COMPOSITIONS AND METHODS FOR THE TARGETING OF PCSK9

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to, and benefit of, U.S. Provisional Application Nos. 63/349,981 filed on June 7, 2022, 63/492,923, filed on March 29, 2023, and 63/505,823, filed on June 2, 2023, the contents of each of which are incorporated by reference herein in their entireties.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0002] The contents of the electronic sequence listing (SCRB_055_01WO_SeqList_ST26.xml; Size: 4,317,702 bytes; and Date of Creation: June 2, 2023) are herein incorporated by reference in their entirety.

BACKGROUND

[0003] In mammals, cholesterol is transported within lipoproteins via emulsification. The lipoprotein particles are classified based on their density: low-density lipoproteins (LDL), very low-density lipoproteins (VLDL), high-density lipoproteins (HDL), and chylomicrons. Surface LDL receptors are internalized during cholesterol absorption. A cell with abundant cholesterol will have its LDL receptor synthesis blocked to prevent new cholesterol in LDL particles from being taken up. Conversely, LDL receptor synthesis is promoted when a cell is deficient in cholesterol. When the process is unregulated, excess LDL particles will travel in the blood without uptake by an LDL receptor. LDL particles in the blood are oxidized and taken up by macrophages, which then become engorged and form foam cells. These foam cells can become trapped in the walls of blood vessels and contribute to atherosclerotic plaque formation, which is one of the main causes of heart attacks, strokes, and other serious medical problems.

[0004] The liver protein proprotein convertase subtilisin/kexin Type 9 (PCSK9) is a secreted, globular, auto-activating serine protease that binds to the low-density lipoprotein receptor (LDL-R) during endocytosis of LDL particles, preventing recycling of the LDL-R to the cell surface and leading to reduction of LDL-cholesterol clearance. PCSK9 binds to the LDL-R (through the EGF-A domain), preventing the conformational change of the receptor-ligand complex, which redirects the LDL-R to the lysosome instead. As the receptor for low-density lipoprotein particles (LDL) typically transports thousands of fat molecules (including cholesterol) per particle within extracellular fluid, blocking or inhibiting the function of PCSK9 to boost LDL-R-

mediated clearance of LDL cholesterol can lower LDL particle concentrations. PCSK9 is expressed mainly in the liver, the intestine, the kidney, and the central nervous system, but is also highly expressed in arterial walls such as endothelium, smooth muscle cells, and macrophages, with a local effect that can regulate vascular homeostasis and atherosclerosis.

[0005] PCSK9 is a member of the proprotein convertase (PC) family and its gene is mutated in ~ 2% to 3% of individuals with familial hypercholesterolemia (FH) (Sepideh Mikaeeli, S., et al. Functional analysis of natural PCSK9 mutants in modern and archaic humans. FEBS J. 2019 Aug 6. doi: 10.1111/febs.15036). Researchers have identified several PCSK9 mutations that cause an inherited form of high cholesterol (hypercholesterolemia). These mutations change a single amino acid in the PCSK9 protein. Researchers describe the mutations responsible for hypercholesterolemia as "gain-of-function" because they appear to enhance the activity of the PCSK9 protein or give the protein a new, atypical function (Blesa, S., et al. A New PCSK9 Gene Promoter Variant Affects Gene Expression and Causes Autosomal Dominant Hypercholesterolemia. J. Clin. Endocrinol. & Metab. 93:3577(2008)). The overactive PCSK9 protein substantially reduces the number of low-density lipoprotein receptors on the surface of liver cells. With fewer receptors to remove low-density lipoproteins from the blood, people with gain-of-function mutations in the PCSK9 gene have very high blood cholesterol levels. Autosomal dominant hypercholesterolemia (ADH) is a genetic disorder characterized by increased low-density lipoprotein (LDL)-cholesterol levels, leading to high risk of premature cardiovascular disease. Approximately 10 mutations in PCSK9 have been identified as a cause of the disease in different populations. All known mutations in PCSK9 causing hypercholesterolemia produce an increase in the enzymatic activity of this protease (Bleasa, S., 2008). In addition, mutations in PCSK9 can lead to autosomal dominant familial hypobetalipoproteinemia, which can lead to hepatic steatosis, cirrhosis, and other disorders.

[0006] The advent of CRISPR/Cas systems, and the programmable nature of these minimal systems, has facilitated their use as a versatile technology for genomic manipulation and engineering. However, current methods of generating PCSK9 protective variants and loss-of-function mutants *in vivo* have been ineffective due to the large number of cells that need to be modified to modulate cholesterol levels. Other concerns involve off-target effects, genome instability, or oncogenic modifications that may be caused by genome editing, as well as a lack of safe delivery modalities for gene-repression systems. Additionally, in certain disease indications, gene silencing, or repression, is preferable to gene editing. The ability to render

CRISPR nucleases such as Cas9 and CasX catalytically-inactive has been demonstrated (WO2020247882A1 and US20200087641A1, incorporated by reference herein), which makes these systems an attractive platform for the generation of fusion proteins with repressor domains capable of gene silencing. While certain repressor systems have been described, there remains a need for additional gene repressor systems that have been optimized and/or offer improvements over earlier generations of gene repressor systems, such as those based on Cas9, for utilization in a variety of therapeutic, diagnostic, and research applications. Thus, there remains a need for improved compositions and methods to regulate PCSK9.

SUMMARY

[0007] The present disclosure provides systems comprising or encoding repressor fusion proteins comprising DNA-binding and linked repressor domains used in the repression and/or epigenetic modification of proprotein convertase subtilisin/kexin Type 9 (*PCSK9*) gene target nucleic acid sequences. In some cases, the repressor fusion protein comprises a DNA-binding protein comprising a zinc finger (ZF) or a transcription-activator-like effector (TALE) protein complementary to the *PCSK9* gene target nucleic acid sequence and one or more linked repressor domains. In some cases, the repressor fusion protein comprises a DNA-binding protein comprising a catalytically-dead CRISPR protein and one or more linked repressor domains, and a guide nucleic acid comprising a targeting sequence complementary to the *PCSK9* gene target nucleic acid sequence. The proteins and guide nucleic acids can be modified for passive entry into target cells and are useful in a variety of methods for repression of PCSK9, which methods are also provided. The present disclosure also provides vectors and lipid nanoparticles (LNP) encoding or encapsulating the repressor fusion proteins and guide nucleic acids components for the delivery of the systems to cells for the transcriptional repression of the PCSK9 target nucleic acid sequence.

[0008] The disclosure provides pharmaceutical compositions comprising the systems, nucleic acids, LNP and vectors described herein.

[0009] The present disclosure also provides methods for treating subjects having a PCSK9-related disease. In some embodiments, the compositions and methods have utility in subjects having a metabolic disorder such as, but not limited to, familial hypercholesterolemia, familial hypobetalipoproteinemia, or elevated cholesterol levels.

[0010] In another aspect, provided herein are systems comprising PCRK9 repressor systems, or vectors comprising or encoding PCSK9 repressor systems for use in the manufacture of a medicament for the treatment of a PCSK9-related disease in a subject in need thereof.

[0011] The present disclosure provides compositions for use in methods of treating subjects having a PCSK9-related disease. In some embodiments, the composition comprises repressor fusion proteins comprising a catalytically-dead CRISPR protein and one or more linked repressor domains, and a guide nucleic acid comprising a targeting sequence complementary to the *PCSK9* gene target nucleic acid sequence for use in the transcriptional repression of *PCSK9* gene target nucleic acid sequences in a subject. In some embodiments, the composition comprises systems, nucleic acids, LNP, vectors and/or pharmaceutical compositions described herein.

[0012] In some embodiments, the *PCSK9* gene comprises one or more mutations, for example amino acid substitutions selected from the group consisting of S127R, D129G, F216L, D374H, and D374Y relative to the sequence of SEQ ID NO: 1823.

[0013] The disclosure provides methods of repressing transcription of a *PCSK9* gene in a population of cells, the method comprising introducing into cells of the population the systems, nucleic acids, LNP, vectors and/or pharmaceutical compositions described herein.

[0014] In some embodiments, the catalytically-dead CRISPR protein and guide nucleic acid for use in the PCSK9 repressor systems comprise catalytically-dead CasX variant proteins and/or CasX variant guide nucleic acids as described herein.

[0015] Further features and advantages of certain embodiments of the present disclosure will become more fully apparent in the following description of embodiments and drawings thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The novel features of the disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the disclosure are utilized, and the accompanying drawings of which:

[0017] FIG. 1 illustrates the schematics of five configurations of long-term repressor protein (LTRP, also referred to herein as “repressor fusion proteins”) fusion proteins with repressor

molecules linked to catalytically-dead CasX. D3A and D3L denote DNA methyltransferase 3 alpha (DNMT3A) and DNMT3A-like protein (DNMT3L), respectively. L1-L4 are linkers. NLS is the nuclear localization signal.

[0018] FIG. 2 illustrates schematics of various configurations of LTRP fusion proteins with the DNMT3A ADD domain incorporated. "D3A ADD", "D3A CD", and "D3L ID" denote the ADD domain of DNMT3A, the catalytic domain of DNMT3A, and the interaction domain of DNMT3L, respectively. L1-L3 are linkers. NLS is the nuclear localization signal.

[0019] FIG. 3 is a dot plot graph showing the correlation between secreted PCSK9 protein and PCSK9 mRNA levels in human hepatocytes that were transiently transfected with LTRPs, as described in Example 1. PCSK9 mRNA levels were normalized to the housekeeping gene RPLP0. Secreted PCSK9 protein levels were normalized to secreted human serum albumin (HSA). Samples were normalized to a non-targeting (NT) control.

[0020] FIG. 4 is a bar plot showing the percentage of mouse Hepa1-6 cells, treated with either dXR1 or LTRP1-ZIM3 mRNA paired with the indicated *PCSK9*-targeting gRNAs, that stained negative for intracellular PCSK9 at day 6, as described in Example 2. Spacer 6.7 targeting the human *PCSK9* locus served as a non-targeting control.

[0021] FIG. 5 is a time course plot showing the percentage of mouse Hepa1-6 cells, treated with dXR1 mRNA paired with the indicated *PCSK9*-targeting gRNAs, that stained negative for intracellular PCSK9 at 6, 13, and 25 days post-delivery, as described in Example 2. Spacer 6.7 targeting the human *PCSK9* locus served as a non-targeting control, and treatment with water served as a negative control.

[0022] FIG. 6 is a time course plot showing the percentage of mouse Hepa1-6 cells, treated with LTRP1-ZIM3 mRNA paired with the indicated *PCSK9*-targeting gRNAs, that stained negative for intracellular PCSK9 at 6, 13, and 25 days post-delivery, as described in Example 2. Spacer 6.7 targeting the human *PCSK9* locus served as a non-targeting control, and treatment with water served as a negative control.

[0023] FIG. 7 is a time course plot showing the percentage of mouse Hepa1-6 cells, treated with IVT-produced LTRP1-ZIM3 vs. LTRP5-ZIM5 mRNA paired with the indicated *PCSK9*-targeting gRNAs, that stained negative for intracellular PCSK9 at the indicated timepoints days post-delivery, as described in Example 2.

[0024] FIG. 8 is a time course plot showing the percentage of mouse Hepa1-6 cells, treated with third-party-produced LTRP1-ZIM3 vs. dCas9-ZNF10-DNMT3A/3L mRNA paired with the

indicated *PCSK9*-targeting gRNAs, that stained negative for intracellular PCSK9 at the indicated timepoints post-delivery, as described in Example 2.

[0025] FIG. 9 is a bar graph showing the quantification of secreted PCSK9 levels at 6, 18, 36, and 87 days post-transfection in Huh7 cells lipofected with mRNA encoding for CasX 676, dXR1, or LTRP5-ADD-ZIM3 when paired with the indicated targeting gRNAs, as described in Example 3. Secreted PCSK9 levels were normalized to total cell count. Naïve, untreated cells served as an experimental control.

[0026] FIG. 10A is a time course plot showing the percentage of mouse Hepa1-6 cells, treated with LTRP5-ZIM3 or LTRP5-ADD-ZIM3 mRNA paired with the *PCSK9*-targeting gRNA with spacer 27.88, that stained negative for intracellular PCSK9 at 4, 12, 18, 24, 41, and 53 days post-delivery, as described in Example 4. A non-targeting (NT) spacer was used as an experimental control.

[0027] FIG. 10B is a time course plot showing the percentage of mouse Hepa1-6 cells, treated with LTRP5-ZIM3 or LTRP5-ADD-ZIM3 mRNA paired with the *PCSK9*-targeting gRNA with spacer 27.94, that stained negative for intracellular PCSK9 at 4, 12, 18, 24, 41, and 53 days post-delivery, as described in Example 4. A non-targeting (NT) spacer was used as an experimental control.

[0028] FIG. 11A is a bar plot showing the quantification of normalized secreted PCSK9 levels at 4 days post-transfection in HepG2 cells lipofected with mRNA encoding for CasX 676, dXR1, or LTRP5-ADD-ZIM3 when paired with the indicated targeting gRNAs, as described in Example 5. Secreted PCSK9 levels were normalized to total cell count. Naïve, untreated cells served as experimental controls.

[0029] FIG. 11B is a bar plot showing the quantification of normalized secreted PCSK9 levels at 4 days post-transfection in Huh7 cells lipofected with mRNA encoding for CasX 676, dXR1, or LTRP5-ADD-ZIM3 when paired with the indicated targeting gRNAs, as described in Example 5. Secreted PCSK9 levels were normalized to total cell count. Naïve, untreated cells served as experimental controls.

[0030] FIG. 11C is a bar plot showing the quantification of normalized secreted PCSK9 levels at 4 days post-transfection in Hep3B cells lipofected with mRNA encoding for CasX 676, dXR1, or LTRP5-ADD-ZIM3 when paired with the indicated targeting gRNAs, as described in Example 5. Secreted PCSK9 levels were normalized to total cell count. Naïve, untreated cells served as experimental controls.

[0031] FIG. 12 is a bar plot showing the quantification of secreted PCSK9 levels at 4, 14, and 27 days post-transfection in Huh7 cells lipofected with mRNA encoding for CasX 676, dXR1, or LTRP5-ADD-ZIM3 when paired with the indicated targeting gRNAs, as described in Example 5. Quantification of secreted PCSK9 levels is shown as relative to the secreted levels detected in the naïve, untreated cells at the day 4 timepoint.

[0032] FIG. 13 is a violin plot showing the distribution of secreted PCSK9 levels in HepG2 cells transfected with CasX 676 mRNA #2 and a gRNA with the indicated *PCSK9*-targeting spacer, as described in Example 6. Naïve, untreated cells and cells transfected with CasX 676 mRNA only served as experimental controls.

[0033] FIG. 14 is a pair of representative western blots showing the levels of pro-PCSK9 and processed PCSK9 protein (top western blot) in HepG2 cells transfected with CasX 676 mRNA and a gRNA with the indicated *PCSK9*-targeting spacer, as described in Example 6. Naïve, untreated cells and cells transfected with CasX 676 mRNA only served as experimental controls. Lysate from HEK293T cells, which do not express the PCSK9 protein, and a cynomolgus macaque recombinant PCSK9 protein control were used as western blot controls. The bottom western blot shows the total protein loading control.

[0034] FIG. 15 is a bar plot showing the western blot quantification for pro-PCSK9, processed PCSK9, and total PCSK9 levels for each of the indicated spacers assessed when transfected with CasX 676 mRNA into HepG2 cells, as described in Example 6. Naïve, untreated cells and cells transfected with CasX 676 mRNA only served as experimental controls. PCSK9 levels were normalized to total PCSK9 levels from the naïve condition.

[0035] FIG. 16A is a schematic illustrating versions 1-3 of chemical modifications made to gRNA scaffold variant 235, as described in Example 7. Structural motifs are highlighted. Standard ribonucleotides are depicted as open circles, and 2'OMe-modified ribonucleotides are depicted as black circles. Phosphorothioate bonds are indicated with * below or beside the bond. For the v2 profile, the addition of three 3' uracils (3'UUU) is annotated with "U"s in the relevant circles.

[0036] FIG. 16B is a schematic illustrating versions 4-6 of chemical modifications made to gRNA scaffold variant 235, as described in Example 7. Structural motifs are highlighted. Standard ribonucleotides are depicted as open circles, and 2'OMe-modified ribonucleotides are depicted as black circles. Phosphorothioate bonds are indicated with * below or beside the bond.

[0037] FIG. 17 is a plot illustrating the quantification of percent knockout of *B2M* in HepG2 cells co-transfected with 100 ng of CasX 491 mRNA and with the indicated doses of end-modified (v1) or unmodified (v0) *B2M*-targeting gRNAs with spacer 7.37, as described in Example 7. Editing level was determined by flow cytometry as the population of cells with loss of surface presentation of the HLA complex due to successful editing at the *B2M* locus.

[0038] FIG. 18 is a schematic illustrating versions 7-9 of chemical modifications made to gRNA scaffold variant 316, as described in Example 7. Structural motifs are highlighted. Standard ribonucleotides are depicted as open circles, and 2'OMe-modified ribonucleotides are depicted as black circles. Phosphorothioate bonds are indicated with * below or beside the bond.

[0039] FIG. 19A is a schematic of gRNA scaffold variant 174 (SEQ ID NO: 1744), as described in Example 7. Structural motifs are highlighted.

[0040] FIG. 19B is a schematic of gRNA scaffold variant 235 (SEQ ID NO: 1745), as described in Example 7. Highlighted structural motifs are the same as in FIG. 19A. The differences between variant 174 and variant 235 lie in the extended stem motif and several single-nucleotide changes (indicated with asterisks). Variant 316 maintains the shorter extended stem from variant 174 but harbors the four substitutions found in scaffold 235.

[0041] FIG. 19C is a schematic of gRNA scaffold variant 316 (SEQ ID NO: 1746), as described in Example 7. Highlighted structural motifs are the same as in FIG. 19A. Variant 316 maintains the shorter extended stem from variant 174 (FIG. 19A) but harbors the four substitutions found in scaffold 235 (FIG. 19B).

[0042] FIG. 20 is a plot displaying a correlation between indel rate (depicted as edit fraction) at the *PCSK9* locus as measured by next-generation sequencing (NGS) (x-axis) and secreted PCSK9 levels (ng/mL) detected by enzyme-linked immunosorbent assay (ELISA) (y-axis) in HepG2 cells lipofected with CasX 491 mRNA and *PCSK9*-targeting gRNAs containing the indicated scaffold variant and spacer combination, as described in Example 7.

[0043] FIG. 21A is a plot depicting the results of an editing assay measured as indel rate detected by NGS at the human *B2M* locus in HepG2 cells treated with the indicated doses of LNPs formulated with CasX 491 mRNA and the indicated *B2M*-targeting gRNA, as described in Example 7.

[0044] FIG. 21B is a plot illustrating the quantification of percent knockout of B2M in HepG2 cells treated with the indicated doses of LNPs formulated with CasX 491 mRNA and the indicated *B2M*-targeting gRNA, as described in Example 7. Editing level was determined by

flow cytometry as population of cells that did not have surface presentation of the HLA complex due to successful editing at the *B2M* locus.

[0045] FIG. 22A is a plot depicting the results of an editing assay measured as indel rate detected by NGS at the mouse *ROSA26* locus in Hepa1-6 cells treated with the indicated doses of LNPs formulated with CasX 676 mRNA #2 and the indicated *ROSA26*-targeting gRNA with either the v1 or v5 modification profile, as described in Example 7.

[0046] FIG. 22B is a plot illustrating the quantification of percent editing measured as indel rate detected by NGS at the *ROSA26* locus in mice treated with LNPs formulated with CasX 676 mRNA #2 and the indicated chemically-modified *ROSA26*-targeting gRNA, as described in Example 7.

[0047] FIG. 23 is a bar graph showing the results of an editing assay measured as indel rate detected by NGS at the mouse *PCSK9* locus in mice treated with LNPs formulated with CasX 676 mRNA #1 and the indicated chemically-modified *PCSK9*-targeting gRNA, as described in Example 7. Untreated mice served as experimental control.

[0048] FIG. 24 is a schematic illustrating versions 1-3 of chemical modifications made to gRNA scaffold variant 316, as described in Example 7. Structural motifs are highlighted. Standard ribonucleotides are depicted as open circles, and 2'OMe-modified ribonucleotides are depicted as black circles. Phosphorothioate bonds are indicated with * below or beside the bond.

[0049] FIG. 25 is a schematic illustrating versions 4-6 of chemical modifications made to gRNA scaffold variant 316, as described in Example 7. Structural motifs are highlighted. Standard ribonucleotides are depicted as open circles, and 2'OMe-modified ribonucleotides are depicted as black circles. Phosphorothioate bonds are indicated with * below or beside the bond.

[0050] FIG. 26 is a bar graph showing the quantification of percent editing measured as indel rate detected by NGS at the mouse *PCSK9* locus in Hepa1-6 cells transfected with the indicated engineered CasX mRNAs and targeting spacers and harvested at 20 hours post-transfection, as described in Example 8.

[0051] FIG. 27A is a diagram of the secondary structure of guide RNA scaffold 235 (SEQ ID NO: 1745), noting the regions with CpG motifs, as described in Example 12. CpG motifs in (1) the pseudoknot stem, (2) the scaffold stem, (3) the extended stem bubble, (4) the extended step, and (5) the extended stem loop are labeled on the structure.

[0052] FIG. 27B is a diagram of the CpG-reducing mutations that were introduced into each of the five regions in the coding sequence of the guide RNA scaffold, as described in Example 12.

The substitute bubble from scaffold 174 has a sequence of AGCUCCCUCUUCGGAGGGAGCA (SEQ ID NO: 3442).

[0053] FIG. 28 provides the results of an editing experiment in which AAV vectors with various CpG-reduced or CpG-depleted guide RNA scaffolds were used to edit the *B2M* locus in induced neurons, as described in Example 12. The AAV vectors were administered at a multiplicity of infection (MOI) of 4e3. The bars show the mean ± the SD of two replicates per sample. "No Tx" indicates a non-transduced control, and "NT" indicates a control with a non-targeting spacer.

[0054] FIG. 29 provides the results of an editing experiment in which AAV vectors with various CpG-reduced or CpG-depleted guide RNA scaffolds were used to edit the *B2M* locus in induced neurons, as described in Example 12. The AAV vectors were administered at an MOI of 3e3. The bars show the mean ± the SD of two replicates per sample. "No Tx" indicates a non-transduced control.

[0055] FIG. 30 provides the results of an editing experiment in which AAV vectors with various CpG-reduced or CpG-depleted guide RNA scaffolds were used to edit the *B2M* locus in induced neurons, as described in Example 12. The AAV vectors were administered at an MOI of 1e3. The bars show the mean ± the SD of two replicates per sample. "No Tx" indicates a non-transduced control.

[0056] FIG. 31 provides the results of an editing experiment in which AAV vectors with various CpG-reduced or CpG-depleted guide RNA scaffolds were used to edit the *B2M* locus in induced neurons, as described in Example 12. The AAV vectors were administered at an MOI of MOI = 3e2. The bars show the mean ± the SD of two replicates per sample. "No Tx" indicates a non-transduced control.

[0057] FIG. 32A presents the results of a time-course experiment comparing beta-2-microglobulin (B2M) repression activities (represented as percentage of HLA-negative cells) of LTRP proteins Nos. 1-3, as described in Example 13. Data are presented as mean with standard deviation, N=3.

[0058] FIG. 32B presents the results of the same time-course experiment shown in FIG. 32A but illustrates the B2M repression activities of LTRP proteins Nos. 1-3 containing the ZIM3-KRAB domain, benchmarked against the same experimental controls, as described in Example 13. Data are presented as mean with standard deviation, N=3.

[0059] FIG. 33A presents the results of a time-course experiment comparing B2M silencing activities (represented as percentage of HLA-negative cells) of LTRP proteins #1, #4, and #5, as described in Example 13. Data are presented as mean with standard deviation, N=3.

[0060] FIG. 33B presents the results of the same time-course experiment shown in FIG. 33A but illustrates the B2M silencing activities of LTRP proteins #1, #4, and #5 containing the ZIM3-KRAB domain, benchmarked against the same experimental controls, as described in Example 13. Data are presented as mean with standard deviation, N=3.

[0061] FIG. 34 is a violin plot of percent CpG methylation for CpG sites around the transcription start site of the *B2M* locus for each indicated experimental condition as described in Example 13.

[0062] FIG. 35 is a dot plot showing the relative activity (average percentage of HLA-negative cells at day 21) versus specificity (percentage of off-target CpG methylation at the *B2M* locus quantified at day 5) for LTRP proteins #1-3, benchmarked against catalytically-active CasX 491 and dCas9-ZNF10-DNMT3A/L, as described in Example 13.

[0063] FIG. 36 is a violin plot of percent CpG methylation for CpG sites downstream of the transcription start site of the *VEGFA* locus for each indicated experimental condition as described in Example 13.

[0064] FIG. 37A is a violin plot of percent CpG methylation for CpG sites around the transcription start site of the *VEGFA* locus for each indicated experimental condition assessing LTRP #1, 4, and 5 with the *B2M*-targeting spacer as described in Example 13.

[0065] FIG. 37B is a violin plot of percent CpG methylation for CpG sites around the transcription start site of the *VEGFA* locus for each indicated experimental condition assessing LTRP #1, 4, and 5 with the non-targeting spacer as described in Example 13.

[0066] FIG. 38 is a scatterplot showing the relative activity (average percentage of HLA-negative cells at day 21) versus specificity (median percentage of off-target CpG methylation at the *VEGFA* locus quantified at day 5) for LTRP proteins #1-5 harboring either the ZNF10- or ZIM-KRAB domain, and the LTRP proteins were benchmarked against catalytically-active CasX 491 and dCas9-ZNF10-DNMT3A/L, as described in Example 13.

[0067] FIG. 39 presents the results of a time-course experiment comparing B2M repression activities (represented as percentage of HLA-negative cells) of the indicated LTRP -ZIM3 and its variants with *B2M*-targeting gRNA using spacer 7.37, as described in Example 14. Data are presented as mean with standard deviation, N=3. CD = catalytic domain of DNMT3A.

[0068] FIG. 40 presents the results of the same time-course experiment shown in FIG. 39 but shows B2M repression activities of the indicated LTRP -ZIM3 variants with *B2M*-targeting gRNA using spacer 7.160, as described in Example 14. Data are presented as mean with standard deviation, N=3.

[0069] FIG. 41 presents the results of the same time-course experiment shown in FIG. 39 but shows B2M repression activities of the indicated LTRP -ZIM3 variants with *B2M*-targeting gRNA using spacer 7.165, as described in Example 14. Data are presented as mean with standard deviation, N=3.

[0070] FIG. 42 presents the results of the same time-course experiment shown in FIG. 39 but shows B2M repression activities of the indicated LTRP -ZIM3 variants with a non-targeting gRNA, as described in Example 14. Data are presented as mean with standard deviation, N=3.

[0071] FIG. 43 is a violin plot of percent CpG methylation for CpG sites downstream of the transcription start site of the *VEGFA* locus for each indicated LTRP -ZIM3 variant for the three *B2M*-targeting gRNA and non-targeting gRNA, as described in Example 14.

[0072] FIG. 44 is a scatterplot showing the relative activity (average percentage of HLA-negative cells at day 21 for spacer 7.160) versus specificity (percentage of off-target CpG methylation at the *VEGFA* locus quantified at day 7 for spacer 7.160) for the indicated LTRP5-ZIM3 variants, as described in Example 14.

[0073] FIG. 45 illustrates the schematics of the various LTRP #5 architectures, where the additional DNMT3A domains were incorporated, as described in Example 14. The additional DNMT3A domains were the ADD domain of DNMT3A ("D3A ADD") and the PWWP domain of DNMT3A ("D3A PWWP"). "D3A endo" encodes for an endogenous sequence that occurs between DNMT3A PWWP and ADD domains. "D3A CD" and "D3L ID" denote the catalytic domain of DNMT3A and the interaction domain of DNMT3L respectively. "L1-L3" are linkers. "NLS" is the nuclear localization signal. See Table 12 for exemplary sequences.

[0074] FIG. 46 illustrates the schematics of the general architectures of the LTRP molecules with the ADD domain for LTRP configuration #1, #4, and #5 tested in Example 15. "D3A ADD", "D3A CD" and "D3L ID" denote the ADD domain of DNMT3A, the catalytic domain of DNMT3A, and the interaction domain of DNMT3L respectively, as described in Example 15. "L1-L4" are linkers. "NLS" is the nuclear localization signal. See Table 17 for exemplary sequences.

[0075] FIG. 47A presents the results of a time-course experiment comparing B2M repression activities (represented as percentage of HLA-negative cells) of LTRPs with the ZIM3-KRAB domain having configuration #1, #4, or #5 with or without the DNMT3A ADD domain when paired with the *B2M*-targeting gRNA with spacer 7.160, as described in Example 15. Data are presented as mean with standard deviation, N=3. "NT" is a gRNA with a non-targeting spacer.

[0076] FIG. 47B is a plot showing the results of the same time-course experiment shown in FIG. 47A but illustrates B2M repression activities for LTRP #5 with the ZNF10 or ZIM3-KRAB domain, with or without the DNMT3A ADD domain, paired with the *B2M*-targeting gRNA with spacer 7.160, as described in Example 15. Data are presented as mean with standard deviation, N=3. "NT" is a gRNA with a non-targeting spacer.

[0077] FIG. 47C is a plot showing the results of the same time-course experiment shown in FIG. 47A but illustrates B2M repression activities for LTRP5-ZIM3 with or without the DNMT3A ADD domain paired with a *B2M*-targeting gRNA with the indicated spacers, as described in Example 15. Data are presented as mean with standard deviation, N=3. "NT" is a gRNA with a non-targeting spacer.

[0078] FIG. 48A is a plot illustrating the results of B2M repression activities on day 27 post-transfection for LTRPs with either the ZNF10 or ZIM3-KRAB domain having configuration #1 with or without the DNMT3A ADD domain for the indicated gRNAs, as described in Example 15. Data are presented as mean with standard deviation, N=3. "NT" is a gRNA with a non-targeting spacer.

[0079] FIG. 48B is a plot illustrating the results of B2M repression activities on day 27 post-transfection for LTRPs with either the ZNF10 or ZIM3-KRAB domain having configuration #4 with or without the DNMT3A ADD domain for the indicated gRNAs, as described in Example 15. Data are presented as mean with standard deviation, N=3. "NT" is a gRNA with a non-targeting spacer.

[0080] FIG. 48C is a plot illustrating the results of B2M repression activities on day 27 post-transfection for LTRPs with either the ZNF10 or ZIM3-KRAB domain having configuration #5 with or without the DNMT3A ADD domain for the indicated gRNAs, as described in Example 15. Data are presented as mean with standard deviation, N=3. "NT" is a gRNA with a non-targeting spacer.

[0081] FIG. 49A is a plot illustrating the results of bisulfite sequencing used to determine off-target methylation at the *VEGFA* locus on day 5 post-transfection for LTRPs with either the

ZNF10 or ZIM3-KRAB domain having configuration #1 with or without the DNMT3A ADD domain for the indicated gRNAs, as described in Example 15. Data are presented as mean percentage of CpG methylation for CpG sites near the *VEGFA* locus; standard error of the mean is also presented; N=3. "NT" is a gRNA with a non-targeting spacer.

[0082] FIG. 49B is a plot illustrating the results of bisulfite sequencing used to determine off-target methylation at the *VEGFA* locus on day 5 post-transfection for LTRPs with either the ZNF10 or ZIM3-KRAB domain having configuration #4 with or without the DNMT3A ADD domain for the indicated gRNAs, as described in Example 15. Data are presented as mean percentage of CpG methylation for CpG sites near the *VEGFA* locus; standard error of the mean is also presented; N=3. "NT" is a gRNA with a non-targeting spacer.

[0083] FIG. 49C is a plot illustrating the results of bisulfite sequencing used to determine off-target methylation at the *VEGFA* locus on day 5 post-transfection for LTRPs with either the ZNF10 or ZIM3-KRAB domain having configuration #5 with or without the DNMT3A ADD domain for the indicated gRNAs, as described in Example 15. Data are presented as mean percentage of CpG methylation for CpG sites near the *VEGFA* locus; standard error of the mean is also presented; N=3. "NT" is a gRNA with a non-targeting spacer.

[0084] FIG. 50A is a dot plot showing the relative activity (average percentage of HLA-negative cells at day 27) versus specificity (percentage of off-target CpG methylation at the *VEGFA* locus quantified at day 5) for the LTRP molecules with the ZIM3-KRAB domain having configurations #1, #4, and #5, for *B2M*-targeting gRNA with spacer 7.160, as described in Example 15.

[0085] FIG. 50B is a dot plot showing the relative activity (average percentage of HLA-negative cells at day 27) versus specificity (percentage of off-target CpG methylation at the *VEGFA* locus quantified at day 5) for the LTRP molecules with the ZNF10-KRAB domain having configurations #1, #4, and #5, for *B2M*-targeting gRNA with spacer 7.160, as described in Example 15.

[0086] FIG. 51A is a dot plot showing the relative activity (average percentage of HLA-negative cells at day 27) versus specificity (percentage of off-target CpG methylation at the *VEGFA* locus quantified at day 5) for the LTRP molecules with the ZIM3-KRAB domain having configurations #1, #4, and #5, for *B2M*-targeting gRNA with spacer 7.37, as described in Example 15.

[0087] FIG. 51B is a dot plot showing the relative activity (average percentage of HLA-negative cells at day 27) versus specificity (percentage of off-target CpG methylation at the *VEGFA* locus quantified at day 5) for the LTRP molecules with the ZNF10-KRAB domain having configurations #1, #4, and #5, for *B2M*-targeting gRNA with spacer 7.37, as described in Example 15.

[0088] FIG. 52A is a dot plot showing the relative activity (average percentage of HLA-negative cells at day 27) versus specificity (percentage of off-target CpG methylation at the *VEGFA* locus quantified at day 5) for the LTRP molecules with the ZIM3-KRAB domain having configurations #1, #4, and #5, for *B2M*-targeting gRNA with spacer 7.165, as described in Example 15.

[0089] FIG. 52B is a dot plot showing the relative activity (average percentage of HLA-negative cells at day 27) versus specificity (percentage of off-target CpG methylation at the *VEGFA* locus quantified at day 5) for the LTRP molecules with the ZNF10-KRAB domain having configurations #1, #4, and #5, for *B2M*-targeting gRNA with spacer 7.165, as described in Example 15.

[0090] FIG. 53 shows the dose response results of the diphtheria toxin titration for cells transduced with either catalytically active CasX editors with gRNAs targeting the gene encoding the Heparin Binding EGF-like Growth Factor (*HBEGF*), *i.e.*, CasX-34.19 and CasX-34.21; a catalytically-dead CasX (dCasX) protein linked to a repressor domain as a fusion protein targeted to *HBEGF* (dXR fusion proteins, *i.e.*, dXR1-34.28); or a non-targeting dXR molecule (CasX-NT or dXR-NT), as described in Example 16. Data represent the mean and standard deviation of two biological replicates.

[0091] FIG. 54 provides violin plots showing the $\log_2$ (fold change) of sequences before and after selection for their ability to support dXR repression of the *HBEGF* locus, as described in Example 17. The plots show the results for the entire library, a negative control set of sequences, a positive control set of known KRAB repressors, the top 1597 enhanced domains tested with $\log_2$(fold change) > 2 and p-values <0.01, and the top 95 enhanced domains tested.

[0092] FIG. 55 shows *B2M* silencing activities (represented as percentage of HLA-negative cells) of dXR proteins with various repressor domains, as described in Example 17. Data are presented as mean with standard deviation, N=3.

[0093] FIG. 56 shows *B2M* silencing activities (represented as percentage of HLA-negative cells) of dXR proteins with various repressor domains, as described in Example 17. Data are presented as mean with standard deviation, N=3.

[0094] FIG. 57A provides the logo of repressor domain motif 1, as described in Example 17.

[0095] FIG. 57B provides the logo of repressor domain motif 2, as described in Example 17.

[0096] FIG. 57C provides the logo of repressor domain motif 3 (SEQ ID NO: 1727), as described in Example 17.

[0097] FIG. 57D provides the logo of repressor domain motif 4 (SEQ ID NO: 1728), as described in Example 17.

[0098] FIG. 57E provides the logo of repressor domain motif 5, as described in Example 17.

[0099] FIG. 57F provides the logo of repressor domain motif 6 (SEQ ID NO: 1729), as described in Example 17.

[00100] FIG. 57G provides the logo of repressor domain motif 7 (SEQ ID NO: 1730), as described in Example 17.

[00101] FIG. 57H provides the logo of repressor domain motif 8, as described in Example 17.

[00102] FIG. 57I provides the logo of repressor domain motif 9, as described in Example 17.

[00103] FIG. 58A provides the logo of alternative repressor domain motif 1 (SEQ ID NO: 2945), as described in Example 19.

[00104] FIG. 58B provides the logo of alternative repressor domain motif 2, as described in Example 19.

[00105] FIG. 58C provides the logo of alternative repressor domain motif 3, as described in Example 19.

[00106] FIG. 58D provides the logo of alternative repressor domain motif 4, as described in Example 19.

[00107] FIG. 58E provides the logo of alternative repressor domain motif 5 (SEQ ID NO: 2946), as described in Example 19.

[00108] FIG. 59 is a plot illustrating percentage of HEK293T cells, transfected with a plasmid encoding the indicated CasX or LTRP:gRNA construct, that expressed B2M six days post-treatment with the DNMT1 inhibitor 5-azadC at varying concentrations, as described in Example 20.

[00109] FIG. 60 is a plot that juxtaposes the quantification of B2M repression in HEK293T cells transfected with a plasmid encoding the indicated CasX or LTRP:gRNA construct and

cultured for 58 days, with the quantification of B2M reactivation upon treatment of transfected cells with 5-azadC, as described in Example 20.

[00110] FIG. 61 is a plot illustrating the percent of secreted PCSK9, normalized to baseline PCSK9 secretion levels, at 4 days post-treatment, for primary cynomolgus macaque (CM) hepatocytes from the BJE lot. CM hepatocytes were treated with the indicated doses of LNPs formulated with CasX 515 or LTRP5-ADD-ZIM3 mRNA and a *PCSK9*-targeting gRNA with spacer 6.1, as described in Example 10. The dashed line represents the lower limit of quantitation (LLOQ).

[00111] FIG. 62 is a plot illustrating the percent of secreted PCSK9, normalized to baseline PCSK9 secretion levels, at 4 days post-treatment, for primary CM hepatocytes from the VDU lot. CM hepatocytes were treated with the indicated doses of LNPs formulated with CasX 515 or LTRP5-ADD-ZIM3 mRNA and a *PCSK9*-targeting gRNA with spacer 6.1, as described in Example 10. The dashed line represents the lower limit of quantitation (LLOQ).

[00112] FIG. 63 is a plot illustrating the percent of secreted PCSK9, normalized to baseline PCSK9 secretion levels, at 11 days post-treatment, for primary CM hepatocytes from the BJE lot. CM hepatocytes were treated with the indicated doses of LNPs formulated with CasX 515 or LTRP5-ADD-ZIM3 mRNA and a *PCSK9*-targeting gRNA with spacer 6.1, as described in Example 10. The dashed line represents the lower limit of quantitation (LLOQ).

[00113] FIG. 64 is a plot illustrating the percent of secreted PCSK9, normalized to baseline PCSK9 secretion levels, at 11 days post-treatment, for primary CM hepatocytes from the VDU lot. CM hepatocytes treated with the indicated doses of LNPs formulated with CasX 515 or LTRP5-ADD-ZIM3 mRNA and a *PCSK9*-targeting gRNA with spacer 6.1, as described in Example 10. The dashed line represents the lower limit of quantitation (LLOQ).

[00114] FIG. 65A is a volcano plot showing the differential gene expression analysis (log2 fold changes (log2FC) of read counts) comparing LTRP5-ADD-ZIM3 paired with a non-targeting (NT) spacer with the untreated, naïve control at 6 days post-transfection. The horizontal dotted line shows the adjusted $p < 0.001$.

[00115] FIG. 65B is a volcano plot showing the differential gene expression analysis (log2FC of read counts) comparing LTRP5-ADD-ZIM3 paired with a non-targeting (NT) spacer with the untreated, naïve control at 26 days post-transfection. The horizontal dotted line shows the adjusted $p < 0.001$, and the vertical lines show the $|\log2FC| > 2$ threshold. Black dots are the identified differentially regulated off-target genes after applying the two significance thresholds.

[00116] FIG. 66A is a volcano plot showing the differential gene expression analysis (log2FC of read counts) comparing LTRP5-ADD-ZIM3 paired with spacer TG-06-154 with the untreated, naïve control at 6 days post-transfection. The horizontal dotted line shows the adjusted $p < 0.001$, and the vertical lines show the $|log2FC| > 2$ threshold. Black dots (except for *PCSK9*) are the identified differentially regulated off-target genes after applying the two significance thresholds.

[00117] FIG. 66B is a volcano plot showing the differential gene expression analysis (log2FC of read counts) comparing LTRP5-ADD-ZIM3 paired with spacer TG-06-154 with the untreated, naïve control at 26 days post-transfection. The horizontal dotted line shows the adjusted $p < 0.001$, and the vertical lines show the $|log2FC| > 2$ threshold. Black dots (except for *PCSK9*) are the identified differentially regulated off-target genes after applying the two significance thresholds.

[00118] FIG. 67A is a volcano plot is a volcano plot showing the differential gene expression analysis (log2FC of read counts) comparing LTRP5-ADD-ZIM3 paired with spacer TG-06-133 with the untreated, naïve control at 6 days post-transfection. The horizontal dotted line shows the adjusted $p < 0.001$, and the vertical lines show the $|log2FC| > 2$ threshold. Black dots (except for *PCSK9*) are the identified differentially regulated off-target genes after applying the two significance thresholds.

[00119] FIG. 67B is a volcano plot showing the differential gene expression analysis (log2FC of read counts) comparing LTRP5-ADD-ZIM3 paired with spacer TG-06-133 with the untreated, naïve control at 26 days post-transfection. The horizontal dotted line shows the adjusted $p < 0.001$, and the vertical lines show the $|log2FC| > 2$ threshold. Black dots (except for *PCSK9*) are the identified differentially regulated off-target genes after applying the two significance thresholds.

[00120] FIG. 68 is a bar graph showing the quantification of percent knockout of B2M in HEK293 cells transfected with CpG-depleted AAV plasmids containing the indicated gRNA scaffolds with spacer 7.37, as described in Example 24. The dotted line indicates ~41% transfection efficiency.

[00121] FIG. 69A is a bar plot showing percent editing at the *AAVS1* locus in human induced neurons (iNs) transduced with AAVs expressing the CasX:gRNA system using the indicated gRNA scaffolds (AAV construct ID # 262-274) at the MOI of 3E4 vg/cell, as described in Example 24.

[00122] FIG. 69B is a bar plot showing percent editing at the *AAVS1* locus in human iNs transduced with AAVs expressing the CasX:gRNA system using the indicated gRNA scaffolds (AAV construct ID # 262-274) at the MOI of 1E4 vg/cell, as described in Example 24.

[00123] FIG. 69C is a bar plot showing percent editing at the *AAVS1* locus in human iNs transduced with AAVs expressing the CasX:gRNA system using the indicated gRNA scaffolds (AAV construct ID # 262-274) at the MOI of 3E3 vg/cell, as described in Example 24.

[00124] FIG. 70A is a bar plot showing the quantification of percent knockout of B2M in HEK293 cells transfected with CpG-depleted AAV plasmids containing the indicated gRNA scaffolds with spacer 7.37 (AAV construct ID # 275-289) at the MOI of 1E4 vg/cell, as described in Example 24.

[00125] FIG. 70B is a bar plot showing the quantification of percent knockout of B2M in HEK293 cells transfected with CpG-depleted AAV plasmids containing the indicated gRNA scaffolds with spacer 7.37 (AAV construct ID # 275-289) at the MOI of 3E3 vg/cell, as described in Example 24.

[00126] FIG. 70C is a bar plot showing the quantification of percent knockout of B2M in HEK293 cells transfected with CpG-depleted AAV plasmids containing the indicated gRNA scaffolds with spacer 7.37 (AAV construct ID # 275-289) at the MOI of 1E3 vg/cell, as described in Example 24.

DETAILED DESCRIPTION

[00127] While exemplary embodiments have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the inventions claimed herein. It should be understood that various alternatives to the embodiments described herein may be employed in practicing the embodiments of the disclosure. It is intended that the claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

[00128] Unless otherwise defined, 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 invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present embodiments, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control.

In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention.

Definitions

[00129] "Hybridizable" or "complementary" are used interchangeably to mean that a nucleic acid (*e.g.*, RNA, DNA) comprises a sequence of nucleotides that enables it to non-covalently bind, *i.e.*, form Watson-Crick base pairs and/or G/U base pairs, "anneal", or "hybridize," to another nucleic acid in a sequence-specific, antiparallel, manner (*i.e.*, a nucleic acid specifically binds to a complementary nucleic acid) under the appropriate *in vitro* and/or *in vivo* conditions of temperature and solution ionic strength. It is understood that the sequence of a polynucleotide need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable; it can have at least about 70%, at least about 80%, or at least about 90%, or at least about 95% sequence identity and still hybridize to the target nucleic acid. Moreover, a polynucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (*e.g.*, a loop structure or hairpin structure, a 'bulge', 'bubble' and the like). Thus, the skilled artisan will understand that while individual bases within a sequence may not be complementary to another sequence, the sequence as a whole is still considered to be complementary.

[00130] A "gene," for the purposes of the present disclosure, includes a DNA region encoding a gene product (*e.g.*, a protein, RNA), as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene may include accessory element sequences including, but not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions. Coding sequences encode a gene product upon transcription or transcription and translation; the coding sequences of the disclosure may comprise fragments and need not contain a full-length open reading frame. A gene can include both the strand that is transcribed as well as the complementary strand containing the anticodons.

[00131] The term "downstream" refers to a nucleotide sequence that is located 3' to a reference nucleotide sequence. In certain embodiments, downstream nucleotide sequences relate to

sequences that follow the starting point of transcription. For example, the translation initiation codon of a gene is located downstream of the start site of transcription.

[00132] The term "upstream" refers to a nucleotide sequence that is located 5' to a reference nucleotide sequence. In certain embodiments, upstream nucleotide sequences relate to sequences that are located on the 5' side of a coding region or starting point of transcription. For example, most promoters are located upstream of the start site of transcription.

[00133] The term “adjacent to” with respect to polynucleotide or amino acid sequences refers to sequences that are next to, or adjoining each other in a polynucleotide or polypeptide. The skilled artisan will appreciate that two sequences can be considered to be adjacent to each other and still encompass a limited amount of intervening sequence, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides or amino acids.

[00134] The term “regulatory element” is used interchangeably herein with the term “regulatory sequence,” and is intended to include promoters, enhancers, and other expression regulatory elements. It will be understood that the choice of the appropriate regulatory element will depend on the encoded component to be expressed (*e.g.*, protein or RNA) or whether the nucleic acid comprises multiple components that require different polymerases or are not intended to be expressed as a fusion protein.

[00135] The term “accessory element” is used interchangeably herein with the term “accessory sequence,” and is intended to include, *inter alia*, polyadenylation signals (poly(A) signal), enhancer elements, introns, posttranscriptional regulatory elements (PTREs), nuclear localization signals (NLS), deaminases, DNA glycosylase inhibitors, additional promoters, factors that stimulate CRISPR-mediated homology-directed repair (*e.g.* in cis or in trans), self-cleaving sequences, and fusion domains, for example a fusion domain fused to a CRISPR protein. It will be understood that the choice of the appropriate accessory element or elements will depend on the encoded component to be expressed (*e.g.*, protein or RNA) or whether the nucleic acid comprises multiple components that require different polymerases or are not intended to be expressed as a fusion protein.

[00136] The term "promoter" refers to a DNA sequence that contains a transcription start site and additional sequences to facilitate polymerase binding and transcription. Exemplary eukaryotic promoters include elements such as a TATA box, and/or B recognition element (BRE) and assists or promotes the transcription and expression of an associated transcribable polynucleotide sequence and/or gene (or transgene). A promoter can be synthetically produced

or can be derived from a known or naturally occurring promoter sequence or another promoter sequence. A promoter can also include a chimeric promoter comprising a combination of two or more heterologous sequences to confer certain properties. A promoter of the present disclosure can include variants of promoter sequences that are similar in composition, but not identical to, other promoter sequence(s) known or provided herein. A promoter can be classified according to criteria relating to the pattern of expression of an associated coding or transcribable sequence or gene operably linked to the promoter, such as constitutive, developmental, tissue-specific, inducible, etc. A promoter can also be classified according to its strength. As used in the context of a promoter, "strength" refers to the rate of transcription of the gene controlled by the promoter. A "strong" promoter means the rate of transcription is high, while a "weak" promoter means the rate of transcription is relatively low.

[00137] A promoter of the disclosure can be a Polymerase II (Pol II) promoter. Polymerase II transcribes all protein coding and many non-coding genes. A representative Pol II promoter includes a core promoter, which is a sequence of about 100 base pairs surrounding the transcription start site, and serves as a binding platform for the Pol II polymerase and associated general transcription factors. The promoter may contain one or more core promoter elements such as the TATA box, BRE, Initiator (INR), motif ten element (MTE), downstream core promoter element (DPE), downstream core element (DCE), although core promoters lacking these elements are known in the art. All Pol II promoters are envisaged as within the scope of the instant disclosure.

[00138] A promoter of the disclosure can be a Polymerase III (Pol III) promoter. Pol III transcribes DNA to synthesize small ribosomal RNAs such as the 5S rRNA, tRNAs, and other small RNAs. Representative Pol III promoters use internal control sequences (sequences within the transcribed section of the gene) to support transcription, although upstream elements such as the TATA box are also sometimes used. All Pol III promoters are envisaged as within the scope of the instant disclosure.

[00139] The term "enhancer" refers to regulatory DNA sequences that, when bound by specific proteins called transcription factors, regulate the expression of an associated gene. Enhancers may be located in the intron of the gene, or 5' or 3' of the coding sequence of the gene. Enhancers may be proximal to the gene (*i.e.*, within a few tens or hundreds of base pairs (bp) of the promoter), or may be located distal to the gene (*i.e.*, thousands of bp, hundreds of thousands

of bp, or even millions of bp away from the promoter). A single gene may be regulated by more than one enhancer, all of which are envisaged as within the scope of the instant disclosure.

[00140] As used herein, a "post-transcriptional regulatory element (PTRE, or TRE)," such as a hepatitis PTRE, refers to a DNA sequence that, when transcribed creates a tertiary structure capable of exhibiting post-transcriptional activity to enhance or promote expression of an associated gene operably linked thereto.

[00141] In the context of the present disclosure and with respect to a gene, "repress", "repression", "repressing", "inhibition of gene expression", "downregulation", and "silencing" are used interchangeably herein to refer to the inhibition or blocking of transcription of a gene or a portion thereof. Accordingly, repression of a gene can result in a decrease in production of a gene product. Examples of gene repression processes which decrease transcription include, but are not limited to, those which inhibit formation of a transcription initiation complex, those which decrease transcription initiation rate, those which decrease transcription elongation rate, those which decrease processivity of transcription and those which antagonize transcriptional activation (by, for example, blocking the binding of a transcriptional activator). Gene repression can constitute, for example, prevention of activation as well as inhibition of expression below an existing level. Transcriptional repression includes both reversible and irreversible inactivation of gene transcription; the latter can result from epigenetic modification of the gene.

[00142] "Repressor" or "repressor domain" are used interchangeably to refer to polypeptide factors that act as regulatory elements on DNA that inhibit, repress, or block transcription of DNA, resulting in repression of gene expression. In the context of the present disclosure, the linking of a repressor domain to DNA-binding protein that can, when bound to the target nucleic acid, prevent transcription from a promoter or otherwise inhibit the expression of a gene. Without wishing to be bound by theory, it is thought that transcriptional repressors can function by a variety of mechanisms, including physically blocking RNA polymerase passage by steric hindrance, altering the polymerase's post-translational modification state, modifying the epigenetic state of the nascent RNA, changing the epigenetic state of the DNA through methylation, changing the epigenetic state of the DNA through histone deacetylation or modulating nucleosome remodeling, or preventing enhancer-promoter interactions, thereby leading to gene silencing or a reduction in the level of gene expression.

[00143] "Long-term repressor protein" or "LTRP" is used interchangeably herein with "repressor fusion protein" and refers to a fusion protein comprising a DNA binding protein (or

DNA binding domain of a protein) fused to one or more domains capable of repressing transcription of a target nucleic acid sequence. Optionally, the repressor fusion proteins of the disclosure may contain additional elements, such as linkers between any of the domains of the fusion protein, nuclear localization signals, nuclear export signals, as well as additional protein domains that confer additional activities upon the repressor fusion protein.

[00144] As used herein a "repressor fusion protein:gRNA system" is a system for transcriptional repression and comprises a repressor fusion protein comprising a catalytically-dead CRISPR protein and one or more linked repressor domains, and a guide nucleic acid (gRNA) that binds to the catalytically-dead CRISPR protein. For clarity, the system also includes any encoding DNA, RNA or vectors and the like that can be used to produce the repressor fusion proteins and gRNA components of the system.

[00145] As used herein, a DNA-binding protein refers to a protein, or domain of a protein, capable of binding to DNA. Exemplary DNA-binding proteins include zinc finger (ZF) proteins, TALEs, and CRISPR proteins. The skilled artisan will appreciate that in multi-functional proteins that are capable of both binding DNA and carrying out another activity such as DNA cleavage, such as, *e.g.*, CRISPR proteins, the DNA binding function can be separated from the other functions of the protein, leading to catalytically-dead DNA binding proteins.

[00146] As used herein a "catalytically-dead CRISPR protein" refers to a CRISPR protein that lacks endonuclease activity. The skilled artisan will appreciate that a CRISPR protein can be catalytically-dead, and still able to carry out additional protein functions, such as DNA binding. Similarly, a "catalytically-dead CasX" refers to a CasX protein that lacks endonuclease activity but is still able to carry out additional protein functions, such as DNA binding.

[00147] "Recombinant," as used herein, means that a particular nucleic acid (DNA or RNA) is the product of various combinations of cloning, restriction, and/or ligation steps resulting in a construct having a structural coding or non-coding sequence distinguishable from endogenous nucleic acids found in natural systems. Generally, DNA sequences encoding the structural coding sequence can be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of synthetic oligonucleotides, to provide a synthetic nucleic acid which is capable of being expressed from a recombinant transcriptional unit contained in a cell or in a cell-free transcription and translation system. Such sequences can be provided in the form of an open reading frame uninterrupted by internal non-translated sequences, or introns, which are typically present in eukaryotic genes. Genomic DNA comprising the relevant sequences can also be used

in the formation of a recombinant gene or transcriptional unit. Sequences of non-translated DNA may be present 5' or 3' from the open reading frame, where such sequences do not interfere with manipulation or expression of the coding regions, and may indeed act to modulate production of a desired product by various mechanisms (see "enhancers" and "promoters", above).

[00148] The term "recombinant polynucleotide" or "recombinant nucleic acid" refers to one which is not naturally occurring, *e.g.*, is made by the artificial combination of two otherwise separated segments of sequence through human intervention. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, *e.g.*, by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a desired combination of functions. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, *e.g.*, by genetic engineering techniques.

[00149] Similarly, the term "recombinant polypeptide" or "recombinant protein" refers to a polypeptide or protein which is not naturally occurring, *e.g.*, is made by the artificial combination of two otherwise separated segments of amino sequence through human intervention. Thus, *e.g.*, a protein that comprises a heterologous amino acid sequence is recombinant.

[00150] As used herein, "lipoprotein", such as VLDL, LDL and HDL, refers to a group of proteins found in the serum, plasma and lymph and are important for lipid transport. The chemical composition of each lipoprotein differs, for example, in that the HDL has a higher proportion of protein versus lipid, whereas the VLDL has a lower proportion of protein versus lipid.

[00151] As used herein, "atherosclerosis" means a hardening of the arteries affecting large and medium-sized arteries and is characterized by the presence of fatty deposits. The fatty deposits are called "atheromas" or "plaques," which consist mainly of cholesterol and other fats, calcium and scar tissue, and damage the lining of arteries.

[00152] As used herein, "coronary heart disease (CHD)" means a narrowing of the small blood vessels that supply blood and oxygen to the heart, which is often a result of atherosclerosis.

[00153] As used herein, "dyslipidemia" refers to a disorder of lipid and/or lipoprotein metabolism, including lipid and/or lipoprotein overproduction or deficiency. Dyslipidemias can be manifested by elevation of lipids such as chylomicron, cholesterol and triglycerides as well as lipoproteins such as low-density lipoprotein (LDL) cholesterol.

[00154] As used herein, "high density lipoprotein-C" or "HDL-C" means cholesterol associated with high-density lipoprotein particles. Concentration of HDL-C in serum (or plasma) is typically quantified in mg/dL or nmol/L. "Serum HDL-C" and "plasma HDL-C" mean HDL-C in serum and plasma, respectively.

[00155] As used herein, "low density lipoprotein-cholesterol (LDL-C)" means cholesterol carried in low density lipoprotein particles. Concentration of LDL-C in serum (or plasma) is typically quantified in mg/dL or nmol/L. "Serum LDL-C" and "plasma LDL-C" mean LDL-C in the serum and plasma, respectively.

[00156] As used herein, "hypercholesterolemia" means a condition characterized by elevated cholesterol or circulating (plasma) cholesterol, LDL-cholesterol and VLDL-cholesterol, as per the guidelines of the Expert Panel Report of the National Cholesterol Educational Program (NCEP) of Detection, Evaluation of Treatment of high cholesterol in adults (*see*, Arch. Int. Med. 148: 36 (1988)).

[00157] As used herein, "hyperlipidemia" or "hyperlipemia" is a condition characterized by elevated serum lipids or circulating (plasma) lipids. This condition manifests an abnormally high concentration of fats. The lipid fractions in the circulating blood are cholesterol, low-density lipoproteins, very low density lipoproteins, chylomicrons and triglycerides. The Fredrickson classification of hyperlipidemias is based on the pattern of TG and cholesterol-rich lipoprotein particles, as measured by electrophoresis or ultracentrifugation and is commonly used to characterize primary causes of hyperlipidemias such as hypertriglyceridemia.

[00158] As used herein, "triglyceride" or "TG" means a lipid or neutral fat consisting of glycerol combined with three fatty acid molecules.

[00159] As used herein, "hypertriglyceridemia" means a condition characterized by elevated triglyceride levels. Its etiology includes primary (*i.e.* genetic causes) and secondary (other underlying causes such as diabetes, metabolic syndrome/insulin resistance, obesity, physical inactivity, cigarette smoking, excess alcohol and a diet very high in carbohydrates) factors or, most often, a combination of both

[00160] As used herein, “diabetes mellitus” or “diabetes” is a syndrome characterized by disordered metabolism and abnormally high blood sugar (hyperglycemia) resulting from insufficient levels of insulin or reduced insulin sensitivity. The characteristic symptoms are excessive urine production (polyuria) due to high blood glucose levels, excessive thirst and increased fluid intake (polydipsia) attempting to compensate for increased urination, blurred vision due to high blood glucose effects on the eye's optics, unexplained weight loss, and lethargy.

[00161] As used herein, “diabetic dyslipidemia” or “type 2 diabetes with dyslipidemia” means a condition characterized by Type 2 diabetes, reduced HDL-C, elevated triglycerides (TG), and elevated small, dense LDL particles.

[00162] As used herein, "lipid nanoparticle" refers to particles having at least one dimension on the order of nanometers (*e.g.*, 1-1,000 nm) comprising one or more lipids (*e.g.*, cationic lipids, non- cationic lipids, and PEG-modified lipids). In some embodiments, lipid nanoparticles are included in a formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, mRNA) to a target site of interest (*e.g.*, cell, tissue, organ, tumor, and the like). In some embodiments, the lipid nanoparticles of the disclosure comprise a nucleic acid. Such lipid nanoparticles typically comprise neutral lipids, charged lipids, steroids and polymer conjugated lipids. In some embodiments, the active agent or therapeutic agent, such as a nucleic acid, may be encapsulated in the lipid portion of the lipid nanoparticle or an aqueous space enveloped by some or all of the lipid portion of the lipid nanoparticle, thereby protecting it from enzymatic degradation or other undesirable effects induced by the mechanisms of the host organism or cells *e.g.* an adverse immune response.

[00163] As used herein, "lipid encapsulated" refers to a lipid nanoparticle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, mRNA), with full encapsulation, partial encapsulation, or both. In an embodiment, the nucleic acid (*e.g.*, mRNA) is fully encapsulated in the lipid nanoparticle.

[00164] As used herein, the term “contacting” means establishing a physical connection between two or more entities. For example, contacting a target nucleic acid with a guide nucleic acid means that the target nucleic acid and the guide nucleic acid are made to share a physical connection; *e.g.*, can hybridize if the sequences share sequence similarity.

[00165] “Dissociation constant”, or “K_d”, are used interchangeably and mean the affinity between a ligand “L” and a protein “P”; *i.e.*, how tightly a ligand binds to a particular protein. It

can be calculated using the formula K_d=[L] [P]/[LP], where [P], [L] and [LP] represent molar concentrations of the protein, ligand and complex, respectively.

[00166] The disclosure provides compositions and methods useful for modifying a target nucleic acid. As used herein “editing” is used interchangeably with “modifying” and "modification" and includes but is not limited to cleaving, nicking, editing, deleting, knocking in, knocking out, and the like. Modifying can also encompass epigenetic modifications to a nucleic acid, or chromatin containing the nucleic acid, such as, but not limited to, changes in DNA methylation, and histone methylation and acetylation.

[00167] By “cleavage” it is meant the breakage of the covalent backbone of a target nucleic acid molecule (e.g., RNA, DNA). Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events.

[00168] The term "knock-down" as used herein refers to reduction in the expression of a gene or its gene product(s). As a result of a gene knock-down, the protein activity or function may be attenuated or the protein levels may be reduced or eliminated.

[00169] A polynucleotide or polypeptide has a certain percent "sequence similarity" or "sequence identity" to another polynucleotide or polypeptide, meaning that, when aligned, that percentage of bases or amino acids are the same, and in the same relative position, when comparing the two sequences. Sequence similarity (sometimes referred to as percent similarity, percent identity, or homology) can be determined in a number of different manners. To determine sequence similarity, sequences can be aligned using the methods and computer programs that are known in the art, including BLAST, available over the world wide web at ncbi.nlm.nih.gov/BLAST. Percent complementarity between particular stretches of nucleic acid sequences within nucleic acids can be determined using any convenient method. Example methods include BLAST programs (basic local alignment search tools) and PowerBLAST programs (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656) or by using the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wis.), *e.g.*, using default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482-489).

[00170] The terms "polypeptide," and "protein" are used interchangeably herein, and refer to a polymeric form of amino acids of any length, which can include coded and non-coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones. The term includes fusion proteins, including, but not limited to, fusion proteins with a heterologous amino acid sequence.

[00171] A "vector" or "expression vector" is a replicon, such as plasmid, phage, virus, or cosmid, to which another DNA segment, *i.e.*, an expression cassette, may be attached so as to bring about the replication or expression of the attached segment in a cell.

[00172] 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.

[00173] As used herein, a "mutation" refers to an insertion, deletion, substitution, duplication, or inversion of one or more amino acids or nucleotides as compared to a wild-type or reference amino acid sequence or to a wild-type or reference nucleotide sequence.

[00174] As used herein the term "isolated" is meant to describe a polynucleotide, a polypeptide, or a cell that is in an environment different from that in which the polynucleotide, the polypeptide, or the cell naturally occurs. An isolated genetically modified host cell may be present in a mixed population of genetically modified host cells.

[00175] A "host cell," as used herein, denotes a eukaryotic cell, a prokaryotic cell, or a cell from a multicellular organism (*e.g.*, a cell line) cultured as a unicellular entity, which eukaryotic or prokaryotic cells are used as recipients for a nucleic acid (*e.g.*, an AAV vector), and include the progeny of the original cell which has been genetically modified by the nucleic acid. It is understood that the progeny of a single cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. A "recombinant host cell" (also referred to as a "genetically modified host cell") is a host cell into which has been introduced a heterologous nucleic acid, *e.g.*, an AAV vector.

[00176] The term "conservative amino acid substitution" refers to the interchangeability in proteins of amino acid residues having similar side chains. For example, a group of amino acids having aliphatic side chains consists of glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains consists of serine and threonine; a group of amino acids having amide-containing side chains consists of asparagine and glutamine; a group

of amino acids having aromatic side chains consists of phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains consists of lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains consists of cysteine and methionine. Exemplary conservative amino acid substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

[00177] As used herein, "treatment" or "treating," are used interchangeably herein and refer to an approach for obtaining beneficial or desired results, including but not limited to a therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying disorder or disease being treated. A therapeutic benefit can also be achieved with the eradication or amelioration of one or more of the symptoms or an improvement in one or more clinical parameters associated with the underlying disease such that an improvement is observed in the subject, notwithstanding that the subject may still be afflicted with the underlying disorder.

[00178] The terms "therapeutically effective amount" and "therapeutically effective dose", as used herein, refer to an amount of a drug or a biologic, alone or as a part of a composition, that is capable of having any detectable, beneficial effect on any symptom, aspect, measured parameter or characteristics of a disease state or condition when administered in one or repeated doses to a subject such as a human or an experimental animal. Such effect need not be absolute to be beneficial.

[00179] As used herein, "administering" means a method of giving a dosage of a compound (*e.g.*, a composition of the disclosure) or a composition (*e.g.*, a pharmaceutical composition) to a subject.

[00180] A "subject" is a mammal. Mammals include, but are not limited to, domesticated animals, non-human primates, humans, dogs, rabbits, mice, rats and other rodents.

[00181] The term "low-density lipoprotein (LDL)" refers to one of the five major groups of lipoprotein, from least dense (lower weight-volume ratio particles) to most dense (larger weight-volume ratio particles): chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), and high-density lipoproteins (HDL). Lipoproteins transfer lipids (fats) around the body in the extracellular fluid thereby facilitating the transfer of fats to the cells body via receptor-mediated endocytosis. An LDL particle is about 220-275 angstroms in diameter.

[00182] "Low-density lipoprotein (LDL) receptor" refers to a receptor protein of 839 amino acids (after removal of 21-amino acid signal peptide) that mediates the endocytosis of cholesterol-rich LDL particles. It is a cell-surface receptor that recognizes the apoprotein B100 and apoE protein found in chylomicron remnants and VLDL remnants (IDL) resulting in the binding and endocytosis of LDL-cholesterol. This process occurs in all nucleated cells, but mainly in the liver which removes approximately 70% of LDL from the circulation. The human LDLR gene is described in part in the NCBI database (ncbi.nlm.nih.gov) as Reference Sequence NG_009060.1, which is incorporated by reference herein.

