24/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF RETINOPATHY
WO 2023/015014 A1	2/9/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF MYOTONIC DYSTROPHY
WO 2023/086842 A1	5/19/2023	GENOME EDITING COMPOSITIONS AND METIIODS FOR TREATMENT OF FUCIIS ENDOTHELIAL CORNEAL DYSTROPHY

Publication No.	Publication Date	Title
WO 2023/096992 A1	6/1/2023	GENOME EDITING COMPOSITIONS AND METHODS FOR TREATMENT OF GLYCOGEN STORAGE DISEASE TYPE 1B
WO 2023/096977 A2	6/1/2023	MODIFIED PRIME EDITING GUIDE RNAS

[00533]In some embodiments, the cargo comprises a prime editing system or a polynucleotide encoding a prime editing system. In some embodiments, the cargo comprises a component of a prime editing system or a polynucleotide encoding a component of a prime editing system.

[00534]Prime editing is a versatile and precise genome editing method that directly writes new genetic information into a specified DNA site using a catalytically impaired Cas fused to an engineered reverse transcriptase, also referred to as a prime editor, which is programmable using a prime editing guide RNA ("pegRNA") that both specifies the target site and encodes the desired edit (*see, e.g.*, Anzalone *et al.*, Nature 2019). Prime editing bypasses the need for DNA donor templates by using a prime editor having nickase or catalytically impaired enzymatic activity.

[00535]A prime editing system comprises a prime editor. The prime editor ("PE") comprises a catalytically impaired Cas protein fused to an engineered reverse transcriptase which can precisely and permanently edit one or more target nucleobases in a target polynucleotide.

[00536]In some embodiments, the prime editor comprises an engineered Moloney murine leukemia virus ("M-MLV") reverse transcriptase ("RT") fused to a Cas-H840A nickase (called "PE2"). In some embodiments, the prime editor comprises an engineered M-MLV RT fused to a Cas9-H840A nickase. In some embodiments, the prime editor comprises an engineered M-MLV RT fused to a Streptococcus pyogenes Cas9 (spCas9)-H840A nickase. PE modifications include increased PAM flexibility to increase the utility of PE2 editing, expanding the coverage of targetable pathogenic variants in the ClinVar database that can now be prime edited to 94.4%.

[00537]In some embodiments, the prime editing system further comprises a prime editing guide RNA ("pegRNA"). In some embodiments, the cargo comprises a pegRNA or a polynucleotide encoding a pegRNA.

[00538]pegRNAs may be designed and synthesized using methods, software, and commercial sources which are well known to those having ordinary skill in the art such that guide RNAs for any given naspDBP or prime editor may be obtained without undue experimentation.

[00539]Reference may be made to the following references providing information and tools for the design, synthesis, modification, and structural configuration of pegRNAs, and guide RNAs in general: (1) Mohr SE, Hu Y, Ewen-Campen B, Housden BE, Viswanatha R, Perrimon N. CRISPR guide RNA design for research applications. FEBS J. 2016 Sep;283(17):3232-8. doi: 10.1111/febs.13777. Epub 2016 Jun 22. PMID: 27276584; PMCID: PMC5014588; (2) Hoberecht L, Perampalam P, Lun A,

Fortin JP. A comprehensive Bioconductor ecosystem for the design of CRISPR guide RNAs across nucleases and technologies. Nat Commun. 2022 Nov 2;13(1):6568. doi: 10.1038/s41467-022-34320-7. PMID: 36323688; PMCID: PMC9630310; (3) Cram D, Kulkarni M, Buchwaldt M, Rajagopalan N, Bhowmik P, Rozwadowski K, Parkin IAP, Sharpe AG, Kagale S. WheatCRISPR: a web-based guide RNA design tool for CRISPR/Cas9-mediated genome editing in wheat. BMC Plant Biol. 2019 Nov 6;19(1):474. doi: 10.1186/s12870-019-2097-z. PMID: 31694550; PMCID: PMC6836449; (4) Pliatsika V, Rigoutsos I. "Off-Spotter": very fast and exhaustive enumeration of genomic lookalikes for designing CRISPR/Cas guide RNAs. Biol Direct. 2015 Jan 29;10:4. doi: 10.1186/s13062-015-0035-z. PMID: 25630343; PMCID: PMC4326336; (5) Hoof JB, Nødvig CS, Mortensen UH. Genome Editing: CRISPR-Cas9. Methods Mol Biol. 2018;1775:119-132. doi: 10.1007/978-1-4939-7804-5_11. PMID: 29876814; (6) Labun K, Krause M, Torres Cleuren Y, Valen E. CRISPR Genome Editing Made Easy Through the CHOPCHOP Website. Curr Protoc. 2021 Apr;1(4):e46. doi: 10.1002/cpz1.46. PMID: 33905612; (7) Lee CM, Davis TH, Bao G. Examination of CRISPR/Cas9 design tools and the effect of target site accessibility on Cas9 activity. Exp Physiol. 2018 Apr 1;103(4):456-460. doi: 10.1113/EP086043. Epub 2017 Apr 12. PMID: 28303677; PMCID: PMC7266697; (8) Ma S, Lv J, Feng Z, Rong Z, Lin Y. Get ready for the CRISPR/Cas system: A beginner's guide to the engineering and design of guide RNAs. J Gene Med. 2021 Nov;23(11):e3377. doi: 10.1002/jgm.3377. Epub 2021 Jul 28. PMID: 34270141; (9) Hiranniramol K, Chen Y, Wang X. CRISPR/Cas9 Guide RNA Design Rules for Predicting Activity. Methods Mol Biol. 2020;2115:351-364. doi: 10.1007/978-1-0716-0290-4_19. PMID: 32006410; (10) Wiles MV, Qin W, Cheng AW, Wang H. CRISPR-Cas9-mediated genome editing and guide RNA design. Mamm Genome. 2015 Oct;26(9-10):501-10. doi: 10.1007/s00335-015-9565-z. Epub 2015 May 20. PMID: 25991564; PMCID: PMC4602062; (11) Creutzburg SCA, Wu WY, Mohanraju P, Swartjes T, Alkan F, Gorodkin J, Staals RHJ, van der Oost J. Good guide, bad guide: spacer sequence-dependent cleavage efficiency of Cas12a. Nucleic Acids Res. 2020 Apr 6;48(6):3228-3243. doi: 10.1093/nar/gkz1240. PMID: 31989168; PMCID: PMC7102956; (12) Heigwer F, Boutros M. Cloud-Based Design of Short Guide RNA (sgRNA) Libraries for CRISPR Experiments. Methods Mol Biol. 2021;2162:3-22. doi: 10.1007/978-1-0716-0687-2_1. PMID: 32926374; (13) Dronina J, Samukaite-Bubniene U, Ramanavicius A. Towards application of CRISPR-Cas12a in the design of modern viral DNA detection tools (Review). J Nanobiotechnology. 2022 Jan 21;20(1):41. doi: 10.1186/s12951-022-01246-7. PMID: 35062978; PMCID: PMC8777428; (14) Krysler AR, Cromwell CR, Tu T, Jovel J, Hubbard BP. Guide RNAs containing universal bases enable Cas9/Cas12a recognition of polymorphic sequences. Nat Commun. 2022 Mar 25;13(1):1617. doi: 10.1038/s41467-022-29202-x. PMID: 35338140; PMCID: PMC8956631; (15) Shin HR, Kweon J, Kim Y. Gene Manipulation Using Fusion Guide RNAs for Cas9 and Cas12a. Methods Mol Biol. 2021;2162:185-193. doi: 10.1007/978-1-0716-0687-2_10. PMID: 32926383; (16) Schubert MS, Thommandru B, Woodley J, Turk R, Yan S, Kurgan G, McNeill MS, Rettig GR. Optimized design parameters for CRISPR Cas9 and Cas12a

homology-directed repair. Sci Rep. 2021 Sep 30;11(1):19482. doi: 10.1038/s41598-021-98965-y. PMID: 34593942; PMCID: PMC8484621; (17) Crone MA, MacDonald JT, Freemont PS, Siciliano V. gDesigner: computational design of synthetic gRNAs for Cas12a-based transcriptional repression in mammalian cells. NPJ Syst Biol Appl. 2022 Sep 16;8(1):34. doi: 10.1038/s41540-022-00241-w. PMID: 36114193; PMCID: PMC9481559; (18) Konstantakos V, Nentidis A, Krithara A, Paliouras G. CRISPR-Cas9 gRNA efficiency prediction: an overview of predictive tools and the role of deep learning. Nucleic Acids Res. 2022 Apr 22;50(7):3616-3637. doi: 10.1093/nar/gkac192. PMID: 35349718; PMCID: PMC9023298; (19) Wang J, Zhang X, Cheng L, Luo Y. An overview and metanalysis of machine and deep learning-based CRISPR gRNA design tools. RNA Biol. 2020 Jan;17(1):13-22. doi: 10.1080/15476286.2019.1669406. Epub 2019 Sep 27. PMID: 31533522; PMCID: PMC6948960; and (20) Cram D, Kulkarni M, Buchwaldt M, Rajagopalan N, Bhowmik P, Rozwadowski K, Parkin IAP, Sharpe AG, Kagale S. WheatCRISPR: a web-based guide RNA design tool for CRISPR/Cas9-mediated genome editing in wheat. BMC Plant Biol. 2019 Nov 6;19(1):474. doi: 10.1186/s12870-019-2097-z. PMID: 31694550; PMCID: PMC6836449; each of which are incorporated herein by reference in their entireties.

[00540]In the specific case of prime editing, in particular, further reference may be made to the following references providing information and tools for the design, synthesis, modification, and structural configuration of pegRNAs: (1) Hsu JY, Grünewald J, Szalay R, Shih J, Anzalone AV, Lam KC, Shen MW, Petri K, Liu DR, Joung JK, Pinello L. PrimeDesign software for rapid and simplified design of prime editing guide RNAs. Nat Commun. 2021 Feb 15;12(1):1034. doi: 10.1038/s41467-021-21337-7. PMID: 33589617; PMCID: PMC7884779; (2) Li Y, Chen J, Tsai SQ, Cheng Y. Easy-Prime: a machine learning-based prime editor design tool. Genome Biol. 2021 Aug 19;22(1):235. doi: 10.1186/s13059-021-02458-0. PMID: 34412673; PMCID: PMC8377858; (3) Zhang W, Petri K, Ma J, Lee H, Tsai CL, Joung JK, Yeh JJ. Enhancing CRISPR prime editing by reducing misfolded pegRNA interactions. bioRxiv [Preprint]. 2023 Aug 15:2023.08.14.553324. doi: 10.1101/2023.08.14.553324. PMID: 37645936; PMCID: PMC10462064; (4) Jin S, Lin Q, Gao Q, Gao C. Optimized prime editing in monocot plants using PlantPegDesigner and engineered plant prime editors (ePPEs). Nat Protoc. 2023 Mar;18(3):831-853. doi: 10.1038/s41596-022-00773-9. Epub 2022 Nov 25. PMID: 36434096; (5) Lin Q, Jin S, Zong Y, Yu H, Zhu Z, Liu G, Kou L, Wang Y, Qiu JL, Li J, Gao C. High-efficiency prime editing with optimized, paired pegRNAs in plants. Nat Biotechnol. 2021 Aug;39(8):923-927. doi: 10.1038/s41587-021-00868-w. Epub 2021 Mar 25. PMID: 33767395; (6) Standage-Beier K, Tekel SJ, Brafman DA, Wang X. Prime Editing Guide RNA Design Automation Using PINE-CONE. ACS Synth Biol. 2021 Feb 19;10(2):422-427. doi: 10.1021/acssynbio.0c00445. Epub 2021 Jan 19. PMID: 33464043; PMCID: PMC7901017; (7) Zhang W, Petri K, Ma J, Lee H, Tsai CL, Joung JK, Yeh JJ. Enhancing CRISPR prime editing by reducing misfolded pegRNA interactions. bioRxiv [Preprint]. 2023 Aug 15:2023.08.14.553324. doi: 10.1101/2023.08.14.553324. PMID: 37645936; PMCID: PMC10462064; (8) Chow RD, Chen JS,

Shen J, Chen S. A web tool for the design of prime-editing guide RNAs. Nat Biomed Eng. 2021 Feb;5(2):190-194. doi: 10.1038/s41551-020-00622-8. Epub 2020 Sep 28. PMID: 32989284; PMCID: PMC7882013; each of which are incorporated herein by reference in their entireties.

[00541]Reference may also be made to the following commercial vendors which synthesize pegRNAs for prime editing applications and provide various tools and instruction for the ordering, design, synthesis, modification, and structural configuration of pegRNAs: GENSCRIPT, SYNTHEGO, TAKARA BIO, INTEGRATED DNA TECHNOLOGIES, LC SCIENCES, HORIZON DISCOVERY; SIGMA-ALDRICH; ORIGENE, and TWIST BIOSCIENCES, among others.

[00542]In addition, pegRNAs may be modified with chemical modifications and/or structural modifications for enhancing various properties thereof, including specificity, stability, and limiting off-target activity. One of ordinary skill in the art will be able to modify a guide RNA with any known modification without undue experimentation. Guide modifications are discussed in the following references: (1) Ke Y, Ghalandari B, Huang S, Li S, Huang C, Zhi X, Cui D, Ding X. 2'-*O*-Methyl modified guide RNA promotes the single nucleotide polymorphism (SNP) discrimination ability of CRISPR-Cas12a systems. Chem Sci. 2022 Feb 1;13(7):2050-2061. doi: 10.1039/d1sc06832f. PMID: 35308857; PMCID: PMC8848812; (2) Allen D, Rosenberg M, Hendel A. Using Synthetically Engineered Guide RNAs to Enhance CRISPR Genome Editing Systems in Mammalian Cells. Front Genome Ed. 2021 Jan 28;2:617910. doi: 10.3389/fgeed.2020.617910. PMID: 34713240; PMCID: PMC8525374; (3) Basila M, Kelley ML, Smith AVB. Minimal 2'-O-methyl phosphorothioate linkage modification pattern of synthetic guide RNAs for increased stability and efficient CRISPR-Cas9 gene editing avoiding cellular toxicity. PLoS One. 2017 Nov 27;12(11):e0188593. doi: 10.1371/journal.pone.0188593. PMID: 29176845; PMCID: PMC5703482; (4) Sakovina L, Vokhtantsev I, Vorobyeva M, Vorobyev P, Novopashina D. Improving Stability and Specificity of CRISPR/Cas9 System by Selective Modification of Guide RNAs with 2'-fluoro and Locked Nucleic Acid Nucleotides. Int J Mol Sci. 2022 Nov 3;23(21):13460. doi: 10.3390/ijms232113460. PMID: 36362256; PMCID: PMC9655745; (5) Shapiro J, Tovin A, Iancu O, Allen D, Hendel A. Chemical Modification of Guide RNAs for Improved CRISPR Activity in CD34+ Human Hematopoietic Stem and Progenitor Cells. Methods Mol Biol. 2021;2162:37-48. doi: 10.1007/978-1-0716-0687-2_3. PMID: 32926376; (6) Filippova J, Matveeva A, Zhuravlev E, Stepanov G. Guide RNA modification as a way to improve CRISPR/Cas9-based genome-editing systems. Biochimie. 2019 Dec;167:49-60. doi: 10.1016/j.biochi.2019.09.003. Epub 2019 Sep 4. PMID: 31493470; (7) Hendel A, Bak RO, Clark JT, Kennedy AB, Ryan DE, Roy S, Steinfeld I, Lunstad BD, Kaiser RJ, Wilkens AB, Bacchetta R, Tsalenko A, Dellinger D, Bruhn L, Porteus MH. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat Biotechnol. 2015 Sep;33(9):985-989. doi: 10.1038/nbt.3290. Epub 2015 Jun 29. PMID: 26121415; PMCID: PMC4729442; (8) Ryan DE, Taussig D, Steinfeld I, Phadnis SM, Lunstad BD, Singh M, Vuong X, Okochi KD, McCaffrey R, Olesiak M, Roy S, Yung CW, Curry B, Sampson JR, Bruhn L,

Dellinger DJ. Improving CRISPR-Cas specificity with chemical modifications in single-guide RNAs. Nucleic Acids Res. 2018 Jan 25;46(2):792-803. doi: 10.1093/nar/gkx1199. Erratum in: Nucleic Acids Res. 2022 Mar 21;50(5):2986. PMID: 29216382; PMCID: PMC5778453; (9) Palumbo CM, Gutierrez-Bujari JM, O'Geen H, Segal DJ, Beal PA. Versatile 3' Functionalization of CRISPR Single Guide RNA. Chembiochem. 2020 Jun 2;21(11):1633-1640. doi: 10.1002/cbic.201900736. Epub 2020 Mar 5. PMID: 31943634; PMCID: PMC7323579; (10) Mullally G, van Aelst K, Naqvi MM, Diffin FM, Karvelis T, Gasiunas G, Siksnys V, Szczelkun MD. 5' modifications to CRISPR-Cas9 gRNA can change the dynamics and size of R-loops and inhibit DNA cleavage. Nucleic Acids Res. 2020 Jul 9;48(12):6811-6823. doi: 10.1093/nar/gkaa477. PMID: 32496535; PMCID: PMC7337959; (12) Lu S, Zhang Y, Yin H. Chimeric DNA-RNA Guide RNA Designs. Methods Mol Biol. 2021;2162:79-85. doi: 10.1007/978-1-0716-0687-2_6. PMID: 32926379; each of which are incorporated by reference herein in their entireties.

[00543]In the specific case of prime editing, pegRNAs may be further modified with chemical modifications and/or structural modifications for enhancing various properties thereof, including specificity, stability, and limiting off-target activity. One of ordinary skill in the art will be able to modify a pegRNA for prime editing with any known modification without undue experimentation. pegRNA modifications are discussed in the following references: (1) Nelson JW, Randolph PB, Shen SP, Everette KA, Chen PJ, Anzalone AV, An M, Newby GA, Chen JC, Hsu A, Liu DR. Engineered pegRNAs improve prime editing efficiency. Nat Biotechnol. 2022 Mar;40(3):402-410. doi: 10.1038/s41587-021-01039-7. Epub 2021 Oct 4. Erratum in: Nat Biotechnol. 2021 Dec 8;: PMID: 34608327; PMCID: PMC8930418; (2) Liu B, Dong X, Cheng H, Zheng C, Chen Z, Rodríguez TC, Liang SQ, Xue W, Sontheimer EJ. A split prime editor with untethered reverse transcriptase and circular RNA template. Nat Biotechnol. 2022 Sep;40(9):1388-1393. doi: 10.1038/s41587-022-01255-9. Epub 2022 Apr 4. PMID: 35379962; each of which are incorporated by reference herein in their entireties.

[00544]In some embodiments, the prime editing system further comprises a second guide RNA targeting the complementary strand, allowing the Cas9 nickase to also nick the non-edited strand (called "PE3"), which biases mismatch DNA repair in favor of the edited sequence. In some embodiments, the second guide RNA is designed to recognize the complementary strand of DNA only after the PE3 edit has occurred (called "PE3b"), which reduces indel formation.

[00545]In some embodiments, the prime editing system comprises an uracil glycosylase inhibitor. In some embodiments, the prime editing system comprises a Cas9 protein fused to an uracil glycosylase inhibitor. In some embodiments, the cargo comprises an uracil glycosylase inhibitor or a polynucleotide encoding an uracil glycosylase inhibitor. In some embodiments, the cargo comprises a Cas9 protein fused to an uracil glycosylase inhibitor or a polynucleotide encoding a Cas9 protein fused to an uracil glycosylase inhibitor.

[00546]Any of the above prime editor embodiments or variants, modifications, or derivatives thereof are contemplated herein to be delivered by the LNP systems disclosed in this specification for gene editing in cells, tissues, and/or organs under *in vitro*, *ex vivo*, or *in vivo* conditions. The various components described herein may be configured and delivered in any suitable manner. Any of the descriptions presented in this section are not intended to be strictly limiting.

[00547]In embodiments, the reverse transcriptase component of the prime editor can be any reverse transcriptase known in the art, or any variant thereof, such as those described in the above published prime editing application or in the scientific literature, such as in: (1) Gao Z, Ravendran S, Mikkelsen NS, Haldrup J, Cai H, Ding X, Paludan SR, Thomsen MK, Mikkelsen JG, Bak RO. A truncated reverse transcriptase enhances prime editing by split AAV vectors. Mol Ther. 2022 Sep 7;30(9):2942-2951. doi: 10.1016/j.ymthe.2022.07.001. Epub 2022 Jul 8. PMID: 35808824; PMCID: PMC9481986; (2) Lan T, Chen H, Tang C, Wei Y, Liu Y, Zhou J, Zhuang Z, Zhang Q, Chen M, Zhou X, Chi Y, Wang J, He Y, Lai L, Zou Q. Mini-PE, a prime editor with compact Cas9 and truncated reverse transcriptase. Mol Ther Nucleic Acids. 2023 Aug 18;33:890-897. doi: 10.1016/j.omtn.2023.08.018. PMID: 37680986; PMCID: PMC10480570; (3) or available biological sequence databases, all of which are incorporated herein by reference.

[00548]In addition, the reverse transcriptase may be a retron reverse transcriptase (retron RT), such as any of those described in: (1) US Patent Application Serial No. 18/087,673; (2) International PCT Application No. PCT/US2023/061038; (3) International Application No. PCT/US2023/072872; (4) Mestre *et al.*, Nucleic Acids Research, Volume 48, Issue 22, 16 December 2020, Pages 12632-12647; (5) Mestre *et al.*, *UG/Abi: "A Highly Diverse Family of Prokaryotic Reverse Transcriptases Associated With Defense Functions,"* doi.org/10.1101/2021.12.02.470933; (6) International Application No. PCT/US2023/016262; (7) International Application No. PCT/US2023/016263; (8) International Application No. PCT/US23/72799; (9) International Application NO. PCT/US2022/079220; (10) International Application No. PCT/US2023/016317; and (10) may particularly be any retron selected from Table A of International Application No. PCT/US2023/072872, or any amino acid sequence having at having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8% or at least 99.9% sequence identity to a polypeptide listed in Table A of International Application No. PCT/US2023/072872. The contents of each of the documents in this paragraph are incorporated herein by reference in their entireties.

Retron editors

[00549]In still other embodiments, the herein disclosed LNPs may be used to encapsulate and deliver a retron editing system. A retron editing system in various embodiments may comprise (a) a retron reverse transcriptase, or a nucleic acid molecule encoding a retron reverse transcriptase, (b) a retron

ncRNA (or a nucleic acid molecule encoding same) comprising a modified msd region to include a sequence that is reverse transcribed to form a single strand template DNA sequence (RT-DNA), (c) a nucleic acid programmable nuclease (e.g., a CRISPR Cas9 or Cas12a), and (d) a guide RNA to target the nuclease to a desired target site.

[00550]Retrons are defined by their unique ability to produce an unusual satellite DNA known as msDNA (multicopy single-stranded DNA). DNA encoding retrons includes a reverse trancriptase (RT)-coding gene (*ret*) and a nucleic acid sequence encoding the non-coding RNA (ncRNA), which contains two contiguous and inverted non-coding sequences referred to as the *msr* and *msd*. The *ret* gene and the non-coding RNA (including the *msr* and *msd*) are transcribed as a single RNA transcript, which becomes folded into a specific secondary structure following post-transcriptional processing. Once translated, the RT binds the RNA template downstream from the *msd* locus, initiating reverse transcription of the RNA towards its 5′ end, assisted by the 2'OH group present in a conserved branching guanosine residue that acts as a primer. Reverse transcription halts before reaching the *msr* locus, and the resulting DNA, the msDNA, remains covalently attached to the RNA template via a 2'-5′ phosphodiester bond and base-pairing between the 3′ ends of the msDNA and the RNA template. The external regions, at the 5′ and 3′ ends of the msd/msr transcript (a1 and a2, respectively) are complementary and can hybridize, leaving the structures located in the *msr* and *msd* regions in internal positions (see FIG. 1A). The *msr* locus, which is not reverse transcribed, forms one to three short stem-loops of variable size, ranging from 3 to 10 base pairs, whereas the *msd* locus folds into a single/double long hairpin with a highly variable long stem of 10-50 bp in length that is also present in the final msDNA form.

[00551]It has recently been reported that retrons may be utilized as a means to provide donor DNA template for HDR-dependent genome editing (e.g., see Lopez et al., "Precise genome editing across kingdoms of life using retron-derived DNA," *Nature Chemical Biology*, December 12, 2021, **18**, pages 199–206 (2022)), however, producing sufficient levels of donor DNA template intracellularly to sufficiently support efficient HDR-dependent editing remains a significant challenge.

[00552]Retrons have previously been described in the scientific literature, including in the context of retron editing. For example, retrons have been described in the following references, each of which are incorporated herein by reference:

Title	Pub.	Journal	Author/s	V	Ps
Recording gene expression order in DNA by CRISPR addition of retron barcodes.	7/27/2022	Nature	Bhattarai-Kline; etal.	608	217-225
Retrons Display Genome Editing Strengths Even CRISPR Might Envy	6/1/2021	Genetic Engineering & Biotechnology News		41	15-15
Retron reverse transcriptase termination and phage	3/16/2022	Nucleic acids research	Palka; et al.	50	3490-3504

Title	Pub.	Journal	Author/s	V	Ps
defense are dependent on host RNase H1.					
Retrons: Complementing CRISPR in Phage Defense.	8/1/2020	The CRISPR journal	Maxwell	3	226-227
Systematic prediction of genes functionally associated with bacterial retrons and classification of the encoded tripartite systems.	12/4/2020	Nucleic acids research	Rodríguez Mestre; et al.	48	12632-12647
Precise genome editing without exogenous donor DNA via retron editing system in human cells.	8/17/2021	Protein & cell	Kong; et al.	12	899-902
Precise genome editing across kingdoms of life using retron-derived DNA.	12/23/2021	Nature chemical biology	Lopez; et al.	18	199-206
Prokaryotic reverse transcriptases: from retroelements to specialized defense systems	5/13/2021	FEMS microbiology reviews	González-Delgado, et al.	45	
A function for retrons.	11/17/2020	Nature reviews. Microbiology	Grant Otto	19	3-3
Retrons and their applications in genome engineering.	10/10/2019	Nucleic acids research	Simon, et al.	47	11007-11019
High-efficiency retron-mediated single-stranded DNA production in plants.	1/1/2022	Synthetic biology (Oxford, England)	Jiang, et al.	7	
Bacterial retrons enable precise gene editing in human cells	1/24/2022	The CRISPR journal	Zhao B, et al.	5	31-39
Like CRISPR, mystery gene editor began as a virus fighter.	11/20/2020	Science (New York, N.Y.)	Pennisi	370	898-899
Bacterial Retrons Function In Anti-Phage Defense.	11/5/2020	Cell	Millman, et al.	183	1551-1561
Multiplex generation, tracking, and functional screening of substitution mutants using a crispr/retron system	5/4/2020	ACS synthetic biology	Lim, et al.	9	1003-1009
Multiple origins of reverse transcriptases linked to CRISPR-Cas systems.	7/11/2019	RNA biology	Toro, et al.	16	1486-1493
Retroelement-Based Genome Editing and Evolution.	10/26/2018	ACS synthetic biology	Simon, et al.	7	2600-2611

[00553]In addition, retrons have previously been described in the patent literature, including in the context of retron editing. For example, retrons have been described in the following references, each of which are incorporated herein by reference:

Publication No.	TITLE
US 2020/0115706 A1	METHOD OF RECORDING MULTIPLEXED BIOLOGICAL INFORMATION INTO A CRISPR ARRAY USING A RETRON
EP 3510151 A4	HIGH-THROUGHPUT PRECISION GENOME EDITING
US 2019/0330619 A1	HIGH-THROUGHPUT PRECISION GENOME EDITING
EP 3510151 A1	HIGH-THROUGHPUT PRECISION GENOME EDITING
WO 2018/191525 A1	METHOD OF RECORDING MULTIPLEXED BIOLOGICAL INFORMATION INTO A CRISPR ARRAY USING A RETRON
US 2018/0127759 A1	DYNAMIC GENOME ENGINEERING
WO 2018/049168 A1	HIGH-THROUGHPUT PRECISION GENOME EDITING
US 2017/0204399 A1	GENOMICALLY-ENCODED MEMORY IN LIVE CELLS
EP 3180430 A1	GENOMICALLY-ENCODED MEMORY IN LIVE CELLS
CA 2488328 C	RETRONS FOR GENE TARGETING
WO 2016/025719 A1	GENOMICALLY-ENCODED MEMORY IN LIVE CELLS
US 8932860 B2	RETRONS FOR GENE TARGETING
EP 1517992 B1	RETRONS FOR GENE TARGETING
AU 2003/233734 C1	RETRONS FOR GENE TARGETING
AU 2003/233734 B2	RETRONS FOR GENE TARGETING
US 2009/0123991 A1	RETRONS FOR GENE TARGETING
US 2005/0250207 A1	RETRONS FOR GENE TARGETING
EP 1517992 A2	RETRONS FOR GENE TARGETING
WO 2003/104470 A3	RETRONS FOR GENE TARGETING
AU 2003/233734 A1	RETRONS FOR GENE TARGETING
CA 2488328 A1	RETRONS FOR GENE TARGETING
WO 2003/104470 A2	RETRONS FOR GENE TARGETING

[00554]In some embodiments, the LNP-based retron editing system can be used for genome editing a desired site. A retron is engineered with a heterologous nucleic acid sequence encoding a donor polynucleotide ("template or donor nucleotide sequence" or "template DNA") suitable for use with nuclease genome editing system. The nuclease is designed to specifically target a location proximal to the desired edit (the nuclease should be designed such that it will not cut the target once the edit is properly installed). The nuclease (*e.g.*, CAS or non-CAS) is linked to the retron, either by direct fusion to the RT or by fusion of the msDNA to the gRNA (only applicable for RNA-guided nucleases). A heterologous nucleic acid sequence is inserted into the retron msd.

[00555]In some embodiments, the heterologous nucleic acid sequence has 10-100 or more bp of homologous nucleic acid sequence to the genome on both sides of the desired edit. The desired edit (insertion, deletion, or mutation) is in between the homologous sequence.

[00556]In some embodiments, donor polynucleotides comprise a sequence comprising an intended genome edit flanked by a pair of homology arms responsible for targeting the donor polynucleotide to the target locus to be edited in a cell. The donor polynucleotide typically comprises a 5′ homology arm that hybridizes to a 5′ genomic target sequence and a 3′ homology arm that hybridizes to a 3′ genomic target sequence. The homology arms are referred to herein as 5′ and 3′ (*i.e.*, upstream and downstream) homology arms, which relate to the relative position of the homology arms to the nucleotide sequence comprising the intended edit within the donor polynucleotide. The 5′ and 3′ homology arms hybridize to regions within the target locus in the genomic DNA to be modified, which are referred to herein as the "5′ target sequence" and "3′ target sequence," respectively.

[00557]The homology arm must be sufficiently complementary for hybridization to the target sequence to mediate homologous recombination between the donor polynucleotide and genomic DNA at the target locus. For example, a homology arm may comprise a nucleotide sequence having at least about 80-100% sequence identity to the corresponding genomic target sequence, including any percent identity within this range, such as at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity thereto, wherein the nucleotide sequence comprising the intended edit can be integrated into the genomic DNA by HDR at the genomic target locus recognized (*i.e.*, having sufficient complementary for hybridization) by the 5′ and 3′ homology arms.

[00558]In some embodiments, the corresponding homologous nucleotide sequences in the genomic target sequence (*i.e.*, the "5′ target sequence" and "3′ target sequence") flank a specific site for cleavage and/or a specific site for introducing the intended edit. The distance between the specific cleavage site and the homologous nucleotide sequences (*e.g.*, each homology arm) can be several hundred nucleotides. In some embodiments, the distance between a homology arm and the cleavage site is 200 nucleotides or less (*e.g.*, 0, 10, 20, 30, 50, 75, 100, 125, 150, 175, and 200 nucleotides). In most cases, a smaller distance may give rise to a higher gene targeting rate. In some embodiments, the donor polynucleotide is substantially identical to the target genomic sequence, across its entire length except for the sequence changes to be introduced to a portion of the genome that encompasses both the specific cleavage site and the portions of the genomic target sequence to be altered.

[00559]A homology arm can be of any length, *e.g.* 10 nucleotides or more, 15 nucleotides or more, 20 nucleotides or more, 50 nucleotides or more, 100 nucleotides or more, 250 nucleotides or more, 300 nucleotides or more, 350 nucleotides or more, 400 nucleotides or more, 450 nucleotides or more, 500 nucleotides or more, 1000 nucleotides (1 kb) or more, 5000 nucleotides (5 kb) or more, 10000 nucleotides (10 kb) or more, *etc*. In some instances, the 5′ and 3′ homology arms are substantially equal in length to one another. However, in some instances the 5′ and 3′ homology arms are not

necessarily equal in length to one another. For example, one homology arm may be 30% shorter or less than the other homology arm, 20% shorter or less than the other homology arm, 10% shorter or less than the other homology arm, 5% shorter or less than the other homology arm, 2% shorter or less than the other homology arm, or only a few nucleotides less than the other homology arm. In other instances, the 5′ and 3′ homology arms are substantially different in length from one another, *e.g.* one may be 40% shorter or more, 50% shorter or more, sometimes 60% shorter or more, 70% shorter or more, 80% shorter or more, 90% shorter or more, or 95% shorter or more than the other homology arm.

[00560]The donor polynucleotide may be used in combination with an RNA-guided nuclease, which is targeted to a particular genomic sequence (*i.e.*, genomic target sequence to be modified) by a guide RNA. A target-specific guide RNA comprises a nucleotide sequence that is complementary to a genomic target sequence, and thereby mediates binding of the nuclease-gRNA complex by hybridization at the target site. For example, the gRNA can be designed with a sequence complementary to the sequence of a minor allele to target the nuclease-gRNA complex to the site of a mutation. The mutation may comprise an insertion, a deletion, or a substitution. For example, the mutation may include a single nucleotide variation, gene fusion, translocation, inversion, duplication, frameshift, missense, nonsense, or other mutation associated with a phenotype or disease of interest. The targeted minor allele may be a common genetic variant or a rare genetic variant. In some embodiments, the gRNA is designed to selectively bind to a minor allele with single base-pair discrimination, for example, to allow binding of the nuclease-gRNA complex to a single nucleotide polymorphism (SNP). In particular, the gRNA may be designed to target disease-relevant mutations of interest for the purpose of genome editing to remove the mutation from a gene. Alternatively, the gRNA can be designed with a sequence complementary to the sequence of a major or wild-type allele to target the nuclease-gRNA complex to the allele for the purpose of genome editing to introduces a mutation into a gene in the genomic DNA of the cell, such as an insertion, deletion, or substitution. Such genetically modified cells can be used, for example, to alter phenotype, confer new properties, or produce disease models for drug screening.

[00561]In some embodiments, the RNA-guided nuclease used for genome modification is a clustered regularly interspersed short palindromic repeats (CRISPR) system Cas nuclease. Any RNA-guided Cas nuclease capable of catalyzing site- directed cleavage of DNA to allow integration of donor polynucleotides by the HDR mechanism can be used in genome editing, including CRISPR system Class 1, Type I, II, or III Cas nucleases; Class 2, Type II nuclease (such as Cas9); a Class 2, Type V nuclease (such as Cpf1), or a Class 2, Type VI nuclease (such as C2c2). Examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas5e (CasD), Cas6, Cas6e, Cas6f, Cas7, Cas8a1, Cas8a2, Cas8b, Cas8c, Cas9 (Csn1 or Csx12), Cas10, Cas10d, CasF, CasG, CasH, Csy1, Csy2, Csy3, Cse1 (CasA), Cse2 (CasB), Cse3 (CasE), Cse4 (CasC), Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4,

Csm5, Csm6, Cmrl, Cmr3, Cmr4, Cmr5, Cmr6, Csbl, Csb2, Csb3, Csxl7, Csxl4, CsxlO, Csxl6, CsaX, Csx3, Csxl, Csxl5, Csfl, Csf2, Csf3, Csf4, and Cul966, and homologs or modified versions thereof.

[00562]In some embodiments, a Class 1, type II CRISPR system Cas9 endonuclease is used. Cas9 nucleases from any species, or biologically active fragments, variants, analogs, or derivatives thereof that retain Cas9 endonuclease activity (*i.e.*, catalyze site-directed cleavage of DNA to generate double-strand breaks) may be used to perform genome modification as described herein. The Cas9 need not be physically derived from an organism but may be synthetically or recombinantly produced. Cas9 sequences from a number of bacterial species are well known in the art and listed in the National Center for Biotechnology Information (NCBI) database. See, for example, NCBI entries for Cas9 from: *Streptococcus pyogenes* (WP 002989955, WP_038434062, WP_011528583); *Campylobacter jejuni* (WP_022552435, YP 002344900), *Campylobacter coli* (WP 060786116); *Campylobacter fetus* (WP 059434633); *Corynebacterium ulcerans* (NC_015683, NC_017317); *Corynebacterium diphtheria* (NC_016782, NC_016786); *Enterococcus faecalis* (WP 033919308); *Spiroplasma syrphidicola* (NC 021284); *Prevotella intermedia* (NC 017861); *Spiroplasma taiwanense* (NC 021846); *Streptococcus iniae* (NC 021314); *Belliella baltica* (NC 018010); *Psychroflexus torquisl* (NC O 18721); *Streptococcus thermophilus* (YP 820832), *Streptococcus mutans* (WP 061046374, WP 024786433); *Listeria innocua* (NP 472073); *Listeria monocytogenes* (WP 061665472); *Legionella pneumophila* (WP 062726656); *Staphylococcus aureus* (WP_001573634); *Francisella tularensis* (WP_032729892, WP_014548420), *Enterococcus faecalis* (WP 033919308); *Lactobacillus rhamnosus* (WP 048482595, WP_032965177); and *Neisseria meningitidis* (WP_061704949, YP_002342100); all of which sequences (as entered by the date of filing of this application) are herein incorporated by reference in their entireties. Any of these sequences or a variant thereof comprising a sequence having at least about 70-100% sequence identity thereto, including any percent identity within this range, such as 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity thereto, can be used for genome editing, as described herein. See also Fonfara *et al.* (2014) Nucleic Acids Res. 42(4):2577-90; Kapitonov *et al.* (2015) J. Bacterid. 198(5): 797-807, Shmakov *et al.* (2015) Mol. Cell. 60(3):385- 397, and Chylinski *et al.* (2014) Nucleic Acids Res. 42(10):6091-6105); for sequence comparisons and a discussion of genetic diversity and phylogenetic analysis of Cas9.

[00563]The genomic target site will typically comprise a nucleotide sequence that is complementary to the gRNA and may further comprise a protospacer adjacent motif (PAM). In some embodiments, the target site comprises 20-30 base pairs in addition to a 3 or more base pair PAM. Typically, the first nucleotide of a PAM can be any nucleotide, while the two or more other nucleotides will depend on the specific Cas9 protein that is chosen. Exemplary PAM sequences are known to those of skill in the art and include, without limitation, NNG, NGN, NAG, and NGG, wherein N represents any nucleotide. In some embodiments, the allele targeted by a gRNA comprises a mutation that creates a PAM within the allele, wherein the PAM promotes binding of the Cas9-gRNA complex to the allele.

[00564]In some embodiments, the gRNA is 5-50 nucleotides, 10-30 nucleotides, 15- 25 nucleotides, 18-22 nucleotides, or 19-21 nucleotides in length, or any length between the stated ranges, including, for example, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 nucleotides in length. The guide RNA may be a single guide RNA comprising crRNA and tracrRNA sequences in a single RNA molecule, or the guide RNA may comprise two RNA molecules with crRNA and tracrRNA sequences residing in separate RNA molecules.

[00565]In another embodiment, the CRISPR nuclease from *Prevotella* and *Francisella* 1 (Cpfl, or Cas12a) is used. Cpfl is another class II CRISPR/Cas system RNA-guided nuclease with similarities to Cas9 and may be used analogously. Unlike Cas9, Cpfl does not require a tracrRNA and only depends on a crRNA in its guide RNA, which provides the advantage that shorter guide RNAs can be used with Cpfl for targeting than Cas9. Cpfl is capable of cleaving either DNA or RNA. The PAM sites recognized by Cpfl have the sequences 5′-YTN-3′ (where "Y" is a pyrimidine and "N" is any nucleobase) or 5′-TTN-3′, in contrast to the G-rich PAM site recognized by Cas9. Cpfl cleavage of DNA produces double-stranded breaks with a sticky-ends having a 4 or 5 nucleotide overhang. For a discussion of Cpfl, see, *e.g.*, Ledford *et al.* (2015) Nature. 526 (7571):17-17, Zetsche *et al.* (2015) Cell. 163 (3):759-771, Murovec *et al.* (2017) Plant Biotechnol. J. 15(8):917-926, Zhang *et al.* (2017) Front. Plant Sci. 8:177, Fernandes *et al.* (2016) Postepy Biochem. 62(3):315-326; herein incorporated by reference.

[00566]C2c1 (Cas12b) is another class II CRISPR/Cas system RNA-guided nuclease that may be used. C2cl, similarly to Cas9, depends on both a crRNA and tracrRNA for guidance to target sites. See, *e.g.*, Shmakov *et al.* (2015) Mol Cell. 60(3):385-397, Zhang *et al.* (2017) Front Plant Sci. 8:177; herein incorporated by reference.

[00567]In yet another embodiment, an engineered RNA-guided Fokl nuclease may be used. RNA-guided Fokl nucleases comprise fusions of inactive Cas9 (dCas9) and the Fokl endonuclease (FokI-dCas9), wherein the dCas9 portion confers guide RNA-dependent targeting on Fokl. For a description of engineered RNA-guided Fold nucleases, see, *e.g.*, Havlicek *et al.* (2017) Mol. Ther. 25(2):342-355, Pan *et al.* (2016) Sci Rep. 6:35794, Tsai *et al.* (2014) Nat Biotechnol. 32(6):569-576; herein incorporated by reference.

[00568]In other embodiments, any other Cas enzymes and variants described in other sections of the application (all incorporated herein) can be used similarly.

[00569]In some embodiments, the RNA-guided nuclease is provided in the form of a protein, optionally where the nuclease is complexed with a gRNA to form a ribonucleoprotein (RNP) complex. In some embodiments, the RNA-guided nuclease is provided by a nucleic acid encoding the RNA-guided nuclease, such as an RNA (*e.g.*, messenger RNA) or DNA (expression vector). In some embodiments, the RNA-guided nuclease and the gRNA are both provided by vectors, such as the vectors and the vector system described in other parts of the application (all incorporated herein by reference). Both can be expressed by a single vector or separately on different vectors. The vectors

encoding the RNA-guided nuclease and gRNA may be included in the vector system comprising the engineered retron *msr* gene, *msd* gene and *ret* gene sequences. In some embodiments, the RNA-guided nuclease is fused to the RT and/or the msDNA.

[00570]The RNP complex may be administered to a subject or delivered into a cell by methods known in the art, such as those described in U.S. Pat. No. 11,390,884, which is incorporated by reference herein in its entirety. In some embodiments, the endonuclease/gRNA ribonucleoprotein (RNP) complexes are delivered to cells by electroporation. Direct delivery of the RNP complex to a subject or cell eliminates the need for expression from nucleic acids (e.g., transfection of plasmids encoding Cas9 and gRNA). It also eliminates unwanted integration of DNA segments derived from nucleic acid delivery (e.g., transfection of plasmids encoding Cas9 and gRNA). An endonuclease/gRNA ribonucleoprotein (RNP) complex usually is formed prior to administration.

[00571]Codon usage may be optimized to further improve production of an RNA-guided nuclease and/or reverse transcriptase (RT) in a particular cell or organism. For example, a nucleic acid encoding an RNA-guided nuclease or reverse transcriptase can be modified to substitute codons having a higher frequency of usage in a yeast cell, a bacterial cell, a human cell, a non-human cell, a mammalian cell, a rodent cell, a mouse cell, a rat cell, or any other host cell of interest, as compared to the naturally occurring polynucleotide sequence. When a nucleic acid encoding the RNA-guided nuclease or reverse transcriptase is introduced into cells, the protein can be transiently, conditionally, or constitutively expressed in the cell.

[00572]In some embodiments, the engineered retron used for genome editing with nuclease genome editing systems can further include accessory or enhancer proteins for recombination. Examples of recombination enhancers can include nonhomologous end joining (NHEJ) inhibitors (e.g., inhibitor of DNA ligase IV, a KU inhibitor (*e.g.*, KU70 or KU80), a DNA-PKc inhibitor, or an artemis inhibitor) and homologous directed repair (HDR) promoters, or both, that can enhance or improve more precise genome editing and/or the efficiency of homologous recombination. In some embodiments, the recombination accessory or enhancers can comprise C-terminal binding protein interacting protein (CtIP), cyclinB2, Rad family members (*e.g.* Rad50, Rad51, Rad52, etc).

[00573]CtIP is a transcription factor containing C2H2 zinc fingers that are involved in early steps of homologous recombination. Mammalian CtIP and its orthologs in other eukaryotes promote the resection of DNA double-strand breaks and are essential for meiotic recombination. HDR may be enhanced by using Cas9 nuclease associated (*e.g.* fused) to an N-terminal domain of CtIP, an approach that forces CtIP to the cleavage site and increases transgene integration by HDR. In some embodiments, an N-terminal fragment of CtIP, called HE for HDR enhancer, may be sufficient for HDR stimulation and requires the CtIP multimerization domain and CDK phosphorylation sites to be active. HDR stimulation by the Cas9-HE fusion depends on the guide RNA used, and therefore the guide RNA will be designed accordingly.

[00574]Using the gene editing system described herein, any target gene or sequence in a host cell can be edited or modified for a desired trait, including but not limited to: Myostatin (*e.g.*, GDF8) to increase muscle growth; Pc POLLED to induce hairlessness; KISS1R to induce bore taint; Dead end protein (dnd) to induce sterility; Nano2 and DDX to induce sterility; CD163 to induce PRRSV resistance; RELA to induce ASFV resilience; CD18 to induce Mannheimia (Pasteurella) haemolytica resilience; NRAMPl to induce tuberculosis resilience; Negative regulators of muscle mass (*e.g.*, Myostatin) to increase muscle mass.

[00575]Any of the above retron editor embodiments or variants, modifications, or derivatives thereof are contemplated herein to be delivered by the LNP systems disclosed in this specification for gene editing in cells, tissues, and/or organs under *in vitro*, *ex vivo*, or *in vivo* conditions. The various components described herein may be configured and delivered in any suitable manner. Any of the descriptions presented in this section are not intended to be strictly limiting.

TnpB editors

[00576]In other embodiments, the herein disclosed LNPs may be used to encapsulate and deliver a TnpB editing system and/or components thereof. A TnpB editing system in various embodiments may comprise (a) a TnpB protein, or a nucleic acid molecule encoding a TnpB protein, (b) a TnpB guide RNA known as an "reRNA" or "right end RNA", and optionally one or more additional components, including (c) an effector domain or otherwise accessory protein, and (d) a DNA template (e.g., a DNA donor for HDR-dependent repair at the TnpB-cut target site.

[00577]In various embodiments, the TnpB protein can be naturally occurring or the TnpB can be an engineered variant thereof and can be used in various applications, including precision gene editing in cells, tissues, organs, or organisms. The TnpB-based gene editing systems comprise a TnpB polypeptide and a nucleic acid component capable of forming a complex with the TnpB polypeptide which directs the complex to a target nucleotide sequence (e.g., a genomic target sequence such as a disease-associated gene). The TnpB gene editing systems contemplated herein may also be modified with one or more additional effector or accessory functions, such as a nuclease, recombinase, ligase, reverse transcriptase, polymerase, deaminase, etc. to provide additional genome editing functionality. In addition, the TnpB gene editing systems contemplated herein can utilize a nuclease-limited or nuclease-deficienty TnpB variant. Normal TnpB nuclease activity cuts both strands of a target DNA, however, TnpB nickases (having only the ability to cut one of the two strands but not both strands) and nuclease-inactive or "dead" TnpB (which does not cut either strand) may also be used into the TnpB systems described herein, particularly when combined with at least another genome editing functionality, such as a deaminase (for base editing functionality) or a reverse transcriptase (for prime editing functionality). Thus, disclosed herein are TnpB systems that may function as nuclease, nickases, or catalytically inactive polynucleotide binding proteins that can be coupled with other functional domains, such as deaminases, recombinase, ligases, polymerases (e.g., reverse transcriptase), nucleases, or reverse transcriptases.

[00578]In one embodiment, the TnpB systems and related compositions may specifically target single-strand or double-strand DNA. In one embodiment, the TnpB system may bind and cleave double-strand DNA. In one embodiment, the TnpB system may bind to double-stranded DNA without introducing a break to either of the strands. In one embodiment, the TnpB polypeptides or nuclease/nucleic acid component complexes may open, disrupting the continuity of one of the two DNA strands, thereby introducing a nick of the double stranded DNA. In an embodiment, and without being bound by theory, the size and configuration of the TnpB systems allows exposure to the non-targeting strand, which may be in single-stranded form, to allow for for the ability to modify, edit, delete or insert polynucleotides on the non-target strand. In an embodiment, this accessibility further allows for enhanced editing outcomes on the target and/or non-target strand, e.g., increased specificity, enhanced editing efficiency.

[00579]In one aspect, embodiments disclosed herein are directed to compositions comprising a TnpB and a reRNA capable of forming a complex with the TnpB and directing site-specific binding of the TnpB to a target sequence on a target polynucleotide.

TnpB polypeptides

[00580]Any TnpB polypeptide may be utilized with the compositions described herein. The below description of various TnpBs which can be used in connection with the presently disclose TnpB editing systems is not meant to be limiting in any way. The TnpB editing systems disclosed herein may comprise a canonical or naturally-occurring TnpB, or any ortholog TnpB protein, or any variant TnpB protein—including any naturally occurring variant, mutant, or otherwise engineered version of TnpB—that is known or which can be made or evolved through a directed evolutionary or otherwise mutagenic process. In various embodiments, the TnpB or TnpB variants can have a nickase activity, i.e., only cleave of strand of the target DNA sequence. In other embodiments, the TnpB or TnpB variants have inactive nucleases, i.e., are "dead" TnpB proteins. Other variant TnpB proteins that may be used are those having a smaller molecular weight than the canonical TnpB (e.g., for easier delivery) or having modified amino acid sequences or substitutions.

[00581] Examples of TnpB proteins are provided as follows; however, these specific examples are not meant to be limiting. The TnpB editing systems of the present disclosure may use any suitable TnpB protein.

[00582]In various embodiments, the TnpB editing systems of the present disclosure may include one or more TnpB polypeptides selected from SEQ ID NOs 53-187, or a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% sequence identity with one or more of the TnpB polypeptides of SEQ ID NOs 53-187.

[00583]In various other embodiments, the TnpB editing systems of the present disclosure may include one or more TnpB polypeptides and reRNAs disclosed in any of the following published applications, or a polypeptide (or reRNA as the case may be) having at least 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% sequence identity with one or more of the TnpB polypeptides or reRNAs disclosed

therein: US2023/0056577; US2023/0051396 A1; US11578313 B2; US2023/0040216 A1; WO2023/015259 A2; US2023/0032369 A1; US2023/0033866 A1; WO2023/004430 A1; US11560555 B2; WO2023/275601 A1; WO2022/253903 A1; WO2022/248607 A2; US2022/0372525 A1; US2022/0348929 A1; US2022/0348925 A1; US11453866 B2; WO2022/173830 A1; WO2022/174144 A1; WO2022/159892 A1; WO2022/150651 A1; US11384344 B2; WO2022/140572 A1; US2022/0195503 A1; WO2022/098923 A1; WO2022/087494 A1; WO2022/086846 A2; WO2022/076425 A1; WO2022/076890 A1; WO2021/257997 A2; WO2021/247924 A1; US2021/0380956 A1; US11180751 B2; WO2021/188729 A1; WO2021/188286 A2; WO2021/183807 A1; WO2021/159020 A2; US2021/0214697 A1; US2021/0166783 A1; WO2021/050601 A1; EP3009511 B2; US2020/0291395 A1; US2020/0239896 A1; WO2019/178428 A1; US2012/0178668 A1; US7608450 B2; US2004/0091856 A1; US2004/0009477 A1; US2003/0134302 A1; US6562958 B1; and WO1999/051766 A1, each of which are incorporated in their entireties by reference.

[00584]In certain example embodiments, the TnpB polypeptides are between 175 and 800 amino acids in size, between 200 and 790 amino acids in size, between 200 and 780 amino acids in size, between 200 and 770 amino acids in size, between 200 and 760 amino acids in size, between 200 and 750 amino acids in size, between 200 and 740 amino acids in size, between 200 and 730 amino acids in size, between 200 and 720 amino acids in size, between 200 and 720 amino acids in size, between 200 and 710 amino acids in size, between 200 and 700 amino acids in size, between 200 and 690 amino acids in size, between 200 and 680 amino acids in size, between 200 and 670 amino acids in size, between 200 and 660 amino acids in size, between 200 and 650 amino acids in size, between 200 and 640 amino acids in size, between 200 and 630 amino acids in size, between 200 and 620 amino acids in size, between 200 and 610 amino acids in size, between 200 and 600 amino acids in size, between 200 and 590 amino acids in size, between 200 and 580 amino acids in size, between 200 and 570 amino acids in size, between 200 and 560 amino acid, between 200 between 550 amino acids, between 200 and 540 amino acids, between 200 and 530 amino acids, between 200 and 520 amino acids, between 200 and 510 amino acids, between 200 and 500 amino acids, between 200 and 490 amino acids, between 200 and 480 amino acids, between 200 and 470 amino acids, between 200 and 460 amino acids, between 200 and 450 amino acids, between 200 and 440 amino acids, between 200 and 430 amino acids, between 200 and 420 amino acids, between 200 and 410 amino acids, between 210 and 500 amino acids, between 220 and 500 amino acids. Between 230 and 500 amino acids, between 240 and 500 amino acids, between 250 and 500 amino acids, between 260 and 500 amino acids, between 270 and 500 amino acids, between 280 and 500 amino acids, between 290 and 500 amino acids, between 300 and 500 amino acids, between 250 and 490 amino acids, between 250 and 480 amino acids, between 250 and 490 amino acids, or between 250 and 600 amino acids. In one embodiment, the TnpB polypeptide is between 300 and 500 amino acids, or between 350 and 450 amino acids.

[00585]In one embodiment, the TnpB polypeptides may comprise a modified naturally occurring protein, functional fragment or truncated version thereof, or a non-naturally occurring protein. In one embodiment, the TnpB polypeptide comprises one or more domains originating from other TnpB polypeptides, more particularly originating from different organisms. In one embodiment, the TnpB polypeptides may be designed by in silico approaches. Examples of in silico protein design have been described in the art and are therefore known to a skilled person.

[00586]The TnpB polypeptides also encompass homologs or orthologs of TnpB polypeptides whose sequences are specifically described herein (such as the sequences of Table A). The terms "ortholog" and "homolog" are well known in the art. By means of further guidance, a "homolog" of a protein as used herein is a protein of the same species which performs the same or a similar function as the protein it is a homolog of. Homologous proteins may but need not be structurally related, or are only partially structurally related. An "ortholog" of a protein as used herein is a protein of a different species which performs the same or a similar function as the protein it is an orthologue of. Orthologous proteins may be, but may not always be, structurally related or are only partially structurally related. In particular embodiments, the homolog or ortholog of a TnpB polypeptide such as referred to herein has a sequence homology or identity of at least 80%, at least 81%, at least 82%, at least 83%, at least 84% at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% with a TnpB polypeptide, more specifically with a TnpB sequence identified in Table A. In particular embodiments, a homolog or ortholog is identified according to its domain structure and/or function. Sequence alignments conducted as described herein, as well as folding studies and domain predictions can aid in the identification of a homolog or ortholog with the structural and functional characteristics identifying TnpB polypeptides, particularly those with conserved residues, including catalytic residues, and domains of TnpB polypeptides.

[00587]In one embodiment, the TnpB polypeptide comprises at least at least one RuvC-like nuclease domain. The RuvC domain may comprise conserved catalytic amino acids indicative of the RuvC catalytic residue. In an example embodiment, the RuvC catalytic residue may be referenced relative to D191, E278, and D361 of the TnpB of *D. radiodurans* or a corresponding amino acid in an aligned sequence. In an aspect, the RuvC domain may comprise multiple subdomains, e.g., RuvC-I, RuvC-II and RuvC-III. The subdomains may be separated by intervening amino acid sequence of the protein.

[00588]In one embodiment, examples of the RuvC domain include any polypeptides a structural similarity and/or sequence similarity to a RuvC domain described in the art. In some examples, the RuvC domain may have an amino acid sequence that share at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% sequence identity with RuvC domains known in the art. One of ordinary skill in the art can modify, substitute, or otherwise alter the activity of the RuvC domain to alter the nuclease activity, such as whether and/or where the nuclease cuts the DNA.

[00589]In embodiments, the TnpB polypeptide has a nuclease activity. In one embodiment, the TnpB and the targeting RNA (e.g., the reRNA) can direct sequence-specific nuclease activity. The cleavage may result in a 5' overhang. The cleavage may occur distal to a target-adjacent motif (TAM), and may occur at the site of the spacer (i.e., the spacer of the reRNA which is complementary to the target sequences) annealing site or 3' of the target sequence. In an aspect, the TnpB cleaves at multiple positions within and beyond the nucleic acid component annealing site. In an aspect, DNA cleavage occurs 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more base pairs distal to the TAM and results in a 5' overhang. In various embodiments, the TnpB has a nuclease activity against single-stranded DNA. In other embodiments, the TnpB has a nuclease activity against double-stranded DNA.

TnpB modifications

[00590]In various aspects, the present disclosure provides one or more modifications of TnpB comprising TnpB fusions, TnpB mutations to increase sufficiency and/or efficiency and modification of TnpB reRNA. In some embodiments, one or more domains of the TnpB are modified, e.g., wedge domain, corresponding to the β-barrel, REC – helical bundle, RuvC – RuvC domain with the inserted helical hairpin (HH) and the zinc-finger domain (ZnF).

[00591]Without intending to be limited to any particular theory, TnpB operates as a homodimer with one DNA molecule and for some orthologs, its ability to form this conformation may be efficacy limiting. Takeda, Satoru N et al. "Structure of the miniature type V-F CRISPR-Cas effector enzyme." *Molecular cell* vol. 81,3 (2021): 558-570.e3.

[00592]Karvelis et al. demonstrated *Deinococcus radiodurans* ISDra2 TnpB to be an RNA-directed nuclease guided by RE-derived RNA (reRNA) to cleave DNA next to the 5' TTGAT transposon associated motif (TAM). Karvelis, T., Druteika, G., Bigelyte, G. *et al.* Transposon-associated TnpB is a programmable RNA-guided DNA endonuclease. *Nature* **599,** 692–696 (2021).

[00593]Without being bound by theory, it is contemplated that TnpB likely operates as a homodimer. Recent studies show that Cas9-Cas9 fusions displayed higher levels of genome modification and a higher proportion of these editing events were precise deletions than are observed for two independent Cas9 nucleases. Bolukbasi, M.F., Liu, P., Luk, K. *et al.* Orthogonal Cas9–Cas9 chimeras provide a versatile platform for genome editing. *Nat Commun* **9,** 4856 (2018).

[00594]Accordingly, in one embodiment, a TnpB is fused to a second TnpB or the like, for example TnpB-TnpB or TnpB-Cas9. Such dual-nuclease formats comprise one TnpB component displaying expanded targeting and/or enhanced specificity and the second TnpB component having nuclease activity. In other preferred embodiments, a TnpB is fused to two or more nuclease proteins.

[00595]The TnpB polypeptide may comprise one or more modifications. As used herein, the term "modified" with regard to a TnpB polypeptide generally refers to a TnpB polypeptide having one or more modifications or mutations (including point mutations, truncations, insertions, deletions, chimeras, fusion proteins, etc.) compared to the wild type counterpart from which it is derived (e.g., from a TnpB sequence from Tables B or C). By derived is meant that the derived enzyme is largely

based, in the sense of having a high degree of sequence or structural homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as known in the art or as described herein.

[00596]The modified proteins, e.g., modified TnpB polypeptide may be catalytically inactive (dead). As used herein, a catalytically inactive or dead nuclease may have reduced, or no nuclease activity compared to a wildtype counterpart nuclease. In some cases, a catalytically inactive or dead nuclease may have nickase activity. In some cases, a catalytically inactive or dead nuclease may not have nickase activity. Such a catalytically inactive or dead nuclease may not make either double-strand or single-strand break on a target polynucleotide but may still bind or otherwise form complex with the target polynucleotide.

[00597]In an embodiment, eukaryotic homologues of bacterial TnpB may be utilized in the present disclosure. These TnpB-like proteins, Fanzor 1 and Fanzor 2, while having a shared amino acid motif in their C-terminal half regions, are variable in their N terminal regions.

[00598]In one embodiment, the modifications of the TnpB polypeptide may or may not cause an altered functionality. By means of example, modifications which do not result in an altered functionality include for instance codon optimization for expression into a particular host, or providing the nuclease with a particular marker (e.g. for visualization). Modifications with may result in altered functionality may also include mutations, including point mutations, insertions, deletions, truncations (including split nucleases), etc., as well as chimeric nucleases (e.g., comprising domains from different orthologues or homologues) or fusion proteins. Fusion proteins may without limitation include, for instance, fusions with heterologous domains or functional accessory domains (e.g., localization signals, catalytic domains, etc.). In one embodiment, various different modifications may be combined (e.g., a mutated nuclease which is catalytically inactive and which further is fused to a functional domain, such as for instance to induce DNA methylation or another nucleic acid modification, such as including without limitation, a break (e.g. by a different nuclease (domain)), a mutation, a deletion, an insertion, a replacement, a ligation, a digestion, a break or a recombination). As used herein, "altered functionality" includes without limitation an altered specificity (e.g., altered target recognition, increased (e.g., "enhanced" TnpB polypeptide) or decreased specificity, or altered TAM recognition), altered activity (e.g., increased or decreased catalytic activity, including catalytically inactive nucleases or nickases), and/or altered stability (e.g., fusions with destabilization domains).

[00599]Examples of all these modifications are known in the art. It will be understood that a "modified" nuclease as referred to herein, and in particular a "modified" TnpB polypeptide or system or complex preferably still has the capacity to interact with or bind to the polynucleic acid (e.g., in complex with the nucleic acid component molecule). Such modified TnpB polypeptide can be combined with the deaminase protein or active domain thereof as described herein.

[00600]In one embodiment, an unmodified TnpB polypeptides may have cleavage activity. In one embodiment, the TnpB polypeptides may direct cleavage of one or both nucleic acid (DNA or RNA) strands at the location of or near a target sequence, such as within the target sequence and/or within the complement of the target sequence or at sequences associated with the target sequence. In one embodiment, the TnpB polypeptides may direct cleavage of one or both DNA or RNA strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs or nucleotides from the first or last nucleotide of a target sequence. In one embodiment, the cleavage may be staggered, i.e., generating sticky ends. In one embodiment, the cleavage is a staggered cut with a 5' overhang. In one embodiment, the cleavage is a staggered cut with a 5' overhang of 1 to 5 or up to 10 nucleotides. In particular embodiments, the TnpB polypeptides cleave DNA strands.

[00601]In one embodiment, a TnpB polypeptide may be mutated with respect to a corresponding wild-type enzyme (e.g., the TnpB polypeptides of Tables B and C) such that the mutated TnpB lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. As a further example, two or more catalytic domains of a TnpB polypeptide (e.g., RuvC) may be mutated to produce a mutated TnpB polypeptide substantially lacking all DNA cleavage activity. In one embodiment, a TnpB polypeptide may be considered to substantially lack all polynucleotide cleavage activity when the polynucleotide cleavage activity of the mutated enzyme is no more than 25%, no more than 10%, no more than 5%, no more than 1%, no more than 0.1%, no more than 0.01% of the nucleic acid cleavage activity of the non-mutated form of the enzyme; an example can be when the nucleic acid cleavage activity of the mutated form is nil or negligible as compared with the non-mutated form.

[00602]In one embodiment, the TnpB polypeptide may comprise one or more modifications resulting in enhanced activity and/or specificity, such as including mutating residues that stabilize the targeted or non-targeted strand. In one embodiment, the altered or modified activity of the engineered TnpB polypeptide comprises increased targeting efficiency or decreased off-target binding. In one embodiment, the altered activity of the engineered TnpB polypeptide comprises modified cleavage activity. In one embodiment, the altered activity comprises increased cleavage activity as to the target polynucleotide loci. In one embodiment, the altered activity comprises decreased cleavage activity as to the target polynucleotide loci. In one embodiment, the altered activity comprises decreased cleavage activity as to off-target polynucleotide loci. In one embodiment, the modified nuclease comprises a modification that alters association of the protein with the nucleic acid molecule comprising RNA, or a strand of the target polynucleotide loci, or a strand of off-target polynucleotide loci.

[00603]In an aspect of the disclosure, the engineered TnpB polypeptide comprises a modification that alters formation of the TnpB polypeptide and related complex. In one embodiment, the altered activity comprises increased cleavage activity as to off-target polynucleotide loci. Accordingly, in one embodiment, there is increased specificity for target polynucleotide loci as compared to off-target

polynucleotide loci. In other embodiments, there is reduced specificity for target polynucleotide loci as compared to off-target polynucleotide loci. In one embodiment, the mutations result in decreased off-target effects (e.g. cleavage or binding properties, activity, or kinetics), such as in case for TnpB polypeptide for instance resulting in a lower tolerance for mismatches between target and the reRNA. Other mutations may lead to increased off-target effects (e.g., cleavage or binding properties, activity, or kinetics). Other mutations may lead to increased or decreased on-target effects (e.g., cleavage or binding properties, activity, or kinetics). In one embodiment, the mutations result in altered (e.g., increased or decreased) activity, association or formation of the functional nuclease complex. Examples mutations include mutation of negative or neutral residues to positively charged residues, or positively charged residues to neutral or neutral residues to negative residues and/or (evolutionary) conserved residues, such as conserved positively charged residues, in order to enhance specificity. In one embodiment, such residues may be mutated to uncharged residues, such as alanine. Because the TnpB polypeptide interacts with guide or bound DNA over the length of the TnpB polypeptide, mutation of residues across the TnpB polypeptide may be utilized for altered activity. In an aspect, the TnpB polypeptide residues for mutation are altered based on amino acid sequence positions of *Deinococcus radiodurans* ISDra2, see, e.g. Karvelis et al., Nature 599, 692-696 (2021).

[0001] Preferably, one or more TnpB comprises one or more mutated residues in the Rec domain and optionally these mutated residues are hydrophobic. Alternatively, one or more TnpB comprises mutated residues in the RuvC domain. Preferably, one or more of the mutated residues typically form a hydrogen bond with another TnpB monomer. More preferably, a combination of the two sets of mutations as described above.

[0002] In yet other embodiments, the TnpB-nuclease fusions are linked using a polypeptide comprising glycine and serine residues or unstructured XTEN protein polymer.

[0003] In other exemplary embodiments, the TnpB-nuclease fusions are linked using an RNA wherein the RNA comprises a guide RNA or a reRNA.

[0004] In further embodiments, the TnpB-nuclease fusions comprise one or more nuclear localization signals selected from but not limited to SV40, c-Myc, NLP-1.

[0005] Also described herein are methods and compositions for increasing the TnpB-mediated editing efficiency. In some aspects, the editing effiency is greater than 70%, at least 70.5%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99%.

[00604]Additionally described herein are methods and compositions for increasing the TnpB-mediated editing specificity. In some aspects, the editing specificity is greater than 70%, at least 70.5%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99%.

TnpB accessory domains/proteins

[00605]In other aspect, the TnpB-based genome perturbation systems may comprise one or more additional accessory proteins having genome modifying functions, including recombinases, invertases, nucleases, polymerases, ligases, deaminases, or reverse transcriptases. In various embodiments, the accessory proteins may be provided separately. In other embodiments, the accessory proteins may be fused to TnpB, optionally with a linker.

[0006] Liu et al. has recently developed base editing as a technology that edits target nucleotides without creating DSBs or relying on HDR. Direct modification of DNA bases by Cas-fused deaminase enzymes allows for C•G to T•A, or A•T to G•C, base pair conversions in a short target window (~5-7 bases) with very high efficiency. Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A. & Liu, D. R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533, 420–424 (2016). Nishida, K. et al. Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. Science 353, aaf8729 (2016). 6. Gaudelli, N. M. et al. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature 551, 464–471 (2017). Kim, Y. B. et al. Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. Nat. Biotechnol.35, 371–376 (2017). 25. Li, X. et al. Base editing with a Cpf1–cytidine deaminase fusion. Nat. Biotechnol.36, 324–327 (2018). Gehrke, J. M. et al. An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities. Nat. Biotechnol. (2018). doi:10.1038/nbt.4199. Rees, H. A. & Liu, D. R. Base editing: precision chemistry on the genome and transcriptome of living cells. Nat. Rev. Genet.1 (2018). doi:10.1038/s41576-018-0059-1.

[0007] Accordingly, in various aspects of the disclosure, the TnpB is fused to a deaminase suitable for base editing. In some embodiments, the deaminase is selected from an adenosine deaminase, *E. coli* tRNA adenosine, or TadA deaminase wherein TadA is engineered for higher efficiency in human cells in comparison to pWT TadA base editor. In certain embodiments, TadA is engineered through directed evolution.

[0008] In certain other embodiments, the deaminase comprises a cytidine deaminase. Preferably, the cytidine deaminase is engineered for higher efficiency in human cells in comparison to wild type cytidine deaminase base editor. In further embodiments, the TnpB genome editing system contains one or more uracil glycosylase inhibitor.

[0009] In yet other embodiments, the TnpB-deaminase fusions are linked using a polypeptide comprising glycine and serine residues or unstructured XTEN protein polymer.

[0010] In further embodiments, the TnpB RuvC domain is mutated wherein the mutation slows cleavage of the target strand or slows the cleavage of the non-target strand. In other embodiments, the TnpB is mutated to be catalytically inactive.

[0011] In certain preferred embodiments one or more deaminase is fused to a TnpB dimer. In certain embodiments, the deaminase is fused to the N-terminus of TnpB. In other embodiments, the deaminase

is fused to the C-terminus of TnpB. In further embodiments, the deaminase is placed in various locations of the TnpB including without limitations: inside the Rec-domain of the TnpB, after the Rec-domain of the TnpB, in the Wedge domain of TnpB, after the Wedge domain of TnpB, in the RuvC domain of TnpB, after the RuvC domain of TnpB, in the Helical hairpin domain of TnpB, after the Helical hairpin domain of TnpB, in the ZnF domain of TnpB, after the Znf domain of TnpB. The present disclosure contemplates placement of the deaminase in and around or near or adjacent to the aforementioned domains.

[0012] In certain alternative embodiments, the TnpB fusion protein is co-expressed with one or more TnpB not fused to a deaminase. In other embodiments, the unfused TnpB is mutated to be catalytically inactive. In other examples, the TnpB fusion contains one or more nuclear localization signals selected or derived from SV40, c-Myc or NLP-1.

[0013] In other exemplary embodiments, the TnpB-deaminase fusions bind to a guide RNA or a reRNA. In instances where the TnpB system is fused to a polypeptide that modulates host-repair. In some examples, the polypeptide is a uracil glycosylase inhibitor. In other examples, the polypeptide inhibits mismatch repair wherein the MMR inhibiting polypeptide is a dominant negative MLH1.

[0014] In various other aspects, one or more TnpB is fused to a reverse transcriptase suitable for prime editing. In some embodiments, the reverse transcriptase comprises M-MLV. In certain embodiments, the M-MLV is an engineered reverse transcriptase variant designed to improve processivity, efficiency, and/or fidelity. In various embodiments, the reverse transcriptase is derived from the human genome or derived from a human endogenous retrovirus.

[00606]In one embodiment, the accessory function that is added or otherwise coupled or attached to a TnpB polypeptide (e.g., deaminase or reverse transcriptase) provides for a TnpB-based system that is capable of performing a specialized function or activity (e.g., base editing or prime editing). For example, the TnpB protein may be fused, operably coupled to, or otherwise associated with one or more heterologous functionals domains. In certain example embodiments, the TnpB protein may be a catalytically dead TnpB protein and/or have nickase activity. A nickase is an TnpB protein that cuts only one strand of a double stranded target. In such embodiments, the catalytically inactive TnpB or nickase provide a sequence specific targeting functionality via the coRNA that delivers the functional domain to or proximate a target sequence.

[00607]It is also contemplated that the TnpB complex as a whole may be associated with two or more functional domains. For example, there may be two or more functional domains associated with the TnpB polypeptide, or there may be two or more functional domains associated with the reRNA component (via one or more adaptor proteins or aptamers), or there may be one or more functional domains associated with the TnpB polypeptide and one or more functional domains associated with the reRNA component.

[00608]In one embodiment, one or more functional domains are associated with a TnpB polypeptide via an adaptor protein, for example as used with the modified guides of Konnerman et al. (Nature 517,

583-588, 29 January 2015). In one embodiment, the one or more functional domains is attached to the adaptor protein so that upon binding of the TnpB polypeptide to reRNA and target, the functional domain is in a spatial orientation allowing for the functional domain to function in its attributed function.

[00609]Exemplary functional accessory domains that may be fused to, operably coupled to, or otherwise associated with an TnpB protein can be or include, but are not limited to a nuclear localization signal (NLS) domain, a nuclear export signal (NES) domain, a translational activation domain, a transcriptional activation domain (e.g. VP64, p65, MyoDl, HSF1, RTA, and SET7/9), a translation initiation domain, a transcriptional repression domain (e.g., a KRAB domain, NuE domain, NcoR domain, and a SID domain such as a SID4X domain), a nuclease domain (e.g., FokI), a histone modification domain (e.g., a histone acetyltransferase), a light inducible/controllable domain, a chemically inducible/controllable domain, a transposase domain, a homologous recombination machinery domain, a recombinase domain, a ligase domain, a topoisomerase domain, a deaminase domain, a polymerase domain (e.g., reverse transcriptase), an integrase domain, and combinations thereof. In an embodiment, the functional domain is an HNH domain, and may be used with a naturally catalytically inactive TnpB protein to engineer a nickase. Methods for generating catalytically dead TnpB or a nickase TnpB can be adapted from approaches in Cas9 proteins, see, for example, WO 2014/204725, Ran et al. Cell. 2013 Sept 12; 154(6): 1380-1389, known in the art and incorporated herein by reference. Briefly, one or more mutations in the catalytic domain of the RuvC domain and/or the HNH domain of the TnpB protein can be introduced that may reduce or abolish NHEJ activity. In an aspect, at least one mutation in the RuvC domain and at least one mutation in the HNH domain is provided. In an embodiment, the TnpB polypeptide comprises a mutation at D191 and/or E278 based on amino acid sequence positions of *Deinococcus* radiodurans ISDra2 (see FIG. 1). In an aspect, the amino acid mutations comprise D191A and/or E278A based on amino acid sequence positions of *Deinococcus radiodurans* ISDra2.

[00610]In one embodiment, the functional domains can have one or more of the following activities: nucleobase deaminse activity, reverse transcriptase activity, retrotransposase activity, transposase activity, integrase activity, recombinase activity, topoisomerase activity, ligase activity, polymerase activity, helicase activity, methylase activity, demethylase activity, translation activation activity, translation initiation activity, translation repression activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, nuclease activity (e.g. VirD2), single-strand RNA cleavage activity, double-strand RNA cleavage activity, single-strand DNA cleavage activity, double-strand DNA cleavage activity, molecular switch activity, chemical inducibility, light inducibility, and nucleic acid binding activity. In one embodiment, the one or more functional domains may comprise epitope tags or reporters. Non-limiting examples of epitope tags include histidine (His) tags, V5 tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Examples of reporters

include, but are not limited to, glutathione- S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) betagalactosidase, beta-glucuronidase, luciferase, green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and auto-fluorescent proteins including blue fluorescent protein (BFP).

[00611]The one or more functional domain(s) may be positioned at, near, and/or in proximity to a terminus of the TnpB protein. In embodiments having two or more functional domains, each of the two can be positioned at or near or in proximity to a terminus of the TnpB protein. In one embodiment, such as those where the functional domain is operably coupled to the effector protein, the one or more functional domains can be tethered or linked via a suitable linker (including, but not limited to, GlySer linkers) to the TnpB protein. When there is more than one functional domain, the functional domains can be same or different. In one embodiment, all the functional domains are the same. In one embodiment, all of the functional domains are different from each other. In one embodiment, at least two of the functional domains are different from each other. In one embodiment, at least two of the functional domains are the same as each other.

[00612]In additional embodiments, the TnpB-deaminase fusion protein is co-expressed with a TnpB not fused to a reverse transcriptase. Preferably, the unfused TnpB is mutated to be catalytically inactive, however, fused TnpB may also be mutated to be catalytically inactive, either or both. Various TnpB-RT fusion protein binds to a truncated reRNA or to a truncated guide RNA. In some embodiments, this maintains DNA binding activity but slows cleavage kinetics or deactivates DNA cleavage partially or entirely. Additional embodiments, include the reverse transcriptase fused to the N-terminus of TnpB or to the C-terminus of TnpB. In further embodiments, the reverse transcriptase is placed inside the Rec-domain of the TnpB, after the Rec-domain of the TnpB, in the Wedge domain of TnpB, after the Wedge domain of TnpB, in the RuvC domain of TnpB, after the RuvC domain of TnpB, in the Helical hairpin domain of TnpBafter the Helical hairpin domain of TnpB, in the ZnF domain of TnpB, after the Znf domain of TnpB.

[00613]Preferably, the TnpB-RT fusion protein is bound to an engineered reRNA wherein the engineered reRNA contains a 5' extension, the engineered reRNA contains a 3' extension, the extensions contain a template for a desired edit, the extension contains homology to the target site, the extension contains homology to the human genome, the extension contains sequence encoding a landing-pad for a homing integrase and/or recombinase. In preferred embodiments, the TnpB-RT fusion protein is fused or cleaved. In certain embodiments, the TnpB-RT system is fused to a polypeptide that modulates host-repair, wherein the polypeptide is a uracil glycosylase inhibitor, wherein the polypeptide inhibits mismatch repair, wherein the MMR inhibiting polypeptide is a dominant negative MLH1.

[00614]In various aspects of the disclosure, the TnpB fused to a transcriptional modulating polypeptide suitable for transcriptional interference, activation or epigenetic editing.

[00615]In some embodiments, the TnpB-transcriptional modulating polypeptide fusions comprise one or more nuclear localization signals selected or derived from SV40, c-Myc or NLP-1.

[00616]In other embodiments, the TnpB-transcriptional modulating polypeptide fusion proteins bind to a truncated guide RNA. In further embodiments, the TnpB-transcriptional modulating polypeptide comprises glycine and serine residues. In yet other embodiments, the TnpB-transcriptional modulating polypeptide are linked to one or more unstructured XTEN protein polymers.

[00617]In various embodiments, the transcriptional modulating polypeptide of the TnpB-transcriptional modulating polypeptide fusion performs histone acetylation or comprises histone acetyltransferase (HAT) p300 activity.

[00618]In other embodiments, the transcriptional modulating polypeptide of the TnpB-transcriptional modulating polypeptide fusion performs histone demethylation or comprises lysine-specific demethylase (LSD1) activity.

[00619]In further embodiments, the transcriptional modulating polypeptide of the TnpB-transcriptional modulating polypeptide fusion performs cystine methylation or comprises one or more activities selected from DNA (cytosine-5)-methyltransferase (DNMT3A), DNA-methyltransferase 3-like (DNMT3L) and MQ1.

[00620]In other embodiments, the transcriptional modulating polypeptide of the TnpB-transcriptional modulating polypeptide fusion performs cystine demethylation or comprises TET1 activity.

[00621]In additional embodiments, the transcriptional modulating peptide of the TnpB-transcriptional modulating polypeptide fusion is a transcriptional repressor or comprises a KRAB domain. Alternatively, the transcriptional modulating peptide of the TnpB-transcriptional modulating polypeptide fusion is a transcriptional activator or comprises one or more activators including without limitation, for example, HS1, VP64 and p65.

[00622]In other embodiments, Where the the transcriptional modulating peptide of the TnpB-transcriptional modulating polypeptide fusion is a repressor or comprises multiple transcriptional modulating peptides. In yet other embodiments, the TnpB of the TnpB-transcriptional modulating polypeptide fusion is mutated to be catalytically inactive.

[00623]In further embodiments, the transcriptional modulating peptides of the TnpB-transcriptional modulating polypeptide fusion are physically coupled through an engineered reRNA wherein the reRNA comprises one or more aptamers.

[00624]In additional embodiments, the transcriptional modulating peptides of the TnpB-transcriptional modulating polypeptide fusion are physically coupled through an engineered guide RNA, wherein the guide RNA contains one or more aptamers.

reRNA

[00625]The TnpB systems herein may further comprise one or more nucleic acid components, which are also referred to herein as reRNA. As reported in Karvelis et al., "Transposon-associated TnpB is a programmable RNA-guided DNA endonuclease," *Nature*, November 25, 2021, Vol. 599, pp. 692-700

(incorporated herein by reference), TnpB is an RNA-guided dsDNA nuclease that forms a complex with a non-coding RNA called "reRNA." The reRNA is a transcript that is generated from the transcription of the IS DNA sequence beginning at a transcription initiation site located within the 3' end of the TnpB coding region and ending at a transcription termination site located in the flanking genomic DNA region that is immediately downstream of the RE of the Insertion Sequence. Thus, the reRNA comprises three regions: (a) a region corresponding to the 3' end of the TnpB coding region, (b) a region corresponding to the RE, and (c) a region corresponding to the flanking genomic DNA immediately downstream of the 3' end of the RE. Regions (a) and (b) generally form a folded scaffold that appears to bind to the TnpB protein. Region (c) functions as a spacer or targeting sequence which allows for the targeting of a TnpB-reRNA complex to a target site to which the region (c) has complementarity to and anneals. Region (c), in various embodiments, can be engineered to be any desired target sequence such that the TnpB-reRNA complex is targeted to a desired target sequence.

[00626]Thus, the reRNA sequence may be predicted from the sequence of the region spanning the 3' end of the TnpB coding region through a flanking region downstream of the RE.

[00627] Computational methods can be used to predict the reRNA sequences for identified TnpB and TnpB-like proteins. As reported in Karvelis et al., "Transposon-associated TnpB is a programmable RNA-guided DNA endonuclease," *Nature*, November 25, 2021, Vol. 599, pp. 692-700, the TnpB protein co-purified with an RNA molecule of about 150 nucleotides long which had a sequence that was derived from the IS and a sequence downstream of the IS.

[00628]In various embodiments, reRNA may be engineered to include RNA, DNA, or combinations of both and include modified and non-canonical nucleotides as described further below. The reRNA can comprise a reprogrammable spacer sequence and a scaffold that interacts with the TnpB polypeptide. reRNA may form a complex with a TnpB polypeptide, and direct sequence-specific binding of the complex to a target sequence of a target polynucleotide. In one example embodiment, the reRNA is a single molecule comprising a scaffold sequence and a spacer sequence. In certain example embodiments, the spacer is 5' of the scaffold sequence. In one example embodiment, the reRNA may further comprise a conserved nucleic acid sequence between the scaffold and spacer portions.

[00629]In embodiments, the reRNA comprises a spacer sequence and a scaffold sequence, e.g. a conserved nucleotide sequence. In embodiments, the reRNA comprises about 45 to about 350 nucleotides, or about 45, 46, 47 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 17, 138, 19, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 11, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175,

176, 177, 178, 179, 180. 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 2340, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 272, 273, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, or 350 nucleotides.

[00630]In embodiments, the reRNA comprises a scaffold sequence, e.g. a conserved nucleotide sequence that binds to the TnpB protein. The scaffold sequence therefore typically comprises conserved regions, with the scaffold comprising about 30 to 200 nucleotides, about 50 to 180, about 80 to 175 nucleotides, or about 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 40, 41, 42, 43, 44, 45, 46, 47 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180 or more nucleotides.

[00631]The reRNA may further comprise a spacer, which can be re-programmed to direct site specific binding to a target sequence of a target polynucleotide. The spacer may also be referred to herein as part of the reRNA scaffold or reRNA, and may comprise an engineered heterologous sequence.

[00632]In one embodiment, the spacer length or targeting sequence length of the reRNA is from 10 to 50 nt. In one embodiment, the spacer length of the oRNA is at least 10, 11, 12, 13, 14, or 15 nucleotides. In one embodiment, the spacer length is from 10 to 40 nuecleotides, from 15 to 30 nt, 15 to 17 nt, e.g., 15, 16, or 17 nt, from 17 to 20 nt, e.g., 17, 18, 19, or 20 nt, from 20 to 24 nt, e.g., 20, 21, 22, 23, or 24 nt, from 23 to 25 nt, e.g., 23, 24, or 25 nt, from 24 to 27 nt, e.g., 24, 25, 26, or 27 nt, from 27 to 30 nt, e.g., 27, 28, 29, or 30 nt, from 30 to 35 nt, e.g., 30, 31, 32, 33, 34, or 35 nt, or 35 nt or longer. In example embodiments, the spacer sequence is 14, 15, 16, 17,18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 40, 41, 42, 43, 44, 45, 46, 47 48, 49, or 50 nt.

[00633] As used herein, the term "spacer" may also be referred to as a "guide sequence" or "targeting sequence" which has complementarity to a target sequence (e.g., a desired target gene in a genome which is desired to be edited). In one embodiment, the degree of complementarity of the spacer sequence to a given target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. In certain example embodiments, the reRNA molecule comprises a spacer sequence that may be designed to have at least

one mismatch with the target sequence, such that a RNA duplex formed between the sequence and the target sequence. Accordingly, the degree of complementarity is less than 99%. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g., the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net).

[00634]The ability of a sequence (within a nucleic acid-targeting reRNA molecule) to direct sequence-specific binding of a nucleic acid-targeting complex to a target nucleic acid sequence may be assessed by any suitable assay. For example, the components of a reRNA system sufficient to form a TnpB-targeting complex, including the reRNA molecule sequence to be tested, may be provided to a host cell having the corresponding target nucleic acid sequence, such as by transfection with vectors encoding the components of the TnpB-targeting complex, followed by an assessment of preferential targeting (e.g., cleavage) within the target nucleic acid sequence. Similarly, cleavage of a target nucleic acid sequence (or a sequence in the vicinity thereof) may be evaluated in a test tube by providing the target nucleic acid sequence, components of a TnpB-targeting complex, including the sequence to be tested and a control sequence different from the test coRNA, and comparing binding or rate of cleavage at or in the vicinity of the target sequence between the test and control reRNA molecule sequence reactions. Other assays are possible, and will occur to those skilled in the art. A spacer sequence, and hence a nucleic acid targeting reRNA may be selected to target any target nucleic acid sequence.

reRNA modifications

[00635] In one embodiment, the reRNA comprises non-naturally occurring nucleic acids and/or non-naturally occurring nucleotides and/or nucleotide analogs, and/or chemical modifications. Preferably, these non-naturally occurring nucleic acids and non-naturally occurring nucleotides are located outside the reRNA sequence. Non-naturally occurring nucleic acids can include, for example, mixtures of naturally and non-naturally occurring nucleotides. Non-naturally occurring nucleotides and/or nucleotide analogs may be modified at the ribose, phosphate, and/or base moiety. In an embodiment of the disclosure, a reRNA component nucleic acid comprises ribonucleotides and non-ribonucleotides. In one such embodiment, a reRNA component comprises one or more ribonucleotides and one or more deoxyribonucleotides. In an embodiment of the disclosure, the reRNA component comprises one or more non-naturally occurring nucleotide or nucleotide analog such as a nucleotide with phosphorothioate linkage, a locked nucleic acid (LNA) nucleotides comprising a methylene bridge between the 2' and 4' carbons of the ribose ring, or bridged nucleic acids (BNA).

[00636]Other examples of modified nucleotides include 2'-O-methyl analogs, 2'-deoxy analogs, or 2'-fluoro analogs. Further examples of modified bases include, but are not limited to, 2-aminopurine, 5-

bromo-uridine, pseudouridine, inosine, 7-methylguanosine. Examples of coRNA chemical modifications include, without limitation, incorporation of 2'-O-methyl (M), 2'-O-methyl 3 'phosphorothioate (MS), S-constrained ethyl(cEt), or 2'-O-methyl 3 'thioPACE (MSP) at one or more terminal nucleotides. Such chemically modified oRNA components can comprise increased stability and increased activity as compared to unmodified oRNA components, though on-target vs. off-target specificity is not predictable. (See, Hendel, 2015, Nat Biotechnol. 33(9):985-9, doi: 10.1038/nbt.3290, published online 29 June 2015 Ragdarm et al., 0215, PNAS, E7110-E7111; Allerson et al., J. Med. Chem. 2005, 48:901-904; Bramsen et al., Front. Genet., 2012, 3:154; Deng et al., PNAS, 2015, 112: 11870-11875; Sharma et al., MedChemComm., 2014, 5: 1454-1471; Hendel et al., Nat. Biotechnol. (2015) 33(9): 985-989; Li et al., Nature Biomedical Engineering, 2017, 1, 0066 D01: 10.1038/s41551-017-0066). In one embodiment, the 5' and/or 3' end of a reRNA component is modified by a variety of functional moieties including fluorescent dyes, polyethylene glycol, cholesterol, proteins, or detection tags. (See Kelly et al., 2016, J. Biotech. 233:74-83). In one embodiment, a reRNA component comprises ribonucleotides in a region that binds to a target sequence and one or more deoxyribonucletides and/or nucleotide analogs in a region that binds to the TnpB polypeptide.

[00637]In an embodiment, deoxyribonucleotides and/or nucleotide analogs are incorporated in engineered reRNA component structures. In one embodiment, 3-5 nucleotides at either the 3' or the 5' end of a reRNA component is chemically modified. In one embodiment, only minor modifications are introduced in the seed region, such as 2'-F modifications. In one embodiment, 2'-F modification is introduced at the 3' end of a reRNA component. In one embodiment, three to five nucleotides at the 5' and/or the 3' end of the reRNA component are chemically modified with 2' -O-methyl (M), 2'-O-methyl 3' phosphorothioate (MS), S-constrained ethyl(cEt), or 2' -O-methyl 3' thioPACE (MSP). Such modification can enhance genome editing efficiency (see Hendel et al., Nat. Biotechnol. (2015) 33(9): 985-989). In one embodiment, all of the phosphodiester bonds of a reRNA component are substituted with phosphorothioates (PS) for enhancing levels of gene disruption. In one embodiment, more than five nucleotides at the 5' and/or the 3' end of the reRNA component are chemically modified with 2'-0-Me, 2'-F or S-constrained ethyl(cEt). Such chemically modified reRNA component can mediate enhanced levels of gene disruption (see Ragdarm et al., 0215, PNAS, E7110-E7111). In an embodiment of the disclosure, a reRNA component is modified to comprise a chemical moiety at its 3' and/or 5' end. Such moieties include, but are not limited to amine, azide, alkyne, thio, dibenzocyclooctyne (DBCO), or Rhodamine. In certain embodiment, the chemical moiety is conjugated to the reRNA component by a linker, such as an alkyl chain. In one embodiment, the chemical moiety of the modified nucleic acid component can be used to attach the reRNA component to another molecule, such as DNA, RNA, protein, or nanoparticles. Such chemically modified reRNA component can be used to identify or enrich cells generically edited by a TnpB polypeptide and related systems (see Lee et al., eLife, 2017, 6:e25312, DOI: 10.7554).

[00638]Other reRNA modifications are described in Kim, D.Y., Lee, J.M., Moon, S.B. *et al.* Efficient CRISPR editing with a hypercompact Cas12f1 and engineered guide RNAs delivered by adeno-associated virus. *Nat Biotechnol* **40**, 94–102 (2022).

[0015] Accordingly, in various aspects of the disclosure, the reRNA are modified in one or more TnpB reRNA. MS1, an internal penta(uridinylate) (UUUUU) sequence in the tracrRNA; MS2, the 3′ terminus of the crRNA; MS3, the 'stem 1' region of the tracrRNA; MS4, the tracrRNA–crRNA complementary region; and MS5, the 'stem 2' region of the tracrRNA.

[00639]Various aspects of the disclosure provide methods and compositions for improved reRNA stability via chemical modifications. Braasch, D. A., Jensen, S., Liu, Y., Kaur, K., Arar, K., White, M. A., et al. (2003). RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry* 42, 7967–7975. Chiu, Y. L., and Rana, T. M. (2003). siRNA function in RNAi: a chemical modification analysis. *RNA* 9, 1034–1048. Behlke, M. A. (2008). Chemical modification of siRNAs for *in vivo* use. *Oligonucleotides*18, 305–319. Bennett, C. F., and Swayze, E. E. (2010). RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu. Rev. Pharmacol. Toxicol.* 50, 259–293. Deleavey, G. F., and Damha, M. J. (2012). Designing chemically modified oligonucleotides for targeted gene silencing. *Chem. Biol.* 19, 937–954. Lennox, K. A., and Behlke, M. A. (2020). Chemical modifications in RNA interference and CRISPR/Cas genome editing reagents. *Methods Mol. Biol.* 2115, 23–55.

[00640]For instance, Hendel et al. improved guideRNA stability by chemically modifying gRNA ends to reduce degradation by exonucleases, RNA nuclease. Hendel, A., Bak, R. O., Clark, J. T., Kennedy, A. B., Ryan, D. E., Roy, S., et al. (2015a). Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat. Biotechnol.* 33, 985–989. Chemical modifications of gRNAs may enable more efficient and safer gene-editing in primary cells suitable for clinical applications.

[00641]A review of types of chemical modifications are provided in the table below. Allen, Daniel et al. "Using Synthetically Engineered Guide RNAs to Enhance CRISPR Genome Editing Systems in Mammalian Cells." *Frontiers in genome editing* vol. 2 617910. 28 Jan. 2021.

Modification(s)	Modification location	Effect on genome editing efficiency
M	Terminal residues	↑#
MS	Terminal residues	↑#
	Spacer (PAM-distal region)	↑*
	Spacer (tracrRNA-binding region)	↑*
	Spacer (Seed region)	↓
MSP	Terminal residues	↑#
cEt	Spacer (PAM-distal region)	↑

	Spacer (tracrRNA-binding)	↑
	Spacer (Seed region)	↓
2'-F	Spacer (PAM-distal region)	↑
	Spacer (tracrRNA-binding)	↑
	Spacer (Seed region)	↓
2'-F + PS	Spacer (PAM-distal region)	↑
	Spacer (tracrRNA-binding)	↑
	Spacer (Seed region)	↓
	Spacer (Seed region, Cas9-non-interacting residues)	↑*
PS	Whole crRNA	↑

* additionally validated in vivo; # additionally validated in human primary cells

2'-O-methyl (M or 2'-O-Me); 2'-O-methyl 3'phosphorothioate (MS); 2'-O-methyl-3'-thioPACE (MSP); S-constrained ethyl (cET); 2'-fluoro (2'-F); phosphorothioate (PS)

[00642]Accordingly, in various embodiments of the present disclosure, the genome editing system comprising TnpB and further comprises one or more chemical modifications selected from, but not limited to the modifications in the above table.

[00643]In exemplary embodiments, chemical modifications to the reRNA include modifications on the ribose rings and phosphate backbone of reRNAs and modifications at the 2′OH include 2′-O-Me, 2′-F, and 2′F-ANA. More extensive ribose modifications include 2′F-4′-Cα-OMe and 2′,4′-di-Cα-OMe combine modification at both the 2′ and 4′ carbons. Phosphodiester modifications include sulfide-based Phosphorothioate (PS) or acetate-based phosphonoacetate alterations. Combinations of the ribose and phosphodiester modifications have given way to formulations such as 2′-O-methyl 3′phosphorothioate (MS), or 2′-O-methyl-3′-thioPACE (MSP), and 2′-O-methyl-3′-phosphonoacetate (MP) RNAs. Locked and unlocked nucleotides such as locked nucleic acid (LNA), bridged nucleic acids (BNA), S-constrained ethyl (cEt), and unlocked nucleic acid (UNA) are examples of sterically hindered nucleotide modifications. Modifications to make a phosphodiester bond between the 2′ and 5′ carbons (2′,5′-RNA) of adjacent RNAs as well as a butane 4-carbon chain link between adjacent RNAs have been described.

[00644]Any of the above TnpB editor embodiments or variants, modifications, or derivatives thereof are contemplated herein to be delivered by the LNP systems disclosed in this specification for gene editing in cells, tissues, and/or organs under *in vitro*, *ex vivo*, or *in vivo* conditions. The various components described herein may be configured and delivered in any suitable manner. Any of the descriptions presented in this section are not intended to be strictly limiting.

Integrase editors (e.g., PASTE)

[00645]In some embodiments, the gene editing system comprises one or more integrase editors. In certain embodiments, the gene editing system comprises a construct enabling programmable addition via site-specific targeting elements (PASTE). In certain embodiments, the gene editing system comprises one or more integrase editors and/or gene editing systems described and disclosed in PCT Publications WO2022087235A1, WO2020191245A1, WO2022060749A1, WO2021188840A1, WO2021138469A1, US Patent Application Publications US20140349400A1, US20210222164A1 or US20150071898A1, each of which is incorporated by reference herein in their entirety. In certain embodiments, the one or more integrase editors comprise CRISPR directed integrases disclosed in Yarnall, M.T.N., Ioannidi, E.I., Schmitt-Ulms, C. et al. Drag-and-drop genome insertion of large sequences without double-strand DNA cleavage using CRISPR-directed integrases. Nat Biotechnol (2022).

Epigenetic editors

In still other embodiments, the LNPs may be used to deliver an epigenetic editing system. Epigenetic editors are generally composed of an epigenetic enzyme or their catalytic domain fused with a user-programmable DNA-binding protein, such as a CRISPR-Cas enzyme or TnpB enzyme. The user-programmable DNA-binding protein (plus a guide RNA in the case of a nucleic acid programmable DNA binding protein) guides the epigenetic enzyme (e.g., a DNA methyltransferase or DNMT) to a specific site (e.g., a CpG island in a promoter region of a gene) in order to induce a change in promoter activity.

[00646]Epigenetic modifications of DNA and histones are known for their multifaceted contributions to transcriptional regulation. As these modifications are faithfully propagated throughout DNA replication, they are considered central players in cellular memory of transcriptional states. Many efforts in the last decade have generated a vast understanding of individual epigenetic modifications and their contribution to transcriptional regulation. Epigenetic editing offers powerful tools to selectively induce epigenetic changes in a genome without altering the sequence of a nucleotide sequence as a means to regulate gene activity. The foundation of epigenetic editing is formed by the ability to generate fusion proteins of epigenetic enzymes or their catalytic domains with programmable DNA-binding platforms such as the clustered regularly interspaced short palindromic repeat (e.g., CRISPR Cas9 or Cas12a) to target these to an endogenous locus of choice. The enzymatic fusion protein then dictates the initial deposited modification while subsequent cross-talk within the local chromatin environment likely influences epigenetic and transcriptional output.

[00647]The following published literature discussing epigenetic editing is incorporated herein by reference each in their entireties.

Gjaltema RAF, Rots MG. Advances of epigenetic editing. Curr Opin Chem Biol. 2020 Aug;57:75-81. Epub 2020 Jun 30. PMID: 32619853.

Kleinstiver BP, Sousa AA, Walton RT, Tak YE, Hsu JY, Clement K, Welch MM, Horng JE, Malagon-Lopez J, Scarfò I, Maus MV, Pinello L, Aryee MJ, Joung JK. Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing. Nat Biotechnol. 2019 Mar;37(3):276-282. Epub 2019 Feb 11. Erratum in: Nat Biotechnol. 2020 Jul;38(7):901. PMID: 30742127; PMCID: PMC6401248.

Rots MG, Jeltsch A. Editing the Epigenome: Overview, Open Questions, and Directions of Future Development. Methods Mol Biol. 2018;1767:3-18. PMID: 29524127.

Liu XS, Jaenisch R. Editing the Epigenome to Tackle Brain Disorders. Trends Neurosci. 2019 Dec;42(12):861-870. Epub 2019 Nov 7. PMID: 31706628.

Waryah CB, Moses C, Arooj M, Blancafort P. Zinc Fingers, TALEs, and CRISPR Systems: A Comparison of Tools for Epigenome Editing. Methods Mol Biol. 2018;1767:19-63. PMID: 29524128.

Xu X, Hulshoff MS, Tan X, Zeisberg M, Zeisberg EM. CRISPR/Cas Derivatives as Novel Gene Modulating Tools: Possibilities and In Vivo Applications. Int J Mol Sci. 2020 Apr 25;21(9):3038. PMID: 32344896; PMCID: PMC7246536.

[00648] In addition, the following published patent literature relating to epigenetic editing is incorporated herein by reference each in their entireties.

Pub Number	**Title**
WO2023283359A2	COMPOSITIONS AND METHODS FOR MODULATING SECRETED FRIZZLED RECEPTOR PROTEIN 1 (SFRP1) GENE EXPRESSION
WO2022226139A1	TISSUE-SPECIFIC NUCLEIC ACID DELIVERY BY MIXED CATIONIC LIPID PARTICLES
WO2022132926A1	TISSUE-SPECIFIC NUCLEIC ACID DELIVERY BY 1,2-DIOLEOYL-3-TRIMETHYLAMMONIUM-PROPANE (DOTAP) LIPID NANOPARTICLES
WO2021183720A1	COMPOSITIONS AND METHODS FOR MODULATING FORKHEAD BOX P3 (FOXP3) GENE EXPRESSION
WO2021061815A1	COMPOSITIONS AND METHODS FOR MODULATING HEPATOCYTE NUCLEAR FACTOR 4-ALPHA (HNF4α) GENE EXPRESSION
WO2021061707A1	COMPOSITIONS AND METHODS FOR MODULATING APOLIPOPROTEIN B (APOB) GENE EXPRESSION
WO2021061698A1	METHODS AND COMPOSITIONS FOR MODULATING FRATAXIN EXPRESSION AND TREATING FRIEDRICH'S ATAXIA

Gene writing

[00649]In some embodiments, the gene editing system is a gene writing system. In certain embodiments, the gene editing system is one described and disclosed in US Patent Application

Publications US2022039681A1 or US20200109398A1, each of which is incorporated by reference herein in their entirety.

[00650]In certain embodiments, the gene editing system is a system for modifying DNA comprising a polypeptide or a nucleic acid encoding a polypeptide capable of target primed reverse transcription, wherein the polypeptide comprises (a) a reverse transcriptase domain and (b) an endonuclease domain, wherein at least one of (a) or (b) is heterologous; and a template RNA comprising (i) a sequence that binds the polypeptide and (ii) a heterologous object sequence. In certain embodiments, the gene editing system is a system for modifying DNA comprising a polypeptide or a nucleic acid encoding a polypeptide capable of target primed reverse transcription, wherein the polypeptide comprises (a) a target DNA binding domain, (b) a reverse transcriptase domain and (c) an endonuclease domain, wherein at least one of (a), (b) or (c) is heterologous, and a template RNA comprising (i) a sequence that binds the polypeptide and (ii) a heterologous object sequence. In certain embodiments, the polypeptide comprises a sequence of at least 50 amino acids having at least 80% identity to a reverse transcriptase domain of a sequence of a polypeptide listed in TABLE 1, TABLE 2, or TABLE 3 of US Patent Application Publication US20200109398A1, which is incorporated by reference in its entirety, including the aforementioned sequence tables.

[00651]In certain embodiments, the reverse transcriptase domain is from a retrovirus or a retrotransposon, such as a LTR-retrotransposon, or a non-LTR retrotransposon. In certain embodiments, the reverse transcriptase is from a non-LTR retrotransposon, wherein the non-LTR retrotransposon is a RLE-type non-LTR retrotransposon from the R2, NeSL, HERO, R4, or CRE clade, or an APE-type non-LTR retrotransposon from the R1, or Tx1 clade. In certain embodiments, the reverse transcriptase domain is from an avian retrotransposase of column 8 of Table 3 of US20200109398A1, or a sequence having at least 70%, identity thereto. In certain embodiments, the reverse transcriptase domain does not comprise an RNA binding domain and the polypeptide comprises an RNA binding domain heterologous to the reverse transcriptase domain, wherein the RNA binding domain is a B-box protein, a MS2 coat protein, a dCas protein, or a UTR binding protein, or a fragment or variant of any of the foregoing.

[00652]In certain embodiments, the endonuclease domain is heterologous to the reverse transcriptase domain, and wherein the endonuclease is a Fok1 nuclease (or a functional fragment thereof), a type-II restriction 1-like endonuclease (RLE-type nuclease), another RLE-type endonuclease, or a Prp8 nuclease. In certain embodiments, the endonuclease domain is heterologous to the reverse transcriptase domain, wherein endonuclease domain contains DNA binding functionality. In certain embodiments, the endonuclease domain is heterologous to the reverse transcriptase domain, and wherein the endonuclease has nickase activity and does not form double stranded breaks.

[00653]In certain embodiments, the polypeptide comprises a DNA binding domain heterologous to the reverse transcriptase domain, and wherein the DNA binding domain is: a zinc-finger element, or a functional fragment thereof; or a TAL effector element, or a functional fragment thereof; a Myb

domain; or a sequence-guided DNA binding element. In certain embodiments, the polypeptide comprises a DNA binding domain heterologous to the reverse transcriptase domain, and wherein the DNA binding element is a sequence-guided DNA binding element, further wherein the sequence-guided DNA binding element is Cas9, Cpf1, or other CRISPR-related protein. In certain embodiments, the polypeptide comprises a DNA binding domain heterologous to the reverse transcriptase domain, and wherein the DNA binding domain is a transcription factor.

[00654]In certain embodiments, the sequence-guided DNA binding element has been altered to have no endonuclease activity. In certain embodiments, the sequence-guided DNA binding element replaces the endonuclease element of the polypeptide. In certain embodiments, the editing system is capable of modifying DNA using reverse transcriptase activity, optionally in the absence of homologous recombination activity.

[00655]In certain embodiments, the gene editing system is a system for modifying DNA comprising:

a) a recombinase polypeptide selected from Rec27 (WP_021170377.1, SEQ ID NO: 1241 of US20220396813A1), Rec35 (WP_134161939.1, SEQ ID NO: 1249 of US20220396813A1), or comprising an amino acid sequence of Table 1 or 2 of US20220396813A1, or an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto, or a nucleic acid encoding the recombinase polypeptide; and

b) a double-stranded insert DNA comprising:

(i) a DNA recognition sequence that binds to the recombinase polypeptide of (a), said DNA recognition sequence having a first parapalindromic sequence and a second parapalindromic sequence, wherein each parapalindromic sequence is about 10-30, 12-27, or 10-15 nucleotides, e.g., about 13 nucleotides, and the first and second parapalindromic sequences together comprise the parapalindromic region of a nucleotide sequence of Table 1 of US20220396813A1, or a nucleotide sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto, or having no more than 1, 2, 3, 4, 5, 6, 7, 8 sequence alterations (e.g., substitutions, insertions, or deletions) relative thereto, and said DNA recognition sequence further comprises a core sequence of about 5-10 nucleotides, e.g., about 8 nucleotides, wherein the core sequence is situated between the first and second parapalindromic sequences, and

(ii) a heterologous object sequence.

Gene inactivating systems

[00656]In some embodiments, the gene editing system comprises a polypeptide or an RNA encoding a polypeptide capable of inducing a double-stranded or single-stranded break in a desired gene, thereby inactivating said gene. In certain embodiments, the gene editing system is one described and disclosed in PCT Publications WO2020028327A1, WO2020069296A1 or WO2020118041A1, each of which is incorporated by reference herein in their entirety. In certain embodiments, the gene editing

system is one described and disclosed in a patent application publication disclosed below, each of which is incorporated by reference herein in their entirety:

Pub Number	Title
WO2022271780A1	METHODS FOR IN VIVO EDITING OF A LIVER GENE
WO2022261292A1	MODIFIED GUIDE RNAS COMPRISING AN INTERNAL LINKER FOR GENE EDITING
WO2022221697A1	LIPID NANOPARTICLE COMPOSITIONS
WO2022221696A1	INHIBITORS OF DNA-DEPENDENT PROTEIN KINASE AND COMPOSITIONS AND USES THEREOF
WO2022221695A1	LIPID NANOPARTICLE COMPOSITIONS
WO2022170194A2	LYMPHOCYTE ACTIVATION GENE 3 (LAG3) COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY
WO2022170193A2	T-CELL IMMUNOGLOBULIN AND MUCIN DOMAIN 3 (TIM3) COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY
WO2022170172A1	NATURAL KILLER CELL RECEPTOR 2B4 COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY
WO2022147133A1	ENGINEERED T CELLS
WO2022140587A1	COMPOSITIONS AND METHODS FOR GENETICALLY MODIFYING CIITA IN A CELL
WO2022140586A2	COMPOSITIONS AND METHODS FOR REDUCING HLA-A IN A CELL
WO2022125982A1	COMPOSITIONS AND METHODS FOR REDUCING MHC CLASS II IN A CELL
WO2022125968A1	POLYNUCLEOTIDES, COMPOSITIONS, AND METHODS FOR GENOME EDITING INVOLVING DEAMINATION
WO2021222287A2	METHODS OF IN VITRO CELL DELIVERY
WO2021220132A1	METHODS AND COMPOSITIONS FOR OCULAR CELL THERAPY
WO2021188840A1	METHODS AND COMPOSITIONS FOR DIRECTED GENOME EDITING
WO2021158858A1	COMPOSITIONS AND METHODS FOR KALLIKREIN (KLKB1) GENE EDITING
WO2021119275A1	MODIFIED GUIDE RNAS FOR GENE EDITING
WO2020219876A1	IONIZABLE AMINE LIPIDS AND LIPID NANOPARTICLES
WO2020198706A1	COMPOSITIONS AND METHODS FOR TTR GENE EDITING AND TREATING ATTR AMYLOIDOSIS COMPRISING A CORTICOSTEROID OR USE THEREOF

Pub Number	Title
WO2020198697A1	COMPOSITIONS AND METHODS COMPRISING A TTR GUIDE RNA AND A POLYNUCLEOTIDE ENCODING AN RNA-GUIDED DNA BINDING AGENT
WO2020198641A2	POLYNUCLEOTIDES, COMPOSITIONS, AND METHODS FOR POLYPEPTIDE EXPRESSION
WO2020118041A1	MODIFIED AMINE LIPIDS
WO2020084580A1	METHODS AND COMPOSITIONS FOR OCULAR CELL THERAPY
WO2020082047A1	COMPOSITIONS AND METHODS FOR TREATING ALPHA-1 ANTITRYPSIN DEFICIENCEY
WO2020082046A2	COMPOSITIONS AND METHODS FOR EXPRESSING FACTOR IX
WO2020082042A2	COMPOSITIONS AND METHODS FOR TRANSGENE EXPRESSION FROM AN ALBUMIN LOCUS
WO2020082041A1	NUCLEIC ACID CONSTRUCTS AND METHODS OF USE
WO2020081613A1	COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY
WO2020072605A1	IONIZABLE AMINE LIPIDS
WO2020069296A1	COMPOSITIONS AND METHODS FOR LACTATE DEHYDROGENASE (LDHA) GENE EDITING
WO2020028327A1	COMPOSITIONS AND METHODS FOR HYDROXYACID OXIDASE 1 (HAO1) GENE EDITING FOR TREATING PRIMARY HYPEROXALURIA TYPE 1 (PH1)
WO2019237069A1	MODIFIED GUIDE RNAS FOR GENE EDITING
WO2019237035A1	COMPOSITIONS AND METHODS FOR IMMUNOONCOLOGY
WO2019067999A1	IN VITRO METHOD OF MRNA DELIVERY USING LIPID NANOPARTICLES
WO2019067992A1	FORMULATIONS
WO2019067910A1	POLYNUCLEOTIDES, COMPOSITIONS, AND METHODS FOR GENOME EDITING
WO2019067872A1	COMPOSITIONS AND METHODS FOR TTR GENE EDITING AND TREATING ATTR AMYLOIDOSIS
WO2019036513A1	STABILIZED NUCLEIC ACIDS ENCODING MESSENGER RIBONUCLEIC ACID (MRNA)
WO2019003193A1	METHODS FOR THE TREATMENT OF DISEASE WITH GENE EDITING SYSTEMS
WO2018175636A2	COMPOSITIONS AND METHODS FOR IMMUNOONCOLOGY

Pub Number	Title
WO2018142364A1	COMPOSITIONS AND METHODS FOR THE TREATMENT OF HEMOGLOBINOPATHIES
WO2018119182A1	COMPOSITIONS AND METHODS FOR TREATING ALPHA-1 ANTITRYPSIN DEFICIENCY
WO2018107028A1	MODIFIED GUIDE RNAS
WO2018053070A1	IMPROVED METHODS FOR ANALYZING EDITED DNA
WO2017185054A1	COMPOSITIONS AND METHODS FOR TREATMENT OF DISEASES ASSOCIATED WITH TRINUCLEOTIDE REPEATS IN TRANSCRIPTION FACTOR FOUR
WO2017173054A1	LIPID NANOPARTICLE FORMULATIONS FOR CRISPR/CAS COMPONENTS
WO2017160752A1	METHODS AND COMPOSITIONS FOR GENE EDITING
WO2017115268A1	COMPOSITIONS AND METHODS FOR THE TREATMENT OF HEMOGLOBINOPATHIES
WO2017093969A1	COMPOSITIONS AND METHODS FOR IMMUNOONCOLOGY

Compositions that increase gene editing efficiency

[00657]In some embodiments, the gene editing system comprises a polypeptide, or a nucleic acid that encodes a polypeptide, that increases gene editing efficiency. In some embodiments, the gene editing system comprises a composition described and disclosed in US Application Publication US20220090064A1, which is incorporated by reference herein in its entirety. In some embodiments, the composition comprises a guide nucleic acid, a Cas9 nickase, and/or a reverse transcriptase. The reverse transcriptase may be fused to the Cas9 nickase. The reverse transcriptase may heterodimerize with the Cas9 nickase. The reverse transcriptase may bind to a guide nucleic acid. The reverse transcriptase may be engineered to increase processivity. The guide nucleic acid may be engineered to facilitate synthesis or editing of a sequence. The guide nucleic acid may comprise a region that binds to another region on the guide nucleic acid to improve gene editing.

[00658]In some embodiments, the composition comprises a Cas 9 nickase and a reverse transcriptase, or one or two polynucleotides encoding the Cas 9 nickase and reverse transcriptase, wherein:

(i) the composition comprises a first polypeptide chain comprising the Cas nickase or a segment of the Cas nickase, and a second polypeptide chain comprising the reverse transcriptase, or the one or two polynucleotides encoding the polypeptide chains, wherein the polypeptide chains comprise leucine zippers that bind one another, or

(ii) the composition comprises a first polypeptide chain comprising a first segment of the Cas nickase, and a second polypeptide chain comprising a second segment of the Cas nickase and the

reverse transcriptase, or the one or two polynucleotides encoding the polypeptide chains, wherein the polypeptide chains comprise inteins that bind one another, the Cas nickase comprises an amino acid sequence at least 80% identical to SEQ ID NO: 32 of US20220090064A1, the first and second polypeptide chains respectively comprise amino acids 1-1124 and 1125-1368 of the Cas nickase, 1-1129 and 1130-1368 of the Cas nickase, 1-1139 and 1140-1368 of the Cas nickase, 1-1167 and 1168-1368 of the Cas nickase, 1-1172 and 1173-1368 of the Cas nickase, or 1-1202 and 1203-1368 of the Cas nickase, and the Cas nickase comprises a mutation at amino acid position 1030 or after amino acid position 1030 with regard to SEQ ID NO: 32 of US20220090064A1, the mutation comprising a point mutation to a cysteine, threonine, alanine, or serine, or an insertion of a cysteine, threonine, alanine, or serine at the C-terminal half of the Cas9 nickase or

(iii) the reverse transcriptase comprises a Moloney leukemia virus reverse transcriptase (mlvRT) comprising an amino acid sequence at least 80% identical to SEQ ID NO: 13 of US20220090064A1 or at least 80% identical to a functional fragment thereof comprising at least 400 amino acids, and a point mutation at amino acid position Q84, L139, Q221, V223, T664, or L671 with regard to SEQ ID NO: 13 of US20220090064A1; wherein the respective SEQ ID NOs are those disclosed in US Application Publication US20220090064A1.

[00659]In certain embodiments, the composition comprises a guide nucleic acid comprising: optionally, a spacer reverse complementary to a first region of a target nucleic acid, wherein the spacer is included in the guide nucleic acid, or the spacer is included in a second, different guide nucleic acid when not included in the guide nucleic acid; a scaffold configured to bind to a Cas nuclease; a reverse transcriptase template encoding a sequence to be reverse transcribed into a first synthesized strand to be inserted into the target nucleic acid; a first strand primer binding site reverse complementary to a second region of the target nucleic acid; and at least one of:

(i) a guide nucleic acid positioning system (GPS) region and a GPS binding site that hybridizes to the GPS region, wherein the GPS region and the GPS binding site are at least 10 nucleotides in length and are at least 60% reverse complementary to each other, and wherein hybridization of the GPS region and the GPS binding site positions the first strand primer binding site closer to the second region of the target nucleic acid,

(ii) a GPS region that hybridizes to a GPS binding site on the second guide nucleic acid, wherein the GPS region and the GPS binding site are at least 10 nucleotides in length and are at least 60% reverse complementary to each other, wherein the second region of the target nucleic acid does not include any part of the first region of the target nucleic acid, and wherein the second region of the target nucleic acid does not include any part of a reverse complement of the first region of the target nucleic acid, and wherein hybridization of the GPS region and the GPS binding site positions the first strand primer binding site closer to the second region of the target nucleic acid, or

(iii) a modification in the reverse transcriptase template that disrupts a track of at least 4 consecutive nucleotides of the same base in the target nucleic acid.

Zinc finger nucleases, TALENS, and meganucleases

[00660]In some embodiments, the gene editing systems contemplated herein may comprise user-programmable DNA binding proteins that bind DNA through a specific amino acid sequence (i.e., are not reliant upon a guide RNA or nucleic acid programmability). Such enzymes include zinc finger nucleases and TALENS.

[00661]In some embodiments, the user-programmable nuclease is or comprises a TALE Nuclease, a TALE nickase, Zinc Finger (ZF) Nuclease, ZF Nickase, meganuclease, or a combination thereof. In some embodiments, the non-CRISPR/Cas sequence-specific nuclease is or includes two, three, four, or more of an independently selected TALE Nuclease, TALE nickase, Zinc Finger (ZF) Nuclease, ZF Nickase, Meganuclease, restriction enzymes or a combination thereof. In some embodiments, the combination is or comprises a TALE Nuclease/a ZF Nuclease; a TALE Nickase/a ZF nickase.

TALENs

[00662]In some embodiments, the non-CRISPR/Cas sequence-specific nuclease is or comprises a TALE Nuclease (Transcription Activator-Like Effector Nucleases (TALEN)). TALENs are restriction enzymes engineered to cut specific target DNA sequences. TALENs comprise a TAL effector (TALE) DNA-binding domain (which binds at or close to the target DNA), fused to a DNA cleavage domain which cuts target DNA. TALEs are engineered to bind to practically any desired DNA sequence. Thus in some embodiments, the TALEN comprises an N-terminal capping region, a DNA binding domain which may comprise at least one or more TALE monomers or half-monomers specifically ordered to target the genomic locus of interest, and a C-terminal capping region, wherein these three parts are arranged in a predetermined N-terminus to C-terminus orientation. Optionally, the TALEN includes at least one or more regulatory or functional protein domains.

[00663]In some embodiments, the TALE monomers or half monomers may be variant TALE monomers derived from natural or wild type TALE monomers but with altered amino acids at positions usually highly conserved in nature, and in particular have a combination of amino acids as RVDs that do not occur in nature, and which may recognize a nucleotide with a higher activity, specificity, and/or affinity than a naturally occurring RVD. The variants may include deletions, insertions and substitutions at the amino acid level, and transversions, transitions and inversions at the nucleic acid level at one or more locations. The variants may also include truncations.

[00664]In some embodiments, the TALE monomer / half monomer variants include homologous and functional derivatives of the parent molecules. In some embodiments, the variants are encoded by polynucleotides capable of hybridizing under high stringency conditions to the parent molecule-encoding wild-type nucleotide sequences.

[00665]In some embodiments, the DNA binding domain of the TALE has at least 5 of more TALE monomers and at least one or more half-monomers specifically ordered or arranged to target a genomic locus of interest. The construction and generation of TALEs or polypeptides of the disclosure may involve any of the methods known in the art.

[00666]Naturally occurring TALEs or "wild type TALEs" are nucleic acid binding proteins secreted by numerous species of proteobacteria. TALEs contain a nucleic acid binding domain composed of tandem repeats of highly conserved monomer polypeptides that are predominantly 33, 34 or 35 amino acids in length and that differ from each other mainly in amino acid positions 12 and 13. A general representation of a TALE monomer which is comprised within the DNA binding domain is Xl-11-(X12X13)-X14-33 or 34 or 35, where the subscript indicates the amino acid position and X represents any amino acid. X12X13 indicate the RVDs. In some polypeptide monomers, the variable amino acid at position 13 is missing or absent and in such monomers, the RVD consists of a single amino acid. In such cases the RVD may be alternatively represented as X*, where X represents X12 and (*) indicates that X13 is absent. The DNA binding domain may comprise several repeats of TALE monomers and this may be represented as (Xl-11-(X12X13)-X14-33 or 34 or 35)z, where z is optionally at least 5-40, such as 10-26.

[00667]The TALE monomers have a nucleotide binding affinity that is determined by the identity of the amino acids in its RVD. Polypeptide monomers with an RVD of NI preferentially bind to adenine (A), monomers with an RVD of NG preferentially bind to thymine (T), monomers with an RVD of HD preferentially bind to cytosine (C), monomers with an RVD of NN preferentially bind to both adenine (A) and guanine (G), monomers with an RVD of IG preferentially bind to T, monomers with an RVD of NS recognize all four base pairs and may bind to A, T, G or C. Thus, the number and order of the polypeptide monomer repeats in the nucleic acid binding domain of a TALE determines its nucleic acid target specificity. The structure and function of TALEs is further described in, for example, Moscou *et al.*, *Science* 326:1501 (2009); Boch *et al.*, *Science* 326:1509-1512 (2009); and Zhang *et al.*, *Nature Biotechnology* 29:149-153 (2011), each of which is incorporated by reference in its entirety.

[00668]In some embodiments, the TALE is a dTALE (or designerTALE), see Zhang *et al.*, Nature Biotechnology 29:149-153 (2011), incorporated herein by reference.

[00669]In some embodiments, the TALE monomer comprises an RVD of IIN or NII that preferentially binds to guanine, and the TALEs have high binding specificity for guanine containing target nucleic acid sequences. In come embodiments, polypeptide monomers having RVDs RN, NN, NK, SN, NH, KN, HN, NQ, HH, RG, KH, RH and SS preferentially bind to guanine. In some embodiments, polypeptide monomers having RVDs RN, NK, NQ, HH, KH, RH, SS and SN preferentially bind to guanine. In some embodiments, polypeptide monomers having RVDs HH, KH, NH, NK, NQ, RH, RN and SS preferentially bind to guanine. In some embodiments, the RVDs that have high binding specificity for guanine are RN, NH RH and KH. In some embodiments, polypeptide monomers having an RVD of NV preferentially bind to adenine and guanine as do monomers having the RVD HN. Monomers having an RVD of NC preferentially bind to adenine, guanine and cytosine, and monomers having an RVD of S (or S*), bind to adenine, guanine, cytosine and thymine with comparable affinity. In more embodiments, monomers having RVDs of H*, HA,

KA, N*, NA, NC, NS, RA, and S* bind to adenine, guanine, cytosine and thymine with comparable affinity. Such polypeptide monomers allow for the generation of degenerative TALEs able to bind to a repertoire of related, but not identical, target nucleic acid sequences.

[00670]In certain embodiments, the TALE polypeptide has a nucleic acid binding domain containing polypeptide monomers arranged in a predetermined N-terminus to C-terminus order such that each polypeptide monomer binds to a nucleotide of a predetermined target nucleic acid sequence, and where at least one of the polypeptide monomers has an RVD of HN or NH and preferentially binds to guanine, an RVD of NV and preferentially binds to adenine and guanine, an RVD of NC and preferentially binds to adenine, guanine and cytosine or an RVD of S and binds to adenine, guanine, cytosine and thymine.

[00671]In some embodiments, each polypeptide monomer of the nucleic acid binding domain that binds to adenine has an RVD of NI, NN, NV, NC or S. In certain embodiments, each polypeptide monomer of the nucleic acid binding domain that binds to guanine has an RVD of HN, NH, NN, NV, NC or S. In certain embodiments, each polypeptide monomer of the nucleic acid binding domain that binds to cytosine has an RVD of HD, NC or S. In some embodiments, each polypeptide monomer that binds to thymine has an RVD of NG or S. In some embodiments, each polypeptide monomer of the nucleic acid binding domain that binds to adenine has an RVD of NI. In certain embodiments, each polypeptide monomer of the nucleic acid binding domain that binds to guanine has an RVD of HN or NH. In certain embodiments, each polypeptide monomer of the nucleic acid binding domain that binds to cytosine has an RVD of HD. In some embodiments, each polypeptide monomer that binds to thymine has an RVD of NG. In certain embodiments, the RVDs that have a specificity for adenine are NI, RI, KI, HI, and SI. In certain embodiments, the RVDs that have a specificity for adenine are HN, SI and RI, most preferably the RVD for adenine specificity is SI. In certain embodiments, the RVDs that have a specificity for thymine are NG, HG, RG and KG. In certain embodiments, the RVDs that have a specificity for thymine are KG, HG and RG, most preferably the RVD for thymine specificity is KG or RG. In certain embodiments, the RVDs that have a specificity for cytosine are HD, ND, KD, RD, HH, YG and SD. In certain embodiments, the RVDs that have a specificity for cytosine are SD and RD.

[00672]FIG. 4B of WO 2012/067428 provides representative RVDs and the nucleotides they target, the entire content of which is hereby incorporated herein by reference.

[00673]In certain embodiments, the variant TALE monomers may comprise any of the RVDs that exhibit specificity for a nucleotide as depicted in FIG. 4A of WO2012/067428. All such TALE monomers allow for the generation of degenerative TALEs able to bind to a repertoire of related, but not identical, target nucleic acid sequences.

[00674]In certain embodiments, the RVD SH may have a specificity for G, the RVD IS may have a specificity for A, and the RVD IG may have a specificity for T.

[00675]In certain embodiments, the RVD NT may bind to G and A. In certain embodiments, the RVD NP may bind to A, T and C. In certain embodiments, at least one selected RVD may be NI, HD, NG, NN, KN, RN, NH, NQ, SS, SN, NK, KH, RH, HH, KI, HI, RI, SI, KG, HG, RG, SD, ND, KD, RD, YG, HN, NV, NS, HA, S*, N*, KA, H*, RA, NA or NC.

[00676]The predetermined N-terminal to C-terminal order of the one or more polypeptide monomers of the nucleic acid or DNA binding domain determines the corresponding predetermined target nucleic acid sequence to which the TALE or polypeptides of the disclosure may bind.

[00677]As used herein the monomers and at least one or more half monomers are "specifically ordered to target" the genomic locus or gene of interest. In plant genomes, the natural TALE-binding sites always begin with a thymine (T), which may be specified by a cryptic signal within the non-repetitive N-terminus of the TALE polypeptide; in some cases this region may be referred to as repeat 0. In animal genomes, TALE binding sites do not necessarily have to begin with a thymine (T) and polypeptides of the disclosure may target DNA sequences that begin with T, A, G or C. The tandem repeat of TALE monomers always ends with a half-length repeat or a stretch of sequence that may share identity with only the first 20 amino acids of a repetitive full length TALE monomer and this half repeat may be referred to as a half-monomer (FIG. 8 of WO 2012/067428). Therefore, it follows that the length of the nucleic acid or DNA being targeted is equal to the number of full monomers plus two (see FIG. 44 of WO 2012/067428).

[00678]In certain embodiments, nucleic acid binding domains are engineered to contain 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more polypeptide monomers arranged in a N-terminal to C-terminal direction to bind to a predetermined 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 nucleotide length nucleic acid sequence.

[00679]In certain embodiments, nucleic acid binding domains are engineered to contain 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or more full length polypeptide monomers that are specifically ordered or arranged to target nucleic acid sequences of length 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 and 28 nucleotides, respectively. In certain embodiments, the polypeptide monomers are contiguous. In some embodiments, half-monomers may be used in the place of one or more monomers, particularly if they are present at the C-terminus of the TALE.

[00680]Polypeptide monomers are generally 33, 34 or 35 amino acids in length. With the exception of the RVD, the amino acid sequences of polypeptide monomers are highly conserved or as described herein, the amino acids in a polypeptide monomer, with the exception of the RVD, exhibit patterns that effect TALE activity, the identification of which may be used in preferred embodiments of the disclosure.

[00681]In certain embodiments, when the DNA binding domain may comprise (Xl-11-X12X13-X14-33 or 34 or 35)z, wherein Xl-11 is a chain of 11 contiguous amino acids, wherein X12X13 is a repeat variable di-residue (RVD), wherein X14-33 or 34 or 35 is a chain of 21, 22 or 23 contiguous amino

acids, wherein z is at least 5 to 26, then the preferred combinations of amino acids are LTLD or LTLA or LTQV at Xl-4, or EQHG or RDHG at positions X30-33 or X31-34 or X32-35. Furthermore, other amino acid combinations of interest in the monomers are LTPD at Xl-4 and NQALE at XI 6-20 and DHG at X32-34 when the monomer is 34 amino acids in length. When the monomer is 33 or 35 amino acids long, then the corresponding shift occurs in the positions of the contiguous amino acids NQALE and DHG. In certain embodiments, NQALE is at X15-19 or X17-21 and DHG is at X31-33 or X33-35.

[00682]In certain embodiments, amino acid combinations of interest in the monomers, are LTPD at Xl-4 and KRALE at X16-20 and AHG at X32-34 or LTPE at Xl-4 and KRALE at XI 6-20 and DHG at X32-34 when the monomer is 34 amino acids in length. When the monomer is 33 or 35 amino acids long, the corresponding shift occurs in the positions of the contiguous amino acids KRALE, AHG and DHG. In certain embodiments, the positions of the contiguous amino acids may be (LTPD at Xl-4 and KRALE at X15-19 and AHG at X31-33) or (LTPE at Xl-4 and KRALE at X15-19 and DHG at X31-33) or (LTPD at Xl-4 and KRALE at X17-21 and AHG at X33-35) or (LTPE at Xl-4 and KRALE at X17-21 and DHG at X33-35).

[00683]In certain embodiments, contiguous amino acids [NGKQALE] are present at positions X14-20 or X13-19 or X15-21. These representative positions put forward various embodiments of the disclosure and provide guidance to identify additional amino acids of interest or combinations of amino acids of interest in all the TALE monomers (see FIGs. 24A-24F, and 25 of WO 2012/067428).

[00684]As used herein the predetermined "N-terminus" to "C terminus" orientation of the N-terminal capping region, the DNA binding domain comprising the repeat TALE monomers and the C-terminal capping region provide structural basis for the organization of different domains in the d-TALEs or polypeptides of the disclosure.

[00685]The entire N-terminal and/or C-terminal capping regions are not necessary to enhance the binding activity of the DNA binding region. Therefore, in certain embodiments, fragments of the N-terminal and/or C-terminal capping regions are included in the TALE polypeptides described herein.

[00686]In certain embodiments, the TALE (including TALEs) polypeptides described herein contain a N-terminal capping region fragment that included at least 10, 20, 30, 40, 50, 54, 60, 70, 80, 87, 90, 94, 100, 102, 110, 117, 120, 130, 140, 147, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260 or 270 amino acids of an N-terminal capping region. In certain embodiments, the N-terminal capping region fragment amino acids are of the C-terminus (the DNA-binding region proximal end) of an N-terminal capping region. N-terminal capping region fragments that include the C-terminal 240 amino acids enhance binding activity equal to the full length capping region, while fragments that include the C-terminal 147 amino acids retain greater than 80% of the efficacy of the full length capping region, and fragments that include the C-terminal 117 amino acids retain greater than 50% of the activity of the full-length capping region.

[00687]In some embodiments, the TALE polypeptides described herein contain a C-terminal capping region fragment that included at least 6, 10, 20, 30, 37, 40, 50, 60, 68, 70, 80, 90, 100, 110, 120, 127, 130, 140, 150, 155, 160, 170, 180 amino acids of a C-terminal capping region. In certain embodiments, the C-terminal capping region fragment amino acids are of the N-terminus (the DNA-binding region proximal end) of a C-terminal capping region. In certain embodiments, C-terminal capping region fragments that include the C-terminal 68 amino acids enhance binding activity equal to the full length capping region, while fragments that include the C-terminal 20 amino acids retain greater than 50% of the efficacy of the full length capping region.

[00688]In certain embodiments, the capping regions of the TALE polypeptides described herein do not need to have identical sequences to the capping region sequences provided herein. Thus, in some embodiments, the capping region of the TALE polypeptides described herein have sequences that are at least 50%, 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical or share identity to the capping region amino acid sequences provided herein. Sequence identity is related to sequence homology. Homology comparisons may be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs may calculate percent (%) homology between two or more sequences and may also calculate the sequence identity shared by two or more amino acid or nucleic acid sequences. In some preferred embodiments, the capping region of the TALE polypeptides described herein have sequences that are at least 95% identical or share identity to the capping region amino acid sequences provided herein.

[00689]Sequence homologies may be generated by any of a number of computer programs known in the art, which include but are not limited to BLAST or FASTA. Suitable computer program for carrying out alignments like the GCG Wisconsin Bestfit package may also be used. Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result. % homology may be calculated over contiguous sequences, *i.e.*, one sequence is aligned with the other sequence and each amino acid or nucleotide in one sequence is directly compared with the corresponding amino acid or nucleotide in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

[00690]Additional sequences for the conserved portions of polypeptide monomers and for N-terminal and C-terminal capping regions are included in the sequences with the following gene accession numbers: AAW59491.1, AAQ79773.2, YP_450163.1, YP_001912778.1, ZP_02242672.1, AAW59493.1, AAY54170.1, ZP_02245314.1, ZP_02243372.1, AAT46123.1, AAW59492.1, YP_451030.1, YP_001915105.1, ZP_02242534.1, AAW77510.1, ACD11364.1, ZP_02245056.1, ZP_02245055.1, ZP_02242539.1, ZP_02241531.1, ZP_02243779.1, AAN01357.1, ZP_02245177.1, ZP_02243366.1, ZP_02241530.1, AAS58130.3, ZP_02242537.1, YP_200918.1, YP_200770.1,

YP_451187.1, YP_451156.1, AAS58127.2, YP_451027.1, UR_451025.1, AAA92974.1, UR_001913755.1, ABB70183.1, UR_451893.1, UR_450167.1, ABY60855.1, UR_200767.1, ZR_02245186.1, ZR_02242931.1, ZR_02242535.1, AAU54169.1, UR_450165.1, UR_001913452.1, AAS58129.3, ACM44927.1, ZR_02244836.1, AAT46125.1, UR_450161.1, ZR_02242546.1, AAT46122.1, UR_451897.1, AAF98343.1, UR_001913484.1, AAY54166.1, UR_001915093.1, UR_001913457.1, ZR_02242538.1, UR_200766.1, UR_453043.1, UR_001915089.1, UR_001912981.1, ZR_02242929.1, UR_001911730.1, UR_201654.1, UR_199877.1, ABB70129.1, UR_451696.1, UR_199876.1, AAS75145.1, AAT46124.1, UR_200914.1, UR 001915101.1, ZR_02242540.1, AAG02079.2, UR_451895.1, YP 451189.1, UR_200915.1, AAS46027.1, UR_001913759.1, UR_001912987.1, AAS58128.2, AAS46026.1, UR_201653.1, UR_202894.1, UR_001913480.1, ZR_02242666.1, R_001912775.1, ZR_02242662.1, AAS46025.1, AAC43587.1, BAA37119.1, NPJ544725.1, AB077779.1, BAA37120.1, ACZ62652.1, BAF46271.1, ACZ62653.1, NPJ544793.1, ABO77780.1, ZR_02243740.1, ZR_02242930.1, AAB69865.1, AAY54168.1, ZR_02245191.1, UR_001915097.1, ZR_02241539.1, UR_451158.1, BAA37121.1, UR_001913182.1, UR_200903.1, ZR_02242528.1, ZR_06705357.1, ZR_06706392.1, ADI48328.1, ZR_06731493.1, ADI48327.1, AB077782.1, ZR 06731656.1, NR_942641.1, AAY43360.1, ZR_06730254.1, ACN39605.1, UR_451894.1, UR_201652.1, UR_001965982.1, BAF46269.1, NPJ544708.1, ACN82432.1, AB077781.1, P14727.2, BAF46272.1, AAY43359.1, BAF46270.1, NR_644743.1, ABG37631.1, AAB00675.1, YP 199878.1, ZR_02242536.1, CAA48680.1, ADM80412.1, AAA27592.1, ABG37632.1, ABP97430.1, ZR_06733167.1, AAY43358.1, 2KQ5_A, BAD42396.1, ABO27075.1, UR_002253357.1, UR_002252977.1, ABO27074.1, ABO27067.1, ABO27072.1, ABO27068.1, UR_003750492.1, ABO27073.1, NR_519936.1, ABO27071.1, AB027070.1, and ABO27069.1, each of which is hereby incorporated by reference.

[00691]In some embodiments, the TALEs described herein also include a nuclear localization signal and/or cellular uptake signal. Such signals are known in the art and may target a TALE to the nucleus and/or intracellular compartment of a cell. Such cellular uptake signals include, but are not limited to, the minimal Tat protein transduction domain which spans residues 47-57 of the human immunodeficiency virus Tat protein.

[00692]In some embodiments, the TALEs described herein include a nucleic acid or DNA binding domain that is a non-TALE nucleic acid or a non-TALE DNA binding domain.

[00693]As used herein the term "non-TALE DNA binding domain" refers to a DNA binding domain that has a nucleic acid sequence corresponding to a nucleic acid sequence which is not substantially homologous to a nucleic acid that encodes for a TALE protein or fragment thereof, *e.g.*, a nucleic acid sequence which is different from a nucleic acid that encodes for a TALE protein and which is derived from the same or a different organism.

[00694]In certain embodiments, the TALEs described herein include a nucleic acid or DNA binding domain that is linked to a non-TALE polypeptide.

[00695]A "non-TALE polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to a TALE protein or fragment thereof, *e.g.*, a protein which is different from a TALE protein and which is derived from the same or a different organism. In this context, the term "linked" is intended include any manner by which the nucleic acid binding domain and the non-TALE polypeptide could be connected to each other, including, for example, through peptide bonds by being part of the same polypeptide chain or through other covalent interactions, such as a chemical linker. The non-TALE polypeptide may be linked, for example to the N-terminus and/or C-terminus of the nucleic acid binding domain, may be linked to a C-terminal or N-terminal cap region, or may be connected to the nucleic acid binding domain indirectly.

[00696]In certain embodiments, the TALEs or polypeptides of the disclosure comprise chimeric DNA binding domains. Chimeric DNA binding domains may be generated by fusing a full TALE (including the N- and C- terminal capping regions) with another TALE or non-TALE DNA binding domain such as zinc finger (ZF), helix-loop-helix, or catalytically-inactivated DNA endonucleases (*e.g.*, EcoRI, meganucleases, etc.), or parts of TALE may be fused to other DNA binding domains. The chimeric domain may have novel DNA binding specificity that combines the specificity of both domains.

[00697]In certain embodiments, the TALE polypeptides of the disclosure include a nucleic acid binding domain linked to the one or more effector domains. In certain embodiments, the effector domain is a nickase or nuclease.

ZFNs

[00698]In certain embodiments, the sequence-specific nuclease is a zinc finger nuclease (ZFN), such as an artificial zinc-finger nuclease having arrays of zinc-finger (ZF) modules to target new DNA-binding sites in a target sequence (*e.g.*, target sequence or target site in the genome). Each zinc finger module in a ZF array targets three DNA bases. A customized array of individual zinc finger domains is assembled into a ZF protein (ZFP). The resulting ZFP can be linked to a functional domain such as a nuclease.

[00699] ZF nucleases (ZFN) may be used as alternative programmable nucleases for use in retron-based editing in place of RNA-guide nucleases. ZFN proteins have been extensively described in the art, for example, in Carroll et al.,"Genome Engineering with Zinc-Finger Nucleases," Genetics, Aug 2011, Vol.188: 773-782; Durai et al.,"Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells," Nucleic Acids Res, 2005, Vol.33: 5978-90; and Gaj et al., "ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering," Trends Biotechnol.2013, Vol.31: 397-405, each of which are incorporated herein by reference in their entireties.

[00700]In certain embodiments, the ZF-linked nuclease is a catalytic domain of the Type IIS restriction enzyme FokI (see Kim *et al.*, *PNAS U.S.A.* 91:883-887, 1994; Kim *et al.*, PNAS U.S.A. 93:1156-1160, 1996, both incorporated herein by reference).

[00701]In certain embodiments, the ZFN comprises paired ZFN heterodimers, resulting in increased cleavage specificity and/or decreased off-target activity. In this embodiment, each ZFN in the heterodimer targets different nucleotide sequences separated by a short spacer (see Doyon *et al.*, *Nat. Methods* 8:74-79, 2011, incorporated herein by reference).

[00702]In certain embodiments, the ZFN comprises a polynucleotide-binding domain (comprising multiple sequence-specific ZF modules) and a polynucleotide cleavage nickase domain.

[00703]In certain embodiments, the ZFs are engineered using libraries of two finger modules. In certain embodiments, strings of two-finger units are used in ZFNs to improve DNA binding specificity from polyzinc finger peptides (see PNAS USA 98: 1437-1441, incorporated herein by reference).

[00704]In certain embodiments, the ZFN has more than 3 fingers. In certain embodiments, the ZFN has 4, 5, or 6 fingers. In certain embodiments, the ZF modules in the ZFN are separated by one or more linkers to improve specificity.

[00705]In certain embodiments, the ZF of the ZFN includes substitutions in the dimer interface of the cleavage domain that prevent homodimerization between ZFs, but allow heterodimers to form.

[00706]In certain embodiments, the ZF of the ZFN has a design that retains activity while suppressing homodimerization.

[00707]In certain embodiments, the ZFN is any one of the ZF nucleases in Table 1 of Carroll *et al.*, Genetics 188(4):773-782, 2011, incorporated herein by reference.

[00708]General principles and guidance for generating ZF, ZF arrays, and ZFN can be found in the art, such as the modular design (where the different modules can be rearranged and assembled into new combinations for new targets) of the ZF or ZF arrays in the ZFN as taught in Carroll *et al.*, *Nat. Protoc.* 1: 1329-1341, 2006 (incorporated herein by reference); the new three-finger sets for engineered ZFs generated by using partially randomized libraries; profiling the DNA-binding specificities of engineered Cys2His2 zinc finger domains using a rapid cell-based method (see *Nucleic Acids Res.* 35: e81, incorporated by reference). ZFs for certain DNA triplets that work well in neighbor combination are described in Sander *et al.*, 2011. Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA) is taught in *Nat. Methods* 8: 67-69). ToolGen describes the individual fingers in their collection that are best behaved in modular assembly (Kim et al., 2011). Preassembled zinc-finger arrays for rapid construction of ZFNs are taught in *Nat. Methods* 8:7.

[00709]Additional, non-limiting ZFs and AFNz that can be adapted for use in the instant disclosure include those described in WO2010/065123, WO2000/041566, WO2003/080809, WO2015/143046, WO2016/183298, WO2013/044008, WO2015/031619, WO2017/136049, WO2016/014794,

WO2017/091512, WO1995/009233, WO2000/023464, WO2000/042219, WO2002/026960, WO2001/083793; US9428756, US9145565, US8846578, US8524874, US6777185, US6599692, US7235354, US6503717, US7491531, US7943553, US7262054, US8680021, US7705139, US7273923, US6780590, US6785613, US7788044, US7177766, US6453242, US6794136, US7358085, US8383766, US7030215, US7013219, US7361635, US7939327, US8772453, US9163245, US7045304, US8313925, US9260726, US6689558, US8466267, US7253273, US7947873, US9388426, US8153399, US8569253, US8524221, US7951925, US9115409, US8772008, US9121072, US9624498, US6979539, US9491934, US6933113, US9567609, US7070934, US9624509, US8735153, US9567573, US6919204, US2002-0081614, US2004-0203064, US2006-0166263, US2006-0292621, US2003-0134318, US2006-0294617, US2007-0287189, US2007-0065931, US2003-0105593, US2003-0108880, US2009-0305402, US2008-0209587, US2013-0123484, US2004-0091991, US2009-0305977, US2008-0233641, US2014-0287500, US2011-0287512, US2009-0258363, US2013-0244332, US2007-0134796, US2010-0256221, US2005-0267061, US2012-0204282, US2012-0252122, US2010-0311124, US2016-0215298, US2008-0031109, US2014-0017214, US2015-0267205, US2004-0235002, US2004-0204345, US2015-0064789, US2006-0063231, US2011-0265198, US2017-0218349, all incorporated herein by reference.

[00710]Polynucleotides and vectors capable of expressing one or more of the ZFNs are also provided herein, which can be part of the vector system of the disclosure. The polynucleotides and vectors can be expressed in a cell, such as a eukaryotic cell, a mammalian cell, or a human cell. Suitable vectors, cells and expression systems are described in greater detail elsewhere herein, and can be suitable for use with the TALEs, the meganucleases, and the CRISPR-Cas nucleases.

Meganucleases

[00711]In some embodiments, the gene editing system comprises meganucleases. Meganucleases are homing endonucleases discovered in yeast that recognize fairly long DNA sequences, and create double-strand breaks that are mended via stimulation of homologous recombination. Meganucleases are sequence-specific endonucleases that use large (recognition sites to generate accurate double-strand breaks (DSBs), promoting efficient gene targeting through homologous recombination (HR). Meganuclease enzymes and editing systems comprising meganucleases have been described in the literature, including the following references, each of which are incorporated herein in their entireties by reference. Khalil AM. The genome editing revolution: review. J Genet Eng Biotechnol. 2020 Oct 29;18(1):68. doi: 10.1186/s43141-020-00078-y. PMID: 33123803; PMCID: PMC7596157. Lanigan TM, Kopera HC, Saunders TL. Principles of Genetic Engineering. Genes (Basel). 2020 Mar 10;11(3):291. doi: 10.3390/genes11030291. PMID: 32164255; PMCID: PMC7140808. Arnould S, Delenda C, Grizot S, Desseaux C, Pâques F, Silva GH, Smith J. The I-CreI meganuclease and its engineered derivatives: applications from cell modification to gene therapy. Protein Eng Des Sel. 2011 Jan;24(1-2):27-31. doi: 10.1093/protein/gzq083. Epub 2010 Nov 3. PMID: 21047873. Pâques F,

Duchateau P. Meganucleases and DNA double-strand break-induced recombination: perspectives for gene therapy. Curr Gene Ther. 2007 Feb;7(1):49-66. doi: 10.2174/156652307779940216. PMID: 17305528. Zekonyte U, Bacman SR, Smith J, Shoop W, Pereira CV, Tomberlin G, Stewart J, Jantz D, Moraes CT. Mitochondrial targeted meganuclease as a platform to eliminate mutant mtDNA in vivo. Nat Commun. 2021 May 28;12(1):3210. doi: 10.1038/s41467-021-23561-7. PMID: 34050192; PMCID: PMC8163834.

[00712]Meganuclease enzymes and editing systems comprising meganucleases have also been described in the patent literature, including the following references, each of which are incorporated herein in their entireties by reference.

Pub Number	**Title**
WO2022226316A1	COMPOSITIONS AND METHODS FOR GENERATING MALE STERILE PLANTS
WO2022226305A1	ENGINEERED MEGANUCLEASES THAT TARGET HUMAN MITOCHONDRIAL GENOMES
WO2022226303A1	ENGINEERED MEGANUCLEASES THAT TARGET HUMAN MITOCHONDRIAL GENOMES
WO2022165111A1	MODULATION OF TGF BETA SIGNALING IN GENETICALLY-MODIFIED EUKARYOTIC CELLS
WO2022104062A1	ENGINEERED MEGANUCLEASES HAVING SPECIFICITY FOR RECOGNITION SEQUENCES IN THE DYSTROPHIN GENE
WO2022076547A1	LIPID NANOPARTICLE COMPOSITIONS
WO2022040582A1	ENGINEERED MEGANUCLEASES HAVING SPECIFICITY FOR A RECOGNITION SEQUENCE IN THE TRANSTHYRETIN GENE
WO2022035793A1	ANTIBODIES AND FRAGMENTS SPECIFIC FOR B-CELL MATURATION ANTIGEN AND USES THEREOF
WO2021231959A1	METHODS FOR IMMUNOTHERAPY
WO2021231954A1	METHODS FOR IMMUNOTHERAPY
WO2021231495A1	TREATMENT OF RETINITIS PIGMENTOSA USING IMPROVED ENGINEERED MEGANUCLEASES
WO2021231259A1	SELF-LIMITING VIRAL VECTORS ENCODING NUCLEASES
WO2021158915A1	RECOMBINANT ADENO-ASSOCIATED VIRUS COMPOSITIONS AND METHODS FOR PRODUCING AND USING THE SAME
WO2021113765A1	OPTIMIZED ENGINEERED MEGANUCLEASES HAVING SPECIFICITY FOR A RECOGNITION SEQUENCE IN THE HEPATITIS B VIRUS GENOME

Pub Number	Title
WO2021113543A1	METHODS FOR CANCER IMMUNOTHERAPY, USING LYMPHODEPLETION REGIMENS AND CD19, CD20 OR BCMA ALLOGENEIC CAR T CELLS
WO2021087305A1	CD20 CHIMERIC ANTIGEN RECEPTORS AND METHODS OF USE FOR IMMUNOTHERAPY
WO2021035170A1	COMPOSITIONS AND METHODS FOR TCR REPROGRAMMING USING FUSION PROTEINS
WO2021035054A1	LYMPHODEPLETION DOSING REGIMENS FOR CELLULAR IMMUNOTHERAPIES
WO2021016608A1	COMPOSITIONS AND METHODS FOR SEQUENTIAL STACKING OF NUCLEIC ACID SEQUENCES INTO A GENOMIC LOCUS
WO2020227534A1	OPTIMIZATION OF ENGINEERED MEGANUCLEASES FOR RECOGNITION SEQUENCES
WO2020206248A1	GENETICALLY-MODIFIED IMMUNE CELLS COMPRISING A MICRORNA-ADAPTED SHRNA (SHRNAMIR)
WO2020206231A1	METHODS OF PREPARING POPULATIONS OF GENETICALLY-MODIFIED IMMUNE CELLS
WO2020146807A1	GENETICALLY-MODIFIED CELLS COMPRISING A MODIFIED TRANSFERRIN GENE
WO2020132659A1	GENETIC MODIFICATION OF THE HYDROXYACID OXIDASE 1 GENE FOR TREATMENT OF PRIMARY HYPEROXALURIA
WO2019200247A1	OPTIMIZED ENGINEERED MEGANUCLEASES HAVING SPECIFICITY FOR A RECOGNITION SEQUENCE IN THE HEPATITIS B VIRUS GENOME
WO2019200122A1	OPTIMIZED ENGINEERED NUCLEASES HAVING SPECIFICITY FOR THE HUMAN T CELL RECEPTOR ALPHA CONSTANT REGION GENE
WO2019089913A1	ENGINEERED NUCLEASES THAT TARGET HUMAN AND CANINE FACTOR VIII GENES AS A TREATMENT FOR HEMOPHILIA A
WO2019070856A1	MODIFIED EPIDERMAL GROWTH FACTOR RECEPTOR PEPTIDES FOR USE IN GENETICALLY-MODIFIED CELLS
WO2019005957A1	GENETICALLY-MODIFIED T CELLS COMPRISING A MODIFIED INTRON IN THE T CELL RECEPTOR ALPHA GENE
WO2018208837A1	NUCLEIC ACID MOLECULES ENCODING AN ENGINEERED ANTIGEN RECEPTOR AND AN INHIBITORY NUCLEIC ACID MOLECULE AND METHODS OF USE THEREOF

Pub Number	Title
WO2018201144A1	METHODS FOR REDUCING DNA-INDUCED CYTOTOXICITY AND ENHANCING GENE EDITING IN PRIMARY CELLS
WO2018195449A1	ENGINEERED MEGANUCLEASES SPECIFIC FOR RECOGNITION SEQUENCES IN THE PCSK9 GENE
WO2018071849A2	ENGINEERED MEGANUCLEASES SPECIFIC FOR RECOGNITION SEQUENCES IN THE HEPATITIS B VIRUS GENOME
WO2018067697A1	CO-STIMULATORY DOMAINS FOR USE IN GENETICALLY-MODIFIED CELLS
WO2017192741A1	ENGINEERED NUCLEASES USEFUL FOR TREATMENT OF HEMOPHILIA A
WO2017112859A1	ENGINEERED MEGANUCLEASES WITH RECOGNITION SEQUENCES FOUND IN THE HUMAN BETA-2 MICROGLOBULIN GENE
WO2017062451A1	GENETICALLY-MODIFIED CELLS COMPRISING A MODIFIED HUMAN T CELL RECEPTOR ALPHA CONSTANT REGION GENE
WO2017062439A1	ENGINEERED MEGANUCLEASES WITH RECOGNITION SEQUENCES FOUND IN THE HUMAN T CELL RECEPTOR ALPHA CONSTANT REGION GENE
WO2017044649A1	TREATMENT OF RETINITIS PIGMENTOSA USING ENGINEERED MEGANUCLEASES
WO2016205825A1	SELF-LIMITING VIRAL VECTORS ENCODING NUCLEASES
WO2016179112A1	PRECISE DELETION OF CHROMOSCOMAL SEQUENCES IN VIVO AND TREATMENT OF NUCLEOTIDE REPEAT EXPANSION DISORDERS USING ENGINEERED NUCLEASES
WO2015138739A2	DYSTROPHIN GENE OXON DELETION USING ENGINEERED NUCLEASES
WO2012167192A2	METHODS AND PRODUCTS FOR PRODUCING ENGINEERED MAMMALIAN CELL LINES WITH AMPLIFIED TRANSGENES
WO2010009147A1	RECOGNITION SEQUENCES FOR I-CREI-DERIVED MEGANUCLEASES AND USES THEREOF
WO2009134714A2	FUSION MOLECULES OF RATIONALLY-DESIGNED DNA-BINDING PROTEINS AND EFFECTOR DOMAINS
WO2009114321A2	RATIONALLY-DESIGNED MEGANUCLEASES FOR MAIZE GENOME ENGINEERING

Pub Number	Title
WO2009076292A2	RATIONALLY-DESIGNED MEGANUCLEASES WITH RECOGNITION SEQUENCES FOUND IN DNASE HYPERSENSITIVE REGIONS OF THE HUMAN GENOME
WO2009059195A2	RATIONALLY-DESIGNED SINGLE-CHAIN MEGANUCLEASES WITH NON-PALINDROMIC RECOGNITION SEQUENCES
WO2007047859A2	RATIONALLY-DESIGNED MEGANUCLEASES WITH ALTERED SEQUENCE SPECIFICITY AND DNA-BINDING AFFINITY
US20130340121A1	Temperature-Dependent Meganuclease Activity

Gene editor accessory proteins

[00713]In other aspects, the gene editing systems described herein may comprise one or more additional accessory proteins having genome modifying functions, including recombinases, invertases, nucleases, polymerases (e.g., reverse transcriptases), ligases, deaminases, transposases, or DNA binding domains. In various embodiments, the accessory proteins may be provided separately. In other embodiments, the accessory proteins may be fused to another component of a given gene editing system, such as a CRISPR-Cas9, through a linker.

Guide RNA components

Guide RNAs

[00714]The present disclosure further provides guide RNAs for use in accordance with the disclosed nucleic acid programmable DNA binding proteins (e.g., Cas9) for use in methods of editing. The disclosure provides guide RNAs that are designed to recognize target sequences. Such gRNAs may be designed to have guide sequences (or "spacers") having complementarity to a target sequence. Such gRNAs may be designed to have not only a guide sequences having complementarity to a target sequence to be edited, but also to have a backbone sequence that interacts specifically with the nucleic acid programmable DNA binding protein.

[00715]In some embodiments, the guide RNA may be 15-100 nucleotides in length and comprise a sequence of at least 10, at least 15, or at least 20 contiguous nucleotides that is complementary to a target nucleotide sequence. The guide RNA may comprise a spacer sequence of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 contiguous nucleotides that is complementary to a target nucleotide sequence. In some cases, the guide sequence has a length in a range of from 17-30 nucleotides (nt) (e.g., from 17-25, 17-22, 17-20, 19-30, 19-25, 19-22, 19-20, 20-30, 20-25, or 20-22 nt). In some cases, the guide sequence has a length in a range of from 17-25 nucleotides (nt) (e.g., from 17-22, 17-20, 19-25, 19-22, 19-20, 20-25, or 20-22 nt). In some cases, the guide sequence has a length of 17 or more nt (e.g., 18 or more, 19 or more, 20 or more, 21 or more, or 22 or more nt; 19 nt, 20 nt, 21 nt, 22 nt, 23 nt, 24 nt, 25 nt, etc.). In some cases, the guide sequence

has a length of 19 or more nt (e.g., 20 or more, 21 or more, or 22 or more nt; 19 nt, 20 nt, 21 nt, 22 nt, 23 nt, 24 nt, 25 nt, etc.). In some cases, the guide sequence has a length of 17 nt. In some cases, the guide sequence has a length of 18 nt. In some cases, the guide sequence has a length of 19 nt. In some cases, the guide sequence has a length of 20 nt. In some cases, the guide sequence has a length of 21 nt. In some cases, the guide sequence has a length of 22 nt. In some cases, the guide sequence has a length of 23 nt.

[00716]In some cases, the spacer sequence has a length of from 15 to 50 nucleotides (e.g., from 15 nucleotides (nt) to 20 nt, from 20 nt to 25 nt, from 25 nt to 30 nt, from 30 nt to 35 nt, from 35 nt to 40 nt, from 40 nt to 45 nt, or from 45 nt to 50 nt).

[00717]A subject guide RNA can interact with a target nucleic acid (e.g., double stranded DNA (dsDNA), single stranded DNA (ssDNA), single stranded RNA (ssRNA), or double stranded RNA (dsRNA)) in a sequence-specific manner via hybridization (i.e., base pairing).

[00718]The guide RNA can be modified to hybridize to any desired target sequence (e.g., while taking the PAM into account, e.g., when targeting a dsDNA target) within a target nucleic acid (e.g., a eukaryotic target nucleic acid such as genomic DNA). In some cases, the percent complementarity between the spacer sequence of the guide and the target site of the target nucleic acid is 60% or more (e.g., 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the spacer and the target site of the target nucleic acid is 80% or more (e.g., 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the spacer and the target site of the target nucleic acid is 90% or more (e.g., 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the spacer and the target site of the target nucleic acid is 100%.

[00719]In some cases, the percent complementarity between the spacer sequence and the target site of the target nucleic acid is 100% over an at least 5-nucleotide contiguous region of the spacer. In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 6-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 7-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 8-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of

the target nucleic acid over an at least 9-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 10-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 11-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 12-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 13-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 14-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 15-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 16-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 17-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 18-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 19-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between

the guide sequence and the target site of the target nucleic acid over an at least 20-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 21-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 22-nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%).

[00720]In some cases, the percent complementarity between the spacer sequence and the target site of the target nucleic acid is 100% over an at least 5-10 nucleotide contiguous region of the spacer. In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 6-11 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 7-12 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 8-13 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 9-14 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 10-15 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 11-16 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 12-17 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the

target site of the target nucleic acid over an at least 13-18 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 14-19 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 15-20 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 16-21 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 17-22 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 18-23 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 19-24 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 20-25 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 21-26 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%). In some cases, the percent complementarity between the guide sequence and the target site of the target nucleic acid over an at least 22-27 nucleotide contiguous region of the spacer is 60% or more (e.g., 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more, or 100%).

[00721]In various embodiments, the guide RNAs may have a scaffold or core region that complexes with a cognate nucleic acid programmable DNA binding protein (e.g., CRISPR Cas9 or Cas12a). In

some cases, a guide scaffold can have two stretches of nucleotides that are complementary to one another and hybridize to form a double stranded RNA duplex (dsRNA duplex). Thus, in some cases, the protein binding segment of a guide RNA includes a dsRNA duplex. In some embodiments, the dsRNA duplex region includes a range of from 5-25 base pairs (bp) (e.g., from 5-22, 5-20, 5-18, 5-15, 5-12, 5-10, 5-8, 8-25, 8-22, 8-18, 8-15, 8-12, 12-25, 12-22, 12-18, 12- 15, 13-25, 13-22, 13-18, 13-15, 14-25, 14-22, 14-18, 14-15, 15-25, 15-22, 15-18, 17-25, 17-22, or 17-18 bp, e.g., 5 bp, 6 bp, 7 bp, 8 bp, 9 bp, 10 bp, etc.). In some cases, the dsRNA duplex region includes a range of from 6-15 base pairs (bp) (e.g., from 6-12, 6-10, or 6-8 bp, e.g., 6 bp, 7 bp, 8 bp, 9 bp, 10 bp, etc.). In some cases, the duplex region includes 5 or more bp (e.g., 6 or more, 7 or more, or 8 or more bp). In some cases, the duplex region includes 6 or more bp (e.g., 7 or more, or 8 or more bp). In some cases, not all nucleotides of the duplex region are paired, and therefore the duplex forming region can include a bulge. The term "bulge" herein is used to mean a stretch of nucleotides (which can be one nucleotide) that do not contribute to a double stranded duplex, but which are surround 5' and 3' by nucleotides that do contribute, and as such a bulge is considered part of the duplex region. In some cases, the dsRNA includes 1 or more bulges (e.g., 2 or more, 3 or more, 4 or more bulges). In some cases, the dsRNA duplex includes 2 or more bulges (e.g., 3 or more, 4 or more bulges). In some cases, the dsRNA duplex includes 1-5 bulges (e.g., 1-4, 1-3, 2-5, 2-4, or 2-3 bulges).

[00722]Thus, in some cases, the stretches of nucleotides that hybridize to one another to form the dsRNA duplex in a guide scaffold region have 70%-100% complementarity (e.g., 75%-100%, 80%-10%, 85%-100%, 90%- 100%, 95%-100% complementarity) with one another. In some cases, the stretches of nucleotides that hybridize to one another to form the dsRNA duplex have 70%-100% complementarity (e.g., 75%-100%, 80%-10%, 85%-100%, 90%-100%, 95%-100% complementarity) with one another. In some cases, the stretches of nucleotides that hybridize to one another to form the dsRNA duplex have 85%-100% complementarity (e.g., 90%-100%, 95%-100% complementarity) with one another. In some cases, the stretches of nucleotides that hybridize to one another to form the dsRNA duplex have 70%-95% complementarity (e.g., 75%-95%, 80%-95%, 85%-95%, 90%-95% complementarity) with one another. In other words, in some cases, the dsRNA duplex includes two stretches of nucleotides that have 70%-100% complementarity (e.g., 75%-100%, 80%-10%, 85%-100%, 90%-100%, 95%-100% complementarity) with one another. In some cases, the dsRNA duplex includes two stretches of nucleotides that have 85%-100% complementarity (e.g., 90%-100%, 95%-100% complementarity) with one another. In some cases, the dsRNA duplex includes two stretches of nucleotides that have 70%-95% complementarity (e.g., 75%-95%, 80%-95%, 85%-95%, 90%-95% complementarity) with one another.

[00723]In various embodiments, the scaffold region of a guide RNA can also include one or more (1, 2, 3, 4, 5, etc.) mutations relative to a naturally occurring scaffold region. For example, in some cases a base pair can be maintained while the nucleotides contributing to the base pair from each segment can be different. In some cases, the duplex region of a subject guide RNA includes more paired bases,

less paired bases, a smaller bulge, a larger bulge, fewer bulges, more bulges, or any convenient combination thereof, as compared to a naturally occurring duplex region (of a naturally occurring guide RNA).

[00724]Examples of various guide RNAs can be found in the art, and in some cases variations similar to those introduced into Cas9 guide RNAs can also be introduced into guide RNAs of the present disclosure (e.g., mutations to the dsRNA duplex region, extension of the 5' or 3' end for added stability for to provide for interaction with another protein, and the like). For example, see Jinek et al., Science. 2012 Aug 17;337(6096):816-21; Chylinski et al., RNA Biol. 2013 May;10(5):726- 37; Ma et al., Biomed Res Int. 2013;2013:270805; Hou et al., Proc Natl Acad Sci U S A. 2013 Sep 24;110(39):15644-9; Jinek et al., Elife. 2013;2:e00471; Pattanayak et al., Nat Biotechnol. 2013 Sep;31(9):839-43; Qi et al, Cell. 2013 Feb 28; 152(5): 1173-83; Wang et al., Cell. 2013 May 9;153(4):910-8; Auer et al., Genome Res. 2013 Oct 31; Chen et al., Nucleic Acids Res. 2013 Nov 1;41(20):el9; Cheng et al., Cell Res. 2013 Oct;23(10):1163-71; Cho et al., Genetics. 2013 Nov;195(3):1177-80; DiCarlo et al., Nucleic Acids Res. 2013 Apr;41(7):4336-43; Dickinson et al., Nat Methods. 2013 Oct;10(10):1028-34; Ebina et al., Sci Rep. 2013;3:2510; Fujii et. al, Nucleic Acids Res. 2013 Nov l;41(20):el87; Hu et al., Cell Res. 2013 Nov;23(ll):1322-5; Jiang et al., Nucleic Acids Res. 2013 Nov l;41(20):el88; Larson et al., Nat Protoc. 2013 Nov;8(l l):2180-96; Mali et. at., Nat Methods. 2013 Oct;10(10):957-63; Nakayama et al., Genesis. 2013 Dec;51(12):835-43; Ran et al., Nat Protoc. 2013 Nov;8(l l):2281-308; Ran et al., Cell. 2013 Sep 12;154(6):1380-9; Upadhyay et al., G3 (Bethesda). 2013 Dec 9;3(12):2233-8; Walsh et al., Proc Natl Acad Sci U S A. 2013 Sep 24;110(39):15514-5; Xie et al., Mol Plant. 2013 Oct 9; Yang et al., Cell. 2013 Sep 12;154(6):1370-9; Briner et al., Mol Cell. 2014 Oct 23;56(2):333-9; and U.S. patents and patent applications: 8,906,616; 8,895,308; 8,889,418; 8,889,356; 8,871,445; 8,865,406; 8,795,965; 8,771,945; 8,697,359; 20140068797; 20140170753; 20140179006; 20140179770; 20140186843; 20140186919; 20140186958; 20140189896; 20140227787; 20140234972; 20140242664; 20140242699; 20140242700; 20140242702; 20140248702; 20140256046; 20140273037; 20140273226; 20140273230; 20140273231; 20140273232; 20140273233; 20140273234; 20140273235; 20140287938; 20140295556; 20140295557; 20140298547; 20140304853; 20140309487; 20140310828; 20140310830; 20140315985; 20140335063; 20140335620; 20140342456; 20140342457; 20140342458; 20140349400; 20140349405; 20140356867; 20140356956; 20140356958; 20140356959; 20140357523; 20140357530; 20140364333; and 20140377868; all of which are hereby incorporated by reference in their entirety.

Guide RNA modifications

[00725]In one embodiment, the guide RNAs (including pegRNAs) contemplated herein comprise non-naturally occurring nucleic acids and/or non-naturally occurring nucleotides and/or nucleotide analogs, and/or chemical modifications. Non-naturally occurring nucleic acids can include, for example, mixtures of naturally and non-naturally occurring nucleotides. Non-naturally occurring

nucleotides and/or nucleotide analogs may be modified at the ribose, phosphate, and/or base moiety. In an embodiment of the disclosure, a guide RNA (including pegRNA) component nucleic acid comprises ribonucleotides and non-ribonucleotides. In one such embodiment, a guide RNA (including pegRNA) component comprises one or more ribonucleotides and one or more deoxyribonucleotides. In an embodiment of the disclosure, the guide RNA (including pegRNA) component comprises one or more non-naturally occurring nucleotide or nucleotide analog such as a nucleotide with phosphorothioate linkage, a locked nucleic acid (LNA) nucleotides comprising a methylene bridge between the 2' and 4' carbons of the ribose ring, or bridged nucleic acids (BNA).

[00726]Other examples of modified nucleotides include 2'-O-methyl analogs, 2'-deoxy analogs, or 2'-fluoro analogs. Further examples of modified bases include, but are not limited to, 2-aminopurine, 5-bromo-uridine, pseudouridine, inosine, 7-methylguanosine. Examples of coRNA chemical modifications include, without limitation, incorporation of 2'-O-methyl (M), 2'-O-methyl 3 'phosphorothioate (MS), S-constrained ethyl(cEt), or 2'-O-methyl 3 'thioPACE (MSP) at one or more terminal nucleotides. Such chemically modified oRNA components can comprise increased stability and increased activity as compared to unmodified oRNA components, though on-target vs. off-target specificity is not predictable. (See, Hendel, 2015, Nat Biotechnol. 33(9):985-9, doi: 10.1038/nbt.3290, published online 29 June 2015 Ragdarm et al., 0215, PNAS, E7110-E7111; Allerson et al., J. Med. Chem. 2005, 48:901-904; Bramsen et al., Front. Genet., 2012, 3:154; Deng et al., PNAS, 2015, 112: 11870-11875; Sharma et al., MedChemComm., 2014, 5: 1454-1471; Hendel et al., Nat. Biotechnol. (2015) 33(9): 985-989; Li et al., Nature Biomedical Engineering, 2017, 1, 0066 D01: 10.1038/s41551-017-0066). In one embodiment, the 5' and/or 3' end of a guide RNA (including pegRNA) component is modified by a variety of functional moieties including fluorescent dyes, polyethylene glycol, cholesterol, proteins, or detection tags. (See Kelly et al., 2016, J. Biotech. 233:74-83). In one embodiment, a guide RNA (including pegRNA) component comprises ribonucleotides in a region that binds to a target sequence and one or more deoxyribonucletides and/or nucleotide analogs in a region that binds to a nucleic acid programmable DNA binding protein (e.g., Cas9 nickase).

[00727]In an embodiment, deoxyribonucleotides and/or nucleotide analogs are incorporated in engineered guide RNA (including pegRNA) component structures. In one embodiment, 3-5 nucleotides at either the 3' or the 5' end of a guide RNA (including pegRNA) component is chemically modified. In one embodiment, only minor modifications are introduced in the seed region, such as 2'-F modifications. In one embodiment, 2'-F modification is introduced at the 3' end of a guide RNA (including pegRNA) component. In one embodiment, three to five nucleotides at the 5' and/or the 3' end of the reRNA component are chemically modified with 2' -O-methyl (M), 2'-O-methyl 3' phosphorothioate (MS), S-constrained ethyl(cEt), or 2' -O-methyl 3' thioPACE (MSP). Such modification can enhance genome editing efficiency (see Hendel et al., Nat. Biotechnol. (2015) 33(9): 985-989). In one embodiment, all of the phosphodiester bonds of a guide RNA (including pegRNA) component are substituted with phosphorothioates (PS) for enhancing levels of gene

disruption. In one embodiment, more than five nucleotides at the 5' and/or the 3' end of the guide RNA (including pegRNA) component are chemically modified with 2'-0-Me, 2'-F or S-constrained ethyl(cEt). Such chemically modified guide RNA (including pegRNA) component can mediate enhanced levels of gene disruption (see Ragdarm et al., 0215, PNAS, E7110-E7111). In an embodiment of the disclosure, a guide RNA (including pegRNA) component is modified to comprise a chemical moiety at its 3' and/or 5' end. Such moieties include, but are not limited to amine, azide, alkyne, thio, dibenzocyclooctyne (DBCO), or Rhodamine. In certain embodiment, the chemical moiety is conjugated to the guide RNA (including pegRNA) component by a linker, such as an alkyl chain. In one embodiment, the chemical moiety of the modified nucleic acid component can be used to attach the guide RNA (including pegRNA) component to another molecule, such as DNA, RNA, protein, or nanoparticles. Such chemically modified guide RNA (including pegRNA) component can be used to identify or enrich cells generically edited by a gene editing system described herein.

[00728]Other guide RNA (including pegRNA) modifications are described in Kim, D.Y., Lee, J.M., Moon, S.B. *et al.* Efficient CRISPR editing with a hypercompact Cas12f1 and engineered guide RNAs delivered by adeno-associated virus. *Nat Biotechnol* **40,** 94–102 (2022).

[0016] Accordingly, in various aspects of the disclosure, the guide RNA (including pegRNA) are modified in one or more locations within the molecule. MS1, an internal penta(uridinylate) (UUUUU) sequence in the tracrRNA; MS2, the 3′ terminus of the crRNA; MS3, the 'stem 1' region of the tracrRNA; MS4, the tracrRNA–crRNA complementary region; and MS5, the 'stem 2' region of the tracrRNA.

[00729]Various aspects of the disclosure provide methods and compositions for improved guide RNA (including pegRNA) stability via chemical modifications. Braasch, D. A., Jensen, S., Liu, Y., Kaur, K., Arar, K., White, M. A., et al. (2003). RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry* 42, 7967–7975. doi: 10.1021/bi0343774. Chiu, Y. L., and Rana, T. M. (2003). siRNA function in RNAi: a chemical modification analysis. *RNA* 9, 1034–1048. doi: 10.1261/rna.5103703. Behlke, M. A. (2008). Chemical modification of siRNAs for *in vivo* use. *Oligonucleotides*18, 305–319. doi: 10.1089/oli.2008.0164. Bennett, C. F., and Swayze, E. E. (2010). RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu. Rev. Pharmacol. Toxicol.* 50, 259–293. doi: 10.1146/annurev.pharmtox.010909.105654. Deleavey, G. F., and Damha, M. J. (2012). Designing chemically modified oligonucleotides for targeted gene silencing. *Chem. Biol.* 19, 937–954. doi: 10.1016/j.chembiol.2012.07.011. Lennox, K. A., and Behlke, M. A. (2020). Chemical modifications in RNA interference and CRISPR/Cas genome editing reagents. *Methods Mol. Biol.* 2115, 23–55. doi: 10.1007/978-1-0716-0290-4_2.

[00730]For instance, Hendel et al. improved guide RNA stability by chemically modifying gRNA ends to reduce degradation by exonucleases, RNA nuclease. Hendel, A., Bak, R. O., Clark, J. T., Kennedy, A. B., Ryan, D. E., Roy, S., et al. (2015a). Chemically modified guide RNAs enhance

CRISPR-Cas genome editing in human primary cells. *Nat. Biotechnol.* 33, 985–989. doi: 10.1038/nbt.3290. Chemical modifications of gRNAs may enable more efficient and safer gene-editing in primary cells suitable for clinical applications.

[00731]A review of types of chemical modifications are provided in Allen, Daniel et al. "Using Synthetically Engineered Guide RNAs to Enhance CRISPR Genome Editing Systems in Mammalian Cells." *Frontiers in genome editing* vol. 2 617910. 28 Jan. 2021, doi:10.3389/fgeed.2020.617910.

[00732]Accordingly, in various embodiments of the present disclosure, the genome editing system comprising a guide RNA (including pegRNA) and further comprises one or more chemical modifications selected from, but not limited to the modifications in the above table.

[00733]In exemplary embodiments, chemical modifications to the guide RNA (including pegRNA) include modifications on the ribose rings and phosphate backbone of guide RNA (including pegRNA) and modifications at the 2′OH include 2′-O-Me, 2′-F, and 2′F-ANA. More extensive ribose modifications include 2′F-4′-Cα-OMe and 2′,4′-di-Cα-OMe combine modification at both the 2′ and 4′ carbons. Phosphodiester modifications include sulfide-based Phosphorothioate (PS) or acetate-based phosphonoacetate alterations. Combinations of the ribose and phosphodiester modifications have given way to formulations such as 2′-O-methyl 3′phosphorothioate (MS), or 2′-O-methyl-3′-thioPACE (MSP), and 2′-O-methyl-3′-phosphonoacetate (MP) RNAs. Locked and unlocked nucleotides such as locked nucleic acid (LNA), bridged nucleic acids (BNA), S-constrained ethyl (cEt), and unlocked nucleic acid (UNA) are examples of sterically hindered nucleotide modifications. Modifications to make a phosphodiester bond between the 2′ and 5′ carbons (2′,5′-RNA) of adjacent RNAs as well as a butane 4-carbon chain link between adjacent RNAs have been described.

E.Additional components and aspects

[00734]In addition to the above LNPs and cargoes, including (A) nucleic acid payloads, (B) linear mRNA payloads, circular mRNA payloads, and (D) gene editing systems, the present disclosure provides additional optional LNP cargo components and tools that may be included as appropriate in the LNP gene editing systems described herein. The following optional components and tools may be combined in any combination as appropriate depending upon the particular gene editing system being delivered by the herein disclosed LNP-based gene editing systems.

F.Gene editing systems and components for treatment of hemoglobinopathies

[00735]In certain embodiments, the present disclosure provides compositions and methods of delivering gene editing tools. Genome editing tools encompass a diverse set of technologies that can make many types of genomic alterations in various contexts. These technologies have evolved over the last couple of decades to provide a range of user-programmable editing tools that include ZFN (zinc finger) nuclease editing systems, meganuclease editing systems, and TALENS (transcription activator-like effector nucleases). The past decade has seen an explosive growth in a new generation of genome editing systems based on components from bacterial immune pathways, including CRISPR

(clustered regularly interspaced short palindromic repeats) and the associated CRISPR-associated proteins (e.g., CRISPR-Cas9) (Jinek *et al.*, "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity," *Science*, Vol. 337 (6096), pp. 816-821), meganuclease editors (Boissel et al., "megaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering," *Nucleic Acids Research* 42: pp. 2591-2601) and bacterial retron systems (Schubert *et al.*, "High-throughput functional variant screens via in vivo production of single-stranded DNA," *PNAS*, April 27, 2021, Vol. 118(18), pp. 1-10). In particular, CRISPR-Cas9 has been derivatized in numerous ways to expand upon its guide RNA-based programmable double-strand cutting activity to form systems ranging from finding alternative CRISPR Cas nuclease enzymes having different PAM requirements and cutting properties (e.g., Cas12a, Cas12f, Cas13a, and Cas13b) *to* base editing (Komor *et al.*, "Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage," *Nature*, May 19, 2016, 533 (7603); pp. 420-424 [cytosine base editors or CBEs] and Gaudelli *et al.*, "Programmable base editing of A-T to G-C in genomic DNA without DNA cleavage," *Nature*, Vol. 551, pp. 464-471 [adenine base editors or ABEs]) *to* prime editing (Anzalone *et al.*, "Search-and-replace genome editing without double-strand breaks or donor DNA," *Nature*, Dec 2019, 576 (7789): pp. 149-157) *to* twin prime editing (Anzalone *et al.*, "Programmable deletion, replacement, integration and inversion of large DNA sequences with twin prime editing," *Nature Biotechnology*, Dec 9, 2021, vol. 40, pp. 731-740) *to* epigenetic editing (Kungulovski and Jeltsch, "Epigenome Editing: State of the Art, Concepts, and Perspective," *Trends in Genetics,* Vol.32, 206, pp. 101-113) *to* CRISPR-directed integrase editing (Yarnell *et al.*, "Drag-and-drop genome insertion of large sequences without double-stranded DNA cleavage using CRISPR-directed integrases," *Nature Biotechnology*, Nov 24, 2022, ("PASTE")), among others. Such editing systems may be implemented for treating diseases, such as hemoglobinopathies.

[00736]While the expansion of genome editing tools has exploded, the development of safe and effective gene editing tool delivery systems has lagged behind. There remain numerous challenges associated with the delivery of gene editing tools—including, but not limited to, CRISPR-Cas9 and alternative Cas nuclease editors, retron editors, base editors, prime editors, twin prime editors, epigenetic editors, and integrase editors—to achieve safe and effective therapeutic application of such tools in cells and patients for treating disease and/or otherwise modifying the nucleotide sequence of a target nucleic acid molecule (e.g., a gene or genome). That said, the use of lipid nanoparticles (LNPs) has emerged as a leading delivery option for the safe, effective, and targeted delivery of gene editing tools to target tissues and cells. However, there remains a need for improved LNPs, including better performing ionizable lipids, that will enhance the targeted delivery of LNP-based gene editing tools. Preferably, such improved LNPs would protect payloads from degradation and clearance while achieving targeted delivery, be suitable for systemic or local delivery, and provide delivery of a wide variety of gene editing tools, such as those mentioned above. In addition, such improved LNP-based therapeutics should exhibit low toxicity and provide an adequate therapeutic index, such that patient

treatment at an effective dose of the LNP minimizes risk to the patient while maximizing therapeutic benefit. The present invention provides these and related advantages.

[00737]In one embodiment, the present disclosure provides pharmaceutical compositions comprising lipid nanoparticles comprising one or more gene editing systems, or components thereof, capable of editing, modifying, or altering a polynucleotide sequence that results in treatment of a hemoglobinopathy.

[00738]In certain embodiments, the gene editing system is a system that corrects the sickle cell mutation by repairing the β-globin gene. In general, strategies of repairing the β-globin gene in order to address a β-hemoglobinopathy are illustrated in the central panel of FIG. 1. In certain embodiments, the gene editing system corrects the sickle cell mutation through homology directed repair. In certain embodiments, the gene editing system corrects the sickle cell mutation through non-homologous end-joining (NHEJ), by targeting aberrant splicing sites in introns I and II, resulting in restoration of functional β-globin transcripts. In certain embodiments, the gene editing system corrects the sickle cell mutation through a single base pair gene correction. In certain embodiments, the gene editing system is a prime editing or base editing system capable of executing a single base pair edit. In certain embodiments, the gene editing system executes a single base pair correction, converting the aberrant thymine to the normal adenine, thereby converting the aberrant valine amino acid in the mutant hemoglobin to the normal glutamic acid. In certain embodiments, the gene editing system executes a single base pair correction, converting the aberrant thymine to cytosine, thereby converting the aberrant valine amino acid in the mutant hemoglobin to alanine, which results in a functional, nonpathogenic hemoglobin variant (HbG Makassar).

[00739]In certain embodiments, the gene editing system is a system that reactivates γ-globin production. In general, strategies of reactivating γ-globin production in order to address a β-hemoglobinopathy are illustrated in the right panel of FIG. 1. One method of treating SCD and beta-Thalassemia is to reactivate production of functional fetal hemoglobin (γ-globin) to compensate for the pathogenic mutant β-globin. In certain embodiments, the gene editing system reactivates γ-globin production by deleting inhibitory sequences, or portions thereof. In certain embodiments, the gene editing system eliminates an HbF inhibitory sequence, or a portion thereof, thereby reactivating γ-globin production. In certain embodiments, the gene editing system juxtaposes the γ-globin promoters to remote enhancer regions. In certain embodiments, the gene editing system targets the HBG1 and/or HBG2 promoters, resulting in disruption of binding sites for γ-globin transcriptional repressors, leading to HbF expression. In certain embodiments, the gene editing system comprises base editors that reproduce HPFH mutations that create binding sites for transcriptional activators (such as KLF1) or disrupt repressor binding sites (such as BCL11A). In certain embodiments, the gene editing system disrupts BCL11A binding sites. In certain embodiments, the gene editing system disrupts the GATA1 motif in the erythroid-specific intronic enhancer of BCL11A. Disruption of

BCL11A can be achieved using nucleases or base editors, and BCL11A knock-down results in reactivation of γ-globin expression.

[00740]In certain embodiments, an LNP of the present disclosure comprises a gene editing system, or a polynucleotide encoding the same, disclosed in any one of the documents cited in Table X1. In certain embodiments, an LNP of the present disclosure comprises a nuclease, or a polynucleotide encoding the same, disclosed in any one of the documents cited in Table X1. In certain embodiments, an LNP of the present disclosure comprises a Cas9 or ortholog thereof, or a polynucleotide encoding the same, disclosed in any one of the documents cited in Table X1. In certain embodiments, an LNP of the present disclosure comprises a Cas12a (formerly known as Cpf1), or Type V protein, or a polynucleotide encoding the same, disclosed in any one of the documents cited in Table X1. In certain embodiments, an LNP of the present disclosure comprises a Cas12a, Type V or Cas9 ortholog protein, or a polynucleotide encoding the same, disclosed in any one of the documents cited in Table X1. In certain embodiments, an LNP of the present disclosure comprises a nickase, or a polynucleotide encoding the same, disclosed in any one of the documents cited in Table X1. In certain embodiments, an LNP of the present disclosure comprises a zinc finger protein, or a polynucleotide encoding the same, disclosed in any one of the documents cited in Table X1. In certain embodiments, an LNP of the present disclosure comprises a TALEN, or a polynucleotide encoding the same, disclosed in any one of the documents cited in Table X1. In certain embodiments, an LNP of the present disclosure comprises a guide RNA, or a portion or segment thereof, disclosed in any one of the documents cited in Table X1. In certain embodiments, an LNP of the present disclosure comprises a spacer sequence disclosed in any one of the documents cited in Table X1.

Table X1. Patent and Patent Application References

US Patents					
11,242,525	11,572,543	11,268,077	11,466,271	9,957,501	10,808,020
11,021,696	10,563,184	11,155,803	10,738,305	11,344,609	10,435,677
11,466,271					
US Patent Application Publications					
US20200339979A1	US20210180091A1	US20200140896A1	US20190010495A1		
US20210254061A1	US20210040506A1	US20230002760A1	US20230018543A1		
US20180273609A1	US20220047637A1	US20190184035A1	US20200255857A1		
US20200399626A1	US20220228142A1	US20180200387A1	US20180030438A1		
US20220401530A1	US20210371858A1	US20190010495A1	US20210180091A1		
US20210008161A1	US20210155927A1				
PCT Publications					
WO2022240787A1	WO2021119040A1	WO2023014839A1	WO2023039440A2		
WO2022120094A2	WO2022155458A1	WO2023019227A1	WO2023019226A1		

WO2022067122A1	WO2023019225A2	WO2021163587A1	WO2024020346A1

[00741]Each reference of Table X1 is incorporated by reference herein in its entirety.

[00742]In certain embodiments, an LNP of the present disclosure comprises a nuclease, or a polynucleotide encoding said nuclease, described or disclosed in PCT Application Publication WO2024020346A1, filed July 17, 2023, which is incorporated by reference herein in its entirety. In certain embodiments, the LNP further comprises a guide RNA that enables an edit for treating sickle cell anemia or beta-thalassemia.

[00743]In certain embodiments, an LNP of the present disclosure comprises (a) a base editor, or a polynucleotide encoding the base editor, wherein the base editor comprises a polynucleotide-programmable nucleotide- binding domain and a deaminase domain; (b) a guide polynucleotide, wherein the guide polynucleotide targets the base editor to a target nucleotide sequence. In certain embodiments, the target nucleotide sequence is a protein coding region responsible for sickle cell anemia or beta-thalassemia. In certain embodiments, the base editor and the guide RNA are any of those described and disclosed in US Application Publication US20210371858A1, which is incorporated by reference herein, in its entirety.

[00744]In certain embodiments, an LNP of the present disclosure comprises a system for changing a cytosine to a thymine in the genome of a cell, comprising a first fusion protein and a second fusion protein, or first and second expression constructs for expressing the first and second fusion proteins, respectively, wherein a) the first fusion protein comprises: i) a first zinc finger protein (ZFP) domain that binds to a first sequence in a target genomic region in the cell, and ii) a first portion of a cytidine deaminase polypeptide, wherein the cytidine deaminase is a toxin-derived deaminase (TDD) comprising an amino acid sequence at least 90% identical to SEQ ID NO: 49, 81, 92, 95, 98, 101, 104, 107, 134, 143, 152, 157, 162, 167, 172, 177, 184, 189, 194, 199, 204, 209, 214, or 219 of PCT Publication WO2022067122A1; b) the second fusion protein comprises: i) a second ZFP domain that binds to a second sequence in the target genomic region, and ii) a second portion of the cytidine deaminase polypeptide; c) the first and second portions lack cytidine deaminase activity on their own; and d) binding of the first fusion protein and the second fusion protein to the target genomic region results in dimerization of the first and second portions, wherein the dimerized portions form an active cytidine deaminase capable of changing a cytosine to a thymine in the target genomic region, optionally wherein the cell is a eukaryotic cell, optionally wherein the eukaryotic cell is a mammalian cell or a plant cell, further optionally wherein the mammalian cell is a human cell.

[00745]In certain embodiments, an LNP of the present disclosure comprises a pair of zinc finger nucleases (ZFNs), the pair comprising the zinc finger proteins (ZFPs) designated 47773 and 47817, wherein 47773 binds to a target site within SEQ ID NO:23 and 47817 binds to a SEQ ID NO:24 of an endogenous human beta-hemoglobin (Hbb) gene comprising a sickle cell mutation, wherein the pair of ZFNs makes a genomic modification in the mutant endogenous Hbb gene and the genomic

modification comprises insertion of a donor sequence such that the sickle cell mutation is corrected to wild-type IIbb, wherein the referenced SEQ ID NOs are those disclosed in US Patent 10,435,677.

[00746]In certain embodiments, an LNP of the present disclosure comprises a guide RNA and a fusion protein comprising a polynucleotide programmable DNA binding domain and an adenosine deaminase domain, wherein the adenosine deaminase domain comprises an arginine (R) or a threonine (T) at amino acid position 147 of the amino acid sequence, wherein the adenosine deaminase domain has at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 2 of US Patent 11,344,609, and wherein said guide RNA targets said fusion protein to effect a deamination of a nucleobase of the HBG1/2 promoter in the cell. In certain embodiments, an LNP of the present disclosure comprises a guide RNA and a fusion protein comprising a polynucleotide programmable DNA binding domain and an adenosine deaminase domain, comprising a serine (S) at amino acid position 82, wherein the adenosine deaminase domain has at least 85% sequence identity to the amino acid sequence of SEQ ID NO: 2 of US Patent 11,142,760. In certain embodiments, the polynucleotide programmable DNA binding domain comprises a Cas9 domain. In certain embodiments, the Cas9 domain comprises a dead Cas9 (dCas9) or a nickase Cas9 (nCas9). In certain embodiments, the Cas9 domain is capable of programmable DNA binding and is selected from the group consisting of a Streptococcus pyogenes Cas9 (SpCas9), Staphylococcus aureus Cas9 (SaCas9), and a Streptococcus thermophilus 1 Cas9 (St1Cas9). In certain embodiments, the Cas9 domain comprises an amino acid sequence having at least 85% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 1 of US Patent 11,142,760. In certain embodiments, the fusion protein is selected from ABE8.1-m, ABE8.2-m, ABE8.8-m, ABE8.9-m, ABE8.10-m, ABE8.11-m, ABE8.12-m, ABE8.13-m, ABE8.15-m, ABE8.16-m, ABE8.20-m, ABE.21-m, ABE8.24-m, ABE8.1-d, ABE8.2-d, ABE8.8-d, ABE8.9-d, ABE8.10-d, ABE8.11-d, ABE8.12-d, ABE8.13-d, ABE8.15-d, ABE8. 16-d, ABE8.20-d, ABE.21-d, and ABE8.24-d. In certain embodiments, an LNP of the present disclosure comprises a guide RNA and a base editor comprising a polynucleotide programmable DNA binding domain and a deaminase domain, or a polynucleotide encoding the base editor, wherein the guide RNA is capable of targeting the polynucleotide programmable DNA binding domain to induce a 10 nucleobase change in a target hemoglobin (HBB) gene or in the promoter region of HBG1/2. In certain embodiments, the base editor and the guide RNA are any of those described and disclosed in PCT Publication WO2021163587A1, which is incorporated by reference herein, in its entirety.

[00747]In certain embodiments, an LNP of the present disclosure comprises a base editor in complex with one or more guide polynucleotides, wherein the base editor comprises a polynucleotide programmable DNA binding domain and an adenosine deaminase domain, and wherein the one or more guide polynucleotides target the base editor to effect an A•T to G•C alteration of a single nucleotide polymorphism (SNP) associated with sickle cell disease. In certain embodiments, the polynucleotide programmable DNA binding domain is a modified Staphylococcus aureus Cas9 (SaCas9), Streptococcus thermophilus 1 Cas9 (St1Cas9), a modified Streptococcus pyogenes Cas9

(SpCas9), or variants thereof. In certain embodiments, the adenosine deaminase is a TadA deaminase. In certain embodiments, the one or more guide RNAs comprises a CRISPR RNA (crRNA) and a trans-encoded small RNA (tracrRNA), wherein the crRNA comprises a nucleic acid sequence complementary to an HBB nucleic acid sequence comprising the SNP associated with sickle cell disease; or wherein the base editor is in complex with a single guide RNA (sgRNA) comprising a nucleic acid sequence complementary to an HBB nucleic acid sequence comprising the SNP associated with sickle cell disease. In certain embodiments, the base editor and the guide RNA are any of those described and disclosed in US Application Publication US20220401530A1, which is incorporated by reference herein, in its entirety.

[00748]In certain embodiments, an LNP of the present disclosure comprises a system for modifying DNA comprising: (a) a polypeptide or a nucleic acid encoding the polypeptide, wherein the polypeptide comprises (i) a reverse transcriptase (RT) domain, (ii) a DNA-binding domain (DBD); and (iii) an endonuclease domain, e.g., a nickase domain; and (b) a template RNA (or DNA encoding the template RNA) comprising (e.g., from 5' to 3') (i) optionally a sequence that binds a target site (e.g., a second strand of a site in a target genome), (ii) optionally a sequence that binds the polypeptide, (iii) a heterologous object sequence, and (iv) a 3' target homology domain, wherein the RT domain comprises a sequence of Table 1 or 3 or a sequence of a reverse transcriptase domain of Table 2, or a sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity thereto, or wherein the RT domain further comprises a number of substitutions relative to the natural sequence, e.g., at least 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 substitutions (referenced tables being those in PCT Publication WO2021178720A2, which is incorporated by reference herein in its entirety). In certain embodiments, an LNP of the present disclosure comprises a system for modifying DNA comprising: (a) a polypeptide or a nucleic acid encoding the polypeptide, wherein the polypeptide comprises (i) a reverse transcriptase (RT) domain, (ii) a DNA-binding domain (DBD); and (iii) an endonuclease domain, e.g., a nickase domain; and (b) a template RNA (etRNA) (or DNA encoding the template RNA) comprising (e.g., from 5' to 3') (i) optionally a sequence that binds a target site (e.g., a second strand of a site in a target genome), (ii) optionally a sequence that binds the polypeptide, (iii) a heterologous object sequence, and (iv) a 3' target homology domain, wherein the system is capable of producing an insertion into the target site of at least 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 nucleotides or wherein the heterologous object sequence is at least 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 120, 140, 160, 180, 200, 500, or 1,000 nts in length. In certain embodiments, the LNP comprises one or more of the systems for modifying DNA disclosed and described in PCT Publication WO2021178720A2, which is incorporated by reference herein in its entirety.

[00749]In certain embodiments, an LNP of the present disclosure comprises a system for correcting an E6V mutation in human beta-globin (HBB) in a cell or population of cells, the system comprising: (a) a Cas9 endonuclease, an mRNA encoding the Cas9 endonuclease, or a recombinant expression

vector comprising a nucleotide sequence encoding the Cas9 endonuclease; (b) a single guide RNA (sgRNA) comprising a spacer sequence corresponding to a target sequence adjacent to a PAM, the target sequence comprising a target site within intron 1 of HBB; and (c) a recombinant vector comprising a nucleic acid for correcting the E6V mutation, the nucleic acid comprising a nucleotide sequence homologous with a region of the HBB gene encoding the E6V mutation, wherein the nucleotide sequence comprises a codon encoding E6. In certain embodiments, the target sequence comprises a nucleotide sequence selected from SEQ ID NO: 1 or SEQ ID NO: 49 of US Application Publication US20220228142A1. In certain embodiments, the codon encoding E6 is selected from GAA and GAG, and wherein the nucleotide sequence of (c) comprises one or more silent mutations relative to the HBB gene.

[00750]In certain embodiments, the nucleotide sequence of (c) comprises a nucleotide sequence having at least 90% sequence identity to a nucleotide sequence selected from SEQ ID NO: 6 or SEQ ID NO: 19 of US Application Publication US20220228142A1. In certain embodiments, the nucleotide sequence of (c) comprises a mutation to delete the PAM. In certain embodiments, the Cas9 endonuclease is a S. pyogenes Cas9 (SpCas9) endonuclease. In certain embodiments, the SpCas9 endonuclease is a high fidelity SpCas9 endonuclease. In certain embodiments, the system comprises the Cas9 endonuclease as a polypeptide, and wherein the system comprises a ribonucleoprotein complex of the sgRNA and the Cas9 endonuclease. In certain embodiments, the system comprises an mRNA encoding the Cas9 endonuclease.

[00751]In certain embodiments, an LNP of the present disclosure comprises:

(a) a S. pyogenes Cas9 endonuclease, an mRNA encoding the S. pyogenes Cas9 endonuclease, or a recombinant expression vector comprising a nucleotide sequence encoding the S. pyogenes Cas9 endonuclease, wherein the S. pyogenes Cas9 endonuclease is a high fidelity Cas9;

(b) a single guide RNA (sgRNA) targeting a target site in an HBB gene, the sgRNA comprising a spacer sequence corresponding to a target sequence consisting of SEQ ID NO: 15 of US Patent Application Publication US20210008161A1; and (c) a recombinant vector comprising a nucleic acid, the nucleic acid comprising from 5' to 3' (i) a nucleotide sequence homologous with a region located upstream of the target region in the HBB gene, (ii) a nucleotide sequence homologous with a region of the HBB gene comprising the target region, and (iii) a nucleotide sequence homologous with a region located downstream of the target region in the HBB gene; wherein the contents of the LNP are capable of executing a double-strand break (DSB) at the target site in the HBB gene and the nucleic acid is exchanged with a homologous nucleotide sequence of the HBB gene. In certain embodiments, the nucleotide sequence of (c)(ii) corrects an E6V mutation in the HBB gene and is homologous with a region of the HBB gene encoding the E6V mutation. In certain embodiments, the nucleotide sequence of (c)(ii) comprises the sequence of SEQ ID NO: 102 of US Patent Application Publication US20210008161A1.

[00752]In certain embodiments, an LNP of the present disclosure comprises a gene editing system disclosed in any one of PCT Publications WO2023019227A1, WO2023019226A1, and WO2023019225A2 which are incorporated by reference herein in their entirety. In certain embodiments the gene editing system is capable of executing a modification in a cell selected from (i) increased expression of one or more tolerogenic factors, (ii) increased expression of CD46, (iii) increased expression of CD59, and (iv) reduced expression of one or more MHC class I molecules and/or one or more MHC class II molecules, wherein the increased expression of (i), (ii), and (iii) and the reduced expression of (iv) is relative to a cell of the same cell type that does not comprise modifications. In certain embodiments, the gene editing system is capable of executing a modification in a cell selected from (a) reduced expression of a MHC class I chain-related protein A (MICA) and/ or a MHC class I chain-related protein B (MICB); (b) increased expression of one or more tolerogenic factors; and (c) reduced expression of one or more major histocompatibility complex class I molecules (MHC class I molecules) and/ or one or more major histocompatibility complex class II molecules (MHC class II molecules), wherein the change in expression is relative to a cell of the same cell type that does not comprise modifications. In certain embodiments the gene editing system is capable of executing a modification in a cell selected from (i) increased expression of one or more tolerogenic factor, (ii) reduced expression of CD 142, and (iii) reduced expression of one or more MHC class I molecules and/or one or more MHC class II molecules, wherein the increased expression of (i) and the reduced expression of (ii) and (iii) is relative to a cell of the same cell type that does not comprise modifications.

[00753]In certain embodiments, an LNP of the present disclosure comprises a template RNA comprising, from 5' to 3': (i) a gRNA spacer that is complementary to a first portion of the human HBB gene, wherein the gRNA spacer has a sequence comprising the core nucleotides of a gRNA spacer sequence of Table 1, and optionally comprises one or more consecutive nucleotides starting with the 3' end of the flanking nucleotides of the gRNA spacer, or wherein the gRNA spacer has a sequence of a spacer chosen from Table A, Table AA, Table B, Table Bl, Tables 5A-5D, Table X4, or Table X4A (referenced tables being those in PCT Publication WO2023039440A2, which is incorporated by reference herein in its entirety); (ii) a gRNA scaffold that binds a gene modifying polypeptide (e.g., binds the Cas domain of the gene modifying polypeptide), (iii) a heterologous object sequence comprising a mutation region to introduce a mutation into (e.g., to correct a mutation in) a second portion of the human HBB gene (wherein optionally the heterologous object sequence comprises, from 5' to 3', a post-edit homology region, a mutation region, and a pre-edit homology region), and (iv) a primer binding site (PBS) sequence comprising at least 5, 6, 7, or 8 bases with 100% identity to a third portion of the human HBB gene. In certain embodiments, an LNP of the present disclosure comprises a template RNA comprising, from 5' to 3': (i) a gRNA spacer that is complementary to a first portion of the human HBB gene, (ii) a gRNA scaffold that binds a gene modifying polypeptide (e.g., binds the Cas domain of the gene modifying polypeptide), (iii) a

heterologous object sequence comprising a mutation region to introduce a mutation into a second portion of the human HBB gene, wherein the heterologous object sequence comprises the core nucleotides of an RT template sequence of Table 3, and optionally comprises one or more consecutive nucleotides starting with the 3' end of the flanking nucleotides of the RT template sequence, or wherein the heterologous object sequence comprises an RT template sequence of Table A, Table AA, Table B, Table Bl, Tables 5A-5D, Table X4, or Table X4A (referenced tables being those in PCT Publication WO2023039440A2, which is incorporated by reference herein in its entirety); and (iv) a PBS sequence comprising at least 5, 6, 7, or 8 bases of 100% identity to a third portion of the human HBB gene.

[00754]In certain embodiments, an LNP of the present disclosure comprises a guide RNA (gRNA) comprises, from 5′ to 3′, [targeting domain]-(SEQ ID NO: 195 or 231), wherein the targeting domain comprises SEQ ID NO: 67, wherein the referred to SEQ ID NOs are those described in US Patent 11,466,271. In certain embodiments, said SEQ ID NO: 195 or 231 is disposed immediately 3′ to said targeting domain. In certain embodiments, the gRNA comprises at the 3′ end, 1, 2, 3, 4, 5, 6 or 7 uracil (U) nucleotides. In certain embodiments, the LNP further comprises a Cas9 or a polynucleotide encoding the same. In certain embodiments, the LNP further comprises a template nucleic acid. In certain embodiments, the Cas9 comprises a sequence of SEQ ID NO: 205 of US Patent 11,466,271, or a sequence with at least 95% homology thereto. In certain embodiments, the Cas9 comprises a sequence selected from SEQ ID NOs: 233-244 of US Patent 11,466,271. In certain embodiments, the gRNA comprises or consists of the sequence of SEQ ID NOs: 174-176 of US Patent 11,466,271. In certain embodiments, an LNP of the present disclosure comprises any gRNA, nuclease and/or template nucleic acid (or a polynucleotide encoding any of the aforementioned) disclosed in US Patent 11,466,271, which is incorporated by reference herein, in its entirety.

[00755]In certain embodiments, an LNP of the present disclosure comprises a gRNA comprising a tracr and crRNA, wherein the crRNA comprises a targeting domain that is complementary with a target sequence of a BCL11A gene (e.g., a human BCL11a gene), a BCL11a enhancer (e.g., a human BCL11a enhancer), or a HFPH region (e.g., a human HPFH region). In certain embodiments, the target sequence is of the BCL11A gene, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 1 to SEQ ID NO: 85 or SEQ ID NO: 400 to SEQ ID NO: 1231 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is of a BCL11a enhancer, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 1232 to SEQ ID NO: 1499 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is of a BCL11a enhancer, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 182 to SEQ ID NO: 277 or SEQ ID NO: 334 to SEQ ID NO: 341 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 341, SEQ ID NO: 246, SEQ ID NO: 248, SEQ ID NO: 247, SEQ ID NO: 245, SEQ ID NO: 249, SEQ ID NO: 244, SEQ ID NO: 199, SEQ ID NO: 251, SEQ ID NO:

250, SEQ ID NO: 334, SEQ ID NO: 185, SEQ ID NO: 186, SEQ ID NO: 336, or SEQ ID NO: 337 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 247, SEQ ID NO: 248, SEQ ID NO: 335, SEQ ID NO: 336, SEQ ID NO: 337, or SEQ ID NO: 338 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 248 or SEQ ID NO: 338 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, SEQ ID NO: 248 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, SEQ ID NO: 338 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is of a BCL11a enhancer, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 278 to SEQ ID NO: 333 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 318, SEQ ID NO: 312, SEQ ID NO: 313, SEQ ID NO: 294, SEQ ID NO: 310, SEQ ID NO: 319, SEQ ID NO: 298, SEQ ID NO: 322, SEQ ID NO: 311, SEQ ID NO: 315, SEQ ID NO: 290, SEQ ID NO: 317, SEQ ID NO: 309, SEQ ID NO: 289, or SEQ ID NO: 281 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, SEQ ID NO: 318 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is of a BCL11a enhancer, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 1596 to SEQ ID NO: 1691 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 1683, SEQ ID NO: 1638, SEQ ID NO: 1647, SEQ ID NO: 1609, SEQ ID NO: 1621, SEQ ID NO: 1617, SEQ ID NO: 1654, SEQ ID NO: 1631, SEQ ID NO: 1620, SEQ ID NO: 1637, SEQ ID NO: 1612, SEQ ID NO: 1656, SEQ ID NO: 1619, SEQ ID NO: 1675, SEQ ID NO: 1645, SEQ ID NO: 1598, SEQ ID NO: 1599, SEQ ID NO: 1663, SEQ ID NO: 1677, or SEQ ID NO: 1626 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is of a HFPH region (e.g., a French HPFH region), and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 86 to SEQ ID NO: 181, SEQ ID NO: 1500 to SEQ ID NO: 1595, or SEQ ID NO: 1692 to SEQ ID NO: 1761 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 100, SEQ ID NO: 165, SEQ ID NO: 113, SEQ ID NO: 99, SEQ ID NO: 112, SEQ ID NO: 98, SEQ ID NO: 1580, SEQ ID NO: 106, SEQ ID NO: 1503, SEQ ID NO: 1589, SEQ ID NO: 160, SEQ ID NO: 1537, SEQ ID NO: 159, SEQ ID NO: 101, SEQ ID NO: 162, SEQ ID NO: 104, SEQ ID NO: 138, SEQ ID NO: 1536, SEQ ID NO: 1539, SEQ ID NO: 1585 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 1505, SEQ ID NO: 1589, SEQ ID NO: 1700, or SEQ ID NO: 1750 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 100,

SEQ ID NO: 165, or SEQ ID NO: 113. In certain embodiments, an LNP of the present disclosure comprises any gRNA, nuclease and/or template nucleic acid (or a polynucleotide encoding any of the aforementioned) disclosed in US Application Publication US20190010495A1, which is incorporated by reference herein, in its entirety.

[00756]In certain embodiments, an LNP of the present disclosure comprises one or more *S. pyogenes* Cas9 deoxyribonucleic acid (DNA) endonucleases and one or two ribonucleic acid (RNA) guides capable of effecting a pair of double-strand breaks (DSBs), the first at a 5' DSB locus and the second at a 3' DSB locus within the δ 3-globin region of human chromosome 1 1 , causing a deletion or inversion of the chromosomal DNA between the 5' DSB locus and the 3' DSB locus that results in increased expression of γ-globin. In certain embodiments, an LNP of the present disclosure comprises one or more *S. pyogenes* Cas9 deoxyribonucleic acid (DNA) endonucleases and one or two ribonucleic acid (RNA) guides capable of effecting a pair of double-strand breaks (DSBs), the first at a 5′ DSB locus and the second at a 3′ DSB locus within the δβ-globin region of human chromosome 11, causing a deletion or inversion of the chromosomal DNA between the 5′ DSB locus and the 3′ DSB locus. In certain embodiments, the first RNA guide comprises a spacer sequence that is complementary to a segment of the 5′ DSB locus, and the second RNA guide comprises a spacer sequence that is complementary to a segment of the 3′ DSB locus. In certain embodiments, the 5′ DSB locus is proximal to the 5′ boundary of an HPFH deletion. In certain embodiments, the 5′ DSB locus is proximal to the 5′ boundary of an HPFH deletion selected from the group consisting of the HPFH-4 deletion, the HPFH-5 deletion, the HPFH-Kenya deletion, the HPFH-Black deletion, the long Corfu deletion, and the short Corfu deletion. In certain embodiments, the 3′ DSB locus is proximal to the 3′ boundary of an HPFH deletion. In certain embodiments, the 3′ DSB locus is proximal to the 3′ boundary of an HPFH deletion selected from the group consisting of the HPFH-4 deletion, the HPFH-5 deletion, the HPFH-Kenya deletion, the HPFH-Black deletion, the long Corfu deletion, and the short Corfu deletion. In certain embodiments, the endonuclease and the guide RNA are any of those described and disclosed in US20180030438A1 and/or US Patent 10,738,305, both of which are incorporated by reference herein, in their entirety. In certain embodiments, the LNP comprises a polynucleotide encoding the endonuclease. In certain embodiments, an LNP of the present disclosure comprises a DNA endonuclease capable of effecting a double-strand break (DSB) at one or more loci within the δβ-globin region of human chromosome 11, causing deletions or insertions of chromosomal DNA at the one or more loci that results in increased expression of γ-globin, thereby increasing the level of HbF in the cell, wherein one of the loci comprises a sequence that is complementary to a nucleic acid sequence of SEQ ID NO: 139 of US Patent 10,738,305. In certain embodiments, the DNA endonuclease is a Cas9 endonuclease and the method comprises introducing into the cell one or more polynucleotides encoding Cas9 and one or more guide RNAs, each comprising a spacer sequence that is complementary to the one or more loci within the β-globin region of human chromosome 11. In certain embodiments, the one or more guide RNA comprises a

spacer sequence that is complementary to positions 51-69 of the nucleic acid sequence of SEQ ID NO: 180 of US Patent 10,738,305. In certain embodiments, the one or more guide RNA comprises a spacer sequence that hybridizes to the same target sequence of the δβ-globin region of human chromosome 11 as a nucleic acid having the nucleic acid sequence of SEQ ID NO: 139 of US Patent 10,738,305.

[00757]In certain embodiments, an LNP of the present disclosure comprises a DNA endonuclease capable of effecting a pair of double-strand breaks (DSBs), the first at a 5′ DSB locus and the second at a 3′ DSB locus within the δβ-globin region of human chromosome 11, causing a deletion or inversion of the chromosomal DNA between the 5′ DSB locus and the 3′ DSB locus. In certain embodiments, the DNA endonuclease is a Cas9 endonuclease, a zinc finger nuclease, a transcription activator-like effector nuclease, a homing endonuclease, a dCas9-FokI nuclease or a MegaTal nuclease. In certain embodiments, the LNP comprises a polynucleotide encoding the endonuclease. In certain embodiments, the LNP comprises two guide RNAs, the first guide RNA comprising a spacer sequence that is complementary to a segment of the 5′ DSB locus, and the second guide RNA comprising a spacer sequence that is complementary to a segment of the 3′ DSB locus. In certain embodiments, both guide RNAs are single-molecule gRNAs. In certain embodiments, the endonuclease and the guide RNA are any of those described and disclosed in US20180200387A1, which is incorporated by reference herein, in its entirety. In certain embodiments, the endonuclease and the guide RNA are any of those described and disclosed in US20180273609A1, which is incorporated by reference herein, in its entirety.

[00758]In certain embodiments, an LNP of the present disclosure comprises one or more base editors, or a polynucleotide encoding the same, and a guide RNA bound to the base editor, wherein the guide RNA (gRNA) comprises a guide sequence that is complementary to a target nucleic acid sequence in the promoter of the HBGl and/or HBG2 gene. In certain embodiments, the guide sequence comprises at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 contiguous nucleobases that are 100% complementary to the target nucleic acid sequence of the promoter. In certain embodiments, the target nucleic acid comprises one of SEQ ID NOs: 838-845 of US20200399626A1. In certain embodiments, the target nucleic acid sequence further comprises 5'- CCT-3' at the 5' end. In certain embodiments, the target nucleic acid sequence comprises SEQ ID NO: 842 of US20200399626A1. In certain embodiments, the guide sequence comprises one of SEQ ID NOs: 846-853 of US20200399626A1. In certain embodiments, the guide sequence comprises one of SEQ ID NOs: 254-291 of US20200399626A1. In certain embodiments, the target nucleic acid comprises one of SEQ ID NOs: 297-302 of US20200399626A1. In certain embodiments, the guide sequence comprises SEQ ID NO: 260 of US20200399626A1. In certain embodiments, the target sequence comprises SEQ ID NO: 303 of US20200399626A1. In certain embodiments, the guide sequence comprises one of SEQ ID NOs: 261-266 of US20200399626A1. In certain embodiments, the target nucleic acid comprises one of SEQ ID NOs: 304-309 of US20200399626A1. In certain embodiments,

the guide sequence comprises SEQ ID NO: 267 of US20200399626A1. In certain embodiments, the target sequence comprises SEQ ID NO: 310 of US20200399626A1. In certain embodiments, the target nucleic acid comprises one of SEQ ID NOs: 297-323 of US20200399626A1. In certain embodiments, the LNP comprises a second guide RNA.

[00759]In certain embodiments, an LNP of the present disclosure comprises one or more deoxyribonucleic acid (DNA) endonucleases capable of effecting two or more single-strand breaks (SSB) or double-strand breaks (DSB), a first SSB or DSB within or near a human beta globin locus on chromosome 11, and a second SSB or DSB within or near one or more of: a BCL11A gene on chromosome 2, and a DNA sequence that encodes a transcriptional control region of the BCL11A gene on chromosome 2; wherein the first SSB or DSB results in one or more of: a permanent deletion or inversion within or near the human beta globin locus on chromosome 11, and a mutation within or near the human beta globin locus on chromosome 11;

[00760]wherein the second SSB or DSB results in one or more of: a permanent deletion or inversion within or near the BCL11A gene on chromosome 2, a mutation within or near the BCL11A gene on chromosome 2, a permanent deletion or inversion within or near the DNA sequence that encodes a transcriptional control region of the BCL11A gene on chromosome 2, and a mutation within or near the DNA sequence that encodes a transcriptional control region of the BCL11A gene on chromosome 2; and wherein the two or more permanent deletions, inversions, or mutations result in an increased expression of γ-globin and an increase of fetal hemoglobin (HbF) in the human cell. In certain embodiments, the permanent deletion within or near the human beta globin locus on chromosome 11 is a hereditary persistence of fetal hemoglobin (HPFH) deletion. In certain embodiments, the HPFH deletion is selected from the group consisting of: a HPFH-4 deletion, a HPFH-5 deletion, a HPFH-Kenya deletion, a HPFH-Black deletion, a large Corfu deletion, a small Corfu deletion, a small deletion, and combinations thereof. In certain embodiments, the HPFH deletion is a 7.2 kb, 3.5 kb, 12.9 kb, 22 kb, or a 13 bp deletion. In certain embodiments, the one or more DNA endonuclease is selected from the group consisting of a zinc finger nuclease, TAL effector nuclease, meganuclease, MegaTAL, Tev-m TALEN, MegaTev, homing endonuclease, FokI, StsI, Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas100, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, or Cpf1 endonuclease, a homolog thereof, a recombination of the naturally occurring molecule thereof, a codon-optimized thereof, modified versions thereof, and combinations thereof. In certain embodiments, the LNP further comprises one or more guide ribonucleic acid (gRNA), optionally comprising one or more of the gRNA sequences provided in Table 2 of US20210180091A1, optionally wherein the one or more gRNAs comprise the sequence of SPY101 in combination with the sequence of any one or more of HPFH5-1, HPFH5-D, HPFH5-T5, HPFH5-T7, Kenya-K5, or Kenya-K1; or optionally wherein the one or more gRNAs comprise the sequence of 1450 in

combination with the sequence of any one or more of HPFH5-1, HPFH5-D, HPFH5-T5, HPFH5-T7, Kenya-K5, or Kenya-K1; or optionally wherein the one or more gRNAs comprise the sequence of SPY101 in combination with the sequence of SD2; or optionally wherein the one or more gRNAs comprise the sequence of 1450 in combination with the sequence of SD2; or optionally wherein the one or more gRNAs comprise the sequence of HPFH5-1, HPFH5-D, HPFH5-T5, HPFH5-T7, Kenya-K5, or Kenya-K1 in combination with the sequence of any one or more of HPFH5-1, HPFH5-D, HPFH5-T5, HPFH5-T7, Kenya-K5, or Kenya-K1. In certain embodiments, the one or more DNA endonucleases and/or gRNAs are selected from those disclosed in US20210180091A1, which is incorporated by reference herein, in its entirety.

[00761]In certain embodiments, an LNP of the present disclosure comprises a genome editing system comprising an RNA-guided nuclease; a first guide RNA; and a second guide RNA, wherein the first and second guide RNAs comprise first and second targeting domains complimentary to first and second sequences on opposite sides of positions of a 13 nt target region of a human HBG1 or HBG2 gene, wherein one or both of the first and second sequences optionally overlaps the 13 nt target region of the human HBG1 or HBG2 gene. In certain embodiments, the genome editing system further comprises a nucleic acid template encoding a deletion of the 13 nt region of a human HBG1 or HBG2 gene. In certain embodiments, the RNA-guided nuclease is an S. pyogenes Cas9. In certain embodiments, the first and second targeting domains are complimentary to sequences immediately adjacent to a protospacer adjacent motif recognized by S. pyogenes Cas9. In certain embodiments, the RNA-guided nuclease is a nickase, and optionally lacks RuvC activity. In certain embodiments, the first targeting domain is complimentary to a sequence within positions c. −1,114 to −114 of a human HBG1 or HBG2 gene. In certain embodiments, the first targeting domain is complimentary to a sequence within positions c. −214 to −114 of a human HBG1 or HBG2 gene. In certain embodiments, one of the first and second targeting domains is complimentary to a sequence within positions c. −102 to −52 of a human HBG1 or HBG2 gene. In certain embodiments, the second targeting domain is complimentary to a sequence within positions c. −102 to −2 of a human HBG1 or HBG2 gene. In certain embodiments, at least one of the first and second targeting domains differ by no more than 3 nucleotides from a targeting domain listed in Table 7 of US Patent Application Publication US20220047637A1. In certain embodiments, the genome editing system comprises first and second RNA-guided nucleases. In certain embodiments, the first and second RNA-guided nucleases are complexed with the first and second guide RNAs, respectively, forming first and second ribonucleoprotein complexes. In certain embodiments, the genome editing system further comprises: a third guide RNA; and optionally a fourth guide RNA, wherein the third and fourth guide RNAs comprise third and fourth targeting domains complimentary to third and fourth sequences on opposite sides of positions of a GATA1 binding motif in BCL11A erythroid enhancer (BCL11Ae) of a human BCL11A gene, wherein one or both of the third and fourth sequences optionally overlaps the GATA1 binding motif in BCL11Ae of the human BCL11A gene. In certain embodiments, the genome editing

system further comprises a nucleic acid template encoding a deletion of the GATA1 binding motif in BCL11Ae. In certain embodiments, the third targeting domain is complimentary to a sequence within 1000 nucleotides upstream of the GATA1 binding motif in BCL11Ae. In certain embodiments, the third targeting domain is complimentary to a sequence within 100 nucleotides upstream of the GATA1 binding motif in BCL11Ae. In certain embodiments, one of the third and fourth targeting domains is complimentary to a sequence within 100 nucleotides downstream of the GATA1 binding motif in BCL11Ae. In certain embodiments, the fourth targeting domain is complimentary to a sequence within 50 nucleotides downstream of the GATA1 binding motif in BCL11Ae. In certain embodiments, at least one of the third and fourth targeting domains differ by no more than 3 nucleotides from a targeting domain listed in Table 9 of US Patent Application Publication US20220047637A1.

[00762]In certain embodiments, an LNP of the present disclosure comprises a guide RNA (gRNA) comprising: a 5′ end and a 3′ end, a DNA extension at the 5′ end, a 2′-O-methyl-3′-phosphorothioate modification at the 3′ end, a targeting domain that is complementary to a target site in a promoter of an HBG gene, and an RNA segment capable of associating with a Cpf1 RNA-guided nuclease. In certain embodiments, the DNA extension comprises a sequence selected from the group consisting of SEQ ID NOs:1235-1250 as disclosed in US Patent Application Publication US20210254061A1. In certain embodiments, the targeting domain comprises a sequence set forth in Table 13, Table 18, or Table 19 of US Patent Application Publication US20210254061A1. In certain embodiments, the target site comprises nucleotides located between Chr 11 (NC_000011.10) 5,249,904-5,249,927 (Table 17, Region 6, of US Patent Application Publication US20210254061A1); Chr 11 (NC_000011.10) 5,254,879-5,254,909 (Table 17, Region 16, of US Patent Application Publication US20210254061A1); or a combination thereof. In certain embodiments, an LNP of the present disclosure comprises a ribonucleoprotein (RNP) complex comprising a Cpf1 variant and a gRNA as described above. In certain embodiments, the Cpf1 variant contains one or more modifications selected from the group consisting of one or more mutations in a wild-type Cpf1 amino acid sequence, one or more mutations in a wild-type Cpf1 nucleic acid sequence, one or more nuclear localization signals, one or more purification tags, and a combination thereof. In certain embodiments, the Cpf1 variant is encoded by a sequence selected from the group consisting of SEQ ID NOs:1000, 1001, 1008-1018, 1032, 1035-39, 1094-1097, and 1107-09 of US Patent Application Publication US20210254061A1. In certain embodiments, the Cpf1 variant is encoded by a sequence selected from the group consisting of SEQ ID NOs:1019-1021 and 1110-1117 of US Patent Application Publication US20210254061A1.

[00763]In certain embodiments, an LNP of the present disclosure comprises a fusion protein comprising a polynucleotide programmable DNA binding domain and at least one adenosine deaminase variant comprising a serine (S) at amino acid position 82 of the following sequence and having at least 85% identity to SEQ ID NO: 4 of US Patent 11,155,803. In certain embodiments,

the adenosine deaminase variant further comprises an alteration at amino acid position 166. In certain embodiments, the adenosine deaminase variant further comprises a T166R alteration. In certain embodiments, the adenosine deaminase variant further comprises one or more of the following alterations: Y147T, Y147R, Q154S, Y123H, and Q154R. In certain embodiments, the adenosine deaminase variant further comprises an alteration or combination of alterations selected from the group consisting of: Y147T and Q154R; Y147T and Q154S; Y147R and Q154S; Q154S; Y147R; Q154R; Y123H; I76Y; Y123H, and Y147T; Y123H, and Y147R; Y123H, and Q154R; Y147R, Q154R, and Y123H; Y147R, Q154R, and I76Y; Y147R, Q154R, and T166R; Y123H, Y147R, Q154R, and I76Y; Y123H, Y147R, and Q154R; and I76Y, Y123H, Y147R, and Q154R. In certain embodiments, the fusion protein further comprises a second adenosine deaminase variant or a second adenosine deaminase. In certain embodiments, the fusion protein is ABE8.5-m, ABE8.14-m, ABE8.15-m, ABE8.16-m, ABE8.17-m, ABE8.18-m, ABE8.19-m, ABE8.20-m, ABE8.22-m, ABE8.23-m, or ABE8.24-m. In certain embodiments, the second adenosine deaminase comprises a wild-type adenosine deaminase. In certain embodiments, the fusion protein is ABE8.5-d, ABE8.14-d, ABE8.15-d, ABE8.16-d, ABE8.17-d, ABE8.18-d, ABE8.19-d, ABE8.20-d, ABE8.22-d, ABE8.23-d, or ABE8.24-d. In certain embodiments, the adenosine deaminase variant is TadA*8.14, TadA*8.15, TadA*8.16, TadA*8.17, TadA*8.18, TadA*8.19, TadA*8.20, TadA*8.22, TadA*8.23, or TadA*8.24. In certain embodiments, the adenosine deaminase variant comprises a deletion of 1, 2, 3, 4, 5, 6, 7, or 8 N-terminal or C-terminal amino acid residues relative to the full-length adenosine deaminase. In certain embodiments, the polynucleotide programmable DNA binding domain comprises SEQ ID NO: 12 of US Patent 11,155,803. In certain embodiments, the polynucleotide programmable DNA binding domain comprises a Cas9 domain. In certain embodiments, the polynucleotide programmable DNA binding domain comprises the SEQ ID NO: 1 of US Patent 11,155,803. In certain embodiments, the Cas9 domain is a Staphylococcus aureus Cas9 (SaCas9), Streptococcus thermophilus 1 Cas9 (St1Cas9), a Streptococcus pyogenes Cas9 (SpCas9), or variants thereof. In certain embodiments, the polynucleotide programmable DNA binding domain is a nuclease inactive or nickase variant. In certain embodiments, the fusion protein comprises a linker between the polynucleotide programmable DNA binding domain and the adenosine deaminase variant. In certain embodiments, the fusion protein comprises one or more nuclear localization signals. In certain embodiments, the adenosine deaminase variant has at least 90% identity to the sequence. In certain embodiments, the adenosine deaminase variant has at least 95% identity to the sequence. In certain embodiments, an LNP of the present disclosure comprises a polynucleotide encoding the fusion protein of any of the above described embodiments.

[00764]In certain embodiments, an LNP of the present disclosure comprises a guide RNA molecule comprising a targeting domain comprising a nucleotide sequence complementary or partially complementary with a target sequence encompassing or proximal to position -175 of a 5' UTR and/or -228 of a transcription start site of HBG1 or HBG2. In certain embodiments, the target sequence is

between -150 and -250 of the proximal promoter regions. In certain embodiments, the targeting domain is configured to guide a nucleobase modification at position -175. In certain embodiments, the nucleobase modification results in a T->C mutation. In certain embodiments, an LNP of the present disclosure comprises a nucleic acid composition, comprising: a nucleotide sequence encoding a guide RNA molecule comprising a targeting domain comprising a nucleotide sequence complementary or partially complementary with a target sequence encompassing or proximal to position - 175 of a proximal promoter region of HBG1 or HBG2. In certain embodiments, the nucleic acid composition further comprises a nucleotide sequence encoding a base editing protein or peptide. In certain embodiments, the base editing protein or peptide is an adenine base editor (ABE). In certain embodiments, the ABE is selected from the group consisting of ABE7.10, ABE8e, and an ABE comprising SpRY. In certain embodiments, the base editing protein or peptide comprises an adenosine deaminase, optionally an AB Emax protein or peptide. In certain embodiments, the base editing protein or peptide is configured to introduce a T->C mutation in or near the target sequence of the guide RNA. In certain embodiments, the nucleic acid is a viral vector genome or an mRNA. In certain embodiments, an LNP of the present disclosure comprises an isolated ribonucleoprotein complex, comprising: a guide RNA molecule comprising a targeting domain comprising a nucleotide sequence complementary or partially complementary with a target sequence encompassing or proximal to position -175 of a proximal promoter region of HBG1 or HBG2, and a base editor protein or peptide. In any of the above embodiments, the base editing system is configured to introduce a T->C mutation at one or more of: HBG2: GRCh/hgl9; chrl 1:5,276,186.; and HBG1: GRCh/hgl9; chrl 1:5,271,262. In certain embodiments, the gRNA and the base editing protein or peptide, or nucleotide sequence encoding the same, is any one disclosed in PCT Application Publication WO2022155458A1, which is incorporated by reference herein, in its entirety.

[00765]In certain embodiments, an LNP of the present disclosure comprises a ribonucleoprotein (RNP) complex comprising:

(a) a Cas9 endonuclease protein; and

(b) a guide RNA (gRNA) comprising a targeting domain comprising a first nucleotide sequence that is complementary or partially complementary with a target domain comprising a second nucleotide sequence that is:

(i) within nucleotides 1-4758 of SEQ ID NO:902, nucleotides 1-4773 of SEQ ID NO:903, or both; or

(ii) complementary to a sequence within nucleotides 1-4758 of SEQ ID NO:902, nucleotides 1-4773 of SEQ ID NO:903, or both;

wherein any of the sequences referenced are those disclosed in US Patent Application Publication US20200255857A1.

[00766]In certain embodiments, the second nucleotide sequence is: (i) within nucleotides 1-2990 of SEQ ID NO:902, nucleotides 1-2914 of SEQ ID NO:903, or both; or (ii) complementary to a

sequence within nucleotides 1-2990 of SEQ ID NO:902, nucleotides 1-2914 of SEQ ID NO:903, or both, wherein any of the sequences referenced are those disclosed in US Patent Application Publication US20200255857A1. In certain embodiments, the second nucleotide sequence is: (i) within nucleotides 2424-3236 of SEQ ID NO:902, nucleotides 2348-3160 of SEQ ID NO:903, or a combination thereof or (ii) complementary to a sequence within nucleotides 2424-3236 of SEQ ID NO:902, nucleotides 2348-3160 of SEQ ID NO:903, or a combination thereof, wherein any of the sequences referenced are those disclosed in US Patent Application Publication US20200255857A1. In certain embodiments, the targeting domain comprises a nucleotide sequence that is identical to, or differs by no more than 1, 2, 3, 4, or 5 nucleotides, from a nucleotide sequence set forth in any of SEQ ID NOs:251-901, wherein any of the sequences referenced are those disclosed in US Patent Application Publication US20200255857A1. In certain embodiments, the gRNA comprises one or more modifications selected from the group consisting of a 2′-acetylation, a 2′-methylation, and a phosphorothioate modification. In certain embodiments, the Cas9 endonuclease protein is selected from the group consisting of a S. pyogenes, S. aureus, and S. thermophilus Cas9 endonuclease protein

[00767]In certain embodiments, an LNP of the present disclosure comprises a gRNA molecule comprising a targeting domain which is complementary with a target domain from the HBB or BCL11A gene. In certain embodiments, an LNP of the present disclosure comprises a nucleotide sequence that encodes a gRNA molecule comprising a targeting domain which is complementary with an SCD target domain in the HBB or BCL11A gene. In certain embodiments, the targeting domain is configured to provide a cleavage event selected from a double strand break and a single strand break, within 500, 400, 300, 200, 100, 50, 25, or 10 nucleotides of an SCD target point position or an SCD target knockout position. In certain embodiments, an LNP of the present disclosure comprises:

(a) a first gRNA molecule comprising a first targeting domain which is complementary with a first target domain located in, or within, a BCL11A gene, wherein the first targeting domain is configured to provide a first cleavage event in a region of the BCL11A gene which is complementary to a sequence that is the same as, or differs by no more than 3 nucleotides from, a sequence selected from the group consisting of SEQ ID NOs:16261 to 16279 of US Patent 11,242,525; and

(b) a Cas9 molecule, or a polynucleotide encoding the same.

[00768]In certain embodiments, the LNP further comprises a second gRNA. In certain embodiments, the second gRNA molecule comprises a second targeting domain which is complementary with a second target domain located in a BCL11A gene, wherein the second targeting domain is configured to provide a second cleavage event in a region of the BCL11A gene, and wherein the second targeting domain comprises a sequence that is the same as, or differs by no more than 3 nucleotides from, a sequence selected from the group consisting of SEQ ID NOs:16261 to 16279 of US Patent 11,242,525. In certain embodiments, the LNP further comprises a third gRNA, and optionally a fourth gRNA.

[00769]In certain embodiments, an LNP of the present disclosure comprises:

1a) a gRNA molecule comprising a targeting domain which is complementary with a target sequence from a target nucleic acid;

1b) one or more Cas9 molecules or one or more nucleic acids encoding one or more Cas9 molecules; and

1c) optionally, a template nucleic acid; or

2a) a nucleic acid encoding the gRNA molecule;

2b) one or more Cas9 molecules or one or more nucleic acids encoding one or more Cas9 molecules; and

2c) optionally, a template nucleic acid.

[00770]In certain embodiments, the one or more Cas9 molecules comprise a first eaCas9 and a second eaCas9. In certain embodiments, the one or more nucleic acids encoding one or more Cas9 molecules comprise one or more nucleic acids encoding a first eaCas9 molecule and a second eaCas9 molecule. In certain embodiments, the composition comprises a first nucleic acid comprising a first promoter operably linked to a sequence encoding the gRNA. In certain embodiments, the LNP comprises a second nucleic acid comprising a second promoter operably linked to a sequence encoding a second gRNA. In certain embodiments, the LNP comprises a first nucleic acid comprising a first promoter operably linked to a sequence encoding the first eaCas9 and a second nucleic acid comprises a second promoter operably linked to a sequence encoding the second eaCas9. In certain embodiments, the targeting domain comprises a core domain and is 15 to 50 nucleotides in length. In certain embodiments, the gRNA has a structure of 5′-targeting domain-first complementarity domain-linking domain-second complementarity domain-proximal domain-3′. In certain embodiments, the first complementarity domain is 5 to 25 nucleotides in length. In certain embodiments, the proximal domain is 5 to 20 nucleotides in length. In certain embodiments, the linking domain is 1 to 5 nucleotides in length. In certain embodiments, the second complementarity domain is complementary to the first complementarity domain. In certain embodiments, any of the above embodiment are formulated such that the gRNA and the one or more Cas9 molecules, or one or more nucleic acids encoding one or more Cas9 molecules, are formulated in separate LNP formulations. In certain embodiments, the contents of the LNP(s) induce exon skipping. In certain embodiments, the gRNA comprises or consists of any guide RNA sequence, or any portion thereof, disclosed in US Application Publication US20210040506A1, which is incorporated by reference herein in its entirety. In certain embodiments, the LNP further comprises a CRISPR Cas9, or a polynucleotide encoding the same, disclosed in US Application Publication US20210040506A1, which is incorporated by reference herein in its entirety.

[00771]In certain embodiments, an LNP of the present disclosure comprises a polypeptide comprising a homing endonuclease (HE) variant that cleaves a target site in the human BCL11A gene, or a polynucleotide encoding such a polypeptide. In certain embodiments, the HE variant is an LAGLIDADG homing endonuclease (LHE) variant. In certain embodiments, the HE variant is a

variant of an LHE selected from the group consisting of: I-AabMI, I-AacMI, I-AniI, I-ApaMI, I-CapIII, I-CapIV, I-CkaMI, I-CpaMI, I-CpaMII, I-CpaMIII, I-CpaMIV, I-CpaMV, I-CpaV, I-CraMI, I-EjeMI, I-GpeMI, I-GpiI, I-GzeMI, I-GzeMII, I-GzeMIII, I-HjeMI, I-LtrII, I-LtrI, I-LtrWI, I-MpeMI, I-MveMI, I-NcrII, I-NcrI, I-NcrMI, I-OheMI, I-OnuI, I-OsoMI, I-OsoMII, I-OsoMIII, I-OsoMIV, I-PanMI, I-PanMII, I-PanMIII, I-PnoMI, I-ScuMI, I-SmaMI, I-SscMI, and I-Vdil41I. In certain embodiments, HE variant comprises one or more amino acid substitutions at amino acid positions selected from the group consisting of: 19, 24, 26, 28, 30, 32, 34, 35, 36, 37, 38, 40, 42, 44, 46, 48, 68, 70, 72, 75, 76, 77, 78, 80, 82, 168, 180, 182, 184, 186, 188, 189, 190, 191, 192, 193, 195, 197, 199, 201, 203, 223, 225, 227, 229, 231, 232, 234, 236, 238, and 240 of an I-OnuI LHE amino acid sequence as set forth in SEQ ID NOs: 1-5 of US Patent Application Publication US20190184035A1, or a biologically active fragment thereof. In certain embodiments, the HE variant comprises the amino acid sequence set forth in any one of SEQ ID NOs: 6-19 of US Patent Application Publication US20190184035A1. In certain embodiments, the LNP further comprises a donor repair template.

[00772]In certain embodiments, an LNP of the present disclosure comprises one or more polynucleotides encoding a zinc finger nuclease (ZFN) or a TAL-effector domain nuclease (TALEN) that binds to a target site in a BCL11A gene as shown in any of SEQ ID Nos:58-61, 65, 74 or 77 of US Patent 10,619,154. In certain embodiments, the encoded nuclease cleaves and genetically modifies the BCL11A gene and expression of the globin gene is altered. In certain embodiments, an LNP of the present disclosure comprises a DNA binding molecule comprising a TALE-effector protein (TALE), or a polynucleotide encoding said TALE, or a single guide RNA that binds to a target site as shown in any of SEQ ID Nos:58-62, 65 or 74-77 of US Patent 10,619,154.

[00773]In certain embodiments, an LNP of the present disclosure comprises SB-mRENH1 mRNAs and SB-mRENH2 mRNAs, which encode a ZFN pair; wherein the ZFN pair is capable of making a genomic modification within an endogenous BCL11A enhancer sequence. In certain embodiments, the SB-mRENH1 mRNA, SB-mRENH2 mRNA, and ZFN pair are one or more disclosed in PCT Application Publication WO2023014839A1, which is incorporated by reference herein in its entirety.

[00774]In certain embodiments, an LNP of the present disclosure comprises:

(i) an endogenous BCL11a enhancer sequence; and

(ii) a pair of TALENs, each TALEN of the pair comprising a FokI cleavage half-domain and a DNA-binding domain comprising a plurality of TALE repeat units, each repeat unit comprising a hypervariable di-residue region (RVD), wherein the RVDs of the TALE repeats units are shown in a single row of as shown in Table 1, Table 2 or Table 4 of US Patent 11,021,696, and further wherein the pair of TALENs is selected from the group consisting of: 102740 and 102741; 102736 and 102737; 102756 and 102757; 102752 and 102753; 102750 and 102751; 102775 and 102774; 102795 and 102794; 102832 and 102833; 102834 and 102835; 102836 and 102837; 102838 and 102839; 102840 and 102841; 102842 and 102843; 102844 and 102845; 102846 and 102847; 102848 and 102849; 102850 and 102851; 102852 and 102853; 102854 and 102855;

102856 and 102857; 102858 and 102859; 102860 and 102861; 102862 and 102863; 102864 and 102865; 102866 and 102867; 102868 and 102869; 102870 and 102871; 102872 and 102873; 102874 and 102875; 102756 and 102757; 102752 and 102753; 102759 and 102751; 102876 and 102877; 102878 and 102879; 102880 and 102881; 102882 and 102883; 102884 and 102885; 102886 and 102887; 102888 and 102889; 102890 and 102891; 102892 and 102893; 102894 and 102895; 102896 and 102897; 102898 and 102899; 102902 and 102903; 102904 and 102905; 102906 and 102907; 102912 and 102913; 102914 and 102915; 102916 and 102917; 102918 and 102919; 102920 and 102921; or 102922 and 102923, wherein the pair of TALENs cleave and genetically modify the BCL11a enhancer sequence. In certain embodiments, the LNP comprises a polynucleotide encoding any of the TALEN pairs described above. In certain embodiments, an LNP of the present disclosure comprises a DNA binding protein comprising a TALE-effector protein (TALE), wherein the TALE protein comprises a plurality of TALE repeat units, each repeat unit comprising a hypervariable diresidue region (RVD), wherein the RVDs of the TALE repeats units are shown in a single row of Table 1, Table 2 or Table 4 of US Patent 11,021,696. In certain embodiments, the LNP comprises a fusion protein comprising the TALE protein and a wild-type or engineered cleavage domain or cleavage half-domain. In certain embodiments, the LNP comprises a polynucleotide encoding the DNA binding protein described above. In certain embodiments, an LNP of the present disclosure comprises a nuclease, or a polynucleotide encoding said nuclease, capable of making genomic modification selected from an insertion, deletion or combination thereof, within an endogenous BCL11a enhancer sequence. In certain embodiments, the genomic modification is within one or more sequences selected from SEQ ID NO: 1, 2 and 3 of US Patent 11,021,696. In certain embodiments, the genomic modification is at or near one or more sequences selected from SEQ ID NOs: 143 to 184 and 232-251 of US Patent 11,021,696. In certain embodiments, the genomic modification is at or near one or more sequences selected from SEQ ID NOs: 4 to 80 and 362 of US Patent 11,021,696. In certain embodiments, the nuclease comprises at least one ZFN or TALEN. In certain embodiments, the nuclease comprises a zinc finger nuclease, the zinc finger nuclease comprising 4, 5, or 6 zinc finger domains comprising a recognition helix and further wherein the zinc finger proteins comprise the recognition helix regions in the order shown in a single row of Table 3 or Table 6 of US Patent 11,021,696.