[00183] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. The contents of WO 2020/247882, filed on June 5, 2020, WO 2020/247883, filed June 5, 2020, WO 2021/050593, filed on September 9, 2020, WO 2021/050601, filed on September 9, 2021, WO 2021/142342, filed on January 8, 2021, WO 2021/113763, filed on December 4, 2020, WO 2021/113769, filed on December 4, 2020, WO 2021/113772, filed on December 4, 2020, WO 2021/188729, filed on December 4, 2020, WO 2022/120095, filed December 2, 2021, WO 2022/120094, filed on December 2, 2021, WO 2022/125843, filed on December 9, 2021, WO 2022/120089, filed on December 2, 2021, WO 2022/261150, filed on June 7, 2022, WO 2023/049742, filed on September 21, 2022, WO 2022/261149, filed on June 7, 2022, and PCT/US2023/067791, filed on June 1, 2023, which disclose CasX variants and gRNA variants, and methods of delivering same, are hereby incorporated by reference in their entirety.

I. General Methods

[00184] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which can be found in such standard textbooks as Molecular Cloning: A Laboratory Manual, 3rd Ed. (Sambrook et al., Harbor Laboratory Press 2001); Short Protocols in Molecular Biology, 4th Ed. (Ausubel et al. eds., John Wiley & Sons 1999); Protein Methods (Bollag et al., John Wiley & Sons 1996); Nonviral Vectors for Gene Therapy (Wagner et al. eds., Academic Press 1999); Viral Vectors (Kaplift & Loewy eds., Academic Press 1995); Immunology Methods Manual (I. Lefkovits ed., Academic Press 1997); and Cell and Tissue Culture: Laboratory Procedures in Biotechnology (Doyle & Griffiths, John Wiley & Sons 1998), the disclosures of which are incorporated herein by reference.

[00185] Where a range of values is provided, it is understood that endpoints are included and that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included.

[00186] 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 invention belongs. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[00187] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

[00188] It will be appreciated that certain features of the disclosure, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. In other cases, various features of the disclosure, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. It is intended that all combinations of the embodiments pertaining to the disclosure are specifically embraced by the present disclosure and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations of the various embodiments and elements thereof are also specifically embraced by the present disclosure and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

II. Systems for Epigenetic Modification and Repression of PCSK9 Genes

[00189] In a first aspect, the present disclosure provides systems comprising or encoding a fusion protein comprising a DNA-binding protein and linked repressor domains capable of binding a target nucleic acid sequence of a *PCSK9* gene targeted for transcriptional repression, silencing, and/or epigenetic modification (collectively, long-term repressor proteins, referred to herein as "LTRP" or "LTRP fusion protein" or "repressor fusion protein"; a reflection of the long-term repression effects that can be achieved on the targeted gene). As used herein, a "system" is used interchangeably with "composition". The disclosure also provides nucleic acids

encoding the systems provided herein. Also provided herein are methods of making the systems, as well as methods of using the systems, including methods of gene repression and/or epigenetic modification and methods of treatment of PCSK9-related diseases.

[00190] In some embodiments, the DNA-binding proteins comprise zinc finger (ZF) or TALE (transcription-activator-like effector) proteins, or DNA binding domains thereof, also referred to herein as a DNA-binding protein, that bind but do not cleave the target nucleic acid. The DNA-binding domain of a TALE is comprised of a tandem array of 33–34 amino acid (aa)-long customizable monomers that theoretically can be assembled to recognize any genetic sequence following a one-repeat-binds-one-base-pair recognition code (Jain, S., et al. TALE outperforms Cas9 in editing heterochromatin target sites. Nat. Commun. 12:606 (2021)). The specificity of TALEs for binding DNA arises from two polymorphic amino acids, the so-called repeat variable diresidues (RVDs) located at positions 12 and 13 of a repeated unit. By re-arranging the repeats, the DNA binding specificities of TALE can be changed at will. Zinc finger proteins are transcription factors, where each finger recognizes 3-4 bases of DNA. By mixing and matching these finger modules, the ZFs can be customized for the sequence to be targeted. Exemplary ZFs that are capable of binding the *PCSK9* gene are described in WO2018049009A2.

[00191] In some embodiments, the DNA-binding protein is a catalytically-dead Class 1 or Class 2 CRISPR protein. Catalytically-dead CRISPR proteins are also referred to in the art as "catalytically inactive" CRISPR proteins. In some embodiments, the Class 2, Type II protein is a catalytically-dead Cas9. In other embodiments, the Class 2 CRISPR protein is selected from the group consisting of a Type II, Type V, or Type VI protein. In one embodiment, the Class 2 Type V protein is selected from the group consisting of Cas12a (Cpf1), Cas12b (C2c1), Cas12c (C2c3), Cas12d (CasY), Cas12e (CasX), Cas12f, Cas12g, Cas12h, Cas12i, Cas12j, Cas12k, Cas14, and/or CasΦ, in each case rendered catalytically-dead by specific mutations, as described herein. In some embodiments, the CasX protein is a catalytically-dead CasX variant (dCasX), wherein the dCasX comprises a sequence selected from the group consisting of SEQ ID NOS: 4-29, or a sequence having at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto, wherein the fusion protein comprising the dCasX retains the ability to form an RNP with a gRNA. In some embodiments, the dCasX comprises a sequence selected from the group consisting of SEQ ID NOS: 4-29. In some embodiments, the dCasX comprises a sequence selected from the group

consisting of SEQ ID NOS: 3281-3441, comprising a RuvC domain with one or more mutations that inactivates the cleavage activity of the RuvC domain, thereby rendering the CasX protein catalytically dead. In a particular embodiment, the dCasX comprises a sequence of SEQ ID NO: 4.

[00192] The CRISPR-based systems further comprise a guide nucleic acid (gNA), for example a guide ribonucleic acid (gRNA) with a targeting sequence complementary to the target sequence of a gene. Upon binding of the target sequence by the CRISPR-based system, (the CRISPR protein and linked repressor domains) and the gRNA, transcription the gene is repressed.

[00193] The present disclosure provides systems for transcriptional repression of a *PCSK9* gene. In some embodiments, the system comprises a repressor fusion protein comprising a catalytically-dead CasX protein and linked repressor domains , and a guide ribonucleic acid (gRNA) comprising a targeting sequence complementary to a target nucleic acid sequence of a *PCSK9* gene targeted for repression, silencing, or downregulation (a repressor fusion protein:gRNA system). In some embodiments, the system comprises nucleic acids encoding the repressor fusion protein, for example a dCasX and linked repressor domains, and gRNA. In some embodiments, the system comprises a repressor fusion protein and a gRNA as gene repressor pairs that are capable of forming a ribonucleoprotein (RNP) complex and binding a *PCSK9* target nucleic acid in a eukaryotic cell. In other cases, the disclosure provides systems of nucleic acids encoding the repressor fusion protein and gRNA, or a gRNA and an mRNA encoding the repressor fusion protein for use in certain particle formulations (*e.g.*, an LNP) described herein.

[00194] Also provided herein are methods of making repressor fusion proteins and gRNAs, as well as methods of using the repressor fusion protein:gRNA systems, including methods of gene repression and/or epigenetic modification, and methods of treatment. The DNA-binding proteins (*e.g.*, dCasX) and linked repressor domain(s) and gRNA components of the systems and their features, as well as the delivery modalities and the methods of using the systems for the repression, down-regulation or silencing of a *PCSK9* gene are described more fully, below.

[00195] The disclosure provides systems specifically designed to repress or silence transcription of the *PCSK9* gene. In some cases, the system is designed to repress transcription of the *PCSK9* gene in eukaryotic cells having a gain of function mutation. In some cases, the system is designed to repress transcription of the wild-type *PCSK9* gene in eukaryotic cells. In

the alternative, the system is designed to repress transcription of a mutant allele of the *PCSK9* gene in eukaryotic cells. Generally, any portion of the *PCSK9* gene can be targeted using the programable systems and methods provided herein, described more fully, herein.

[00196] The *PCSK9* gene encodes proprotein convertase subtilisin/kexin Type 9 ("PCSK9"), a protein that binds to the receptor for low-density lipoprotein particles (LDL) for transport of LDL into the cell. The *PCSK9* gene encompasses the sequence that spans chr1:55,039,476-55,064,853 of the human genome (GRCh38/hg38) (the notation refers to the chromosome 1 (chr1), starting at the 55,039,476 bp to 55,064,853 bp on chromosome 1 (Homo sapiens Updated Annotation Release 109.20190905, GRCh38.p13) (NCBI). The human *PCSK9* gene is described in part in the NCBI database (ncbi.nlm.nih.gov) as Reference Sequence NG_009061.1, which is incorporated by reference herein. The *PCSK9* locus has 12 exons that produces an mRNA of 3636 bp encoding a 692-amino acid protein that, following its synthesis, undergoes an autocatalytic cleavage reaction that clips off the prodomain, resulting in an activated protein having 540 amino acids. The prodomain remains attached to the catalytic and resistin-like domains, likely because the prodomain serves as a chaperone and facilitates folding and secretion (Seidah, NG et al., Proc Natl Acad Sci USA 100(3):928 (2003)). The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation (Seidah NG, et al.). This protein, also called neural apoptosis regulated convertase, is a serine protease belonging to the protease K subfamily of subtilases.

[00197] The human PCSK9 gene (HGNC:20001) encodes a protein (Q8NBP7) having the sequence

MGTVSSRRSWWPLPLLLLLLLLLGPAGARAQEDEDGDYEELVLALRSEEDGLAEAPEHGTTATFHRCAK
DPWRLPGTYVVVLKEETHLSQSERTARRLQAQAARRGYLTKILHVFHGLLPGFLVKMSGDLLELALKLP
HVDYIEEDSSVFAQSIPWNLERITPPRYRADEYQPPDGGSLVEVYLLDTSIQSDHREIEGRVMVTDFEN
VPEEDGTRFHRQASKCDSHGTHLAGVVSGRDAGVAKGASMRSLRVLNCQGKGTVSGTLIGLEFIRKSQL
VQPVGPLVVLLPLAGGYSRVLNAACQRLARAGVVLVTAAGNFRDDACLYSPASAPEVITVGATNAQDQP
VTLGTLGTNFGRCVDLFAPGEDIIGASSDCSTCFVSQSGTSQAAAHVAGIAAMMLSAEPELTLAELRQR
LIHFSAKDVINEAWFPEDQRVLTPNLVAALPPSTHGAGWQLFCRTVWSAHSGPTRMATAVARCAPDEEL
LSCSSFSRSGKRRGERMEAQGGKLVCRAHNAFGGEGVYAIARCCLLPQANCSVHTAPPAEASMGTRVHC
HQQGHVLTGCSSHWEVEDLGTHKPPVLRPRGQPNQCVGHREASIHASCCHAPGLECKVKEHGIPAPQEQ
VTVACEEGWTLTGCSALPGTSHVLGAYAVDNTCVVRSRDVSTTGSTSEGAVTAVAICCRSRHLAQASQE
LQ (SEQ ID NO: 1823).

III. Catalytically-dead Proteins for Use in the Repressor Systems

[00198] In some embodiments, the DNA-binding proteins for use in the fusion proteins, systems and methods of the disclosure are zinc finger (ZF) or TALE (transcription-activator-like effector) proteins that can bind, but not cleave, a *PCSK9* target nucleic acid.

[00199] In some embodiments, the DNA-binding protein is a catalytically-dead Class 1 or Class 2 CRISPR protein. In one embodiment, the Class 2, Type II protein is a catalytically-dead Cas9. In another embodiment, the Class 2 CRISPR protein is selected from the group consisting of a Type II, Type V, or Type VI protein. In one embodiment, the Class 2 CRISPR Type V protein is selected from the group consisting of Cas12a (Cpf1), Cas12b (C2c1), Cas12c (C2c3), Cas12d (CasY), Cas12e (CasX), Cas12f, Cas12g, Cas12h, Cas12i, Cas12j, Cas12k, Cas14, and/or CasΦ, in each case rendered catalytically-dead by specific mutations, as described herein. In some embodiments, the CasX protein is a catalytically-dead CasX variant (dCasX).

[00200] The term "CasX protein", as used herein, refers to a family of proteins, and encompasses all naturally-occurring CasX proteins ("reference CasX"), as well as engineered CasX proteins with multiple sequence modifications, in addition to those rendering the CasX catalytically-dead (dCasX), that possess one or more improved characteristics relative to a reference CasX protein, described more fully, below. CasX proteins of the disclosure comprise the following domains: a non-target strand binding (NTSB) domain, a target strand loading (TSL) domain, a helical I domain, a helical II domain, an oligonucleotide binding domain (OBD), and a RuvC domain, and, in some cases, domains can be further divided into subdomains, as listed in Table 1.

[00201] In the context of the present disclosure, the CasX for use in the repressor fusion proteins, systems and methods are catalytically-dead (dCasX); achieved by mutations introduced at select locations in the RuvC sequence, described below.

a. Reference CasX Proteins

[00202] The disclosure provides naturally-occurring CasX proteins (referred to herein as a "reference CasX protein"), which were subsequently modified to create the engineered dCasX of the disclosure. For example, reference CasX proteins can be isolated from naturally occurring prokaryotes, such as *Deltaproteobacteria*, *Planctomycetes*, or *Candidatus Sungbacteria* species. A reference CasX protein (interchangeably referred to herein as a reference CasX polypeptide) is a Class 2, Type V CRISPR/Cas endonuclease belonging to the CasX (interchangeably referred

to as Cas12e) family of proteins that interacts with a guide RNA to form a ribonucleoprotein (RNP) complex.

[00203] In some cases, a reference CasX protein is isolated or derived from *Deltaproteobacter* having a sequence of:

```
   1 MEKRINKIRK KLSADNATKP VSRSGPMKTL LVRVMTDDLK KRLEKRRKKP EVMPQVISNN
  61 AANNLRMLLD DYTKMKEAIL QVYWQEFKDD HVGLMCKFAQ PASKKIDQNK LKPEMDEKGN
 121 LTTAGFACSQ CGQPLFVYKL EQVSEKGKAY TNYFGRCNVA EHEKLILLAQ LKPEKDSDEA
 181 VTYSLGKFGQ RALDFYSIHV TKESTHPVKP LAQIAGNRYA SGPVGKALSD ACMGTIASFL
 241 SKYQDIIIEH QKVVKGNQKR LESLRELAGK ENLEYPSVTL PPQPHTKEGV DAYNEVIARV
 301 RMWVNLNLWQ KLKLSRDDAK PLLRLKGFPS FPVVERRENE VDWWNTINEV KKLIDAKRDM
 361 GRVFWSGVTA EKRNTILEGY NYLPNENDHK KREGSLENPK KPAKRQFGDL LLYLEKKYAG
 421 DWGKVFDEAW ERIDKKIAGL TSHIEREEAR NAEDAQSKAV LTDWLRAKAS FVLERLKEMD
 481 EKEFYACEIQ LQKWYGDLRG NPFAVEAENR VVDISGFSIG SDGHSIQYRN LLAWKYLENG
 541 KREFYLLMNY GKKGRIRFTD GTDIKKSGKW QGLLYGGGKA KVIDLTFDPD DEQLIILPLA
 601 FGTRQGREFI WNDLLSLETG LIKLANGRVI EKTIYNKKIG RDEPALFVAL TFERREVVDP
 661 SNIKPVNLIG VDRGENIPAV IALTDPEGCP LPEFKDSSGG PTDILRIGEG YKEKQRAIQA
 721 AKEVEQRRAG GYSRKFASKS RNLADDMVRN SARDLFYHAV THDAVLVFEN LSRGFGRQGK
 781 RTFMTERQYT KMEDWLTAKL AYEGLTSKTY LSKTLAQYTS KTCSNCGFTI TTADYDGMLV
 841 RLKKTSDGWA TTLNNKELKA EGQITYYNRY KRQTVEKELS AELDRLSEES GNNDISKWTK
 901 GRRDEALFLL KKRFSHRPVQ EQFVCLDCGH EVHADEQAAL NIARSWLFLN SNSTEFKSYK
 961 SGKQPFVGAW QAFYKRRLKE VWKPNA (SEQ ID NO: 1).
```

[00204] In some cases, a reference CasX protein is isolated or derived from *Planctomycetes* having a sequence of:

```
   1 MQEIKRINKI RRRLVKDSNT KKAGKTGPMK TLLVRVMTPD LRERLENLRK KPENIPQPIS
  61 NTSRANLNKL LTDYTEMKKA ILHVYWEEFQ KDPVGLMSRV AQPAPKNIDQ RKLIPVKDGN
 121 ERLTSSGFAC SQCCQPLYVY KLEQVNDKGK PHTNYFGRCN VSEHERLILL SPHKPEANDE
 181 LVTYSLGKFG QRALDFYSIH VTRESNHPVK PLEQIGGNSC ASGPVGKALS DACMGAVASF
 241 LTKYQDIILE HQKVIKKNEK RLANLKDIAS ANGLAFPKIT LPPQPHTKEG IEAYNNVVAQ
 301 IVIWVNLNLW QKLKIGRDEA KPLQRLKGFP SFPLVERQAN EVDWWDMVCN VKKLINEKKE
 361 DGKVFWQNLA GYKRQEALLP YLSSEEDRKK GKKFARYQFG DLLLHLEKKH GEDWGKVYDE
 421 AWERIDKKVE GLSKHIKLEE ERRSEDAQSK AALTDWLRAK ASFVIEGLKE ADKDEFCRCE
 481 LKLQKWYGDL RGKPFAIEAE NSILDISGFS KQYNCAFIWQ KDGVKKLNLY LIINYFKGGK
 541 LRFKKIKPEA FEANRFYTVI NKKSGEIVPM EVNFNFDDPN LIILPLAFGK RQGREFIWND
 601 LLSLETGSLK LANGRVIEKT LYNRRTRQDE PALFVALTFE RREVLDSSNI KPMNLIGIDR
 661 GENIPAVIAL TDPEGCPLSR FKDSLGNPTH ILRIGESYKE KQRTIQAAKE VEQRRAGGYS
 721 RKYASKAKNL ADDMVRNTAR DLLYYAVTQD AMLIFENLSR GFGRQGKRTF MAERQYTRME
 781 DWLTAKLAYE GLPSKTYLSK TLAQYTSKTC SNCGFTITSA DYDRVLEKLK KTATGWMTTI
 841 NGKELKVEGQ ITYYNRYKRQ NVVKDLSVEL DRLSEESVNN DISSWTKGRS GEALSLLKKR
 901 FSHRPVQEKF VCLNCGFETH ADEQAALNIA RSWLFLRSQE YKKYQTNKTT GNTDKRAFVE
 961 TWQSFYRKKL KEVWKPAV (SEQ ID NO: 2).
```

[00205] In some cases, a reference CasX protein is isolated or derived from *Candidatus Sungbacteria* having a sequence of

```
   1 MDNANKPSTK SLVNTTRISD HFGVTPGQVT RVFSFGIIPT KRQYAIIERW FAAVEAARER
  61 LYGMLYAHFQ ENPPAYLKEK FSYETFFKGR PVLNGLRDID PTIMTSAVFT ALRHKAEGAM
 121 AAFHTNHRRL FEEARKKMRE YAECLKANEA LLRGAADIDW DKIVNALRTR LNTCLAPEYD
 181 AVIADFGALC AFRALIAETN ALKGAYNHAL NQMLPALVKV DEPEEAEESP RLRFFNGRIN
 241 DLPKFPVAER ETPPDTETII RQLEDMARVI PDTAEILGYI HRIRHKAARR KPGSAVPLPQ
 301 RVALYCAIRM ERNPEEDPST VAGHFLGEID RVCEKRRQGL VRTPFDSQIR ARYMDIISFR
 361 ATLAHPDRWT EIQFLRSNAA SRRVRAETIS APFEGFSWTS NRTNPAPQYG MALAKDANAP
```

```
421 ADAPELCICL SPSSAAFSVR EKGGDLIYMR PTGGRRGKDN PGKEITWVPG SFDEYPASGV
481 ALKLRLYFGR SQARRMLTNK TWGLLSDNPR VFAANAELVG KKRNPQDRWK LFFHMVISGP
541 PPVEYLDFSS DVRSRARTVI GINRGEVNPL AYAVVSVEDG QVLEEGLLGK KEYIDQLIET
601 RRRISEYQSR EQTPPRDLRQ RVRHLQDTVL GSARAKIHSL IAFWKGILAI ERLDDQFHGR
661 EQKIIPKKTY LANKTGFMNA LSFSGAVRVD KKGNPWGGMI EIYPGGISRT CTQCGTVWLA
721 RRPKNPGHRD AMVVIPDIVD DAAATGFDNV DCDAGTVDYG ELFTLSREWV RLTPRYSRVM
781 RGTLGDLERA IRQGDDRKSR QMLELALEPQ PQWGQFFCHR CGFNGQSDVL AATNLARRAI
841 SLIRRLPDTD TPPTP (SEQ ID NO: 3).
```

b. Catalytically-dead CasX Variant Proteins

[00206] In the repressor fusion proteins and systems comprising same of the disclosure, the CasX protein is catalytically-dead (dCasX) in that it is unable to cleave DNA, but retains the ability to bind a target nucleic acid when complexed with a guide RNA (gRNA). The present disclosure provides catalytically-dead variants (interchangeably referred to herein as "dCasX variant" or "dCasX variant protein"), wherein the catalytically-dead CasX variants comprise multiple modifications in select domains relative to the catalytically-dead versions of sequences of SEQ ID NOS:1-3 (described, *supra*). An exemplary catalytically-dead CasX protein comprises one or more mutations in the active site of the RuvC domain of the CasX protein. In some embodiments, a catalytically-dead reference CasX protein comprises substitutions at residues 672, 769 and/or 935 with reference to SEQ ID NO: 1. In some embodiments, a catalytically-dead reference CasX protein comprises substitutions of D672A, E769A and/or D935A with reference to SEQ ID NO: 1. In other embodiments, a catalytically-dead reference CasX protein comprises substitutions at amino acids 659, 756 and/or 922 with reference to SEQ ID NO: 2. In some embodiments, a catalytically-dead reference CasX protein comprises D659A, E756A and/or D922A substitutions with reference to of SEQ ID NO: 2. An exemplary RuvC domain of the dCasX of the disclosure comprises amino acids 661-824 and 935-986 of SEQ ID NO: 1, or amino acids 648-812 and 922-978 of SEQ ID NO: 2, with one or more amino acid modifications relative to said RuvC cleavage domain sequence, wherein the dCasX variant exhibits one or more improved characteristics compared to the reference dCasX. In further embodiments, a catalytically-dead CasX variant protein comprises deletions of all or part of the RuvC domain of the reference CasX protein. It will be understood that the same foregoing substitutions or deletions can similarly be introduced into CasX variants known in the art, resulting in a dCasX variant (*see*, *e.g.*, WO2022120095A1 and US11,560,555, incorporated by reference herein, for exemplary sequences).

[00207] In some embodiments, the dCasX variant with linked repressor domains exhibits at least one improved characteristic compared to the reference dCasX protein with linked repressor

domains configured in a comparable fashion. All dCasX variants that improve one or more functions or characteristics of the dCasX variant protein with linked repressor domain compared to a reference dCasX protein with linked repressor domain described herein are envisaged as being within the scope of the disclosure. In some embodiments, the modification is a mutation in one or more amino acids of the reference dCasX other than those rendering the dCasX catalytically-dead. For example, dCasX variants can comprise one or more amino acid substitutions, insertions, deletions, or swapped domains, or any combinations thereof, relative to a reference dCasX protein sequence. Any amino acid can be substituted for any other amino acid in the substitutions described herein. The substitution can be a conservative substitution (*e.g.*, a basic amino acid is substituted for another basic amino acid). The substitution can be a non-conservative substitution (*e.g.*, a basic amino acid is substituted for an acidic amino acid or vice versa). For example, a proline in a reference dCasX protein can be substituted for any of arginine, histidine, lysine, aspartic acid, glutamic acid, serine, threonine, asparagine, glutamine, cysteine, glycine, alanine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine or valine to generate a dCasX variant protein of the disclosure. In some embodiments, the dCasX variant exhibits an improved characteristic compared to a reference dCasX. Exemplary improved characteristics of the dCasX variant embodiments include, but are not limited to improved folding of the variant, increased binding affinity to the gRNA, increased binding affinity to the target nucleic acid, improved ability to utilize a greater spectrum of PAM sequences in the repression and/or binding of target nucleic acid, improved unwinding of the target DNA, increased target strand loading, increased binding of the non-target strand of DNA, improved protein stability, increased ability to complex with gRNA, improved protein:gRNA (RNP) complex stability, and, with linked repressor domains and when complexed as an RNP, increased repressor activity, improved repressor specificity for the target nucleic acid, decreased off-target repression, increased percentage of a eukaryotic genome that can be efficiently repressed and/or epigenetically modified. In some embodiments, an improved characteristic of the dCasX variant is at least about 1.1 to about 100,000-fold improved relative to the reference dCasX protein. In some embodiments, an improved characteristic of the dCasX variant is at least about 1.1 to about 10,000-fold improved, at least about 1.1 to about 1,000-fold improved, at least about 1.1 to about 500-fold improved, at least about 1.1 to about 400-fold improved, at least about 1.1 to about 300-fold improved, at least about 1.1 to about 200-fold improved, at least about 1.1 to about 100-fold improved, at least about 1.1 to about 50-fold improved, at least about

1.1 to about 40-fold improved, at least about 1.1 to about 30-fold improved, at least about 1.1 to about 20-fold improved, at least about 1.1 to about 10-fold improved, at least about 1.1 to about 9-fold improved, at least about 1.1 to about 8-fold improved, at least about 1.1 to about 7-fold improved, at least about 1.1 to about 6-fold improved, at least about 1.1 to about 5-fold improved, at least about 1.1 to about 4-fold improved, at least about 1.1 to about 3-fold improved, at least about 1.1 to about 2-fold improved, at least about 1.1 to about 1.5-fold improved, at least about 1.5 to about 3-fold improved, at least about 1.5 to about 4-fold improved, at least about 1.5 to about 5-fold improved, at least about 1.5 to about 10-fold improved, at least about 5 to about 10-fold improved, at least about 10 to about 20-fold improved, at least 10 to about 30-fold improved, at least 10 to about 50-fold improved or at least 10 to about 100-fold improved relative to the reference dCasX protein. In some embodiments, an improved characteristic of the dCasX variant is at least about 10 to about 1000-fold improved relative to the reference dCasX protein. Additional disclosure on improved characteristics is described herein, below.

[00208] In other embodiments, the modification is a substitution of one or more domains of the reference dCasX with one or more domains from a different CasX. In some embodiments, insertion includes the insertion of a part or all of a domain from a different CasX protein. Mutations can be placed in any one or more domains of the dCasX variant, and may include, for example, deletion of part or all of one or more domains, or one or more amino acid substitutions, deletions, or insertions in any domain. The domains of dCasX proteins include the non-target strand binding (NTSB) domain, the target strand loading (TSL) domain, the helical I domain, the helical II domain, the oligonucleotide binding domain (OBD), and the RuvC DNA cleavage domain, which can further comprise subdomains, described below.

[00209] Suitable mutagenesis methods for generating dCasX variant proteins of the disclosure may include, for example, Deep Mutational Evolution (DME), deep mutational scanning (DMS), error prone PCR, cassette mutagenesis, random mutagenesis, staggered extension PCR, gene shuffling, or domain swapping. In some embodiments, the dCasX variants are designed, for example by selecting one or more desired mutations in a reference dCasX. In certain embodiments, the activity of a reference dCasX protein is used as a benchmark against which the activity of one or more dCasX variants are compared, thereby measuring improvements in function of the dCasX variants.

[00210] In some embodiments, the dCasX variant protein comprises between 700 and 1200 amino acids, between 800 and 1100 amino acids or between 900 and 1000 amino acids.

[00211] The dCasX and linked repressor domains of the disclosure have an enhanced ability to efficiently bind target nucleic acid, when complexed with a gRNA as an RNP, utilizing and binding to a PAM TC motif, including PAM sequences selected from TTC, ATC, GTC, or CTC, compared to an RNP of a reference dCasX protein and reference gRNA in a comparable assay system. In the foregoing, the PAM sequence is located at least 1 nucleotide 5' to the non-target strand of the protospacer having identity with the targeting sequence of the gRNA.

[00212] In some embodiments, an RNP comprising the dCasX variant protein with linked repressor domains and a gRNA of the disclosure, at a concentration of 20 pM or less, is capable of binding a double stranded DNA target with an efficiency of at least 70%, at least 80%, at least 85%, at least 90% or at least 95%. In one embodiment, an RNP of a dCasX variant with linked repressor domains and a gRNA variant exhibits greater binding of a target sequence in the target nucleic acid compared to an RNP comprising a reference dCasX protein with linked repressor domains and a reference gRNA in a comparable assay system, wherein the PAM sequence of the target nucleic acid is TTC. In another embodiment, an RNP of a dCasX variant with linked repressor domains and gRNA variant exhibits greater binding affinity of a target sequence in the target nucleic acid compared to an RNP comprising a reference dCasX protein with linked repressor domains and a reference gRNA in a comparable assay system, wherein the PAM sequence of the target nucleic acid is ATC. In another embodiment, an RNP of a dCasX variant with linked repressor domains and gRNA variant exhibits greater binding affinity of a target sequence in the target nucleic acid compared to an RNP comprising a reference dCasX protein with linked repressor domains and a reference gRNA in a comparable assay system, wherein the PAM sequence of the target nucleic acid is CTC. In another embodiment, an RNP of a dCasX variant with linked repressor domains and gRNA variant exhibits greater binding affinity of a target sequence in the target nucleic acid compared to an RNP comprising a reference dCasX protein with linked repressor domains and a reference gRNA in a comparable assay system, wherein the PAM sequence of the target nucleic acid is GTC. In the foregoing embodiments, the increased binding affinity for the one or more PAM sequences is at least 1.5-fold greater or more compared to the binding affinity of an RNP of any one of the reference dCasX proteins (modified from SEQ ID NOS: 1-3) with linked repressor domains and the gRNA of SEQ ID NOS: 1731-1743 of Table 6 for the PAM sequences.

c. dCasX Variant Proteins with Domains from Multiple Source Proteins

[00213] Also contemplated within the scope of the disclosure are chimeric dCasX proteins. As used herein, a "chimeric dCasX" protein refers to both a dCasX protein containing at least two domains from different sources, as well a dCasX protein containing at least one domain that itself is chimeric. Accordingly, in some embodiments, a chimeric dCasX protein is one that includes at least two domains isolated or derived from different sources, such as from two different naturally occurring CasX proteins, (e.g., from two different CasX reference proteins), or from two different engineered CasX proteins. In some embodiments, the helical I-I domain and NTSB domain of the dCasX variant derived from SEQ ID NO: 2 is replaced with the corresponding helical I-I and NTSB sequences from SEQ ID NO: 1, resulting in a chimeric dCasX protein. As another example of the foregoing, the chimeric RuvC domain comprises amino acids 660 to 823 of SEQ ID NO: 1 and amino acids 921 to 978 of SEQ ID NO: 2. As an alternative example of the foregoing, a chimeric RuvC domain comprises amino acids 647 to 810 of SEQ ID NO: 2 and amino acids 934 to 986 of SEQ ID NO: 1.

[00214] In other embodiments, the chimeric dCasX protein is one that contains at least one domain that is a chimeric domain, e.g., in some embodiments, part of a domain comprises a substitution from a different CasX protein (from a reference CasX protein, or another engineered CasX protein). In some embodiments, the at least one chimeric domain can be any of the NTSB, TSL, helical I, helical II, OBD or RuvC domains as described herein. In some embodiments, the helical I-I domain (sometimes referred to as helical I-a) of the dCasX variant derived from SEQ ID NO: 2 is replaced with the corresponding helical I-I sequence from SEQ ID NO: 1, resulting in a chimeric dCasX protein.

[00215] Sequences of Table 2 having the NTSB domain and helical I-II domain from SEQ ID NO: 1 and a helical I-I domain from SEQ ID NO: 2 include dCasX 491, 515, 516, 518-520, 522-527, 532, 593, 676 (with a L169K substitution in the NTSB domain), and 812 (see Table 2 for SEQ ID NOS). Coordinates of CasX domains in the reference CasX proteins of SEQ ID NO: 1 and SEQ ID NO: 2 are provided in Table 1 below. The skilled artisan will understand that the domain boundaries indicated in Table 1 below are approximate, and that protein fragments whose boundaries differ from those given in the table below by 1, 2, or 3 amino acids may have the same activity as the domains described below. In some embodiments, the disclosure provides the CasX proteins of SEQ ID NOS: 3281-3441, or 3444-3446 having the NTSB domain and helical I-II domain from SEQ ID NO: 1 and a helical I-I domain from SEQ ID NO: 2, wherein

the CasX have additional amino acid changes (i.e., 1, 2, 3, 4, or 5 mismatches) at select locations relative to the domains of the reference CasX, and that are rendered catalytically dead by introducing one or more mutations that inactivates the cleavage activity of the RuvC domain.

Table 1: Domain coordinates in Reference CasX proteins

Domain Name	Coordinates in SEQ ID NO: 1*	Coordinates in SEQ ID NO: 2*
OBD-I	1-55	1-57
helical I-I	56-99	58-101
NTSB	100-190	102-191
helical I-II	191-331	192-332
helical II	332-508	333-500
OBD-II	509-659	501-646
RuvC-I	660-823	647-810
TSL	824-933	811-920
RuvC-II	934-986	921-978

* amino acid position

[00216] In some embodiments, a dCasX variant protein utilized in the fusion proteins of the disclosure comprises a sequence of SEQ ID NOS: 4-29 as set forth in Table 2. In other embodiments, a dCasX variant protein utilized in the fusion proteins of the disclosure comprises a sequence at least 70% identical, at least 75% identical, at least 80% identical, at least 81% identical, at least 82% identical, at least 83% identical, at least 84% identical, at least 85% identical, at least 86% identical, at least 86% identical, at least 87% identical, at least 88% identical, at least 89% identical, at least 89% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, at least 99% identical, at least 99.5% identical to a sequence of SEQ ID NOS: 4-29 as set forth in Table 2. In a particular embodiment, the dCasX variant protein utilized in the fusion protein of the gene repressor systems of the disclosure comprises a sequence of SEQ ID NO: 4 (dCasX 491). In another particular embodiment, the dCasX variant protein utilized in the fusion protein of the gene repressor systems of the disclosure comprises a sequence of SEQ ID NO: 6 (dCasX 515). In another particular embodiment, the dCasX variant protein utilized in the fusion protein of the gene repressor systems of the disclosure comprises a sequence of SEQ ID NO: 29 (dCasX 812).

Table 2: dCasX Variant Sequences

SEQ ID NO	dCasX	Amino Acid Sequence
4	dCasX491	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVLE KLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSWT KGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKYQ TNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
5	dCasX514	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTIHTSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
6	dCasX515	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD 7KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLY LIINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRV LEKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISS WTKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKK YQTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
7	dCasX516	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT

SEQ ID NO	dCasX	Amino Acid Sequence
		IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNHNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVLE KLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSWT KGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKYQ TNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
8	dCasX517	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGAPVGKALSDACMG TIASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAY NEVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKK LINEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGE DWGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEA DKDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLY LIINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
9	dCasX518	RQEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPI SNTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMD EKGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEK DSDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMG TIASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAY NEVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKK LINEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGE DWGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEA DKDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLY LIINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
10	dCasX519	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD

SEQ ID NO	dCasX	Amino Acid Sequence
		KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHIQLRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
11	dCasX520	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTTQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVLE KLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSWT KGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKYQ TNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
12	dCasX522	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKRSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVLE KLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSWT KGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKYQ TNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
13	dCasX523	QEIKRINKIRRRLVKDSNTKKAGKTYPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK

SEQ ID NO	dCasX	Amino Acid Sequence
		KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVLE KLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSWT KGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKYQ TNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
14	dCasX524	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTIHSADYDRVLE KLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSWT KGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKYQ TNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
15	dCasX525	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAATQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVLE KLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSWT KGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKYQ TNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
16	dCasX526	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAA KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVLE KLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSWT KGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKYQ

SEQ ID NO	dCasX	Amino Acid Sequence
		TNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
17	dCasX527	QEIKRINKIRRRLVKDSNTKKAGKTRGPMKTLLVRVMTPDLRERLENLRKKPENIPQPI SNTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMD EKGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEK DSDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMG TIASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAY NEVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKK LINEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGE DWGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEA DKDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLY LIINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
18	dCasX528	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASYPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
19	dCasX529	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASNPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
20	dCasX530	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE

SEQ ID NO	dCasX	Amino Acid Sequence
		KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGWGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRV LEKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISS WTKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKK YQTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
21	dCasX531	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGYGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRV LEKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISS WTKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKK YQTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
22	dCasX532	QEIKRINKIRRRLVKDSNTKKAGKTRGPMKTLLVRVMTPDLRERLENLRKKPENIPQPI SNTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMD EKGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEK DSDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMG TIASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAY NEVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKK LINEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGE DWGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEA DKDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLY LIINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRV LEKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISS WTKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKK YQTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
23	dCasX533	QEIKRINKIRRRLVKDSNTKKAGKTRGPMKTLLVRVMTPDLRERLENLRKKPENIPQPI SNTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMD EKGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEK DSDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASYPVGKALSDACMG TIASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAY NEVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKK

SEQ ID NO	dCasX	Amino Acid Sequence
		LINEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGE DWGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEA DKDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLY LIINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRV LEKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISS WTKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKK YQTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
24	dCasX535	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASSPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
25	dCasX593	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRWWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
26	dCasX668	QEIKRINKIRRRLVKDSNTKKAGKTRGPMKTLLVRVMTPDLRERLENLRKKPENIPQPI SNTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMD EKGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEK DSDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASSPVGKALSDACMG TIASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAY NEVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKK LINEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGE DWGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEA DKDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLY LIINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG

SEQ ID NO	dCasX	Amino Acid Sequence
		KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRV LEKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISS WTKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKK YQTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
27	dCasX672	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLIKLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASSPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
28	dCasX676	QEIKRINKIRRRLVKDSNTKKAGKTRGPMKTLLVRVMTPDLRERLENLRKKPENIPQPI SNTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMD EKGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLIKLAQLKPEK DSDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASSPVGKALSDACMG TIASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAY NEVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKGFPSFPLVERQANEVDWWDMVCNVKK LINEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGE DWGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEA DKDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLY LIINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFG KRQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSS NIKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQA KKEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQG KRTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRV LEKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISS WTKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKK YQTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV
29	dCasX812	QEIKRINKIRRRLVKDSNTKKAGKTGPMKTLLVRVMTPDLRERLENLRKKPENIPQPIS NTSRANLNKLLTDYTEMKKAILHVYWEEFQKDPVGLMSRVAQPASKKIDQNKLKPEMDE KGNLTTAGFACSQCGQPLFVYKLEQVSEKGKAYTNYFGRCNVAEHEKLILLAQLKPEKD SDEAVTYSLGKFGQRALDFYSIHVTKESTHPVKPLAQIAGNRYASGPVGKALSDACMGT IASFLSKYQDIIIEHQKVVKGNQKRLESLRELAGKENLEYPSVTLPPQPHTKEGVDAYN EVIARVRMWVNLNLWQKLKLSRDDAKPLLRLKKFPSFPLVERQANEVDWWDMVCNVKKL INEKKEDGKVFWQNLAGYKRQEALRPYLSSEEDRKKGKKFARYQLGDLLLHLEKKHGED WGKVYDEAWERIDKKVEGLSKHIKLEEERRSEDAQSKAALTDWLRAKASFVIEGLKEAD KDEFCRCELKLQKWYGDLRGKPFAIEAENSILDISGFSKQYNCAFIWQKDGVKKLNLYL IINYFKGGKLRFKKIKPEAFEANRFYTVINKKSGEIVPMEVNFNFDDPNLIILPLAFGK RQGREFIWNDLLSLETGSLKLANGRVIEKTLYNRRTRQDEPALFVALTFERREVLDSSN IKPMNLIGVARGENIPAVIALTDPEGCPLSRFKDSLGNPTHILRIGESYKEKQRTIQAK KEVEQRRAGGYSRKYASKAKNLADDMVRNTARDLLYYAVTQDAMLIFANLSRGFGRQGK RTFMAERQYTRMEDWLTAKLAYEGLPSKTYLSKTLAQYTSKTCSNCGFTITSADYDRVL

SEQ ID NO	dCasX	Amino Acid Sequence
		EKLKKTATGWMTTINGKELKVEGQITYYNRYKRQNVVKDLSVELDRLSEESVNNDISSW TKGRSGEALSLLKKRFSHRPVQEKFVCLNCGFETHAAEQAALNIARSWLFLRSQEYKKY QTNKTTGNTDKRAFVETWQSFYRKKLKEVWKPAV

[00217] In some embodiments, a dCasX comprises a sequence selected from the group consisting of SEQ ID NOS: 4-29, or a sequence having or a sequence having at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto. In some embodiments, a dCasX comprises a sequence selected from the group consisting of SEQ ID NOS: 4-29. In some embodiments, a dCasX comprises a sequence selected from the group consisting of SEQ ID NOS: 3281-3441 and 3444-3446, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto, wherein the sequence further comprises one or mutations in the RuvC domain that render the dCasX capable of binding DNA, but is otherwise catalytically dead. In some embodiments, the one or more mutations are in the RuvC domain and render the RuvC catalytically inactive (i.e. not capable of cleaving DNA). In some embodiments, the one or more mutations comprise D659A, E756A and/or D922A substitutions corresponding to a sequence of SEQ ID NO: 2. The repressor fusion protein comprising the dCasX retains the ability to form an RNP with a gRNA. In some embodiments, the repressor fusion protein comprising the dCasX retains one or more functions of a CasX protein, including but not limited to, affinity for the gRNA, binding to the target nucleic acid, specificity for the target nucleic acid, unwinding of the target nucleic acid, target strand loading, or any combination thereof.

d. **Affinity for the gRNA**

[00218] In some embodiments, a dCasX with linked repressor domains has improved affinity for the gRNA relative to a reference dCasX protein, leading to the formation of the ribonucleoprotein complex. Increased affinity of the repressor fusion protein for the gRNA may, for example, result in a lower K_d for the generation of a RNP complex, which can, in some cases, result in a more stable ribonucleoprotein complex formation. In some embodiments, the K_d of a repressor fusion protein for a gRNA is increased relative to a reference dCasX protein and linked repressor domains by a factor of at least about 1.1, at least about 1.2, at least about

1.3, at least about 1.4, at least about 1.5, at least about 1.6, at least about 1.7, at least about 1.8, at least about 1.9, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, 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, or at least about 100. In some embodiments, the dCasX variant has about 1.1 to about 10-fold increased binding affinity to the gRNA compared to the catalytically-dead variant of reference CasX protein of SEQ ID NO: 2.

[00219] In some embodiments, increased affinity of the dCasX with linked repressor domains for the gRNA results in increased stability of the ribonucleoprotein complex when delivered to mammalian cells, including *in vivo* delivery to a subject. This increased stability can affect the function and utility of the complex in the cells of a subject, as well as result in improved pharmacokinetic properties in blood, when delivered to a subject. In some embodiments, increased affinity of the repressor fusion protein, and the resulting increased stability of the ribonucleoprotein complex, allows for a lower dose of the repressor fusion protein to be delivered to the subject or cells while still having the desired activity; for example *in vivo* or *in vitro* gene repression and/or epigenetic modification. The increased ability to form RNP and keep them in stable form can be assessed using *in vitro* assays known in the art.

[00220] In some embodiments, a higher affinity (tighter binding) of a dCasX variant protein and linked repressor domain to a gRNA allows for a greater amount of repression and/or epigenetic modification events when both the dCasX variant protein and the gRNA remain in an RNP complex. Increased repression events can be assessed using assays described herein.

[00221] Methods of measuring repressor fusion protein binding affinity for a gRNA include *in vitro* methods using purified an repressor fusion protein and a gRNA. The binding affinity for the repressor fusion protein can be measured by fluorescence polarization if the gRNA or the repressor fusion protein is tagged with a fluorophore. Alternatively, or in addition, binding affinity can be measured by biolayer interferometry, electrophoretic mobility shift assays (EMSAs), or filter binding. Additional standard techniques to quantify absolute affinities of RNA binding proteins such as the reference dCasX and variant proteins of the disclosure for specific gRNAs such as reference gRNAs and variants thereof include, but are not limited to, isothermal calorimetry (ITC), and surface plasmon resonance (SPR).

e. Improved Specificity for a Target Nucleic Acid Sequence

[00222] In some embodiments, a repressor fusion protein comprising a dCasX variant protein with linked repressor domains has improved specificity for a target nucleic acid sequence that is complementary to the targeting sequence of the gRNA relative to a reference dCasX protein with linked repressor domains. As used herein, "specificity," sometimes referred to as "target specificity," refers to the degree to which a CRISPR/Cas system ribonucleoprotein complex binds off-target sequences that are similar, but not identical to the target nucleic acid sequence; *e.g.*, a repressor fusion protein RNP with a higher degree of specificity would exhibit reduced off-target methylation of sequences relative to an RNP of a reference dCasX with linked repressor domains. The specificity, and the reduction of potentially deleterious off-target effects, of repressor fusion proteins can be vitally important in order to achieve an acceptable therapeutic index for use in mammalian subjects.

[00223] Without wishing to be bound by theory, it is possible that amino acid changes in the helical I and II domains that increase the specificity of the repressor fusion protein for the target nucleic acid strand can increase the specificity of the repressor fusion protein for the target nucleic acid overall. In some embodiments, amino acid changes that increase specificity of repressor fusion proteins for target nucleic acid may also result in decreased affinity of repressor fusion proteins for DNA, but the overall benefit and safety of the composition is enhanced.

f. Repressor fusion proteins with Additional Heterologous Proteins

[00224] Also contemplated within the scope of the disclosure are repressor fusion proteins comprising one or more heterologous proteins fused to the repressor fusion protein. This includes repressor fusion proteins comprising N-terminal or C-terminal fusions to a heterologous protein or domain thereof. In some embodiments, the repressor fusion protein is fused to one or more proteins or domains thereof that has a different activity of interest.

[00225] In some cases, a heterologous polypeptide (a fusion partner) for use with a repressor fusion protein provides for subcellular localization, *i.e.*, the heterologous polypeptide contains a subcellular localization sequence (*e.g.*, a nuclear localization signal (NLS) for targeting to the nucleus, a sequence to keep the fusion protein out of the nucleus, a nuclear export sequence (NES), a sequence to keep the fusion protein retained in the cytoplasm, a mitochondrial localization signal for targeting to the mitochondria, a chloroplast localization signal for targeting to a chloroplast, an ER retention signal, and the like).

[00226] In some cases, a repressor fusion protein includes (is fused to) a nuclear localization signal (NLS). In some cases, a repressor fusion protein is fused to 2 or more, 3 or more, 4 or more, or 5 or more 6 or more, 7 or more, 8 or more NLSs. In some cases, one or more NLSs (2 or more, 3 or more, 4 or more, or 5 or more NLSs) are positioned at or near (*e.g.*, within 50 amino acids of) the N-terminus and/or the C-terminus of the repressor fusion protein. In some cases, one or more NLSs (2 or more, 3 or more, 4 or more, or 5 or more NLSs) are positioned at or near (*e.g.*, within 50 amino acids of) the N-terminus of the repressor fusion protein. In some cases, one or more NLSs (2 or more, 3 or more, 4 or more, or 5 or more NLSs) are positioned at or near (*e.g.*, within 50 amino acids of) the C-terminus of the repressor fusion protein. In some cases, one or more NLSs (3 or more, 4 or more, or 5 or more NLSs) are positioned at or near (*e.g.*, within 50 amino acids of) both the N-terminus and the C-terminus of the repressor fusion protein. In some cases, a single NLS is positioned at the N-terminus and a single NLS is positioned at the C-terminus of the repressor fusion protein. The person of ordinary skill in the art will understand that an NLS at or near the N- or C-terminus of a protein can be within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids of the N- or C-terminus. In some embodiments, the NLS linked to the N-terminus of the dCasX or the repressor fusion protein are identical to the NLS linked to the C-terminus. In other embodiments, the NLS linked to the N-terminus of the dCasX or the repressor fusion protein are different to the NLS linked to the C-terminus. Representative configurations of repressor fusion proteins with NLS are shown in FIG. 1 and FIG. 2. In some embodiments, NLSs suitable for use with a repressor fusion protein in the systems of the disclosure comprise sequences having at least about 85%, at least about 90%, or at least about 95% identity or are identical to sequences derived from: the NLS of the simian virus 40 (SV40) virus large T-antigen, having the amino acid sequence PKKKRKV (SEQ ID NO: 30); the NLS from nucleoplasmin (*e.g.*, the nucleoplasmin bipartite NLS with the sequence KRPAATKKAGQAKKKK (SEQ ID NO: 31); the c-MYC NLS having the amino acid sequence PAAKRVKLD (SEQ ID NO: 32) or RQRRNELKRSP (SEQ ID NO: 33). In some embodiments, the NLS linked to the N-terminus of the repressor fusion protein is selected from the group consisting of the N-terminal sequences as set forth in Table 3. In some embodiments, the NLS linked to the C-terminus of the repressor fusion protein is selected from the group consisting of the C-terminal sequences as set forth in Table 4. In some embodiments, NLSs suitable for use with a repressor fusion protein in the systems of the disclosure include sequences having at least about 80%, at least about 90%, or at least about 95% identity or are identical to

one or more sequences of Table 3 or Table 4. The skilled artisan will understand that Tables 3 and 4 present NLS sequences as N-terminal or C-terminal as exemplary embodiments. Any of the NLS in Table 3 or 4 can be fused to either the N or C terminal of a repressor fusion protein described herein.

Table 3: N-terminal NLS Amino Acid Sequences

NLS Amino Acid Sequence*	SEQ ID NO
PKKKRKVSR	34
PKKKRKVGGSPKKKRKVGGSPKKKRKVGGSPKKKRKVSR	35
PKKKRKVGGSPKKKRKVGGSPKKKRKVGGSPKKKRKVGGSPKKKRKVGGSPKKKRKVSR	36
PAAKRVKLDSR	37
PAAKRVKLDGGSPAAKRVKLDSR	38
PAAKRVKLDGGSPAAKRVKLDGGSPAAKRVKLDGGSPAAKRVKLDSR	39
PAAKRVKLDGGSPAAKRVKLDGGSPAAKRVKLDGGSPAAKRVKLDGGSPAAKRVKLDGGSPAAKRVKLDSR	40
KRPAATKKAGQAKKKKSR	41
KRPAATKKAGQAKKKKGGSKRPAATKKAGQAKKKKSR	42
PAAKRVKLDGGSPKKKRKVSR	43
PAAKKKKLDGGSPKKKRKVSR	44
PAAKKKKLDSR	45
PAAKKKKLDGGSPAAKKKKLDGGSPAAKKKKLDSR	46
PAAKKKKLDGGSPAAKKKKLDGGSPAAKKKKLDGGSPAAKKKKLDSR	47
PAKRARRGYKCSR	48
PAKRARRGYKCGSPAKRARRGYKCSR	49
PRRKREESR	50
PYRGRKESR	51
PLRKRPRRSR	52
PLRKRPRRGSPLRKRPRRSR	53
PAAKRVKLDGGKRTADGSEFESPKKKRKVGGS	54
PAAKRVKLDGGKRTADGSEFESPKKKRKVPPPPG	55
PAAKRVKLDGGKRTADGSEFESPKKKRKVGIHGVPAAPG	56

NLS Amino Acid Sequence*	SEQ ID NO
PAAKRVKLDGG**KRTADGSEFESPKKKRKV**GGGSGGGSPG	57
PAAKRVKLDGG**KRTADGSEFESPKKKRKV**PGGGSGGGSPG	58
PAAKRVKLDGG**KRTADGSEFESPKKKRKV**AEAAAKEAAAKEAAAKAPG	59
PAAKRVKLDGGS**PKKKRKV**GGS	60
PAAKRVKLDPPP**PKKKRKV**PG	61
PAAKRVKLDPG	62
PAAKRVKLDGGGSGGGSGGGS	63
PAAKRVKLDPPP	64
PAAKRVKLDGGGSGGGSGGGSPPP	65
PKKKRKVPPP	66
PKKKRKVGGS	67

*Residues in **bold** are NLS residues, while unbolded residues are linkers.

Table 4: C-terminal NLS Amino Acid Sequences

NLS Amino Acid Sequence	SEQ ID NO
GS**PKKKRKV**GGS**PKKKRKV**GGS**PKKKRKV**GGS**PKKKRKV**	68
GS**PKKKRKV**GGS**PKKKRKV**GGS**PKKKRKV**GGS**PKKKRKV**GGS**PKKKRKV**GGS**PKKK RKV**	69
GS**PAAKRVKLD**GGS**PAAKRVKLD**	70
GS**PAAKRVKLD**GGS**PAAKRVKLD**GGS**PAAKRVKLD**GGS**PAAKRVKLD**	71
GS**KRPAATKKAGQAKKKK**	72
KRPAATKKAGQAKKKKGGS**KRPAATKKAGQAKKKK**	73
GS**KLGPRKATGRW**GS	74
GS**KRKGSPERGERKRHW**GS	75
GS**PKKKRKV**GSGS**KRPAATKKAGQAKKKK**LE	76
GP**KRTADSQHSTPPKTKRKV**EFE**PKKKRKV**	77
GGGSGGGS**KRTADSQHSTPPKTKRKV**EFE**PKKKRKV**	78
AEAAAKEAAAKEAAAKA**KRTADSQHSTPPKTKRKV**EFE**PKKKRKV**	79
GPPKKKRKVGGS**KRTADSQHSTPPKTKRKV**EFE**PKKKRKV**	80
GPAEAAAKEAAAKEAAAKA**PAAKRVKLD**	81

NLS Amino Acid Sequence	SEQ ID NO
GPGGGSGGGSGGGS**PAAKRVKLD**	82
GP**PAAKRVKLD**	83
VGS**KRPAATKKAGQAKKKK**	84
TGGGPGGGAAAGSGS**PKKKRKV**GSGS**KRPAATKKAGQAKKKK**LE	85
TGGGPGGGAAAGSGS**PKKKRKV**GSGS	86
PPP**PKKKRKV**PPP	87
GGS**PKKKRKV**PPP	88
PPP**PKKKRKV**	89
GGS**PKKKRKV**	90
GGS**PKKKRKV**GGSGGSGGS	91
GGS**PKKKRKV**GGSPKKKRKV	92
GGSGGSGGS**PKKKRKV**GGS**PKKKRKV**	93
VGGGSGGGSGGGS**PAAKRVKLD**	94
VPPP**PAAKRVKLD**	95
VPPPGGGSGGGSGGGS**PAAKRVKLD**	96
VGS**PAAKRVKLD**	97

[00227] In some embodiments, the one or more NLSs are linked to the repressor fusion protein or to adjacent NLS with a linker peptide wherein the linker peptide is selected from the group consisting of SR, GS, GP, VGS, GGS, (G)n (SEQ ID NO: 98), (GS)n (SEQ ID NO: 99), (GSGGS)n (SEQ ID NO: 100), (GGSGGS)n (SEQ ID NO: 101), (GGGS)n (SEQ ID NO: 102), GGSG (SEQ ID NO: 103), GGSGG (SEQ ID NO: 104), GSGSG (SEQ ID NO: 105), GSGGG (SEQ ID NO: 106), GGGSG (SEQ ID NO: 107), GSSSG (SEQ ID NO: 108), GPGP (SEQ ID NO: 109), GGP, PPP, VPPP, PPAPPA (SEQ ID NO: 110), PPPG (SEQ ID NO: 111), PPPGPPP (SEQ ID NO: 112), PPP(GGGS)n (SEQ ID NO: 113), (GGGS)nPPP (SEQ ID NO: 114), AEAAAKEAAAKEAAAKA (SEQ ID NO: 115), VPPPGGGSGGGSGGGS (SEQ ID NO: 116), TGGGPGGGAAAGSGS (SEQ ID NO: 117), GGGSGGGSGGGSPPP (SEQ ID NO: 118), TPPKTKRKVEFE (SEQ ID NO: 119), GGSGGGS (SEQ ID NO: 120), GSGSGGG (SEQ ID NO: 121), SSGNSNANSRGPSFSSGLVPLSLRGSH (SEQ ID NO: 122), GGPSSGAPPPSGGSPAGSPTSTEEGTSESATPESGPGTSTEPSEGSAPGSPAGSPTSTEEGT

STEPSEGSAPGTSTEPSE (SEQ ID NO: 123), and GGSGGG (SEQ ID NO: 124), where n is 1 to 5.

[00228] In general, NLS (or multiple NLSs) are of sufficient strength to drive accumulation of a LTRP fusion protein in the nucleus of a eukaryotic cell. Detection of accumulation in the nucleus may be performed by any suitable technique. For example, a detectable marker may be fused to a LTRP fusion protein such that location within a cell may be visualized. Cell nuclei may also be isolated from cells, the contents of which may then be analyzed by any suitable process for detecting protein, such as immunohistochemistry, Western blot, or enzyme activity assay. Accumulation in the nucleus may also be determined indirectly.

IV. Repressor Domains

[00229] In some embodiments, the disclosure provides repressor fusion proteins and systems comprising same, the repressor fusion proteins comprising a DNA-binding protein linked to multiple repressor domains (repressor fusion proteins), wherein the system is capable of binding to a target nucleic acid of *PCSK9* and repressing transcription of a PCSK9 target nucleic acid, including by epigenetic modification of the target nucleic acid. Exemplary DNA-binding proteins for use in the repressor fusion proteins include zinc finger (ZF), TALE (transcription-activator-like effector) proteins, and DNA-binding proteins such as catalytically-dead CRISPR proteins.

[00230] In some embodiments, the disclosure provides repressor fusion proteins comprising a catalytically-dead CRISPR protein, such as a dCasX, linked to multiple repressor domains that, when complexed with a guide ribonucleic acid (gRNA) comprising a targeting sequence complementary to a target nucleic acid sequence of *PCSK9*, wherein the system is capable of binding to a target nucleic acid of *PCSK9* and repressing transcription and/or epigenetic modification of a *PCSK9* target nucleic acid. Examples of gene repression processes which decrease transcription include, but are not limited to, those which inhibit formation of a transcription initiation complex, those which decrease transcription initiation rate, those which decrease transcription elongation rate, those which decrease processivity of transcription and those which antagonize transcriptional activation (by, for example, blocking the binding of a transcriptional activator). Gene repression can constitute, for example, prevention of activation as well as inhibition of expression below an existing level. Transcriptional repression includes both reversible and irreversible inactivation of gene transcription; the latter can result from epigenetic modification of the target nucleic acid.

[00231] Amongst repressor domains that have the ability to repress, or silence genes, the Krüppel-associated box (KRAB) repressor domain is amongst the most powerful in human genome systems (Alerasool, N., et al. An efficient KRAB domain for CRISPRi applications. Nat. Methods 17:1093 (2020)). KRAB domains are present in approximately 400 human zinc finger protein-based transcription factors that upon binding of the linked dCasX to the target nucleic acid, is capable of recruiting additional repressor domains such as, but not limited to, Trim28 (also known as Kap1 or Tif1-beta) that, in turn, assembles a protein complex with chromatin regulators such as CBX5/HP1α and SETDB1 that induce repression of transcription of the gene, but do so in a limited, temporal fashion. Representative, non-limiting examples of KRAB domains suitable for use in the systems of the disclosure include ZIM3 (SEQ ID NO: 128) and ZNF10 (SEQ ID NO: 129). The disclosure provides additional repressor domains that are from human and non-human sources that have been found to result in enhanced activity compared to ZIM3 and ZNF10 when incorporated in a repressor fusion proteins, described herein.

[00232] In some embodiments, the disclosure provides systems in which the modification imparted by use of the repressor fusion protein:gRNA system is epigenetic, and hence the silencing of the PCSK9 gene is heritable by mechanisms other than by replication of a target nucleic acid that has been edited. As used herein "epigenetic modification" means a modification to either DNA or histones associated with DNA, other than a change in the DNA sequence itself (*e.g.*, a substitution, deletion or rearrangement), wherein the modification is either a direct modification by a component of the system or is indirect by the recruitment of one or more additional cellular components, but in which the DNA target nucleic acid sequence itself is not edited to change the sequence. For example, DNA methyltransferase 3A (DNMT3A) (or its catalytic domain) directly modifies the DNA by methylating it, whereas KRAB recruits KAP-1/TIF1β corepressor complexes that act as potent transcriptional repressors and can further recruit factors associated with DNA methylation and formation of repressive chromatin, such as heterochromatin protein 1 (HP1), histone deacetylases and histone methyltransferases (Ying, Y., et al. The Krüppel-associated box repressor domain induces reversible and irreversible regulation of endogenous mouse genes by mediating different chromatin states. Nucleic Acids Res. 43(3): 1549 (2015)). Further, the catalytically inactive DNMT3L cofactor helps establish a heritable methylation pattern after DNA replication, together with endogenous DNMT1 of the cell. The ATRX-DNMT3-DNMT3L domain (ADD) of DNMT3A is known to have two key

functions: 1) it allosterically regulates the catalytic activity of DNMT3A by serving as a methyltransferase auto-inhibitory domain, and 2) it specifically interacts with histone H3 tails that are unmethylated at lysine (K)4, leading to the preferential methylation of DNA bound to chromatin H3 tails that are unmethylated at K4 (Zhang, Y., et al. Chromatin methylation activity of Dnmt3a and Dnmt3a/3L is guided by interaction of the ADD domain with the histone H3 tail. Nucleic Acids Research 38:4246 (2010)).

[00233] In some embodiments, the repressor fusion protein (or the mRNA encoding the repressor fusion protein) comprises a DNA-binding protein linked to a first, second, third, and fourth repressor domain, wherein each of the repressor domains are different. In some embodiments, the DNA-binding protein is a TALE that can bind but not cleave the target nucleic acid. In some embodiments, the DNA-binding protein is a zinc-finger protein that can bind but not cleave the target nucleic acid. In some embodiments, the DNA-binding protein is a catalytically dead CRISPR protein that can bind but not cleave the target nucleic acid. In some embodiments, the repressor fusion protein (or the mRNA encoding the repressor) comprises a catalytically-dead CasX sequence, a first repressor domain (herein after referred to as "RD1"), a DNMT3A catalytic domain (herein after referred to as "DNMT3A") as the second domain, a DNMT3L interaction domain (herein after referred to as "DNMT3L") as the third domain, and an ATRX-DNMT3-DNMT3L domain (herein after referred to as "ADD") as the fourth domain. In some embodiments, the ADD is fused to the N-terminus of the DNMT3A. In some embodiments, the repressor fusion protein comprises a first and a second NLS and one or more linker peptides described herein, and the fusion protein is capable of forming an RNP with a gRNA of the system that binds to the target nucleic acid.

[00234] It has been discovered that the use of the foregoing domains, when configured in select orientations relative to a dCasX in a repressor fusion protein, results in pronounced epigenetic modification of a *PCSK9* target nucleic acid when complexed with a gRNA with a targeting sequence complementary to defined regions of the *PCSK9* gene, and that the combination of the repressor domains work in synchrony, resulting in an additive or synergistic effect on transcriptional silencing of the targeted gene, depending on the configuration. In one embodiment of the foregoing, the dCasX of the repressor fusion protein comprises a sequence selected from the group consisting of SEQ ID NOS: 4-29, or a sequence having at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at

least about 99% identity thereto. In another embodiment of the foregoing, the first repressor domain (RD1) of the repressor fusion protein comprises a sequence selected from the group consisting of SEQ ID NOS: 128-1726, or a sequence having at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% identity thereto. In another embodiment of the foregoing, the RD1 of the repressor fusion protein comprises a sequence selected from the group consisting of SEQ ID NOS: 130-224 or a sequence having at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% identity thereto. In another embodiment of the foregoing, the first repressor domain (RD1) of the repressor fusion protein comprises a sequence selected from the group consisting of SEQ ID NOS: 130-138 or a sequence having at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% identity thereto. In another embodiment of the foregoing, the RD1 of the repressor fusion protein comprises a sequence selected from the group consisting of SEQ ID NOS: 135 or a sequence having at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% identity thereto. In another embodiment of the foregoing, the RD1 of the repressor fusion protein comprises a sequence selected from the group consisting of SEQ ID NOS: 131 or a sequence having at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% identity thereto. In another embodiment of the foregoing, the second repressor domain of the repressor fusion protein is a DNMT3A, comprising a sequence of SEQ ID NO: 126, or a sequence having at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% identity thereto. In another embodiment of the foregoing, the third repressor domain of the repressor fusion protein is a DNMT3L, comprising a sequence of SEQ ID NO: 127, or a sequence having at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%

identity thereto. In another embodiment of the foregoing, the fourth repressor domain of the repressor fusion protein is an ADD, comprising a sequence of SEQ ID NO: 125, or a sequence having at least about 80%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% identity thereto. In a surprising finding, it has been discovered that the addition of the ADD to the repressor fusion proteins comprising the RD1, DNMT3A, and DNMT3L greatly enhances or increases the long-term repression and/or epigenetic modification of the target nucleic acid, as well as the specificity of the repression, in comparison to repressor fusion proteins lacking the ADD. Exemplary data for the improved repression and specificity of repressor fusion proteins comprising the ADD are presented in the Examples. Exemplary configurations of repressor fusion proteins comprising the ADD are presented in FIG. 2.

[00235] In some embodiments, the present disclosure provides a system of an repressor fusion protein comprising a first, a second, a third, and a fourth repressor domain operably linked to a dCasX comprising the sequence of SEQ ID NO: 4, or a sequence having at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto, wherein the RD1 comprises one or more motifs selected from the group consisting of a) $PX_1X_2X_3X_4X_5X_6EX_7$, wherein X_1 is A, D, E, or N, X_2 is L or V, X_3 is I or V, X_4 is S, T, or F, X_5 is H, K, L, Q, R or W, X_6 is L or M, and X_7 is G, K, Q, or R; b) $X_1X_2X_3X_4GX_5X_6X_7X_8X_9$, wherein X_1 is L or V, X_2 is A, G, L, T or V, X_3 is A, F, or S, X_4 is L or V, X_5 is C, F, H, I, L or Y, X_6 is A, C, P, Q, or S, X_7 is A, F, G, I, S, or V, X_8 is A, P, S, or T, and X_9 is K or R; c) $QX_1X_2LYRX_3VMX_4$ (SEQ ID NO: 1727), wherein X_1 is K or R, X_2 is A, D, E, G, N, S, or T, X_3 is D, E, or S, and X_4 is L or R; d) $X_1X_2X_3FX_4DVX_5X_6X_7FX_8X_9X_{10}X_{11}$ (SEQ ID NO: 1728), wherein X_1 is A, L, P, or S, X_2 is L or V, X_3 is S or T, X_4 is A, E, G, K, or R, X_5 is A or T, X_6 is I or V, X_7 is D, E, N, or Y, X_8 is S or T, X_9 is E, P, Q, R, or W, X_{10} is E or N, and X_{11} is E or Q; e) $X_1X_2X_3PX_4X_5X_6X_7X_8X_9X_{10}$, wherein X_1 is E, G, or R, X_2 is E or K, X_3 is A, D, or E, X_4 is C or W, X_5 is I, K, L, M, T, or V, X_6 is I, L, P, or V, X_7 is D, E, K, or V, X_8 is E, G, K, P, or R, X_9 is A, D, R, G, K, Q, or V, and X_{10} is D, E, G, I, L, R, S, or V; f) $LYX_1X_2VMX_3EX_4X_5X_6X_7X_8X_9X_{10}$ (SEQ ID NO: 1729), wherein X_1 is K or R, X_2 is D or E, X_3 is L, Q, or R, X_4 is N or T, X_5 is F or Y, X_6 is A, E, G, Q, R, or S, X_7 is H, L, or N, X_8 is L or V,

X_9 is A, G, I, L, T, or V, and X_{10} is A, F, or S; g) $FX_1DVX_2X_3X_4FX_5X_6X_7EWX_8$ (SEQ ID NO: 1730), wherein X_1 is A, E, G, K, or R, X_2 is A, S, or T, X_3 is I or V, X_4 is D, E, N, or Y, X_5 is S or T, X_6 is E, L, P, Q, R, or W, X_7 is D or E, and X_8 is A, E, G, Q, or R; h) $X_1PX_2X_3X_4X_5X_6LEX_7X_8X_9X_{10}X_{11}X_{12}$, wherein X_1 is K or R, X_2 is A, D, E, or N, X_3 is I, L, M, or V, X_4 is I or V, X_5 is F, S, or T, X_6 is H, K, L, Q, R, or W, X_7 is K, Q, or R, X_8 is E, G, or R, X_9 is D, E, or K, X_{10} is A, D, or E, X_{11} is L or P, and X_{12} is C or W; and i) $X_1LX_2X_3X_4QX_5X_6$, wherein X_1 is C, H, L, Q, or W, X_2 is D, G, N, R, or S, X_3 is L, P, S, or T, X_4 is A, S, or T, X_5 is K or R, and X_6 is A, D, E, K, N, S, or T; or comprises a first and a second motif wherein the first amino acid sequence motif comprises a) $LYX_1X_2VMX_3EX_4X_5X_6X_7X_8X_9X_{10}$ (SEQ ID NO: 1729), wherein (i) X_1 is K or R, (ii) X_2 is D or E, (iii) X_3 is L, Q, or R, (iv) X_4 is N or T, (v) X_5 is F or Y, (vi) X_6 is A, E, G, Q, R, or S, (vii) X_7 is H, L, or N, (viii) X_8 is L or V, (ix) X_9 is A, G, I, L, T, or V, and (x) X_{10} is A, F, or S; and b) the second amino acid sequence motif comprises $FX_1DVX_2X_3X_4FX_5X_6X_7EWX_8$ (SEQ ID NO: 1730), wherein (i) X_1 is A, E, G, K, or R, (ii) X_2 is A, S, or T, (iii) X_3 is I or V, (iv) X_4 is D, E, N, or Y, (iv) X_5 is S or T, (v) X_6 is E, L, P, Q, R, or W, (vi) X_7 is D or E, and (vii) X_8 is A, E, G, Q, or R; or comprises an amino acid sequence motif selected from the group consisting of: a) DVAVYFSPEEWGCL (SEQ ID NO: 2945); b) $X_1X_2X_3QX_4X_5LY$, wherein (i) X_1 is A, D, G, N, R, or S, (ii) X_2 is P, S, or T, (iii) X_3 is A, S, or T, (iv) X_4 is K or R, and (v) X_5 is A, D, K, N, S, or T; c) $X_1KPX_2X_3X_4X_5X_6$, wherein (i) X_1 is A, P, or S, (ii) X_2 is A, D, or E, (iii) X_3 is L, M, or V, (iv) X_4 is I or V, (v) X_5 is F, S, or T, and (vi) X_6 is H, K, L, Q, R, or W; d) $LEX_1X_2X_3X_4X_5X_6$, wherein (i) X_1 is E, K, Q or R, (ii) X_2 is E, G, or R, (iii) X_3 is A, D, E, or K, (iv) X_4 is A, D, or E, (v) X_5 is L or P, and (vi) X_6 is C or W; and e) $X_1VMLEX_2YX_3X_4X_5X_6SX_7X_8X_9$ (SEQ ID NO: 2946), wherein (i) X_1 is D or E, (ii) X_2 is N or T, (iii) X_3 is A, E, G, Q, R, or S, (iv) X_4 is H or N, (v) X_5 is L, M, or V, (vi) X_6 is A, L, or V, (vii) X_7 is L or V, (ix) X_8 is A, G, or V, and (x) X_9 is C, F, or L, and the second repressor domain is a DNMT3A sequence comprises the sequence of SEQ ID NO: 126, or sequence variants having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto, the third repressor is a DNMT3L comprising the sequence of SEQ ID NO: 127, or a sequence variant having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto,

and the fourth repressor is an ADD comprising the sequence of SEQ ID NO: 125, or a sequence variant having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto, wherein the fusion protein comprises one or more linker peptides described herein, and wherein the fusion protein is capable of forming an RNP with a gRNA of the system that binds to the target nucleic acid. In some embodiments of the foregoing, the RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 130-1726, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto, and the second repressor domain is a DNMT3A sequence comprises the sequence of SEQ ID NO: 126, or sequence variants having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto, the third repressor is a DNMT3L comprising the sequence of SEQ ID NO: 127, or a sequence variant having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto, and the fourth repressor is an ADD comprising the sequence of SEQ ID NO: 125, or a sequence variant having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto, wherein the fusion protein comprises one or more linker peptides described herein, and wherein the fusion protein is capable of forming an RNP with a gRNA of the system that binds to the target nucleic acid. In other embodiments of the foregoing, the first RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 130-224, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto. In other embodiments of the foregoing, the first RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 130-138, or a sequence having at least about 70%, at least about 80%, at least about 85%,

at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto. In other embodiments of the foregoing, the first RD1 comprises the sequence of SEQ ID NO: 135, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto. In other embodiments of the foregoing, the first RD1 comprises the sequence of SEQ ID NO: 131, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90% at least about 91%, at least about 92%, at least about 93% at least about 94% at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity thereto. In the foregoing embodiments, the fusion protein can comprise a first and a second NLS comprising a sequence selected from the group consisting of SEQ ID NOS: 30-97, and one or more linker peptides comprising a sequence selected from the group consisting of SEQ ID NOS: 98-124, as set forth in Table 5. In the foregoing embodiments of the paragraph, the repressor fusion protein is capable of forming an RNP complex with a gRNA of the system that is capable of binding to the gene target nucleic acid.

[00236] The skilled artisan will understand that RD1 proteins comprising the motifs described supra, with one or more conservative substitutions to the motif, may also function as RD1 domains and are envisaged as within the scope of the instant disclosure.

[00237] In some embodiments, the repressor fusion protein comprises, from N- to C-terminus, an RD1, an ADD, a DNMT3A, a DNMT3L, and a DNA-binding protein. In some embodiments, the repressor fusion protein comprises, from N- to C-terminus, an RD1, an ADD, a DNMT3A, a DNMT3L, and a catalytically-dead CRISPR protein. In some embodiments, the repressor fusion protein comprises, from N- to C-terminus, an RD1, an ADD, a DNMT3A, a DNMT3L, and a dCasX.

[00238] In some embodiments, the repressor fusion protein comprises, from N- to C-terminus, an ADD, a DNMT3A, a DNMT3L, an RD1, and a DNA-binding protein. In some embodiments, the repressor fusion protein comprises, from N- to C-terminus, an ADD, a DNMT3A, a DNMT3L, an RD1, and a catalytically-dead CRISPR protein. In some embodiments, the repressor fusion protein comprises, from N- to C-terminus, an ADD, a DNMT3A, a DNMT3L, an RD1, and a dCasX.

[00239] In some embodiments, the repressor fusion protein has a configuration of, N-terminal to C-terminal of NLS-ADD-DNMT3A-DNMT3L-dCasX-RD1-NLS, NLS-dCasX-RD1-NLS-ADD-DNMT3A-DNMT3L, NLS- dCasX- ADD-DNMT3A- DNMT3L- RD1-NLS), NLS-RD-ADD-DNMT3A-DNMT3L- dCasX-NLS, or NLS-ADD-DNMT3A-DNMT3L-RD1-dCasX-NLS. In any of the foregoing, a linker peptide may be inserted between one or more of the ADD, DNMT3A, DNMT3L, RD1 or dCasX domains.

[00240] In some embodiments, the repressor fusion protein has a configuration of, N-terminal to C-terminal, of configuration 1 (NLS-ADD-DNMT3A-Linker2-DNMT3L-Linker1-Linker3A-dCasX-Linker3B-RD1-NLS), configuration 2 (NLS-Linker3A-dCasX-Linker3B-RD1-NLS-Linker1-ADD-DNMT3A-Linker2-DNMT3L), configuration 3 (NLS-Linker3A-dCasX-Linker1-ADD-DNMT3A-Linker2-DNMT3L-Linker3B-RD1-NLS), configuration 4 (NLS-RD1-Linker3A-ADD-DNMT3A-Linker2-DNMT3L-Linker1-dCasX-Linker3B-NLS), or configuration 5 (NLS-ADD-DNMT3A-Linker2-DNMT3L-Linker3A-RD1-Linker1-dCasX-Linker3B-NLS). In some embodiments, the repressor fusion protein has a configuration of, N-terminal to C-terminal, of configuration 1' (NLS-DNMT3A-Linker2-DNMT3L-Linker1-Linker3A-dCasX-Linker3B-RD1-NLS), configuration 2' (NLS-Linker3A-dCasX-Linker3B-RD1-NLS-Linker1-DNMT3A-Linker2-DNMT3L), configuration 3' (NLS-Linker3A-dCasX-Linker1-DNMT3A-Linker2-DNMT3L-Linker3B-RD1-NLS), configuration 4' (NLS-RD1-Linker3A-DNMT3A-Linker2-DNMT3L-Linker1-dCasX-Linker3B-NLS), or configuration 5' (NLS-DNMT3A-Linker2-DNMT3L-Linker3A-RD1-Linker1-dCasX-Linker3B-NLS). The skilled artisan will appreciate that configurations 1'-5' correspond to configurations 1-5 without the ADD domain. In some embodiments of the system, the fusion protein components of the system are configured as schematically portrayed in FIG. 1 or FIG. 2. In the foregoing embodiment of configurations 1-5 or 1'- 5', the NLS comprise a sequence selected from the group consisting of SEQ ID NOS: 30-97 and the linker sequences are independently selected from the group consisting of SEQ ID NOS: 98-124 as set forth in Table 5. In some embodiments, the linker sequences are independently selected from the group consisting of SEQ ID NOS 120-123. In some embodiments, Linker 1 comprises a sequence of SEQ ID NOS: 123. In some embodiments, Linker 2 comprises a sequence of SEQ ID NO: 122. In some embodiments, Linker 3A and/or Linker 3B comprise a sequence of SEQ ID NO: 120. In some embodiments, Linker 4 comprises a sequence of SEQ ID NO: 121.

Table 5: Exemplary linker amino acid sequences for LTRP fusion proteins

Amino Acid Sequence*	SEQ ID NO
(G)n	98
(GS)n	99
(GSGGS)n	100
(GGSGGS)n	101
(GGGS)n	102
GGSG	103
GGSGG	104
GSGSG	105
GSGGG	106
GGGSG	107
GSSSG	108
GPGP	109
PPAPPA	110
PPPG	111
PPPGPPP	112
PPP(GGGS)n	113
(GGGS)nPPP	114
AEAAAKEAAAKEAAAKA	115
VPPPGGGSGGGSGGGS	116
TGGGPGGGAAAGSGS	117
GGGSGGGSGGGSPPP	118
TPPKTKRKVEFE	119
GGSGGGS	120
GSGSGGG	121
SSGNSNANSRGPSFSSGLVPLSLRGSH	122
GGPSSGAPPPSGGSPAGSPTSTEEGTSESATPESGPGTSTE PSEGSAPGSPAGSPTSTEEGTSTEPSEGSAPGTSTEPSE	123
GGSGGG	124

* n is 1 to 5

[00241] In some embodiments of the repressor fusion proteins and systems comprising same, the repressor fusion protein comprises a DNA-binding protein, a first, second, third, and fourth repressor domain configured as a configuration selected from the group consisting of configuration 1, configuration 2, configuration 3, configuration 4, configuration 5, configuration 1', configuration 2', configuration 3,' configuration 4', and configuration 5', described *supra*, upon binding of an RNP of the repressor fusion protein and the gRNA with a targeting sequence complementary to the *PCSK9* target nucleic acid in a cell, the target nucleic acid is epigenetically-modified and transcription of the *PCSK9* gene is repressed. In some

embodiments, transcription of the *PCSK9* gene is repressed by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least 99%, when assayed in an *in vitro* assay, including cell-based assays, when compared to untreated cells or cells treated with a comparable system comprising a non-targeting spacer. Most preferably, *PCSK9* gene repression results in complete inhibition of gene expression, such that no gene product is detectable. In some embodiments, transcription of the *PCSK9* gene of at least about 1%, at least about 2%, at least about 3%, at least about 4%, at least about 5%, at least about 6%, at least about 7%, at least about 8%, at least about 9%, or at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60% or more of cells of a population targeted by the repressor fusion protein:gRNA system are repressed.

[00242] In some embodiments, the repression of transcription of the *PCSK9* gene is sustained for at least about 8 hours, at least about 1 day, at least about 7 days, at least 2 weeks, at least about 3 weeks, at least about 1 month, or at least about 2 months, when assayed in an *in vitro* assay, including cell-based assays. In some embodiments, the repression of transcription of the PCSK9 gene is sustained for at least about 7 days, at least 2 weeks, at least about 3 weeks, at least about 1 month, at least about 2 months, at least about 3 months, at least about 4 months, at least about 5 months, or at least about 6 months in targeted cells of a subject when the composition is administered as a therapeutically effective dose, wherein the subject is selected from the group consisting of mouse, rat, pig, non-human primate, and human. In a particular embodiment, repressor fusion proteins configurations 4 and 5, or 4' and 5', when used in the repressor fusion protein:gRNA system, result in less off-target methylation or off-target activity in an *in vitro* assay compared to configuration 1. In some embodiments, use of the repressor fusion protein configurations 4 and 5, or 4' and 5', when used in a repressor fusion protein:gRNA system, results in off-target methylation or off-target activity that is less than about 10%, less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, less that 0.5%, or less than 0.1% in the cells.

a. **mRNA compositions encoding LTRP fusion proteins**

[00243] In another aspect, the disclosure relates to messenger RNA (mRNA) compositions comprising sequences that encode DNA-binding protein (*e.g.*, dCasX) and linked repressor

domain fusion proteins (repressor fusion proteins) of the disclosure. The mRNA compositions can be used in the repressor fusion protein:gRNA systems of the disclosure, and in certain delivery formulations; *e.g.*, particles such as lipid nanoparticles (LNP). In some embodiments, the compositions have been designed to result in one or more of improved expression, reduced immunogenicity, increased stability, and enhanced manufacturability of the repressor fusion protein relative to repressor fusion proteins encoded by unmodified mRNAs. In some embodiments, the repressor fusion proteins are designed to result in heritable repression, wherein the repression of the *PCKS9* gene persists for at least 1, 2, 3, 4, 5, or 6 or more cell divisions. In some embodiments, the repressor fusion proteins result in repression of transcription of the *PCSK9* gene that is sustained for at least about 8 hours, at least about 1 day, at least about 7 days, at least 2 weeks, at least about 3 weeks, at least about 1 month, or at least about 2 months, when assayed in an in vitro assay. The disclosure also provides methods utilized to design the compositions, and formulations to deliver the compositions.

[00244] Modifications to an mRNA sequence can affect mRNA stability, protein translation and expression levels, and immunogenicity, and therefore can have a significant impact on the efficacy of mRNA-based delivery. Optimization of coding sequences and untranslated regions (UTRs) may be particularly significant when delivering an mRNA encoding a protein of interest, as opposed to a DNA template that would be transcribed into an mRNA. DNA templates are long-lived, can replicate, and can produce many RNA transcripts over their lifetimes. For DNA templates, efficiency of transcription and pre-mRNA processing are major determinants of protein expression levels. In contrast, mRNAs generally have a much shorter half-life, on the order of hours, as they are vulnerable to degradation in the cytoplasm, and cannot produce more copies of themselves. As such, mRNA stability and translation efficiency are determinants of protein expression levels for mRNA-based delivery, and the specific sequences of UTRs and coding sequences that dictate mRNA stability and translation efficiency can therefore be enhanced to improve the efficacy of mRNA-based delivery.

[00245] In some embodiments, the disclosure provides an mRNA encoding dCasX 515 (SEQ ID NO: 6) for incorporation into an mRNA encoding a repressor fusion protein, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or having at least about 99% sequence identity thereto. In some embodiments, the disclosure provides an mRNA encoding dCasX 812 (SEQ ID NO: 29) for incorporation into an mRNA encoding a repressor

fusion protein, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or having at least about 99% sequence identity thereto. In some embodiments, the disclosure provides an mRNA sequence encoding dCasX 491 (SEQ ID NO: 4) for incorporation into an mRNA encoding a repressor fusion protein of the disclosure, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or having at least about 99% sequence identity thereto. In some embodiments, the disclosure provides an mRNA encoding dCasX 676 (SEQ ID NO: 28) for incorporation into an mRNA encoding a repressor fusion protein, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or having at least about 99% sequence identity thereto. In some embodiments, the disclosure provides an mRNA encoding a repressor fusion protein comprising dCasX 491 comprising a sequence of SEQ ID NO: 3122.

[00246] Various naturally-occurring or modified nucleosides may be used to produce mRNA according to the present disclosure. In some embodiments, an mRNA is or comprises natural nucleosides (*e.g.*, adenosine, guanosine, cytidine, uridine); nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, pseudouridine, (*e.g.*, N-1-methyl-pseudouridine), 2-thiouridine, and 2-thiocytidine); chemically modified bases; biologically modified bases (*e.g.*, methylated bases); intercalated bases; modified sugars (*e.g.*, 2′-fluororibose, ribose, 2′-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (*e.g.*, phosphorothioates and 5′-N-phosphoramidite linkages). In some embodiments, the mRNA comprises one or more nonstandard nucleotide residues. The nonstandard nucleotide residues may include, *e.g.*, 5-methyl-cytidine ("5 mC"), pseudouridine ("ψU"), and/or 2-thio-uridine ("2sU"). In a particular embodiment, one or more of the uridine residues of the mRNA of the disclosure are replaced with N1-methyl-pseudouridine. See, *e.g.*, U.S. Pat. No. 8,278,036 or WO2011012316, incorporated by reference herein, for a discussion of such residues and their incorporation into mRNA. In some embodiments, the mRNA encoding CasX 515 has N1-methyl-pseudouridine

nucleosides replacing one or more, or all uridines in the sequence. In some embodiments, the mRNA encoding CasX 812 has N1-methyl-pseudouridine nucleosides replacing one or more, or all uridines in the sequence.

[00247] In some embodiments, the mRNA sequence encoding the repressor fusion protein comprises a 5' UTR and a 3' UTR sequence. The person of ordinary skill in the art will be able to select appropriate UTR sequences. In some embodiments, the 3' UTR comprises a sequence of SEQ ID NOS: 3189, 3205-3209 or 3278. In some embodiments, the 5' UTR comprises a sequence of SEQ ID NOS: 3200-3204 or 3274.

V. Guide Nucleic Acids of the Systems

[00248] In another aspect, the disclosure relates to guide ribonucleic acids (gRNA) comprising a scaffold and a linked targeting sequence complementary to (and are therefore able to hybridize with) a target nucleic acid sequence of a *PCSK9* gene that have utility in repression of transcription of the PCSK9 target nucleic acid in a eukaryotic cell. As used herein, the term "gRNA" covers naturally-occurring molecules and gRNA variants, including chimeric gRNA variants comprising domains from different gRNA. gRNAs of the disclosure comprise a scaffold and a targeting sequence complementary to a target nucleic acid of a cell.

[00249] In some embodiments, the disclosure provides systems comprising an mRNA encoding a repressor fusion protein comprising a dCasX protein, and one or more gRNAs as a repressor fusion protein:gRNA system designed, upon expression of the dCasX protein in a transfected cell, to form a ribonucleoprotein (RNP) complex with the gRNA. The RNP targets and binds to specific locations in the target nucleic acid sequence of the cell for repression of transcription. The gRNA provides target specificity to the RNP complex by including a targeting sequence (or "spacer") comprising a nucleotide sequence that is complementary to a sequence of the target nucleic acid sequence. The repressor fusion protein of the system provides the site-specific activity, such as the binding and repression of the target sequence, and is guided to a target site (*e.g.*, stabilized at a target site) within a target nucleic acid sequence by virtue of its association with the gRNA in the RNP.

[00250] Embodiments of gRNAs and formulations of mRNAs and gRNAs for use in the repression and/or epigenetic modification of *PCSK9* target nucleic acids are described herein, below.

a. Reference gRNA and gRNA variants

[00251] As used herein, a “reference gRNA" refers to a CRISPR guide ribonucleic acid comprising a wild-type sequence of a naturally-occurring gRNA. In some embodiments, a gRNA scaffold of the disclosure may be subjected to one or more mutagenesis methods, such as the mutagenesis methods described in WO2022120095A1 and WO2020247882A1, incorporated by reference herein, which may include Deep Mutational Evolution (DME), deep mutational scanning (DMS), error prone PCR, cassette mutagenesis, random mutagenesis, staggered extension PCR, gene shuffling, domain swapping, or chemical modification to generate one or more gRNA variants with enhanced or varied properties relative to the gRNA scaffold that was modified. The activity of the gRNA scaffold from which a gRNA variant was derived may be used as a benchmark against which the activity of the gRNA variant is compared, thereby measuring improvements in function or other characteristics of the gRNA scaffold.

[00252] Table 6 provides the sequences of reference gRNA tracr and scaffold sequences. In some embodiments, the disclosure provides gRNA sequences wherein the gRNA has a scaffold comprising a sequence having one or more nucleotide modifications relative to a reference gRNA sequence of any one of SEQ ID NOS: 1731-1743 of Table 6.

Table 6: Reference gRNA tracr and scaffold sequences

SEQ ID NO.	Nucleotide Sequence
1731	ACAUCUGGCGCGUUUAUUCCAUUACUUUGGAGCCAGUCCCAGCGACUAUGUCG UAUGGACGAAGCGCUUAUUUAUCGGAGAGAAACCGAUAAGUAAAACGCAUCAA AG
1732	UACUGGCGCUUUUAUCUCAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGU AUGGGUAAAGCGCUUAUUUAUCGGAGAGAAAUCCGAUAAAUAAGAAGCAUCAA AG
1733	ACAUCUGGCGCGUUUAUUCCAUUACUUUGGAGCCAGUCCCAGCGACUAUGUCG UAUGGACGAAGCGCUUAUUUAUCGGAGA
1734	ACAUCUGGCGCGUUUAUUCCAUUACUUUGGAGCCAGUCCCAGCGACUAUGUCG UAUGGACGAAGCGCUUAUUUAUCGG
1735	UACUGGCGCUUUUAUCUCAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGU AUGGGUAAAGCGCUUAUUUAUCGGAGA
1736	UACUGGCGCUUUUAUCUCAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGU AUGGGUAAAGCGCUUAUUUAUCGG
1737	GUUUACACACUCCCUCUCAUAGGGU
1738	GUUUACACACUCCCUCUCAUGAGGU
1739	UUUUACAUACCCCCUCUCAUGGGAU

SEQ ID NO.	Nucleotide Sequence
1740	GUUUACACACUCCCUCUCAUGGGGG
1741	CCAGCGACUAUGUCGUAUGG
1742	GCGCUUAUUUAUCGGAGAGAAAUCCGAUAAAUAAGAAGC
1743	GGCGCUUUUAUCUCAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGUAUGG GUAAAGCGCUUAUUUAUCGGA

b. **gRNA Domains and their Functions**

[00253] The gRNAs of the disclosure comprise two segments: a targeting sequence and a protein-binding segment. The targeting segment of a gRNA includes a nucleotide sequence (referred to interchangeably as a spacer, a targeter, or a targeting sequence) that is complementary to (and therefore hybridizes with) a specific sequence (a target site) within the target nucleic acid sequence (*e.g.*, a strand of a double stranded target DNA, a target ssRNA, a target ssDNA, etc.), described more fully below. The targeting sequence of a gRNA is capable of binding to a target nucleic acid sequence, including, in the context of the present disclosure, a coding sequence, a complement of a coding sequence, a non-coding sequence, and to accessory elements. The protein-binding segment (or "activator" or "protein-binding sequence") of the gRNA interacts with (*e.g.*, binds to) a CasX protein as a complex, forming an RNP (described more fully, below). As used herein, "scaffold" refers to all parts to the guide with the exception of the targeting sequence, which is comprised of several regions, described more fully, below. The properties and characteristics of CasX gRNA, both wild-type and variants, are described in WO2020247882A1, US20220220508A1, and WO2022120095A1, incorporated by reference herein.

[00254] In the case of a reference gRNA, the gRNA occurs naturally as a dual guide RNA (dgRNA), wherein the targeter and the activator portions each have a duplex-forming segment that have complementarity with one another and hybridize to one another to form a double stranded duplex (dsRNA duplex for a gRNA). The term "targeter" or "targeter RNA" is used herein to refer to a crRNA-like molecule (crRNA: "CRISPR RNA") of a CasX dual guide RNA (and therefore of a CasX single guide RNA when the "activator" and the "targeter" are linked together, *e.g.*, by intervening nucleotides). The crRNA has a 5' region that anneals with the tracrRNA followed by the nucleotides of the targeting sequence. In the case of the gRNA for use in the systems of the disclosure, the scaffolds are designed such that the activator and targeter portions are covalently linked to one another (rather than hybridizing to one another) and

comprise a single molecule, and can be referred to as a "single-molecule gRNA," "single guide RNA", a "single-molecule guide RNA," a "one-molecule guide RNA", or a "sgRNA". The gRNA variants of the disclosure for use in the systems are all single molecule versions.

[00255] Collectively, the assembled gRNAs of the disclosure comprise distinct structured regions, or domains: the RNA triplex, the scaffold stem loop, the extended stem loop, the pseudoknot, and the targeting sequence that, in the embodiments of the disclosure is specific for a target nucleic acid and is located on the 3' end of the gRNA. The RNA triplex, the scaffold stem loop, the pseudoknot and the extended stem loop, together with the unstructured triplex loop that bridges portions of the triplex, together, are referred to as the "scaffold" of the gRNA. In some cases, the scaffold stem further comprises a bubble. In other cases, the scaffold further comprises a triplex loop region. In still other cases, the scaffold further comprises a 5' unstructured region. In some embodiments, the gRNA scaffolds of the disclosure for use in the repressor fusion protein:gRNA systems comprise a scaffold stem loop having the sequence of CCAGCGACUAUGUCGUAGUGG (SEQ ID NO: 1822), or a sequence with at least 1, 2, 3, 4 or 5 mismatches thereto.

[00256] Each of the structured domains are critical to establish the global RNA fold of the guide and retain functionality of the guide; particularly the ability to properly complex with the dCasX protein. For example, the guide scaffold stem interacts with the helical I domain of dCasX protein, while residues within the triplex, triplex loop, and pseudoknot stem interact with the OBD of the dCasX protein. Together, these interactions confer the ability of the guide to bind and form an RNP with the dCasX that retains stability, while the spacer (or targeting sequence) directs and defines the specificity of the RNP for binding a specific sequence of DNA.

[00257] Site-specific binding of a target nucleic acid sequence (*e.g.*, genomic DNA) by the dCasX protein can occur at one or more locations (*e.g.*, a sequence of a target nucleic acid) determined by base-pairing complementarity between the targeting sequence of the gRNA and the target nucleic acid sequence. Thus, for example, the gRNA of the disclosure have sequences complementarity to and therefore can hybridize with the target nucleic acid that is adjacent to a sequence complementary to a TC protospacer adjacent motif (PAM) motif or a PAM sequence, such as ATC, CTC, GTC, or TTC. Because the targeting sequence of a guide sequence hybridizes with a sequence of a target nucleic acid sequence, a targeting sequence can be modified by a user to hybridize with a specific target nucleic acid sequence, so long as the location of the PAM sequence is considered. In some embodiments, for design of a targeting

sequence, the target nucleic acid comprises a PAM sequence located 5' of the targeting sequence with at least a single nucleotide separating the PAM from the first nucleotide of the target nucleic acid complementary to that of the targeting sequence. In some embodiments, the PAM is located on the non-targeted strand of the target region, *i.e.,* the strand that is complementary to the target nucleic acid. In some embodiments, the targeting sequence of the gRNA is complementary to a target nucleic acid sequence one nucleotide from an ATC PAM sequence. In some embodiments, the targeting sequence of the gRNA is complementary to a target nucleic acid sequence one nucleotide from an CTC PAM sequence. In some embodiments, the targeting sequence of the gRNA is complementary to a target nucleic acid sequence one nucleotide from an GTC PAM sequence. In some embodiments, the targeting sequence of the gRNA is complementary to a target nucleic acid sequence one nucleotide from an TTC PAM sequence. By selection of the targeting sequences of the gRNA, defined regions of the target nucleic acid sequence or sequences bracketing a particular location within the target nucleic acid can be repressed using the repressor fusion protein:gRNA systems described herein. In some embodiments, the targeting sequence of the gRNA has between 15 and 20 consecutive nucleotides. In some embodiments, the targeting sequence has 15, 16, 17, 18, 19, and 20 consecutive nucleotides. In some embodiments, the targeting sequence consists of 20 consecutive nucleotides. In some embodiments, the targeting sequence consists of 19 consecutive nucleotides. In some embodiments, the targeting sequence consists of 18 consecutive nucleotides. In some embodiments, the targeting sequence consists of 17 consecutive nucleotides. In some embodiments, the targeting sequence consists of 16 consecutive nucleotides. In some embodiments, the targeting sequence consists of 15 consecutive nucleotides. By selection of the targeting sequences of the gRNA, defined regions of the target nucleic acid sequence can be repressed and/or epigenetically modified using the repressor fusion protein:gRNA systems described herein.

[00258] The gene repressor systems of the present disclosure can be designed to target any region of, or proximal to, a *PCSK9* gene or region of a *PCSK9* gene for which repression of transcription is sought. When the entirety of the gene is to be repressed, designing a guide with a targeting sequence complementary to a sequence encompassing or proximal to the transcription start site (TSS) is contemplated by the disclosure. The TSS selection occurs at different positions within the promoter region, depending on promoter sequence and initiating-substrate concentration. The core promoter serves as a binding platform for the transcription machinery,

which comprises Pol II and its associated general transcription factors (GTFs) (Haberle, V. et al. Eukaryotic core promoters and the functional basis of transcription initiation (Nat Rev Mol Cell Biol. 19(10):621 (2018)). Variability in TSS selection has been proposed to involve DNA 'scrunching' and 'anti-scrunching,' the hallmarks of which are: (i) forward and reverse movement of the RNA polymerase leading edge, but not trailing edge, relative to DNA, and (ii) expansion and contraction of the transcription bubble. In some embodiments, the target nucleic acid sequence bound by an RNP of the repressor fusion protein:gRNA system is within 1 kb of a transcription start site (TSS) in the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by an RNP of the system is within 20 bp, 50 bp, 100 bp, 150 bp, 200 bp, 250 bp, 500 bps, 1 kb, or 1.5 kb upstream of a TSS of the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by an RNP of the system is within 20 bp, 50 bp, 100 bp, 150 bp, 200 bp, 250 bp, 500 bps, 1 kb, or 1.5 kb downstream of a TSS of the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by an RNP of the system is within 1.5 kb upstream to 1.5 downstream, 1kb upstream to 1 kb downstream, 500 bps upstream to 500 bps downstream, or 300 bps upstream to 300 bps downstream, or 100 bps upstream to 100 bps downstream of a TSS of the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by an RNP of the system is within 20 bp, 50 bp, 100 bp, 150 bp, 200 bp, 250 bp, 500 bps, 1 kb, or 1.5 kb of an enhancer of the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by an RNP of the system of the disclosure is within 1 kb 3' to a 5' untranslated region of the *PCSK9* gene. In other embodiments, the target nucleic acid sequence bound by an RNP of the system is within the open reading frame of the *PCSK9* gene, inclusive of introns (if any). In some embodiments, the targeting sequence of a gRNA of the system of the disclosure is designed to be specific for an exon of the *PCSK9* gene. In a particular embodiment, the targeting sequence of a gRNA of the system of the disclosure is designed to be specific for exon 1 of the *PCSK9* gene. In other embodiments, the targeting sequence of a gRNA of the system of the disclosure is designed to be specific for an intron of the *PCSK9* gene. In other embodiments, the targeting sequence of the gRNA of the system of the disclosure is designed to be specific for an intron-exon junction of the *PCSK9* gene. In other embodiments, the targeting sequence of the gRNA of the system of the disclosure is designed to be specific for a regulatory element of the *PCSK9* gene. In other embodiments, the targeting sequence of the gRNA of the system of the disclosure is designed to be complementary to a sequence of an intergenic region of the *PCSK9* gene. In other embodiments, the targeting sequence of a gRNA of the system of

the disclosure is specific for a junction of the exon, an intron, and/or a regulatory element of the *PCSK9* gene. In those cases where the targeting sequence is specific for a regulatory element, such regulatory elements include, but are not limited to promoter regions, enhancer regions, intergenic regions, 5' untranslated regions (5' UTR), 3' untranslated regions (3' UTR), conserved elements, and regions comprising cis-regulatory elements. The promoter region is intended to encompass nucleotides within 5 kb of the initiation point of the encoding sequence or, in the case of gene enhancer elements or conserved elements, can be thousands of bp, hundreds of thousands of bp, or even millions of bp away from the encoding sequence of the *PCSK9* gene. In the foregoing, the targets are those in which the encoding *PCSK9* gene of the target is intended to be repressed such that the *PCSK9* gene product is not expressed or is expressed at a lower level in a cell. In some embodiments, upon binding of the RNP of the system of the disclosure to the binding location of the target nucleic acid, the system is capable of repressing transcription of the *PCSK9* gene 5' to the binding location of the RNP. In other embodiments, upon binding of the RNP of the system to the binding location of the target nucleic acid, the system is capable of repressing transcription of the *PCSK9* gene 3' to the binding location of the RNP.

[00259] In some embodiments, the target nucleic acid comprises a PAM sequence located 5' of the targeting sequence with at least a single nucleotide separating the PAM from the first nucleotide of the targeting sequence. In some embodiments, the PAM is located on the non-targeted strand of the target region, *i.e.* the strand that is complementary to the target nucleic acid. Representative, but non-limiting examples of targeting sequences to wild-type *PCSK9* nucleic acid are presented as SEQ ID NOS: 1824-2944, and are shown below as Table 7, representing targeting sequences for *PCSK9* target nucleic acid for linkage to the gRNA scaffolds of the disclosure; *e.g.*, gRNA 174, 235, 316, or chemically-modified versions thereof. In some embodiments, the targeting sequence of the gRNA comprises a sequence having at least about 65%, at least about 75%, at least about 85%, or at least about 95% identity to a sequence selected from the group consisting of SEQ ID NOS: 1824-2944. In some embodiments, the PAM sequence is TTC. In some embodiments, a targeting sequences for a TTC PAM comprises SEQ ID NOS: 1824-2944, or a sequence that is at least 50% identical, at least 55% identical, at least 60% identical, at least 65% identical, at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, or at least

99% identical to SEQ ID NOS: 1824-2944. In some embodiments, a targeting sequence for a TTC PAM is selected from the group consisting of SEQ ID NOS: 1824-2944.

[00260] In some embodiments, the targeting sequence of the gRNA for use in the repressor fusion protein:gRNA systems of the disclosure comprises a sequence selected from the group consisting of SEQ ID NO: 1824-2545. In a particular embodiment, the targeting sequence of the gRNA for use in the repressor fusion protein:gRNA systems of the disclosure consists of a sequence selected from the group consisting of SEQ ID NOS: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714. In some embodiments, the targeting sequence consists of SEQ ID NO: 1834. In some embodiments, the targeting sequence consists of SEQ ID NO: 2009. In some embodiments, the targeting sequence consists of SEQ ID NO: 2341. In some embodiments, the targeting sequence consists of SEQ ID NO: 1841. In some embodiments, the targeting sequence consists of SEQ ID NO: 1842. In some embodiments, the targeting sequence consists of SEQ ID NO: 1844. In some embodiments, the targeting sequence consists of SEQ ID NO: 1845. In some embodiments, the targeting sequence consists of SEQ ID NO: 2672. In some embodiments, the targeting sequence consists of SEQ ID NO: 1884. In some embodiments, the targeting sequence consists of SEQ ID NO: 1851. In some embodiments, the targeting sequence consists of SEQ ID NO: 1849. In some embodiments, the targeting sequence consists of SEQ ID NO: 1852. In some embodiments, the targeting sequence consists of SEQ ID NO: 1853. In some embodiments, the targeting sequence consists of SEQ ID NO: 1855. In some embodiments, the targeting sequence consists of SEQ ID NO: 1856. In some embodiments, the targeting sequence consists of SEQ ID NO: 1857. In some embodiments, the targeting sequence consists of SEQ ID NO: 1858. In some embodiments, the targeting sequence consists of SEQ ID NO: 1859. In some embodiments, the targeting sequence consists of SEQ ID NO: 1860. In some embodiments, the targeting sequence consists of SEQ ID NO: 1862. In some embodiments, the targeting sequence consists of SEQ ID NO: 1863. In some embodiments, the targeting sequence consists of SEQ ID NO: 1867. In some embodiments, the targeting sequence consists of SEQ ID NO: 1869. In some embodiments, the targeting sequence consists of SEQ ID NO: 1870. In some embodiments, the targeting sequence consists of SEQ ID NO: 1872. In some embodiments, the targeting sequence consists of SEQ ID NO: 1875. In some embodiments, the targeting sequence consists of SEQ ID NO: 1830. In any of the foregoing, the targeting sequence may have 1, 2, 3, 4, or 5 nucleotides removed from the 3' end of the targeting sequence.

Table 7: Targeting Sequences Specific to *PCSK9*

SEQ ID NO:	PAM Sequence
1824-2944	TTC

Table 8: Exemplary Targeting Sequences of *PCSK9*

SEQ ID NO:	PAM Sequence
1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714	TTC

c. **gRNA Modifications**

[00261] In another aspect, the disclosure relates to gRNAs (sometimes referred to as gRNA variants herein) which comprise modifications relative to a reference gRNA from which the gRNAs were derived. The gRNAs can be used in the systems of the disclosure. In some embodiments, a gRNA variant comprises one or more nucleotide substitutions, insertions, deletions, or swapped or replaced domains relative to a reference gRNA sequence that improve a characteristic relative to the reference gRNA. Exemplary regions for modifications and swapped regions or domains include the RNA triplex, the pseudoknot, the scaffold stem loop, and the extended stem loop. In some embodiments, the gRNA variant comprises at least a first swapped region from a different gRNA, resulting in a chimeric gRNA. A representative example of such a chimeric gRNA is guide 316 (SEQ ID NO: 1746), in which the extended stem loop of gRNA scaffold 235 is replaced with the extended stem loop of gRNA scaffold 174, wherein the resulting 316 variant retains the ability to form an RNP with an repressor fusion protein and exhibits an improved characteristic compared to the parent 235, when assessed in an *in vitro* or *in vivo* assay under comparable conditions.

[00262] All gRNAs that have one or more improved functions, characteristics, or add one or more new functions when the gRNA scaffold variant is compared to a gRNA scaffold from which it was derived, while retaining the functional properties of being able to complex with the repressor fusion protein and guide the ribonucleoprotein holo RNP complex to the target nucleic acid are envisaged as within the scope of the disclosure. In some embodiments, the gRNA has an improved characteristic selected from the group consisting of increased pseudoknot stem stability, increased triplex region stability, increased scaffold stem stability, extended stem stability, reduced off-target folding intermediates, increased binding affinity to a repressor fusion protein, and increased repression activity when complexed with a repressor fusion protein, or

any combination thereof. In some cases of the foregoing, the improved characteristic is assessed in an *in vitro* assay, including the assays of the Examples. In other cases of the foregoing, the improved characteristic is assessed in vivo.

[00263] Table 9 provides exemplary gRNA variant scaffold sequences for the generation of the gRNAs. The gRNAs can be used in the repressor fusion protein:gRNA systems of the disclosure. In some embodiments, the gRNA variant scaffold comprises any one of the sequences listed in Table 9, 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 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99% sequence identity thereto, wherein the gRNA variant retains the ability to form an RNP with a dCasX of the disclosure. In other embodiments, the gRNA variant scaffold comprises any one of the sequences listed in Table 9, wherein the gRNA variant retains the ability to form an RNP with an repressor fusion protein of the disclosure. It will be understood that in those embodiments wherein a vector comprises a DNA encoding sequence for a gRNA, that thymine (T) bases can be substituted for the uracil (U) bases of any of the gRNA sequence embodiments described herein. In some embodiments, the disclosure provides gRNA variants of Table 9 that are chemically-modified, described below.

Table 9: gRNA Scaffold Sequences

SEQ ID NO:	Scaffold variant ID	Nucleotide sequence
1744	174	ACUGGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGACUAU GUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAAAG
1745	235	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAU GUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUCCGUAAGAGGCAU CAGAG
1746	316	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAU GUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGAG

[00264] Additional gRNA scaffold variants contemplated for use in the gRNAs, and in the repressor fusion protein:gRNA systems of the disclosure are selected from the group consisting of SEQ ID NOS: 1747-1821.

d. **gRNA Scaffold 316**

[00265] Guide scaffolds can be made by several methods, including recombinantly or by solid-phase RNA synthesis. However, the length of the scaffold can affect the manufacturability when

using solid-phase RNA synthesis, with longer lengths resulting in increased manufacturing costs, decreased purity and yield, and higher rates of synthesis failure. For use in particle formulations, such as lipid nanoparticle (LNP) formulations, solid-phase RNA synthesis of the scaffold is preferred to generate the quantities needed for commercial development. While previous experiments had identified gRNA scaffold 235 as having enhanced properties relative to gRNA scaffold 174, its increased length (in nucleotides) rendered its use for LNP formulations problematic due to synthetic manufacturing constraints. Accordingly, alternative sequences were sought. In some embodiments, the disclosure provides gRNA variant scaffolds having improved manufacturability compared to the gRNA scaffold from which it was derived. In some embodiments, the disclosure provides a gRNA wherein the gRNA scaffold and linked targeting sequence has a sequence that is less than about 115 nucleotides, less than about 110 nucleotides, or less than about 100 nucleotides.

[00266] In some embodiments, a gRNA scaffold was designed wherein the scaffold 174 (SEQ ID NO: 1744) sequence was modified by introducing one or more mutations at positions selected from the group consisting of U11, U24, A29, and A87. In some embodiments, the gRNA comprises a sequence of SEQ ID NO: 1744, or a sequence having at least about 70% sequence identity thereto, comprising an extended stem loop sequence of SEQ ID NO: 49739 and one or more mutations at positions selected from the group consisting of U11, U24, A29, and A87. In one embodiment of the foregoing, the mutations consist of U11C, U24C, A29C, and A87G, resulting in the sequence of SEQ ID NO: 1746.

[00267] In another embodiment, the 316 gRNA scaffold was designed wherein the scaffold 235 sequence was modified by a domain swap in which the extended stem loop of scaffold 174 replaced the extended stem loop of the 235 scaffold, resulting in the chimeric gRNA scaffold 316 (SEQ ID NO: 1746), having 89 nucleotides, compared with the 99 nucleotides of gRNA scaffold 235. The resulting 316 scaffold had the further advantage in that the extended stem loop does not contain CpG motifs; an enhanced property conferring reduced potential to elicit an immune response. In some embodiments, the shorter sequence length of the 316 scaffold confers the improvements of a higher fidelity in the ability to create the guide synthetically with the correct and complete sequence, as well as an enhanced ability to be successfully incorporated into an LNP. In some embodiments, the disclosure provides gRNA 316 variants that are chemically-modified, described below.

e. **Chemically-modified gRNAs**

[00268] In some embodiments, the gRNAs have one or more chemical modifications. In some embodiments, the chemical modification is the addition of a 2'O-methyl group to one or more nucleotides of the sequence. In some embodiments, the chemical modification is substitution of a phosphorothioate bond between two or more nucleosides of the sequence. In some embodiments, the first 1, 2, or 3 nucleosides of the 5' end of the scaffold (*i.e.*, A, C, and U in the case of gRNA 174, 235, and 316) are modified by the addition of a 2'O-methyl group and each of the modified nucleosides is linked to the adjoining nucleoside by a phosphorothioate bond. Similarly, the last 1, 2, or 3 nucleotides of the 3' end of the targeting sequence linked to the 3' end of the scaffold are similarly modified. In some embodiments, the disclosure provides gRNA with chemical modifications selected from the group consisting of the sequences of SEQ ID NOS: 2948-2956, 2958-2966, and 2968-2976, as set forth in Table 25, or a sequence having 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 some embodiments, the gRNA with chemical modifications comprises a scaffold of SEQ ID NOS: 2948-2956, 2958-2966, and 2968-2976, i.e., a sequence of SEQ ID NOS: 2948-2956, 2958-2966, and 2968-2976 without the spacer represented in the foregoing sequences as undefined nucleotides. The skilled artisan will understand the 20 3' terminal undefined sequences in the foregoing represent non-targeting sequences, and can be substituted with any suitable targeting sequence complementary to a target nucleic acid of the *PCSK9* gene; for example a targeting sequence selected from the group consisting of SEQ ID NOS: 1824-2944. In some embodiments, the chemically modified gRNA comprises the sequence of SEQ ID NO: 2968. A schematic of the structure of gRNA variants 174, 235, and 316 are shown in FIGS. 19A-19C, respectively. In some embodiments, the gRNA with chemical modifications exhibit improved stability compared to gRNA without chemical modifications.

f. **Complex Formation with Repressor Fusion Proteins**

[00269] Upon delivery or expression of the components of the system in a target cell, the gRNA variant is capable of complexing as an RNP with a repressor fusion protein comprising a catalytically-dead CRISPR protein and binding to the target nucleic acid of the *PCSK9* gene. In some embodiments, a gRNA variant has an improved ability to form an RNP complex with a repressor fusion protein when compared to a reference gRNA or another gRNA variant from which it was derived. Improving ribonucleoprotein complex formation may, in some

embodiments, improve the efficiency with which functional RNPs are assembled. In some embodiments, greater than 90%, greater than 93%, greater than 95%, greater than 96%, greater than 97%, greater than 98% or greater than 99% of RNPs comprising a gRNA variant and its targeting sequence are competent for gene repression of a target nucleic acid.

VI. Polynucleotides and Vectors

[00270] In another aspect, the present disclosure relates to polynucleotides encoding the repressor fusion proteins, and, in some embodiments, gRNAs, that have utility in the repression and epigenetic modification of the *PCSK9* gene.

[00271] A repressor fusion protein or an mRNA encoding the repressor fusion protein of the disclosure may be prepared by *in vitro* synthesis, using conventional methods as known in the art. Various commercial synthetic apparatuses are available, for example, automated synthesizers by Applied Biosystems, Inc., Beckman, etc. By using synthesizers, naturally occurring amino acids or nucleotides (as applicable) may be substituted with unnatural amino acids or nucleotides. The particular sequence and the manner of preparation will be determined by convenience, economics, purity required, and the like. A gRNA can also be produced synthetically; for example by use of a T7 RNA polymerase system known in the art.

[00272] The repressor fusion protein and/or the gRNA may also be prepared by recombinantly producing a polynucleotide sequence coding for the repressor or gRNA of any of the embodiments described herein using standard recombinant techniques known in the art and incorporating the encoding gene into an expression vector appropriate for a host cell. For production of the encoded repressor fusion protein and/or gRNA, the methods include transforming an appropriate host cell with an expression vector comprising the encoding polynucleotide, and culturing the host cell under conditions causing or permitting the resulting repressor or gRNA to be expressed or transcribed in the transformed host cell, which are recovered by methods described herein or by standard purification methods known in the art, or as described in the Examples. Standard recombinant techniques in molecular biology are used to make the polynucleotides and expression vectors of the present disclosure.

[00273] A repressor fusion protein and/or a gRNA of the disclosure may also be isolated and purified in accordance with conventional methods of recombinant synthesis. A lysate may be prepared of the expression host and the lysate purified using high performance liquid chromatography (HPLC), exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. For the most part, the compositions which are

used will comprise 50% or more by weight of the desired product, more usually 75% or more by weight, preferably 95% or more by weight, and for therapeutic purposes, usually 99.5% or more by weight, in relation to contaminants related to the method of preparation of the product and its purification. Usually, the percentages will be based upon total protein. Thus, in some cases, a repressor fusion protein or gRNA of the present disclosure is at least 80% pure, at least 85% pure, at least 90% pure, at least 95% pure, at least 98% pure, or at least 99% pure (*e.g.*, free of contaminants or other macromolecules, etc.).

[00274] Additionally, the disclosure provides vectors comprising polynucleotides encoding the repressor fusion proteins and, in some cases, the gRNAs described herein. In some cases, the vectors are utilized for the expression and recovery of the CasX and gRNA components of the repressor fusion protein:gRNA system. In other cases, the vectors are utilized for the delivery of the encoding polynucleotides to target cells for the repression and/or epigenetic modification of the target nucleic acid, as described more fully, below. In some embodiments, sequences encoding the repressor fusion protein and the gRNA are encoded by the same vector. In some embodiments, sequences encoding the repressor fusion protein and a gRNA are encoded by sequences on different vectors. Suitable vectors are described, for example, in WO2022120095A1 and WO2020247882A1, incorporated by reference herein. As described in WO2022120095A1 and WO2020247882A1, depending on the host/vector system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector.

[00275] In some embodiments, the disclosure provides polynucleotide sequences encoding repressor fusion proteins, including the repressor fusion proteins of SEQ ID NOS: 3131-3132 as set forth in Table 20, or sequences 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%, or at least about 99% sequence identity thereto. In some embodiments, the disclosure provides an isolated polynucleotide sequence encoding a gRNA variant. In some embodiments, the disclosure provides polynucleotides encoding a gRNA comprising a scaffold sequence of SEQ ID NOS: 1744-1746 and 2947-2976, 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, wherein the expressed gRNA variant retains the

ability to form an RNP with an repressor fusion protein. In some embodiments, the disclosure provides polynucleotide sequences encoding gRNAs comprising targeting sequences of SEQ ID NOS: 1824-2944, or sequences having at least about 65%, at least about 75%, at least about 85%, or at least about 95% identity thereto. In some embodiments, the disclosure provides polynucleotide sequences encoding gRNAs comprising targeting sequences of SEQ ID NOS: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714, or sequences having at least about 65%, at least about 75%, at least about 85%, or at least about 95% identity thereto.

[00276] In some embodiments, the disclosure relates to methods to produce polynucleotide sequences encoding the repressor fusion proteins or the gRNAs, including variants thereof, as well as methods to express the proteins or RNA transcribed by the polynucleotide sequences. In general, the methods include producing a polynucleotide sequence coding for the repressor fusion protein or the gRNA of any of the embodiments described herein and incorporating the encoding gene into an expression vector. In some embodiments, the vector is designed for transduction of cells for repression and/or epigenetic modification of the *PCSK9* target nucleic acid. Such vectors can include a retroviral vector, a lentiviral vector, an adenoviral vector, an adeno-associated viral (AAV) vector, a herpes simplex virus (HSV) vector, a plasmid, a minicircle, a nanoplasmid, a DNA vector, and an RNA vector. In other embodiments, the expression vector is designed for production of a repressor fusion protein, mRNA encoding the repressor fusion protein, or gRNA in either a cell-free system or in a host cell. For production of the encoded repressor fusion protein or the gRNA of any of the embodiments described herein in a host cell, the methods include transforming an appropriate host cell with an expression vector comprising the encoding polynucleotide, and culturing the host cell under conditions causing or permitting the resulting repressor fusion protein or the gRNA of any of the embodiments described herein to be expressed or transcribed in the transformed host cell, thereby producing the repressor fusion protein or the gRNA, which are recovered by methods described herein (*e.g.*, in the Examples, below) or by standard purification methods known in the art. Standard recombinant techniques in molecular biology are used to make the polynucleotides and expression vectors of the present disclosure.

[00277] In accordance with the disclosure, nucleic acid sequences that encode the repressor fusion protein or the gRNA of any of the embodiments described herein are used to generate recombinant DNA molecules that direct the expression in appropriate host cells. Several cloning strategies are suitable for performing the present disclosure, many of which are used to generate

a construct that comprises a gene coding for a composition of the present disclosure, or its complement. In some embodiments, the cloning strategy is used to create a gene that encodes a construct that comprises nucleotides encoding the repressor fusion protein or the gRNA that is used to transform a host cell for expression of the composition.

[00278] In one approach, a construct is first prepared containing the DNA sequence encoding a repressor fusion protein or a gRNA. Exemplary methods for the preparation of such constructs are described in the Examples. The construct is then used to create an expression vector suitable for transforming a host cell, such as a prokaryotic or eukaryotic host cell for the expression and recovery of the protein construct, in the case of the repressor fusion protein, or the gRNA. Where desired, the host cell is an *E. coli*. In other embodiments, the host cell is a eukaryotic cell. The eukaryotic host cell can be selected from Baby Hamster Kidney fibroblast (BHK) cells, human embryonic kidney 293 (HEK293), human embryonic kidney 293T (HEK293T), NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells, hybridoma cells, NIH3T3 cells, CV-1 (simian) in Origin with SV40 genetic material (COS), HeLa, Chinese hamster ovary (CHO), yeast cells, or other eukaryotic cells known in the art suitable for the production of recombinant products. Exemplary methods for the creation of expression vectors, the transformation of host cells and the expression and recovery of the repressor fusion protein or the gRNA are described in the Examples.

[00279] The gene encoding the repressor fusion protein or the gRNA construct can be made in one or more steps, either fully synthetically or by synthesis combined with enzymatic processes, such as restriction enzyme-mediated cloning, PCR and overlap extension, including methods more fully described in the Examples. The methods disclosed herein can be used, for example, to ligate sequences of polynucleotides encoding the various components into a gene of a desired sequence. Genes encoding polypeptide compositions are assembled from oligonucleotides using standard techniques of gene synthesis.

[00280] In some embodiments, the nucleotide sequence encoding an repressor fusion protein is codon optimized. This type of optimization can entail a mutation of an encoding nucleotide sequence to mimic the codon preferences of the intended host organism or cell while encoding the same protein. Thus, the codons can be changed, but the encoded protein remains unchanged. For example, if the intended target cell of the repressor fusion protein was a human cell, a human codon-optimized repressor fusion protein-encoding nucleotide sequence could be used. As another non-limiting example, if the intended host cell were a mouse cell, then a mouse

codon-optimized repressor fusion protein-encoding nucleotide sequence could be generated. The gene design can be performed using algorithms that optimize codon usage and amino acid composition appropriate for the host cell utilized in the production of the repressor fusion protein or the gRNA. In one method of the disclosure, a library of polynucleotides encoding the components of the constructs is created and then assembled, as described above. The resulting genes are then assembled and the resulting genes used to transform a host cell and produce and recover the repressor fusion protein or the gRNA compositions for evaluation of its properties or for use in the modification of the PCSK9 target nucleic acid, as described herein.

[00281] In some embodiments, a nucleotide sequence encoding a gRNA is operably linked to a control element, *e.g.*, a transcriptional control element, such as a promoter. In some embodiments, a nucleotide sequence encoding a repressor fusion protein is operably linked to a control element, *e.g.*, a transcriptional control element, such as a promoter. In some cases, the promoter is a constitutively active promoter. In some cases, the promoter is a regulatable promoter. In some cases, the promoter is an inducible promoter. In some cases, the promoter is a tissue-specific promoter. In some cases, the promoter is a cell type-specific promoter. In some cases, the transcriptional control element (*e.g.*, the promoter) is functional in a targeted cell type or targeted cell population. For example, in some cases, the transcriptional control element can be functional in eukaryotic cells, *e.g.*, hepatocytes or a liver sinusoidal endothelial cell.

[00282] Non-limiting examples of Pol II promoters operably linked to the polynucleotide encoding the repressor fusion protein of the disclosure include, but are not limited to EF-1alpha, EF-1alpha core promoter, Jens Tornoe (JeT), promoters from cytomegalovirus (CMV), CMV immediate early (CMVIE), CMV enhancer, herpes simplex virus (HSV) thymidine kinase, early and late simian virus 40 (SV40), the SV40 enhancer, long terminal repeats (LTRs) from retrovirus, mouse metallothionein-I, adenovirus major late promoter (Ad MLP), CMV promoter full-length promoter, the minimal CMV promoter, the chicken β-actin promoter (CBA), CBA hybrid (CBh), chicken β-actin promoter with cytomegalovirus enhancer (CB7), chicken beta-Actin promoter and rabbit beta-Globin splice acceptor site fusion (CAG), the rous sarcoma virus (RSV) promoter, the HIV-Ltr promoter, the hPGK promoter, the HSV TK promoter, a 7SK promoter, the Mini-TK promoter, the human synapsin I (SYN) promoter which confers neuron-specific expression, beta-actin promoter, super core promoter 1 (SCP1), the Mecp2 promoter for selective expression in neurons, the minimal IL-2 promoter, the Rous sarcoma virus enhancer/promoter (single), the spleen focus-forming virus long terminal repeat (LTR) promoter,

the TBG promoter, promoter from the human thyroxine-binding globulin gene (Liver specific), the PGK promoter, the human ubiquitin C promoter (UBC), the UCOE promoter (Promoter of HNRPA2B1-CBX3), the synthetic CAG promoter, the Histone H2 promoter, the Histone H3 promoter, the U1a1 small nuclear RNA promoter (226 nt), the U1a1 small nuclear RNA promoter (226 nt), the U1b2 small nuclear RNA promoter (246 nt) 26, the GUSB promoter, the CBh promoter, rhodopsin (Rho) promoter, silencing-prone spleen focus forming virus (SFFV) promoter, a human H1 promoter (H1), a POL1 promoter, the TTR minimal enhancer/promoter, the b-kinesin promoter, mouse mammary tumor virus long terminal repeat (LTR) promoter, the human eukaryotic initiation factor 4A (EIF4A1) promoter, the ROSA26 promoter, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter, tRNA promoters, and truncated versions and sequence variants of the foregoing. In a particular embodiment, the Pol II promoter is EF-1alpha, wherein the promoter enhances transfection efficiency, the transgene transcription or expression of the CRISPR nuclease, the proportion of expression-positive clones and the copy number of the episomal vector in long-term culture.

[00283] Non-limiting examples of Pol III promoters operably linked to the polynucleotide encoding the gRNA variants of the disclosure include, but are not limited to U6, mini U6, U6 truncated promoters,7SK, and H1 variants, BiH1 (Bidirectional H1 promoter), BiU6, Bi7SK, BiH1 (Bidirectional U6, 7SK, and H1 promoters), gorilla U6, rhesus U6, human 7SK, human H1 promoters, and truncated versions and sequence variants thereof. In the foregoing embodiment, the pol III promoter enhances the transcription of the gRNA. In a particular embodiment, the Pol III promoter is U6, wherein the promoter enhances expression of the gRNA. In another particular embodiment, the promoter linked to the gene encoding the tropism factor is CMV promoter. Experimental details and data for the use of such promoters are provided in the Examples.

[00284] Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art, as it related to controlling expression. The expression vector may also contain a ribosome binding site for translation initiation, and a transcription terminator. The expression vector may also include appropriate sequences for amplifying expression. The expression vector may also include nucleotide sequences encoding protein tags (*e.g.*, 6xHis tag, hemagglutinin tag, fluorescent protein, *etc.*) that can be fused to the repressor fusion protein, thus resulting in a chimeric protein that are used for purification or detection.

[00285] Recombinant expression vectors of the disclosure can also comprise elements that facilitate robust expression of the proteins and the gRNAs of the disclosure. For example, recombinant expression vectors can include one or more of a polyadenylation signal (poly(A)), an intronic sequence or a post-transcriptional regulatory element such as a woodchuck hepatitis post-transcriptional regulatory element (WPRE). Exemplary poly(A) sequences include hGH poly(A) signal (short), HSV TK poly(A) signal, synthetic polyadenylation signals, SV40 poly(A) signal, β-globin poly(A) signal and the like (for example, SEQ ID NO: 3459). A person of ordinary skill in the art will be able to select suitable elements to include in the recombinant expression vectors described herein.

[00286] The polynucleotides encoding the repressor fusion protein or the gRNA sequences can be individually cloned into an expression vector. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art, as it relates to controlling expression, *e.g.*, for repressing expression and/or epigenetic modification of the PCSK9 gene. The expression vector may also contain a ribosome binding site for translation initiation and a transcription terminator. The expression vector may also include appropriate sequences for amplifying expression.

[00287] The nucleic acid sequence is inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan. Such techniques are well known in the art and well described in the scientific and patent literature. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage that may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, *i.e.*, a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. Once introduced into a suitable host cell, expression of the repressor fusion protein can be determined using any nucleic acid or protein

assay known in the art. For example, the presence of transcribed mRNA of the repressor fusion protein can be detected and/or quantified by conventional hybridization assays (*e.g.*, Northern blot analysis), amplification procedures (*e.g.,* RT-PCR), SAGE (U.S. Pat. No. 5,695,937), and array-based technologies (see *e.g.*, U.S. Pat. Nos. 5,405,783, 5,412,087 and 5,445,934), using probes complementary to any region of CasX polynucleotide.

[00288] In some embodiments, a vector is created for the transcription of the repressor fusion protein gene and expression and recovery of the resulting encoding mRNA. In some embodiments, the mRNA is generated by *in vitro* transcription (IVT) using a PCR product or linearized plasmid DNA template and a T7 RNA polymerase, wherein the plasmid contains a T7 promoter. If using a PCR product, DNA sequences encoding candidate mRNAs will be cloned into a plasmid containing a T7 promoter, wherein the plasmid DNA template will be linearized and then used to perform IVT reactions for expression of the mRNA. Exemplary methods for the generation of such vectors and the production and recovery of the mRNA are provided in the Examples, below.

VII. Particles for Delivery of Repressor Fusion Proteins

[00289] In another aspect, the present disclosure provides particle compositions for delivery of the repressor fusion proteins to cells or to subjects for the modification of the *PCSK9* gene. In some embodiments, the particle composition delivers a repressor fusion protein:gRNA system, e.g., when the repressor fusion protein comprises a catalytically dead CRISPR protein such as a dCasX, to cells or to subjects for the repression of the *PCSK9* gene. In some embodiments, the disclosure provides synthetic nanoparticles that encapsulate gRNA variants and mRNAs encoding a repressor fusion proteins comprising a dCasX protein of any of the embodiments described herein. In some embodiments, materials for the creation of biodegradable polymeric nanoparticles (PNP) include polylactide, poly (lactic-co-glycolic acid) (PLGA), poly(ethyl cyanoacrylate), poly(butyl cyanoacrylate), poly(isobutyl cyanoacrylate), and poly(isohexyl cyanoacrylate), polyglutamic acid (PGA), poly (ε-caprolactone) (PCL), cyclodextrin, and natural polymers for instance chitosan, albumin, gelatin, and alginate are the most utilized polymers for the synthesis of PNP (Production and clinical development of nanoparticles for gene delivery. Molecular Therapy-Methods & Clinical Development 3:16023; doi:10.1038 (2016)). In some embodiments, the disclosure provides virus-like particles for delivery of the repressor fusion proteins comprising a dCasX protein and gRNA variants (*see*, WO2021113772A1, incorporated by reference herein). In other embodiments, the disclosure provides lipid nanoparticles that

encapsulate gRNA variants and mRNAs encoding repressor fusion proteins comprising a dCasX protein of any of the embodiments described herein, described more fully, below.

a. **Lipid Nanoparticles (LNP)**

[00290] In another aspect, the present disclosure provides lipid nanoparticles (LNP) for delivery of the repressor fusion protein:gRNA systems of the disclosure to cells or to subjects for the transcriptional repression of the *PCSK9* gene. In some embodiments, the LNPs of the disclosure are tissue- or organ-specific (*e.g.*, the liver), have excellent biocompatibility, and can deliver the systems with high efficiency, and thus can be usefully used for the repression of the *PCSK9* gene.

[00291] In their native forms, nucleic acid polymers are unstable in biological fluids and cannot penetrate into the cytoplasm of target cells, thus requiring delivery systems. Lipid nanoparticles (LNP) have proven useful for both the protection and delivery of nucleic acids to tissues and cells. Furthermore, the use of mRNA in LNPs to encode the CRISPR nuclease eliminates the possibility of undesirable genome integration compared to DNA vectors. Moreover, mRNA efficiently translates into protein in both mitotic and non-mitotic cells, as it does not require to enter into the nucleus since it exerts its function in the cytoplasmic compartment. LNPs as a delivery platform offers the additional advantage of being able to co-formulate both the mRNA encoding the nuclease and the gRNA into single LNP particles.

[00292] Accordingly, in various embodiments, the disclosure encompasses lipid nanoparticles and compositions that may be used for a variety of purposes, including the delivery of encapsulated or associated (*e.g.*, complexed) therapeutic agents such as nucleic acids to cells, both *in vitro* and *in vivo*. In certain embodiments, the disclosure encompasses methods of treating or preventing diseases or disorders in a subject in need thereof by contacting the subject with a lipid nanoparticle that encapsulates or is associated with a suitable therapeutic agent complexed through various physical, chemical or electrostatic interactions between one or more of the lipid components used in the compositions to make LNPs. In some embodiments, the suitable therapeutic agent comprises a repressor fusion protein:gRNA system as described herein.

[00293] In certain embodiments, the lipid nanoparticles are useful for the delivery of nucleic acids, including, *e.g.*, the mRNA encoding the repressor fusion proteins of the disclosure, and the gRNA variants of the disclosure, including the sequences of SEQ ID NOS: 1744-1746 and 2947-2976. In some embodiments, the present disclosure provides LNP in which the gRNA and

mRNA encoding the repressor fusion proteins are incorporated into single LNP particles. In other embodiments, the present disclosure provides LNP in which the gRNA and mRNA encoding the repressor fusion proteins are incorporated into separate populations of LNPs, which can be formulated together in varying ratios for administration. In some embodiments, the mRNA for incorporation into the LNP of the disclosure encode any of the repressor fusion proteins described herein. In some embodiments, the gRNA for use in the LNP comprises a sequence of SEQ ID NOS: 1744-1746 and 2947-2976.