[00775]In certain embodiments, an LNP of the present disclosure comprises a zinc finger nuclease, or a polynucleotide encoding the same, comprising the amino acid sequence as shown in SEQ ID NO:29 or SEQ ID NO:31 of US Patent 10,563,184. In certain embodiments, the LNP comprises an isolated zinc finger nuclease (ZFN), or a polynucleotide encoding the same, comprising left and right ZFNs, the left ZFN comprising the amino acid sequence as shown in SEQ ID NO:29 and the right ZFN comprising the amino acid sequence as shown in SEQ ID NO:31, both sequences being those disclosed in US Patent 10,563,184. In certain embodiments, the left ZFN is encoded a polynucleotide

according to SEQ ID NO:28 and the right ZFN is encoded by a polynucleotide according to SEQ ID NO:30, both sequences being those disclosed in US Patent 10,563,184. In certain embodiments, the zinc finger nuclease is capable of executing an indel in a BCL11A locus. In certain embodiments, the indel occurs in a BCL11A enhancer sequence. In certain embodiments, the indel comprises inserting an exogenous sequence into an endogenous BCL11A enhancer sequence.

[00776]In certain embodiments, an LNP of the present disclosure comprises a zinc finger protein comprising 4, 5 or 6 fingers designated F1 to F4, F1 to F5 or F1 to F6, each finger comprising a recognition helix region that recognizes a target subsite wherein the protein is selected from the group consisting of:

(i) a protein comprising the recognition helix regions as follows:

F1: (SEQ ID NO: 7); F2: (SEQ ID NO: 5); F3: (SEQ ID NO: 2); and F4: (SEQ ID NO: 6);

(ii) a protein comprising the recognition helix regions as follows:

F1: (SEQ ID NO: 2); F2: (SEQ ID NO: 3); F3: (SEQ ID NO: 8); F4: (SEQ ID NO: 5); F5: (SEQ ID NO: 2); and F6: (SEQ ID NO: 6);

(iii) a protein comprising the recognition helix regions as follows:

F1: (SEQ ID NO: 2); F2: (SEQ ID NO: 9); F3: (SEQ ID NO: 10) or (SEQ ID NO: 11); F4: (SEQ ID NO: 5); F5: (SEQ ID NO: 2); and F6: (SEQ ID NO: 6);

(iv) a protein comprising the recognition helix regions as follows:

F1: (SEQ ID NO: 13); F2: (SEQ ID NO: 14); F3: (SEQ ID NO: 15); F4: (SEQ ID NO: 18); and F5: (SEQ ID NO: 17);

(v) a protein comprising the recognition helix regions as follows:

F1: (SEQ ID NO: 19); F2: (SEQ ID NO: 20); F3: (SEQ ID NO: 15); F4: (SEQ ID NO: 25); and F5: (SEQ ID NO: 17);

(vi) a protein comprising the recognition helix regions as follows:

F1: (SEQ ID NO: 13); F2: (SEQ ID NO: 22); F3: (SEQ ID NO: 15) or (SEQ ID NO:24); F4: (SEQ ID NO: 23); and F5: (SEQ ID NO: 17);

(vii) a protein comprising the recognition helix regions as follows:

F1: (SEQ ID NO: 13); F2: (SEQ ID NO: 20); F3: (SEQ ID NO: 15) or (SEQ ID NO: 24); F4: (SEQ ID NO: 21); and F5: (SEQ ID NO: 17); and

(viii) a protein comprising the recognition helix regions as follows:

F1: (SEQ ID NO: 13); F2: (SEQ ID NO: 14); F3: (SEQ ID NO: 24); F4: (SEQ ID NO: 16); and F5: (SEQ ID NO: 17);

wherein each of the above SEQ ID NOs correspond with those of US Patent 10,808,020.

[00777]In certain embodiments, the LNP comprises a polynucleotide encoding said zinc finger protein. In certain embodiments, the LNP comprises a fusion protein comprising the zinc finger protein and a functional domain, or a polynucleotide encoding the same. In certain embodiments, the

functional domain is a transcriptional activation domain, a transcriptional repression domain, or a cleavage domain.

[00778]In certain embodiments, an LNP of the present disclosure comprises:

a) a protospacer adjacent motif (PAM) sequence;

b) a trans-activating CRISPR RNA (tracrRNA) sequence; and

c) a single guide RNA selected from the group consisting of SEQ ID NOS: 1-94 disclosed in US Patent 11,572,543.

[00779]In certain embodiments, an LNP of the present disclosure further comprises a DNA-targeting endonuclease, or a polynucleotide encoding the same. In certain embodiments, the DNA-targeting endonuclease is a Cas protein. In certain embodiments, the Cas protein is a Cas9.

[00780]In certain embodiments, an LNP of the present disclosure comprises a single guide RNA that is:

a. complementary to the plus or minus strand of the human chromosome 2 at location 60725424 to 60725688 (+55 functional region);

b. complementary to the plus or minus strand of the human chromosome 2 at location 60722238 to 60722466 (+58 functional region); or

c. complementary to the plus or minus strand of the human chromosome 2 at location 60718042 to 60718186 (+62 functional region)

wherein the human chromosome 2 is that according to UCSC Genome Browser hg 19 human genome assembly, and

wherein the nucleic acid sequence excludes the entire human chromosome 2 and also excludes the genomic DNA sequence on the human chromosome 2 from location 60,716,189 to 60,728,612.

[00781]In certain embodiments, an LNP of the present disclosure comprises an sgRNA targeting a BCL11A genomic region from 0-15 nucleotides upstream of the CTTCCT sequence to 0-15 nucleotides downstream of the CTTCCT sequence in the chromosome 2 in the hematopoietic stem cell is introduced into said hematopoietic stem cells, wherein the BCL11A genomic region is located in a region from chr2:60495236 to chr2:60495271, as described in US Patent Application Publication US20200339979A1. In certain embodiments, the sgRNA construct comprises a nucleotide sequence selected from any one of SEQ ID NOs: 3-25, as disclosed in US Patent Application Publication US20200339979A1. In certain embodiments, the sgRNA comprise a 2'-OMe and or an internucleotide 3'-thio modification. In certain embodiments, the sgRNA comprises a chemical modification comprising a 2′-O-methyl modification of the first one, two and/or three bases at the 5′ end and/or the last base at the 3′ end of a nucleotide sequence. In certain embodiments, the LNP further comprises a gene editing technology. In certain embodiments, the LNP further comprises a zinc finger nuclease-based gene editing technology, a TALEN gene editing technology, or a CRISPR/Cas gene editing technology. In certain embodiments, the LNP further comprises one or

more of a basal medium, a growth factor, an antagonist or inhibitor of a progesterone receptor or a glucocorticoid receptor.

[00782]In certain embodiments, an LNP of the present disclosure comprises a CRISPR/Cas genome modification system and a BCL11A specific guide RNA, as disclosed in PCT Application Publication No. WO2022240787A1. In certain embodiments, the BCL11A specific guide RNA comprises BCL11A sgRNA 1617 having a sequence of: CTAACAGTTGCTTTTATCAC. In certain embodiments, LNPs comprising the aforementioned payload are suitable for treating subjects having an SNP polymorphism at site rsl 14518452. In certain embodiments, the SNP polymorphism is guanine (G) on the 5' to 3' strand.

[00783]In certain embodiments, an LNP of the present disclosure comprises a Class 2 Type V CRISPR protein, or a polynucleotide encoding the same, and a first guide ribonucleic acid (gRNA), wherein the gRNA comprises a targeting sequence complementary to a target nucleic acid sequence comprising a polypyrimidine tract-binding protein 1 (BCL11 A) gene. In certain embodiments, the gRNA comprises a targeting sequence complementary to a target nucleic acid sequence selected from the group consisting of a. a BCL11A intron; b. a BCLUA exon; c. a BCL11 A intron-exon junction; d. a BCL11 A regulatory element; and e. an intergenic region. In certain embodiments, the gRNA is a single-molecule gRNA (sgRNA) or a dual-molecule gRNA (dgRNA). In certain embodiments, the targeting sequence of the gRNA comprises a sequence selected from the group consisting of SEQ ID NOS: 272-2100 and 2286- 26789 of PCT Application Publication No. WO2022120094A2. In certain embodiments, the targeting sequence has 1, 2, 3, 4, or 5 nucleotides removed from the 3' end of the sequence. In certain embodiments, the targeting sequence of the gRNA is complementary to a sequence of a BCL11A exon. In certain embodiments, the targeting sequence of the gRNA is complementary to a sequence selected from the group consisting of a BCL11A exon 1 sequence, BCL11A exon 2 sequence, BCL11A exon 3 sequence, BCL11A exon 4 sequence, BCL11A exon 5 sequence, BCL11A exon 6 sequence, BCL11A exon 7 sequence, BCL11A exon 8 sequence, and a BCL11A exon 9 sequence. In certain embodiments, the targeting sequence of the gRNA is complementary to a sequence of a BCL11 A regulatory element. In certain embodiments, the targeting sequence of the gRNA is complementary to a sequence of a promoter of the BCL11 A gene. In certain embodiments, the targeting sequence of the gRNA is complementary to a sequence of an enhancer regulatory element. In certain embodiments, the targeting sequence of the gRNA is complementary to a sequence that comprises a GATA1 erythroid-specific enhancer binding site (GATA1) of the BCL11A gene. In certain embodiments, the targeting sequence of the gRNA is complementary to a sequence that is 5' to the GATA1 binding site of the BCL11 A gene. In certain embodiments, the targeting sequence of the gRNA comprises a sequence of selected from SEQ ID NOs: 22, 23, 2949, 2948, 15747, or 15748 of PCT Application Publication No. WO2022120094A2 or a sequence having at least 90% or 95% sequence identity thereto. In certain embodiments, the LNP further comprises a second gRNA, wherein the second gRNA has a targeting sequence

complementary to a different or overlapping portion of the BCL11 A target nucleic acid compared to the targeting sequence of the gRNA of the first gRNA. In certain embodiments, the targeting sequence of the second gRNA is complementary to a sequence of the target nucleic acid that is 5' or 3' to the GATA1 binding site sequence. In certain embodiments, the first and the second gRNA each have a targeting sequence complementary to a sequence within the promoter of the BCL11 A gene. In certain embodiments, the first or second gRNA has a scaffold comprising a sequence selected from the group consisting of SEQ ID NOS: 2238-2285, 26794- 26839 and 27219-27265 of PCT Application Publication No. WO2022120094A2, or a sequence having at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% sequence identity thereto. In certain embodiments, the first or second gRNA has a scaffold comprising or consisting of a sequence selected from the group consisting of SEQ ID NOs: 2238-2285, 26794- 26839 and 27219-27265 of PCT Application Publication No. WO2022120094A2. In certain embodiments, the Class 2 Type V CRISPR protein is a CasX variant protein comprising a sequence selected from the group consisting of SEQ ID NOS: 59, 72-99, 101-148, and 26908-27154 of PCT Application Publication No. WO2022120094A2, or a sequence having at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 95%, or at least about 95%, or at least about 96% , or at least about 97%, or at least about 98%, or at least about 99% sequence identity thereto. In certain embodiments, the CasX variant protein consists of a sequence selected from the group consisting of SEQ ID NOS: 126, 27043, 27046, 27050 of PCT Application Publication No. WO2022120094A2. In certain embodiments, the CasX variant protein comprises at least one modification relative to a reference CasX protein having a sequence selected from SEQ ID NOS: 1-3 of PCT Application Publication No. WO2022120094A2. In certain embodiments, the at least one modification comprises at least one amino acid substitution, deletion, or substitution in a domain of the CasX variant protein relative to the reference CasX protein.

[00784]In certain embodiments, an LNP of the present disclosure comprises one or more single-molecule guide RNA (sgRNAs), wherein at least one of the one or more sgRNAs comprises (a) the nucleic acid sequence of any one of SEQ ID NOs: 13-18 of US Patent No. 11,268,077 or (b) a spacer sequence comprising an RNA sequence corresponding to any one of SEQ ID NOs: 6-8 of US Patent No. 11,268,077 or the nucleic acid sequence of any one of SEQ ID NOs: 19-21 of US Patent No. 11,268,077. In certain embodiments, the LNP further comprises one or more DNA endonucleases, or polynucleotides encoding the same. In certain embodiments, the one or more DNA endonucleases comprises a Cas9 endonuclease, or polynucleotides encoding the same. In certain embodiments, the one or more DNA endonucleases each comprise, at the N-terminus, the C-terminus, or both the N-terminus and C-terminus, one or more nuclear localization signals (NLSs), optionally wherein the one or more DNA endonucleases each comprise two NLSs, one NLS located at the N-terminus and the second NLS located at the C-terminus, further optionally wherein the one or more NLSs is a SV40

NLS. In certain embodiments, the sgRNA comprises the nucleic acid sequence of any one of SEQ ID NO: 13-18 of US Patent No. 11,268,077 and the DNA endonuclease is a S. pyogenes Cas9 comprising a N-terminus SV40 NLS and a C-terminus SV40 NLS.

[00785]In certain embodiments, an LNP of the present disclosure comprises one or more deoxyribonucleic acid (DNA) endonucleases capable of effecting two or more single-strand breaks (SSB) or double-strand breaks (DSB), a first SSB or DSB within or near a human beta globin locus on chromosome 11, and a second SSB or DSB within or near one or more of: a BCL11A gene on chromosome 2, and a DNA sequence that encodes a transcriptional control region of the BCL11A gene on chromosome 2. In certain embodiments, the first SSB or DSB results in one or more of: a permanent deletion or inversion within or near the human beta globin locus on chromosome 11, and a mutation within or near the human beta globin locus on chromosome 11. In certain embodiments, the second SSB or DSB results in one or more of: a permanent deletion or inversion within or near the BCL11A gene on chromosome 2, a mutation within or near the BCL11A gene on chromosome 2, a permanent deletion or inversion within or near the DNA sequence that encodes a transcriptional control region of the BCL11A gene on chromosome 2, and a mutation within or near the DNA sequence that encodes a transcriptional control region of the BCL11A gene on chromosome 2. In certain embodiments, the two or more permanent deletions, inversions, or mutations result in an increased expression of γ-globin and an increase of fetal hemoglobin (HbF) in the human cell. Such endonucleases are generally described in US Patent Application Publication US20210180091A1, which is incorporated by reference herein in its entirety. In certain embodiments, the LNP further comprises one or more guide RNA (gRNA). In certain embodiments, the one or more gRNA sequences are selected from those provided in US Patent Application Publication US20210180091A1 Table 2, optionally wherein the one or more gRNAs comprise the sequence of SPY101 in combination with the sequence of any one or more of HPFH5-1, HPFH5-D, HPFH5-T5, HPFH5-T7, Kenya-K5, or Kenya-K1; or optionally wherein the one or more gRNAs comprise the sequence of 1450 in combination with the sequence of any one or more of HPFH5-1, HPFH5-D, HPFH5-T5, HPFH5-T7, Kenya-K5, or Kenya-K1; or optionally wherein the one or more gRNAs comprise the sequence of SPY101 in combination with the sequence of SD2; or optionally wherein the one or more gRNAs comprise the sequence of 1450 in combination with the sequence of SD2; or optionally wherein the one or more gRNAs comprise the sequence of HPFH5-1, HPFH5-D, HPFH5-T5, HPFH5-T7, Kenya-K5, or Kenya-K1 in combination with the sequence of any one or more of HPFH5-1, HPFH5-D, HPFH5-T5, HPFH5-T7, Kenya-K5, or Kenya-K1.

[00786]In certain embodiments, an LNP of the present disclosure comprises a guide RNA (gRNA) molecule comprising, from 5′ to 3′, [targeting domain]-SEQ ID NO: 195, wherein the targeting domain comprises SEQ ID NO: 67, wherein the referenced sequences are those disclosed in US Patent 11,466,271. In certain embodiments, said SEQ ID NO: 195 is disposed immediately 3′ to said targeting domain. In certain embodiments, an LNP of the present disclosure comprises a guide RNA

(gRNA) molecule comprising, from 5′ to 3′, [targeting domain]-SEQ ID NO: 231, wherein the targeting domain comprises SEQ ID NO: 67, wherein the referenced sequences are those disclosed in US Patent 11,466,271. In certain embodiments, said SEQ ID NO: 231 is disposed immediately 3′ to said targeting domain. In certain embodiments, the gRNA further comprises, at the 3′ end, 1, 2, 3, 4, 5, 6 or 7 uracil (U) nucleotides. In certain embodiments, the LNP further comprises a CRISPR system capable of utilizing the gRNA to form an indel at or near a target sequence complementary to the targeting domain of the gRNA. In certain embodiments, the indel is formed in a region selected from the HBG1 promoter region, the HBG2 promoter region. In certain embodiments the CRISPR system comprises a Cas9. In certain embodiments, the gRNA comprises a sequence selected from SEQ ID NOs: 174, 175 and 176 of US Patent 11,466,271. In certain embodiments, the Cas9 molecule comprises a sequence selected from SEQ ID NOs: 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, and 244.

[00787]In certain embodiments, the LNP comprises a gRNA comprising a tracr and crRNA, wherein the crRNA comprises a targeting domain that:

a) is complementary with a target sequence within the genomic nucleic acid sequence at Chrl 1:5,250,094-5,250,237, - strand, hg38;

b) is complementary with a target sequence within the genomic nucleic acid sequence at Chrl l:5,255,022-5,255,164, - strand, hg38;

c) is complementary with a target sequence of a non-deletional HFPH region (e.g., a human nondeletional HPFH region);

d) is complementary with a target sequence within the genomic nucleic acid sequence at Chrl 1:5,249,833 to Chrl 1:5,250,237, - strand, hg38;

e) is complementary with a target sequence within the genomic nucleic acid sequence at Chrl 1:5,254,738 to Chrl 1:5,255, 164, - strand, hg38;

f) is complementary with a target sequence within the genomic nucleic acid sequence at Chrl 1 : 5,249,833-5,249,927, - strand, hg38;

g) is complementary with a target sequence within the genomic nucleic acid sequence at Chrl 1 : 5,254,738-5,254,851, - strand, hg38;

h) is complementary with a target sequence within the genomic nucleic acid sequence at Chrl 1:5,250, 139-5,250,237, - strand, hg38; or

i) combinations thereof.

[00788]In certain embodiments, the gRNA comprises a sequence of any one of SEQ ID NOs. 1-72 of US Patent 11,466,271, or a fragment thereof.

[00789]In certain embodiments, an LNP of the present disclosure comprises a gene editing system capable of targeting the HBG1 promoter (Chrl 1 :5,249,833-5,250,237 according to hg38) and/or HBG2 promoter (Chrl 1 :5,254,738- 5,255,164 according to hg38). In certain embodiments, the gene editing system targets a targeting locus selected from the HPFH region, the AAVS1 locus, the

BCL11a gene, and the BCL11a enhancer region. In certain embodiments, the target locus is: a) the +55 region of the BCL1 1a enhancer (Chr2:60497676-60498941 according to hg38); b) the +58 region of the BCL1 1a enhancer (Chr2:60494251-60495546 according to hg38); or c) the +62 region of the BCL1 1a enhancer (Chr2:60490409-60491734 according to hg38). In certain embodiments, the gene editing system comprises a) a zinc finger nuclease (ZFN) system; b) a TALEN system; c) a meganuclease system; or d) a CRISPR system. In certain embodiments, the gene editing system comprises a CRISPR system comprising a gRNA molecule comprising a targeting domain complementary to any one of SEQ ID NO: 1 to 161, and 197 of PCT Publication WO2017/077394, or any one of SEQ ID NO: 1 to 135 of PCT Publication WO2016/182917. In certain embodiments, the gene editing system comprises a ZFN system comprising a targeting domain complementary to any one of SEQ ID NO: 63-80 and 232-251 of PCT Publication WO2015/073683. In certain embodiments, the gene editing system comprises a TALEN system comprising a targeting domain complementary to any one of SEQ ID NO: 7-11, 16-62, and 143-184 of PCT Publication WO2015/073683. In certain embodiments, the target sequence is a target sequence identified in Table 6 of US Application Publication US20200140896A1, and the gene editing system is a CRISPPv gene editing system comprising a gRNA molecule comprising a targeting domain listed in Table 6 of US Application Publication US20200140896A1. All patent application publication references in the above paragraph are incorporated by reference herein in their entirety.

[00790]In certain embodiments, an LNP of the present disclosure comprises a gRNA molecule comprising a tracr and crRNA, wherein the crRNA comprises a targeting domain that is complementary with a target sequence of a BCL11A gene (e.g., a human BCL11a gene), a BCL11a enhancer (e.g., a human BCL11a enhancer), or a HFPH region (e.g., a human HPFH region). In certain embodiments, the target sequence is of the BCL11A gene, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 1 to SEQ ID NO: 85 or SEQ ID NO: 400 to SEQ ID NO: 1231 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is of a BCL11a enhancer, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 1232 to SEQ ID NO: 1499 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is of a BCL11a enhancer, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 182 to SEQ ID NO: 277 or SEQ ID NO: 334 to SEQ ID NO: 341 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 341, SEQ ID NO: 246, SEQ ID NO: 248, SEQ ID NO: 247, SEQ ID NO: 245, SEQ ID NO: 249, SEQ ID NO: 244, SEQ ID NO: 199, SEQ ID NO: 251, SEQ ID NO: 250, SEQ ID NO: 334, SEQ ID NO: 185, SEQ ID NO: 186, SEQ ID NO: 336, or SEQ ID NO: 337 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 247, SEQ ID NO: 248, SEQ ID NO: 335, SEQ ID NO: 336, SEQ ID NO: 337, or SEQ ID NO: 338 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain

comprises, e.g., consists of, any one of SEQ ID NO: 248 or SEQ ID NO: 338 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is a BCL11a enhancer, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 278 to SEQ ID NO: 333.

[00791]In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 318, SEQ ID NO: 312, SEQ ID NO: 313, SEQ ID NO: 294, SEQ ID NO: 310, SEQ ID NO: 319, SEQ ID NO: 298, SEQ ID NO: 322, SEQ ID NO: 311, SEQ ID NO: 315, SEQ ID NO: 290, SEQ ID NO: 317, SEQ ID NO: 309, SEQ ID NO: 289, or SEQ ID NO: 281 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, SEQ ID NO: 318 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is of a BCL11a enhancer, and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 1596 to SEQ ID NO: 1691 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 1683, SEQ ID NO: 1638, SEQ ID NO: 1647, SEQ ID NO: 1609, SEQ ID NO: 1621, SEQ ID NO: 1617, SEQ ID NO: 1654, SEQ ID NO: 1631, SEQ ID NO: 1620, SEQ ID NO: 1637, SEQ ID NO: 1612, SEQ ID NO: 1656, SEQ ID NO: 1619, SEQ ID NO: 1675, SEQ ID NO: 1645, SEQ ID NO: 1598, SEQ ID NO: 1599, SEQ ID NO: 1663, SEQ ID NO: 1677, or SEQ ID NO: 1626 of US Application Publication US20190010495A1. In certain embodiments, the target sequence is of a HFPH region (e.g., a French HPFH region), and the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 86 to SEQ ID NO: 181, SEQ ID NO: 1500 to SEQ ID NO: 1595, or SEQ ID NO: 1692 to SEQ ID NO: 1761 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 100, SEQ ID NO: 165, SEQ ID NO: 113, SEQ ID NO: 99, SEQ ID NO: 112, SEQ ID NO: 98, SEQ ID NO: 1580, SEQ ID NO: 106, SEQ ID NO: 1503, SEQ ID NO: 1589, SEQ ID NO: 160, SEQ ID NO: 1537, SEQ ID NO: 159, SEQ ID NO: 101, SEQ ID NO: 162, SEQ ID NO: 104, SEQ ID NO: 138, SEQ ID NO: 1536, SEQ ID NO: 1539, or SEQ ID NO: 1585 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 1505, SEQ ID NO: 1589, SEQ ID NO: 1700, or SEQ ID NO: 1750 of US Application Publication US20190010495A1. In certain embodiments, the targeting domain comprises, e.g., consists of, any one of SEQ ID NO: 100, SEQ ID NO: 165, or SEQ ID NO: 113 of US Application Publication US20190010495A1. In certain embodiments, the gRNA comprises or consists of any guide RNA sequence, or any portion thereof, disclosed in US Application Publication US20190010495A1, which is incorporated by reference herein in its entirety.

[00792]In certain embodiments, an LNP of the present disclosure comprises an RNP complex comprising a first gRNA and a Cpf1 RNA-guided nuclease or a modified Cpf1 RNA-guided nuclease, wherein RNP complex is capable of generating indels in a population of unmodified CD34+ or hematopoietic stem cells. In certain embodiments, the first gRNA comprises a 5' end and a 3' end, a

DNA extension at the 5' end, and a 2'-O-methyl-3'-phosphorothioate modification at the 3' end. In certain embodiments, the DNA extension comprises a sequence set forth in SEQ ID NOs:1235-1250 of PCT Publication WO2021119040A1. In certain embodiments, the first gRNA comprises a targeting domain comprising SEQ ID NO:1254 of PCT Publication WO2021119040A1. In certain embodiments, the gRNA comprises SEQ ID NO:1051 of PCT Publication WO2021119040A1. In certain embodiments, the modified Cpf1 comprises SEQ ID NO:1097 of PCT Publication WO2021119040A1.

[00793]In certain embodiments, an LNP of the present disclosure comprises a gRNA molecule comprising a targeting domain that is complementary to a target domain from a gene expressed in a eukaryotic cell, wherein the gRNA molecule comprises a 3′ polyA tail comprising fewer than 30 adenine nucleotides. In certain embodiments, the polyA tail comprises fewer than 25 adenine nucleotides. In certain embodiments, the gRNA molecule lacks a 5′ triphosphate group. In certain embodiments, the gRNA molecule includes a 5′ cap. In certain embodiments, the gRNA molecule comprises a targeting domain and the 5′ end of the targeting domain includes a 5′ cap. In certain embodiments, the gRNA molecule contains one or more nucleotides that stabilize the gRNA molecule against nuclease degradation. In certain embodiments, the gRNA molecule contains one or more modified uridines. In certain embodiments, the gRNA molecule contains one or more modified adenosines. In certain embodiments, the gRNA molecule contains one or more modified cytidines. In certain embodiments, the gRNA molecule contains one or more modified guanosines. In certain embodiments, the phosphate backbone of the gRNA is modified. In certain embodiments, the phosphate backbone of the gRNA is modified with a phosphorothioate group. In certain embodiments, the gRNA comprises or consists of any guide RNA sequence, or any portion thereof, disclosed in US Application Publication US20230002760A1, which is incorporated by reference herein in its entirety. In certain embodiments, the LNP further comprises a CRISPR Cas9, or a polynucleotide encoding the same, disclosed in US Application Publication US20230002760A1, which is incorporated by reference herein in its entirety.

[00794]In certain embodiments, an LNP of the present disclosure comprises a first gRNA, a first enzymatically active Cas9 (eaCas9) (or a polynucleotide encoding the same), a second gRNA and a second eaCas9 (or a polynucleotide encoding the same). In certain embodiments, the first gRNA molecule and the first eaCas9 molecule associate with a target gene and generate a first single strand cleavage event on a first strand of the target gene, and the second gRNA molecule and the second eaCas9 molecule associate with the target gene and generate a second single strand cleavage event on a second strand of the target gene, thereby forming a double strand break having a first overhang and a second overhang. In certain embodiments, the first and second overhang are repaired by gene conversion using an endogenous homologous region. In certain embodiments, the first eaCas9 and/or the second eaCas9 are a nickase, optionally the same nickase or a different nickase. In certain embodiments, the first gRNA, the first eaCas9 (or a polynucleotide encoding the same), the second

gRNA and the second caCas9 (or a polynucleotide encoding the same) are capable of correcting a mutation, such as but not limited to a mutation in an endogenous hemoglobin beta (HBB) target gene. In certain embodiments, the first gRNA molecule is a gRNA molecule comprising any one of SEQ ID NOs: 387-485, 6803-6871, or 16010-16256, and wherein the second gRNA molecule is a gRNA molecule comprising any one of SEQ ID NOs: 387-485, 6803-6871, or 16010-16256, wherein the sequences are those disclosed in US Patent Application Publication US20230018543A1, which is incorporated by reference herein in its entirety.

[00795]In certain embodiments, an LNP of the present disclosure comprises a gene editing system described and disclosed in US Patent Application Publication US20210155927A1. In certain embodiments, the LNP comprises:

a) a ribonucleoprotein (RNP) complex comprising:

i) a class 2 CRISPR/Cas effector polypeptide, or a nucleic acid comprising a nucleotide sequence encoding the class 2 CRISPR/Cas effector polypeptide; and

ii) a guide RNA; and

b) a donor DNA template comprising a nucleotide sequence that provides for correction of the SCD-associated SNP in the globin gene.

[00796]In certain embodiments, the class 2 CRISPR/Cas effector polypeptide is selected from a type II, type V, or type VI CRISPR/Cas effector polypeptide, a Cpf1 protein, a C2c1 protein, a C2c3 protein, a C2c2 protein, a Cas12 enzyme, or a Cas13 enzyme or nucleic acid encoding any of the aforementioned proteins. In certain embodiments, the CRISPR/Cas guide RNA is a Cas9 guide RNA.

IV. Encoded products of payload mRNA (e.g., antigens and therapeutic proteins)

A. Polypeptides, peptides, and proteins

[00797]The LNP-based RNA vaccines and therapeutics described herein comprise one or more RNA payloads (e.g., linear or circular mRNA) which may comprise one or more coding regions that encode one or more products of interest. The one or more coding regions may encode a polypeptide, peptide and/or protein. As used herein, the term "polypeptide" generally refers to polymers of amino acids linked by peptide bonds and embraces "protein" and "peptides." Polypeptides for the present disclosure include all polypeptides, proteins and/or peptides known in the art. Non-limiting categories of polypeptides include antigens, antibodies, antibody fragments, cytokines, peptides, hormones, enzymes, oxidants, antioxidants, synthetic polypeptides, and chimeric polypeptides, receptor, enzymes, hormones, transcription factors, ligands, membrane transporters, structural proteins, nucleases, or a component, variant or fragment (e.g., a biologically active fragment) thereof.

[00798]As used herein, the term "peptide" generally refers to shorter polypeptides of about 50 amino acids or less. Peptides with only two amino acids may be referred to as "dipeptides." Peptides with only three amino acids may be referred to as "tripeptides." Polypeptides generally refer to polypeptides with from about 4 to about 50 amino acids. Peptides may be obtained via any method

known to those skilled in the art. In some embodiments, peptides may be expressed in culture. In some embodiments, peptides may be obtained via chemical synthesis (e.g., solid phase peptide synthesis).

[00799]In some embodiments, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest), e.g., the originator constructs and benchmark constructs described herein, may encode a simple protein which upon hydrolysis yields the amino acids and occasionally small carbohydrate compounds. Non-limiting examples of simple proteins include albumins, albuminoids, globulins, glutelins, histones and protamines.

[00800]In some embodiments, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest), e.g., the originator constructs and benchmark constructs described herein, may encode a simple protein associated with a non-protein. Non-limiting examples of conjugated proteins include, glycoproteins, hemoglobins, lecithoproteins, nucleoproteins, and phosphoproteins.

[00801]In some embodiments, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest), e.g., the originator constructs and benchmark constructs described herein, may encode a protein that is derived from a simple or conjugated protein by chemical or physical means. Non-limiting examples of derived proteins include denatured proteins and peptides.

[00802]In some embodiments, the polypeptide, protein or peptide may be unmodified.

[00803]In some embodiments, the polypeptide, protein or peptide may be modified. Types of modifications include, but are not limited to, phosphorylation, glycosylation, acetylation, ubiquitylation/sumoylation, methylation, palmitoylation, quinone, amidation, myristoylation, pyrrolidone carboxylic acid, hydroxylation, phosphopantetheine, prenylation, GPI anchoring, oxidation, ADP-ribosylation, sulfation, S-nitrosylation, citrullination, nitration, gamma-carboxyglutamic acid, formylation, hypusine, topaquinone (TPQ), bromination, lysine topaquinone (LTQ), tryptophan tryptophylquinone (TTQ), iodination, and cysteine tryptophylquinone (CTQ). In some aspects, the polypeptide, protein or peptide may be modified by a post-transcriptional modification which can affect its structure, subcellular localization, and/or function.

[00804]In some embodiments, the polypeptide, protein or peptide may be modified using phosphorylation. Phosphorylation, or the addition of a phosphate group to serine, threonine, or tyrosine residues, is one of most common forms of protein modification. Protein phosphorylation plays an important role in fine tuning the signal in the intracellular signaling cascades.

[00805]In some embodiments, the polypeptide, protein or peptide may be modified using ubiquitination which is the covalent attachment of ubiquitin to target proteins. Ubiquitination-mediated protein turnover has been shown to play a role in driving the cell cycle as well as in protein-degradation-independent intracellular signaling pathways.

[00806]In some embodiments, the polypeptide, protein or peptide may be modified using acetylation and methylation which can play a role in regulating gene expression. As a non-limiting example, the acetylation and methylation could mediate the formation of chromatin domains (e.g., euchromatin and heterochromatin) which could have an impact on mediating gene silencing.

[00807]In some embodiments, the polypeptide, protein or peptide may be modified using glycosylation.Glycosylation is the attachment of one of a large number of glycan groups and is a modification that occurs in about half of all proteins and plays a role in biological processes including, but not limited to, embryonic development, cell division, and regulation of protein structure. The two main types of protein glycosylation are N-glycosylation and O-glycosylation. For N-glycosylation the glycan is attached to an asparagine and for O-glycosylation the glycan is attached to a serine or threonine.

[00808]In some embodiments, the polypeptide, protein or peptide may be modified using sumoylation. Sumoylation is the addition of SUMOs (small ubiquitin-like modifiers) to proteins and is a post-translational modification similar to ubiquitination.

[00809]In other embodiments, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest), e.g., the originator constructs and benchmark constructs described herein, may encode a therapeutic protein, such as those exemplified below.

[00810]In other embodiments, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest), e.g., the originator constructs and benchmark constructs described herein, may encode vaccine antigen, such as those exemplified below. As used herein, a "vaccine antigen" is a biological preparation that improves immunity to a particular disease or infectious agent. According to the present disclosure, one or more vaccine antigens currently being marketed or in development may be encoded by the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest) described herein of the present disclosure.

B. Therapeutic proteins

[00811]In various embodiments, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest) may encode a therapeutic protein. Therapeutic proteins mediate a variety of effects in a host cell or a subject in order to treat a disease or ameliorate the signs and symptoms of a disease. For example, a therapeutic protein can replace a protein that is deficient or abnormal, augment the function of an endogenous protein, provide a novel function to a cell (e.g., inhibit or activate an endogenous cellular activity, or act as a delivery agent for another therapeutic compound (e.g., an antibody-drug conjugate). Therapeutic proteins may be useful for the treatment of the following diseases and conditions: bacterial infections, viral infections, parasitic infections, cell proliferation disorders, cancer, genetic disorders, and autoimmune disorders, among others, or utilized to treat conditions or diseases in many therapeutic areas such as, but not limited to, blood, cardiovascular, CNS, poisoning (including antivenoms), dermatology, endocrinology, genetic,

genitourinary, gastrointestinal, musculoskeletal, oncology, and immunology, respiratory, sensory and anti-infective. As used herein, a "therapeutic protein" refers to a protein that, when administered to a cell has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[00812]The RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest) may encode a therapeutic protein selected from any of several categories including, but not limited to, biologics, antibodies, vaccines, cell penetrating peptides, secreted proteins, plasma membrane proteins, cytoplasmic or cytoskeletal proteins, intracellular membrane bound proteins, nuclear proteins, proteins associated with human disease, targeting moieties or those proteins encoded by the human genome for which no therapeutic indication has been identified but which nonetheless have utility in areas of research and discovery. As used herein, a "biologic" is a polypeptide-based molecule produced by the methods provided herein and which may be used to treat, cure, mitigate, prevent, or diagnose a serious or life-threatening disease or medical condition. Biologics, according to the present disclosure include, but are not limited to, allergenic extracts (e.g., for allergy shots and tests), blood components, gene therapy products, human tissue or cellular products used in transplantation, vaccines, monoclonal antibodies, cytokines, growth factors, enzymes, thrombolytics, and immunomodulators, among others.

[00813]In various embodiments, the therapeutic proteins produced by the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest) may be a cell-penetrating polypeptide. As used herein, a "cell-penetrating polypeptide" refers to a polypeptide which may facilitate the cellular uptake of molecules. A cell-penetrating polypeptide of the present disclosure may contain one or more detectable labels. The polypeptides may be partially labeled or completely labeled throughout. The mRNA may encode the detectable label completely, partially or not at all. The cell-penetrating peptide may also include a signal sequence. As used herein, a "signal sequence" refers to a sequence of amino acid residues bound at the amino terminus of a nascent protein during protein translation. The signal sequence may be used to signal the secretion of the cell-penetrating polypeptide.

[00814]The cell-penetrating polypeptide encoded by the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest) described herein may form a complex after being translated. The complex may comprise a charged protein linked, e.g., covalently linked, to the cell-penetrating polypeptide.

[00815]In one embodiment, the cell-penetrating polypeptide encoded by the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest) described herein may comprise a first domain and a second domain. The first domain may comprise a supercharged polypeptide. The second domain may comprise a protein-binding partner. As used herein, "protein-binding partner" includes, but is not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. The cell-penetrating polypeptide may further comprise an

intracellular binding partner for the protein-binding partner. The cell-penetrating polypeptide may be capable of being secreted from a cell where the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more encoded products of interest) described herein may be introduced. The cell-penetrating polypeptide may also be capable of penetrating the first cell.

[00816]In one embodiment, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) described herein may encode a cell-penetrating polypeptide which may comprise a protein-binding partner. The protein binding partner may include, but is not limited to, an antibody, a supercharged antibody or a functional fragment. The RNA payloads may be introduced into the cell where a cell-penetrating polypeptide comprising the protein- binding partner is introduced.

[00817]In another embodiment, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) described herein may encode therapeutic proteins have organelle-sorting sequences. Human and other eukaryotic cells are subdivided by membranes into many functionally distinct compartments. Each membrane-bound compartment, or organelle, contains different proteins essential for the function of the organelle. The cell uses "sorting signals" which are amino acid motifs located within the protein, to target proteins to particular cellular organelles. One type of sorting signal, called a signal sequence, a signal peptide, or a leader sequence, directs a class of proteins to an organelle called the endoplasmic reticulum (ER). Proteins targeted to the ER by a signal sequence can be released into the extracellular space as a secreted protein. Similarly, proteins residing on the cell membrane can also be secreted into the extracellular space by proteolytic cleavage of a "linker" holding the protein to the membrane. While not wishing to be bound by theory, the molecules of the present disclosure may be used to exploit the cellular trafficking described above. As such, in some embodiments of the disclosure, therapeutic proteins are expressed as secreted proteins. In one embodiment, these may be used in the manufacture of large quantities of valuable human gene products.

[00818]In some embodiments of the disclosure, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) described herein may encode a protein of the plasma membrane.

[00819]In some embodiments of the disclosure, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) described herein may encode a cytoplasmic or cytoskeletal protein.

[00820]In some embodiments of the disclosure, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) described herein may encode an intracellular membrane bound protein.

[00821]In some embodiments of the disclosure, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) described herein may encode a nuclear protein.

[00822]Examples of therapeutic proteins that may be encoded by the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) described herein can include hormones and growth and differentiation factors including, without limitation, insulin, glucagon, growth hormone (GH), parathyroid hormone (PTH), growth hormone releasing factor (GRF), follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), vascular endothelial growth factor (VEGF), angiopoietins, angiostatin, granulocyte colony stimulating factor (GCSF), erythropoietin (EPO), connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), any one of the transforming growth factor alpha superfamily, including TGFα, activins, inhibins, or any of the bone morphogenic proteins (BMP) BMPs 1-15, any one of the heregluin/neuregulin/ARIA/neu differentiation factor (NDF) family of growth factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophies NT-3 and NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), neurturin, agrin, any one of the family of semaphorins/collapsins, netrin-1 and netrin-2, hepatocyte growth factor (HGF), ephrins, noggin, sonic hedgehog and tyrosine hydroxylase.

[00823]Examples of additional therapeutic proteins that may be encoded by the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) described herein can include, but are not limited to proteins that regulate the immune system including, without limitation, cytokines and lymphokines such as thrombopoietin (TPO), interleukins (IL) IL-1 through IL-25 (including IL-2, IL-4, IL-12 and IL-18), monocyte chemoattractant protein, leukemia inhibitory factor, granulocyte-macrophage colony stimulating factor, Fas ligand, tumor necrosis factors alpha and beta., interferons (alpha, beta, and gamma), stem cell factor, flk-2/flt3 ligand. Gene products produced by the immune system are also useful in the present disclosure. These include, without limitations, immunoglobulins IgG, IgM, IgA, IgD and IgE, chimeric immunoglobulins, humanized antibodies, single chain antibodies, T cell receptors, chimeric T cell receptors, single chain T cell receptors, class I and class II MHC molecules, as well as engineered immunoglobulins and MHC molecules. Useful gene products also include complement regulatory proteins such as complement regulatory proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF), CR1, CF2 and CD59.

[00824]Examples of still further therapeutic proteins that may be encoded by the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) described herein can include, in some embodiments; receptors for the hormones, growth factors, cytokines, lymphokines, regulatory proteins and immune system proteins; receptors for cholesterol regulation and/or lipid modulation, including the low density lipoprotein (LDL) receptor, high density lipoprotein (HDL) receptor, the very low density lipoprotein (VLDL) receptor, and scavenger receptors. The disclosure also encompasses the use of gene products such as members of the steroid

hormone receptor superfamily including glucocorticoid receptors and estrogen receptors, Vitamin D receptors and other nuclear receptors. In addition, useful gene products include transcription factors such as jun, fos, max, mad, serum response factor (SRF), AP-1, AP-2, myb, MyoD and myogenin, ETS-box containing proteins, TFE3, E2F, ATFL, ATF2, ATF3, ATF4, ZF5, NFAT, CREB, HNF-4, C/EBP, SP1, CCAAT-box binding proteins, interferon regulation factor (IRF-1), Wilms tumor protein, ETS-binding protein, STAT, GATA-box binding proteins, e.g., GATA-3, and the forkhead family of winged helix proteins.

[00825]In some embodiments, useful heterologous nucleic acid sequence products include, carbamoyl synthetase I, ornithine transcarbamylase, arginosuccinate synthetase, arginosuccinate lyase, arginase, fumarylacetacetate hydrolase, phenylalanine hydroxylase, alpha-1 antitrypsin, glucose-6-phosphatase, porphobilinogen deaminase, cystathione beta-synthase, branched chain ketoacid decarboxylase, albumin, isovaleryl-coA dehydrogenase, propionyl CoA carboxylase, methyl malonyl CoA mutase, glutaryl CoA dehydrogenase, insulin, beta-glucosidase, pyruvate carboxylate, hepatic phosphorylase, phosphorylase kinase, glycine decarboxylase, H-protein, T-protein, a cystic fibrosis transmembrane regulator (CFTR) sequence, and a dystrophin cDNA sequence. Still other useful gene products include enzymes useful in enzyme replacement therapy, and which are useful in a variety of conditions resulting from deficient activity of enzyme. For example, enzymes containing mannose-6-phosphate may be utilized in therapies for lysosomal storage diseases (e.g., a suitable gene includes that encoding β-glucuronidase (GUSB)).

Exemplary therapeutic proteins

[00826]In some embodiments, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) and associated compositions and methods described herein provide for the delivery of one or more therapeutic proteins chosen from the proteins listed in Table (IV), or the delivery of one or more homologs of the therapeutic proteins of Table (IV).

Table (IV) – *Exemplary therapeutic proteins encoded by RNA payloads*

A1E959, Odontogenic ameloblast associated protein, ODAM
A1KZ92, Peroxidasin like protein, PXDN L
A1L453, Serine protease 38, PRSS38
A1L4H1, Soluble scavenger receptor cysteine-rich domain-containing protein, SSC5D
A2RUU4, Colipase-like protein 1, CLPSL1
A2VDF0, Fucose mutarotase, FUOM
A2VEC9, SCO-spondin, SSPO
A3KMH 1, von Willebrand factor A domain-containing protein 8, VWA8
A4D0S4, Laminin subunit beta-4, LAMB
A4D1T9, Probable inactive serine protease 37, PRSS37
A5D8T8, C-type lectin domain family 18 member A, CLEC18A
A6NC86, phospholipase A2 inhibitor and Ly6/PLAUR domain-containing protein, PINLYP
A6NCI4, von Willebrand factor A domain containing protein 3A, VWA3A
A6ND01, Probable folate receptor delta, FOLR4
A6NE02, BTB/POZ domain-containing protein 17, BTBD17
A6NEF6, Growth hormone 1, GH1
A6NF02, NPIP-like protein, LOC730153
A6NFB4, HCG 1749481, isoform CRA_k, CSH1
A6NFZ4, Protein FAM24A, FAM24A
A6NG13, Glycosyltransferase 54 domain-containing Protein
A6NGN9, IgLON family member 5, IGLON5
A6NHN0, Otolin-1, OTOL1
A6NHN6, Nuclear pore complex-interacting protein-like 2, NPIPL2
A6NI73 Leukocyte immunoglobulin-like receptor subfamily A member 5, LILRA5
A6NIT4 Chorionic somatomammotropin hormone 2 isoform 2, CSH2
A6NJ69 IgA-inducing protein homolog, *IGIP*
A6NKQ9 Choriogonadotropin subunit beta variant 1 CGB1
A6NMZ7 Collagen alpha-6(VI) chain COL6A6
A6NNS2 Dehydrogenase/reductase SDR family DHRS7C member 7C
A6XGL2 Insulin A chain INS
A8K0G1 Protein Wnt WNT7B
A8K2U0 Alpha-2-macroglobulin-like protein 1 A2ML1
A8K7I4 Calcium-activated chloride channel CLCA1 regulator 1
A8MTL9 Serpin-like protein HMSD HMSD
A8MV23 Serpin E3, SERPINE3
A8MZH6 Oocyte-secreted protein 1 homolog, OOSP1
A8TX70 Collagen alpha-5(VI) chain, COL6A5
B0ZBE8 Natriuretic peptide, NPPA
B1A4G9 Somatotropin, GH1
B1A4H2 HCG 1749481, isoform CRA_d, CSH1
B1A4H9 Chorionic somatomammotropin hormone, CSH2
B1AJZ6 Protein Wnt, WNT4
B1AKI9 Isthmin-1, ISM1
B2RN N3 Complement Clq and tumor necrosis F9B factor-related protein 9B, C1QTN
B2RUY7 von Willebrand factor C domain-containing protein 2-like, VWC2L
B3GLJ2 Prostate and testis expressed protein 3, PATE3

B4DI03 SECll-like 3 (S. cerevisiae), SEC11L3
B4DJF9 Protein Wn,t WNT4
B4DUL4 SECll-like 1 (S. cerevisiae), isoform CRA_d, SEC11L1
B5MCC8 Protein Wnt, WNT10B
B8A595 Protein Wnt,WNT7B
B8A597 Protein Wnt, WNT7B
B8A598 Protein Wnt, WNT7B
B9A064 Immunoglobulin lambda-like polypeptide5, IGLL5
C9J3H3 Protein Wnt, WNT10B
C9J8I8 Protein Wnt, WNT5A
C9JAF2 Insulin-like growth factor II Ala-25 Del, IGF2
C9JCI2 Protein Wnt, WNT10B
C9JL84 HERV-H LTR-associating protein 1, HHLA1
C9JN R5 Insulin A chain, INS
C9JU I2 Protein Wnt, WNT2
D6RF47 Protein Wnt, WNT8A
D6RF94 Protein Wnt, WNT8A
E2RYF7 Protein PBMUCL2, HCG22
E5RFR1 PEN K(114-133), PENK
E7EM L9 Serine protease 44, PRSS44
E7EPC3 Protein Wnt, WNT9B
E7EVP0 Nociceptin, PNOC
E9PD02 Insulin-like growth factor 1, IGF1
E9PH60 Protein Wnt, WNT16
E9PJ L6 Protein Wnt, WNT11
F5GYM2 Protein Wnt, WNT5B
F5H034 Protein Wnt, WNT5B
F5H364 Protein Wnt, WNT5B
F5H7Q6 Protcin Wnt, WNT5B
F8WCM5 Protein INS-IGF2, INS-IGF2
F8WDR1 Protein Wnt, WNT2
H0Y663 Protein Wnt, WNT4
H0YK72 Signal peptidase complex catalytic subunit, SEC11A
H0YK83 Signal peptidase complex catalytic subunit, SEC11A
H0YM39 Chorionic somatomammotropin hormone, CSH2
H0YMT7 Chorionic somatomammotropin hormone, CSH1
H0YN 17 Chorionic somatomammotropin hormone, CSH2
H0YNA5 Signal peptidase complex catalytic subunit SEC11A
H0YNG3 Signal peptidase complex catalytic subunit SEC11A
H0YNX5 Signal peptidase complex catalytic subunit SEC11A
H7BZB8 Protein Wnt. WNT10A
H9KV56 Choriogonadotropin subunit beta variant 2, CGB2
I3L0L8 Protein Wnt. WNT9B
J3KNZ1 Choriogonadotropin subunit beta variant 1, CGB1
J3KP00 Choriogonadotropin subunit beta. CGB7
J3QT02 Choriogonadotropin subunit beta variant 1, CGB1
000175 C-C motif chemokine 24, CCL24
000182 Galectin-9, LGALS9
000187 Mannan-binding lectin serine protease 2, MASP2
000230 Cortistatin, CORT
000253 Agouti-related protein, AGRP

000270 12-(S)-hydroxy-5,8,10,14-eicosatetraenoic acid receptor, GPR31
000292 Left-right determination factor 2, LEFTY2
000294 Tubby-related protein 1, TULP1
000295 Tubby-related protein 2, TULP2
000300 Tumor necrosis factor receptor superfamily member 11B, TNFRSF11B
000339 Matrilin-2, MATN2
000391 Sulfhydryl oxidase 1, QSOX1
000468 Agrin, AGRN
000515 Ladinin-1, LAD1
000533 Processed neural cell adhesion molecule Ll-like protein, CHL1
000584 Ribonuclease T2, RNASET2
000585 C-C motif chemokine 21, CCL21
000602 Ficolin-1, FCN1
000622 Protein CYR61, CYR61
000626 MDC(5-69), CCL22
000634 Netrin-3, NTN3
000744 Protein Wnt-10b, WNT10B
000755 Protein Wnt-7a, WNT7A
014498 Immunoglobulin superfamily containing leucine-rich repeat protein, ISLR
014511 Pro-neuregulin-2, membrane-bound isoform, NRG2
014594 Neurocan core protein, NCAN
014625 C-X-C motif chemokine 11, CXCL11
014638 Ectonucleotide pyrophosphatase/phosphodiesterase family member 3, ENPP3
014656 Torsin-IA, TOR1A
014657 Torsin-IB, TOR1B
014786 Neuropilin-1, NRP1
014788 Tumor necrosis factor ligand superfamily member 11, membrane form, TNFSF11
014791 Apolipoprotein LI, APOL1
014793 Growth/differentiation factor 8, MSTN
014904 Protein Wnt-9a, WNT9A
014905 Protein Wnt-9b, WNT9B
014944 Proepiregulin, EREG
014960 Leukocyte cell-derived chemotaxin-2, LECT2
015018 Processed PDZ domain-containing protein 2, PDZD2
015041 Semaphorin-3E, SEMA3E
015072 A disintegrin and metalloproteinase with thrombospondin motifs 3, ADAMTS3
015123 Angiopoietin-2, ANGPT2
015130 Neuropeptide FF, NPFF
015197 Ephrin type-B receptor 6, EPHB6
015230 Laminin subunit alpha-5, LAMA5
015232 Matrilin-3, MATN3
015240 Neuroendocrine regulatory peptide-1, VGF
015263 Beta-defensin 4A, DEFB4A
015335 Chondroadherin, CHAD
015393 Transmembrane protease serine 2 catalytic chain, TMPRSS2
015444 C-C motif chemokine 25, CCL25
015467 C-C motif chemokine 16, CCL16
015496 Group 10 secretory phospholipase A2, PLA2G10
015520 Fibroblast growth factor 10, FGF10
015537 Retinoschisin, RSI
043157 Plexin-Bl, PLXN B1
043184 Disintegrin and metalloproteinase domain-containing protein 12, ADAM12

043240 Kallikrein-10, KLK10
043278 Kunitz-type protease inhibitor 1, SPINT1
043320 Fibroblast growth factor 16, FGF16
043323 Desert hedgehog protein C-product, DHH
043405 Cochlin, COCH
043508 Tumor necrosis factor ligand superfamily member 12, membrane form, TNFSF12
043555 Progonadoliberin-2, GN RH2
043557 Tumor necrosis factor ligand superfamily member 14, soluble form, TNFSF14
043692 Peptidase inhibitor 15, PI15
043699 Sialic acid-binding Ig-like lectin 6, SIGLEC6
043820 Hyaluronidase-3, HYAL3
043827 Angiopoietin-related protein 7, ANGPTL7
043852 Calumenin, CALU
043854 EGF-like repeat and discoidin l-like domain-containing protein 3, EDIL3
043866 CD5 antigen-like, CD5L
043897 Tolloid-like protein 1, TLL1
043915 Vascular endothelial growth factor D, FIGF
043927 C-X-C motif chemokine 13, CXCL13
060218 Aldo-keto reductase family 1 member, AKR1B10
060235 Transmembrane protease serine 11D, TMPRSS11D
060258 Fibroblast growth factor 17, FGF17
060259 Kallikrein-8, KLK8
060383 Growth/differentiation factor 9, GDF9
060469 Down syndrome cell adhesion molecule, DSCAM
060542 Persephin, PSPN
060565 Gremlin-1, GREM1
060575 Serine protease inhibitor Kazal-type 4, SPIN K4
060676 Cystatin-8, CST8
060687 Sushi repeat-containing protein, SRPX2
060844 Zymogen granule membrane protein 16, ZG16
060882 Matrix metalloproteinase-20, MMP20
060938 Keratocan, KERA
075015 Low affinity immunoglobulin gamma Fc region receptor lll-B, FCGR3B
075077 Disintegrin and metalloproteinase domain-containing protein 23, ADAM23
075093 Slit homolog 1 protein, SLIT1
075094 Slit homolog 3 protein, SLIT3
075095 Multiple epidermal growth factor-like domains protein 6, MEGF6
075173 A disintegrin and metalloproteinase with thrombospondin motifs 4, ADAMTS4
075200 Nuclear pore complex-interacting protein-like 1, NPIPL1
075339 Cartilage intermediate layer protein 1, CI CILP
075354 Ectonucleoside triphosphate diphosphohydrolase 6, ENTPD6
075386 Tubby-related protein 3, TULP3
075398 Deformed epidermal autoregulatory factor 1 homolog, DEAF1
075443 Alpha-tectorin, TECTA
075445 Usherin, USH2A
075462 Cytokine receptor-like factor 1, CRLF1
075487 Glypican-4, GPC4

075493 Carbonic anhydrase-related protein 11, CA11
075594 Peptidoglycan recognition protein 1, PGLYRP1
075596 C-type lectin domain family 3 member A, CLEC3A
075610 Left-right determination factor 1, LEFTY1
075629 Protein CREG1, CREG1
075636 Ficolin-3, FCN3
075711 Scrapie-responsive protein 1, SCRG1
075715 Epididymal secretory glutathione peroxidase, GPX5
075718 Cartilage-associated protein, CRTAP
075829 Chondrosurfactant protein, LECT1
075830 Serpin 12, SERPINI2
075882 Attractin, ATRN
075888 Tumor necrosis factor ligand superfamily member 13, TNFSF13
075900 Matrix metalloproteinase-23, MMP23A
075951 Lysozyme-like protein 6, LYZL6
075973 Clq-related factor, C1QL1
076038 Secretagogin, SCGN
076061 Stanniocalcin-2, STC2
076076 WNTl-inducible-signaling pathway protein 2, WISP2
076093 Fibroblast growth factor 18, FGF18
076096 Cystatin-F, CST7
094769 Extracellular matrix protein 2, ECM2
094813 Slit homolog 2 protein C-product, SLIT2
094907 Dickkopf-related protein 1, DKK1
094919 Endonuclease domain-containing 1 protein, ENDOD1
094964 N-terminal form, SOGA1
095025 Semaphorin-3D, SEMA3D
095084 Serine protease 23, PRSS23
095150 Tumor necrosis factor ligand superfamily member 15, TNFSF15
095156 Neurexophilin-2, NXPH2
095157 Neurexophilin-3, NXPH3
095158 Neurexophilin-4 ,NXPH4
095388 WNTl-inducible-signaling pathway protein 1, WISP1
095389 WNTl-inducible-signaling pathway protein 3, WISP3
095390 Growth/differentiation factor 11, GDF11
095393 Bone morphogenetic protein 10, BMP10
095399 Urotensin-2, UTS2
095407 Tumor necrosis factor receptor superfamily member 6B, TNFRSF6B
095428 Papilin, PAPLN
095445 Apolipoprotein M, APOM
095450 A disintegrin and metalloproteinase with thrombospondin motifs 2, ADAMTS2
095460 Matrilin-4, MATN4
095467 LHAL tetrapeptide, GNAS
095631 Netrin-1, NTN1
095633 Follistatin-related protein 3, FSTL3
095711 Lymphocyte antigen 86, LY86
095715 C-X-C motif chemokine 14, CXCL14
095750 Fibroblast growth factor 19, FGF19
095760 Interleukin-33, IL33
095813 Cerberus, CER1
095841 Angiopoietin-related protein 1, ANGPTL1
095897 Noelin-2, OLFM2
095925 Eppin, EPPIN
095965 Integrin beta-like protein 1, ITGBL1

095967 EGF-containing fibulin-like extracellular matrix protein 2, EFEMP2
095968 Secretoglobin family ID member 1, SCGB1D1
095969 Secretoglobin family ID member 2, SCGB1D2
095970 Leucine-rich glioma-inactivated protein 1, LGI1
095972 Bone morphogenetic protein 15, BMP15
095994 Anterior gradient protein 2 homolog, AGR2
095998 Interleukin-18-binding protein, IL18BP
096009 Napsin-A, NAPSA
096014 Protein Wnt-11, WNT11
P00450 Ceruloplasmin CP
P00451 Factor Villa light chain F8
P00488 Coagulation factor XIII A chain, F13A1
P00533 Epidermal growth factor receptor, EGFR
P00709 Alpha-lactalbumin LA LB A
P00734 Prothrombin F2
P00738 Haptoglobin beta chain HP
P00739 Haptoglobin-related protein HPR
P00740 Coagulation factor IXa heavy chain F9
P00742 Factor X heavy chain F10
P00746 Complement factor D CFD
P00747 Plasmin light chain B PLG
P00748 Coagulation factor XI la light chain F12
P00749 Urokinase-type plasminogen activator long chain A, PLAU
P00750 Tissue-type plasminogen activator, PLAT
P00751 Complement factor B Ba fragment, CFB
P00797 Renin, REN
P00973 2'-5'-oligoadenylate synthase 1, OAS1
P00995 Pancreatic secretory trypsin inhibitor, SPIN K1
P01008 Antithrombin-lll, SERPINC1
P01009 Alpha-l-antitrypsin, SERPINA1
P01011 Alpha-l-antichymotrypsin His-Pro-less, SERPINA3
P01019 Angiotensin-1, AGT
P01023 Alpha-2-macroglobulin, A2M
P01024 Acylation stimulating protein, C3
P01031 Complement C5 beta chain, C5
P01033 Metalloproteinase inhibitor 1, TIM P1
P01034 Cystatin-C, CST3
P01036 Cystatin-S, CST4
P01037 Cystatin-SN, CST1
P01042 Kininogen-1 light chain, KNG1
P01127 Platelet-derived growth factor subunit B, PDGFB
P01135 Transforming growth factor alpha, TGFA
P01137 Transforming growth factor beta-1, TGFB1
P01138 Beta-nerve growth factor, NGF
P01148 Gonadoliberin-1, GN RH 1
P01160 Atrial natriuretic factor, NPPA
P01178 Oxytocin, OXT
P01185 Vasopressin-neurophysin 2-copeptin, AVP
P01189 Corticotropin, POMC
P01210 PEN K(237-258), PENK
P01213 Alpha-neoendorphin, PDYN
P01215 Glycoprotein hormones alpha chain, CGA
P01222 Thyrotropin subunit beta, TSHB
P01225 Follitropin subunit beta, FSHB
P01229 Lutropin subunit beta, LHB

P01233 Choriogonadotropin subunit beta, CGB8
P01236 Prolactin, PRL
P01241 Somatotropin, GH1
P01242 Growth hormone variant, GH2
P01243 Chorionic somatomammotropin hormone, CSH2
P01258 Katacalcin, CALCA
P01266 Thyroglobulin, TG
P01270 Parathyroid hormone, PTH
P01275 Glucagon, GCG
P01282 Intestinal peptide, PHM-27 VIP
P01286 Somatoliberin, GH RH
P01298 Pancreatic prohormone, PPY
P01303 C-flanking peptide of N PY, NPY
P01308 Insulin, INS
P01344 Insulin-like growth factor, II IGF2
P01350 Big gastrin, GAST
P01374 Lymphotoxin-alpha, LTA
P01375 C-domain 1, TNF
P01562 Interferon alpha-1/13 IFNA1
P01563 Interferon alpha-2 IFNA2
P01566 Interferon alpha-10 IFNA10
P01567 Interferon alpha-7 IFNA7
P01568 Interferon alpha-21 IFNA21
P01569 Interferon alpha-5 IFNA5
P01570 Interferon alpha-14 IFNA14
P01571 Interferon alpha-17 IFNA17
P01574 Interferon beta IFNB1
P01579 Interferon gamma IFNG
P01583 Interleukin-1 alpha ILIA
P01584 Interleukin-1 beta IL1B
P01588 Erythropoietin EPO
P01591 Immunoglobulin J chain IGJ
P01732 T-cell surface glycoprotein CD8 alpha CD8A chain
P01833 Polymeric immunoglobulin receptor PIGR
P01857 Ig gamma-1 chain C region IGHG1
P01859 Ig gamma-2 chain C region IGHG2
P01860 Ig gamma-3 chain C region IGHG3
P01861 Ig gamma-4 chain C region IGHG4
P01871 Ig mu chain C region IGHM
P01880 Ig delta chain C region IGHD
P02452 Collagen alpha-1(I) chain COL1A1
P02458 Chondrocalcin COL2A1
P02461 Collagen alpha-1(III) chain COL3A1
P02462 Collagen alpha-1(IV) chain COL4A1
P02647 Apolipoprotein A-I APOA1
P02649 Apolipoprotein E APOE
P02652 Apolipoprotein A-II APOA2
P02654 Apolipoprotein C-I APOC1
P02655 Apolipoprotein C-II APOC2
P02656 Apolipoprotein C-III APOC3
P02671 Fibrinogen alpha chain FGA
P02675 Fibrinopeptide B FGB
P02679 Fibrinogen gamma chain FGG
P02741 C-reactive protein CRP
P02743 Serum amyloid P-component(1-203) APCS
P02745 Complement C1q subcomponent subunit A, C1QA
P02746 Complement C1q subcomponent subunit B, C1QB
P02747 Complement C1q subcomponent subunit C, C1QC
P02748 Complement component C9b C9
P02749 Beta-2-glycoprotein 1 APOH

P02750 Leucine-rich alpha-2-glycoprotein LRG1
P02751 Ugl-Y2 FN 1
P02753 Retinol-binding protein 4 RBP4
P02760 Trypstatin AMBP
P02763 Alpha-l-acid glycoprotein 1 ORM1
P02765 Alpha-2-HS-glycoprotein chain A AHSG
P02766 Transthyretin TTR
P02768 Serum albumin ALB
P02771 Alpha-fetoprotein AFP
P02774 Vitamin D-binding protein GC
P02775 Connective tissue-activating peptide III PPBP
P02776 Platelet factor 4 PF4
P02778 CXCL10(l-73) CXCL10
P02786 Transferrin receptor protein 1 TFRC
P02787 Serotransferrin TF
P02788 Lactoferroxin-C LTF
P02790 Hemopexin HPX
P02808 Statherin STATH
P02810 Salivary acidic proline-rich phosphoprotein 1/2 PRH2
P02812 Basic salivary proline-rich protein 2 PRB2
P02814 Peptide D1A SMR3B
P02818 Osteocalcin BGLAP
P03950 Angiogenin ANG
P03951 Coagulation factor Xla heavy chain Fll
P03952 Plasma kallikrein KLKB1
P03956 27 kDa interstitial collagenase MMP1
P03971 Muellerian-inhibiting factor AMH
P03973 Antileukoproteinase SLPI
P04003 C4b-binding protein alpha chain C4BPA
P04004 Somatomedin-B VTN
P04054 Phospholipase A2 PLA2G1B
P04085 Platelet-derived growth factor subunit A PDGFA
P04090 Relaxin A chain RLN2
P04114 Apolipoprotein B-100 APOB
P04118 Colipase CLPS
P04141 Granulocyte-macrophage colony- stimulating factor CSF2
P04155 Trefoil factor 1 TFF1
P04180 Phosphatidylcholine-sterol acyltransferase LCAT
P04196 Histidine-rich glycoprotein HRG
P04217 Alpha-lB-glycoprotein A1BG
P04275 von Willebrand antigen 2 VWF
P04278 Sex hormone-binding globulin SHBG
P04279 Alpha-inhibin-31 SEMG1
P04280 Basic salivary proline-rich protein 1 PRB1
P04628 Proto-oncogene Wnt-1 WNT1
P04745 Alpha-amylase 1 AMY1A
P04746 Pancreatic alpha-amylase AMY2A
P04808 Prorelaxin HI RLN1
P05000 Interferon omega-1 IFNW1
P05013 Interferon alpha-6 IFNA6
P05014 Interferon alpha-4 IFNA4
P05015 Interferon alpha-16 IFNA16
P05019 Insulin-like growth factor 1 IGF1
P05060 GAWK peptide CHGB
P05090 Apolipoprotein D APOD
P05109 Protein S100-A8 S100A8
P05111 Inhibin alpha chain INHA
P05112 Interleukin-4 IL4
P05113 Interleukin-5 IL5
P05120 Plasminogen activator inhibitor 2 SERPINB2

P05121 Plasminogen activator inhibitor 1 SERPINE1
P05154 Plasma serine protease inhibitor SERPINA5
P05155 Plasma protease CI inhibitor SERPING1
P05156 Complement factor 1 heavy chain CFI
P05160 Coagulation factor XIII B chain F13B
P05161 Ubiquitin-like protein ISG15 ISG15
P05230 Fibroblast growth factor 1 FGF1
P05231 Interleukin-6 IL6
P05305 Big endothelin-1 EDN1
P05408 C-terminal peptide SCG5
P05451 Lithostathine-l-alpha REG1A
P05452 Tetranectin CLEC3B
P05543 Thyroxine-binding globulin SERPINA7
P05814 Beta-casein CSN2
P05997 Collagen alpha-2(V) chain COL5A2
P06276 Cholinesterase BCHE
P06307 Cholecystokinin-12 CCK
P06396 Gelsolin GSN
P06681 Complement C2 C2
P06702 Protein S100-A9 S100A9
P06727 Apolipoprotein A-IV APOA4
P06734 Low affinity immunoglobulin epsilon Fc receptor soluble form, FCER2
P06744 Glucose-6-phosphate isomerase GPI
P06850 Corticoliberin CRH
P06858 Lipoprotein lipase LPL
P06881 Calcitonin gene-related peptide 1 CALCA
P07093 Glia-derived nexin SERPINE2
P07098 Gastric triacylglycerol lipase LIPF
P07225 Vitamin K-dependent protein S PROS1
P07237 Protein disulfide-isomerase P4HB
P07288 Prostate-specific antigen KLK3
P07306 Asialoglycoprotein receptor 1 ASGR1
P07355 Annexin A2 ANXA2
P07357 Complement component C8 alpha chain C8A
P07358 Complement component C8 beta chain C8B
P07360 Complement component C8 gamma C8G chain
P07477 Alpha-trypsin chain 2 PRSS1
P07478 Trypsin-2 PRSS2
P07492 Neuromedin-C GRP
P07498 Kappa -casein CSN3
P07585 Decorin DCN
P07911 Uromodulin UMOD
P07942 Laminin subunit beta-1 LAMB1
P07988 Pulmonary surfactant-associated protein B, SFTPB
P07998 Ribonuclease pancreatic RNASE1
P08118 Beta-microseminoprotein MSM B
P08123 Collagen alpha-2(I) chain COL1A2
P08185 Corticosteroid-binding globulin SERPINA6
P08217 Chymotrypsin-like elastase family member 2A, CELA2A
P08218 Chymotrypsin-like elastase family member 2B, CELA2B
P08253 72 kDa type IV collagenase MMP2
P08254 Stromelysin-1 MMP3
P08294 Extracellular superoxide dismutase [Cu-SOD3Zn]
P08476 Inhibin beta A chain IN HBA
P08493 Matrix Gla protein MGP
P08572 Collagen alpha -2(IV) chain COL4A2

P08581 Hepatocyte growth factor receptor MET
P08603 Complement factor H CFH
P08620 Fibroblast growth factor 4 FGF4
P08637 Low affinity immunoglobulin gamma Fc region receptor lll-A, FCGR3A
P08697 Alpha-2-antiplasmin, SERPINF2
P08700 lnterleukin-3 IL3
P08709 Coagulation factor VII F7
P08833 Insulin-like growth factor-binding protein 1, IGFBP1
P08887 lnterleukin-6 receptor subunit alpha IL6R
P08949 Neuromedin-B-32 NMB
P08F94 Fibrocystin PKH D1
P09038 Fibroblast growth factor 2 FGF2
P09228 Cystatin-SA CST2
P09237 Matrilysin MMP7
P09238 Stromelysin-2 MMP10
P09341 Growth-regulated alpha protein CXCL1
P09382 Galectin-1 LGALS1
P09466 Glycodelin PAEP
P09486 SPARC SPARC
P09529 Inhibin beta B chain IN HBB
P09544 Protein Wnt-2 WNT2
P09603 Processed macrophage colony-stimulating factor 1, CSF1
P09681 Gastric inhibitory polypeptide GIP
P09683 Secretin SCT
P09919 Granulocyte colony-stimulating factor CSF3
P0C091 FRASl-related extracellular matrix FREM3 protein 3
P0C0L4 C4d-A C4A
P0C0L5 Complement C4-B alpha chain C4B
P0C0P6 Neuropeptide S NPS
P0C7L1 Serine protease inhibitor Kazal-type 8 SPIN K8
P0C862 Complement Clq and tumor necrosis factor-related protein 9A, C1QTN F9
P0C8F1 Prostate and testis expressed protein 4 PATE4
POCGOl Gastrokine-3 GKN3P
P0CG36 Cryptic family protein IB CFC1B
P0CG37 Cryptic protein CFC1
P0CJ68 Humanin-like protein 1, MTRN R2L1
P0CJ69 Humanin-like protein 2, MTRN R2L2
P0CJ70 Humanin-like protein 3, MTRN R2L3
P0CJ71 Humanin-like protein 4, MTRN R2L4
P0CJ72 Humanin-like protein 5, MTRN R2L5
P0CJ73 Humanin-like protein 6, MTRN R2L6
P0CJ74 Humanin-like protein 7, MTRN R2L7
P0CJ75 Humanin-like protein 8, MTRN R2L8
P0CJ76 Humanin-like protein 9, MTRN R2L9
P0CJ77 Humanin-like protein 10, MTRN R2L10
P0DJD7 Pepsin A-4, PGA4
P0DJD8 Pepsin A-3, PGA3
P0DJD9 Pepsin A-5, PGA5
P0DJI8 Amyloid protein A, SAA1
P0DJI9 Serum amyloid A-2 protein, SAA2
P10082 Peptide YY(3-36), PYY
P10092 Calcitonin gene-related peptide 2, CALCB
P10124 Serglycin SRGN
P10145 MDNCF-a IL8
P10147 MIP-l-alpha(4-69) CCL3
P10163 Peptide P-D PRB4
P10451 Osteopontin SPP1
P10599 Thioredoxin TXN

P10600 Transforming growth factor beta-3 TGFB3
P10643 Complement component C7 C7
P10645 Vasostatin-2 CHGA
P10646 Tissue factor pathway inhibitor TFPI
P10720 Platelet factor 4 variant(4-74) PF4V1
P10745 Retinol-binding protein 3 RBP3
P10767 Fibroblast growth factor 6 FGF6
P10909 Clusterin alpha chain CLU
P10912 Growth hormone receptor GHR
P10915 Hyaluronan and proteoglycan link protein 1, HAPLN 1
P10966 T-cell surface glycoprotein CD8 beta chain, CD8B
P10997 Islet amyloid polypeptide IAPP
P11047 Laminin subunit gamma-1 LAMC1
P11150 Hepatic triacylglycerol lipase UPC
P11226 Mannose-binding protein C MBL2
P11464 Pregnancy-specific beta-l-glycoprotein 1 PSG1
P11465 Pregnancy-specific beta-l-glycoprotein 2 PSG2
P11487 Fibroblast growth factor 3 FGF3
P11597 Cholesteryl ester transfer protein CETP
P11684 Uteroglobin SCGB1A1
P11686 Pulmonary surfactant-associated protein C, SFTPC
P12034 Fibroblast growth factor 5 FGF5
P12107 Collagen alpha-l(XI) chain COL11A1
P12109 Collagen alpha-l(VI) chain COL6A1
P12110 Collagen alpha-2(VI) chain COL6A2
P12111 Collagen alpha-3(VI) chain COL6A3
P12259 Coagulation factor V F5
P12272 PTHrP[l-36] PTHLH
P12273 Prolactin-inducible protein PIP
P12544 Granzyme A GZMA
P12643 Bone morphogenetic protein 2 BMP2
P12644 Bone morphogenetic protein 4 BMP4
P12645 Bone morphogenetic protein 3 BMP3
P12724 Eosinophil cationic protein RNASE3
P12821 Angiotensin-converting enzyme, soluble ACE form
P12838 Neutrophil defensin 4 DEFA4
P12872 Motilin MLN
P13232 Interleukin-7 IL7
P13236 C-C motif chemokine 4 CCL4
P13284 Gamma-interferon-inducible lysosomal IFI30 thiol reductase
P13500 C-C motif chemokine 2 CCL2
P13501 C-C motif chemokine 5 CCL5
P13521 Secretogranin-2, SCG2
P13591 Neural cell adhesion molecule 1, NCAM 1
P13611 Versican core protein, VCAN
P13671 Complement component C6, C6
P13688 Carcinoembryonic antigen-related cell adhesion molecule 1, CEACAM1
P13725 Oncostatin-M, OSM
P13726 Tissue factor F3
P13727 Eosinophil granule major basic protein, PRG2
P13942 Collagen alpha-2(XI) chain, COL11A2
P13987 CD59 glycoprotein CD59
P14138 Endothelin-3 EDN3
P14174 Macrophage migration inhibitory factor MIF
P14207 Folate receptor beta FOLR2
P14222 Perforin-1 PRF1

P14543 Nidogen-1, NIDI
P14555 Phospholipase A2, membrane associated, PLA2G2A
P14625 Endoplasmin, HSP90B1
P14735 Insulin-degrading enzyme IDE
P14778 Interleukin-1 receptor type 1, soluble IL1R1 form
P14780 82 kDa matrix metalloproteinase-9, MMP9
P15018 Leukemia inhibitory factor LIF
P15085 Carboxypeptidase Al CPA1
P15086 Carboxypeptidase B CPB1
P15151 Poliovirus receptor PVR
P15169 Carboxypeptidase N catalytic chain CPN1
P15248 Interleukin-9 IL9
P15291 N-acetyllactosamine synthase B4GALT1
P15309 PAPf39 ACPP
P15328 Folate receptor alpha FOLR1
P15374 Ubiquitin carboxyl-terminal hydrolase isozyme L3, UCHL3
P15502 Elastin ELN
P15509 Granulocyte-macrophage colony-stimulating factor receptor subunit alpha, CSF2RA
P15515 Histatin-1 HTN1
P15516 His3-(31-51)-peptide HTN3
P15692 Vascular endothelial growth factor A VEGFA
P15814 Immunoglobulin lambda-like polypeptide 1, IGLL1
P15907 Beta-galactoside alpha-2,6-sialyltransferase 1, ST6GAL1
P15941 Mucin-1 subunit beta MUC1
P16035 Metalloproteinase inhibitor 2, TIM P2
P16112 Aggrecan core protein 2, ACAN
P16233 Pancreatic triacylglycerol lipase, PNLIP
P16442 Histo-blood group ABO system ABO transferase
P16471 Prolactin receptor PRLR
P16562 Cysteine-rich secretory protein 2, CRISP2
P16619 C-C motif chemokine 3-like 1, CCL3L1
P16860 BN P(3-29) NPPB
P16870 Carboxypeptidase E CPE
P16871 Interleukin-7 receptor subunit alpha IL7R
P17213 Bactericidal permeability-increasing BPI protein
P17538 Chymotrypsinogen B, CTRBl
P17931 Galectin-3 LGALS3
P17936 Insulin-like growth factor-binding protein 3, IGFBP3
P17948 Vascular endothelial growth factor receptor 1, FLT1
P18065 Insulin-like growth factor-binding protein 2, IGFBP2
P18075 Bone morphogenetic protein 7 BMP7
P18428 Lipopolysaccharide-binding protein LBP
P18509 PACAP-related peptide ADCYAP1
P18510 Interleukin-1 receptor antagonist protein IL1RN
P18827 Syndecan-1 SDC1
P19021 Peptidylglycine alpha-hydroxylating monooxygenase, PAM
P19235 Erythropoietin receptor, EPOR
P19438 Tumor necrosis factor-binding protein 1, TNFRSF1A
P19652 Alpha-l-acid glycoprotein 2, ORM2

P19801 Amiloride-sensitive amine oxidase ABP1
P19823 Inter-alpha-trypsin inhibitor heavy chain H2, ITIH2
P19827 Inter-alpha-trypsin inhibitor heavy chain H1, ITIH1
P19835 Bile salt-activated lipase CEL
P19875 C-X-C motif chemokine 2 CXCL2
P19876 C-X-C motif chemokine 3 CXCL3
P19883 Follistatin FST
P19957 Elafin PI3
P19961 Alpha-amylase 2B AMY2B
P20061 Transcobalamin-1 TCN 1
P20062 Transcobalamin-2 TCN2
P20142 Gastricsin PGC
P20155 Serine protease inhibitor Kazal-type 2 SPIN K2
P20231 Tryptase beta-2 TPSB2
P20333 Tumor necrosis factor receptor superfamily member IB, TNFRSF1B
P20366 Substance P TAC1
P20382 Melanin-concentrating hormone PMCH
P20396 Thyroliberin TRH
P20742 Pregnancy zone protein PZP
P20774 Mimecan OGN
P20783 Neurotrophin-3 NTF3
P20800 Endothelin-2 EDN2
P20809 Interleukin-11 IL11
P20827 Ephrin-Al EFNA1
P20849 Collagen alpha-l(IX) chain COL9A1
P20851 C4b-binding protein beta chain C4BPB
P20908 Collagen alpha-l(V) chain COL5A1
P21128 Poly(U)-specific endoribonuclease ENDOU
P21246 Pleiotrophin PTN
P21583 Kit ligand KITLG
P21741 Midkine MDK
P21754 Zona pellucida sperm-binding protein 3 ZP3
P21781 Fibroblast growth factor 7 FGF7
P21802 Fibroblast growth factor receptor 2 FGFR2
P21810 Biglycan BGN
P21815 Bone sialoprotein 2 IBSP
P21860 Receptor tyrosine-protein kinase erbB-3 ERBB3
P21941 Cartilage matrix protein MATN 1
P22003 Bone morphogenetic protein 5 BMP5
P22004 Bone morphogenetic protein 6 BMP6
P22079 Lactoperoxidase LPO
P22105 Tenascin-X TNXB
P22301 Interleukin-10 IL10
P22303 Acetylcholinesterase ACHE
P22352 Glutathione peroxidase 3 GPX3
P22362 C-C motif chemokine 1 CCL1
P22455 Fibroblast growth factor receptor 4 FGFR4
P22466 Galanin message-associated peptide GAL
P22692 Insulin-like growth factor-binding protein 4, IGFBP4
P22749 Granulysin GN LY
P22792 Carboxypeptidase N subunit 2 CPN2
P22891 Vitamin K-dependent protein Z PROZ
P22894 Neutrophil collagenase MMP8
P23142 Fibulin-1 FBLN 1
P23280 Carbonic anhydrase 6 CA6
P23352 Anosmin-1 KALI
P23435 Cerebellin-1 CBLN1

P23560 Brain-derived neurotrophic factor BDN F
P23582 C-type natriuretic peptide NPPC
P23946 Chymase CMA1
P24043 Laminin subunit alpha-2 LAMA2
P24071 Immunoglobulin alpha Fc receptor FCAR
P24347 Stromelysin-3 MMP11
P24387 Corticotropin-releasing factor-binding CRHBP protein
P24592 Insulin-like growth factor-binding protein 6, IGFBP6
P24593 Insulin-like growth factor-binding protein 5, IGFBP5
P24821 Tenascin TNC
P24855 Deoxyribonuclease-1 DNASE1
P25067 Collagen alpha-2(VIII) chain COL8A2
P25311 Zinc-alpha-2-glycoprotein AZGP1
P25391 Laminin subunit alpha-1 LAMA1
P25445 Tumor necrosis factor receptor FAS superfamily member 6
P25940 Collagen alpha-3(V) chain COL5A3
P25942 Tumor necrosis factor receptor CD40 superfamily member 5
P26022 Pentraxin-related protein PTX3 PTX3
P26927 Hepatocyte growth factor-like protein MST1 beta chain
P27169 Serum paraoxonase/arylesterase 1 PON1
P27352 Gastric intrinsic factor GIF
P27487 Dipeptidyl peptidase 4 membrane form DPP4
P27539 Embryonic growth/differentiation factor 1, GDF1
P27658 Vastatin COL8A1
P27797 Calreticulin CALR
P27918 Properdin CFP
P28039 Acyloxyacyl hydrolase AOAH
P28300 Protein-lysine 6-oxidase LOX
P28325 Cystatin-D CST5
P28799 Granulin-1 GRN
P29122 Proprotein convertase subtilisin/kexin type 6, PCSK6
P29279 Connective tissue growth factor CTGF
P29320 Ephrin type-A receptor 3 EPHA3
P29400 Collagen alpha-5(IV) chain COL4A5
P29459 Interleukin-12 subunit alpha IL12A
P29460 Interleukin-12 subunit beta IL12B
P29508 Serpin B3 SERPINB3
P29622 Kallistatin SERPINA4
P29965 CD40 ligand, soluble form CD40LG
P30990 Neurotensin/neuromedin N NTS
P31025 Lipocalin-1 LCN 1
P31151 Protein S100-A7 S100A7
P31371 Fibroblast growth factor 9 FGF9
P31431 Syndecan-4 SDC4
P31947 14-3-3 protein sigma SFN
P32455 Interferon-induced guanylate-binding GBP1 protein 1
P32881 Interferon alpha-8 IFNA8
P34096 Ribonuclease 4 RNASE4
P34130 Neurotrophin-4 NTF4
P34820 Bone morphogenetic protein 8B BMP8B
P35030 Trypsin-3 PRSS3
P35052 Secreted glypican-1 GPC1
P35070 Betacellulin BTC
P35225 Interleukin-13 IL13
P35247 Pulmonary surfactant-associated protein D, SFTPD
P35318 ADM ADM

P35542 Serum amyloid A-4 protein SAA4
P35555 Fibrillin-1 FBN1
P35556 Fibrillin-2 FBN2
P35625 Metalloproteinase inhibitor 3 TIM P3
P35858 Insulin-like growth factor-binding protein complex acid labile subunit, IGFALS
P35916 Vascular endothelial growth factor FLT4 receptor 3
P35968 Vascular endothelial growth factor KDR receptor 2
P36222 Chitinase-3-like protein 1 CHI3L1
P36952 Serpin B5 SERPINB5
P36955 Pigment epithelium-derived factor SERPINF1
P36980 Complement factor H-related protein 2 CFHR2
P39059 Collagen alpha-l (XV) chain COL15A1
P39060 Collagen alpha-l (XVIII) chain COL18A1
P39877 Calcium-dependent phospholipase A2 PLA2G5
P39900 Macrophage metalloelastase MMP12
P39905 Glial cell line-derived neurotrophic factor GDNF
P40225 Thrombopoietin THPO
P40967 M-alpha PMEL
P41159 Leptin LEP
P41221 Protein Wnt-5a WNT5A
P41222 Prostaglandin-H2 D-isomerase PTGDS
P41271 Neuroblastoma suppressor of NBL1 tumorigenicity 1
P41439 Folate receptor gamma FOLR3
P42127 Agouti-signaling protein ASIP
P42702 Leukemia inhibitory factor receptor LIFR
P42830 ENA-78(9-78) CXCL5
P43026 Growth/differentiation factor 5 GDF5
P43251 Biotinidase BTD
P43652 Afamin AFM
P45452 Collagenase 3 MMP13
P47710 Casoxin-D CSN1S1
P47929 Galectin-7 LGALS7B
P47972 Neuronal pentraxin-2 NPTX2
P47989 Xanthine oxidase XDH
P47992 Lymphotactin XCL1
P48023 Tumor necrosis factor ligand superfamily FASLG member 6, membrane form
P48052 Carboxypeptidase A2 CPA2
P48061 Stromal cell-derived factor 1 CXCL12
P48304 Lithostathine-l-beta REG1B
P48307 Tissue factor pathway inhibitor 2 TFPI2
P48357 Leptin receptor LEPR
P48594 Serpin B4 SERPINB4
P48645 Neuromedin-U-25 NMU
P48740 Mannan-binding lectin serine protease 1 MASP1
P48745 Protein NOV homolog NOV
P48960 CD97 antigen subunit beta CD97
P49223 Kunitz-type protease inhibitor 3 SPINT3
P49747 Cartilage oligomeric matrix protein COM P
P49763 Placenta growth factor PGF
P49765 Vascular endothelial growth factor B VEGFB
P49767 Vascular endothelial growth factor C VEGFC
P49771 Fms-related tyrosine kinase 3 ligand FLT3LG
P49862 Kallikrein-7 KLK7

P49863 Granzyme K GZMK
P49908 Selenoprotein P SEPP1
P49913 Antibacterial protein FALL-39 CAMP
P50607 Tubby protein homolog TUB
P51124 Granzyme M GZMM
P51512 Matrix metalloproteinase-16 MMP16
P51654 Glypican-3 GPC3
P51671 Eotaxin CCL11
P51884 Lumican LUM
P51888 Prolargin PRELP
P52798 Ephrin-A4 EFNA4
P52823 Stanniocalcin-1 STC1
P53420 Collagen alpha-4(IV) chain COL4A4
P53621 Coatomer subunit alpha COPA
P54108 Cysteine-rich secretory protein 3 CRISP3
P54315 Pancreatic lipase-related protein 1 PNLIPRP1
P54317 Pancreatic lipase-related protein 2 PNLIPRP2
P54793 Arylsulfatase F ARSF
P55000 Secreted Ly-6/uPAR-related protein 1 SLURP1
P55001 Microfibrillar-associated protein 2 MFAP2
P55056 Apolipoprotein C-IV APOC4
P55058 Phospholipid transfer protein PLTP
P55075 Fibroblast growth factor 8 FGF8
P55081 Microfibrillar-associated protein 1 MFAP1
P55083 Microfibril-associated glycoprotein 4 MFAP4
P55107 Bone morphogenetic protein 3B GDF10
P55145 Mesencephalic astrocyte-derived MAN F neurotrophic factor
P55259 Pancreatic secretory granule membrane GP2 major glycoprotein, GP2
P55268 Laminin subunit beta-2 LAMB2
P55773 CCL23(30-99) CCL23
P55774 C-C motif chemokine 18 CCL18
P55789 FAD-linked sulfhydryl oxidase ALR GFER
P56703 Proto-oncogene Wnt-3 WNT3
P56704 Protein Wnt-3a WNT3A
P56705 Protein Wnt-4 WNT4
P56706 Protein Wnt-7b WNT7B
P56730 Neurotrypsin PRSS12
P56851 Epididymal secretory protein E3-beta EDDM3B
P56975 Neuregulin-3 NRG3
P58062 Serine protease inhibitor Kazal-type 7 SPIN K7
P58215 Lysyl oxidase homolog 3 LOXL3
P58294 Prokineticin-1 PROK1
P58335 Anthrax toxin receptor 2 ANTXR2
P58397 A disintegrin and metalloproteinase with thrombospondin motifs 12, ADAMTS12
P58417 Neurexophilin-1 NXPH1
P58499 Protein FAM3B FAM3B
P59510 A disintegrin and metalloproteinase with thrombospondin motifs 20, ADAMTS20
P59665 Neutrophil defensin 1 DEFA1B
P59666 Neutrophil defensin 3 DEFA3
P59796 Glutathione peroxidase 6 GPX6
P59826 BPI fold-containing family B member 3 BPIFB3
P59827 BPI fold-containing family B member 4 BPIFB4
P59861 Beta-defensin 131 DEFB131

P60022 Beta-defensin 1 DEFB1
P60153 Inactive ribonuclease-like protein 9 RNASE9
P60827 Complement Clq tumor necrosis factor-C1QTN F8 related protein 8
P60852 Zona pellucida sperm-binding protein 1 ZP1
P60985 Keratinocyte differentiation-associated KRTDAP protein
P61109 Kidney androgen-regulated protein KAP
P61278 Somatostatin-14 SST
P61366 Osteocrin OSTN
P61626 Lysozyme C LYZ
P61769 Beta-2-microglobulin B2M
P61812 Transforming growth factor beta-2 TGFB2
P61916 Epididymal secretory protein El NPC2
P62502 Epididymal-specific lipocalin-6 LCN6
P62937 Peptidyl-prolyl cis-trans isomerase A PPIA
P67809 Nuclease-sensitive element-binding YBX1 protein 1
P67812 Signal peptidase complex catalytic SEC11A subunit SEC11A
P78310 Coxsackievirus and adenovirus receptor CXADR
P78333 Secreted glypican-5 GPC5
P78380 Oxidized low-density lipoprotein receptor 1, OLR1
P78423 Processed fractalkine CX3CL1
P78509 Reelin RELN
P78556 CCL20(2-70) CCL20
P80075 MCP-2(6-76) CCL8
P80098 C-C motif chemokine 7 CCL7
P80108 Phosphatidylinositol-glycan-specific phospholipase D, GPLD1
P80162 C-X-C motif chemokine 6 CXCL6
P80188 Neutrophil gelatinase-associated lipocalin LCN2
P80303 Nucleobindin-2 NUCB2
P80511 Calcitermin S100A12
P81172 Hepcidin-25 HAMP
P81277 Prolactin-releasing peptide PRLH
P81534 Beta-defensin 103 DEFB103A
P81605 Dermcidin DCD
P82279 Protein crumbs homolog 1 CRB1
P82987 ADAMTS-like protein 3 ADAMTSL3
P83105 Serine protease HTRA4 HTRA4
P83110 Serine protease HTRA3 HTRA3
P83859 Orexigenic neuropeptide QRFP QRFP
P98088 Mucin-5AC MUC5AC
P98095 Fibulin-2 FBLN2
P98160 Basement membrane-specific heparan HSPG2 sulfate proteoglycan core protein
P98173 Protein FAM3A, FAM3A
Q00604 Norrin NDP
Q00796 Sorbitol dehydrogenase SORD
Q00887 Pregnancy-specific beta-l-glycoprotein 9, PSG9
Q00888 Pregnancy-specific beta-l-glycoprotein 4, PSG4
Q00889 Pregnancy-specific beta-l-glycoprotein 6, PSG6
Q01523 HD5(56-94), DEFA5
Q01524 Defensin-6, DEFA6
Q01955 Collagen alpha-3(IV) chain, COL4A3
Q02297 Pro-neuregulin-1, membrane-bound NRG1 isoform

Q02325 Plasminogen-like protein B PLGLB1
Q02383 Semenogelin-2 SEMG2
Q02388 Collagen alpha-l(VII) chain COL7A1
Q02505 Mucin-3A MUC3A
Q02509 Otoconin-90 OC90
Q02747 Guanylin GUCA2A
Q02763 Angiopoietin-1 receptor TEK
Q02817 Mucin-2 MUC2
Q02985 Complement factor H-related protein 3, CFHR3
Q03167 Transforming growth factor beta receptor type 3, TGFBR3
Q03403 Trefoil factor 2, TFF2
Q03405 Urokinase plasminogen activator surface receptor, PLAUR
Q03591 Complement factor H-related protein 1, CFHR1
Q03692 Collagen alpha-l(X) chain, COL10A1
Q04118 Basic salivary proline-rich protein 3, PRB3
Q04756 Hepatocyte growth factor activator short chain, HGFAC
Q04900 Sialomucin core protein 24 CD164
Q05315 Eosinophil lysophospholipase CLC
Q05707 Collagen alpha-l(XIV) chain COL14A1
Q05996 Processed zona pellucida sperm-binding ZP2
protein 2
Q06033 Inter-alpha-trypsin inhibitor heavy chain ITIH3
H3
Q06141 Regenerating islet-derived protein 3-alpha, REG3A
Q06828 Fibromodulin FMOD
Q07092 Collagen alpha-l(XVI) chain, COL16A1
Q07325 C-X-C motif chemokine 9, CXCL9
Q07507 Dermatopontin DPT
Q075Z2 Binder of sperm protein homolog 1, BSPH1
Q07654 Trefoil factor 3, TFF3
Q07699 Sodium channel subunit beta-1, SCN1B
Q08345 Epithelial discoidin domain-containing receptor 1, DDR1
Q08380 Galectin-3-binding protein LGALS3BP
Q08397 Lysyl oxidase homolog 1 LOXL1
Q08431 Lactadherin MFGE8
Q08629 Testican-1 SPOCK1
Q08648 Sperm-associated antigen 11B SPAG11B
Q08830 Fibrinogen-like protein 1 FGL1
Q10471 Polypeptide N-acetylgalactosaminyltransferase 2, GALNT2
Q10472 Polypeptide N-acetylgalactosaminyltransferase 1, GALNT1
Q11201 CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 1, ST3GAL1
Q11203 CMP-N-acetylneuraminate-beta-1,4-galactoside alpha-2,3-sialyltransferase 3, ST3GAL3
Q11206 CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 4, ST3GAL4
Q12794 Hyaluronidase-1, HYAL1
Q12805 EGF-containing fibulin-like extracellular matrix protein 1, EFEMP1
Q12836 Zona pellucida sperm-binding protein 4 ZP4

Q12841 Follistatin-related protein 1, FSTL1
Q12904 Aminoacyl tRNA synthase complex-interacting multifunctional protein 1, AIMP1
Q13018 Soluble secretory phospholipase A2 receptor, PLA2R1
Q13072 B melanoma antigen 1, BAGE
Q13093 Platelet-activating factor acetylhydrolase, PLA2G7
Q13103 Secreted phosphoprotein 24 SPP2
Q13162 Peroxiredoxin-4 PRDX4
Q13201 Platelet glycoprotein la* MMRN 1
Q13214 Semaphorin-3B SEMA3B
Q13219 Pappalysin-1 PAPPA
Q13231 Chitotriosidase-1 CHIT1
Q13253 Noggin NOG
Q13261 Interleukin-15 receptor subunit alpha IL15RA
Q13275 Semaphorin-3F SEMA3F
Q13291 Signaling lymphocytic activation molecule SLAMF1
Q13316 Dentin matrix acidic phosphoprotein 1 DMP1
Q13361 Microfibrillar-associated protein 5 MFAP5
Q13410 Butyrophilin subfamily 1 member Al BTN 1A1
Q13421 Mesothelin, cleaved form MSLN
Q13429 Insulin-like growth factor 1 IGF-I
Q13443 Disintegrin and metalloproteinase ADAM9 domain-containing protein 9
Q13519 Neuropeptide 1 PNOC
Q13751 Laminin subunit beta-3 LAMB3
Q13753 Laminin subunit gamma-2 LAMC2
Q13790 Apolipoprotein F APOF
Q13822 Ectonucleotide ENPP2 pyrophosphatase/phosphodiesterase family member 2
Q14031 Collagen alpha-6(IV) chain COL4A6
Q14050 Collagen alpha-3(IX) chain COL9A3
Q14055 Collagen alpha-2(IX) chain COL9A2
Q14112 Nidogen-2 NID2
Q14114 Low-density lipoprotein receptor-related protein 8, LRP8
Q14118 Dystroglycan DAG1
Q14314 Fibroleukin FGL2
Q14393 Growth arrest-specific protein 6 GAS6
Q14406 Chorionic somatomammotropin CSHL1 hormone-like 1
Q14507 Epididymal secretory protein E3-alpha EDDM3A
Q14508 WAP four-disulfide core domain protein 2 WFDC2
Q14512 Fibroblast growth factor-binding protein 1, FGFBP1
Q14515 SPARC-like protein 1, SPARCL1
Q14520 Hyaluronan-binding protein 2 27 kDa light HABP2 chain
Q14563 Semaphorin-3A, SEMA3A
Q14623 Indian hedgehog protein IHH
Q14624 Inter-alpha-trypsin inhibitor heavy chain H4, ITIH4
Q14667 UPF0378 protein KIAA0100, KIAA0100
Q14703 Membrane-bound transcription factor site-1 protease, MBTPS1
Q14766 Latent-transforming growth factor beta-binding protein 1, LTBP1

Q14767 Latent-transforming growth factor beta-binding protein 2, LTBP2
Q14773 Intercellular adhesion molecule 4, ICAM4
Q14993 Collagen alpha-l(XIX) chain, COL19A1
Q14CN2 Calcium-activated chloride channel regulator 4, 110 kDa form, CLCA4
Q15046 Lysine-tRNA ligase KARS
Q15063 Periostin POSTN
Q15109 Advanced glycosylation end product-specific receptor, AGER
Q15113 Procollagen C-endopeptidase enhancer 1, PCOLCE
Q15166 Serum paraoxonase/lactonase 3, PON3
Q15195 Plasminogen-like protein A, PLGLA
Q15198 Platelet-derived growth factor receptor like protein, PDGFRL
Q15223 Poliovirus receptor-related protein 1, PVRL1
Q15238 Pregnancy-specific beta-l-glycoprotein 5, PSG5
Q15363 Transmembrane emp24 domain-containing protein 2, TMED2
Q15375 Ephrin type-A receptor 7, EPHA7
Q15389 Angiopoietin-1, ANGPT1
Q15465 Sonic hedgehog protein, SHH
Q15485 Ficolin-2, FCN2
Q15517 Corneodesmosin, CDSN
Q15582 Transforming growth factor-beta-induced protein ig-h3, TGFBI
Q15661 Tryptase alpha/beta-1, TPSAB1
Q15726 Metastin, KISS1
Q15782 Chitinase-3-like protein 2, CHI3L2
Q15828 Cystatin-M, CST6
Q15846 Clusterin-like protein 1, CLU L1
Q15848 Adiponectin, ADIPOQ
Q16206 Protein disulfide-thiol oxidoreductase, ENOX2
Q16270 Insulin-like growth factor-binding protein 7, IGFBP7
Q16363 Laminin subunit alpha-4, LAMA4
Q16378 Proline-rich protein 4, PRR4
Q16557 Pregnancy-specific beta-l-glycoprotein 3, PSG3
Q16568 CART(42-89), CARTPT
Q16610 Extracellular matrix protein 1, ECM 1
Q16619 Cardiotrophin-1, CTF1
Q16623 Syntaxin-1A, STX1A
Q16627 HCC-l(9-74), CCL14
Q16651 Prostasin light chain, PRSS8
Q16661 Guanylate cyclase C-activating peptide 2, GUCA2B
Q16663 CCL15(29-92), CCL15
Q16674 Melanoma-derived growth regulatory protein, MIA
Q16769 Glutaminyl-peptide cyclotransferase QPCT
Q16787 Laminin subunit alpha-3 LAM A3
Q16842 CMP-N-acetylneuraminate-beta-galactosamide-alpha-2,3-sialyltransferase 2, ST3GAL2
Q17RR3 Pancreatic lipase-related protein 3, PNLIPRP3
Q17RW2 Collagen alpha-l(XXIV) chain, COL24A1
Q17RY6 Lymphocyte antigen 6K LY6K
Q1L6U9 Prostate-associated microseminoprotein MSMP

Q1W4C9 Serine protease inhibitor Kazal-type 13 SPIN K13
Q1ZYL8 Izumo sperm-egg fusion protein 4 IZUM04
Q29960 HLA class 1 histocompatibility antigen Cw-16 alpha chain, HLA-C
Q2I0M5 R-spondin-4 RSP04
Q2L4Q9 Serine protease 53 PRSS53
Q2MKA7 R-spondin-1 RSPOl
Q2MV58 Tectonic-1 TCTN1
Q2TAL6 Brorin VWC2
Q2UY09 Collagen alpha-l(XXVIII) chain COL28A1
Q2VPA4 Complement component receptor 1-like CR1L protein
Q2WEN9 Carcinoembryonic antigen-related cell CEACAM16 adhesion molecule 16
Q30KP8 Beta-defensin 136 DEFB136
Q30KP9 Beta-defensin 135 DEFB135
Q30KQ1 Beta-defensin 133 DEFB133
Q30KQ2 Beta-defensin 130 DEFB130
Q30KQ4 Beta-defensin 116 DEFB116
Q30KQ5 Beta-defensin 115 DEFB115
Q30KQ6 Beta-defensin 114 DEFB114
Q30KQ7 Beta-defensin 113 DEFB113
Q30KQ8 Beta-defensin 112 DEFB112
Q30KQ9 Beta-defensin 110 DEFB110
Q30KR1 Beta-defensin 109 DEFB109P1
Q32P28 Prolyl 3-hydroxylase 1 LEPRE1
Q3B7J2 Glucose-fructose oxidoreductase domain- containing protein 2, GFOD2
Q3SY79 Protein Wnt WNT3A
Q3T906 N-acetylglucosamine-1-phosphotransferase subunits alpha/beta, GN PTAB
Q495T6 Membrane metallo-endopeptidase-like 1 MMEL1
Q49AH0 Cerebral dopamine neurotrophic factor CDN F
Q4G0G5 Secretoglobin family 2B member 2 SCGB2B2
Q4G0M1 Protein FAM 132B FAM132B
Q4LDE5 Sushi, von Willebrand factor type A, and pentraxin domain-containing protein 1, EGF SVEP1
Q4QY38 Beta-defensin 134, DEFB134
Q4VAJ4 Protein Wnt, WNT10B
Q4W5P6 Protein TMEM155, TMEM155
Q4ZHG4 Fibronectin type III domain-containing FN DC1 protein 1
Q53H76 Phospholipase Al member A PLA1A
Q53RD9 Fibulin-7, FBLN7
Q53S33 BolA-like protein 3, BOLA3
Q5BLP8 Neuropeptide-like protein C4orf48, C4orf48
Q5DT21 Serine protease inhibitor Kazal-type 9, SPIN K9
Q5EBL8 PDZ domain-containing protein 11, PDZD11
Q5FYB0 Arylsulfatase J, ARSJ
Q5FYB1 Arylsulfatase 1, ARSI
Q5GAN3 Ribonuclease-like protein 13, RNASE13
Q5GAN4 Ribonuclease-like protein 12, RNASE12

Q5GAN6 Ribonuclease-like protein 10, RNASE10
Q5GFL6 von Willebrand factor A domain-containing protein 2, VWA2
Q5H8A3 Neuromedin-S, NMS
Q5H8C1 FRASl-related extracellular matrix protein 1, FREM 1
Q5IJ48 Protein crumbs homolog 2, CRB2
Q5J5C9 Beta-defensin 121, DEFB121
Q5JS37 NH L repeat-containing protein 3, NHLRC3
Q5JTB6 Placenta-specific protein 9, PLAC9
Q5JU69 Torsin-2A, TOR2A
Q5JXM2 Methyltransferase-like protein 24, METTL24
Q5JZY3 Ephrin type-A receptor 10, EPHA10
Q5K4E3 Polyserase-2, PRSS36
Q5SRR4 Lymphocyte antigen 6 complex locus protein G5c, LY6G5C
Q5T1H1 Protein eyes shut homolog EYS
Q5T4F7 Secreted frizzled-related protein 5 SFRP5
Q5T4W7 Artemin ARTN
Q5T7M4 Protein FAM 132A FAM132A
Q5TEH8 Protein Wnt WNT2B
Q5TIE3 von Willebrand factor A domain-containing protein 5B1, VWA5B1
Q5UCC4 ER membrane protein complex subunit 10, EMC10
Q5VST6 Abhydrolase domain-containing protein FAM 108B1, FAM108B1
Q5VTL7 Fibronectin type III domain-containing FNDC7 protein 7
Q5VUM1 UPF0369 protein C6orf57, C6orf57
Q5VV43 Dyslexia-associated protein KIAA0319, KIAA0319
Q5VWW1 Complement Clq-like protein 3, C1QL3
Q5VXI9 Lipase member N LIPN
Q5VXJ0 Lipase member K LIPK
Q5VXM 1 CUB domain-containing protein 2, CDCP2
Q5VYX0 Renalase RN LS
Q5VYY2 Lipase member M LIPM
Q5W186 Cystatin-9 CST9
Q5W5W9 Regulated endocrine-specific protein 18 RESP18
Q5XG92 Carboxylesterase 4A CES4A
Q63HQ2 Pikachurin EGFLAM
Q641Q3 Meteorin-like protein METRN L
Q66K79 Carboxypeptidase Z CPZ
Q685J3 Mucin-17 MUC17
Q68BL7 Olfactomedin-like protein 2A OLFML2A
Q68BL8 Olfactomedin-like protein 2B OLFML2B
Q68DV7 E3 ubiquitin-protein ligase RNF43 RN F43
Q6B9Z1 Insulin growth factor-like family member 4, IGFL4
Q6BAA4 Fc receptor-like B FCRLB
Q6E0U4 Dermokine DMKN
Q6EMK4 Vasorin VASN
Q6FHJ7 Secreted frizzled-related protein 4 SFRP4
Q6GPI1 Chymotrypsin B2 chain B CTRB2
Q6GTS8 Probable carboxypeptidase PM20D1, PM20D1

Q6H9L7 Isthmin-2 ISM2
Q6IE36 Ovostatin homolog 2, OVOS2
Q6IE37 Ovostatin homolog 1, OVOS1
Q6IE38 Serine protease inhibitor Kazal-type 14, SPIN K14
Q6ISS4 Leukocyte-associated immunoglobulin-like receptor 2, LAIR2
Q6JVE5 Epididymal-specific lipocalin-12, LCN 12
Q6JVE6 Epididymal-specific lipocalin-10, LCN 10
Q6JVE9 Epididymal-specific lipocalin-8, LCN8
Q6KF10 Growth/differentiation factor 6, GDF6
Q6MZW2 Follistatin-related protein 4, FSTL4
Q6NSX1 Coiled-coil domain-containing protein 70, CCDC70
Q6NT32 Carboxylesterase 5A, CES5A
Q6NT52 Choriogonadotropin subunit beta variant 2, CGB2
Q6NU I6 Chondroadherin-like protein, CHADL
Q6NUJ1 Saposin A-like PSAPL1
Q6P093 Arylacetamide deacetylase-like 2 AADACL2
Q6P4A8 Phospholipase B-like 1 PLBD1
Q6P5S2 UPF0762 protein C6orf58 C6orf58
Q6P988 Protein notum homolog NOTU M
Q6PCB0 von Willebrand factor A domain-containing protein 1, VWA1
Q6PDA7 Sperm-associated antigen 11A SPAG11A
Q6PEW0 Inactive serine protease 54 PRSS54
Q6PEZ8 Podocan-like protein 1 PODNL1
Q6PKH6 Dehydrogenase/reductase SDR family member 4-like 2, DHRS4L2
Q6Q788 Apolipoprotein A-V APOA5
Q6SPF0 Atherin SAM D1
Q6UDR6 Kunitz-type protease inhibitor 4 SPINT4
Q6U RK8 Testis, prostate and placenta-expressed protein, TEPP
Q6UW01 Cerebellin-3 CBLN3
Q6UW10 Surfactant-associated protein 2 SFTA2
Q6UW15 Regenerating islet-derived protein 3-gamma, REG3G
Q6UW32 Insulin growth factor-like family member 1, IGFL1
Q6UW78 UPF0723 protein Cllorf83, Cllorf83
Q6UW88 Epigen EPGN
Q6UWE3 Colipase-like protein 2, CLPSL2
Q6UWF7 NXPE family member 4, NXPE4
Q6UWF9 Protein FAM 180A, FAM180A
Q6UWM5 GLIPRl-like protein 1, GLIPR1L1
Q6UWN8 Serine protease inhibitor Kazal-type 6, SPIN K6
Q6UWP2 Dehydrogenase/reductase SDR family DHRS11 member 11
Q6UWP8 Supra basin SBSN
Q6UWQ5 Lysozyme-like protein 1 LYZL1
Q6UWQ7 Insulin growth factor-like family member 2, IGFL2
Q6UWR7 Ectonucleotide ENPP6 pyrophosphatase/phosphodiesterase family member 6 soluble form
Q6UWT2 Adropin ENHO
Q6UWU2 Beta-galactosidase-l-like protein GLB1L
Q6UWW0 Lipocalin-15 LCN 15
Q6UWX4 HH IP-like protein 2 HHIPL2

Q6UWY0 Arylsulfatase K ARSK
Q6UWY2 Serine protease 57 PRSS57
Q6UWY5 Olfactomedin-like protein 1 OLFML1
Q6UX06 Olfactomedin-4 OLFM4
Q6UX07 Dehydrogenase/reductase SDR family DHRS13 member 13
Q6UX39 Amelotin, AMTN
Q6UX46 Protein FAM 150B, FAM150B
Q6UX73 UPF0764 protein C16orf89, C16orf89
Q6UXB0 Protein FAM 131A, FAM131A
Q6UXB1 Insulin growth factor-like family member 3, IGFL3
Q6UXB2 VEGF co-regulated chemokine 1, CXCL17
Q6UXF7 C-type lectin domain family 18 member B, CLEC18B
Q6UXH0 Hepatocellular carcinoma-associated protein TD26, C19orf80
Q6UXH1 Cysteine-rich with EGF-like domain protein 2, CRELD2
Q6UXH8 Collagen and calcium-binding domain-containing protein 1, EGF CCBE1
Q6UXH9 Inactive serine protease PAMR1, PAMR1
Q6UXI7 Vitrin, VIT
Q6UXI9 Nephronectin, NPNT
Q6UXN2 Trem-like transcript 4 protein, TREML4
Q6UXS0 C-type lectin domain family 19 member A CLEC19A
Q6UXT8 Protein FAM 150A, FAM150A
Q6UXT9 Abhydrolase domain-containing protein 15, ABHD15
Q6UXV4 Apolipoprotein O-like, APOOL
Q6UXX5 Inter-alpha-trypsin inhibitor heavy chain H6, ITIH6
Q6UXX9 R-spondin-2, RSP02
Q6UY14 ADAMTS-like protein 4, ADAMTSL4
Q6UY27 Prostate and testis expressed protein 2, PATE 2
Q6W4X9 Mucin-6, MUC6
Q6WN34 Chordin-like protein 2, CHRDL2
Q6WRI0 Immunoglobulin superfamily member 10, IGSF10
Q6X4U4 Sclerostin domain-containing protein 1, SOSTDC1
Q6X784 Zona pellucida-binding protein 2, ZPBP2
Q6XE38 Secretoglobin family ID member 4, SCGB1D4
Q6XPR3 Repetin, RPTN
Q6XZB0 Lipase member 1, LIPI
Q6ZMM2 ADAMTS-like protein 5, ADAMTSL5
Q6ZMP0 Thrombospondin type-1 domain-containing protein 4, THSD4
Q6ZNF0 Iron/zinc purple acid phosphatase-like protein, PAPL
Q6ZRI0 Otogelin, OTOG
Q6ZRP7 Sulfhydryl oxidase 2, QSOX2
Q6ZWJ8 Kielin/chordin-like protein, KCP
Q75N90 Fibrillin-3, FBN3
Q765I0 Urotensin-2B, UTS2D
Q76B58 Protein FAM5C, FAM5C
Q76LX8 A disintegrin and metalloproteinase with thrombospondin motifs 13, ADAMTS13
Q76M96 Coiled-coil domain-containing protein 80, CCDC80

Q7L1S5 Carbohydrate sulfotransferase 9, CHST9
Q7L513 Fc receptor-like A, FCRLA
Q7L8A9 Vasohibin-1, VASH1
Q7RTM1 Otopetrin-1, OTOP1
Q7RTW8 Otoancorin, OTOA
Q7RTY5 Serine protease 48, PRSS48
Q7RTY7 Ovochymase-1, OVCH1
Q7RTZ1 Ovochymase-2, OVCH2
Q7Z304 MAM domain-containing protein 2, MAMDC2
Q7Z3S9 Notch homolog 2 N-terminal-like protein, NOTCH2NL
Q7Z4H4 Intermedin-short, ADM2
Q7Z4P5 Growth/differentiation factor 7, GDF7
Q7Z4R8 UPF0669 protein C6orfl20, C6orfl20
Q7Z4W2 Lysozyme-like protein 2, LYZL2
Q7Z5A4 Serine protease 42, PRSS42
Q7Z5A7 Protein FAM 19A5, FAM19A5
Q7Z5A8 Protein FAM 19A3, FAM19A3
Q7Z5A9 Protein FAM 19A1, FAM19A1
Q7Z5J1 Hydroxysteroid 11-beta-dehydrogenase 1-like protein, HSD11B1L
Q7Z5L0 Vitelline membrane outer layer protein 1 homolog, VMOl
Q7Z5L3 Complement Clq-like protein 2, C1QL2
Q7Z5L7 Podocan, PODN
Q7Z5P4 17-beta-hydroxysteroid dehydrogenase 13, HSD17B13
Q7Z5P9 Mucin-19, MUC19
Q7Z5Y6 Bone morphogenetic protein 8A, BMP8A
Q7Z7B7 Beta-defensin 132, DEFB132
Q7Z7B8 Beta-defensin 128, DEFB128
Q7Z7C8 Transcription initiation factor TFIID subunit 8, TAF8
Q7Z7H5 Transmembrane emp24 domain-containing protein 4, TMED4
Q86SG7 Lysozyme g-like protein 2, LYG2
Q86SI9 Protein CEI, C5orf38
Q86TE4 Leucine zipper protein 2, LUZP2
Q86TH1 ADAMTS-like protein 2, ADAMTSL2
Q86U17 Serpin All, SERPINA11
Q86UU9 Endokinin-A, TAC4
Q86UW8 Hyaluronan and proteoglycan link protein 4, HAPLN4
Q86UX2 Inter-alpha-trypsin inhibitor heavy chain H5, ITIH5
Q86V24 Adiponectin receptor protein 2, ADIPOR2
Q86VB7 Soluble CD163, CD163
Q86VR8 Four-jointed box protein 1, FJX1
Q86WD7 Serpin A9, SERPINA9
Q86WN2 Interferon epsilon, IFNE
Q86WS3 Placenta-specific 1-like protein, PLAC1L
Q86X52 Chondroitin sulfate synthase 1, CHSY1
Q86XP6 Gastrokine-2, GKN2
Q86XS5 Angiopoietin-related protein 5, ANGPTL5
Q86Y27 B melanoma antigen 5, BAGE5
Q86Y28 B melanoma antigen 4, BAGE4
Q86Y29 B melanoma antigen 3, BAGE3
Q86Y30 B melanoma antigen 2, BAGE2
Q86Y38 Xylosyltransferase 1, XYLT1
Q86Y78 Ly6/PLAUR domain-containing protein 6, LYPD6
Q86YD3 Transmembrane protein 25, TMEM25

Q86YJ6 Threonine synthase-like 2, THNSL2
Q86YW7 Glycoprotein hormone beta-5, GPHB5
Q86Z23 Complement Clq-like protein 4, C1QL4
Q8IU57 Interleukin-28 receptor subunit alpha, IL28RA
Q8IUA0 WAP four-disulfide core domain protein 8, WFDC8
Q8IUB2 WAP four-disulfide core domain protein 3, WFDC3
Q8IUB3 Protein WFDCIOB, WFDCIOB
Q8IUB5 WAP four-disulfide core domain protein 13, WFDC13
Q8IUH2 Protein CREG2, CREG2
Q8IUK5 Plexin domain-containing protein 1, PLXDC1
Q8IUL8 Cartilage intermediate layer protein 2, C2 CILP2
Q8IUX7 Adipocyte enhancer-binding protein 1, AEBP1
Q8IUX8 Epidermal growth factor-like protein 6, EGFL6
Q8IVL8 Carboxypeptidase 0, CPO
Q8IVN8 Somatomedin-B and thrombospondin type-1 domain-containing protein, SBSPON
Q8IVW8 Protein spinster homolog 2, SPNS2
Q8IW75 Serpin A12, SERPINA12
Q8IW92 Beta-galactosidase-l-like protein 2, GLB1L2
Q8IWL1 Pulmonary surfactant-associated protein A2, SFTPA2
Q8IWL2 Pulmonary surfactant-associated protein A1, SFTPA1
Q8IWV2 Contactin-4, CNTN4
Q8IWY4 Signal peptide, CU B and EGF-like domain- containing protein 1, SCU BE1
Q8IX30 Signal peptide, CU B and EGF-like domain- containing protein 3, SCU BE3
Q8IXA5 Sperm acrosome membrane-associated protein 3, membrane form, SPACA3
Q8IXB1 DnaJ homolog subfamily C member 10, DNAJC10
Q8IXL6 Extracellular serine/threonine protein kinase Fam20C, FAM20C
Q8IYD9 Lung adenoma susceptibility protein 2, LAS2
Q8IYP2 Serine protease 58, PRSS58
Q8IYS5 Osteoclast-associated immunoglobulin-like receptor, OSCAR
Q8IZC6 Collagen alpha-l(XXVII) chain, COL27A1
Q8IZJ3 C3 and PZP-like alpha-2-macroglobulin domain-containing protein 8, CPAMD8
Q8IZN7 Beta-defensin 107, DEFB107B
Q8N0V4 Leucine-rich repeat LGI family member 2, LGI2
Q8N104 Beta-defensin 106, DEFB106B
Q8N119 Matrix metalloproteinase-21, MMP21
Q8N129 Protein canopy homolog 4, CN PY4
Q8N135 Leucine-rich repeat LGI family member 4, LGI4
Q8N145 Leucine-rich repeat LGI family member 3, LGI3
Q8N158 Glypican-2, GPC2
Q8N1E2 Lysozyme g-like protein 1, LYG1
Q8N2E2 von Willebrand factor D and EGF domain- containing protein, VWDE
Q8N2E6 Prosalusin, TOR2A