[00294] The lipid nanoparticles and systems of certain embodiments of the disclosure may be used to induce expression of a desired protein both in vitro and in vivo by contacting cells with a lipid nanoparticle comprising one or more novel ionizable cationic lipids or permanently charged cationic lipids described herein, wherein the lipid nanoparticle encapsulates or is associated with a nucleic acid that is expressed to produce the desired protein (e.g., a messenger RNA encoding the CasX protein). In some embodiments, the lipid nanoparticles and systems may be used to decrease the expression of the PCKS9 target gene both in vitro and in vivo by contacting cells with a lipid nanoparticle comprising one or more novel ionizable/cationic lipids described herein, wherein the lipid nanoparticle encapsulates or is associated with nucleic acids of the CasX:gRNA system that reduces target gene expression. The lipid nanoparticles and systems of embodiments of the disclosure may also be used for co-delivery of different nucleic acids (e.g., mRNA, gRNA, siRNA, saRNA, mcDNA and plasmid DNA) separately or in combination, such as may be useful to provide an effect requiring colocalization of different nucleic acids (e.g. mRNA encoding for a suitable gene modifying enzyme and gRNA for targeting of the target nucleic acid).

[00295] In some embodiments, LNPs and LNP compositions described herein include at least one cationic lipid, at least one conjugated lipid, at least one steroid or derivative thereof, at least one helper lipid, or any combination thereof. Alternatively, the lipid compositions of the disclosure can include an ionizable lipid, such as an ionizable cationic lipid, a helper lipid (usually a phospholipid), cholesterol, and a polyethylene glycol-lipid conjugate (PEG-lipid) to improve the colloidal stability in biological environments by, for example, reducing a specific absorption of plasma proteins and forming a hydration layer over the nanoparticles. Such lipid compositions can be formulated at typical mole ratios of 50:10:37-39:1-3 or 20-50:8-65:15-70:1-3.0 of IL:HL:Sterol: PEG-lipid, with variations made to include or exclude one or more of the

components to the traditional 4-component system in the LNP and to adjust individual properties.

[00296] The LNPs and LNP compositions of the present disclosure are configured to protect and deliver an encapsulated payload of the systems of the disclosure to tissues and cells, both *in vitro* and in vivo. Various embodiments of the LNPs and LNP compositions of the present disclosure are described in further detail herein.

Cationic lipid

[00297] In some embodiments, the LNPs and LNP compositions of the present disclosure include at least one cationic lipid. The term "cationic lipid," refers to a lipid species that has a net positive charge. In some embodiments, the cationic lipid is an ionizable cationic lipid that has a net positive charge at a selected pH<pKa of the ionizable lipid. In some embodiments, the ionizable cationic lipid has a pKa less than about 7 such that the LNPs and LNP compositions achieve efficient encapsulation of the payload at a relatively low pH below the pKa of the respective lipid. In some embodiments, the cationic lipid has a pKa of about 5 to about 8, about 5.5 to about 7.5, about 6 to about 7, or about 6.5 to about 7. In some embodiments, the cationic lipid may be protonated at a pH below the pKa of the cationic lipid, and it may be substantially neutral at a pH over the pKa. The LNPs and LNP compositions may be safely delivered to a target organ (for example, the liver, lung, heart, spleen, as well as to tumors) and/or cell(hepatocyte, LSEC, cardiac cell, cancer cell, etc.) in vivo, and during endocytosis, exhibit a positive charge when pH drops below the ionizable lipid pKa to release the encapsulated payload through electrostatic interaction with an anionic lipids of the endosomal membrane.

[00298] Early formulations of LNP utilizing permanently cationic lipids resulted in LNPs with positive surface charge that proved toxic in vivo, plus were rapidly cleared by phagocytic cells. By changing to ionizable cationic lipids bearing tertiary amines, especially those with pKa < 7, results in LNP achieving efficient encapsulation of nucleic acid polymers at low pH by interacting electrostatically with the negative charges of the phosphate backbone of mRNA, that also result in largely neutral systems at physiological pH values, thus alleviating problems associated with permanently-charged cationic lipids.

[00299] As used herein, "ionizable lipid" means an amine-containing lipid which can be easily protonated, and, for example, it may be a lipid of which charge state changes depending on the surrounding pH. The ionizable lipid may be protonated (positively charged) at a pH below the pKa of a cationic lipid, and it may be substantially neutral at a pH over the pKa. In one example,

the LNP may comprise a protonated ionizable lipid and/or an ionizable lipid showing neutrality. In some embodiments, the LNP has a pKa of 5 to 8, 5.5 to 7.5, 6 to 7, or 6.5 to 7. The pKa of the LNP is important for in vivo stability and release of the nucleic acid payload of the LNP in the target cell or organ. In some embodiments, the LNP having the foregoing pKa ranges may be safely delivered to a target organ (for example, the liver, lung, heart, spleen, as well as to tumors) and/or target cell (hepatocyte, LSEC, cardiac cell, cancer cell, etc.) in vivo, and inside endosomes, exhibit a positive charge to release the encapsulated payload through electrostatic interaction with an anionic lipids of the endosome membrane.

[00300] The ionizable lipid is an ionizable compound having characteristics similar to lipids generally, and through electrostatic interaction with a nucleic acid (for example, an mRNA of the disclosure), may play a role of encapsulating the nucleic acid payloads within the LNP with high efficiency.

[00301] According to the type of the amine and the tail group comprised in the ionizable lipid, (i) the nucleic acid encapsulation efficiency, (ii) PDI (polydispersity index) and/or (iii) the nucleic acid delivery efficiency to tissue and/or cells constituting an organ (for example, hepatocytes or liver sinusoidal endothelial cells in the liver) of the LNP may be different. In certain embodiments, the ionizable lipid is an ionizable cationic lipid, and comprises from about 25 mol % to about 66 mol % of the total lipid present in the particle.

[00302] The LNP comprising an ionizable lipid comprising an amine may have one or more kinds of the following characteristics: (1) the ability to encapsulate a nucleic acid with high efficiency; (2) uniform size of prepared particles (or having a low PDI value); and/or (3) excellent nucleic acid delivery efficiency to organs such as liver, lung, heart, spleen, bone marrow, as well as to tumors, and/or cells constituting such organs (for example, hepatocytes, LSEC, cardiac cells, cancer cells, etc.).

[00303] In particular embodiments, the cationic lipid form plays a crucial role both in nucleic acid encapsulation through electrostatic interactions and intracellular release by disrupting endosomal membranes. The nucleic acid payloads are encapsulated within the LNP by the ionic interactions they form with the positively charged cationic lipid. Non-limiting examples of ionizable cationic lipid components utilized in the LNP of the disclosure are selected from DLin-MC3-DMA (heptatriaconta-6,9,28,31-tetraen-19-yl4-(dimethylamino)butanoate), DLin- KC2-DMA (2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane), and TNT (1,3,5-triazinane-2,4,6-trione) and TT (N1,N3,N5-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide). Non-limiting

examples of helper lipids utilized in the LNP of the disclosure are selected from DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), POPC (2-Oleoyl-1- palmitoyl-sn-glycero-3-phosphocholine) and DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) DOPG, 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), sphingolipid, and ceramide. Cholesterol and PEG-DMG ((R)-2,3- bis(octadecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate), PEG-DSG (1,2-Distearoyl-rac-glycero-3-methylpolyoxyethylene glycol 2000), or DSPE-PEG2k (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]), are components utilized in the LNP of the disclosure for the stability, circulation, and size of the LNP.

[00304] In some embodiments, the cationic lipid in the LNP of the disclosure comprises a tertiary amine. In some embodiments, the tertiary amine includes alkyl chains connected to N of the tertiary amine with ether linkages. In some embodiments, the alkyl chains comprise C12-C30 alkyl chains having 0 to 3 double bonds. In some embodiments, the alkyl chains comprise C16-C22 alkyl chains. In some embodiments, the alkyl chains comprise C18 alkyl chains. A number of cationic lipids and related analogs have been described in U.S. Patent Publication Nos. 20060083780, 20060240554, 20110117125, 20190336608, 20190381180 and 20200121809; U.S. Pat. Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; 5,785,992; 9,738,593; 10,106,490; 10,166,298; 10,221,127; and 11,219,634; and PCT Publication No. WO 96/10390, the disclosures of which are herein incorporated by reference in their entirety.

[00305] In some embodiments, the cationic lipid in the LNP of the disclosure may comprise, for example, one or more ionizable cationic lipids wherein the ionizable cationic lipid is a dialkyl lipid. In other embodiments, the ionizable cationic lipid is a tetraalkyl lipid.

[00306] In some embodiments, the cationic lipid in the LNP of the disclosure is selected from 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-

morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3-(N—(N′,N′-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5′-(cholest-5-en-3-beta-oxy)-3′-oxapentoxy)-3-dimethyl-1-(cis,cis-9′,1-2′-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N′-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N′-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), and any combination of the forgoing.

[00307] In some embodiments, the cationic lipid in the LNP of the disclosure is selected from heptatriaconta-6,9,28,31-tetraen-19-yl4-(dimethylamino)butanoate (DLin-MC3-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin- KC2-DMA), (1,3,5-triazinane-2,4,6-trione) (TNT), N1,N3,N5-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide (TT), and any combination of the forgoing.

[00308] In some embodiments, the N/P ratio (nitrogen from the cationic/ionizable lipid and phosphate from the nucleic acid) in the LNP of the disclosure is in the range of is about 3:1 to 7:1, or about 4:1 to 6:1, or is 3:1, or is 4:1, or is 5:1, or is 6:1, or is 7:1, or is 8:1, or is 9:1.

Conjugated Lipid

[00309] In some embodiments, the LNPs and LNP compositions of the present disclosure include at least one conjugated lipid. In some embodiments, the conjugated lipid may be selected from a polyethyleneglycol (PEG)-lipid conjugate, a polyamide (ATTA)-lipid conjugate, a

cationic-polymer-lipid conjugate (CPL), and any combination of the foregoing. In some cases, conjugated lipids can inhibit aggregation of the LNPs of the disclosure.

[00310] In some embodiments, the conjugated lipid of the LNP of the disclosure comprises a pegylated lipid. The terms “polyethyleneglycol (PEG)-lipid conjugate,” “pegylated lipid” "lipid-PEG conjugate", "lipid-PEG", "PEG-lipid", "PEG-lipid", or "lipid-PEG" are used interchangeably herein and refer to a lipid attached to a polyethylene glycol (PEG) polymer which is a hydrophilic polymer. The pegylated lipid contributes to the stability of the LNPs and LNP compositions and reduces aggregation of the LNPs. In other embodiments, the lipid of the LNP comprises peptide modified PEG lipids that are used for targeting cell surface receptors Ex: DSPE-PEG-RGD, DSPE-PEG-Transferrin, DSPE-PEG-cholesterol.

[00311] As the PEG-lipid can form the surface lipid, the size of the LNP can be readily varied by varying the proportion of surface (PEG) lipid to the core (ionizable cationic) lipids. In some embodiments, the PEG-lipid of the LNP of the disclosure can be varied from ~1 to 5 mol% to modify particle properties such as size, stability, and circulation time.

[00312] The lipid-PEG conjugate contributes to the particle stability in serum of the nanoparticle within the LNP, and plays a role of preventing aggregation between nanoparticles. In addition, the lipid-PEG conjugate may protect nucleic acids, such as mRNAs encoding the repressor fusion proteins of the disclosure, or gRNAs of the disclosure, from degrading enzymes during in vivo delivery of the nucleic acids and enhance the stability of the nucleic acids in vivo and increase the half-life of the delivered nucleic acids encapsulated in the nanoparticle. Examples of PEG-lipid conjugates include, but are not limited to, PEG-DAG conjugates, PEG-DAA conjugates, and mixtures thereof. In certain embodiments, the PEG-lipid conjugate is selected from the group consisting of a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, a PEG-phospholipid conjugate, a PEG-ceramide (PEG-Cer) conjugate, and a mixture thereof.

[00313] In some embodiments, the pegylated lipid of the LNP of the disclosure is selected from a PEG-ceramide, a PEG-diacylglycerol, a PEG-dialkyloxypropyl, a PEG-dialkoxypropylcarbamate, a PEG-phosphatidylethanoloamine, a PEG-phospholipid, a PEG-succinate diacylglycerol, and any combination of the foregoing.

[00314] In some embodiments, the pegylated lipid of the LNP of the disclosure is a PEG-dialkyloxypropyl. In some embodiments, the pegylated lipid is selected from PEG-didecyloxypropyl (C10), PEG-dilauryloxypropyl (C12), PEG-dimyristyloxypropyl (C14), PEG-

dipalmityloxypropyl (C16), PEG-distearyloxypropyl (C18), and any combination of the foregoing.

[00315] In other embodiments, the lipid-PEG conjugate of the LNP of the disclosure may be PEG bound to phospholipid such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramide (PEG-CER, ceramide-PEG conjugate, ceramide-PEG, cholesterol or PEG conjugated to derivative thereof, PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPC, PEG-DSPE(DSPE-PEG), and a mixture thereof, and for example, may be C16-PEG2000 ceramide (N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]}), DMG-PEG 2000, 14:0 PEG2000 PE.

[00316] In some embodiments, the pegylated lipid of the LNP of the disclosure is selected from 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol, 4-O-(2′,3′-di(tetradecanoyloxy)propyl-1-O-(ω-methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), ω-methoxy(polyethoxy)ethyl-N-(2,3-di(tetradecanoxy)propyl)carbamate, 2,3-di(tetradecanoxy)propyl-N-(ω-methoxy(polyethoxy)ethyl)carbamate, and any combination of the foregoing.

[00317] In some embodiments, the pegylated lipid of the LNP of the disclosure is selected from mPEG2000-1,2-di-O-alkyl-sn3-carbomoylglyceride (PEG-C-DOMG), 1-[8′-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3′,6′-dioxaoctanyl]carbamoyl-w-methyl-poly(ethylene glycol) (2 KPEG-DMG), and any combination of the foregoing.

[00318] In some embodiments, the PEG is directly attached to the lipid of the pegylated lipid. In other embodiments, the PEG is attached to the lipid of the pegylated lipid by a linker moiety selected from an ester-free linker moiety or an ester-containing linker moiety. Non-limiting examples of the ester-free linker moiety include amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulfide (-S-S-), ether (-O-), succinyl (-(O)CCH2CH2C(O)-), succinamidyl (-NHC(O)CH2CH2C(O)NH-), ether, disulfide and combinations thereof. For example, the linker may contain a carbamate linker moiety and an amido linker moiety. Non-limiting examples of the ester-containing linker moiety include carbonate (-OC(O)O-), succinoyl, phosphate ester (-O-(O)POH-O-), sulfonate ester, and combinations thereof.

[00319] The PEG moiety of the pegylated lipid of the LNP of the disclosure described herein may have an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain embodiments, the PEG moiety has an average molecular weight of from about 750

daltons to about 5,000 daltons, about 1,000 daltons to about 4,000 daltons, about 1,500 daltons to about 3,000 daltons, about 750 daltons to about 3,000 daltons, or about 1750 daltons to about 2,000 daltons.

[00320] In some embodiments, the conjugated lipid (*e.g.*, pegylated lipid) comprises from about 1 mol % to about 60 mol %, from about 2 mol % to about 50 mol %, from about 5 mol % to about 40 mol %, or from about 5 mol % to about 20 mol % of the total lipid present in the LNPs and/or LNP compositions. In certain embodiments, the conjugated lipid comprises from about 0.5 mol % to about 3 mol % of the total lipid present in the particle.

[00321] In additional embodiments, the conjugated lipid (*e.g.*, pegylated lipid) of the LNP of the disclosure comprises at least about 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 mol %, or an intermediate range of any of the foregoing, of the total lipid present in the LNPs and/or LNP compositions.

[00322] For the lipid in the lipid-PEG conjugate of the LNP of the disclosure, any lipid capable of binding to polyethyleneglycol may be used without limitation, and the phospholipid and/or cholesterol which are other elements of the LNP may be also used. In some embodiments, the lipid in the lipid-PEG conjugate may be ceramide, dimyristoylglycerol (DMG), succinoyl-diacylglycerol (s-DAG), distearoylphosphatidylcholine (DSPC), distearoylphosphatidylethanolamine (DSPE), or cholesterol, but not limited thereto.

[00323] In the lipid-PEG conjugate of the LNP of the disclosure, the PEG may be directly conjugated to the lipid or linked to the lipid via a linker moiety. Any linker moiety suitable for binding PEG to the lipid may be used, and for example, includes an ester-free linker moiety and an ester-containing linker moiety. The ester-free linker moiety includes not only amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulfide (-S-S-), ether (-O-), succinyl (-(O)CCH2CH2C(O)-), succinamidyl (-NHC(O)CH2CH2C(O)NH-), ether, disulfide but also combinations thereof (for example, a linker containing both a carbamate linker moiety and an amido linker moiety), but not limited thereto. The ester-containing linker moiety includes for example, carbonate (-OC(O)O-), succinoyl, phosphate ester (-O-(O)POH-O-), sulfonate ester, and combinations thereof, but not limited thereto.

Steroids

[00324] In some embodiments, the LNPs and LNP compositions of the present disclosure include at least one steroid or derivative thereof. In some embodiments, the steroid comprises

cholesterol. In some embodiments, the LNPs and LNP compositions comprise a cholesterol derivative selected from cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2′-hydroxyethyl ether, cholesteryl-4′-hydroxybutyl ether, and any combination of the foregoing.

[00325] In some embodiments, the steroid (*e.g.*, cholesterol) of the LNP of the disclosure comprises from about 1 mol % to about 65 mol %, from about 2 mol % to about 50 mol %, from about 5 mol % to about 40 mol %, or from about 5 mol % to about 20 mol % of the total lipid present in the LNPs and/or LNP compositions. In other embodiments, the steroid (*e.g.*, cholesterol) of the LNP of the disclosure comprises at least about 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 mol %, or an intermediate range of any of the foregoing, of the total lipid present in the LNPs and/or LNP compositions.

Helper lipid/Helper lipid or Structural Lipid

[00326] In some embodiments, the LNPs and LNP compositions of the present disclosure include at least one helper lipid. In some embodiments, the helper lipid is non-cationic lipid selected from an anionic lipid, a neutral lipid, or both. In some embodiments, the helper lipid comprises at least one phospholipid. In some embodiments, the phospholipid is selected from an anionic phospholipid, a neutral phospholipid, or both. The phospholipid of the elements of the LNPs and LNP compositions can play a role in covering and protecting a core of the LNP formed by interaction of the cationic lipid and nucleic acid in the LNP, and may facilitate cell membrane permeation and endosomal escape during intracellular delivery of the nucleic acid by binding to the phospholipid bilayer of a target cell. A phospholipid which can promote fusion of the LNP to a cell may include without limitation, any of the phospholipids selected from the group described below.

[00327] In some embodiments, the LNPs and LNP compositions comprise at least one phospholipid selected from, but not limited to, dipalmitoyl-phosphatidylcholine (DPPC), distearoyl-phosphatidylcholine (DSPC), dioleoyl-phosphatidylethanolamine (DOPE), dioleoyl-phosphatidylcholine (DOPC), dioleoyl-phosphatidylglycerol (DOPG), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dipalmitoyl-phosphatidylethanolamine (DPPE), dipalmitoyl-phosphatidylglycerol (DPPG), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), egg phosphatidylcholine (EPC),

phosphatidylethanolamine (PE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (DOPS), 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine], and any combination of the foregoing. In one example, the LNP comprising DOPE may be effective in mRNA delivery (excellent drug delivery efficacy).

[00328] In some embodiments, the helper lipid (*e.g.*, phospholipid) of the LNP of the disclosure comprises from about 1 mol % to about 60 mol %, from about 2 mol % to about 50 mol %, from about 5 mol % to about 40 mol %, or from about 5 mol % to about 20 mol % of the total lipid present in the LNPs and/or LNP compositions. In other embodiments, the helper lipid (*e.g.*, phospholipid) of the LNP of the disclosure comprises at least about 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 mol %, or an intermediate range of any of the foregoing, of the total lipid present in the LNPs and/or LNP compositions.

[00329] It will be appreciated that the total lipid present in the LNPs and/or LNP compositions comprises the lipids as individual or in combination of the cationic lipid or ionizable cationic lipid, the conjugated lipid, (e.g., pegylated lipid), the peptide conjugated PEG lipid, the steroid (e.g., cholesterol), peptide conjugated-structural lipid (Ex: DSPE-cRGD) and the structural lipid (e.g., phospholipid), leading from LNP formulation containing one to multi-component but not limited to one, two, three, four or five components in an LNP formulation.

[00330] The LNPs and/or LNP compositions may be prepared by dissolving the total lipids (or a portion thereof) in an organic solvent (e.g., ethanol) followed by mixing through a micromixer with the payload (e.g., nucleic acids of the systems) dissolved in an acidic buffer (e.g., pH between 1.0-6.5). At this pH the ionizable cationic lipid is positively charged and interacts with the negatively-charged nucleic acid polymers. The resulting nanostructures containing the nucleic acids are then converted to neutral LNPs when dialyzed against a neutral buffer which also includes removal of the organic solvent (e.g., ethanol) during the exchange of LNPs into physiologically relevant buffer. The LNPs and/or LNP compositions thus formed have a distinct electron-dense nanostructured core where the cationic lipids are organized into inverted micelles around the encapsulated payload, as opposed to traditional bilayer liposomal structures. In another embodiment, the LNP may form a bleb-like structure with nucleic acids in aqueous pockets along the non-electron dense lipid core.

b. **Lipid nanoparticle properties**

[00331] The LNPs and/or LNP compositions may be prepared by dissolving the total lipids (or a portion thereof) in an organic solvent (e.g., ethanol) followed by mixing through a micromixer with the payload (e.g., nucleic acids of the systems) dissolved in an acidic buffer (e.g., pH between 1.0-6.5). At this pH the ionizable cationic lipid is positively charged and interacts with the negatively-charged nucleic acid polymers. The resulting nanostructures containing the nucleic acids are then converted to neutral LNPs when dialyzed against a neutral buffer which also includes removal of the organic solvent (e.g., ethanol) during the exchange of LNPs into physiologically relevant buffer. The LNPs and/or LNP compositions thus formed have a distinct electron-dense nanostructured core where the cationic lipids are organized into inverted micelles around the encapsulated payload, as opposed to traditional bilayer liposomal structures. In another embodiment, the LNP may form a bleb-like structure with nucleic acids in aqueous pockets along the non-electron dense lipid core.

[00332] In some embodiments, the LNPs and/or LNP compositions of the disclosure comprise cationic lipid : helper lipid (*e.g.*, phospholipid) : steroid (*e.g.*, cholesterol) : conjugated lipid, (*e.g.*, pegylated lipid) at a molar ratio of 20 to 50:10 to 30:30 to 60:0.5 to 5, at a molar ratio of 25 to 45:10 to 25:40 to 50:0.5 to 3, at a molar ratio of 25 to 45:10 to 20:40 to 55:0.5 to 3, or at a molar ratio of 25 to 45:10 to 20:40 to 55:1.0 to 1.5.

[00333] In some embodiments, the LNPs and/or LNP compositions of the disclosure have a total lipid : payload ratio (mass/mass) of from about 1 to about 100. In some embodiments, the total lipid : payload ratio is about 1 to about 50, from about 2 to about 25, from about 3 to about 20, from about 4 to about 15, or from about 5 to about 10. In some embodiments, the total lipid : payload ratio is about 5 to about 15, *e.g.*, about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or an intermediate range of any of the foregoing.

[00334] In certain embodiments, the LNPs of the disclosure comprise a total lipid: nucleic acid mass ratio of from about 5:1 to about 15:1. In some embodiments, the weight ratio of the cationic lipid and nucleic acid comprised in the LNP may be 1 to 20:1, 1 to 15:1, 1 to 10:1, 5 to 20:1, 5 to 15:1, 5 to 10:1, 7.5 to 20:1, 7.5 to 15:1, or 7.5 to 10:1.

[00335] In some embodiments, the LNP of the disclosure may comprise the cationic lipid of 20 to 50 parts by weight, the phospholipid of 10 to 30 parts by weight, cholesterol of 20 to 60 parts by weight (or 20 to 60 parts by weight), and lipid-PEG conjugate of 0.1 to 10 parts by weight (or 0.25 to 10 parts by weight, 0.5 to 5 parts by weight). Alternatively, the LNP may comprise the

cationic lipid of 20 to 50 % by weight, phospholipid of 10 to 60 % by weight, cholesterol of 20 to 60 % by weight (or 30 to 60 % by weight), and lipid-PEG conjugate of 0.1 to 10 % by weight (or 0.25 to 10 % by weight, 0.5 to 5 % by weight) based on the total nanoparticle weight. As a further alternative, the LNP may comprise the cationic lipid of 25 to 50 % by weight, phospholipid of 10 to 20 % by weight, cholesterol of 35 to 55 % by weight, and lipid-PEG conjugate of 0.1 to 10 % by weight (or 0.25 to 10 % by weight, 0.5 to 5 % by weight), based on the total nanoparticle weight.

[00336] In some embodiments, the LNPs of the present disclosure have a mean diameter of from about 20 to 200 nm, 20 to 180 nm, 20 to 170 nm, 20 to 150 nm, 20 to 120 nm, 20 to 100 nm, 20 to 90 nm, 30 to 200 nm, 30 to 180 nm, 30 to 170 nm, 30 to 150 nm, 30 to 120 nm, 30 to 100 nm, 30 to 90 nm, 40 to 200 nm, 40 to 180 nm, 40 to 170 nm, 40 to 150 nm, 40 to 120 nm, 40 to 100 nm, 40 to 90 nm, 40 to 80 nm, 40 to 70 nm, 50 to 200 nm, 50 to 180 nm, 50 to 170 nm, 50 to 150 nm, 50 to 120 nm, 50 to 100 nm, 50 to 90 nm, 60 to 200 nm, 60 to 180 nm, 60 to 170 nm, 60 to 150 nm, 60 to 120 nm, 60 to 100 nm, 60 to 90 nm, 70 to 200 nm, 70 to 180 nm, 70 to 170 nm, 70 to 150 nm, 70 to 120 nm, 70 to 100 nm, 70 to 90 nm, 80 to 200 nm, 80 to 180 nm, 80 to 170 nm, 80 to 150 nm, 80 to 120 nm, 80 to 100 nm, 80 to 90 nm, 90 to 200 nm, 90 to 180 nm, 90 to 170 nm, 90 to 150 nm, 90 to 120 nm, or 90 to 100 nm, or an intermediate range of any of the foregoing.

[00337] In some embodiments, the LNPs and/or LNP compositions of the disclosure have a positive charge at acidic pH and may encapsulate the payload (e.g., therapeutic agent) through electrostatic interaction produced by negative charges of the payload (e.g., therapeutic agent). The term “encapsulation,” refers to the mixture of lipids surrounding and embedding the payload (e.g., therapeutic agent) at physiological conditions, forming the LNPs. The term “encapsulation efficiency,” as used herein is the percent amount of payload (e.g., therapeutic agent) encapsulated by the LNPs. It is a measure of payload (e.g., therapeutic agent) in bulk before disruption of LNPs divided by the total amount of payload (e.g., therapeutic agent) measured in bulk post-disruption of LNPs using a surfactant based reagent such as 1-2% Triton X-100. The encapsulation efficiency of the LNPs and/or LNP compositions may be 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 94% or more, or 95% or more. In other embodiments, the encapsulation efficiency of the LNPs and/or LNP compositions is about 80% to 99%, about 85% to 98%, about 88% to 95%, about 90% to 95%, or the payload (e.g., nucleic acids of the systems) may be fully encapsulated within the lipid

portion of the LNPs compositions, and thereby protected from enzymatic degradation. In some embodiments, the payload (e.g., therapeutic agent) is not substantially degraded after exposure of the LNPs and/or LNP compositions to a nuclease at 37° C. for at least about 20, 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In some embodiments, the payload (e.g., nucleic acids of the systems) is complexed with the lipid portion of the LNPs and/or LNP compositions. The LNPs and/or LNP compositions of the present disclosure are non-toxic to mammals such as humans.

[00338] The term "fully encapsulated" indicates that the payload (*e.g.*, the nucleic acids of the system) in the LNPs and/or LNP compositions is not significantly degraded after exposure to conditions that significantly degrade free DNA, RNA, or protein. In a fully encapsulated system, less than about 25%, more preferably less than about 10%, and most preferably less than about 5% of the payload (*e.g.*, nucleic acids of the system) in the LNPs and/or LNP compositions is degraded by conditions that would degrade 100% of a non-encapsulated payload. "Fully encapsulated" also indicates that the LNPs and/or LNP compositions are serum-stable, and do not decompose into their component parts immediately upon exposure to serum proteins post in vivo administration and protects the cargo until endosomal escape and release into cytoplasm of the cell.

[00339] In some embodiments, the amount of the LNPs and/or LNP compositions having the payload (*e.g.*, therapeutic agent), encapsulated therein is from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, %, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or an intermediate range of any of the foregoing.

[00340] In some embodiments, the amount of the payload (*e.g.*, the nucleic acids), encapsulated within the LNPs and/or LNP compositions is from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about

30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, %, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or an intermediate range of any of the foregoing.

[00341] In some embodiments, the nucleic acids of the disclosure, such as the mRNA encoding the repressor fusion protein, and/or the gRNA, may be provided in a solution to be mixed with a lipid solution such that the nucleic acids may be encapsulated in the lipid nanoparticles. A suitable nucleic acid solution may be any aqueous solution containing the nucleic acid to be encapsulated at various concentrations. For example, a suitable nucleic acid solution may contain the nucleic acid (or nucleic acids) at a concentration of or greater than about 0.01 mg/ml, 0.05 mg/ml, 0.06 mg/ml, 0.07 mg/ml, 0.08 mg/ml, 0.09 mg/ml, 0.1 mg/ml, 0.15 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 1.25 mg/ml, 1.5 mg/ml, 1.75 mg/ml, or 2.0 mg/ml. In some embodiments, the nucleic acid comprises an mRNA encoding a repressor fusion protein, and a suitable mRNA solution may contain the mRNA at a concentration ranging from about 0.01-2.0 mg/ml, 0.01-1.5 mg/ml, 0.01-1.25 mg/ml, 0.01-1.0 mg/ml, 0.01-0.9 mg/ml, 0.01-0.8 mg/ml, 0.01-0.7 mg/ml, 0.01-0.6 mg/ml, 0.01-0.5 mg/ml, 0.01-0.4 mg/ml, 0.01-0.3 mg/ml, 0.01-0.2 mg/ml, 0.01-0.1 mg/ml, 0.05-1.0 mg/ml, 0.05-0.9 mg/ml, 0.05-0.8 mg/ml, 0.05-0.7 mg/ml, 0.05-0.6 mg/ml, 0.05-0.5 mg/ml, 0.05-0.4 mg/ml, 0.05-0.3 mg/ml, 0.05-0.2 mg/ml, 0.05-0.1 mg/ml, 0.1-1.0 mg/ml, 0.2-0.9 mg/ml, 0.3-0.8 mg/ml, 0.4-0.7 mg/ml, or 0.5-0.6 mg/ml. In some embodiments, a suitable mRNA solution may contain an mRNA at a concentration up to about 5.0 mg/ml, 4.0 mg/ml, 3.0 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.9 mg/ml, 0.8 mg/ml, 0.7 mg/ml, 0.6 mg/ml, 0.5 mg/ml, 0.4 mg/ml, 0.3 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.04 mg/ml, 0.03 mg/ml, 0.02 mg/ml, 0.01 mg/ml, or 0.05 mg/ml. In some embodiments, a suitable gRNA solution may contain an gRNA at a concentration up to about 5.0 mg/ml, 4.0 mg/ml, 3.0 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.9 mg/ml, 0.8 mg/ml, 0.7 mg/ml, 0.6 mg/ml, 0.5 mg/ml, 0.4 mg/ml, 0.3 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.04 mg/ml, 0.03 mg/ml, 0.02 mg/ml, 0.01 mg/ml, or 0.05 mg/ml.

[00342] In some embodiments, the LNP may have an average diameter of 20nm to 200nm, 20 to 180nm, 20nm to 170nm, 20nm to 150nm, 20nm to 120nm, 20nm to 100nm, 20nm to 90nm,

30nm to 200nm, 30 to 180nm, 30nm to 170nm, 30nm to 150nm, 30nm to 120nm, 30nm to 100nm, 30nm to 90nm, 40nm to 200nm, 40 to 180nm, 40nm to 170nm, 40nm to 150nm, 40nm to 120nm, 40nm to 100nm, 40nm to 90nm, 40nm to 80nm, 40nm to 70nm, 50nm to 200nm, 50 to 180nm, 50nm to 170nm, 50nm to 150nm, 50nm to 120nm, 50nm to 100nm, 50nm to 90nm, 60nm to 200nm, 60 to 180nm, 60nm to 170nm, 60nm to 150nm, 60nm to 120nm, 60nm to 100nm, 60nm to 90nm, 70nm to 200nm, 70 to 180nm, 70nm to 170nm, 70nm to 150nm, 70nm to 120nm, 70nm to 100nm, 70nm to 90nm, 80nm to 200nm, 80 to 180nm, 80nm to 170nm, 80nm to 150nm, 80nm to 120nm, 80nm to 100nm, 80nm to 90nm, 90nm to 200nm, 90 to 180nm, 90nm to 170nm, 90nm to 150nm, 90nm to 120nm, or 90nm to 100nm for easy introduction into liver tissue, hepatocytes and/or LSEC (liver sinusoidal endothelial cells). The LNP may be sized for easy introduction into organs or tissues, including but not limited to liver, lung, heart, spleen, as well as to tumors. When the size of the LNP is smaller than the above range, it can be difficult to maintain stability as the surface area of the LNP is excessively increased, and thus delivery to the target tissue and/or drug effect may be reduced. The LNP may specifically target liver tissue. Without wishing to be bound by theory, it is thought that one mechanism by which LNP may be used to deliver therapeutic agents is through the imitation of the metabolic behaviors of natural lipoproteins, and so LNP may be usefully delivered to a subject through the lipid metabolism processes carried out by the liver. During the delivery of therapeutic agents to hepatocytes or and/or LSEC (liver sinusoidal endothelial cells), the diameter of the fenestrae leading from the sinusoidal lumen to the hepatocytes and LSEC is about 140 nm in mammals and about 100 nm in humans, so the LNP composition for therapeutic agent delivery having LNPs with a diameter in the above ranges may have excellent delivery efficiency to hepatocytes and LSEC when compared to LNP having the diameter outside the above range.

[00343] According to one example, the LNPs of the LNP composition may comprise the ionizable cationic lipid : phospholipid : cholesterol : lipid-PEG conjugate in the range described above or at a molar ratio of 20 to 50:10 to 30:30 to 60:0.5 to 5, at a molar ratio of 25 to 45:10 to 25:40 to 50:0.5 to 3, at a molar ratio of 25 to 45:10 to 20:40 to 55:0.5 to 3, or at a molar ratio of 25 to 45:10 to 20:40 to 55:1.0 to 1.5. The LNP comprising components at a molar ratio in the above range may have excellent delivery efficiency of therapeutic agents specific to cells of target organs.

[00344] In certain aspects, the LNP exhibit a positive charge under the acidic pH condition by showing a pKa of 5 to 8, 5.5 to 7.5, 6 to 7, or 6.5 to 7, and may encapsulate a nucleic acid with high efficiency by easily forming a complex with a nucleic acid through electrostatic interaction with a therapeutic agent such as a nucleic acid showing a negative charge. In such cases, the LNP may be usefully used as a composition for intracellular or *in vivo* delivery of a therapeutic agent (for example, nucleic acid).

[00345] Herein, "encapsulate" or "encapsulation" refers to incorporation of a therapeutic agent efficiently inside a lipid envelope , i.e., by surrounding it by the particle surface and/or embedding it within the particle interior made of various lipids that self-assemble when the polarity of the solvent surrounding them is increased. The encapsulation efficiency means the content of the therapeutic agent encapsulated in the LNP relative the total therapeutic agent content measured per given volume of the LNP formulation measured post-disruption of the LNPs.

[00346] The encapsulation of the nucleic acids of the composition in the LNP may be 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 94% or more, or 95% or more of LNP in the composition encapsulate nucleic acids. In some embodiments, the encapsulation of the nucleic acids of the composition in the LNP is such that between 80% to 99%, between 80% to 97%, between 80% to 95%, between 85% to 95%, between 87% to 95%, between 90% to 95%, between 91% or more to 95% or less, 91% or more to 94% or less, over 91% to 95% or less, 92% to 99%, between 92% to 97%, or between 92% to 95% of the LNP in the composition encapsulate nucleic acids. In some embodiments, the mRNA encoding the repressor fusion protein and/or a gRNA of any of the embodiments of the disclosure are fully encapsulated in the LNP.

[00347] The target organs to which a nucleic acid is delivered by the LNP include, but are not limited to the liver, lung, heart, spleen, as well as to tumors. The LNP according to one example is liver tissue-specific and has excellent biocompatibility and can deliver the nucleic acids of a composition with high efficiency, and thus it can be usefully used in related technical fields such as lipid nanoparticle-mediated gene therapy. In a particular embodiment, the target cell to which the nucleic acids are delivered by the LNP according to one example may be a hepatocyte and/or LSEC *in vivo*. In other embodiments, the disclosure provides LNP formulated for delivery of the nucleic acids of the embodiments to cells *ex vivo*.

[00348] The disclosure provides a pharmaceutical composition comprising a plurality of LNPs comprising nucleic acids, such as mRNA encoding repressor fusion protein and/or a gRNA variant described herein, and a pharmaceutically acceptable carrier.

[00349] In certain embodiments, the LNP comprising the nucleic acid(s) has an electron dense core.

[00350] The disclosure provides LNP comprising one or more nucleic acids comprising: (a) an mRNA encoding the repressor fusion protein, and/or a gRNA variant described herein; (b) one or more cationic lipids or ionizable cationic lipids or salts thereof comprising from about 20 mol % to about 60 mol % of the total lipid present in the LNP; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the LNP; and (d) one or more conjugated lipids that inhibit aggregation of LNPs comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle. In another embodiment, the disclosure provides LNP comprising one or more nucleic acids comprising: (a) an mRNA encoding the repressor fusion protein, and/or a gRNA variant described herein; (b) one or more cationic lipids or ionizable cationic lipids or salts thereof comprising from about 22 mol % to about 85 mol % of the total lipid present in the LNP; (c) one or more non-cationic/phospholipids comprising from about 10 mol % to about 70 mol % of the total lipid present in the LNP; (d) 15 mol % to about 50 mol % sterol, and (d) 1 mol % to about 5 mol % lipid-PEG or lipid-PEG-peptide in the particle. In certain embodiments the repressor fusion protein mRNA and gRNA may be present in the same nucleic acid-lipid particle, or they may be present in different nucleic acid-lipid particles.

[00351] The disclosure provides LNP comprising one or more nucleic acids comprising: (a) an mRNA encoding the repressor fusion proteins described herein; (b) a cationic lipid or a salt thereof comprising from about 52 mol % to about 62 mol % of the total lipid present in the LNP; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the LNP; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the LNP. In particular embodiments, the formulation is a four-component system comprising about 1.4 mol % PEG-lipid conjugate (*e.g.*, PEG2000-C-DMA), about 57.1 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, about 7.1 mol % DPPC (or DSPC), and about 34.3 mol % cholesterol (or derivative thereof).

[00352] In other embodiments, the LNP comprising one or more nucleic acids comprises: (a) an mRNA encoding the repressor fusion proteins and/or a gRNA of any of the embodiments described herein; (b) a cationic lipid or a salt thereof comprising from about 46.5 mol % to about 66.5 mol % of the total lipid present in the LNP; (c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the LNP; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the LNP. In particular embodiments, the formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-lipid conjugate (*e.g.*, PEG2000-C-DMA), about 61.5 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, and about 36.9 mol % cholesterol (or derivative thereof).

[00353] Additional formulations are described in PCT Publication No. WO 09/127060 and US patent publication numbers US 2011/0071208 A1 and US 2011/0076335 A1, the disclosures of which are herein incorporated by reference in their entirety.

[00354] In other embodiments, the LNP comprising one or more nucleic acids comprises: (a) an mRNA encoding the repressor fusion protein and a gRNA of any of the embodiments described herein; (b) one or more cationic lipid or ionizable cationic lipids or salts thereof comprising from about 2 mol % to about 50 mol % of the total lipid present in the LNP; (c) one or more non-cationic lipid or ionizable cationic lipids comprising from about 5 mol % to about 90 mol % of the total lipid present in the LNP; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 20 mol % of the total lipid present in the LNP.

[00355] In other embodiments, the LNP comprising one or more nucleic acids comprises: (a) an mRNA encoding the repressor fusion protein and a gRNA of any of the embodiments described herein; (b) a cationic lipid or a salt thereof comprising from about 30 mol % to about 50 mol % of the total lipid present in the LNP; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 47 mol % to about 69 mol % of the total lipid present in the LNP; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 3 mol % of the total lipid present in the LNP. In particular embodiments, the formulation is a four-component system which comprises about 2 mol % PEG-lipid conjugate (*e.g.*, PEG2000-C-DMA), about 40 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, about 10 mol % DPPC (or DSPC), and about 48 mol % cholesterol (or derivative thereof).

[00356] In other embodiments, the LNP comprising one or more nucleic acids comprises: (a) an mRNA encoding the repressor fusion protein and a gRNA of any of the embodiments described herein; (b) one or more cationic lipid or ionizable cationic lipids or salts thereof comprising from about 50 mol % to about 65 mol % of the total lipid present in the LNP; (c) one or more non-cationic lipid or ionizable cationic lipids comprising from about 25 mol % to about 45 mol % of the total lipid present in the LNP; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 5 mol % to about 10 mol % of the total lipid present in the LNP.

[00357] In other embodiments, the LNP comprising one or more nucleic acids comprises: (a) an mRNA encoding the repressor fusion protein and a gRNA of any of the embodiments described herein; (b) a cationic lipid or a salt thereof comprising from about 50 mol % to about 60 mol % of the total lipid present in the LNP; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 35 mol % to about 45 mol % of the total lipid present in the LNP; and (d) a PEG-lipid conjugate comprising from about 5 mol % to about 10 mol % of the total lipid present in the LNP.

[00358] In certain embodiments, the non-cationic lipid mixture in the formulation comprises: (i) a phospholipid of from about 10 mol % to about 70 mol % of the total lipid present in the LNP; (ii) cholesterol or a derivative thereof of from about 15 mol % to about 50 mol % of the total lipid present in the LNP; and 1-5% lipid-PEG or lipid-PEG-peptide. In particular embodiments, the formulation is a four-component system which comprises about 7 mol % PEG-lipid conjugate (*e.g.*, PEG750-C-DMA), about 54 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, about 7 mol % DPPC (or DSPC), and about 32 mol % cholesterol (or derivative thereof).

[00359] In other embodiments, the LNP comprising one or more nucleic acids comprises: (a) an mRNA encoding the repressor fusion protein and/or a gRNA of any of the embodiments described herein; (b) a cationic lipid or a salt thereof comprising from about 55 mol % to about 65 mol % of the total lipid present in the LNP; (c) cholesterol or a derivative thereof comprising from about 30 mol % to about 40 mol % of the total lipid present in the LNP; and (d) a PEG-lipid conjugate comprising from about 5 mol % to about 10 mol % of the total lipid present in the LNP. In particular embodiments, the formulation is a three-component system which is phospholipid-free and comprises about 7 mol % PEG-lipid conjugate (*e.g.*, PEG750-C-DMA),

about 58 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, and about 35 mol % cholesterol (or derivative thereof).

[00360] In other embodiments, the LNP comprising one or more nucleic acids comprises: (a) an mRNA encoding the repressor fusion protein and/or a gRNA of any of the embodiments described herein; (b) a cationic lipid or a salt thereof comprising from about 48 mol % to about 62 mol % of the total lipid present in the LNP; (c) a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises about 7 mol % to about 17 mol % of the total lipid present in the LNP, and wherein the cholesterol or derivative thereof comprises about 25 mol % to about 40 mol % of the total lipid present in the LNP; and (d) a PEG-lipid conjugate comprising from about 0.5 mol % to about 3.0 mol % of the total lipid present in the LNP.

VIII. Systems and Methods for Repression of PCSK9 Target Nucleic Acids

[00361] In another aspect, the present disclosure provides systems comprising a repressor fusion protein comprising a catalytically dead CRISPR protein, and one or more gRNAs (repressor fusion protein:gRNA system), for use in repressing a target nucleic acid of a *PCSK9* gene in a population of cells. The systems provided herein are useful for various applications, including as therapeutics, diagnostics, and for research. To effect the methods of the disclosure, resulting in repression or silencing of the *PCSK9* gene, provided herein are programmable repressor fusion protein:gRNA systems. The programmable nature of the systems provided herein allows for the precise targeting to achieve the desired effect at one or more regions of predetermined interest in the *PCSK9* gene target nucleic acid. In some embodiments, it may be desirable to reduce or eliminate expression of the PCSK9 protein in a subject comprising mutations, for example dominant mutations leading to hypercholesterolemia or familial or autosomal dominant hypercholesterolemia. In some embodiments, it may be desirable to reduce or eliminate expression of the PCSK9 protein in a subject with elevated cholesterol levels that is not the result of mutations in the *PCSK9* gene.

[00362] In some embodiments, the disclosure provides systems specifically designed for use in the methods to repress or silence transcription the target nucleic acid of a *PCSK9* gene in eukaryotic cells; either in vitro, ex vivo, or in vivo in a subject. Generally, any portion of the gene can be targeted using the programmable systems and methods provided herein. In one embodiment, the disclosure provides for a method of repressing a target nucleic acid sequence of a *PCSK9* gene in a population of cells, the method comprising introducing into each cell of the

population: i) a repressor fusion protein:gRNA system comprising a repressor fusion protein and a gRNA of any of the embodiments described herein; ii) a nucleic acid encoding the repressor fusion protein and gRNA of any of the embodiments described herein; iii) a vector selected from the group consisting of a retroviral vector, a lentiviral vector, an adenoviral vector, an adeno-associated viral (AAV) vector, and a herpes simplex virus (HSV) vector, and comprising the nucleic acid of (iv), above; v) an LNP or a synthetic nanoparticle comprising a gRNA and a mRNA encoding the repressor fusion protein; or vi) combinations of two or more of (i) to (v), wherein transcription of the target nucleic acid sequence of the cells targeted by the gRNA is repressed by the repressor fusion protein. In some embodiments of the method, contacting cells with a repressor fusion protein:gRNA system of the embodiments results in repression of the *PCSK9* target nucleic acid of at least about 1%, at least about 2%, at least about 3%, at least about 4%, at least about 5%, at least about 6%, at least about 7%, at least about 8%, at least about 9%, or at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60% or more of the cells of the population. In some embodiments of the method, the *PCSK9* gene in the cells of the population is repressed or silenced such that expression of the *PCSK9* protein is decreased by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% in comparison to a cell where the *PCSK9* gene has not been targeted. In some embodiments, repression of transcription of the *PCSK9* gene of the cells of the population is sustained for at least about 8 hours, at least about 1 day, at least about 7 days, at least 2 weeks, at least about 3 weeks, at least about 1 month, or at least about 2 months, when assayed in an in vitro assay. In some embodiments, repression of transcription of the *PCSK9* gene of the cells of the population is heritable, wherein the repression of the PCKS9 gene persists for at least 1, 2, 3, 4, 5, or 6 or more cell divisions.

[00363] In some embodiments of the method, the repression of the cell occurs *in vitro*. In some embodiments of the method, the repression of the cell occurs *ex vivo*. In some embodiments, repression occurs *in vitro* inside of the cell prior to introducing the cell into a subject. In some embodiments, the cell is autologous or allogeneic with respect to the subject. In some embodiments of the method, the repression of the cell occurs *in vivo* in a subject administered a repressor fusion protein of any of the embodiments disclosed herein. In some embodiments of the method, the cell is a eukaryotic cell. In some embodiments of the method, the eukaryotic cell is selected from the group consisting of a rodent cell, a mouse cell, a rat cell, a primate cell, and

a non-human primate cell. In some embodiments of the method, the eukaryotic cell is a human cell. In some embodiments of the method, the cell is an embryonic stem cell, an induced pluripotent stem cell, a germ cell, a fibroblast, an oligodendrocyte, a glial cell, a hematopoietic stem cell, a neuron progenitor cell, a neuron, an astrocyte, a muscle cell, a bone cell, a hepatocyte, a pancreatic cell, a retinal cell, a cancer cell, a T-cell, a B-cell, an NK cell, a fetal cardiomyocyte, a myofibroblast, a mesenchymal stem cell, an autotransplanted expanded cardiomyocyte, an adipocyte, a totipotent cell, a pluripotent cell, a blood stem cell, a myoblast, a bone marrow cell, a mesenchymal cell, a parenchymal cell, an epithelial cell, an endothelial cell, a mesothelial cell, fibroblasts, osteoblasts, chondrocytes, a hematopoietic stem cell, a bone-marrow derived progenitor cell, a myocardial cell, a skeletal cell, a fetal cell, an undifferentiated cell, a multi-potent progenitor cell, a unipotent progenitor cell, a monocyte, a cardiac myoblast, a skeletal myoblast, a macrophage, a capillary endothelial cell, a xenogeneic cell, an allogenic cell, or a post-natal stem cell.

[00364] In some embodiments, the disclosure provides a method for reversing the repression of the *PCSK9* gene in a population of cells resulting from the repressor fusion protein:gRNA systems. In some embodiments, the repression is reversible by introducing into cells of the population an inhibitor of DNMT. In some embodiments of the method, the repression is reversible by use of a cytidine analog inhibitor of DNMT. In some embodiments, the repression is reversible by use of an inhibitor selected from the group consisting of azacytidine, decitabine, clofarabine, and zebularine. In some embodiments, the repression is reversible by use of an inhibitor at a concentration of 0.1 μM to 40 μM, or any intermediate concentration. In some embodiments, the method comprises administrations of a therapeutically effective dose of the inhibitor of DNMT to a subject treated with a system of the disclosure, thereby reversing the repression of *PCSK9* by the system.

[00365] In some embodiments, the repressor fusion protein:gRNA systems comprise a repressor fusion protein comprising a sequence of SEQ ID NOS: 3131-3132 as set forth in Table 20, or a sequence at least 60% identical, at least 70% identical, at least 80% identical, at least 81% identical, at least 82% identical, at least 83% identical, at least 84% identical, at least 85% identical, at least 86% identical, at least 86% identical, at least 87% identical, at least 88% identical, at least 89% identical, at least 89% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, at least 99%

identical, or at least 99.5% identical thereto, a gRNA scaffold comprising a sequence of SEQ ID NOS: 1744-1746 or 2947-2976, or a sequence at least 65% identical, at least 70% identical, at least 75% identical, at least 80% identical, at least 81% identical, at least 82% identical, at least 83% identical, at least 84% identical, at least 85% identical, at least 86% identical, at least 86% identical, at least 87% identical, at least 88% identical, at least 89% identical, at least 89% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, at least 99% identical, at least 99.5% identical thereto, and the gRNA comprises a targeting sequence of SEQ ID NOS: 1824-2944 or a sequence at least 65% identical, at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, or at least 95% identical thereto and having between 15 and 20 amino acids. In some embodiments of the system, the gRNA comprises a targeting sequence of SEQ ID NOS: 1824-2944, as set forth in Table 7. In a particular embodiment, the repressor fusion protein of the system comprises a sequence selected from the group consisting of SEQ ID NOS: 3131-3132, the gRNA scaffold comprises a sequence selected from the group consisting of SEQ ID NOS: 1744-1746 and 2947-2976, and the targeting sequence of the gRNA of the repressor fusion protein:gRNA system is selected from the group consisting of the sequence of SEQ ID NOS: 1824-2545. In a particular embodiment, the repressor fusion protein comprises a sequence selected from the group consisting of the sequences of SEQ ID NOS: 3131-3132, the gRNA scaffold comprises a sequence selected from the group consisting of SEQ ID NOS: 1744-1746 and 2947-2976, and the targeting sequence of the gRNA is selected from the group consisting of the sequence of SEQ ID NOS: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714 as set forth in Table 8. In a particular embodiment, wherein the systems are formulated in LNP, the repressor fusion protein comprises a sequence selected from the group consisting of SEQ ID NOS: 3131-3132, and is encoded by an mRNA, the gRNA scaffold comprises a sequence of SEQ ID NO: 1746, and the targeting sequence of the gRNA is selected from the group consisting of SEQ ID NOS: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714.

[00366] In some embodiments, the systems comprise an mRNA comprising one or more sequences selected from the group consisting of SEQ ID NOS: 3105, 3109, and 3115-3128, or sequences at least 60% identical, at least 70% identical, at least 80% identical, at least 81% identical, at least 82% identical, at least 83% identical, at least 84% identical, at least 85% identical, at least 86% identical, at least 86% identical, at least 87% identical, at least 88%

identical, at least 89% identical, at least 89% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, at least 99% identical, or at least 99.5% identical thereto. In some embodiments, the systems comprise an mRNA comprising one or more sequences selected from the group consisting of SEQ ID NOS: 3105, 3109, and 3115-3128. In a particular embodiment, the systems are formulated in LNP that encapsulate the mRNA sequence comprising one or more sequences selected from the group consisting of SEQ ID NOS: 3105, 3109, and 3115-3128, a gRNA selected from the group consisting of SEQ ID NOS: 2948- 2956, 2958-2966, and 2968-2976, and the targeting sequence of the gRNA is selected from the group consisting of the sequence of SEQ ID NOS: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714. In some embodiments, the mRNA comprises a sequence wherein at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99%, or 100% of uridine nucleosides of the sequence are replaced with N1-methylpseudouridine. In some embodiments, the mRNA further comprises a 5' untranslated region (UTR) and a 3' untranslated region (UTR).

[00367] In one embodiment of the method, the system is introduced into the cells using LNP comprising mRNA encoding the repressor fusion protein and gRNA variant of any of the embodiments disclosed herein. In some embodiments, the LNP comprises an mRNA encoding the repressor fusion protein comprising one or more sequences selected from the group consisting of SEQ ID NOS: 3105, 3109, and 3115-3128, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or having at least about 99% sequence identity thereto. In some embodiments of the foregoing, the LNP comprises a gRNA variant of the disclosure having a targeting sequence complementary to the *PCSK9* target nucleic acid. In some embodiments, the LNP comprises an mRNA encoding an repressor fusion protein, wherein the mRNA comprises a sequence selected from SEQ ID NOS: 3129-3130. In some embodiments, the LNP comprises gRNA variant scaffold 174 (SEQ ID NO: 1744). In some embodiments, the LNP comprises gRNA variant scaffold 235 (SEQ ID NO: 1745). In some embodiments, the LNP comprises gRNA variant scaffold 316 (SEQ ID NO: 1746). In some embodiments, the LNP comprises gRNA variant scaffold 316 with chemical modifications, including modifications set forth in the sequences of SEQ ID NOS: 2968-2976, or a sequence having 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 a particular embodiment, the LNP comprises an mRNA encoding a repressor fusion protein comprising the dCasX 491 (SEQ ID NO: 4) and gRNA variant 316 with chemical modifications selected from the group consisting of SEQ ID NOS: 2968-2976, or a sequence having 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, with a linked targeting sequence selected from the group consisting of the sequences of SEQ ID NOS: 1824-2944 that are chemically modified. In a particular embodiment, the LNP comprises an mRNA encoding a repressor fusion protein comprising the dCasX 491 (SEQ ID NO: 4) and gRNA variant scaffold 316 with chemical modifications comprising the sequence of SEQ ID NO: 2968, with a linked targeting sequence selected from the group consisting of the sequences of SEQ ID NOS: 1824-2944 that are chemically modified. In some embodiments of the method, the cells to be modified are selected from the group consisting of rodent cells, mouse cells, rat cells, and non-human primate cells. In other embodiments of the method, the cells to be modified are human cells. In some embodiments of the method, the transcriptional repression of the population of cells occurs *in vivo* in a subject, wherein the subject is selected from the group consisting of a rodent, a mouse, a rat, a non-human primate, and a human. In some embodiments of the methods, the modified cell is a hepatocyte, or a cell of the intestine, the kidney, the central nervous system, a smooth muscle cell, macrophage or a cell of arterial walls such as the endothelium.

[00368] The LNP can be administered by a route of administration selected from the group consisting of intravenous, intraarterial, intraportal vein injection, intraperitoneal, intramuscular, intracerebroventricular, intracisternal, intrathecal, intracranial, intralumbar, intraocular, subcutaneous, and oral routes.

[00369] In some embodiments of the method of repression of a *PCSK9* gene, the gene repressor systems of the present disclosure can be designed to target any region of, or proximal to, a *PCSK9* gene or region of a *PCSK9* gene for which repression of transcription is sought. When the entirety of the gene is to be repressed, designing a guide with a targeting sequence complementary to a sequence encompassing or proximal to the transcription start site (TSS) is contemplated by the disclosure. The TSS selection occurs at different positions within the promoter region, depending on promoter sequence and initiating-substrate concentration. The core promoter serves as a binding platform for the transcription machinery, which comprises Pol

II and its associated general transcription factors (GTFs) (Haberle, V. et al. Eukaryotic core promoters and the functional basis of transcription initiation (Nat Rev Mol Cell Biol. 19(10):621 (2018)). Variability in TSS selection has been proposed to involve DNA 'scrunching' and 'anti-scrunching,' the hallmarks of which are: (i) forward and reverse movement of the RNA polymerase leading edge, but not trailing edge, relative to DNA, and (ii) expansion and contraction of the transcription bubble. In some embodiments, the target nucleic acid sequence bound by an RNP of the repressor fusion protein:gRNA system is within 1 kb of a transcription start site (TSS) of the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by an RNP of the system is within 20 bp, 50 bp, 100 bp, 150 bp, 200 bp, 250 bp, 500 bps, or 1 kb upstream of a TSS of the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by an RNP of the system is within 20 bp, 50 bp, 100 bp, 150 bp, 200 bp, 250 bp, 500 bps or 1 kb downstream of a TSS of the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by an RNP of the system is within 500 bps upstream to 500 bps downstream, or 300 bps upstream to 300 bps downstream of a TSS of the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by an RNP of the system is within 20 bp, 50 bp, 100 bp, 150 bp, 200 bp, 250 bp, 500 bps, or 1 kb of an enhancer of the *PCSK9* gene. In some embodiments, the target nucleic acid sequence bound by a repressor fusion protein:gRNA RNP is within 1 kb 3' to a 5' untranslated region of the *PCSK9* gene. In other embodiments, the target nucleic acid sequence bound by an RNP of the system is within the open reading frame of the PCSK9 gene, inclusive of introns (if any). In some embodiments, the targeting sequence of a gRNA of the system is designed to be specific for an exon of the *PCSK9* gene. In a particular embodiment, the targeting sequence of a gRNA of the system is designed to be specific for exon 1 of the *PCSK9* gene. In other embodiments, the targeting sequence of a gRNA of the system is designed to be specific for an intron of the *PCSK9* gene. In other embodiments, the targeting sequence of the gRNA of the system is designed to be specific for an intron-exon junction of the *PCSK9* gene. In other embodiments, the targeting sequence of the gRNA of the system is designed to be specific for a regulatory element of the *PCSK9* gene . In other embodiments, the targeting sequence of the gRNA of the system is designed to be complementary to a sequence of an intergenic region of the *PCSK9* gene. In other embodiments, the targeting sequence of a gRNA of the system is specific for a junction of the exon, an intron, and/or a regulatory element of the *PCSK9* gene. In those cases where the targeting sequence is specific for a regulatory element, such regulatory elements include, but are not limited to promoter regions, enhancer

regions, intergenic regions, 5' untranslated regions (5' UTR), 3' untranslated regions (3' UTR), conserved elements, and regions comprising cis-regulatory elements. In some embodiments, the targeting sequence of a gRNA of the system is complementary to the gene target nucleic acid sequence within 1 kb of an enhancer of the *PCSK9* gene. In some embodiments, the targeting sequence of a gRNA of the system is complementary to the gene target nucleic acid sequence within the 3' untranslated region of the *PCSK9* gene. The promoter region is intended to encompass nucleotides within 5 kb of the initiation point of the encoding sequence or, in the case of gene enhancer elements or conserved elements, can be thousands of bp, hundreds of thousands of bp, or even millions of bp away from the encoding sequence of the *PCSK9* gene. In the foregoing, the targets are those in which the encoding *PCSK9* gene is intended to be repressed and/or epigenetically modified such that the *PCSK9* gene product is not expressed or is expressed at a lower level in a cell. In some embodiments, upon binding of the RNP of the system to the binding location of the target nucleic acid, the system is capable of repressing transcription of the *PCSK9* gene 5' to the binding location of the RNP. In other embodiments, upon binding of the RNP of the system to the binding location of the target nucleic acid, the system is capable of repressing transcription of the *PCSK9* gene 3' to the binding location of the RNP.

[00370] The systems and methods described herein can be used in a variety of cells associated with disease, *e.g.*, cells of the liver, the intestine, the kidney, the central nervous system, smooth muscle cells, macrophages or cells of arterial walls, in which the *PCSK9* gene is to be repressed or silenced. This approach, therefore, could be used for applications in a subject with a PCSK9-related disorder such as, but not limited to autosomal dominant hypercholesterolemia (ADH), hypercholesterolemia, elevated total cholesterol levels, hyperlipidemia, elevated low-density lipoprotein (LDL) levels, elevated LDL-cholesterol levels, reduced high-density lipoprotein levels, liver steatosis, coronary heart disease, ischemia, stroke, peripheral vascular disease, thrombosis, type 2 diabetes, high elevated blood pressure, atherosclerosis, obesity, Alzheimer's disease, neurodegeneration, age-related macular degeneration (AMD), or a combination thereof.

IX. Therapeutic Methods

[00371] The present disclosure provides methods of treating a PCSK9-related disease or disorder in a subject in need thereof, including but not limited to autosomal dominant hypercholesterolemia (ADH), hypercholesterolemia, elevated total cholesterol levels, elevated low-density lipoprotein (LDL) levels, reduced high-density lipoprotein levels, liver steatosis,

atherosclerotic cardiovascular disease, and coronary artery disease, ischemia, stroke, peripheral vascular disease, thrombosis, type 2 diabetes, high elevated blood pressure, obesity, Alzheimer's disease, neurodegeneration, age-related macular degeneration (AMD), or a combination thereof. In some embodiments, the methods of the disclosure can prevent, treat and/or ameliorate a PCSK9-related disease or disorder of a subject by the administering to the subject of a composition of the disclosure. In some embodiments, the *PCSK9*-related disease is autosomal dominant hypercholesterolemia (ADH), hypercholesterolemia, elevated total cholesterol levels, hyperlipidemia, elevated low-density lipoprotein (LDL) levels, elevated LDL-cholesterol levels, reduced high-density lipoprotein levels, liver steatosis, coronary heart disease, ischemia, stroke, peripheral vascular disease, thrombosis, type 2 diabetes, high elevated blood pressure, atherosclerosis, obesity, aortic stenosis, elevated PCSK9 levels, or a combination thereof. In some embodiments, the composition administered to the subject further comprises pharmaceutically acceptable carrier, diluent or excipient.

[00372] In some cases, the PCSK9 gene of the subject to be treated by the methods of the disclosure is wild-type, but the subject nevertheless has hypercholesterolemia, elevated total cholesterol levels, hyperlipidemia, elevated low-density lipoprotein (LDL) levels, elevated LDL-cholesterol levels, reduced high-density lipoprotein levels, liver steatosis, coronary heart disease, ischemia, stroke, peripheral vascular disease, thrombosis, type 2 diabetes, high elevated blood pressure, atherosclerosis, obesity, aortic stenosis, elevated PCSK9 levels, or a combination thereof. In such cases, the methods of the disclosure can prevent, treat and/or ameliorate a PCSK9-related disease or disorder of a subject by the administering to the subject of a composition of the disclosure.

[00373] In some cases, one or both alleles of the *PCSK9* gene of the subject comprises a mutation. In some cases, the PCSK9-related disease or disorder mutation is a gain of function mutation, including, but not limited to mutations encoding amino acid substitutions selected from the group consisting of S127R, D129G, F216L, D374H, and D374Y relative to the sequence of SEQ ID NO: 1823. In other cases, the PCSK9-related disorder mutation is a loss of function mutation including, but not limited to mutations encoding amino acid substitutions selected from the group consisting of R46L, G106R, Y142X, N157K, R237W and C679X relative to the sequence of SEQ ID NO: 1823.

[00374] In some embodiments, the disclosure provides methods of treating a PCSK9 or related disease or disorder in a subject in need thereof comprising repressing or silencing a PCSK9 gene

in a cell of the subject, the method comprising contacting said cells with a therapeutically effective dose of: i) a repressor fusion protein:gRNA system comprising a repressor fusion protein and a gRNA; ii) a nucleic acid encoding the repressor fusion protein and gRNA of any of the embodiments described herein; iii) an LNP or a synthetic nanoparticle comprising a gRNA and a mRNA encoding the repressor fusion protein of any one of the embodiments described herein; or iv) combinations of two or more of (i) to (iii), wherein the target nucleic acid sequence of the cells targeted by the gRNA is repressed or silenced by the repressor fusion protein. In some embodiments of the method, contacting cells with a repressor fusion protein:gRNA system results in repression of the *PCSK9* target nucleic acid of at least about 1%, at least about 2%, at least about 3%, at least about 4%, at least about 5%, at least about 6%, at least about 7%, at least about 8%, at least about 9%, or at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60% or more of the cells of the targeted organ. In some embodiments of the method, the *PCSK9* gene in the cells of the targeted organ are repressed such that expression of the PCSK9 protein is decreased by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% in comparison to an untreated cell. In some embodiments of the method, contacting cells of the targeted organ with a repressor fusion protein:gRNA system results in heritable repression of the *PCSK9* target nucleic acid in the cells. The cell of the treated subject can be a eukaryotic cell selected from the group consisting of a rodent cell, a mouse cell, a rat cell, a primate cell, and a non-human primate cell. In some embodiments, the eukaryotic cell of the treated subject is a human cell. In some embodiments, the cell is a cell involved in the production of LDL, including but not limited to a hepatocyte, or a cell of the intestine, the kidney, the central nervous system, a smooth muscle cell, macrophage, a retinal cell, or cell of arterial walls such as the endothelium. In some embodiments, the cell is an eye cell. In some embodiments of the methods of treating a PCSK9-related disorder in a subject, the subject is selected from the group consisting of mouse, rat, pig, non-human primate, and human.

[00375] A number of therapeutic strategies have been used to design the systems for use in the methods of treatment of a subject with a PCSK9-related disease or disorder. In some embodiments, the disclosure provides a method of treatment of a subject having a PCSK9-related disease or disorder, the method comprising administering to the subject a repressor fusion protein:gRNA composition according to a treatment regimen comprising one or more

consecutive doses using a therapeutically effective dose. In some embodiments of the treatment regimen, the therapeutically effective dose of the composition is administered as a single dose. In other embodiments of the treatment regimen, the therapeutically effective dose is administered to the subject as two or more doses over a period of at least two weeks, or at least one month, or at least two months, or at least three months, or at least four months, or at least five months, or at least six months. In some embodiments of the treatment regiment, the effective doses are administered by a route selected from the group consisting of intravenous, intraportal vein injection, intraperitoneal, intramuscular, subcutaneous, intraocular, and oral routes.

[00376] In some embodiments, the disclosure provides systems of a repressor fusion protein and gRNA of any of the embodiments described herein for use in a method of treatment of a PCSK9-related disease or disorder, wherein a therapeutically effective dose of the composition is administered to a subject. In some embodiments, the composition comprises a repressor fusion protein of SEQ ID NOS: 3131-3132 as set forth in Table 20, or a sequence at least 60% identical, at least 70% identical, at least 80% identical, at least 81% identical, at least 82% identical, at least 83% identical, at least 84% identical, at least 85% identical, at least 86% identical, at least 86% identical, at least 87% identical, at least 88% identical, at least 89% identical, at least 89% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, at least 99% identical, or at least 99.5% identical thereto, the gRNA scaffold comprises a sequence selected from the group consisting of SEQ ID NOS: 1744-1746, as set forth in Table 9, and SEQ ID NOS: 2947-2976, or a sequence at least 65% identical, at least 70% identical, at least 75% identical, at least 80% identical, at least 81% identical, at least 82% identical, at least 83% identical, at least 84% identical, at least 85% identical, at least 86% identical, at least 86% identical, at least 87% identical, at least 88% identical, at least 89% identical, at least 89% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, at least 99% identical, at least 99.5% identical thereto, and the gRNA comprises a targeting sequence a sequence selected from the group consisting of SEQ ID NOS: 1824-2944 or a sequence at least 65% identical, at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, or at least 95% identical thereto and having between 15 and 20

amino acids. In some embodiments, the gRNA comprises a targeting sequence selected from the group consisting of SEQ ID NOS: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714, or a sequence having at least about 65%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95% identity thereto.

[00377] In some embodiments, the composition is administered in an LNP formulation. In some embodiments, the disclosure provides repressor fusion proteins and gRNA compositions for use in the manufacture of a medicament for use in the treatment of a PCSK9-related disease or disorder in a subject wherein repression of a *PCSK9* leads to the amelioration of the disease or prevention of symptoms or clinical findings associated with the disease or disorder.

[00378] In some embodiments, the administering to a subject with a PCSK9-related disease or disorder of the therapeutically effective amount of the repressor fusion protein:gRNA modality, to repress or silence expression of *PCSK9*, leads to the prevention or amelioration of the underlying PCSK9-related disorder or disorder such that an improvement is observed in the subject, notwithstanding that the subject may still be afflicted with the underlying disorder. In some embodiments, the repressor fusion protein:gRNA modality comprises LNP comprising a gRNA and an mRNA encoding a repressor fusion protein and a guide ribonucleic acid disclosed herein. In some embodiments, the administration of the therapeutically effective amount of the repressor fusion protein:gRNA modality leads to an improvement in at least one clinically-relevant endpoint including, but not limited to percent change from baseline in LDL-cholesterol, decrease in plaque atheroma volume, reduction in in coronary plaque, reduction in atherosclerotic cardiovascular disease (ASCVD), cardiovascular death, nonfatal myocardial infarction, ischemic stroke, nonfatal stroke, coronary revascularization, unstable angina, or visual acuity. In some embodiments, the administration of the therapeutically effective amount of the repressor fusion protein:gRNA modality leads to an improvement in at least two clinically-relevant endpoints. In some embodiments, the subject is selected from mouse, rat, pig, dog, non-human primate, and human. In some embodiments, the subject is human.

[00379] In some embodiments, the methods of treatment further comprise administering a chemotherapeutic agent wherein the agent is effective in lowering LDL levels. Such agents include, but are not limited to, statins, niacin, fibrates, or anti-PCSK9 antibody drugs.

[00380] Methods of obtaining samples from treated subjects for analysis to determine the effectiveness of the treatment, such as body fluids or tissues, and methods of preparation of the samples to allow for analysis are well known to those skilled in the art. Methods for analysis of

RNA and protein levels are discussed above and are well known to those skilled in the art. The effects of treatment can also be assessed by measuring biomarkers associated with the *PCSK9* gene expression in the aforementioned fluids, tissues or organs, collected from an animal contacted with one or more compounds of the disclosure, by routine clinical methods known in the art. Biomarkers of PCSK9 disorders include, but are not limited to, PCSK9 levels, low-density lipoprotein (LDL-cholesterol), apolipoprotein B, non-HDL cholesterol, triglycerides and lipoprotein a, soluble CD40 ligand, osteopontin (OPN), osteoprotegerin (OPG), matrix metalloproteinases (MMP) and myeloperoxidase (MPOP), wherein the concentration of the marker is compared to concentrations known to be physiologically normal or in subjects not having a PCSK9 disorder.

[00381] Several mouse models expressing mutant forms of PCSK9 exist and are suitable for evaluating the methods of treatment. Transgenic mouse models of PCSK9-related disorders include knock-in mouse models having hPCSK9 (Carreras, A. *In vivo* genome and base editing of a human PCSK9 knock-in hypercholesterolemic mouse model. MC Biology 17:4 (2019); Herbert B., et al. Increased secretion of lipoproteins in transgenic mice expressing human D374Y PCSK9 under physiological genetic control. Arterioscler Thromb Vasc Biol. 30(7):1333 (2010)).

[00382] In some embodiments, the method of treating a PCSK9-related disease or disorder in a subject comprises pretreating the subject with a therapeutic agent that increases hepatic LDL receptor (LDLR) expression. In some embodiments, the therapeutic agent is a PCSK9 inhibitor, such as a monoclonal antibody, nucleic acid-based agent, or a small molecule. Exemplary therapeutic agents include, but are not limited to, evolocumab, inclisiran, alirocumab, and MK-0616. Without wishing to be bound by theory or mechanism, it is believed that the pretreatment with an inhibitor of PCSK9, may lead to an increase in hepatic LDL receptor (LDLR) expression that, in turn, may facilitate the uptake of the LNP comprising the CasX:gRNA composition that is subsequently administered to the subject. By increasing the hepatic cell uptake of the LNP, it is expected that editing of the *PCSK9* gene will be enhanced such that an improvement in the PCSK9-related disorder would be attained.

X. Pharmaceutical Compositions, Kits, and Articles of Manufacture

[00383] In some embodiments, the disclosure provides pharmaceutical compositions comprising: i) a repressor fusion protein and a gRNA of the disclosure comprising a targeting

sequence specific for a *PCSK9* gene; ii) one or more nucleic acids encoding the repressor and the gRNA of (i); iii) an LNP or synthetic nanoparticle comprising a gRNA and an mRNA encoding a repressor fusion protein, together with one or more pharmaceutically suitable excipients. In some embodiments, the pharmaceutical composition is formulated for a route of administration selected from the group consisting of intravenous, intraportal vein injection, intraperitoneal, intramuscular, subcutaneous, intraocular, and oral routes. In one embodiment, the pharmaceutical composition is in a liquid form or a frozen form. In another embodiment, the pharmaceutical composition is in a pre-filled syringe for a single injection. In another embodiment, the pharmaceutical composition is in solid form, for example the pharmaceutical composition is lyophilized.

[00384] In other embodiments, provided herein are kits comprising a repressor fusion protein and one or a plurality of CasX gRNA of any of the embodiments of the disclosure comprising a targeting sequence specific for a PCSK9 gene and a suitable container (for example a tube, vial or plate).

[00385] In other embodiments, provided herein are kits comprising an LNP formulation encapsulating an mRNA encoding a repressor fusion protein and one or a plurality of CasX gRNA of any of the embodiments of the disclosure comprising a targeting sequence specific for a PCSK9 gene, and a suitable container (for example a tube, vial or plate). In exemplary embodiments, a kit of the disclosure comprises any one of the repressor fusion proteins disclosed herein and a gRNA scaffold of any one of SEQ ID NOS: 1744-1746 and 2947-2976.

[00386] In some embodiments, the kit comprises a gRNA or a vector encoding a gRNA, wherein the gRNA comprises a scaffold sequence selected from the group consisting of SEQ ID NOS: 1744-1746 and 2947-2976, and a targeting sequence selected from the group consisting of SEQ ID NOS: 1824-2944 as set forth in Table 7. In some embodiments, the gRNA scaffold comprises a sequence selected from the group consisting of SEQ ID NOS: 2948-2956, 2958-2966, and 2968-2976, and a targeting sequence selected from the group consisting of SEQ ID NOS: 1824-2944 as set forth in Table 7. In some embodiments, the gRNA scaffold comprises a sequence selected from the group consisting of SEQ ID NOS: 2948-2956, 2958-2966, and 2968-2976, and a targeting selected from the group consisting of the sequences of SEQ ID NOs: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714.

[00387] In certain embodiments, provided herein are kits comprising a repressor fusion protein and gRNA repressor pair comprising any one of the repressor fusion proteins disclosed herein,

and a gRNA variant comprising a scaffold sequence selected from the group consisting of SEQ ID NOS: 1744-1746 as set forth in Table 9 and a targeting sequence selected from the group consisting of SEQ ID NOS: 1824-2944 as set forth in Table 7. In some embodiments, the gRNA of the gene repression pair comprises a scaffold sequence selected from the group consisting of SEQ ID NOS: 2947-2976 and a targeting sequence of any one of SEQ ID NOS: 1824-2944 as set forth in Table 7. In some embodiments, the gRNA of the gene repression pair comprises a scaffold sequence selected from the group consisting of SEQ ID NOS: 2948-2956, 2958-2966, and 2968-2976, and a targeting selected from the group consisting of the sequences of SEQ ID NOs: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714.

[00388] In some embodiments, the kit further comprises a buffer, a nuclease inhibitor, a protease inhibitor, a liposome, a therapeutic agent, a label, a label visualization reagent, instructions for use, or any combination of the foregoing. In some embodiments, the kit further comprises a pharmaceutically acceptable carrier, diluent or excipient.

[00389] In some embodiments, the kit comprises appropriate control compositions for gene repression applications, and instructions for use.

[00390] In some embodiments, the kit comprises a vector comprising a sequence encoding a repressor fusion protein of the disclosure and a CasX gRNA of the disclosure.

[00391] The following Examples are merely illustrative and are not meant to limit any aspects of the present disclosure in any way.

EXAMPLES

Example 1: Use of repressor fusion proteins to repress *PCSK9* in human cells

[0392] Fusing chromatin remodelers and DNA methyltransferases to CasX to reduce expression of target genes is an alternative approach to conventional gene editing for reducing expression of proteins associated with certain diseases and disorders. Experiments were performed to transiently express long-term repressor proteins (LTRPs, also referred to interchangeably herein as repressor fusion proteins) in HepG2 cells to reduce PCSK9 levels without the use of permanent genome editing.

Materials and Methods:

[0393] Spacer (also referred to as targeting sequence) design: Spacers 1KB upstream of the *PCSK9* promoter and through *PCSK9* Exon 1 were chosen manually based on availability of TTC PAM sequences. Spacers in Intron 1, Exon 5, and Intron 5 were selected based on the availability of TTC PAMs in regions identified as hypomethylated in human livers.

[0394] Lentiviral plasmid constructs comprising sequences encoding an LTRP protein having the #1 configuration (as diagrammed in FIG. 1), guide scaffold variant 174, and the *PCSK9*-targeting spacers listed in Table 10 were generated using standard molecular cloning techniques. Cloned and sequence-validated constructs were midi-prepped and subjected to quality assessment prior to transfection into HepG2 cells. Plasmid constructs were transfected using Viafect transfection reagent according to the manufacturer's instructions.

[0395] HepG2s were grown in DMEM F/12 media supplemented with 10% FBS and 1% Pen/Strep and were kept in the growth phase.

[0396] mRNA was isolated using Zymo Quick-RNA kit and Reverse Transcription was performed using Thermo High-Capacity RNA-to-cDNA™ Kit according to the manufacturer instructions.

[0397] Secreted PCSK9 and human serum albumin levels were assessed via PerkinElmer/Cisbio cat#63ADK050PEH and HSA ELISA: Perkin Elmer/Cisbio cat#6FHSAPEG respectively.

Table 10: Sequences of LTRP-specific spacers targeting the human *PCSK9* locus.

Spacer ID	PAM	Spacer DNA sequence	SEQ ID NO:	Spacer RNA sequence	SEQ ID NO:	Targeting region
6.1	TTC	GAGGAGGACGGCCTGGCCGA	2977	GAGGAGGACGGCCUGGCCGA	1834	Exon 1
6.4	TTC	GCCAGGCCGTCCTCCTCGGA	2978	GCCAGGCCGUCCUCCUCGGA	1836	Exon 1

Spacer ID	PAM	Spacer DNA sequence	SEQ ID NO:	Spacer RNA sequence	SEQ ID NO:	Targeting region
6.5	TTC	GTGCTCGGGTGCTTCGGCCA	2979	GUGCUCGGGUGCUUCGGCCA	1837	Exon 1
6.112	TTC	CTTGGCAGTTGAGCACGCGC	2980	CUUGGCAGUUGAGCACGCGC	2223	Exon 5
6.117	TTC	ACTTTGTTTGCAAAGACCTC	2981	ACUUUGUUUGCAAAGACCUC	1838	Promoter
6.118	TTC	GAGTGAAATGGCCTGCTCTG	2982	GAGUGAAAUGGCCUGCUCUG	1839	Promoter
6.119	TTC	GAGCAGGCCATTTCACTCGG	2983	GAGCAGGCCAUUUCACUCGG	1840	Promoter
6.120	TTC	CTCGGAATCTGCTGTGCATC	2984	CUCGGAAUCUGCUGUGCAUC	1841	Promoter
6.121	TTC	GGAAGGGCTGTCGATACTGG	2985	GGAAGGGCUGUCGAUACUGG	1842	Promoter
6.122	TTC	CCTTTGTTTCTTCCCAGTAT	2986	CCUUUGUUUCUUCCCAGUAU	1883	Promoter
6.123	TTC	TCCCAGTATCGACAGCCCTT	2987	UCCCAGUAUCGACAGCCCUU	1843	Promoter
6.124	TTC	CAGTATCGACAGCCCTTCCA	2988	CAGUAUCGACAGCCCUUCCA	1844	Promoter
6.125	TTC	AGAAAGAGCAAGCCTCATGT	2989	AGAAAGAGCAAGCCUCAUGU	1845	Promoter
6.126	TTC	CCTTTTCATCCTCCTGCCTG	2990	CCUUUUCAUCCUCCUGCCUG	2672	Promoter
6.127	TTC	TCCTCCTGCCTGGTACACAA	2991	UCCUCCUGCCUGGUACACAA	1884	Promoter
6.128	TTC	AGAAATCAACTGGACAAGCA	2992	AGAAAUCAACUGGACAAGCA	1846	Promoter
6.129	TTC	TTTTACACACCATGTTCAAG	2993	UUUUACACACCAUGUUCAAG	2675	Promoter
6.130	TTC	ATTTGCAAAGATTCCTTTTA	2994	AUUUGCAAAGAUUCCUUUUA	2677	Promoter
6.131	TTC	TGAACATGGTGTGTAAAAGG	2995	UGAACAUGGUGUGUAAAAGG	1847	Promoter
6.132	TTC	AGAAGATTCAATTTGCAAAG	2996	AGAAGAUUCAAUUUGCAAAG	1848	Promoter
6.133	TTC	ATGGTAGGCACAAGCTCAGC	2997	AUGGUAGGCACAAGCUCAGC	1849	Promoter
6.134	TTC	GAATTCTATGGTAGGCACAA	2998	GAAUUCUAUGGUAGGCACAA	1850	Promoter
6.135	TTC	GGAAAGCTGAGCTTGTGCCT	2999	GGAAAGCUGAGCUUGUGCCU	1851	Promoter
6.136	TTC	GGTTTTAAGTTTGCAAAGAC	3000	GGUUUUAAGUUUGCAAAGAC	2683	Promoter
6.137	TTC	GAATGTACCTATATGACGTC	3001	GAAUGUACCUAUAUGACGUC	1885	Promoter
6.138	TTC	AGGGATTTATACTACAAAGA	3002	AGGGAUUUAUACUACAAAGA	1852	Promoter
6.139	TTC	AGGAGCAGCTAGTTGGTAAG	3003	AGGAGCAGCUAGUUGGUAAG	1853	Promoter
6.140	TTC	AAACTTAGCCTGGACCCCCT	3004	AAACUUAGCCUGGACCCCCU	1854	Promoter
6.141	TTC	ACTGGCCTTAACCTGGCAGC	3005	ACUGGCCUUAACCUGGCAGC	1855	Promoter
6.142	TTC	TTCCACTGGCCTTAACCTGG	3006	UUCCACUGGCCUUAACCUGG	1856	Promoter
6.143	TTC	GAATCAATCCTACTGTGGAC	3007	GAAUCAAUCCUACUGUGGAC	1857	Promoter
6.144	TTC	GTGGGCAGCGAGGAGTCCAC	3008	GUGGGCAGCGAGGAGUCCAC	1858	Promoter
6.145	TTC	TGGGTCCACCTTGTCTCCTG	3009	UGGGUCCACCUUGUCUCCUG	1859	Promoter
6.146	TTC	GAAGTCTCACTGGTCAGCAG	3010	GAAGUCUCACUGGUCAGCAG	1860	Promoter
6.147	TTC	GTGTTTCCTGGGTCCACCTT	3011	GUGUUUCCUGGGUCCACCUU	1861	Promoter
6.148	TTC	GCCGGGCCCACCTTTTCAGT	3012	GCCGGGCCCACCUUUUCAGU	2694	Promoter
6.149	TTC	AGCCCAGTTAGGATTTGGGA	3013	AGCCCAGUUAGGAUUUGGGA	1862	Promoter
6.150	TTC	TCCCTCTGCGCGTAATCTGA	3014	UCCCUCUGCGCGUAAUCUGA	1863	Promoter
6.151	TTC	CTCTGCGCGTAATCTGACGC	3015	CUCUGCGCGUAAUCUGACGC	1864	Promoter
6.152	TTC	GCCTCGCCCTCCCCAAACAG	3016	GCCUCGCCCUCCCCAAACAG	1865	Promoter
6.153	TTC	GTTAATGTTTAATCAGATAG	3017	GUUAAUGUUUAAUCAGAUAG	1866	Promoter
6.154	TTC	AGGGTGTGGGTGCTTGACGC	3018	AGGGUGUGGGUGCUUGACGC	1867	Promoter
6.155	TTC	GCAGCGACGTCGAGGCGCTC	3019	GCAGCGACGUCGAGGCGCUC	1868	Exon 1
6.156	TTC	GTTCAGGGTCTGAGCCTGGA	3020	GUUCAGGGUCUGAGCCUGGA	2702	Exon 1
6.157	TTC	GGGTCTGAGCCTGGAGGAGT	3021	GGGUCUGAGCCUGGAGGAGU	1869	Exon 1
6.158	TTC	GGAGCAGGGCGCGTGAAGGG	3022	GGAGCAGGGCGCGUGAAGGG	1870	Exon 1
6.159	TTC	GCGCGCCCCTTCACGCGCCC	3023	GCGCGCCCCUUCACGCGCCC	1871	Exon 1
6.160	TTC	CGCGCCCTGCTCCTGAACTT	3024	CGCGCCCUGCUCCUGAACUU	1872	Exon 1
6.161	TTC	GCTCCTGCACAGTCCTCCCC	3025	GCUCCUGCACAGUCCUCCCC	1873	Exon 1
6.167	TTC	CACTGAATAGCGCAGCCGCA	3026	CACUGAAUAGCGCAGCCGCA	1874	Intron 1
6.168	TTC	GTGGGAAGGTTCGCGGGGTT	3027	GUGGGAAGGUUCGCGGGGUU	1875	Intron 1
6.169	TTC	CGGGGTTGGGAGACCCGGAG	3028	CGGGGUUGGGAGACCCGGAG	1876	Intron 1
6.170	TTC	TCGGCCTCCGGGTCTCCCAA	3029	UCGGCCUCCGGGUCUCCCAA	1877	Intron 1
6.171	TTC	CAGTACGTTCCAGGCATTCA	3030	CAGUACGUUCCAGGCAUUCA	1878	Intron 1

Spacer ID	PAM	Spacer DNA sequence	SEQ ID NO:	Spacer RNA sequence	SEQ ID NO:	Targeting region
6.172	TTC	GCTGAAACAGATGGAATACT	3031	GCUGAAACAGAUGGAAUACU	1879	Intron 1
6.173	TTC	ATCTGTTTCAGCCGAAGAAA	3032	AUCUGUUUCAGCCGAAGAAA	1922	Intron 1
6.174	TTC	TTTCTTCGGCTGAAACAGAT	3033	UUUCUUCGGCUGAAACAGAU	1923	Intron 1
6.175	TTC	GCCGAAGAAAAGAACCAGCT	3034	GCCGAAGAAAAGAACCAGCU	1924	Intron 1
6.176	TTC	CGAGGCCCATTGGCGTCCTT	3035	CGAGGCCCAUUGGCGUCCUU	1926	Intron 1
6.177	TTC	TCTCACTAGCTGTGGTGCTT	3036	UCUCACUAGCUGUGGUGCUU	1934	Intron 1
6.178	TTC	GTTGACCATGAGTGAACTTA	3037	GUUGACCAUGAGUGAACUUA	1938	Intron 1
6.179	TTC	CTGGCTCTGCGGCAGAGGCT	3038	CUGGCUCUGCGGCAGAGGCU	2225	Intron 5
6.180	TTC	TCTGCACTCGTGGCCACTGG	3039	UCUGCACUCGUGGCCACUGG	2226	Intron 5
6.181	TTC	TCATCTGCACTCGTGGCCAC	3040	UCAUCUGCACUCGUGGCCAC	2228	Intron 5
6.182	TTC	AGACTGTGACTACATTTAGT	3041	AGACUGUGACUACAUUUAGU	2235	Intron 5
6.183	TTC	TCAACTATTTAGCAGCTACG	3042	UCAACUAUUUAGCAGCUACG	2240	Intron 5
6.184	TTC	CAGCGAGTTCCCCAGCTTGA	3043	CAGCGAGUUCCCCAGCUUGA	2242	Intron 5
6.185	TTC	GCCCTGAGACTTTCCTACAG	3044	GCCCUGAGACUUUCCUACAG	2246	Intron 5
6.186	TTC	GCCCCATCAGGTGACCCCTT	3045	GCCCCAUCAGGUGACCCCUU	2248	Intron 5
6.187	TTC	GGAACTGACCTGACTGAGCC	3046	GGAACUGACCUGACUGAGCC	2249	Intron 5

Results:

[0398] HepG2 cells were transiently transfected with LTRP fusion proteins in configuration #1 with the targeting spacers listed in Table 10 and were subsequently selected with puromycin. Puromycin-resistant cells were allowed to expand in culture. After 4 weeks of culture, the media was collected to measure secreted PCSK9 levels, and mRNA was analyzed. Secreted PCSK9 levels were normalized to secreted human serum albumin to control for differences in cell number in different wells, and the results are presented as dot plots in FIG. 3. Compared to the non-targeting (NT) control, ~ 60% of the constructs demonstrated reduction of PCSK9 mRNA and protein levels, while ~20% of constructs repressed PCSK9 by greater than 50%.

Example 2: Demonstration that use of LTRP fusion proteins can induce silencing of the endogenous *PCSK9* locus in mouse Hepa 1-6 cells

[0399] Experiments were performed to demonstrate the ability of LTRP fusion proteins to induce durable repression of the endogenous *PCSK9* locus in mouse Hepa1-6 liver cells, when delivered as mRNA co-transfected with a targeting gRNA.

Materials and Methods:

Experiment #1: dXR1 vs. LTRP #1 in Hepa1-6 cells when delivered as mRNA

Generation of dXR1 and LTRP #1 mRNA:

[0400] mRNA encoding dXR1, a dCasX fused to a ZIM3-KRAB domain, or LTRP #1 (configuration #1 in FIG. 1) containing the ZIM3-KRAB (hereafter known as dXR1 and

LTRP1-ZIM3 respectively) domain was generated by in vitro transcription (IVT) either in-house or via a third-party. Briefly, constructs encoding for a 5'UTR region, dXR1 or LTRP1-ZIM3 harboring the ZIM3-KRAB domain with flanking SV40 NLSes, and a 3'UTR region were generated and cloned into a plasmid containing a T7 promoter and 80-nucleotide poly(A) tail. These constructs also contained a 2x FLAG sequence. Sequences encoding the dXR1 and LTRP1-ZIM3 molecules were codon-optimized using a codon utilization table, in addition to using a publicly available codon optimization tool and adjusting parameters such as GC content as needed. For in-house in vitro transcription (IVT), the resulting plasmid was linearized prior to use for IVT reactions, which were carried out with CleanCap® AG and N1-methyl-pseudouridine. IVT reactions were then subjected to DNase digestion and oligodT purification on-column. For experiment #1, the DNA sequences encoding the dXR1 and LTRP1-ZIM3 molecules are listed in Table 11. The corresponding mRNA sequences encoding the dXR1 and LTRP1-ZIM3 mRNAs are listed in Table 12. The protein sequences of the dXR1 and LTRP1-ZIM3 are shown in Table 13.

Table 11: Encoding sequences of the dXR1 and LTRP1-ZIM3 mRNA molecules assessed in experiment #1 of this example*.

dXR or LTRP ID	Component	DNA sequence or SEQ ID NO:
dXR1 (codon-optimized)	5'UTR	3047
	START codon + NLS + linker	3048
	dCasX491	3049
	Linker + buffer sequence	3050
	ZIM3-KRAB	3051
	Buffer sequence + NLS	3052
	Tag	3053
	STOP codon + buffer sequence	3054
	3'UTR	3055
	Buffer sequence	TCTAG
	Poly(A) tail	3057
LTRP #1 (codon-optimized)	5'UTR	3047
	START codon + NLS + buffer sequence + linker	3058
	START codon + DNMT3A catalytic domain	3059
	Linker	3060
	DNMT3L interaction domain	3061
	Linker	3062
	dCasX491	3049
	Linker	3050

dXR or LTRP ID	Component	DNA sequence or SEQ ID NO:
	ZIM3-KRAB	3051
	Buffer sequence + NLS	3052
	Tag	3053
	STOP codons + buffer sequence	3054
	3'UTR	3055
	Buffer sequence	TCTAG
	Poly(A) tail	3057

*Components are listed in a 5' to 3' order within the constructs

Table 12: Full-length RNA sequences of dXR1 and LTRP1-ZIM3 mRNA molecules assessed in experiment #1 of this example. Modification 'mψ' = N1-methyl-pseudouridine.

dXR or LTRP ID	SEQ ID NO	RNA Sequence
dXR1	3063	AAAmψAAGAGAGAAAAGAAGAGmψAAGAAGAAAmψAmψAAGAGCCACCAmψGGCCCCm ψAAGAAGAAGCGmψAAAGmψGAGCCGGGGCGGCAGCGGCGGCGGCAGCGCCCAGGAGA mψmψAAACGGAmψCAACAAGAmψCAGAAGAAGACmψmψGmψGAAAGACAGCAACACCA AGAAGGCCGGCAAGACAGGCCCCAmψGAAAACCCmψGCmψGGmψmψAGAGmψGAmψGA CACCCGAmψCmψGAGAGAGCGGCmψGGAAAACCmψGAGAAAGAAGCCmψGAAAAmψAm ψCCCCCAGCCCAmψCAGCAAmψACAmψCmψAGAGCCAACCmψGAAmψAAGCmψGCmψG ACCGAmψmψACACCGAAAmψGAAGAAGGCGAmψCCmψGCAmψGmψGmψACmψGGGAAG AGmψmψCCAGAAGGACCCmψGmψGGGCCmψGAmψGAGCCGGGmψGGCCCAGCCmψGCC AGCAAGAAGAmψCGAmψCAGAACAAGCmψGAAACCmψGAGAmψGGACGAGAAGGGCAA CCmψGACCACCGCCGGCmψmψmψGCCmψGCmψCmψCAGmψGmψGGCCAGCCCCmψGmψ mψCGmψGmψACAAGCmψGGAGCAGGmψGmψCmψGAGAAGGGCAAGGCmψmψACACCAA CmψACmψmψCGGACGGmψGCAAmψGmψGGCCGAGCACGAAAAGCmψGAmψCCmψGCmψ GGCCCAGCmψGAAGCCCGAGAAGGAmψAGCGACGAAGCCGmψGACAmψAmψAGCCmψG GGAAAGmψmψmψGGGCAGAGGGCCCmψGGAmψmψmψCmψACAGCAmψmψCAmψGmψGA CCAAGGAGmψCCACCCACCCCGmψGAAGCCCCmψGGCCCAGAmψCGCCGGAAACAGAm ψACGCCmψCCGGACCmψGmψGGGAAAGGCCCmψGAGCGACGCAmψGmψAmψGGGCACA AmψCGCCmψCCmψmψCCmψGmψCmψAAGmψACCAGGACAmψCAmψCAmψCGAACACCA GAAGGmψGGmψGAAGGGCAACCAGAAGAGACmψGGAGAGCCmψGCGGGAGCmψGGCCG GCAAGGAAAACCmψGGAAmψACCCmψAGCGmψGACCCmψGCCACCmψCAGCCmψCACA CCAAGGAGGGCGmψmψGAmψGCCmψACAACGAAGmψGAmψCGCCCGGGmψGCGAAmψG mψGGGmψGAACCmψGAACCmψGmψGGCAGAAGCmψGAAGCmψAAGCAGAGAmψGAmψG CCAAGCCmψCmψGCmψGAGACmψGAAGGGAmψmψCCCmψmψCCmψmψmψCCmψCmψGG mψCGAGAGACAGGCCAACGAAGmψGGACmψGGmψGGGACAmψGGmψGmψGmψAACGmψ GAAGAAGCmψGAmψCAACGAGAAAAAGGAGGAmψGGCAAGGmψGmψmψmψmψGGCAGA AmψCmψGGCmψGGCmψACAAGAGACAGGAAGCCCmψGAGACCAmψACCmψGAGCAGCG AGGAAGAmψCGGAAGAAGGGAAAGAAAmψmψCGCmψCGGmψACCAGCmψGGGCGACCm ψGCmψGCmψGCACCmψGGAAAAGAAGCACGGCGAGGACmψGGGGAAAGGmψGmψACGA CGAGGCCmψGGGAGCGGAmψmψGACAAGAAAGmψGGAAGGCCmψGAGCAAGCACAmψC AAGCmψGGAAGAGGAACGGAGAAGCGAGGACGCCCAGAGCAAGGCCGCCCmψGACCGA CmψGGCmψGCGGGCmψAAGGCCAGCmψmψCGmψGAmψCGAGGGCCmψGAAGGAGGCCG ACAAGGACGAGmψmψCmψGCAGAmψGCGAGCmψGAAGCmψGCAGAAGmψGGmψACGGG GACCmψGCGGGGAAAGCCCmψmψCGCCAmψCGAAGCCGAGAACAGCAmψCCmψGGACA mψCAGCGGCmψmψCAGCAAGCAGmψACAACmψGmψGCCmψmψCAmψCmψGGCAGAAGG ACGGCGmψGAAGAAGCmψGAACCmψGmψACCmψGAmψCAmψCAACmψACmψmψCAAGG GCGGCAAGCmψGCGGmψmψCAAGAAGAmψCAAACCmψGAAGCCmψmψCGAAGCCAACA

dXR or LTRP ID	SEQ ID NO	RNA Sequence
		GAmψmψCmψACACCGmψGAmψCAACAAAAAGAGCGGCGAGAmψCGmψGCCCAmψGGAG GmψGAACmψmψCAACmψmψCGACGACCCCAACCmψGAmψCAmψCCmψGCCmψCmψGGC Cmψmψmψ GGCAAGAGACAGGGCAGAGAAmψmψCAmψCmψGGAACGACCmψGCmψGmψC CCmψGGAAACCGGCAGCCmψGAAGCmψGGCCAACGGAAGAGmψGAmψCGAGAAGACAC mψGmψACAACAGAAGAACCCGGCAGGAmψGAGCCmψGCCCmψGmψmψCGmψGGCCCmψ GACCmψmψCGAGCGGCGGGAGGmψCCmψGGACmψCCmψCCAAmψAmψCAAACCAAmψG AACCmψGAmψCGGCGmψGGCAAGAGGCGAAAACAmψCCCCGCCGmψGAmψCGCCCmψG ACCGACCCCGAGGGCmψGCCCACmψGAGCCGGmψmψmψAAGGAmψAGCCmψGGGAAAC CCAACCCACAmψCCmψGAGAAmψCGGCGAGAGCmψAmψAAGGAGAAGCAGCGGACCAm ψCCAGGCCAAGAAGGAGGmψGGAGCAGCGGAGAGCCGGCGGCmψACAGCCGGAAGmψA CGCCAGCAAAGCCAAGAAmψCmψGGCAGACGAmψAmψGGmψGAGAAACACCGCmψAGA GAmψCmψGCmψGmψACmψACGCCGmψGACCCAGGAmψGCCAmψGCmψGAmψCmψmψCG CCAACCmψGAGCCGGGGCmψmψCGGCCGGCAGGGCAAGCGGACCmψmψCAmψGGCCGA GAGACAGmψACACACGGAmψGGAGGACmψGGCmψGACCGCCAAGCmψGGCCmψACGAG GGCCmψGAGCAAGACCmψACCmψGmψCCAAGACACmψGGCCCAGmψACACCmψCCAAG ACAmψGCAGCAACmψGmψGGGmψmψmψACCAmψCACCAGCGCCGACmψACGACAGGGm ψGCmψGGAGAAGCmψGAAGAAGACAGCAACAGGCmψGGAmψGACCACAAmψmψAACGG CAAGGAGCmψGAAGGmψGGAGGGCCAGAmψmψACCmψACmψACAACAGAmψACAAGAG ACAGAACGmψAGmψCAAGGACCmψGmψCCGmψCGAGCmψGGAmψAGACmψGAGCGAAG AAmψCmψGmψGAACAACGACAmψCmψCCmψCCmψGGACAAAGGGCAGAAGCGGAGAAG CmψCmψGAGCCmψCCmψGAAGAAAAGAmψmψCmψCCCAmψAGACCCGmψGCAGGAGAA GmψmψCGmψGmψGCCmψGAACmψGCGGCmψmψCGAGACACACGCAGCCGAGCAAGCCG CCCmψGAACAmψCGCCAGAmψCCmψGGCmψGmψmψCCmψGCGGAGCCAGGAGmψACAA GAAAmψACCAGACAAACAAGACAACCGGCAACACCGAmψAAGAGAGCCmψmψCGmψCG AGACCmψGGCAGmψCCmψmψmψmψACCGGAAGAAGCmψmψAAGGAGGmψGmψGGAAAC CmψGCCGmψGCGGmψCmψGGCGGAmψCmψGGCGGAGGCmψCCACAAGCAmψGAACAAC mψCCCAGGGCAGAGmψGACCmψmψCGAGGACGmψGACCGmψGAAmψmψmψmψACACAG GGAGAGmψGGCAGAGACmψGAACCCCGAGCAGAGAAACCmψGmψACCGGGAmψGmψGA mψGCmψGGAAAACmψACAGCAAmψCmψGGmψGmψCCGmψGGGCCAGGGCGAGACCACA AAGCCmψGACGmψGAmψCCmψGCGmψCmψGGAGCAGGGCAAGGAACCCmψGGCmψGGA GGAGGAGGAGGmψGCmψGGGAAGCGGACGGGCCGAGAAGAACGGCGACAmψCGGCGGA CAGAmψCmψGGAAGCCmψAAGGACGmψGAAAGAAAGCCmψGACCAGCCCCAAGAAAAA GAGAAAAGmψCGACmψACAAGGAmψGACGAmψGACAAGGACmψACAAGGAmψGACGAC GACAAGmψAAmψAGAmψAAGCGGCCGCmψmψAAmψmψAAGCmψGCCmψmψCmψGCGGG GCmψmψGCCmψmψCmψGGCCAmψGCCCmψmψCmψmψCmψCmψCCCmψmψGCACCmψGm ψACCmψCmψmψGGmψCmψmψmψGAAmψAAAGCCmψGAGmψAGGAAGmψcmψagaaaaa aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa aaaaaaaaaaaaaaaa
LTRP #1	3064	AAAmψAAGAGAGAAAAGAAGAGmψAAGAAGAAAmψAmψAAGAGCCACCAmψGGCCCCm ψAAGAAGAAGCGmψAAAGmψGAGCCGGGmψGAACGGCAGCGGCAGCGGCGGCGGCAmψ GAACCACGACCAGGAGmψmψCGACCCCCCmψAAGGmψGmψACCCmψCCCGmψCCCCGC CGAGAAGAGAAAGCCCAmψCCGGGmψCCmψGAGCCmψGmψmψCGAmψGGCAmψCGCCA CCGGmψCmψGCmψGGmψGCmψGAAGGACCmψGGGCAmψCCAGGmψGGAmψAGGmψACA mψmψGCCmψCCGAGGmψGmψGCGAGGACmψCCAmψCACCGmψGGGAAmψGGmψGCGmψ CAmψCAGGGCAAGAmψCAmψGmψACGmψGGGCGACGmψGCGGAGCGmψGACACAGAAG CAmψAmψCCAGGAGmψGGGGCCCmψmψmψCGACCmψGGmψGAmψCGGCGGCAGCCCmψ mψGCAAmψGACCmψGAGCAmψCGmψGAACCCAGCCCGGAAGGGCCmψGmψACGAGGGA ACCGGCAGACmψGmψmψCmψmψCGAGmψmψmψmψACAGACmψGCmψGCACGACGCCCG GCCmψAAGGAAGGCGACGACCGGCCCmψmψCmψmψmψmψGGCmψGmψmψCGAGAAmψG mψGGmψGGCCAmψGGGAGmψCAGCGACAAGCGGGAmψAmψmψAGCCGGmψmψCCmψGG AGAGCAACCCCGmψGAmψGAmψCGAmψGCCAAGGAAGmψGAGCGCCGCCCACCGGGCC AGAmψACmψmψCmψGGGGCAAmψCmψGCCmψGGCAmψGAACAGACCCCmψGGCCAGCA CCGmψGAACGACAAGCmψGGAGCmψGCAGGAGmψGCCmψGGAGCACGGCCGGAmψCGC CAAGmψmψCAGCAAGGmψGAGAACCAmψCACCACCCGAAGCAACAGCAmψCAAACAAG GCAAGGACCAGCACmψmψmψCCmψGmψGmψmψCAmψGAACGAGAAGGAGGACAmψCCm

dXR or LTRP ID	SEQ ID NO	RNA Sequence
		ψGmψGGmψGmψACCGAGAmψGGAGAGAGmψGmψmψCGGGmψmψCCCAGmψCCACmψAC ACAGAmψGmψCAGCAACAmψGmψCmψAGACmψGGCCAGACAGAGACmψGCmψGGGAAG AAGCmψGGmψCCGmψCCCmψGmψGAmψCAGACACCmψGmψmψCGCCCCmψCmψGAAGG AGmψACmψmψCGCCmψGCGmψGAGCAGCGGCAACAGCAACGCCAACAGCCGGGGCCCC AGCmψmψCmψCmψAGCGGCCmψGGmψGCCACmψGmψCCCmψGAGAGGGAGCCACAmψG GGCCCCAmψGGAGAmψCmψACAAAACCGmψGAGCGCCmψGGAAGCGGCAGCCmψGmψG CGCGmψGCmψGAGCCmψGmψmψmψCGGAAmψAmψCGAmψAAAGmψCCmψGAAAAGCCm ψGGGAmψmψCCmψGGAGAGCGGCmψCmψGGCmψCCGGCGGmψGGCACCCmψGAAGmψA CGmψGGAGGAmψGmψGACAAACGmψGGmψCAGACGGGAmψGmψGGAGAAGmψGGGGCC CCmψmψCGAmψCmψGGmψGmψACGGCAGCACCCAACCCCmψGGGCAGCmψCmψmψGmψ GACCGGmψGCCCmψGGCmψGGmψACAmψGmψmψmψCAGmψmψCCACCGGAmψCCmψGC AGmψACGCCCmψGCCGAGACAGGAGmψCCCAGCGGCCAmψmψCmψmψmψmψGGAmψmψ mψmψCAmψGGACAACmψmψGCmψGCmψGACCGAGGAmψGACCAGGAAACmψACCACmψ CGGmψmψCCmψGCAGACCGAAGCCGmψGACCCmψGCAGGACGmψGAGAGGCCGGGACm ψACCAGAACGCCAmψGCGGGmψGmψGGmψCCAACAmψCCCmψGGACmψGAAAAGCAAG CACGCACCmψCmψGACCCCmψAAAGAAGAGGAGmψACCmψGCAGGCCCAGGmψGCGGA GCAGAAGCAAGCmψGGACGCCCCmψAAGGmψGGAmψCmψGCmψGGmψGAAGAAmψmψG CCmψCCmψGCCCCmψGAGAGAGmψACmψmψCAAGmψAmψmψmψCAGCCAGAAmψAGmψ CmψGCCCCmψGGGCGGCCCAAGCAGCGGCGCCCCmψCCmψCCCAGCGGCGGCAGCCCA GCCGGCmψCCCCAACCmψCmψACCGAGGAGGGCACCmψCmψGAGmψCCGCCACCCCCG AGAGCGGCCCmψGGCACCmψCCACCGAGCCCAGCGAGGGCAGCGCACCCGGCAGCCCm ψGCCGGCAGCCCCACCmψCCACAGAGGAGGGAACCAGCACCGAGCCCAGCGAAGGCAG CGCCCCAGGCACCAGCACCGAGCCmψAGmψGAGGGCGGCmψCmψGGCGGCGGCAGCGC CCAGGAGAmψmψAAACGGAmψCAACAAGAmψCAGAAGAAGACCmψmψGmψGAAAGACAG CAACACCAAGAAGGCCGGCAAGACAGGCCCCAmψGAAAACCCmψGCmψGGmψmψAGAG mψGAmψGACACCCGAmψCmψGAGAGAGCGGCmψGGAAAACCmψGAGAAAGAAGCCmψG AAAAmψAmψCCCCCAGCCCAmψCAGCAAmψACAmψCmψAGAGCCAACCmψGAAmψAAG CmψGCmψGACCGAmψmψACACCGAAAmψGAAGAAGGCGAmψCCmψGCAmψGmψGmψAC mψGGGAAGAGmψmψCCAGAAGGACCCmψGmψGGGCCmψGAmψGAGCCGGGmψGGCCCA GCCmψGCCAGCAAGAAGAmψCGAmψCAGAACAAGCmψGAAACCmψGAGAmψGGACGAG AAGGGCAACCmψGACCACCGCCGGCmψmψmψGCCmψGCmψCmψCAGmψGmψGGCCAGC CCCmψGmψmψCGmψGmψACAAGCmψGGAGCAGGmψGmψCmψGAGAAGGGCAAGGCmψm ψACACCAACmψACmψmψCGGACGGmψGCAAmψGmψGGCCGAGCACGAAAAGCmψGAmψ CCmψGCmψGGCCCAGCmψGAAGCCCGAGAAGGAmψAGCGACGAAGCCGmψGACAmψAm ψAGCCmψGGGAAAGmψmψmψGGGCAGAGGGCCCmψGGAmψmψmψCmψACAGCAmψmψC AmψGmψGACCAAGGAGmψCCACCCACCCCGmψGAAGCCCCmψGGCCCAGAmψCGCCGG AAACAGAmψACGCCmψCCGGACCmψGmψGGGAAAGGCCCmψGAGCGACGCAmψGmψAm ψGGGCACAAmψCGCCmψCCmψmψCCmψGmψCmψAAGmψACCAGGACAmψCAmψCAmψC GAACACCAGAAGGmψGGmψGAAGGGCAACCAGAAGAGACmψGGAGAGCCmψGCGGGAG CmψGGCCGGCAAGGAAAACCmψGGAAmψACCCmψAGCGmψGACCCmψGCCACCmψCAG CCmψCACACCAAGGAGGGCGmψmψGAmψGCCmψACAACGAAGmψGAmψCGCCCGGGmψ GCGAAmψGmψGGGmψGAACCmψGAACCmψGmψGGCAGAAGCmψGAAGCmψAAGCAGAG AmψGAmψGCCAAGCCmψCmψGCmψGAGACmψGAAGGGAmψmψCCCmψmψCCmψmψmψC CmψCmψGGmψCGAGAGACAGGCCAACGAAGmψGGACmψGGmψGGGACAmψGGmψGmψG mψAACGmψGAAGAAGCmψGAmψCAACGAGAAAAAGGAGGAmψGGCAAGGmψGmψmψmψ mψGGCAGAAmψCmψGGCmψGGCmψACAAGAGACAGGAAGCCCmψGAGACCAmψACCmψ GAGCAGCGAGGAAGAmψCGGAAGAAGGGAAAGAAAmψmψCGCmψCGGmψACCAGCmψG GGCGACCmψGCmψGCmψGCACCmψGGAAAAGAAGCACGGCGAGGACmψGGGGAAAGGm ψGmψACGACGAGGCCmψGGGAGCGGAmψmψGACAAGAAAGmψGGAAGGCCmψGAGCAA GCACAmψCAAGCmψGGAAGAGGAACGGAGAAGCGAGGACGCCCAGAGCAAGGCCGCCC mψGACCGACmψGGCmψGCGGGCmψAAGGCCAGCmψmψCGmψGAmψCGAGGGCCmψGAA GGAGGCCGACAAGGACGAGmψmψCmψGCAGAmψGCGAGCmψGAAGCmψGCAGAAGmψG GmψACGGGGACCmψGCGGGGAAAGCCCmψmψCGCCAmψCGAAGCCGAGAACAGCAmψC CmψGGACAmψCAGCGGCmψmψCAGCAAGCAGmψACAACmψGmψGCCmψmψCAmψCmψG GCAGAAGGACGGCGmψGAAGAAGCmψGAACCmψGmψACCmψGAmψCAmψCAACmψACm

dXR or LTRP ID	SEQ ID NO	RNA Sequence
		ψmψCAAGGGCGGCAAGCmψGCGGmψmψCAAGAAGAmψCAAACCmψGAAGCCmψmψCGA AGCCAACAGAmψmψCmψACACCGmψGAmψCAACAAAAAGAGCGGCGAGAmψCGmψGCC CAmψGGAGGmψGAACmψmψCAACmψmψCGACGACCCCAACCmψGAmψCAmψCCmψGCC mψCmψGGCCmψmψmψGGCAAGAGACAGGGCAGAGAAmψmψCAmψCmψGGAACGACCmψ GCmψGmψCCCmψGGAAACCGGCAGCCmψGAAGCmψGGCCAACGGAAGAGmψGAmψCGA GAAGACACmψGmψACAACAGAAGAACCCGGCAGGAmψGAGCCmψGCCCmψGmψmψCGm ψGGCCCmψGACCmψmψCGAGCGGCGGGAGGmψCCmψGGACmψCCmψCCAAmψAmψCAA ACCAAmψGAACCmψGAmCGGCGmψGGCAAGAGGCGAAAACAmψCCCCGCCGmψGAmψC GCCCmψGACCGACCCCGAGGGCmψGCCCACmψGAGCCGGmψmψmψAAGGAmψAGCCmψ GGGAAACCCAACCCACAmψCCmψGAGAAmψCGGCGAGAGCmψAmψAAGGAGAAGCAGC GGACCAmψCCAGGCCAAGAAGGAGGmψGGAGCAGCGGAGAGCCGGCGGCmψACAGCCG GAAGmψACGCCAGCAAAGCCAAGAAmψCmψGGCAGACGAmψAmψGGmψGAGAAACACC GCmψAGAGAmψCmψGCmψGmψACmψACGCCGmψGACCCAGGAmψGCCAmψGCmψGAmψ CmψmψCGCCAACCmψGAGCCGGGGCmψmψCGGCCGGCAGGGCAAGCGGACCmψmψCAm ψGGCCGAGAGACAGmψACACACGGAmψGGAGGACmψGGCmψGACCGCCAAGCmψGGCC mψACGAGGGCCmψGAGCAAGACCmψACCmψGmψCCAAGACACmψGGCCCAGmψACACC mψCCAAGACAmψGCAGCAACmψGmψGGGmψmψmψACCAmψCACCAGCGCCGACmψACG ACAGGGmψGCmψGGAGAAGCmψGAAGAAGACAGCAACAGGCmψGGAmψGACCACAAmψ mψAACGGCAAGGAGCmψGAAGGmψGGAGGGCCAGAmψmψACCmψACmψACAACAGAmψ ACAAGAGACAGAACGmψAGmψCAAGGACCmψGmψCCGmψCGAGCmψGGAmψAGACmψG AGCGAAGAAmψCmψGmψGAACAACGACAmψCmψCCmψCCmψGGACAAAGGGCAGAAGC GGAGAAGCmψCmψGAGCCmψCCmψGAAGAAAAGAmψmψCmψCCCAmψAGACCCGmψGC AGGAGAAGmψmψCGmψGmψGCCmψGAACmψGCGGCmψmψCGAGACACACGCAGCCGAG CAAGCCGCCCmψGAACAmψCGCCAGAmψCCmψGGCmψGmψmψCCmψGCGGAGCCAGGA GmψACAAGAAAmψACCAGACAAACAAGACAACCGGCAACACCGAmψAAGAGAGCCmψm ψCGmψCGAGACCmψGGCAGmψCCmψmψmψmψACCGGAAGAAGCmψmψAAGGAGGmψGm ψGGAAACCmψGCCGmψGCGGmψCmψGGCGGAmψCmψGGCGGAGGCmψCCACAAGCAmψ GAACAACmψCCCAGGGCAGAGmψGACCmψmψCGAGGACGmψGACCGmψGAAmψmψmψm ψACACAGGGAGAGmψGGCAGAGACmψGAACCCCGAGCAGAGAAACCmψGmψACCGGGA mψGmψGAmψGCmψGGAAAACmψACAGCAAmψCmψGGmψGmψCCGmψGGGCCAGGGCGA GACCACAAAGCCmψGACGmψGAmψCCmψGCGmψCmψGGAGCAGGGCAAGGAACCCmψG GCmψGGAGGAGGAGGAGGmψGCmψGGGAAGCGGACGGGCCGAGAAGAACGGCGACAmψ CGGCGGACAGAmψCmψGGAAGCCmψAAGGACGmψGAAAGAAAGCCmψGACCAGCCCCA AGAAAAAGAGAAAAGmψCGACmψACAAGGAmψGACGAmψGACAAGGACmψACAAGGAm ψGACGACGACAAGmψAAmψAGAmψAAGCGGCCGCmψmψAAmψmψAAGCmψGCCmψmψC mψGCGGGGCmψmψGCCmψmψCmψGGCCAmψGCCCmψmψCmψmψCmψCmψCCCmψmψGC ACCmψGmψACCmψCmψmψGGmψCmψmψmψGAAmψAAAGCCmψGAGmψAGGAAGmψcmψ agaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa aaaaaaaaaaaaaaaaaaaaaaa

Table 13: Full-length protein sequences of dXR1 and LTRP1-ZIM3 molecules assessed in experiment #1 of this example.

dXR or LTRP ID	Amino acid sequence SEQ ID NO:
dXR1	3065
LTRP #1	3066

Synthesis of gRNAs:

[0401] In experiment #1, gRNAs targeting the *PCSK9* locus were designed using gRNA scaffold 174 and chemically synthesized using the v1 modification profile (as described in Example 7, below). Spacers were designed in proximity to the *PCSK9* promoter. The sequences of the *PCSK9*-targeting spacers and the resulting chemically-modified gRNAs are listed in Table 14.

Table 14: Sequences of spacers targeting the *PCSK9* locus and chemically-modified gRNAs used in this example. Chemical modifications: * = phosphorothioate bond; m = 2'OMe modification.

gRNA ID (scaffold-variant spacer)	Target	Targeting spacer sequence (RNA)	SEQ ID NO:	Full gRNA sequence (RNA)	SEQ ID NO:
174-6.7	human *PCSK9*	UCCUGGCUUCCUGGUGAAGA	2008	mA*mC*mU*GGCGCUUUUAUCUGAUU ACUUUGAGAGCCAUCACCAGCGACUA UGUCGUAGUGGGUAAAGCUCCCUCUU CGGAGGGAGCAUCAAAGUCCUGGCUU CCUGGUGAmA*mG*mA	3074
174-27.1	mouse *PCSK9*	GCCUCGCCCUCCCCAGACAG	3067	mA*mC*mU*GGCGCUUUUAUCUGAUU ACUUUGAGAGCCAUCACCAGCGACUA UGUCGUAGUGGGUAAAGCUCCCUCUU CGGAGGGAGCAUCAAAGGCCUCGCCC UCCCCAGAmC*mA*mG	3075
174-27.88	mouse *PCSK9*	CGCUACCUGCCUAAACUUUG	3068	mA*mC*mU*GGCGCUUUUAUCUGAUU ACUUUGAGAGCCAUCACCAGCGACUA UGUCGUAGUGGGUAAAGCUCCCUCUU CGGAGGGAGCAUCAAAGCGCUACCUG CCUAAACUmU*mU*mG	3076
174-27.92	mouse *PCSK9*	CCCUCCAACAAUAUUAACUA	3069	mA*mC*mU*GGCGCUUUUAUCUGAUU ACUUUGAGAGCCAUCACCAGCGACUA UGUCGUAGUGGGUAAAGCUCCCUCUU CGGAGGGAGCAUCAAAGCCCUCCAAC AAUAUUAAmC*mU*mA	3077
174-27.93	mouse *PCSK9*	GGGGUCUCCCAGCCACCCCU	3070	mA*mC*mU*GGCGCUUUUAUCUGAUU ACUUUGAGAGCCAUCACCAGCGACUA UGUCGUAGUGGGUAAAGCUCCCUCUU CGGAGGGAGCAUCAAAGGGGGUCUCC CAGCCACCmC*mC*mU	3078
174-27.94	mouse *PCSK9*	CCCCUCUUAAUCCCCACUCC	3071	mA*mC*mU*GGCGCUUUUAUCUGAUU ACUUUGAGAGCCAUCACCAGCGACUA UGUCGUAGUGGGUAAAGCUCCCUCUU CGGAGGGAGCAUCAAAGCCCCUCUUA AUCCCCACmU*mC*mC	3079
174-27.100	mouse *PCSK9*	CUCUCUCUUUCUGAGGCUAG	3072	mA*mC*mU*GGCGCUUUUAUCUGAUU ACUUUGAGAGCCAUCACCAGCGACUA UGUCGUAGUGGGUAAAGCUCCCUCUU CGGAGGGAGCAUCAAAGCUCUCUCUU UCUGAGGCmU*mA*mG	3080

174-27.103	mouse *PCSK9*	UAAUCUCCAUCCUCGUCCUG	3073	mA*mC*mU*GGCGCUUUUAUCUGAUU ACUUUGAGAGCCAUCACCAGCGACUA UGUCGUAGUGGGUAAAGCUCCCUCUU CGGAGGGAGCAUCAAAGUAAUCUCCA UCCUCGUCmC*mU*mG	3081

Transfection of mRNA and gRNA into Hepa1-6 cells and intracellular PCSK9 staining:

[0402] Seeded Hepa1-6 cells treated with the NATE™ inhibitor were lipofected with 300 ng of mRNA encoding dXR1 or LTRP1-ZIM3 (Table 12) and 150 ng of a *PCSK9*-targeting gRNA (Table 14). Seven different gRNAs spanning the promoter region of the mouse *PCSK9* locus were tested, in addition to a non-targeting sequence complementary to the human *PCSK9* gene (Table 14). Cells were harvested at 6, 13, and 25 days after transfection to measure intracellular levels of the PCSK9 protein using an intracellular flow cytometry staining protocol. Briefly, cells were fixed using 4% paraformaldehyde in PBS, permeabilized, and stained using a mouse anti-PCSK9 primary antibody (R&D Systems), followed by a fluorescent goat anti-mouse IgG secondary antibody (Thermo Fisher). Fluorescence levels were measured using the Attune™ NxT flow cytometer, and data were analyzed using the FlowJo™ software. Cell populations were gated using the non-targeting gRNA as a negative control.

Experiment #2: LTRP #1 vs. LTRP #5 in Hepa1-6 cells when delivered as mRNA

Generation of mRNA:

[0403] mRNA encoding LTRP #1 or LTRP #5 (configuration #5 in FIG. 1) containing the ZIM3-KRAB domain (hereafter known as LTRP1-ZIM3 or LTRP5-ZIM3 respectively; configurations are diagrammed in FIG. 1) was generated by IVT in-house using plasmid-based PCR templates. Briefly, PCR was performed on plasmids encoding LTRP #1 or LTRP #5 harboring the ZIM3-KRAB domain with flanking NLSes with a forward primer containing a T7 promoter and reverse primer encoding a 120-nucleotide poly(A) tail. These constructs also contained a 2x FLAG sequence. DNA sequences encoding these molecules are listed in Table 15. The resulting PCR templates were used for IVT reactions, which were carried out with CleanCap® AG and N1-methyl-pseudouridine. IVT reactions were then subjected to DNase digestion and on-column oligo dT purification. Full-length RNA sequences encoding the LTRP mRNAs are listed in Table 16.

[0404] As experimental controls, mRNA encoding catalytically-active CasX 491 was also similarly generated by IVT using a PCR template as described. Generation of mRNAs encoding LTRP1-ZIM3 and dCas9-ZNF10-DNMT3A/3L, a catalytically-dead Cas9 fused to both the ZNF10-KRAB domain and DNMT3A/L domains, by IVT by a third-party was performed as

described above for experiment #1.

Table 15: Encoding sequences of the LTRP1-ZIM3 and LTRP5-ZIM3 mRNA molecules assessed in experiment #2 of this example*.