Q8N2S1 Latent-transforming growth factor beta-binding protein 4, LTBP4
Q8N302 Angiogenic factor with G patch and FHA domains 1, AGGF1
Q8N307 Mucin-20, MUC20
Q8N323 NXPE family member 1, NXPE1
Q8N387 Mucin-15, MUC15
Q8N3Z0 Inactive serine protease 35, PRSS35
Q8N436 Inactive carboxypeptidase-like protein X2, CPXM2
Q8N474 Secreted frizzled-related protein 1, SFRP1
Q8N475 Follistatin-related protein 5, FSTL5
Q8N4F0 BPI fold-containing family B member 2, BPIFB2
Q8N4T0 Carboxypeptidase A6, CPA6
Q8N5W8 Protein FAM24B, FAM24B
Q8N687 Beta-defensin 125, DEFB125
Q8N688 Beta-defensin 123, DEFB123
Q8N690 Beta-defensin 119, DEFB119
Q8N6C5 Immunoglobulin superfamily member 1, IGSF1
Q8N6C8 Leukocyte immunoglobulin-like receptor subfamily A member 3, LILRA3
Q8N6G6 ADAMTS-like protein 1, ADAMTSL1
Q8N6Y2 Leucine-rich repeat-containing protein 17, LRRC17
Q8N729 Neuropeptide W-23, NPW
Q8N8U9 BM P-binding endothelial regulator protein, BMPER
Q8N907 DAN domain family member 5, DAND5
Q8NAT1 Glycosyltransferase-like domain-containing protein 2, GTDC2
Q8NAU1 Fibronectin type III domain-containing protein 5, FN DC5
Q8N B37 Parkinson disease 7 domain-containing protein 1, PDDC1
Q8N BI3 Draxin, DRAXIN
Q8N BM8 Prenylcysteine oxidase-like, PCYOX1L
Q8NBP7 Proprotein convertase subtilisin/kexin type 9, PCSK9
Q8N BQ5 Estradiol 17-beta-dehydrogenase 11, HSD17B11
Q8N BV8 Synaptotagmin-8, SYT8
Q8NCC3 Group XV phospholipase A2, PLA2G15
Q8NCF0 C-type lectin domain family 18 member C, CLEC18C
Q8NCW5 NAD(P)H-hydrate epimerase, APOA1BP
Q8N DA2 Hemicentin-2, HMCN2
Q8N DX9 Lymphocyte antigen 6 complex locus protein G5b, LY6G5B
Q8N DZ4 Deleted in autism protein 1, C3orf58
Q8N EB7 Acrosin-binding protein, ACRBP
Q8NES8 Beta-defensin 124, DEFB124
Q8N ET1 Beta-defensin 108B, DEFB108B
Q8N EX5 Protein WFDC9, WFDC9
Q8N EX6 Protein WFDC11, WFDC11
Q8N F86 Serine protease 33, PRSS33
Q8N FM7 Interleukin-17 receptor D, IL17RD
Q8N FQ5 BPI fold-containing family B member 6, BPIFB6
Q8N FQ6 BPI fold-containing family C protein, BPIFC

Q8N FU4 Follicular dendritic cell secreted peptide, FDCSP
Q8N FW1 Collagen alpha-l(XXII) chain, COL22A1
Q8NG35 Beta-defensin 105, DEFB105B
Q8NG41 Neuropeptide B-23, NPB
Q8N HW6 Otospiralin, OTOS
Q8NI99 Angiopoietin-related protein 6, ANGPTL6
Q8TAA1 Probable ribonuclease 11, RNASE11
Q8TAG5 V-set and transmembrane domain-containing protein 2A, VSTM2A
Q8TAL6 Fin bud initiation factor homolog, FIBIN
Q8TAT2 Fibroblast growth factor-binding protein 3, FGFBP3
Q8TAX7 Mucin-7, MUC7
Q8TB22 Spermatogenesis-associated protein 20, SPATA20
Q8TB73 Protein NDNF, NDNF
Q8TB96 T-cell immunomodulatory protein, ITFG1
Q8TC92 Protein disulfide-thiol oxidoreductase, ENOX1
Q8TCV5 WAP four-disulfide core domain protein 5, WFDC5
Q8TD06 Anterior gradient protein 3 homolog, AGR3
Q8TD33 Secretoglobin family 1C member 1, SCGB1C1
Q8TD46 Cell surface glycoprotein CD200 receptor 1, CD200R1
Q8TDE3 Ribonuclease 8, RNASE8
Q8TDF5 Neuropilin and tolloid-like protein 1, NETOl
Q8TDL5 BPI fold-containing family B member 1, BPIFB1
Q8TE56 A disintegrin and metalloproteinase with thrombospondin motifs 17, ADAMTS17
Q8TE57 A disintegrin and metalloproteinase with thrombospondin motifs 16, ADAMTS16
Q8TE58 A disintegrin and metalloproteinase with thrombospondin motifs 15, ADAMTS15
Q8TE59 A disintegrin and metalloproteinase with thrombospondin motifs 19, ADAMTS19
Q8TE60 A disintegrin and metalloproteinase with thrombospondin motifs 18, ADAMTS18
Q8TE99 Acid phosphatase-like protein 2 ,ACPL2
Q8TER0 Sushi, nidogen and EGF-like domain-containing protein 1, SNED1
Q8TEU8 WAP, kazal, immunoglobulin, kunitz and NTR domain-containing protein 2, WFIKKN2
Q8WTQ1 Beta-defensin 104, DEFB104B
Q8WTR8 Netrin-5, NTN5
Q8WTU2 Scavenger receptor cysteine-rich domain- containing group B protein, SRCRB4D
Q8WU66 Protein TSPEAR, TSPEAR
Q8WUA8 Tsukushin, TSKU
Q8WUF8 Protein FAM 172A, FAM172A
Q8WUJ1 Neuferricin, CYB5D2
Q8WUY1 UPF0670 protein THEM6, THEM6
Q8WVN6 Secreted and transmembrane protein 1, SECTM1
Q8WVQ1 Soluble calcium-activated nucleotidase 1, CANT1
Q8WWA0 lntelectin-1, ITLN1

Q8WWG1 Neuregulin-4, NRG4
Q8WWQ2 Inactive heparanase-2, HPSE2
Q8WWU7 Intelectin-2, ITLN2
Q8WWY7 WAP four-disulfide core domain protein 12, WFDC12
Q8WWY8 Lipase member H, LIPH
Q8WWZ8 Oncoprotein-induced transcript 3 protein, OIT3
Q8WX39 Epididymal-specific lipocalin-9, LCN9
Q8WXA2 Prostate and testis expressed protein 1 PATE1 Secretogranin-3 SCG3, Q8WXD2
Q8WXF3 Relaxin-3 A chain, RLN3
Q8WXI7 Mucin-16, MUC16
Q8WXQ8 Carboxypeptidase A5, CPA5
Q8WXS8 A disintegrin and metalloproteinase with thrombospondin motifs 14, ADAMTS14
Q92484 Acid sphingomyelinase-like phosphodiesterase 3a, SMPDL3A
Q92485 Acid sphingomyelinase-like phosphodiesterase 3b, SMPDL3B
Q92496 Complement factor H-related protein 4, CFHR4
Q92520 Protein FAM3C, FAM3C
Q92563 Testican-2, SPOCK2
Q92583 C-C motif chemokine 17, CCL17
Q92626 Peroxidasin homolog, PXDN
Q92743 Serine protease HTRA1, HTRA1
Q92752 Tenascin-R, TNR
Q92765 Secreted frizzled-related protein 3, FRZB
Q92819 Hyaluronan synthase 2, HAS2
Q92820 Gamma-glutamyl hydrolase, GGH
Q92824 Proprotein convertase subtilisin/kexin type 5, PCSK5
Q92832 Protein kinase C-binding protein NELL1, NELL1
Q92838 Ectodysplasin-A, membrane form, EDA
Q92874 Deoxyribonuclease-1-like 2, DNASE1L2
Q92876 Kallikrein-6, KLK6
Q92913 Fibroblast growth factor 13, FGF13
Q92954 Proteoglycan 4 C-terminal part, PRG4
Q93038 Tumor necrosis factor receptor superfamily member 25, TNFRSF25
Q93091 Ribonuclease K6, RNASE6
Q93097 Protein Wnt-2b, WNT2B
Q93098 Protein Wnt-8b, WNT8B
Q95460 Major histocompatibility complex class 1-related gene protein, MR1
Q969D9 Thymic stromal lymphopoietin, TSLP
Q969E1 Liver-expressed antimicrobial peptide 2, LEAP2
Q969H8 UPF0556 protein C19orf10, C19orf10
Q969Y0 NXPE family member 3, NXPE3
Q96A54 Adiponectin receptor protein 1, ADIPOR1
Q96A83 Collagen alpha-1(XXVI) chain, EMID2
Q96A84 EMI domain-containing protein 1, EMID1
Q96A98 Tuberoinfundibular peptide of 39 residues, PTH2
Q96A99 Pentraxin-4, PTX4
Q96BH3 Epididymal sperm-binding protein 1, ELSPBP1
Q96BQ1 Protein FAM3D, FAM3D
Q96CG8 Collagen triple helix repeat-containing protein 1, CTHRC1
Q96DA0 Zymogen granule protein 16 homolog B, ZG16B

Q96DN2 von Willebrand factor C and EGF domain- containing protein, VWCE
Q96DR5 BPI fold-containing family A member 2, BPIFA2
Q96DR8 Mucin-like protein 1, MUCL1
Q96DX4 RING finger and SPRY domain-containing protein 1, RSPRY1
Q96EE4 Coiled-coil domain-containing protein 126, CCDC126
Q96GS6 Abhydrolase domain-containing protein, FAM108A1
Q96GW7 Brevican core protein, BCAN
Q96HF1 Secreted frizzled-related protein 2, SFRP2
Q96I82 Kazal-type serine protease inhibitor domain-containing protein 1, KAZALD1
Q96ID5 Immunoglobulin superfamily member 21, IGSF21
Q96II8 Leucine-rich repeat and calponin homology domain-containing protein 3, LRCH3
Q96IY4 Carboxypeptidase B2, CPB2
Q96JB6 Lysyl oxidase homolog 4, LOXL4
Q96JK4 HH IP-like protein 1, HHIPL1
Q96KN2 Beta-Ala-His dipeptidase, CNDP1
Q96KW9 Protein SPACA7, SPACA7
Q96KX0 Lysozyme-like protein 4, LYZL4
Q96L15 Ecto-ADP-ribosyltransferase 5, ART5
Q96LB8 Peptidoglycan recognition protein 4, PGLYRP4
Q96LB9 Peptidoglycan recognition protein ,3 PGLYRP3
Q96LC7 Sialic acid-binding Ig-like lectin 10, SIGLEC10
Q96LR4 Protein FAM 19A4, FAM19A4
Q96MK3 Protein FAM20A, FAM20A
Q96MS3 Glycosyltransferase 1 domain-containing protein 1, GLT1D1
Q96NY8 Processed poliovirus receptor-related protein 4, PVRL4
Q96NZ8 WAP, kazal, immunoglobulin, kunitz and NTR domain-containing protein 1, WFIKKN1
Q96NZ9 Proline-rich acidic protein 1, PRAP1
Q96P44 Collagen alpha-l(XXI) chain, COL21A1
Q96PB7 Noelin-3, OLFM3
Q96PC5 Melanoma inhibitory activity protein 2, MIA2
Q96PD5 N-acetylmuramoyl-L-alanine amidase, PGLYRP2
Q96PH6 Beta-defensin 118, DEFB118
Q96PL1 Secretoglobin family 3A member 2, SCGB3A2
Q96PL2 Beta-tectorin, TECTB
Q96QH8 Sperm acrosome-associated protein 5, SPACA5
Q96QR1 Secretoglobin family 3A member 1, SCGB3A1
Q96QU1 Protocadherin-15, PCDH15
Q96QV1 Hedgehog-interacting protein, HHIP
Q96RW7 Hemicentin-1, HMCN 1
Q96S42 Nodal homolog, NODAL
Q96S86 Hyaluronan and proteoglycan link protein 3, HAPLN3
Q96SL4 Glutathione peroxidase 7, GPX7
Q96SM3 Probable carboxypeptidase XI, CPXM 1
Q96T91 Glycoprotein hormonc alpha-2, GPHA2

Q99062 Granulocyte colony-stimulating factor receptor, CSF3R
Q99102 Mucin-4 alpha chain, MUC4
Q99217 Amelogenin, X isoform, AMELX
Q99218 Amelogenin, Y isoform, AM ELY
Q99435 Protein kinase C-binding protein NELL2, NELL2
Q99470 Stromal cell-derived factor 2, SDF2
Q99542 Matrix metalloproteinase-19, MMP19
Q99574 Neuroserpin, SERPINI1
Q99584 Protein S100-A13, S100A13
Q99616 C-C motif chemokine 13, CCL13
Q99645 Epiphycan, EPYC
Q99674 Cell growth regulator with EF hand domain protein 1, CGREF1
Q99715 Collagen alpha-l(XII) chain, COL12A1
Q99727 Metalloproteinase inhibitor 4, TIM P4
Q99731 C-C motif chemokine 1,9 CCL19
Q99748 Neurturin, NRTN
Q99935 Proline-rich protein 1, PROL1
Q99942 E3 ubiquitin-protein ligase RN F5, RNF5
Q99944 Epidermal growth factor-like protein 8, EGFL8
Q99954 Submaxillary gland androgen-regulated protein 3A, SMR3A
Q99969 Retinoic acid receptor responder protein 2, RARRES2
Q99972 Myocilin, MYOC
Q99983 Osteomodulin, OMD
Q99985 Semaphorin-3C, SEMA3C
Q99988 Growth/differentiation factor 15, GDF15
Q9BPW4 Apolipoprotein L4, APOL4
Q9BQ08 Resistin-like beta, RETN LB
Q9BQ16 Testican-3, SPOCK3
Q9BQ51 Programmed cell death 1 ligand 2, PDCD1LG2
Q9BQB4 Sclerostin, SOST
Q9BQI4 Coiled-coil domain-containing protein 3, CCDC3
Q9BQP9 BPI fold-containing family A member 3, BPIFA3
Q9BQR3 Serine protease 27, PRSS27
Q9BQY6 WAP four-disulfide core domain protein 6, WFDC6
Q9BRR6 ADP-dependent glucokinase, ADPGK
Q9BS86 Zona pellucida-binding protein 1, ZPBP
Q9BSG0 Protease-associated domain-containing protein 1, PRADC1
Q9BSG5 Retbindin, RTBDN
Q9BT30 Probable alpha-ketoglutarate-dependent dioxygenase ABH7, ALKBH7
Q9BT56 Spexin, C12orf39
Q9BT67 NEDD4 family-interacting protein 1, NDFIP1
Q9BTY2 Plasma alpha-L-fucosidase, FUCA2
Q9BU40 Chordin-like protein 1, CH RDL1
Q9BUD6 Spondin-2, SPON2
Q9BUN1 Protein MENT, MENT
Q9BUR5 Apolipoprotein 0, APO0
Q9BV94 ER degradation-enhancing alpha-mannosidase-like 2, EDEM2
Q9BWP8 Collectin-11, COLEC11
Q9BWS9 Chitinase domain-containing protein 1, CHID1
Q9BX67 Junctional adhesion molecule C, JAM3
Q9BX93 Group XI 1 B secretory phospholipase A2- like protein, PLA2G12B

Q9BXI9 Complement Clq tumor necrosis factor-related protein 6, C1QTN F6
Q9BXJ0 Complement Clq tumor necrosis factor-related protein 5, C1QTN F5
Q9BXJ1 Complement Clq tumor necrosis factor-related protein 1, C1QTN F1
Q9BXJ2 Complement Clq tumor necrosis factor-related protein 7, C1QTN F7
Q9BXJ3 Complement Clq tumor necrosis factor-related protein 4, C1QTN F4
Q9BXJ4 Complement Clq tumor necrosis factor-related protein 3, C1QTN F3
Q9BXJ5 Complement Clq tumor necrosis factor-related protein 2, C1QTN F2
Q9BXN1 Asporin, ASPN
Q9BXP8 Pappalysin-2, PAPPA2
Q9BXR6 Complement factor H-related protein 5, CFHR5
Q9BXS0 Collagen alpha-l(XXV) chain, COL25A1
Q9BXX0 EMILIN-2, EMILIN2
Q9BXY4 R-spondin-3, RSP03
Q9BY15 EGF-like module-containing mucin-like hormone receptor-like 3 subunit beta, EMR3
Q9BY50 Signal peptidase complex catalytic SEC11C subunit, SEC11C
Q9BY76 Angiopoietin-related protein 4, ANGPTL4
Q9BYF1 Processed angiotensin-converting enzyme 2, ACE2
Q9BYJ0 Fibroblast growth factor-binding protein, FGFBP2
Q9BYW3 Beta-defensin 126, DEFB126
Q9BYX4 Interferon-induced helicase C domain-containing protein 1, IFIH1
Q9BYZ8 Regenerating islet-derived protein 4, REG4
Q9BZ76 Contactin-associated protein-like 3, CNTNAP3
Q9BZG9 Ly-6/neurotoxin-like protein 1, LYNX1
Q9BZJ3 Tryptase delta, TPSD1
Q9BZM1 Group XI IA secretory phospholipase A2, PLA2G12A
Q9BZM2 Group IIF secretory phospholipase A2, PLA2G2F
Q9BZM5 NKG2D ligand 2, ULBP2
Q9BZP6 Acidic mammalian chitinase, CHIA
Q9BZZ2 Sialoadhesin, SIGLECl
Q9C0B6 Protein FAM5B, FAM5B
Q9GZM7 Tubulointerstitial nephritis antigen-like, TINAGL1
Q9GZN4 Brain-specific serine protease 4, PRSS22
Q9GZP0 Platelet-derived growth factor D, receptor-binding form, PDGFD
Q9GZT5 Protein Wnt-10a ,WNT10A
Q9GZU5 Nyctalopin, NYX
Q9GZV7 Hyaluronan and proteoglycan link protein 2, HAPLN2
Q9GZV9 Fibroblast growth factor 23, FGF23
Q9GZX9 Twisted gastrulation protein homolog 1, TWSG1
Q9GZZ7 GDN F family receptor alpha-4, GFRA4
Q9GZZ8 Extracellular glycoprotein lacritin, LACRT

Q9H0B8 Cysteine-rich secretory protein LCCL domain-containing 2, CRISPLD2
Q9H106 Signal-regulatory protein delta, SIRPD
Q9H114 Cystatin-like 1, CSTL1
Q9H173 Nucleotide exchange factor SIL1, SIL1
Q9H1E1 Ribonuclease 7, RNASE7
Q9H1F0 WAP four-disulfide core domain protein 10A, WFDCIOA
Q9H1J5 Protein Wnt-8a, WNT8A
Q9H1J7 Protein Wnt-5b, WNT5B
Q9H1M3 Beta-defensin 129, DEFB129
Q9H1M4 Beta-defensin 127, DEFB127
Q9H1Z8 Augurin, C2orf40
Q9H239 Matrix metalloproteinase-28, MMP28
Q9H2A7 C-X-C motif chemokine 16, CXCL16
Q9H2A9 Carbohydrate sulfotransferase 8, CHST8
Q9H2R5 Kallikrein-15, KLK15
Q9H2X0 Chordin, CHRD
Q9H2X3 C-type lectin domain family 4 member M, CLEC4M
Q9H306 Matrix metalloproteinase-27, MMP27
Q9H324 A disintegrin and metalloproteinase with thrombospondin motifs 10, ADAMTS10
Q9H336 Cysteine-rich secretory protein LCCL domain-containing, CRISPLD1 1
Q9H3E2 Sorting nexin-25, SNX25
Q9H3R2 Mucin-13, MUC13
Q9H3U7 SPARC-related modular calcium-binding protein 2, SMOC2
Q9H3Y0 Peptidase inhibitor R3H DM L, R3HDML
Q9H4A4 Aminopeptidase B, RN PEP
Q9H4F8 SPARC-related modular calcium-binding protein 1, SMOC1
Q9H4G1 Cystatin-9-like, CST9L
Q9H5V8 CUB domain-containing protein 1, CDCP1
Q9H6B9 Epoxide hydrolase 3, EPHX3
Q9H6E4 Coiled-coil domain-containing protein 134, CCDC134
Q9H741 UPF0454 protein C12orf49, C12orf49
Q9H772 Gremlin-2, GREM2
Q9H7Y0 Deleted in autism-related protein 1, CXorf36
Q9H8L6 Multimerin-2, MMRN2
Q9H9S5 Fukutin-related protein, FKRP
Q9HAT2 Sialate O-acetylesterase, SIAE
Q9HB40 Retinoid-inducible serine carboxypeptidase, SCPEP1
Q9HB63 Netrin-4, NTN4
Q9HBJ0 Placenta-specific protein 1, PLAC1
Q9HC23 Prokineticin-2, PROK2
Q9HC57 WAP four-disulfide core domain protein 1, WFDC1
Q9HC73 Cytokine receptor-like factor 2, CRLF2
Q9HC84 Mucin-5B, MUC5B
Q9HCB6 Spondin-1, SPON 1
Q9HCQ7 Neuropeptide NPSF, NPVF
Q9HCT0 Fibroblast growth factor 22, FGF22
Q9HD89 Resistin, RETN
Q9NNX1 Tuftelin, TUFT1
Q9NNX6 CD209 antigen, CD209
Q9NP55 BPI fold-containing family A member 1, BPIFA1
Q9NP70 Amcloblastin, AMBN
Q9NP95 Fibroblast growth factor 20, FGF20

Q9NP99 Triggering receptor expressed on myeloid cells 1, TREM1
Q9NPA2 Matrix metalloproteinase-25, MMP25
Q9NPE2 Neugrin, NGRN
Q9NPH0 Lysophosphatidic acid phosphatase type 6, ACP6
Q9NPH6 Odorant-binding protein 2b, OBP2B
Q9NQ30 Endothelial cell-specific molecule 1, ESM1
Q9NQ36 Signal peptide, CU B and EGF-like domain- containing protein 2, SCU BE2
Q9NQ38 Serine protease inhibitor Kazal-type 5, SPIN K5
Q9NQ76 Matrix extracellular phosphoglycoprotein, MEPE
Q9NQ79 Cartilage acidic protein 1, CRTAC1
Q9NR16 Scavenger receptor cysteine-rich type 1 protein M160, CD163L1
Q9NR23 Growth/differentiation factor 3, GDF3
Q9NR71 Neutral ceramidase, ASAH2
Q9NR99 Matrix-remodeling-associated protein 5, MXRA5
Q9N RA1 Platelet-derived growth factor C, PDGFC
Q9N RC9 Otoraplin, OTOR
Q9N RE1 Matrix metalloproteinase-26, MMP26
Q9N RJ3 C-C motif chemokine 28, CCL28
Q9N RM1 Enamelin, ENAM
Q9N RN5 Olfactomedin-like protein 3, OLFML3
Q9N RR1 Cytokine-like protein 1, CYTL1
Q9NS15 Latent-transforming growth factor beta-binding protein 3, LTBP3
Q9NS62 Thrombospondin type-1 domain-containing protein 1, THSD1
Q9NS71 Gastrokine-1, GKN1
Q9NS98 Semaphorin-3G, SEMA3G
Q9NSA1 Fibroblast growth factor 21, FGF21
Q9NT22 EMILIN-3, EMILIN3
Q9NTU7 Cerebellin-4, CBLN4
Q9NVR0 Kelch-like protein 11, KLHL11
Q9NWH7 Spermatogenesis-associated protein 6, SPATA6
Q9NXC2 Glucose-fructose oxidoreductase domain- containing protein 1, GFOD1
Q9NY56 Odorant-binding protein 2a, OBP2A
Q9NY84 Vascular non-inflammatory molecule 3, VN N3
Q9NZ20 Group 3 secretory phospholipase A2, PLA2G3
Q9NZC2 Triggering receptor expressed on myeloid cells 2, TREM2
Q9NZK5 Adenosine deaminase CECR1, CECR1
Q9NZK7 Group ME secretory phospholipase A2, PLA2G2E
Q9NZP8 Complement Clr subcomponent-like protein, C1RL
Q9NZV1 Cysteine-rich motor neuron 1 protein, CRIM1
Q9NZW4 Dentin sialoprotein, DSPP
Q9P0G3 Kallikrein-14, KLK14
Q9P0W0 Interferon kappa, IFNK
Q9P218 Collagen alpha-l(XX) chain, COL20A1
Q9P2C4 Transmembrane protein 181, TMEM181
Q9P2K2 Thioredoxin domain-containing protein 16, TXN DC16
Q9P2N4 A disintegrin and metalloproteinase with thrombospondin motifs 9, ADAMTS9
Q9UBC7 Galanin-like peptide, GALP

Q9U BD3 Cytokine SCM-1 beta, XCL2
Q9U BD9 Cardiotrophin-like cytokine factor 1, CLCF1
Q9U BM4 Opticin, OPTC
Q9UBP4 Dickkopf-related protein 3, DKK3
Q9U BQ6 Exostosin-like 2, EXTL2
Q9UBR5 Chemokine-like factor, CKLF
Q9UBS5 Gamma-aminobutyric acid type B receptor subunit 1, GABBR1
Q9U BT3 Dickkopf-related protein 4 short form, DKK4
Q9U BU2 Dickkopf-related protein 2, DKK2
Q9U BU3 Ghrelin-28, GHRL
Q9U BV4 Protein Wnt-16, WNT16
Q9U BX5 Fibulin-5, FBLN5
Q9U BX7 Kallikrein-11, KLK11
Q9UEF7 Klotho, KL
Q9UFP1 Protein FAM 198A, FAM198A
Q9UGM3 Deleted in malignant brain tumors 1 protein, DMBT1
Q9UGM5 Fetuin-B, FETUB
Q9UGP8 Translocation protein homolog, SEC63
Q9UHF0 Neurokinin-B, TAC3
Q9UHF1 Epidermal growth factor-like protein 7, EGFL7
Q9U HG2 ProSAAS, PCSK1N
Q9UHI8 A disintegrin and metalloproteinase with thrombospondin motifs 1, ADAMTS1
Q9UHL4 Dipeptidyl peptidase 2, DPP7
Q9UI42 Carboxypeptidase A4, CPA4
Q9UIG4 Psoriasis susceptibility 1 candidate gene 2 protein, PSORS1C2
Q9UIK5 Tomoregulin-2, TMEFF2
Q9UIQ6 Leucyl-cystinyl aminopeptidase, pregnancy serum form, LN PEP
Q9UJA9 Ectonucleotide pyrophosphatase/phosphodiesterase family member 5, ENPP5
Q9UJH8 Meteorin, METRN
Q9UJJ9 N-acetylglucosamine-1-phosphotransferase subunit gamma, GN PTG
Q9UJW2 Tubulointerstitial nephritis antigen, TINAG
Q9UK05 Growth/differentiation factor 2, GDF2
Q9UK55 Protein Z-dependent protease inhibitor, SERPINA10
Q9UK85 Dickkopf-like protein 1, DKKL1
Q9UKJ1 Paired immunoglobulin-like type 2 receptor alpha, PILRA
Q9UKP4 A disintegrin and metalloproteinase with thrombospondin motifs 7, ADAMTS7
Q9UKP5 A disintegrin and metalloproteinase with thrombospondin motifs 6, ADAMTS6
Q9UKQ2 Disintegrin and metalloproteinase domain-containing protein 28, ADAM28
Q9UKQ9 Kallikrein-9, KLK9
Q9UKR0 Kallikrein-12, KLK12
Q9UKR3 Kallikrein-13, KLK13
Q9U KU9 Angiopoietin-related protein 2, ANGPTL2
Q9UKZ9 Procollagen C-endopeptidase enhancer 2, PCOLCE2
Q9UL52 Transmembrane protease serine HE non- catalytic chain, TMPRSS11E
Q9ULC0 Endomucin, EMCN
Q9ULI3 Protein HEG homolog 1, HEG1
Q9U LZ1 Apelin-13, APLN

Q9U LZ9 Matrix metalloproteinase-17, MMP17
Q9UM21 Alpha-l,3-mannosyl-glycoprotein 4-beta- N-acetylglucosaminyltransferase A, soluble form, MGAT4A
Q9UM22 Mammalian ependymin-related protein 1, EPDR1
Q9UM73 ALK tyrosine kinase receptor, ALK
Q9UM D9 97 kDa linear IgA disease antigen, COL17A1
Q9UMX5 Neudesin, NENF
Q9UN73 Protocadherin alpha-6, PCDHA6
Q9UNA0 A disintegrin and metalloproteinase with thrombospondin motifs 5, ADAMTS5
Q9UN I1 Chymotrypsin-like elastase family member 1, CELA1
Q9UN K4 Group IID secretory phospholipase A2, PLA2G2D
Q9UP79 A disintegrin and metalloproteinase with thrombospondin motifs 8, ADAMTS8
Q9UPZ6 Thrombospondin type-1 domain-containing protein 7A, THSD7A
Q9UQ72 Pregnancy-specific beta-l-glycoprotein 11, PSG11
Q9UQ74 Pregnancy-specific beta-l-glycoprotein 8, PSG8
Q9UQC9 Calcium-activated chloride channel regulator 2, CLCA2
Q9UQE7 Structural maintenance of chromosomes protein 3, SMC3
Q9UQP3 Tenascin-N, TNN
Q9Y223 UDP-N-acetylglucosamine 2-epimerase, GN E
Q9Y240 C-type lectin domain family 11 member A, CLEC11A
Q9Y251 Heparanase 8 kDa subunit, HPSE
Q9Y258 C-C motif chemokine 26, CCL26
Q9Y264 Angiopoietin-4, ANGPT4
Q9Y275 Tumor necrosis factor ligand superfamily member 13b, membrane form, TNFSF13B
Q9Y287 BRI2 intracellular domain, ITM2B
Q9Y2E5 Epididymis-specific alpha-mannosidase, MAN2B2
Q9Y334 von Willebrand factor A domain-containing protein 7, VWA7
Q9Y337 Kallikrein-5, KLK5
Q9Y3B3 Transmembrane emp24 domain-containing protein 7, TMED7
Q9Y3E2 BolA-like protein 1, BOLA1
Q9Y426 C2 domain-containing protein 2, C2CD2
Q9Y4K0 Lysyl oxidase homolog 2, LOXL2
Q9Y4X3 C-C motif chemokine 27, CCL27
Q9Y5C1 Angiopoietin-related protein 3, ANGPTL3
Q9Y5I2 Protocadherin alpha-10, PCDHA10
Q9Y5I3 Protocadherin alpha-1, PCDHA1
Q9Y5K2 Kallikrein-4, KLK4
Q9Y5L2 Hypoxia-inducible lipid droplet-associated protein, HILPDA
Q9Y5Q5 Atrial natriuretic peptide-converting enzyme, CORIN
Q9Y5R2 Matrix metalloproteinase-24, MMP24
Q9Y5U5 Tumor necrosis factor receptor superfamily member 18, TNFRSF18
Q9Y5W5 Wnt inhibitory factor, WIF1
Q9Y5X9 Endothelial lipase, LIPG
Q9Y625 Secreted glypican-6, GPC6

Q9Y646 Carboxypeptidase Q, CPQ
Q9Y6C2 EMILIN-1, EMILINl
Q9Y6F9 Protein Wnt-6, WNT6
Q9Y6I9 Testis-expressed sequence 264 protein, TEX264
Q9Y6L7 Tolloid-like protein 2, TLL2
Q9Y6N3 Calcium-activated chloride channel regulator family member 3, CLCA3P
Q9Y6N6 Laminin subunit gamma-3, LAMC3
Q9Y6R7 IgGFc-binding protein, FCGBP
Q9Y6Y9 Lymphocyte antigen 96, LY96
Q9Y6Z7 Collectin-10, COLEC10

[00827]For each of the above therapeutic proteins, provided are the (a) the Uniprot ID, (b) the protein name, and (c) the gene name.

Antibodies

[00828]In some embodiments, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) and associated compositions and methods described herein provide for the delivery of one or more antibodies or functional fragment thereof, as outlined below.

[00829]As used herein, the term "antibody" is referred to in the broadest sense and specifically covers various embodiments including, but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies formed from at least two intact antibodies), and antibody fragments (e.g., diabodies) so long as they exhibit a desired biological activity (e.g., "functional"). Antibodies are primarily amino acid-based molecules which are monomeric or multimeric polypeptides which comprise at least one amino acid region derived from a known or parental antibody sequence and at least one amino acid region derived from a non-antibody sequence. The antibodies may comprise one or more modifications (including, but not limited to the addition of sugar moieties, fluorescent moieties, chemical tags, etc.). For the purposes herein, an "antibody" may comprise a heavy and light variable domain as well as an Fc region.

[00830]The encoded products of interest of the herein disclosed RNA payloads may comprise or may encode polypeptides that form one or more functional antibodies.

[00831]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may comprise or may encode polypeptides that form or function as any antibody including, but not limited to, antibodies that are known in the art and/or antibodies that are commercially available which may be therapeutic, diagnostic, or for research purposes. Additionally, the encoded products of interest of the herein disclosed RNA payloads may comprise or may encode fragments of such antibodies or antibodies such as, but not limited to, variable domains or complementarity determining regions (CDRs).

[00832]As used herein, the term "native antibody" refers to a usually heterotetrameric glycoprotein of about 150,000 Daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Genes encoding antibody heavy and light chains are known and segments making up each have been well

characterized and described (Matsuda, F. et al., 1998. The Journal of Experimental Medicine. 188(11); 2151-62 and Li, A. et al., 2004. Blood. 103(12: 4602-9, the content of each of which are herein incorporated by reference in their entirety). Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. As used herein, the term "light chain" refers to a component of an antibody from any vertebrate species assigned to one of two clearly distinct types, called kappa and lambda based on amino acid sequences of constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2.

[00833]As used herein, the term “variable domain” refers to specific antibody domains found on both the antibody heavy and light chains that differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. Variable domains comprise hypervariable regions. As used herein, the term “hypervariable region” refers to a region within a variable domain comprising amino acid residues responsible for antigen binding. The amino acids present within the hypervariable regions determine the structure of the complementarity determining regions (CDRs) that become part of the antigen-binding site of the antibody. As used herein, the term “CDR” refers to a region of an antibody comprising a structure that is complimentary to its target antigen or epitope. Other portions of the variable domain, not interacting with the antigen, are referred to as framework (FW) regions. The antigen-binding site (also known as the antigen combining site or paratope) comprises the amino acid residues necessary to interact with a particular antigen. The exact residues making up the antigen-binding site are typically elucidated by co-crystallography with bound antigen, however computational assessments can also be used based on comparisons with other antibodies (Strohl, W.R. Therapeutic Antibody Engineering. Woodhead Publishing, Philadelphia PA. 2012. Ch. 3, p47-54, the contents of which are herein incorporated by reference in their entirety). Determining residues making up CDRs may include the use of numbering schemes including, but not limited to, those taught by Kabat [Wu, T.T. et al., 1970, JEM, 132(2):211-50 and Johnson, G. et al., 2000, Nucleic Acids Res. 28(1): 214-8, the contents of each of which are herein incorporated by reference in their entirety], Chothia [Chothia and Lesk, J. Mol. Biol. 196, 901 (1987), Chothia et al., Nature 342, 877 (1989) and Al-Lazikani, B. et al.,

1997, J. Mol. Biol. 273(4):927-48, the contents of each of which are herein incorporated by reference in their entirety], Lefranc (Lefranc, M.P. et al., 2005, Immunome Res. 1:3) and Honegger (Honegger, A. and Pluckthun, A. 2001. J. Mol. Biol. 309(3):657-70, the contents of which are herein incorporated by reference in their entirety).

[00834] V_H and V_L domains each have three CDRs. V_L CDRs are referred to herein as CDR-L1, CDR-L2 and CDR-L3, in order of occurrence when moving from N- to C- terminus along the variable domain polypeptide. V_H CDRs are referred to herein as CDR-H1, CDR-H2, and CDR-H3, in order of occurrence when moving from N- to C-terminus along the variable domain polypeptide. Each of CDRs have favored canonical structures with the exception of the CDR-H3, which comprises amino acid sequences that may be highly variable in sequence and length between antibodies resulting in a variety of three-dimensional structures in antigen-binding domains. In some cases, CDR-H3s may be analyzed among a panel of related antibodies to assess antibody diversity.

[00835] Various methods of determining CDR sequences are known in the art and may be applied to known antibody sequences. The system described by Kabat, also referred to as "numbered according to Kabat," "Kabat numbering," "Kabat definitions," and "Kabat labeling," provides an unambiguous residue numbering system applicable to any variable domain of an antibody, and provides precise residue boundaries defining the three CDRs of each chain. (Kabat et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, Md. (1987) and (1991), the contents of which are incorporated by reference in their entirety). Kabat CDRs and comprise about residues 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3) in the light chain variable domain, and 31-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3) in the heavy chain variable domain. Chothia and coworkers found that certain sub-portions within Kabat CDRs adopt nearly identical peptide backbone conformations, despite having great diversity at the level of amino acid sequence. (Chothia et al. (1987) J. Mol. Biol. 196: 901-917; and Chothia et al. (1989) Nature 342: 877-883, the contents of each of which is herein incorporated by reference in its entirety). These CDRs can be referred to as "Chothia CDRs," "Chothia numbering," or "numbered according to Chothia," and comprise about residues 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3) in the light chain variable domain, and 26-32 (CDR1), 52-56 (CDR2) and 95-102 (CDR3) in the heavy chain variable domain. Mol. Biol. 196:901-917 (1987). The system described by MacCallum, also referred to as "numbered according to MacCallum," or "MacCallum numbering" comprises about residues 30-36 (CDR1), 46-55 (CDR2) and 89-96 (CDR3) in the light chain variable domain, and 30-35 (CDR1), 47-58 (CDR2) and 93-101 (CDR3) in the heavy chain variable domain. (MacCallum et al. ((1996) J. Mol. Biol. 262(5):732-745), the contents of which is herein incorporated by reference in its entirety). The system described by AbM, also referred to as "numbering according to AbM," or "AbM numbering" comprises about residues 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3) in the light chain variable

domain, and 26-35 (CDR1), 50-58 (CDR2) and 95-102 (CDR3) in the heavy chain variable domain. The IMGT (INTERNATIONAL IMMUNOGENETICS INFORMATION SYSTEM) numbering of variable regions can also be used, which is the numbering of the residues in an immunoglobulin variable heavy or light chain according to the methods of the IIMGT (Lefranc, M.-P., "The IMGT unique numbering for immunoglobulins, T cell Receptors and Ig-like domains", The Immunologist, 7, 132-136 (1999), and is herein incorporated by reference in its entirety by reference). As used herein, "IMGT sequence numbering" or "numbered according to IMTG," refers to numbering of the sequence encoding a variable region according to the IMGT. For the heavy chain variable domain, when numbered according to IMGT, the hypervariable region ranges from amino acid positions 27 to 38 for CDR1, amino acid positions 56 to 65 for CDR2, and amino acid positions 105 to 117 for CDR3. For the light chain variable domain, when numbered according to IMGT, the hypervariable region ranges from amino acid positions 27 to 38 for CDR1, amino acid positions 56 to 65 for CDR2, and amino acid positions 105 to 117 for CDR3.

[00836]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode antibodies which have been produced using methods known in the art such as, but are not limited to immunization and display technologies (e.g., phage display, yeast display, and ribosomal display), hybridoma technology, heavy and light chain variable region cDNA sequences selected from hybridomas or from other sources,

[00837]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode antibodies which were developed using any naturally occurring or synthetic antigen. As used herein, an "antigen" is an entity which induces or evokes an immune response in an organism. An immune response is characterized by the reaction of the cells, tissues and/or organs of an organism to the presence of a foreign entity. Such an immune response typically leads to the production by the organism of one or more antibodies against the foreign entity, e.g., antigen or a portion of the antigen. As used herein, "antigens" also refer to binding partners for specific antibodies or binding agents in a display library.

[00838]As used herein, the term "monoclonal antibody" refers to an antibody obtained from a population of substantially homogeneous cells (or clones), i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variants that may arise during production of the monoclonal antibodies, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen

[00839]The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. The monoclonal antibodies herein include "chimeric" antibodies

(immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies.

[00840]As used herein, the term "humanized antibody" refers to a chimeric antibody comprising a minimal portion from one or more non-human (e.g., murine) antibody source(s) with the remainder derived from one or more human immunoglobulin sources. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from the hypervariable region from an antibody of the recipient are replaced by residues from the hypervariable region from an antibody of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and/or capacity.

[00841]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode antibody mimetics. As used herein, the term "antibody mimetic" refers to any molecule which mimics the function or effect of an antibody and which binds specifically and with high affinity to their molecular targets. In some embodiments, antibody mimetics may be monobodies, designed to incorporate the fibronectin type III domain (Fn3) as a protein scaffold. In some embodiments, antibody mimetics may be those known in the art including, but are not limited to affibody molecules, affilins, affitins, anticalins, avimers, Centyrins, DARPINS™, fynomers, Kunitz domains, and domain peptides. In other embodiments, antibody mimetics may include one or more non-peptide regions.

Antibody fragments and variants

[00842]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode antibody fragments which comprise antigen binding regions from full-length antibodies. Non-limiting examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments, diabodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site. Also produced is a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen. Compounds and/or compositions of the present disclosure may comprise one or more of these fragments.

[00843]In some embodiments, the Fc region may be a modified Fc region wherein the Fc region may have a single amino acid substitution as compared to the corresponding sequence for the wild-type Fc region, wherein the single amino acid substitution yields an Fc region with preferred properties to those of

the wild-type Fc region. Non-limiting examples of Fc properties that may be altered by the single amino acid substitution include bind properties or response to pH conditions

[00844] As used herein, the term "Fv" refers to an antibody fragment comprising the minimum fragment on an antibody needed to form a complete antigen binding site. These regions consist of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. Fv fragments can be generated by proteolytic cleavage, but are largely unstable. Recombinant methods are known in the art for generating stable Fv fragments, typically through insertion of a flexible linker between the light chain variable domain and the heavy chain variable domain to form a single chain Fv (scFv) or through the introduction of a disulfide bridge between heavy and light chain variable domains.

[00845]As used herein, the term "single chain Fv" or "scFv" refers to a fusion protein of V_H and V_L antibody domains, wherein these domains are linked together into a single polypeptide chain by a flexible peptide linker. In some embodiments, the Fv polypeptide linker enables the scFv to form the desired structure for antigen binding. In some embodiments, scFvs are utilized in conjunction with phage display, yeast display or other display methods where they may be expressed in association with a surface member (e.g. phage coat protein) and used in the identification of high affinity peptides for a given antigen.

[00846]As used herein, the term "antibody variant" refers to a modified antibody (in relation to a native or starting antibody) or a biomolecule resembling a native or starting antibody in structure and/or function (e.g., an antibody mimetic). Antibody variants may be altered in their amino acid sequence, composition, or structure as compared to a native antibody. Antibody variants may include, but are not limited to, antibodies with altered isotypes (e.g., IgA, IgD, IgE, IgG_1, IgG_2, IgG_3, IgG_4, or IgM), humanized variants, optimized variants, multispecific antibody variants (e.g., bispecific variants), and antibody fragments.

Multispecific antibodies

[00847]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode antibodies that bind more than one epitope. As used herein, the terms "multibody" or "multispecific antibody" refer to an antibody wherein two or more variable regions bind to different epitopes. The epitopes may be on the same or different targets. In certain embodiments, a multispecific antibody is a "bispecific antibody," which recognizes two different epitopes on the same or different antigens.

[00848]In some embodiments, multi-specific antibodies may be prepared by the methods used by BIOATLA® and described in International Patent publication WO201109726, the contents of which are herein incorporated by reference in their entirety. First a library of homologous, naturally occurring antibodies is generated by any method known in the art (i.e., mammalian cell surface display), then screened by FACSAria or another screening method, for multi-specific antibodies that specifically bind to two or more target antigens. In some embodiments, the identified multi-specific antibodies are further

evolved by any method known in the art, to produce a set of modified multi-specific antibodies. These modified multi-specific antibodies are screened for binding to the target antigens. In some embodiments, the multi-specific antibody may be further optimized by screening the evolved modified multi-specific antibodies for optimized or desired characteristics.

[00849]In some embodiments, multi-specific antibodies may be prepared by the methods used by BIOATLA® and described in Unites States Publication No. US20150252119, the contents of which are herein incorporated by reference in their entirety. In one approach, the variable domains of two parent antibodies, wherein the parent antibodies are monoclonal antibodies are evolved using any method known in the art in a manner that allows a single light chain to functionally complement heavy chains of two different parent antibodies. Another approach requires evolving the heavy chain of a single parent antibody to recognize a second target antigen. A third approach involves evolving the light chain of a parent antibody so as to recognize a second target antigen. Methods for polypeptide evolution are described in International Publication WO2012009026, the contents of which are herein incorporated by reference in their entirety, and include as non-limiting examples, Comprehensive Positional Evolution (CPE), Combinatorial Protein Synthesis (CPS), Comprehensive Positional Insertion (CPI), Comprehensive Positional Deletion (CPD), or any combination thereof. The Fc region of the multi-specific antibodies described in United States Publication No. US20150252119 may be created using a knob-in-hole approach, or any other method that allows the Fc domain to form heterodimers. The resultant multi-specific antibodies may be further evolved for improved characteristics or properties such as binding affinity for the target antigen.

Bispecific antibodies

[00850]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode bispecific antibodies. As used herein, the term "bispecific antibody" refers to an antibody capable of binding two different antigens. Such antibodies typically comprise regions from at least two different antibodies. Such antibodies typically comprise antigen-binding regions from at least two different antibodies. For example, a bispecific monoclonal antibody (BsMAb, BsAb) is an artificial protein composed of fragments of two different monoclonal antibodies, thus allowing the BsAb to bind to two different types of antigen.

[00851]In some cases, the encoded products of interest of the herein disclosed RNA payloads may encode bispecific antibodies comprising antigen-binding regions from two different anti-tau antibodies. For example, such bispecific antibodies may comprise binding regions from two different antibodies

[00852]Bispecific antibody frameworks may include any of those described in Riethmuller, G., 2012. *Cancer Immunity*. 12:12-18; Marvin, J.S. *et al.*, 2005. *Acta Pharmacologica Sinica*. 26(6):649-58; and

Schaefer, W. *et al.*, 2011. *PNAS.* 108(27):11187-92, the contents of each of which are herein incorporated by reference in their entirety.

[00853]New generations of BsMAb, called "trifunctional bispecific" antibodies, have been developed. These consist of two heavy and two light chains, one each from two different antibodies, where the two Fab regions (the arms) are directed against two antigens, and the Fc region (the foot) comprises the two heavy chains and forms the third binding site.

[00854]Of the two paratopes that form the tops of the variable domains of a bispecific antibody, one can be directed against a target antigen and the other against a T-lymphocyte antigen like CD3. In the case of trifunctional antibodies, the Fc region may additionally bind to a cell that expresses Fc receptors, like a macrophage, a natural killer (NK) cell or a dendritic cell. In sum, the targeted cell is connected to one or two cells of the immune system, which subsequently destroy it.

[00855]Other types of bispecific antibodies have been designed to overcome certain problems, such as short half-life, immunogenicity and side-effects caused by cytokine liberation. They include chemically linked Fabs, consisting only of the Fab regions, and various types of bivalent and trivalent single-chain variable fragments (scFvs), fusion proteins mimicking the variable domains of two antibodies. The furthest developed of these newer formats are the bi-specific T-cell engagers (BiTEs) and mAb2's, antibodies engineered to contain an Fcab antigen-binding fragment instead of the Fc constant region.

[00856]Using molecular genetics, two scFvs can be engineered in tandem into a single polypeptide, separated by a linker domain, called a "tandem scFv" (tascFv). TascFvs have been found to be poorly soluble and require refolding when produced in bacteria, or they may be manufactured in mammalian cell culture systems, which avoids refolding requirements but may result in poor yields. Construction of a tascFv with genes for two different scFvs yields a "bispecific single-chain variable fragments" (bis-scFvs). Only two tascFvs have been developed clinically by commercial firms; both are bispecific agents in active early phase development by Micromet for oncologic indications, and are described as "Bispecific T-cell Engagers (BiTE)." Blinatumomab is an anti-CD19/anti-CD3 bispecific tascFv that potentiates T-cell responses to B-cell non-Hodgkin lymphoma in Phase 2. MT110 is an anti-EP-CAM/anti-CD3 bispecific tascFv that potentiates T-cell responses to solid tumors in Phase 1.

[00857]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode antibodies comprising a single antigen-binding domain. These molecules are extremely small, with molecular weights approximately one-tenth of those observed for full-sized mAbs. Further antibodies may include "nanobodies" derived from the antigen-binding variable heavy chain regions (V_{HH}s) of heavy chain antibodies found in camels and llamas, which lack light chains.

[00858]Disclosed and claimed in PCT Publication WO2014144573 (the contents of which are herein incorporated by reference in its entirety) to Memorial Sloan-Kettering Cancer Center are multimerization

technologies for making dimeric multispecific binding agents (*e.g.*, fusion proteins comprising antibody components) with improved properties over multispecific binding agents without the capability of dimerization.

[00859]In some cases, the encoded products of interest of the herein disclosed RNA payloads may encode tetravalent bispecific antibodies (TetBiAbs as disclosed and claimed in PCT Publication WO2014144357, the contents of which are herein incorporated in its entirety). TetBiAbs feature a second pair of Fab fragments with a second antigen specificity attached to the C-terminus of an antibody, thus providing a molecule that is bivalent for each of the two antigen specificities. The tetravalent antibody is produced by genetic engineering methods, by linking an antibody heavy chain covalently to a Fab light chain, which associates with its cognate, co-expressed Fab heavy chain.

[00860]In some aspects, the encoded products of interest of the herein disclosed RNA payloads may encode biosynthetic antibodies as described in U.S. Patent No. 5,091,513 (the contents of which are herein incorporated by reference in their entirety). Such antibody may include one or more sequences of amino acids constituting a region which behaves as a biosynthetic antibody binding site (BABS). The sites comprise 1) non-covalently associated or disulfide bonded synthetic V_H and V_L dimers, 2) V_H-V_L or V_L-V_H single chains wherein the V_H and V_L are attached by a polypeptide linker, or 3) individuals V_H or V_L domains. The binding domains comprise linked CDR and FR regions, which may be derived from separate immunoglobulins. The biosynthetic antibodies may also include other polypeptide sequences which function, *e.g.*, as an enzyme, toxin, binding site, or site of attachment to an immobilization media or radioactive atom. Methods are disclosed for producing the biosynthetic antibodies, for designing BABS having any specificity that can be elicited by in vivo generation of antibody, and for producing analogs thereof.

[00861]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode antibodies with antibody acceptor frameworks taught in U.S. Patent No. 8,399,625. Such antibody acceptor frameworks may be particularly well suited accepting CDRs from an antibody of interest. In some cases, CDRs from anti-tau antibodies known in the art or developed according to the methods presented herein may be used.

Miniaturized Antibody

[00862]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a "miniaturized" antibody. Among the best examples of mAb miniaturization are the small modular immunopharmaceuticals (SMIPs) from Trubion Pharmaceuticals. These molecules, which can be monovalent or bivalent, are recombinant single-chain molecules containing one V_L, one V_H antigen-binding domain, and one or two constant "effector" domains, all connected by linker domains. Presumably, such a molecule might offer the advantages of increased tissue or tumor penetration claimed

by fragments while retaining the immune effector functions conferred by constant domains. At least three "miniaturized" SMIPs have entered clinical development. TRU-015, an anti-CD20 SMIP developed in collaboration with Wyeth, is the most advanced project, having progressed to Phase 2 for rheumatoid arthritis (RA). Earlier attempts in systemic lupus erythrematosus (SLE) and B cell lymphomas were ultimately discontinued. Trubion and Facet Biotechnology are collaborating in the development of TRU-016, an anti-CD37 SMIP, for the treatment of CLL and other lymphoid neoplasias, a project that has reached Phase 2. Wyeth has licensed the anti-CD20 SMIP SBI-087 for the treatment of autoimmune diseases, including RA, SLE, and possibly multiple sclerosis, although these projects remain in the earliest stages of clinical testing.

Diabodies

[00863]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode diabodies. As used herein, the term "diabody" refers to a small antibody fragment with two antigen-binding sites. Diabodies comprise a heavy chain variable domain V_H connected to a light chain variable domain V_L in the same polypeptide chain. By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites.

[00864]Diabodies are functional bispecific single-chain antibodies (bscAb). These bivalent antigen-binding molecules are composed of non-covalent dimers of scFvs, and can be produced in mammalian cells using recombinant methods. (*See*, *e.g.*, Mack et al., *Proc. Natl. Acad. Sci.*, 92: 7021-7025, 1995). Few diabodies have entered clinical development. An iodine-123-labeled diabody version of the anti-CEA chimeric antibody cT84.66 has been evaluated for pre-surgical immunoscintigraphic detection of colorectal cancer in a study sponsored by the Beckman Research Institute of the City of Hope (Clinicaltrials.gov NCT00647153).

Unibody

[00865]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a "unibody," in which the hinge region has been removed from IgG4 molecules. While IgG4 molecules are unstable and can exchange light-heavy chain heterodimers with one another, deletion of the hinge region prevents heavy chain-heavy chain pairing entirely, leaving highly specific monovalent light/heavy heterodimers, while retaining the Fc region to ensure stability and half-life in vivo. This configuration may minimize the risk of immune activation or oncogenic growth, as IgG4 interacts poorly with FcRs and monovalent unibodies fail to promote intracellular signaling complex formation. These contentions are, however, largely supported by laboratory, rather than clinical, evidence. Other antibodies may be "miniaturized" antibodies, which are compacted 100 kDa antibodies.

Intrabodies

[00866]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode intrabodies. The term "intrabody" refers to a form of antibody that is not secreted from a cell in which it is produced, but instead targets one or more intracellular proteins. Intrabodies may be used to affect a multitude of cellular processes including, but not limited to intracellular trafficking, transcription, translation, metabolic processes, proliferative signaling, and cell division. In some embodiments, methods of the present disclosure may include intrabody-based therapies. In some such embodiments, variable domain sequences and/or CDR sequences disclosed herein may be incorporated into one or more constructs for intrabody-based therapy. For example, intrabodies may target one or more glycated intracellular proteins or may modulate the interaction between one or more glycated intracellular proteins and an alternative protein.

[00867]More than two decades ago, intracellular antibodies against intracellular targets were first described (Biocca, Neuberger and Cattaneo *EMBO J.* 9: 101-108, 1990, the contents of which are herein incorporated by reference in their entirety). The intracellular expression of intrabodies in different compartments of mammalian cells allows blocking or modulation of the function of endogenous molecules (Biocca, *et al.*, *EMBO J.* 9: 101-108, 1990; Colby et al., Proc. Natl. Acad. Sci. U.S.A. 101: 17616-21, 2004, the contents of which are herein incorporated by reference in their entirety). Intrabodies can alter protein folding, protein-protein, protein-DNA, protein-RNA interactions and protein modification. They can induce a phenotypic knockout and work as neutralizing agents by direct binding to the target antigen, by diverting its intracellular trafficking or by inhibiting its association with binding partners. They have been largely employed as research tools and are emerging as therapeutic molecules for the treatment of human diseases such as viral pathologies, cancer and misfolding diseases. The fast-growing bio-market of recombinant antibodies provides intrabodies with enhanced binding specificity, stability, and solubility, together with lower immunogenicity, for their use in therapy.

[00868]In some embodiments, intrabodies have advantages over interfering RNA (iRNA); for example, iRNA has been shown to exert multiple non-specific effects, whereas intrabodies have been shown to have high specificity and affinity to target antigens. Furthermore, as proteins, intrabodies possess a much longer active half-life than iRNA. Thus, when the active half-life of the intracellular target molecule is long, gene silencing through iRNA may be slow to yield an effect, whereas the effects of intrabody expression can be almost instantaneous. Lastly, it is possible to design intrabodies to block certain binding interactions of a particular target molecule, while sparing others.

[00869]Intrabodies are often single chain variable fragments (scFvs) expressed from a recombinant nucleic acid molecule and engineered to be retained intracellularly (*e.g.*, retained in the cytoplasm, endoplasmic reticulum, or periplasm). Intrabodies may be used, for example, to ablate the function of a

protein to which the intrabody binds. The expression of intrabodies may also be regulated through the use of inducible promoters in the nucleic acid expression vector comprising the intrabody. Intrabodies may be produced for use in the viral genomes of the disclosure using methods known in the art, such as those disclosed and reviewed in: Marasco *et al.*, 1993 *Proc. Natl. Acad. Sci. USA*, 90: 7889-7893; Chen *et al.*, 1994, *Hum. Gene Ther.* 5:595-601; Chen *et al.*, 1994, *Proc. Natl. Acad. Sci. USA*, 91: 5932-5936; Maciejewski *et al.*, 1995, *Nature Med.*, 1: 667-673; Marasco, 1995, *Immunotech*, 1: 1-19; Mhashilkar, *et al.*, 1995, *EMBO J.* 14: 1542-51; Chen *et al.*, 1996, *Hum. Gene Therap.*, 7: 1515-1525; Marasco, *Gene Ther.* 4:11-15, 1997; Rondon and Marasco, 1997, *Annu. Rev. Microbiol.* 51:257-283; Cohen, *et al.*, 1998, *Oncogene* 17:2445-56; Proba *et al.*, 1998, *J. Mol. Biol.* 275:245-253; Cohen *et al.*, 1998, *Oncogene* 17:2445-2456; Hassanzadeh, *et al.*, 1998, *FEBS Lett.* 437:81-6; Richardson *et al.*, 1998, *Gene Ther.* 5:635-44; Ohage and Steipe, 1999, *J. Mol. Biol.* 291:1119-1128; Ohage *et al.*, 1999, *J. Mol. Biol.* 291:1129-1134; Wirtz and Steipe, 1999, *Protein Sci.* 8:2245-2250; Zhu *et al.*, 1999, *J. Immunol. Methods* 231:207-222; Arafat *et al.*, 2000, *Cancer Gene Ther.* 7:1250-6; der Maur *et al.*, 2002, *J. Biol. Chem.* 277:45075-85; Mhashilkar *et al.*, 2002, *Gene Ther.* 9:307-19; and Wheeler *et al.*, 2003, *FASEB J.* 17: 1733-5; and references cited therein). In particular, a CCR5 intrabody has been produced by Steinberger *et al.*, 2000, *Proc. Natl. Acad. Sci. USA* 97:805-810). *See* generally Marasco, WA, 1998, "Intrabodies: Basic Research and Clinical Gene Therapy Applications" Springer: New York; and for a review of scFvs, *see* Pluckthun in "The Pharmacology of Monoclonal Antibodies," 1994, vol. 113, Rosenburg and Moore *eds.* Springer-Verlag, New York, pp. 269-315; the contents of each of which are each incorporated by reference in their entireties.

[00870]Sequences from donor antibodies may be used to develop intrabodies. Intrabodies are often recombinantly expressed as single domain fragments such as isolated V_H and V_L domains or as a single chain variable fragment (scFv) antibody within the cell. For example, intrabodies are often expressed as a single polypeptide to form a single chain antibody comprising the variable domains of the heavy and light chains joined by a flexible linker polypeptide. Intrabodies typically lack disulfide bonds and are capable of modulating the expression or activity of target genes through their specific binding activity. Single chain antibodies can also be expressed as a single chain variable region fragment joined to the light chain constant region.

[00871]As is known in the art, an intrabody can be engineered into recombinant polynucleotide vectors to encode sub-cellular trafficking signals at its N or C terminus to allow expression at high concentrations in the sub-cellular compartments where a target protein is located. For example, intrabodies targeted to the endoplasmic reticulum (ER) are engineered to incorporate a leader peptide and, optionally, a C-terminal ER retention signal. Intrabodies intended to exert activity in the nucleus are engineered to include a nuclear localization signal. Lipid moieties are joined to intrabodies in order to tether the intrabody to the

cytosolic side of the plasma membrane. Intrabodies can also be targeted to exert function in the cytosol. For example, cytosolic intrabodies are used to sequester factors within the cytosol, thereby preventing them from being transported to their natural cellular destination.

[00872]There are certain technical challenges with intrabody expression. In particular, protein conformational folding and structural stability of the newly-synthesized intrabody within the cell is affected by reducing conditions of the intracellular environment.

[00873]Intrabodies of the disclosure may be promising therapeutic agents for the treatment of misfolding diseases, including Tauopathies, prion diseases, Alzheimer's, Parkinson's, and Huntington's, because of their virtually infinite ability to specifically recognize the different conformations of a protein, including pathological isoforms, and because they can be targeted to the potential sites of aggregation (both intra- and extracellular sites). These molecules can work as neutralizing agents against amyloidogenic proteins by preventing their aggregation, and/or as molecular shunters of intracellular traffic by rerouting the protein from its potential aggregation site.

Maxibodies

[00874]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a maxibody (bivalent scFV fused to the amino terminus of the Fc (CH2-CH3 domains) of IgG.

Chimeric Antigen Receptors (CARs)

[00875]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a chimeric antigen receptors (CARs) which when transduced into immune cells (e.g., T cells and NK cells), can re-direct the immune cells against the target (e.g., a tumor cell) which expresses a molecule recognized by the extracellular target moiety of the CAR.

[00876]As used herein, the term "chimeric antigen receptor (CAR)" refers to a synthetic receptor that mimics TCR on the surface of T cells. In general, a CAR is composed of an extracellular targeting domain, a transmembrane domain/region and an intracellular signaling/activation domain. In a standard CAR receptor, the components: the extracellular targeting domain, transmembrane domain and intracellular signaling/activation domain, are linearly constructed as a single fusion protein. The extracellular region comprises a targeting domain/moiety (e.g., a scFv) that recognizes a specific tumor antigen or other tumor cell-surface molecules. The intracellular region may contain a signaling domain of TCR complex (e.g., the signal region of CD3ζ), and/or one or more costimulatory signaling domains, such as those from CD28, 4-1BB (CD137) and OX-40 (CD134). For example, a "first-generation CAR" only has the CD3ζ signaling domain, whereas in an effort to augment T-cell persistence and proliferation, costimulatory intracellular domains are added, giving rise to second generation CARs having a CD3ζsignal domain plus one costimulatory signaling domain, and third generation CARs having CD3ζ

signal domain plus two or more costimulatory signaling domains. A CAR, when expressed by a T cell, endows the T cell with antigen specificity determined by the extracellular targeting moiety of the CAR. In some aspects you could add one or more elements such as homing and suicide genes to develop a more competent and safer architecture of CAR (so called the fourth generation CAR).

[00877]In some embodiments, the extracellular targeting domain is joined through the hinge (also called space domain or spacer) and transmembrane regions to an intracellular signaling domain. The hinge connects the extracellular targeting domain to the transmembrane domain which transverses the cell membrane and connects to the intracellular signaling domain. The hinge may need to be varied to optimize the potency of CAR transformed cells toward cancer cells due to the size of the target protein where the targeting moiety binds, and the size and affinity of the targeting domain itself. Upon recognition and binding of the targeting moiety to the target cell, the intracellular signaling domain leads to an activation signal to the CAR T cell, which is further amplified by the "second signal" from one or more intracellular costimulatory domains. The CAR T cell, once activated, can destroy the target cell.

[00878]In some embodiments, the CAR may be split into two parts, each part is linked a dimerizing domain, such that an input that triggers the dimerization promotes assembly of the intact functional receptor. Wu and Lim reported a split CAR in which the extracellular CD19 binding domain and the intracellular signaling element are separated and linked to the FKBP domain and the FRB* (T2089L mutant of FKBP-rapamycin binding) domain that heterodimerize in the presence of the rapamycin analog AP21967. The split receptor is assembled in the presence of AP21967 and together with the specific antigen binding, activates T cells (Wu et al., Science, 2015, 625(6258): aab4077, the contents of which are herein incorporated by reference in its entirety).

[00879]In some embodiments, the CAR may be designed as an inducible CAR which has an incorporation of a Tet-On inducible system to a CD19 CAR construct. The CD19 CAR is activated only in the presence of doxycycline (Dox). Sakemura reported that Tet-CD19CAR T cells in the presence of Dox were equivalently cytotoxic against CD19+ cell lines and had equivalent cytokine production and proliferation upon CD19 stimulation, compared with conventional CD19CAR T cells (Sakemura et al., Cancer Immuno. Res., 2016, Jun 21, Epub; the contents of which is herein incorporated by reference in its entirety). The dual systems provide more flexibility to turn-on and off of the CAR expression in transduced T cells.

[00880]In some embodiments, the cargo or payload may be or may encode a first generation CAR, or a second generation CAR, or a third generation CAR, or a fourth generation CAR. In some embodiments, the cargo or payload may be or may encode a full CAR construct composed of the extracellular domain, the hinge and transmembrane domain and the intracellular signaling region. In other embodiments, the cargo or payload may be or may encode a component of the full CAR construct including an extracellular

targeting moiety, a hinge region, a transmembrane domain, an intracellular signaling domain, one or more co-stimulatory domain, and other additional elements that improve CAR architecture and functionality including but not limited to a leader sequence, a homing element and a safety switch, or the combination of such components.

[00881]In some embodiments, the CROI of the herein disclosed RNA payloads may encode a tunable CARs. The reversible on-off switch mechanism allows management of acute toxicity caused by excessive CAR-T cell expansion. The ligand conferred regulation of the CAR may be effective in offsetting tumor escape induced by antigen loss, avoiding functional exhaustion caused by tonic signaling due to chronic antigen exposure and improving the persistence of CAR expressing cells in vivo. The tunable CAR may be utilized to down regulate CAR expression to limit on target on tissue toxicity caused by tumor lysis syndrome. Down regulating the expression of the CARs following anti-tumor efficacy may prevent (1) On target off tumor toxicity caused by antigen expression in normal tissue. (2) antigen independent activation in vivo.

Extracellular targeting domain / CARs

[00882]In some embodiments, the extracellular target moiety of a CAR may be any agent that recognizes and binds to a given target molecule, for example, a neoantigen on tumor cells, with high specificity and affinity. The target moiety may be an antibody and variants thereof that specifically binds to a target molecule on tumor cells, or a peptide aptamer selected from a random sequence pool based on its ability to bind to the target molecule on tumor cells, or a variant or fragment thereof that can bind to the target molecule on tumor cells, or an antigen recognition domain from native T-cell receptor (TCR) (e.g., CD4 extracellular domain to recognize HIV infected cells), or exotic recognition components such as a linked cytokine that leads to recognition of target cells bearing the cytokine receptor, or a natural ligand of a receptor.

[00883]In some embodiments, the targeting domain of a CAR may be a Ig NAR, a Fab fragment, a Fab' fragment, a F(ab)'2 fragment, a F(ab)'3 fragment, Fv, a single chain variable fragment (scFv), a bis-scFv, a (scFv)2, a minibody, a diabody, a triabody, a tetrabody, a disulfide stabilized Fv protein (dsFv), a unibody, a nanobody, or an antigen binding region derived from an antibody that specifically recognizes a target molecule, for example a tumor specific antigen (TSA). In one embodiment, the targeting moiety is a scFv antibody. The scFv domain, when it is expressed on the surface of a CAR T cell and subsequently binds to a target protein on a cancer cell, is able to maintain the CAR T cell in proximity to the cancer cell and to trigger the activation of the T cell. A scFv can be generated using routine recombinant DNA technology techniques and is discussed in the present disclosure.

[00884]In some embodiments, the targeting moiety of a CAR construct may be an aptamer such as a peptide aptamer that specifically binds to a target molecule of interest. The peptide aptamer may be selected from a random sequence pool based on its ability to bind to the target molecule of interest.

[00885]In some embodiments, the targeting moiety of a CAR construct may be a natural ligand of the target molecule, or a variant and/or fragment thereof capable of binding the target molecule. In some aspects, the targeting moiety of a CAR may be a receptor of the target molecule, for example, a full length human CD27, as a CD70 receptor, may be fused in frame to the signaling domain of CD3 ζ forming a CD27 chimeric receptor as an immunotherapeutic agent for CD70-positive malignancies.

[00886]In some embodiments, the targeting moiety of a CAR may recognize a tumor specific antigen (TSA), for example a cancer neoantigen which is restrictedly expressed on tumor cells.

[00887]As non-limiting examples, the CAR of the present disclosure may comprise the extracellular targeting domain capable of binding to a tumor specific antigen selected from 5T4, 707-AP, A33, AFP (alpha-fetoprotein), AKAP-4 (A kinase anchor protein 4), ALK, α5β1-integrin, androgen receptor, annexin II, alpha- actinin-4, ART-4, B1, B7H3, B7H4, BAGE (B melanoma antigen), BCMA, BCR-ABL fusion protein, beta-catenin, BKT-antigen, BTAA, CA-I (carbonic anhydrase I), CA50 (cancer antigen 50), CA125, CA15-3, CA195, CA242, calretinin, CAIX (carbonic anhydrase), CAMEL (cytotoxic T-lymphocyte recognized antigen on melanoma), CAM43, CAP-1, Caspase-8/m, CD4, CD5, CD7, CD19, CD20, CD22, CD23, CD25, CD27/m, CD28, CD30, CD33, CD34, CD36, CD38, CD40/CD154, CD41, CD44v6, CD44v7/8, CD45,CD49f, CD56, CD68\KP1, CD74, CD79a/CD79b, CD103, CD123, CD133, CD138, CD171, cdc27/m, CDK4 (cyclin dependent kinase 4), CDKN2A, CDS, CEA (carcinoembryonic antigen), CEACAM5, CEACAM6, chromogranin, c-Met, c-Myc, coa-1, CSAp, CT7, CT10, cyclophilin B, cyclin B1, cytoplasmic tyrosine kinases, cytokeratin, DAM-10, DAM-6, dek-can fusion protein, desmin, DEPDC1 (DEP domain containing 1), E2A-PRL, EBNA, EGF-R (epidermal growth factor receptor), EGP-1(epithelial glycoprotein -1) (TROP-2), EGP-2, EGP-40, EGFR (epidermal growth factor receptor), EGFRvIII, EF-2, ELF2M, EMMPRIN, EpCAM (epithelial cell adhesion molecule), EphA2, Epstein Barr virus antigens, Erb (ErbB1; ErbB3; ErbB4), ETA (epithelial tumor antigen), ETV6-AML1 fusion protein, FAP (fibroblast activation protein), FBP (folate-binding protein), FGF-5, folate receptor, FOS related antigen 1, fucosyl GM1, G250, GAGE (GAGE-1; GAGE-2), galectin, GD2 (ganglioside), GD3, GFAP (glial fibrillary acidic protein), GM2 (oncofetal antigen-immunogenic-1; OFA-I-1), GnT-V, Gp100, H4-RET, HAGE (helicase antigen), HER-2/neu, HIFs (hypoxia inducible factors), HIF-1, HIF-2, HLA-A2, HLA-A*0201-R170I, HLA-Al l, HMWMAA, Hom/Mel-40, HSP70-2M (Heat shock protein 70), HST-2, HTgp-175, hTERT (or hTRT), human papillomavirus-E6/human papillomavirus-E7 and E6, iCE (immune-capture EIA), IGF-1R, IGH-IGK, IL-2R, IL-5, ILK (integrin-linked kinase), IMP3 (insulin-like growth factor II mRNA-binding protein 3), IRF4 (interferon regulatory factor 4), KDR (kinase insert

domain receptor), KIAA0205, KRAB-zinc finger protein (KID)-3; KID31, KSA (17-1A), K-ras, LAGE, LCK, LDLR/FUT (LDLR-fucosyltransferaseAS fusion protein), LeY (Lewis Y), MAD-CT-1, MAGE (tyrosinase, melanoma-associated antigen) (MAGE-1; MAGE-3), melan-A tumor antigen (MART), MART-2/Ski, MC1R (melanocortin 1 receptor), MDM2, mesothelin, MPHOSPH1, MSA(muscle-specific actin), mTOR (mammalian targets of rapamycin), MUC-1, MUC-2, MUM-1 (melanoma associated antigen (mutated) 1), MUM-2, MUM-3, Myosin/m, MYL-RAR, NA88-A, N-acetylglucosaminyltransferase, neo-PAP, NF-KB (nuclear factor-kappa B), neurofilament, NSE (neuron-specific enolase), Notch receptors, NuMa, N-Ras, NY-BR-1, NY- CO-1, NY-ESO-1, Oncostatin M, OS-9, OY-TES1, p53 mutants, p190 minor bcr-abl, pl5(58), pl85erbB2, pl80erbB-3, PAGE (prostate associated gene), PAP (prostatic acid phosphatase), PAX3, PAX5, PDGFR (platelet derived growth factor receptor), cytochrome P450 involved in piperidine and pyrrolidine utilization (PIPA), Pml-RAR alpha fusion protein, PR-3 (proteinase 3), PSA (prostate specific antigen), PSM, PSMA (Prostate stem cell antigen), PRAME (preferentially expressed antigen of melanoma), PTPRK, RAGE (renal tumor antigen), Raf (A-Raf, B-Raf and C-Raf), Ras, receptor tyrosine kinases, RCAS1, RGSS, ROR1 (receptor tyrosine kinase-like orphan receptor 1), RU1, RU2, SAGE, SART-1, SART-3, SCP-1, SDCCAG16, SP-17 (sperm protein 17), src-family, SSX (synovial sarcoma X breakpoint)-1, SSX-2(HOM-MEL-40), SSX-3, SSX-4, SSX-5, STAT-3, STAT-5, STAT-6, STEAD, STn, survivin, syk-ZAP70, TA-90 (Mac-2 binding protein\cyclophilin C-associated protein), TAAL6, TACSTD1 (tumor associated calcium signal transducer 1), TACSTD2, TAG-72-4, TAGE, TARP (T cell receptor gamma alternate reading frame protein), TEL/AML1 fusion protein, TEM1, TEM8 (endosialin or CD248), TGFβ, TIE2, TLP, TMPRSS2 ETS fusion gene, TNF-receptor (TNF-α receptor, TNF-β receptor; or TNF-γ receptor), transferrin receptor, TPS, TRP-1 (tyrosine related protein 1), TRP-2, TRP-2/INT2, TSP-180, VEGF receptor, WNT, WT-1 (Wilm's tumor antigen) and XAGE.

[00888]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a CAR which comprises a universal immune receptor which has a targeting moiety capable of binding to a labelled antigen.

[00889]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a CAR which comprises a targeting moiety capable of binding to a pathogen antigen.

[00890]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a CAR which comprises a targeting moiety capable of binding to non-protein molecules such as tumor-associated glycolipids and carbohydrates.

[00891]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a CAR which comprises a targeting moiety capable of binding to a component within the tumor microenvironment including proteins expressed in various tumor stroma cells including tumor

associated macrophages (TAMs), immature monocytes, immature dendritic cells, immunosuppressive CD4+CD25+ regulatory T cells (Treg) and MDSCs.

[00892]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a CAR which comprises a targeting moiety capable of binding to a cell surface adhesion molecule, a surface molecule of an inflammatory cell that appears in an autoimmune disease, or a TCR causing autoimmunity. As non-limiting examples, the targeting moiety of the present disclosure may be a scFv antibody that recognizes a tumor specific antigen (TSA), for example scFvs of antibodies SS, SS1 and HN1 that specifically recognize and bind to human mesothelin, scFv of antibody of GD2, a CD19 antigen binding domain, a NKG2D ligand binding domain, human anti-mesothelin scFvs, an anti-CS1 binding agent, an anti-BCMA binding domain, anti-CD19 scFv antibody, GFR alpha 4 antigen binding fragments, anti-CLL-1 (C-type lectin-like molecule 1) binding domains, CD33 binding domains, a GPC3 (glypican-3) binding domain, a GFR alpha4 (Glycosyl-phosphatidylinositol (GPI)-linked GDNF family α -receptor 4 cell-surface receptor) binding domain, CD123 binding domains, an anti-ROR1 antibody or fragments thereof, scFvs specific to GPC-3, scFv for CSPG4, and scFv for folate receptor alpha.

Intracellular signaling domain / CARs

[00893]The intracellular domain of a CAR fusion polypeptide, after binding to its target molecule, transmits a signal to the immune effector cell, activating at least one of the normal effector functions of immune effector cells, including cytolytic activity (e.g., cytokine secretion) or helper activity. Therefore, the intracellular domain comprises an "intracellular signaling domain" of a T cell receptor (TCR).

[00894]In some aspects, the entire intracellular signaling domain can be employed. In other aspects, a truncated portion of the intracellular signaling domain may be used in place of the intact chain as long as it transduces the effector function signal.

[00895]In some embodiments, the intracellular signaling domain may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs (ITAMs). Examples of ITAM containing cytoplasmic signaling sequences include those derived from TCR CD3zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d. In one example, the intracellular signaling domain is a CD3 zeta (CD3ζ) signaling domain.

[00896]In some embodiments, the intracellular region further comprises one or more costimulatory signaling domains which provide additional signals to the immune effector cells. These costimulatory signaling domains, in combination with the signaling domain can further improve expansion, activation, memory, persistence, and tumor-eradicating efficiency of CAR engineered immune cells (e.g., CAR T cells). In some cases, the costimulatory signaling region contains 1, 2, 3, or 4 cytoplasmic domains of one or more intracellular signaling and /or costimulatory molecules. The costimulatory signaling domain may be the intracellular/cytoplasmic domain of a costimulatory molecule, including but not limited to CD2,

CD7, CD27, CD28, 4-1BB (CD137), OX40 (CD134), CD30, CD40, ICOS (CD278), GITR (glucocorticoid-induced tumor necrosis factor receptor), LFA-1 (lymphocyte function-associated antigen-1), LIGHT, NKG2C, B7-H3. In one example, the costimulatory signaling domain is derived from the cytoplasmic domain of CD28. In another example, the costimulatory signaling domain is derived from the cytoplasmic domain of 4-1BB (CD137). In another example, the co-stimulatory signaling domain may be an intracellular domain of GITR as taught in U.S. Pat. NO.: 9, 175, 308; the contents of which are incorporated herein by reference in its entirety.

[00897]In some embodiments, the intracellular region may comprise a functional signaling domain from a protein selected from the group consisting of an MHC class I molecule, a TNF receptor protein, an immunoglobulin-like protein, a cytokine receptor, an integrin, a signaling lymphocytic activation protein (SLAM) such as CD48, CD229, 2B4, CD84, NTB-A, CRACC, BLAME,CD2F-10, SLAMF6, SLAMF7, an activating NK cell receptor, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, IL-15Ra, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, NKD2C SLP76, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, CD270 (HVEM), GADS, SLP-76, PAG/Cbp, CD19a, a ligand that specifically binds with CD83, DAP 10, TRIM, ZAP70, Killer immunoglobulin receptors (KIRs) such as KIR2DL1, KIR2DL2/L3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1/S1, KIR3DL2, KIR3DL3, and KIR2DP1; lectin related NK cell receptors such as Ly49, Ly49A, and Ly49C.

[00898]In some embodiments, the intracellular signaling domain of the present disclosure may contain signaling domains derived from JAK-STAT. In other embodiments, the intracellular signaling domain of the present disclosure may contain signaling domains derived from DAP-12 (Death associated protein 12) (Topfer et al., Immunol., 2015, 194: 3201-3212; and Wang et al., Cancer Immunol., 2015, 3: 815-826). DAP-12 is a key signal transduction receptor in NK cells. The activating signals mediated by DAP-12 play important roles in triggering NK cell cytotoxicity responses toward certain tumor cells and virally infected cells. The cytoplasmic domain of DAP12 contains an Immunoreceptor Tyrosine-based Activation Motif (ITAM). Accordingly, a CAR containing a DAP12-derived signaling domain may be used for adoptive transfer of NK cells.

Transmembrane domains / CARs

[00899]In some embodiments, the CAR may comprise a transmembrane domain. As used herein, the term "Transmembrane domain (TM)" refers broadly to an amino acid sequence of about 15 residues in length which spans the plasma membrane. The transmembrane domain may include at least 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 amino acid residues and spans the plasma membrane. In some embodiments, the transmembrane domain may be derived either from a natural or from a synthetic source. The transmembrane domain of a CAR may be derived from any naturally membrane-bound or transmembrane protein. For example, the transmembrane region may be derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD3 epsilon, CD4, CD5, CD8, CD8α, CD9, CD16, CD22, CD33, CD28, CD37, CD45, CD64, CD80, CD86, CD134, CD137, CD152, or CD154.

[00900]Alternatively, the transmembrane domain of the present disclosure may be synthetic. In some aspects, the synthetic sequence may comprise predominantly hydrophobic residues such as leucine and valine.

[00901]In some embodiments, the transmembrane domain may be selected from the group consisting of a CD8α transmembrane domain, a CD4 transmembrane domain, a CD 28 transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, and a human IgG4 Fc region.

[00902]In some embodiments, the CAR may comprise an optional hinge region (also called spacer). A hinge sequence is a short sequence of amino acids that facilitates flexibility of the extracellular targeting domain that moves the target binding domain away from the effector cell surface to enable proper cell/cell contact, target binding and effector cell activation. The hinge sequence may be positioned between the targeting moiety and the transmembrane domain. The hinge sequence can be any suitable sequence derived or obtained from any suitable molecule. The hinge sequence may be derived from all or part of an immunoglobulin (e.g., IgGl, IgG2, IgG3, IgG4) hinge region, i.e., the sequence that falls between the CHI and CH2 domains of an immunoglobulin, e.g., an IgG4 Fc hinge, the extracellular regions of type 1 membrane proteins such as CD8α CD4, CD28 and CD7, which may be a wild-type sequence or a derivative. Some hinge regions include an immunoglobulin CH3 domain or both a CH3 domain and a CH2 domain. In certain embodiments, the hinge region may be modified from an IgG1, IgG2, IgG3, or IgG4 that includes one or more amino acid residues, for example, 1, 2, 3, 4 or 5 residues, substituted with an amino acid residue different from that present in an unmodified hinge.

[00903]In some embodiments, the CAR may comprise one or more linkers between any of the domains of the CAR. The linker may be between 1-30 amino acids long. In this regard, the linker may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acids in length. In other embodiments, the linker may be flexible.

[00904]In some embodiments, the components including the targeting moiety, transmembrane domain and intracellular signaling domains may be constructed in a single fusion polypeptide. The fusion polypeptide may be the payload of an effector module of the disclosure.

[00905]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a CD19 specific CAR targeting different B cell malignancies and HER2-specific CAR targeting sarcoma, glioblastoma, and advanced Her2-positive lung malignancy.

Tandem CAR (TanCAR)

[00906]In some embodiments, the CAR may be a tandem chimeric antigen receptor (TanCAR) which is able to target two, three, four, or more tumor specific antigens. In some aspects, The CAR is a bispecific TanCAR including two targeting domains which recognize two different TSAs on tumor cells. The bispecific TanCAR may be further defined as comprising an extracellular region comprising a targeting domain (e.g., an antigen recognition domain) specific for a first tumor antigen and a targeting domain (e.g., an antigen recognition domain) specific for a second tumor antigen. In other aspects, the CAR is a multispecific TanCAR that includes three or more targeting domains configured in a tandem arrangement. The space between the targeting domains in the TanCAR may be between about 5 and about 30 amino acids in length, for example, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30 amino acids.

Split CARs

[00907]In some embodiments, the CAR components including the targeting moiety, transmembrane domain and intracellular signaling domains may be split into two or more parts such that it is dependent on multiple inputs that promote assembly of the intact functional receptor. As a non-limiting example, the split CAR consists of two parts that assemble in a small molecule-dependent manner; one part of the receptor features an extracellular antigen binding domain (e.g., scFv) and the other part has the intracellular signaling domains, such as the CD3ζ intracellular domain.

[00908]In other aspects, the split parts of the CAR system can be further modified to increase signal. As a non-limiting example, the second part of cytoplasmic fragment may be anchored to the plasma membrane by incorporating a transmembrane domain (e.g., CD8α transmembrane domain) to the construct. An additional extracellular domain may also be added to the second part of the CAR system, for instance an extracellular domain that mediates homo-dimerization. These modifications may increase receptor output activity, i.e., T cell activation.

[00909]In some embodiments, the two parts of the split CAR system contain heterodimerization domains that conditionally interact upon binding of a heterodimerizing small molecule. As such, the receptor components are assembled in the presence of the small molecule, to form an intact system which can then be activated by antigen engagement. Any known heterodimerizing components can be incorporated into a

split CAR system. Other small molecule dependent heterodimerization domains may also be used, including, but not limited to, gibberellin-induced dimerization system (GID1-GAI), trimethoprim-SLF induced ecDHFR and FKBP dimerization and ABA (abscisic acid) induced dimerization of PP2C and PYL domains. The dual regulation using inducible assembly (e.g., ligand dependent dimerization) and degradation (e.g., destabilizing domain induced CAR degradation) of the split CAR system may provide more flexibility to control the activity of the CAR modified T cells.

Switchable CARs

[00910]In some embodiments, the CAR may be a switchable CAR which is a controllable CARs that can be transiently switched on in response to a stimulus (e.g. a small molecule). In this CAR design, a system is directly integrated in the hinge domain that separate the scFv domain from the cell membrane domain in the CAR. Such system is possible to split or combine different key functions of a CAR such as activation and costimulation within different chains of a receptor complex, mimicking the complexity of the TCR native architecture. This integrated system can switch the scFv and antigen interaction between on/off states controlled by the absence/presence of the stimulus.

Reversible CARs

[00911]In some embodiments, the CAR may be a reversible CAR system. In this CAR architecture, a LID domain (ligand-induced degradation) is incorporated into the CAR system. The CAR can be temporarily down-regulated by adding a ligand of the LID domain.

Inhibitory CARs (iCARs)

[00912]In some embodiments, the CAR may be an inhibitory CAR. Inhibitory CAR (iCAR) refers to a bispecific CAR design wherein a negative signal is used to enhance the tumor specificity and limit normal tissue toxicity. This design incorporates a second CAR having a surface antigen recognition domain combined with an inhibitory signal domain to limit T cell responsiveness even with concurrent engagement of an activating receptor. This antigen recognition domain is directed towards a normal tissue specific antigen such that the T cell can be activated in the presence of first target protein, but if the second protein that binds to the iCAR is present, the T cell activation is inhibited.

[00913]As a non-limiting example, iCARs against Prostate specific membrane antigen (PMSA) based on CTLA4 and PD1 inhibitory domains demonstrated the ability to selectively limit cytokine secretion, cytotoxicity and proliferation induced by T cell activation.

Chimeric switch receptors

[00914]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads encodes a chimeric switch receptor which can switch a negative signal to a positive signal. As used herein, the term "chimeric switch receptor" refers to a fusion protein comprising a first extracellular domain and a second transmembrane and intracellular domain, wherein the first domain includes a

negative signal region and the second domain includes a positive intracellular signaling region. In some aspects, the fusion protein is a chimeric switch receptor that contains the extracellular domain of an inhibitory receptor on T cell fused to the transmembrane and cytoplasmic domain of a co-stimulatory receptor. This chimeric switch receptor may convert a T cell inhibitory signal into a T cell stimulatory signal.

[00915]As a non-limiting example, the chimeric switch receptor may comprise the extracellular domain of PD-1 fused to the transmembrane and cytoplasmic domain of CD28. In some aspects, extracellular domains of other inhibitory receptors such as CTLA-4, LAG-3, TIM-3, KIRs and BTLA may also be fused to the transmembrane and cytoplasmic domain derived from costimulatory receptors such as CD28, 4-1BB, CD27, OX40, CD40, GTIR and ICOS.

[00916]In some embodiments, chimeric switch receptors may include recombinant receptors comprising the extracellular cytokine-binding domain of an inhibitory cytokine receptor (e.g., IL-13 receptor α (IL-13Rα1), IL-10R, and IL-4Rα) fused to an intracellular signaling domain of a stimulatory cytokine receptor such as IL-2R (IL-2Ra, IL-2Rβ and IL-2Rgamma) and IL-7Rα. One example of such chimeric cytokine receptor is a recombinant receptor containing the cytokine-binding extracellular domain of IL-4Rα linked to the intracellular signaling domain of IL-7Rα.

[00917]In some embodiments, the chimeric switch receptor may be a chimeric TGFβ receptor. The chimeric TGFβ receptor may comprise an extracellular domain derived from a TGFβ receptor such as TGFβ receptor 1, TGFβ receptor 2, TGFβ receptor 3, or any other TGFβ receptor or variant thereof; and a non- TGFβ receptor intracellular domain. The non-TGFβ receptor intracellular domain may be the intracellular domain or fragment thereof derived from TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, CD28, 4-1BB (CD137), OX40 (CD134), CD3zeta, CD40, CD27, or a combination thereof.

Activation-conditional CAR

[00918]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode an activation-conditional chimeric antigen receptor, which is only expressed in an activated immune cell. The expression of the CAR may be coupled to activation conditional control region which refers to one or more nucleic acid sequences that induce the transcription and/or expression of a sequence e.g., a CAR under its control. Such activation conditional control regions may be promoters of genes that are upregulated during the activation of the immune effector cell e.g. IL2 promoter or NFAT binding sites.

CAR targeting to tumor cells with specific proteoglycan markers

[00919]In some embodiments, the encoded products of interest of the herein disclosed RNA payloads may encode a CAR that targets specific types of cancer cells. Human cancer cells and metastasis may

express unique and otherwise abnormal proteoglycans, such as polysaccharide chains (e.g., chondroitin sulfate (CS), dermatan sulfate (DS or CSB), heparan sulfate (HS) and heparin). Accordingly, the CAR may be fused with a binding moiety that recognizes cancer associated proteoglycans. In one example, a CAR may be fused with VAR2CSA polypeptide (VAR2-CAR) that binds with high affinity to a specific type of chondroitin sulfate A (CSA) attached to proteoglycans. The extracellular ScFv portion of the CAR may be substituted with VAR2CSA variants comprising at least the minimal CSA binding domain, generating CARs specific to chondroitin sulfate A (CSA) modifications. Alternatively, the CAR may be fused with a split-protein binding system to generate a spy-CAR, in which the scFv portion of the CAR is substituted with one portion of a split-protein binding system such as SpyTag and Spy-catcher and the cancer-recognition molecules (e.g. scFv and or VAR2-CSA) are attached to the CAR through the split-protein binding system.

CAR Treatment Methods

[00920]In some embodiments, an LNP of the present disclosure comprising an RNA encoding a CAR can be used to treat a disease. The present disclosure contemplates a method of treating a disease in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an LNP or pharmaceutical composition comprising an LNP disclosed and described herein. In certain embodiments, the LNP comprises an RNA encoding a CAR protein described herein. In certain embodiments, the LNP delivers the RNA encoding the CAR protein to a T cell. In certain embodiments, the LNP delivers the RNA encoding the CAR protein to an NK cell. In certain embodiments, the LNP delivers the RNA encoding the CAR protein to both a T cell and an NK cell. Interestingly, LNPs of the present disclosure have been found to demonstrate high delivery to both T cells and NK cells (see Example 9). Methods and compositions of the present disclosure can enable simultaneous generation of CAR T cells and CAR NK cells, in vivo, increasing the therapeutic applications and efficacy over other delivery systems that target only one variety of immune cell. In certain embodiments, the disease is a tumor or cancer.

C. Vaccine antigens

[00921]In some embodiments, the RNA payloads (e.g., linear and/or circular mRNA payloads encoding one or more products of interest) and associated compositions and methods described herein provide for the delivery of a wide variety of antigens, e.g., virus antigens, cancer antigens, and/or infectious disease antigens.

[00922]In certain embodiments, the antigen is a protein (including recombinant proteins), polypeptide, or peptide (including synthetic peptides). The compositions provided herein can contain one or more antigens (e.g., at least two, three, four, five, or six antigens).

[00923]In specific embodiments, antigens can be selected from the group consisting of the following: (a) polypeptides suitable to induce an immune response against cancer cells; (b) polypeptides suitable to induce an immune response against infectious diseases; (c) polypeptides suitable to induce an immune response against allergens; and (d) polypeptides suitable to induce an immune response in farm animals or pets.

[00924]In certain embodiments, the compositions of the disclosure can be used in combination with an immunoregulatory therapy to target either activating receptors or inhibitory receptors. See, e.g., Mellman et al., 2013, Nature 480:480-489. The immunoregulatory therapy can be, for example, a T cell engaging agent selected from agonistic antibodies which bind to human OX40, to GITR, to CD27, or to 4-IBB, and T-cell bispecific antibodies (e.g. T cell-engaging BiTE™ antibodies CD3-CD19, CD3-EpCam, CD3-EGFR), IL-2 (Proleukin), Interferon (IFN) alpha, antagonizing antibodies which bind to human CTLA-4 (e.g. ipilimumab), to PD-1, to PD-L1, to TIM-3, to BTLA, to VISTA, to LAG-3, or to CD25.

[00925]Exemplary antigens include those from a pathogen (e.g. virus, bacterium, parasite, fungus) and tumors (especially tumor-associated antigens or "tumor markers"). Other exemplary antigens include autoantigens.

[00926]In some embodiments, the antigen or antigenic determinant is one that is useful for the prevention of infectious disease. Such treatment will be useful to treat a wide variety of infectious diseases affecting a wide range of hosts, preferably human, but including cow, sheep, pig, dog, cat, and other mammalian species and non-mammalian species. Thus, antigens or antigenic determinants selected for the compositions will be well known to those in the medical art.

[00927]Examples of antigens or antigenic determinants include the following: SARS-CoV-2 spike protein, coronavirus spike proteins and/or envelope proteins, the RSV F or G antigens, Chlamydia antigens such as the Major outer membrane protein (mOMP), the Dengue type 1 to 4 envelope proteins, the HIV antigens gp140 and gp160; the influenza antigens hemagglutinin, M2 protein, and neuraminidase; hepatitis B surface antigen or core; and circumsporozoite protein of malaria, or fragments thereof. Other antigens include those antigen polypeptides listed in Tables 6-18 of U.S. Patent No. 10,709,779, or any variant thereof having at least 80% , 85%, 90%, 95%, or 99% or up to 100% sequence identity with any of the polypeptide antigens of Tables 6-18 of U.S. Patent No. 10,709,779 (which is incorporated herein by reference).

[00928]Appropriate antigens for use with this LNP technology may be derived from, but not limited to, pathogenic bacterial, fungal, or viral

organisms, Streptococcus species, Candida species, Brucella species, Salmonella species, Shigella species , Pseudomonas species, Bordetella species, Clostridium species, Norwalk virus, Bacillus anthracis, Mycobacterium tuberculosis, human immunodeficiency virus (HIV), Chlamydia species, human

Papillomaviruses, Influenza virus, Paramyxovirus species, Herpes virus, Cytomegalovirus, Varicella-Zoster virus, Epstein-Barr virus, Hepatitis viruses, Plasmodium species, Trichomonas species, Ebola, sexually transmitted disease agents, viral encephalitis agents, protozoan disease agents, fungal disease agents, cancer cells, or mixtures thereof. Other appropriate molecules incorporated in the nanoparticle vaccines may include self-antigens, adhesins, or surface exposed cell signaling receptors or ligands. A variety of diseases and disorders may be treated by the LNP RNA delivery systems described herein, including: inflammatory diseases, infectious diseases, cancer, genetic disorders, organ transplant rejection, autoimmune diseases and immunological disorders.

[00929]Examples of infectious disease include, but are not limited to, viral infectious diseases, such as AIDS, Respiratory Syncytial Virus (RSV), Chickenpox (Varicella), Common cold, Cytomegalovirus Infection, Colorado tick fever, Dengue fever, Ebola hemorrhagic fever, Hand, foot and mouth disease, Hepatitis, Herpes simplex, Herpes zoster, HPV, Influenza (Flu), Lassa fever, Measles, Marburg hemorrhagic fever, Infectious mononucleosis, Mumps, Norovirus, Poliomyelitis, Progressive multifocal leukencephalopathy, Rabies, Rubella, SARS, Smallpox (Variola), Viral encephalitis, Viral gastroenteritis, Viral meningitis, Viral pneumonia, West Nile disease and Yellow fever; bacterial infectious diseases, such as Anthrax, Bacterial Meningitis, Botulism, Brucellosis, Campylobacteriosis, Cat Scratch Disease, Cholera, Diphtheria, Epidemic Typhus, Gonorrhea, Impetigo, Legionellosis, Leprosy (Hansen's Disease), Leptospirosis, Listeriosis, Lyme disease, Melioidosis, Rheumatic Fever, MRSA infection, Nocardiosis, Pertussis (Whooping Cough), Plague, Pneumococcal pneumonia, Psittacosis, Q fever, Rocky Mountain Spotted Fever (RMSF), Salmonellosis, Scarlet Fever, Shigellosis, Syphilis, Tetanus, Trachoma, Tuberculosis, Tularemia, Typhoid Fever, Typhus and Urinary Tract Infections; parasitic infectious diseases, such as African trypanosomiasis, Amebiasis, Ascariasis, Babesiosis, Chagas Disease, Clonorchiasis, Cryptosporidiosis, Cysticercosis, Diphyllobothriasis, Dracunculiasis, Echinococcosis, Enterobiasis, Fascioliasis, Fasciolopsiasis, Filariasis, Free-living amebic infection, Giardiasis, Gnathostomiasis, Hymenolepiasis, Isosporiasis, Kalaazar, Leishmaniasis, Malaria, Metagonimiasis, Myiasis, Onchocerciasis, Pediculosis, Pinworm Infection, Scabies, Schistosomiasis, Taeniasis, Toxocariasis, Toxoplasmosis, Trichinellosis, Trichinosis, Trichuriasis, Trichomoniasis and Trypanosomiasis; fungal infectious disease, such as Aspergillosis, Blastomycosis, Candidiasis, Coccidioidomycosis, Cryptococcosis, Histoplasmosis, Tinea pedis (Athlete's Foot) and Tinea cruris; prion infectious diseases, such as Alpers' disease, Fatal Familial Insomnia, Gerstmann-Straussler-Scheinker syndrome, Kuru and Variant Creutzfeldt-Jakob disease.

[00930]Examples of cancers include, but are not limited to breast cancer; biliary tract cancer; bladder cancer; brain cancer including glioblastomas; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; hematological neoplasms including acute

lymphocytic and myelogenous leukemia, e.g., B Cell CLL; T-cell acute lymphoblastic leukemia/lymphoma; hairy cell leukemia; chronic myelogenous leukemia, multiple myeloma; AIDS-associated leukemias and adult T-cell leukemia/lymphoma; intraepithelial neoplasms including Bowen's disease and Paget's disease; liver cancer; lung cancer; lymphomas including Hodgkin's disease and lymphocytic lymphomas; neuroblastomas; oral cancer including squamous cell carcinoma; ovarian cancer including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells; pancreatic cancer; prostate cancer; rectal cancer; sarcomas including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma, and osteosarcoma; skin cancer including melanoma, Merkel cell carcinoma, Kaposi's sarcoma, basal cell carcinoma, and squamous cell cancer; testicular cancer including germinal tumors such as seminoma, non-seminoma (teratomas, choriocarcinomas), stromal tumors, and germ cell tumors; thyroid cancer including thyroid adenocarcinoma and medullar carcinoma; and renal cancer including adenocarcinoma and Wilms tumor.

[00931]Any antigen associated with any of the diseases or conditions provided herein can be used in the compositions and methods described herein. These include antigens associated with cancer, infections or infectious disease or degenerative or non-autoimmune disease. Antigens associated with HIV, malaria, leischmaniasis, a human filovirus infection, a togavirus infection, a alphavirus infection, an arenavirus infection, a bunyavirus infection, a flavivirus infection, a human papillomavirus infection, a human influenza A virus infection, a hepatitis B infection or a hepatitis C infection are also included.

[00932]Examples of cancer antigens include HER 2 (pi 85), CD20, CD33, GD3 ganglioside, GD2 ganglioside, carcinoembryonic antigen (CEA), CD22, milk mucin core protein, TAG-72, Lewis A antigen, ovarian associated antigens such as OV-TL3 and MOvl8, high Mr melanoma antigens recognized by antibody 9.2.27, HMFG-2, SM-3, B72.3, PR5C5, PR4D2, and the like. Further examples include MAGE, MART-1/Melan-A, gplOO, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), FAP, cyclophilin b, Colorectal associated antigen (CRC)—C017-1A/GA733, Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, amll, prostatic acid phosphatase (PAP), Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, pro state-specific membrane antigen (PSMA), T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (e.g., MAGE-I or MAGE-II families) (e.g., MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5), GAGE-family of tumor antigens (e.g., GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinasc, p53, MUC family, HER2/ncu, p21ras, RCAS1, a-fetoprotein, E-cadherin, a-catenin, β-catenin and γ-catenin, pl20ctn, gplOOPmell 17, PRAME, NY-ESO-

1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papilloma virus proteins, Smad family of tumor antigens, lmp-1, PIA, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7, CD20 and c-erbB-2.

[00933]In another embodiment, antigens associated with infection or infectious disease are associated with any of the infectious agents provided herein. In one embodiment, the infectious agent is a virus of the Adenoviridae, Picornaviridae, Herpesviridae, Hepadnaviridae, Flaviviridae, Retroviridae, Orthomyxoviridae, Paramyxoviridae, Papillomaviridae, Rhabdoviridae, Togaviridae or Paroviridae family. In still another embodiment, the infectious agent is adenovirus, coxsackievirus, hepatitis A virus, poliovirus, Rhinovirus, Herpes simplex virus, Varicella-zoster virus, Epstein-barr virus, Human cytomegalovirus, Human herpesvirus, Hepatitis B virus, Hepatitis C virus, yellow fever virus, dengue virus, West Nile virus, HIV, Influenza virus, Measles virus, Mumps virus, Parainfluenza virus, Respiratory syncytial virus, Human metapneumovirus, Human papillomavirus, Rabies virus, Rubella virus, Human bocarivus or Parvovirus B19. In yet another embodiment, the infectious agent is a bacteria of the Bordetella, Borrelia, Brucella, Campylobacter, Chlamydia and Chlamydophila, Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter, Legionella, Leptospira, Listeria, Mycobacterium, Mycoplasma, Neisseria, Pseudomonas, Rickettsia, Salmonella, Shigella, Staphylococcus, Streptococcus, Treponema Vibrio or Yersinia genus. In a further embodiment, the infectious agent is Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia trachomatis, Chlamydophila psittaci, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitides, Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae or Yersinia pestis. In another embodiment, the infectious agent is a fungus of the Candida, Aspergillus, Cryptococcus, Histoplasma, Pneumocystis or Stachybotrys genus. In still another embodiment, the infectious agent is C. albicans, Aspergillus fumigatus, Aspergillus flavus, Cryptococcus neoformans, Cryptococcus laurentii, Cryptococcus albidus, Cryptococcus gattii, Histoplasma capsulatum, Pneumocystis jirovecii or Stachybotrys chartarum.

[00934]In yet another embodiment, the antigen associated with infection or infectious disease is one that comprises VI, VII, E1A, E3-19K, 52K, VP1, surface antigen, 3A protein, capsid protein, nucleocapsid, surface projection, transmembrane proteins, UL6, UL18, UL35, UL38, UL19, early antigen, capsid antigen, Pp65, gB, p52, latent nuclear antigen-1, NS3, envelope protein, envelope protein E2 domain, gpl20, p24, lipopeptides Gag (17-35), Gag (253-284), Nef (66-97), Nef (116-145), Pol (325-355), neuraminidase, nucleocapsid protein, matrix protein, phosphoprotein, fusion protein, hemagglutinin, hemagglutinin-neuraminidase, glycoprotein, E6, E7, envelope lipoprotein or non-structural protein (NS). In another embodiment, the antigen comprises pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (PRN), fimbriae (FIM 2/3), VlsE; DbpA, OspA, Hia, PrpA, MltA, L7/L12, D15, 0187, VirJ, Mdh, AfuA, L7/L12, out membrane protein, LPS, antigen type A, antigen type B, antigen type C, antigen type D, antigen type E, FliC, FliD, Cwp84, alpha-toxin, theta-toxin, fructose 1,6-biphosphate-aldolase (FBA), glyceraldehydes-3-phosphate dehydrogenase (GPD), pyruvate:ferredoxin oxidoreductase (PFOR), elongation factor-G (EF-G), hypothetical protein (HP), T toxin, Toxoid antigen, capsular polysaccharide, Protein D, Mip, nucleoprotein (NP), RD1, PE35, PPE68, EsxA, EsxB, RD9, EsxV, Hsp70, lipopolysaccharide, surface antigen, Sp1, Sp2, Sp3, Glycerophosphodiester Phosphodiesterase, outer membrane protein, chaperone-usher protein, capsular protein (Fl) or V protein. In yet another embodiment, the antigen is one that comprises capsular glycoprotein, Yps3P, Hsp60, Major surface protein, MsgC1, MsgC3, MsgC8, MsgC9 or SchS34.

[00935]As a non-limiting example, the polynucleotides (e.g., linear or circular mRNA payloads of the LNP delivery systems described herein) encoding an immunogen may be delivered to cells to trigger multiple innate response pathways (see International Pub. No. WO2012006377 and US Patent Publication No. US20130177639; herein incorporated by reference in its entirety). As another non-limiting example, the polynucleotides of the LNP delivery systems described herein encoding an immunogen may be delivered to a vertebrate in a dose amount large enough to be immunogenic to the vertebrate (see International Pub. No. WO2012006372 and WO2012006369 and US Publication No. US20130149375 and US20130177640; the contents of each of which are herein incorporated by reference in their entirety).

[00936]A non-limiting list of infectious diseases that the polynucleotide vaccines may treat includes, viral infectious diseases such as Covid-19 (SARS-CoV-2), AIDS (HIV), HIV resulting in mycobacterial infection, AIDS related Cacheixa, AIDS related Cytomegalovirus infection, HIV-associated nephropathy, Lipodystrophy, AID related cryptococcal meningitis, AIDS related neutropaenia, Pneumocysitis jiroveci (Pneumocystis carinii) infections, AID related toxoplasmosis, hepatitis A, B, C, D or E, herpes, herpes zoster (chicken pox), German measles (rubella virus), yellow fever, dengue fever etc. (flavi viruses), flu (influenza viruses), haemorrhagic infectious diseases (Marburg or Ebola viruses), bacterial infectious diseases such as Legionnaires' disease (Legionella), gastric ulcer (Helicobacter), cholera (Vibrio), E.

coli infections, staphylococcal infections, salmonella infections or streptococcal infections, tetanus (Clostridium tetani), protozoan infectious diseases (malaria, sleeping sickness, leishmaniasis, toxoplasmosis, i.e. infections caused by plasmodium, trypanosomes, leishmania and toxoplasma), diphtheria, leprosy, measles, pertussis, rabies, tetanus, tuberculosis, typhoid, varicella, diarrheal infections such as Amoebiasis, Clostridium difficile-associated diarrhea (CDAD), Cryptosporidiosis, Giardiasis, Cyclosporiasis and Rotaviral gastroenteritis, encephalitis such as Japanese encephalitis, Wester equine encephalitis and Tick-borne encephalitis (TBE), fungal skin diseases such as candidiasis, onychomycosis, Tinea captis/scal ringworm, Tinea corporis/body ringworm, Tinea cruris/jock itch, sporotrichosis and Tinea pedis/Athlete's foot, Meningitis such as Haemophilus influenza type b (Hib), Meningitis, viral, meningococcal infections and pneumococcal infection, neglected tropical diseases such as Argentine haemorrhagic fever, Leishmaniasis, Nematode/roundworm infections, Ross river virus infection and West Nile virus (WNV) disease, Non-HIV STDs such as Trichomoniasis, Human papillomavirus (HPV) infections, sexually transmitted chlamydial diseases, Chancroid and Syphilis, Non-septic bacterial infections such as cellulitis, lyme disease, MRSA infection, pseudomonas, staphylococcal infections, Boutonneuse fever, Leptospirosis, Rheumatic fever, Botulism, Rickettsial disease and Mastoiditis, parasitic infections such as Cysticercosis, Echinococcosis, Trematode/Fluke infections, Trichinellosis, Babesiosis, Hypodermyiasis, Diphyllobothriasis and Trypanosomiasis, respiratory infections such as adenovirus infection, aspergillosis infections, avian (H5N1) influenza, influenza, RSV infections, severe acute respiratory syndrome (SARS), sinusitis, Legionellosis, Coccidioidomycosis and swine (H1N1) influenza, sepsis such as bacteraemia, sepsis/septic shock, sepsis in premature infants, urinary tract infection such as vaginal infections (bacterial), vaginal infections (fungal) and gonococcal infection, viral skin diseases such as B19 parvovirus infections, warts, genital herpes, orofacial herpes, shingles, inner ear infections, fetal cytomegalovirus syndrome, foodborn illnesses such as brucellosis (Brucella species), Clostridium perfringens (Epsilon toxin), E. Coli O157:H7 (Escherichia coli), Salmonellosis (Salmonella species), Shingellosis (Shingella), Vibriosis and Listeriosis, bioterrorism and potential epidemic diseases such as Ebola haemorrhagic fever, Lassa fever, Marburg haemorrhagic fever, plague, Anthrax Nipah virus disease, Hanta virus, Smallpox, Glanders (Burkholderia mallei), Melioidosis (Burkholderia pseudomallei), Psittacosis (Chlamydia psittaci), Q fever (Coxiella burnetii), Tularemia (Fancisella tularensis), rubella, mumps and polio.

[00937]The LNP delivery systems described herein may be utilized in various settings depending on the prevalence of the infection or the degree or level of unmet medical need. As a non-limiting example, the LNP delivery systems disclosed herein may be utilized to treat and/or prevent influenza infection, i.e. diseases and conditions related to influenza virus infection (seasonal and pandemic).

[00938]The LNP delivery systems described herein may be utilized in various settings depending on the prevalence of the infection or the degree or level of unmet medical need. As a non-limiting example, the LNP delivery systems disclosed herein may be utilized to treat and/or prevent coronavirus infection, i.e. diseases and conditions related to coronavirus virus infection (seasonal and pandemic).

[00939]The LNP delivery systems described herein may be utilized in various settings depending on the prevalence of the infection or the degree or level of unmet medical need. As a non-limiting example, the LNP delivery systems disclosed herein may be utilized to treat and/or prevent SARS-CoV-2 infection, i.e. diseases and conditions related to SARS-CoV-2 virus infection (seasonal and pandemic), e.g., Covid-19.

D. Signal peptides

[00940]In some embodiments, the polypeptide products (e.g., vaccine antigens and/or therapeutic proteins) of the RNA payload disclosed herein may comprise a signal peptide. Signal peptides, comprising the about N-terminal 15-60 amino acids of proteins, are typically needed for the translocation across the membrane on the secretory pathway and thus universally control the entry of most proteins both in eukaryotes and prokaryotes to the secretory pathway. Signal peptides generally include three regions: an N-terminal region of differing length, which usually comprises positively charged amino acids, a hydrophobic region, and a short carboxy-terminal peptide region. In eukaryotes, the signal peptide of a nascent precursor protein (pre-protein) directs the ribosome to the rough endoplasmic reticulum (ER) membrane and initiates the transport of the growing peptide chain across it. The signal peptide is not responsible for the final destination of the mature protein, however. Secretory proteins devoid of further address tags in their sequence are by default secreted to the external environment. Signal peptides are cleaved from precursor proteins by an endoplasmic reticulum (ER)-resident signal peptidase or they remain uncleaved and function as a membrane anchor.

[00941]In various embodiments, the polypeptide products (e.g., vaccine antigens and/or therapeutic proteins) of the RNA payload disclosed herein may comprise artificial signal peptides, wherein the signal peptide coding sequence is operably linked to and is in frame with the coding sequence of the encoded polypeptide product. Thus, polypeptides produced by the RNA payload disclosed herein may comprise a fusion with a signal peptide (either naturally occurring signal peptide or an artificial signal peptide). The signal peptide can be fused to either the N-terminus or the C-terminus of an encoded polypeptide product produced by an RNA payload disclosed herein. In some embodiments, a signal peptide can be fused to both the N-terminus and the C-terminus of an encoded polypeptide product produced by an RNA payload disclosed herein.

[00942]Any signal peptide that is known in the art to facilitate targeting of a protein to ER for processing and/or targeting of a protein to the cell membrane may be used in accordance with the present disclosure.

[00943]A signal peptide may have a length of about 10-60 amino acids. For example, a signal peptide may have a length of about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 amino acids. In some embodiments, a signal peptide may have a length of 20-60, 25-60, 30-60, 35-60, 40-60, 45-60, 50-60, 55-60, 15-55, 20-55, 25-55, 30-55, 35-55, 40-55, 45-55, 50-55, 15-50, 20-50, 25-50, 30-50, 35-50, 40-50, 45-50, 15-45, 20-45, 25-45, 30-45, 35-45, 40-45, 15-40, 20-40, 25-40, 30-40, 35-40, 15-35, 20-35, 25-35, 30-35, 15-30, 20-30, 25-30, 15-25, 20-25, or 15-20 amino acids.

E. Fusion proteins

[00944]In some embodiments, the polypeptide products (e.g., vaccine antigens and/or therapeutic proteins) of the RNA payload disclosed herein may be in the form of a fusion protein. Thus, the encoded polypeptides may include two or more proteins (e.g., protein and/or protein fragment) joined together, e.g., by a linker. In some embodiments, the fusion partner can provide an additional function to the encode polypeptide product, such as, but not limited to intracellular targeting, signaling, enzymatic function, stability, scaffolds, enhanced immunogenicity (in the case where the polypeptide encoded by the RNA payload is an antigen). The disclosure contemplates that the polypeptide products (e.g., vaccine antigens and/or therapeutic proteins) of the RNA payload disclosed herein may be fused to any useful fusion partner known in the art.

F. Scaffold Moieties

[00945]In various embodiments, the RNA payloads of the LNP delivery systems described herein encode fusion proteins which comprise antigens linked to scaffold moieties. In some embodiments, such scaffold moieties impart desired properties to an antigen encoded by a nucleic acid of the disclosure. For example scaffold proteins may improve the immunogenicity of an antigen, e.g., by altering the structure of the antigen, altering the uptake and processing of the antigen, and/or causing the antigen to bind to a binding partner.

[00946]In some embodiments, the scaffold moiety is a protein that can self-assemble into protein nanoparticles that are highly symmetric, stable, and structurally organized, with diameters of 10-150 nm, a highly suitable size range for optimal interactions with various cells of the immune system. In one embodiment, viral proteins or virus-like particles can be used to form stable nanoparticle structures. Examples of such viral proteins are known in the art. For example, in some embodiments, the scaffold moiety is a hepatitis B surface antigen (HBsAg). HBsAg forms spherical particles with an average

diameter of ~22 nm and which lacked nucleic acid and hence are non-infectious (Lopez-Sagaseta, J. et al. Computational and Structural Biotechnology Journal 14 (2016) 58-68). In some embodiments, the scaffold moiety is a hepatitis B core antigen (HBcAg) self-assembles into particles of 24-31 nm diameter, which resembled the viral cores obtained from HBV-infected human liver. HBcAg produced in self-assembles into two classes of differently sized nanoparticles of 300 Å and 360 Å diameter, corresponding to 180 or 240 protomers. In some embodiments an antigen is fused to HBsAG or HBcAG to facilitate self-assembly of nanoparticles displaying the antigen.

[00947]In another embodiment, bacterial protein platforms may be used. Non-limiting examples of these self-assembling proteins include ferritin, lumazine and encapsulin.

[00948]Ferritin is a protein whose main function is intracellular iron storage. Ferritin is made of 24 subunits, each composed of a four-alpha-helix bundle, that self-assemble in a quaternary structure with octahedral symmetry (Cho K. J. et al. J Mol Biol. 2009; 390:83-98). Several high-resolution structures of ferritin have been determined, confirming that Helicobacter pylori ferritin is made of 24 identical protomers, whereas in animals, there are ferritin light and heavy chains that can assemble alone or combine with different ratios into particles of 24 subunits (Granier T. et al. J Biol Inorg Chem. 2003; 8:105-111; Lawson D. M. et al. Nature. 1991; 349:541-544). Ferritin self-assembles into nanoparticles with robust thermal and chemical stability. Thus, the ferritin nanoparticle is well-suited to carry and expose antigens.

[00949]Lumazine synthase (LS) is also well-suited as a nanoparticle platform for antigen display. LS, which is responsible for the penultimate catalytic step in the biosynthesis of riboflavin, is an enzyme present in a broad variety of organisms, including archaea, bacteria, fungi, plants, and eubacteria (Weber S. E. Flavins and Flavoproteins. Methods and Protocols, Series: Methods in Molecular Biology. 2014). The LS monomer is 150 amino acids long, and consists of beta-sheets along with tandem alpha-helices flanking its sides. A number of different quaternary structures have been reported for LS, illustrating its morphological versatility: from homopentamers up to symmetrical assemblies of 12 pentamers forming capsids of 150 Å diameter. Even LS cages of more than 100 subunits have been described (Zhang X. et al. J Mol Biol. 2006; 362:753-770).

[00950]Encapsulin, a novel protein cage nanoparticle isolated from thermophile Thermotoga maritima, may also be used as a platform to present antigens on the surface of self-assembling nanoparticles. Encapsulin is assembled from 60 copies of identical 31 kDa monomers having a thin and icosahedral T=1 symmetric cage structure with interior and exterior diameters of 20 and 24 nm, respectively (Sutter M. et al. Nat Struct Mol Biol. 2008, 15: 939-947). Although the exact function of encapsulin in T. maritima is not clearly understood yet, its crystal structure has been recently solved and its function was postulated as a cellular compartment that encapsulates proteins such as DyP (Dye decolorizing peroxidase) and Flp

(Ferritin like protein), which are involved in oxidative stress responses (Rahmanpour R. et al. FEBS J. 2013, 280: 2097-2104).

G. Linkers and Cleavable Peptides

[00951]In some embodiments, the mRNA payloads of the disclosure encode more than one polypeptide, referred to herein as fusion proteins. In some embodiments, the mRNA further encodes a linker located between at least one or each domain of the fusion protein. The linker can be, for example, a cleavable linker or protease-sensitive linker. In some embodiments, the linker is selected from the group consisting of F2A linker, P2A linker, T2A linker, E2A linker, and combinations thereof. This family of self-cleaving peptide linkers, referred to as 2A peptides, has been described in the art (see for example, Kim, J. H. et al. (2011) PLoS ONE 6:e18556). In some embodiments, the linker is an F2A linker. In some embodiments, the linker is a GGGS linker (SEQ ID NO: 188). In some embodiments, the fusion protein contains three domains with intervening linkers, having the structure: domain-linker-domain-linker-domain.

[00952]Cleavable linkers known in the art may be used in connection with the disclosure. Exemplary such linkers include: F2A linkers, T2A linkers, P2A linkers, E2A linkers (See, e.g., WO2017127750). The skilled artisan will appreciate that other art-recognized linkers may be suitable for use in the constructs of the disclosure (e.g., encoded by the nucleic acids of the disclosure). The skilled artisan will likewise appreciate that other polycistronic constructs (mRNA encoding more than one antigen/polypeptide separately within the same molecule) may be suitable for use as provided herein.