LTRP ID	Component	DNA sequence SEQ ID NO:
LTRP #1 - ZIM3-KRAB	5'UTR	3082
	START codon + NLS + linker	3083
	START codon + DNMT3A catalytic domain	3084
	Linker	3085
	DNMT3L interaction domain	3086
	Linker	3087
	Linker + buffer	3088
	dCasX491	3089
	Linker + buffer	3090
	ZIM3-KRAB	3091
	Buffer + NLS	3092
	Tag	3093
	Buffer	3094
	Poly(A) tail	3095
LTRP #5 - ZIM3-KRAB	5'UTR	3082
	START codon + NLS + buffer	3096
	START codon + DNMT3A catalytic domain	3084
	Linker	3085
	DNMT3L interaction domain	3086
	Linker	3097
	ZIM3-KRAB	3091
	Linker	3087
	dCasX491	3089
	Linker + buffer	3090
	NLS	3098
	Tag	3093
	Buffer	3094
	Poly(A) tail	3095

*Components are listed in a 5' to 3' order within the constructs

Table 16: Full-length RNA sequences of LTRP1-ZIM3 and LTRP5-ZIM3 mRNA

molecules assessed in experiment #2 of this example. Modification 'mψ' = N1-methyl-pseudouridine

LTRP ID	SEQ ID NO	RNA Sequence
LTRP #1 - ZIM3-KRAB	3099	GACCGGCCGCCACCAmψGGCCCCAAAGAAGAAGCGGAAGGmψCmψCmψAGAGmψmψA ACGGAmψCAGGCmψCmψGGAGGmψGGAAmψGAACCAmψGACCAGGAAmψmψmψGACC CCCCAAAGGmψmψmψACCCACCmψGmψGCCAGCmψGAGAAGAGGAAGCCCAmψCCGC GmψGCmψGmψCmψCmψCmψmψmψGAmψGGGAmψmψGCmψACAGGGCmψCCmψGGmψG CmψGAAGGACCmψGGGCAmψCCAAGmψGGACCGCmψACAmψCGCCmψCCGAGGmψGm ψGmψGAGGACmψCCAmψCACGGmψGGGCAmψGGmψGCGGCACCAGGGAAAGAmψCAm ψGmψACGmψCGGGGACGmψCCGCAGCGmψCACACAGAAGCAmψAmψCCAGGAGmψGG GGCCCAmψmψCGACCmψGGmψGAmψmψGGAGGCAGmψCCCmψGCAACGACCmψCmψC CAmψmψGmψCAACCCmψGCCCGCAAGGGACmψmψmψAmψGAGGGmψACmψGGCCGCC mψCmψmψCmψmψmψGAGmψmψCmψACCGCCmψCCmψGCAmψGAmψGCGCGGCCCAAG GAGGGAGAmψGAmψCGCCCCmψmψCmψmψCmψGGCmψCmψmψmψGAGAAmψGmψGGm ψGGCCAmψGGGCGmψmψAGmψGACAAGAGGGACAmψCmψCGCGAmψmψmψCmψmψGA GmψCmψAACCCCGmψGAmψGAmψmψGACGCCAAAGAAGmψGmψCmψGCmψGCACACA GGGCCCGmψmψACmψmψCmψGGGGmψAACCmψmψCCmψGGCAmψGAACAGGCCmψmψ mψGGCAmψCCACmψGmψGAAmψGAmψAAGCmψGGAGCmψGCAAGAGmψGmψCmψGGA GCACGGCAGAAmψAGCCAAGmψmψCAGCAAAGmψGAGGACCAmψmψACCACCAGGmψ CAAACmψCmψAmψAAAGCAGGGCAAAGACCAGCAmψmψmψCCCCGmψCmψmψCAmψG AACGAGAAGGAGGACAmψCCmψGmψGGmψGCACmψGAAAmψGGAAAGGGmψGmψmψm ψGGCmψmψCCCCGmψCCACmψACACAGACGmψGmψCCAACAmψGAGCCGCmψmψGGC GAGGCAGAGACmψGCmψGGGCCGGmψCGmψGGAGCGmψGCCGGmψCAmψCCGCCACC mψCmψmψCGCmψCCGCmψGAAGGAAmψAmψmψmψmψGCmψmψGmψGmψGmψCmψAGC GGCAAmψAGmψAACGCmψAACAGCCGCGGGCCGAGCmψmψCAGCAGCGGCCmψGGmψ GCCGmψmψAAGCmψmψGCGCGGCAGCCAmψAmψGGGCCCmψAmψGGAGAmψAmψACA AGACAGmψGmψCmψGCAmψGGAAGAGACAGCCAGmψGCGGGmψACmψGAGCCmψCmψ mψCAGAAACAmψCGACAAGGmψACmψAAAGAGmψmψmψGGGCmψmψCmψmψGGAAAG CGGmψmψCmψGGmψmψCmψGGGGGAGGAACGCmψGAAGmψACGmψGGAAGAmψGmψC ACAAAmψGmψCGmψGAGGAGGGACGmψGGAGAAAmψGGGGCCCCmψmψmψGACCmψG GmψGmψACGGCmψCGACGCAGCCCCmψAGGCAGCmψCmψmψGmψGAmψCGCmψGmψC CCGGCmψGGmψACAmψGmψmψCCAGmψmψCCACCGGAmψCCmψGCAGmψAmψGCGCm ψGCCmψCGCCAGGAGAGmψCAGCGGCCCmψmψCmψmψCmψGGAmψAmψmψCAmψGGA CAAmψCmψGCmψGCmψGACmψGAGGAmψGACCAAGAGACAACmψACCCGCmψmψCCm ψmψCAGACAGAGGCmψGmψGACCCmψCCAGGAmψGmψCCGmψGGCAGAGACmψACCA GAAmψGCmψAmψGCGGGmψGmψGGAGCAACAmψmψCCAGGGCmψGAAGAGCAAGCAm ψGCGCCCCmψGACCCCAAAGGAAGAAGAGmψAmψCmψGCAAGCCCAAGmψCAGAAGC AGGAGCAAGCmψGGACGCCCCGAAAGmψmψGACCmψCCmψGGmψGAAGAACmψGCCm ψmψCmψCCCGCmψGAGAGAGmψACmψmψCAAGmψAmψmψmψmψmψCmψCAAAACmψC ACmψmψCCmψCmψmψGGAGGGCCGAGCmψCmψGGCGCACCCCCACCAAGmψGGAGGG mψCmψCCmψGCCGGGmψCCCCAACAmψCmψACmψGAAGAAGGCACCAGCGAAmψCCG CAACGCCCGAGmψCAGGCCCmψGGmψACCmψCCACAGAACCAmψCmψGAAGGmψAGm ψGCGCCmψGGmψmψCCCCAGCmψGGAAGCCCmψACmψmψCCACCGAAGAAGGCACGm ψCAACCGAACCAAGmψGAAGGAmψCmψGCCCCmψGGGACCAGCACmψGAACCAmψCm ψGAGGGCGGmψmψCCGGCGGAGGAAGCGCmψCAAGAGAmψCAAGAGAAmψCAACAAG AmψCAGAAGGAGACmψGGmψCAAGGACAGCAACACAAAGAAGGCCGGCAAGACAGGC CCCAmψGAAAACCCmψGCmψCGmψCAGAGmψGAmψGACCCCmψGACCmψGAGAGAGC GGCmψGGAAAACCmψGAGAAAGAAGCCCGAGAACAmψCCCmψCAGCCmψAmψCAGCA ACACCAGCAGGGCCAACCmψGAACAAGCmψGCmψGACCGACmψACACCGAGAmψGAA GAAAGCCAmψCCmψGCACGmψGmψACmψGGGAAGAGmψmψCCAGAAAGACCCCGmψG GGCCmψGAmψGAGCAGAGmψmψGCmψCAGCCmψGCCAGCAAGAAGAmψCGACCAGAA CAAGCmψGAAGCCCGAGAmψGGACGAGAAGGGCAAmψCmψGACCACAGCCGGCmψmψ mψGCCmψGCmψCmψCAGmψGmψGGCCAGCCmψCmψGmψmψCGmψGmψACAAGCmψGG AACAGGmψGmψCCGAGAAAGGCAAGGCCmψACACCAACmψACmψmψCGGCAGAmψGm ψAACGmψGGCCGAGCACGAGAAGCmψGAmψmψCmψGCmψGGCCCAGCmψGAAACCmψ

LTRP ID	SEQ ID NO	RNA Sequence
		GAGAAGGACmψCmψGAmψGAGGCCGmψGACCmψACAGCCmψGGGCAAGmψmψmψGGA CAGAGAGCCCmψGGACmψmψCmψACAGCAmψCCACGmψGACCAAAGAAAGCACACAC CCCGmψGAAGCCCCmψGGCmψCAGAmψCGCCGGCAAmψAGAmψACGCCmψCmψGGAC CmψGmψGGGCAAAGCCCmψGmψCCGAmψGCCmψGCAmψGGGAACAAmψCGCCAGCmψ mψCCmψGAGCAAGmψACCAGGACAmψCAmψCAmψCGAGCACCAGAAGGmψGGmψCAA GGGCAACCAGAAGAGACmψGGAAAGCCmψGAGGGAGCmψGGCCGGCAAAGAGAACCm ψGGAAmψACCCCAGCGmψGACCCmψGCCmψCCmψCAGCCmψCACACAAAAGAAGGCG mψGGACGCCmψACAACGAAGmψGAmψCGCCAGAGmψGAGAAmψGmψGGGmψCAACCm ψGAACCmψGmψGGCAGAAGCmψGAAACmψGmψCCAGGGACGACGCCAAGCCmψCmψG CmψGAGACmψGAAGGGCmψmψCCCmψAGCmψmψCCCmψCmψGGmψGGAAAGACAGGC CAAmψGAAGmψGGAmψmψGGmψGGGACAmψGGmψCmψGCAACGmψGAAGAAGCmψGA mψCAACGAGAAGAAAGAGGAmψGGCAAGGmψmψmψmψCmψGGCAGAACCmψGGCCGG CmψACAAGAGACAAGAAGCCCmψGAGGCCmψmψACCmψGAGCAGCGAAGAGGACCGG AAGAAGGGCAAGAAGmψmψCGCCAGAmψACCAGCmψGGGCGACCmψGCmψGCmψGCA CCmψGGAAAAGAAGCACGGCGAGGACmψGGGGCAAAGmψGmψACGAmψGAGGCCmψG GGAGAGAAmψCGACAAGAAGGmψGGAAGGCCmψGAGCAAGCACAmψmψAAGCmψGGA AGAGGAAAGAAGGAGCGAGGACGCCCAAmψCmψAAAGCCGCmψCmψGACCGAmψmψG GCmψGAGAGCCAAGGCCAGCmψmψmψGmψGAmψCGAGGGCCmψGAAAGAGGCCGACA AGGACGAGmψmψCmψGCAGAmψGCGAGCmψGAAGCmψGCAGAAGmψGGmψACGGCGA mψCmψGAGAGGCAAGCCCmψmψCGCCAmψmψGAGGCCGAGAACAGCAmψCCmψGGAC AmψCAGCGGCmψmψCAGCAAGCAGmψACAACmψGCGCCmψmψCAmψmψmψGGCAGAA AGACGGCGmψCAAGAAACmψGAACCmψGmψACCmψGAmψCAmψCAAmψmψACmψmψC AAAGGCGGCAAGCmψGCGGmψmψCAAGAAGAmψCAAACCCGAGGCCmψmψCGAGGCm ψAACAGAmψmψCmψACACCGmψGAmψCAACAAAAAGmψCCGGCGAGAmψCGmψGCCC AmψGGAAGmψGAACmψmψCAACmψmψCGACGACCCCAACCmψGAmψmψAmψCCmψGC CmψCmψGGCCmψmψCGGCAAGAGACAGGGCAGAGAGmψmψCAmψCmψGGAACGAmψC mψGCmψGAGCCmψGGAAACCGGCmψCmψCmψGAAGCmψGGCCAAmψGGCAGAGmψGA mψCGAGAAAACCCmψGmψACAACAGGAGAACCAGACAGGACGAGCCmψGCmψCmψGm ψmψmψGmψGGCCCmψGACCmψmψCGAGAGAAGAGAGGmψGCmψGGACAGCAGCAACA mψCAAGCCCAmψGAACCmψGAmψCGGCGmψGGCCCGGGGCGAGAAmψAmψCCCmψGC mψGmψGAmψCGCCCmψGACAGACCCmψGAAGGAmψGCCCACmψGAGCAGAmψmψCAA GGACmψCCCmψGGGCAACCCmψACACACAmψCCmψGAGAAmψCGGCGAGAGCmψACA AAGAGAAGCAGAGGACAAmψCCAGGCCAAGAAAGAGGmψGGAACAGAGAAGAGCCGG CGGAmψACmψCmψAGGAAGmψACGCCAGCAAGGCCAAGAAmψCmψGGCCGACGACAm ψGGmψCCGAAACACCGCCAGAGAmψCmψGCmψGmψACmψACGCCGmψGACACAGGAC GCCAmψGCmψGAmψCmψmψCGCGAAmψCmψGAGCAGAGGCmψmψCGGCCGGCAGGGC AAGAGAACCmψmψmψAmψGGCCGAGAGGCAGmψACACCAGAAmψGGAAGAmψmψGGC mψCACAGCmψAAACmψGGCCmψACGAGGGACmψGAGCAAGACCmψACCmψGmψCCAA AACACmψGGCCCAGmψAmψACCmψCCAAGACCmψGCAGCAAmψmψGCGGCmψmψCAC CAmψCACCAGCGCCGACmψACGACAGAGmψGCmψGGAAAAGCmψCAAGAAAACCGCC ACCGGCmψGGAmψGACCACCAmψCAACGGCAAAGAGCmψGAAGGmψmψGAGGGCCAG AmψCACCmψACmψACAACAGGmψACAAGAGGCAGAACGmψCGmψGAAGGAmψCmψGA GCGmψGGAACmψGGACAGACmψGAGCGAAGAGAGCGmψGAACAACGACAmψCAGCAG CmψGGACAAAGGGCAGAmψCAGGCGAGGCmψCmψGAGCCmψGCmψGAAGAAGAGGmψ mψmψAGCCACAGACCmψGmψGCAAGAGAAGmψmψCGmψGmψGCCmψGAACmψGCGGC mψmψCGAGACACACGCCGCmψGAACAGGCmψGCCCmψGAACAmψmψGCCAGAAGCmψ GGCmψGmψmψCCmψGAGAAGCCAAGAGmψACAAGAAGmψACCAGACCAACAAGACCA CCGGCAACACCGACAAGAGGGCCmψmψmψGmψGGAAACCmψGGCAGAGCmψmψCmψA CAGAAAAAAGCmψGAAAGAAGmψCmψGGAAGCCCGCCGmψGCGAmψCGGGCGGmψmψ CCGGCGGAGGmψmψCCACmψAGmψAmψGAACAAmψmψCCCAGGGAAGAGmψGACCmψ mψCGAGGAmψGmψCACmψGmψGAACmψmψCACCCAGGGGGAGmψGGCAGCGGCmψGA AmψCCCGAACAGAGAAACmψmψGmψACAGGGAmψGmψGAmψGCmψGGAGAAmψmψAC AGCAACCmψmψGmψCmψCmψGmψGGGACAAGGGGAAACCACCAAACCCGAmψGmψGA mψCmψmψGAGGmψmψGGAACAAGGAAAGGAGCCAmψGGmψmψGGAGGAAGAGGAAGm ψGCmψGGGAAGmψGGCCGmψGCAGAAAAAAAmψGGGGACAmψmψGGAGGGCAGAmψm ψmψGGAAGCCAAAGGAmψGmψGAAAGAGAGmψCmψCACmψAGmψCCAAAAAAGAAGA

LTRP ID	SEQ ID NO	RNA Sequence
		GAAAGGmψAGAmψmψACAAAGAmψGACGAmψGACAAAGACmψACAAGGAmψGAmψGA mψGAmψAAGGGAmψCCGGCmψGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAA
LTRP #5 - ZIM3-KRAB	3100	GACCGGCCGCCACCAmψGGCCCCAAAGAAGAAGCGGAAGGmψCmψCmψAGAAmψGAA CCAmψGACCAGGAAmψmψmψGACCCCCCAAAGGmψmψmψACCCACCmψGmψGCCAGC mψGAGAAGAGGAAGCCCAmψCCGCGmψGCmψGmψCmψCmψCmψmψmψGAmψGGGAmψ mψGCmψACAGGGCmψCCmψGGmψGCmψGAAGGACCmψGGGCAmψCCAAGmψGGACCG CmψACAmψCGCCmψCCGAGGmψGmψGmψGAGGACmψCCAmψCACGGmψGGGCAmψGG mψGCGGCACCAGGGAAAGAmψCAmψGmψACGmψCGGGGACGmψCCGCAGCGmψCACA CAGAAGCAmψAmψCCAGGAGmψGGGGCCCAmψmψCGACCmψGGmψGAmψmψGGAGGC AGmψCCCmψGCAACGACCmψCmψCCAmψmψGmψCAACCCmψGCCCGCAAGGGACmψm ψmψAmψGAGGGmψACmψGGCCGCCmψCmψmψCmψmψmψGAGmψmψCmψACCGCCmψC CmψGCAmψGAmψGCGCGGCCCAAGGAGGGAGAmψGAmψCGCCCCmψmψCmψmψCmψG GCmψCmψmψmψGAGAAmψGmψGGmψGGCCAmψGGGCGmψmψAGmψGACAAGAGGGAC AmψCmψCGCGAmψmψmψCmψmψGAGmψCmψAACCCCGmψGAmψGAmψmψGACGCCAA AGAAGmψGmψCmψGCmψGCACACAGGGCCCGmψmψACmψmψCmψGGGGmψAACCmψm ψCCmψGGCAmψGAACAGGCCmψmψmψGGCAmψCCACmψGmψGAAmψGAmψAAGCmψG GAGCmψGCAAGAGmψGmψCmψGGAGCACGGCAGAAmψAGCCAAGmψmψCAGCAAAGm ψGAGGACCAmψmψACCACCAGGmψCAAACmψCmψAmψAAAGCAGGGCAAAGACCAGC AmψmψmψCCCCGmψCmψmψCAmψGAACGAGAAGGAGGACAmψCCmψGmψGGmψGCAC mψGAAAmψGGAAAGGGmψGmψmψmψGGCmψmψCCCCGmψCCACmψACACAGACGmψG mψCCAACAmψGAGCCGCmψmψGGCGAGGCAGAGACmψGCmψGGGCCGGmψCGmψGGA GCGmψGCCGGmψCAmψCCGCCACCmψCmψmψCGCmψCCGCmψGAAGGAAmψAmψmψm ψmψGCmψmψGmψGmψGmψCmψAGCGGCAAmψAGmψAACGCmψAACAGCCGCGGGCCG AGCmψmψCAGCAGCGGCCmψGGmψGCCGmψmψAAGCmψmψGCGCGGCAGCCAmψAmψ GGGCCCmψAmψGGAGAmψAmψACAAGACAGmψGmψCmψGCAmψGGAAGAGACAGCCA GmψGCGGGmψACmψGAGCCmψCmψmψCAGAAACAmψCGACAAGGmψACmψAAAGAGm ψmψmψGGGCmψmψCmψmψGGAAAGCGGmψmψCmψGGmψmψCmψGGGGGAGGAACGCm ψGAAGmψACGmψGGAAGAmψGmψCACAAAmψGmψCGmψGAGGAGGGACGmψGGAGAA AmψGGGGCCCCmψmψmψGACCmψGGmψGmψACGGCmψCGACGCAGCCCCmψAGGCAG CmψCmψmψGmψGAmψCGCmψGmψCCCGGCmψGGmψACAmψGmψmψCCAGmψmψCCAC CGGAmψCCmψGCAGmψAmψGCGCmψGCCmψCGCCAGGAGAGmψCAGCGGCCCmψmψC mψmψCmψGGAmψAmψmψCAmψGGACAAmψCmψGCmψGCmψGACmψGAGGAmψGACCA AGAGACAACmψACCCGCmψmψCCmψmψCAGACAGAGGCmψGmψGACCCmψCCAGGAm ψGmψCCGmψGGCAGAGACmψACCAGAAmψGCmψAmψGCGGGmψGmψGGAGCAACAmψ mψCCAGGGCmψGAAGAGCAAGCAmψGCGCCCCmψGACCCCAAAGGAAGAAGAGmψAm ψCmψGCAAGCCCAAGmψCAGAAGCAGGAGCAAGCmψGGACGCCCCGAAAGmψmψGAC CmψCCmψGGmψGAAGAACmψGCCmψmψCmψCCCGCmψGAGAGAGmψACmψmψCAAGm ψAmψmψmψmψmψCmψCAAAACmψCACmψmψCCmψCmψmψGGCGGmψmψCCGGCGGAG GAAmψGAACAAmψmψCCCAGGGAAGAGmψGACCmψmψCGAGGAmψGmψCACmψGmψG AACmψmψCACCCAGGGGGAGmψGGCAGCGGCmψGAAmψCCCGAACAGAGAAACmψmψ GmψACAGGGAmψGmψGAmψGCmψGGAGAAmψmψACAGCAACCmψmψGmψCmψCmψGm ψGGGACAAGGGGAAACCACCAAACCCGAmψGmψGAmψCmψmψGAGGmψmψGGAACAA GGAAAGGAGCCAmψGGmψmψGGAGGAAGAGGAAGmψGCmψGGGAAGmψGGCCGmψGC AGAAAAAAAmψGGGGACAmψmψGGAGGGCAGAmψmψmψGGAAGCCAAAGGAmψGmψG AAAGAGAGmψCmψCGGAGGGCCGAGCmψCmψGGCGCACCCCCACCAAGmψGGAGGGm ψCmψCCmψGCCGGGmψCCCCAACAmψCmψACmψGAAGAAGGCACCAGCGAAmψCCGC AACGCCCGAGmψCAGGCCCmψGGmψACCmψCCACAGAACCAmψCmψGAAGGmψAGmψ GCGCCmψGGmψmψCCCCAGCmψGGAAGCCCmψACmψmψCCACCGAAGAAGGCACGmψ CAACCGAACCAAGmψGAAGGAmψCmψGCCCCmψGGGACCAGCACmψGAACCAmψCmψ GAGCAAGAGAmψCAAGAGAAmψCAACAAGAmψCAGAAGGAGACmψGGmψCAAGGACA GCAACACAAAGAAGGCCGGCAAGACAGGCCCCAmψGAAAACCCmψGCmψCGmψCAGA GmψGAmψGACCCCmψGACCmψGAGAGAGCGGCmψGGAAAACCmψGAGAAAGAAGCCC GAGAACAmψCCCmψCAGCCmψAmψCAGCAACACCAGCAGGGCCAACCmψGAACAAGC mψGCmψGACCGACmψACACCGAGAmψGAAGAAAGCCAmψCCmψGCACGmψGmψACmψ

LTRP ID	SEQ ID NO	RNA Sequence
		GGGAAGAGmψmψCCAGAAAGACCCCGmψGGGCCmψGAmψGAGCAGAGmψmψGCmψCA GCCmψGCCAGCAAGAAGAmψCGACCAGAACAAGCmψGAAGCCCGAGAmψGGACGAGA AGGGCAAmψCmψGACCACAGCCGGCmψmψmψGCCmψGCmψCmψCAGmψGmψGGCCAG CCmψCmψGmψmψCGmψGmψACAAGCmψGGAACAGGmψGmψCCGAGAAAGGCAAGGCC mψACACCAACmψACmψmψCGGCAGAmψGmψAACGmψGGCCGAGCACGAGAAGCmψGA mψmψCmψGCmψGGCCCAGCmψGAAACCmψGAGAAGGACmψCmψGAmψGAGGCCGmψG ACCmψACAGCCmψGGGCAAGmψmψmψGGACAGAGAGCCCmψGGACmψmψCmψACAGC AmψCCACGmψGACCAAAGAAAGCACACACCCCGmψGAAGCCCCmψGGCmψCAGAmψC GCCGGCAAmψAGAmψACGCCmψCmψGGACCmψGmψGGGCAAAGCCCmψGmψCCGAmψ GCCmψGCAmψGGGAACAAmψCGCCAGCmψmψCCmψGAGCAAGmψACCAGGACAmψCA mψCAmψCGAGCACCAGAAGGmψGGmψCAAGGGCAACCAGAAGAGACmψGGAAAGCCm ψGAGGGAGCmψGGCCGGCAAAGAGAACCmψGGAAmψACCCCAGCGmψGACCCmψGCC mψCCmψCAGCCmψCACACAAAAGAAGGCGmψGGACGCCmψACAACGAAGmψGAmψCG CCAGAGmψGAGAAmψGmψGGGmψCAACCmψGAACCmψGmψGGCAGAAGCmψGAAACm ψGmψCCAGGGACGACGCCAAGCCmψCmψGCmψGAGACmψGAAGGGCmψmψCCCmψAG CmψmψCCCmψCmψGGmψGGAAAGACAGGCCAAmψGAAGmψGGAmψmψGGmψGGGACA mψGGmψCmψGCAACGmψGAAGAAGCmψGAmψCAACGAGAAGAAAGAGGAmψGGCAAG GmψmψmψmψCmψGGCAGAACCmψGGCCGGCmψACAAGAGACAAGAAGCCCmψGAGGC CmψmψACCmψGAGCAGCGAAGAGGACCGGAAGAAGGGCAAGAAGmψmψCGCCAGAmψ ACCAGCmψGGGCGACCmψGCmψGCmψGCACCmψGGAAAAGAAGCACGGCGAGGACmψ GGGGCAAAGmψGmψACGAmψGAGGCCmψGGGAGAGAAmψCGACAAGAAGGmψGGAAG GCCmψGAGCAAGCACAmψmψAAGCmψGGAAGAGGAAAGAAGGAGCGAGGACGCCCAA mψCmψAAAGCCGCmψCmψGACCGAmψmψGGCmψGAGAGCCAAGGCCAGCmψmψmψGm ψGAmψCGAGGGCCmψGAAAGAGGCCGACAAGGACGAGmψmψCmψGCAGAmψGCGAGC mψGAAGCmψGCAGAAGmψGGmψACGGCGAmψCmψGAGAGGCAAGCCCmψmψCGCCAm ψmψGAGGCCGAGAACAGCAmψCCmψGGACAmψCAGCGGCmψmψCAGCAAGCAGmψAC AACmψGCGCCmψmψCAmψmψmψGGCAGAAAGACGGCGmψCAAGAAACmψGAACCmψG mψACCmψGAmψCAmψCAAmψmψACmψmψCAAAGGCGGCAAGCmψGCGGmψmψCAAGA AGAmψCAAACCCGAGGCCmψmψCGAGGCmψAACAGAmψmψCmψACACCGmψGAmψCA ACAAAAAGmψCCGGCGAGAmψCGmψGCCCAmψGGAAGmψGAACmψmψCAACmψmψCG ACGACCCCAACCmψGAmψmψAmψCCmψGCCmψCmψGGCCmψmψCGGCAAGAGACAGG GCAGAGAGmψmψCAmψCmψGGAACGAmψCmψGCmψGAGCCmψGGAAACCGGCmψCmψ CmψGAAGCmψGGCCAAmψGGCAGAGmψGAmψCGAGAAAACCCCmψGmψACAACAGGAG AACCAGACAGGACGAGCCmψGCmψCmψGmψmψmψGmψGGCCCmψGACCmψmψCGAGA GAAGAGAGGmψGCmψGGACAGCAGCAACAmψCAAGCCCAmψGAACCmψGAmψCGGCG mψGGCCCGGGGCGAGAAmψAmψCCCmψGCmψGmψGAmψCGCCCmψGACAGACCCmψG AAGGAmψGCCCACmψGAGCAGAmψmψCAAGGACmψCCCmψGGGCAACCCmψACACAC AmψCCmψGAGAAmψCGGCGAGAGCmψACAAAGAGAAGCAGAGGACAAmψCCAGGCCA AGAAAGAGGmψGGAACAGAGAAGAGCCGGCGGAmψACmψCmψAGGAAGmψACGCCAG CAAGGCCAAGAAmψCmψGGCCGACGACAmψGGmψCCGAAACACCGCCAGAGAmψCmψ GCmψGmψACmψACGCCGmψGACACAGGACGCCAmψGCmψGAmψCmψmψCGCGAAmψC mψGAGCAGAGGCmψmψCGGCCGGCAGGGCAAGAGAACCmψmψmψAmψGGCCGAGAGG CAGmψACACCAGAAmψGGAAGAmψmψGGCmψCACAGCmψAAACmψGGCCmψACGAGG GACmψGAGCAAGACCmψACCmψGmψCCAAAACACmψGGCCCAGmψAmψACCmψCCAA GACCmψGCAGCAAmψmψGCGGCmψmψCACCAmψCACCAGCGCCGACmψACGACAGAG mψGCmψGGAAAAGCmψCAAGAAAACCGCCACCGGCmψGGAmψGACCACCAmψCAACG GCAAAGAGCmψGAAGGmψmψGAGGGCCAGAmψCACCmψACmψACAACAGGmψACAAG AGGCAGAACGmψCGmψGAAGGAmψCmψGAGCGmψGGAACmψGGACAGACmψGAGCGA AGAGAGCGmψGAACAACGACAmψCAGCAGCmψGGACAAAGGGCAGAmψCAGGCGAGG CmψCmψGAGCCmψGCmψGAAGAAGAGGmψmψmψAGCCACAGACCmψGmψGCAAGAGA AGmψmψCGmψGmψGCCmψGAACmψGCGGCmψmψCGAGACACACGCCGCmψGAACAGG CmψGCCCmψGAACAmψmψGCCAGAAGCmψGGCmψGmψmψCCmψGAGAAGCCAAGAGm ψACAAGAAGmψACCAGACCAACAAGACCACCGGCAACACCGACAAGAGGGCCmψmψm ψGmψGGAAACCmψGGCAGAGCmψmψCmψACAGAAAAAAGCmψGAAAGAAGmψCmψGG AAGCCCGCCGmψGCGAmψCGGGCGGmψmψCCGGCGGAGGmψmψCCACmψAGmψCCAA AAAAGAAGAGAAAGGmψAGAmψmψACAAAGAmψGACGAmψGACAAAGACmψACAAGG

LTRP ID	SEQ ID NO	RNA Sequence
		AmψGAmψGAmψGAmψAAGGGAmψCCGGCmψGAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

[0405] For experiment #2, synthesis of *PCSK9*-targeting gRNAs was performed as described above for experiment #1, and the sequences of the targeting spacers are listed in Table 14. For pairing with dCas9-ZNF10-DNMT3A/3L, targeting spacers were as follows: 1) 7.148 (*B2M*, as non-targeting control; CGCGAGCACAGCUAAGGCCA; SEQ ID NO: 3101), 27.126 (*PCSK9*; CACGCCACCCCGAGCCCCAU; SEQ ID NO: 3102), and 27.128 (*PCSK9*; CAGCCUGCGCGUCCACGUGA; SEQ ID NO: 3103).

[0406] Transfection of mRNA and gRNA into Hepa1-6 cells and intracellular PCSK9 staining:

[0407] Seeded Hepa1-6 cells treated with the NATE™ inhibitor were lipofected with 300 ng of mRNA encoding LTRP1-ZIM3, LTRP5-ZIM3, catalytically-active CasX 491, or dCas9-ZNF10-DNMT3A/3L, and 150 ng of *PCSK9*-targeting gRNA (Table 14). Intracellular levels of PCSK9 protein were measured at 7, 14, 21, 36, and 71 days post-transfection using an intracellular staining protocol as described earlier for experiment #1.

Results:

[0408] In experiment #1, mRNAs encoding dXR1 or LTRP1-ZIM3 were co-transfected with a *PCSK9*-targeting gRNA into mouse Hepa1-6 cells to assess their ability to induce PCSK9 knockdown by silencing the mouse *PCSK9* locus. The quantification of the resulting PCSK9 knockdown is shown in FIGS. 4-6. The data demonstrate that at day 6, use of six out of seven gRNAs targeting the mouse *PCSK9* locus with LTRP1-ZIM3 mRNA resulted in >50% knockdown of intracellular PCSK9, with the leading spacer 27.94 achieving >80% repression level (FIG. 4). A similar trend was observed with use of dXR1 mRNA at day 6, although the degree of repression was less substantial when paired with certain spacers, such as spacer 27.92 and 27.100 (FIG. 4). The results also demonstrate that use of LTRP1-ZIM3 mRNA led to sustained repression of the *PCSK9* locus through at least 25 days, with use of the top two spacers 27.94 and 27.88 showing the strongest permanence in silencing *PCSK9* (FIG. 6). However, the PCSK9 repression mediated by dXR1 that was observed at day 6 reverted to similar levels of PCSK9 as detected with the non-targeting control (spacer 6.7) by day 13; such transient repression was noticeable for all gRNAs assayed that targeted the *PCSK9* gene (FIG. 5).

[0409] In experiment #2, mRNAs encoding LTRP1-ZIM3 or LTRP5-ZIM3, dCas9-ZNF10-DNMT3A/3L, or catalytically active CasX 491 were co-transfected with a *PCSK9*-targeting gRNA into mouse Hepa1-6 cells to assess their ability to induce PCSK9 knockdown by silencing the mouse *PCSK9* locus. The quantification of the resulting PCSK9 repression is shown in FIGS. 7-8. The data demonstrate that delivery of IVT-produced LTRP1-ZIM3 or LTRP5-ZIM3 mRNA resulted in comparable levels of sustained PCSK9 knockdown when paired with a targeting gRNA with the top spacer 27.94 (~40% knockdown by day 71), while use of an alternative spacer 27.88 did not result in as effective of a sustained PCSK9 knockdown by day 71 (~12%) (FIG. 7). Furthermore, third-party-produced mRNA encoding LTRP1-ZIM3 and dCas9-ZNF10-DNMT3A/3L led to similar levels of durable PCSK9 knockdown when paired with gRNAs containing various spacers, with use of spacer 27.94 still resulting in the highest level of PCSK9 repression (FIG. 8).

[0410] These experiments demonstrate that LTRP molecules, having different configurations, can induce heritable silencing of an endogenous locus in a mouse liver cell line. Meanwhile, as anticipated, use of dXR constructs result in efficient repression of the target locus at early timepoints, but their use does not lead to durable silencing. These findings also show that dXR and LTRP molecules (of different configurations) can be delivered as mRNA and co-transfected with a targeting gRNA to cells, indicating that the transient nature of the delivered payload is still sufficient to induce silencing.

Example 3: Assessment of spacers in achieving repression of the *PCSK9* locus in human hepatocyte cells when paired with an LTRP5-ADD molecule

[0411] Experiments were performed to demonstrate that multiple spacers with the TTC recognition motif, when paired with an LTRP molecule in configuration #5 (LTRP5; diagrammed in FIG. 2) containing the ADD domain, can induce durable repression of a therapeutically-relevant endogenous locus in human cells. Specifically, an initial proof-of-concept experiment was performed in human Huh7 cells to evaluate a subset of spacers that exhibit sequence conservation to the non-human primate (NHP) genome to identify leading spacers for testing in future in vivo NHP studies.

Materials and Methods:

Computational selection of PCSK9-targeting spacers for experimental testing with an LTRP5 molecule containing the ADD domain:

[0412] To determine potential LTRP-specific spacers throughout the human *PCSK9* locus, a target search region was defined as starting at 5KB upstream of the transcription start site (TSS) through 5KB downstream of the transcription stop site. Spacers were determined based on the availability of TTC PAMs; consequently, a total of 1,121 TTC spacers were identified throughout the target *PCSK9* locus. These spacers were then functionally annotated by overlaying key genomic features based on their positioning, i.e., determining whether the putative spacer targeted an exon, an intron, or a candidate cis-regulatory element (cCRE), within the promoter region, and/or overlapped with a common site of genetic variation (e.g., SNPs). To narrow down and determine an initial group of spacers for experimental screening, the extracted spacers were subjected to a set of filtering criteria. Firstly, non-specific spacers were excluded by removing spacers with off-target sites that contain up to one base pair mismatch with the on-target site. Furthermore, spacers containing the following mononucleotide repeats were excluded: thymine nucleotide repeats greater than four base pairs (bp) in length or adenine, guanine, or cytosine nucleotide repeats greater than 5 bp in length. Next, from this filtered set, spacers with more than one off-target site containing mismatches in the last four nucleotides of the spacer were excluded. Lastly, spacers that targeted >2KB upstream of the TSS and >2KB downstream of the transcription stop site were excluded. This resulted in a filtered set of 722 TTC spacers (SEQ ID NO: 1824-2545). From this filtered set of 722 spacers, spacers that were TSS-proximal (within 1100 bp upstream and downstream of the TSS) were selected for experimental assessment, resulting in the identification of 67 TTC spacers. Two additional spacers, TG-06-354 and TG-06-352, positioned beyond the 1100 bp threshold window, were also selected for inclusion. The sequences of the resulting 69 TTC spacers are shown in Table 17.

Table 17: RNA sequences of the 69 TTC spacers targeting the human *PCSK9* locus. Bolded spacers were spacers having sequence consensus between human and non-human primate genomes and were assessed in this example.

Spacer ID	Spacer RNA sequence	SEQ ID NO:
TG-06-342	AAUUACAGGCAACAGGAAGG	1824
TG-06-343	CCCCAUGUAAGAGAGGAAGU	1825
TG-06-344	CAGUUUCUGCCUCGCCGCGG	1826
TG-06-345	GCCUCGCCGCGGCACAGGUG	1827
TG-06-346	**CCCACCUGUGCCGCGGCGAG**	**1828**

Spacer ID	Spacer RNA sequence	SEQ ID NO:
TG-06-347	CUCCUUCACCCACCUGUGCC	1829
TG-06-348	AGGCAUUCACUCCUUCACCC	1830
TG-06-349	CUGUGCCUGGUGCAGUUCCC	1831
TG-06-350	GUGUCAUAAAGAAAUUGCCU	1832
TG-06-351	UUAUGACACAGAACUCAUGC	1833
TG-06-001	**GAGGAGGACGGCCUGGCCGA**	**1834**
TG-06-002	**ACCGCUGCGCCAAGGUGCGG**	**1835**
TG-06-004	GCCAGGCCGUCCUCCUCGGA	1836
TG-06-005	GUGCUCGGGUGCUUCGGCCA	1837
TG-06-117	ACUUUGUUUGCAAAGACCUC	1838
TG-06-118	GAGUGAAAUGGCCUGCUCUG	1839
TG-06-119	GAGCAGGCCAUUUCACUCGG	1840
TG-06-120	CUCGGAAUCUGCUGUGCAUC	1841
TG-06-121	GGAAGGGCUGUCGAUACUGG	1842
TG-06-123	UCCCAGUAUCGACAGCCCUU	1843
TG-06-124	CAGUAUCGACAGCCCUUCCA	1844
TG-06-125	AGAAAGAGCAAGCCUCAUGU	1845
TG-06-128	AGAAAUCAACUGGACAAGCA	1846
TG-06-131	**UGAACAUGGUGUGUAAAAGG**	**1847**
TG-06-132	**AGAAGAUUCAAUUUGCAAAG**	**1848**
TG-06-133	**AUGGUAGGCACAAGCUCAGC**	**1849**
TG-06-134	**GAAUUCUAUGGUAGGCACAA**	**1850**
TG-06-135	GGAAAGCUGAGCUUGUGCCU	1851
TG-06-138	AGGGAUUUAUACUACAAAGA	1852
TG-06-139	AGGAGCAGCUAGUUGGUAAG	1853
TG-06-140	AAACUUAGCCUGGACCCCCU	1854
TG-06-141	ACUGGCCUUAACCUGGCAGC	1855
TG-06-142	UUCCACUGGCCUUAACCUGG	1856
TG-06-143	GAAUCAAUCCUACUGUGGAC	1857
TG-06-144	GUGGGCAGCGAGGAGUCCAC	1858
TG-06-145	UGGGUCCACCUUGUCUCCUG	1859
TG-06-146	**GAAGUCUCACUGGUCAGCAG**	**1860**
TG-06-147	**GUGUUUCCUGGGUCCACCUU**	**1861**
TG-06-149	AGCCCAGUUAGGAUUUGGGA	1862
TG-06-150	UCCCUCUGCGCGUAAUCUGA	1863
TG-06-151	CUCUGCGCGUAAUCUGACGC	1864
TG-06-152	**GCCUCGCCCUCCCCAAACAG**	**1865**
TG-06-153	GUUAAUGUUUAAUCAGAUAG	1866

Spacer ID	Spacer RNA sequence	SEQ ID NO:
TG-06-154	**AGGGUGUGGGUGCUUGACGC**	**1867**
TG-06-155	GCAGCGACGUCGAGGCGCUC	1868
TG-06-157	GGGUCUGAGCCUGGAGGAGU	1869
TG-06-158	GGAGCAGGGCGCGUGAAGGG	1870
TG-06-159	GCGCGCCCCUUCACGCGCCC	1871
TG-06-160	CGCGCCCUGCUCCUGAACUU	1872
TG-06-161	GCUCCUGCACAGUCCUCCCC	1873
TG-06-167	**CACUGAAUAGCGCAGCCGCA**	**1874**
TG-06-168	GUGGGAAGGUUCGCGGGGUU	1875
TG-06-169	CGGGGUUGGGAGACCCGGAG	1876
TG-06-170	UCGGCCUCCGGGUCUCCCAA	1877
TG-06-171	CAGUACGUUCCAGGCAUUCA	1878
TG-06-172	GCUGAAACAGAUGGAAUACU	1879
TG-06-249	AAACCAAAUCGGAACCCACU	1880
HS-6-147	UGUUGCCUGUAAUUGGAAUU	1881
HS-6-149	CCUUCCUGUUGCCUGUAAUU	1882
TG-06-122	CCUUUGUUUCUUCCCAGUAU	1883
TG-06-127	UCCUCCUGCCUGGUACACAA	1884
TG-06-137	GAAUGUACCUAUAUGACGUC	1885
TG-06-188	CCCCGGCCUCCCAUCCCUAC	1886
TG-06-243	**CUUGGCACGAUCUUGGGGAC**	**1887**
TG-06-250	GAUUUGGUUUGGAAAACAUG	1888
TG-06-251	CUCCAGGCCCUCCACCCUCC	1889
HS-6-159	CACCCCGCCCCUGUCUCGGG	1890
TG-06-354	**CCCCUGCCCCUUCAGCUGGU**	**1925**
TG-06-352	**UCCCUCACCAAUUACCCCUC**	**1910**

Note, for spacer nomenclature throughout these Examples, dashes and periods are used interchangeably. Thus, spacer "06-146" is the same as spacer "6.146."

Assessment of PCSK9 secretion levels for select PCSK9-targeting spacers having sequence conservation with the non-human primate genome:

[0413] Of the 69 TTC spacers identified, 15 spacers that exhibited sequence conservation between human and non-human primate genomes (bolded spacers in Table 17) were initially tested to assess their effect on PCSK9 secretion levels.

[0414] mRNA encoding the following molecules were generated by IVT following similar methods as described in Example 2: 1) a catalytically-active CasX 676 (as described in Example 5), 2) dXR1 (as described in Example 2), and 3) LTRP5-ADD-ZIM3 (as described in Example 4). The DNA and mRNA sequences for CasX 676 are shown in Tables 21 and 22; the DNA and

mRNA sequences for dXR1 are shown in Tables 11 and 12; the DNA and mRNA sequences for LTRP5-ADD-ZIM3 are shown in Tables 18 and 19.

[0415] gRNAs containing NHP-conserved spacers targeting the *PCSK9* locus (bolded spacers in Table 17) were designed using gRNA scaffold 316 and chemically synthesized. Furthermore, a *B2M*-targeting gRNA was used as a non-targeting control, while spacer TG-06-138 (also known as spacer 6.138; SEQ ID NO: 1852) was used to pair with dXR1, and spacer TG-06-001 (also known as spacer 6.1; SEQ ID NO: 1834) was used to pair with CasX 676. Spacer TG-06-157 (also known as spacer 6.157; SEQ ID NO: 1869), which is not an NHP-conserved spacer, was included as a positive control given its demonstrated efficacy in sustaining repression of the *PCSK9* locus, which is shown in Example 5, below.

[0416] To assess PCSK9 secretion, seeded Huh7 cells were transfected with mRNA encoding a catalytically-active CasX 676, dXR1, or LTRP5-ADD-ZIM3 and a gRNA with scaffold 316 and spacer targeting either the *B2M* or *PCSK9* locus. Media supernatant was harvested at 6, 18, 36, and 87 days post-transfection to assess level of PCSK9 secretion by ELISA. Levels of PCSK9 secretion were normalized to total cell count. As an additional control, PCSK9 secretion was also measured in the media supernatant harvested from wells containing untreated, naïve cells.

Results:

[0417] Quantification of normalized PCSK9 secretion level for Huh7 cells transfected with mRNA encoding catalytically-active CasX 676, dXR1, or LTRP5-ADD-ZIM3 with an NHP-conserved gRNA targeting the *PCSK9* locus at the four timepoints is shown FIG. 9. The data demonstrate that use of most NHP-conserved spacers with LTRP5-ADD-ZIM3 resulted in sustained repression through 36 days post-transfection when compared with control conditions, i.e., naïve, untreated cells, cells treated with dXR1, and cells treated with the non-targeting control (using spacer 7.37 targeting the *B2M* locus). Specifically, in comparison to the PCSK9 secretion level observed with use of spacer 6.1 paired with CasX 676, use of TSS-proximal spacers TG-06-147, TG-06-167, TG-06-133, TG-06-146, and TG-06-154 paired with LTRP5-ADD-ZIM3 resulted in similar or further reduced level of sustained repression through day 36 (FIG. 9). Interestingly, use of TG-06-352, which is positioned beyond the 1100 bp threshold window designated here as "TSS-proximal", also resulted in effective repression through day 36 (FIG. 9). Similar to the findings observed in Example 5, treatment with LTRP5-ADD-ZIM3 with spacer 6.157 resulted in sustained repression of secreted PCSK9 levels, while treatment

with dXR1 and spacer 6.138 resulted in transient repression. Furthermore, treatment with any of the three mRNA molecules with spacer 7.37 targeting the *B2M* locus did not affect PCSK9 secretion (FIG. 9). However, by 87 days post-transfection, only use of spacers TG-06-157, TG-06-154, TG-06-167, and TG-06-243 paired with LTRP5-ADD-ZIM3 resulted in similar or further reduced level of sustained repression when compared to use of spacer 6.1 paired with CasX 676 (FIG. 9).

[0418] These results demonstrate that delivery of mRNA encoding an LTRP molecule with the ADD domain with the appropriate PCSK9-targeting gRNA can result in sustained repression of an endogenous target locus in human cells. Furthermore, these experiments revealed that several human spacers having consensus sequence with the non-human primate species achieved strong phenotypic effects from targeting a therapeutically-relevant locus, supporting the potential use of these select spacers in preclinical efficacy studies utilizing non-human primate models.

Example 4: Demonstration that inclusion of the ADD domain into an LTRP molecule enhances repression of an endogenous locus in mouse Hepa1-6 cells

[0419] Experiments were performed to demonstrate that incorporation of the ADD domain into an LTRP molecule enhances the ability of LTRPs to induce durable repression of an endogenous locus in mouse Hepa1-6 liver cells, when delivered as mRNA co-transfected with a targeting gRNA.

Materials and Methods:

Generation of LTRP #5 mRNA:

[0420] mRNA encoding two variants of the LTRP #5 molecule were generated by in vitro transcription (IVT): 1) an LTRP #5 molecule containing the ZIM3-KRAB domain (hereafter known as LTRP5-ZIM3) and 2) a LTRP5-ZIM3 containing the DNMT3A-ADD domain (hereafter known as LTRP5-ADD-ZIM3). Briefly, constructs encoding for a 5'UTR region, LTRP5-ZIM3 or LTRP5-ADD-ZIM3 with flanking SV40 NLSes, and a 3'UTR region were generated and cloned into a plasmid containing a T7 promoter and 79-nucleotide poly(A) tail. Sequences encoding the LTRP5-ZIM3 or LTRP5-ADD-ZIM3 molecules were codon-optimized using a codon utilization table, in addition to using a publicly available codon optimization tool and adjusting parameters such as GC content as needed. The DNA sequences encoding the

LTRP5-ZIM3 and LTRP5-ADD-ZIM3 mRNAs are listed in Table 18. The corresponding mRNA sequences and protein sequences are listed in Table 19 and Table 20 respectively.

Table 18: Encoding DNA and RNA sequences of the LTRP5-ZIM3 and LTRP5-ADD-ZIM3 mRNA molecules assessed in this example*.

LTRP molecule	Component	DNA sequence or SEQ ID NO	RNA sequence or SEQ ID NO
LTRP5-ZIM3	5'UTR	3047	3115
	START codon + NLS + linker	3104	3116
	START codon + DNMT3A catalytic domain	3059	3117
	Linker	3060	3118
	DNMT3L interaction domain	3061	3119
	Linker	3105	3105
	ZIM3-KRAB	3051	3120
	Linker	3106	3121
	dCasX491	3049	3122
	Buffer + linker	3107	3123
	NLS + STOP codon + buffer sequence	3108	3124
	3'UTR	3055	3125
	Buffer sequence	TCTAG	UCUAG
	Poly(A) tail	3109	3109
LTRP5-ADD-ZIM3	5'UTR	3047	3115
	START codon + NLS + linker	3104	3116
	START codon + DNMT3A ADD domain	3111	3127
	DNMT3A catalytic domain	3112	3128
	Linker	3060	3118
	DNMT3L interaction domain	3061	3119
	Linker	3105	3105
	ZIM3-KRAB	3051	3120
	Linker	3106	3121
	dCasX491	3049	3122
	Buffer + linker	3107	3123

LTRP molecule	Component	DNA sequence or SEQ ID NO	RNA sequence or SEQ ID NO
	NLS + STOP codon + buffer sequence	3108	3124
	3'UTR	3055	3125
	Buffer sequence	3056	3126
	Poly(A) tail	3109	3109

*Components are listed in a 5' to 3' order within the constructs

Table 19: Full-length RNA sequences of LTRP5-ZIM3 and LTRP5-ADD-ZIM3 mRNA molecules assessed in this example. Modification 'mψ' = N1-methyl-pseudouridine.

LTRP molecule	SEQ ID NO	RNA Sequence
LTRP5-ZIM3	3129	AAAmψAAGAGAGAAAAGAAGAGmψAAGAAGAAAmψAmψAAGAGCCACCAmψGGC CCCmψAAGAAGAAGCGmψAAAGmψGAGCCGGAmψGAACCACGACCAGGAGmψmψ CGACCCCCCmψAAGGmψGmψACCCmψCCCGmψCCCCGCCGAGAAGAGAAAGCCC AmψCCGGGmψCCmψGAGCCmψGmψmψCGAmψGGCAmψCGCCACCGGmψCmψGCm ψGGmψGCmψGAAGGACCmψGGGCAmψCCAGGmψGGAmψAGGmψACAmψmψGCCm ψCCGAGGmψGmψGCGAGGACmψCCAmψCACCGmψGGGAAmψGGmψGCGmψCAmψ CAGGGCAAGAmψCAmψGmψACGmψGGGCGACGmψGCGGAGCGmψGACACAGAAG CAmψAmψCCAGGAGmψGGGGCCCmψmψmψCGACCmψGGmψGAmψCGGCGGCAGC CCmψmψGCAAmψGACCmψGAGCAmψCGmψGAACCCAGCCCGGAAGGGCCmψGmψ ACGAGGGAACCGGCAGACmψGmψmψCmψmψCGAGmψmψmψmψACAGACmψGCmψ GCACGACGCCCGGCCmψAAGGAAGGCGACGACCGGCCCmψmψCmψmψmψmψGGC mψGmψmψCGAGAAmψGmψGGmψGGCCAmψGGGAGmψCAGCGACAAGCGGGAmψA mψmψAGCCGGmψmψCCmψGGAGAGCAACCCCGmψGAmψGAmψCGAmψGCCAAGG AAGmψGAGCGCCGCCCACCGGGCCAGAmψACmψmψCmψGGGGCAAmψCmψGCCm ψGGCAmψGAACAGACCCCmψGGCCAGCACCGmψGAACGACAAGCmψGGAGCmψG CAGGAGmψGCCmψGGAGCACGGCCGGAmψCGCCAAGmψmψCAGCAAGGmψGAGA ACCAmψCACCACCCGAAGCAACAGCAmψCAAACAAGGCAAGGACCAGCACmψmψ mψCCmψGmψGmψmψCAmψGAACGAGAAGGAGGACAmψCCmψGmψGGmψGmψACC GAGAmψGGAGAGAGmψGmψmψCGGGmψmψCCCAGmψCCACmψACACAGAmψGmψ CAGCAACAmψGmψCmψAGACmψGGCCAGACAGAGACmψGCmψGGGAAGAAGCmψ GGmψCCGmψCCCmψGmψGAmψCAGACACCmψGmψmψCGCCCCmψCmψGAAGGAG mψACmψmψCGCCmψGCGmψGAGCAGCGGCAACAGCAACGCCAACAGCCGGGGCC CCAGCmψmψCmψCmψAGCGGCCmψGGmψGCCACmψGmψCCCmψGAGAGGGAGCC ACAmψGGGCCCCAmψGGAGAmψCmψACAAAACCGmψGAGCGCCmψGGAAGCGGC AGCCmψGmψGCGCGmψGCmψGAGCCmψGmψmψmψCGGAAmψAmψCGAmψAAAGm ψCCmψGAAAAGCCmψGGGAmψmψCCmψGGAGAGCGGCmψCmψGGCmψCCGGCGG mψGGCACCCmψGAAGmψACGmψGGAGGAmψGmψGACAAACGmψGGmψCAGACGG GAmψGmψGGAGAAGmψGGGGCCCCmψmψCGAmψCmψGGmψGmψACGGCAGCACC CAACCCCmψGGGCAGCmψCmψmψGmψGACCGGmψGCCCmψGGCmψGGmψACAmψ Gmψmψmψ CAGmψmψCCACCGGAmψCCmψGCAGmψACGCCCmψGCCGAGACAGGA GmψCCCAGCGGCCAmψmψCmψmψmψmψGGAmψmψmψmψCAmψGGACAACmψmψG CmψGCmψGACCGAGGAmψGACCAGGAAACmψACCACmψCGGmψmψCCmψGCAGA CCGAAGCCGmψGACCCmψGCAGGACGmψGAGAGGCCGGGACmψACCAGAACGCC AmψGCGGGmψGmψGGmψCCAACAmψCCCmψGGACmψGAAAAGCAAGCACGCACC mψCmψGACCCCmψAAAGAAGAGGAGmψACCmψGCAGGCCCAGGmψGCGGAGCAG

LTRP molecule	SEQ ID NO	RNA Sequence
		AAGCAAGCmψGGACGCCCCmψAAGGmψGGAmψCmψGCmψGGmψGAAGAAmψmψG CCmψCCmψGCCCCmψGAGAGAGmψACmψmψCAAGmψAmψmψmψCAGCCAGAAmψ AGmψCmψGCCCCmψGGGAGGCAGCGGCGGCGGCAmψGAACAACmψCCCAGGGCA GAGmψGACCmψmψCGAGGACGmψGACCGmψGAAmψmψmψmψACACAGGGAGAGm ψGGCAGAGACmψGAACCCCGAGCAGAGAAACCmψGmψACCGGGAmψGmψGAmψG CmψGGAAAACmψACAGCAAmψCmψGGmψGmψCCGmψGGGCCAGGGCGAGACCAC AAAGCCmψGACGmψGAmψCCmψGCGmψCmψGGAGCAGGGCAAGGAACCCmψGGC mψGGAGGAGGAGGAGGmψGCmψGGGAAGCGGACGGGCCGAGAAGAACGGCGACA mψCGGCGGACAGAmψCmψGGAAGCCmψAAGGACGmψGAAAGAAAGCCmψGGGCG GCCCAAGCAGCGGCGCCCCmψCCmψCCCAGCGGCGGCAGCCCAGCCGGCmψCCC CAACCmψCmψACCGAGGAGGGCACCmψCmψGAGmψCCGCCACCCCCGAGAGCGG CCCmψGGCACCmψCCACCGAGCCCAGCGAGGGCAGCGCACCCGGCAGCCCmψGC CGGCAGCCCCACCmψCCACAGAGGAGGGAACCAGCACCGAGCCCAGCGAAGGCA GCGCCCCAGGCACCAGCACCGAGCCmψAGmψGAGCAGGAGAmψmψAAACGGAmψ CAACAAGAmψCAGAAGAAGACmψmψGmψGAAAGACAGCAACACCAAGAAGGCCG GCAAGACAGGCCCCAmψGAAAACCCmψGCmψGGmψmψAGAGmψGAmψGACACCC GAmψCmψGAGAGAGCGGCmψGGAAAACCmψGAGAAAGAAGCCmψGAAAAmψAmψ CCCCCAGCCCAmψCAGCAAmψACAmψCmψAGAGCCAACCmψGAAmψAAGCmψGC mψGACCGAmψmψACACCGAAAmψGAAGAAGGCGAmψCCmψGCAmψGmψGmψACm ψGGGAAGAGmψmψCCAGAAGGACCCmψGmψGGGCCmψGAmψGAGCCGGGmψGGC CCAGCCmψGCCAGCAAGAAGAmψCGAmψCAGAACAAGCmψGAAACCmψGAGAmψ GGACGAGAAGGGCAACCmψGACCACCGCCGGCmψmψmψGCCmψGCmψCmψCAGm ψGmψGGCCAGCCCCmψGmψmψCGmψGmψACAAGCmψGGAGCAGGmψGmψCmψGA GAAGGGCAAGGCmψmψACACCAACmψACmψmψCGGACGGmψGCAAmψGmψGGCC GAGCACGAAAAGCmψGAmψCCmψGCmψGGCCCAGCmψGAAGCCCGAGAAGGAmψ AGCGACGAAGCCGmψGACAmψAmψAGCCmψGGGAAAGmψmψmψGGGCAGAGGGC CCmψGGAmψmψmψCmψACAGCAmψmψCAmψGmψGACCAAGGAGmψCCACCCACC CCGmψGAAGCCCCmψGGCCCAGAmψCGCCGGAAACAGAmψACGCCmψCCGGACC mψGmψGGGAAAGGCCCmψGAGCGACGCAmψGmψAmψGGGCACAAmψCGCCmψCC mψmψCCmψGmψCmψAAGmψACCAGGACAmψCAmψCAmψCGAACACCAGAAGGmψ GGmψGAAGGGCAACCAGAAGAGACmψGGAGAGCCmψGCGGGAGCmψGGCCGGCA AGGAAAACCmψGGAAmψACCCmψAGCGmψGACCCmψGCCACCmψCAGCCmψCAC ACCAAGGAGGGCGmψmψGAmψGCCmψACAACGAAGmψGAmψCGCCCGGGmψGCG AAmψGmψGGGmψGAACCmψGAACCmψGmψGGCAGAAGCmψGAAGCmψAAGCAGA GAmψGAmψGCCAAGCCmψCmψGCmψGAGACmψGAAGGGAmψmψCCCmψmψCCmψ mψmψCCmψCmψGGmψCGAGAGACAGGCCAACGAAGmψGGACmψGGmψGGGACAm ψGGmψGmψGmψAACGmψGAAGAAGCmψGAmψCAACGAGAAAAAGGAGGAmψGGC AAGGmψGmψmψmψmψGGCAGAAmψCmψGGCmψGGCmψACAAGAGACAGGAAGCC CmψGAGACCAmψACCmψGAGCAGCGAGGAAGAmψCGGAAGAAGGGAAAGAAAmψ mψCGCmψCGGmψACCAGCmψGGGCGACCmψGCmψGCmψGCACCmψGGAAAAGAA GCACGGCGAGGACmψGGGGAAAGGmψGmψACGACGAGGCCmψGGGAGCGGAmψm ψGACAAGAAAGmψGGAAGGCCmψGAGCAAGCACAmψCAAGCmψGGAAGAGGAAC GGAGAAGCGAGGACGCCCAGAGCAAGGCCGCCCmψGACCGACmψGGCmψGCGGG CmψAAGGCCAGCmψmψCGmψGAmψCGAGGGCCmψGAAGGAGGCCGACAAGGACG AGmψmψCmψGCAGAmψGCGAGCmψGAAGCmψGCAGAAGmψGGmψACGGGGACCm ψGCGGGGAAAGCCCmψmψCGCCAmψCGAAGCCGAGAACAGCAmψCCmψGGACAm ψCAGCGGCmψmψCAGCAAGCAGmψACAACmψGmψGCCmψmψCAmψCmψGGCAGA AGGACGGCGmψGAAGAAGCmψGAACCmψGmψACCmψGAmψCAmψCAACmψACmψ mψCAAGGGCGGCAAGCmψGCGGmψmψCAAGAAGAmψCAAACCmψGAAGCCmψmψ CGAAGCCAACAGAmψmψCmψACACCGmψGAmψCAACAAAAAGAGCGGCGAGAmψ CGmψGCCCAmψGGAGGmψGAACmψmψCAACmψmψCGACGACCCCAACCmψGAmψ CAmψCCmψGCCmψCmψGGCCmψmψmψGGCAAGAGACAGGGCAGAGAAmψmψCAm ψCmψGGAACGACCmψGCmψGmψCCCmψGGAAACCGGCAGCCmψGAAGCmψGGCC AACGGAAGAGmψGAmψCGAGAAGACACmψGmψACAACAGAAGAACCCGGCAGGA mψGAGCCmψGCCCmψGmψmψCGmψGGCCCmψGACCmψmψCGAGCGGCGGGAGGm ψCCmψGGACmψCCmψCCAAmψAmψCAAACCAAmψGAACCmψGAmψCGGCGmψGG

LTRP molecule	SEQ ID NO	RNA Sequence
		CAAGAGGCGAAAACAmψCCCCGCCGmψGAmψCGCCCmψGACCGACCCCGAGGGC mψGCCCACmψGAGCCGGmψmψmψAAGGAmψAGCCmψGGGAAACCCAACCCACAm ψCCmψGAGAAmψCGGCGAGAGCmψAmψAAGGAGAAGCAGCGGACCAmψCCAGGC CAAGAAGGAGGmψGGAGCAGCGGAGAGCCGGCGGCmψACAGCCGGAAGmψACGC CAGCAAAGCCAAGAAmψCmψGGCAGACGAmψAmψGGmψGAGAAACACCGCmψAG AGAmψCmψGCmψGmψACmψACGCCGmψGACCCAGGAmψGCCAmψGCmψGAmψCm ψmψCGCCAACCmψGAGCCGGGGCmψmψCGGCCGGCAGGGCAAGCGGACCmψmψC AmψGGCCGAGAGACAGmψACACACGGAmψGGAGGACmψGGCmψGACCGCCAAGC mψGGCCmψACGAGGGCCmψGAGCAAGACCmψACCmψGmψCCAAGACACmψGGCC CAGmψACACCmψCCAAGACAmψGCAGCAACmψGmψGGGmψmψmψACCAmψCACC AGCGCCGACmψACGACAGGGmψGCmψGGAGAAGCmψGAAGAAGACAGCAACAGG CmψGGAmψGACCACAAmψmψAACGGCAAGGAGCmψGAAGGmψGGAGGGCCAGAm ψmψACCmψACmψACAACAGAmψACAAGAGACAGAACGmψAGmψCAAGGACCmψG mψCCGmψCGAGCmψGGAmψAGACmψGAGCGAAGAAmψCmψGmψGAACAACGACA mψCmψCCmψCCmψGGACAAAGGGCAGAAGCGGAGAAGCmψCmψGAGCCmψCCmψ GAAGAAAAGAmψmψCmψCCCAmψAGACCCGmψGCAGGAGAAGmψmψCGmψGmψG CCmψGAACmψGCGGCmψmψCGAGACACACGCAGCCGAGCAAGCCGCCCmψGAAC AmψCGCCAGAmψCCmψGGCmψGmψmψCCmψGCGGAGCCAGGAGmψACAAGAAAm ψACCAGACAAACAAGACAACCGGCAACACCGAmψAAGAGAGCCmψmψCGmψCGA GACCmψGGCAGmψCCmψmψmψmψACCGGAAGAAGCmψmψAAGGAGGmψGmψGGA AACCmψGCCGmψGCGGmψCmψGGCGGAmψCmψGGCGGAGGCmψCCACCAGCCCC AAGAAAAAGAGAAAAGmψCmψAAmψAGAmψAAGCmψGCCmψmψCmψGCGGGGCm ψmψGCCmψmψCmψGGCCAmψGCCCmψmψCmψmψCmψCmψCCCmψmψGCACCmψG mψACCmψCmψmψGGmψCmψmψmψGAAmψAAAGCCmψGAGmψAGGAAGmψCmψAG AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAA
LTRP5-ADD-ZIM3	3130	AAAmψAAGAGAGAAAAGAAGAGmψAAGAAGAAAmψAmψAAGAGCCACCAmψGGC CCCmψAAGAAGAAGCGmψAAAGmψGAGCCGGAmψGGAACGCCmψCGmψCmψACG AGGmψGCGGCAGAAGmψGCAGAAACAmψCGAGGACAmψCmψGCAmψCmψCCmψG CGGAmψCmψCmψGAACGmψGACCCmψGGAGCACCCACmψGmψmψCAmψCGGCGG CAmψGmψGCCAGAACmψGmψAAAAACmψGmψmψmψmψCmψGGAGmψGmψGCCmψ AmψCAAmψACGACGAmψGACGGCmψACCAGAGCmψACmψGCACCAmψCmψGmψm ψGCGGCGGAAGAGAGGmψGCmψGAmψGmψGmψGGAAAmψAACAACmψGCmψGCC GGmψGCmψmψCmψGCGmψGGAAmψGCGmψGGACCmψGCmψGGmψGGGCCCCGGC GCCGCCCAGGCCGCmψAmψmψAAGGAAGAmψCCmψmψGGAACmψGCmψACAmψG mψGCGGCCACAAGGGCACAmψACGGCCmψGCmψGAGACGGAGAGAGGACmψGGC CmψAGCAGACmψGCAGAmψGmψmψCmψmψCGCCAAmψAACCACGACCAGGAGmψ mψCGACCCCCCmψAAGGmψGmψACCCmψCCCGmψCCCCGCCGAGAAGAGAAAGC CCAmψCCGGGmψCCmψGAGCCmψGmψmψCGAmψGGCAmψCGCCACCGGmψCmψG CmψGGmψGCmψGAAGGACCmψGGGCAmψCCAGGmψGGAmψAGGmψACAmψmψGC CmψCCGAGGmψGmψGCGAGGACmψCCAmψCACCGmψGGGAAmψGGmψGCGmψCA mψCAGGGCAAGAmψCAmψGmψACGmψGGGCGACGmψGCGGAGCGmψGACACAGA AGCAmψAmψCCAGGAGmψGGGGCCCmψmψmψCGACCmψGGmψGAmψCGGCGGCA GCCCmψmψGCAAmψGACCmψGAGCAmψCGmψGAACCCAGCCCGGAAGGGCCmψG mψACGAGGGAACCGGCAGACmψGmψmψCmψmψCGAGmψmψmψmψACAGACmψGC mψGCACGACGCCCGGCCmψAAGGAAGGCGACGACCGGCCCmψmψCmψmψmψmψG GCmψGmψmψCGAGAAmψGmψGGmψGGCCAmψGGGAGmψCAGCGACAAGCGGGAm ψAmψmψAGCCGGmψmψCCmψGGAGAGCAACCCCGmψGAmψGAmψCGAmψGCCAA GGAAGmψGAGCGCCGCCCACCGGGCCAGAmψACmψmψCmψGGGGCAAmψCmψGC CmψGGCAmψGAACAGACCCCmψGGCCAGCACCGmψGAACGACAAGCmψGGAGCm ψGCAGGAGmψGCCmψGGAGCACGGCCGGAmψCGCCAAGmψmψCAGCAAGGmψGA GAACCAmψCACCACCCGAAGCAACAGCAmψCAAACAAGGCAAGGACCAGCACmψ mψmψCCmψGmψGmψmψCAmψGAACGAGAAGGAGGACAmψCCmψGmψGGmψGmψA CCGAGAmψGGAGAGAGmψGmψmψCGGGmψmψCCCAGmψCCACmψACACAGAmψG mψCAGCAACAmψGmψCmψAGACmψGGCCAGACAGAGACmψGCmψGGGAAGAAGC mψGGmψCCGmψCCCmψGmψGAmψCAGACACCmψGmψmψCGCCCCmψCmψGAAGG

LTRP molecule	SEQ ID NO	RNA Sequence
		AGmψACmψmψCGCCmψGCGmψGAGCAGCGGCAACAGCAACGCCAACAGCCGGGG CCCCAGCmψmψCmψCmψAGCGGCCmψGGmψGCCACmψGmψCCCmψGAGAGGGAG CCACAmψGGGCCCCAmψGGAGAmψCmψACAAAACCGmψGAGCGCCmψGGAAGCG GCAGCCmψGmψGCGCGmψGCmψGAGCCmψGmψmψmψCGGAAmψAmψCGAmψAAA GmψCCmψGAAAAGCCmψGGGAmψmψCCmψGGAGAGCGGCmψCmψGGCmψCCGGC GGmψGGCACCCmψGAAGmψACGmψGGAGGAmψGmψGACAAACGmψGGmψCAGAC GGGAmψGmψGGAGAAGmψGGGGCCCCmψmψCGAmψCmψGGmψGmψACGGCAGCA CCCAACCCCmψGGGCAGCmψCmψmψGmψGACCGGmψGCCCmψGGCmψGGmψACA mψGmψmψmψCAGmψmψCCACCGGAmψCCmψGCAGmψACGCCCmψGCCGAGACAG GAGmψCCCAGCGGCCAmψmψCmψmψmψmψGGAmψmψmψmψCAmψGGACAACmψm ψGCmψGCmψGACCGAGGAmψGACCAGGAAACmψACCACmψCGGmψmψCCmψGCA GACCGAAGCCGmψGACCCmψGCAGGACGmψGAGAGGCCGGGACmψACCAGAACG CCAmψGCGGGmψGmψGGmψCCAACAmψCCCmψGGACmψGAAAAGCAAGCACGCA CCmψCmψGACCCCmψAAAGAAGAGGAGmψACCmψGCAGGCCCAGGmψGCGGAGC AGAAGCAAGCmψGGACGCCCCmψAAGGmψGGAmψCmψGCmψGGmψGAAGAAmψm ψGCCmψCCmψGCCCCmψGAGAGAGmψACmψmψCAAGmψAmψmψmψCAGCCAGAA mψAGmψCmψGCCCCmψGGGAGGCAGCGGCGGCGGCAmψGAACAACmψCCCAGGG CAGAGmψGACCmψmψCGAGGACGmψGACCGmψGAAmψmψmψmψACACAGGGAGA GmψGGCAGAGACmψGAACCCCGAGCAGAGAAACCmψGmψACCGGGAmψGmψGAm ψGCmψGGAAAACmψACAGCAAmψCmψGGmψGmψCCGmψGGGCCAGGGCGAGACC ACAAAGCCmψGACGmψGAmψCCmψGCGmψCmψGGAGCAGGGCAAGGAACCCmψG GCmψGGAGGAGGAGGAGGmψGCmψGGGAAGCGGACGGGCCGAGAAGAACGGCGA CAmψCGGCGGACAGAmψCmψGGAAGCCmψAAGGACGmψGAAAGAAAGCCmψGGG CGGCCCAAGCAGCGGCGCCCCmψCCmψCCCAGCGGCGGCAGCCCAGCCGGCmψC CCCAACCmψCmψACCGAGGAGGGCACCmψCmψGAGmψCCGCCACCCCCGAGAGC GGCCCmψGGCACCmψCCACCGAGCCCAGCGAGGGCAGCGCACCCGGCAGCCCmψ GCCGGCAGCCCCACCmψCCACAGAGGAGGGAACCAGCACCGAGCCCAGCGAAGG CAGCGCCCCAGGCACCAGCACCGAGCCmψAGmψGAGCAGGAGAmψmψAAACGGA mψCAACAAGAmψCAGAAGAAGACmψmψGmψGAAAGACAGCAACACCAAGAAGGC CGGCAAGACAGGCCCCAmψGAAAACCCmψGCmψGGmψmψAGAGmψGAmψGACAC CCGAmψCmψGAGAGAGCGGCmψGGAAAACCmψGAGAAAGAAGCCmψGAAAAmψA mψCCCCCAGCCCAmψCAGCAAmψACAmψCmψAGAGCCAACCmψGAAmψAAGCmψ GCmψGACCGAmψmψACACCGAAAmψGAAGAAGGCGAmψCCmψGCAmψGmψGmψA CmψGGGAAGAGmψmψCCAGAAGGACCCmψGmψGGGCCmψGAmψGAGCCGGGmψG GCCCAGCCmψGCCAGCAAGAAGAmψCGAmψCAGAACAAGCmψGAAACCmψGAGA mψGGACGAGAAGGGCAACCmψGACCACCGCCGGCmψmψmψGCCmψGCmψCmψCA GmψGmψGGCCAGCCCCmψGmψmψCGmψGmψACAAGCmψGGAGCAGGmψGmψCmψ GAGAAGGGCAAGGCmψmψACACCAACmψACmψmψCGGACGGmψGCAAmψGmψGG CCGAGCACGAAAAGCmψGAmψCCmψGCmψGGCCCAGCmψGAAGCCCGAGAAGGA mψAGCGACGAAGCCGmψGACAmψAmψAGCCmψGGGAAAGmψmψmψGGGCAGAGG GCCCmψGGAmψmψmψCmψACAGCAmψmψCAmψGmψGACCAAGGAGmψCCACCCA CCCCGmψGAAGCCCCmψGGCCCAGAmψCGCCGGAAACAGAmψACGCCmψCCGGA CCmψGmψGGGAAAGGCCCmψGAGCGACGCAmψGmψAmψGGGCACAAmψCGCCmψ CCmψmψCCmψGmψCmψAAGmψACCAGGACAmψCAmψCAmψCGAACACCAGAAGG mψGGmψGAAGGGCAACCAGAAGAGACmψGGAGAGCCmψGCGGGAGCmψGGCCGG CAAGGAAAACCmψGGAAmψACCCmψAGCGmψGACCCmψGCCACCmψCAGCCmψC ACACCAAGGAGGGCGmψmψGAmψGCCmψACAACGAAGmψGAmψCGCCCGGGmψG CGAAmψGmψGGGmψGAACCmψGAACCmψGmψGGCAGAAGCmψGAAGCmψAAGCA GAGAmψGAmψGCCAAGCCmψCmψGCmψGAGACmψGAAGGGAmψmψCCCmψmψCC mψmψmψCCmψCmψGGmψCGAGAGACAGGCCAACGAAGmψGGACmψGGmψGGGAC AmψGGmψGmψGmψAACGmψGAAGAAGCmψGAmψCAACGAGAAAAAGGAGGAmψG GCAAGGmψGmψmψmψmψGGCAGAAmψCmψGGCmψGGCmψACAAGAGACAGGAAG CCCmψGAGACCAmψACCmψGAGCAGCGAGGAAGAmψCGGAAGAAGGGAAAGAAA mψmψCGCmψCGGmψACCAGCmψGGGCGACCmψGCmψGCmψGCACCmψGGAAAAG AAGCACGGCGAGGACCmψGGGGAAAGGmψGmψACGACGAGGCCmψGGGAGCGGAm ψmψGACAAGAAAGmψGGAAGGCCmψGAGCAAGCACAmψCAAGCmψGGAAGAGGA

LTRP molecule	SEQ ID NO	RNA Sequence
		ACGGAGAAGCGAGGACGCCCAGAGCAAGGCCGCCCmψGACCGACmψGGCmψGCG GGCmψAAGGCCAGCmψmψCGmψGAmψCGAGGGCCmψGAAGGAGGCCGACAAGGA CGAGmψmψCmψGCAGAmψGCGAGCmψGAAGCmψGCAGAAGmψGGmψACGGGGAC CmψGCGGGGAAAGCCCmψmψCGCCAmψCGAAGCCGAGAACAGCAmψCCmψGGAC AmψCAGCGGCmψmψCAGCAAGCAGmψACAACmψGmψGCCmψmψCAmψCmψGGCA GAAGGACGGCGmψGAAGAAGCmψGAACCmψGmψACCmψGAmψCAmψCAACmψAC mψmψCAAGGGCGGCAAGCmψGCGGmψmψCAAGAAGAmψCAAACCmψGAAGCCmψ mψCGAAGCCAACAGAmψmψCmψACACCGmψGAmψCAACAAAAAGAGCGGCGAGA mψCGmψGCCCAmψGGAGGmψGAACmψmψCAACmψmψCGACGACCCCAACCmψGA mψCAmψCCmψGCCmψCmψGGCCmψmψmψGGCAAGAGACAGGGCAGAGAAmψmψC AmψCmψGGAACGACCmψGCmψGmψCCCmψGGAAACCGGCAGCCmψGAAGCmψGG CCAACGGAAGAGmψGAmψCGAGAAGACACmψGmψACAACAGAAGAACCCGGCAG GAmψGAGCCmψGCCCmψGmψmψCGmψGGCCCmψGACCmψmψCGAGCGGCGGGAG GmψCCmψGGACmψCCmψCCAAmψAmψCAAACCAAmψGAACCmψGAmψCGGCGmψ GGCAAGAGGCGAAAACAmψCCCCGCCGmψGAmψCGCCCmψGACCGACCCCGAGG GCmψGCCCACmψGAGCCGGmψmψmψAAGGAmψAGCCmψGGGAAACCCAACCCAC AmψCCmψGAGAAmψCGGCGAGAGCmψAmψAAGGAGAAGCAGCGGACCAmψCCAG GCCAAGAAGGAGGmψGGAGCAGCGGAGAGCCGGCGGCmψACAGCCGGAAGmψAC GCCAGCAAAGCCAAGAAmψCmψGGCAGACGAmψAmψGGmψGAGAAACACCGCmψ AGAGAmψCmψGCmψGmψACmψACGCCGmψGACCCAGGAmψGCCAmψGCmψGAmψ CmψmψCGCCAACCmψGAGCCGGGGCmψmψCGGCCGGCAGGGCAAGCGGACCmψm ψCAmψGGCCGAGAGACAGmψACACACGGAmψGGAGGACmψGGCmψGACCGCCAA GCmψGGCCmψACGAGGGCCmψGAGCAAGACCmψACCmψGmψCCAAGACACmψGG CCCAGmψACACCmψCCAAGACAmψGCAGCAACmψGmψGGGmψmψmψACCAmψCA CCAGCGCCGACmψACGACAGGGmψGCmψGGAGAAGCmψGAAGAAGACAGCAACA GGCmψGGAmψGACCACAAmψmψAACGGCAAGGAGCmψGAAGGmψGGAGGGCCAG AmψmψACCmψACmψACAACAGAmψACAAGAGACAGAACGmψAGmψCAAGGACCm ψGmψCCGmψCGAGCmψGGAmψAGACmψGAGCGAAGAAmψCmψGmψGAACAACGA CAmψCmψCCmψCCmψGGACAAAGGGCAGAAGCGGAGAAGCmψCmψGAGCCmψCC mψGAAGAAAAGAmψmψCmψCCCAmψAGACCCGmψGCAGGAGAAGmψmψCGmψGm ψGCCmψGAACmψGCGGCmψmψCGAGACACACGCAGCCGAGCAAGCCGCCCmψGA ACAmψCGCCAGAmψCCmψGGCmψGmψmψCCmψGCGGAGCCAGGAGmψACAAGAA AmψACCAGACAAACAAGACAACCGGCAACACCGAmψAAGAGAGCCmψmψCGmψC GAGACCmψGGCAGmψCCmψmψmψmψACCGGAAGAAGCmψmψAAGGAGGmψGmψG GAAACCmψGCCGmψGCGGmψCmψGGCGGAmψCmψGGCGGAGGCmψCCACCAGCC CCAAGAAAAAGAGAAAAGmψCmψAAmψAGAmψAAGCmψGCCmψmψCmψGCGGGG CmψmψGCCmψmψCmψGGCCAmψGCCCmψmψCmψmψCmψCmψCCCmψmψGCACCm ψGmψACCmψCmψmψGGmψCmψmψmψGAAmψAAAGCCmψGAGmψAGGAAGmψCmψ AGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAA

Table 20: Full-length protein sequences of LTRP5-ZIM3 and LTRP5-ADD-ZIM3 mRNA molecules assessed in this example

LTRP molecule	Amino acid sequence SEQ ID NO
LTRP5-ZIM3	3131
LTRP5-ADD-ZIM3	3132

Synthesis of gRNAs:

[0421] Two gRNAs targeting the mouse *PCSK9* locus were designed using gRNA scaffold 316 and chemically synthesized. *PCSK9*-targeting spacers 27.88 and 27.94 (sequences listed in Table 14) were assessed in this example. As shown in Example 2, use of spacer 27.88 was less effective in achieving PCSK9 knockdown than use of spacer 27.94.