H. Functional domains

[00953]In some embodiments, the polypeptides encoded by the RNA payloads described herein may further comprise additional sequences or functional domains. For example, the antigen polypeptides of the present disclosure may comprise one or more linker sequences. In some embodiments, the antigen polypeptide may comprise a polypeptide tag, such as an affinity tag (chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST), SBP-tag, Strep-tag, AviTag, Calmodulin-tag); solubilization tag; chromatography tag (polyanionic amino acid tag, such as FLAG-tag); epitope tag (short peptide sequences that bind to high-affinity antibodies, such as V5-tag, Myc-tag, VSV-tag, Xpress tag, E-tag, S-tag, and HA-tag); fluorescence tag (e.g., GFP). In some embodiments, the antigen may comprise an amino acid tag, such as one or more lysines, histidines, or glutamates, which can be added to the polypeptide sequences (e.g., at the N-terminal or C-terminal ends). Lysines can be used to increase peptide solubility or to allow for biotinylation. Protein and amino acid tags are peptide sequences genetically grafted onto a recombinant protein. Sequence tags are attached to proteins for various purposes, such as peptide purification, identification, or localization, for use in various applications including, for example, affinity purification, protein array, western blotting, immunofluorescence, and

immunoprecipitation. Such tags are subsequently removable by chemical agents or by enzymatic means, such as by specific proteolysis or intein splicing.

[00954]Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g., C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

I. Codon optimization

[00955]The LNP-based RNA vaccines and therapeutics described herein may comprise one or more RNA payloads (e.g., linear or circular mRNA) having nucleotide sequences which may be codon optimized.

[00956]For example, a nucleotide sequence (e.g., as part of an RNA payload) encoding an antigen of the disclosure is codon optimized. Codon optimization methods are known in the art. For example, a protein encoding sequence of any one or more of the sequences provided herein may be codon optimized. Codon optimization, in some embodiments, may be used to match codon frequencies in target and host organisms to ensure proper folding; bias GC content to increase mRNA stability or reduce secondary structures; minimize tandem repeat codons or base runs that may impair gene construction or expression; customize transcriptional and translational control regions; insert or remove protein trafficking sequences; remove/add post translation modification sites in encoded protein (e.g., glycosylation sites); add, remove or shuffle protein domains; insert or delete restriction sites; modify ribosome binding sites and mRNA degradation sites; adjust translational rates to allow the various domains of the protein to fold properly; or reduce or eliminate problem secondary structures within the polynucleotide. Codon optimization tools, algorithms and services are known in the art—non-limiting examples include services from GeneArt (Life Technologies), DNA2.0 (Menlo Park Calif.) and/or proprietary methods. In some embodiments, the protein encoding sequence is optimized using optimization algorithms.

[00957]In some embodiments, a codon optimized sequence shares less than 95% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding an antigen). In some embodiments, a codon optimized sequence shares less than 90% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding an antigen). In some embodiments, a codon optimized sequence shares less than 85% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding an antigen). In some embodiments, a codon optimized sequence shares less than 80% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding an antigen). In some embodiments, a codon optimized sequence

shares less than 75% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding an antigen).

[00958]In some embodiments, a codon optimized sequence shares between 65% and 85% (e.g., between about 67% and about 85% or between about 67% and about 80%) sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding an Influenza antigen). In some embodiments, a codon optimized sequence shares between 65% and 75% or about 80% sequence identity to a naturally-occurring or wild-type sequence (e.g., a naturally-occurring or wild-type mRNA sequence encoding an Influenza antigen).

[00959]In some embodiments, a codon-optimized sequence encodes an antigen that is as immunogenic as, or more immunogenic than (e.g., at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 100%, or at least 200% more), than an antigen encoded by a non-codon-optimized) sequence.

[00960]When transfected into mammalian cells, the modified mRNA payloads have a stability of between 12-18 hours, or greater than 18 hours, e.g., 24, 36, 48, 60, 72, or greater than 72 hours.

[00961]In some embodiments, a codon optimized RNA may be one in which the levels of G/C are enhanced. The G/C-content of nucleic acid molecules (e.g., mRNA) may influence the stability of the RNA. RNA having an increased amount of guanine (G) and/or cytosine (C) residues may be functionally more stable than RNA containing a large amount of adenine (A) and thymine (T) or uracil (U) nucleotides. As an example, WO02/098443 discloses a pharmaceutical composition containing an mRNA stabilized by sequence modifications in the translated region. Due to the degeneracy of the genetic code, the modifications work by substituting existing codons for those that promote greater RNA stability without changing the resulting amino acid. The approach is limited to coding regions of the RNA.

V. LNP pharmaceutical compositions

[00962]The LNP-based RNA vaccines, RNA therapeutics and pharmaceutical compositions thereof described herein can be formulate using one or more excipients to: (1) increase stability; (2) increase cell transfection or transduction; (3) permit the sustained or delayed expression of the payload; (4) alter the biodistribution (e.g., target the viral particle to specific tissues or cell types); (5) increase the translation of encoded protein; (6) alter the release profile of encoded protein; and/or (7) allow for regulatable expression of an RNA payload expression product.

[00963]Formulations can include, without limitation, saline, liposomes, lipid nanoparticles, polymers, peptides, proteins, cells transfected with viral vectors (e.g., for transfer or transplantation into a subject) and combinations thereof.

[00964]Formulations of the LNP-based RNA vaccines, RNA therapeutics and pharmaceutical compositions thereof described herein may be prepared by any method known or hereafter developed in the art of pharmacology. As used herein the term "pharmaceutical composition" refers to compositions

comprising at least one active ingredient and optionally one or more pharmaceutically acceptable excipients.

[00965]In general, such preparatory methods include the step of associating the active ingredient (e.g., encapsulated LNP with an mRNA payload expressing a protein of interest) with an excipient and/or one or more other accessory ingredients. As used herein, the phrase "active ingredient" can refer to an LNP encapsulated with a payload mRNA, as well as to the mRNA payload construct itself, including originator constructs and benchmark construct as described herein.

[00966]Formulations of the encapsulated LNPs, the payload mRNA constructs, and pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-dose unit.

[00967]A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" refers to a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[00968]In some embodiments, a pharmaceutically acceptable excipient may be at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use for humans and for veterinary use. In some embodiments, an excipient may be approved by United States Food and Drug Administration. In some embodiments, an excipient may be of pharmaceutical grade. In some embodiments, an excipient may meet the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[00969]Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure may vary, depending upon the identity, size, and/or condition of the subject being treated and further depending upon the route by which the composition is to be administered. For example, the composition may comprise between 0.1% and 99% (w/w) of the active ingredient. By way of example, the composition may comprise between 0.1% and 100%, e.g., between 0.5 and 50%, between 1-30%, between 5-80%, or at least 80% (w/w) active ingredient.

[00970]Excipients, as used herein, include, but are not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents,

thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[00971]Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, *etc.*, and/or combinations thereof.

[00972]In some embodiments, formulations described herein may comprise at least one inactive ingredient. As used herein, the term "inactive ingredient" refers to one or more agents that do not contribute to the activity of the active ingredient of the pharmaceutical composition included in formulations. In some embodiments, all, none or some of the inactive ingredients which may be used in the formulations of the present disclosure may be approved by the US Food and Drug Administration (FDA).

[00973]In one embodiment, the formulations described herein comprise at least one inactive ingredient such as, but not limited to, 1,2,6-Hexanetriol; 1,2-Dimyristoyl-Sn-Glycero-3-(Phospho-S-(1-Glycerol)); 1,2-Dimyristoyl-Sn-Glycero-3-Phosphocholine; 1,2-Dioleoyl-Sn-Glycero-3-Phosphocholine; 1,2-Dipalmitoyl-Sn-Glycero-3-(Phospho-Rac-(1-Glycerol)); 1,2-Distearoyl-Sn-Glycero-3-(Phospho-Rac-(1-Glycerol)); 1,2-Distearoyl-Sn-Glycero-3-Phosphocholine; 1-O-Tolylbiguanide; 2-Ethyl-1,6-Hexanediol; Acetic Acid; Acetic Acid, Glacial; Acetic Anhydride; Acetone; Acetone Sodium Bisulfite; Acetylated Lanolin Alcohols; Acetylated Monoglycerides; Acetylcysteine; Acetyltryptophan, DL-; Acrylates Copolymer; Acrylic Acid-Isooctyl Acrylate Copolymer; Acrylic Adhesive 788; Activated Charcoal; Adcote 72A103; Adhesive Tape; Adipic Acid; Aerotex Resin 3730; Alanine; Albumin Aggregated; Albumin Colloidal; Albumin Human; Alcohol; Alcohol, Dehydrated; Alcohol, Denatured; Alcohol, Diluted; Alfadex; Alginic Acid; Alkyl Ammonium Sulfonic Acid Betaine; Alkyl Aryl Sodium Sulfonate; Allantoin; Allyl .Alpha.-Ionone; Almond Oil; Alpha-Terpineol; Alpha-Tocopherol; Alpha-Tocopherol Acetate, Dl-; Alpha-Tocopherol, Dl-; Aluminum Acetate; Aluminum Chlorhydroxy Allantoinate; Aluminum Hydroxide; Aluminum Hydroxide - Sucrose, Hydrated; Aluminum Hydroxide Gel; Aluminum Hydroxide Gel F 500; Aluminum Hydroxide Gel F 5000; Aluminum Monostearate; Aluminum Oxide; Aluminum Polyester; Aluminum Silicate; Aluminum Starch Octenylsuccinate; Aluminum Stearate;

Aluminum Subacetate; Aluminum Sulfate Anhydrous; Amerchol C; Amerchol-Cab; Aminomethylpropanol; Ammonia; Ammonia Solution; Ammonia Solution, Strong; Ammonium Acetate; Ammonium Hydroxide; Ammonium Lauryl Sulfate; Ammonium Nonoxynol-4 Sulfate; Ammonium Salt Of C-12-C-15 Linear Primary Alcohol Ethoxylate; Ammonium Sulfate; Ammonyx; Amphoteric-2; Amphoteric-9; Anethole; Anhydrous Citric Acid; Anhydrous Dextrose; Anhydrous Lactose; Anhydrous Trisodium Citrate; Aniseed Oil; Anoxid Sbn; Antifoam; Antipyrine; Apaflurane; Apricot Kernel Oil Peg-6 Esters; Aquaphor; Arginine; Arlacel; Ascorbic Acid; Ascorbyl Palmitate; Aspartic Acid; Balsam Peru; Barium Sulfate; Beeswax; Beeswax, Synthetic; Beheneth-10; Bentonite; Benzalkonium Chloride; Benzenesulfonic Acid; Benzethonium Chloride; Benzododecinium Bromide; Benzoic Acid; Benzyl Alcohol; Benzyl Benzoate; Benzyl Chloride; Betadex; Bibapcitide; Bismuth Subgallate; Boric Acid; Brocrinat; Butane; Butyl Alcohol; Butyl Ester Of Vinyl Methyl Ether/Maleic Anhydride Copolymer (125000 Mw); Butyl Stearate; Butylated Hydroxyanisole; Butylated Hydroxytoluene; Butylene Glycol; Butylparaben; Butyric Acid; C20-40 Pareth-24; Caffeine; Calcium; Calcium Carbonate; Calcium Chloride; Calcium Gluceptate; Calcium Hydroxide; Calcium Lactate; Calcobutrol; Caldiamide Sodium; Caloxetate Trisodium; Calteridol Calcium; Canada Balsam; Caprylic/Capric Triglyceride; Caprylic/Capric/Stearic Triglyceride; Captan; Captisol; Caramel; Carbomer 1342; Carbomer 1382; Carbomer 934; Carbomer 934p; Carbomer 940; Carbomer 941; Carbomer 980; Carbomer 981; Carbomer Homopolymer Type B (Allyl Pentaerythritol Crosslinked); Carbomer Homopolymer Type C (Allyl Pentaerythritol Crosslinked); Carbon Dioxide; Carboxy Vinyl Copolymer; Carboxymethylcellulose; Carboxymethylcellulose Sodium; Carboxypolymethylene; Carrageenan; Carrageenan Salt; Castor Oil; Cedar Leaf Oil; Cellulose; Cellulose, Microcrystalline; Cerasynt-Se; Ceresin; Ceteareth-12; Ceteareth-15; Ceteareth-30; Cetearyl Alcohol/Ceteareth-20; Cetearyl Ethylhexanoate; Ceteth-10; Ceteth-2; Ceteth-20; Ceteth-23; Cetostearyl Alcohol; Cetrimonium Chloride; Cetyl Alcohol; Cetyl Esters Wax; Cetyl Palmitate; Cetylpyridinium Chloride; Chlorobutanol; Chlorobutanol Hemihydrate; Chlorobutanol, Anhydrous; Chlorocresol; Chloroxylenol; Cholesterol; Choleth; Choleth-24; Citrate; Citric Acid; Citric Acid Monohydrate; Citric Acid, Hydrous; Cocamide Ether Sulfate; Cocamine Oxide; Coco Betaine; Coco Diethanolamide; Coco Monoethanolamide; Cocoa Butter; Coco-Glycerides; Coconut Oil; Coconut Oil, Hydrogenated; Coconut Oil/Palm Kernel Oil Glycerides, Hydrogenated; Cocoyl Caprylocaprate; Cola Nitida Seed Extract; Collagen; Coloring Suspension; Corn Oil; Cottonseed Oil; Cream Base; Creatine; Creatinine; Cresol; Croscarmellose Sodium; Crospovidone; Cupric Sulfate; Cupric Sulfate Anhydrous; Cyclomethicone; Cyclomethicone/Dimethicone Copolyol; Cysteine; Cysteine Hydrochloride; Cysteine Hydrochloride Anhydrous; Cysteine, Dl-; D&C Red No. 28; D&C Red No. 33; D&C Red No. 36; D&C Red No. 39; D&C Yellow No. 10; Dalfampridine; Daubert 1-5 Pestr (Matte) 164z; Decyl Methyl Sulfoxide; Dehydag Wax Sx; Dehydroacetic Acid; Dehymuls E; Denatonium Benzoate; Deoxycholic

Acid; Dextran; Dextran 40; Dextrin; Dextrose; Dextrose Monohydrate; Dextrose Solution; Diatrizoic Acid; Diazolidinyl Urea; Dichlorobenzyl Alcohol; Dichlorodifluoromethane; Dichlorotetrafluoroethane; Diethanolamine; Diethyl Pyrocarbonate; Diethyl Sebacate; Diethylene Glycol Monoethyl Ether; Diethylhexyl Phthalate; Dihydroxyaluminum Aminoacetate; Diisopropanolamine; Diisopropyl Adipate; Diisopropyl Dilinoleate; Dimethicone 350; Dimethicone Copolyol; Dimethicone Mdx4-4210; Dimethicone Medical Fluid 360; Dimethyl Isosorbide; Dimethyl Sulfoxide; Dimethylaminoethyl Methacrylate - Butyl Methacrylate - Methyl Methacrylate Copolymer; Dimethyldioctadecylammonium Bentonite; Dimethylsiloxane/Methylvinylsiloxane Copolymer; Dinoseb Ammonium Salt; Dipalmitoylphosphatidylglycerol, Dl-; Dipropylene Glycol; Disodium Cocoamphodiacetate; Disodium Laureth Sulfosuccinate; Disodium Lauryl Sulfosuccinate; Disodium Sulfosalicylate; Disofenin; Divinylbenzene Styrene Copolymer; Dmdm Hydantoin; Docosanol; Docusate Sodium; Duro-Tak 280-2516; Duro-Tak 387-2516; Duro-Tak 80-1196; Duro-Tak 87-2070; Duro-Tak 87-2194; Duro-Tak 87-2287; Duro-Tak 87-2296; Duro-Tak 87-2888; Duro-Tak 87-2979; Edetate Calcium Disodium; Edetate Disodium; Edetate Disodium Anhydrous; Edetate Sodium; Edetic Acid; Egg Phospholipids; Entsufon; Entsufon Sodium; Epilactose; Epitetracycline Hydrochloride; Essence Bouquet 9200; Ethanolamine Hydrochloride; Ethyl Acetate; Ethyl Oleate; Ethylcelluloses; Ethylene Glycol; Ethylene Vinyl Acetate Copolymer; Ethylenediamine; Ethylenediamine Dihydrochloride; Ethylene-Propylene Copolymer; Ethylene-Vinyl Acetate Copolymer (28% Vinyl Acetate); Ethylene-Vinyl Acetate Copolymer (9% Vinylacetate); Ethylhexyl Hydroxystearate; Ethylparaben; Eucalyptol; Exametazime; Fat, Edible; Fat, Hard; Fatty Acid Esters; Fatty Acid Pentaerythriol Ester; Fatty Acids; Fatty Alcohol Citrate; Fatty Alcohols; Fd&C Blue No. 1; Fd&C Green No. 3; Fd&C Red No. 4; Fd&C Red No. 40; Fd&C Yellow No. 10 (Delisted); Fd&C Yellow No. 5; Fd&C Yellow No. 6; Ferric Chloride; Ferric Oxide; Flavor 89-186; Flavor 89-259; Flavor Df-119; Flavor Df-1530; Flavor Enhancer; Flavor Fig 827118; Flavor Raspberry Pfc-8407; Flavor Rhodia Pharmaceutical No. Rf 451; Fluorochlorohydrocarbons; Formaldehyde; Formaldehyde Solution; Fractionated Coconut Oil; Fragrance 3949-5; Fragrance 520a; Fragrance 6.007; Fragrance 91-122; Fragrance 9128-Y; Fragrance 93498g; Fragrance Balsam Pine No. 5124; Fragrance Bouquet 10328; Fragrance Chemoderm 6401-B; Fragrance Chemoderm 6411; Fragrance Cream No. 73457; Fragrance Cs-28197; Fragrance Felton 066m; Fragrance Firmenich 47373; Fragrance Givaudan Ess 9090/1c; Fragrance H-6540; Fragrance Herbal 10396; Fragrance Nj-1085; Fragrance P O Fl-147; Fragrance Pa 52805; Fragrance Pera Derm D; Fragrance Rbd-9819; Fragrance Shaw Mudge U-7776; Fragrance Tf 044078; Fragrance Ungerer Honeysuckle K 2771; Fragrance Ungerer N5195; Fructose; Gadolinium Oxide; Galactose; Gamma Cyclodextrin; Gelatin; Gelatin, Crosslinked; Gelfoam Sponge; Gellan Gum (Low Acyl); Gelva 737; Gentisic Acid; Gentisic Acid Ethanolamide; Gluceptate Sodium; Gluceptate Sodium Dihydrate; Gluconolactone; Glucuronic Acid; Glutamic Acid, Dl-;

Glutathione; Glycerin; Glycerol Ester Of Hydrogenated Rosin; Glyceryl Citrate; Glyceryl Isostearate; Glyceryl Laurate; Glyceryl Monostearate; Glyceryl Oleate; Glyceryl Oleate/Propylene Glycol; Glyceryl Palmitate; Glyceryl Ricinoleate; Glyceryl Stearate; Glyceryl Stearate - Laureth-23; Glyceryl Stearate/Peg Stearate; Glyceryl Stearate/Peg-100 Stearate; Glyceryl Stearate/Peg-40 Stearate; Glyceryl Stearate-Stearamidoethyl Diethylamine; Glyceryl Trioleate; Glycine; Glycine Hydrochloride; Glycol Distearate; Glycol Stearate; Guanidine Hydrochloride; Guar Gum; Hair Conditioner (18n195-1m); Heptane; Hetastarch; Hexylene Glycol; High Density Polyethylene; Histidine; Human Albumin Microspheres; Hyaluronate Sodium; Hydrocarbon; Hydrocarbon Gel, Plasticized; Hydrochloric Acid; Hydrochloric Acid, Diluted; Hydrocortisone; Hydrogel Polymer; Hydrogen Peroxide; Hydrogenated Castor Oil; Hydrogenated Palm Oil; Hydrogenated Palm/Palm Kernel Oil Peg-6 Esters; Hydrogenated Polybutene 635-690; Hydroxide Ion; Hydroxyethyl Cellulose; Hydroxyethylpiperazine Ethane Sulfonic Acid; Hydroxymethyl Cellulose; Hydroxyoctacosanyl Hydroxystearate; Hydroxypropyl Cellulose; Hydroxypropyl Methylcellulose 2906; Hydroxypropyl-Beta-cyclodextrin; Hypromellose 2208 (15000 Mpa.S); Hypromellose 2910 (15000 Mpa.S); Hypromelloses; Imidurea; Iodine; Iodoxamic Acid; Iofetamine Hydrochloride; Irish Moss Extract; Isobutane; Isoceteth-20; Isoleucine; Isooctyl Acrylate; Isopropyl Alcohol; Isopropyl Isostearate; Isopropyl Myristate; Isopropyl Myristate - Myristyl Alcohol; Isopropyl Palmitate; Isopropyl Stearate; Isostearic Acid; Isostearyl Alcohol; Isotonic Sodium Chloride Solution; Jelene; Kaolin; Kathon Cg; Kathon Cg II; Lactate; Lactic Acid; Lactic Acid, Dl-; Lactic Acid, L-; Lactobionic Acid; Lactose; Lactose Monohydrate; Lactose, Hydrous; Laneth; Lanolin; Lanolin Alcohol - Mineral Oil; Lanolin Alcohols; Lanolin Anhydrous; Lanolin Cholesterols; Lanolin Nonionic Derivatives; Lanolin, Ethoxylated; Lanolin, Hydrogenated; Lauralkonium Chloride; Lauramine Oxide; Laurdimonium Hydrolyzed Animal Collagen; Laureth Sulfate; Laureth-2; Laureth-23; Laureth-4; Lauric Diethanolamide; Lauric Myristic Diethanolamide; Lauroyl Sarcosine; Lauryl Lactate; Lauryl Sulfate; Lavandula Angustifolia Flowering Top; Lecithin; Lecithin Unbleached; Lecithin, Egg; Lecithin, Hydrogenated; Lecithin, Hydrogenated Soy; Lecithin, Soybean; Lemon Oil; Leucine; Levulinic Acid; Lidofenin; Light Mineral Oil; Light Mineral Oil (85 Ssu); Limonene, (+/-)-; Lipocol Sc-15; Lysine; Lysine Acetate; Lysine Monohydrate; Magnesium Aluminum Silicate; Magnesium Aluminum Silicate Hydrate; Magnesium Chloride; Magnesium Nitrate; Magnesium Stearate; Maleic Acid; Mannitol; Maprofix; Mebrofenin; Medical Adhesive Modified S-15; Medical Antiform A-F Emulsion; Medronate Disodium; Medronic Acid; Meglumine; Menthol; Metacresol; Metaphosphoric Acid; Methanesulfonic Acid; Methionine; Methyl Alcohol; Methyl Gluceth-10; Methyl Gluceth-20; Methyl Gluceth-20 Sesquistearate; Methyl Glucose Sesquistearate; Methyl Laurate; Methyl Pyrrolidone; Methyl Salicylate; Methyl Stearate; Methylboronic Acid; Methylcellulose (4000 Mpa.S); Methylcelluloses; Methylchloroisothiazolinone; Methylene Blue; Methylisothiazolinone; Methylparaben; Microcrystalline

Wax; Mineral Oil; Mono and Diglyceride; Monostearyl Citrate; Monothioglycerol; Multisterol Extract; Myristyl Alcohol; Myristyl Lactate; Myristyl-.Gamma.-Picolinium Chloride; N-(Carbamoyl-Methoxy Peg-40)-1,2-Distearoyl-Cephalin Sodium; N,N-Dimethylacetamide; Niacinamide; Nioxime; Nitric Acid; Nitrogen; Nonoxynol Iodine; Nonoxynol-15; Nonoxynol-9; Norflurane; Oatmeal; Octadecene-1/Maleic Acid Copolymer; Octanoic Acid; Octisalate; Octoxynol-1; Octoxynol-40; Octoxynol-9; Octyldodecanol; Octylphenol Polymethylene; Oleic Acid; Oleth-10/Oleth-5; Oleth-2; Oleth-20; Oleyl Alcohol; Oleyl Oleate; Olive Oil; Oxidronate Disodium; Oxyquinoline; Palm Kernel Oil; Palmitamine Oxide; Parabens; Paraffin; Paraffin, White Soft; Parfum Creme 45/3; Peanut Oil; Peanut Oil, Refined; Pectin; Peg 6-32 Stearate/Glycol Stearate; Peg Vegetable Oil; Peg-100 Stearate; Peg-12 Glyceryl Laurate; Peg-120 Glyceryl Stearate; Peg-120 Methyl Glucose Dioleate; Peg-15 Cocamine; Peg-150 Distearate; Peg-2 Stearate; Peg-20 Sorbitan Isostearate; Peg-22 Methyl Ether/Dodecyl Glycol Copolymer; Peg-25 Propylene Glycol Stearate; Peg-4 Dilaurate; Peg-4 Laurate; Peg-40 Castor Oil; Peg-40 Sorbitan Diisostearate; Peg-45/Dodecyl Glycol Copolymer; Peg-5 Oleate; Peg-50 Stearate; Peg-54 Hydrogenated Castor Oil; Peg-6 Isostearate; Peg-60 Castor Oil; Peg-60 Hydrogenated Castor Oil; Peg-7 Methyl Ether; Peg-75 Lanolin; Peg-8 Laurate; Peg-8 Stearate; Pegoxol 7 Stearate; Pentadecalactone; Pentaerythritol Cocoate; Pentasodium Pentetate; Pentetate Calcium Trisodium; Pentetic Acid; Peppermint Oil; Perflutren; Perfume 25677; Perfume Bouquet; Perfume E-1991; Perfume Gd 5604; Perfume Tana 90/42 Scba; Perfume W-1952-1; Petrolatum; Petrolatum, White; Petroleum Distillates; Phenol; Phenol, Liquefied; Phenonip; Phenoxyethanol; Phenylalanine; Phenylethyl Alcohol; Phenylmercuric Acetate; Phenylmercuric Nitrate; Phosphatidyl Glycerol, Egg; Phospholipid; Phospholipid, Egg; Phospholipon 90g; Phosphoric Acid; Pine Needle Oil (Pinus Sylvestris); Piperazine Hexahydrate; Plastibase-50w; Polacrilin; Polidronium Chloride; Poloxamer 124; Poloxamer 181; Poloxamer 182; Poloxamer 188; Poloxamer 237; Poloxamer 407; Poly(Bis(P-Carboxyphenoxy)Propane Anhydride):Sebacic Acid; Poly(Dimethylsiloxane/Methylvinylsiloxane/Methylhydrogensiloxane) Dimethylvinyl Or Dimethylhydroxy Or Trimethyl Endblocked; Poly(Dl-Lactic-Co-Glycolic Acid), (50:50; Poly(Dl-Lactic-Co-Glycolic Acid), Ethyl Ester Terminated, (50:50; Polyacrylic Acid (250000 Mw); Polybutene (1400 Mw); Polycarbophil; Polyester; Polyester Polyamine Copolymer; Polyester Rayon; Polyethylene Glycol 1000; Polyethylene Glycol 1450; Polyethylene Glycol 1500; Polyethylene Glycol 1540; Polyethylene Glycol 200; Polyethylene Glycol 300; Polyethylene Glycol 300-1600; Polyethylene Glycol 3350; Polyethylene Glycol 400; Polyethylene Glycol 4000; Polyethylene Glycol 540; Polyethylene Glycol 600; Polyethylene Glycol 6000; Polyethylene Glycol 8000; Polyethylene Glycol 900; Polyethylene High Density Containing Ferric Oxide Black (<1%); Polyethylene Low Density Containing Barium Sulfate (20-24%); Polyethylene T; Polyethylene Terephthalates; Polyglactin; Polyglyceryl-3 Oleate; Polyglyceryl-4 Oleate; Polyhydroxyethyl Methacrylate; Polyisobutylene; Polyisobutylene (1100000 Mw);

Polyisobutylene (35000 Mw); Polyisobutylene 178-236; Polyisobutylene 241-294; Polyisobutylene 35-39; Polyisobutylene Low Molecular Weight; Polyisobutylene Medium Molecular Weight; Polyisobutylene/Polybutene Adhesive; Polylactide; Polyols; Polyoxyethylene - Polyoxypropylene 1800; Polyoxyethylene Alcohols; Polyoxyethylene Fatty Acid Esters; Polyoxyethylene Propylene; Polyoxyl 20 Cetostearyl Ether; Polyoxyl 35 Castor Oil; Polyoxyl 40 Hydrogenated Castor Oil; Polyoxyl 40 Stearate; Polyoxyl 400 Stearate; Polyoxyl 6 And Polyoxyl 32 Palmitostearate; Polyoxyl Distearate; Polyoxyl Glyceryl Stearate; Polyoxyl Lanolin; Polyoxyl Palmitate; Polyoxyl Stearate; Polypropylene; Polypropylene Glycol; Polyquaternium-10; Polyquaternium-7 (70/30 Acrylamide/Dadmac; Polysiloxane; Polysorbate 20; Polysorbate 40; Polysorbate 60; Polysorbate 65; Polysorbate 80; Polyurethane; Polyvinyl Acetate; Polyvinyl Alcohol; Polyvinyl Chloride; Polyvinyl Chloride-Polyvinyl Acetate Copolymer; Polyvinylpyridine; Poppy Seed Oil; Potash; Potassium Acetate; Potassium Alum; Potassium Bicarbonate; Potassium Bisulfite; Potassium Chloride; Potassium Citrate; Potassium Hydroxide; Potassium Metabisulfite; Potassium Phosphate, Dibasic; Potassium Phosphate, Monobasic; Potassium Soap; Potassium Sorbate; Povidone Acrylate Copolymer; Povidone Hydrogel; Povidone K17; Povidone K25; Povidone K29/32; Povidone K30; Povidone K90; Povidone K90f; Povidone/Eicosene Copolymer; Povidones; Ppg-12/Smdi Copolymer; Ppg-15 Stearyl Ether; Ppg-20 Methyl Glucose Ether Distearate; Ppg-26 Oleate; Product Wat; Proline; Promulgen D; Promulgen G; Propane; Propellant A-46; Propyl Gallate; Propylene Carbonate; Propylene Glycol; Propylene Glycol Diacetate; Propylene Glycol Dicaprylate; Propylene Glycol Monolaurate; Propylene Glycol Monopalmitostearate; Propylene Glycol Palmitostearate; Propylene Glycol Ricinoleate; Propylene Glycol/Diazolidinyl Urea/Methylparaben/Propylparben; Propylparaben; Protamine Sulfate; Protein Hydrolysate; Pvm/Ma Copolymer; Quaternium-15; Quaternium-15 Cis-Form; Quaternium-52; Ra-2397; Ra-3011; Saccharin; Saccharin Sodium; Saccharin Sodium Anhydrous; Safflower Oil; Sd Alcohol 3a; Sd Alcohol 40; Sd Alcohol 40-2; Sd Alcohol 40b; Sepineo P 600; Serine; Sesame Oil; Shea Butter; Silastic Brand Medical Grade Tubing; Silastic Medical Adhesive,Silicone Type A; Silica, Dental; Silicon; Silicon Dioxide; Silicon Dioxide, Colloidal; Silicone; Silicone Adhesive 4102; Silicone Adhesive 4502; Silicone Adhesive Bio-Psa Q7-4201; Silicone Adhesive Bio-Psa Q7-4301; Silicone Emulsion; Silicone/Polyester Film Strip; Simethicone; Simethicone Emulsion; Sipon Ls 20np; Soda Ash; Sodium Acetate; Sodium Acetate Anhydrous; Sodium Alkyl Sulfate; Sodium Ascorbate; Sodium Benzoate; Sodium Bicarbonate; Sodium Bisulfate; Sodium Bisulfite; Sodium Borate; Sodium Borate Decahydrate; Sodium Carbonate; Sodium Carbonate Decahydrate; Sodium Carbonate Monohydrate; Sodium Cetostearyl Sulfate; Sodium Chlorate; Sodium Chloride; Sodium Chloride Injection; Sodium Chloride Injection, Bacteriostatic; Sodium Cholesteryl Sulfate; Sodium Citrate; Sodium Cocoyl Sarcosinate; Sodium Desoxycholate; Sodium Dithionite; Sodium Dodecylbenzenesulfonate; Sodium Formaldehyde Sulfoxylate; Sodium Gluconate;

Sodium Hydroxide; Sodium Hypochlorite; Sodium Iodide; Sodium Lactate; Sodium Lactate, L-; Sodium Laureth-2 Sulfate; Sodium Laureth-3 Sulfate; Sodium Laureth-5 Sulfate; Sodium Lauroyl Sarcosinate; Sodium Lauryl Sulfate; Sodium Lauryl Sulfoacetate; Sodium Metabisulfite; Sodium Nitrate; Sodium Phosphate; Sodium Phosphate Dihydrate; Sodium Phosphate, Dibasic; Sodium Phosphate, Dibasic, Anhydrous; Sodium Phosphate, Dibasic, Dihydrate; Sodium Phosphate, Dibasic, Dodecahydrate; Sodium Phosphate, Dibasic, Heptahydrate; Sodium Phosphate, Monobasic; Sodium Phosphate, Monobasic, Anhydrous; Sodium Phosphate, Monobasic, Dihydrate; Sodium Phosphate, Monobasic, Monohydrate; Sodium Polyacrylate (2500000 Mw); Sodium Pyrophosphate; Sodium Pyrrolidone Carboxylate; Sodium Starch Glycolate; Sodium Succinate Hexahydrate; Sodium Sulfate; Sodium Sulfate Anhydrous; Sodium Sulfate Decahydrate; Sodium Sulfite; Sodium Sulfosuccinated Undecyclenic Monoalkylolamide; Sodium Tartrate; Sodium Thioglycolate; Sodium Thiomalate; Sodium Thiosulfate; Sodium Thiosulfate Anhydrous; Sodium Trimetaphosphate; Sodium Xylenesulfonate; Somay 44; Sorbic Acid; Sorbitan; Sorbitan Isostearate; Sorbitan Monolaurate; Sorbitan Monooleate; Sorbitan Monopalmitate; Sorbitan Monostearate; Sorbitan Sesquioleate; Sorbitan Trioleate; Sorbitan Tristearate; Sorbitol; Sorbitol Solution; Soybean Flour; Soybean Oil; Spearmint Oil; Spermaceti; Squalane; Stabilized Oxychloro Complex; Stannous 2-Ethylhexanoate; Stannous Chloride; Stannous Chloride Anhydrous; Stannous Fluoride; Stannous Tartrate; Starch; Starch 1500, Pregelatinized; Starch, Corn; Stearalkonium Chloride; Stearalkonium Hectorite/Propylene Carbonate; Stearamidoethyl Diethylamine; Steareth-10; Steareth-100; Steareth-2; Steareth-20; Steareth-21; Steareth-40; Stearic Acid; Stearic Diethanolamide; Stearoxytrimethylsilane; Steartrimonium Hydrolyzed Animal Collagen; Stearyl Alcohol; Sterile Water For Inhalation; Styrene/Isoprene/Styrene Block Copolymer; Succimer; Succinic Acid; Sucralose; Sucrose; Sucrose Distearate; Sucrose Polyesters; Sulfacetamide Sodium; Sulfobutylether .Beta.-Cyclodextrin; Sulfur Dioxide; Sulfuric Acid; Sulfurous Acid; Surfactol Qs; Tagatose, D-; Talc; Tall Oil; Tallow Glycerides; Tartaric Acid; Tartaric Acid, Dl-; Tenox; Tenox-2; Tert-Butyl Alcohol; Tert-Butyl Hydroperoxide; Tert-Butylhydroquinone; Tetrakis(2-Methoxyisobutylisocyanide)Copper(I) Tetrafluoroborate; Tetrapropyl Orthosilicate; Tetrofosmin; Theophylline; Thimerosal; Threonine; Thymol; Tin; Titanium Dioxide; Tocopherol; Tocophersolan; Total parenteral nutrition, lipid emulsion; Triacetin; Tricaprylin; Trichloromonofluoromethane; Trideceth-10; Triethanolamine Lauryl Sulfate; Trifluoroacetic Acid; Triglycerides, Medium Chain; Trihydroxystearin; Trilaneth-4 Phosphate; Trilaureth-4 Phosphate; Trisodium Citrate Dihydrate; Trisodium Hedta; Triton 720; Triton X-200; Trolamine; Tromantadine; Tromethamine (TRIS); Tryptophan; Tyloxapol; Tyrosine; Undecylenic Acid; Union 76 Amsco-Res 6038; Urea; Valine; Vegetable Oil; Vegetable Oil Glyceride, Hydrogenated; Vegetable Oil, Hydrogenated; Versetamide; Viscarin; Viscose/Cotton; Vitamin E; Wax, Emulsifying;

Wecobee Fs; White Ceresin Wax; White Wax; Xanthan Gum; Zinc; Zinc Acetate; Zinc Carbonate; Zinc Chloride; and Zinc Oxide.

[00974]In some embodiments, formulations disclosed herein may include cations or anions. The formulations include metal cations such as, but not limited to, Zn^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, and combinations thereof. As a non-limiting example, formulations may include polymers and complexes with a metal cation.

[00975]Formulations of the disclosure may also include one or more pharmaceutically acceptable salts. As used herein, "pharmaceutically acceptable salts" refers to derivatives of the disclosed compounds wherein the parent compound is modified by converting an existing acid or base moiety to its salt form (e.g., by reacting the free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, acetic acid, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzene sulfonic acid, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. The pharmaceutically acceptable salts of the present disclosure include the conventional non-toxic salts of the parent compound formed, for example, from non-toxic inorganic or organic acids.

[00976]Solvates may be prepared by crystallization, recrystallization, or precipitation from a solution that includes organic solvents, water, or a mixture thereof. Examples of suitable solvents are ethanol, water (for example, mono-, di-, and tri-hydrates), *N*-methylpyrrolidinone (NMP), dimethyl sulfoxide (DMSO), *N*,*N*′-dimethylformamide (DMF), *N*,*N*′-dimethylacetamide (DMAC), 1,3-dimethyl-2-imidazolidinone (DMEU), 1,3-dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU), acetonitrile (ACN), propylene glycol, ethyl acetate, benzyl alcohol, 2-pyrrolidone, benzyl benzoate, and the like. When water is the solvent, the solvate is referred to as a "hydrate."

[00977]In some embodiments, the payloads are encapsulated in nanoparticles (e.g., LNPs) for delivery. In embodiments, a nanoparticle can include an ionizable lipid, a phospholipid, a PEG lipid, and a structural lipid. In certain embodiments, the lipid component of the nanoparticle composition includes about 30 mol % to about 60 mol % ionizable lipid, about 0 mol % to about 30 mol % phospholipid, about 18.5 mol % to about 48.5 mol % structural lipid, and about 0 mol% to about 10 mol% of PEG lipid, provided that the total mol % does not exceed 100%. In certain embodiments, the lipid component of the nanoparticle composition includes about 20 mol % to about 45 mol % ionizable lipid, about 30 mol % to about 60 mol % phospholipid, about 10 mol % to about 30 mol % structural lipid, and about 0 mol% to about 10 mol% of PEG lipid, provided that the total mol % does not exceed 100%. In some embodiments, the lipid component of the nanoparticle composition includes about 35 mol % to about 55 mol % ionizable lipid, about 5 mol % to about 25 mol % phospholipid, about 30 mol % to about 40 mol % structural lipid, and about 0 mol % to about 10 mol % of PEG lipid, provided that the total mol % does not exceed 100%. In some embodiments, the lipid component of the nanoparticle composition includes about 30 mol % to about 40 mol % ionizable lipid, about 35 mol % to about 45 mol % phospholipid, about 20 mol % to about 30 mol % structural lipid, and about 0.5 mol % to about 5 mol % of PEG lipid, provided that the total mol % does not exceed 100%. In a particular embodiment, the lipid component includes about 50 mol % ionizable lipid, about 10 mol % phospholipid, about 38.5 mol % structural lipid, and about 1.5 mol% of PEG lipid. In another particular embodiment, the lipid component includes about 40 mol % ionizable lipid, about 20 mol % phospholipid, about 38.5 mol % structural lipid, and about 1.5 mol % of PEG lipid. In another particular embodiment, the lipid component includes about 48.5 mol % ionizable lipid, about 10 mol % phospholipid, about 40 mol % structural lipid, and about 1.5 mol % of PEG lipid. In another particular embodiment, the lipid component includes about 48.5 mol % ionizable lipid, about 10 mol % phospholipid, about 39 mol % structural lipid, and about 2.5 mol % of PEG lipid. In another particular embodiment, the lipid component includes about 33 mol % ionizable lipid, about 40 mol % phospholipid, about 25 mol % structural lipid, and about 2 mol % of PEG lipid. In some embodiments, the phospholipid is DOPE or DSPC. In some embodiments, the phospholipid is DSPC. In some embodiments, the phospholipid is a sphingolipid. In some embodiments, the phospholipid is sphingomyelin. In other embodiments, the PEG lipid is PEG-DMG (eg. PEG2K-DMG). In other embodiments, the PEG lipid is PEG-DSPE (eg. PEG2K-DSPE). In other embodiments, the PEG lipid is PEG-DMPE (eg. PEG2K-DMPE). In other embodiments, the structural lipid is cholesterol. In other embodiments, the PEG lipid is PEG-DMG and/or the structural lipid is cholesterol. In some embodiments, the PEG lipids is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is DSPC. In some embodiments, the PEG lipids is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is sphingomyelin. In some embodiments, the PEG lipids is PEG-DMG, the structural lipid is

cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In certain embodiments, the LNP comprises about 33mol% ionizable lipid (eg. at least one ionizable lipid of Formula AX described herein), about 40mol% of a sphingolipid, about 25mol% cholesterol and about 2mol% PEG2K-DMG. In some embodiments, the PEG lipids is PEG2K-DSPE, the structural lipid is cholesterol, and the phospholipid is DSPC. In some embodiments, the PEG lipids is PEG2K-DSPE, the structural lipid is cholesterol, and the phospholipid is sphingomyelin. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 20mol% of a sphingolipid, about 20mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 43mol% ionizable lipid, about 15mol% of a sphingolipid, about 15mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 25mol% of a sphingolipid, about 15mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In certain embodiments, the LNP comprises about 33mol% ionizable lipid, about 15mol% of a sphingolipid, about 25mol% of a non-sphingolipid phospholipid, about 25mol% cholesterol and about 2mol% of a PEGylated lipid. In some embodiments, the PEG lipid is PEG2K-DSPE, the structural lipid is cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In some embodiments, the PEG lipid is PEG2K-DMG, the structural lipid is cholesterol, and the phospholipid is a mixture of DSPC and sphingomyelin. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 40mol% cholesterol and about 1.5mol% PEG2K-DSPE. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 40mol% cholesterol and about 1.5mol% PEG2K-DMG. In certain embodiments, the LNP comprises about 48.5mol% ionizable lipid, about 10mol% of a phospholipid (such as DSPC), about 39mol% cholesterol and about 2.5mol% PEG2K-DSPE. The amount of active agent in a nanoparticle composition may depend on the size, composition, desired target and/or application, or other properties of the nanoparticle composition as well as on the properties of the active agent. For example, the amount of active agent useful in a nanoparticle composition may depend on the size, sequence, and other characteristics of the active agent. The relative amounts of active agent and other elements (e.g., lipids) in a nanoparticle composition may also vary. In some embodiments, the wt/wt ratio of the lipid component to a payload in a nanoparticle composition is from about 5:1 to about 60:1, such as 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, and 60:1. The amount of a payload in a nanoparticle composition may, for example, be measured using absorption spectroscopy (e.g., ultraviolet-visible spectroscopy).

[00978]In some embodiments, a nanoparticle composition of the present disclosure is formulated to provide a specific N:P ratio. The N:P ratio of the composition refers to the molar ratio of nitrogen atoms

in one or more lipids to the number of phosphate groups in an RNA active agent (e.g., a linear or circular mRNA payload). In general, a lower N:P ratio is preferred. The one or more polynucleotides, lipids, and amounts thereof is selected to provide an N:P ratio from about 2:1 to about 30:1, such as 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 12:1, 14:1, 16:1, 18:1, 20:1, 22:1, 24:1, 26:1, 28:1, or 30:1. In certain embodiments, the N:P ratio is from about 2:1 to about 8:1. In other embodiments, the N:P ratio is from about 5:1 to about 8:1. For example, the N:P ratio is about 5.0:1, about 5.5:1, about 5.67:1, about 6.0:1, about 6.5:1, or about 7.0:1. In some embodiments, a nanoparticle composition of the present disclosure is formulated to provide a specific IL:P ratio, referring to the molar ratio of ionizable lipid (as a whole compound) to the number of phosphate groups in an RNA active agent. The one or more polynucleotides, lipids, and amounts thereof is selected to provide an IL:P ratio from about 2:1 to about 30:1, such as 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 12:1, 14:1, 16:1, 18:1, 20:1, 22:1, 24:1, 26:1, 28:1, or 30:1. In certain embodiments, the IL:P ratio is from about 2:1 to about 8:1. In other embodiments, the IL:P ratio is from about 5:1 to about 8:1. For example, the IL:P ratio is about 5.0:1, about 5.5:1, about 5.67:1, about 6.0:1, about 6.5:1, about 7.0:1, or about 7.5:1.

[00979]The characteristics of a nanoparticle composition may depend on the components thereof. For example, a nanoparticle composition including cholesterol as a structural lipid may have different characteristics than a nanoparticle composition that includes a different structural lipid. Similarly, the characteristics of a nanoparticle composition may depend on the absolute or relative amounts of its components. For instance, a nanoparticle composition including a higher molar fraction of a phospholipid may have different characteristics than a nanoparticle composition including a lower molar fraction of a phospholipid. Characteristics may also vary depending on the method and conditions of preparation of the nanoparticle composition. Nanoparticle compositions may be characterized by a variety of methods. For example, microscopy (e.g., transmission electron microscopy or scanning electron microscopy) may be used to examine the morphology and size distribution of a nanoparticle composition. Dynamic light scattering or potentiometry (e.g., potentiometric titrations) may be used to measure Zeta potentials. Dynamic light scattering may also be utilized to determine particle sizes. Instruments such as the Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK) may also be used to measure multiple characteristics of a nanoparticle composition, Such as particle size, polydispersity index, and Zeta potential.

[00980]In some embodiments, the mean size of a nanoparticle composition is between 10s of nm and 100s of nm, e.g., measured by dynamic light scattering (DLS). For example, the mean size may be from about 40 nm to about 150 nm, such as about 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm. In some embodiments, the mean size of a nanoparticle composition is from about

50 nm to about 100 nm, from about 50 nm to about 90 nm, from about 50 nm to about 80 nm, from about 50 nm to about 70 nm, from about 50 nm to about 60 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 60 nm to about 70 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, from about 80 nm to about 100 nm, from about 80 nm to about 90 nm, or from about 90 nm to about 100 nm. In certain embodiments, the mean size of a nanoparticle composition is from about 70 nm to about 100 nm. In a particular embodiment, the mean size is about 80 nm. In other embodiments, the mean size is about 100 nm.

[00981]In some embodiments, the LNPs of the present disclosure can be characterized by their shape. In some embodiments, the LNPs are essentially spherical. In some embodiments, the LNPs are essentially rod-shaped (i.e., cylindrical). In some embodiments, the LNPs are essentially disk shaped.

[00982]A nanoparticle composition may be relatively homogenous. A polydispersity index may be used to indicate the homogeneity of a nanoparticle composition, e.g., the particle size distribution of the nanoparticle compositions. A small (e.g., less than 0.3) polydispersity index generally indicates a narrow particle size distribution. A nanoparticle composition may have a polydispersity index from about 0 to about 0.25, such as 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.21, 0.22, 0.23, 0.24, or 0.25.

[00983]The Zeta potential of a nanoparticle composition may be used to indicate the electrokinetic potential of the composition. For example, the Zeta potential may describe the surface charge of a nanoparticle composition. Nanoparticle compositions with relatively low charges, positive or negative, are generally desirable, as more highly charged species may interact undesirably with cells, tissues, and other elements in the body. In some embodiments, the Zeta potential of a nanoparticle composition is from about -10 mV to about +20 mV, from about -10 mV to about +15 mV, from about -10 mV to about +10 mV, from about -10 mV to about +5 mV, from about -10 mV to about 0 mV, from about -10 mV to about -5 mV, from about -5 mV to about +20 mV, from about -5 mV to about +15 mV, from about -5 mV to about +10 mV, from about -5 mV to about +5 mV, from about -5 mV to about 0 mV, from about 0 mV, to about +20 mV, from about 0 mV to about +15 mV, from about 0 mV to about +10 mV, from about 0 mV to about +5 mV, from about +5 mV to about +20 mV, from about +5 mV, to about +15 mV, or from about +5 mV to about +10 mV.

[00984]The efficiency of encapsulation of a payload describes the amount of payload that is encapsulated or otherwise associated with a nanoparticle composition after preparation, relative to the initial amount provided. The encapsulation efficiency is desirably high (e.g., close to 100%). The encapsulation efficiency may be measured, for example, by comparing the amount of payload in a solution containing the nanoparticle composition before and after breaking up the nanoparticle composition with one or more

organic solvents or detergents. Fluorescence may be used to measure the amount of free payload in a solution. For the nanoparticle compositions described herein, the encapsulation efficiency of a therapeutic and/or prophylactic may be at least 50%, for example 50%, 55%, 60%. 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the encapsulation efficiency is at least 80%. In certain embodiments, the encapsulation efficiency is at least 90%.

[00985]Lipids and their method of preparation are disclosed in, e.g., U.S. Patent Nos. 8,569,256, 5,965,542 and U.S. Patent Publication Nos. 2016/0199485, 2016/0009637, 2015/0273068, 2015/0265708, 2015/0203446, 2015/0005363, 2014/0308304, 2014/0200257, 2013/086373, 2013/0338210, 2013/0323269, 2013/0245107, 2013/0195920, 2013/0123338, 2013/0022649, 2013/0017223, 2012/0295832, 2012/0183581, 2012/0172411, 2012/0027803, 2012/0058188, 2011/0311583, 2011/0311582, 2011/0262527, 2011/0216622, 2011/0117125, 2011/0091525, 2011/0076335, 2011/0060032, 2010/0130588, 2007/0042031, 2006/0240093, 2006/0083780, 2006/0008910, 2005/0175682, 2005/017054, 2005/0118253, 2005/0064595, 2004/0142025, 2007/0042031, 1999/009076 and PCT Pub. Nos. WO 99/39741, WO 2017/117528, WO 2017/004143, WO 2017/075531, WO 2015/199952, WO 2014/008334, WO 2013/086373, WO 2013/086322, WO 2013/016058, WO 2013/086373, WO2011/141705, and WO 2001/07548 and Semple et. al, Nature Biotechnology, 2010, 28, 172-176, the full disclosures of which are herein incorporated by reference in their entirety for all purposes.

[00986]A nanoparticle composition may include any substance useful in pharmaceutical compositions. For example, the nanoparticle composition may include one or more pharmaceutically acceptable excipients or accessory ingredients such as, but not limited to, one or more solvents, dispersion media, diluents, dispersion aids, suspension aids, granulating aids, disintegrants, fillers, glidants, liquid vehicles, binders, surface active agents, isotonic agents, thickening or emulsifying agents, buffering agents, lubricating agents, oils, preservatives, and other species. Excipients such as waxes, butters, coloring agents, coating agents, flavorings, and perfuming agents may also be included. Pharmaceutically acceptable excipients are well known in the art (see for example Remington's The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro: Lippincott, Williams & Wilkins, Baltimore, Md., 2006).

[00987]In some embodiments, provided herein are pharmaceutical compositions comprising: a) at least one lipid nanoparticle comprising at least one Lipid of the Disclosure; and b) at least one nucleobase editing system. In some embodiments, the nucleobase editing system comprises a CRISPR-Cas gene editing system. In some embodiments, the nucleobase editing system comprises a prime editing system or components thereof. In some embodiments, the nucleobase editing system comprises a retron editing system, a TnpB editing system, an integrase editing system, an epigenetic editing system, a gene writing

system, a gene inactivating system, a zinc finger nuclease, a TALE Nuclease, a TALE nickase, Zinc Finger (ZF) Nuclease, ZF Nickase, meganuclease, or a combination thereof.

[00988]In some embodiments, provided herein are pharmaceutical compositions comprising: a) at least one lipid nanoparticle comprising at least one Lipid of the Disclosure; and b) at least one nucleic acid encoding a therapeutic protein. In some embodiments, at least one nucleic acid encoding a therapeutic protein is an mRNA or a circular RNA (oRNA). In some embodiments, the therapeutic protein is a CAR or TCR complex protein. In some embodiments, the CAR or TCR complex protein comprises an antigen binding domain specific for an antigen selected from the group: CD 19, CD123, CD22, CD30, CD171, CS-1, C-type lectin-like molecule- 1, CD33, epidermal growth factor receptor variant III (EGFRvIII), disialoganglioside GD2, disaloganglioside GD3, TNF receptor family member, B cell maturation antigen (BCMA), Tn antigen ((Tn Ag) or (GalNAca-Ser/Thr)), prostate- specific membrane antigen (PSMA), Receptor tyrosine kinase-like orphan receptor 1 (ROR1), Fms-Like Tyrosine Kinase 3 (FLT3), Tumor-associated glycoprotein 72 (TAG72), CD38, CD44v6, Carcinoembryonic antigen (CEA), Epithelial cell adhesion molecule (EPCAM), B7H3 (CD276), KIT (CD 117), Interleukin- 13 receptor subunit alpha-2, mesothelin, Interleukin 11 receptor alpha (IL-1 lRa), prostate stem cell antigen (PSCA), Protease Serine 21, vascular endothelial growth factor receptor 2 (VEGFR2), Lewis(Y) antigen, CD24, Platelet-derived growth factor receptor beta (PDGFR-beta), Stage-specific embryonic antigen-4 (SSEA-4), CD20, Folate receptor alpha, HER2, HER3, Mucin 1, cell surface associated (MUC1), epidermal growth factor receptor (EGFR), neural cell adhesion molecule (NCAM), Prostase, prostatic acid phosphatase (PAP), elongation factor 2 mutated (ELF2M), Ephrin B2, fibroblast activation protein alpha (FAP), insulin-like growth factor 1 receptor (IGF-I receptor), carbonic anhydrase IX (CAIX), Proteasome (Prosome, Macropain) Subunit, Beta Type, 9 (LMP2), glycoprotein 100 (gplOO), oncogene fusion protein consisting of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl), tyrosinase, ephrin type- A receptor 2 (EphA2), Fucosyl GM1, sialyl Lewis adhesion molecule (sLe), ganglioside GM3, transglutaminase 5 (TGS5), high molecular weight-melanoma-associated antigen (HMWMAA), o-acetyl-GD2 ganglioside (0AcGD2), Folate receptor beta, tumor endothelial marker 1 (TEM1/CD248), tumor endothelial marker 7 -related (TEM7R), claudin 6 (CLDN6), claudin 18.2 (CLDN18.2), thyroid stimulating hormone receptor (TSHR), G protein-coupled receptor class C group 5, member D (GPRC5D), chromosome X open reading frame 61 (CXORF61), CD97, and CD179a.

[00989]In some embodiments, the pharmaceutical compositions disclosed herein further comprise: i) at least one structural lipid; ii) at least one phospholipid or non-ionizable lipid and/or zwitterionic lipid; and iii) at least one PEGylated lipid.

[00990]In some embodiments, the structural lipid is selected from cholesterol, fecosterol, fucosterol, beta sitosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, cholic acid,

sitostanol, litocholic acid, tomatine, ursolic acid, alpha-tocopherol, Vitamin D3, Vitamin D2, Calcipotriol, botulin, lupeol, oleanolic acid, beta-sitosterol-acetate and any combinations thereof.

[00991]In some embodiments, the phospholipid is selected from 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1.2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocho line (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sodium (S)-2-ammonio-3-((((R)-2-(oleoyloxy)-3-(stearoyloxy)propoxy)oxidophosphoryl)oxy)propanoate (L-α-phosphatidylserine; Brain PS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE), 1,2-Dielaidoyl-sn-phosphatidylethanolamine (DEPE), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), 1,2-dioleoyl-sn-glycero-3-phosphate (18:1 PA; DOPA), ammonium bis((S)-2-hydroxy-3-(oleoyloxy)propyl) phosphate (18:1 DMP; LBPA), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol) (DOPI; 18:1 PI), 1,2-distearoyl-sn-glycero-3-phospho-L-serine (18:0 PS), 1,2-dilinoleoyl-sn-glycero-3-phospho-L-serine (18:2 PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (16:0-18:1 PS; POPS), 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (18:0-18:1 PS), 1-stearoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (18:0-18:2 PS), 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:1 Lyso PS), 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:0 Lyso PS), and sphingomyelin.

[00992]In some embodiments, the non-ionizable lipid is a phospholipid selected from the group consisting of Egg Sphingomyelin (Egg SM / ESM / (2S,3R,E)-3-hydroxy-2-palmitamidooctadec-4-en-1-

yl (2-(trimethylammonio)ethyl) phosphate), Brain or Porcine Sphingomyelin (Brain SM / (2S,3R,E)-3-hydroxy-2-stearamidooctadec-4-en-1-yl (2-(trimethylammonio)ethyl) phosphate), Milk or Bovine Sphingomyelin (Milk SM / (2S,3R,E)-3-hydroxy-2-tricosanamidooctadec-4-en-1-yl (2-(trimethylammonio)ethyl) phosphate), 28:0 SM (N-octacosanoyl-D-erythro-sphingosylphosphorylcholine), 14:0 SM (N-myristoyl-D-erythro-sphingosylphosphorylcholine), 16:1 SM (N-palmitoleoyl-D-erythro-sphingosylphosphorylcholine), 12:0 Dihydro SM (N-lauroyl-D-erythro-sphinganylphosphorylcholine), Lyso SM (Sphingosylphosphorylcholine), Lyso SM (Sphingosylphosphorylcholine), Lyso SM (dihydro) (Sphinganine Phosphorylcholine), 24:1 SM (N-nervonoyl-D-erythro-sphingosylphosphorylcholine), 24:0 SM (N-lignoceroyl-D-erythro-sphingosylphosphorylcholine), 18:1 SM (N-oleoyl-D-erythro-sphingosylphosphorylcholine), 18:0 SM (N-stearoyl-D-erythro-sphingosylphosphorylcholine), 17:0 SM (N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine), 16:0 SM (N-palmitoyl-D-erythro-sphingosylphosphorylcholine), 12:0 SM (N-lauroyl-D-erythro-sphingosylphosphorylcholine), 06:0 SM (N-hexanoyl-D-erythro-sphingosylphosphorylcholine), 02:0 SM (N-acetyl-D-erythro-sphingosylphosphorylcholine), 3-O-methyl Lyso SM (3-O-methyl-spingosylphosphorylcholine), 3-O-methyl-N-methyl Lyso SM (3-O-methyl-N-methyl-spingosylphosphorylcholine), and 3-N-methyl Lyso SM (3-N-methyl-spingosylphosphorylcholine).

[00993]In some embodiments, the PEGylated lipid is selected from (R)-2,3-bis(octadecyloxy)propyl-1-(methoxypoly(ethyleneglycol)2000)propylcarbamate, PEG-S-DSG, PEG-S-DMG, PEG-PE, PEG-PAA, PEG-OH DSPE C18, PEG-DSPE, PEG-DSG, PEG-DPG, PEG-DOMG, PEG-DMPE Na, PEG-DMPE, PEG-DMG2000, PEG-DMG C14, PEG-DMG 2000, PEG-DMG, PEG-DMA, PEG-Ceramide C16, PEG-C-DOMG, PEG-c-DMOG, PEG-c-DMA, PEG-cDMA, PEGA, PEG750-C-DMA, PEG400, PEG2k-DMG, PEG2k-C11, PEG2000-PE, PEG2000P, PEG2000-DSPE, PEG2000-DOMG, PEG2000-DMG, PEG2000-C-DMA, PEG2000, PEG200, PEG(2k)-DMG, PEG DSPE C18, PEG DMPE C14, PEG DLPE C12, PEG Click DMG C14, PEG Click C12, PEG Click C10, N(Carbonyl-methoxypolyethylenglycol-2000)-l,2-distearoyl-sn-glycero3-phosphoethanolamine, Myrj52, mPEG-PLA, MPEG-DSPE, mPEG3000-DMPE, MPEG-2000-DSPE, MPEG2000-DSPE, mPEG2000-DPPE, mPEG2000-DMPE, mPEG2000-DMG, mDPPE-PEG2000, l,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG2000, HPEG-2K-LIPD, Folate PEG-DSPE, DSPE-PEGMA 500, DSPE-PEGMA, DSPE-PEG6000, DSPE-PEG5000, DSPE-PEG2K-NAG, DSPE-PEG2k, DSPE-PEG2000maleimide, DSPE-PEG2000, DSPE-PEG, DSG-PEGMA, DSG-PEG5000, DPPE-PEG-2K, DPPE-PEG, DPPE-mPEG2000, DPPE-mPEG, DPG-PEGMA, DOPE-PEG2000, DMPE-PEGMA, DMPE-PEG2000, DMPE-Peg, DMPE-mPEG2000, DMG-PEGMA, DMG-PEG2000, DMG-PEG, distearoyl-glycerol-polyethyleneglycol, Cl8PEG750, CI8PEG5000, CI8PEG3000, CI8PEG2000, CI6PEG2000, CI4PEG2000, C18-PEG5000, C18PEG,

C16PEG, C16 mPEG (polyethylene glycol) 2000 Ceramide, C14-PEG-DSPE200, C14-PEG2000, C14PEG2000, C14-PEG 2000, C14-PEG, C14PEG, 14:0-PEG2KPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG2000, (R)-2,3-bis(octadecyloxy)propyl-1-(methoxypoly(ethyleneglycol)2000)propylcarbamate, (PEG)-C-DOMG, PEG-C-DMA, and DSPE-PEG-X.

[00994]In some embodiments, the LNP further comprises at least one additional lipid component selected from 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine (18:3 PC), Acylcarnosine (AC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), N-oleoyl-sphingomyelin (SPM) (C18:l), N-lignoceryl SPM (C24:0), N-nervonoylshphingomyelin (C24:l), Cardiolipin (CL), l,2-bis(tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine (DC8-9PC), dicetyl phosphate (DCP), dihexadecyl phosphate (DCP1), 1,2-Dipalmitoylglycerol-3-hemisuccinate (DGSucc), short-chain bis-n-heptadecanoyl phosphatidylcholine (DHPC), dihexadecoyl-phosphoethanolamine (DHPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), l,2-dilauroyl-sn-glycero-3-PE (DLPE), dimyristoyl glycerol hemisuccinate (DMGS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleyloxybenzylalcohol (DOBA), 1,2-dioleoylglyceryl-3-hemisuccinate (DOGHEMS), N-[2-(2-{2-[2-(2,3-Bis-octadec-9-enyloxy-propoxy)-ethoxy]-ethoxy}-ethoxy)-ethyl]-3-(3,4,5-dihydroxy-6-hydroxymethyl-1etrahydro-pyran-2-ylsulfanyl)-propionamide (DOGP4αMan), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), histaminedistearoylglycerol (HDSG), 1,2-Dipalmitoylglycerol-hemisuccinate-Nα-Histidinyl-Hemisuccinate (HistSuccDG), N-(5'-hydroxy-3'-oxypentyl)-10-12-pentacosadiynamide (h-Pegi-PCDA), 2-[l-hexyloxyethyl]-2-devinylpyropheophorbide-a (HPPH), hydrogenatedsoybeanphosphatidylcholine (HSPC), 1,2-Dipalmitoylglycerol-O-α-histidinyl-Nα-hemisuccinate (IsohistsuccDG), mannosialized dipalmitoylphosphatidylethanolamine (ManDOG), l,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (MCC-PE), 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16:0 PE), 1-myristoyl-2-hydroxy-sn-glyccro-phosphocholinc (MHPC), a thiol-rcactivc malcimidc hcadgroup lipid c.g.1,2-diolcoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)but-yramid (MPB-PE), Nervonic Acid (NA),

sodium cholate (NaChol), l,2-dioleoyl-sn-glycero-3-[phosphoethanolamine-N-dodecanoyl (NC12-DOPE), 1-oleoyl-2-cholesteryl hemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), phosphatidylethanolamine lipid (PE), PE lipid conjugated with polyethylene glycol(PEG) (e.g., polyethylene glycol-distearoylphosphatidylethanolamine lipid (PEG-PE)), phosphatidylglycerol (PG), partially hydrogenated soy phosphatidylchloline (PHSPC), phosphatidylinositol lipid (PI), phosphotidylinositol-4-phosphate (PIP), palmitoyloleoylphosphatidylcholine (POPC), phosphatidylethanolamine (POPE), palmitoyloleyolphosphatidylglycerol (POPG), phosphatidylserine (PS), lissamine rhodamineB-phosphatidylethanolamine lipid (Rh-PE), purifiedsoy-derivedmixtureofphospholipids (SIOO), phosphatidylcholine (SM), 18-1-trans-PE,1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), soybean phosphatidylcholine (SPC), sphingomyelins (SPM), alpha,alpha-trehalose-6,6'-dibehenate (TDB), l,2-dielaidoyl-sn-glycero-3-phophoethanolamine (transDOPE), ((23S,5R)-3-(bis(hexadecyloxy)methoxy)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methylmethylphosphate, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleyl-sn-glycero-3-phosphoethanolamine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 16-O-monomethyl PE, 16-O-dimethyl PE, and dioleylphosphatidylethanolamine.

[00995]In some embodiments, (a) the PEG lipid is PEG2k-DMG or PEG2k-DSPE or a mixture thereof; (b) the structural lipid is cholesterol; and (c) the phospholipid, non-ionizable lipid or zwitterionic lipid is a sphingolipid or DSPC or a mixture thereof.

[00996]In some embodiments, lipid component of the nanoparticle comprises: (a) about 2 mol% of PEG lipid; (b) about 25 mol% structural lipid; (c) about 40 mol% phospholipid, non-ionizable lipid or zwitterionic lipid; and (d) about 33 mol% of a Lipid of the Disclosure.

[00997]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 2.5 mol% of PEG lipid; (b) about 39 mol% structural lipid; (c) about 10 mol% phospholipid, non-ionizable lipid or zwitterionic lipid; and (d) about 48.5 mol% of a Lipid of the Disclosure.

[00998]In some embodiments, the lipid component of the nanoparticle comprises: (a) about 1.5 mol% of PEG lipid; (b) about 40 mol% structural lipid; (c) about 10 mol% phospholipid, non-ionizable lipid or zwitterionic lipid; and (d) about 48.5 mol% of a Lipid of the Disclosure.

VI. Routes of Administration

[00999]The LNP-based RNA vaccines, RNA therapeutics and pharmaceutical compositions thereof described herein may be administered by any delivery route which results in a therapeutically effective outcome. These include, but are not limited to, enteral (into the intestine), gastroenteral, epidural (into the

dura mater), oral (by way of the mouth), transdermal, intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin), intradermal (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intravenous bolus, intravenous drip, intra-arterial (into an artery), intramuscular (into a muscle), intracardiac (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraparenchymal (into brain tissue), intraperitoneal (infusion or injection into the peritoneum), intravesical infusion, intravitreal (through the eye), intracavernous injection (into a pathologic cavity) intracavitary (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), transvaginal, insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), ear drops, auricular (in or by way of the ear), buccal (directed toward the cheek), conjunctival, cutaneous, dental (to a tooth or teeth), electro-osmosis, endocervical, endosinusial, endotracheal, extracorporeal, hemodialysis, infiltration, interstitial, intra-abdominal, intra-amniotic, intra-articular, intrabiliary, intrabronchial, intrabursal, intracartilaginous (within a cartilage), intracaudal (within the cauda equine), intracisternal (within the cisterna magna cerebellomedularis), intracorneal (within the cornea), dental intracoronal, intracoronary (within the coronary arteries), intracorporus cavernosum (within the dilatable spaces of the corporus cavernosa of the penis), intradiscal (within a disc), intraductal (within a duct of a gland), intraduodenal (within the duodenum), intradural (within or beneath the dura), intraepidermal (to the epidermis), intraesophageal (to the esophagus), intragastric (within the stomach), intragingival (within the gingivae), intraileal (within the distal portion of the small intestine), intralesional (within or introduced directly to a localized lesion), intraluminal (within a lumen of a tube), intralymphatic (within the lymph), intramedullary (within the marrow cavity of a bone), intrameningeal (within the meninges), intramyocardial (within the myocardium), intraocular (within the eye), intraovarian (within the ovary), intrapericardial (within the pericardium), intrapleural (within the pleura), intraprostatic (within the prostate gland), intrapulmonary (within the lungs or its bronchi), intrasinal (within the nasal or periorbital sinuses), intraspinal (within the vertebral column), intrasynovial (within the synovial cavity of a joint), intratendinous (within a tendon), intratesticular (within the testicle), intrathecal (within the cerebrospinal fluid at any level of the cerebrospinal axis), intrathoracic (within the thorax), intratubular (within the tubules of an organ), intratumor (within a tumor), intratympanic (within the aurus media), intravascular (within a vessel or vessels), intraventricular (within a ventricle), iontophoresis (by means of electric current where ions of soluble salts migrate into the tissues of the body), irrigation (to bathe or flush open wounds or body cavities), laryngeal (directly upon the larynx), nasogastric (through the nose and into the stomach), occlusive dressing technique (topical route administration which is then covered by a dressing which

occludes the area), ophthalmic (to the external eye), oropharyngeal (directly to the mouth and pharynx), parenteral, percutaneous, periarticular, peridural, perineural, periodontal, rectal, respiratory (within the respiratory tract by inhaling orally or nasally for local or systemic effect), retrobulbar (behind the pons or behind the eyeball), soft tissue, subarachnoid, subconjunctival, submucosal, topical, transplacental (through or across the placenta), transtracheal (through the wall of the trachea), transtympanic (across or through the tympanic cavity), ureteral (to the ureter), urethral (to the urethra), vaginal, caudal block, diagnostic, nerve block, biliary perfusion, cardiac perfusion, photopheresis, and spinal.

[001000]In some embodiments, compositions may be administered in a way which allows them to cross the blood-brain barrier, vascular barrier, or other epithelial barrier. The originator constructs, benchmark constructs, and targeting systems may be administered in any suitable form, either as a liquid solution or suspension, as a solid form suitable for liquid solution or suspension in a liquid solution. The originator constructs, benchmark constructs, and targeting systems may be formulated with any appropriate and pharmaceutically acceptable excipient.

[001001]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be delivered to a subject via a single route administration.

[001002]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be delivered to a subject via a multi-site route of administration. A subject may be administered at 2, 3, 4, 5, or more than 5 sites.

[001003]In some embodiments, a subject may be administered the originator constructs, benchmark constructs, and targeting systems using a bolus infusion.

[001004]In some embodiments, a subject may be administered originator constructs, benchmark constructs, and targeting systems using sustained delivery over a period of minutes, hours, or days. The infusion rate may be changed depending on the subject, distribution, formulation or another delivery parameter.

[001005]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be delivered by intramuscular delivery route. Non-limiting examples of intramuscular administration include an intravenous injection or a subcutaneous injection.

[001006]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be delivered by oral administration. Non-limiting examples of oral delivery include a digestive tract administration and a buccal administration.

[001007]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be delivered by intraocular delivery route. A non-limiting example of intraocular delivery include an intravitreal injection.

[001008]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be delivered by intranasal delivery route. Non-limiting examples of intranasal delivery include nasal drops or nasal sprays.

[001009]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be administered to a subject by peripheral injections. Non-limiting examples of peripheral injections include intraperitoneal, intramuscular, intravenous, conjunctival, or joint injection.

[001010]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be delivered by injection into the cerebrospinal fluid. Non-limiting examples of delivery to the cerebrospinal fluid include intrathecal and intracerebroventricular administration.

[001011]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be delivered by systemic delivery. As a non-limiting example, the systemic delivery may be by intravascular administration.

[001012]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be administered to a subject by intracranial delivery.

[001013]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be administered to a subject by intraparenchymal administration.

[001014]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be administered to a subject by intramuscular administration.

[001015]In some embodiments, the originator constructs, benchmark constructs, and targeting systems are administered to a subject and transduce muscle of a subject. As a non-limiting example, the originator constructs, benchmark constructs, and targeting systems are administered by intramuscular administration.

[001016]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be administered to a subject by intravenous administration.

[001017]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be administered to a subject by subcutaneous administration.

[001018]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be administered to a subject by topical administration.

[001019]In some embodiments, the originator constructs, benchmark constructs, and targeting systems may be delivered by more than one route of administration.

[001020]The originator constructs, benchmark constructs, and targeting systems described herein may be co-administered in conjunction with one or more originator constructs, benchmark constructs, targeting systems, or therapeutic agents or moieties.

[001021]In some embodiments, pharmaceutical compositions and/or formulations described herein may be administered parenterally. Liquid dosage forms for oral and parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof. In other embodiments, surfactants are included such as hydroxypropylcellulose.

[001022]Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[001023]Injectable formulations may be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[001024]In order to prolong the effect of active ingredients, it is often desirable to slow the absorption of active ingredients from subcutaneous or intramuscular injections. This may be accomplished by the use of liquid suspensions of crystalline or amorphous material with poor water solubility. The rate of absorption of active ingredients depends upon the rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer

employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

[001025]In some embodiments, pharmaceutical compositions and/or formulations described herein may be formulated for administration topically. The skin may be an ideal target site for delivery as it is readily accessible. Three routes are commonly considered to deliver pharmaceutical compositions and/or formulations described herein to the skin: (i) topical application (*e.g.* for local/regional treatment and/or cosmetic applications); (ii) intradermal injection (*e.g.* for local/regional treatment and/or cosmetic applications); and (iii) systemic delivery (*e.g.* for treatment of dermatologic diseases that affect both cutaneous and extracutaneous regions).

[001026]In some embodiments, pharmaceutical compositions and/or formulations described herein may be delivered using a variety of dressings (e.g., wound dressings) or bandages (e.g., adhesive bandages) for conveniently and/or effectively carrying out methods described herein. Typically dressing or bandages may comprise sufficient amounts of pharmaceutical compositions and/or formulations described herein to allow users to perform multiple treatments.

[001027]Dosage forms for topical and/or transdermal administration may include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants and/or patches. Generally, active ingredients are admixed under sterile conditions with pharmaceutically acceptable excipients and/or any needed preservatives and/or buffers. Additionally, contemplated herein is the use of transdermal patches, which often have the added advantage of providing controlled delivery of pharmaceutical compositions and/or formulations described herein to the body. Such dosage forms may be prepared, for example, by dissolving and/or dispensing pharmaceutical compositions and/or formulations described herein in the proper medium. Alternatively, or additionally, rates may be controlled by either providing rate controlling membranes and/or by dispersing pharmaceutical compositions and/or formulations described herein in a polymer matrix and/or gel.

[001028]Formulations suitable for topical administration include, but are not limited to, liquid and/or semi liquid preparations such as liniments, lotions, oil in water and/or water in oil emulsions such as creams, ointments and/or pastes, and/or solutions and/or suspensions.

[001029]Topically-administrable formulations may, for example, comprise from about 1% to about 10% (w/w) active ingredient, although the concentration of active ingredient may be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

[001030]In some embodiments, pharmaceutical compositions and/or formulations described herein may be prepared, packaged, and/or sold in formulations suitable for ophthalmic and/or otic administration.

Such formulations may, for example, be in the form of eye and/or ear drops including, for example, a 0.1/1.0% (w/w) solution and/or suspension of the active ingredient in aqueous and/or oily liquid excipients. Such drops may further comprise buffering agents, salts, and/or one or more other of any additional ingredients described herein. Other ophthalmically-administrable formulations which are useful include those which comprise active ingredients in microcrystalline form and/or in liposomal preparations. Subretinal inserts may also be used as forms of administration.

[001031]In some embodiments, pharmaceutical compositions and/or formulations described herein may be administered orally. Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, an active ingredient is mixed with at least one inert, pharmaceutically acceptable excipient such as sodium citrate or dicalcium phosphate and/or fillers or extenders (*e.g.* starches, lactose, sucrose, glucose, mannitol, and silicic acid), binders (*e.g.* carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia), humectants (*e.g.* glycerol), disintegrating agents (*e.g.* agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate), solution retarding agents (*e.g.* paraffin), absorption accelerators (*e.g.* quaternary ammonium compounds), wetting agents (*e.g.* cetyl alcohol and glycerol monostearate), absorbents (*e.g.* kaolin and bentonite clay), and lubricants (*e.g.* talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate), and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may comprise buffering agents.

[001032]In some embodiments, pharmaceutical compositions and/or formulations described herein are formulated in depots for extended release.

[001033]In some embodiments, pharmaceutical compositions and/or formulations described herein are spatially retained within or proximal to target tissues. Provided are methods of providing pharmaceutical compositions and/or formulations described herein to target tissues of mammalian subjects by contacting target tissues (which comprise one or more target cells) with pharmaceutical compositions and/or formulations described herein under conditions such that they are substantially retained in target tissues, meaning that at least 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the composition is retained in the target tissues. Advantageously, retention is determined by measuring the amount of pharmaceutical compositions and/or formulations described herein that enter one or more target cells. For example, at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.9%, 99.99%, or greater than 99.99% of pharmaceutical compositions and/or formulations described herein administered to subjects are present intracellularly at a period of time following administration. For example, intramuscular injection to mammalian subjects may be performed using aqueous compositions comprising an active ingredient and one or more

transfection reagents, and retention is determined by measuring the amount of active ingredient present in muscle cells.

[001034]In some embodiments, provided are methods for delivering pharmaceutical compositions and/or formulations described herein to target tissues of mammalian subjects, by contacting target tissues (comprising one or more target cells) with pharmaceutical compositions and/or formulations described herein under conditions such that they are substantially retained in such target tissues. Pharmaceutical compositions and/or formulations described herein comprise enough active ingredient such that the effect of interest is produced in at least one target cell. In some embodiments, pharmaceutical compositions and/or formulations described herein generally comprise one or more cell penetration agents, although "naked" formulations (such as without cell penetration agents or other agents) are also contemplated, with or without pharmaceutically acceptable carriers.

[001035]In some embodiments, pharmaceutical compositions and/or formulations described herein may be prepared, packaged, and/or sold in formulations suitable for pulmonary administration. In some embodiments, such administration is via the buccal cavity. In some embodiments, formulations may comprise dry particles comprising active ingredients. In such embodiments, dry particles may have a diameter in the range from about 0.5 nm to about 7 nm or from about 1 nm to about 6 nm. In some embodiments, formulations may be in the form of dry powders for administration using devices comprising dry powder reservoirs to which streams of propellant may be directed to disperse such powder. In some embodiments, self-propelling solvent/powder dispensing containers may be used. In such embodiments, active ingredients may be dissolved and/or suspended in low-boiling propellant in sealed containers. Such powders may comprise particles wherein at least 98% of the particles by weight have diameters greater than 0.5 nm and at least 95% of the particles by number have diameters less than 7 nm. Alternatively, at least 95% of the particles by weight have a diameter greater than 1 nm and at least 90% of the particles by number have a diameter less than 6 nm. Dry powder compositions may include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

[001036]Low boiling propellants generally include liquid propellants having a boiling point of below 65 °F at atmospheric pressure. Generally, propellants may constitute 50% to 99.9% (w/w) of the composition, and active ingredient may constitute 0.1% to 20% (w/w) of the composition. Propellants may further comprise additional ingredients such as liquid non-ionic and/or solid anionic surfactant and/or solid diluent (which may have particle sizes of the same order as particles comprising active ingredients).

[001037]Pharmaceutical compositions formulated for pulmonary delivery may provide active ingredients in the form of droplets of solution and/or suspension. Such formulations may be prepared, packaged, and/or sold as aqueous and/or dilute alcoholic solutions and/or suspensions, optionally sterile, comprising

active ingredients, and may conveniently be administered using any nebulization and/or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, and/or a preservative such as methylhydroxybenzoate. Droplets provided by this route of administration may have an average diameter in the range from about 0.1 nm to about 200 nm.

[001038]In some embodiments, pharmaceutical compositions and/or formulations described herein may be administered nasally and/or intranasal. In some embodiments, formulations described herein useful for pulmonary delivery may also be useful for intranasal delivery. In some embodiments, formulations for intranasal administration comprise a coarse powder comprising the active ingredient and having an average particle from about 0.2 μm to 500 μm. Such formulations are administered in the manner in which snuff is taken, *i.e.* by rapid inhalation through the nasal passage from a container of the powder held close to the nose.

[001039]Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of active ingredient, and may comprise one or more of the additional ingredients described herein. A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets and/or lozenges made using conventional methods, and may, for example, 0.1% to 20% (w/w) active ingredient, the balance comprising an orally dissolvable and/or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise powders and/or an aerosolized and/or atomized solutions and/or suspensions comprising active ingredients. Such powdered, aerosolized, and/or aerosolized formulations, when dispersed, may comprise average particle and/or droplet sizes in the range of from about 0.1 nm to about 200 nm, and may further comprise one or more of any additional ingredients described herein.

[001040]In some embodiments, pharmaceutical compositions and/or formulations described herein may be administered rectally and/or vaginally. Compositions for rectal or vaginal administration are typically suppositories which can be prepared by mixing compositions with suitable non-irritating excipients such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active ingredient.

VII. Methods of use (including vaccination and treatment)

[001041]The LNP-based RNA vaccines and therapeutics and compositions described herein may be used to deliver a protein of interest (e.g., an antigen or therapeutic protein) to a cell, tissue, or organ. In addition, the LNP-based RNA vaccines and therapeutics and compositions described herein may be used to deliver an immunogenic protein of interest to a subject in need as a means for vaccination against an

infectious disease (e.g., virus or bacteria) or cancer. Still further, the LNP-based RNA vaccines and therapeutics and compositions described herein may be used to deliver a therapeutic protein of interest to a subject in need as a means for treating a patient for a disease, such as a disease of the CNS or muscle, or for treating cancer.

A. Methods of producing polypeptides in cells

[001042] The present disclosure provides methods of producing a polypeptide of interest in a mammalian cell. Methods of producing polypeptides involve contacting a cell with an LNP-based RNA vaccine or therapeutic and/or a formulation or composition thereof as described herein. Upon contacting the cell with the lipid nanoparticle, the mRNA may be taken up and translated in the cell to produce the polypeptide of interest, e.g., an antigen or therapeutic protein.

[001043] In general, the step of contacting a mammalian cell with a LNP including an mRNA encoding a polypeptide of interest may be performed in vivo, ex vivo, in culture, or in vitro. The amount of lipid nanoparticle contacted with a cell, and/or the amount of mRNA therein, may depend on the type of cell or tissue being contacted, the means of administration, the physiochemical characteristics of the lipid nanoparticle and the mRNA (e.g., size, charge, and chemical composition) therein, and other factors. In general, an effective amount of the lipid nanoparticle will allow for efficient polypeptide production in the cell. Metrics for efficiency may include polypeptide translation (indicated by polypeptide expression), level of mRNA degradation, and immune response indicators.

[001044] The step of contacting an LNP including an mRNA with a cell may involve or cause transfection. A phospholipid including in the lipid component of a LNP may facilitate transfection and/or increase transfection efficiency, for example, by interacting and/or fusing with a cellular or intracellular membrane. Transfection may allow for the translation of the mRNA within the cell.

[001045] In some embodiments, the lipid nanoparticles described herein is used therapeutically. For example, an mRNA included in an LNP may encode a therapeutic polypeptide (e.g., in a translatable region) and produce the therapeutic polypeptide upon contacting and/or entry (e.g., transfection) into a cell. In other embodiments, an mRNA included in a LNP may encode a polypeptide that may improve or increase the immunity of a subject. In some embodiments, an mRNA may encode a granulocyte-colony stimulating factor or trastuzumab.

[001046] In some embodiments, an mRNA included in an LNP may encode a recombinant polypeptide that may replace one or more polypeptides that is substantially absent in a cell contacted with the lipid nanoparticle. The one or more substantially absent polypeptides may be lacking due to a genetic mutation of the encoding gene or a regulatory pathway thereof. Alternatively, a recombinant polypeptide produced by translation of the mRNA may antagonize the activity of an endogenous protein present in, on the surface of, or secreted from the cell. An antagonistic recombinant polypeptide may be desirable to combat

deleterious effects caused by activities of the endogenous protein, such as altered activities or localization caused by mutation. In another alternative, a recombinant polypeptide produced by translation of the mRNA may indirectly or directly antagonize the activity of a biological moiety present in, on the surface of, or secreted from the cell. Antagonized biological moieties may include, but are not limited to, lipids (e.g., cholesterol), lipoproteins (e.g., low density lipoprotein), nucleic acids, carbohydrates, and small molecule toxins. Recombinant polypeptides produced by translation of the mRNA may be engineered for localization within the cell, such as within a specific compartment such as the nucleus, or may be engineered for secretion from the cell or for translocation to the plasma membrane of the cell.

[001047]In some embodiments, contacting a cell with an LNP including an mRNA may reduce the innate immune response of a cell to an exogenous nucleic acid. A cell may be contacted with a first lipid nanoparticle including a first amount of a first exogenous mRNA including a translatable region and the level of the innate immune response of the cell to the first exogenous mRNA may be determined. Subsequently, the cell may be contacted with a second composition including a second amount of the first exogenous mRNA, the second amount being a lesser amount of the first exogenous mRNA compared to the first amount. Alternatively, the second composition may include a first amount of a second exogenous mRNA that is different from the first exogenous mRNA. The steps of contacting the cell with the first and second compositions may be repeated one or more times. Additionally, efficiency of polypeptide production (e.g., translation) in the cell may be optionally determined, and the cell may be re-contacted with the first and/or second composition repeatedly until a target protein production efficiency is achieved.

B.Therapeutic methods using LNPs described herein

[001048]Provided herein are therapeutic methods for treating a disease or disorder by using the LNP-based RNA compositions described herein to deliver one or more therapeutic agents encoded on a payload RNA (e.g., linear or circular mRNA payload) to a cell, organ, or tissue. Delivery may be *in vitro* or *ex vivo* to cells, or to a cell, tissue, or organ *in vivo*.

[001049]Delivery of a therapeutic and/or prophylactic to a cell involves administering a composition of the disclosure that comprises a LNP encapsulated with a payload RNA (e.g., a linear or circular RNA) that encodes a therapeutic and/or prophylactic, where administration of the composition involves contacting the cell with the composition. In some embodiments, a protein, cytotoxic agent, radioactive ion, chemotherapeutic agent, or nucleic acid (such as an RNA, e.g., mRNA) is delivered to a cell or organ. In some embodiments, the mRNA payload itself may be regarded as the therapeutic and/or prophylactic as it encodes a therapeutic and/or prophylactic. Upon contacting a cell with the lipid nanoparticle, a translatable mRNA may be translated in the cell to produce a polypeptide of interest. However, mRNAs that are substantially not translatable may also be delivered to cells. Substantially non-

translatable mRNAs may be useful as vaccines and/or may sequester translational components of a cell to reduce expression of other species in the cell.

[001050]In some embodiments, an LNP may target a particular type or class of cells (e.g., cells of a particular organ or system thereof). In some embodiments, a LNP including an RNA payload coding for a therapeutic and/or prophylactic of interest is specifically delivered to a mammalian liver, kidney, spleen, femur, or lung. "Specific delivery" to a particular class of cells, an organ, or a system or group thereof implies that a higher proportion of lipid nanoparticles including a therapeutic and/or prophylactic are delivered to the destination (e.g., tissue) of interest relative to other destinations, e.g., upon administration of an LNP to a mammal. In some embodiments, specific delivery may result in a greater than 2-fold, 5-fold, 10-fold, 15-fold, or 20-fold increase in the amount of therapeutic and/or prophylactic per 1 g of tissue of the targeted destination (e.g., tissue of interest, such as a liver) as compared to another destination (e.g., the spleen). In some embodiments, the tissue of interest is selected from the group consisting of a liver, kidney, a lung, a spleen, a femur, vascular endothelium in vessels (e.g., intra-coronary or intra-femoral) or kidney, and tumor tissue (e.g., via intratumoral injection).

[001051]As another example of targeted or specific delivery, an mRNA that encodes a protein-binding partner (e.g., an antibody or functional fragment thereof, a scaffold protein, or a peptide) or a receptor on a cell surface may be included in an LNP. An mRNA may additionally or instead be used to direct the synthesis and extracellular localization of lipids, carbohydrates, or other biological moieties. Alternatively, other therapeutics and/or prophylactics or elements (e.g., lipids or ligands) of an LNP may be selected based on their affinity for particular receptors (e.g., low density lipoprotein receptors) such that a LNP may more readily interact with a target cell population including the receptors. In some embodiments, ligands may include, but are not limited to, members of a specific binding pair, antibodies, monoclonal antibodies, Fv fragments, single chain Fv (scFv) fragments, Fab' fragments, F(ab')2 fragments, single domain antibodies, camelized antibodies and fragments thereof, humanized antibodies and fragments thereof, and multivalent versions thereof; multivalent binding reagents including mono- or bi-specific antibodies such as disulfide stabilized Fv fragments, scFv tandems, diabodies, tribodies, or tetrabodies; and aptamers, receptors, and fusion proteins.

[001052]In some embodiments, a ligand is a surface-bound antibody, which can permit tuning of cell targeting specificity. This is especially useful since highly specific antibodies can be raised against an epitope of interest for the desired targeting site. In some embodiments, multiple antibodies are expressed on the surface of a cell, and each antibody can have a different specificity for a desired target. Such approaches can increase the avidity and specificity of targeting interactions.

[001053]A ligand can be selected, e.g., by a person skilled in the biological arts, based on the desired localization or function of the cell. In some embodiments an estrogen receptor ligand, such as tamoxifen,

can target cells to estrogen-dependent breast cancer cells that have an increased number of estrogen receptors on the cell surface. Other non-limiting examples of ligand/receptor interactions include CCR1 (e.g., for treatment of inflamed joint tissues or brain in rheumatoid arthritis, and/or multiple sclerosis), CCR7, CCR8 (e.g., targeting to lymph node tissue), CCR6, CCR9, CCR10 (e.g., to target to intestinal tissue), CCR4, CCR10 (e.g., for targeting to skin), CXCR4 (e.g., for general enhanced transmigration), HCELL (e.g., for treatment of inflammation and inflammatory disorders, bone marrow), Alpha4beta7 (e.g., for intestinal mucosa targeting), and VLA-4NCAM-1 (e.g., targeting to endothelium). In general, any receptor involved in targeting (e.g., cancer metastasis) can be harnessed for use in the methods and compositions described herein.

[001054]Targeted cells may include, but are not limited to, hepatocytes, epithelial cells, hematopoietic cells, epithelial cells, endothelial cells, lung cells, bone cells, stem cells, mesenchymal cells, neural cells, cardiac cells, adipocytes, vascular smooth muscle cells, cardiomyocytes, skeletal muscle cells, beta cells, pituitary cells, synovial lining cells, ovarian cells, testicular cells, fibroblasts, B cells, T cells, reticulocytes, leukocytes, granulocytes, and tumor cells.

[001055]In some embodiments, an LNP may target hepatocytes. Apolipoproteins such as apolipoprotein E (apoE) have been shown to associate with neutral or near neutral lipid-containing lipid nanoparticles in the body, and are known to associate with receptors such as low-density lipoprotein receptors (LDLRs) found on the surface of hepatocytes. Thus, an LNP including a lipid component with a neutral or near neutral charge that is administered to a subject may acquire apoE in a subject's body and may subsequently deliver a therapeutic and/or prophylactic (e.g., an RNA) to hepatocytes including LDLRs in a targeted manner.

[001056]Lipid nanoparticles described herein are useful for treating a disease, disorder, or condition. In particular, such compositions are useful in treating a disease, disorder, or condition characterized by missing or aberrant protein or polypeptide activity. In some embodiments, a formulation of the disclosure that comprises an LNP including an mRNA encoding a missing or aberrant polypeptide is administered or delivered to a cell. Subsequent translation of the mRNA may produce the polypeptide, thereby reducing or eliminating an issue caused by the absence of or aberrant activity caused by the polypeptide. Because translation may occur rapidly, the methods and compositions may be useful in the treatment of acute diseases, disorders, or conditions such as sepsis, stroke, and myocardial infarction. A therapeutic and/or prophylactic included in an LNP may also be capable of altering the rate of transcription of a given species, thereby affecting gene expression.

[001057]Diseases, disorders, and/or conditions characterized by dysfunctional or aberrant protein or polypeptide activity for which a composition may be administered include, but are not limited to, rare diseases, infectious diseases (as both vaccines and therapeutics), cancer and proliferative diseases, genetic

diseases (e.g., cystic fibrosis), muscle-related conditions, autoimmune diseases, diabetes, neurodegenerative diseases, cardio- and reno-vascular diseases, and metabolic diseases. Multiple diseases, disorders, and/or conditions may be characterized by missing (or substantially diminished such that proper protein function does not occur) protein activity. Such proteins may not be present, or they may be essentially non-functional. A specific example of a dysfunctional protein is the missense mutation variants of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which produce a dysfunctional protein variant of CFTR protein, which causes cystic fibrosis. The present disclosure provides a method for treating such diseases, disorders, and/or conditions in a subject by administering a LNP including an RNA and a lipid component including a PEGylated lipid compound disclosed herein, a phospholipid (optionally unsaturated), optionally a second PEGylated lipid, and a structural lipid, wherein the RNA may be an mRNA encoding a polypeptide that antagonizes or otherwise overcomes an aberrant protein activity present in the cell of the subject.

Treatment of CNS disorders

[001058]Provided herein are therapeutic methods for treating a CNS disease or disorder by using the LNP-based RNA compositions described herein to deliver one or more therapeutic agents encoded on a payload RNA (e.g., linear or circular mRNA payload) to a cell, organ, or tissue of the central nervous system. Delivery may be *in vitro* or *ex vivo* to cells, or to a cell, tissue, or organ *in vivo*.

[001059]The present disclosure provides, among other things, improved methods and compositions for efficient LNP delivery of mRNA payloads (e.g., circular mRNA and/or linear mRNA), encoding a therapeutic protein, to neurons and other cell types of the CNS. In various embodiments, the mRNA-encapsulated nanoparticles (e.g., LNPs) can be administered directly into the CNS space (e.g., via intrathecal administration) and effectively penetrate neuronal cell membrane, resulting in intracellular delivery of mRNA in neurons in the brain and/or spinal cord.

[001060]Thus, in one aspect, the disclosure provides methods of LNP delivery of mRNA payloads (e.g., circular mRNA and/or linear mRNA) to the central nervous system (CNS). In some embodiments, an inventive method according to the present disclosure includes administering intrathecally to a subject in need of delivery a composition comprising an mRNA encoding a protein, encapsulated within a liposome such that the administering of the composition results in the intracellular delivery of mRNA in neurons in the brain and/or spinal cord.

[001061]In some embodiments, the LNP-encapsulated mRNA therapeutics described comprising one or more mRNA payloads (e.g., linear or circular mRNA) are delivered to neurons located within the brain. In some embodiments, the mRNA payloads are delivered to neurons located within the spinal cord. In some embodiments, the mRNA payloads are delivered to motor neurons. In some embodiments, the mRNA payloads are delivered to upper motor neurons and/or lower motor neurons. In some

embodiments, the motor neurons are located within the anterior horn and/or dorsal root ganglia of the spinal cord.

[001062] In other embodiments, the present disclosure provides, among other things, methods and compositions for effective delivery of messenger RNA (mRNA) to the central nervous system (CNS). In particular, the present disclosure provides methods and compositions for administering intrathecally to a subject in need of delivery a composition comprising an mRNA encoding a protein, encapsulated within an LNP described herein, such that the administering of the composition results in the intracellular delivery of mRNA in neurons in the brain and/or spinal cord. The present disclosure is particularly useful for the treatment of CNS diseases, disorders or conditions, such as spinal muscular atrophy.

[001063]The present disclosure can be used to deliver any mRNA to the central nervous system. In particular, the present disclosure is useful to deliver mRNA that encodes a protein associated with or implicated in a CNS disease, disorder or condition. As used herein, a "CNS disease, disorder or condition" refers to a disease, disorder or condition affecting one or more neuronal functions of the central nervous system (i.e., the brain and/or spinal cord). In some embodiments, a CNS disease, disorder or condition may be caused by a protein deficiency or dysfunction in neurons of the CNS (i.e., the brain and/or spinal cord).

[001064]Exemplary CNS diseases, disorders or conditions include, but are not limited to, Acid Lipase Disease, Acid Maltase Deficiency, Acquired Epileptiform Aphasia, Acute Disseminated Encephalomyelitis, ADHD, Adie's Pupil, Adie's Syndrome, Adrenoleukodystrophy, Agnosia, Aicardi Syndrome, Aicardi-Goutieres Syndrome Disorder, Alexander Disease, Alpers' Disease, Alternating Hemiplegia, Alzheimer's Disease, Amyotrophic Lateral Sclerosis (ALS), Anencephaly, Aneurysm, Angelman Syndrome, Angiomatosis, Anoxia, Antiphospholipid Syndrome, Aphasia, Apraxia, Arachnoiditis, Arnold-Chiari Malformation, Asperger Syndrome, Ataxia, Ataxia Telangiectasia, Ataxias and Cerebellar or Spinocerebellar Degeneration, Attention Deficit-Hyperactivity Disorder, Autism, Autonomic Dysfunction, Barth Syndrome, Batten Disease, Becker's Myotonia, Behcet's Disease, Bell's Palsy, Bernhardt-Roth Syndrome, Binswanger's Disease, Bloch-Sulzberger Syndrome, Bradbury-Eggleston Syndrome, Brown-Sequard Syndrome, Bulbospinal Muscular Atrophy, CADASIL, Canavan Disease, Causalgia, Cavernomas, Cavernous Angioma, Central Cervical Cord Syndrome, Central Cord Syndrome, Central Pontine Myelinolysis, Ceramidase Deficiency, Cerebellar Degeneration, Cerebellar Hypoplasia, Cerebral Beriberi, Cerebral Gigantism, Cerebral Palsy, Cerebro-Oculo-Facio-Skeletal Syndrome (COFS), Cholesterol Ester Storage Disease, Chorea, Choreoacanthocytosis, Chronic Inflammatory Demyelinating Polyneuropathy (CIDP), Chronic Orthostatic Intolerance, Cockayne Syndrome Type II, Coffin Lowry Syndrome, Colpocephaly, Congenital Myasthenia, Corticobasal Degeneration, Cranial Arteritis, Cree encephalitis, Creutzfeldt-Jakob Disease, Cushing's Syndrome,

Cytomegalic Inclusion Body Disease, Dancing Eyes-Dancing Feet Syndrome, Dandy-Walker Syndrome, Dawson Disease, De Morsier's Syndrome, Dejerine-Klumpke Palsy, Dentate Cerebellar Ataxia, Dentatorubral Atrophy, Dermatomyositis, Developmental Dyspraxia, Devic's Syndrome, Diffuse Sclerosis, Dravet Syndrome, Dysautonomia, Dysgraphia, Dyslexia, Dysphagia, Dyspraxia, Dyssynergia Cerebellaris Myoclonica, Dyssynergia Cerebellaris Progressiva, Fabry Disease, Fahr's Syndrome, Familial Dysautonomia, Familial Hemangioma, Familial Idiopathic Basal Ganglia Calcification, Familial Periodic Paralyses, Familial Spastic Paralysis, Farber's Disease, Fibromuscular Dysplasia, Fisher Syndrome, Floppy Infant Syndrome, Friedreich's Ataxia, Gaucher Disease, Generalized Gangliosidoses, Gerstmann's Syndrome, Gerstmann-Straussler-Scheinker Disease, Giant Axonal Neuropathy, Giant Cell Arteritis, Giant Cell Inclusion Disease, Globoid Cell Leukodystrophy, Glossopharyngeal Neuralgia, Glycogen Storage Disease, Guillain-Barré Syndrome, Hallervorden-Spatz Disease, Hemicrania Continua, Hemiplegia Alterans, Hereditary Spastic Paraplegia, Heredopathia Atactica Polyneuritiformis, Holmes-Adie syndrome, Holoprosencephaly, Hughes Syndrome, Huntington's Disease, Hydranencephaly, Hydromyelia, Hypercortisolism, Immune-Mediated, Encephalomyelitis, Inclusion Body Myositis, Incontinentia Pigmenti, Infantile Hypotonia, Infantile Neuroaxonal Dystrophy, Acid Storage Disease, Iniencephaly, Isaac's Syndrome, Joubert Syndrome, Kearns-Sayre Syndrome, Kennedy's Disease, Kinsbourne syndrome, Kleine-Levin Syndrome, Klippel-Feil Syndrome, Klippel-Trenaunay Syndrome (KTS), Klüver-Bucy Syndrome, Korsakoffs Amnesic Syndrome, Krabbe Disease, Kugelberg-Welander Disease, Lambert-Eaton Myasthenic Syndrome, Landau-Kleffner Syndrome, Lateral, Femoral Cutaneous Nerve Entrapment, Lateral Medullary Syndrome, Leigh's Disease, Lennox-Gastaut Syndrome, Lesch-Nyhan Syndrome, Levine-Critchley Syndrome, Lewy Body Dementia, Lipoid Proteinosis, Lissencephaly, Locked-In Syndrome, Lou Gehrig's Disease, Lupus - Neurological Sequelae, Lyme Disease, Machado-Joseph Disease, Macrencephaly, Melkersson-Rosenthal Syndrome, Menkes Disease, Meralgia Paresthetica, Metachromatic Leukodystrophy, Microcephaly, Miller Fisher Syndrome, Moebius Syndrome, Multiple Sclerosis, Muscular Dystrophy, Myasthenia Gravis, Myelinoclastic Diffuse Sclerosis, Narcolepsy, Neuroacanthocytosis, Neurofibromatosis, Neuroleptic Malignant Syndrome, Neurosarcoidosis, Niemann-Pick Disease, Ohtahara Syndrome, Olivopontocerebellar Atrophy, Opsoclonus Myoclonus, O'Sullivan-McLeod Syndrome, Pantothenate Kinase-Associated Neurodegeneration, Paraneoplastic Syndromes, Paresthesia, Parkinson's Disease, Paroxysmal Choreoathetosis, Paroxysmal Hemicrania, Parry-Romberg, Pelizaeus-Merzbacher Disease, Pena Shokeir II Syndrome, Periventricular Leukomalacia, Phytanic Acid Storage Disease, Pick's Disease, Piriformis Syndrome, Polymyositis, Pompe Disease, Post-Polio Syndrome, Primary Dentatum Atrophy, Primary Lateral Sclerosis, Primary Progressive Aphasia, Prion Diseases, Progressive Hemifacial Atrophy, Progressive Locomotor Ataxia, Progressive Multifocal Leukoencephalopathy, Progressive Sclerosing

Poliodystrophy, Progressive Supranuclear Palsy, Prosopagnosia, Ramsay Hunt Syndrome I, Ramsay Hunt Syndrome II, Rasmussen's Encephalitis, Refsum Disease, Rett Syndrome, Reye's Syndrome, Riley-Day Syndrome, Sandhoff Disease, Schilder's Disease, Seitelberger Disease, Severe Myoclonic Epilepsy of Infancy (SMEI), Shy-Drager Syndrome, Sjögren's Syndrome, Spasticity, Spina Bifida, Spinal Muscular Atrophy, Spinocerebellar Atrophy, Spinocerebellar Degeneration, Steele-Richardson-Olszewski Syndrome, Striatonigral Degeneration, Sturge-Weber Syndrome, Tardive Dyskinesia, Tay-Sachs Disease, Thoracic Outlet Syndrome, Thyrotoxic Myopathy, Tic Douloureux, Todd's Paralysis, Trigeminal Neuralgia, Tropical Spastic Paraparesis, Troyer Syndrome, Von Economo's Disease, Von Hippel-Lindau Disease (VHL), Von Recklinghausen's Disease, Wallenberg's Syndrome, Werdnig-Hoffman Disease, Wemicke-Korsakoff Syndrome, West Syndrome, Whipple's Disease, Williams Syndrome, Wilson Disease, Wolman's Disease, X-Linked Spinal and Bulbar Muscular Atrophy and Zellweger Syndrome.

Motor Neuron Diseases

[001065]In some embodiments, a CNS disease, disorder or condition is a disease, disorder or condition that affects one or more functions of motor neurons, which is also referred to as a motor neuron disease. In some embodiments, a motor neuron disease may be caused by a protein deficiency or dysfunction in motor neurons of the CNS (i.e., the brain and/or spinal cord). As used herein, the term "motor neurons" refer to those neurons that control voluntary muscle activity. Typically, motor neurons include upper motor neurons and lower motor neurons. As used herein, the term "upper motor neuron" refers to motor neurons that originate in the motor region of the cerebral cortex or the brain stem and carry motor information down to the final common pathway. Upper motor neurons also referred to as "corticospinal neurons". Typically, upper motor neurons refer to any motor neurons that are not directly responsible for stimulating the target muscle. As used herein, the term "lower motor neuron" refers to the motor neurons connecting the brainstem and spinal cord to muscle fibers. In other words, lower motor neurons bring the nerve impulses from the upper motor neurons out to the muscles. Typically, a lower motor neuron's axon terminates on an effector (muscle). Lower motor neurons include "spinal neuron" and "Anterior horn cells".

[001066]Exemplary motor neuron diseases, disorders or conditions include, but are limited to, Amyotrophic Lateral Sclerosis (ALS), Primary Lateral Sclerosis (PLS), Pseudobulbar Pasly, Hereditary Spastic Paraplegia, Progressive Muscular Atrophy (PMA), Progressive Bulbar Palsy (PBP), Distal Hereditary Motor Neuropathies, and Spinal Muscular Atrophies.

[001067]In some embodiments, a motor neuron disease, disorder or condition is a form of spinal muscular atrophy. The family of spinal muscular atrophies are a genetically and clinically heterogeneous group of rare debilitating disorders characterized by degeneration of the lower motor neurons. Degeneration of the cells within the lower motor neurons, which are also known as the anterior horn cells

of the spinal cord, leads to a loss of motor function resulting in atrophy and excessive wasting of various muscle groups within the body. Diseases that comprise the family can be divided into Proximal, Distal, Autosomal Recessive Proximal and Localized spinal muscular atrophies. However, given that protein deficiencies are the major cause of the various forms of spinal muscular atrophy, each disease member is usually classified according to the gene associated with the condition.

[001068]Diseases with a CNS component

[001069]In some embodiments, a CNS disease, disorder or condition is a disease with a CNS component. Typically, a disease with a CNS component is caused by a protein deficiency in one or more tissues, including both CNS and peripheral tissues, of the body, resulting in one or more CNS etiology and/or symptoms. For example, in some embodiments, a protein deficiency may result in the excess accumulation of an intracellular and/or extracellular component such as: glucosaminoglycans (GAGs), lipids, plaque (i.e.; Beta-amyloid) or protein. Thus, in some embodiments, a disease with a CNS component is a lysosomal storage disease caused by a deficiency in a lysosomal enzyme, which results in the excess accumulation of glucosaminoglycans (GAGs) in both the CNS and peripheral tissues.

[001070]In some embodiments, lysosomal storage diseases can be treated having CNS etiology and/or symptoms include, but are not limited to, aspartylglucosaminuria, cholesterol ester storage disease, Wolman disease, cystinosis, Danon disease, Fabry disease, Farber lipogranulomatosis, Farber disease, fucosidosis, galactosialidosis types I/II, Gaucher disease types I/II/III, globoid cell leukodystrophy, Krabbe disease, glycogen storage disease II, Pompe disease, GM1-gangliosidosis types I/II/III, GM2-gangliosidosis type I, Tay Sachs disease, GM2-gangliosidosis type II, Sandhoff disease, GM2-gangliosidosis, α-mannosidosis types I/II, beta-mannosidosis, metachromatic leukodystrophy, mucolipidosis type I, sialidosis types I/II, mucolipidosis types II /III, I-cell disease, mucolipidosis type IIIC pseudo-Hurler polydystrophy, mucopolysaccharidosis type I, mucopolysaccharidosis type II, mucopolysaccharidosis type IIIA, Sanfilippo syndrome, mucopolysaccharidosis type IIIB, mucopolysaccharidosis type IIIC, mucopolysaccharidosis type IIID, mucopolysaccharidosis type IVA, Morquio syndrome, mucopolysaccharidosis type IVB, mucopolysaccharidosis type VI, mucopolysaccharidosis type VII, Sly syndrome, mucopolysaccharidosis type IX, multiple sulfatase deficiency, neuronal ceroid lipofuscinosis, CLN1 Batten disease, CLN2 Batten diseae, Niemann-Pick disease types A/B, Niemann-Pick disease type C1, Niemann-Pick disease type C2, pycnodysostosis, Schindler disease types I/II, Gaucher disease and sialic acid storage disease.

[001071]A detailed review of the genetic etiology, clinical manifestations, and molecular biology of the lysosomal storage diseases are detailed in Scriver et al., eds., The Metabolic and Molecular Basis of Inherited Disease, 7.sup.th Ed., Vol. II, McGraw Hill, (1995).

[001072]In various embodiments, the present disclosure may be used to deliver an mRNA encoding a protein that is deficient in any of the CNS diseases, disorders or conditions described herein. In some embodiments, the present disclosure may be used to deliver an mRNA encoding a protein that is deficient in a motor neuron disease. In particular embodiments, the present disclosure may be used to deliver an mRNA encoding a protein that is deficient in Spinal muscular atrophy (SMA), e.g., SMN1, which is described in detail below. In some embodiments, the present disclosure may be used to deliver an mRNA encoding a lysosomal enzyme that is deficient in a lysosomal storage disease with a CNS component. disclosureIn some embodiments, an mRNA suitable for the disclosure may encoded a wild-type or naturally occurring amino acid sequence. In some embodiments, an mRNA suitable for the disclosure may be a wild-type or naturally occurring sequence. In some embodiments, an mRNA suitable for the disclosure may be a codon-optimized sequence. In some embodiments, an mRNA suitable for the disclosure may encode an amino acid sequence having substantial homology or identify to the wild-type or naturally-occurring amino acid protein sequence (e.g., having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% sequence identity to the wild-type or naturally-occurring sequence).

Survival of Motor Neuron

[001073]In some embodiments, the methods and compositions provided by the present disclosure are used to deliver an mRNA encoding a Survival of Motor Neuron protein to the CNS for treatment of spinal muscular atrophy (SMA).

[001074]A suitable SMN mRNA encodes any full length, fragment or portion of a SMN protein which can be substituted for naturally-occurring SMN protein activity or rescue one or more phenotypes or symptoms associated with spinal muscular atrophy. The mRNA sequence for human Survival of Motor Neuron-1 (hSMN-1) and corresponding amino acid sequence of a typical wild-type or naturally occurring hSMN-1 protein are known in the art.

[001075]Thus, in some embodiments, a suitable mRNA for the present disclosure is a wild-type hSMN-2 mRNA sequence. In some embodiments, a suitable mRNA may be a codon optimized hSMN-1 mRNA sequence.

[001076]Human SMN-1 gene may undergo alternative processing and transcriptional modification to produce alternative splice isoforms. For example, there are five known hSMN-1 splice isoforms: hSMN-1 isoform b, c, e, f and g. Human SMN-2 gene can also undergo alternative processing and transcriptional modification to produce alternative splice isoforms. There are four known hSMN-2 splice isoforms: hSMN-2 isoform a, b, c and d. In some embodiments, the present disclosure is used to deliver an mRNA encoding an hSMN-1 isoform (e.g., isoform b, c, e, f, or g). In some embodiments, the present disclosure is used to deliver an mRNA encoding an hSMN-2 isoform (e.g., isoform a, b, c or d). The nucleotide and amino acid sequence of the hSMN-1 and hSMN-2 isoforms are known in the art. Thus, in some

embodiments, the present disclosure can be used to deliver an mRNA encoding an hSMN-1 isoform or an hSMN-2 protein or an isoform thereof. In some embodiments, an mRNA suitable for the disclosure may be a wild-type or naturally occurring hSMN-1 or hSMN-2 isoform sequence. In some embodiments, an mRNA suitable for the disclosure may be a codon-optimized hSMN-1 or hSMN-2 isoform sequence. In some embodiments, an mRNA suitable for the disclosure may encode an amino acid sequence having substantial homology or identify to the wild-type or naturally-occurring hSMN-1 or hSMN-2 isoform sequence (e.g., having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% sequence identity to the wild-type or naturally-occurring hSMN-1 or hSMN-2 isoform sequence).

Intrathecal delivery

[001077] In some embodiments, mRNA loaded LNPs are delivered to the CNS by injecting into the cerebrospinal fluid (CSF) of a subject in need of treatment. In some embodiments, intrathecal administration is used for injecting mRNA or mRNA loaded nanoparticles to the CSF. As used herein, intrathecal administration (also referred to as intrathecal injection) refers to an injection into the spinal canal (intrathecal space surrounding the spinal cord). Various techniques may be used including, without limitation, lateral cerebroventricular injection through a burrhole or cistemal or lumbar puncture or the like. Exemplary methods are described in Lazorthes et al. Advances in Drug Delivery Systems and Applications in Neurosurgery, 143-192 and Omaya et al., Cancer Drug Delivery, 1: 169-179, the contents of which are incorporated herein by reference.

[001078]According to the present disclosure, mRNA or mRNA loaded nanoparticles may be injected at any region surrounding the spinal canal. In some embodiments, mRNA or mRNA loaded nanoparticles are injected into the lumbar area or the cisterna magna or intraventricularly into a cerebral ventricle space. As used herein, the term "lumbar region" or "lumbar area" refers to the area between the third and fourth lumbar (lower back) vertebrae and, more inclusively, the L2-S1 region of the spine. Typically, intrathecal injection via the lumbar region or lumber area is also referred to as "lumbar IT delivery" or "lumbar IT administration." The term "cisterna magna" refers to the space around and below the cerebellum via the opening between the skull and the top of the spine. Typically, intrathecal injection via cisterna magna is also referred to as "cisterna magna delivery." The term "cerebral ventricle" refers to the cavities in the brain that are continuous with the central canal of the spinal cord. Typically, injections via the cerebral ventricle cavities are referred to as intravetricular Cerebral (ICV) delivery.

Delivery to neurons and other cell types in the brain and/or spinal cord

[001079]The disclosure provides methods and compositions for the delivery of mRNA to various neurons and other cell types in the brain and/or spinal cord. In some embodiments, mRNA encoding a therapeutic protein is delivered to various cells in the brain including, but not limited to, neurons, glial cells, perivascular cells and/or meningeal cells. In particular, the methods and compositions for delivery to

neurons results in delivery of mRNA in various neurons and other cell types affected by a CNS disease and/or deficiency, or various neurons and other cell types in which the deficient protein associated with the CNS disease is normally expressed. In some embodiments, the herein described methods result in delivery of mRNA in various neurons and other cell types in the CNS in which there is a detectable or abnormally high amount of enzyme substrate, for example stored in the cellular lysosomes of the tissue, in patients suffering from or susceptible to the lysosomal storage disease. In some embodiments, the methods disclosed herein result in delivery of mRNA in various neurons and other cell types that display disease-associated pathology, symptom, or feature. For example, mRNA may be delivered to neurons or other cell types that are deteriorating, degenerating or undergoing apoptosis such as those neurons or non-neuronal cells associated with neurodenegrative diseases (e.g., Alzheimer's disease, Parkinson's disease, and Huntington's disease) or motor neurons associated with motor neuron diseases (e.g., Amyotrophic Lateral Sclerosis (ALS), Primary Lateral Sclerosis (PLS), Pseudobulbar Pasly, Hereditary Spastic Paraplegia, Progressive Muscular Atrophy (PMA), Progressive Bulbar Palsy (PBP), Distal Hereditary Motor Neuropathies, and Spinal Muscular Atrophies).

[001080]In some embodiments, mRNA is delivered to neurons and/or non-neuronal cells located within the brain. In some embodiments, mRNA is delivered to neurons and/or non-neuronal cells located within the spinal cord. In some embodiments, mRNA is delivered to motor neurons. In some embodiments, the mRNA is delivered to upper motor neurons and/or lower motor neurons. In some embodiments, the motor neurons are located within the anterior horn and/or dorsal root ganglia of the spinal cord.

[001081]In some embodiments, mRNA is delivered intracellularly in various neurons and other cell types in the brain and/or spinal cord. In some embodiments, mRNA is delivered to the axons of neurons. In some embodiments, mRNA delivery according to the present disclosure results in intracellular expression of the protein encoded by the mRNA within cytosol of the neurons. In some embodiments, mRNA delivery according to the present disclosure results in expression of the protein encoded by the mRNA in subcellular compartment of the neurons, e.g., lysosomes, mitochondria, transmembrane, and the like. In some embodiments, mRNA delivery according to the present disclosure results in expression of the protein encoded by the mRNA and secretion extracellularly from the neurons.

Brain

[001082] In general, methods according to the present disclosure can be used to deliver mRNA and encoded protein to neurons and other cell types in various regions of the brain. Typically, brain can be divided into different regions, layers and tissues. For example, meningeal tissue is a system of membranes which envelops the central nervous system, including the brain. The meninges contain three layers, including dura mater, arachnoid mater, and pia mater. In general, the primary function of the meninges

and of the cerebrospinal fluid is to protect the central nervous system. In some embodiments, mRNA and the encoded protein is delivered to neurons or non-neuronal cells in one or more layers of the meninges.

[001083]The brain has three primary subdivisions, including the cerebrum, cerebellum, and brain stem. The cerebral hemispheres, which are situated above most other brain structures, are covered with a cortical layer. Underneath the cerebrum lies the brainstem, which resembles a stalk on which the cerebrum is attached. At the rear of the brain, beneath the cerebrum and behind the brainstem, is the cerebellum.

[001084]The diencephalon, which is located near the midline of the brain and above the mesencephalon, contains the thalamus, metathalamus, hypothalamus, epithalamus, prethalamus, and pretectum. The mesencephalon, also called the midbrain, contains the tectum, tegumentum, ventricular mesocoelia, and cerebral peduncels, the red nucleus, and the cranial nerve III nucleus. The mesencephalon is associated with vision, hearing, motor control, sleep/wake, alertness, and temperature regulation.

[001085]In some embodiments, mRNA and the encoded protein is delivered to neurons and/or non-neuronal cells of one or more tissues of the cerebellum. In certain embodiments, the targeted one or more tissues of the cerebellum are selected from the group consisting of tissues of the molecular layer, tissues of the Purkinje cell layer, tissues of the Granular cell layer, cerebellar peduncles, and combination thereof. In some embodiments, mRNA and the encoded protein is delivered to one or more deep tissues of the cerebellum including, but not limited to, tissues of the Purkinje cell layer, tissues of the Granular cell layer, deep cerebellar white matter tissue (e.g., deep relative to the Granular cell layer), and deep cerebellar nuclei tissue.

[001086]In some embodiments, mRNA and the encoded protein is delivered to one or more tissues of the brainstem.

In some embodiments, mRNA and encoded protein is delivered to various brain tissues including, but not limited to, gray matter, white matter, periventricular areas, piaarachnoid, meninges, neocortex, cerebellum, deep tissues in cerebral cortex, molecular layer, caudate/putamen region, midbrain, deep regions of the pons or medulla, and combinations thereof. In some embodiments, mRNA and encoded protein is delivered to oligodendrocytes of deep white matter.

Spinal Cord

[001087] In some embodiments, the methods according to the present disclosure can be used to deliver mRNA and encoded protein to neurons and other cell types in various regions of the spinal cord. In general, regions or tissues of the spinal cord can be characterized based on the depth of the tissues. For example, spinal cord tissues can be characterized as surface or shallow tissues, mid-depth tissues, and/or deep tissues.