[0422] Transfection of mRNA and gRNA into Hepa1-6 cells and intracellular PCSK9 staining were performed as described in Example 2. Briefly, each well of seeded Hepa1-6 cells was transfected with 300 ng of mRNA encoding LTRP5-ZIM3 or LTRP5-ADD-ZIM3 and 150 ng of *PCSK9*-targeting gRNA with spacer 27.88 or 27.94. Intracellular levels of PCSK9 protein were measured at various timepoints, up to day 53 post-transfection using an intracellular staining protocol as described earlier in Example 2. A non-targeting gRNA was used as an experimental control.

Results:

[0423] To determine the effects of incorporating the ADD domain into an LTRP molecule on activity, i.e., inducing more durable repression of an endogenous locus in vitro, mRNAs encoding LTRP5-ZIM3 or LTRP5-ADD-ZIM3 were co-transfected with a *PCSK9*-targeting gRNA into Hepa1-6 cells. The quantification of the resulting PCSK9 knockdown is shown in FIGS. 10A-10B. The data demonstrate a noticeable improvement in achieving PCSK9 knockdown when the cells were treated with LTRP5-ADD-ZIM3 than with LTRP5-ZIM3, and this improvement was more pronounced when using a *PCSK9*-targeting gRNA containing the weaker spacer 27.88. Further supporting the data discussed in Example 2, use of spacer 27.94 resulted in more durable repression than use of spacer 27.88 by day 53 when paired with either the LTRP5-ZIM3 or LTRP5-ADD-ZIM3 molecule (FIG. 10B). As expected, use of the non-targeting spacer did not result in PCSK9 knockdown.

[0424] These experiments demonstrate that use of LTRP constructs with the ADD domain can result in increased durable repression of an endogenous locus in cells compared to constructs without the ADD domain. Furthermore, these findings show that LTRP molecules with the ADD domain can be delivered as mRNA and co-transfected with a targeting gRNA to cells to induce effective silencing.

Example 5: Demonstration that mRNA encoding for LTRPs containing the ADD domain can induce repression of an endogenous locus in multiple human cell lines

[0425] Experiments were performed to demonstrate that mRNA encoding for LTRPs containing the ADD domain can induce long-term repression of an endogenous target locus in various human cell lines, when delivered as mRNA co-transfected with a targeting gRNA.

Materials and Methods

Generation of mRNA:

[0426] mRNA encoding the following molecules were generated by IVT following similar methods as described in Example 2: 1) a catalytically-active CasX 676, 2) dXR1 (as described in Example 2), and 3) LTRP5-ADD-ZIM3 (as described in Example 4). Sequences encoding these molecules were codon-optimized using a codon utilization table, in addition to using a publicly available codon optimization tool and adjusting parameters such as GC content. The DNA and mRNA sequences encoding the catalytically-active CasX 676 are shown in Table 21 and Table 22 respectively. The DNA and mRNA sequences encoding for dXR1 are shown in Table 11 and Table 12 respectively. The DNA and mRNA sequences encoding for LTRP5-ADD-ZIM3 are shown in Table 18 and Table 19 respectively.

Table 21: Encoding sequences of the catalytically-active CasX 676 mRNA molecule assessed in this example*.

CasX mRNA ID	Component (ID)	Description	DNA sequence or SEQ ID NO:
CasX 676 mRNA	5'UTR	TriLink	3047
	START codon + c-MYC NLS		3133
	CasX 676		3134
	c-MYC NLS + STOP codons		3135
	3'UTR	Mouse *HBA*	3055
	XbaI restriction site (partial)		TCTAG
	Poly(A) tail		3057

*Components are listed in a 5' to 3' order within the constructs

Table 22: Full-length RNA sequences of catalytically-active CasX 676 mRNA molecule assessed in this example. Modification 'mψ' = N1-methyl-pseudouridine.

CasX mRNA	SEQ ID NO	RNA Sequence
CasX 676 mRNA	3136	AAAmψAAGAGAGAAAAGAAGAGmψAAGAAGAAAmψAmψAAGAGCCACCAmψGGCCCCmψGCmψG CCAAGAGAGmψGAAGCmψGGAmψAGCAGACAGGAGAmψCAAGCGGAmψmψAAmψAAAAmψmψCG

CasX mRNA	SEQ ID NO	RNA Sequence
		GAGAAGACmψGGmψGAAGGAmψmψCmψAACACAAAGAAGGCmψGGCAAGACACGGGGCCCmψAm ψGAAGACACmψGCmψGGmψGAGAGmψGAmψGACACCCGACCmψGAGAGAAAGACmψGGAAAACC mψGAGAAAGAAGCCmψGAGAAmψAmψCCCCCAGCCCAmψCAGCAACACAAGCCGGGCCAACCmψ GAAmψAAGCmψGCmψGACCGACmψACACCGAAAmψGAAGAAGGCCAmψCCmψGCACGmψGmψAm ψmψGGGAAGAGmψmψCCAGAAAGACCCAGmψCGGCCmψGAmψGAGCAGAGmψGGCmψCAGCCmψ GCCAGCAAGAAGAmψCGAmψCAGAACAAGCmψGAAGCCCGAAAmψGGACGAGAAGGGGAACCmψ GACAACCGCCGGCmψmψmψGCCmψGmψAGCCAGmψGCGGCCAGCCCCmψGmψmψmψGmψGmψAC AAACmψGGAACAGGmψGAGCGAAAAGGGCAAGGCmψmψACACGAAmψmψACmψmψCGGCAGAmψ GCAACGmψGGCCGAGCACGAGAAGCmψGAmψCAAGCmψGGCCCAGCmψGAAGCCmψGAGAAGGA mψAGCGAmψGAGGCAGmψGACAmψAmψmψCCCmψGGGCAAGmψmψCGGACAGCGGGCCCmψGGA mψmψmψmψmψAmψmψCCAmψmψCAmψGmψGACCAAGGAAmψCCACCCACCCCGmψCAAGCCmψC mψmψGCCCAAAmψmψGCCGGCAACAGAmψACGCCmψCCAGCCCCGmψGGGCAAGGCCCmψGAGC GACGCCmψGmψAmψGGGCACCAmψCGCCAGCmψmψCCmψGmψCmψAAGmψACCAGGACAmψmψA mψCAmψCGAGCACCAGAAGGmψGGmψGAAGGGCAACCAGAAGAGACmψGGAGAGCCmψGCGCGA GCmψGGCCGGCAAGGAAAACCmψGGAGmψAmψCCmψAGCGmψGACCCmψGCCmψCCmψCAGCCm ψCAmψACAAAGGAGGGCGmψGGAmψGCCmψACAACGAAGmψGAmψCGCCCGGGmψGCGGAmψGm ψGGGmψGAACCmψGAAmψCmψGmψGGCAGAAGCmψGAAGCmψGmψCmψAGAGACGACGCCAAGC CCCmψGCmψGAGACmψGAAGGGCmψmψCCCCAGCmψmψCCCmψCmψGGmψGGAGAGACAGGCAA AmψGAAGmψGGACmψGGmψGGGACAmψGGmψGmψGmψAACGmψGAAGAAGCmψGAmψCAAmψGA GAAGAAGGAGGACGGCAAAGmψGmψmψCmψGGCAGAAmψCmψGGCCGGCmψACAAGCGmψCAGG AGGCCCmψGCGGCCCmψACCmψGAGCAGCGAGGAAGACAGAAAGAAGGGCAAGAAGmψmψCGCC CGGmψAmψCAGCmψGGGGGACCmψGCmψGCmψGCACCmψCGAGAAGAAGCACGGCGAAGACCmψG GGGGAAGGmψGmψACGAmψGAGGCCmψGGGAGCGGAmψCGAmψAAGAAGGmψGGAGGGCCmψGA GCAAGCACAmψCAAGCmψGGAGGAGGAACGGAGAmψCmψGAGGACGCCCAGAGCAAGGCCGCCC mψGACCGACmψGGCmψGAGAGCCAAGGCCAGCmψmψCGmψCAmψCGAGGGGCmψGAAGGAGGCC GACAAGGACGAGmψmψCmψGCCGGmψGCGAACmψGAAGCmψGCAGAAGmψGGmψACGGAGAmψC mψGAGAGGCAAACCmψmψmψCGCCAmψCGAGGCCGAGAACAGCAmψCCmψGGACAmψCAGCGGC mψmψCAGCAAGCAGmψACAACmψGCGCCmψmψmψAmψmψmψGGCAGAAGGACGGAGmψGAAGAA GCmψGAACCmψGmψACCmψGAmψCAmψCAACmψAmψmψmψCAAGGGCGGCAAGCmψGAGAmψmψ CAAGAAGAmψCAAGCCmψGAAGCCmψmψCGAGGCCAACAGAmψmψCmψACACCGmψGAmψmψAA CAAGAAAAGCGGAGAGAmψCGmψGCCAAmψGGAAGmψGAACmψmψCAACmψmψCGACGACCCmψ AACCmψGAmψCAmψCCmψGCCCCmψGGCAmψmψmψGGCAAGCGGCAGGGCAGAGAGmψmψCAmψ CmψGGAACGACCmψGCmψGmψCmψCmψGGAGACCGGCAGCCmψGAAGCmψGGCCAACGGCAGAG mψGAmψCGAGAAGACACmψGmψACAACAGACGAACCAGACAAGACGAGCCCGCCCmψGmψmψmψ GmψGGCCCmψGACCmψmψCGAGAGAAGAGAGGmψGCmψGGACAGCAGCAAmψAmψCAAGCCmψA mψGAACCmψGAmψCGGCGmψGGACCGGGGCGAGAACAmψCCCmψGCCGmψGAmψCGCCCmψmψA CCGACCCCGAGGGAmψGCCCmψCmψGAGCCGGmψmψmψAAAGACAGCCmψGGGCAACCCmψACC CACAmψCCmψGAGAAmψmψGGCGAGmψCCmψACAAGGAGAAGCAGAGAACCAmψCCAGGCCAAG AAGGAGGmψGGAGCAGCGGCGGGCmψGGCGGCmψACmψCCCGGAAGmψACGCCAGCAAGGCCAA GAACCmψGGCCGACGACAmψGGmψmψAGAAAmψACCGCCAGAGACCCmψCCmψGmψACmψACGCm ψGmψGACCCAGGACGCCAmψGCmψGAmψCmψmψCGAGAACCmψGAGCAGAGGCmψmψCGGCAGA CAGGGCAAGAGAACCmψmψCAmψGGCCGAGAGACAGmψACACCCGGAmψGGAGGACmψGGCmψG ACCGCCAAGCmψGGCCmψACGAGGGCCmψGCCCmψCmψAAGACCmψACCmψGmψCCAAGACCmψ mψGGCACAGmψACACCAGCAAGACAmψGCmψCmψAACmψGCGGCmψmψCACAAmψCACGAGCGC CGACmψACGACCGGGmψGCmψGGAGAAACmψGAAGAAGACCGCCACAGGCmψGGAmψGACCACC AmψmψAACGGCAAGGAGCmψGAAGGmψGGAGGGCCAGAmψCACCmψACmψACAACAGGmψACAA ACGGCAGAACGmψGGmψGAAGGACCmψGAGCGmψGGAACmψGGAmψAGACmψGAGCGAGGAAAG CGmψAAACAAmψGACAmψCAGCAGCmψGGACCAAGGGCCGGAGCGGCGAGGCCCmψGAGCCmψG CmψGAAGAAGAGAmψmψCmψCCCACAGACCAGmψGCAGGAGAAGmψmψCGmψGmψGmψCmψGAA CmψGCGGCmψmψCGAGACCCACGCCGACGAGCAAGCCGCCCmψGAACAmψCGCCCGGmψCmψmψ GGCmψmψmψmψCCmψGCGGAGCCAGGAGmψACAAGAAGmψACCAGACAAACAAGACCACAGGCA ACACAGACAAGAGAGCCmψmψCGmψCGAGACCmψGGCAGAGCmψmψCmψACAGAAAGAAGCmψG AAGGAGGmψGmψGGAAGCCmψGCCGmψGGGAAGCCCCGCmψGCCAAGAGAGmψGAAGCmψGGAC mψAAmψAGAmψAAGCmψGCCmψmψCmψGCGGGGCmψmψGCCmψmψCmψGGCCAmψGCCCmψmψC mψmψCmψCmψCCCmψmψGCACCmψGmψACCmψCmψmψGGmψCmψmψmψGAAmψAAAGCCmψGAG

CasX mRNA	SEQ ID NO	RNA Sequence
		mψAGGAAGmψCmψAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Synthesis of gRNAs:

[0427] gRNAs targeting the human *PCSK9* locus were designed using gRNA scaffold 316 and chemically synthesized. Furthermore, a *B2M*-targeting gRNA was used as an experimental control. Sequences of targeting spacers as assessed in this example as listed in Table 23.

Table 23: Sequences of spacers assessed in this example.

Spacer ID	Target	Targeting spacer sequence (RNA)	SEQ ID NO
7.37	human *B2M*	GGCCGAGAUGUCUCGCUCCG	3137
6.1	human *PCSK9*	GAGGAGGACGGCCUGGCCGA	1834
6.125	human *PCSK9*	AGAAAGAGCAAGCCUCAUGU	1845
6.138	human *PCSK9*	AGGGAUUUAUACUACAAAGA	1852
6.153	human *PCSK9*	GUUAAUGUUUAAUCAGAUAG	1866
6.157	human *PCSK9*	GGGUCUGAGCCUGGAGGAGU	1869
6.172	human *PCSK9*	GCUGAAACAGAUGGAAUACU	1879
6.181	human *PCSK9*	UCAUCUGCACUCGUGGCCAC	2228

Transfection of mRNA and gRNA into HepG2 cells, Hep3B cells, and Huh7 cells and ELISA to assess PCSK9 secretion:

[0428] The following three human hepatocyte cancer cell lines were used in this experiment: HepG2 cells, Hep3B cells, and Huh7 cells. ~15,000 cells of each cell line were seeded per well; the next day, seeded cells were transfected with mRNA encoding a catalytically-active CasX 676, dXR1, or LTRP5-ADD-ZIM3 and a gRNA with scaffold 316 and spacer targeting either the *B2M* or *PCSK9* locus (see Table 23 for specific spacers and sequences). Media supernatant was harvested at 4 days post-transfection to assess level of PCSK9 secretion by ELISA, and levels of PCSK9 secretion were normalized to total cell count and illustrated in FIGS. 11A-11C. Culturing of treated Huh7 cells continued, and media supernatant was harvested at 14 and 27 days post-transfection for measuring PCSK9 secretion by ELISA. As an additional experimental control, PCSK9 secretion was also measured in the media supernatant harvested from wells containing untreated, naïve cells.

Results:

[0429] HepG2 cells, Hep3B cells, and Huh7 cells were transfected with mRNA encoding catalytically-active CasX 676, dXR1, or LTRP5-ADD-ZIM3 with a gRNA targeting either the *B2M* or the *PCSK9* locus, and secreted PCSK9 levels were measured. Quantification of normalized PCSK9 secretion levels for each condition at 4 days post-transfection is depicted in FIGS. 11A-11C. The data demonstrate that the most efficient knockdown of PCSK9 secretion by CasX 676, dXR1, or LTRP5-ADD-ZIM3 was observed in Huh7 cells, while HepG2 cells did not exhibit as efficient of a knockdown of secreted PCSK9 levels (FIG. 11A-11C). Meanwhile, Hep3B cells demonstrated low PCSK9 secretion levels overall, illustrating that, of the cell lines used, the Hep3B cell line is the least amenable to treatment to induce and demonstrate PCSK9 repression (FIG. 11A-11C).

[0430] Culturing of treated Huh7 cells continued up to day 27 post-transfection, and PCSK9 secretion was measured at day 14 and day 27. The bar plot in FIG. 12 shows the quantification results of PCSK9 repression at day 4, day 14, and day 27 timepoints displayed as PCSK9 knockdown relative to the levels detected for the naïve control at the day 4 timepoint. The data demonstrate that treatment of Huh7 cells with LTRP5-ADD-ZIM3 with gRNAs having spacers 6.138 and 6.157 resulted in the most effective repression of PCSK9 secretion, and this repression was sustained through day 27 post-transfection (FIG. 12). Similarly, sustained knockdown was observed when Huh7 cells were treated with catalytically-active CasX 676 with spacer 6.1. While treatment with dXR1 and spacer 6.138 resulted in an initial strong repression at day 4, this repressive effect was transient as PCSK9 secretion levels returned to baseline levels at day 14 and day 27 post-transfection (FIG. 12). As anticipated, treatment with any of the three mRNA molecules with spacer 7.37 targeting the *B2M* locus did not affect PCSK9 secretion levels during this time course experiment.

[0431] These experiments demonstrate that use of LTRP molecules with the ADD domain with the appropriate targeting spacer can result in long-term silencing of an endogenous target locus in various human cell lines. These findings also show that LTRP molecules with the ADD domain can be co-delivered as mRNA with a targeting gRNA to cells to induce repression.

Example 6: Demonstration that use of certain *PCSK9*-targeting spacers can result in undesired intracellular PCSK9 retention

[0432] Secretory proteins that cannot properly fold are consequently retained in the endoplasmic reticulum (ER) to be ultimately targeted for proteasomal degradation. However,

excessive protein accumulation in the ER could cause ER stress. PCSK9 is initially synthesized as a zymogen (known as pro-PCSK9) that undergoes autocatalytic cleavage during maturation in the ER into an inactive secretory protein (also known as mature or processed PCSK9). Furthermore, certain gain-of-function mutations in the *PCSK9* gene resulting in hypercholesterolemia have been shown to be associated with intracellular PCSK9 retention in the ER (Benjannet S et al. NARC-1/PCSK9 and its natural mutants: zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. *J. Biol. Chem.* 279:48865–48875 (2004); Park SW et al., Post-transcriptional regulation of low density lipoprotein receptor protein by proprotein convertase subtilisin/kexin type 9a in mouse liver. *J. Biol. Chem.* 279:50630–50638 (2004); Uribe KB et al. A Systematic Approach to Assess the Activity and Classification of PCSK9 Variants. *Int. J. Mol. Sci.* 22:13602 (2021)), an indication that targeting certain regions of the *PCSK9* locus may result in undesired intracellular retention. As a result, experiments were performed to demonstrate that use of certain *PCSK9*-targeting spacers can result in unwanted increase in intracellular PCSK9 levels that may possibly cause unexpected consequences such as abnormal ER stress.

Materials and Methods:

[0433] In vitro transcription of CasX 676 mRNA #2 (sequence listed in Table 28, below) was performed as described in Example 7, below. Guide RNAs using scaffold 316 and a *PCSK9*-targeting spacer were synthesized with a v1 modification profile (as discussed in Example 7). Spacers 6.1, 6.8, 6.86, 6.114, 6.197, and 6.203 (sequences listed in Table 24) were assessed for intracellular PCSK9 retention.

Table 24: Sequences of human *PCSK9*-targeting spacers

Spacer ID	Spacer DNA sequence	SEQ ID NO:	Spacer RNA sequence	SEQ ID NO:
6.1	GAGGAGGACGGCCTGGCCGA	2977	GAGGAGGACGGCCUGGCCGA	1834
6.8	TGGCTTCCTGGTGAAGATGA	3139	UGGCUUCCUGGUGAAGAUGA	2009
6.86	TGGTGAAGATGAGTGGCGAC	3464	UGGUGAAGAUGAGUGGCGAC	3466
6.114	TCCCAGGCCTGGAGTTTATT	3141	UCCCAGGCCUGGAGUUUAUU	2291
6.197	AGGTCATCACAGTTGGGGCC	3465	AGGUCAUCACAGUUGGGGCC	3467
6.203	CCAGGAGTGGGAAGCGGCGG	3144	CCAGGAGUGGGAAGCGGCGG	2341

In vitro delivery of CasX mRNA and gRNA via transfection:

[0434] To determine whether use of certain *PCSK9*-targeting spacers would result in potential intracellular PCSK9 retention, ~50,000 HepG2 cells were seeded per well in a 96-well plate.

CasX 676 mRNA #2 was transfected into HepG2 cells with a *PCSK9*-targeting gRNA using lipofectamine. After a media change, the following were harvested two days post-transfection: 1) media supernatant to measure secreted PCSK9 protein levels by ELISA; and 2) transfected cells for western blotting analysis to evaluate intracellular PCSK9 levels. Harvested cells were subjected to whole cell lysate extraction for western blotting analysis. Briefly, extracted protein samples were resolved by SDS-PAGE followed by immunoblotting to analyze levels of pro-PCSK9 and processed PCSK9, which were quantified by densitometry. Secreted PCSK9 levels in the media supernatant were analyzed using the BioLegend® ELISA MAX™ kit following the manufacturer's instructions. Naïve, untreated cells and cells transfected with CasX 676 mRNA #2 only served as two experimental controls.

Results:

[0435] Following transfection of HepG2 cells with CasX 676 mRNA #2 and a *PCSK9*-targeting gRNA, secreted PCSK9 levels in the media supernatant were quantified by ELISA, and the results are shown in FIG. 13. The data demonstrate that transfection of HepG2s cells with CasX 676 mRNA and the *PCSK9*-targeting gRNAs resulted in reduced secreted PCSK9 levels to varying degrees. Of the spacers tested, use of spacers 6.1, 6.8, 6.86, and 6.203 resulted in nearly 50% reduction in secreted levels compared to cells transfected with CasX 676 mRNA only (FIG. 13).

[0436] Intracellular levels of PCSK9 were also evaluated in the transfected HepG2 cells. FIG. 14 is a western blot analysis of PCSK9 protein levels, along with the total protein loading control, in the transfected HepG2 cells, and FIG. 15 is a bar plot illustrating the densitometry quantification for pro-PCSK9, processed PCSK9, and total PCSK9 protein levels normalized to total PCSK9 levels from the naïve condition. The data show that of the PCSK9-targeting spacers assessed, only use of spacer 6.1 did not result in substantially increased pro-PCSK9 levels, therefore indicating that use of spacer 6.1 did not increase intracellular protein levels (FIGS. 14-15). While use of spacer 6.203 also did not noticeably increase intracellular protein levels, its use resulted in increased processed PCSK9 levels (FIGS. 14-15), appearing to contradict findings of its effects to reduce secreted PCSK9 levels (FIG. 13) when compared to the either the naïve or CasX mRNA only control. This apparent contradictory effect observed by use of spacer 6.203 indicates that retention of processed PCSK9 may be involved in the mechanism by which use of spacer 6.203 decreases PCSK9 secretion.

[0437] The results demonstrate that although use of certain *PCSK9*-targeting spacers would result in decreased secreted PCSK9 levels, there is a possibility for some of these seemingly effective spacers to exhibit potentially undesired characteristics, such as increased intracellular protein retention. Therefore, the findings from these experiments indicate the use of assessing increased intracellular protein retention as a potential safety criterion to identify effective targeting spacers for therapeutic use.

Example 7: Design and assessment of modified gRNAs in improving editing when delivered together with CasX mRNA in vitro and in vivo

[0438] Experiments were performed to identify new gRNA variant sequences and demonstrate that chemical modifications of these gRNA variants enhance the editing efficiency of the CasX:gRNA system when delivered in vitro in conjunction with CasX mRNA.

Materials and Methods:

Synthesis of gRNAs:

[0439] All gRNAs tested in this example were chemically-synthesized and were derived from gRNA scaffolds 174, 235, and 316. The sequences of gRNA scaffolds 174, 235, and 316 and their chemical modification profiles are listed in Table 25. The sequences of the resulting gRNAs, including spacers targeting *PCSK9*, *B2M*, or *ROSA26*, and their chemical modification profiles assayed in this example are listed in Table 26. A schematic of the structure of gRNA scaffold variants 174, 235, and 316 are shown in FIGS. 19A-19C, respectively, and the sites of chemical modifications of the gRNA variants are shown schematically in FIGS. 16A, 16B, 18, 24, and 25.

Table 25. Sequences of gRNA scaffolds with their different chemical modification profiles (denoted by version number), where "NNNNNNNNNNNNNNNNNNNN" is a spacer placeholder. Chemical modifications: * = phosphorothioate bond; m = 2'OMe modification

gRNA scaffold (version)	gRNA sequence	SEQ ID NO:
174 (v0)	ACUGGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCCUCUUCGGAGGGAGCAUCAAAGNNNNNNNNNNNNNNNNNNNN	2947
174 (v1)	mA*mC*mU*GGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCCUCUUCGGAGGGAGCAUCAAAGNNNNNNNNNNNNNNNNNNmN*mN*mN	2948
174 (v2)	mA*mC*mU*GGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCCUCUUCGGAGGGAGCAUCAAAGNNNNNNNNNNNNNNNNNNNNNN*mU*mU*mU	2949

gRNA scaffold (version)	gRNA sequence	SEQ ID NO:
174 (v3)	mA*mC*mU*mGmGmCmGmCmUmUmUmUmAmUmCmUmGmAmUUACUUUGmAmGmAmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUmCmGUAGUGmGmGmUmAmAmAmGmCmUmCmCmCmUmCmUmUmUmCmGmGmAmGmGmGmAmGmCmAmUmCmAAAGNNNNNNNNNNNNNNNNmN*mN*mN	2950
174 (v4)	mA*mC*mU*mGmGmCmGmCUUUUmAmUmCmUmGmAmUUACUUUGmAmGmAmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUmCmGUAGUGmGmGmUmAmAmAmGmCmUmCmCmCmUmCmUmUmUmCmGmGmAmGmGmGmAmGmCmAmUCAAAGNNNNNNNNNNNNNNNNNmN*mN*mN	2951
174 (v5)	mA*mC*mU*GGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGAmCmUAUmGmUmCmGUAGUGGGUAAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCAUCAAAGNNNNNNNNNNNNNNNNNmN*mN*mN	2952
174 (v6)	mA*mC*mU*GGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCAUCAAAGNNNNNNNNNNNNNNNNNmN*mN*mN	2953
174 (v7)	mA*mC*mU*GGmCGmCmUUUUAmUmCUGAUUACUUUGmAmGAGCCmAmUmCmAmCCAGCmGmAmCmUAUmGmUmCmGUAGUGGmGmUAmAmAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCmAmUCAAAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2954
174 (v8)	mA*mC*mU*GGCGCUUUUAUCUGAUUACUUUGAGAGCCmAmUmCmAmCCAGCmGmAmCmUAUmGmUmCmGUAGUGGmGmUmAmAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCmAmUCAAAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2955
174 (v9)	mA*mC*mU*GGmCmGCmUUUUAmUmCUGAUUACUUUGmAmGAGCCAUCACCAGCmGmAmCmUAUmGmUmCmGUAGUGGGUAAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCAUCAAAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2956
235 (v0)	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUCCGUAAGAGGCAUCAGAGNNNNNNNNNNNNNNNNNNNN	2957
235 (v1)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUCCGUAAGAGGCAUCAGAGNNNNNNNNNNNNNNNNmN*mN*mN	2958
235 (v2)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUCCGUAAGAGGCAUCAGAGNNNNNNNNNNNNNNNNNN*mU*mU*mU	2959
235 (v3)	mA*mC*mU*mGmGmCmGmCmUmUmCmUmAmUmCmUmGmAmUUACUCUGmAmGmCmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUmCmGUAGUGmGmGmUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUmCmAGAGNNNNNNNNNNNNNNNNNmN*mN*mN	2960
235 (v4)	mA*mC*mU*mGmGmCmGmCUUCUmAmUmCmUmGmAmUUACUCUGmAmGmCmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUmCmGUAGUGmGmGmUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUCAGAGNNNNNNNNNNNNNNNNNmN*mN*mN	2961
235 (v5)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGAmCmUAUmGmUmCmGUAGUGGGUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUCAGAGNNNNNNNNNNNNNNNNNmN*mN*mN	2962
235 (v6)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUCAGAGNNNNNNNNNNNNNNNNNmN*mN*mN	2963
235 (v7)	mA*mC*mU*GGmCGmCmUUCUAmUmCUGAUUACUCUGmAmGCGCCmAmUmCmAmCCAGCmGmAmCmUAUmGmUmCmGUAGUGGmGmUmAmAAmGmCmCmGmCmUmUmAmCmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUCAGAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2964
235 (v8)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCmAmUmCmAmCCAGCmGmAmCmUAUmGmUmCmGUAGUGGmGmUmAmAAmGmCmCmGmCmUmUmAmCmGmGm	2965

gRNA scaffold (version)	gRNA sequence	SEQ ID NO:
	AmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUCAGAGNNNNNNNNNNNNNNNNN*mN*mN*mN	
235 (v9)	mA*mC*mU*GGmCGmCmUUCUAmUmCUGAUUACUCUGmAmGCGCCAUCACCAGCmGmAmCmUAUmGmUmCmGUAGUGGGUAAAmGmCmCmGmCmUmUmAmCmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCAUCAGAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2966
316 (v0)	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGAGNNNNNNNNNNNNNNNNNN	2967
316 (v1)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGAGNNNNNNNNNNNNNNNN*mN*mN*mN	2968
316 (v2)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGAGNNNNNNNNNNNNNNNNNN*mU*mU*mU	2969
316 (v3)	mA*mC*mU*mGmGmCmGmCmUmUmCmUmAmUmCmUmGmAmUUACUCUGmAmGmCmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUmCmGUAGUGmGmGmUmAmAmAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmCmAmUmCmAmUmCmAGAGNNNNNNNNNNNNNNN*mN*mN*mN	2970
316 (v4)	mA*mC*mU*mGmGmCmGmCUUCUmAmUmCmUmGmAmUUACUCUGmAmGmCmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUmCmGUAGUGmGmGmUmAmAmAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmCmAmUmCmAmUCAGAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2971
316 (v5)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGAmCmUAUmGmUmCmGUAGUGGGUAAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCAUCAGAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2972
316 (v6)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCAUCAGAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2973
316 (v7)	mA*mC*mU*GGmCGmCmUUCUAmUmCUGAUUACUCUGmAmGCGCCmAmUmCmAmCCAGCmGmAmCmUAUmGmUmCmGUAGUGGmGmUmAmAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmCmAmUmCmAmUCAGAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2974
316 (v8)	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCmAmUmCmAmCCAGCmGmAmCmUAUmGmUmCmGUAGUGGmGmUmAmAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmCmAmUmCmAmUCAGAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2975
316 (v9)	mA*mC*mU*GGmCGmCmUUCUAmUmCUGAUUACUCUGmAmGCGCCAUCACCAGCmGmAmCmUAUmGmUmCmGUAGUGGGUAAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCAUCAGAGNNNNNNNNNNNNNNNNN*mN*mN*mN	2976

Table 26: Sequences of gRNAs with their different chemical modification profiles (denoted by version number) assayed in this example. Chemical modifications: * = phosphorothioate bond; m = 2'Ome modification

gRNA ID (scaffold variant-spacer)	Target	gRNA sequence	SEQ ID NO:
174-6.7 (v0)	human *PCSK9*	ACUGGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAAAGUCCUGGCUUCCUGGUGAAGA	3145
174-6.7	human	mA*mC*mU*GGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACC	3074

gRNA ID (scaffold variant-spacer)	Target	gRNA sequence	SEQ ID NO:
(v1)	*PCSK9*	AGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGC AUCAAAGUCCUGGCUUCCUGGUGAmA*mG*mA	
174-6.8 (v0)	human *PCSK9*	ACUGGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGAC UAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAAA GUGGCUUCCUGGUGAAGAUGA	3146
174-6.8 (v1)	human *PCSK9*	mA*mC*mU*GGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACC AGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGC AUCAAAGUGGCUUCCUGGUGAAGAmU*mG*mA	3147
174-7.9 (v0)	human *B2M*	ACUGGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGAC UAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAAA GGUGUAGUACAAGAGAUAGAA	3148
174-7.9 (v1)	human *B2M*	mA*mC*mU*GGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACC AGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGC AUCAAAGGUGUAGUACAAGAGAUAmG*mA*mA	3149
316-6.7 (v0)	human *PCSK9*	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGAC UAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGA GUCCUGGCUUCCUGGUGAAGA	3150
316-6.7 (v1')	human *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACC AGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGC AUCAGAGUCCUGGCUUCCUGGUGAmA*mG*mA	3151
316-6.8 (v0)	human *PCSK9*	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGAC UAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGA GUGGCUUCCUGGUGAAGAUGA	3152
316-6.8 (v1')	human *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACC AGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGC AUCAGAGUGGCUUCCUGGUGAAGAmU*mG*mA	3153
316-7.9 (v0)	human *B2M*	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGAC UAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGA GGUGUAGUACAAGAGAUAGAA	3154
316-7.9 (v1')	human *B2M*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACC AGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGC AUCAGAGGUGUAGUACAAGAGAUAmG*mA*mA	3155
174-7.37 (v0)	human *B2M*	ACUGGCGCUUUUAUCUgAUUACUUUGAGAGCCAUCACCAGCGAC UAUGUCGUAgUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAAA GGGCCGAGAUGUCUCGCUC	3156
174-7.37 (v1*)	human *B2M*	mA*mC*mU*GGCGCUUUUAUCUgAUUACUUUGAGAGCCAUCACC AGCGACUAUGUCGUAgUGGGUAAAGCUCCCUCUUCGGAGGGAGC AUCAAAGGGCCGAGAUGUCUCG*mC*mU*mC	3157
235-6.7 (v0)	human *PCSK9*	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGAC UAUGUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUCCGUAAG AGGCAUCAGAGUCCUGGCUUCCUGGUGAAGA	3158

gRNA ID (scaffold variant-spacer)	Target	gRNA sequence	SEQ ID NO:
235-6.7 (v1)	human *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACC AGCGACUAUGUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUC CGUAAGAGGCAUCAGAGUCCUGGCUUCCUGGUGAmA*mG*mA	3159
235-6.7 (v2)	human *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACC AGCGACUAUGUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUC CGUAAGAGGCAUCAGAGUCCUGGCUUCCUGGUGAAGAU*mU*mU *mU	3160
235-6.7 (v3)	human *PCSK9*	mA*mC*mU*mGmGmCmGmCmUmUmCmUmAmUmCmUmGmAmUUAC UCUGmAmGmCmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUm CmGUAGUGmGmGmUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmA mCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUmCmA GAGUCCUGGCUUCCUGGUGAmA*mG*mA	3161
235-6.7 (v4)	human *PCSK9*	mA*mC*mU*mGmGmCmGmCUUCUmAmUmCmUmGmAmUUACUCUG mAmGmCmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUmCmGU AGUGmGmGmUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmAmCmU mUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUCAGAGUCC UGGCUUCCUGGUGAmA*mG*mA	3162
235-6.7 (v5)	human *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACC AGCGAmCmUAUmGmUmCmGUAGUGGGUmAmAmAmGmCmCmGmCm UmUmAmCmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAm GmGmCAUCAGAGUCCUGGCUUCCUGGUGAmA*mG*mA	3163
235-6.7 (v6)	human *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACC AGCGACUAUGUCGUAGUGGGUmAmAmAmGmCmCmGmCmUmUmAm CmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCA UCAGAGUCCUGGCUUCCUGGUGAmA*mG*mA	3164
235-6.8 (v0)	human *PCSK9*	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGAC UAUGUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUCCGUAAG AGGCAUCAGAGUGGCUUCCUGGUGAAGAUGA	3165
235-6.8 (v1)	human *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACC AGCGACUAUGUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUC CGUAAGAGGCAUCAGAGUGGCUUCCUGGUGAAGAmU*mG*mA	3166
235-6.8 (v2)	human *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACC AGCGACUAUGUCGUAGUGGGUAAAGCCGCUUACGGACUUCGGUC CGUAAGAGGCAUCAGAGUGGCUUCCUGGUGAAGAUGA*mU*mU* mU	3167
235-6.8 (v3)	human *PCSK9*	mA*mC*mU*mGmGmCmGmCmUmUmCmUmAmUmCmUmGmAmUUAC UCUGmAmGmCmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUm CmGUAGUGmGmGmUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmA mCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUmCmA GAGUGGCUUCCUGGUGAAGAmU*mG*mA	3168
235-6.8 (v4)	human *PCSK9*	mA*mC*mU*mGmGmCmGmCUUCUmAmUmCmUmGmAmUUACUCUG mAmGmCmGmCmCmAmUmCmAmCmCAGCGAmCmUAUmGmUmCmGU AGUGmGmGmUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmAmCmU mUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCmAmUCAGAGUGG CUUCCUGGUGAAGAmU*mG*mA	3169

gRNA ID (scaffold variant-spacer)	Target	gRNA sequence	SEQ ID NO:
235-6.8 (v5)	human PCSK9	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGAmCmUAUmGmUmCmGUAGUGGGUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCAUCAGAGUGGCUUCCUGGUGAAGAmU*mG*mA	3170
235-6.8 (v6)	human PCSK9	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUmAmAmAmGmCmCmGmCmUmUmAmCmGmGmAmCmUmUmCmGmGmUmCmCmGmUmAmAmGmAmGmGmCAUCAGAGUGGCUUCCUGGUGAAGAmU*mG*mA	3171
316-27.107 (v0)	mouse PCSK9	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGAGCUGGCUUCUUGGUGAAGAUG	3172
316-27.107 (v1)	mouse *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGAGCUGGCUUCUUGGUGAAG*mA*mU*mG	3173
316-27.107 (v7)	mouse PCSK9	mA*mC*mU*GGmCGmCmUUCUAmUmCUGAUUACUCUGmAmGCGCCmAmUmCmAmCCAGCmGmAmCmUAUmGmUmCmGUAGUGGmGmUmAmAAmGmCmUmCmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCmAmUCAGAGCUGGCUUCUUGGUGAAG*mA*mU*mG	3174
316-27.107 (v8)	mouse PCSK9	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCmAmUmCmAmCCAGCmGmAmCmUAUmGmUmCmGUAGUGGmGmUmAmAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCmAmUCAGAGCUGGCUUCUUGGUGAAG*mA*mU*mG	3175
316-27.107 (v9*)	mouse PCSK9	mA*mC*mU*GGmCGmCmUUCUAmUmCUGAUUACUCUGmAmGCGCCAUCACCAGCmGmAmCmUAUmGmUmCmGUAGUGGGUAAAmGmCmUmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCAUCAGAGCUGGCUUCUUGGUGAA*mG*mA*mU*mG	3176
174-35.2 (v0)	*ROSA26*	ACUGGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAAAGAGAAGAUGGGCGGGAGUCUU	3177
174-35.2 (v2)	*ROSA26*	mA*mC*mU*GGCGCUUUUAUCUGAUUACUUUGAGAGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAAAGAGAAGAUGGGCGGGAGUCUU*mU*mU*mU	3178
316-35.2 (v0)	*ROSA26*	ACUGGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGAGAGAAGAUGGGCGGGAGUCUU	3179
316-35.2 (v1)	*ROSA26*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGAGGGAGCAUCAGAGAGAAGAUGGGCGGGAGU*mC*mU*mU	3180
316-35.2 (v5)	*ROSA26*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUCACCAGCGAmCmUAUmGmUmCmGUAGUGGGUAAAmGmCmUmCmCmCmCmUmCmUmUmCmGmGmAmGmGmGmAmGmCAUCAGAGAGAAGAUGGGCGGGAGU*mC*mU*mU	3181

Note that gRNAs annotated with a v1' design contain one less phosphorothioate bond on the 3' end of the gRNA. gRNAs annotated with v1* contain one extra phosphorothioate bond on the 3'end of the gRNA. gRNAs annotated with a v9* contain an extra phosphorothioate bond on the 3' end of the gRNA.

Biochemical characterization of gRNA activity:

[0440] Target DNA oligonucleotides with fluorescent moieties on the 5' ends were purchased commercially (sequences listed in Table 27). Double-stranded DNA (dsDNA) targets were formed by mixing the oligos in a 1:1 ratio in 1x cleavage buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM TCEP, 5% glycerol, 10 mM $MgCl_2$), following by heating to 95°C for 10 minutes, and then allowing the solution to cool to room temperature. CasX ribonucleoproteins (RNPs) were reconstituted with CasX 491 and the indicated gRNAs at a final concentration of 1 μM with 1.2-fold excess of the indicated gRNA in 1x cleavage buffer. RNPs were allowed to form at 37°C for 10 minutes.

[0441] The effects of various structural and chemical modifications to the gRNA scaffold on the cleavage rate of CasX 491 RNPs were determined. Cleavage reactions were prepared with final RNP concentrations of 200 nM and final target concentrations of 10 nM, and reactions were carried out at 16°C and initiated by the addition of the labeled target DNA substrate (Table 27). Aliquots of reactions were taken at 0.25, 0.5, 1, 2, 5, and 10 minutes and quenched by adding an equal volume of 95% formamide with 20 mM EDTA. Samples were denatured at 95°C for 10 minutes and resolved on a 10% urea-PAGE gel. Gels were imaged on a Typhoon™ laser-scanner platform and quantified using ImageQuant™ TL 8.2 image analysis software (Cytiva™). The apparent first-order rate constant of non-target strand cleavage ($k_{cleave-}$) was determined for each CasX:gRNA combination.

[0442] To determine the competent fraction formed by each gRNA, cleavage reactions were prepared with final RNP concentrations of 100 nM and final target concentrations of 100 nM. Reactions were carried out at 37°C and initiated by the addition of the labeled target substrate (Table 27). Aliquots were taken at 0.5, 1, 2, 5, 10, and 30 minutes and quenched by adding an equal volume of 95% formamide with 25 mM EDTA. Samples were denatured by heating at 95°C for 10 minutes and resolved on a 10% urea-PAGE gel. Gels were imaged and quantified as above. CasX was assumed to act as a single-turnover enzyme under the assayed conditions, as indicated by the observation that sub-stoichiometric amounts of enzyme would fail to cleave a greater-than-stoichiometric amount of target substrate even under extended time-scales, and

instead would approach a plateau that scaled with the amount of enzyme present. Thus, the fraction of target substrate cleaved over long time-scales by an equimolar amount of RNP would be indicative of the fraction of RNP that was properly formed and active for cleavage. The cleavage traces were fitted with a biphasic rate model, as the cleavage reaction clearly deviated from monophasic under this concentration regime. The plateau of each fit was determined and reported as the active fraction for each RNP in Table 30.

Table 27: Sequences of target DNA substrate oligonucleotides with fluorescent moieties on the 5' ends used for biochemical characterization of gRNA activity. /700/ = IRDye700; /800/ = IRDye800

DNA substrate	Sequence
6.7/6.8 target top strand (SEQ ID NO: 3182)	/700/catgtcttccatggccttcttcctggcttcctggtgaagatgagtggcgacctg ctggag
6.7/6.8 target bottom strand (SEQ ID NO: 3183)	/800/ctccagcaggtcgccactcatcttcaccaggaagccaggaagaaggccatggaa gacatg

In vitro transcription of CasX mRNA:

[0443] DNA templates encoding for CasX 491 (see Table 28 for encoding sequences) used for in vitro transcription were generated by PCR using forward primers containing a T7 promoter, followed by agarose gel extraction of the appropriately sized DNA. 25 ng/µL final concentration of template DNA was used in each in vitro transcription reaction that was carried out following the manufacturer's recommended protocol with slight modifications. Following in vitro transcription reaction incubation for 2-3 hours at 37°C, which were carried out with CleanCap® AG and N1-methyl-pseudouridine, DNAse digestion of template DNA and column-based purification using the Zymo RNA miniprep kit were performed. The poly(A) tail was added using *E. coli* PolyA Polymerase following the manufacturer's protocol, followed by column-based purification as stated above. Poly(A) tailed in vitro transcribed RNA was eluted in RNAse free water, analyzed on an Agilent TapeStation for integrity, and flash frozen prior to storage at -80°C.

Table 28: Encoding sequences of the CasX mRNA molecules assessed in this example*.

CasX 491 mRNA ID	Component (ID)	DNA sequence or SEQ ID NO:
CasX 491 mRNA #1	5'UTR	3082
	START codon + c-MYC NLS + linker	3184
	CasX 491	3185
	Linker + c-MYC NLS	3186
	P2A mScarlet + STOP codon	3187
CasX 676 mRNA #2	5'UTR	3047
	START codon + c-MYC NLS	3133
	CasX 676	3134
	c-MYC NLS + STOP codons	3135
	3'UTR	3055
	XbaI restriction site (partial)	TCTAG
	Poly(A) tail	3057

*Components are listed in a 5' to 3' order within the constructs

In vitro delivery of gRNA and CasX mRNA via transfection:

[0444] Editing at the *PCSK9* locus and consequential effects on secreted PCSK9 levels were assessed for conditions using CasX 491 mRNA co-delivered with a *PCSK9*-targeting gRNA with scaffold variant 174 compared to conditions where a *PCSK9*-targeting gRNA with scaffold variant 316 was used. 100 ng of in vitro transcribed mRNA coding for CasX 491 with a P2A and mScarlet fluorescent protein was transfected into HepG2 cells with version 1 (v1) of gRNAs 174-6.7, 174-6.8, 316-6.7, and 316-6.8 (see Table 26) using lipofectamine. After a media change, the following were harvested at 28 hours post-transfection: 1) transfected cells were harvested for editing assessment at the *PCSK9* locus by NGS (next generation sequencing); and 2) media supernatant was harvested to measure secreted PCSK9 protein levels by ELISA. For editing analysis by NGS, amplicons were amplified from 200 ng of extracted gDNA with a set of primers targeting the *PCSK9* locus and processed by NGS (described below). Secreted PCSK9 levels in the media supernatant were also analyzed using a fluorescence resonance energy transfer-based immunoassay from CISBio following the manufacturer's instructions. Here, a gRNA using scaffold 174 with spacer 7.37 (v0; see Table 26), which targeted the endogenous *B2M* (beta-2-microglobulin) locus, served as the non-targeting (NT) control. These results are shown in FIG. 20.

[0445] To compare the editing potency of version 0 (v0) and version 1 (v1) of *B2M*-targeting gRNAs, ~6E4 HepG2 hepatocytes were seeded per well of a 96-well plate. 24 hours later, seeded cells were co-transfected using lipofectamine with 100 ng of in vitro transcribed mRNA coding for CasX 491 and different doses (1, 5, or 50 ng) of either v0 or v1 of the *B2M*-targeting gRNA containing scaffold variant 174 and spacer 7.37 (see Table 26). Six days post-transfection, cells were harvested for B2M protein expression analysis via immunostaining of the B2M-dependent HLA protein, followed by flow cytometry using the Attune™ NxT flow cytometer. These results are shown in FIG. 17.

[0446] V1 through v6 variants of chemically-modified *PCSK9*-targeting gRNAs (Table 26) were assessed for their effects on editing potency and consequential effects on secreted PCSK9 levels in vitro. Briefly, 100 ng of in vitro transcribed mRNA coding for CasX variant 491 and a P2A and mScarlet fluorescent protein was transfected into HepG2 cells with 50 ng of the indicated chemically-modified gRNA using lipofectamine. After a media change, the following were harvested at 28 hours post-transfection: 1) transfected cells for editing assessment at the *PCSK9* locus by NGS as described above; and 2) media supernatant to measure secreted PCSK9 protein levels by ELISA, as described above. Here, a *B2M*-targeting gRNA was used as a non-targeting control. These results are shown in Table 31.

NGS processing and analysis:

[0447] Genomic DNA (gDNA) from harvested cells were extracted using the Zymo Quick-DNA Miniprep Plus kit following the manufacturer's instructions. Target amplicons were formed by amplifying regions of interest from ~50-200 ng of extracted gDNA with a set of primers targeting the human *PCSK9* locus. These gene-specific primers contained an additional sequence at the 5′ ends to introduce Illumina reads 1 and 2 sequences. Further, they contained a 16-nucleotide random sequence that functioned as a unique molecular identifier (UMI). The quality and quantification of the amplicon was assessed using a Fragment Analyzer DNA analyzer kit (Agilent, dsDNA 35-1500 bp). Amplicons were sequenced on the Illumina MiSeq™ according to the manufacturer's instructions. Raw fastq sequencing files were processed by trimming for quality and adapter sequences and merging read 1 and read 2 into a single insert sequence; insert sequences were then analyzed by the CRISPResso2 (v 2.0.29) program. The percentage of reads modified in a window around the 3' end of the spacer was determined. The activity of the CasX molecule was quantified as the total percent of reads that contain insertions, substitutions, and/or deletions anywhere within this window for each.

Formulations of lipid nanoparticles (LNPs):

[0448] CasX mRNA and gRNA were encapsulated into LNPs using GenVoy-ILM™ lipids on the Precision NanoSystems Inc. (PNI) Ignite™ Benchtop System and following the manufacturer's guidelines. GenVoy-ILM™ lipids are manufactured by PNI, with a proprietary composition of ionizable lipid:DSPC:cholesterol:stabilizer at 50:10:37.5:2.5 mol%.

[0449] Briefly, to formulate LNPs, equal mass ratios of CasX mRNA and gRNA were diluted in PNI Formulation Buffer, pH 4.0. GenVoy-ILM™ was diluted 1:1 in anhydrous ethanol. mRNA/gRNA co-formulations were performed using a predetermined N/P ratio. The RNA and lipids were run through a PNI laminar flow cartridge at a predetermined flow rate ratio (RNA:Genvoy-ILM™) on the PNI Ignite™ Benchtop System. After formulation, the LNPs were diluted in PBS, pH 7.4, to decrease the ethanol concentration and increase the pH, which increases the stability of the particles. Buffer exchange of the mRNA/sgRNA-LNPs was achieved by overnight dialysis into PBS, pH 7.4, at 4°C using 10k Slide-A-Lyzer™ Dialysis Cassettes (Thermo Scientific™). Following dialysis, the mRNA/gRNA-LNPs were concentrated to > 0.5 mg/mL using 100 kDa Amicon®-Ultra Centrifugal Filters (Millipore) and then filter-sterilized. Formulated LNPs were analyzed on a Stunner (Unchained Labs) to determine their diameter and polydispersity index (PDI). Encapsulation efficiency and RNA concentration was determined by RiboGreen™ assay using Invitrogen's Quant-iT™ Ribogreen™ RNA assay kit. LNPs were used in various experiments as described herein to deliver CasX mRNA and gRNA to target cells and tissue.

Delivery of LNPs encapsulating CasX mRNA and targeting gRNAs in vitro:

[0450] ~50,000 HepG2 cells, cultured in DMEM/F-12 media containing 10% FBS and 1% PenStrep, were seeded per well in a 96-well plate. The next day, seeded cells were treated with varying concentrations of LNPs, which were prepared in six 2-fold serial dilutions starting at 250 ng. These LNPs were formulated to encapsulate CasX 491 mRNA and a *B2M*-targeting gRNA incorporating either scaffold variant 174 or 316 with spacer 7.9 (v1; see Table 26). Media was changed 24 hours after LNP treatment, and cells were cultured for six additional days prior to harvesting for gDNA extraction for editing assessment at the *B2M* locus by NGS and B2M protein expression analysis via HLA immunostaining, followed by flow cytometry using the Attune NxT flow cytometer. Briefly, for editing assessment, amplicons were amplified from 200 ng of extracted gDNA with primers targeting the human *B2M* locus and processed by NGS using

similar methods as described in Example 7.The results of these assays are shown in FIGS. 21A and 21B.

[0451] ~20,000 mouse Hepa1-6 hepatocytes were seeded per well in a 96-well plate. The following day, seeded cells were treated with varying concentrations of LNPs, which were prepared in eight 2-fold serial dilutions starting at 1000 ng. These LNPs were formulated to encapsulate CasX 676 mRNA #2 (see Table 28) and a *ROSA26*-targeting gRNA incorporating scaffold variant 316 with spacer 35.2 (v1 or 5; see Table 26). Media was changed 24 hours post-treatment with LNPs, and cells were cultured for seven additional days prior to harvesting for gDNA extraction for editing assessment at the *ROSA26* locus by NGS. Briefly, amplicons were amplified from extracted gDNA with primers targeting the mouse *ROSA26* locus and processed by NGS using similar methods as described in Example 7. The results of this experiment are shown in FIG. 22A.

Delivery of LNPs encapsulating CasX mRNA and targeting gRNA in vivo:

[0452] To assess the effects of using v1 and v5 of scaffold 316 in vivo, CasX 676 mRNA #2 (see Table 28) and a *ROSA26*-targeting gRNA using scaffold 316 with spacer 35.2 (v1 or v5; see Table 26) were encapsulated within the same LNP using a 1:1 mass ratio for mRNA:gRNA. Formulated LNPs were buffer-exchanged to PBS for in vivo injection. Briefly, LNPs were administered intravenously through the retro-orbit sinus into 4-week old C57BL/6 mice. Mice were observed for five minutes after injection to ensure recovery from anesthesia before being placed into their home cage. Naïve, uninjected animals served as experimental controls. Six days post-administration, mice were euthanized, and the liver tissue was harvested for gDNA extraction using the Zymo Research Quick DNA/RNA Miniprep kit following the manufacturer's instructions. Target amplicons were then amplified from the extracted gDNA with a set of primers targeting the mouse *ROSA26* locus and processed using similar methods as described in Example 7 for editing assessment by NGS. The results of this experiment are shown in FIG. 22B.

[0453] To compare the effects of using v7, v8, and v9 of scaffold 316 on editing at the *PCSK9* locus *in vivo*, CasX 676 mRNA #1 (see Table 29 for sequences) and a *PCSK9*-targeting gRNA using scaffold 316 with spacer 27.107 (v1, v7, v8, or v9; see Table 26), were encapsulated within the same LNP using a 1:1 mass ratio for mRNA:gRNA for each gRNA. LNPs were administered retro-orbitally into 6-week old C57BL/6 mice, as described above, and mice were euthanized seven days post-injection to harvest liver tissue for gDNA extraction for editing

assessment by NGS at the *PCSK9* locus. The results of this experiment are shown in FIG. 23.

Table 29: Encoding sequences of CasX 676 mRNA #1 molecule

CasX ID	Component (ID)	Description	SEQ ID NO:
CasX 676 mRNA #1	5'UTR	hHBA	3188
	START codon + c-MYC NLS		3133
	CasX 676		3134
	c-MYC NLS + STOP codons		3135
	3'UTR	hHBA	3189
	Poly(A) tail		3057

*Components are listed in a 5' to 3' order within the constructs

Results:

Assessing the effects of various chemical modifications on gRNA activity:

[0454] Several studies involving Cas9 have demonstrated that chemical modifications of the gRNA resulted in significantly improved editing activity when delivered with Cas9 mRNA. Following delivery of Cas9 mRNA and gRNA into target cells, unprotected gRNA is susceptible to degradation during the mRNA translation process. Addition of chemical modifications such as 2'O-methyl (2'Ome) groups and phosphorothioate bonds can reduce the susceptibility of the gRNA to cellular Rnases, but also have the potential to disrupt folding of the gRNA and its interactions with the CRISPR-Cas protein. Given the lack of structural similarity between CasX and Cas9, as well as their respective gRNAs, appropriate chemical modification profiles must be designed and validated *de novo*. Using published structures of wild-type CasX from *Deltaproteobacteria* (PDB codes 6NY1, 6NY2, and 6NY3) as reference, residues that appeared potentially amenable to modification were selected. However, the published structures were of a wild type CasX ortholog and gRNA distinct from the species used as the basis for the engineered variants presented here, and they also lacked the resolution to confidently determine interactions between protein side-chains and the RNA backbone. These limitations introduced a significant amount of ambiguity into determining which nucleotides might be safely modified. As a result, six profiles of chemical modifications (denoted as versions) were designed for initial testing, and these six profiles are illustrated in FIGS. 16A and 16B. The v1 profile was designed as a simple end-protected structure, where the first and last three nucleotides were modified with 2'Ome and phosphorothioate bonds. In the v2 profile, 3'UUU tail was added to mimic the termination

sequence used in cellular transcription systems and to move the modified nucleotides outside of the region of the spacer involved in target recognition. The v3 profile included the end protection as in v1, as well as the addition of 2'Ome modifications at all nucleotides identified to be potentially modifiable based on structural analysis. The v4 profile was modeled based on v3, but with all the modifications in the triplex region removed, as this structure was predicted to be more sensitive to any perturbation of the RNA helical structure and backbone flexibility. The v5 profile maintained chemical modifications in the scaffold stem and extended stem regions, while the v6 profile harbored modifications only in the extended stem. The extended stem is a region that would become fully exposed to solvent in the RNP and is amenable to replacement by other hairpin structures, and therefore presumably relatively insensitive to chemical modifications.

[0455] The minimally modified v1 gRNA was initially assessed against an unmodified gRNA (v0) to determine the potential benefit of such chemical modifications on editing when the gRNA was co-delivered with CasX mRNA to target cells. Modified (v1) and unmodified (v0) *B2M*-targeting gRNAs with spacer 7.37 were co-transfected with CasX mRNA into HepG2 cells, and editing at the *B2M* locus was measured by loss of surface presentation of the B2M-dependent HLA complex, as detected by flow cytometry (FIG. 17). The data demonstrate that use of the v1 gRNA resulted in substantially greater loss of B2M expression compared to the levels seen with v0 gRNA across the various doses, thereby confirming that end modifications of the gRNA increased CasX-mediated editing activity upon delivery of the CasX mRNA and gRNA.

[0456] The broader set of gRNA chemical modification profiles were assessed using *PCSK9*-targeting gRNAs using scaffold variant 235 and spacers 6.7 and 6.8 to determine whether the additional chemical modifications would be able to support the formation of active RNPs. In vitro cleavage assays described above were performed to determine k_{cleave} and fraction competence for these engineered gRNAs harboring the various chemical modification profiles. The results from these in vitro cleavage assays are shown in Table 30. The data demonstrate that gRNAs with the v3 profiles exhibited no activity, an indication that the addition of some chemical modifications significantly interfered with RNP formation or activity. Adding v4 chemical modifications resulted in a reasonable cleavage rate in the excess RNP condition, but exhibited very low fraction competence. The difference between v3 and v4 modifications confirmed that modifications in the triplex region prevented the formation of any active RNP, either due to the inability of the gRNA to fold properly or a disruption in the gRNA-protein

interactions. The reduced fraction competence resulting from appending v4 modifications suggest that while the gRNA was able to successfully assemble with the CasX protein to form a cleavage-competent RNP, a large majority of the gRNA was misfolded, or that the appended chemical modifications reduced the affinity of the gRNA for the CasX protein and impeded the efficiency of RNP formation. Application of the v5 or v6 profiles resulted in competent fractions that were comparable to, but slightly lower than, those obtained for reactions using the v1 and v2 modifications. While the k_{cleave} values were relatively consistent between v5 and v6 gRNAs, both v5 and v6 gRNAs achieved nearly half of the k_{cleave} values for v1 and v2 gRNAs. The reduced k_{cleave} value for v6 gRNA was particularly surprising, given the lack of expected interaction between the gRNA and CasX protein in the modified extended stem. However, for both v5 and v6 gRNAs, it is possible that the reduced flexibility of the gRNA, resulting from the 2'Ome modifications, inhibited structural changes in the RNP required for efficient cleavage, or that the modified initial base-pairs of the hairpin involved in CasX protein interaction had been negatively impacted by the inclusion of the 2'Ome groups.

Table 30: Parameters of cleavage activity assessed for CasX RNPs with the various *PCSK9*-targeting gRNAs using scaffold 235 and harboring the indicated chemical modification profile, denoted by version number.

gRNA (scaffold variant-spacer, version no.)	k_{cleave} (min^{-1})	Fraction competence
235-6.7, v1	0.901	0.398
235-6.8, v1	1.36	0.398
235-6.7, v2	0.454	0.386
235-6.8, v2	2.03	0.361
235-6.7, v3	0	0
235-6.8, v3	0	0
235-6.7, v4	0.434	0.031
235-6.8, v4	0.257	0.005
235-6.7, v5	0.506	0.313
235-6.8, v5	0.680	0.388

gRNA (scaffold variant-spacer, version no.)	k_{cleave} (min^{-1})	Fraction competence
235-6.7, v6	0.462	0.346
235-6.8, v6	0.715	0.325

[0457] The chemically-modified *PCSK9*-targeting gRNAs based on scaffold 235 were subsequently assessed for editing in a cell-based assay. CasX mRNA and chemically modified *PCSK9*-targeting gRNAs were co-transfected into HepG2 cells using lipofectamine. Editing levels were measured by indel rate at the *PCSK9* locus by NGS and secreted PCSK9 levels by ELISA, and the data are displayed in Table 31. The data demonstrate that use of v3 and v4 gRNAs resulted in minimal editing activity at the *PCSK9* locus, consistent with findings from the biochemical in vitro cleavage assays shown in Table 30. Meanwhile, use of v5 and v6 gRNAs resulted in editing levels, measured by indel rate and PCSK9 secretion, that were slightly lower than the levels attained with use of v1 and v2 gRNAs (Table 31). Specifically, the results show that use of v1 and v2 gRNAs, which harbored end modifications, resulted in ~80-85% editing at the *PCSK9* locus, indicating that adding chemical modifications to the gRNA ends was sufficient to achieve efficient editing with CasX. While the data demonstrate that use of v5 and v6 gRNAs resulted in efficient editing in vitro, near-saturating levels of editing were observed with use of the v1 gRNA in this experiment where a single dose of the gRNA was transfected. As a result, the use of a single dose rendered it challenging to assess clearly the effects of the chemical modifications on editing under guide-limiting conditions. Therefore, profiles v1 and v5 were chosen for further testing, as v1 contains the simplest modification profile, and v5 is the most heavily modified profile whose application demonstrated robust activity in vitro (Tables 30 and 31).

Table 31: Editing levels measured by indel rate at *PCSK9* locus by NGS and secreted PCSK9 levels by ELISA in HepG2 cells co-transfected with CasX 491 mRNA and various

chemically-modified *PCSK9*-targeting gRNAs using scaffold 235 and either spacer 6.7 or 6.8.