[001088]In some embodiments, mRNA and the encoded protein is delivered to one or more surface or shallow tissues of the spinal cord. In some embodiments, a targeted surface or shallow tissue of the spinal cord contains pia mater and/or the tracts of white matter.

[001089]In some embodiments, mRNA and the encoded protein is delivered to one or more deep tissues of the spinal cord. In some embodiments, a targeted deep tissue of the spinal cord contains spinal cord grey matter and/or ependymal cells.

[001090]The disclosure will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the disclosure. All literature citations are incorporated by reference.

Treatment of muscle disorders

[001091]Provided herein are therapeutic methods for treating muscle diseases or disorders by using the LNP-based RNA compositions described herein to deliver one or more therapeutic agents encoded on a payload RNA (e.g., linear or circular mRNA payload) to a cell, tissue, or organ having muscle. Delivery may be *in vitro* or *ex vivo* to cells, or to a cell, tissue, or organ *in vivo*.

[001092]The mRNA payloads of the herein disclosed LNP-based RNA therapeutics may delivery one or more therapeutic proteins for treating a muscle condition, which may include the following diseases:

(A) Muscular dystrophies;

(B) Myopathies;

(C) Motor neuron diseases;

(D) Ion channel diseases;

(E) Mitochondrial diseases;

(F) Neuromuscular junction diseases; and

(G) Peripheral nerve diseases.

[001093]With regard to (A) muscular dystrophies, this category of muscular diseases can encompass the following specific disorders:

Becker muscular dystrophy (BMD); Congenital muscular dystrophies (CMD); Bethlem CMD; Fukuyama CMD; Muscle-eye-brain diseases (MEBs); Rigid spine syndromes; Ullrich CMD; Walker-Warburg syndromes (WWS); Duchenne muscular dystrophy (DMD); Emery-Dreifuss muscular dystrophy (EDMD); Facioscapulohumeral muscular dystrophy (FSHD); Limb-girdle muscular dystrophies (LGMD); Myotonic dystrophy (DM); or Oculopharyngeal muscular dystrophy (OPMD).

[001094]With regard to (B) myopathies, this category of muscular diseases can encompass the following specific disorders:

Congenital myopathies; Cap myopathies; Centronuclear myopathies; Congenital myopathies with fiber type disproportion; Core myopathies; Central core disease; Multiminicore myopathies; Myosin

storage myopathies; Myotubular myopathy; Nemaline myopathies; Distal myopathies; GNE myopathy/Nonaka myopathy/hereditary inclusion-body myopathy (HIBM); Laing distal myopathy; Markesbery-Griggs late-onset distal myopathy; Miyoshi myopathy; Udd myopathy/tibial muscular dystrophy; VCP Myopathy / IBMPFD; Vocal cord and pharyngeal distal myopathy; Welander distal myopathy; Endocrine myopathies; Hyperthyroid myopathy; Hypothyroid myopathy; Inflammatory myopathies; Dermatomyositis; Inclusion-body myositis; Polymyositis; Metabolic myopathies; Acid maltase deficiency (AMD, Pompe disease); Carnitine deficiency; Carnitine palmitoyltransferase deficiency; Debrancher enzyme deficiency (Cori disease, Forbes disease); Lactate dehydrogenase deficiency; Myoadenylate deaminase deficiency; Phosphofructokinase deficiency (Tarui disease); Phosphoglycerate kinase deficiency; Phosphoglycerate mutase deficiency; Phosphorylase deficiency (McArdle disease); Myofibrillar myopathies (MFM); or Scapuloperoneal myopathy.

[001095]With regard to (C) motor neuron diseases, this category of muscular diseases can encompass the following specific disorders:

ALS (amyotrophic lateral sclerosis);

Spinal-bulbar muscular atrophy (SBMA); or

Spinal muscular atrophy (SMA).

[001096]With regard to (D) ion channel diseases, this category of muscular diseases can encompass the following specific disorder:

Andersen-Tawil syndrome; Hyperkalemic periodic paralysis; Hypokalemic periodic paralysis; Myotonia congenita; Becker myotonia; Thomsen myotonia; Paramyotonia congenita; or Potassium-aggravated myotonia.

[001097]With regard to (E) mitochondrial diseases, this category of muscular diseases can encompass the following specific disorder:

Friedreich's ataxia (FA); Mitochondrial myopathies; Kearns-Sayre syndrome (KSS); Leigh syndrome (subacute necrotizing encephalomyopathy); Mitochondrial DNA depletion syndromes; Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS); Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE); Myoclonus epilepsy with ragged red fibers (MERRF); Neuropathy, ataxia and retinitis pigmentosa (NARP); Pearson syndrome; or Progressive external opthalmoplegia (PEO).

[001098]With regard to (F) neuromuscular junction diseases, this category of muscular diseases can encompass the following specific disorder:

Congenital myasthenic syndromes (CMS);

Lambert-Eaton myasthenic syndrome (LEMS); or

Myasthenia gravis (MG).

[001099]With regard to (G) peripheral nerve diseases, this category of muscular diseases can encompass the following specific disorder:

Charcot-Marie-Tooth disease (CMT); or

Giant axonal neuropathy (GAN).

[001100]In some embodiments, the compositions may be used to treat a range of muscle-related disorders, including, but not limited to, Duchenne muscular dystrophy, limb girdle muscle disease, and spinal muscular atrophy, as well as other muscle tissue related diseases. Exemplary muscle tissue related diseases include but are not limited to Acid Maltase Deficiency (AMD), Amyotrophic Lateral Sclerosis (ALS), Andersen-Tawil Syndrome, Becker Muscular Dystrophy (BMD), Becker Myotonia Congenita, Bethlem Myopathy, Bulbospinal Muscular Atrophy (Spinal-Bulbar Muscular Atrophy), Carnitine Deficiency, Carnitine Palmityl Transferase Deficiency (CPT Deficiency), Central Core Disease (CCD), Centronuclear Myopathy, Charcot-Marie-Tooth Disease (CMT), Congenital Muscular Dystrophy (CMD), Congenital Myasthenic Syndromes (CMS), Congenital Myotonic Dystrophy, Cori Disease (Debrancher Enzyme Deficiency), Debrancher Enzyme Deficiency, Dejerine-Sottas Disease (DSD), Dermatomyositis (DM), Distal Muscular Dystrophy (DD), Duchenne Muscular Dystrophy (DMD), Dystrophia Myotonica (Myotonic Muscular Dystrophy), Emery-Dreifuss Muscular Dystrophy (EDMD), Endocrine Myopathies, Eulenberg Disease (Paramyotonia Congenita), Facioscapulohumeral Muscular Dystrophy (FSH or FSHD), Finnish (Tibial) Distal Myopathy, Forbes Disease (Debrancher Enzyme Deficiency), Friedreich's Ataxia (FA), Fukuyama Congenital Muscular Dystrophy, Glycogenosis Type 10, Glycogenosis Type 11, Glycogenosis Type 2, Glycogenosis Type 3, Glycogenosis Type 5, Glycogenosis Type 7, Glycogenosis Type 9, Gowers-Laing Distal Myopathy, Hauptmann-Thanheuser MD (Emery-Dreifuss Muscular Dystrophy), Hereditary Inclusion-Body Myositis, Hereditary Motor and Sensory Neuropathy (Charcot-Marie-Tooth Disease), Hyperthyroid Myopathy, Hypothyroid Myopathy, Inclusion-Body Myositis (IBM), Inherited Myopathies, Integrin-Deficient Congenital Muscular Dystrophy, Kennedy Disease (Spinal-Bulbar Muscular Atrophy), Kugelberg-Welander Disease (Spinal Muscular Atrophy), Lactate Dehydrogenase Deficiency, Lambert-Eaton Myasthenic Syndrome (LEMS), Limb-Girdle Muscular Dystrophy (LGMD), Lou Gehrig's Disease (Amyotrophic Lateral Sclerosis), McArdle Disease (Phosphorylase Deficiency), Merosin-Deficient Congenital Muscular Dystrophy, Metabolic Diseases of Muscle, Mitochondrial Myopathy, Miyoshi Distal Myopathy, Motor Neurone Disease, Muscle-Eye-Brain Disease, Myasthenia Gravis (MG), Myoadenylate Deaminase Deficiency, Myofibrillar Myopathy, Myophosphorylase Deficiency, Myotonia Congenita (MC), Myotonic Muscular Dystrophy (MMD), Myotubular Myopathy (MTM or MM), Nemaline Myopathy, Nonaka Distal Myopathy, Oculopharyngeal Muscular Dystrophy (OPMD), Paramyotonia Congenita, Pearson Syndrome, Periodic Paralysis, Peroneal Muscular Atrophy (Charcot-Marie-Tooth Disease), Phosphofructokinase Deficiency, Phosphoglycerate

Kinase Deficiency, Phosphoglycerate Mutase Deficiency, Phosphorylase Deficiency, Phosphorylase Deficiency, Polymyositis (PM), Pompe Disease (Acid Maltase Deficiency), Progressive External Ophthalmoplegia (PEO), Rod Body Disease (Nemaline Myopathy), Spinal Muscular Atrophy (SMA), Spinal-Bulbar Muscular Atrophy (SBMA), Steinert Disease (Myotonic Muscular Dystrophy), Tarui Disease (Phosphofructokinase Deficiency), Thomsen Disease (Myotonia Congenita), Ullrich Congenital Muscular Dystrophy, Walker-Warburg Syndrome (Congenital Muscular Dystrophy), Welander Distal Myopathy, Werdnig-Hoffmann Disease (Spinal Muscular Atrophy), and ZASP-Related Myopathy.

[001101]The present disclosure provides, among other things, improved methods and compositions for efficient LNP delivery of mRNA payloads (e.g., circular mRNA and/or linear mRNA), encoding a therapeutic protein, to muscles. In various embodiments, the mRNA-encapsulated nanoparticles (e.g., LNPs) can be administered directly into a muscle (e.g., via intramuscular administration) and effectively penetrate muscle cell membrane, resulting in intracellular delivery of mRNA in muscle cells. Other delivery modes are also contemplated.

[001102]Further, the present disclosure provides methods for treating one more or muscle diseases or conditions by administering an effective amount of the LNP-based RNA therapeutics described herein. In various embodiments, the RNA payload of the LNP-based RNA therapeutics may encode one more therapeutic polypeptides.

[001103]For example, the LNP-based RNA therapeutics and pharmaceutical compositions thereof described herein may be used to treat muscle atrophy, which is both a disease state and an aging process condition that diminishes the quality of life. There has been a high demand for therapeutics that increase lean mass while abrogating the need for special dietary and exercise requirements that are usually unattainable in people with active muscle wasting. Therefore, in some embodiments, a nanomedicine approach capable of increasing skeletal muscle mass and restricting body fat accumulation is disclosed herein. The therapeutic modality is based on LNP-based nanoparticles that deliver mRNA payloads (e.g., linear or circular mRNA) that encode a therapeutic protein for treating muscle wasting, such as follistatin.

[001104]By "muscle cell" or "muscle tissue" is meant a cell or group of cells derived from muscle of any kind (for example, skeletal muscle and smooth muscle, e.g. from the digestive tract, urinary bladder, blood vessels or cardiac tissue). Such muscle cells may be differentiated or undifferentiated, such as myoblasts, myocytes, myotubes, cardiomyocytes and cardiomyoblasts. Since muscle tissue is readily accessible to the circulatory system, a protein produced and secreted by muscle cells and tissue in vivo will logically enter the bloodstream for systemic delivery, thereby providing sustained, therapeutic levels of protein secretion from muscle.

[001105]In some embodiments, exemplary polypeptides include neuroprotective polypeptides and anti-angiogenic polypeptides. Suitable polypeptides include, but are not limited to, glial derived

neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF-2), nurturin, ciliary neurotrophic factor (CNTF), nerve growth factor (NGF; e.g., nerve growth factor-.beta.), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), neurotrophin-6 (NT-6), epidermal growth factor (EGF), pigment epithelium derived factor (PEDF), a Wnt polypeptide, soluble Flt-1, angiostatin, endostatin, VEGF, an anti-VEGF antibody, a soluble VEGFR, Factor VIII (FVIII), Factor IX (FIX), and a member of the hedgehog family (sonic hedgehog, Indian hedgehog, and desert hedgehog, etc.).

[001106]The delivered RNA payload directs the body (e.g., hepatic cellular machinery) to produce the protein encoded in the delivered linear or circular mRNA.

[001107]The disclosure further relates to methods of treating muscle disease, and/or modulating muscle mass in a subject by administering an effective amount of the LNP-based RNA therapeutics and/or pharmaceutical compositions thereof described herein to a subject in need. In various embodiments, the RNA payloads of the LNP-based RNA therapeutics and/or pharmaceutical compositions thereof may encode one or more the therapeutic polypeptides disclosed herein.

[001108]In embodiments, the LNP-based methods include slowing the loss of, increasing, and/or maintaining lean muscle mass in a subject (e.g., a human and/or mammalian subject). In some examples, the LNP-based methods include treating a subject with a muscle-wasting disease and/or muscle atrophy. In embodiments, the LNP-based methods include administering to a subject an effective amount (e.g., a therapeutically effective amount) of any LNP-based composition disclosed herein. In embodiments, administering to the subject the mRNA polymer complex or the drug delivery system slows the loss of, increases, and/or maintains lean muscle mass in a subject and/or treats acute or chronic muscle atrophy and/or a muscle-wasting disease. The methods can include selecting a subject in need of augmented or maintained muscle growth, such as a subject with a muscle-wasting disease or muscle atrophy or a healthy subject, for example, to enhance athletic performance. Any type of subject can be selected, including human or mammalian subjects.

[001109]In some examples, the subject can have a muscle-wasting disease and/or acute or chronic muscle atrophy. The subject can have any muscle-wasting disease and/or any condition associated with acute or chronic muscle atrophy. In some examples, the subject can have sarcopenia, cachexia, cancer, congestive heart failure, renal failure, chronic obstructive pulmonary disease, severe burns, an inflammatory muscle disease, myasthenia gravis, neuropathy, polio, multiple sclerosis, anorexia nervosa, human immunodeficiency virus, acquired immune deficiency syndrome, osteomalacia, herniated disk, hypercalicemia, kwashiorkor, Creutzfeldt-Jakob disease, bovine spongiform encephalopathy, diabetes, amyotrophic lateral sclerosis, necrotizing vasculitis, abetalipoproteinemia,

malabsorption syndrome, Legg Calvé-Perthes disease, muscular dystrophy, polymyositis, Guillain-Barre syndrome, and/or osteoarthritis.

[001110]In some particular embodiments, the LNP-based compositions may comprise an RNA payload that encodes one or more polypeptides that may be delivered as a substitute for a defective endogenous muscle related protein that is associated with a particular muscle disorder. For example, the composition herein may be used to deliver a gene responsible for one of the neuromuscular diseases listed herein, preferably selected from the group comprising Duchenne muscular dystrophy and Becker muscular dystrophy (DMD gene), Limb-girdle muscular dystrophies (LGMDs) (CAPN3, DYSF, FKRP, AN05 genes and others), Spinal muscular atrophy (SMN1 , ASAH1 genes) and Amyotrophic lateral sclerosis (SOD1, ALS2, SETX, FUS, ANG, TARDBP, FIG4, OPTN and others), Myotubular myopathy (MTM1 gene), Centronuclear myopathies (MTM1, DNM2, BIN1 genes), Nemaline myopathies (ACTA1, KLHL40, KLHL41, KBTBD13 genes), Selenoprotein N-related myopathy (SEPN1 gene), Congenital myasthenia (ColQ, CHRNE , RAPSN, DOK7, MUSK genes), Pompe disease (GAA gene), Glycogen storage disease III (GSD3) (AGL gene), Myotonic dystrophy type 1 (DMPK gene) and type 2 (CNBP/ZNF9 gene); Hereditary paraplegia (SPAST) and Charcot-Marie-Tooth, Type 4B1 (MTMR2). In some more preferred embodiments, the target gene is selected from the group consisting of : DMD, CAPN3, DYSF, FKRP, AN05, MTM1, DNM2, BIN1, ACTA1, KLHL40, KLHL41, KBTBD13, TPM3, TPM2, TNNT1, CFL2, LMOD3, SEPN1, GAA, AGL , SMN1, and ASAH1 genes.

[001111]The LNP compositions described may be used to treat (i) myopathies, such as muscular dystrophies, including congenital muscular dystrophies; (ii) spinal muscular atrophies (SMAs) and motor neuron diseases; (iii) Myotonic syndrome, in particular myotonic dystrophy type 1 and type 2; (iv) Hereditary motor and sensory neuropathies; (v) Hereditary paraplegia and Hereditary ataxia; (vi) Congenital myasthenic syndromes, in particular muscular dystrophies including congenital muscular dystrophies, congenital myasthenic syndromes , and spinal muscular atrophies (SMAs) and motor neuron diseases.

[001112]In some embodiments, the LNP-based RNA payload compositions may be used to deliver a desired polynucleotide that will restore the function of a protein that is associated with a muscle disease, muscular dystrophy, including congenital muscular dystrophy affecting at least the nervous system, selected from the group consisting of: FKTN, POMT1, POMT2, POMGNT1, POMGNT2, LMNA, ISPD, GMPPB, LARGE, LAMA2, TRIM 32, and B3GALNT2.

[001113]The compositions may also used to treat dystrophinopathies, which are a spectrum of X-linked muscle diseases caused by pathogenic variants in DMD gene, which encodes the protein

dystrophin. Dystrophinopathies comprises Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD) and DMD-associated dilated cardiomyopathy.

[001114]The compositions may also be used to treat the Limb-girdle muscular dystrophies (LGMDs), which are a group of disorders that are clinically similar to DMD but occur in both sexes as a result of autosomal recessive and autosomal dominant inheritance. Limb-girdle dystrophies are caused by mutation of genes that encode sarcoglycans and other proteins associated with the muscle cell membrane, which interact with dystrophin. The term LGMD1 refers to genetic types showing dominant inheritance (autosomal dominant), whereas LGMD2 refers to types with autosomal recessive inheritance. Pathogenic variants at more than 50 loci have been reported (LGMD1A to LGMD1G; LGMD2A to LGMD2W). Calpainopathy (LGMD2A) is caused by mutation of the gene CAPN3 with more than 450 pathogenic variants described. Contributing genes to LGMD phenotype include: anoctamin 5 (. AN05), blood vessel epicardial substance (BYES), calpain 3 (CAPN3), caveolin 3 (CAV3), CDP-L-ribitol pyrophosphorylase A (CRPPA), dystroglycan 1 (DAG1), desmin (DES), DnaJ heat shock protein family (Hsp40) homolog, subfamily B, member 6 (DNAJB6), dysferlin (DYSF), fukutin related protein (FKRP), fukutin (FKT), GDP-mannose pyrophosphorylase B (GMPPB), heterogeneous nuclear ribonucleoprotein D like (HNRNPDL), LIM zinc finger domain containing 2 (LIMS2), lain A:C (LMNA), myotilin (MYOT), plectin (PLEC), protein O-glucosyltransferase 1 (PLOGLUT1), protein O-linked mannose N-acetylglucosaminyltransferase 1 (beta 1,2-) (POMGNT1), protein O-mannose kinase (POMK), protein O-mannosyltransferase 1 (POMT1), protein O-mannosyltransferase 2 (POMT2), sarcoglycan alpha (SGCA), sarcoglycan beta (SGCB), sarcoglycan delta (SGCD), sarcoglycan gamma (SGCG), titin-cap (TCAP), transportin 3 (TNP03), torsin 1A interacting protein (TOR1AIP1), trafficking protein particle complex 11 (TRAPPC11), tripartite motif containing 32 (TRIM 32) and titin (TTN). Major contributing genes to LGMD phenotype include CAPN3, DYSF, FKRP and AN 05 (Babi Ramesh Reddy Nallamilli et ah, Annals of Clinical and Translational Neurology, 2018, 5, 1574-1587.

[001115]The compositions may also be used to treat certain neurological disorders caused by Dysferlin, which is involved in neurological disorders including multiple sclerosis (Hochmeister et ah, J. Neuropathol. Exp. Neurol., 2006 Sep;65(9):855-65); Alzheimer (Galvin et ah, Acta Neuropathol., 2006 Dec;112(6):665-71 and choreic movement (Takahashi T, et al,, Mov. Disord., 2006, Sep;21(9): 1513-5).

[001116]Spinal muscular atrophy is a genetic disorder caused by mutations in the Survival Motor Neuron 1 (SMN1) gene which is characterized by weakness and wasting (atrophy) in muscles used

for movement. Mutations in ASAH1 gene lead to SMA-PME (spinal muscular atrophy with progressive myoclonic epilepsy). Compositions may be used to deliver replacement SMN1 protein.

[001117]The composition disclosed here may also be used to treat X-linked X-linked myotubular myopathy is a genetic disorder caused by mutations in the myotubularin (MTM1) gene which affects muscles used for movement (skeletal muscles) and occurs almost exclusively in males. This condition is characterized by muscle weakness (myopathy) and decreased muscle tone (hypotonia).

[001118]Pompe disease is a genetic disorder caused by mutations in the acid alpha- glucosidase (GAA) gene. Mutations in the GAA gene prevent acid alpha-glucosidase from breaking down glycogen effectively, which allows this sugar to build up to toxic levels in lysosomes. This buildup damages organs and tissues throughout the body, particularly the muscles, leading to the progressive signs and symptoms of Pompe disease.

[001119]Glycogen storage disease III (GSD3) is an autosomal recessive metabolic disorder caused by homozygous or compound heterozygous mutation in the Amylo-Alpha-1, 6-Glucosidase, 4-Alpha-Glucanotransferase (AGL) gene which encodes the glycogen debrancher enzyme and associated with an accumulation of abnormal glycogen with short outer chains. Clinically, patients with GSD III present in infancy or early childhood with hepatomegaly, hypoglycemia, and growth retardation. Muscle weakness in those with Ilia is minimal in childhood but can become more severe in adults; some patients develop cardiomyopathy. The compositions of the disclosure can be used to supplement with a healthy version of AGS.

[001120]The methods can include administering an LNP-based composition described herein in any amount (e.g., any concentration and/or dose). The administered amount (e.g., concentration and/or dose) of the LNP-based compositions described herein will depend on the subject being treated, the severity of the affliction, and the manner of administration and is best left to the judgment of the prescribing clinician. The herein described compositions can be administered at any concentration. Exemplary concentrations include at least about 10, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 ng/ml or about 10-1000, 25-500, 50-250, 100-500, 300-700, 400-800, 600-1000, 10-50, 50-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400-450, 450-500, 500-550, 600-650, 650-700, 700-750, 750-800, 800-850, 850-900, 900-950, or 950-1000 ng/ml or about 250 or 500 ng/ml. The herein described compositions can be administered at any dose. Exemplary doses include at least about 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, or 5 mg/kg or about 0.05-1, 0.1-2, 0.5-5, 1-5, 2-4, 3-5, 0.05-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5, 0.5-0.6, 0.6-0.7, 0.7-0.8, 0.8-0.9, 0.9-1, 1-2, or 2-5 mg/kg or about 0.5 mg/kg.

[001121]The methods can include administering a herein described composition for treating a muscle disease over any timeframe. The timeframe of administration will depend on the subject

being treated, the severity of the affliction, and the manner of administration and is best left to the judgment of the prescribing clinician. For example, the herein described compositions can be injected or infused over any length of time and/or the herein described compositions can be administered (e.g., by injection or infusion) in any amount of doses over any amount of time. In some examples, the herein disclosed LNP compositions are infused, such as at least over about 1 minute, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 1.5 hours, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, or 8 hours or about 1-15 minutes, 2-30 minutes, 5-60 minutes, 15-90 minutes, 30-120 minutes, 5-10 minutes, 10-15 minutes, 15-30 minutes, 30-45 minutes, 45 minutes-1 hour, 1-1.5 hours, 1.5-2 hours, 2-3 hours, 3-4 hours, 4-5 hours, 5-6 hours, 6-7 hours, or 7-8 hours. In some examples, multiple doses of the herein disclosed LNP compositions can be administered (e.g., by injection or infusion, such as by subcutaneous injection), for example, at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18, 20, 25, 30, 35, 40, 45, or 50 doses or about 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9, 9-10, 10-12, 12-15, 15-18, 18-20, 20-25, 25-30, 30-35, 35-40, 40-45, or 45-50 doses or about 1 dose, 16 doses, or 18 doses. Exemplary frequencies for administering one or more doses of the LNP compositions described herein include administration (e.g., injection or infusions, such as subcutaneous injection) at least about every day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 30 days, 60 days, or 180 days or about every 1-7 days, 3-10 days, 5-14 days, 21-42 days, 28-60 days, 1 day-2 days, 2-3 days, 3-4 days, 4-5 days, 5-6 days, 6-7 days, 7-8 days, 8-9 days, 9-10 days, 10-30 days, 30-60 days, or 60-180 days or about every 3 days. Exemplary timespans for administering herein disclosed compositions include administering the doses over at least about 1 day, 3 days, 9 days, 18 days, 30 days, 60 days, 180 days, 1 year, 2 years, 3 years, or 4 years or about 1 day-3 days, 3-9 days, 9-18 days, 18-30 days, 30-60 days, 60-180 days, 180 days-1 year, 1-2 years, 2-3 years, or 3-4 years or about 1 days or 60 days. In some examples, the mRNA polymer complex or drug delivery system can be administered (e.g., by subcutaneous injection, such as every 3 days) indefinitely, such as to a subject with chronic muscle atrophy and/or a chronic muscle-wasting disease.

[001122]In embodiments, the methods include assessing biocompatibility (e.g., efficacy, therapeutic response, toxicity, inflammation, side effects, and/or elimination), such as before, with, and/or after administration of a composition disclosed herein. In some examples, efficacy and/or therapeutic response is assessed. Assays for efficacy and/or therapeutic response are known in the art. Any efficacy and/or therapeutic response assay can be used. Exemplary efficacy and/or therapeutic response assays include measuring levels of molecules involved in forming muscle mass, such as follistatin, myostatin, and/or activin A levels. Levels of molecules involved in forming muscle mass can be measured in any fluid or tissue, such as in a urine or blood sample (e.g., whole blood, serum,

and/or plasma). Any method of measuring such molecules can be used (e.g., immunohistochemistry, IHC). In some examples, efficacy and/or therapeutic response is assessed by measuring physiological changes, such as changes in body fat and/or muscle mass. Any method of measuring physiological changes can be used (e.g., Correa-de-Araujo, Front Physiol., 8:87, 2017, incorporated herein by reference). In some examples, measurements in an efficacy and/or therapeutic response assay can be compared to measurements and/or expected measurements in a control subject (e.g., a subject that was not administered a herein disclosed LNP composition).

[001123]In some examples, the methods herein include delivery a composition described herein to a muscle in order to increase in the levels (e.g., blood levels, such as blood serum levels) of a therapeutic protein (e.g., follistatin - an endogenous glycoprotein that promotes growth and repair of skeletal muscle by sequestering inhibitory ligands of the transforming growth factor-β superfamily and may therefore have therapeutic potential for neuromuscular diseases) of a subject by at least about 0.5-, 1-, 1.5-, 2-, 2.5-, 3-, 3.5-, 4-, 4.5-, 5-, 5.5-, 6-, 6.5-, 7-, 7.5-, or 8-fold or about 0.5- to 1-fold, 1- to 1.5-fold, 1.5- to 2-fold, 2- to 2.5-fold, 2.5- to 3-fold, 3- to 3.5-fold, 3.5- to 4-fold, 4- to 4.5-fold, 4.5- to 5-fold, 5- to 5.5-fold, 5.5- to 6-fold, 6- to 6.5-fold, 6.5- to 7-fold, 7- to 7.5-fold, or 7.5- to 8-fold or about 1.8-, 2.1-, or 2.4-fold (e.g., after administration of a composition described herein, such as at least about 2, 4, 8, 12, 24, 48, 72, 96, or 120 hours or about 2-4, 4-8, 8-12, 12-24, 24-48, 48-72, 72-96, or 96-120 hours or about 8 or 24 hours after administration). In some examples, the methods herein include increasing the blood levels (e.g., blood serum levels) of fola therapeutic protein (e.g. follistatin) for at least about 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days or about 1 hour-2 hours, 2-4 hours, 4-8 hours, 8-12 hours, 12 hours-1 day, 1-2 days, 2-3 days, 3-4 days, 4-5 days, 5-6 days, or 6-7 days or at least about 3 days after administration of an effective dose of an LNP composition described herein.

[001124]In some examples, the methods herein include decreasing in the blood levels (e.g., blood serum levels) a muscle protein (e.g., myostatin and/or activin A) by at least about 0.5-, 1-, 1.5-, 2-, 2.5-, 3-, 3.5-, 4-, 4.5-, 5-, 5.5-, 6-, 6.5-, 7-, 7.5-, or 8-fold or about 0.5- to 1-fold, 1- to 1.5-fold, 1.5- to 2-fold, 2- to 2.5-fold, 2.5- to 3-fold, 3- to 3.5-fold, 3.5- to 4-fold, 4- to 4.5-fold, 4.5- to 5-fold, 5- to 5.5-fold, 5.5- to 6-fold, 6- to 6.5-fold, 6.5- to 7-fold, 7- to 7.5-fold, or 7.5- to 8-fold or about 1.8-fold (e.g., after administration of a composition described herein an mRNA polymer complex or a drug delivery system disclosed herein, such as at least about 2, 4, 8, 12, 24, 48, 72, 96, or 120 hours or about 2-4, 4-8, 8-12, 12-24, 24-48, 48-72, 72-96, or 96-120 hours after administration of a disclosed mRNA polymer complex or drug delivery system, for example, about 0.5 mg/kg thereof).

[001125]In some examples, the methods herein include inducing physiological changes in a muscle, such as changes in body fat and/or muscle mass, in a subject, following the administration of an effective amount of an LNP composition described herein.

[001126]In some examples, side effects and/or toxicity is assessed. Side effect and toxicity assays are known in the art (see, e.g., Mendell et al., Mol Ther., 23(1):192-201, 2015; Haidet et al., Proc Natl Acad Sci USA, 105(11):4318-22, 2008; Rodina-Klapac et al., Muscle Nerve., 39(3):283-96, 2009). Any assays for side effects and toxicity can be used. Exemplary toxicity assays include measuring molecules associated with toxicity (e.g., toxicity-associated biomarkers), such as molecules associated with liver, kidney, muscle, heart, and/or major organ toxicity, in a subject and comparing the measured values to those expected from a control subject without toxicity.

[001127]In some examples, toxicity assays can include liver toxicity assays, such as measuring alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transferase (AST), gamma-glutamyl transferase (GGT), and/or bilirubin levels (see, e.g., Giannini et al., CMAJ, 172(3): 367-379, 2005, incorporated herein by reference). Levels of ALP, ALT, AST, GGT, and bilirubin typically increase under conditions of liver toxicity. In some examples, the methods include administration of an mRNA polymer complex or a drug delivery system disclosed herein without an increase (such as without a statistically significant increase) in ALP, ALT, AST, GGT, and/or bilirubin (e.g., the disclosed mRNA polymer complex or drug delivery system is non-toxic). In other examples, an increase in ALP, ALT, AST, GGT, and/or bilirubin is within clinically acceptable limits.

[001128]In some examples, toxicity assays can include kidney toxicity assays, such as measuring blood urea nitrogen (BUN), creatinine levels, urinary proteins with enzymatic activity, proteinuria, kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), cytokines, clusterin, osteopontin, and/or type IV collagen (see, e.g., Kim and Moon, Biomol Ther (Seoul), 20(3): 268-272, 2012, incorporated herein by reference). Levels of BUN, creatinine, urinary proteins with enzymatic activity, proteinuria, KIM-1, NGAL, cytokines, clusterin, oseopontin, and/or type IV collagen typically increase under conditions of kidney toxicity. In some examples, the methods include administration of an mRNA polymer complex or a drug delivery system disclosed herein without an increase (such as without a statistically significant increase) in BUN, creatinine, urinary proteins with enzymatic activity, proteinuria, KIM-1, NGAL, cytokines, clusterin, oseopontin, and/or type IV collagen (e.g., the disclosed mRNA polymer complex or drug delivery system is non-toxic). In other examples, an increase in BUN, creatinine, urinary proteins with enzymatic activity, proteinuria, KIM-1, NGAL, cytokines, clusterin, oseopontin, and/or type IV collagen is within clinically acceptable limits.

[001129]In some examples, toxicity assays can include muscle and/or heart toxicity assays, such as measuring creatine kinase (CK), AST, skeletal troponin I (Tnni1, Tnni2), skeletal troponin T (Tnnt1, Tnnt3), creatinine kinase protein M, parvalbumin (Pvalb), myosin light chain 3 (My13), fatty acid-binding protein 3 (Fabp3), aldolase A (Aldoa), and/or myoglobin, including myoglobinuria (see, e.g., Campion et al., Expert Opin Drug Metab Toxicol, 9(11), doi:10.1517/17425255.2013.827170, 2013, incorporated herein by reference). Levels of CK, AST, Tnni1 (or Tnni2), Tnnt1 (or Tnnt3), creatinine kinase protein M, Pvalb, My13, Fabp3, Aldoa, and/or myoglobin typically increase under conditions of muscle or heart toxicity. In some examples, the methods include administration of an mRNA polymer complex or a drug delivery system disclosed herein without an increase (such as without a statistically significant increase) in CK, AST, Tnni1 (or Tnni2), Tnnt1 (or Tnnt3), creatinine kinase protein M, Pvalb, My13, Fabp3, Aldoa, and/or myoglobin (e.g., the disclosed mRNA polymer complex or drug delivery system is non-toxic). In other examples, an increase in CK, AST, Tnni1 (or Tnni2), Tnnt1 (or Tnnt3), creatinine kinase protein M, Pvalb, My13, Fabp3, Aldoa, and/or myoglobin is within clinically acceptable limits.

[001130]In some examples, toxicity assays can include major organ toxicity assays, such as measuring blood protein and/or electrolyte levels (e.g., in whole blood, serum, and/or plasma). In general, changes in electrolyte levels and increases in protein levels are associated with major organ toxicity. In some examples, the methods include administration of an mRNA polymer complex or a drug delivery system disclosed herein without a change in electrolyte levels and/or an increase in protein levels (such as without a statistically significant increase) (e.g., the disclosed mRNA polymer complex or drug delivery system is non-toxic). Other indicia of toxicity (e.g., toxicity-associated biomarkers) are possible (see, e.g., Campion et al., Expert Opin Drug Metab Toxicol, 9(11), doi:10.1517/17425255.2013.827170, 2013; Anadón et al., Chapter 34. Biomarkers of drug toxicity. Biomarkers in Toxicology, 593-607, 2014, both of which are incorporated herein by reference). In other examples, changes in electrolyte levels and/or protein levels is within clinically acceptable limits.

[001131]In some examples, inflammation is assessed. Inflammation assays are known in the art. Any inflammation assay can be used. Exemplary inflammation assays include measuring molecules associated with inflammation (e.g., inflammation-associated molecules), such as expression of inflammatory genes, in a subject and comparing the measured values to those expected from a control subject without inflammation. In some examples, inflammation assays include measuring expression of tumor necrosis factor (TNF), interleukin (IL)-6, IL-1b, C-reactive protein (CRP), and/or adenomatous polyposis coli (APC) genes. In some examples, the methods include administration of an mRNA polymer complex or a drug delivery system disclosed herein without a

change (such as without a statistically significant change) in TNF, IL-6, IL-1b, CRP, and/or APC gene expression (e.g., the disclosed mRNA polymer complex or drug delivery system is non-inflammatory). Expression levels of other inflammatory genes can also be assessed (see, e.g., Cooper et al., Genome Biol., 6(1): R5, 2005; Newton and Dixit, Cold Spring Harb Perspect Biol, 4(3):pii: a006049, 2012, both of which are incorporated herein by reference).

C. Vaccination methods using LNPs described herein

[001132] The compositions described herein may also be used as immunogenic compositions and/or vaccines. For example, an LNP composition described here is capable of delivering an RNA payload that encodes one or more vaccine antigens and/or immunogenic polypeptides to a subject which generate an antigen-specific immune response.

[001133] In some embodiments, the antigen-specific immune response is characterized by measuring an anti-viral antigenic polypeptide antibody titer produced in a subject administered an influenza RNA (e.g., mRNA) vaccine as provided herein. An antibody titer is a measurement of the amount of antibodies within a subject, for example, antibodies that are specific to a particular antigen (e.g., an anti-influenza antigenic polypeptide) or epitope of an antigen. Antibody titer is typically expressed as the inverse of the greatest dilution that provides a positive result. Enzyme-linked immunosorbent assay (ELISA) is a common assay for determining antibody titers, for example.

[001134] In some embodiments, an antibody titer is used to assess whether a subject has had an infection or to determine whether immunizations are required. In other embodiments an antibody titer is used to assess the effectiveness of a vaccine after administering same to a subject. In some embodiments, an antibody titer is used to determine the strength of an autoimmune response, to determine whether a booster immunization is needed, to determine whether a previous vaccine was effective, and to identify any recent or prior infections. In accordance with the present disclosure, an antibody titer may be used to determine the strength of an immune response induced in a subject by an LNP composition described herein.

[001135] In some embodiments, when using the LNP compositions described herein, the anti-antigen antibody titer produced in a subject is increased by at least 1 log relative to a control. For example, anti-antigen antibody titer produced in a subject after having been administered an LNP composition described herein may be increased by at least 1.5, at least 2, at least 2.5, or at least 3 log relative to a control. In some embodiments, the anti-antigen antibody titer produced in the subject is increased by 1, 1.5, 2, 2.5 or 3 log relative to a control. In some embodiments, the anti-antigen antibody titer produced in the subject is increased by 1-3 log relative to a control. For example, the anti-antigen antibody titer produced in a subject may be increased by 1-1.5, 1-2, 1-2.5, 1-3, 1.5-2, 1.5-2.5, 1.5-3, 2-2.5, 2-3, or 2.5-3 log relative to a control.

[001136]In some embodiments, an effective amount of an LNP-based RNA (e.g., mRNA) vaccine disclosed herein is a dose that is reduced compared to the standard of care dose of a recombinant protein vaccine. A "standard of care," as provided herein, refers to a medical or psychological treatment guideline and can be general or specific. "Standard of care" specifies appropriate treatment based on scientific evidence and collaboration between medical professionals involved in the treatment of a given condition. It is the diagnostic and treatment process that a physician/clinician should follow for a certain type of patient, illness or clinical circumstance. A "standard of care dose," as provided herein, refers to the dose of a recombinant or purified virus protein vaccine, or a live attenuated or inactivated virus vaccine, that a physician/clinician or other medical professional would administer to a subject to treat or prevent a virus infection, while following the standard of care guideline for treating or preventing influenza, or an influenza-related condition.

[001137]In some embodiments, an effective amount of an LNP-based RNA vaccine composition described herein is a dose equivalent to an at least 2-fold reduction in a standard of care dose of a recombinant or purified influenza protein vaccine. For example, an effective amount of an LNP-based RNA vaccine composition described herein may be a dose equivalent to an at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, or at least 10-fold reduction in a standard of care dose of a recombinant or purified influenza protein vaccine. In some embodiments, an effective amount of an LNP-based RNA vaccine composition described herein is a dose equivalent to an at least at least 100-fold, at least 500-fold, or at least 1000-fold reduction in a standard of care dose of a recombinant or purified influenza protein vaccine. In some embodiments, an effective amount of an LNP-based RNA vaccine composition described herein is a dose equivalent to a 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 20-, 50-, 100-, 250-, 500-, or 1000-fold reduction in a standard of care dose of a recombinant or purified influenza protein vaccine. In some embodiments, an effective amount of an LNP-based RNA vaccine composition described herein is a dose equivalent to a 2-fold to 1000-fold (e.g., 2-fold to 100-fold, 10-fold to 1000-fold) reduction in the standard of care dose of a recombinant or purified influenza protein vaccine.

[001138]In some embodiments, the effective amount of an LNP-based RNA vaccine composition described herein is a dose equivalent to a 2 to 1000-, 2 to 900-, 2 to 800-, 2 to 700-, 2 to 600-, 2 to 500-, 2 to 400-, 2 to 300-, 2 to 200-, 2 to 100-, 2 to 90-, 2 to 80-, 2 to 70-, 2 to 60-, 2 to 50-, 2 to 40-, 2 to 30-, 2 to 20-, 2 to 10-, 2 to 9-, 2 to 8-, 2 to 7-, 2 to 6-, 2 to 5-, 2 to 4-, 2 to 3-, 3 to 1000-, 3 to 900-, 3 to 800-, 3 to 700-, 3 to 600-, 3 to 500-, 3 to 400-, 3 to 3 to 00-, 3 to 200-, 3 to 100-, 3 to 90-, 3 to 80-, 3 to 70-, 3 to 60-, 3 to 50-, 3 to 40-, 3 to 30-, 3 to 20-, 3 to 10-, 3 to 9-, 3 to 8-, 3 to 7-, 3 to 6-, 3 to 5-, 3 to 4-, 4 to 1000-, 4 to 900-, 4 to 800-, 4 to 700-, 4 to 600-, 4 to 500-, 4 to 400-, 4 to 4 to 00-, 4 to 200-, 4 to 100-, 4 to 90-, 4 to 80-, 4 to 70-, 4 to 60-, 4 to 50-, 4 to 40-, 4 to 30-, 4 to 20-, 4 to 10-, 4 to 9-, 4 to 8-, 4 to 7-, 4 to 6-, 4 to

5-, 4 to 4-, 5 to 1000-, 5 to 900-, 5 to 800-, 5 to 700-, 5 to 600-, 5 to 500-, 5 to 400-, 5 to 300-, 5 to 200-, 5 to 100-, 5 to 90-, 5 to 80-, 5 to 70-, 5 to 60-, 5 to 50-, 5 to 40-, 5 to 30-, 5 to 20-, 5 to 10-, 5 to 9-, 5 to 8-, 5 to 7-, 5 to 6-, 6 to 1000-, 6 to 900-, 6 to 800-, 6 to 700-, 6 to 600-, 6 to 500-, 6 to 400-, 6 to 300-, 6 to 200-, 6 to 100-, 6 to 90-, 6 to 80-, 6 to 70-, 6 to 60-, 6 to 50-, 6 to 40-, 6 to 30-, 6 to 20-, 6 to 10-, 6 to 9-, 6 to 8-, 6 to 7-, 7 to 1000-, 7 to 900-, 7 to 800-, 7 to 700-, 7 to 600-, 7 to 500-, 7 to 400-, 7 to 300-, 7 to 200-, 7 to 100-, 7 to 90-, 7 to 80-, 7 to 70-, 7 to 60-, 7 to 50-, 7 to 40-, 7 to 30-, 7 to 20-, 7 to 10-, 7 to 9-, 7 to 8-, 8 to 1000-, 8 to 900-, 8 to 800-, 8 to 700-, 8 to 600-, 8 to 500-, 8 to 400-, 8 to 300-, 8 to 200-, 8 to 100-, 8 to 90-, 8 to 80-, 8 to 70-, 8 to 60-, 8 to 50-, 8 to 40-, 8 to 30-, 8 to 20-, 8 to 10-, 8 to 9-, 9 to 1000-, 9 to 900-, 9 to 800-, 9 to 700-, 9 to 600-, 9 to 500-, 9 to 400-, 9 to 300-, 9 to 200-, 9 to 100-, 9 to 90-, 9 to 80-, 9 to 70-, 9 to 60-, 9 to 50-, 9 to 40-, 9 to 30-, 9 to 20-, 9 to 10-, 10 to 1000-, 10 to 900-, 10 to 800-, 10 to 700-, 10 to 600-, 10 to 500-, 10 to 400-, 10 to 300-, 10 to 200-, 10 to 100-, 10 to 90-, 10 to 80-, 10 to 70-, 10 to 60-, 10 to 50-, 10 to 40-, 10 to 30-, 10 to 20-, 20 to 1000-, 20 to 900-, 20 to 800-, 20 to 700-, 20 to 600-, 20 to 500-, 20 to 400-, 20 to 300-, 20 to 200-, 20 to 100-, 20 to 90-, 20 to 80-, 20 to 70-, 20 to 60-, 20 to 50-, 20 to 40-, 20 to 30-, 30 to 1000-, 30 to 900-, 30 to 800-, 30 to 700-, 30 to 600-, 30 to 500-, 30 to 400-, 30 to 300-, 30 to 200-, 30 to 100-, 30 to 90-, 30 to 80-, 30 to 70-, 30 to 60-, 30 to 50-, 30 to 40-, 40 to 1000-, 40 to 900-, 40 to 800-, 40 to 700-, 40 to 600-, 40 to 500-, 40 to 400-, 40 to 300-, 40 to 200-, 40 to 100-, 40 to 90-, 40 to 80-, 40 to 70-, 40 to 60-, 40 to 50-, 50 to 1000-, 50 to 900-, 50 to 800-, 50 to 700-, 50 to 600-, 50 to 500-, 50 to 400-, 50 to 300-, 50 to 200-, 50 to 100-, 50 to 90-, 50 to 80-, 50 to 70-, 50 to 60-, 60 to 1000-, 60 to 900-, 60 to 800-, 60 to 700-, 60 to 600-, 60 to 500-, 60 to 400-, 60 to 300-, 60 to 200-, 60 to 100-, 60 to 90-, 60 to 80-, 60 to 70-, 70 to 1000-, 70 to 900-, 70 to 800-, 70 to 700-, 70 to 600-, 70 to 500-, 70 to 400-, 70 to 300-, 70 to 200-, 70 to 100-, 70 to 90-, 70 to 80-, 80 to 1000-, 80 to 900-, 80 to 800-, 80 to 700-, 80 to 600-, 80 to 500-, 80 to 400-, 80 to 300-, 80 to 200-, 80 to 100-, 80 to 90-, 90 to 1000-, 90 to 900-, 90 to 800-, 90 to 700-, 90 to 600-, 90 to 500-, 90 to 400-, 90 to 300-, 90 to 200-, 90 to 100-, 100 to 1000-, 100 to 900-, 100 to 800-, 100 to 700-, 100 to 600-, 100 to 500-, 100 to 400-, 100 to 300-, 100 to 200-, 200 to 1000-, 200 to 900-, 200 to 800-, 200 to 700-, 200 to 600-, 200 to 500-, 200 to 400-, 200 to 300-, 300 to 1000-, 300 to 900-, 300 to 800-, 300 to 700-, 300 to 600-, 300 to 500-, 300 to 400-, 400 to 1000-, 400 to 900-, 400 to 800-, 400 to 700-, 400 to 600-, 400 to 500-, 500 to 1000-, 500 to 900-, 500 to 800-, 500 to 700-, 500 to 600-, 600 to 1000-, 600 to 900-, 600 to 800-, 600 to 700-, 700 to 1000-, 700 to 900-, 700 to 800-, 800 to 1000-, 800 to 900-, or 900 to 1000-fold reduction in the standard of care dose of a recombinant protein vaccine.

[001139]In some embodiments, the effective amount is a dose equivalent to (or equivalent to an at least) 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 20-, 30-, 40-, 50-, 60-, 70-, 80-, 90-, 100-, 110-, 120-, 130-, 140-, 150-, 160-, 170-, 1280-, 190-, 200-, 210-, 220-, 230-, 240-, 250-, 260-, 270-, 280-, 290-, 300-, 310-, 320-, 330-, 340-, 350-, 360-, 370-, 380-, 390-, 400-, 410-, 420-, 430-, 440-, 450-, 4360-, 470-, 480-, 490-, 500-, 510-,

520-, 530-, 540-, 550-, 560-, 5760-, 580-, 590-, 600-, 610-, 620-, 630-, 640-, 650-, 660-, 670-, 680-, 690-, 700-, 710-, 720-, 730-, 740-, 750-, 760-, 770-, 780-, 790-, 800-, 810-, 820-, 830-, 840-, 850-, 860-, 870-, 880-, 890-, 900-, 910-, 920-, 930-, 940-, 950-, 960-, 970-, 980-, 990-, or 1000-fold reduction in the standard of care dose of a recombinant influenza protein vaccine

[001140]In some embodiments, the effective amount of an LNP-based RNA vaccine composition described herein is a total dose of 50-1000 μg. In some embodiments, the effective amount of an i LNP-based RNA vaccine composition described herein is a total dose of 50-1000, 50-900, 50-800, 50-700, 50-600, 50-500, 50-400, 50-300, 50-200, 50-100, 50-90, 50-80, 50-70, 50-60, 60-1000, 60-900, 60-800, 60-700, 60-600, 60-500, 60-400, 60-300, 60-200, 60-100, 60-90, 60-80, 60-70, 70-1000, 70-900, 70-800, 70-700, 70-600, 70-500, 70-400, 70-300, 70-200, 70-100, 70-90, 70-80, 80-1000, 80-900, 80-800, 80-700, 80-600, 80-500, 80-400, 80-300, 80-200, 80-100, 80-90, 90-1000, 90-900, 90-800, 90-700, 90-600, 90-500, 90-400, 90-300, 90-200, 90-100, 100-1000, 100-900, 100-800, 100-700, 100-600, 100-500, 100-400, 100-300, 100-200, 200-1000, 200-900, 200-800, 200-700, 200-600, 200-500, 200-400, 200-300, 300-1000, 300-900, 300-800, 300-700, 300-600, 300-500, 300-400, 400-1000, 400-900, 400-800, 400-700, 400-600, 400-500, 500-1000, 500-900, 500-800, 500-700, 500-600, 600-1000, 600-900, 600-900, 600-700, 700-1000, 700-900, 700-800, 800-1000, 800-900, or 900-1000 μg. In some embodiments, the effective amount of an LNP-based RNA vaccine composition described herein is a total dose of 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000 μg. In some embodiments, the effective amount is a dose of 25-500 μg administered to the subject a total of two times. In some embodiments, the effective amount of an LNP-based RNA vaccine composition described herein is a dose of 25-500, 25-400, 25-300, 25-200, 25-100, 25-50, 50-500, 50-400, 50-300, 50-200, 50-100, 100-500, 100-400, 100-300, 100-200, 150-500, 150-400, 150-300, 150-200, 200-500, 200-400, 200-300, 250-500, 250-400, 250-300, 300-500, 300-400, 350-500, 350-400, 400-500 or 450-500 μg administered to the subject a total of two times. In some embodiments, the effective amount of an LNP-based RNA vaccine composition described herein is a total dose of 25, 50, 100, 150, 200, 250, 300, 350, 400, 450, or 500 μg administered to the subject a total of two times.

[001141]In some embodiments, the antigen specific immune response induced by the LNP-based RNA vaccine composition described herein in a subject is the production of antibodies specific to an expressed antigen. In some embodiments, such antibodies are capable of neutralizing the corresponding virus of the antigen in an infected host. In some embodiments, the antigen specific immune response induced by the LNP-based RNA vaccine composition described herein in a subject is antigen-specific T-cell response. Such T-cell response may provide immunity to the immunized animal (e.g., mice or human) against future infections by the virus of the expressed antigen.

[001142]The LNP-based vaccines disclosed herein may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited, to intradermal, intramuscular, and/or subcutaneous administration. The present disclosure provides methods comprising administering RNA vaccines to a subject in need thereof. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like. influenza RNA vaccines compositions are typically formulated in dosage unit form for ease of administration and uniformity of dosage.

[001143]It will be understood, however, that the total daily usage of influenza RNA vaccines compositions may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective, prophylactically effective, or appropriate imaging dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[001144]In some embodiments, the LNP-based vaccines disclosed compositions may be administered at dosage levels sufficient to deliver 0.0001 mg/kg to 100 mg/kg, 0.001 mg/kg to 0.05 mg/kg, 0.005 mg/kg to 0.05 mg/kg, 0.001 mg/kg to 0.005 mg/kg, 0.05 mg/kg to 0.5 mg/kg, 0.01 mg/kg to 50 mg/kg, 0.1 mg/kg to 40 mg/kg, 0.5 mg/kg to 30 mg/kg, 0.01 mg/kg to 10 mg/kg, 0.1 mg/kg to 10 mg/kg, or 1 mg/kg to 25 mg/kg, of subject body weight per day, one or more times a day, per week, per month, etc. to obtain the desired therapeutic, diagnostic, prophylactic, or imaging effect (see e.g., the range of unit doses described in International Publication No WO2013078199, herein incorporated by reference in its entirety). The desired dosage may be delivered three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, every four weeks, every 2 months, every three months, every 6 months, etc. In certain embodiments, the desired dosage may be delivered using multiple administrations (e.g., two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations). When multiple administrations are employed, split dosing regimens such as those described herein may be used. In exemplary embodiments, influenza RNA vaccines compositions may be administered at dosage levels sufficient to deliver 0.0005 mg/kg to 0.01 mg/kg, e.g., about 0.0005 mg/kg to about 0.0075 mg/kg, e.g., about 0.0005 mg/kg, about 0.001 mg/kg, about 0.002 mg/kg, about 0.003 mg/kg, about 0.004 mg/kg or about 0.005 mg/kg.

[001145]In some embodiments, the LNP-based vaccine compositions may be administered once or twice (or more) at dosage levels sufficient to deliver 0.025 mg/kg to 0.250 mg/kg, 0.025 mg/kg to 0.500 mg/kg, 0.025 mg/kg to 0.750 mg/kg, or 0.025 mg/kg to 1.0 mg/kg.

[001146]In some embodiments, the LNP-based vaccine compositions may be administered twice (e.g., Day 0 and Day 7, Day 0 and Day 14, Day 0 and Day 21, Day 0 and Day 28, Day 0 and Day 60, Day 0 and Day 90, Day 0 and Day 120, Day 0 and Day 150, Day 0 and Day 180, Day 0 and 3 months later, Day 0 and 6 months later, Day 0 and 9 months later, Day 0 and 12 months later, Day 0 and 18 months later, Day 0 and 2 years later, Day 0 and 5 years later, or Day 0 and 10 years later) at a total dose of or at dosage levels sufficient to deliver a total dose of 0.0100 mg, 0.025 mg, 0.050 mg, 0.075 mg, 0.100 mg, 0.125 mg, 0.150 mg, 0.175 mg, 0.200 mg, 0.225 mg, 0.250 mg, 0.275 mg, 0.300 mg, 0.325 mg, 0.350 mg, 0.375 mg, 0.400 mg, 0.425 mg, 0.450 mg, 0.475 mg, 0.500 mg, 0.525 mg, 0.550 mg, 0.575 mg, 0.600 mg, 0.625 mg, 0.650 mg, 0.675 mg, 0.700 mg, 0.725 mg, 0.750 mg, 0.775 mg, 0.800 mg, 0.825 mg, 0.850 mg, 0.875 mg, 0.900 mg, 0.925 mg, 0.950 mg, 0.975 mg, or 1.0 mg. Higher and lower dosages and frequency of administration are encompassed by the present disclosure. For example, an influenza RNA vaccine composition may be administered three or four times.

[001147]In some embodiments, the LNP-based vaccine compositions may be administered twice (e.g., Day 0 and Day 7, Day 0 and Day 14, Day 0 and Day 21, Day 0 and Day 28, Day 0 and Day 60, Day 0 and Day 90, Day 0 and Day 120, Day 0 and Day 150, Day 0 and Day 180, Day 0 and 3 months later, Day 0 and 6 months later, Day 0 and 9 months later, Day 0 and 12 months later, Day 0 and 18 months later, Day 0 and 2 years later, Day 0 and 5 years later, or Day 0 and 10 years later) at a total dose of or at dosage levels sufficient to deliver a total dose of 0.010 mg, 0.025 mg, 0.100 mg or 0.400 mg.

[001148]In some embodiments, the LNP-based vaccines for use in a method of vaccinating a subject is administered to the subject in a single dosage of between 10 μg/kg and 400 μg/kg of the nucleic acid vaccine in an effective amount to vaccinate the subject. In some embodiments the RNA vaccine for use in a method of vaccinating a subject is administered to the subject in a single dosage of between 10 μg and 400 μg of the nucleic acid vaccine in an effective amount to vaccinate the subject. In some embodiments, an influenza RNA (e.g., mRNA) vaccine for use in a method of vaccinating a subject is administered to the subject in a single dosage of 10 μg. In some embodiments, an influenza RNA vaccine for use in a method of vaccinating a subject is administered to the subject in a single dosage of 2 μg. In some embodiments, an influenza RNA vaccine for use in a method of vaccinating a subject is administered to the subject in two dosages of 10 μg. In some embodiments, an influenza RNA vaccine for use in a method of vaccinating a subject is administered the subject two dosages of 2 μg.

[001149]An the LNP-based vaccine pharmaceutical compositions described herein can be formulated into a dosage form described herein, such as an intranasal, intratracheal, or injectable (e.g., intravenous, intraocular, intravitreal, intramuscular, intradermal, intracardiac, intraperitoneal, and subcutaneous).

[001150]The LNP delivery systems described herein may be utilized in various settings depending on the prevalence of the infection or the degree or level of unmet medical need. As a non-limiting example, the LNP delivery systems disclosed herein may be utilized to treat and/or prevent influenza infection, i.e. diseases and conditions related to influenza virus infection (seasonal and pandemic).

[001151]The LNP delivery systems described herein may be utilized in various settings depending on the prevalence of the infection or the degree or level of unmet medical need. As a non-limiting example, the LNP delivery systems disclosed herein may be utilized to treat and/or prevent coronavirus infection, i.e. diseases and conditions related to coronavirus virus infection (seasonal and pandemic).

[001152]The LNP delivery systems described herein may be utilized in various settings depending on the prevalence of the infection or the degree or level of unmet medical need. As a non-limiting example, the LNP delivery systems disclosed herein may be utilized to treat and/or prevent SARS-CoV-2 infection, i.e. diseases and conditions related to SARS-CoV-2 virus infection (seasonal and pandemic), e.g., Covid-19.

D.Treating/preventing infections and/or their associated conditions

[001153]In other embodiments, the LNP-based RNA compositions described herein may be used to protect, treat, or cure infections arising from contact with an infectious agent, which can include bacteria, viruses, fungi, protozoa, and parasites.

[001154]In one embodiment, provided are the LNP-based RNA compositions described herein and their use for treating or preventing a microbial infection (e.g., a bacterial infection) and/or a disease, disorder, or condition associated with a microbial or viral infection, or a symptom thereof, in a subject, by administering an LNP-based RNA composition described herein one or more polynucleotide encoding an anti-microbial polypeptide. The administration may be in combination with an anti-microbial agent (e.g., an anti-bacterial agent), e.g., an anti-microbial polypeptide or a small molecule anti-microbial compound described herein. The anti-microbial agents include, but are not limited to, anti-bacterial agents, anti-viral agents, anti-fungal agents, anti-protozoal agents, anti-parasitic agents, and anti-prion agents.

Treating conditions associated with bacterial infections

[001155]Diseases, disorders, or conditions which may be associated with bacterial infections which may be treated using the LNP-based RNA compositions described herein include, but are not limited to one or more of the following: abscesses, actinomycosis, acute prostatitis, Aeromonas hydrophila, annual ryegrass toxicity, anthrax, bacillary peliosis, bacteremia, bacterial gastroenteritis, bacterial meningitis, bacterial pneumonia, bacterial vaginosis, bacterium-related cutaneous conditions, bartonellosis, BCG-oma, botryomycosis, botulism, Brazilian purpuric fever, Brodie abscess, brucellosis, Buruli ulcer,

campylobacteriosis, caries, Carrion's disease, cat scratch disease, cellulitis, chlamydia infection, cholera, chronic bacterial pro statitis, chronic recurrent multifocal osteomyelitis, clostridial necrotizing enteritis, combined periodontic-endodontic lesions, contagious bovine pleuropneumonia, diphtheria, diphtheritic stomatitis, ehrlichiosis, erysipelas, piglottitis, erysipelas, Fitz-Hugh-urtis syndrome, flea-borne spotted fever, foot rot (infectious pododermatitis), Garre's sclerosing osteomyelitis, Gonorrhea, Granuloma inguinale, human granulocytic anaplasmosis, human monocytotropic ehrlichiosis, hundred days' cough, impetigo, late congenital syphilitic oculopathy, legionellosis, Lemierre's syndrome, leprosy (Hansen's Disease), leptospirosis, listeriosis, Lyme disease, lymphadenitis, melioidosis, meningococcal disease, meningococcal septicaemia, methicillin-resistant Staphylococcus aureus (MRSA) infection, Mycobacterium avium-intracellulare (MAI), mycoplasma pneumonia, necrotizing fasciitis, nocardiosis, noma (cancrum oris or gangrenous stomatitis), omphalitis, orbital cellulitis, osteomyelitis, overwhelming post-splenectomy infection (OPSI), ovine brucellosis, pasteurellosis, periorbital cellulitis, pertussis (whooping cough), plague, pneumococcal pneumonia, Pott disease, proctitis, pseudomonas infection, psittacosis, pyaemia, pyomyositis, Q fever, relapsing fever (typhinia), rheumatic fever, Rocky Mountain spotted fever (RMSF), rickettsiosis, salmonellosis, scarlet fever, sepsis, serratia infection, shigellosis, southern tick-associated rash illness, staphylococcal scalded skin syndrome, streptococcal pharyngitis, swimming pool granuloma, swine brucellosis, syphilis, syphilitic aortitis, tetanus, toxic shock syndrome (TSS), trachoma, trench fever, tropical ulcer, tuberculosis, tularemia, typhoid fever, typhus, urogenital tuberculosis, urinary tract infections, vancomycin-resistant Staphylococcus aureus infection, Waterhouse-Friderichsen syndrome, pseudotuberculosis (Yersinia) disease, and yersiniosis.

Treating bacterial pathogens

[001156]The LNP-based RNA compositions described herein may be administered to treat infections by bacterial pathogens.

[001157]The bacterium described herein can be a Gram-positive bacterium or a Gram-negative bacterium. Bacterial pathogens include, but are not limited to, Acinetobacter baumannii, Bacillus anthracis, Bacillus subtilis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia trachomatis, Chlamydophila psittaci, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, coagulase Negative Staphylococcus, Corynebacterium diphtheria, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, enterotoxigenic Escherichia coli (ETEC), enteropathogenic E. coli, E. coli O157:H7, Enterobacter sp., Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Klebsiella pneumoniae, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Moraxella catarralis, Mycobacterium leprae, Mycobacterium tuberculosis, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitides, Preteus mirabilis, Proteus sps.,

Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella typhi, Salmonella typhimurium, Serratia marcesens, Shigella flexneri, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus mutans, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, and Yersinia pestis.

[001158]Bacterial pathogens may also include bacteria that cause resistant bacterial infections, for example, clindamycin-resistant Clostridium difficile, fluoroquinolon-resistant Clostridium difficile, methicillin-resistant Staphylococcus aureus (MRSA), multidrug-resistant Enterococcus faecalis, multidrug-resistant Enterococcus faecium, multidrug-resistance Pseudomonas aeruginosa, multidrug-resistant Acinetobacter baumannii, and vancomycin-resistant Staphylococcus aureus (VRSA).

Co-administered antibiotic combinations

[001159]The LNP-based RNA compositions described herein may be co-administered with one or more antibiotics and/or antibacterial agents.

Antibacterial Agents

[001160]Anti-bacterial agents that may be co-administered with LNP-based RNA compositions include, but are not limited to, aminoglycosides (e.g., amikacin (AMIKIN®), gentamicin (GARAMYCIN®), kanamycin (KANTREX®), neomycin (MYCIFRADIN®), netilmicin (NETROMYCIN®), tobramycin (NEBCIN®), Paromomycin (HUMATIN®)), ansamycins (e.g., geldanamycin, herbimycin), carbacephem (e.g., loracarbef (LORABID®), Carbapenems (e.g., ertapenem (INVANZ®), doripenem (DORIBAX®), imipenem/cilastatin (PRIMAXIN®), meropenem (MERREM®), cephalosporins (first generation) (e.g., cefadroxil (DURICEF®), cefazolin (ANCEF®), cefalotin or cefalothin (KEFLIN®), cefalexin (KEFLEX®), cephalosporins (second generation) (e.g., cefaclor (CECLOR®), cefamandole (MANDOL®), cefoxitin (MEFOXIN®), cefprozil (CEFZIL®), cefuroxime (CEFTIN®, ZINNAT®)), cephalosporins (third generation) (e.g., cefixime (SUPRAX®), cefdinir (OMNICEF®, CEFDIEL®), cefditoren (SPECTRACEF®), cefoperazone (CEFOBID®), cefotaxime (CLAFORAN®), cefpodoxime (VANTIN®), ceftazidime (FORTAZ®), ceftibuten (CEDAX®), ceftizoxime (CEFIZOX®), ceftriaxone (ROCEPHIN®)), cephalosporins (fourth generation) (e.g., cefepime (MAXIPIME®)), cephalosporins (fifth generation) (e.g., ceftobiprole (ZEFTERA®)), glycopeptides (e.g., teicoplanin (TARGOCID®), vancomycin (VANCOCIN®), telavancin (VIBATIV®)), lincosamides (e.g., clindamycin (CLEOCIN®), lincomycin (LINCOCIN®)), lipopeptide (e.g., daptomycin (CUBICIN®)), macrolides (e.g., azithromycin (ZITHROMAX®, SUMAMED®, ZITROCIN®), clarithromycin (BIAXIN®), dirithromycin (DYNABAC®), erythromycin (ERYTHOCIN®, ERYTHROPED®), roxithromycin, troleandomycin (TAO®), telithromycin (KETEK®), spectinomycin (TROBICIN®)), monobactams (e.g., aztreonam (AZACTAM®)), nitrofurans (e.g., furazolidone (FUROXONE®), nitrofurantoin (MACRODANTIN®, MACROBID®)), penicillins (e.g., amoxicillin (NOVAMOX®, AMOXIL®), ampicillin (PRINCIPEN®),

azlocillin, carbenicillin (GEOCILLIN®), cloxacillin (TEGOPEN®), dicloxacillin (DYNAPEN®), flucloxacillin (FLOXAPEN®), mezlocillin (MEZLIN®), methicillin (STAPHCILLIN®), nafcillin (UNIPEN®), oxacillin (PROSTAPHLIN®), penicillin G (PENTIDS®), penicillin V (PEN-VEE-K®), piperacillin (PIPRACIL®), temocillin (NEGABAN®), ticarcillin (TICAR®)), penicillin combinations (e.g., amoxicillin/clavulanate (AUGMENTIN®), ampicillin/sulbactam (UNASYN®), piperacillin/tazobactam (ZOSYN®), ticarcillin/clavulanate (TIMENTIN®)), polypeptides (e.g., bacitracin, colistin (COLY-MYCIN-S®), polymyxin B, quinolones (e.g., ciprofloxacin (CIPRO®, CIPROXIN®, CIPROBAY®), enoxacin (PENETREX®), gatifloxacin (TEQUIN®), levofloxacin (LEVAQUIN®), lomefloxacin (MAXAQUIN®), moxifloxacin (AVELOX®), nalidixic acid (NEGGRAM®), norfloxacin (NOROXIN®), ofloxacin (FLOXIN®, OCUFLOX®), trovafloxacin (TROVAN®), grepafloxacin (RAXAR®), sparfloxacin (ZAGAM®), temafloxacin (OMNIFLOX®)), sulfonamides (e.g., mafenide (SULFAMYLON®), sulfonamidochrysoidine (PRONTOSIL®), sulfacetamide (SULAMYD®, BLEPH-10®), sulfadiazine (MICRO-SULFON®), silver sulfadiazine (SILVADENE®), sulfamethizole (THIOSULFIL FORTE®), sulfamethoxazole (GANTANOL®), sulfanilimide, sulfasalazine (AZULFIDINE®), sulfisoxazole (GANTRISIN®), trimethoprim (PROLOPRIM®), TRIMPEX®), trimethoprim-sulfamethoxazole (co-trimoxazole) (TMP-SMX) (BACTRIM®, SEPTRA®)), tetracyclines (e.g., demeclocycline (DECLOMYCIN®), doxycycline (VIBRAMYCIN®), minocycline (MINOCIN®), oxytetracycline (TERRAMYCIN®), tetracycline (SUMYCIN®, ACHROMYCIN® V, STECLIN®)), drugs against mycobacteria (e.g., clofazimine (LAMPRENE®), dapsone (AVLOSULFON®), capreomycin (CAPASTAT®), cycloserine (SEROMYCIN®), ethambutol (MYAMBUTOL®), ethionamide (TRECATOR®), isoniazid (I.N.H.®), pyrazinamide (ALDINAMIDE®), rifampin (RIFADIN®, RIMACTANE®), rifabutin (MYCOBUTIN®), rifapentine (PRIFTIN®), streptomycin), and others (e.g., arsphenamine (SALVARSAN®), chloramphenicol (CHLOROMYCETIN®), fosfomycin (MONUROL®), fusidic acid (FUCIDIN®), linezolid (ZYVOX®), metronidazole (FLAGYL®), mupirocin (BACTROBAN®), platensimycin, quinupristin/dalfopristin (SYNERCID®), rifaximin (XIFAXAN®), thiamphenicol, tigecycline (TIGACYL®), tinidazole (TINDAMAX®, FASIGYN®)).

Treating conditions associated with viral infection

[001161]In another embodiment, provided are methods for treating or preventing a viral infection and/or a disease, disorder, or condition associated with a viral infection, or a symptom thereof, in a subject, by administering an LNP-based RNA composition to a subject infected with or who is suspected of being infected with a virus.

[001162] In various other embodiments, a LNP-based RNA composition may be co-administered with one or more polynucleotides encoding an anti-viral polypeptide, e.g., an LNP-based RNA composition

described herein in combination with an anti-viral agent, e.g., an anti-viral polypeptide or a small molecule anti-viral agent described herein.

[001163]Diseases, disorders, or conditions associated with viral infections which may be treated using the LNP-based RNA compositions disclosed herein, but are not limited to, acute febrile pharyngitis, pharyngoconjunctival fever, epidemic keratoconjunctivitis, infantile gastroenteritis, Coxsackie infections, infectious mononucleosis, Burkitt lymphoma, acute hepatitis, chronic hepatitis, hepatic cirrhosis, hepatocellular carcinoma, primary HSV-1 infection (e.g., gingivostomatitis in children, tonsillitis and pharyngitis in adults, keratoconjunctivitis), latent HSV-1 infection (e.g., herpes labialis and cold sores), primary HSV-2 infection, latent HSV-2 infection, aseptic meningitis, infectious mononucleosis, Cytomegalic inclusion disease, Kaposi sarcoma, multicentric Castleman disease, primary effusion lymphoma, AIDS, influenza, Reye syndrome, measles, postinfectious encephalomyelitis, Mumps, hyperplastic epithelial lesions (e.g., common, flat, plantar and anogenital warts, laryngeal papillomas, epidermodysplasia verruciformis), cervical carcinoma, squamous cell carcinomas, croup, pneumonia, bronchiolitis, common cold, Poliomyelitis, Rabies, bronchiolitis, pneumonia, influenza-like syndrome, severe bronchiolitis with pneumonia, German measles, congenital rubella, Varicella, and herpes zoster.

Viral infections treatable by the LNP compositions

[001164]In another embodiment, provided are methods for treating or preventing a viral infection and/or a disease, disorder, or condition associated with a viral infection, or a symptom thereof, in a subject, by administering an LNP-based RNA composition to a subject infected with or who is suspected of being infected with a virus. The viral pathogen can be any viral infectious agent.

[001165]Examples of viral infectious agents include, but are not limited to, adenovirus; Herpes simplex, type 1; Herpes simplex, type 2; encephalitis virus, papillomavirus, Varicella-zoster virus; Epstein-barr virus; Human cytomegalovirus; Human herpesvirus, type 8; Human papillomavirus; BK virus; JC virus; Smallpox; polio virus, Hepatitis B virus; Human bocavirus; Parvovirus B19; Human astrovirus; Norwalk virus; coxsackievirus; hepatitis A virus; poliovirus; rhinovirus; Severe acute respiratory syndrome virus; Hepatitis C virus; yellow fever virus; dengue virus; West Nile virus; Rubella virus; Hepatitis E virus; Human immunodeficiency virus (HIV); Influenza virus, type A or B; Guanarito virus; Junin virus; Lassa virus; Machupo virus; Sabia virus; Crimean-Congo hemorrhagic fever virus; Ebola virus; Marburg virus; Measles virus; Mumps virus; Parainfluenza virus; Respiratory syncytial virus; Human metapneumovirus; Hendra virus; Nipah virus; Rabies virus; Hepatitis D; Rotavirus; Orbivirus; Coltivirus; Hantavirus, Middle East Respiratory Coronavirus; Chikungunya virus or Banna virus.

Antiviral agents

[001166]Exemplary anti-viral agents include, but are not limited to, abacavir (ZIAGEN®), abacavir/lamivudine/zidovudine (Trizivir®), aciclovir or acyclovir (CYCLOVIR®, HERPEX®,

ACIVIR®, ACIVIRAX®, ZOVIRAX®, ZOVIR®), adefovir (Preveon®, Hepsera®), amantadine (SYMMETREL®), amprenavir (AGENERASE®), ampligen, arbidol, atazanavir (REYATAZ®), boceprevir, cidofovir, darunavir (PREZISTA®), delavirdine (RESCRIPTOR®), didanosine (VIDEX®), docosanol (ABREVA®), edoxudine, efavirenz (SUSTIVA®, STOCRIN®), emtricitabine (EMTRIVA®), emtricitabine/tenofovir/efavirenz (ATRIPLA®), enfuvirtide (FUZEON®), entecavir (BARACLUDE®, ENNAVIR®), famciclovir (FAMVIR®), fomivirsen (VITRAVENE®), fosamprenavir (LEXIVA®, TELZIR®), foscarnet (FOSCAVIR®), fosfonet, ganciclovir (CYTOVENE®, CYMEVENE®, VITRASERT®), GS 9137 (ELVITEGRAVIR®), imiquimod (ALDARA®, ZYCLARA®, BESELNA®), indinavir (CRIXIVAN®), inosine, inosine pranobex (IMUNOVIR®), interferon type I, interferon type II, interferon type III, kutapressin (NEXAVIR®), lamivudine (ZEFFIX®, HEPTOVIR®, EPIVIR®), lamivudine/zidovudine (COMBIVIR®), lopinavir, loviride, maraviroc (SELZENTRY®, CELSENTRI®), methisazone, MK-2048, moroxydine, nelfinavir (VIRACEPT®), nevirapine (VIRAMUNE®), oseltamivir (TAMIFLU®), peginterferon alfa-2a (PEGASYS®), penciclovir (DENAVIR®), peramivir, pleconaril, podophyllotoxin (CONDYLOX®), raltegravir (ISENTRESS®), ribavirin (COPEGUs®, REBETOL®, RIBASPHERE®, VILONA® AND VIRAZOLE®), rimantadine (FLUMADINE®), ritonavir (NORVIR®), pyramidine, saquinavir (INVIRASE®, FORTOVASE®), stavudine, tea tree oil (melaleuca oil), tenofovir (VIREAD®), tenofovir/emtricitabine (TRUVADA®), tipranavir (APTIVUS®), trifluridine (VIROPTIC®), tromantadine (VIRU-MERZ®), valaciclovir (VALTREX®), valganciclovir (VALCYTE®), vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir (RELENZA®), and zidovudine (azidothymidine (AZT), RETROVIR®, RETROVIS®).

Conditions Associated with Fungal Infections

[001167]Diseases, disorders, or conditions associated with fungal infections which may be treated using the LNP-based RNA compositions described herein include, but are not limited to, aspergilloses, blastomycosis, candidasis, coccidioidomycosis, cryptococcosis, histoplasmosis, mycetomas, paracoccidioidomycosis, and tinea pedis. Furthermore, persons with immuno-deficiencies are particularly susceptible to disease by fungal genera such as Aspergillus, Candida, Cryptoccocus, Histoplasma, and Pneumocystis. Other fungi can attack eyes, nails, hair, and especially skin, the so-called dermatophytic fungi and keratinophilic fungi, and cause a variety of conditions, of which ringworms such as athlete's foot are common. Fungal spores are also a major cause of allergies, and a wide range of fungi from different taxonomic groups can evoke allergic reactions in some people.

Fungal pathogens

[001168]Fungal pathogens include, but are not limited to, Ascomycota (e.g., Fusarium oxysporum, Pneumocystis jirovecii, Aspergillus spp., Coccidioides immitis/posadasii, Candida albicans), Basidiomycota (e.g., Filobasidiella neoformans, Trichosporon), Microsporidia (e.g., Encephalitozoon

cuniculi, Enterocytozoon bieneusi), and Mucoromycotina (e.g., Mucor circinelloides, Rhizopus oryzae, Lichtheimia corymbifera).

Anti fungal agents

[001169]The LNP-based RNA compositions described herein may be co-administered with one or more anti-fungal agents.

[001170]Exemplary anti-fungal agents include, but are not limited to, polyene antifungals (e.g., natamycin, rimocidin, filipin, nystatin, amphotericin B, candicin, hamycin), imidazole antifungals (e.g., miconazole (MICATIN®, DAKTARIN®), ketoconazole (NIZORAL®, FUNGORAL®, SEBIZOLE®), clotrimazole (LOTRIMIN®, LOTRIMIN® AF, CANESTEN®), econazole, omoconazole, bifonazole, butoconazole, fenticonazole, isoconazole, oxiconazole, sertaconazole (ERTACZO®), sulconazole, tioconazole), triazole antifungals (e.g., albaconazole fluconazole, itraconazole, isavuconazole, ravuconazole, posaconazole, voriconazole, terconazole), thiazole antifungals (e.g., abafungin), allylamines (e.g., terbinafine (LAMISIL®), naftifine (NAFTIN®), butenafine (LOTRIMIN® Ultra)), echinocandins (e.g., anidulafungin, caspofungin, micafungin), and others (e.g., polygodial, benzoic acid, ciclopirox, tolnaftate (TINACTIN®, DESENEX®, AFTATE®), undecylenic acid, flucytosine or 5-fluorocytosine, griseofulvin, haloprogin, sodium bicarbonate, allicin).

Conditions associated with protozoal infection

[001171]Diseases, disorders, or conditions associated with protozoal infections which may be treated using the LNP-based RNA compositions of the disclosure include, but are not limited to, amoebiasis, giardiasis, trichomoniasis, African Sleeping Sickness, American Sleeping Sickness, leishmaniasis (Kala-Azar), balantidiasis, toxoplasmosis, malaria, acanthamoeba keratitis, and babesiosis.

Protozoan Pathogens

[001172]Protozoal pathogens include, but are not limited to, Entamoeba histolytica, Giardia lambila, Trichomonas vaginalis, Trypanosoma brucei, T. cruzi, Leishmania donovani, Balantidium coli, Toxoplasma gondii, Plasmodium spp., and Babesia microti. These protozoal pathogens may be treatable with the LNP-based RNA compositions.

Anti-Protozoan Agents

[001173]Exemplary anti-protozoal agents include, but are not limited to, eflornithine, furazolidone (FUROXONE®, DEPENDAL-M®), melarsoprol, metronidazole (FLAGYL®), ornidazole, paromomycin sulfate (HUMATIN®), pentamidine, pyrimethamine (DARAPRIM®), and tinidazole (TINDAMAX®, FASIGYN®).

Conditions associated with parasitic infection

[001174]Diseases, disorders, or conditions associated with parasitic infections which may be treated using the LNP-based RNA compositions of the disclosure include, but are not limited to, acanthamoeba

keratitis, amoebiasis, ascariasis, babesiosis, balantidiasis, baylisascariasis, chagas disease, clonorchiasis, cochliomyia, cryptosporidiosis, diphyllobothriasis, dracunculiasis, echinococcosis, elephantiasis, enterobiasis, fascioliasis, fasciolopsiasis, filariasis, giardiasis, gnathostomiasis, hymenolepiasis, isosporiasis, katayama fever, leishmaniasis, lyme disease, malaria, metagonimiasis, myiasis, onchocerciasis, pediculosis, scabies, schistosomiasis, sleeping sickness, strongyloidiasis, taeniasis, toxocariasis, toxoplasmosis, trichinosis, and trichuriasis.

Parasitic Pathogens

[001175]Parasitic pathogens include, but are not limited to, Acanthamoeba, Anisakis, Ascaris lumbricoides, botfly, Balantidium coli, bedbug, Cestoda, chiggers, Cochliomyia hominivorax, Entamoeba histolytica, Fasciola hepatica, Giardia lamblia, hookworm, Leishmania, Linguatula serrata, liver fluke, Loa boa, Paragonimus, pinworm, Plasmodium falciparum, Schistosoma, Strongyloides stercoralis, mite, tapeworm, Toxoplasma gondii, Trypanosoma, whipworm, Wuchereria bancrofti.

Anti-Parasitic Agents

[001176]Exemplary anti-parasitic agents include, but are not limited to, antinematodes (e.g., mebendazole, pyrantel pamoate, thiabendazole, diethylcarbamazine, ivermectin), anticestodes (e.g., niclosamide, praziquantel, albendazole), antitrematodes (e.g., praziquantel), antiamoebics (e.g., rifampin, amphotericin B), and antiprotozoals (e.g., melarsoprol, eflornithine, metronidazole, tinidazole).

E.Cancer

[001177]In various embodiments, the LNP-based RNA therapeutics and pharmaceutical compositions thereof described herein may be used to treat cancer.

[001178]A "cancer" refers to a broad group of various diseases characterized by the uncontrolled growth of abnormal cells in the body. Unregulated cell division and growth results in the formation of malignant tumors that invade neighboring tissues and may also metastasize to distant parts of the body through the lymphatic system or bloodstream. A "cancer" or "cancer tissue" may include a tumor. Examples of cancers that may be treated by the methods disclosed herein include, but are not limited to, cancers of the immune system including lymphoma, leukemia, myeloma, and other leukocyte malignancies. In some embodiments, the methods disclosed herein may be used to reduce the tumor size of a tumor derived from, for example, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, multiple myeloma, Hodgkin's Disease, non-Hodgkin's lymphoma (NHL), primary mediastinal large B cell lymphoma (PMBC), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), transformed follicular lymphoma, splenic marginal zone lymphoma (SMZL), cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, cancer of the urethra,

cancer of the penis, chronic or acute leukemia, acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia (ALL) (including non T cell ALL), chronic lymphocytic leukemia (CLL), solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, epidermoid cancer, squamous cell cancer, T cell lymphoma, environmentally induced cancers including those induced by asbestos, other B cell malignancies, and combinations of said cancers. In some embodiments, the methods disclosed herein may be used to reduce the tumor size of a tumor derived from, for example, sarcomas and carcinomas, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, Kaposi's sarcoma, sarcoma of soft tissue, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, hepatocellular carcinomna, lung cancer, colorectal cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma (for example adenocarcinoma of the pancreas, colon, ovary, lung, breast, stomach, prostate, cervix, or esophagus), sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, bladder carcinoma, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, carcinoma of the renal pelvis, CNS tumors (such as a glioma, astrocytoma, medulloblastoma, craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma and retinoblastoma). The particular cancer may be responsive to chemo- or radiation therapy or the cancer may be refractory. A refractor cancer refers to a cancer that is not amendable to surgical intervention and the cancer is either initially unresponsive to chemo- or radiation therapy or the cancer becomes unresponsive over time.

F.Autoimmune Diseases and other diseases that can be treated through administration of an RNA encoding a CAR

[001179]In some embodiments, provided herein is a method of treating an autoimmune disorder in a subject by introducing or administering an effective amount of a pharmaceutical composition described herein including at least one lipid nanoparticle described herein.

[001180]In some embodiments, provided herein is a method of treating cancer in a subject by introducing or administering an effective amount of a pharmaceutical composition described herein including at least one lipid nanoparticle described herein.

[001181]In some embodiments, the RNA construct (such as an mRNA or circular RNA) encodes a CAR, the CARs have biological activity, e.g., ability to recognize an antigen, e.g., CD19, HER2, or BCMA,

such that the CAR, when expressed by a cell, is able to mediate an immune response against the cell expressing the antigen, e.g., CD19, HER2, or BCMA, for which the CAR is specific.

[001182]Adoptive T-cell immunotherapy is a rapidly growing field, in particular in cancer treatments. In general, chimeric antigen receptor (CAR) T cell or "CAR-T" engagement of CD19-expressing cancer cells results in T-cell activation, proliferation and secretion of inflammatory cytokines and chemokines resulting in tumor cell lysis. However, while CAR-T therapies have become an important tool in cancer treatments, they have toxic side effects and involve complex procedures. Treatment with CAR-T can lead to a large and rapid release of cytokines into the blood and can cause cytokine release syndrome (CRS) or CAR-T cell-related encephalopathy syndrome (CRES), also referred to as neurotoxicity associated with CAR-T. CRS is the most common and well-described toxicity associated with CAR-T therapy, occurring in over 90% of patients at any grade and is characterized by high fever, hypotension, hypoxia and/ or multiple organ toxicity and can lead to death. Neurotoxicity is characterized by damage to nervous tissue that can cause tremors, encephalopathy, dizziness or seizures. Additionally, prior to infusion, the patients generally undergo lymphodepletion. Lymphodepletion is known to increase CAR-T cell expansion and enhanced efficacy of infused CAR-T cells by, for example, altering the tumor phenotype and microenvironment. However, lymphodepletion agents often cause side effects to the patients. For example, lymphodepletion can cause neutropenia, anemia, thrombocytopenia, and immunosuppression, leading to a greater risk of infection, along with other toxicities. In addition to the toxicities associated with targeted CAR-T therapies, there are procedures, specialized equipment, and costs involved in producing the modified lymphocytes. CAR-T therapies require an assortment of protocols to isolate, genetically modify, and selectively expand the redirected cells before infusing them back into the patient.

[001183]In some embodiments, the subject has a cancer selected from the group consisting of: acute myeloid leukemia (AML); alveolar rhabdomyosarcoma; B cell malignancies; bladder cancer (e.g., bladder carcinoma); bone cancer; brain cancer (e.g., medulloblastoma and glioblastoma multiforme); breast cancer; cancer of the anus, anal canal, or anorectum; cancer of the eye; cancer of the intrahepatic bile duct; cancer of the joints; cancer of the neck; gallbladder cancer; cancer of the pleura; cancer of the nose, nasal cavity, or middle ear; cancer of the oral cavity; cancer of the vulva; chronic lymphocytic leukemia; chronic myeloid cancer; colon cancer; esophageal cancer, cervical cancer; fibrosarcoma; gastrointestinal carcinoid tumor; head and neck cancer (e.g., head and neck squamous cell carcinoma); Hodgkin lymphoma; hypopharynx cancer; kidney cancer; larynx cancer; leukemia; liquid tumors; lipoma; liver cancer; lung cancer (e.g., non-small cell lung carcinoma, lung adenocarcinoma, and small cell lung carcinoma); lymphoma; mesothelioma; mastocytoma; melanoma; multiple myeloma; nasopharynx cancer; non-Hodgkin lymphoma; B-chronic lymphocytic leukemia; hairy cell leukemia; Burkitt's lymphoma; ovarian cancer; pancreatic cancer; cancer of the peritoneum; cancer of the omentum;

mesentery cancer; pharynx cancer; prostate cancer; rectal cancer; renal cancer; skin cancer; small intestine cancer; soft tissue cancer; solid tumors; synovial sarcoma; gastric cancer; teratoma; testicular cancer; thyroid cancer; and ureter cancer.

[001184]In some embodiments, the subject has an autoimmune disorder. In some embodiments, the subject has an autoimmune disorder selected from scleroderma, Grave's disease, Crohn's disease, Sjogren's disease, multiple sclerosis, Hashimoto's disease, psoriasis, myasthenia gravis, autoimmune polyendocrinopathy syndromes, Type I diabetes mellitus (TIDM), autoimmune gastritis, autoimmune uveoretinitis, polymyositis, colitis, thyroiditis, and the generalized autoimmune diseases typified by human Lupus.

[001185]In some embodiments, the subject has an autoimmune disease or disorder that is B-cell mediated. In some embodiments, the subject has lupus, e.g., systemic lupus erythematosus (SLE), cutaneous lupus erythematosus (CLE), lupus nephritis (LN), neonatal lupus, drug-induced lupus.

[001186]In some embodiments, the subject has antisynthetase syndrome, multifocal motor neuropathy, myasthenia gravis, neuromyelitis optica, pemphigus vulgaris, and/or systemic sclerosis.

[001187]In a compassionate-use anti-CD19 CAR T cell therapy for refractory systemic lupus erythematosus, autologous T cells from five SLE patients "were transduced with a lentiviral anti-CD19 CAR vector, expanded and reinfused into the patients after lymphodepletion with fludarabine and cyclophosphamide. CAR T cells expanded in vivo, led to deep depletion of B cells, improvement of clinical symptoms and normalization of laboratory parameters including seroconversion of anti-double-stranded DNA antibodies. Remission of SLE according to DORIS criteria was achieved in all five patients after 3 months and the median (range) Systemic Lupus Erythematosus Disease Activity Index score after 3 months was 0 (2)." Mackensen et al., Anti-CD19 CAR T cell therapy for refractory systemic lupus erythematosus, Nature Medicine (2022); *see also* Nunez et al., Cytokine and reactivity profiles in SLE patients following anti-CD19 CART therapy, Molecular Therapy (2023); the contents of both of which are hereby incorporated by reference in their entireties.

G.Co-therapy with another therapeutic agent

[001188]In other embodiments, LNP-based RNA therapeutics and pharmaceutical compositions thereof described herein may be co-administered in conjunction with another agent for treating a disease (e.g., muscle atrophy and/or muscle-wasting disease, disease of the CNS, or cancer). Such additional agents are as follows: agents that treat conditions related to acute or chronic muscle atrophy and/or a muscle-wasting disease, such as sarcopenia, cachexia (e.g., megestrol, Megace®, Megace ES®, somatropin, Serostim®, and Norditropin FlexPro Pen®), cancer (e.g., carboplatin, Adriamycin®, Adrucil®, etoposide, fluorouracil, doxorubicin, Paraplatin®, Cosmegen®, cyclophosphamide, Ethyol®, Leukeran®, Vincasar PFS®, vincristine, Etopophos®, Oncovin®, Toposar®, Hycamtin®, Ifex®, ifosfamide, Mustargen®,

Velban®, VePesid®, vinblastine, amifostine, chlorambucil, dactinomycin, mechlorethamine, Tepadina®, thiotepa, topotecan, and fludarabine), congestive heart failure (e.g., Furosemide®, Lasix®, carvedilol, Coreg®, spironolactone, Lisinopril®, digoxin, Metoprolol Succinate ER®, Aldactone®, Accupril®, metoprolol, isosorbide mononitrate, Coumadin®, Cardizem®, warfarin, Altace®, amlodipine, Lanoxin®, Norvasc®, Toprol-XL®, Nitrostat®, Diovan®, Prinivil®, Zestril®, diltiazem, hydralazine, nitroglycerin, Ramipril®, Apresoline®, bisoprolol, enalapril, torsemide, Vasotec®, isosorbide dinitrate, Lotensin®, allopurinol, Atacand®, Coreg®, valsartan, benazepril, Cardizem CD®, Cartia XT®, Digox®, Entresto®, Qbrelis®, quinapril, Cardizem LA®, CaroSpir®, Demadex®, Digitek®, Dilacor®, Dilt-XR®, Diltia XT®, Diltzac®, eplerenone, Jantoven®, Matzim LA®, nifedipine, Nitrolingual Pumpspray®, NitroQuick®, Taztia XT®, Tiadylt ER®, Tiazac®, candesartan, captopril, Clinacort®, dobutamine, hydrochlorothiazide/lisinopril, Isordil, Kenalog-40®, Lanoxicaps®, milrinone, Minitran®, Monoket®, Nitrek®, Nitro-Bid®, Nitro-Dur®, Nitro-Time®, Nitrocot®, Nitrol Appli-Kit®, NitroMist®, Nitro TD Patch-A®, Prexxartan®, Transderm-Nitro®, triamcinolone, amiloride, BiDil®, Capoten®, Corlanor®, Dobutrex®, hydrochlorothiazide/spironolactone, Inspra®, ivabradine, Midamor®, Minipress®, perindopril, prazosin, Prinzide®, sacubitril/valsartan, trandolapril, Zestoretic®, Aceon®, Aldactazide®, amiloride/hydrochlorothiazide, Capozide®, Capozide®, Capozide 25/25®, Capozide 50/15®, Capozide 50/25®, captopril/hydrochlorothiazide, Cardene®, Cardene IV®, Cardene SR®, Dilatrate-SR®, enalapril/hydrochlorothiazide, fosinopril, hydralazine/isosorbide dinitrate, Isochron®, IsoDitrate®, Isordil Titradose®, Mavik®, Moduretic 5-50®, moexipril, Monopril®, Natrecor®, nesiritide, nicardipine, Nipride RTU®, Nitropress®, nitroprusside, Primacor®, Univasc®, and Vaseretic®), renal failure (e.g., furosemide, Lasix®, Demadex®, Edecrin®, torsemide, Sodium Edecrin®, and ethacrynic acid), chronic obstructive pulmonary disease (e.g., Symbicort®, prednisone, montelukast, Breo Ellipta®, Daliresp®, Anoro Ellipta®, budesonide/formoterol, Tudorza Pressair®, Rayos®, aclidinium, fluticasone/vilanterol, Incruse Ellipta®, umeclidinium/vilanterol, roflumilast, Stiolto Respimat®, guaifenesin/theophylline, levalbuterol, olodaterol/tiotropium, dyphylline, olodaterol, Striverdi Respimat®, umeclidinium, Xopenex HFA®, Xopenex®, fluticasone/umeclidinium/vilanterol, Trelegy Ellipta®, and Xopenex Concentrate®), severe burns (e.g., silver sulfadiazine, Silvadene®, lidocaine, Xylocaine Jelly®, Bactine®, Dermoplast®, AneCream®, Solarcaine Burn Relief®, Albuminar-25®, Aloe Vera Burn Relief Spray with Lidocaine®, Lidocream®, Nupercainal®, SSD®, Xylocaine Topical®, Garamycin®, Thermazene®, Albutein®, AneCream with Tegaderm®, benzocaine, CidalEaze®, DermacinRx Lido V Pak®, Eha Lotion®, LidaMantle®, Lidopac®, Lidopin®, LidoRx®, LidoRxKit®, Lidotrans®, Lidovex®, Lidozion®, Lidozol®, Medi-Quik Spray®, RadiaGuard®, Regenecare HA Spray®, Senatec®, Sulfamylon®, Topicaine®, Vancocin®, Bionect®, gentamicin, vancomycin, mafenide, albumin human, dibucaine, Flexbumin®, Human Albumin Grifols®, Nebcin®, sodium hyaluronate, Tobi®, Tobramycin®, Vancocin

HCl®, Vancocin HCl Pulvules®, Albuminar-5®, Albuminar-20®, SSD AF®, Albuked®, Albuked 5®, Albuked 25®, Albumin-ZLB®, Alburx®, Buminate®, Hylira®, IPM Wound®, Kedbumin®, Plasbumin®, Plasbumin-5®, Plasbumin-25®, RadiaPlex®, Solarcaine First Aid Medicated Spray®, and Xclair®), an inflammatory muscle disease, myasthenia gravis (e.g., Mestinon®, pyridostigmine, Mestinon Timespan®, azathioprine, mycophenolate mofetil, Prostigmin®, neostigmine, Prostigmin Bromide®, Regonol®, immune globulin intravenous, Soliris®, ephedrine, and eculizumab), neuropathy, polio (e.g., amantadine), multiple sclerosis (e.g., Copaxone®, Gilenya®, Ampyra®, Tysabri®, Tecfidera®, Aubagio®, Rebif®, Avonex®, Betaseron®, Decadron®, Prednisone®, Avonex Pen®, glatiramer, interferon beta-1a, fingolimod, dalfampridine, Novantrone®, dimethyl fumarate, teriflunomide, Acthar®, dexamethasone, Extavia®, natalizumab, Imuran®, Dexamethasone Intensol®, interferon beta-1b, prednisolone, Plegridy®, Prelone®, Rebif Rebidose®, valacyclovir, azathioprine, Lemtrada®, ocrelizumab, alemtuzumab, corticotropin, cyclophosphamide, Glatopa®, H.P. Acthar Gel®, mitoxantrone, peginterferon beta-1a, Azasan®, cladribine, daclizumab, De-Sone LA®, Dexpak Taperpak®, Millipred®, Millipred DP®, mycophenolate mofetil, Ocrevus®, Orapred®, PediaPred®, Veripred 20®, and Zinbryta®), anorexia nervosa (e.g., olanzapine and cyproheptadine), human immunodeficiency virus/acquired immune deficiency syndrome (e.g., non-nucleoside reverse transcriptase inhibitors, nucleoside analog reverse transcriptase inhibitors, and protease inhibitors), osteomalacia (e.g., vitamin D2, Drisdol®, ergocalciferol, Calciferol®, Calcidol®, Posture®, Ridactate®, calcium lactate, and calcium phosphate, tribasic), herniated disk, hypercalcemia, kwashiorkor, Creutzfeldt-Jakob disease or bovine spongiform encephalopathy (e.g., methylene blue, cefotaxime, and Claforan®), diabetes (e.g., Tresiba®, insulin degludec, insulin aspart/insulin degludec, and Ryzodeg 70/30®), amyotrophic lateral sclerosis (e.g., Rilutek®, riluzole, edaravone, and Radicava®), necrotizing vasculitis, abetalipoproteinemia, malabsorption syndrome, Legg-Calvé-Perthes disease, polymyositis (e.g., prednisone), Guillain-Barre syndrome, osteoarthritis (e.g., paracetamol, nonsteroidal anti-inflammatory drugs, antacid, COX-2 selective inhibitors, and glucocorticoids), and/or muscular dystrophy (e.g., deflazacort, eteplirsen, Emflaza®, Exondys 51®, mexiletine, phenytoin, procainamide, and nusinersen), such as Duchenne, Becker congenital, distal, myotonic, oculopharyngeal, Limb-Girdle, facioscapulohumeral, and/or Emery-Dreifuss muscular dystrophy.

[001189]In other embodiments, the LNP-based RNA therapeutics and pharmaceutical compositions thereof described herein may be co-administered with a co-stimulatory molecule in order to achieve an immune response. An "immune response" refers to the action of a cell of the immune system (for example, T lymphocytes, B lymphocytes, natural killer (NK) cells, macrophages, eosinophils, mast cells, dendritic cells and neutrophils) and soluble macromolecules produced by any of these cells or the liver (including Abs, cytokines, and complement) that results in selective targeting, binding to, damage to,

destruction of, and/or elimination from a vertebrate's body of invading pathogens, cells or tissues infected with pathogens, cancerous or other abnormal cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

[001190]A "costimulatory signal," as used herein, refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to a T cell response, such as, but not limited to, proliferation and/or upregulation or down regulation of key molecules.

[001191]A "costimulatory ligand," as used herein, includes a molecule on an antigen presenting cell that specifically binds a cognate co-stimulatory molecule on a T cell. Binding of the costimulatory ligand provides a signal that mediates a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A costimulatory ligand induces a signal that is in addition to the primary signal provided by a stimulatory molecule, for instance, by binding of a T cell receptor (TCR)/CD3 complex with a major histocompatibility complex (MHC) molecule loaded with peptide. A co-stimulatory ligand may include, but is not limited to, 3/TR6, 4-IBB ligand, agonist or antibody that binds Toll ligand receptor, B7-1 (CD80), B7-2 (CD86), CD30 ligand, CD40, CD7, CD70, CD83, herpes virus entry mediator (HVEM), human leukocyte antigen G (HLA-G), ILT4, immunoglobulin-like transcript (ILT) 3, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), ligand that specifically binds with B7-H3, lymphotoxin beta receptor, MHC class I chain-related protein A (MICA), MHC class I chain-related protein B (MICB), 0X40 ligand, PD-L2, or programmed death (PD) LI. A co-stimulatory ligand includes, without limitation, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as, but not limited to, 4-1BB, B7-H3, CD2, CD27, CD28, CD30, CD40, CD7, ICOS, ligand that specifically binds with CD83, lymphocyte function-associated antigen-1 (LFA-1), natural killer cell receptor C (NKG2C), OX40, PD-1, or tumor necrosis factor superfamily member 14 (TNFSF14 or LIGHT).

[001192]A "costimulatory molecule" is a cognate binding partner on a T cell that specifically binds with a costimulatory ligand, thereby mediating a costimulatory response by the T cell, such as, but not limited to, proliferation. Costimulatory molecules include, but are not limited to, A "costimulatory molecule" is a cognate binding partner on a T cell that specifically binds with a costimulatory ligand, thereby mediating a costimulatory response by the T cell, such as, but not limited to, proliferation. Costimulatory molecules include, but are not limited to, 4-1BB/CD137, B7-H3, BAFFR, BLAME (SLAMF8), BTLA, CD 33, CD 45, CD100 (SEMA4D), CD103, CD134, CD137, CD154, CD16, CD160 (BY55), CD 18, CD19, CD19a, CD2, CD22, CD247, CD27, CD276 (B7-H3), CD28, CD29, CD3 (alpha; beta; delta; epsilon; gamma; zeta), CD30, CD37, CD4, CD4, CD40, CD49a, CD49D, CD49f, CD5, CD64, CD69, CD7, CD80, CD83 ligand, CD84, CD86, CD8alpha, CD8bcta, CD9, CD96 (Tactilc), CD1-1a, CD1-1b, CD1-1c, CD1-1d, CDS, CEACAM1, CRT AM, DAP-10, DNAM1 (CD226), Fc gamma receptor, GADS, GITR, HVEM

(LIGHTR), IA4, ICAM-1, ICAM-1, ICOS, Ig alpha (CD79a), IL2R beta, IL2R gamma, IL7R alpha, integrin, ITGA4, ITGA4, ITGA6, IT GAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB2, ITGB7, ITGB1, KIRDS2, LAT, LFA-1, LFA-1, LIGHT, LIGHT (tumor necrosis factor superfamily member 14; TNFSF14), LTBR, Ly9 (CD229), lymphocyte function-associated antigen-1 (LFA-1 (CD1 la/CD18), MHC class I molecule, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), 0X40, PAG/Cbp, PD-1, PSGL1, SELPLG (CD162), signaling lymphocytic activation molecule, SLAM (SLAMF1; CD150; IPO-3), SLAMF4 (CD244; 2B4), SLAMF6 (NTB-A; Ly108), SLAMF7, SLP-76, TNF, TNFr, TNFR2, Toll ligand receptor, TRANCE/RANKL, VLA1, or VLA-6, or fragments, truncations, or combinations thereof.

H.Hemoglobinopathies

[001193]In various embodiments, the LNP-based therapeutics and pharmaceutical compositions thereof described herein may be used to treat hemoglobinopathies. In certain embodiments, the LNPs of the present disclosure can be used to treat sickle cell disease (SCD). In certain embodiments, the LNPs of the present disclosure can be used to treat beta thalassemia (β-Thal). As discussed elsewhere herein, the LNPs of the present disclosure can be used to treat hemoglobinopathies by delivering a gene editing system capable of correcting the HBB mutation or reactivating expression of fetal hemoglobin.

[001194]Provided herein are methods of treating a hemoglobinopathy in a patient in need thereof, the method comprising administering to the patient a therapeutically effective amount of a pharmaceutical composition of the present disclosure, comprising an LNP comprising a gene editing system of the present disclosure. In certain embodiments, the pharmaceutical composition is administered intravenously.

[001195]Also provided herein are uses of pharmaceutical compositions of the present disclosure for the manufacture of a medicament for treating hemoglobinopathies. Further provided herein are pharmaceutical compositions for use in treating hemoglobinopathies. Additionally provided herein are uses of pharmaceutical compositions of the present disclosure for the manufacture of a medicament for treating hemoglobinopathies, wherein the medicament is prepared to be administered in a dosage regime effective to treat the hemoglobinopathy.

Correction of HBB Mutation

[001196]In certain embodiments, the gene editing system treats the hemoglobinopathy by repairing the mutant β-globin gene. In general, strategies of repairing the β-globin gene in order to address a β-hemoglobinopathy are illustrated in the central panel of FIG. 1.

Homology Directed Repair (HDR)

[001197]In certain embodiments, the gene editing system corrects the β-globin mutation through homology directed repair.

[001198]In one aspect, the present disclosure provides a method of treating a β-hemoglobinopathy, the method comprising administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid of the present disclosure; and

b) at least one gene editing system that corrects a β-globin mutation through homology directed repair.

[001199]In certain embodiments, the gene editing system comprises a CRISPR-Cas9 capable of removing the mutant HBB exon. In certain embodiments, the gene editing system comprises a nickase capable of removing the mutant HBB exon. In certain embodiments, the gene editing system comprises a TALEN capable of removing the mutant HBB exon. In certain embodiments, the gene editing system comprises a ZFN capable of removing the mutant HBB exon. In certain embodiments, the gene editing system comprises a donor template comprising a corrected or wild type HBB exon. In certain embodiments, the at least one gene editing system that corrects a β-globin mutation through homology directed repair is one described in a reference in Table X1.

Non-homologous End-joining (NHEJ)

[001200]In certain embodiments, the gene editing system corrects the β-globin mutation through non-homologous end-joining (NHEJ), by targeting aberrant splicing sites in introns I and II, resulting in restoration of functional β-globin transcripts.

[001201]In one aspect, the present disclosure provides a method of treating a β-hemoglobinopathy, the method comprising administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid of the present disclosure; and

b) at least one gene editing system that corrects a β-globin mutation through non-homologous end-joining (NHEJ).

[001202]In certain embodiments, the gene editing system comprises a CRISPR-Cas9 capable of targeting aberrant splicing sites in an intron of a mutated HBB. In certain embodiments, the gene editing system comprises a TALEN capable of targeting aberrant splicing sites in an intron of a mutated HBB. In certain embodiments, the at least one gene editing system that corrects a β-globin mutation through non-homologous end-joining (NHEJ) is one described in a reference in Table X1.

Single Base Pair Correction

[001203]In certain embodiments, the gene editing system corrects the β-globin mutation through a single base pair gene correction. In certain embodiments, the gene editing system is a prime editing or base

editing system capable of executing a single base pair edit. In certain embodiments, the gene editing system executes a single base pair correction in a SCD mutation, converting the aberrant thymine to the normal adenine, thereby converting the aberrant valine amino acid in the mutant hemoglobin to the wild-type glutamic acid. In certain embodiments, the gene editing system executes a single base pair correction, converting the aberrant thymine to cytosine, thereby converting the aberrant valine amino acid in the mutant hemoglobin to alanine, which results in a functional, nonpathogenic hemoglobin variant (HbG Makassar).

[001204]In one aspect, the present disclosure provides a method of treating a β-hemoglobinopathy, the method comprising administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid of the present disclosure; and

b) at least one gene editing system comprising a base editor capable of executing a single base pair edit in a mutant HBB gene.

[001205]In certain embodiments, the gene editing system comprises an adenine base editor. In certain embodiments, the gene editing system comprises a base editor that converts aberrant thymine to the normal adenine, thereby correcting a mutant HBB to wild-type HBB. In certain embodiments, the gene editing system comprises a base editor that converts an aberrant thymine to cytosine resulting in HbG Makassar. In certain embodiments, the gene editing system comprises a base editor described in a reference in Table X1.

[001206]In one aspect, the present disclosure provides a method of treating a β-hemoglobinopathy, the method comprising administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid of the present disclosure; and

b) at least one gene editing system comprising a prime editor capable of executing a single base pair edit in a mutant HBB gene.

[001207]In certain embodiments, the gene editing system comprises a prime editor that converts aberrant thymine to the normal adenine, thereby correcting a mutant HBB to wild-type HBB. In certain embodiments, the gene editing system comprises a prime editor that converts an aberrant thymine to cytosine resulting in HbG Makassar. In certain embodiments, the gene editing system comprises a prime editor described in a reference in Table X1.

Reactivation of γ-globin production

[001208]In certain embodiments, the gene editing system reactivates γ-globin production. One method of treating SCD and beta-Thalassemia is to reactivate production of functional fetal hemoglobin (γ-globin) to compensate for the pathogenic mutant β-globin. This mutation occurs naturally and is known as hereditary persistence of fetal hemoglobin (HPFH). β-globin mutations can be treated by artificially inducing a second mutation that mimics HPFH in a patient. In general, strategies of reactivating production of functional fetal hemoglobin (γ-globin) in order to address a β-hemoglobinopathy are illustrated in the right panel of FIG. 1.

Disruption of HbF inhibitory sequences

[001209]In certain embodiments, the gene editing system reactivates γ-globin production by deleting or disrupting inhibitory sequences, or portions thereof. In certain embodiments, the gene editing system eliminates an HbF inhibitory sequence, or a portion thereof, thereby reactivating γ-globin production.

[001210]In one aspect, the present disclosure provides a method of treating a β-hemoglobinopathy, the method comprising administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid of the present disclosure; and

b) at least one gene editing system comprising at least one nuclease and one guide RNA, capable of deleting at least a portion of the HbF inhibitory sequence.

[001211]In certain embodiments, the gene editing system comprises a CRISPR/Cas9. In certain embodiments, the gene editing system comprises a CRISPR/Cas12a. In certain embodiments, the gene editing system comprises multiple guide RNAs targeting multiple points on the HbF inhibitor sequence, thereby enabling a large deletion. In certain embodiments, the gene editing system juxtaposes the γ-globin promoters to remote enhancer regions. In certain embodiments, the gene editing system comprises a gene editing system for deleting at least a portion of the HbF inhibitor sequence described in a reference in Table X1.

Disruption of HBG1 and/or HBG2 promoters

[001212]In certain embodiments, the gene editing system targets the HBG1 and/or HBG2 promoters, resulting in disruption of binding sites for γ-globin transcriptional repressors, leading to HbF expression.

[001213]In one aspect, the present disclosure provides a method of treating a β-hemoglobinopathy, the method comprising administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid of the present disclosure; and

b) at least one gene editing system comprising at least one nuclease and one guide RNA, capable of deleting or disrupting at least a portion of the HBG1 and/or HBG2 promoter sequences.

[001214]In certain embodiments, the gene editing system comprises a CRISPR/Cas9. In certain embodiments, the gene editing system comprises a CRISPR/Cas12a. In certain embodiments, the gene editing system comprises one or more base editors capable of disrupting the HBG1/2 promoters. In certain embodiments, the gene editing system comprises multiple guide RNAs targeting multiple points on the HBG1 and/or HBG2 promoters. In certain embodiments, the gene editing system comprises a gene editing system for deleting or disrupting at least a portion of the HBG1 and/or HBG2 promoter sequences described in a reference in Table X1. In certain embodiments, the gene editing system is one disclosed or described in one of US Patent 11,466,271; US Application Publications US20200140896A1; US20220047637A1; and US20200399626A1; and PCT Publications WO2017/077394; WO2016/182917; WO2015/073683; WO2022/155458A1; and WO2021/163587A1.

Disruption of GATA1 Binding Site

[001215]In certain embodiments, the gene editing system disrupts the GATA1 motif in the erythroid-specific intronic enhancer of BCL11A.

[001216]In one aspect, the present disclosure provides a method of treating a β-hemoglobinopathy, the method comprising administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid of the present disclosure; and

b) at least one gene editing system comprising at least one nuclease and one guide RNA, capable of deleting or disrupting at least a portion of the GATA1 binding site in the BCL11A enhancer.

[001217]In certain embodiments, the gene editing system comprises a CRISPR/Cas9. In certain embodiments, the gene editing system comprises a CRISPR/Cas12a. In certain embodiments, the gene editing system comprises one or more base editors. In certain embodiments, the gene editing system comprises a gene editing system for deleting or disrupting at least a portion of the GATA1 binding site described in a reference in Table X1.

Disruption of BCL11A Binding Site

[001218]In certain embodiments, the gene editing system comprises base editors that reproduce HPFH mutations by disrupting repressor binding sites (such as BCL11A). In certain embodiments, the gene editing system disrupts BCL11A binding sites.

[001219]In one aspect, the present disclosure provides a method of treating a β-hemoglobinopathy, the method comprising administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid of the present disclosure; and

b) at least one gene editing system comprising at least one nuclease and one guide RNA, capable of deleting or disrupting at least a portion of the BCL11A binding site.

[001220]In certain embodiments, the gene editing system comprises a CRISPR/Cas9. In certain embodiments, the gene editing system comprises a CRISPR/Cas12a. In certain embodiments, the gene editing system comprises one or more base editors. In certain embodiments, the gene editing system comprises a gene editing system for deleting or disrupting at least a portion of the BCL11A binding site described in a reference in Table X1.

Creation of Transcriptional Activator Binding Site

[001221]In certain embodiments, the gene editing system creates transcriptional activator binding sites. In certain embodiments, the gene editing system comprises base editors that reproduce HPFH mutations by creating binding sites for transcriptional activators (such as KLF1).

[001222]In one aspect, the present disclosure provides a method of treating a β-hemoglobinopathy, the method comprising administering to a patient in need thereof a therapeutically effective amount of a pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid of the present disclosure; and

b) at least one gene editing system comprising at least one nuclease and one guide RNA, capable of creating or activating a binding site for a transcriptional activator that promotes fetal hemoglobin production.

[001223]In certain embodiments, the gene editing system comprises a CRISPR/Cas9. In certain embodiments, the gene editing system comprises a CRISPR/Cas12a. In certain embodiments, the gene editing system comprises one or more base editors. In certain embodiments, the gene editing system comprises a gene editing system capable of creating or activating a binding site for a transcriptional activator that promotes fetal hemoglobin production described in a reference in Table X1. In certain embodiments, the gene editing system comprises one or more components disclosed in Zhou, et al. KLF1 regulates BCL11A expression and γ- to β-globin gene switching. Nat. Genet. 2010, 42, 742–744.

DEFINITIONS

[001224]For convenience, certain terms employed in the specification, examples, and appended claims are collected here. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs.

[001225]As used herein, the following terms and phrases are intended to have the following meanings:

A / an

[001226]The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

About

[001227]As used herein, the term "about" means acceptable variations within 20%, within 10% and within 5% of the stated value. In certain embodiments, "about" can mean a variation of +/-1%, 2%, 3%, 4%, 5%, 10% or 20%. Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the disclosure, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[001228]In addition, it is to be understood that any particular embodiment of the present disclosure that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the disclosure (e.g., any antibiotic, therapeutic or active ingredient; any method of production; any method of use; etc.) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[001229]It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the disclosure in its broader aspects.

Adjuvant

[001230]As used herein, "adjuvant" means an agent that does not constitute a specific antigen, but modifies (Th1/Th2), boosts the strength and longevity of an immune response, and/or broadens the immune response to a concomitantly administered antigen.

Administration

[001231]The term "administration" or "administering" as used herein includes all means of introducing the compounds or the pharmaceutical compositions to the subject in need thereof, including but not limited to, oral, intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, inhalation, buccal, ocular, sublingual, vaginal, rectal and the like. Administration of the compound or the composition is suitably parenteral. For example, the compounds or the composition can be preferentially administered intravenously, but can also be administered intraperitoneally or via inhalation like is currently used in the

clinic for liposomal amikacin in the treatment of mycobacterium avium (see Shirley et al., Amikacin Liposome Inhalation Suspension: A Review in Mycobacterium avium Complex Lung Disease. Drugs. 2019 Apr; 79(5):555-562).

Aliphatic

[001232]The term "aliphatic" or "aliphatic group," as used herein, means a straight-chain (i.e., unbranched) or branched, substituted or unsubstituted hydrocarbon chain that is completely saturated or that contains one or more units of unsaturation, or a monocyclic hydrocarbon or bicyclic hydrocarbon that is completely saturated or that contains one or more units of unsaturation, but which is not aromatic (also referred to herein as "carbocycle," "cycloaliphatic" or "cycloalkyl"), that has a single point of attachment to the rest of the molecule or multiple points of attachment to the rest of the molecule, as would be readily apparent to a person of ordinary skill in the art based on the context of the described molecule. In some embodiments, aliphatic groups contain 1-6 aliphatic carbon atoms. In some embodiments, aliphatic groups contain 1-5 aliphatic carbon atoms. In other embodiments, aliphatic groups contain 1-4 aliphatic carbon atoms. In still other embodiments, aliphatic groups contain 1-3 aliphatic carbon atoms, and in yet other embodiments, aliphatic groups contain 1-2 aliphatic carbon atoms. In some embodiments, "cycloaliphatic" (or "carbocycle" or "cycloalkyl") refers to a monocyclic, bicyclic, or polycyclic C_3-C_{14} hydrocarbon that is completely saturated or that contains one or more units of unsaturation, but which is not aromatic, that has a single point of attachment to the rest of the molecule. Exemplary aliphatic groups include, but are not limited to, linear or branched, substituted or unsubstituted alkyl, alkenyl, alkynyl groups and hybrids thereof such as (cycloalkyl)alkyl, (cycloalkenyl)alkyl or (cycloalkyl)alkenyl. Examples of bicyclic and polycyclic cycloalkyls include bridged, fused, and spirocyclic carbocyclyls.

Alkenyl

[001233]As used herein, "alkenyl" means a straight chain, cyclic or branched aliphatic hydrocarbon having the specified number of carbon atoms and one or more double bonds including but not limited to diene, triene and tetraene unsaturated aliphatic hydrocarbons. The terms "alkenyl" and "alkynyl", refer to unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double or triple bond respectively. In one embodiment, the alkenyl contains one double bond. In another embodiment, the alkenyl contains two double bonds. In another embodiment, the alkenyl contains three double bonds.

Alkenylenyl

[001234]The term "alkenylenyl" as used herein refers to a divalent radical of an alkenyl group. In one embodiment, the alkenylenyl is a divalent form of a $C_{2\text{-}12}$ alkenyl, i.e., a C_2-C_{12} alkenylenyl. In one embodiment, the alkenylenyl is a divalent form of a $C_{2\text{-}6}$ alkenyl, i.e., a C_2-C_{10} alkenylenyl. In one embodiment, the alkenylenyl is a divalent form of a $C_{2\text{-}14}$ alkenyl, i.e., a C_2-C_8 alkenylenyl. In one

embodiment, the alkylenyl is a divalent form of an unsubstituted C_{2-6} alkenyl, i.e., a C_2-C_6 alkenylenyl. In another embodiment, the alkylenyl is a divalent form of an unsubstituted C_{2-4} alkyl, i.e., a C_2-C_4 alkenylenyl. Nonlimiting exemplary alkenylenyl groups include CH=CH-, CH_2CH=CH-, CH_2CH_2CH=$CHCH_2$-, and CH_2CH=$CHCH_2CH$=$CHCH_2CH_2$-.

Alkoxyl

[001235]The terms "alkoxyl" or "alkoxy" as used herein refers to an alkyl group, as defined above, having an oxygen radical attached thereto. Representative alkoxyl groups include methoxy, ethoxy, propyloxy, and tert-butoxy. An "ether" is two hydrocarbons covalently linked by an oxygen. Accordingly, the substituent of an alkyl that renders that alkyl an ether is or resembles an alkoxyl, such as can be represented by one of -O-alkyl, -O-alkenyl, and -O-alkynyl. Aroxy can be represented by –O-aryl or O-heteroaryl, wherein aryl and heteroaryl are as defined below. The alkoxy and aroxy groups can be substituted as described above for alkyl.

Alkyl

[001236]As used herein, “alkyl” means a straight chain, cyclic or branched saturated aliphatic hydrocarbon having the specified number of carbon atoms.

Alkylenyl

[001237]The term "alkylenyl" as used herein refers to a divalent radical of a straight-chain or branched-chain alkyl group. In one embodiment, the alkylenyl is a divalent form of a C_{1-12} alkyl, i.e., a C_1-C_{12} alkylenyl. In one embodiment, the alkylenyl is a divalent form of a C_{2-6} alkyl, i.e., a C_1-C_{10} alkylenyl. In one embodiment, the alkylenyl is a divalent form of a C_{2-14} alkyl, i.e., a C_1-C_8 alkylenyl. In one embodiment, the alkylenyl is a divalent form of an unsubstituted C_{1-6} alkyl, i.e., a C_1-C_6 alkylenyl. In another embodiment, the alkylenyl is a divalent form of an unsubstituted C_{1-4} alkyl, i.e., a C_1-C_4 alkylenyl. Nonlimiting exemplary alkylenyl groups include CH_2-, CH_2CH_2-, $CH_2CH_2CH_2$-, $CH_2CH(CH_3)CH_2$-, -$CH_2(CH_2)_2CH_2$-, $CH(CH_2)_3CH_2$-, and $CH_2(CH_2)_4CH_2$-.

Alkylthio

[001238]The term "alkylthio" refers to an alkyl group, as defined above, having a sulfur radical attached thereto. In some embodiments, the "alkylthio" moiety is represented by one of -S-alkyl, -S-alkenyl, and -S-alkynyl. Representative alkylthio groups include methylthio, and ethylthio. The term "alkylthio" also encompasses cycloalkyl groups, alkene and cycloalkene groups, and alkyne groups. "Arylthio" refers to aryl or heteroaryl groups. Alkylthio groups can be substituted as defined above for alkyl groups.

Aralkyl

[001239]The term "aralkyl," as used herein, refers to an alkyl group substituted with an aryl group (e.g., an aromatic or heteroaromatic group).

Aryl

[001240]"Aryl", as used herein, refers to C_5-C_{10}-membered aromatic, heterocyclic, fused aromatic, fused heterocyclic, biaromatic, or bihetereocyclic ring systems. Broadly defined, "aryl", as used herein, includes 5-, 6-, 7-, 8-, 9-, and 10-membered single-ring aromatic groups that may include from zero to four heteroatoms, for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. Those aryl groups having heteroatoms in the ring structure may also be referred to as "aryl heterocycles" or "heteroaromatics". The aromatic ring can be substituted at one or more ring positions with one or more substituents including, but not limited to, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxyl, amino (or quaternized amino), nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, ester, heterocyclyl, aromatic or heteroaromatic moieties, -CF_3, -CN; and combinations thereof.

[001241]The term "aryl" also includes polycyclic ring systems having two or more cyclic rings in which two or more carbons are common to two adjoining rings (i.e., "fused rings") wherein at least one of the rings is aromatic, e.g., the other cyclic ring or rings can be cycloalkyls, cycloalkenyls, cycloalkynyls, aryls and/or heterocycles. Examples of heterocyclic rings include, but are not limited to, benzimidazolyl, benzofuranyl, benzothiofuranyl, benzothiophenyl, benzoxazolyl, benzoxazolinyl, benzthiazolyl, benztriazolyl, benztetrazolyl, benzisoxazolyl, benzisothiazolyl, benzimidazolinyl, carbazolyl, 4aH carbazolyl, carbolinyl, chromanyl, chromenyl, cinnolinyl, decahydroquinolinyl, 2*H*,6*H*-1,5,2-dithiazinyl, dihydrofuro[2,3 b]tetrahydrofuran, furanyl, furazanyl, imidazolidinyl, imidazolinyl, imidazolyl, 1*H*-indazolyl, indolenyl, indolinyl, indolizinyl, indolyl, 3*H*-indolyl, isatinoyl, isobenzofuranyl, isochromanyl, isoindazolyl, isoindolinyl, isoindolyl, isoquinolinyl, isothiazolyl, isoxazolyl, methylenedioxyphenyl, morpholinyl, naphthyridinyl, octahydroisoquinolinyl, oxadiazolyl, 1,2,3-oxadiazolyl, 1,2,4-oxadiazolyl, 1,2,5-oxadiazolyl, 1,3,4-oxadiazolyl, oxazolidinyl, oxazolyl, oxindolyl, pyrimidinyl, phenanthridinyl, phenanthrolinyl, phenazinyl, phenothiazinyl, phenoxathinyl, phenoxazinyl, phthalazinyl, piperazinyl, piperidinyl, piperidonyl, 4-piperidonyl, piperonyl, pteridinyl, purinyl, pyranyl, pyrazinyl, pyrazolidinyl, pyrazolinyl, pyrazolyl, pyridazinyl, pyridooxazole, pyridoimidazole, pyridothiazole, pyridinyl, pyridyl, pyrimidinyl, pyrrolidinyl, pyrrolinyl, 2H-pyrrolyl, pyrrolyl, quinazolinyl, quinolinyl, 4*H*-quinolizinyl, quinoxalinyl, quinuclidinyl, tetrahydrofuranyl, tetrahydroisoquinolinyl, tetrahydroquinolinyl, tetrazolyl, 6*H*-1,2,5-thiadiazinyl, 1,2,3-thiadiazolyl, 1,2,4-thiadiazolyl, 1,2,5-thiadiazolyl, 1,3,4-thiadiazolyl, thianthrenyl, thiazolyl, thienyl, thienothiazolyl, thienooxazolyl, thienoimidazolyl, thiophenyl and xanthenyl. One or more of the rings can be substituted as defined above for "aryl".

Analogs

[001242]As used herein, "analogs" is meant to include polypeptide variants which differ by one or more amino acid alterations, for example, substitutions, additions or deletions of amino acid residues that still maintain one or more of the properties of the parent or starting polypeptide.

Antibodies

[001243]As used herein, the term "antibody" is referred to in the broadest sense and specifically covers various embodiments including, but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies formed from at least two intact antibodies), and antibody fragments (e.g., diabodies) so long as they exhibit a desired biological activity (e.g., "functional"). Antibodies are primarily amino-acid based molecules but may also comprise one or more modifications (including, but not limited to the addition of sugar moieties, fluorescent moieties, chemical tags, etc.). Non-limiting examples of antibodies or fragments thereof include VH and VL domains, scFvs, Fab, Fab', F(ab')2, Fv fragment, diabodies, linear antibodies, single chain antibody molecules, multispecific antibodies, bispecific antibodies, intrabodies, monoclonal antibodies, polyclonal antibodies, humanized antibodies, codon-optimized antibodies, tandem scFv antibodies, bispecific T-cell engagers, mAb2 antibodies, chimeric antigen receptors (CAR), tetravalent bispecific antibodies, biosynthetic antibodies, native antibodies, miniaturized antibodies, unibodies, maxibodies, antibodies to senescent cells, antibodies to conformers, antibodies to disease specific epitopes, or antibodies to innate defense molecules.

Antigen

[001244]As defined herein, the term "antigen" or "antibody generator" ("Ag") refers to a composition, for example, a substance or agent which causes an immune response in an organism, e.g., causes the immune response of the organism to produce antibodies against the substance or agent, in particular, which provokes an adaptive immune response in an organism. Antigens can be any immunogenic substance including, in particular, proteins, polypeptides, polysaccharides, nucleic acids, lipids and the like. Exemplary antigens are derived from infectious agents. Such agents can include parts or subunits of infectious agents, for example, coats, coat components, e.g., coat protein or polypeptides, surface components, e.g., surface proteins or polypeptides, capsule components, cell wall components, flagella, fimbrae, and/or toxins or toxoids) of infectious agents, for example, bacteria, viruses, and other microorganisms. Certain antigens, for example, lipids and/or nucleic acids are antigenic, preferably, when combined with proteins and/or polysaccharides.

Approximately

[001245]As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term

"approximately" or "about" refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

Associated with

[001246]As used herein, the terms "associated with," "conjugated," "linked," "attached," and "tethered," when used with respect to two or more moieties, means that the moieties are physically associated or connected with one another, either directly or via one or more additional moieties that serves as a linking agent, to form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, e.g., physiological conditions. An "association" need not be strictly through direct covalent chemical bonding. It may also suggest ionic or hydrogen bonding or a hybridization based connectivity sufficiently stable such that the "associated" entities remain physically associated.

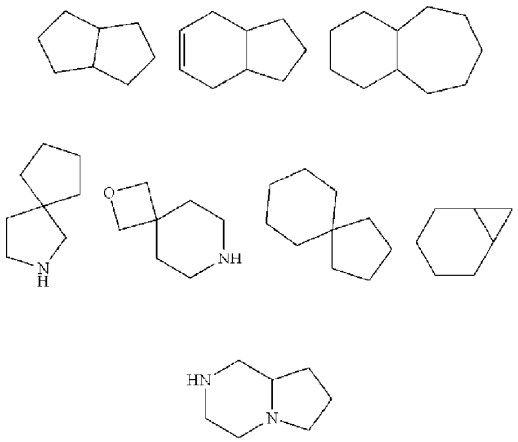
Associated

[001247]As used herein, the terms "associated with," "conjugated," "linked," "attached," and "tethered," when used with respect to two or more moieties, means that the moieties are physically associated or connected with one another, either directly or via one or more additional moieties that serves as a linking agent, to form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, e.g., physiological conditions. An "association" need not be strictly through direct covalent chemical bonding. It may also suggest ionic or hydrogen bonding or a hybridization based connectivity sufficiently stable such that the "associated" entities remain physically associated.

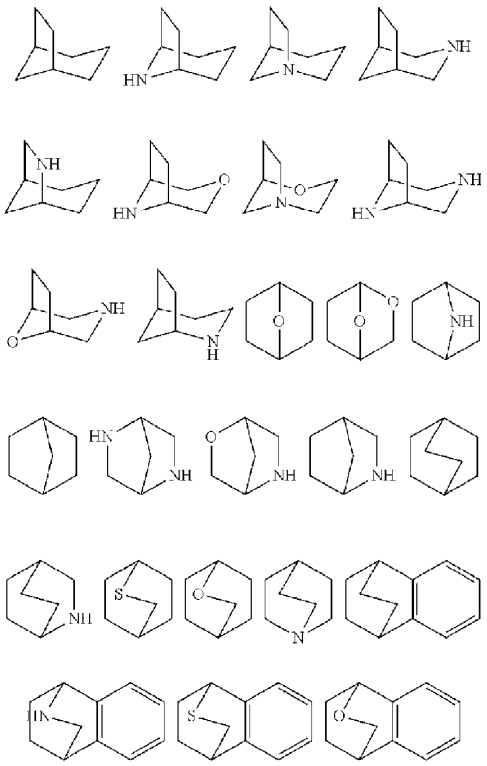
Bicyclic Ring

[001248]As used herein, the term "bicyclic ring" or "bicyclic ring system" refers to any bicyclic ring system, i.e. carbocyclic or heterocyclic, saturated or having one or more units of unsaturation, having one or more atoms in common between the two rings of the ring system. Thus, the term includes any permissible ring fusion, such as ortho-fused or spirocyclic. As used herein, the term "heterobicyclic" is a subset of "bicyclic" that requires that one or more heteroatoms are present in one or both rings of the bicycle. Such heteroatoms may be present at ring junctions and are optionally substituted, and may be selected from nitrogen (including N-oxides), oxygen, sulfur (including oxidized forms such as sulfones and sulfonates), phosphorus (including oxidized forms such as phosphonates and phosphates), boron, etc. In some embodiments, a bicyclic group has 7-12 ring members and 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. As used herein, the term "bridged bicyclic" refers to any bicyclic ring system, i.e. carbocyclic or heterocyclic, saturated or partially unsaturated, having at least one

bridge. As defined by IUPAC, a "bridge" is an unbranched chain of atoms or an atom or a valence bond connecting two bridgeheads, where a "bridgehead" is any skeletal atom of the ring system which is bonded to three or more skeletal atoms (excluding hydrogen). In some embodiments, a bridged bicyclic group has 7-12 ring members and 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. Such bridged bicyclic groups are well known in the art and include those groups set forth below where each group is attached to the rest of the molecule at any substitutable carbon or nitrogen atom. Unless otherwise specified, a bridged bicyclic group is optionally substituted with one or more substituents as set forth for aliphatic groups. Additionally or alternatively, any substitutable nitrogen of a bridged bicyclic group is optionally substituted. Exemplary bicyclic rings include:



[001249]Exemplary bridged bicyclics include:



Biologically active

[001250]As used herein, the phrase "biologically active" refers to a characteristic of any substance that has activity in a biological system and/or organism. For instance, a substance that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active. In particular embodiments, a polynucleotide of the present disclosure may be considered biologically active if even a portion of the polynucleotides is biologically active or mimics an activity considered biologically relevant.

Binding domain

[001251]By "binding domain" it is meant a protein domain that is able to bind non-covalently to another molecule. A binding domain can bind to, for example, a DNA molecule (a DNA-binding domain), an RNA molecule (an RNA-binding domain) and/or a protein molecule (a protein-binding domain). In the case of a protein having a protein-binding domain, it can in some cases bind to itself (to form homodimers, homotrimers, etc.) and/or it can bind to one or more regions of a different protein or proteins.

Bulge

[001252]As used herein, the term "bulge" refers to a small region of unpaired base(s) that interrupts a "stem" of base-paired nucleotides. The bulge may comprise one or two single-stranded or unbase-paired nucleotides joined at both ends by base-paired nucleotides of the stem. The bulge can be symmetrical (*viz.*, the two unbase-paired single-stranded regions have the same number of nucleotides), or asymmetrical (*viz.*, the unbase-paired single stranded region(s) have different or unequal numbers of nucleotides), or there is only one unbase-paired nucleotide on one strand. A bulge can be described as A/B (such as a "2/2 bulge," or a "1/0 bulge") wherein A represents the number of unpaired nucleotides on the upstream strand of the stem, and B represents the number of unpaired nucleotides on the downstream strand of the stem. An upstream strand of a bulge is more 5' to a downstream strand of the bulge in the primary nucleotide sequence.

CARs

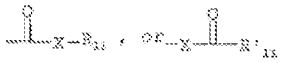
[001253]As used herein, the term "chimeric antigen receptor" or "CAR" refers to an artificial chimeric protein comprising at least one antigen specific targeting region (ASTR), a transmembrane domain and an intracellular signaling domain, wherein the antigen specific targeting region comprises a full-length antibody or a fragment thereof. Any molecule that is capable of binding a target antigen with high affinity can be used in the ASTR of a CAR. The CAR may optionally have an extracellular spacer domain and/or a co-stimulatory domain. A CAR may also be used to generate a cytotoxic cell carrying the CAR.

Carbocycle

[001254]The term "carbocycle," as used herein, refers to an aromatic or non-aromatic ring in which each atom of the ring is carbon.

Carbonyl

[001255]The term "carbonyl" is art-recognized and includes such moieties as can be represented by the general formula:



[001256]wherein X is a bond or represents an oxygen or a sulfur, and R_{11} represents a hydrogen, an alkyl, a cycloalkyl, an alkenyl, a cycloalkenyl, or an alkynyl, R'_{11} represents a hydrogen, an alkyl, a cycloalkyl, an alkenyl, a cycloalkenyl, or an alkynyl. Where X is an oxygen and R_{11} or R'_{11} is not hydrogen, the formula represents an "ester". Where X is an oxygen and R_{11} is as defined above, the moiety is referred to herein as a carboxyl group, and particularly when R_{11} is a hydrogen, the formula represents a "carboxylic acid". Where X is an oxygen and R'_{11} is hydrogen, the formula represents a "formate". In general, where the oxygen atom of the above formula is replaced by sulfur, the formula represents a "thiocarbonyl" group. Where X is a sulfur and R_{11} or R'_{11} is not hydrogen, the formula represents a "thioester." Where X

is a sulfur and R_{11} is hydrogen, the formula represents a "thiocarboxylic acid." Where X is a sulfur and R'_{11} is hydrogen, the formula represents a "thioformate." On the other hand, where X is a bond, and R_{11} is not hydrogen, the above formula represents a "ketone" group. Where X is a bond, and R_{11} is hydrogen, the above formula represents an "aldehyde" group.

Cargo or payload

[001257]As used herein, the term "cargo" or "payload" can refer to one or more molecules or structures encompassed in a delivery vehicle for delivery to or into a cell or tissue. Non-limiting examples of cargo can include a nucleic acid (e.g., mRNA, such as a linear or a circular mRNA), a polypeptide, a peptide, a protein, a liposome, a label, a tag, a small chemical molecule, a large biological molecule, and any combinations thereof.

Cationic lipid

[001258]As used herein, "cationic lipid" refers to any of a number of lipid species which carry a net positive charge at a selective pH, such as physiological pH.

cDNA

[001259]As used herein, the term "cDNA" refers to a strand of DNA copied from an RNA template, e.g., by a reverse transcriptase.

Circular RNA

[001260]As used herein, the terms "circular RNA" or "circRNA" or "oRNA" equivalently refer to a RNA that forms a circular structure through covalent or non-covalent bonds.

Co-administration

[001261]As used herein the term "co-administration" or "co-administering" refers to administration of the LNP adjuvant and an agonist or antigen concurrently, i.e., simultaneously in time, or sequentially, i.e., administration of an LNP adjuvant, followed by administration of the agonist or antigen. That is, after administration of the LNP adjuvant, the agonist or antigen can be administered substantially immediately after the LNP adjuvant or the agonist or antigen can be administered after an effective time period after the LNP adjuvant; the effective time period is the amount of time given for realization of maximum benefit from the administration of the LNP adjuvant. An effective time period can be determined experimentally and can be generally within 1, 2, 3, 5, 10, 15, 20, 25, 30, 45 or 60 minutes.

Complementary

[001262]As used herein, the term "complementary" refers to the ability of polynucleotides to form base pairs with one another. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands. Complementary polynucleotide strands can form base pair in the Watson-Crick manner (e.g., A to T, A to U, C to G), or in any other manner that allows for the formation of duplexes. As persons skilled in the art are aware, when using RNA as opposed to DNA, uracil rather

than thymine is the base that is considered to be complementary to adenosine. However, when a U is denoted in the context of the present disclosure, the ability to substitute a T is implied, unless otherwise stated. Perfect complementarity or 100% complementarity refers to the situation in which each nucleotide unit of one polynucleotide strand can form hydrogen bond with a nucleotide unit of a second polynucleotide strand. Less than perfect complementarity refers to the situation in which some, but not all, nucleotide units of two strands can form hydrogen bond with each other. For example, for two 20-mers, if only two base pairs on each strand can form hydrogen bond with each other, the polynucleotide strands exhibit 10% complementarity. In the same example, if 18 base pairs on each strand can form hydrogen bonds with each other, the polynucleotide strands exhibit 90% complementarity. As used herein, the term "substantially complementary" means that the siRNA has a sequence (e.g., in the antisense strand) which is sufficient to bind the desired target mRNA, and to trigger the RNA silencing of the target mRNA.

Compound or structure

[001263]The term "compound" or "structure," as used herein, is meant to include all stereoisomers, geometric isomers, tautomers, and isotopes of the structures depicted. The compounds or structures described herein can be asymmetric (e.g., having one or more stereocenters). All stereoisomers, such as enantiomers and diastereomers, are intended unless otherwise indicated. Compounds of the present disclosure that contain asymmetrically substituted carbon atoms can be isolated in optically active or racemic forms. Methods on how to prepare optically active forms from optically active starting materials are known in the art, such as by resolution of racemic mixtures or by stereoselective synthesis. Many geometric isomers of olefins, C=N double bonds, and the like can also be present in the compounds described herein, and all such stable isomers are contemplated in the present disclosure. Cis and trans geometric isomers of the compounds of the present disclosure are described and may be isolated as a mixture of isomers or as separated isomeric forms.

[001264]Compounds or structures of the present disclosure also include tautomeric forms. Tautomeric forms result from the swapping of a single bond with an adjacent double bond and the concomitant migration of a proton. Tautomeric forms include prototropic tautomers which are isomeric protonation states having the same empirical formula and total charge. Examples prototropic tautomers include ketone – enol pairs, amide – imidic acid pairs, lactam – lactim pairs, amide – imidic acid pairs, enamine – imine pairs, and annular forms where a proton can occupy two or more positions of a heterocyclic system, such as, 1H- and 3H-imidazole, 1H-, 2H- and 4H- 1,2,4-triazole, 1H- and 2H- isoindole, and 1H- and 2H-pyrazole. Tautomeric forms can be in equilibrium or sterically locked into one form by appropriate substitution.

[001265]Compounds or structures of the present disclosure also include all of the isotopes of the atoms occurring in the intermediate or final compounds. "Isotopes" refers to atoms having the same atomic number but different mass numbers resulting from a different number of neutrons in the nuclei. For example, isotopes of hydrogen include tritium and deuterium.

[001266]In some embodiments, a straight chain or branched chain alkyl has 30 or fewer carbon atoms in its backbone (e.g., C_1-C_{30} for straight chains, C_3-C_{30} for branched chains), 20 or fewer, 12 or fewer, or 7 or fewer. Likewise, in some embodiments cycloalkyls have from 3-10 carbon atoms in their ring structure, e.g., have 5, 6 or 7 carbons in the ring structure. The term "alkyl" (or "lower alkyl") as used throughout the specification, examples, and claims is intended to include both "unsubstituted alkyls" and "substituted alkyls", the latter of which refers to alkyl moieties having one or more substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents include, but are not limited to, halogen, hydroxyl, carbonyl (such as a carboxyl, alkoxycarbonyl, formyl, or an acyl), thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), alkoxyl, phosphoryl, phosphate, phosphonate, a hosphinate, amino, amido, amidine, imine, cyano, nitro, azido, sulfhydryl, alkylthio, sulfate, sulfonate, sulfamoyl, sulfonamido, sulfonyl, heterocyclyl, aralkyl, or an aromatic or heteroaromatic moiety.

[001267]Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to ten carbons, or from one to six carbon atoms in its backbone structure. Likewise, "lower alkenyl" and "lower alkynyl" have similar chain lengths. In some embodiments, alkyl groups are lower alkyls. In some embodiments, a substituent designated herein as alkyl is a lower alkyl.

Comprising / comprises

[001268]As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are present in a given embodiment, yet open to the inclusion of unspecified elements.

Conservative amino acid substitution

[001269]As used herein the term “conservative amino acid substitution” refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are

additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

Consisting essentially of

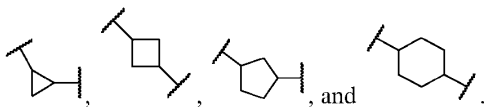
[001270]As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the disclosure.

Consisting of

[001271]The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

Cycloalkylenyl

[001272]The term "cycloalkylenyl" as used herein refers to a divalent radical of a cycloalkyl group. In one embodiment, the cycloalkylenyl is a divalent form of a C_{3-8} cycloalkyl, i.e., a C_3-C_8 cycloalkylenyl. Nonlimiting exemplary cycloalkylenyl groups include:

, and .

[001273]It will be understood by those skilled in the art that the moieties substituted on the hydrocarbon chain can themselves be substituted, if appropriate. For instance, the substituents of a substituted alkyl may include halogen, hydroxy, nitro, thiols, amino, azido, imino, amido, phosphoryl (including phosphonate and phosphinate), sulfonyl (including sulfate, sulfonamido, sulfamoyl and sulfonate), and silyl groups, as well as ethers, alkylthios, carbonyls (including ketones, aldehydes, carboxylates, and esters), $-CF_3$, -CN and the like. Cycloalkyls can be substituted in the same manner.

Delivery

[001274]As used herein, "delivery" refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload.

Derivative

[001275]The present disclosure provides several types of compositions that are polynucleotide or polypeptide based, including variants and derivatives. These include, for example, substitutional, insertional, deletion and covalent variants and derivatives. The term "derivative" is used synonymously with the term "variant" but generally refers to a molecule that has been modified and/or changed in any way relative to a reference molecule or starting molecule.

[001276]As such, polynucleotides encoding peptides or polypeptides containing substitutions, insertions and/or additions, deletions and covalent modifications with respect to reference sequences, in particular the polypeptide sequences disclosed herein, are included within the scope of this disclosure. For example, sequence tags or amino acids, such as one or more lysines, can be added to peptide sequences (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide detection, purification or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g., C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

DNA/RNA

[001277]As used herein, the term "RNA" or "RNA molecule" or "ribonucleic acid molecule" refers to a polymer of ribonucleotides; the term "DNA" or "DNA molecule" or "deoxyribonucleic acid molecule" refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally, e.g., by DNA replication and transcription of DNA, respectively; or be chemically synthesized. DNA and RNA can be single-stranded (i.e., ssRNA or ssDNA, respectively) or multi-stranded (e.g., double stranded, i.e., dsRNA and dsDNA, respectively). The term "mRNA" or "messenger RNA", as used herein, refers to a single stranded RNA that encodes the amino acid sequence of one or more polypeptide chains.

DNA-guided nuclease or nucleic acid programmable nuclease

[001278]As used herein, an “DNA-guided nuclease” is a type of “programmable nuclease,” and a specific type of “nucleic acid-guided nuclease.” Equivalent terms for purposes of this disclosure include “nucleic acid programmable nuclease.” An example of a DNA-guided nuclease is reported in Varshney et al., DNA-guided genome editing using structure-guided endonucleases, Genome Biology, 2016, 17(1), 187, which may be used in the context of the present disclosure and is incorporated herein by reference. As used herein, the term “DNA-guided nuclease” or “DNA-guided endonuclease” refers to a nuclease that associates covalently or non-covalently with a guide RNA thereby forming a complex between the guide RNA and the DNA-guided nuclease. The guide RNA comprises a spacer sequence which comprises a nucleotide sequence having complementarity with a strand of a target DNA sequence. Thus, the DNA-guided nuclease is indirectly guided or programmed to localize to a specific site in a DNA molecule through its association with the guide RNA, which directly binds or anneals to a strand of the target DNA through its complementarity region via Watson-Crick base-pairing. A “nucleic acid programmable

DNA-guided DNA binding protein or nucleic acid programmable DNA binding protein

[001279]As used herein, an "DNA-guided DNA binding protein" or "nucleic acid programmable DNA binding protein" has a similar meaning as a "DNA-guided nuclease" except that a "DNA binding protein" may include a nuclease but is not required to have a nuclease activity. By contrast to a nuclease, a nuclease domain (e.g., FokI) could be fused to a DNA binding protein top give rise to a DNA-guided nuclease as a fusion protein.

DNA regulatory sequences

[001280]As used herein, the terms "DNA regulatory sequences," "control elements," and "regulatory elements," can be used interchangeably herein to refer to transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, protein degradation signals, and the like, that provide for and/or regulate transcription of a non-coding sequence (e.g., guide RNA) or a coding sequence and/or regulate translation of a mRNA into an encoded polypeptide.

Domain

[001281]As used herein when referring to polypeptides the term "domain" refers to a motif of a polypeptide having one or more identifiable structural or functional characteristics or properties (e.g., binding capacity, serving as a site for protein-protein interactions).

Donor template DNA or template DNA

[001282]By a "donor template DNA" or "donor DNA" or "template DNA" it is meant a single-stranded or double-stranded DNA to be incorporated at a site cleaved by a programmable nuclease (e.g., a CRISPR/Cas effector protein; a TALEN; a ZFN; a meganuclease) (e.g., after dsDNA cleavage, after nicking a target DNA, after dual nicking a target DNA, and the like). The donor DNA can contain sufficient homology to a genomic sequence at the target site, e.g. 70%, 80%, 85%, 90%, 95%, or 100% homology with the nucleotide sequences flanking the target site, e.g., within about 200 bases or less of the target site, e.g., within about 190 bases or less of the target site, e.g., within about 180 bases or less of the target site, e.g., within about 170 bases or less of the target site, e.g., within about 160 bases or less of the target site, e.g., within about 150 bases or less of the target site, e.g., within about 140 bases or less of the target site, e.g., within about 130 bases or less of the target site, e.g., within about 120 bases or less of the target site, e.g., within about 110 bases or less of the target site, e.g., within about 100 bases or less of the target site, e.g., within about 90 bases or less of the target site, e.g., within about 80 bases or less of the target site, e.g., within about 70 bases or less of the target site, e.g., within about 60 bases or less of the target site, e.g., 50 bases or less of the target site, e.g., within about 30 bases, within about 15 bases, within about 10 bases, within about 5 bases, or immediately flanking the target site, to support integration into the cut site. In certain embodiments, integration of the donor template DNA occurs by way of homology-directed repair between the donor and the genomic sequence to which it bears homology.

Encapsulate

[001283]The terms "encapsulation" and "entrapped," as used herein, refer to the incorporation or association of the mRNA, DNA, siRNA or other nucleic acid pharmaceutical agent in or with a lipidic nanoparticle. As used herein, the term "encapsulated" refers to complete encapsulation or partial encapsulation. A siRNA may be capable of selectively knocking down or down regulating expression of a gene of interest. For example, an siRNA could be selected to silence a gene associated with a particular disease, disorder, or condition upon administration to a subject in need thereof of a nanoparticle composition including the siRNA. A siRNA may comprise a sequence that is complementary to an mRNA sequence that encodes a gene or protein of interest.

Encapsulation efficiency

[001284]As used herein, "encapsulation efficiency" refers to the amount of a therapeutic and/or prophylactic that becomes part of a nanoparticle composition, relative to theinitial total amount of therapeutic and/or prophylactic used in the preparation of a nanoparticle composition. For example, if 97 mg of a polynucleotide are encapsulated in a nanoparticle composition out of a total 100 mg of therapeutic and/or prophylactic initially provided to the composition, the encapsulation efficiency may be given as 97%. As used herein, "encapsulation" may refer to complete, substantial, or partial enclosure, confinement, surrounding, or encasement.

[001285]Throughout the disclosure, chemical substituents described in Markush structures are represented by variables. Where a variable is given multiple definitions as applied to different Markush formulas in different sections of the disclosure, it is to be understood that each definition should only apply to the applicable formula in the appropriate section of the disclosure.

Encode

[001286]As used herein the term "encode" refers broadly to any process whereby the information in a polymeric macromolecule is used to direct the production of a second molecule that is different from the first. The second molecule may have a chemical structure that is different from the chemical nature of the first molecule.

Exosome

[001287]As used herein, the term "exosomes" refer to small membrane bound vesicles with an endocytic origin. Without wishing to be bound by theory, exosomes are generally released into an extracellular environment from host/progenitor cells post fusion of multivesicular bodies the cellular plasma membrane. As such, exosomes can include components of the progenitor membrane in addition to designed components (*e.g.* engineered TnpB editing system). Exosome membranes are generally lamellar, composed of a bilayer of lipids, with an aqueous inter-nanoparticle space.

Features

[001288]"Features" when referring to polypeptide or polynucleotide are defined as distinct amino acid sequence-based or nucleotide-based components of a molecule respectively. Features of the polypeptides encoded by the polynucleotides include surface manifestations, local conformational shape, folds, loops, half-loops, domains, half-domains, sites, termini or any combination thereof.

Fusion

[001289]The term "fusion" as used herein as applied to a nucleic acid or polypeptide refers to two components that are defined by structures derived from different sources. For example, where "fusion" is used in the context of a fusion polypeptide (e.g., a fusion Cas9-RT protein), the fusion polypeptide includes amino acid sequences that are derived from different polypeptides. A fusion polypeptide may comprise either modified or naturally-occurring polypeptide sequences (e.g., a first amino acid sequence from a modified or unmodified Cas9-RT protein; and a second amino acid sequence from a modified or unmodified protein other than a Cas9-RT protein, etc.). Similarly, "fusion" in the context of a polynucleotide encoding a fusion polypeptide includes nucleotide sequences derived from different coding regions (e.g., a first nucleotide sequence encoding a modified or unmodified Cas9-RT protein; and a second nucleotide sequence encoding a polypeptide other than a Cas9-RT protein).

Fusion Polypeptide

[001290]The term "fusion polypeptide" refers to a polypeptide which is made by the combination (i.e., "fusion") of two otherwise separated segments of amino acid sequence, usually through human intervention.

Formulation

[001291]As used herein, a "formulation" includes at least one compound, substance, entity, moiety, cargo or payload and a delivery agent.

Fragment

[001292]A "fragment," as used herein, refers to a portion. For example, fragments of proteins may comprise polypeptides obtained by digesting full-length protein isolated from cultured cells.

Halogen

[001293]As used herein, "halogen" means Br, Cl, F and I.

Heteroalkyl

[001294]The term "heteroalkyl", as used herein, refers to straight or branched chain, or cyclic carbon-containing radicals, or combinations thereof, containing at least one heteroatom. Suitable heteroatoms include, but are not limited to, O, N, Si, P, Se, B, and S, wherein the phosphorous and sulfur atoms are optionally oxidized, and the nitrogen heteroatom is optionally quaternized. Heteroalkyls can be substituted as defined above for alkyl groups.

Heteroatom

[001295]The term "heteroatom" as used herein means an atom of any element other than carbon or hydrogen. Examples of heteroatoms are boron, nitrogen, oxygen, phosphorus, sulfur and selenium. Other useful heteroatoms include silicon and arsenic.

Heterocyclyl

[001296]As used herein, "heterocyclyl" or "heterocycle" means a 4- to 14-membered aromatic or nonaromatic heterocycle containing from 1 to 4 heteroatoms selected from the group consisting of O, N and S, and includes bicyclic groups. "Heterocyclyl" therefore includes, the following: benzoimidazolyl, benzofuranyl, benzofurazanyl, benzopyrazolyl, benzotriazolyl, benzothiophenyl, benzoxazolyl, carbazolyl, carbolinyl, cinnolinyl, furanyl, imidazolyl, indolinyl, indolyl, indolazinyl, indazolyl, isobenzofuranyl, isoindolyl, isoquinolyl, isothiazolyl, isoxazolyl, naphthpyridinyl, oxadiazolyl, oxazolyl, oxazoline, isoxazoline, oxetanyl, pyranyl, pyrazinyl, pyrazolyl, pyridazinyl, pyridopyridinyl, pyridazinyl, pyridyl, pyrimidyl, pyrrolyl, quinazolinyl, quinolyl, quinoxalinyl, tetrahydropyranyl, tetrazolyl, tetrazolopyridyl, thiadiazolyl, thiazolyl, thienyl, triazolyl, azetidinyl, 1,4-dioxanyl, hexahydroazepinyl, piperazinyl, piperidinyl, pyrrolidinyl, morpholinyl, thiomorpholinyl, dihydrobenzoimidazolyl, dihydrobenzofuranyl, dihydrobenzothiophenyl, dihydrobenzoxazolyl, dihydrofuranyl, dihydroimidazolyl, dihydroindolyl, dihydroisooxazolyl, dihydroisothiazolyl, dihydrooxadiazolyl, dihydrooxazolyl, dihydropyrazinyl, dihydropyrazolyl, dihydropyridinyl, dihydropyrimidinyl, dihydropyrrolyl, dihydroquinolinyl, dihydrotetrazolyl, dihydrothiadiazolyl, dihydrothiazolyl, dihydrothienyl, dihydrotriazolyl, dihydroazetidinyl, methylenedioxybenzoyl, tetrahydrofuranyl, and tetrahydrothienyl, and N-oxides thereof all of which are optionally substituted with one to three substituents selected from R″.

Heterocycle

[001297]"Heterocycle" or "heterocyclic," as used herein, refers to a cyclic radical attached via a ring carbon or nitrogen of a monocyclic, bicyclic, or polycyclic ring containing 3-14 ring atoms, for example, from 5-6 ring atoms, consisting of carbon and one to four heteroatoms each selected from the group consisting of non-peroxide oxygen, sulfur, and N(Y) wherein Y is absent or is H, O, (C_1-C_{10}) alkyl, phenyl or benzyl, and optionally containing 1-3 double bonds and optionally substituted with one or more substituents. Examples of heterocyclic rings include, but are not limited to, benzimidazolyl, benzofuranyl, benzothiofuranyl, benzothiophenyl, benzoxazolyl, benzoxazolinyl, benzthiazolyl, benztriazolyl, benztetrazolyl, benzisoxazolyl, benzisothiazolyl, benzimidazolinyl, carbazolyl, 4a*H*-carbazolyl, carbolinyl, chromanyl, chromenyl, cinnolinyl, decahydroquinolinyl, 2*H*,6*H*-1,5,2-dithiazinyl, dihydrofuro[2,3-*b*]tetrahydrofuran, furanyl, furazanyl, imidazolidinyl, imidazolinyl, imidazolyl, 1*H*-indazolyl, indolenyl, indolinyl, indolizinyl, indolyl, 3H-indolyl, isatinoyl, isobenzofuranyl, isochromanyl,

isoindazolyl, isoindolinyl, isoindolyl, isoquinolinyl, isothiazolyl, isoxazolyl, methylenedioxyphenyl, morpholinyl, naphthyridinyl, octahydroisoquinolinyl, oxadiazolyl, 1,2,3-oxadiazolyl, 1,2,4-oxadiazolyl, 1,2,5-oxadiazolyl, 1,3,4-oxadiazolyl, oxazolidinyl, oxazolyl, oxepanyl, oxetanyl, oxindolyl, pyrimidinyl, phenanthridinyl, phenanthrolinyl, phenazinyl, phenothiazinyl, phenoxathinyl, phenoxazinyl, phthalazinyl, piperazinyl, piperidinyl, piperidonyl, 4-piperidonyl, piperonyl, pteridinyl, purinyl, pyranyl, pyrazinyl, pyrazolidinyl, pyrazolinyl, pyrazolyl, pyridazinyl, pyridooxazole, pyridoimidazole, pyridothiazole, pyridinyl, pyridyl, pyrimidinyl, pyrrolidinyl, pyrrolinyl, 2H-pyrrolyl, pyrrolyl, quinazolinyl, quinolinyl, 4*H*-quinolizinyl, quinoxalinyl, quinuclidinyl, tetrahydrofuranyl, tetrahydroisoquinolinyl, tetrahydropyranyl, tetrahydroquinolinyl, tetrazolyl, 6*H*-1,2,5-thiadiazinyl, 1,2,3-thiadiazolyl, 1,2,4-thiadiazolyl, 1,2,5-thiadiazolyl, 1,3,4-thiadiazolyl, thianthrenyl, thiazolyl, thienyl, thienothiazolyl, thienooxazolyl, thienoimidazolyl, thiophenyl and xanthenyl. Heterocyclic groups can optionally be substituted with one or more substituents at one or more positions as defined above for alkyl and aryl, for example, halogen, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, amino, nitro, sulfhydryl, imino, amido, phosphate, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, ketone, aldehyde, ester, a heterocyclyl, an aromatic or heteroaromatic moiety, -CF3, and -CN. Examples of bicyclic or polycyclic heterocycles include bridged, fused, and spirocyclic heterocycles.

Homology

[001298]As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, e.g. between polynucleotide molecules (e.g. DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical or similar. The term "homologous" necessarily refers to a comparison between at least two sequences (polynucleotide or polypeptide sequences). In accordance with the disclosure, two polynucleotide sequences are considered to be homologous if the polypeptides they encode are at least about 50%, 60%, 70%, 80%, 90%, 95%, or even 99% for at least one stretch of at least about 20 amino acids. In some embodiments, homologous polynucleotide sequences are characterized by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. For polynucleotide sequences less than 60 nucleotides in length, homology is determined by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. In accordance with the disclosure, two protein sequences are considered to be homologous if the proteins are at least about 50%, 60%, 70%, 80%, or 90% identical for at least one stretch of at least about 20 amino acids. Homology implies that the compared sequences diverged in evolution from a common origin. The term “homolog” refers to a first amino acid sequence or nucleic acid sequence (e.g., gene (DNA or RNA) or protein sequence) that is related to a second amino acid sequence or nucleic acid sequence by descent from a

common ancestral sequence. The term "homolog" may apply to the relationship between genes and/or proteins separated by the event of speciation or to the relationship between genes and/or proteins separated by the event of genetic duplication. "Orthologs" are genes (or proteins) in different species that evolved from a common ancestral gene (or protein) by speciation. Typically, orthologs retain the same function in the course of evolution. "Paralogs" are genes (or proteins) related by duplication within a genome. Orthologs retain the same function in the course of evolution, whereas paralogs evolve new functions, even if these are related to the original one.

[001299]Homology implies that the compared sequences diverged in evolution from a common origin. The term "homolog" refers to a first amino acid sequence or nucleic acid sequence (e.g., gene (DNA or RNA) or protein sequence) that is related to a second amino acid sequence or nucleic acid sequence by descent from a common ancestral sequence. The term "homolog" may apply to the relationship between genes and/or proteins separated by the event of speciation or to the relationship between genes and/or proteins separated by the event of genetic duplication. "Orthologs" are genes (or proteins) in different species that evolved from a common ancestral gene (or protein) by speciation. Typically, orthologs retain the same function in the course of evolution. "Paralogs" are genes (or proteins) related by duplication within a genome. Orthologs retain the same function in the course of evolution, whereas paralogs evolve new functions, even if these are related to the original one.

Homology-directed repair

[001300]As used herein, "homology-directed repair (HDR)" refers to the specialized form DNA repair that takes place, for example, during repair of double-strand breaks in cells. This process requires nucleotide sequence homology, uses a "donor" molecule to template repair of a "target" molecule (i.e., the one that experienced the double-strand break), and leads to the transfer of genetic information from the donor to the target. Homology-directed repair may result in an alteration of the sequence of the target molecule (e.g., insertion, deletion, mutation), if the donor polynucleotide differs from the target molecule and part or all of the sequence of the donor polynucleotide is incorporated into the targeted polynucleotide sequence.

Identity

[001301]The term "identity" refers to the overall relatedness between polymeric molecules, for example, between polynucleotide molecules (e.g. DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two polynucleic acid sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at

least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleic acid sequences can be determined using methods such as those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; each of which is incorporated herein by reference. For example, the percent identity between two nucleic acid sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4:11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleic acid sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix. Methods commonly employed to determine percent identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., SIAM J Applied Math., 48:1073 (1988); incorporated herein by reference. Techniques for determining identity are codified in publicly available computer programs. Exemplary computer software to determine homology between two sequences include, but are not limited to, GCG program package, Devereux, J., et al., Nucleic Acids Research, 12(1), 387 (1984)), BLASTP, BLASTN, and FASTA Altschul, S. F. et al., J. Molec. Biol., 215, 403 (1990)).

Immunogenic

[001302]As used herein, the term "immunogenic" refers to a potential to induce an immune response to a substance. An immune response may be induced when an immune system of an organism or a certain type of immune cell is exposed to an immunogenic substance. The term "non-immunogenic" refers to a lack of or absence of an immune response above a detectable threshold to a substance. No immune response is detected when an immune system of an organism or a certain type of immune cell is exposed to a non-immunogenic substance. In some embodiments, a non-immunogenic circular polyribonucleotide as provided herein, does not induce an immune response above a pre-determined threshold when measured by an immunogenicity assay. In some embodiments, no innate immune response is detected

when an immune system of an organism or a certain type of immune cell is exposed to a non-immunogenic circular polyribonucleotide as provided herein. In some embodiments, no adaptive immune response is detected when an immune system of an organism or a certain type of immune cell is exposed to a non-immunogenic circular polyribonucleotide as provided herein.

Ionizable lipid

[001303]As used herein "ionizable lipid" refers to any of a number of lipid species that carry a net positive charge at a selected pH.

IRES

[001304]As used herein, the term "internal ribosome entry site" or "IRES" refers to an RNA sequence or structural element ranging in size form 10 nucleotides to 1,000 nucleotides or more which is capable of initiating translation of a polypeptide in the absence of a normal RNA cap structure. In other words, IRES are sequences that can recruit ribosomes and allow cap-independent translation, which can link two coding sequences in one bicistronic vector and allow the translation of both proteins. See Kozak M, "A second look at cellular mRNA sequences said to function as internal ribosome entry sites," *Nucleic Acids Research*, 2005, Vol.33: pp.6593–6602 (incorporated herein by reference).

Linker

[001305]As used herein, the term"linker" refers to a molecule linking or joining two other molecules or moieties. The linker can be an amino acid sequence in the case of a linker joining two fusion proteins. For example, a TnpB protein can be fused to an accessory protein (e.g., a deaminase, nuclease, ligase, reverse transcriptase, recombinase, etc.) by an amino acid linker sequence. The linker can also be a nucleotide sequence in the case of joining two nucleotide sequences together. For example, in the instant case, a reRNA at its 5′ and/or 3′ ends may be linked by a nucleotide sequence linker to one or more other functional nucleic acid molecules, such as guide RNAs or HDR donor molecules. In other embodiments, the linker is an organic molecule, group, polymer, or chemical moiety. In some embodiments, the linker is 5-100 amino acids in length, for example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 30-35, 35-40, 40- 45, 45-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-150, or 150-200 amino acids in length. Longer or shorter linkers are also contemplated.

Lipid conjugate

[001306]The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, polysarcosine (see e.g. WO2021191265A1 which is herein incorporated by reference in its entirety for all purposes), polyamide oligomers (e.g., ATTA-lipid conjugates), PEG-lipid conjugates, such as PEG coupled to di alkyl oxy propyls, PEG coupled to diacylglycerols, PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, PEG conjugated to ceramides (see, e.g., U.S. Pat. No. 5,885,613, the disclosure of which is herein incorporated

by reference in its entirety for all purposes), cationic PEG lipids, and mixtures thereof. PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, e.g., non-ester containing linker moieties and ester-containing linker moieties. In preferred embodiments, non-ester containing linker moieties are used.

Lipid nanoparticle (LNP)

[001307]The term "lipid nanoparticle", or "LNP", refers to particles having a diameter of from about 5 to 500 nm. In some embodiments, lipid nanoparticle refers to any lipid composition that can be used to deliver a prophylactic product, preferably vaccine antigens, including, but not limited to, liposomes or vesicles, wherein an aqueous volume is encapsulated by amphipathic lipid bilayers (e.g., single; unilamellar or multiple; multilamellar), or, in other embodiments, wherein the lipids coat an interior comprising a prophylactic product, or lipid aggregates or micelles, wherein the lipid encapsulated therapeutic product is contained within a relatively disordered lipid mixture. Except where noted, the lipid nanoparticle does not need to have antigen incorporated therein and may be used to deliver a prophylactic product when in the same formulation.

[001308]In some embodiments, the active agent (e.g., RNA payload encoding a polypeptide, such as an antigen or therapeutic protein) is encapsulated into the LNP. In some embodiments, the active agent can be an anionic compound, for example, but not limited to DNA, RNA, natural and synthetic oligonucleotides (including antisense oligonucleotides, interfering RNA and small interfering RNA), nucleoprotein, peptide, nucleic acid, ribozyme, DNA- containing nucleoprotein, such as an intact or partially deproteinated viral particles (virions), oligomeric and polymeric anionic compounds other than DNA (for example, acid polysaccharides and glycoproteins)). In some embodiments, the active agent can be intermixed with an adjuvant.

[001309]In a LNP vaccine product described herein, the active agent is generally contained in the interior of the LNP. In some embodiments, the active agent comprises a nucleic acid (e.g., a circular or linear mRNA). Typically, water soluble nucleic acids are condensed with cationic lipids or polycationic polymers in the interior of the particle and the surface of the particle is enriched in neutral lipids or PEG-lipid derivatives. Additional ionizable cationic lipid may also be at the surface and respond to acidification in the environment by becoming positively charged, facilitating endosomal escape. Lipid components of the herein disclosed LNPs are described herein.

[001310]Release of nucleic acids from LNP formulations, among other characteristics such as liposomal clearance and circulation half-life, can be modified by the presence of polyethylene glycol and/or sterols (e.g., cholesterol) or other potential additives in the LNP, as well as the overall chemical structure, including pKa of any ionizable cationic lipid included as part of the formulation.

Liposome

[001311]As used herein "liposome" generally refers to a vesicle composed of lipids (e.g., amphiphilic lipids) arranged in one or more spherical bilayers or bilayers.

Modified

[001312]As used herein "modified" refers to a changed state or structure of a molecule. Molecules may be modified in many ways including chemically, structurally, and functionally.

Modulating

[001313]By the term "modulating," as used herein, is meant mediating a detectable increase or decrease in the level of a response in a subject compared with the level of a response in the subject in the absence of a treatment or compound, and/or compared with the level of a response in an otherwise identical but untreated subject. The term encompasses perturbing and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, preferably, a human.

Naturally-occurring

[001314]The term "naturally-occurring" or "unmodified" or "wild type" as used herein as applied to a nucleic acid, a polypeptide, a cell, or an organism, refers to a nucleic acid, polypeptide, cell, or organism that is found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism that can be isolated from a source in nature is naturally occurring.

Non-homologous end joining

[001315]As used herein, "non-homologous end joining (NHEJ)" refers to the repair of double-strand breaks in DNA by direct ligation of the break ends to one another without the need for a homologous template (in contrast to homology-directed repair, which requires a homologous sequence to guide repair). NHEJ often results in the loss (deletion) of nucleotide sequence near the site of the double-strand break.

Nuclear localization sequence (NLS)

[001316]As used herein, the term"nuclear localization sequence" or"NLS" refers to an amino acid sequence that promotes import of a protein (e.g., a RNA-guided nuclease) into the cell nucleus, for example, by nuclear transport. Nuclear localization sequences are known in the art. For example, NLS sequences are described in Plank et al., international PCT application, PCT/EP2000/011690, filed November 23, 2000, published as WO/2001/038547 on May 31, 2001, the contents of which are incorporated herein by reference for its disclosure of exemplary nuclear localization sequences.

Nuclease

[001317]"Nuclease" and "endonuclease" are used interchangeably herein to mean an enzyme which possesses catalytic activity for nucleic acid cleavage (e.g., ribonuclease activity (ribonucleic acid cleavage), deoxyribonuclease activity (deoxyribonucleic acid cleavage), etc.).

Nucleic acid

[001318]As used herein, the term "nucleic acid" or "nucleic acid molecule" or "nucleic acid sequence" or "polynucleotide" generally refer to deoxyribonucleic or ribonucleic oligonucleotides in either single- or double-stranded form. The term may (or may not) encompass oligonucleotides containing known analogues of natural nucleotides. The term also may (or may not) encompass nucleic acid-like structures with synthetic backbones, see, *e.g.*, Eckstein, 1991; Baserga et ah, 1992; Milligan, 1993; WO 97/03211; WO 96/39154; Mata, 1997; Strauss-Soukup, 1997; and Samstag, 1996. The term encompasses both ribonucleic acid (RNA) and DNA, including cDNA, genomic DNA, synthetic, synthesized (*e.g.*, chemically synthesized) DNA, and/or DNA (or RNA) containing nucleic acid analogs. The nucleotides Adenine (A), Thymine (T), Guanine (G) and Cytosine (C) also may (or may not) encompass nucleotide modifications, *e.g.*, methylated and/or hydroxylated nucleotides, *e.g.*, Cytosine (C) encompasses 5-methylcytosine and 5- hydroxymethylcytosine.

Nucleic acid loop

[001319]As used herein, the term "loop" in the polynucleotide refers to a single stranded stretch of one or more nucleotides, such as 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides, wherein the most 5' nucleotide and the most 3' nucleotide of the loop are each linked to a base-paired nucleotide in a stem.

Nucleic acid stem

[001320]As used herein, the term "stem" refers to two or more base pairs, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs, formed by inverted repeat sequences connected at a "tip," where the more 5' or "upstream" strand of the stem bends to allows the more 3' or "downstream"strand to base-pair with the upstream strand. The number of base pairs in a stem is the "length" of the stem. The tip of the stem is typically at least 3 nucleotides, but can be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more nucleotides. Larger tips with more than 5 nucleotides are also referred to as a "loop." An otherwise continuous stem may be interrupted by one or more bulges as defined herein. The number of unpaired nucleotides in the bulge(s) are not included in the length of the stem. The position of a bulge closest to the tip can be described by the number of base pairs between the bulge and the tip (*e.g.*, the bulge is 4 bps from the tip). The position of the other bulges (if any) further away from the tip can be described by the number of base pairs in the stem between the bulge in question and the tip, excluding any unpaired bases of other bulges in between.

Nitro

[001321]As used herein, the term "nitro" means $-NO_2$; the term "halogen" designates -F, -Cl, -Br or -I; the term "sulfhydryl" means -SH; the term "hydroxyl" means -OH; and the term "sulfonyl" means $-SO_2-$.

Non-cationic lipid

[001322]As used herein "non-cationic lipid" refers to any neutral, zwitterionic or anionic lipid.

Open reading frame

[001323]An "open reading frame" is a continuous stretch of DNA beginning with a start codon (e.g., methionine (ATG)), and ending with a stop codon (e.g., TAA, TAG or TGA) and encodes a polypeptide.

Orthologs

[001324]As used herein, "orthologs" refers to genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution. Identification of orthologs is critical for reliable prediction of gene function in newly sequenced genomes.

pegRNA

[001325]As used herein, the terms "prime editing guide RNA" or "pegRNA" or "PEgRNA" or "extended guide RNA" refer to a specialized form of a guide RNA that has been modified to include one or more additional sequences for implementing the prime editing methods and compositions described herein. As described herein, the prime editing guide RNA comprise one or more "extended regions" of nucleic acid sequence. The extended region comprises a "DNA synthesis template" which encodes (by the polymerase of the prime editor) a single-stranded DNA which, in turn, has been designed to be (a) homologous with the endogenous target DNA to be edited, and (b) which comprises at least one desired nucleotide change (e.g., a transition, a transversion, a deletion, or an insertion) to be introduced or integrated into the endogenous target DNA. The extended region may also comprise other functional sequence elements, such as, but not limited to, a "primer binding site" and a "spacer or linker" sequence, or other structural elements, such as, but not limited to aptamers, stem loops, hairpins, toe loops (e.g., a 3′ toeloop), or an RNA-protein recruitment domain (e.g., MS2 hairpin). As used herein the "primer binding site" comprises a sequence that hybridizes to a single-strand DNA sequence having a 3′ end generated from the nicked DNA of the R-loop.

PE1

[001326]As used herein, "PE1" refers to a PE complex comprising a fusion protein comprising Cas9(H840A) and a wild type MMLV RT having the following structure: [NLS]-[Cas9(H840A)]-[linker]-[MMLV_RT(wt)] + a desired pegRNA, wherein the PE fusion has the amino acid sequence of SEQ ID NO: 189.

PE2

[001327]As used herein, "PE2" refers to a PE complex comprising a fusion protein comprising Cas9(H840A) and a variant MMLV RT having the following structure: [NLS]-[Cas9(H840A)]-[linker]-[MMLV_RT(D200N)(T330P)(L603W)(T306K)(W313F)] + a desired pegRNA, wherein the PE fusion has the amino acid sequence of SEQ ID NO: 190.

Pharmaceutically acceptable salt

[001328]The term "pharmaceutically acceptable salt" refers to a relatively non-toxic, inorganic or organic acid addition salt of a compound of the present disclosure which salt possesses the desired pharmacological activity.

Pharmaceutically acceptable carrier, diluent, or excipient

[001329]As used herein, the term "pharmaceutically acceptable carrier, diluent or excipient" includes without limitation any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans or domestic animals. Various aspects and embodiments are described in further detail in the following subsections.

Pharmaceutical composition

[001330]As used herein the term "pharmaceutical composition" refers to compositions comprising at least one active ingredient (e.g., an LNP encapsulated with a mRNA payload) and optionally one or more pharmaceutically acceptable excipients.

PEG

[001331]As used herein "PEG" means any polyethylene glycol or other polyalkylene ether polymer.

Peptide

[001332]As used herein, "peptide" is less than or equal to 50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

PolyA tail

[001333]A "polyA tail" is a region of mRNA that is downstream, e.g., directly downstream (i.e., 3′), from the 3′ UTR that contains multiple, consecutive adenosine monophosphates. A polyA tail may contain 10 to 300 adenosine monophosphates. For example, a polyA tail may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290 or 300 adenosine monophosphates. In some embodiments, a polyA tail contains 50 to 250 adenosine monophosphates. In a relevant biological setting (e.g., in cells, in vivo) the poly(A) tail functions to protect mRNA from enzymatic degradation, e.g., in the cytoplasm, and aids in transcription termination, export of the mRNA from the nucleus and translation.

Polyamine

[001334]As used herein, "polyamine" means compounds having two or more amino groups. Examples include putrescine, cadaverine, spermidine, and spermine.

Polypeptide variant

[001335]The term "polypeptide variant" refers to molecules which differ in their amino acid sequence from a native or reference sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence, as compared to a native or reference sequence. Ordinarily, variants possess at least 50% identity to a native or reference sequence. In some embodiments, variants share at least 80%, or at least 90% identity with a native or reference sequence.

Prime editor

[001336]The term "prime editor (PE)" refers to the polypeptide or polypeptide components involved in prime editing, or any polynucleotide(s) encoding the polypeptide or polypeptide components. In various embodiments, a prime editor includes a polypeptide domain having DNA binding activity and a polypeptide domain having DNA polymerase activity.

Protein fragment, function protein domains, homologous proteins

[001337]As recognized by those skilled in the art, protein fragments, functional protein domains, and homologous proteins are also considered to be within the scope of polypeptides of interest. For example, provided herein is any protein fragment (meaning a polypeptide sequence at least one amino acid residue shorter than a reference polypeptide sequence but otherwise identical) of a reference protein 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or greater than 100 amino acids in length. In another example, any protein that includes a stretch of 20, 30, 40, 50, or 100 amino acids which are 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% identical to any of the sequences described herein can be utilized in accordance with the disclosure. In some embodiments, a polypeptide includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein.

RNA

[001338]The term "RNA" is a well-known term of art that refers to ribonucleic acid.

RNA-guided nuclease

[001339]As used herein, an "RNA-guided nuclease" is a type of "programmable nuclease," and a specific type of "nucleic acid-guided nuclease." As used herein, the term "RNA-guided nuclease" or "RNA-guided endonuclease" refers to a nuclease that associates covalently or non-covalently with a guide RNA thereby forming a complex between the guide RNA and the RNA-guided nuclease. The guide RNA comprises a spacer sequence which comprises a nucleotide sequence having complementarity with a strand of a target DNA sequence. Thus, the RNA-guided nuclease is indirectly guided or programmed to localize to a specific site in a DNA molecule through its association with the guide RNA, which directly binds or anneals to a strand of the target DNA through its complementarity region via Watson-Crick base-pairing.

Sequence identity

[001340]As used herein, the term "sequence identity" refers to the overall relatedness between polymeric molecules, *e.g.*, between polynucleotide molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two polynucleotide sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). For example, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleotide sequences can be determined using methods such as those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; each of which is incorporated herein by reference. For example, the percent identity between two nucleotide sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4:11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleotide sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna. CMP matrix. Methods commonly employed to determine percent identity between sequences include, but are not limited to those disclosed in Carillo, H. and Lipman, D., SIAM J Applied Math., 48:1073 (1988); incorporated herein by reference. Techniques for determining identity are codified in publicly available computer programs. Exemplary computer software to determine homology between two sequences include, but are not limited to, GCG program package, Devereux, J., et al., Nucleic Acids Research, 12(1), 387 (1984)), BLASTP, BLASTN, and FASTA Altschul, S. F. et al., J. Molec. Biol., 215, 403 (1990).

Spacer

[001341]As used herein the term "spacer" refers to a region of a polynucleotide or polypeptide ranging from 1 residue to hundreds or thousands of residues separating two other elements in a sequence. The sequence of the spacer can be defined or random. A spacer sequence is typically non-coding but may be a coding sequence.

Stereoisomers

[001342]The compounds of the disclosure may contain one or more chiral centers and, therefore, exist as stereoisomers. The term "stereoisomers" when used herein consist of all enantiomers or diastereomers. These compounds may be designated by the symbols "R" or "S," depending on the configuration of substituents around the stereogenic carbon atom, but the skilled artisan will recognize that a structure may denote a chiral center implicitly. These compounds may also be designated by "(+)" and "(-)" based on their optical rotation properties. The presently described compounds encompasses various stereoisomers of these compounds and mixtures thereof. Mixtures of enantiomers or diastereomers may be designated by the symbol "(±)" in nomenclature, but the skilled artisan will recognize that a structure may denote a chiral center implicitly.

Structural lipid

[001343]As used herein "structural lipid" refers to sterols and lipids containing sterol moieties.

Subject

[001344]As used herein, the term"subject" refers to an individual organism, for example, an individual mammal or plant. In some embodiments, the subject is a human. In some embodiments, the subject is a non-human mammal. In some embodiments, the subject is a non-human primate. In some embodiments, the subject is a rodent. In some embodiments, the subject is a sheep, a goat, a cattle, a cat, or a dog. In some embodiments, the subject is a vertebrate, an amphibian, a reptile, a fish, an insect, a fly, or a nematode. In some embodiments, the subject is a research animal. In some embodiments, the subject is genetically engineered, e.g., a genetically engineered non-human subject. The subject may be of either sex and at any stage of development. The terms "individual," "subject," "host," and "patient," used interchangeably herein.

Substituted

[001345]The term "substituted" as used herein, refers to all permissible substituents of the compounds described herein. In the broadest sense, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. Illustrative substituents include, but are not limited to, halogens, hydroxyl groups, or any other organic groupings containing any number of carbon atoms, for example, 1-14 carbon atoms, and optionally include one or more heteroatoms such as oxygen, sulfur, or nitrogen grouping in linear,

branched, or cyclic structural formats. Representative substituents include alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, phenyl, substituted phenyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, halo, hydroxyl, alkoxy, substituted alkoxy, phenoxy, substituted phenoxy, aroxy, substituted aroxy, alkylthio, substituted alkylthio, phenylthio, substituted phenylthio, arylthio, substituted arylthio, cyano, isocyano, substituted isocyano, carbonyl, substituted carbonyl, carboxyl, substituted carboxyl, amino, substituted amino, amido, substituted amido, sulfonyl, substituted sulfonyl, sulfonic acid, phosphoryl, substituted phosphoryl, phosphonyl, substituted phosphonyl, polyaryl, substituted polyaryl, C_3-C_{20} cyclic, substituted C_3-C_{20} cyclic, heterocyclic, substituted heterocyclic, aminoacid, peptide, and polypeptide groups.

[001346]As described herein, compounds of the present disclosure may contain "optionally substituted" moieties. In general, the term "substituted", whether preceded by the term "optionally" or not, means that one or more hydrogens of the designated moiety are replaced with a suitable substituent. Unless otherwise indicated, an "optionally substituted" group may have a suitable substituent at each substitutable position of the group, and when more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at every position. Combinations of substituents envisioned by this disclosure are preferably those that result in the formation of stable or chemically feasible compounds. The term "stable", as used herein, refers to compounds that are not substantially altered when subjected to conditions to allow for their production, detection, and, in certain embodiments, their recovery, purification, and use for one or more of the purposes disclosed herein.

[001347]Suitable monovalent substituents on a substitutable carbon atom of an "optionally substituted" group are independently halogen; —$(CH_2)_{0-4}R^\circ$; —$(CH_2)_{0-4}OR^\circ$; —$O(CH_2)_{0-4}R^\circ$, —O—$(CH_2)_{0-4}C(O)OR^\circ$; —$(CH_2)_{0-4}CH(OR^\circ)_2$; —$(CH_2)_{0-4}SR^\circ$; —$(CH_2)_{0-4}Ph$, which may be substituted with R°; —$(CH_2)_{0-4}O(CH_2)_{0-1}Ph$ which may be substituted with R°; —CH=CHPh, which may be substituted with R°; —$(CH_2)_{0-4}O(CH_2)_{0-1}$-pyridyl which may be substituted with R°; —NO_2; —CN; —N_3; —$(CH_2)_{0-4}N(R^\circ)_2$; —$(CH_2)_{0-4}N(R^\circ)C(O)R^\circ$; —$N(R^\circ)C(S)R^\circ$; —$(CH_2)_{0-4}N(R^\circ)C(O)NR^\circ_2$; —$N(R^\circ)C(S)NR^\circ_2$; —$(CH_2)_{0-4}N(R^\circ)C(O)OR^\circ$; —$N(R^\circ)N(R^\circ)C(O)R^\circ$; —$N(R^\circ)N(R^\circ)C(O)NR^\circ_2$; —$N(R^\circ)N(R^\circ)C(O)OR^\circ$; —$(CH_2)_{0-4}C(O)R^\circ$; —$C(S)R^\circ$; —$(CH_2)_{0-4}C(O)OR^\circ$; —$(CH_2)_{0-4}C(O)SR^\circ$; —$(CH_2)_{0-4}C(O)OSiR^\circ_3$; —$(CH_2)_{0-4}OC(O)R^\circ$; —$OC(O)(CH_2)_{0-4}SR^\circ$, SC(S)SR°; —$(CH_2)_{0-4}SC(O)R^\circ$; —$(CH_2)_{0-4}C(O)NR^\circ_2$; —$C(S)NR^\circ_2$; —C(S)SR°; —SC(S)SR°, —$(CH_2)_{0-4}OC(O)NR^\circ_2$; —$C(O)N(OR^\circ)R^\circ$; —$C(O)C(O)R^\circ$; —$C(O)CH_2C(O)R^\circ$; —$C(NOR^\circ)R^\circ$; —$(CH_2)_{0-4}SSR^\circ$; —$(CH_2)_{0-4}S(O)_2R^\circ$; —$(CH_2)_{0-4}S(O)_2OR^\circ$; —$(CH_2)_{0-4}OS(O)_2R^\circ$; —$S(O)_2NR^\circ_2$; —$(CH_2)_{0-4}S(O)R^\circ$; —$N(R^\circ)S(O)_2NR^\circ_2$; —$N(R^\circ)S(O)_2R^\circ$; —$N(OR^\circ)R^\circ$; —$C(NH)NR^\circ_2$; —$P(O)_2R^\circ$; —$P(O)R^\circ_2$; —$OP(O)R^\circ_2$; —$OP(O)(OR^\circ)_2$; SiR°_3; —(C_{1-4} straight or branched alkylene)O—$N(R^\circ)_2$; or —(C_{1-4} straight or branched alkylene)C(O)O—$N(R^\circ)_2$, wherein each R° may be substituted as

defined below and is independently hydrogen, C_{1-6} aliphatic, —CH_2Ph, —$O(CH_2)_{0-1}Ph$, —CH_2-(5-6 membered heteroaryl ring), or a 5-6-membered saturated, partially unsaturated, or aryl ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur, or, notwithstanding the definition above, two independent occurrences of R°, taken together with their intervening atom(s), form a 3-12-membered saturated, partially unsaturated, or aryl mono- or bicyclic ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur, which may be substituted as defined below.

[001348]Suitable monovalent substituents on R° (or the ring formed by taking two independent occurrences of R° together with their intervening atoms), are independently halogen, —$(CH_2)_{0-2}R^\bullet$, -(halo$R^\bullet$), —$(CH_2)_{0-2}OH$, —$(CH_2)_{0-2}OR^\bullet$, —$(CH_2)_{0-2}CH(OR^\bullet)_2$; —O(halo$R^\bullet$), —CN, —$N_3$, —$(CH_2)_{0-2}C(O)R^\bullet$, —$(CH_2)_{0-2}C(O)OH$, —$(CH_2)_{0-2}C(O)OR^\bullet$, —$(CH_2)_{0-2}SR^\bullet$, —$(CH_2)_{0-2}SH$, —$(CH_2)_{0-2}NH_2$, —$(CH_2)_{0-2}NHR^\bullet$, —$(CH_2)_{0-2}NR^\bullet_2$, —$NO_2$, —$SiR^\bullet_3$, —$OSiR^\bullet_3$, —C(O)$SR^\bullet$, —($C_{1-4}$ straight or branched alkylene)C(O)$OR^\bullet$, or —$SSR^\bullet$ wherein each $R^\bullet$ is unsubstituted or where preceded by "halo" is substituted only with one or more halogens, and is independently selected from C_{1-4} aliphatic, —CH_2Ph, —$O(CH_2)_{0-1}Ph$, or a 5-6-membered saturated, partially unsaturated, or aryl ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. Suitable divalent substituents on a saturated carbon atom of R° include =O and =S.

[001349]Suitable divalent substituents on a saturated carbon atom of an "optionally substituted" group include the following: =O, =S, =NNR^*_2, =NNHC(O)R*, =NNHC(O)OR*, =$NNHS(O)_2R^*$, =NR*, =NOR*, —$O(C(R^*_2))_{2-3}O$—, or —$S(C(R^*_2))_{2-3}S$—, wherein each independent occurrence of R* is selected from hydrogen, C_{1-6} aliphatic which may be substituted as defined below, or an unsubstituted 5-6-membered saturated, partially unsaturated, or aryl ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. Suitable divalent substituents that are bound to vicinal substitutable carbons of an "optionally substituted" group include: —$O(CR^*_2)_{2-3}O$—, wherein each independent occurrence of R* is selected from hydrogen, C_{1-6} aliphatic which may be substituted as defined below, or an unsubstituted 5-6-membered saturated, partially unsaturated, or aryl ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[001350]Suitable substituents on the aliphatic group of R* include halogen, —$R^\bullet$, -(halo$R^\bullet$), —OH, —$OR^\bullet$, —O(halo$R^\bullet$), —CN, —C(O)OH, —C(O)$OR^\bullet$, —NH_2, —$NHR^\bullet$, —$NR^\bullet_2$, or —NO_2, wherein each $R^\bullet$ is unsubstituted or where preceded by "halo" is substituted only with one or more halogens, and is independently C_{1-4} aliphatic, —CH_2Ph, —$O(CH_2)_{0-1}Ph$, or a 5-6-membered saturated, partially unsaturated, or aryl ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[001351]Suitable substituents on a substitutable nitrogen of an "optionally substituted" group include —$R^\dagger$, —$NR^\dagger_2$, —C(O)$R^\dagger$, —C(O)$OR^\dagger$, —C(O)C(O)$R^\dagger$, —$C(O)CH_2C(O)R^\dagger$, —$S(O)_2R^\dagger$, —$S(O)_2NR^\dagger_2$, —C(S)$NR^\dagger_2$, —C(NH)$NR^\dagger_2$, or —$N(R^\dagger)S(O)_2R^\dagger$; wherein each $R^\dagger$ is independently hydrogen, C_{1-6} aliphatic

which may be substituted as defined below, unsubstituted —OPh, or an unsubstituted 5-6-membered saturated, partially unsaturated, or aryl ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur, or, notwithstanding the definition above, two independent occurrences of $R^{\dagger}$, taken together with their intervening atom(s) form an unsubstituted 3-12-membered saturated, partially unsaturated, or aryl mono- or bicyclic ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[001352]Suitable substituents on the aliphatic group of $R^{\dagger}$ are independently halogen, —$R^{\bullet}$, -(halo$R^{\bullet}$), —OH, —$OR^{\bullet}$, —O(halo$R^{\bullet}$), —CN, —C(O)OH, —C(O)$OR^{\bullet}$, —NH_2, —$NHR^{\bullet}$, —$NR^{\bullet}{}_2$, or —NO_2, wherein each $R^{\bullet}$ is unsubstituted or where preceded by "halo" is substituted only with one or more halogens, and is independently C_{1-4} aliphatic, —CH_2Ph, —$O(CH_2)_{0-1}Ph$, or a 5-6-membered saturated, partially unsaturated, or aryl ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

[001353]Heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valences of the heteroatoms. It is understood that "substitution" or "substituted" includes the implicit proviso that such substitution is in accordance with permitted valence of the substituted atom and the substituent, and that the substitution results in a stable compound, i.e., a compound that does not spontaneously undergo transformation, for example, by rearrangement, cyclization, or elimination.

[001354]In a broad aspect, the permissible substituents include acyclic and cyclic, branched and unbranched, carbocyclic and heterocyclic, aromatic and nonaromatic substituents of organic compounds. Illustrative substituents include, for example, those described herein. The permissible substituents can be one or more and the same or different for appropriate organic compounds. The heteroatoms such as nitrogen may have hydrogen substituents and/or any permissible substituents of organic compounds described herein which satisfy the valencies of the heteroatoms.

[001355]In various embodiments, the substituent is selected from alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cyano, cycloalkyl, ester, ether, formyl, halogen, haloalkyl, heteroaryl, heterocyclyl, hydroxyl, ketone, nitro, phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid, sulfonamide, and thioketone, each of which optionally is substituted with one or more suitable substituents. In some embodiments, the substituent is selected from alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cycloalkyl, ester, ether, formyl, haloalkyl, heteroaryl, heterocyclyl, ketone, phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid, sulfonamide, and thioketone, wherein each of the alkoxy, aryloxy, alkyl, alkenyl, alkynyl, amide, amino, aryl, arylalkyl, carbamate, carboxy, cycloalkyl, ester, ether, formyl, haloalkyl, heteroaryl, heterocyclyl, ketone,

phosphate, sulfide, sulfinyl, sulfonyl, sulfonic acid, sulfonamide, and thioketone can be further substituted with one or more suitable substituents.

[001356]Examples of substituents include, but are not limited to, halogen, azide, alkyl, aralkyl, alkenyl, alkynyl, cycloalkyl, hydroxyl, alkoxyl, amino, nitro, sulfhydryl, imino, amido, phosphonate, phosphinate, carbonyl, carboxyl, silyl, ether, alkylthio, sulfonyl, sulfonamido, ketone, aldehyde, thioketone, ester, heterocyclyl, –CN, aryl, aryloxy, perhaloalkoxy, aralkoxy, heteroaryl, heteroaryloxy, heteroarylalkyl, heteroaralkoxy, azido, alkylthio, oxo, acylalkyl, carboxy esters, carboxamido, acyloxy, aminoalkyl, alkylaminoaryl, alkylaryl, alkylaminoalkyl, alkoxyaryl, arylamino, aralkylamino, alkylsulfonyl, carboxamidoalkylaryl, carboxamidoaryl, hydroxyalkyl, haloalkyl, alkylaminoalkylcarboxy, aminocarboxamidoalkyl, cyano, alkoxyalkyl, perhaloalkyl, arylalkyloxyalkyl, and the like. In some embodiments, the substituent is selected from cyano, halogen, hydroxyl, and nitro.

Substantially

[001357]As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

Target or target nucleic acid

[001358]A "target" or "target nucleic acid" as used herein is a polynucleotide (e.g., DNA such as genomic DNA) that includes a site ("target site" or "target sequence") targeted by a nucleic acid programmable DNA binding protein of the present disclosure. The target sequence is the sequence to which the guide sequence of a subject nucleic acid programmable DNA binding protein will hybridize. For a double stranded target nucleic acid, the strand of the target nucleic acid that is complementary to and hybridizes with the guide RNA is referred to as the "complementary strand" or "target strand"; while the strand of the target nucleic acid that is complementary to the "target strand" (and is therefore not complementary to the guide RNA) is referred to as the "non-target strand" or "non-complementary strand."

Terminus

[001359]As used herein the terms "termini" or "terminus" when referring to polypeptides or polynucleotides refers to an extremity of a polypeptide or polynucleotide respectively. Such extremity is not limited only to the first or final site of the polypeptide or polynucleotide but may include additional amino acids or nucleotides in the terminal regions. Polypeptide-based molecules may be characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH2)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Proteins are in some cases

made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These proteins have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

Treating

[001360]The terms "treat," "treating," and "treatment," as used herein, refer to therapeutic or preventative measures such as those described herein.

Unmodified

[001361]As used herein, "unmodified" refers to any substance, compound or molecule prior to being changed in any way. Unmodified may, but does not always, refer to the wild type or native form of a biomolecule. Molecules may undergo a series of modifications whereby each modified molecule may serve as the "unmodified" starting molecule for a subsequent modification.

Upstream and downstream

[001362]As used herein, the terms "upstream" and "downstream" are terms of relativity that define the linear position of at least two elements located in a nucleic acid molecule (whether single or double-stranded) that is orientated in a 5′-to-3′ direction. A first element is said to be upstream of a second element in a nucleic acid molecule where the first element is positioned somewhere that is 5′ to the second element. Conversely, a first element is downstream of a second element in a nucleic acid molecule where the first element is positioned somewhere that is 3′ to the second element.

Vaccine

[001363]As used herein, the phrase "vaccine" refers to a biological preparation that improves immunity in the context of a particular disease, disorder or condition.

Vector

[001364]As used herein, a "vector" is any molecule or moiety which transports, transduces or otherwise acts as a carrier of a heterologous molecule. Vectors of the present disclosure may be produced recombinantly and may be based on and/or may comprise viral parent or reference sequence. Such parent or reference viral sequences may serve as an original, second, third or subsequent sequence for engineering vectors. In non-limiting examples, such parent or reference viral sequences may comprise any one or more of the following sequences: a polynucleotide sequence encoding a polypeptide or multi-polypeptide, which sequence may be wild-type or modified from wild-type and which sequence may encode full-length or partial sequence of a protein, protein domain, or one or more subunits of a protein; a polynucleotide comprising a modulatory or regulatory nucleic acid which sequence may be wild-type or modified from wild-type; and a transgene that may or may not be modified from wild-type sequence . These viral sequences may serve as either the "donor" sequence of one or more codons (at the nucleic

acid level) or amino acids (at the polypeptide level) or "acceptor" sequences of one or more codons (at the nucleic acid level) or amino acids (at the polypeptide level). The details of one or more embodiments of the disclosure are set forth in the accompanying description below. Although any materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, the preferred materials and methods are now described. Other features, objects and advantages of the disclosure will be apparent from the description. In the description, the singular forms also include the plural unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. In the case of conflict, the present description will control.

3′ untranslated region

[001365]A "3′ untranslated region" (UTR) refers to a region of an mRNA that is directly downstream (i.e., 3′) from the stop codon (i.e., the codon of an mRNA transcript that signals a termination of translation) that does not encode a polypeptide.

5′ untranslated region

[001366]A "5′ untranslated region" (UTR) refers to a region of an mRNA that is directly upstream (i.e., 5′) from the start codon (i.e., the first codon of an mRNA transcript translated by a ribosome) that does not encode a polypeptide.

[001367]The details of one or more embodiments of the disclosure are set forth in the accompanying description below. Although any materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, the preferred materials and methods are now described. Other features, objects and advantages of the disclosure will be apparent from the description. In the description, the singular forms also include the plural unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. In the case of conflict, the present description will control.

[001368]As used herein, the following abbreviations and initialisms have the indicated meanings:

MC3	4-(dimethylamino)-butanoic acid, (10Z,13Z)-1-(9Z,12Z)-9,12-octadecadien-1-yl-10,13-nonadecadien-1-yl ester
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DMG	1,2-Dimyristoyl-rac-glycero-3-methanol
DOMG-PEG	R-3-[(ω-methoxy-poly(ethyleneglycol))carbamoyl)]-1,2-dimyristyloxypropyl-3-amine
DLPE	1,2-Dilauroyl-sn-Glycero-3-Phosphoethanolamine
DMPE	1,2-Dimyristoyl-sn-Glycero-3-Phosphoethanolamine

DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine
DDAB	Didodecyldimethylammonium bromide
EPC	1,2-dioleoyl-sn-glycero-3-ethylphosphocholine
14PA	1,2-dimyristoyl-sn-glycero-3-phosphate
18BMP	bis(monooleoylglycero)phosphate
DODAP	1,2-dioleoyl-3-dimethylammonium-propane
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
C12-200	1,1'-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol)

EQUIVALENTS AND SCOPE

[001369]Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the disclosure described herein. The scope of the present disclosure is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[001370]In the claims, articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The disclosure includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The disclosure includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[001371]Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the disclosure, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[001372]In addition, it is to be understood that any particular embodiment of the present disclosure that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded

even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the disclosure (e.g., any nucleic acid or protein encoded thereby; any method of production; any method of use; etc.) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[001373]All cited sources, for example, references, publications, databases, database entries, and art cited herein, are incorporated into this application by reference, even if not expressly stated in the citation. In case of conflicting statements of a cited source and the instant application, the statement in the instant application shall control.

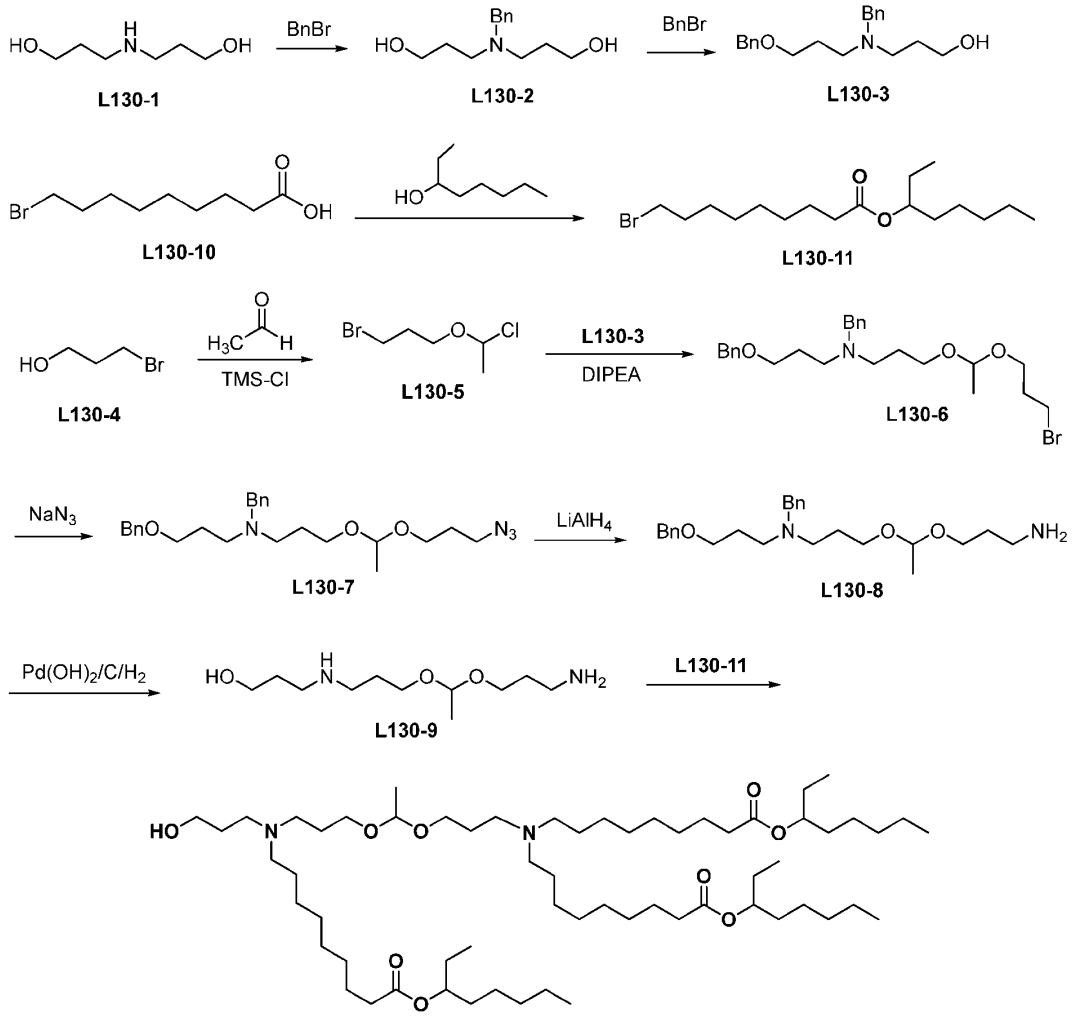
EXAMPLES

[001374]The following examples represent exemplary aspects of the present disclosure.

EXAMPLE 1: Synthesis of Exemplary Ionizable Lipids

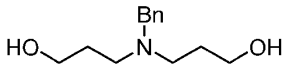
[001375]The following synthetic scheme contemplated by the present disclosure can be used to achieve the synthesis of exemplary ionizable lipids contemplated here that may be useful in forming the LNP-based RNA vaccine and therapeutics of the present disclosure. Additional lipids were prepared as reported in PCT Publications WO2023044343A1, WO2023044333A1, WO2023122752A1, WO2024044728A1, and WO2023196931A1, and PCT Application PCT/US2024/019990 (each of which is incorporated by reference herein in their entirety) using appropriate starting materials and procedures as would be apparent to a person of ordinary skill in the art.

Synthetic Scheme for Compound TL-1



Compound TL-1

Synthesis of 3,3'-(benzylazanediyl)bis(propan-1-ol) (L130-2)

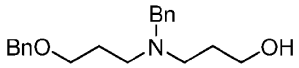


L130-2

[001376]A mixture of dihydroxylamine **L130-1** (3.0 g, 22.5 mmol), benzyl bromide (4.6 g, 26.9 mmol), potassium iodide (3.8 g, 22.5 mmol) and anhydrous potassium carbonate (8.2 g, 67.5 mmol) in anhydrous acetonitrile (30 mL) was refluxed for 20h. After completion of the reaction, solvent was removed under vacuum. To the residue was added 30 g of flash silica along with dichloromethane (30 mL) and the

contents were stirred well to get a uniform mixture. Solvent was removed under vacuum. The residue was loaded on to an empty flash cartridge, which was then attached to flash purification system loaded with 120 g flash silica column and purified by flash chromatography (SiO_2: dichloromethane/methanol (with 1 % ammonium hydroxide) 0-10 %) to get **L130-2** (2.4 g, 48 %) as a clear oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ; 7.39-7.26 (m, 5H), 3.70 (t, *J* = 6.5 Hz, 4H), 3.57 (s, 2H), 3.48 (bs, 2H), 2.63 (t, *J* = 6.5 Hz, 4H), 1.81-1.73 (m, 4H); CIMS m/z $[M+H]^+$ 224.48.

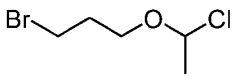
Synthesis of 3-(benzyl(3-(benzyloxy)propyl)amino)propan-1-ol (L130-3)



L130-3

[001377]A solution of **L130-2** (21 g, 94.13 mmol) in anhydrous THF (60 mL) was cooled to 0 °C. Sodium hydride (3.93 g of 60% in mineral oil, 98.25 mmol) was added in small portions and the reaction mixture was stirred under nitrogen atmosphere for 15 min. Benzyl bromide (14 g, 81.85 mmol) was added dropwise over 20 min. The reaction was stirred at 0 °C for 1h and allowed to warm to room temperature and stirred for 8h. After completion of the reaction, saturated ammonium chloride (300 mL) was added dropwise, and the contents were stirred for 10 min. The reaction solvents were removed under vacuum to give a residue which was taken in dichloromethane followed by addition of about 30 g of flash silica. The contents were stirred well to get a uniform mixture. Solvent was removed from this mixture under vacuum. The residue was loaded into an empty flash cartridge, which was then attached to flash purification system loaded with 300 g flash silica column and was purified by flash chromatography (SiO_2: dichloromethane/methanol 0-10%) to get **L130-3** (14.2 g, 55%) as slightly yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.36-7.23 (m, 10H), 4.43 (s, 2H), 3.73 (t, *J* = 6.5 Hz, 2H), 3.58 (s, 2H), 3.47 (t, *J* = 6.5 Hz, 2H), 2.66 (t, *J* = 6.5 Hz, 2H), 2.56 (t, *J* = 6.5 Hz, 2H), 1.88-1.80 (m, 2H), 1.76-1.69 (m, 2H); CIMS m/z $[M+H]^+$ 313.48.

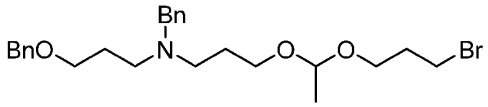
Synthesis of 1-bromo-3-(1-chloroethoxy)propane (L130-5)



L130-5

[001378]A mixture of bromopropanol (3.79 g, 27.04 mmol) and TMSCl (49.35 g, 58 mL) cooled in ice water bath was stirred for 15 min under nitrogen atmosphere. To this was added acetaldehyde (1.0 g, 22.70 mmol) and the reaction mixture was stirred under nitrogen at room temperature for 4 h. All volatile components were removed under vacuum to provide crude **L130-5** (3.08 g, 67%), which was used for next step of reaction without further purification; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.69 (q, 1H), 4.05-3.99 (m, 1H), 3.58 – 3.53 (m, 1H), 3.52-3.46 (m, 2H), 2.19-2.07 (m, 2H), 1.78 (d, *J* = 8 Hz, 3H).

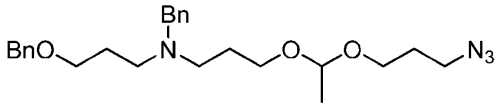
Synthesis of N-benzyl-3-(benzyloxy)-N-(3-(1-(3-bromopropoxy)ethoxy)propyl)propan-1-amine (L130-6)



L130-6

[001379]A solution of **L130-5** (3.08 g, 15.3 mmol) in anhydrous dichloromethane (20 mL) was added dropwise over 10 min to a well stirred mixture of **L130-3** (2.4 g, 7.65 mmol) and DIPEA (4.95 g, 38.28 mmol) in dichloromethane (30 mL). The reaction mixture was stirred at room temperature for 18h. After completion of the reaction, solvent and volatiles were removed under vacuum and the residue was purified by flash chromatography (SiO_2: hexane/ethyl acetate 0-10%) to get **L130-6** (2.40 g, 65%) as clear oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.39-7.22 (m, 10H), 4.60 (q, 1H), 4.45 (s, 2H), 3.70-3.40 (m, 10H), 2.54-2.49 (m, 4H), 2.10-1.98 (m, 2H), 1.81-1.71 (m, 4H), 1.25 (d, J = 8 Hz, 3H); CIMS m/z $[M+H]^+$ 478.51.

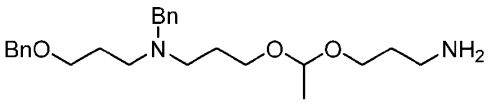
Synthesis of 3-(1-(3-azidopropoxy)ethoxy)-N-benzyl-N-(3-(benzyloxy)propyl)propan-1-amine (L130-7)



L130-7

[001380]A mixture of **L130-6** (2.40 g, 5.099 mmol) and sodium azide (3.3 g, 50.99 mmol) in DMSO (1.2 mL) was stirred at 90-100 °C under nitrogen atmosphere for 18h. Reaction was monitored by NMR. Upon confirmation of completion of reaction, the reaction mixture was loaded into silica cartridge and purified by column chromatography using 40g column (0 to 100% ethyl acetate: Hexane) to obtain compound **L130-7** as clear oil (2.2 g, 75%); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.39-7.22 (m, 10H), 4.59 (q, 1H), 4.45 (s, 2H), 3.62-3.33 (m, 10H), 2.56-2.47 (m, 4H), 1.85-1.71 (m, 6H), 1.24 (d, J = 8 Hz, 3H); CIMS m/z $[M+H]^+$ 441.61.

Synthesis of 3-(1-(3-aminopropoxy)ethoxy)-N-benzyl-N-(3-(benzyloxy)propyl)propan-1-amine (L130-8)

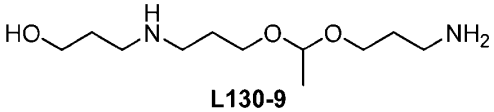


L130-8

[001381]A solution of **L130-7** (1.65 g, 3.74 mmol) in anhydrous THF (20 mL) was cooled to 0 °C. Lithium aluminum hydride (7.5 mL of 1M solution in THF, 7.5 mmol) was added in small portions and

the reaction mixture was stirred under nitrogen atmosphere at 0 ° C for 90 min, then allowed to stir at room temperature for 18h. After completion of the reaction, sodium sulfate decahydrate (10.0 g) was added in small portions and the contents were stirred for 10 min. The reaction solvents were removed under vacuum to give residue. This residue was taken in dichloromethane and was loaded on to a silica cartridge which was then attached to flash purification system loaded with 40 g flash silica column and was purified by flash chromatography (SiO_2: dichloromethane/methanol 0-10%, 1% NH_4OH) to get **L130-8** (1.15 g, 74%) as an oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.38-7.21 (m, 10H), 4.45 (q, 1H), 4.45 (s, 2H), 3.63-3.38 (m, 8H), 2.77 (t, *J* = 8 Hz, 2H), 2.57-2.47 (m, 4H), 1.84-1.64 (m, 6H), 1.24 (d, *J* = 8 Hz, 3H); CIMS m/z $[M+H]^+$ 415.68.

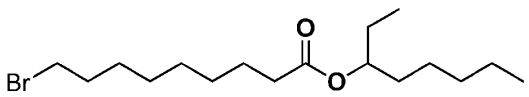
Synthesis of 3-((3-(1-(3-aminopropoxy)ethoxy)propyl)amino)propan-1-ol (L130-9)



L130-9

[001382]To a solution of **L130-8** (1.8 g, 4.34 mmol) in ethanol (80 mL) at room temperature and under nitrogen was added $Pd(OH)_2$ (1.69 g, 10 % on Carbon) and KOH (48 mg). The reaction mixture was evacuated and flushed with hydrogen gas (3 times) and then stirred under hydrogen atmosphere under rubber bladder pressure at room temperature for 24h. The reaction was monitored by mass and TLC. When the starting material mass (415) disappeared and *N*-Bn intermediate (325) showed up as a major peak, the reaction mixture was degassed and flushed with nitrogen and filtered through Celite. The Celite cake was then washed with methanol (200 mL) and the combined filtrates were concentrated on rotavapor. This oily residue was resubjected to hydrogenolysis reaction using the same amount of $Pd(OH)_2$ and ethanol under hydrogen atmosphere at room temperature for 24h. The reaction was monitored by MS. Upon completion of the reaction, the contents were filtered through Celite. The Celite cake was washed with methanol (200 mL). The filtrate was concentrated under reduced pressure and the residue was purified by reverse phase column chromatography (water: ACN (0-100%) followed by ACN: MeOH (0-100%) to give pure **L130-9** (730 mg, 72%); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.66 (q, 1H), 3.81 (t, *J* = 8 Hz, 2H), 3.66-3.63 (m, 2H), 3.52-3.45 (m, 2H), 2.88 (t, *J* = 7 Hz, 2H), 2.81 (t, *J* = 7 Hz, 2H), 2.72 (t, *J* = 7 Hz, 2H), 1.79-1.66 (m, 6H), 1.30 (d, *J* = 8 Hz, 3H); CIMS m/z $[M+H]^+$ 235.38.

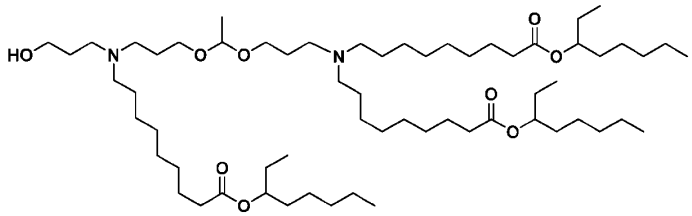
Synthesis of octan-3-yl 9-bromononanoate (L130-11)



L130-11

[001383]To a solution of acid **L130-10** (15.4 g, 65 mmol) in dichloromethane (120 mL) under nitrogen was added EDC (19.0 g, 100 mmol), DMAP (6.1 g, 50 mmol). After completion of addition, the mixture was stirred at room temperature for 15 min. The alcohol (6.5 g, 50 mmol) was added in, and the reaction mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with dichloromethane and water, and the resulting phases were separated. The aqueous phase was extracted again with EtOAc (150 mL). Combined organic extracts were washed with H_2O (150 mL x 2) and dried over anhydrous $MgSO_4$. Filtration and concentration provided crude product which was purified by flash column chromatography (SiO_2: 0 to 10% ethyl acetate in hexanes gradient) to yield **L130-11** as colorless oil (9.45 g, 54%); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 4.8 (m, 1H), 3.51 (t, *J* = 6.87, 1H), 3.38 (t, *J* = 6.87, 1H), 2.27 (t, *J* = 7.33, 2H), 1.72-1.85 (m, 2H), 1.47-1.62 (m, 6H), 1.38-1.44 (m, 2H), 1.26-1.3 (m, 12H), 084-0.87 (m, 6H).

Synthesis of di(octan-3-yl) 10-(3-hydroxypropyl)-15-methyl-20-(9-(octan-3-yloxy)-9-oxononyl)-14,16-dioxa-10,20-diazanonacosanedioate (Compound TL-1)

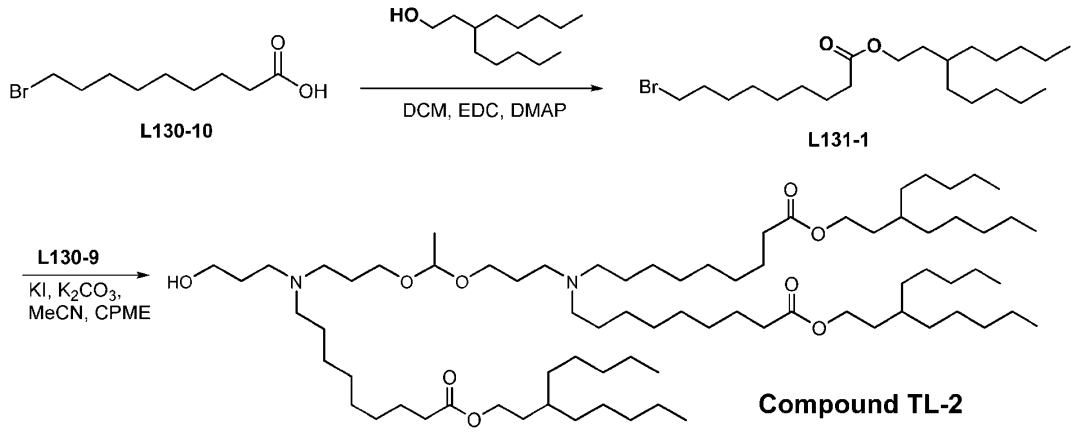


Compound TL-1

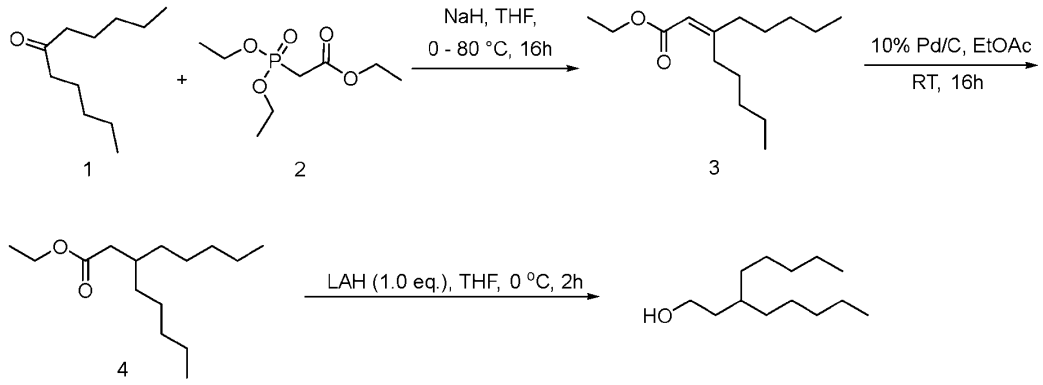
[001384]A mixture of **L130-9** (330 mg, 1.408 mmol), **L130-11** (2.36 g, 5.63 mmol), potassium iodide (350.6 mg, 2.11 mmol) and anhydrous potassium carbonate (1.56 g, 11.26 mmol) in cyclopentyl methyl ether and anhydrous acetonitrile (1:1, 10 mL) was refluxed under nitrogen 24h. After completion of the reaction, solvents were removed under vacuum. To the residue was added 10 g of flash silica along with triethylamine (5 mL), dichloromethane (5 mL), and the contents were stirred well to get a uniform mixture. Solvent was removed from this mixture under vacuum. The residue was loaded into an empty flash cartridge, which was then attached to flash purification system loaded with 20g flash silica column (pre-neutralized with 2% triethylamine in hexane) and was purified by flash chromatography (SiO_2: Hexane (contains 2 % triethylamine)/EtOAc 0-100 %) to get Compound **TL-1** (828 mg, 47 %) as a clear oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ; 4.86-4.78 (m, 3H), 4.66 (q, 1H), 3.79 (t, *J* = 8 Hz, 2H), 3.66-3.63 (m, 2H), 3.46-3.39 (m, 2H), 2.65 (t, *J* = 7 Hz, 2H), 2.59-2.33 (m, 10H), 2.29 (t, *J* = 7 Hz, 6H), 1.79-1.37 (m, 24H), 1.36-1.18 (m, 51H), 0.91-0.85 (m, 18H); CIMS m/z $[M+H]^+$ 1039.9; Analytical HPLC column: Agela Durashell C18, 4.6×50 mm, 3 μm (Catalog No. DC930505-0), mobile phase A: acetonitrile with 0.1% trifluoroacetic acid, mobile phase B: water with 0.1% trifluoroacetic acid, use gradient: A in B 70% to 100% in 5 min, then 100% for 10 min. Flow rate: 1mL/min, column temperature: 20±2 °C, detector:

ELSD, t_R = 5.72 min, purity: 99.35 %; UPLC column: Waters Aquity UPLC CSHTM, C18, 1.7 µm, 3.0×150 mmol, (Part No. 186005302), mobile phase A: acetonitrile with 0.1% trifluoroacetic acid, mobile phase B: water with 0.1% trifluoroacetic acid, use gradient: A in B 5% to 95% in 15 min, flow rate: 1mL/min, column temperature: 20±2 °C, detector: CAD, t_R = 8.31 min, purity: 92.19%.

Synthetic Scheme for Compound TL-2



Synthesis of 3-pentyloctanoic acid



Synthesis of ethyl 3-pentyl-2-octenoate (3)

[001385]To a cooled stirred solution of ethyl (diethoxyphosphoryl)acetate **1** (98.7 g, 3 eq., 440 mmol) in tetrahydrofuran (250 mL) was added sodium hydride (55-60% wt. in mineral oil) (14.1 g, 2 eq., 294 mmol) lot wise over 20 minutes and the mixture was stirred at room temperature until gas evolution ceased (approximately 1h). The reaction mixture was cooled to 0 °C and 6-undecanone **2** was added (25 g, 1.0 eq., 147 mmol) dropwise. The reaction mixture was gradually warmed to room temperature and allowed to stir at 80 °C for 16h. The progress of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was cooled to room temperature and quenched with ice cold water (250 mL). The aqueous phase was extracted with diethyl ether (150 mL X 2), and the organic layer was

washed with brine (250 mL), dried with sodium sulfate, concentrated under reduced pressure to get the crude. The crude material was purified by silica gel chromatography using 0-3% ethyl acetate in heptane as eluent to afford ethyl 3-pentyl-2-octenoate **3** (24 g, 68.0%) as clear liquid. A second 25 g batch was prepared by using the same procedure. ***ELSD analysis:*** Purity 93.0%, Calculated $C_{15}H_{28}O_2$ = 240.21; Observed = 241.30 (m/z, $M+H^+$).

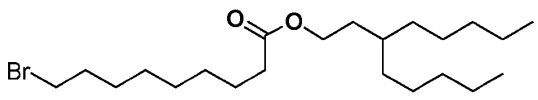
Synthesis of ethyl 3-pentyloctanoate (4)

[001386]To a stirred solution of ethyl 3-pentyl-2-octenoate **3** (25 g, 1 eq., 104 mmol) in ethyl acetate (0.3 L) at room temperature, 10% Pd/C (50% wet) (25 g, 11.74 mmol) was added. The reaction mixture was stirred for 16h under hydrogen pressure (150 psi). The progress of the reaction was monitored by TLC. After the starting material was consumed, the reaction mixture was filtered through celite bed and was washed with ethyl acetate. The filtrate was collected and concentrated under reduced pressure to give ethyl 3-pentyloctanoate **4** (20.0 g, 79.3%) as light-yellow liquid. ***^{1}H NMR (400 MHz, $CDCl_3$):*** δ 4.14-4.12 (m, 2H), 2.21 (d, *J* = 6.9 Hz, 2H), 1.88-1.80 (m, 1H), 1.31-1.20 (m, 19H), 0.87 (t, *J* = 6.7 Hz, 6H).

Synthesis of 3-pentyloctan-1-ol

[001387] To a stirred solution of ethyl 3-pentyloctanoate **4** (70 g, 1 eq., 289 mmol) in tetrahydrofuran (460 mL) was added a solution of LAH (144 mL, 1 eq., 289 mmol) (2M in THF) slowly under nitrogen atmosphere at 0 °C. The resulting mixture was stirred at 0 °C for 2h. The progress of the reaction was monitored by TLC. After the starting material was consumed, the reaction was quenched by dropwise addition of 75 mL aqueous sodium hydroxide solution (15% aqueous solution). Then, water (75 mL) and ethyl acetate (1000 mL) were added into the reaction mixture slowly at 0 - 5 °C. The reaction mixture was stirred for 30 min at the same temperature and added sodium sulphate (250 g). The mixture was filtered and the solid was washed with ethyl acetate (500 mL x 3). The combined filtrate was concentrated under reduced pressure to give 3-pentyl-1-octanol **10** (50 g, 86.4%) as a colorless oil. ^{1}H NMR (400 MHz; $CDCl_3$): δ 3.65 (t, *J* = 6.8 Hz, 2H), 1.54-1.49 (m, 2H), 1.42 (bs, 1H), 1.32-1.22 (m, 16H), 0.88 (t, *J* = 6.8 Hz, 6H).

Synthesis of 3-pentyloctyl 9-bromononanoate (L131-1)

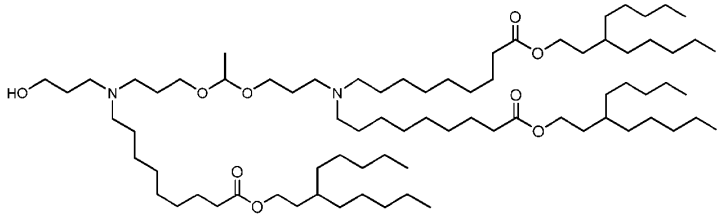


L131-1

[001388]To a solution of acid **L130-10** (7.5 g, 31.6 mmol) in dichloromethane (100 mL) under nitrogen was added EDC (12.6 g, 65.7 mmol), and DMAP (2.2 g, 18.0 mmol). After completion of addition, the mixture was stirred at room temperature for 15 min. 3-pentyloctan-1-ol (6.0 g, 30.0 mmol) was added, and the reaction mixture was stirred at room temperature for 20 h. The reaction mixture was diluted with

dichloromethane and water, and the resulting phases were separated. The aqueous phase was extracted again with EtOAc (150 mL). Combined organic extracts were washed with H_2O (150 mL x 2) and dried over anhydrous $MgSO_4$. Filtration and concentration provided crude product which was purified by flash column chromatography (SiO_2: 0 to 10% ethyl acetate in hexanes gradient) to yield **L131-1** as colorless oil (9.2 g, 75%); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 4.06 (t, *J* = 6.8 Hz, 2H), 3.39 (t, *J* = 6.8 Hz, 2H), 2.27 (t, *J* = 7.6 Hz, 2H), 1.86-1.78 (m, 2H), 1.65-1.51 (m, 2H), 1.47-1.33 (m, 2H), 1.32-1.16 (m, 25H), 0.87 (t, *J* = 6.8 Hz, 6H).

Synthesis of bis(3-pentyloctyl) 10-(3-hydroxypropyl)-15-methyl-20-(9-oxo-9-((3-pentyloctyl)oxy)nonyl)-14,16-dioxa-10,20-diazanonacosanedioate (Compound TL-2)

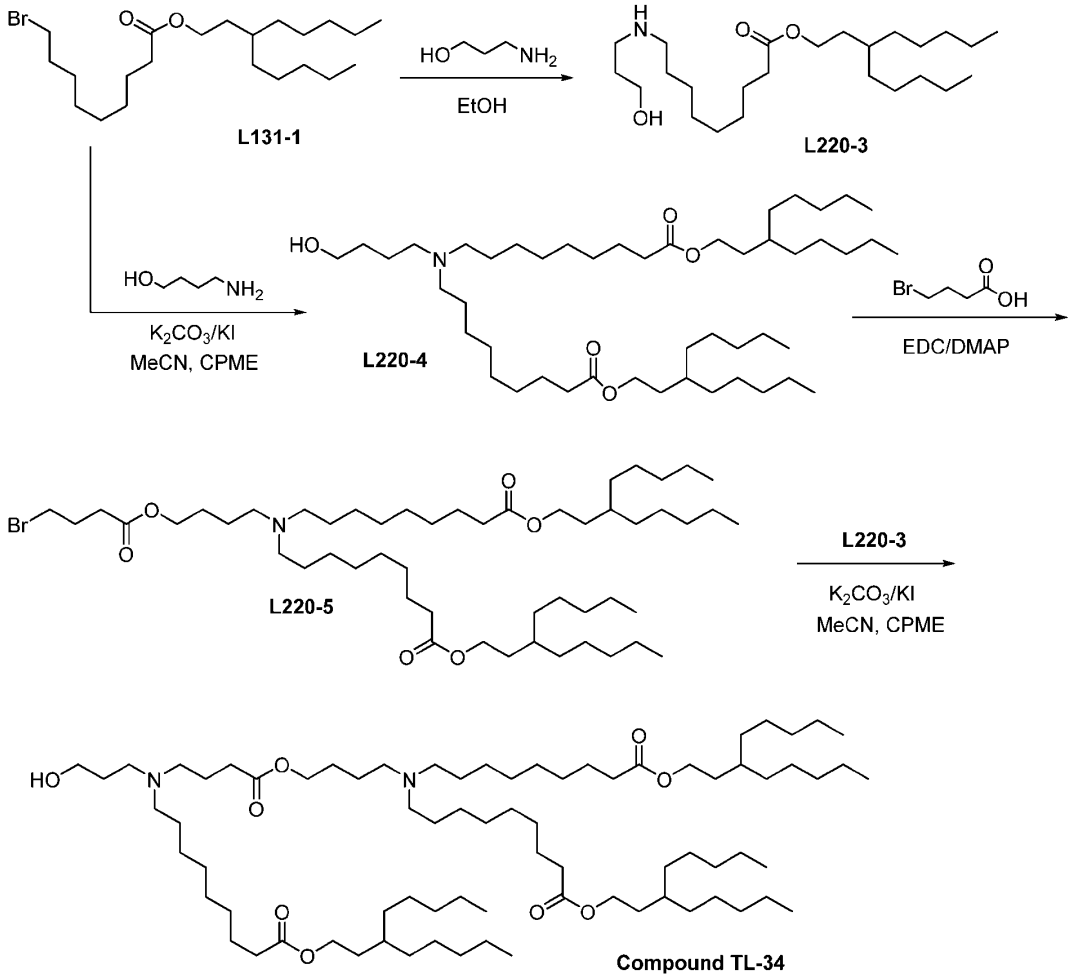


Compound TL-2

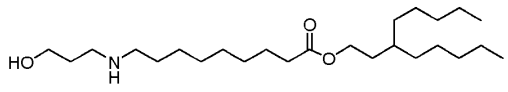
[001389]To a mixture of **L130-9** (330 mg, 1.408 mmol), **L131-1** (2.36 g, 5.63 mmol), potassium iodide (350.6 mg, 2.11 mmol) and anhydrous potassium carbonate (1.56 g, 11.26 mmol) were taken in a mixture of cyclopentyl methyl ether and anhydrous acetonitrile (1:1, 10 mL) and the reaction mixture was refluxed under nitrogen 24h. After completion of the reaction, solvents were removed under vacuum. To the residue was added 10 g of flash silica along with triethylamine (5 mL), dichloromethane (5 mL) and the contents were stirred well to get a uniform mixture. Solvent was removed from this mixture under vacuum. The residue was loaded on to an empty flash cartridge, which was then attached to flash purification system loaded with 20 g flash silica column (pre-neutralized with 2% triethylamine in hexane) and was purified by flash chromatography (SiO_2: Hexane (contains 2 % triethylamine)/EtOAc 0-100 %) to get Compound **TL-2** (828 mg, 47 %) as a clear oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ; 4.66 (q, 1H), 4.08 (t, *J* = 8 Hz, 6H), 3.78 (t, *J* = 8 Hz, 2H), 3.66-3.56 (m, 2H), 3.48-3.37 (m, 2H), 2.65 (t, *J* = 7 Hz, 2H), 2.59-2.35 (m, 10H), 2.29 (t, *J* = 7 Hz, 6H), 1.81-1.52 (m, 18H), 1.53-1.35 (m, 8H), 1.35-1.18 (m, 76H), 0.91-0.85 (m, 18H); CIMS m/z $[M+H]^+$ 1250.09; Analytical HPLC column: Agela Durashell C18, 4.6×50 mm, 3 µm (Catalog No. DC930505-0), mobile phase A: acetonitrile with 0.1% trifluoroacetic acid, mobile phase B: water with 0.1% trifluoroacetic acid, use gradient: A in B 70% to 100% in 5 min, then 100% for 10 min. Flow rate: 1mL/min, column temperature: 20±2 °C, detector: ELSD, t_R = 7.75 min, purity: > 99%; UPLC column: Waters Aquity UPLC CSHTM, C18, 1.7 µm, 3.0×150 mmol, (Part No. 186005302), mobile phase A: acetonitrile with 0.1% trifluoroacetic acid, mobile phase B: water with

0.1% trifluoroacetic acid, use gradient: A in B 5% to 95% in 15 min, flow rate: 1mL/min, column temperature: 20±2 °C, detector: CAD, t_R = 11.31 min, purity: 96.38%.

Synthetic Scheme for Compound TL-34



Synthesis of 3-pentyloctyl 9-((3-hydroxypropyl)amino)nonanoate (L220-3)

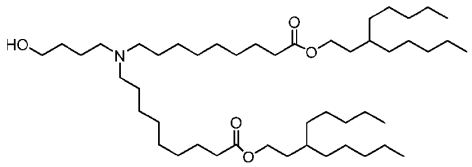


L220-3

[001390]L131-1 (3.0 g, 7.2 mmol; see synthesis of **TL-2**) and 3-aminopropan-1-ol (5.4 g, 72 mmol) were dissolved in ethanol (30 mL). The reaction mixture was refluxed at 80 °C for 24 h. When TLC showed the completion of the reaction (20% MeOH in DCM + 2% NH4OH, Rf = 0.5), the mixture was cooled to room temperature. The solvent was evaporated, and the crude product was purified by silica gel column

(0 - 20% MeOH in DCM + 2% NH_4OH) to afford **L220-3** (2.2 g, 74 %) as light-yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.06 (t, 2H), 3.80 (t, 2H), 2.86 (t, 2H), 2.58 (t, 2H), 2.26 (t, 2H), 1.72-1.63 (m, 2H), 1.63-1.50 (m, 4H), 1.49-1.34 (m, 3H), 1.34-1.13 (m, 24H), 0.87 (t, 6H). CIMS m/z $[M+H]^+$ 414.3.

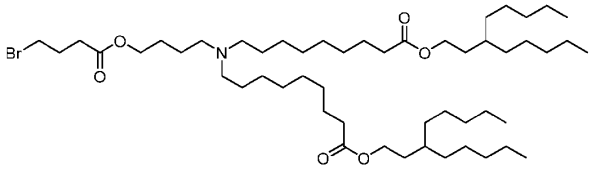
Synthesis of bis(3-pentyloctyl) 9,9'-((4-hydroxybutyl)azanediyl)dinonanoate (L220-4)



L220-4

[001391]L220-2 (5.0 g, 12 mmol), 4-aminobutan-1-ol (0.5 g, 5.6 mmol), potassium carbonate (3.0 g, 22 mmol) and potassium iodide (0.93 g, 6 mmol) were dissolved in acetonitrile (50 mL) and cyclopentyl methyl ether (50 mL). The reaction mixture was refluxed at 90 °C for 68 h. When TLC showed the completion of the reaction (10% MeOH in DCM + 1% NH_4OH, R_f = 0.7), the mixture was cooled to room temperature and filtered through a filter paper. The solvent was evaporated, and the crude product was purified by silica gel column (0 - 10% MeOH in DCM + 1% NH_4OH) to afford **L220-4** (3.4 g, 80 %) as light-yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.07 (t, 4H), 3.55 (bs, 2H), 2.57-2.40 (m, 6H), 2.26 (t, 4H), 1.74-1.43 (m, 16H), 1.43-1.35 (m, 2H), 1.35-1.01 (m, 48H), 0.87 (t, 12H).

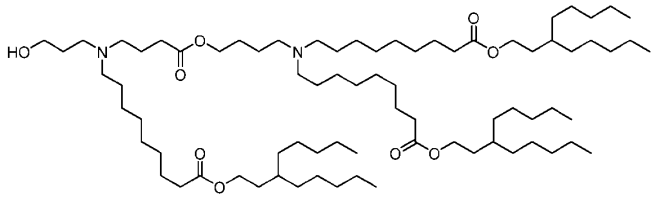
Synthesis of bis(3-pentyloctyl) 9,9'-((4-((4-bromobutanoyl)oxy)butyl)azanediyl)dinonanoate (L220-5)



L220-5

[001392]L220-4 (2.4 g, 3.1 mmol), 4-bromobutanoic acid (0.63 g, 3.8 mmol), EDC (1.5 g, 7.8 mmol) and DMAP (77 mg, 0.63 mmol) were stirred in dichloromethane (50 mL) at 20 °C for 20 h. TLC (R_f = 0.7, 10% MeOH in DCM with 1% NH_4OH) showed the reaction was not complete. The reaction mixture was concentrated and purified by silica gel column (0-10% MeOH in DCM with 1% NH_4OH) to afford pure **L220-5** (1.4 g, 49 %) as clear oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.17-4.00 (m, 6H), 3.47 (t, 2H), 3.00 (bs, 6H), 2.30-2.22 (m, 6H), 2.02-1.66 (m, 6H), 1.66-1.47 (m, 10H), 1.43-1.12 (m, 52H), 0.87 (t, 12H). CIMS m/z $[M+H]^+$ 914.6, 916.6.

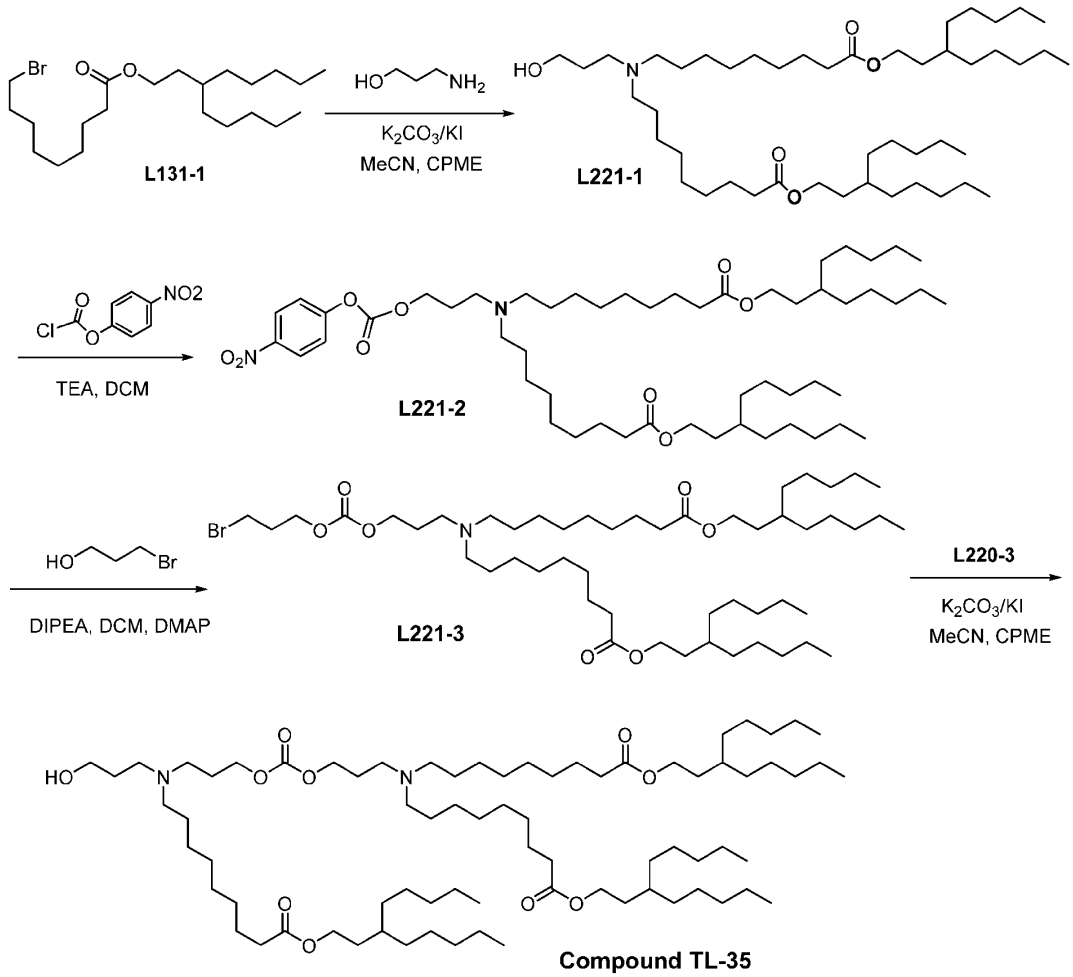
Synthesis of bis(3-pentyloctyl) 9,9'-((4-((4-((3-hydroxypropyl)(9-oxo-9-((3-pentyloctyl)oxy) nonyl)amino)butanoyl)oxy)butyl)azanediyl)dinonanoate (Compound TL-34)



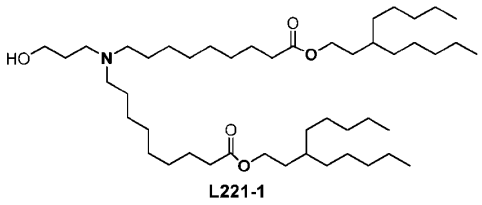
TL-34

[001393]L220-3 (0.92 g, 2.2 mmol), **L220-5** (1.7 g, 1.9 mmol), potassium iodide (0.31 g, 1.9 mmol) and potassium carbonate (1.0 g, 7.3 mmol) were dissolved in acetonitrile (20 mL) and cyclopentyl methyl ether (20 mL). The reaction mixture was refluxed at 95 °C for 72 h. The mixture was cooled to room temperature and filtered through a filter paper. The solvent was evaporated, and the crude product was purified by silica gel column (0 - 10% MeOH in DCM + 1% NH_4OH) to afford **TL-34** (384 mg, 17 %) as light-yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.06 (t, 8H), 3.77 (t, 2H), 2.63 (t, 2H), 2.48-2.22 (m, 18H), 1.78 (t, 2H), 1.71-1.34 (m, 25H), 1.34-1.17 (m, 74H), 0.87 (t, 18H); CIMS m/z $[M+H]^+$ 1248.6; Analytical HPLC column: Agilent Zorbax SB-C18, 5 μm, 4.6×150 mm, mobile phase A: acetonitrile with 0.1% trifluoroacetic acid, mobile phase B: water with 0.1% trifluoroacetic acid, use gradient: A in B 70% to 100% in 5 min, then 100% for 10 min. Flow rate: 1mL/min, column temperature: 20±2 °C, detector: ELSD, t_R = 8.76 min, purity: 99.4%; UPLC column: Thermo Scientific Hypersil GOLD C18, mobile phase A: acetonitrile with 0.1% trifluoroacetic acid, mobile phase B: water with 0.1% trifluoroacetic acid, use gradient: A in B 70% to 100% in 5 min, then 100% for 15 min. Flow rate: 1 mL/min, column temperature: 20±2 °C, detector: CAD, t_R = 11.79 min, purity: 95.4%.

Synthetic Scheme for Compound TL-35



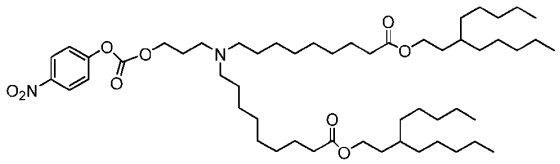
Synthesis of bis(3-pentyloctyl) 9,9'-((3-hydroxypropyl)azanediyl)dinonanoate (L221-1)



[001394]L131-1 (6.0 g, 14 mmol), 3-aminopropan-1-ol (0.5 g, 6.7 mmol), potassium iodide (1.1 g, 6.7 mmol) and potassium carbonate (4.1 g, 30 mmol) were dissolved in acetonitrile (50 mL) and cyclopentyl methyl ether (50 mL). The reaction mixture was refluxed at 90 °C for 48 h. When TLC showed the completion of the reaction (10% MeOH in DCM + 1% NH_4OH, R_f = 0.7), the mixture was cooled to room

temperature and filtered through a filter paper. The solvent was evaporated, and the crude product was purified by silica gel column (0 - 10% MeOH in DCM + 1% NH_4OH) to afford **L221-1** (4.1 g, 82 %) as light-yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.08 (t, 4H), 3.78 (t, 2H), 2.66 (t, 2H), 2.43 (t, 4H), 2.26 (t, 4H), 1.73-1.35 (m, 18H), 1.34-1.11 (m, 46H), 0.87 (t, 12H).

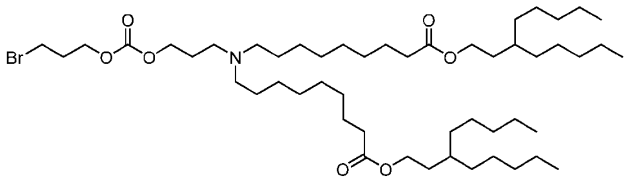
Synthesis of bis(3-pentyloctyl) 9,9'-((3-(((4-nitrophenoxy)carbonyl)oxy)propyl) azanediyl) dinonanoate (L221-2)



L221-2

[001395]L221-1 (500 mg, 665 μmol) and triethylamine (671 mg, 6.65 mmol) were dissolved in dichloromethane (4 mL). A dichloromethane (1mL) solution of 4-nitrophenyl chloroformate (201 mg, 997 μmol) was added to the above mixture slowly while stirring at room temperature. The reaction was stirring at room temperature for 20 h. When TLC (EA/hexane = 1: 1, R_f = 0.6) showed completion of the reaction. The solvent was evaporated, and crude mixture was purified by silica gel column (0 - 50% EA in hexane) to afford **L221-2** (500 mg, 82%) as yellow oil; CIMS m/z $[M+H]^+$ 917.7.

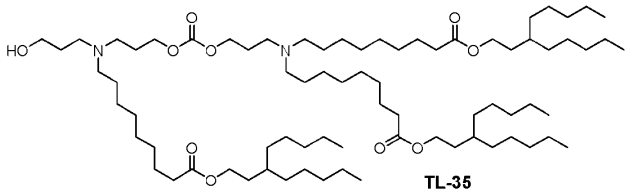
Synthesis of bis(3-pentyloctyl) 9,9'-((3-(((3-bromopropoxy)carbonyl)oxy)propyl) azanediyl) dinonanoate (L221-3)



L221-3

[001396]L221-2 (100 mg, 109 μmol), 3-bromopropan-1-ol (75.8 mg, 545 μmol) and DIPEA (282 mg, 2.18 mmol) were dissolved in dichloromethane (2.0 mL). DMAP (13.3 mg, 109 μmol) was then added to the mixture. The reaction was stirred at 20 °C for 22 h. The reaction mixture was concentrated and purified by silica gel column (0 - 50% EA in hexane) to afford **L221-3** (50 mg, 50%) as colorless oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.24 (t, 2H), 4.13 (t, 2H), 4.04 (t, 4H), 3.52 (t, 6H), 3.45 (t, 2H), 2.46 (t, 2H), 2.34 (t, 4H), 2.28-2.13 (m, 6H), 1.76 (t, 2H), 1.63-1.48 (m, 6H), 1.43-1.12 (m, 50H), 0.85 (t, 12H). CIMS m/z $[M+H]^+$ 916.6, 918.6.

Synthesis of bis(3-pentyloctyl) 10-(3-hydroxypropyl)-15-oxo-20-(9-oxo-9-((3-pentyloctyl) oxy)nonyl)-14,16-dioxa-10,20-diazanonacosanedioate (TL-35)



TL-35

[001397]L221-3 (230 mg, 251 μmol), **L220-3** (207 mg, 501 μmol), potassium iodide (41.6 mg, 251 μmol), and potassium carbonate (139 mg, 1.00 mmol) were added to acetonitrile (2.0 mL) and cyclopentyl methyl ether (2.0 mL). The mixture was refluxed at 95 °C for 18 h. TLC showed only partial conversion of starting material (10% MeOH in DCM + 1% NH_4OH, R_f = 0.7). The reaction mixture was cooled to room temperature and filtered through filter paper. The solvent was evaporated, and the crude product was purified by silica gel column (0 - 10% MeOH in DCM + 1% NH_4OH) to afford **TL-35** (95 mg, 30 %) as yellow oil; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.16 (q, 4H), 4.06 (t, 6H), 3.77 (t, 2H), 2.71-2.34 (m, 10H), 2.26 (t, 6H), 1.96-1.78 (m, 2H), 1.72-1.64 (m, 2H), 1.64-1.34 (m, 20H), 1.34-1.12 (m, 77H), 0.87 (t, 18H); CIMS m/z $[M+H]^+$ 1250.3; Analytical HPLC column: Agilent Zorbax SB-C18, 5 μm, 4.6×150 mm, mobile phase A: acetonitrile with 0.1% trifluoroacetic acid, mobile phase B: water with 0.1% trifluoroacetic acid, use gradient: A in B 70% to 100% in 5 min, then 100% for 10 min. Flow rate: 1mL/min, column temperature: 20±2 °C, detector: ELSD, t_R = 8.02 min, purity: 97.2%; UPLC column: Thermo Scientific Hypersil GOLD C18, mobile phase A: acetonitrile with 0.1% trifluoroacetic acid, mobile phase B: water with 0.1% trifluoroacetic acid, use gradient: A in B 70% to 100% in 5 min, then 100% for 15 min. Flow rate: 1 mL/min, column temperature: 20±2 °C, detector: CAD, t_R = 11.45 min, purity: 91.0%.

EXAMPLE 2A: LNP Formulations A – Biodistribution

[001398]Ionizable lipids, DSPC, cholesterol, and PEG2K-DMG are dissolved in pure ethanol at about a 48.5:10:39:2.5 mol% ratio with a total lipid concentration of about 10.8 mM. A 0.10 mg/mL mRNA solution is prepared using acidic buffer (pH 4.0-5.0) containing mRNAs encoding human erythropoietin (hEPO) and firefly luciferase (fLuc) (1:2 ratio). The nucleotide and lipid solutions are mixed at a 3:1 volume ratio using a NanoAssemblr microfluidic system at a 12 mL/min total flow rate resulting in rapid mixing and self-assembly of LNPs. Formulations are further dialyzed against PBS (pH 7.4) overnight at 4 °C, concentrated using centrifugal filtration and filtered (0.2 μm pore size). The particle size and polydispersity index (PDI) of formulations are measured by dynamic light scattering (DLS) using a Zetasizer Ultra (Malvern Panalytical). RNA encapsulation efficiency (EE%) is determined by Ribogreen assay.

EXAMPLE 2B: LNP Formulations B - Biodistribution

[001399]Ionizable lipids, DSPC, cholesterol, and PEG2K-DSPE are dissolved in pure ethanol at about a 48.5:10:40:1.5 mol% ratio with a total lipid concentration of about 10.8 mM. A 0.10 mg/mL mRNA solution is prepared using acidic buffer (pH 4.0-5.0) containing mRNAs encoding firefly luciferase (fLuc) or Cre recombinase (Cre) mRNA. The nucleotide and lipid solutions are mixed at a 3:1 volume ratio using the NanoAssemblr microfluidic system at a 12 mL/min total flow rate resulting in rapid mixing and self-assembly of LNPs. Formulations are further dialyzed against PBS (pH 7.4) overnight at 4 °C, concentrated using centrifugal filtration and filtered (0.2 μm pore size). The particle size and polydispersity index (PDI) of formulations are measured by dynamic light scattering (DLS) using a Zetasizer Ultra (Malvern Panalytical). RNA encapsulation efficiency (EE%) is determined by Ribogreen assay.

EXAMPLE 2C: LNP Formulations C - Vaccine formulations

[001400]Ionizable lipids, DSPC, cholesterol, and PEG2K-DMG are dissolved in pure ethanol at about a 48.5:10:40:1.5 mol% ratio (formulations comprising ionizable Lipids of the Disclosure) or about a 50:10:38.5:1.5 mol% ratio (for SM102 lipid control formulations) with a total lipid concentration of about 10.8 mM. A 0.10 mg/mL RNA solution is prepared using acidic buffer (pH 4.0-5.0) containing circular RNAs (oRNA) or linear mRNA encoding COVID spike protein. The nucleotide and lipid solutions are mixed at a 3:1 volume ratio using the NanoAssemblr microfluidic system at a 12 mL/min total flow rate resulting in rapid mixing and self-assembly of LNPs. Formulations are further dialyzed against cryobuffer overnight at 4 °C, concentrated using centrifugal filtration and filtered (0.2 μm pore size). The formulations are then stored at -80°C until use. The particle size and polydispersity index (PDI) of formulations are measured by dynamic light scattering (DLS) using a Zetasizer Ultra (Malvern Panalytical). RNA encapsulation efficiency (EE%) is determined by Ribogreen assay.

EXAMPLE 2D: LNP Formulations D – Bone Marrow Distribution

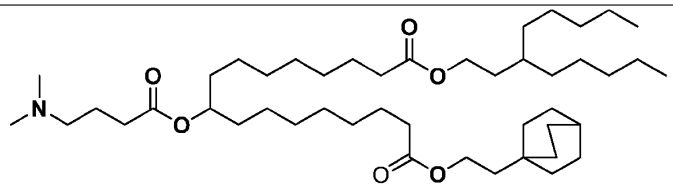
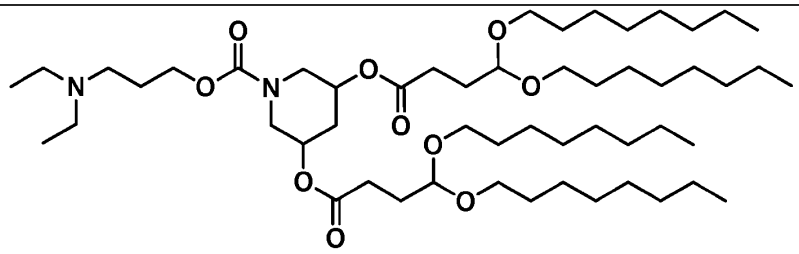
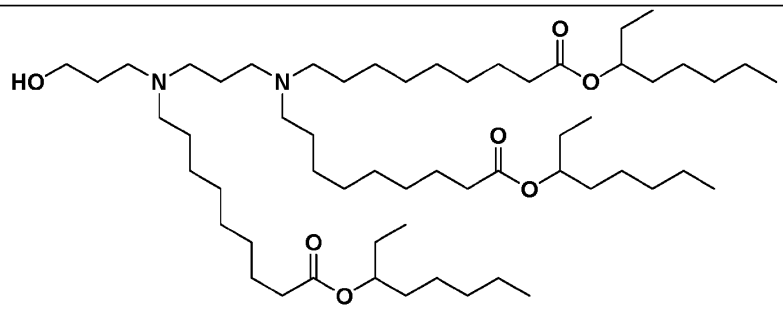
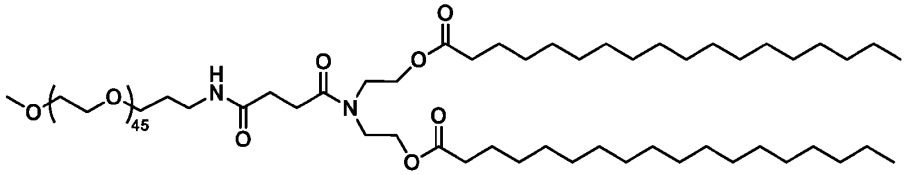
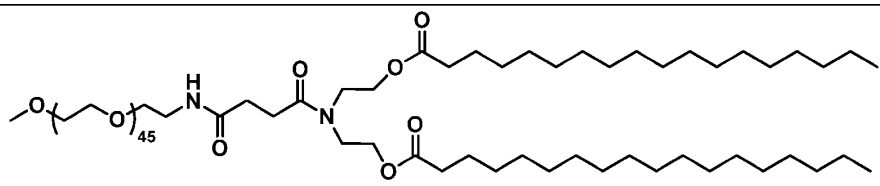
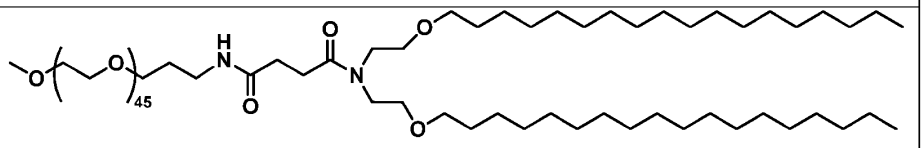
[001401]Ionizable lipids, phospholipid, cholesterol, PEG-lipid were dissolved in pure ethanol at the specified mol% ratio (Table 2D) with a total lipid concentration of ~10.8 mM. 0.10 mg/mL mRNA solution was prepared using acidic buffer (pH 4.0-5.0) containing mRNAs encoding VHH and Cas9 in a 2:1 ratio. The nucleotide and lipid solutions were mixed at a 3:1 volume ratio using the NanoAssemblr microfluidic system at a 135 mL/min total flow rate resulting in rapid mixing and self-assembly of LNPs. Formulations were further dialyzed against PBS (pH 7.4) overnight at 4 °C, and buffer exchanged into a sucrose-containing Tris-HCl cryoprotectant buffer for subsequent storage at -80°C. The individual particle sizes of formulations was measured by dynamic light scattering (DLS) using a Zetasizer Ultra (Malvern Panalytical). RNA encapsulation efficiency was determined by Ribogreen assay.

Table 2D: LNP Formulations

Form.	IL	PL	PEG	Sterol	N:P / Buffer	Size (nm)	PDI	ee%
F-1	C66	DSPC	DSPE-PEG2k	Chol	6	96.45	0.07	87.6
	48.5	10	2	39.5	X			
F-2	I-C	DSPC	DSPE-PEG2k	Chol	6	86.1	0.11	94.3
	48.5	10	2	39.5	X			
F-3	TL-2	DSPC	DSPE-PEG2k	Chol	6	110.7	0.05	93.6
	48.5	10	2	39.5	X			
F-10	CX-8	DSPC	DSPE-PEG2k	Chol	6	96.8	0.23	95.7
	48.5	10	2	39.5	X			
F-13	TL-1	DSPC	DSPE-PEG2k	Chol	8	133.8	0.10	82.8
	48.5	10	2	39.5	Y			
F-14	C66	DSPC	PL-9A	Chol	6	92.4	0.07	91.3
	48.5	10	2	39.5	X			
F-15	TL-2	DSPC	PL-9A	Chol	6	119.3	0.06	92.7
	48.5	10	2	39.5	X			
F-18	TL-2	DSPC	PEG-DMG2k	Chol	8	134.5	0.02	83.1
	48.5	10	2	39.5	Y			
F-19	TL-2	DSPC	PL-9	Chol	6	119.8	0.05	91.1
	48.5	10	2	39.5	X			
F-20	C66	DSPC	DPPE-PEG2k	Chol	6	82.3	0.12	92.0
	48.5	10	2	39.5	X			
F-21	TL-2	DSPC	DPPE-PEG2k	Chol	6	108.8	0.03	93.5
	48.5	10	2	39.5	X			
F-24	TL-2	DSPC	PEG-DSG2k	Chol	6	114.2	0.07	91.8
	48.5	10	2	39.5	X			
F-25	TL-2	DSPC	PL-10A	Chol	6	115.7	0.04	93.1
	48.5	10	2	39.5	X			

Buffer X: 25 mM Sodium Acetate, pH 5.0; **Buffer Y**: 50 mM Citrate, pH 4.0; **C66 =** Compound C66 of WO2023122752A1; **CX-8** = Compound CX-8 of WO2023196931A1; **I-C** = Compound of Formula I-C of WO2023086365A1; **PL-9A** = Compound PL-9A of WO2024044728A1; **PL-9** = Compound PL-9 of WO2024044728A1; **PL-10A** = Compound PL-10A of WO2024044728A1

Cmpd	Reference Compound Structure

C66	
CX-8	
I-C	
PL-9A	
PL-9	
PL-10A	

EXAMPLE 2E: LNP Formulations E – BCL11A editing payload

[001402]Ionizable lipids, DSPC, cholesterol, and a PEG lipid were dissolved in pure ethanol at the specified mol% ratios with a total lipid concentration of 10.8 mM. A 0.10 mg/mL mRNA solution was

prepared using acidic buffer (pH 4.0-5.0) containing mRNA encoding Cas9 mRNA (SEQ ID NO: 53) and gRNA targeting BCL11a erythroid enhancer (SEQ ID NO: 54) in a 1:1 molar ratio. The nucleotide and lipid solutions were mixed at a 3:1 volume ratio using the NanoAssemblr microfluidic system at a 12 mL/min total flow rate resulting in rapid mixing and self-assembly of LNPs. Formulations were further dialyzed against PBS (pH 7.4) overnight at 4 °C, concentrated using centrifugal filtration and filtered (0.2 µm pore size). The particle size and polydispersity index (PDI) of formulations was measured by dynamic light scattering (DLS) using a Zetasizer Ultra (Malvern Panalytical). RNA encapsulation efficiency (EE%) was determined by Ribogreen assay.

Table 2E: LNP Formulations

Form	**IL (mol%)**	**PL (mol%)**	**SL (mol%)**	**PEG-L (mol%)**	**Size (Z-avg nm)**	**PDI**	**EE (%)**	**Buffer**
F-Q1*	S-1 (48.5%)	DSPC (10%)	Chol (39.5%)	DPPE-PEG2k (2%)	80.1	0.146	97.7	X
F-Q2	C66 (48.5%)	DSPC (10%)	Chol (39.5%)	DSPE-PEG2k (2%)	80	0.074	97	X
F-Q3	TL-2 (48.5%)	DSPC (10%)	Chol (39.5%)	DSPE-PEG2k (2%)	91.3	0.036	91.7	X
F-Q4	S-1 (48.5%)	DSPC (10%)	Chol (39.5%)	DPPE-PEG2k (2%)	64.3	0.095	96	X
F-Q5	TL-2 (48.5%)	DSPC (10%)	Chol (39.5%)	PL-9A (2%)	98.8	0.016	97.8	X
F-Q6	S-1 (48.5%)	DSPC (10%)	Chol (39.5%)	PL-9A (2%)	66.8	0.056	98.4	X

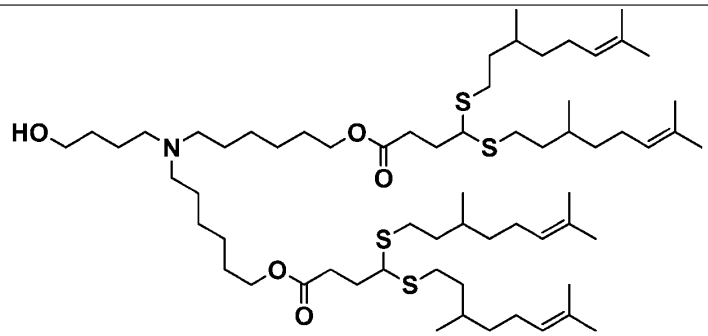
Buffer X: 25 mM Sodium Acetate, pH 5.0; Buffer Y: 50 mM Citrate, pH 4.0

a – N:P = 9; b – N:P = 8; unless otherwise specified all other formulations N:P = 6

FQ1* - Formulated without gRNA as a negative control

S-1 = Compound S-1 of PCT Application PCT/US2024/019990; **C66 =** Compound C66 of WO2023122752A1

Cmpd	**Reference Compound Structure**

S-1	

SEQ ID NO:	Sequence and Description
53	c*u*a*ACAGUUGCUUUUAUCACGUUUUAGAgcuagaaauagcAAGUUAAAAU AAGGCUAGUCCGUUAUCaacuugaaaaaguggcaccgagucggugcu*u*u*u BCL11A gRNA: A, G, U, C = unmodified RNA nucleotide; a, g, u, c = 2'-O-Methyl-nucleotide; * = Phosphorothioate linkage
54	AGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAUAUAAGACCCCGG CGCCGCCACCAUGGACAAGAAAUACUCCAUCGGACUGGACAUCGGAAC CAACUCCGUGGGAUGGGCCGUGAUUACGGACGAGUACAAAGUGCCUUC UAAAAAGUUCAAAGUGCUCGGCAACACCGACCGGCACUCCAUCAAGAA GAACCUCAUUGGAGCACUGCUGUUUGACUCGGGGGAAACUGCUGAAGC GACUCGGCUGAAGAGAACGGCACGCCGCCGGUACACCCGGAGGAAGAA UAGAAUCUGCUACCUUCAAGAAAUCUUUUCCAACGAAAUGGCCAAAGU CGACGACAGCUUCUUCCACCGCCUGGAAGAGUCGUUCCUCGUGGAAGA GGACAAGAAGCACGAGAGACA Cas9 mRNA: All “U” = N-1-Methylpsuedouridine

EXAMPLE 2H: LNP formulation H – Cas9 + b2m gRNA

[001403]Ionizable lipids, phospholipid, cholesterol, PEG-lipid were dissolved in pure ethanol at the specified mol% ratio (Table 2H) with a total lipid concentration of ~10.8 mM. 0.10 mg/mL mRNA solution was prepared using acidic buffer (pH 4.0-5.0) containing mRNAs encoding Cas9 and gRNA targeting beta 2 microglobulin (SEQ ID NO: 51) in a 1:1 molar ratio. The nucleotide and lipid solutions were mixed at a 3:1 volume ratio using the NANOASSEMBLR microfluidic system at a 12 mL/min total flow rate resulting in rapid mixing and self-assembly of LNPs. Formulations were further dialyzed against

PBS (pH 7.4) overnight at 4 °C, concentrated using centrifugal filtration and filtered (0.2 µm pore size). The particle size and polydispersity index (PDI) of formulations was measured by dynamic light scattering (DLS) using a ZETASIZER ULTRA (Malvern Panalytical). RNA encapsulation efficiency (EE%) was determined by Ribogreen assay.

Table 2H: LNP Formulations

Form.	IL	PL	PEG	Sterol	N:P / Buffer	Size (nm)	PDI	ee%
F-81	C66	DSPC	DPPE-PEG2k	Chol	6	82.36	0.07	88.6
	48.5	10	2	39.5	X			
F-82	TL-2	DSPC	DPPE-PEG2k	Chol	6	104	0.019	90.8
	48.5	10	2	39.5	X			
F-83	S-1	DSPC	DPPE-PEG2k	Chol	6	72.95	0.002	92.0
	48.5	10	2	39.5	Y			
F-84	S-1	DSPC	PL-9A	Chol	6	81.34	0.017	95.1
	48.5	10	2	39.5	Y			

Buffer X: 25 mM Sodium Acetate, pH 5.0; **Buffer Y**: 50 mM Citrate, pH 4.0; TL-2: an ionizable lipid comprising two protonatable tertiary amine groups and at least two biodegradable linkers.

Beta 2 microglobulin gRNA (SEQ ID NO: 51) – GACAAGCACCAGAAAGACCA

EXAMPLE 3: LNP Delivery of VHH mRNA to HSCs – BALB/c mice

[001404]LNP formulations were prepared as described in Example 2D. Each formulation was administered to BALB/c mice on Day 1, injected via tail vein. 16-24 h post injection, animals were euthanized by CO_2 inhalation, and spleens, femurs, and tibiae/fibulae were harvested. In one study, two mice dosed with formulation F-18 experienced adverse events. At the time of filing, it was not yet determined whether the adverse events were test article related. Harvested spleens were dissociated into single cell suspension of splenocytes using the gentleMACS Octo Dissociator with Heaters with the Mouse Spleen Dissociation Kit per manufacturer's instructions. Dissociated splenocytes were then passed through a 70µm filter and washed with 1x PBS containing 2mM EDTA and 0.5% BSA. Red blood cells were lysed using ACK Lysing Buffer and washed twice with 1x PBS + 2mM EDTA + 0.5% BSA, passing the cell suspension through an additional 70µm filter prior to the last wash. Following final wash, cells were resuspended in 1x PBS + 2mM EDTA + 0.5% BSA and counted. Cells were diluted, plated, and stained for flow cytometry. Bone marrow (BM) cells were harvested, passed through a 70µm filter, and washed with 1x PBS + 2mM EDTA + 0.5% BSA. Red blood cells were lysed using ACK Lysing Buffer and washed twice with 1x PBS + 2mM EDTA + 0.5% BSA, passing the cell suspension through an additional 70µm filter prior to the last wash. Following final wash, cells were resuspended in 1x PBS + 2mM EDTA + 0.5% BSA and counted. Cells were diluted, plated, and stained for flow cytometry.

Briefly, cells were stained with Live/Dead Fixable Aqua, incubated with Fc block (splenocytes) or labeled CD16/32 antibody (bone marrow) for 5min at 4°C and surface antibody stains (panel shown below in Table 3B). Cells were then washed and filtered and acquired on cytometer (ThermoFisher Attune NXT or Sony ID7000 Cytometer) equipped with a high-throughput autosampler. Analysis performed using Flowjo (BD V10.8.1). Cells were identified with markers in Tables 3A. Flow cytometry was used to define long-term hematopoietic stem cells (LT-HSC) based on the following: Viable, lineage negative, Sca-1+, c-Kit+, CD150+, CD48-. The percentage of LT-HSCs and LSK cells expressing VHH is reported below in Table 3C. Several LNP formulations demonstrated high delivery (>40%) to HSPCs. Specifically, high delivery by many of the LNP formulations to LT-HSCs strongly suggests that delivery of gene editing systems in vivo can be used to treat diseases related to hematopoietic stem cells, such as hemoglobinopathies, in a subject in need thereof.

Table 3A: Definition of Cell Subsets in Mouse BM by Flow Cytometry used in Example 3

Cell Type:	**Surface Marker Phenotype:**
LSK	L/DAqua-Lineage-Sca1+cKit+
LT-HSC	L/DAqua-Lineage-Sca1+cKit+CD150+CD48-

*Lineage is defined as including CD4, CD8a, CD11b, B220, GR1, Ter119.

Table 3B: Bone marrow surface antibody stains used in Example 3

Staining Step	**Target**	**Clone**	**Target**	**Clone**
Live/Dead	Viability	N/A		
Surface Marker	CD16/32	S17011E	CD150	TC15-12F12.2
	cKit	2B8	CD4	GK1.5
	CD135	A2F10	CD8a	53–6.7
	CD34	RAM34	CD11b	M1/70
	CD48	HM48-1	B220	RA3-6B2
	Sca1	D7	Gr1	RB6-8C5
	CD127	A7R34	Ter119	TER119

Table 3C: Percentage of cells expressing VHH mRNA

Form	**LSK**	**LT-HSC**
F-1	**	***
F-2	***	****
F-3	***	****

F-10	**	***
F-13	*	*
F-14	***	****
F-15	**	***
F-18	***	***
F-19	*	**
F-20	****	*****
F-21	****	*****
F-24	**	**
F-25	**	**

Key: * < 5%; 5% ≤ ** < 25%; 25% ≤ *** < 50%; 50% ≤ **** < 75%; 75% ≤ ***** < 100%

LSK = Lin-Sca1+c-Kit+ hematopoietic stem cells; LT-HSC = Long Term hematopoietic stem cells

EXAMPLE 4: LNP Delivery of VHH mRNA to HSCs – Humanized Mice

[001405]LNP formulations were prepared as described in Example 2D. Each formulation was administered to NBSGW mice that had previously been humanized by injection of human CD34+ cells. Animals were 12-20 weeks post-engraftment. LNPs were administered to mice on Day 1 injected via tail vein. 16-24 h post injection, animals were euthanized by CO_2 inhalation, and spleens, femurs, and tibiae/fibulae were harvested and processed as described in Example 3. Cells were diluted, plated, and stained for flow cytometry using surface antibody stains (panel shown below in Table 4B). Cells were then washed and filtered and acquired on cytometer (ThermoFisher Attune NXT or Sony ID7000 Cytometer) equipped with a high-throughput autosampler. Analysis performed using Flowjo (BD V10.8.1). Cells were identified with markers in Tables 4A. The percentage of LT-HSCs and HSPCs expressing VHH is reported below in Table 4C.

Table 4A: Definition of Cell Subsets in Mouse BM by Flow Cytometry used in Example 4

Cell Type:	**Surface Marker Phenotype:**
HSPC	L/DAqua-hCD45+Lineage-CD34+
LT-HSC	L/DAqua-hCD45+Lineage-CD34+CD38-CD90+CD45RA-

*Lineage is defined as including CD3, CD8, CD11b, CD11c, CD14, CD19, CD56.

Table 4B: Bone marrow surface antibody stains used in Example 4

Staining Step	**Target**	**Clone**	**Target**	**Clone**
Live/Dead	Viability	N/A		

Surface Marker	VHH-GPI	96A3F5	CD235a	HI264
	Mouse CD45	30-F11	CD3	SP34-2
	CD38	OKT10	CD8	SK1
	Human CD45	HI30	CD11b	ICRF44
	CD34	561	CD11c	3.9
	CD45RA	5H9	CD14	M5E2
	CD71	M-A712	CD19	HIB19
	CD90	5E10	CD56	5.1H11

Table 4C: Percentage of cells of various types expressing VHH mRNA

Form	HSPC	LT-HSC
F-1	***	***
F-15	****	*****
F-20	***	*****
F-21	*****	*****

Key: * < 5%; 5% ≤ ** < 25%; 25% ≤ *** < 50%; 50% ≤ **** < 75%; 75% ≤ ***** < 100%

HSPC = Hematopoietic stem and progenitor cells; LT-HSC = Long Term hematopoietic stem cells

EXAMPLE 5: LNP editing in CD34+ Bone marrow progenitor cells – Humanized mice

LNP/mRNA formulations were prepared as described in Example 2E. Hu-CD34+ NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl, Jackson Laboratory 005557) were injected via tail vein with the indicated dosage of Cas9 mRNA plus BCL11A gRNA (Biospring) formulated in LNP formulations F-Q2 through F-Q6 in a total volume of 5mL/kg. Control animals received LNP formulation F-Q1 that only contained Cas9 mRNA, lacking the gRNA. 5 days post injection, animals were euthanized by CO_2 inhalation, and spleens, femurs, and tibiae/fibulae were harvested. Bone marrow was removed from bones and red blood cells were lysed using ACK Lysing Buffer and then washed twice with 1x PBS + 2mM EDTA + 0.5% BSA, passing the cell suspension through a 70µm filter prior to the last wash. Following final wash, cells were resuspended in EasySep™ Buffer (StemCell 20144) and counted. Cells were diluted and plated (500 thousand per well) in a 96-well bottom plate for pre-enrichment purity check (Table 5C). Remaining cells had their concentration adjusted to 1e8 cells/ml in EASYSEP™ Buffer (STEMCELL TECHNOLOGIES). Selection cocktail (StemCell 17856) was added to all samples at a concentration of 100ul/ml and samples were incubated at room temperature for 10 minutes. RAPIDSPHERES™ was added to all samples at a concentration of 75µl/ml and samples were incubated at room temperature for 5

minutes. Samples were transferred to Falcon Round-Bottom Polystyrene Tubes (Corning 352052) during incubation and then placed in an EasySep™ magnet (StemCell 18000) for 3 minutes at room temperature. Supernatant was removed by inverting the tubes. Enriched cells were washed, counted, and plated in 96-well plate. Genomic DNA was isolated for downstream NGS analysis. Remaining enriched cells and flow through were purity checked by flow cytometry. Briefly, cells were stained in 1x PBS with Live/Dead Fixable Aqua (Invitrogen L34966) at 1:1000 for 10min at room temperature. Cells were then washed twice with Cell Staining Buffer (BioLegend 420201) and incubated with Fc block for 5min at 4°C and surface antibody stains either in full or FMO (fluorescence minus one) master mixes (panels and dilutions shown below in Tables 12A) added on top of the Fc Block for an additional 30min at 4°C. Cells were then washed and fixed. Cells were acquired on cytometer (Sony ID7000 Cell Analyzer: Cat # LE-ID7000C with UV/V/B/YG/R lasers). Single stain controls were made using UltraComp eBeads (ThermoFisher 01-3333-41) and ArC Amine Reactive Compensation Bead Kit (ThermoFisher A10346). Analysis was performed using Flowjo (BD V10.10.0). Cells were identified with the markers in Tables 12B.

[001406]CD34 purity was checked post enrichment, >95% purity was observed in all groups (Table 5C). NGS analysis of the enriched CD34+ HSPCs showed that Indels were detected in erythroid specific enhancer location with all LNP treatments that contained Cas9 mRNA plus BCL11A gRNA (Table 5D).Spleens were harvested 5 days after dosing and flash frozen. Tissue was lysed with lysis master mix, containing TL Buffer, 1M DTT, and Proteinase K solution, shaking at 55°C overnight. RNAse was added to samples and after centrifugation the supernatants were collected for genomic DNA isolation (MICROGEM PREPGEM UNIVERSAL™ kit following manufacturer's protocol) and downstream NGS analysis. Indel percentage was measured (Table 5E). Low (less than 1%) indel percentages were measured in splenocytes in all groups, suggesting low off-target editing.

Table 5A: Human Bone Marrow antibodies used in Example 5

Staining Step:	**Target**	**Fluorophore**	**Clone**	**Manufacturer**	**Cat No.**	**Usage per test (dilution)**
Live/Dead	Viability	Aqua	N/A	Invitrogen	L34966	1000
Surface	Super Bright Staining Buffer			eBioscience	SB-4401-75	20
	Mouse CD45	PerCP-Cy5.5	BL3	30-F11	Biolegend	200
	Human CD45	APC/Fire750	RL3	HI30	Biolegend	25
	CD34	BV421	VL1	561	Biolegend	20
	CD3	AF700	RL2	SP34-2	BD	20

	CD8	AF700	RL2	SK1	BioLegend	100
	CD11b	AF700	RL2	ICRF44	Biolegend	20
	CD11c	AF700	RL2	3.9	BioLegend	20
	CD14	AF700	RL2	M5E2	Biolegend	100
	CD19	AF700	RL2	HIB19	Biolegend	20
	CD56	AF700	RL2	5.1H11	Biolegend	20
	CD235a	AF700	RL2	HI264	Biolegend	20

Table 5B: Definition of Cell Subsets in NHP BM aspirate by Flow Cytometry used in Example 5

Cell Type:	**Surface Marker Phenotype:**
Total CD34+	L/DAqua-Mouse CD45-Human CD45+CD34+

Table 5C: Percentage of human CD34+ cells pre- and post-enrichment.

Form	**Dose (mg/kg)**	**Total CD34+ Cells**	
		Pre enrichment	**Enriched CD34+**
F-Q1	1.5	8.14	95.63
F-Q2	3.0	11.23	98.2
F-Q3	2.0	11.97	97
F-Q4	3.0	13.1	97.6
F-Q5	2.0	10.92	95.8
F-Q6	3.0	12.3	98.13

Table 5D: Percentage of Indels at the BCL11a erythroid enhancer location in purified human CD34+ cells (Average of 3 animals)

Form	**Dose (mg/kg)**	**Indel%**
F-Q1	1.5	0.02
F-Q2	3.0	0.64
F-Q3	2.0	2.86
F-Q4	3.0	1.25
F-Q5	2.0	3.2
F-Q6	3.0	2.05

Table 5E: Percentage of Indels at the BCL11a erythroid specific enhancer location in splenocytes (Average of 3 animals)

Form	Dose (mg/kg)	Indel%
F-Q1	1.5	0.04
F-Q2	3.0	0.15
F-Q3	2.0	0.26
F-Q4	3.0	0.13
F-Q5	2.0	0.13
F-Q6	3.0	0.1

EXAMPLE 6: LNP editing with mouse b2m gRNA – WT mice

[001407]B6 mice (C57BL/6J, JACKSON LABORATORY 000664) were treated with anti-NK1.1 antibody (VWR 76556-306) by intraperitoneal injection 2 days before and 1 day post LNP injection (LNP/mRNA formulations were prepared as described in Example 2H). For LNP treated groups, mice were injected via tail vein with 2mpk Cas9 mRNA plus beta 2 microglobulin gRNA formulated in LNP formulations F-80 through F-84 in a total volume of 5mL/kg. Control animals received LNPs that contained Cas9 mRNA and non targeting gRNA. 5 days post injection, animals were euthanized by CO_2 inhalation, and spleens, femurs, and tibiae/fibulae were harvested. Bone marrow was treated with ACK red blood cell Lysing Buffer and then washed twice with 1x PBS + 2mM EDTA + 0.5% BSA, passing the cell suspension through a 70µm filter prior to the last wash. Following the final wash, cells were resuspended in EasySepTM Buffer (StemCell 20144) and stained in 1x PBS with Live/Dead Fixable Aqua (INVITROGEN L34966) for 10min at room temperature. Cells were then washed and stained with surface antibody master mix (panels and dilutions shown below in Table 6A) for 30 min at 4°C. Cells were then washed, fixed and acquired on cytometer (SONY ID7000 Cell Analyzer: Cat # LE-ID7000C with UV/V/B/YG/R lasers). Single stain controls were made using UltraComp eBeads (THERMOFISHER 01-3333-41) and ArC Amine Reactive Compensation Bead Kit (THERMOFISHER A10346). Analysis was performed using FLOWJO software (BD V10.10.0). Cells were identified with markers in Table 6B. Results are reported in Table 6C.

Table 6A: Human Bone Marrow antibodies used in Example 6

Staining Step:	Target	Fluorophore	Clone	Manufacturer	Cat No.	Usage per test (dilution)
Live/Dead	Viability	Aqua	N/A	INVITROGEN	L34966	1000

Surface	Super Bright Staining Buffer			eBioscience	SB-4401-75	20
	H-2k^b	AF488	AF6-88.5	BIOLEGEND	116510	200
	CD34	AF647	RAM34	BD	560230	100
	CD48	APC/Fire750	HM48-1	BIOLEGEND	103446	100
	Sca-1	BV421	D7	BIOLEGEND	108128	200
	cKit	BV605	2B8	BIOLEGEND	105847	200
	CD150	BV711	TC15-12F12.2	BIOLEGEND	115941	100
	B2M	PE	A16041A	BIOLEGEND	154504	100
	CD4	PE/Cy7	GK1.5	BIOLEGEND	100422	200
	CD8a	PE/Cy7	53–6.7	BIOLEGEND	100722	200
	CD11b	PE/Cy7	M1/70	BIOLEGEND	101216	200
	B220	PE/Cy7	RA3-6B2	BIOLEGEND	103222	200
	Gr1	PE/Cy7	RB6-8C5	BIOLEGEND	108416	200
	Ter119	PE/Cy7	TER119			

Table 6B: Definition of Cell Subsets in NHP BM aspirate by Flow Cytometry used in Example 6

Cell Type:	**Surface Marker Phenotype:**
LSK	L/DAqua-Lineage-Sca-1+c-kit+
LT-HSC	L/DAqua-Lineage-Sca-1+c-kit+CD150+CD48-

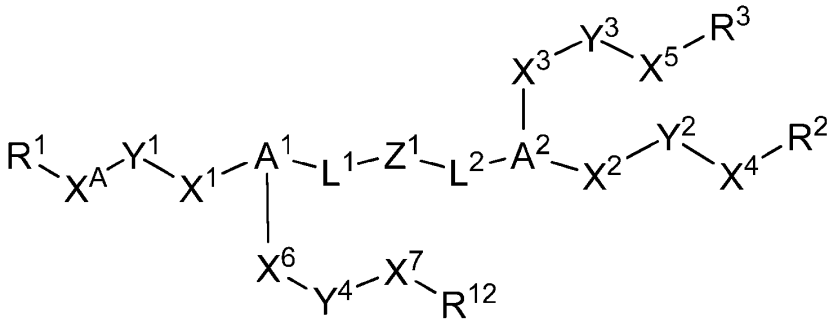
*Lineage is defined as including: CD4, CD8, CD11b, CD220, GR1, TER199

Table 6C: Average percentage of H-2Kb negative cells in HSPC and HSCs (n=3)

Formulation	**Dose (mg/kg)**	**LSK**	**LT-HSC**
PBS	-	0	0
F-81	2.0	0.01	0
F-82	2.0	3.65	11.7
F-83	2.0	0.86	2.67
F-84	2.0	0.29	1.73

CLAIMS

1. A compound of Formula (TL'')



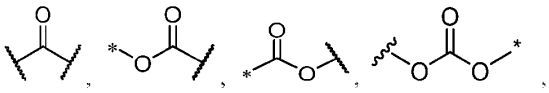
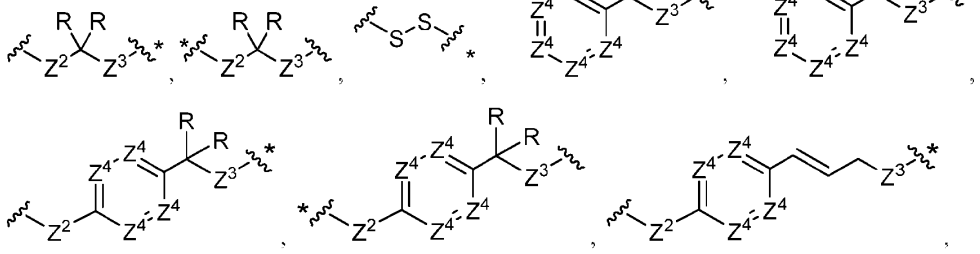
(TL''),

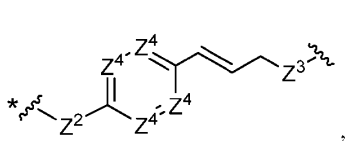
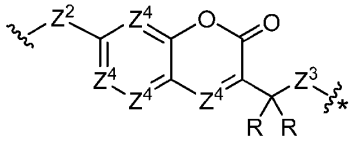
or a pharmaceutically acceptable salt thereof, wherein:

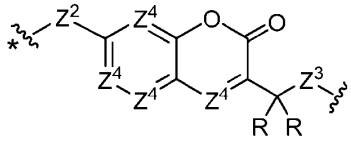
A^1 is selected from CH and N;

A^2 is selected from CH and N;

L^1 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

Z^1 is selected from the group consisting of: , 



, and


, wherein the bond marked with an "*" is attached to L^1;

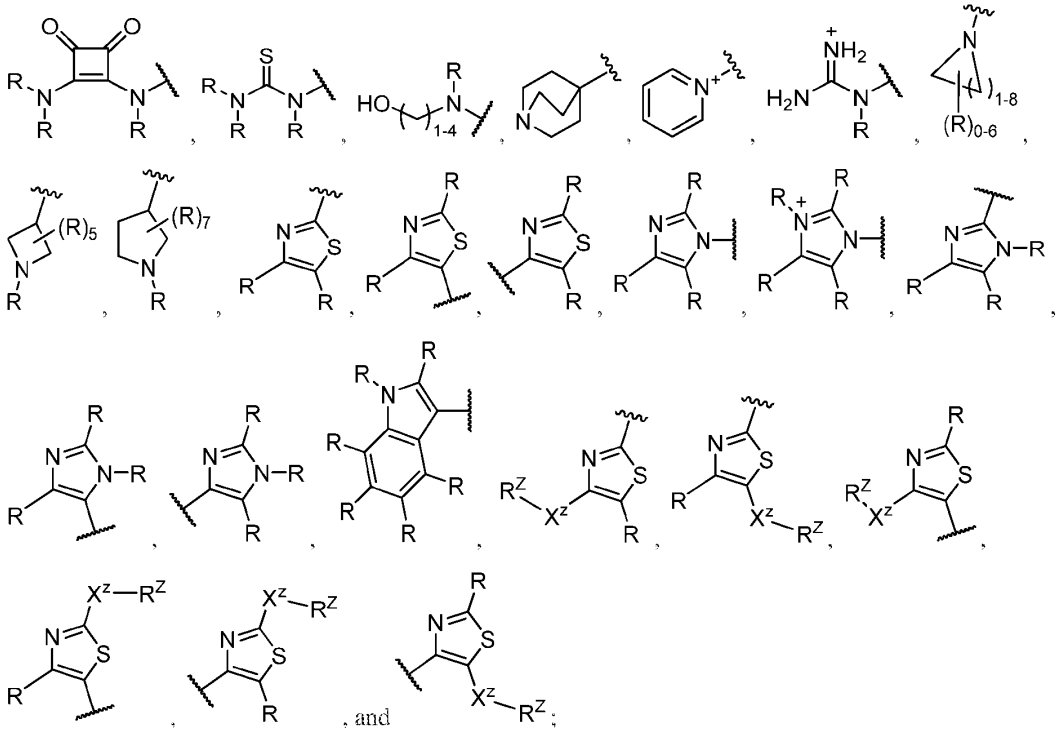
Z^2 is selected from the group consisting of -O-, -NR-, and -S-;

Z^3 is selected from the group consisting of a bond, -O-, -NR-, -S-, -OC(O)-, -C(O)O-, -NRC(O)-, -C(O)NR-,-NRC(O)O-, or -OC(O)NR-;

each Z^4 is independently selected from =CR- and =N-;

L^2 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^1 is selected from the group consisting of -OH, -OAc, $-NR_2$,


, and

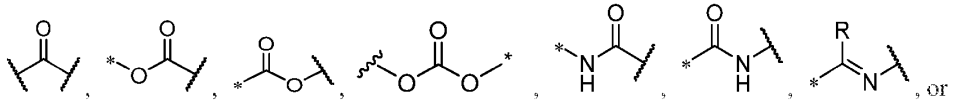
each R is independently -H or C_1-C_6 aliphatic;

X^Z is a bond or optionally substituted C_1-C_6 aliphatic;

R^Z is NR_2 or OH;

X^1 and X^A are each independently a bond or optionally substituted C_1-C_6 aliphatic;

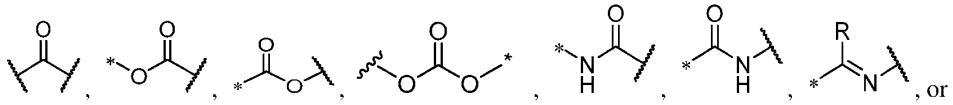
Y^1 is selected from the group consisting of



, and bond; wherein the bond marked with an "*" is attached to X^1;

each of X^2, X^3, and X^6 is independently a bond or optionally substituted C_1-C_{12} aliphatic;

each of Y^2, Y^3, and Y^4 is independently selected from the group consisting of



; wherein the bond marked with an "*" is attached to X^2, X^3, of X^6, as appropriate;

each of X^4, X^5, and X^7 is independently optionally substituted C_1-C_6 aliphatic;

R^2 is -CH(OR^6)(OR^7), -CH(SR^6)(SR^7), -CH(R^6)(R^7), -R^{10}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{10}, wherein one or more methylene linkages of R^2 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^3 is -CH(OR^8)(OR^9), -CH(SR^8)(SR^9), -CH(R^8)(R^9), -R^{11}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{11}, wherein one or more methylene linkages of R^3 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^{12} is -CH(OR^{13})(OR^{14}), -CH(SR^{13})(SR^{14}), -CH(R^{13})(R^{14}), -R^{15}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{15}, wherein one or more methylene linkages of R^{12} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

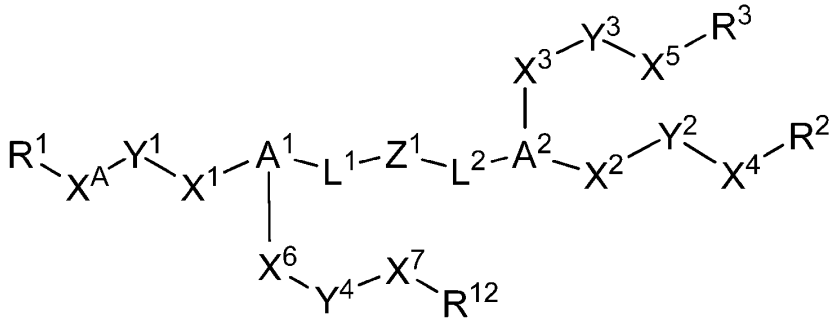
R^6 and R^7 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{10}, or optionally substituted -C_1-C_{14} aliphatic-R^{10}; wherein one or more methylene linkages of R^6 and R^7 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^8 and R^9 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{11}, or optionally substituted -C_1-C_{14} aliphatic-R^{11}; wherein one or more methylene linkages of R^8 and R^9 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^{13} and R^{14} are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{15}, or optionally substituted -C_1-C_{14} aliphatic-R^{15}; wherein one or more methylene linkages of R^{13} and R^{14} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

each of R^{10}, R^{11}, and R^{15} is independently an optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic C_4-C_{14} cycloalkyl or optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic 4-14 membered heterocyclyl, or two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic C_4-C_{14} cycloalkyl or optionally substituted bridged bicyclic or multicyclic 4-14 membered heterocyclyl.

2. The compound of claim A, wherein the compound is of Formula (TL):



(TL),

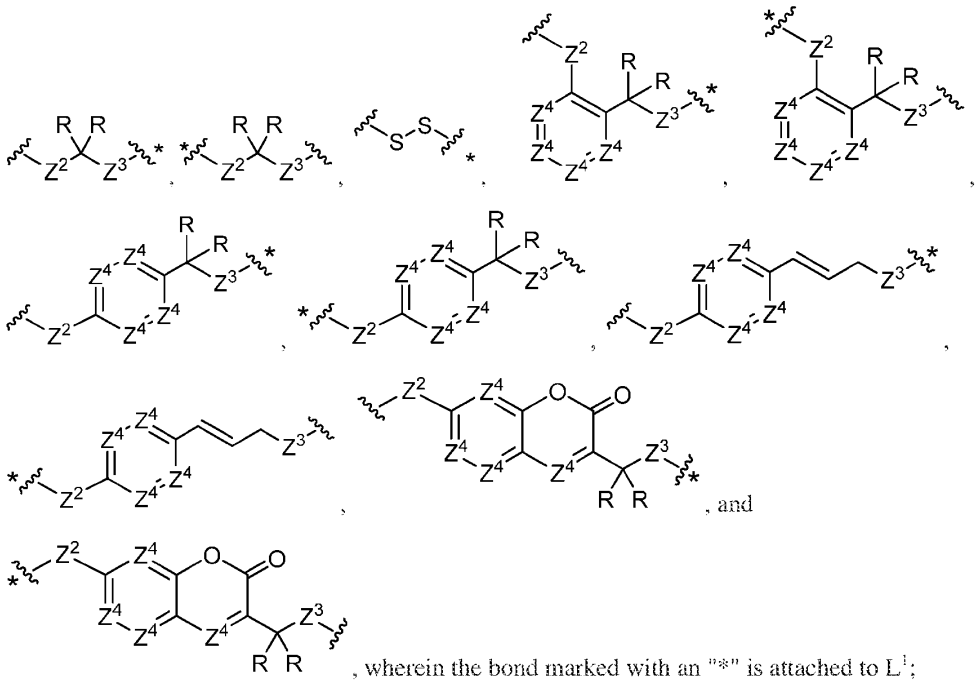
or a pharmaceutically acceptable salt thereof, wherein:

A^1 is selected from CH and N;

A^2 is selected from CH and N;

L^1 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

Z^1 is selected from the group consisting of



, wherein the bond marked with an "*" is attached to L^1;

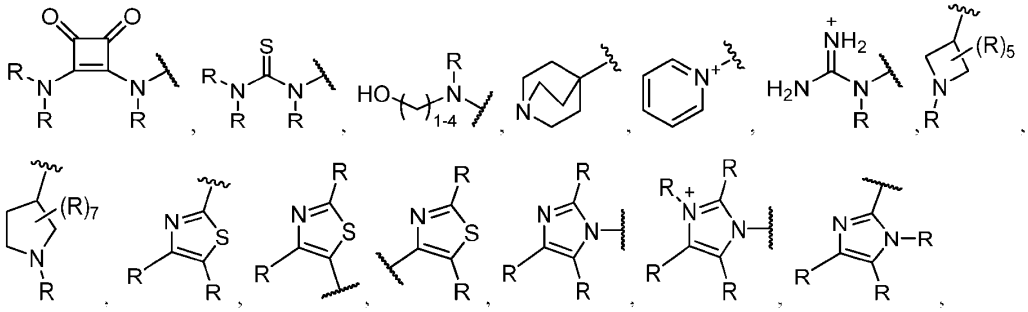
Z^2 is selected from the group consisting of -O-, -NR-, and -S-;

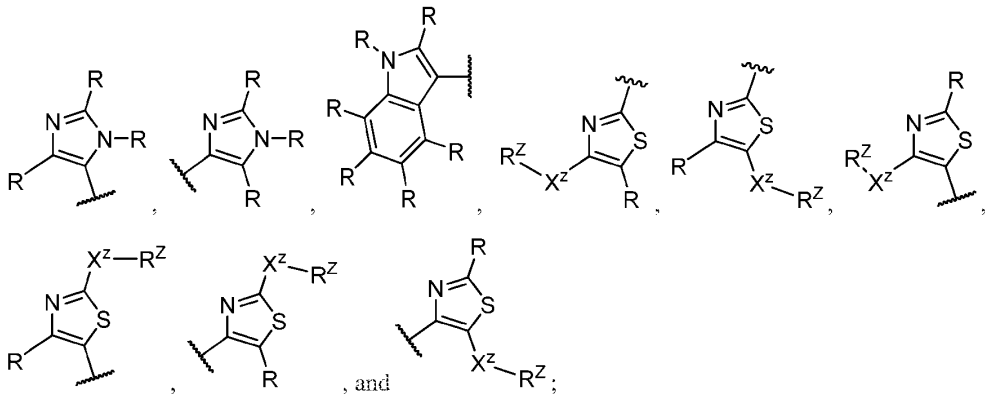
Z^3 is selected from the group consisting of a bond, -O-, -NR-, -S-, -OC(O)-, -C(O)O-, -NRC(O)-, -C(O)NR-,-NRC(O)O-, or -OC(O)NR-;

each Z^4 is independently selected from =CR- and =N-;

L^2 is optionally substituted C_1-C_8 aliphatic, wherein one or more methylene linkages of L^1 are each optionally and independently replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^1 is selected from the group consisting of -OH, -OAc, $-NR_2$,





, and ;

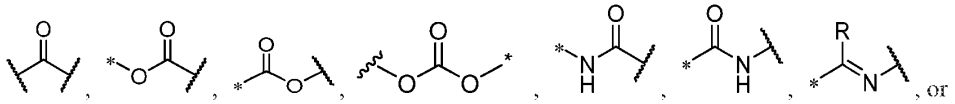
each R is independently -H or C_1-C_6 aliphatic;

X^Z is a bond or optionally substituted C_1-C_6 aliphatic;

R^Z is NR_2 or OH;

X^1 and X^A are each independently a bond or optionally substituted C_1-C_6 aliphatic;

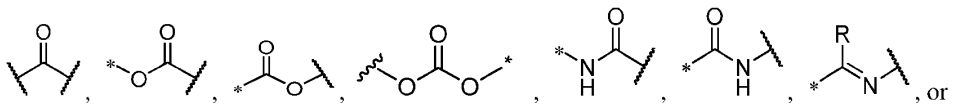
Y^1 is selected from the group consisting of



, and bond; wherein the bond marked with an "*" is attached to X^1;

each of X^2, X^3, and X^6 is independently a bond or optionally substituted C_1-C_{12} aliphatic;

each of Y^2, Y^3, and Y^4 is independently selected from the group consisting of



; wherein the bond marked with an "*" is attached to X^2, X^3, of X^6, as appropriate;

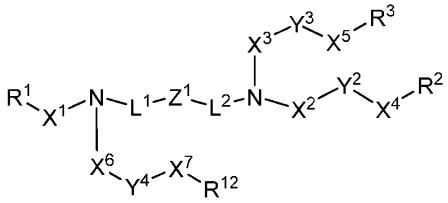
each of X^4, X^5, and X^7 is independently optionally substituted C_1-C_6 aliphatic;

R^2 is -CH(OR^6)(OR^7), -CH(SR^6)(SR^7), -CH(R^6)(R^7), -R^{10}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{10}, wherein one or more methylene linkages of R^2 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;

R^3 is -CH(OR^8)(OR^9), -CH(SR^8)(SR^9), -CH(R^8)(R^9), -R^{11}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{11}, wherein one or more methylene linkages of R^3 are each

optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;
R^{12} is -CH(OR^{13})(OR^{14}), -CH(SR^{13})(SR^{14}), -CH(R^{13})(R^{14}), -R^{15}, optionally substituted C_5-C_{18} aliphatic, or optionally substituted C_1-C_{14} aliphatic-R^{15}, wherein one or more methylene linkages of R^{12} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;
R^6 and R^7 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{10}, or optionally substituted -C_1-C_{14} aliphatic-R^{10}; wherein one or more methylene linkages of R^6 and R^7 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;
R^8 and R^9 are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{11}, or optionally substituted -C_1-C_{14} aliphatic-R^{11}; wherein one or more methylene linkages of R^8 and R^9 are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;
R^{13} and R^{14} are each independently optionally substituted -C_1-C_{14} aliphatic, -R^{15}, or optionally substituted -C_1-C_{14} aliphatic-R^{15}; wherein one or more methylene linkages of R^{13} and R^{14} are each optionally and independently replaced with an optionally substituted C_3-C_8 cycloalkylenyl, phenyl, -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-;
each of R^{10}, R^{11}, and R^{15} is independently an optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic C_4-C_{14} cycloalkyl or optionally substituted cylic, bicyclic, bridged bicyclic, multicyclic or bridged multicyclic 4-14 membered heterocyclyl, or two R^{10}, R^{11} or R^{15} are taken together form an optionally substituted bridged bicyclic or multicyclic C_4-C_{14} cycloalkyl or optionally substituted bridged bicyclic or multicyclic 4-14 membered heterocyclyl.

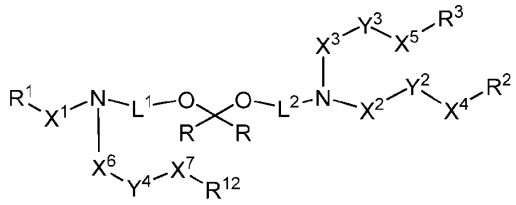
3. The compound of claim 2, wherein the compound is of Formula (TL-A)



(TL-A),

or a pharmaceutically acceptable salt thereof.

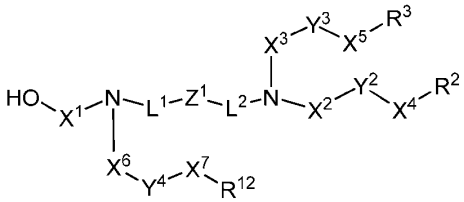
4. The compound of claim 2, wherein the compound is of Formula (TL-B):



(TL-B),

or a pharmaccutically accptablc salt thrcof.

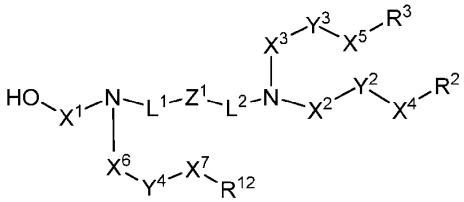
5. The compound of claim 2, wherein the compound is of Formula (TL-B'):



(TL-B'),

or a pharmaceutically acceptable salt thereof.

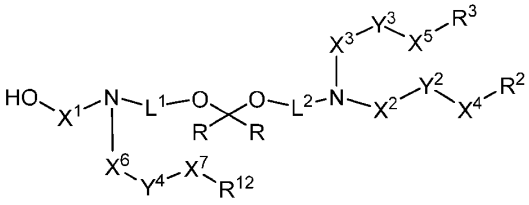
6. The compound of claim 2, wherein the compound is of Formula (TL-C):



(TL-C),

or a pharmaccutically accptablc salt thrcof.

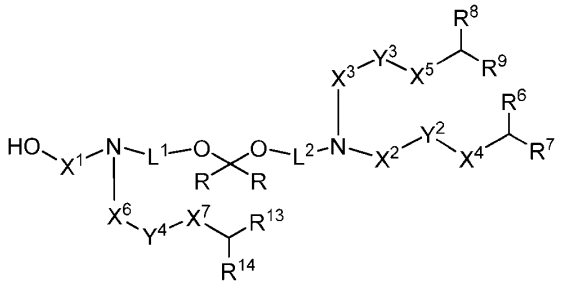
7. The compound of claim 2, wherein the compound is of Formula (TL-D):



(TL-D),

or a pharmaceutically acceptable salt thereof.

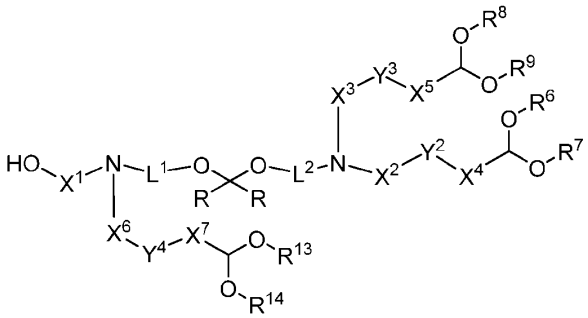
8. The compound of claim 2, wherein the compound is of Formula (TL-E):



(TL-E),

or a pharmaceutically acceptable salt thereof.

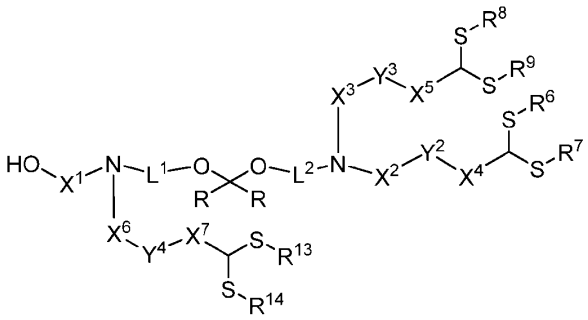
9. The compound of claim 2, wherein the compound is of Formula (TL-E'):



(TL-E'),

or a pharmaceutically acceptable salt thereof.

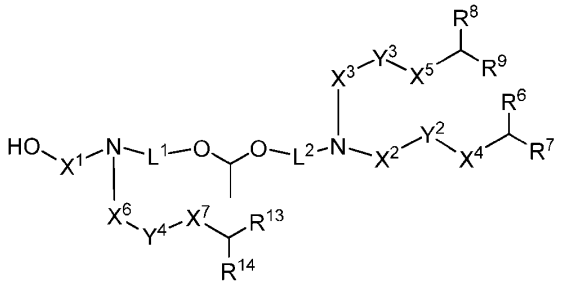
10. The compound of claim 2, wherein the compound is of Formula (TL-E''):



(TL-E''),

or a pharmaceutically acceptable salt thereof.

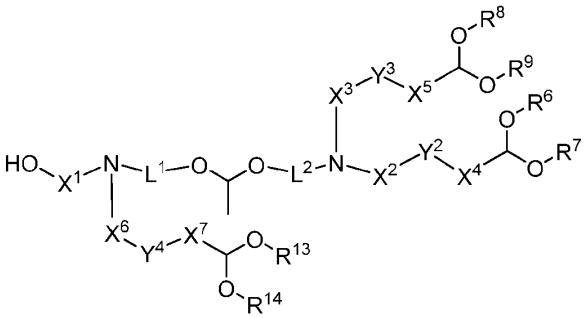
11. The compound of claim 2, wherein the compound is of Formula (TL-F):



(TL-F),

or a pharmaceutically acceptable salt thereof.

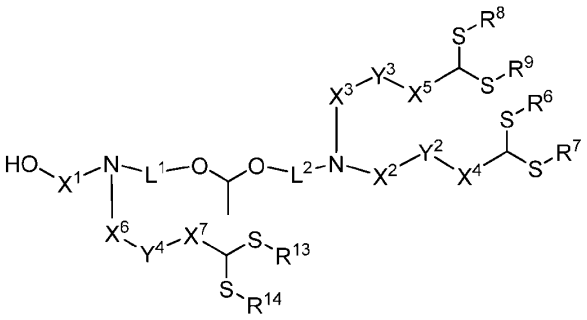
12. The compound of claim 2, wherein the compound is of Formula (TL-F'):



(TL-F'),

or a pharmaceutically acceptable salt thereof.

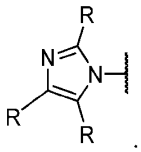
13. The compound of claim 2, wherein the compound is of Formula (TL-F''):



(TL-F''),

or a pharmaceutically acceptable salt thereof.

14. The compound of any one of claims 1-4, wherein R^1 is -OH.

15. The compound of any one of claims 1-4, wherein R^1 is .

16. The compound of any one of claims 1-4, wherein R^1 is .

17. The compound of any of claims 2-16, wherein X^1 is a bond.

18. The compound of any of claims 2-16, wherein X^1 is optionally substituted C_2-C_4 alkylene.

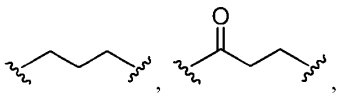
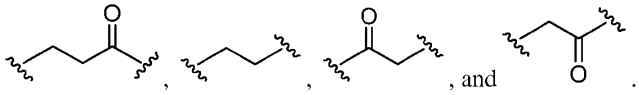
19. The compound of claim 17, wherein X^1 is selected from the group consisting of -$(CH_2)_2$-, -$(CH_2)_3$- and -$(CH_2)_4$-.

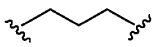
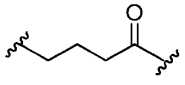
20. The compound of claim 19, wherein X^1 is -$(CH_2)_2$-.

21. The compound of claim 19, wherein X^1 is -$(CH_2)_3$-.

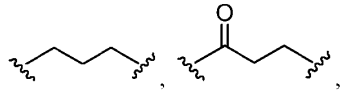
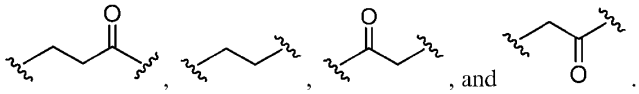
22. The compound of claim 19, wherein X^1 is -$(CH_2)_4$-.

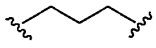
23. The compound of any one of claims 1-22, wherein L^1 is unsubstituted C_1-C_8 aliphatic, wherein one methylene linkage of L^1 is optionally replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-.

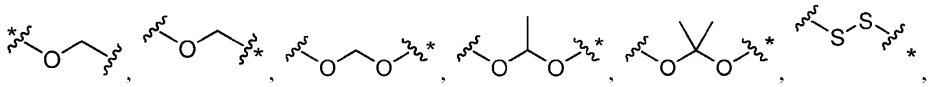
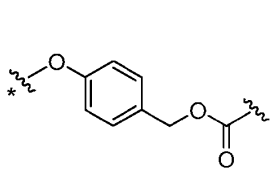
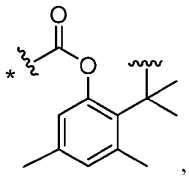
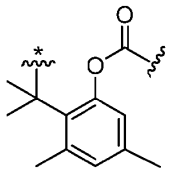
24. The compound of claim 23, wherein L^1 is selected from , , and .

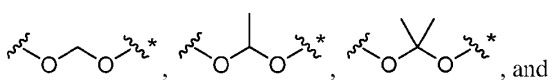
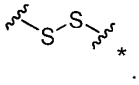
25. The compound of claim 23, wherein The compound of claim 22, wherein L^1 , or .

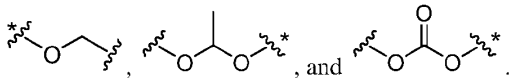
26. The compound of any one of claims 1-25, wherein L^2 is unsubstituted C_1-C_8 aliphatic, wherein one methylene linkage of L^1 is optionally replaced with -O-, -NH-, -S-, -SS-, -C(O)-, -OC(O)O-, -OC(O)-, -NHC(O)-, or -C(O)O-.

27. The compound of claim 26, wherein L^2 is selected from , , and .

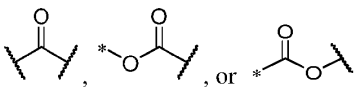
28. The compound of claim 27, wherein L^2 is .

29. The compound of any one of the preceding claims, wherein Z^1 is selected from , , , and , wherein the bond marked with an "*" is attached to L^1.

30. The compound of any one of the preceding claims, wherein Z^1 is selected from , and .

31. The compound of any one of the preceding claims, wherein Z^1 is selected from .

32. The compound of any one of the preceding claims wherein Y^1 is a bond.

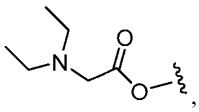
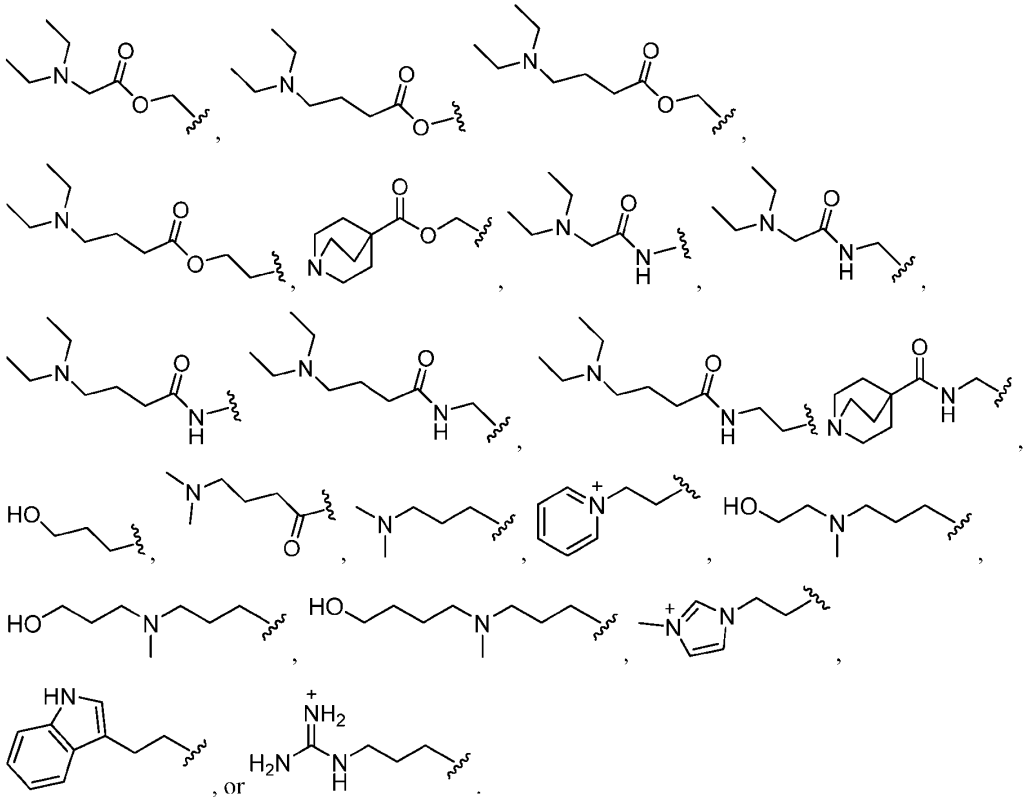
33. The compound of any one of the preceding claims, wherein Y^1 is .

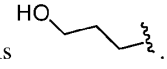
34. The compound of any one of the preceding claims, wherein Y^1 is a bond.

35. The compound of any one of the preceding claims, wherein X^A is a bond.

36. The compound of any one of the preceding claims, wherein X^A is optionally substituted C_2-C_4 alkylene.

37. The compound any one of the preceding claims, wherein X^A is selected from the group consisting of -$(CH_2)_2$-, -$(CH_2)_3$- and -$(CH_2)_4$-.

38. The compound of any one of the preceding claims, wherein -X^1-Y^1-X^A-R^1 is , , or .

39. The compound of any one of the preceding claims, wherein -X^1-Y^1-X^A-R^1 is .

40. The compound of any one of the preceding claims, wherein X^2 is an optionally substituted C_4-C_{10}

alkylene.

41. The compound of any one of the preceding claims, wherein X^2 is an unsubstituted C_4-C_{10} alkylene.

42. The compound of any one of the preceding claims, wherein X^2 is an unsubstituted C_8 alkylene.

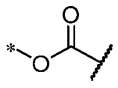
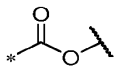
43. The compound of any one of the preceding claims, wherein X^3 is an optionally substituted C_4-C_{10} alkylene.

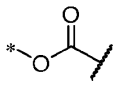
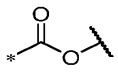
44. The compound of any one of the preceding claims, wherein X^3 is an unsubstituted C_4-C_{10} alkylene.The compound of any one of the preceding claims, wherein X^3 is an unsubstituted C_8 alkylene.

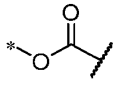
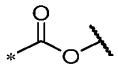
45. The compound of any one of the preceding claims, wherein X^6 is an optionally substituted C_4-C_{10} alkylene.

46. The compound of any one of the preceding claims, wherein X^6 is an unsubstituted C_4-C_{10} alkylene.

47. The compound of any one of the preceding claims, wherein X^6 is an unsubstituted C_8 alkylene.

48. The compound of any one of the preceding claims, wherein Y^2 is  or .

49. The compound of any one of the preceding claims, wherein Y^3 is  or .

50. The compound of any one of the preceding claims, wherein Y^4 is  or .

51. The compound of any one of the preceding claims, wherein X^4 is an optionally substituted C_2-C_5 alkylene.

52. The compound of any one of the preceding claims, wherein X^4 is an unsubstituted C_2-C_5 alkylene.

53. The compound of any one of the preceding claims, wherein X^5 is an optionally substituted C_2-C_5 alkylene.

54. The compound of any one of the preceding claims, wherein X^5 is an unsubstituted C_2-C_5 alkylene.

55. The compound of any one of the preceding claims, wherein X^5 is an unsubstituted C_2 alkylene.

56. The compound of any one of the preceding claims, wherein X^7 is an optionally substituted C_2-C_5 alkylene.

57. The compound of any one of the preceding claims, wherein X^7 is an unsubstituted C_2-C_5 alkylene.The compound of any one of the preceding claims, wherein X^7 is an unsubstituted C_2 alkylene.

58. The compound of any one of the preceding claims, wherein R^2 is $-CH(R^6)(R^7)$.

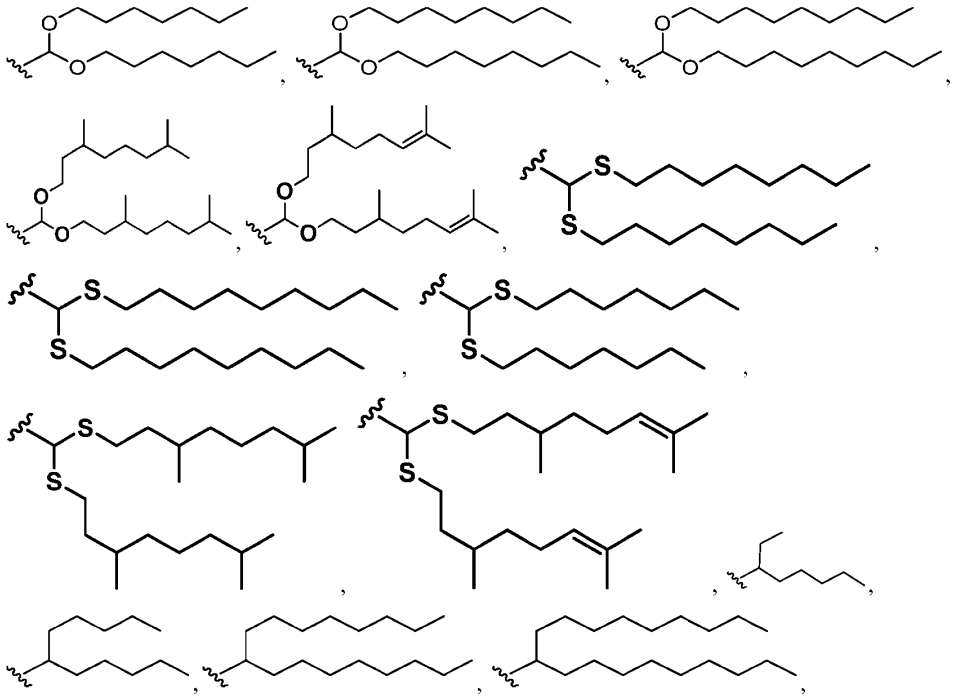
59. The compound of any one of the preceding claims, wherein R^2 is $-CH(OR^6)(OR^7)$.

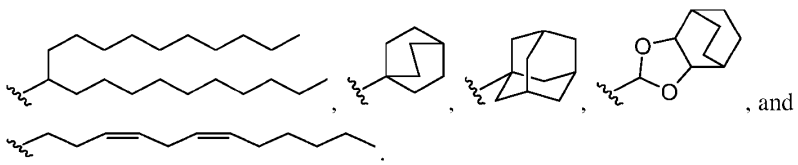
60. The compound of any one of the preceding claims, wherein R^2 is $-CH(SR^6)(SR^7)$.

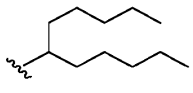
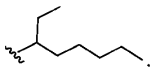
61. The compound of any one of the preceding claims, wherein R^2 is $-R^{10}$.

62. The compound of any one of the preceding claims, wherein R^2 is optionally substituted C_1-C_{14} aliphatic-R^{10}.

63. The compound of any one of the preceding claims, wherein R^2 is selected from





64. The compound of any of the preceding claims, wherein R^2 is  or .

65. The compound of any one of the preceding claims, wherein R^3 is $-CH(R^8)(R^9)$.

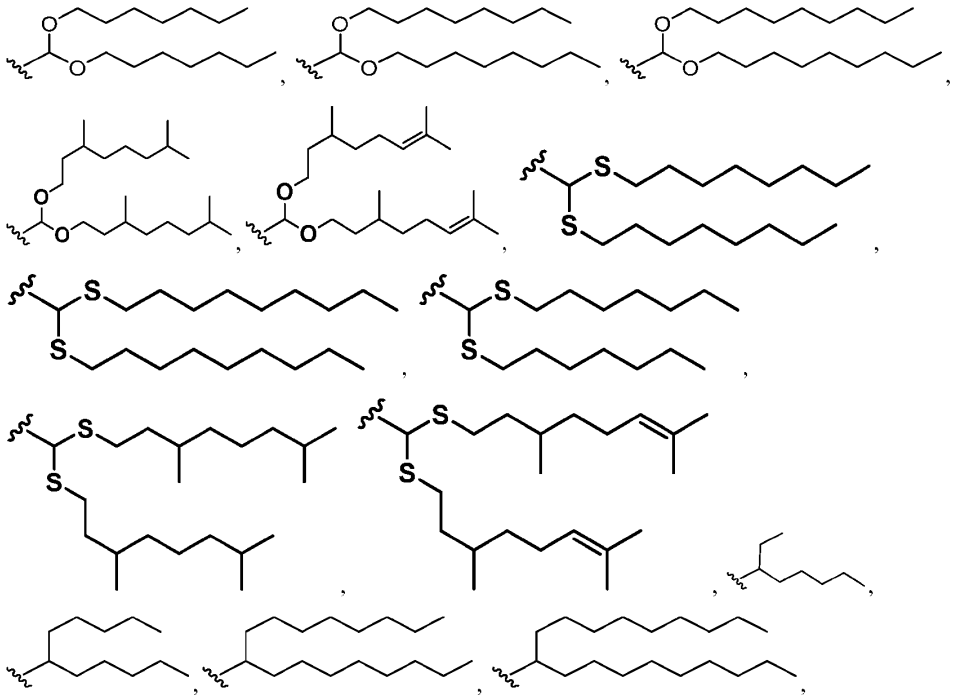
66. The compound of any one of the preceding claims, wherein R^3 is $-CH(OR^8)(OR^9)$.

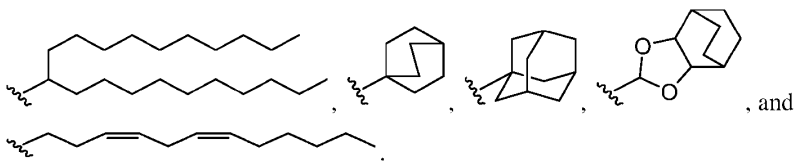
67. The compound of any one of the preceding claims, wherein R^3 is $-CH(SR^8)(SR^9)$.

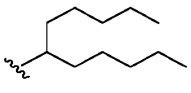
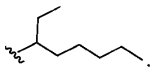
68. The compound of any one of the preceding claims, wherein R^3 is $-R^{11}$.

69. The compound of any one of the preceding claims, wherein R^3 is optionally substituted C_1-C_{14} aliphatic-R^{11}.

70. The compound of any one of the preceding claims, wherein R^3 is selected from




, and

71. The compound of any of the preceding claims, wherein R^3 is  or .

72. The compound of any one of the preceding claims, wherein R^{12} is $-CH(R^{13})(R^{14})$.

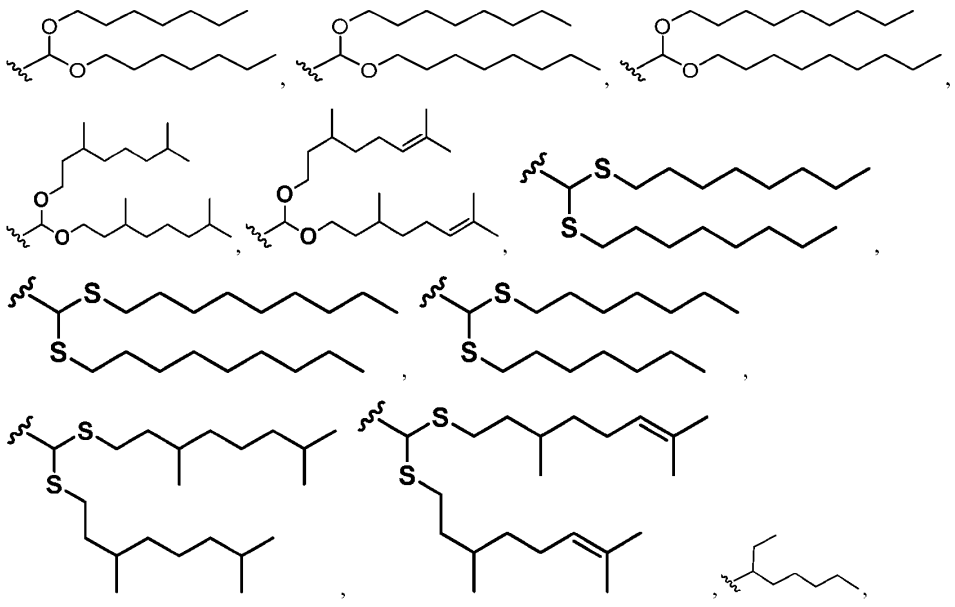
73. The compound of any one of the preceding claims, wherein R^{12} is $-CH(OR^{13})(OR^{14})$.

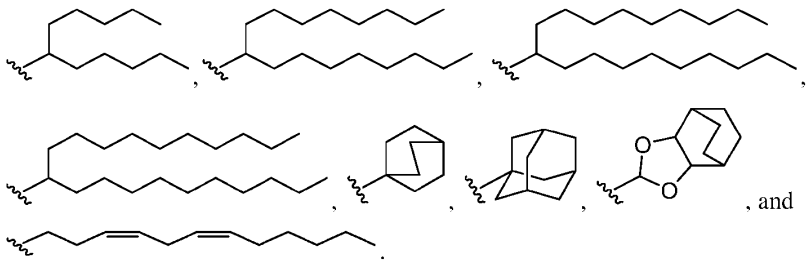
74. The compound of any one of the preceding claims, wherein R^{12} is $-CH(SR^{13})(SR^{14})$.

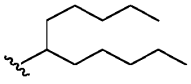
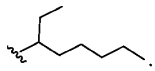
75. The compound of any one of the preceding claims, wherein R^{12} is $-R^{15}$.

76. The compound of any one of the preceding claims, wherein R^{12} is optionally substituted C_1-C_{14} aliphatic-R^{15}.

77. The compound of any one of the preceding claims, wherein R^{12} is selected from





78. The compound of any of the preceding claims, wherein R^3 is  or .

79. A compound selected from the group consisting of those disclosed in Table (I) or a pharmaceutically acceptable salt therefore.

80. A pharmaceutical composition comprising:
a) at least one lipid nanoparticle comprising at least one compound of any one of claims 1-79; and
b) at least one nucleobase editing system.

81. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises a CRISPR-Cas gene editing system.

82. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises a prime editing system or components thereof.

83. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises a retron editing system.

84. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises a TnpB editing system.

85. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises an integrase editing system.

86. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises an integrase editing system.

87. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises an epigenetic editing system.

88. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises a gene writing system.

89. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises a gene inactivating system.

90. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises zinc finger nuclease.

91. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises a TALE Nuclease, a TALE nickase, Zinc Finger (ZF) Nuclease, ZF Nickase, meganuclease, or a combination thereof.

92. The pharmaceutical composition of claim 80, wherein the nucleobase editing system comprises a meganuclease.

93. The pharmaceutical composition of any one of claims 80-92, wherein the at least one lipid nanoparticle further comprises:

i)at least one structural lipid;

ii) at least one phospholipid; and

iii)at least one PEGylated lipid.

94. The pharmaceutical composition of any one of claims 80-93, wherein the at least one structural lipid is selected from cholesterol, fecosterol, fucosterol, beta sitosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, cholic acid, sitostanol, litocholic acid, tomatine, ursolic acid, alpha-tocopherol, Vitamin D3, Vitamin D2, Calcipotriol, botulin, lupeol, oleanolic acid, beta-sitosterol-acetate and any combinations thereof.

95. The pharmaceutical composition of any one of claims 80-94, wherein the at least one phospholipid is selected from 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1.2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocho line (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-

didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sodium (S)-2-ammonio-3-((((R)-2-(oleoyloxy)-3-(stearoyloxy)propoxy)oxidophosphoryl)oxy)propanoate (L-α-phosphatidylserine; Brain PS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE), 1,2-Dielaidoyl-sn-phosphatidylethanolamine (DEPE), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), 1,2-dioleoyl-sn-glycero-3-phosphate (18:1 PA; DOPA), ammonium bis((S)-2-hydroxy-3-(oleoyloxy)propyl) phosphate (18:1 DMP; LBPA), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol) (DOPI; 18:1 PI), 1,2-distearoyl-sn-glycero-3-phospho-L-serine (18:0 PS), 1,2-dilinoleoyl-sn-glycero-3-phospho-L-serine (18:2 PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (16:0-18:1 PS; POPS), 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (18:0-18:1 PS), 1-stearoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (18:0-18:2 PS), 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:1 Lyso PS), 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:0 Lyso PS), and sphingomyelin.

96. T The pharmaceutical composition of any one of claims 80-95, wherein the at least one PEGylated lipid is selected from those depicted in Table (I-X).

97. The pharmaceutical composition of any one of claims 80-95, wherein the at least one PEGylated lipid is selected from (R)-2,3-bis(octadecyloxy)propyl-1-(methoxypoly(ethyleneglycol)2000)propylcarbamate, PEG-S-DSG, PEG-S-DMG, PEG-PE, PEG-PAA, PEG-OH DSPE C18, PEG-DSPE, PEG-DSG, PEG-DPG, PEG-DOMG, PEG-DMPE Na, PEG-DMPE, PEG-DMG2000, PEG-DMG C14, PEG-DMG 2000, PEG-DMG, PEG-DMA, PEG-Ceramide C16, PEG-C-DOMG, PEG-c-DMOG, PEG-c-DMA, PEG-cDMA, PEGA, PEG750-C-DMA, PEG400, PEG2k-DMG, PEG2k-C11, PEG2000-PE, PEG2000P, PEG2000-DSPE, PEG2000-DOMG, PEG2000-DMG, PEG2000-C-DMA, PEG2000, PEG200, PEG(2k)-DMG, PEG DSPE C18, PEG

DMPE C14, PEG DLPE C12, PEG Click DMG C14, PEG Click C12, PEG Click C10, N(Carbonyl-methoxypolyethylenglycol-2000)-l,2-distearoyl-sn-glycero3-phosphoethanolamine, Myrj52, mPEG-PLA, MPEG-DSPE, mPEG3000-DMPE, MPEG-2000-DSPE, MPEG2000-DSPE, mPEG2000-DPPE, mPEG2000-DMPE, mPEG2000-DMG, mDPPE-PEG2000, l,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG2000, HPEG-2K-LIPD, Folate PEG-DSPE, DSPE-PEGMA 500, DSPE-PEGMA, DSPE-PEG6000, DSPE-PEG5000, DSPE-PEG2K-NAG, DSPE-PEG2k, DSPE-PEG2000maleimide, DSPE-PEG2000, DSPE-PEG, DSG-PEGMA, DSG-PEG5000, DPPE-PEG-2K, DPPE-PEG, DPPE-mPEG2000, DPPE-mPEG, DPG-PEGMA, DOPE-PEG2000, DMPE-PEGMA, DMPE-PEG2000, DMPE-Peg, DMPE-mPEG2000, DMG-PEGMA, DMG-PEG2000, DMG-PEG, distearoyl-glycerol-polyethyleneglycol, Cl8PEG750, CI8PEG5000, CI8PEG3000, CI8PEG2000, CI6PEG2000, CI4PEG2000, C18-PEG5000, C18PEG, C16PEG, C16 mPEG (polyethylene glycol) 2000 Ceramide, C14-PEG-DSPE200, C14-PEG2000, C14PEG2000, C14-PEG 2000, C14-PEG, C14PEG, 14:0-PEG2KPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG2000, (R)-2,3-bis(octadecyloxy)propyl-1-(methoxypoly(ethyleneglycol)2000)propylcarbamate, (PEG)-C-DOMG, PEG-C-DMA, and DSPE-PEG-X.

98. The pharmaceutical composition of any one of claims 80-96, wherein the LNP further comprises at least one additional lipid component selected from 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine (18:3 PC), Acylcarnosine (AC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), N-oleoyl-sphingomyelin (SPM) (C18:l), N-lignoceryl SPM (C24:0), N-nervonoylshphingomyelin (C24:l), Cardiolipin (CL), l,2-bis(tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine (DC8-9PC), dicetyl phosphate (DCP), dihexadecyl phosphate (DCP1), 1,2-Dipalmitoylglycerol-3-hemisuccinate (DGSucc), short-chain bis-n-heptadecanoyl phosphatidylcholine (DHPC), dihexadecoyl-phosphoethanolamine (DHPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), l,2-dilauroyl-sn-glycero-3-PE (DLPE), dimyristoyl glycerol hemisuccinate (DMGS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleyloxybenzylalcohol (DOBA), 1,2-dioleoylglyceryl-3-hemisuccinate (DOGHEMS), N-[2-(2-{2-[2-(2,3-Bis-octadec-9-enyloxy-propoxy)-ethoxy]-ethoxy}-ethoxy)-ethyl]-3-(3,4,5-1rihydroxy-6-hydroxymethyl-1etrahydro-pyran-2-ylsulfanyl)-propionamide (DOGP4αMan), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE),

dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), histaminedistearoylglycerol (HDSG), 1,2-Dipalmitoylglycerol-hemisuccinate-Nα-Histidinyl-Hemisuccinate (HistSuccDG), N-(5'-hydroxy-3'-oxypentyl)-10-12-pentacosadiynamide (h-Pegi-PCDA), 2-[l-hexyloxyethyl]-2-devinylpyropheophorbide-a (HPPH), hydrogenatedsoybeanphosphatidylcholine (HSPC), 1,2-Dipalmitoylglycerol-O-α-histidinyl-Nα-hemisuccinate (IsohistsuccDG), mannosialized dipalmitoylphosphatidylethanolamine (ManDOG), l,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (MCC-PE), 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16:0 PE), 1-myristoyl-2-hydroxy-sn-glycero-phosphocholine (MHPC), a thiol-reactive maleimide headgroup lipid e.g.1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)but-yramid (MPB-PE), Nervonic Acid (NA), sodium cholate (NaChol), l,2-dioleoyl-sn-glycero-3-[phosphoethanolamine-N-dodecanoyl (NC12-DOPE), 1-oleoyl-2-cholesteryl hemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), phosphatidylethanolamine lipid (PE), PE lipid conjugated with polyethylene glycol(PEG) (e.g., polyethylene glycol-distearoylphosphatidylethanolamine lipid (PEG-PE)), phosphatidylglycerol (PG), partially hydrogenated soy phosphatidylchloline (PHSPC), phosphatidylinositol lipid (PI), phosphotidylinositol-4-phosphate (PIP), palmitoyloleoylphosphatidylcholine (POPC), phosphatidylethanolamine (POPE), palmitoyloleyolphosphatidylglycerol (POPG), phosphatidylserine (PS), lissamine rhodamineB-phosphatidylethanolamine lipid (Rh-PE), purifiedsoy-derivedmixtureofphospholipids (SIOO), phosphatidylcholine (SM), 18-1-trans-PE,1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), soybean phosphatidylcholine (SPC), sphingomyelins (SPM), alpha,alpha-trehalose-6,6'-dibehenate (TDB), l,2-dielaidoyl-sn-glycero-3-phophoethanolamine (transDOPE), ((23S,5R)-3-(bis(hexadecyloxy)methoxy)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methylmethylphosphate, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleyl-sn-glycero-3-phosphoethanolamine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 16-O-monomethyl PE, 16-O-dimethyl PE, and dioleylphosphatidylethanolamine.

99. A method of delivering a nucleobase editing system to a subject in need thereof, the method

comprising administering to the subject the pharmaceutical composition of any one of claims 80-98.

100. The pharmaceutical composition of any one of claims 80-99 for use as a medicament.

101. Use of a pharmaceutical composition of any one of claims 80-99 for the manufacture of a medicament for delivery of a nucleobase editing system.

102. A lipid nanoparticle (LNP) comprising a compound of any one of claims 1-79, or a pharmaceutically acceptable salt thereof.

103. The LNP of claim 102, further comprising:

(a) a PEG-lipid

(b) a structural lipid; and

(c) a non-ionizable lipid and/or a zwitterionic lipid.

104. The LNP of claim 103, wherein the lipid nanoparticle further comprises an additional ionizable lipid, besides a compound of Formula (TL).

105. The LNP of claim 103 or 104, wherein the PEG-lipid is selected from those depicted in Table (I-X).

106. The LNP of claim 103 or 104, wherein the PEG-lipid is selected from the group consisting of PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPC, and PEG-DSPE.

107. The LNP of any one of claims 103-106, wherein the structural lipid is selected from the group consisting of cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, ursolic acid, an alpha-tocopherol.

108. The LNP of any one of claims 103-107, wherein the non-ionizable lipid is a phospholipid selected from the group consisting of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1.2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocho line (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-

phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sodium (S)-2-ammonio-3-((((R)-2-(oleoyloxy)-3-(stearoyloxy)propoxy)oxidophosphoryl)oxy)propanoate (L-α-phosphatidylserine; Brain PS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), 1,2-dioleoyl-sn-glycero-3-phosphate (18:1 PA; DOPA), ammonium bis((S)-2-hydroxy-3-(oleoyloxy)propyl) phosphate (18:1 DMP; LBPA), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol) (DOPI; 18:1 PI), 1,2-distearoyl-sn-glycero-3-phospho-L-serine (18:0 PS), 1,2-dilinoleoyl-sn-glycero-3-phospho-L-serine (18:2 PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (16:0-18:1 PS; POPS), 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (18:0-18:1 PS), 1-stearoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (18:0-18:2 PS), 1-oleoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:1 Lyso PS), 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:0 Lyso PS), and sphingomyelin.

109. The LNP of any one of claims 103-108, further comprising a targeting moiety.

110. The LNP of claim 109, wherein the targeting moiety is an antibody or a fragment thereof.

111. The LNP of any one of claims 103-110, further comprising an active agent.

112. The LNP of claim 111, wherein the active agent is a nucleic acid.

113. The LNP of claim 112, wherein the nucleic acid is a ribonucleic acid.

114. The LNP of claim 113, wherein the ribonucleic acid is at least one ribonucleic acid selected from the group consisting of a small interfering RNA (siRNA), an asymmetrical interfering RNA (aiRNA), a microRNA (miRNA), a Dicer-substrate RNA (dsRNA), a small hairpin RNA (shRNA), a messenger RNA (mRNA), and a long non-coding RNA (lncRNA).

115. The LNP of claim 114, wherein the nucleic acid is a messenger RNA (mRNA) or a circular RNA.

116. The LNP of claim 115, wherein the mRNA includes an open reading frame encoding a cancer antigen.

117. The LNP of claim 115, wherein the mRNA includes an open reading frame encoding an immune checkpoint modulator.

118. The LNP of any one of claims 115-117, wherein the mRNA includes at least one motif selected from the group consisting of a stem loop, a chain terminating nucleoside, a polyA sequence, a polyadenylation signal, and a 5' cap structure.

119. The LNP of claim 112, wherein the nucleic acid is suitable for a genome editing technique.

120. The LNP of claim 119, wherein the genome editing technique is clustered regularly interspaced short palindromic repeats (CRISPR) or transcription activator-like effector nuclease (TALEN).

121. The LNP of claim 112, wherein the nucleic acid is at least one nucleic acid suitable for a genome editing technique selected from the group consisting of a CRISPR RNA (crRNA), a trans-activating crRNA (tracrRNA), a single guide RNA (sgRNA), and a DNA repair template.

122. The LNP of claim 115, wherein the mRNA is at least 30 nucleotides in length.

123. The LNP of claim 115, wherein the mRNA is at least 300 nucleotides in length.

124. A pharmaceutical composition comprising a LNP of any one of claims 103-123, and a pharmaceutically acceptable carrier.

125. The pharmaceutical composition of claim 124, formulated for intravenous or intramuscular administration.

126. The pharmaceutical composition of claim 124, which is formulated for intravenous administration.

127. A method for delivering a nucleic acid to a cell comprising contacting the cell with a LNP of any one of claims 103-123 or a pharmaceutical composition of any one of claims 99-101.

128. A method for treating a disease characterized by a deficiency of a functional protein, the method comprising administering to a subject having the disease, a LNP formulation comprising a LNP of any one of claims 103-123, wherein the mRNA encodes the functional protein or a protein having the

same biological activity as the functional protein.

129. A method for treating a disease characterized by overexpression of a polypeptide, comprising administering to a subject having the disease a LNP formulation comprising a LNP of any one of claims 103-123 and a siRNA, wherein the siRNA targets expression of the overexpressed polypeptide.

130. A pharmaceutical composition comprising:

a) at least one lipid nanoparticle (LNP) comprising at least one ionizable lipid selected from those in any one of claims 1-79; and

b) at least one gene editing system capable of editing, modifying or altering a polynucleotide sequence that results in treatment of a hemoglobinopathy.

131. The pharmaceutical composition of claim 129, wherein the at least one gene editing system comprises a gene editing system that corrects a sickle cell mutation by repairing the β-globin gene.

132. The pharmaceutical composition of any one of claims 129-130, wherein the at least one gene editing system comprises a gene editing system that corrects a sickle cell mutation through homology directed repair.

133. The pharmaceutical composition of any one of claims 129-130, wherein the at least one gene editing system comprises a gene editing system that corrects a sickle cell mutation through non-homologous end-joining (NHEJ), targeting aberrant splicing sites in introns I and II, resulting in restoration of functional β-globin transcripts.

134. The pharmaceutical composition of any one of claims 129-130, wherein the at least one gene editing system comprises a gene editing system that corrects a sickle cell mutation through a single base pair gene correction.

135. The pharmaceutical composition of any one of claims 129-130, wherein the at least one gene editing system comprises a gene editing system that corrects a sickle cell mutation by executing a single base pair correction, converting the aberrant thymine to adenine or cytosine.

136.. The pharmaceutical composition of claim 129-130, wherein the at least one gene editing system comprises a gene editing system that reactivates γ-globin production.

137. The pharmaceutical composition of any one of claims 129 and 135, wherein the at least one gene

editing system comprises a gene editing system that eliminates an HbF inhibitory sequence, or a portion thereof.

138. The pharmaceutical composition of any one of claims 129 and 135, wherein the at least one gene editing system comprises a gene editing system that targets HBG1 and/or HBG2 promoters, resulting in disruption of binding sites for γ-globin transcriptional repressors.

139. The pharmaceutical composition of any one of claims 129 and 135, wherein the at least one gene editing system comprises a gene editing system that disrupts BCL11A binding sites.

140. The pharmaceutical composition of any one of claims 129 and 135, wherein the at least one gene editing system comprises a gene editing system that disrupts the GATA1 motif in the erythroid-specific intronic enhancer of BCL11A.

141. The pharmaceutical composition of any one of the previous claims, wherein the at least one gene editing system comprises a gene editing system, or components thereof, disclosed in any one of:

US Patents					
11,242,525	11,572,543	11,268,077	11,466,271	9,957,501	10,808,020
11,021,696	10,563,184	11,155,803	10,738,305	11,344,609	10,435,677
11,466,271					

US Patent Application Publications			
US20200339979A1	US20210180091A1	US20200140896A1	US20190010495A1
US20210254061A1	US20210040506A1	US20230002760A1	US20230018543A1
US20180273609A1	US20220047637A1	US20190184035A1	US20200255857A1
US20200399626A1	US20220228142A1	US20180200387A1	US20180030438A1
US20220401530A1	US20210371858A1	US20190010495A1	US20210180091A1
US20210008161A1	US20210155927A1		

PCT Publications			
WO2022240787A1	WO2021119040A1	WO2023014839A1	WO2023039440A2
WO2022120094A2	WO2022155458A1	WO2023019227A1	WO2023019226A1
WO2022067122A1	WO2023019225A2	WO2021163587A1	WO2024020346A1

142. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a CRISPR-Cas gene editing system or components thereof, or a polynucleotide encoding

the same.

143. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a Cas9 nuclease, or a polynucleotide encoding the same.

144. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a Cas12a nuclease, or a polynucleotide encoding the same.

145. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a prime editing system or components thereof, or a polynucleotide encoding the same.

146. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a retron editing system or components thereof, or a polynucleotide encoding the same.

147. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a TnpB editing system or components thereof, or a polynucleotide encoding the same.

148. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises an integrase editing system or components thereof, or a polynucleotide encoding the same.

149. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a base editing system or components thereof, or a polynucleotide encoding the same.

150. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises an epigenetic editing system or components thereof, or a polynucleotide encoding the same.

151. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a gene writing system or components thereof, or a polynucleotide encoding the same.

152. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a gene inactivating system or components thereof, or a polynucleotide encoding the same.

153. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises zinc finger nuclease or components thereof, or a polynucleotide encoding the same.

154. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a TALE Nuclease, a TALE nickase, Zinc Finger (ZF) Nuclease, ZF Nickase,

meganuclease, or a combination thereof, or components thereof, or a polynucleotide encoding any of the same.

155. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises a meganuclease, or a polynucleotide encoding the same.

156. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises at least one guide RNA (gRNA).

157. The pharmaceutical composition of any one of the preceding claims, wherein the gene editing system comprises at least one guide RNA, or a portion thereof, disclosed in any one of:

US Patents					
11,242,525	11,572,543	11,268,077	11,466,271	9,957,501	10,808,020
11,021,696	10,563,184	11,155,803	10,738,305	11,344,609	10,435,677
11,466,271					
US Patent Application Publications					
US20200339979A1	US20210180091A1	US20200140896A1	US20190010495A1		
US20210254061A1	US20210040506A1	US20230002760A1	US20230018543A1		
US20180273609A1	US20220047637A1	US20190184035A1	US20200255857A1		
US20200399626A1	US20220228142A1	US20180200387A1	US20180030438A1		
US20220401530A1	US20210371858A1	US20190010495A1	US20210180091A1		
US20210008161A1	US20210155927A1				
PCT Publications					
WO2022240787A1	WO2021119040A1	WO2023014839A1	WO2023039440A2		
WO2022120094A2	WO2022155458A1	WO2023019227A1	WO2023019226A1		
WO2022067122A1	WO2023019225A2	WO2021163587A1	WO2024020346A1		

158. The pharmaceutical composition of any one of the preceding claims, wherein the at least one lipid nanoparticle further comprises:

i) at least one structural lipid;

ii) at least one phospholipid; and

iii) at least one PEGylated lipid.

159. The pharmaceutical composition of any one of the preceding claims, wherein the at least one structural lipid is selected from cholesterol, fecosterol, fucosterol, beta sitosterol, sitosterol,

ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, cholic acid, sitostanol, litocholic acid, tomatine, ursolic acid, alpha-tocopherol, Vitamin D3, Vitamin D2, Calcipotriol, botulin, lupeol, oleanolic acid, beta-sitosterol-acetate and any combinations thereof.

160. The pharmaceutical composition of any one of the preceding claims, wherein the at least one phospholipid is selected from 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1.2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocho line (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sodium (S)-2-ammonio-3-((((R)-2-(oleoyloxy)-3-(stearoyloxy)propoxy)oxidophosphoryl)oxy)propanoate (L-α-phosphatidylserine; Brain PS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE), 1,2-Dielaidoyl-sn-phosphatidylethanolamine (DEPE), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), 1,2-dioleoyl-sn-glycero-3-phosphate (18:1 PA; DOPA), ammonium bis((S)-2-hydroxy-3-(oleoyloxy)propyl) phosphate (18:1 DMP; LBPA), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol) (DOPI; 18:1 PI), 1,2-distearoyl-sn-glycero-3-phospho-L-serine (18:0 PS), 1,2-dilinoleoyl-sn-glycero-3-phospho-L-serine (18:2 PS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (16:0-18:1 PS; POPS), 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (18:0-18:1 PS), 1-stearoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (18:0-18:2 PS), 1-oleoyl-2-hydroxy-

sn-glycero-3-phospho-L-serine (18:1 Lyso PS), 1-stearoyl-2-hydroxy-sn-glycero-3-phospho-L-serine (18:0 Lyso PS), and sphingomyelin.

161. The pharmaceutical composition of any one of the preceding claims, wherein the at least one PEGylated lipid is selected from (R)-2,3-bis(octadecyloxy)propyl-1-(methoxypoly(ethyleneglycol)2000)propylcarbamate, PEG-S-DSG, PEG-S-DMG, PEG-PE, PEG-PAA, PEG-OH DSPE C18, PEG-DSPE, PEG-DSG, PEG-DPG, PEG-DOMG, PEG-DMPE Na, PEG-DMPE, PEG-DMG2000, PEG-DMG C14, PEG-DMG 2000, PEG-DMG, PEG-DMA, PEG-Ceramide C16, PEG-C-DOMG, PEG-c-DMOG, PEG-c-DMA, PEG-cDMA, PEGA, PEG750-C-DMA, PEG400, PEG2k-DMG, PEG2k-C11, PEG2000-PE, PEG2000P, PEG2000-DSPE, PEG2000-DOMG, PEG2000-DMG, PEG2000-C-DMA, PEG2000, PEG200, PEG(2k)-DMG, PEG DSPE C18, PEG DMPE C14, PEG DLPE C12, PEG Click DMG C14, PEG Click C12, PEG Click C10, N(Carbonyl-methoxypolyethylenglycol-2000)-l,2-distearoyl-sn-glycero3-phosphoethanolamine, Myrj52, mPEG-PLA, MPEG-DSPE, mPEG3000-DMPE, MPEG-2000-DSPE, MPEG2000-DSPE, mPEG2000-DPPE, mPEG2000-DMPE, mPEG2000-DMG, mDPPE-PEG2000, l,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG2000, HPEG-2K-LIPD, Folate PEG-DSPE, DSPE-PEGMA 500, DSPE-PEGMA, DSPE-PEG6000, DSPE-PEG5000, DSPE-PEG2K-NAG, DSPE-PEG2k, DSPE-PEG2000maleimide, DSPE-PEG2000, DSPE-PEG, DSG-PEGMA, DSG-PEG5000, DPPE-PEG-2K, DPPE-PEG, DPPE-mPEG2000, DPPE-mPEG, DPG-PEGMA, DOPE-PEG2000, DMPE-PEGMA, DMPE-PEG2000, DMPE-Peg, DMPE-mPEG2000, DMG-PEGMA, DMG-PEG2000, DMG-PEG, distearoyl-glycerol-polyethyleneglycol, Cl8PEG750, CI8PEG5000, CI8PEG3000, CI8PEG2000, CI6PEG2000, CI4PEG2000, C18-PEG5000, C18PEG, C16PEG, C16 mPEG (polyethylene glycol) 2000 Ceramide, C14-PEG-DSPE200, C14-PEG2000, C14PEG2000, C14-PEG 2000, C14-PEG, C14PEG, 14:0-PEG2KPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG2000, (R)-2,3-bis(octadecyloxy)propyl-1-(methoxypoly(ethyleneglycol)2000)propylcarbamate, (PEG)-C-DOMG, PEG-C-DMA, and DSPE-PEG-X.

162. The pharmaceutical composition of any one of the preceding claims, wherein the LNP further comprises at least one additional lipid component selected from 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine (18:3 PC), Acylcarnosine (AC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), N-oleoyl-sphingomyelin (SPM) (C18:l), N-lignoceryl SPM (C24:0), N-nervonoylshphingomyelin (C24:l), Cardiolipin (CL), l,2-bis(tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine (DC8-9PC), dicetyl phosphate (DCP), dihexadecyl phosphate (DCP1), 1,2-Dipalmitoylglycerol-3-hemisuccinate (DGSucc), short-chain bis-n-heptadecanoyl phosphatidylcholine (DHPC), dihexadecoyl-

phosphoethanolamine (DHPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), l,2-dilauroyl-sn-glycero-3-PE (DLPE), dimyristoyl glycerol hemisuccinate (DMGS), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphoethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG), dioleyloxybenzylalcohol (DOBA), 1,2-dioleoylglyceryl-3-hemisuccinate (DOGHEMS), N-[2-(2-{2-[2-(2,3-Bis-octadec-9-enyloxy-propoxy)-ethoxy]-ethoxy}-ethoxy)-ethyl]-3-(3,4,5-1rihydroxy-6-hydroxymethyl-1etrahydro-pyran-2-ylsulfanyl)-propionamide (DOGP4αMan), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), dioleoyl-phosphatidylethanolamine4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dioleoylphosphatidylglycerol (DOPG), 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), a cell-fusogenicphospholipid (DPhPE), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylglycerol (DPPG), dipalmitoylphosphatidylserine (DPPS), distearoylphosphatidylcholine (DSPC), distearoyl-phosphatidyl-ethanolamine (DSPE), distearoyl phosphoethanolamineimidazole (DSPEI), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), egg phosphatidylcholine (EPC), histaminedistearoylglycerol (HDSG), 1,2-Dipalmitoylglycerol-hemisuccinate-Nα-Histidinyl-Hemisuccinate (HistSuccDG), N-(5'-hydroxy-3'-oxypentyl)-10-12-pentacosadiynamide (h-Pegi-PCDA), 2-[l-hexyloxyethyl]-2-devinylpyropheophorbide-a (HPPH), hydrogenatedsoybeanphosphatidylcholine (HSPC), 1,2-Dipalmitoylglycerol-O-α-histidinyl-Nα-hemisuccinate (IsohistsuccDG), mannosialized dipalmitoylphosphatidylethanolamine (ManDOG), l,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-[4-(p-maleimidomethyl)cyclohexane-carboxamide] (MCC-PE), 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16:0 PE), 1-myristoyl-2-hydroxy-sn-glycero-phosphocholine (MHPC), a thiol-reactive maleimide headgroup lipid e.g.1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl)but-yramid (MPB-PE), Nervonic Acid (NA), sodium cholate (NaChol), l,2-dioleoyl-sn-glycero-3-|phosphoethanolamine-N-dodecanoyl (NC12-DOPE), 1-oleoyl-2-cholesteryl hemisuccinoyl-sn-glycero-3-phosphocholine (OChemsPC), phosphatidylethanolamine lipid (PE), PE lipid conjugated with polyethylene glycol(PEG) (e.g., polyethylene glycol-distearoylphosphatidylethanolamine lipid (PEG-PE)), phosphatidylglycerol (PG), partially hydrogenated soy phosphatidylchloline (PHSPC), phosphatidylinositol lipid (PI), phosphotidylinositol-4-phosphate (PIP), palmitoyloleoylphosphatidylcholine (POPC), phosphatidylethanolamine (POPE), palmitoyloleyolphosphatidylglycerol (POPG), phosphatidylserine (PS), lissamine rhodamineB-phosphatidylethanolamine lipid (Rh-PE), purifiedsoy-derivedmixtureofphospholipids (SIOO), phosphatidylcholine (SM), 18-1-trans-PE,1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), soybean phosphatidylcholine (SPC), sphingomyclins (SPM), alpha,alpha-trehalose-6,6'-dibehenate (TDB), l,2-dielaidoyl-sn-glycero-3-phophoethanolamine (transDOPE), ((23S,5R)-3-

(bis(hexadecyloxy)methoxy)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)tetrahydrofuran-2-yl)methylmethylphosphate, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleyl-sn-glycero-3-phosphoethanolamine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 16-O-monomethyl PE, 16-O-dimethyl PE, and dioleylphosphatidylethanolamine.

163. The pharmaceutical composition of any one of the preceding claims, wherein the LNP further comprises one or more targeting moieties.

164. The pharmaceutical composition of any one of the preceding claims, wherein the lipid nanoparticle comprises:

(a) a PEG lipid selected from PEG2k-DMG or PEG2k-DSPE or a mixture thereof;

(b) cholesterol;

(c) a phospholipid selected from sphingolipid or DSPC or a mixture thereof.

165. The pharmaceutical composition of any one of the preceding claims, wherein the lipid nanoparticle comprises:

(a) about 0 mol% to about 10 mol% of PEG lipid;

(b) about 0 mol% to about 30 mol% structural lipid;

(c) about 20 mol% to about 45 mol% phospholipid; and

(d) about 30 mol% to about 60 mol% of at least one ionizable lipid of any one of claims 1-79.

166. The pharmaceutical composition of any one of the preceding claims, wherein the lipid nanoparticle comprises:

(a) about 1 mol% to about 2 mol% of PEG lipid;

(b) about 25 mol% to about 40 mol% structural lipid;

(c) about 20 mol% to about 45 mol% phospholipid; and

(d) about 30 mol% to about 60 mol% of at least one ionizable lipid of any one of claims 1-79.

167. The pharmaceutical composition of any one of the preceding claims, wherein the lipid nanoparticle comprises:

(a) about 2 mol% of PEG lipid;

(b) about 25 mol% structural lipid;

(c) about 40 mol% phospholipid; and

(d) about 33 mol% of at least one ionizable lipid of any one of claims 1-79.

168. The pharmaceutical composition of any one of the preceding claims, wherein the lipid nanoparticle comprises:

(a) about 2.5 mol% of PEG lipid;

(b) about 39 mol% structural lipid;

(c) about 10 mol% phospholipid; and

(d) about 48.5 mol% of at least one ionizable lipid of any one of claims 1-79.

169. The pharmaceutical composition of any one of the preceding claims, wherein the lipid nanoparticle comprises:

(a) about 1.5 mol% of PEG lipid;

(b) about 40 mol% structural lipid;

(c) about 10 mol% phospholipid; and

(d) about 48.5 mol% of at least one ionizable lipid of any one of claims 1-79.

170. The pharmaceutical composition of any one of the preceding claims, wherein the lipid nanoparticle comprises an mRNA or a circRNA encoding a CRISPR nuclease and a guide RNA capable of enacting a genetic edit that disrupts the GATA1 motif in the erythroid-specific intronic enhancer of BCL11A.

171. The pharmaceutical composition of any one of the preceding claims, wherein the lipid nanoparticle comprises an mRNA or a circRNA encoding a CRISPR nuclease and a guide RNA capable of enacting a genetic edit that disrupts BCL11A binding sites.

172. A method of treating a hemoglobinopathy in a patient in need thereof, the method comprising administering to the subject a pharmaceutical composition of any one of the preceding claims.

173. The pharmaceutical composition of any of the preceding claims for use as a medicament in the treatment of a hemoglobinopathy.

174. Use of a pharmaceutical composition of any of the preceding claims for the manufacture of a medicament for delivery of a gene editing system capable of treating a hemoglobinopathy.