Experimental condition	Indel rate (edit fraction)		Secreted PCSK9 (ng/mL)	
	Mean	Stdev	Mean	Stdev
CasX mRNA only	0.0021	0.003	52	14
235-6.7, v1	0.83	0.0058	18	5.7
235-6.7, v2	0.79	0.0071	21	4
235-6.7, v3	0.024	0.02	48	19
235-6.7, v4	0.12	0.006	34	5.5
235-6.7, v5	0.73	0.023	21	9
235-6.7, v6	0.75	0.0069	22	8.8
235-6.8, v1	0.85	0.017	16	4.4
235-6.8, v2	0.83	0.0028	20	1.5
235-6.8, v3	0.023	0.0027	39	2.7
235-6.8, v4	0.088	0.0086	42	10
235-6.8, v5	0.77	0.017	19	1.6
235-6.8, v6	0.78	0.014	24	6.9
Non-targeting ctrl	0.0019	0.0026	42	12

[0458] The v1 and v5 profiles were further tested in another cell-based assay to assess their effects on editing efficiency. LNPs were formulated to co-encapsulate CasX 676 mRNA #2 and v1 and v5 chemically-modified *ROSA26*-targeting gRNAs using the newly-designed gRNA scaffold 316 (described further in the following sub-section). The "v5" profile was modified slightly for application to the 316 scaffold. Three 2' Ome modifications in the non-base-paired region immediately 5' of the extended stem were removed to restrict modifications to the two stemloop regions. Hepa1-6 hepatocytes were treated with the resulting LNPs at various doses and harvested eight days post-treatment to assess editing at the *ROSA26* locus, measured as indel rate detected by NGS (FIG. 22A). The data demonstrate that treatment with LNPs delivering the

v5 *ROSA26*-targeting gRNA resulted in markedly lower editing levels across the range of doses compared to the levels achieved with the v1 counterpart (FIG. 22A). There are several possible explanations for the differences in relative activity observed with use of v5 gRNA in FIG. 22A relative to that observed in Table 31. The first and most likely possible explanation is that the single dose used to achieve editing shown in Table 31 was too high to measure differences in activity accurately between use of v5 gRNA and v1 gRNA. It is also possible that the removal of the modifications outside the stemloop motifs in the 316 version of v5 negatively impacted guide activity. While it is possible that these modifications provide stability benefits that outweigh an activity cost imparted by the stemloop modifications, this seems unlikely given that increasing levels of modification have so far resulted in decreased activity. A final possible explanation is that the modifications in the v5 profile might negatively impact LNP formulation or behavior through differential interactions between the modified nucleotide backbone and the ionizable lipid of the LNP, potentially resulting in less efficient gRNA encapsulation or in less efficient gRNA release following internalization.

[0459] LNPs co-encapsulating the CasX mRNA #2 and v1 and v5 chemically-modified *ROSA26*-targeting gRNAs based on scaffold 316 were further tested *in vivo*. FIG. 22B shows the results of the editing assay as percent editing measured as indel rate at the *ROSA26* locus. The data demonstrate that use of the v5 gRNA resulted in ~5-fold lower editing compared to that achieved with use of the v1 gRNA, under more relevant testing conditions of *in vivo* LNP delivery. These findings support the reduced cleavage rate observed biochemically for the v5 gRNA in Table 30, an indication that the v5 modifications have interfered with some aspect of CasX activity. Given the consistent decrease in activity detected in v5 and v6 profiles (Table 30), the reduced editing may be attributed to modifications in the extended stem region. Although the extended stem of the gRNA has minimal interactions with the CasX protein, it is possible that addition of 2'Ome groups at the first base-pair disrupted either the CasX protein-gRNA interactions or the complex RNA fold where the extended stem meets the pseudoknot and triplex regions. More specifically, inclusion of the 2'Ome groups might have adversely affected the basal base-pairs of the gRNA extended stem and residues R49, K50, and K51 of the CasX protein. Finally, structural studies of CasX have suggested that flexibility of the gRNA is required for efficient DNA cleavage (Liu J, *et al*, CasX enzymes comprise a distinct family of RNA-guided genome editors. *Nature* 566:218-223 (2019); Tsuchida CA, *et al*, Chimeric CRISPR-CasX enzymes and guide RNAs for improved genome editing activity. *Mol Cell* 82(6):

1199-1209 (2022)). Thus, the addition of the 2'Ome groups throughout the extended stem might have enforced a more rigid A-form helical structure and prevented the needed flexibility for the gRNA for efficient cleavage. Furthermore, it is possible that the additional modifications in the scaffold stem in the v5 and v6 profiles might be detrimental to activity, though this is currently unclear given the limited comparisons between the v5 and v6 profiles.

[0460] Additional modification profiles were designed with the goal of enhancing gRNA stability while mitigating the adverse effects on RNP cleavage activity. Using recently published structures of wild-type CasX from *Planctomycetes* (PDB codes 7WAY, 7WAZ, 7WB0, 7WB1), which has a higher homology to the engineered CasX variants being assessed, additional chemical modification profiles for gRNAs were designed and are illustrated in FIG. 18. These profiles illustrate the addition of 2'Ome groups and phosphorothioate bonds to a newly-designed gRNA scaffold variant, which is described in the ensuing sub-section. These new gRNA chemical modification profiles were designed based on the initial data demonstrating sufficient editing activity observed in Table 31 with use of the v5 gRNA that suggested that modifications to the extended stem and scaffold stem regions would not negatively impact activity. The v7 profile was designed to include 2'Ome at residues likely to be modifiable throughout the gRNA structure, which excluded the triplex region, given the dramatic negative effects of adding such modifications observed earlier with the v3 profile. More conservative profiles, v8 and v9, were also designed, as illustrated in FIG. 18. For the v8 construct, modifications were removed in the pseudoknot and triplex loop region, but were retained in the scaffold stem, extended stem, and their flanking single-stranded regions, in addition to the 5' and 3' termini. For the v9 profile, modifications were removed in the single-stranded regions flanking the stemloops, but were retained in the stemloops themselves, in addition to the pseudoknot, triplex loop, and 5' and 3' termini. The additional chemical modification profiles v7, v8, and v9 of the newly designed gRNA scaffold variant 316 (discussed further below) were assessed in vivo at the *PCSK9* locus. The results of the editing assay in vivo quantified as percent editing at the *PCSK9* locus measured as indel rate as detected NGS are illustrated in FIG. 23. Despite the fact that low editing efficiency was detected overall, the data demonstrate that use of v7, v8, and v9 gRNAs resulted in lower editing levels at the *PCSK9* locus compared to the indel rate achieved with use of the v1 gRNA (FIG. 23). Given the findings in FIGS. 22A-22B showing inferior editing activity attained with the v5 gRNA, it is unsurprising that v7, v8, and v9 profiles similarly demonstrated comparatively lower editing activity. As illustrated in FIG. 18, the v7, v8, and v9

profiles include modifications throughout the extended stem region, which might have interfered with RNP activity.

Comparison of gRNA scaffold variant 174 and 316 using an in vitro cleavage assay:

[0461] Previous work had established gRNA scaffold variant 235 as a top-performing scaffold variant across multiple delivery conditions. However, the longer length of scaffold 235 (119 bp, when using a 20 bp spacer) relative to gRNAs including scaffold 174 (109 bp, when using a 20 bp spacer) increased the difficulty of solid-phase RNA synthesis, which would result in increased manufacturing costs, decreased purity and yield, and higher rates of synthesis failures. To address these issues but retain the improved activity of using scaffold variant 235, a chimeric gRNA scaffold was designed primarily on the basis of the scaffold 235 sequence, but the extended stemloop of scaffold 235 was replaced with the shorter extended stemloop of scaffold variant 174 (FIGS. 19A-19C). The resulting chimeric scaffold, named scaffold 316, was synthesized in parallel with scaffold 174 and *PCSK9*-targeting spacers 6.7 and 6.8, and *B2M*-targeting spacer 7.9 harboring the v1 chemical modification profile, with 2'OMe and phosphorothioate bonds on the first and last three nucleotides of all gRNAs (see Table 26). Scaffold variant 174 was chosen as the comparator rather than variant 235 because variant 174 was the best previously characterized scaffold with the same length as variant 316.

[0462] In vitro cleavage activity was assessed for gRNAs with scaffold 174 and 316 and spacers 6.7 and 6.8. Cleavage assays were carried out with 20-fold excess RNP over a matching dsDNA target. Cleavage rates were quantified for all four guides, and the results are shown in Table 32. The data demonstrate that in the context of spacer 6.7, use of either scaffold 174 or 316 resulted in similar cleavage rates, with scaffold 316 resulting in marginally faster cleavage than that achieved with scaffold 174. In the context of spacer 6.8, the difference in cleavage activity was more pronounced: CasX RNPs using scaffold 316 were able to cleave DNA nearly twice as quickly as CasX RNPs using scaffold 174 (Table 32).

[0463] Assays were also performed with equimolar amounts of RNP and DNA target over a longer time course to assess the fraction of expected RNP active for cleavage. As the CasX RNP is essentially single-turnover over the tested timescale, and the concentrations used are expected to be substantially higher than the K_D of the DNA-binding reaction, the amount of cleaved DNA should approximate the amount of active RNP. For either spacer 6.7 or 6.8, the active fraction of CasX RNPs incorporating scaffold 316 was 25-30% higher than for CasX RNPs using scaffold 174 (Table 32). These data suggest that a higher fraction of gRNA using scaffold 316 was

properly folded for association with the CasX protein, or that the gRNA using scaffold 316 was able to associate more strongly with the CasX protein. Compared to scaffold 174, scaffold 316 harbors mutations expected to stabilize the pseudoknot and triplex structures required for proper gRNA folding. The increased stability of these motifs in particular, which were more likely to misfold than the simple hairpins found elsewhere in the gRNA structure, might result in a slightly higher fraction of the gRNAs folding into an active conformation.

Table 32: Parameters of cleavage activity assessed for CasX RNPs with gRNAs containing scaffold variant 174 or 316 with the version 1 (v1) chemical modification profile.

gRNA (scaffold variant-spacer)	k_{cleave} (min^{-1})	Fraction competence
174-6.7, v1	0.236	0.194
174-6.8, v1	0.142	0.165
316-6.7, v1	0.264	0.244
316-6.8, v1	0.272	0.213

Comparison of gRNA scaffold variant 174 and 316 in a cell-based assay:

[0464] An editing assessment using gRNA scaffold variant 174 compared to variant 316 was performed in a cell-based assay. CasX 491 mRNA and the version 1 (v1) of PCSK9-targeting gRNAs using spacers 6.7 and 6.8 were lipofected into HepG2 cells. Treated cells were harvested 28 hours post-transfection for analysis of editing levels at the PCSK9 locus by NGS and secreted PCSK9 levels by ELISA, and the data are presented in FIG. 20. The data demonstrate that use of any of the PCSK9-targeting gRNA tested resulted in efficient editing at the PCSK9 locus and substantial reduction in PCSK9 secretion compared to the non-targeting control using the B2M-targeting gRNA. The results also show that use of scaffold 316 resulted in more effective editing at the PCSK9 locus than that observed with use of scaffold 174 (~10 percentage point increase in editing rate achieved with scaffold 316 over scaffold 174). This finding is further supported by the ELISA results, such that use of scaffold 316 resulted in more effective reduction of PCSK9 secretion compared to that achieved with use of scaffold 174.

[0465] Scaffold variants 174 and 316 were also assessed in an editing assay where LNPs were formulated to co-encapsulate CasX 491 mRNA and B2M-targeting gRNA harboring either scaffold variant. HepG2 cells were treated with the resulting LNPs at various doses and

harvested seven days post-treatment to assess editing at the B2M locus, measured as indel rate detected by NGS (FIG. 21A) and loss of surface presentation of the B2M-dependent HLA complex, as detected by flow cytometry (FIG. 21B). The results from both assays demonstrate that treatment with LNPs to deliver the B2M-targeting gRNA using scaffold 316 resulted in higher editing potency at the B2M locus compared to LNPs delivering the gRNA using scaffold 174 at each dose (FIGS. 21A and 21B). Specifically, at the highest dose of 250 ng, use of scaffold 316 resulted in an editing level that was nearly two-fold higher than the level attained with using scaffold 174. This substantial increase in editing efficacy when using scaffold 316 versus scaffold 174, compared to the comparatively modest difference in activity observed from the in vitro cleavage assays, might be attributed to the destabilization of gRNA structure and folding during LNP formulation. The low pH conditions and association of cationic lipids during LNP formulation could adversely affect parts of the gRNA structure and result in unfolding. Consequently, it would be necessary for the gRNA to refold quickly in the cytoplasm upon delivery, both to bind the CasX protein to form the RNP and to evade Rnase degradation. The stability-increasing mutations in scaffold 316 compared to scaffold 174 might provide a substantial benefit in supporting proper gRNA refolding in the cytoplasm after LNP delivery, while the deliberate folding protocol carried out for the gRNA prior to biochemical experiments likely reduced the impact of these mutations.

Example 8: Demonstration that altering the UTR sequences of the engineered CasX mRNA can affect CasX-mediated editing

[0466] 5’ and 3’UTRs are essential and required for efficient translation of mRNA. Here, experiments were performed to demonstrate that altering the 5’ and 3’ UTR sequences of the engineered CasX mRNA affects CasX-mediated editing at a target locus when CasX mRNA and targeting gRNAs were delivered in vitro via transfection.

Materials and Methods:

IVT of CasX mRNA:

[0467] CasX 676 mRNA was generated by IVT. Briefly, constructs encoding for a 5’UTR region, a codon-optimized CasX 676 with flanking c-MYC NLSes, and a 3’UTR region were cloned into a plasmid containing a T7 promoter and 80-nucleotide poly(A) tail. The resulting plasmid was linearized prior to use for IVT reactions, which were carried out with CleanCap® AG and N1-methyl-pseudouridine. For the 5’ cap, the CleanCap® AG contains a

m7G(5')ppp(5')mAG structure, where "m7G" denotes N^7-methylguanosine, "mA" denotes 2'O-methyladenosine, and (5')ppp(5') denotes a 5' to 5' triphosphate bridge. An extra guanine nucleotide was incorporated following the CleanCap® AG to enhance transcription initiation, resulting in the incorporation of m7G(5')ppp(5')mAGG as the full 5' cap structure. As discussed below in Example 9, the substitution of the uridine ribonucleoside to N1-methyl-pseudouridine improves mRNA performance and reduces mRNA immunogenicity.

[0468] IVT reactions were subsequently subjected to Dnase digestion to remove template DNA and purification using an oligo-dT column. In this example, two configurations of CasX 676 mRNAs were generated for assessment in vitro. The encoding sequences of the two CasX mRNA configurations are detailed in Table 33. Full-length RNA sequences encoding the CasX mRNA with the chemical modifications are listed in Table 34.

Table 33: Encoding sequences of the two CasX mRNA molecules assessed in this example*.

CasX mRNA ID	Component (ID)	Description	DNA sequence or SEQ ID NO:
CasX 676 mRNA #1	5'UTR	Human *HBA*	3188
	START codon + c-MYC NLS		3133
	CasX 676		3134
	c-MYC NLS + STOP codons		3135
	3'UTR	Human *HBA*	3189
	Poly(A) tail		3057
CasX 676 mRNA #2	5'UTR	Synthetic (TriLink)	3047
	START codon + c-MYC NLS		3133
	CasX 676		3134
	c-MYC NLS + STOP codons		3135
	3'UTR	Mouse *HBA*	3055
	XbaI restriction site (partial)		TCTAG
	Poly(A) tail		3057

*Components are listed in a 5' to 3' order within the constructs

Table 34: Full-length RNA sequences of CasX mRNA molecules assessed in this example. The 5' cap (m7G(5')ppp(5')mAG), discussed in the example herein, is not shown in the table. Modification 'mψ' = N1-methyl-pseudouridine

CasX mRNA	SEQ ID NO	RNA Sequence
CasX 676 mRNA #1	3190	ACmψCmψmψCmψGGmψCCCCACAGACmψCAGAGAGAACCCGCCACCAmψGGCCCCmψGCmψGCCAAGAGAGmψGAAGCmψGGAmψAGCAGACAGGAGAmψCAAGCGGAmψmψAAmψAAAAmψmψCGGAGAAGACmψGGmψGAAGGAmψmψCmψAACACAAAGAAGGCmψGGCAAGACACGGGGCCCmψAmψGAAGACACmψGCmψGGmψGAGAGmψGAmψGACACCCGACCmψGAGAGAAAGACmψGGAAAACCmψGAGAAAGAAGCCmψGAGAAmψAmψCCCCCAGCCCAmψCAGCAACACAAGCCGGGCCAACCmψGAAmψAAGCmψGCmψGACCGACmψACACCGAAAmψGAAGAAGGCCAmψCCmψGCACGmψGmψAmψmψGGGAAGAGmψmψCCAGAAAGACCCAGmψCGGCCmψGAmψGAGCAGAGmψGGCmψCAGCCmψGCCAGCAAGAAGAmψCGAmψCAGAACAAGCmψGAAGCCCGAAAmψGGACGAGAAGGGGAACCmψGACAACCGCCGGCmψmψmψGCCmψGmψAGCCAGmψGCGGCCAGCCCCmψGmψmψmψGmψGmψACAAACmψGGAACAGGmψGAGCGAAAAGGGCAAGGCmψmψACACGAAmψmψACmψmψCGGCAGAmψGCAACGmψGGCCGAGCACGAGAAGCmψGAmψCAAGCmψGGCCCAGCmψGAAGCCmψGAGAAGGAmψAGCGAmψGAGGCAGmψGACAmψAmψmψCCCmψGGGCAAGmψmψCGGACAGCGGGCCCmψGGAmψmψmψmψAmψmψCCAmψmψCAmψGmψGACCAAGGAAmψCCACCCACCCCGmψCAAGCCmψCmψmψGCCCAAAmψmψGCCGGCAACAGAmψACGCCmψCCAGCCCCGmψGGGCAAGGCCCmψGAGCGACGCCmψGmψAmψGGGCACCAmψCGCCAGCmψmψCCmψGmψCmψAAGmψACCAGGACAmψmψAmψCAmψCGAGCACCAGAAGGmψGGmψGAAGGGCAACCAGAAGAGACmψGGAGAGCCmψGCGCGAGCmψGGCCGGCAAGGAAAACCmψGGAGmψAmψCCmψAGCGmψGACCCmψGCCmψCCmψCAGCCmψCAmψACAAAGGAGGGCGmψGGAmψGCCmψACAACGAAGmψGAmψCGCCCGGGmψGCGGAmψGmψGGGmψGAACCmψGAAmψCmψGmψGGCAGAAGCmψGAAGCmψGmψCmψAGAGACGACGCCAAGCCCCmψGCmψGAGACmψGAAGGGCmψmψCCCCAGCmψmψCCCmψCmψGGmψGGAGAGACAGGCAAAmψGAAGmψGGACmψGGmψGGGACAmψGGmψGmψGmψAACGmψGAAGAAGCmψGAmψCAAmψGAGAAGAAGGAGGACGGCAAAGmψGmψmψCmψGGCAGAAmψCmψGGCCGGCmψACAAGCGmψCAGGAGGCCCmψGCGGCCCmψACCmψGAGCAGCGAGGAAGACAGAAAGAAGGGCAAGAAGmψmψCGCCCGGmψAmψCAGCmψGGGGGACCmψGCmψGCmψGCACCmψCGAGAAGAAGCACGGCGAAGACmψGGGGGAAGGmψGmψACGAmψGAGGCCmψGGGAGCGGAmψCGAmψAAGAAGGmψGGAGGGCCmψGAGCAAGCACAmψCAAGCmψGGAGGAGGAACGGAGAmψCmψGAGGACGCCCAGAGCAAGGCCGCCCmψGACCGACmψGGCmψGAGAGCCAAGGCCAGCmψmψCGmψCAmψCGAGGGGCmψGAAGGAGGCCGACAAGGACGAGmψmψCmψGCCGGmψGCGAACmψGAAGCmψGCAGAAGmψGGmψACGGAGAmψCmψGAGAGGCAAACCmψmψmψCGCCAmψCGAGGCCGAGAACAGCAmψCCmψGGACAmψCAGCGGCmψmψCAGCAAGCAGmψACAACmψGCGCCmψmψmψAmψmψmψGGCAGAAGGACGGAGmψGAAGAAGCmψGAACCmψGmψACCmψGAmψCAmψCAACmψAmψmψmψCAAGGGCGGCAAGCmψGAGAmψmψCAAGAAGAmψCAAGCCmψGAAGCCmψmψCGAGGCCAACAGAmψmψCmψACACCGmψGAmψmψAACAAGAAAAGCGGAGAGAmψCGmψGCCAAmψGGAAGmψGAACmψmψCAACmψmψCGACGACCCmψAACCmψGAmψCAmψCCmψGCCCCmψGGCAmψmψmψGGCAAGCGGCAGGGCAGAGAGmψmψCAmψCmψGGAACGACCmψGCmψGmψCmψCmψGGAGACCGGCAGCCmψGAAGCmψGGCCAACGGCAGAGmψGAmψCGAGAAGACACmψGmψACAACAGACGAACCAGACAAGACGAGCCCGCCCmψGmψmψmψGmψGGCCCmψGACCmψmψCGAGAGAAGAGAGGmψGCmψGGACAGCAGCAAmψAmψCAAGCCmψAmψGAACCmψGAmψCGGCGmψGGACCGGGGCGAGAACAmψCCCmψGCCGmψGAmψCGCCCmψmψACCGACCCCGAGGGAmψGCCCmψCmψGAGCCGGmψmψmψAAAGACAGCCmψGGGCAACCCmψACCCACAmψCCmψGAGAAmψmψGGCGAGmψCCmψACAAGGAGAAGCAGAGAACCAmψCCAGGCCAAGAAGGAGGmψGGAGCAGCGGCGGGCmψGGCGGCmψACmψCCCGGAAGmψACGCCAGCAAGGCCAAGAACCmψGGCCGACGACAmψGGmψmψAGAAAmψACCGCCAGAGACCmψCCmψGmψACmψACGCmψGmψGACCCAGGACGCCAmψGCmψGAmψCmψmψCGAGAACCmψGAGCAGAGGCmψmψCGGCAGACAGGGCAAGAGAACCmψmψCAmψGGCCGAGAGACAGmψACACCCGGAmψGGAGGACmψGGCmψGACCGCCAAGCmψGGCCmψACGAGGGCCmψGCCCmψCmψAAGACCmψACCmψGmψCCAAGACCmψmψGGCACAGmψACACCAGCAAGACAmψGCmψCmψAACmψGCGGCmψmψCACAAmψCACGAGCGCCGACmψACGACCGGGmψGCmψGGAGAAACmψGAAGAAGACCGCCACAGGCmψGGAmψGACCACCAmψmψAACGGCAAGGAGCmψGAAGGmψGGAGGGCCAGAmψCACCmψACmψACAA	

CasX mRNA	SEQ ID NO	RNA Sequence
		CAGGmψACAAACGGCAGAACGmψGGmψGAAGGACCmψGAGCGmψGGAACmψGGAmψAGAC mψGAGCGAGGAAAGCGmψAAACAAmψGACAmψCAGCAGCmψGGACCAAGGGCCGGAGCGG CGAGGCCCmψGAGCCmψGCmψGAAGAAGAGAmψmψCmψCCCACAGACCAGmψGCAGGAGA AGmψmψCGmψGmψGmψCmψGAACmψGCGGCmψmψCGAGACCCACGCCGACGAGCAAGCCG CCCmψGAACAmψCGCCCGGmψCmψmψGGCmψmψmψmψCCmψGCGGAGCCAGGAGmψACAA GAAGmψACCAGACAAACAAGACCACAGGCAACACAGACAAGAGAGCCmψmψCGmψCGAGA CCmψGGCAGAGCmψmψCmψACAGAAAGAAGCmψGAAGGAGGmψGmψGGAAGCCmψGCCGm ψGGGAAGCCCCGCmψGCCAAGAGAGmψGAAGCmψGGACmψAAmψAGAmψAAGCmψGGAGC CmψCGGmψGGCCAmψGCmψmψCmψmψGCCCCmψmψGGGCCmψCCCCCCAGCCCCmψCCmψ CCCCmψmψCCmψGCACCCGmψACCCCCGmψGGmψCmψmψmψGAAmψAAAGmψCmψGAGmψ GGGCGGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAA
CasX 676 mRNA #2	3136	*See Table 22 for sequence*

Synthesis of gRNAs:

[0469] In this example, gRNAs targeting the mouse *PCSK9* locus were designed using gRNA scaffold 174 with a v1 modification profile (see Example 7) and chemically synthesized. The sequences of the PCSK9-targeting spacers are listed in Table 35.

Table 35: Sequences of spacers targeting the mouse PCSK9 locus assayed in this example

Spacer ID	Target	RNA sequence	SEQ ID NO:
27.103	mouse *PCSK9*	UAAUCUCCAUCCUCGUCCUG	3073
27.105	mouse *PCSK9*	CCAAGAAGCCAGGGAAGAGG	3192
27.106	mouse *PCSK9*	ACAUAUCUUUUAUGACCUCU	3193
27.107	mouse *PCSK9*	CUGGCUUCUUGGUGAAGAUG	3194
27.108	mouse *PCSK9*	UGGUGAAGAUGAGCAGUGAC	3195
27.116	mouse *PCSK9*	GCCGUUGCUCCAAGGUAUGG	3196
27.117	mouse *PCSK9*	UUCUUGGGGAUCAGGAGGCC	3197

Transfection of CasX mRNA and gRNA into mouse Hepa1-6 cells in vitro:

[0470] Editing at the mouse *PCSK9* locus was assessed by delivering in vitro transcribed CasX mRNA (CasX mRNA #1 or CasX mRNA #2; see Table 33) and synthesized gRNAs targeting *PCSK9* into Hepa1-6 cells via transfection. Briefly, each well of 20,000 Hepa1-6 cells were lipofected with in vitro transcribed mRNA coding for CasX 676 and a *PCSK9*-targeting gRNA. After a media change, transfected cells were harvested at 20 hours post-transfection for editing assessment at the *PCSK9* locus by NGS as described previously in Example 4. As

experimental controls, individual transfections of CasX mRNA #1 and CasX mRNA #2 without gRNAs were performed.

Results:

[0471] CasX-mediated editing at the mouse *PCSK9* locus was used to evaluate the effects of incorporating different 5' and 3' UTRs into the engineered CasX mRNA. The plot in FIG. 26 shows the quantification of percent editing measured as indel rate at the *PCSK9* locus in mouse Hepa1-6 cells transfected with CasX 676 mRNA #1 or CasX 676 mRNA #2 with the indicated *PCSK9*-targeting gRNAs. The data demonstrate that for all targeting spacers tested in this experiment, CasX mRNA #2 consistently exhibited higher editing levels at the mouse *PCSK9* locus compared to editing levels achieved by CasX mRNA #1. Specifically, the highest level of editing rate achieved was with spacer 27.116, where use of CasX mRNA #2 resulted in ~35% editing efficiency compared to ~20% editing level by CasX mRNA #1 (FIG. 26).

[0472] The results demonstrate that altering the 5'UTR and 3'UTR sequences of the CasX mRNA can affect the editing activity of CasX at a target locus in a cell-based assay.

Example 9: Design and assessment of codon-optimized CasX mRNA on editing efficiency when delivered together with targeting gRNAs in vitro

[0473] mRNA sequence and associated modifications can have a significant impact on the efficacy of mRNA-based delivery. Modified nucleotides, including those that encode the 5' cap structure, are important determinants of mRNA stability, translatability, and immunogenicity. Here, for all designed and tested CasX mRNAs, a "Cap 1" structure was used, which included a 5' m7G in a 5'-5' triphosphate linkage to an initiating nucleotide with a 2'Ome modification. This structure, similar to the "Cap 0" structure lacking the 2'Ome modification, promotes efficient translation, and has reduced immunogenicity compared to the "Cap 0" structure. Furthermore, the use of modified nucleobases can reduce immunogenicity of the mRNA. Here, the N1-methyl-pseudouridine was used to substitute the uridine ribonucleoside for all in vitro transcription reactions, since published studies have demonstrated that the N1-methyl-pseudouridine substantially enhances mRNA performance and reduces mRNA immunogenicity. The modifications are expected to result in reduced immunogenicity and higher translation rates *in vivo*, potentially by avoiding activation of RIG-I, a primary cytosolic sensor for double-stranded RNA, which is a common contaminant of *in vitro* transcribed mRNA.

[0474] Optimization of the poly(A) tail will also be explored. The poly(A) tail is required for translation and mRNA stability, with longer tails being associated with a longer mRNA half-life. Polyadenylation can be carried out post-transcriptionally with a poly(A) polymerase, but this results in variable tail lengths and adds a step to the mRNA production process. mRNA productions were conducted using plasmids containing a template 80A-tail, terminating with a Type IIS restriction site to allow for run-off transcription, as constructs with plasmids containing a template 120A-tail were unstable during propagation in *E. coli*, often resulting in clones with significant reductions in tail length. Alternate plasmids were also cloned with a SphI restriction site between two-60A stretches, since published studies have demonstrated that similar constructs were more stable during subcloning and amplification in *E. coli* and produced mRNA with equivalent activity in mammalian cells. These alternate versions will be compared for activity using an *in vitro* editing assay across a range of CasX mRNA and gRNA doses to determine the consequential effects on editing activity. The sequences of the poly(A) tails described herein are listed in Table 36.

[0475] The sequences encoding the 5' and 3' UTRs, as well as the codons used for the CasX protein-coding sequences, are also critical for effective translation. UTRs were selected from annotated human gene transcripts based on genes (e.g., those encoding the α-globin, β-globin proteins) previously characterized to have high mRNA stability, as well as genes expected or previously demonstrated to be particularly well-expressed in the liver (i.e., genes encoding for the following proteins: albumin, complement 3, and cytochrome P450 2E1). The sequences of the 5' and 3' UTRs from these various genes are listed in Table 36. For the 3' UTR, concatenations of individual 3' UTRs were also tested. These constructs were cloned into plasmids containing a T7 promoter, CasX variant 515 or 676, and a poly(A) tail. To isolate the effects of individual 5' and 3' UTRs, each UTR was cloned into a construct that contained either the 3' or 5' α-globin UTR, respectively. IVTs will be performed and purified by binding to poly(dT) beads to capture full-length transcripts. The resulting mRNAs will initially be assessed by co-transfection with a *B2M*-targeting gRNA into HepG2 cells using a range of doses. Editing efficiency will be determined by HLA-immunostaining and flow cytometry as described in Example 7. The best-performing individual UTRs will be combined into various configurations, formulated into LNP, and tested in primary human hepatocytes and in mice.

[0476] Alternate codon optimizations are also being explored. In addition to the CasX codon optimization used for other delivery modalities, new versions were designed by building a codon

usage table based on ribosomal protein codon usage and rebalancing CasX codon usage to match. In addition to potential improvements to the translation rate, this also effectively results in depletion of uracil bases, which may reduce immunogenicity. This codon optimization was also used for production of mRNAs. Additional codon usages have been designed using a variety of available codon optimization tools, adjusting settings as needed to achieve a range of GC content levels. These codon optimizations will be tested under a similar experimental design used for testing UTRs as described above, and the leading codon-optimized CasX candidates will be combined with leading UTR candidates to generate new CasX leads for further validation.

Table 36: List of encoding DNA sequences for the indicated elements used for the generation and optimization of CasX mRNA.

Description	Encoding Sequence	SEQ ID NO:
Poly(A) tails		
A_{80}	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAA	3057
A_{120}	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAA	3198
A_{60}SphIA_{60}	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAGCATGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAA	3199
5' UTRs		
α-globin	ACTCTTCTGGTCCCCACAGACTCAGAGAGAACCC	3200
β-globin	ACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACA	3201
Albumin	CTAGCTTTTCTCTTCTGTCAACCCCACACGCCTTT	3202
Cytochrome P450 2E1 (CYP2E1)	CTCCCGGGCTGGCAGCAGGGCCCCAGC	3203
Complement 3 (C3)	ACTCCTCCCCATCCTCTCCCTCTGTCCCTCTGTCCCTCTGACCCTGCACTGTCCC	3204
3' UTRs		
α-globin	GCTGGAGCCTCGGTGGCCATGCTTCTTGCCCCTTGGGCCTCCCCCCAGCCCCTCCTC CCCTTCCTGCACCCGTACCCCCGTGGTCTTTGAATAAAGTCTGAGTGGGCGGCA	3189

Description	Encoding Sequence	SEQ ID NO:
Albumin	CATCACATTTAAAAGCATCTCAGCCTACCATGAGAATAAGAGAAAGAAAATGAAGAT CAAAAGCTTATTCATCTGTTTTTCTTTTTCGTTGGTGTAAAGCCAACACCCTGTCTA AAAAACATAAATTTCTTTAATCATTTTGCCTCTTTTCTCTGTGCTTCAATTAATAAA AAATGGAAAGAATCTAATAGAGTGGTACAGCACTGTTATTTTTCAAAGATGTGTTGC TATCCTGAAAATTCTGTAGGTTCTGTGGAAGTTCCAGTGTTCTCTCTTATTCCACTT CGGTAGAGGATTTCTAGTTTCTTGTGGGCTAATTAAATAAATCATTAATACTCTTCT AAGTTATGGATTATAAACATTCAAAATAATATTTTGACATTATGATAATTCTGAATA AAAGAACAAAAACCA	3205
Albumin (truncated)	GCATCACATTTAAAAGCATCTCAGCCTACCATGAGAATAAGAGAAAGAAAATGAAGA TCAATAGCTTATTCATCTCTTTTTCTTTTTCGTTGGTGTAAAGCCAACACCCTGTCT AAAAAACATAAATTTCTTTAATCATTTTGCCTCTTTTCTCTGTGCTTCAATTAATAA AAAATGGAAAGAACCTAGATCT	3206
β-globin	GCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAAC TACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTAATAAAA AACATTTATTTTCATTGCAA	3207
α-globin + β-globin	GCTGGAGCCTCGGTGGCCATGCTTCTTGCCCCTTGGGCCTCCCCCCAGCCCCTCCTC CCCTTCCTGCACCCGTACCCCCGTGGTCTTTGAATAAAGTCTGAGTGGGCGGCAGCT CGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAACTAC TAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTAATAAAAAAC ATTTATTTTCATTGCAA	3208
β-globin + α-globin	GCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAAC TACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCTGCCTAATAAAA AACATTTATTTTCATTGCAAGCTGGAGCCTCGGTGGCCATGCTTCTTGCCCCTTGGG CCTCCCCCCAGCCCCTCCTCCCCTTCCTGCACCCGTACCCCCGTGGTCTTTGAATAA AGTCTGAGTGGGCGGCA	3209

Example 10: Proof-of-concept experiment demonstrating delivery of LTRP mRNA and targeting gRNA via LNPs to achieve repression of target locus in vitro

[0477] Experiments were performed to assess whether delivery of lipid nanoparticles (LNPs) encapsulating LTRP mRNA and a *PCSK9*-targeting gRNA could induce durable repression of the target *PCSK9* locus in a cell-based assay. LNPs encapsulating an mRNA encoding for catalytically-active CasX 515 were formulated and included for comparison.

Materials and Methods:

Generation of mRNAs:

[0478] mRNA encoding the following molecules were generated by IVT following similar methods as described in Example 2: 1) a catalytically-active CasX 515 and 2) LTRP5-ADD-ZIM3 (as described in Example 4). Briefly, for the generation of CasX 515, constructs encoding for a synthetic 5'UTR, a codon-optimized CasX 515 with flanking c-MYC NLSes, and a 3'UTR derived from the mouse hemoglobin alpha (mHBA) were cloned into a plasmid containing a T7

promoter and 79-nucleotide poly(A) tail. The resulting plasmid was linearized prior to use for IVT reactions, which were performed as similarly described in Example 2. The DNA and mRNA sequences encoding the catalytically-active CasX 515 are shown in Table 37 and Table 38 respectively. The DNA and mRNA sequences encoding for LTRP5-ADD-ZIM3 are shown in Table 18 and Table 19 respectively.

Table 37: Encoding sequences of the catalytically-active CasX 515 mRNA molecule assessed in this example*.

CasX mRNA ID	Component (ID)	Description	DNA sequence or SEQ ID NO:
CasX 515 mRNA	5'UTR	Synthetic (TriLink)	3274
	START codon + c-MYC NLS		3275
	CasX 515		3276
	c-MYC NLS + STOP codon		3277
	3'UTR	Mouse *HBA*	3278
	XbaI restriction site (partial)		TCTAG
	Poly(A) tail		3279

*Components are listed in a 5' to 3' order within the constructs

Table 38: Full-length RNA sequences of catalytically-active CasX 515 mRNA molecule assessed in this example. Modification 'mψ' = N1-methyl-pseudouridine.

CasX mRNA	SEQ ID NO	RNA Sequence
CasX 515 mRNA	3280	AAAmψAAGAGAGAAAAGAAGAGmψAAGAAGAAAmψAmψAAGAGCCACCAmψGGCCCCmψGCmψGCCAA GAGAGmψGAAGCmψGGAmψAGCAGACAGGAGAmψCAAGCGGAmψmψAAmψAAAAmψmψCGGAGAAGAC mψGGmψGAAGGAmψmψCmψAACACAAAGAAGGCmψGGCAAGACAGGCCCmψAmψGAAGACACmψGCmψ GGmψGAGAGmψGAmψGACACCCGACCmψGAGAGAAAGACmψGGAAAACCmψGAGAAAGAAGCCmψGAG AAmψAmψCCCCCAGCCCAmψCAGCAACACAAGCCGGGCCAACCmψGAAmψAAGCmψGCmψGACCGACm ψACACCGAAAmψGAAGAAGGCCAmψCCmψGCACGmψGmψAmψmψGGGAAGAGmψmψCCAGAAAGACCC AGmψCGGCCmψGAmψGAGCAGAGmψGGCmψCAGCCmψGCCAGCAAGAAGAmψCGAmψCAGAACAAGCm ψGAAGCCCGAAAmψGGACGAGAAGGGGAACCmψGACAACCGCCGGCmψmψmψGCCmψGmψAGCCAGmψ GCGGCCAGCCCCmψGmψmψmψGmψGmψACAAACmψGGAACAGGmψGAGCGAAAAGGGCAAGGCmψmψA CACGAAmψmψACmψmψCGGCAGAmψGCAACGmψGGCCGAGCACGAGAAGCmψGAmψCCmψGCmψGGCC CAGCmψGAAGCCmψGAGAAGGAmψAGCGAmψGAGGCAGmψGACAmψAmψmψCCCmψGGGCAAGmψmψC GGACAGCGGGCCCmψGGAmψmψmψmψmψAmψmψCCAmψmψCAmψGmψGACCAAGGAAmψCCACCCACC CCGmψCAAGCCmψCmψmψGCCCAAAmψmψGCCGGCAACAGAmψACGCCAGCGGCCCCGmψGGGCAAGG CCCmψGAGCGACGCCmψGmψAmψGGGCACCAmψCGCCAGCmψmψCCmψGmψCmψAAGmψACCAGGACA mψmψAmψCAmψCGAGCACCAGAAGGmψGGmψGAAGGGCAACCAGAAGAGACmψGGAGAGCCmψGCGCG AGCmψGGCCGGCAAGGAAAACCmψGGAGmψAmψCCmψAGCGmψGACCCmψGCCmψCCmψCAGCCmψCA

		mψACAAAGGAGGGCGmψGGAmψGCCmψACAACGAAGmψGAmψCGCCCGGGmψGCGGAmψGmψGGGmψG AACCmψGAAmψCmψGmψGGCAGAAGCmψGAAGCmψGmψCmψAGAGACGACGCCAAGCCCCmψGCmψGA GACmψGAAGGGCmψmψCCCCAGCmψmψCCCmψCmψGGmψGGAGAGACAGGCAAAmψGAAGmψGGACmψ GGmψGGGACAmψGGmψGmψGmψAACGmψGAAGAAGCmψGAmψCAAmψGAGAAGAAGGAGGACGGCAAA GmψGmψmψCmψGGCAGAAmψCmψGGCCGGCmψACAAGCGmψCAGGAGGCCCmψGCGGCCCmψACCmψG AGCAGCGAGGAAGACAGAAAGAAGGGCAAGAAGmψmψCGCCCGGmψAmψCAGCmψGGGGGACCmψGCm ψGCmψGCACCmψCGAGAAGAAGCACGGCGAAGACmψGGGGGAAGGmψGmψACGAmψGAGGCCmψGGGA GCGGAmψCGAmψAAGAAGGmψGGAGGGCCmψGAGCAAGCACAmψCAAGCmψGGAGGAGGAACGGAGAm ψCmψGAGGACGCCCAGAGCAAGGCCGCCCmψGACCGACmψGGCmψGAGAGCCAAGGCCAGCmψmψCGm ψCAmψCGAGGGGCmψGAAGGAGGCCGACAAGGACGAGmψmψCmψGCCGGmψGCGAACmψGAAGCmψGC AGAAGmψGGmψACGGAGAmψCmψGAGAGGCAAACCmψmψmψCGCCAmψCGAGGCCGAGAACAGCAmψC CmψGGACAmψCAGCGGCmψmψCAGCAAGCAGmψACAACmψGCGCCmψmψmψAmψmψmψGGCAGAAGGA CGGAGmψGAAGAAGCmψGAACCmψGmψACCmψGAmψCAmψCAACmψAmψmψmψCAAGGGCGGCAAGCm ψGAGAmψmψCAAGAAGAmψCAAGCCmψGAAGCCmψmψCGAGGCCAACAGAmψmψCmψACACCGmψGAm ψmψAACAAGAAAAGCGGAGAGAmψCGmψGCCAAmψGGAAGmψGAACmψmψCAACmψmψCGACGACCCm ψAACCmψGAmψCAmψCCmψGCCCCmψGGCAmψmψmψGGCAAGCGGCAGGGCAGAGAGmψmψCAmψCmψ GGAACGACCmψGCmψGmψCmψCmψGGAGACCGGCAGCCmψGAAGCmψGGCCAACGGCAGAGmψGAmψC GAGAAGACACmψGmψACAACAGACGAACCAGACAAGACGAGCCCGCCCmψGmψmψmψGmψGGCCCmψG ACCmψmψCGAGAGAAGAGAGGmψGCmψGGACAGCAGCAAmψAmψCAAGCCmψAmψGAACCmψGAmψCG GCGmψGGACCGGGGCGAGAACAmψCCCmψGCCGmψGAmψCGCCCmψmψACCGACCCCGAGGGAmψGCC CmψCmψGAGCCGGmψmψmψAAAGACAGCCmψGGGCAACCCmψACCCACAmψCCmψGAGAAmψmψGGCG AGmψCCmψACAAGGAGAAGCAGAGAACCAmψCCAGGCCAAGAAGGAGGmψGGAGCAGCGGCGGGCmψG GCGGCmψACmψCCCGGAAGmψACGCCAGCAAGGCCAAGAACCmψGGCCGACGACAmψGGmψmψAGAAA mψACCGCCAGAGACCmψCCmψGmψACmψACGCmψGmψGACCCAGGACGCCAmψGCmψGAmψCmψmψCG AGAACCmψGAGCAGAGGCmψmψCGGCAGACAGGGCAAGAGAACCmψmψCAmψGGCCGAGAGACAGmψA CACCCGGAmψGGAGGACmψGGCmψGACCGCCAAGCmψGGCCmψACGAGGGCCmψGCCCmψCmψAAGAC CmψACCmψGmψCCAAGACCmψmψGGCACAGmψACACCAGCAAGACAmψGCmψCmψAACmψGCGGCmψm ψCACAAmψCACGAGCGCCGACmψACGACCGGGmψGCmψGGAGAAACmψGAAGAAGACCGCCACAGGCm ψGGAmψGACCACCAmψmψAACGGCAAGGAGCmψGAAGGmψGGAGGGCCAGAmψCACCmψACmψACAAC AGGmψACAAACGGCAGAACGmψGGmψGAAGGACCmψGAGCGmψGGAACmψGGAmψAGACmψGAGCGAG GAAAGCGmψAAACAAmψGACAmψCAGCAGCmψGGACCAAGGGCCGGAGCGGCGAGGCCCmψGAGCCmψ GCmψGAAGAAGAGAmψmψCmψCCCACAGACCAGmψGCAGGAGAAGmψmψCGmψGmψGmψCmψGAACmψ GCGGCmψmψCGAGACCCACGCCGACGAGCAAGCCGCCCmψGAACAmψCGCCCGGmψCmψmψGGCmψmψ mψmψCCmψGCGGAGCCAGGAGmψACAAGAAGmψACCAGACAAACAAGACCACAGGCAACACAGACAAG AGAGCCmψmψCGmψCGAGACCmψGGCAGAGCmψmψCmψACAGAAAGAAGCmψGAAGGAGGmψGmψGGA AGCCmψGCCGmψGGGAAGCCCCGCmψGCCAAGAGAGmψGAAGCmψGGACmψAAGCmψGCCmψmψCmψG CGGGGCmψmψGCCmψmψCmψGGCCAmψGCCCmψmψCmψmψCmψCmψCCCmψmψGCACCmψGmψACCmψ CmψmψGGmψCmψmψmψGAAmψAAAGCCmψGAGmψAGGAAGmψCmψAGAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Synthesis of gRNAs:

[0479] *PCSK9*-targeting gRNAs were designed using gRNA scaffold 316 and spacer 6.1 (sequence listed in Table 24) and chemically synthesized. The sequence of the PCSK9-targeting gRNA with the v1 modification profile (as described in Example 7 above) is listed in Table 39. A schematic of the sites of chemical modifications for a 'v1' profile of the gRNA scaffold variant 316 is shown in FIG. 24.

Table 39: Sequences of chemically modified the gRNA targeting the human *PCSK9* locus assayed in this example.

gRNA ID (scaffold variant-spacer)	Target	gRNA sequence	SEQ ID NO
316-6.1 (v1)	human *PCSK9*	mA*mC*mU*GGCGCUUCUAUCUGAUUACUCUGAGCGCCAUC ACCAGCGACUAUGUCGUAGUGGGUAAAGCUCCCUCUUCGGA GGGAGCAUCAGAGGAGGAGGACGGCCUGGC*mC*mG*mA	3447

[0480] LNP formulations were generated with the LNP lipids as listed in Table 40, using methods described in Example 11, below.

Delivery of LNPs encapsulating LTRP or CasX mRNA and targeting gRNA into primary cynomolgus macaque (CM) hepatocytes:

[0481] Two lots (termed BJE and VDU) of primary CM hepatocytes derived from two different donors (product number: M003055-P; BioIVT), were used to assess LTRP-mediated repression or CasX-mediated editing at the *PCSK9* locus when delivered by LNPs. For each lot, ~50,000 cells, cultured in Williams' E complete media, were seeded per well in a 96-well plate. The next day, seeded cells were treated with varying concentrations of LNPs, which were prepared in six 3-fold serial dilutions starting at 1,200 ng/100 µL. These LNPs were formulated to co-encapsulate CasX 515 or LTRP5-ADD-ZIM3 mRNA and a *PCSK9*-targeting gRNA incorporating scaffold variant 316 with spacer 6.1 (v1; see Table 39). The LNP formulations tested in this example are shown in Table 40. Media was changed one day after LNP treatment, and cells were cultured for additional days prior to harvesting the media supernatant to measure PCSK9 secretion levels at the day 4 and day 11 timepoints. Briefly, PCSK9 secretion levels were measured by ELISA using the BioLegend® ELISA MAX™ kit following the manufacturer's instructions. To ensure accuracy in quantifying PCSK9 secretion levels, a standard curve was constructed using recombinant CM PCSK9 protein as a reference (Cynomolgus PCSK9 protein, from Acro Biosystems). Baseline PCSK9 secretion levels (ng/mL) were also quantified for untreated primary CM hepatocytes (for each lot) at day 4.

Table 40: LNP formulations tested in this example.

LNP lipid	Encapsulated mRNA	Encapsulated gRNA
GenVoy-ILM™	CasX 515	

MC3		gRNA scaffold 316 with spacer 6.1 (v1)
MC3	LTRP5-ADD-ZIM3	
ALC-0315		
SM-102		

Results:

[0482] Two lots of primary CM hepatocytes were treated with five different LNPs, which co-encapsulated either CasX 515 or LTRP5-ADD-ZIM3 and a *PCSK9*-targeting gRNA using spacer 6.1, at various doses. The media supernatant was harvested 4 and 11 days post-treatment to assess effects on PCSK9 secretion (FIGS. 61-64). The results in FIGS. 61-64 demonstrate that either LTRP5-ADD-ZIM3 or CasX mRNA and the targeting gRNA could be co-encapsulated within the various LNPs, and were delivered to target cells where they reduced secreted PCSK9 levels. Dose-dependent reduction in secreted PCSK9 levels was observed for all formulated LNPs in both lots of primary CM hepatocytes at 4 days post-treatment (FIGS. 61-62). By 11 days post-treatment, dose-dependent reduction in secreted PCSK9 levels was observed for all formulated LNPs in both lots, with the exception of MC3-encapsulating LTRP5-ADD-ZIM3 and the targeting gRNA (FIGS. 63-64). Secreted PCSK9 levels will be examined at longer timepoints to determine further the effects of each LNP formulation on decreasing PCSK9 secretion.

[0483] The results from this experiment show that LTRP mRNA, as well as CasX mRNA, and targeting gRNA can be co-encapsulated within LNPs to be delivered to target cells to induce silencing of a target endogenous locus.

Example 11: Formulation of lipid nanoparticles (LNPs) to deliver dXR or LTRP mRNA and gRNA payloads to target cells and tissue

[0484] As described in Example 10, experiments were performed to encapsulate dXR or LTRP mRNA and gRNA into LNPs for delivery to target cells and tissues. The following example provides the methods used for formulating LNPs with various LNP lipids.

For experiments that used GenVoy-ILM™-based LNPs:

[0485] dXR or LTRP mRNA and gRNA were encapsulated into LNPs using GenVoy-ILM™ lipids using the Precision NanoSystems Inc. (PNI) Ignite™ Benchtop System, following the manufacturer's guidelines. GenVoy-ILM™ lipids are a composition of ionizable lipid:DSPC:cholesterol:stabilizer at 50:10:37.5:2.5 mol%. Briefly, to formulate LNPs, equal

mass ratios of dXR or LTRP mRNA and gRNA were diluted in PNI Formulation Buffer, pH 4.0. GenVoy-ILM™ lipids were diluted 1:1 in anhydrous ethanol. mRNA/gRNA co-formulations were generated using a predetermined N/P ratio. The RNA and lipids were run through a PNI laminar flow cartridge at a predetermined flow rate ratio on the PNI Ignite™ Benchtop System. After formulation, the LNPs were diluted in PBS, pH 7.4, to decrease the ethanol concentration and increase the pH, which would increase the stability of the particles. Buffer exchange of the mRNA/sgRNA-LNPs was achieved by overnight dialysis into PBS, pH 7.4, at 4°C using 10k Slide-A-Lyzer™ Dialysis Cassettes (Thermo Scientific™). Following dialysis, the mRNA/gRNA-LNPs was concentrated to > 0.5 mg/mL using 100 kDa Amicon®-Ultra Centrifugal Filters (Millipore) and then filter-sterilized. Formulated LNPs were analyzed on a Stunner (Unchained Labs) to determine their diameter and polydispersity index (PDI). Encapsulation efficiency and RNA concentration was determined by RiboGreen™ assay using Invitrogen's Quant-iT™ RiboGreen™ RNA assay kit.

[0486] For experiments that used ALC-0315 (6-((2-hexyldecanoyl)oxy)-N-(6-((2-hexyldecanoyl)oxy)hexyl)-N-(4-hydroxybutyl)hexan-1-aminium), SM-102 (8-[(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino]-octanoic acid, 1-octylnonyl ester), and MC3 (DLin-MC3-DMA) -based LNPs:

[0487] dXR or LTRP mRNA and gRNA were encapsulated into LNPs using ALC-0315, SM-102, or MC3-based lipid mix using a custom-made T-mixer micro mixing device, at a flow rate of 20 mL/min and 3:1 mixing ratio of aqueous to organic phase. For all three lipid mixes, the composition was the following: ionizable lipid:DSPC:cholesterol:DMG-PEG2000 at 50:10:38.5:1.5 mol%. Briefly, to formulate LNPs, equal mass ratios of dXR or LTRP mRNA and gRNA were diluted in 25 mM sodium acetate, pH 4.0. The lipid mix was made at a 10 mM concentration in anhydrous ethanol. mRNA/gRNA co-formulations were generated using a predetermined N/P ratio. The RNA and lipids are run through a custom-made T-mixer device at a predetermined flow rate ratio using syringe pump infusers. After formulation, the LNPs were dialyzed into PBS, pH 7.4, to decrease the ethanol concentration and increase the pH, which increases the stability of the particles. Buffer exchange of the mRNA/sgRNA-LNPs was achieved by overnight dialysis into PBS, pH 7.4, at 4°C using 10k Slide-A-Lyzer™ Dialysis or Cassettes (Thermo Scientific™) or 12-14 kDa dialysis tubing (Repligen). Following dialysis, the mRNA/gRNA-LNPs was concentrated to > 0.2 mg/mL using 30-100 kDa Amicon®-Ultra Centrifugal Filters (Millipore) and then sterile-filtered using Acrodisc PES membrane filters.

Formulated LNPs were analyzed on a Malvern Zetasizer to determine their diameter and polydispersity index (PDI). Encapsulation efficiency and RNA concentration was determined by RiboGreen™ assay using Invitrogen's Quant-iT™ RiboGreen™ RNA assay kit.

[0488] The GenVoy-ILM™, ALC-0315, SM-102, and MC3 LNPs described above were used in various experiments to deliver XR or LTRP mRNA and gRNA to target cells, and can further be used for delivery to target tissues.

Example 12: CpG-depletion of DNA encoding the guide RNA scaffold improves CasX-mediated editing *in vitro*

[0489] Pathogen-associated molecular patterns (PAMPs), such as unmethylated CpG motifs, are small molecular motifs conserved within a class of microbes. They are recognized by toll-like receptors (TLRs) and other pattern recognition receptors in eukaryotes and often induce a non-specific immune activation. In the context of gene therapy, therapeutics containing PAMPs are often not as well-tolerated and are rapidly cleared from the patient given the strong immune response triggered, which ultimately leads to reduced therapeutic efficacy. CpG motifs are short single-stranded DNA sequences containing the dinucleotide CG. When these CpG motifs are unmethylated, they act as PAMPs and therefore stimulate the immune response. In this example, experiments were performed to deplete CpG motifs in the guide scaffold coding sequence in the context of an AAV construct encoding CasX variant 491, guide scaffold variant 235, and spacer 7.37 targeting the endogenous B2M (beta-2-microglobulin) locus, and test the effect of CpG-depletion in the guide scaffold on editing of the B2M locus *in vitro*.

Materials and methods:

Design of CpG-depleted guide scaffolds:

[0490] Nucleotide substitutions were rationally-designed to replace native CpG motifs within the base gRNA scaffold variant (gRNA scaffold 235) with the intent to preserve editing activity while reducing scaffold immunogenicity. It was believed that as many CpG-motifs as possible should be removed from the scaffold coding sequence in order to sufficiently reduce immunogenicity. Scaffold 235 contains a total of eight CpG elements; six of which are predicted to basepair and form complementary strands of a double-stranded secondary structure (see FIG. 27A). Therefore, the six basepairing CpGs forming three pairs were mutated in concert to maintain important secondary structures. This reduced the number of independent CpG-containing regions to five (three pairs and two single CpGs) to be considered independently for

CpG-removal. Specifically, mutations were designed in (1) the pseudoknot stem, (2) the scaffold stem, (3) the extended stem bubble, (4) the extended step, and (5) the extended stem loop, as diagrammed in FIG. 27B and described in detail below.

[0491] In the pseudoknot stem (region 1), the CpG pair was flipped to a GpC to minimize the alteration of the base composition and sequence. Based on previous experiments involving replacing individual base pairs, it was anticipated that this mutation was not likely to be detrimental to the structure and function of the guide RNA scaffold.

[0492] Similarly, in the scaffold stem (region 2) the CpG pair was flipped to a GpC to minimize the alteration of the base composition and sequence. It was anticipated that this mutation was likely to be detrimental to the structure and function of the guide RNA scaffold because strong sequence conservation was seen in this region in previous experiments mutating individual bases or base pairs. This strong sequence conservation is likely due to the scaffold stem loop being important in interacting with the CasX protein as well as in the formation of a triplex structural element with the pseudoknot region.

[0493] In the extended stem bubble (region 3) the single CpG was removed by one of three strategies. First, the bubble was deleted by mutating CG->C. Second, the bubble was resolved to restore ideal basepairing by mutating CG->CT. Third, the entire extended stem loop was replaced with the extended stem loop of scaffold 174. Note that, by itself, the replacement of the extended stem loop with that of scaffold 174 recapitulates scaffold 316, which has previously been shown to edit efficiently. There are no CpG motifs in the extended stem loop of scaffold 174. Therefore, replacing the extended stem loop with that of scaffold 174 also removes the CpG motif in the extended stem (region 4). Based on previous experiments showing the relative robustness of the extended stem to small changes, it was anticipated that mutating the extended stem bubble was moderately likely to be detrimental to the structure and function of the guide RNA scaffold.

[0494] In the extended stem (region 4), the CpG pair could not be flipped to GpC without generating additional CpG motifs. Therefore, the CpGs were changed to a GG and a complementary CC motif. Similar to region 3, based on the relative robustness of the extended stem to small changes, it was anticipated that this mutation was not likely to be detrimental to the structure and function of the guide RNA scaffold.

[0495] Finally, the extended stem loop (region 5) was mutated in one of three ways that were designed based on previous experiments examining the stability of the stem loop. In particular,

several variations of the stem loop had previously been shown to have similar stability levels, and some of these variations of the stem loop do not contain CpGs. Based on these findings, first, the loop was replaced with a new loop with a CUUG sequence. Second, the loop was replaced with a new loop with a GAAA sequence. Since the GAAA loop replacement would generate a novel CpG adjacent to the loop, it was combined with a C->G base swap and the corresponding G->C base swap on the complementary strand, ultimately resulting in a CUUCGG->GGAAAC exchange. Third, the loop was mutated by the insertion of an A to interrupt the CpG motif and thereby increase the size of the loop from 4 to 5 bases. It was anticipated that randomly mutating the extended stem loop would likely have detrimental effects on secondary structure stability and hence on editing. However, relying on previously confirmed sequences was believed to have a lower risk associated with a replacement.

[0496] To generate guide RNA scaffolds encoded by DNA with reduced CpG levels, the mutations described above were combined in various configurations. Table 41, below, summarizes combinations of the mutations that were used. In Table 41, a 0 indicates that no mutation was introduced to a given region, a 1, 2, or 3 indicates that a mutation was introduced in that region, as diagrammed in FIG. 27B, and n/a indicates not applicable. Specifically, for region 1, the pseudoknot stem, a 1 indicates that a CG->GC mutation was introduced. For region 2, the scaffold stem, a 1 indicates that a CG->GC mutation was introduced. For region 3, the extended stem bubble, a 1 indicates that the bubble was removed by the deletion of the G and A bases that form the bubble, a 2 indicates that the bubble was resolved by a CG->CU mutation that allows for basepairing between the A and U bases, and a 3 indicates that the extended stem loop was replaced with the extended step loop from guide scaffold 174. For region 4, the extended stem, a 1 indicates that a CG->GC mutation was introduced. For region 5, the extended stem loop, a 1 indicates that the loop was replaced from UUCG->CUUG, a 2 indicates that the loop was replaced along with a basepair adjacent to the loop, from CUUCGG->GGAAAC, and a 3 indicates that an A was inserted between the C and the G.

Table 41: Summary of mutations for CpG-reduction and depletion in guide scaffold 235

Scaffold ID	Region 1 (Pseudoknot stem)	Region 2 (Scaffold stem)	Region 3 (Extended stem bubble)	Region 4 (Extended stem)	Region 5 (Extended stem loop)
320	1	0	0	1	0
321	1	0	1	1	0
322	1	0	2	1	0

323	1	0	3	n/a	0
324	1	0	1	1	1
325	1	0	2	1	1
326	1	0	3	n/a	1
327	1	0	1	1	2
328	1	0	2	1	2
329	1	0	3	n/a	2
330	1	0	1	1	3
331	1	0	2	1	3
332	1	0	3	n/a	3
334	1	1	2	1	1
335	1	1	3	n/a	1
336	1	1	1	1	2
337	1	1	2	1	2
338	1	1	3	n/a	2
339	1	1	1	1	3
340	1	1	2	1	3
341	1	1	3	n/a	3
235	0	0	0	0	0

[0497] Table 42, below, lists the DNA sequences encoding the designed CpG-reduced or depleted guide scaffolds.

Table 42: DNA sequences encoding CpG-reduced or depleted guide RNA scaffolds

Scaffold ID	SEQ ID NO
320	3210
321	3211
322	3212
323	3213
324	3214
325	3215
326	3216
327	3217
328	3218
329	3219
330	3220
331	3221
332	3222
333	3223
334	3224

Scaffold ID	SEQ ID NO
335	3225
336	3226
337	3227
338	3228
339	3229
340	3230
341	3231

Generation of CpG-depleted AAV plasmids:

[0498] The CpG-reduced or depleted gRNA scaffolds were tested in the context of AAV vectors that were otherwise CpG-depleted, with the exception of the AAV2 ITRs. Specifically, nucleotide substitutions to replace native CpG motifs in AAV components were designed in silico based on homologous nucleotide sequences from related species for the following elements: the murine U1a snRNA (small nuclear RNA) gene promoter, the bGHpA (bovine growth hormone polyadenylation) sequence, and the human U6 promoter. The coding sequence for CasX 491 was codon-optimized for CpG depletion. All resulting sequences (Tables 42 and 43) were ordered as gene fragments with the appropriate overhangs for cloning and isothermal assembly to replace individually the corresponding elements of the existing base AAV plasmid (construct ID 183). Spacer 7.37 (GGCCGAGAUGUCUCGCUCCG; SEQ ID NO: 3137), which targets the endogenous B2M gene, was used for the experiments discussed in this example. The first time that the experiment was performed ("N=1"), a sample with the non-targeting spacer 0.0 was also included as a control (CGAGACGUAAUUACGUCUCG, SEQ ID NO: 3232; see FIG. 28).

[0499] The resulting AAV constructs were generated using standard molecular cloning techniques. Cloned and sequence-validated plasmid constructs were midi-prepped for subsequent nucleofection and AAV vector production. The sequences of the additional components of AAV constructs, with the exception of sequences encoding the gRNAs (Table 41), are listed in Table 43.

Table 43: Sequences of AAV elements (5'-3' in AAV construct)

Element	DNA sequence SEQ ID NO:
AAV2 5' ITR	3233
CpG-depleted U1a promoter	3234

Element	DNA sequence SEQ ID NO:
CpG-depleted cMycNLS-CasX491-cMycNLS	3235
CpG-depleted bGH-polyA sequence	3236
CpG-depleted U6 promoter	3237
See sgRNA sequences in Table 42, above.	
AAV2 3' ITR	3238

AAV production:

[0500] Suspension-adapted HEK293T cells, maintained in FreeStyle 293 media, were seeded in 20-30 mL of media at 1.5E6 cells/mL on the day of transfection. Endotoxin-free pAAV plasmids with the transgene flanked by ITR repeats were co-transfected with plasmids supplying the adenoviral helper genes for replication and AAV rep/cap genome using PEI Max (Polysciences) in serum-free Opti-MEM media. Three days later, cultures were centrifuged to separate the supernatant from the cell pellet, and the AAV particles were collected, concentrated, and filtered following standard procedures.

[0501] To determine the viral genome (vg) titer, 1 µL from crude lysate viruses was digested with DNase and ProtK, followed by quantitative PCR. 5 µL of digested virus was used in a 25 µL qPCR reaction composed of IDT primetime master mix and a set of primer and 6'FAM/Zen/IBFQ probe (IDT) designed to amplify a 62 bp-fragment located in the AAV2-ITR. An AAV ITR plasmid was used as reference standards to calculate the titer (vg/mL) of viral samples.

AAV transduction of induced neurons in vitro:

[0502] 24 hours prior to transduction, 50,000 induced neurons per well were seeded on Matrigel-coated 96-well plates. AAVs expressing the CasX:gRNA system with various versions of the guide scaffold were then diluted in neuronal plating media and added to cells. The first time that the experiment was performed ("N=1"), cells were transduced at a multiplicity of infection (MOI) of 4e3 viral genomes (vg)/cell (see FIG. 28). Seven days post-plating, induced neurons were transduced with virus diluted in fresh feeding media. Eight days post-transduction, cells were lifted using lysis buffer, 4-well replicates were pooled per experimental condition, and genomic DNA (gDNA) was harvested and prepared for editing analysis at the B2M locus using next generation sequencing (NGS). The second time that the experiment was performed ("N=2"), cells were transduced at an MOI of 3e3 vg/cell, 1e3 vg/cell, or 3e2 vg/cell (see FIG.

29, FIG. 30, and FIG. 31). Seven days post-plating, induced neurons were transduced with virus diluted in fresh feeding media. Seven days post-transduction, cells were lifted using lysis buffer, 2-well replicates were pooled per experimental condition, and gDNA was harvested and prepared for editing analysis at the B2M locus using NGS. Samples that were not transduced with AAV were included as controls.

NGS processing and analysis:

[0503] Genomic DNA (gDNA) from harvested cells were extracted using the Zymo Quick-DNA Miniprep Plus kit following the manufacturer's instructions. Target amplicons were formed by amplifying regions of interest from 200 ng of extracted gDNA with a set of primers specific to the human B2M gene. These gene-specific primers contained an additional sequence at the 5′ end to introduce an Illumina adapter and a 16-nucleotide unique molecule identifier. Amplified DNA products were purified with the Ampure XP DNA cleanup kit. Quality and quantification of the amplicon were assessed using a Fragment Analyzer DNA Analysis kit (Agilent, dsDNA 35-1500bp). Amplicons were sequenced on the Illumina Miseq according to the manufacturer's instructions. Raw fastq files from sequencing were quality-controlled and processed using cutadapt v2.1, flash2 v2.2.00, and CRISPResso2 v2.0.29. Each sequence was quantified for containing an insertion or deletion (indel) relative to the reference sequence, in a window around the 3′ end of the spacer (30 bp window centered at –3 bp from 3′ end of spacer). CasX activity was quantified as the total percent of reads that contain insertions, substitutions, and/or deletions anywhere within this window for each sample.

Results:

[0504] Mutations were introduced into the guide scaffold 235 in order to reduce the CpG content of the DNA sequence coding the guide scaffold. Surprisingly, compared to scaffold 235, all of the CpG-reduced and CpG-depleted scaffold variants produced higher levels of editing in induced neurons. This was the case with two independent repeats of the experiment (with the results from the first repeat of the experiment shown in FIG. 28, and the results of the second repeat of the experiment shown in FIGS. 29-31), and across multiple MOIs (see FIGS. 30-31). The enhanced level of editing was surprising because the goal of reducing CpG content was to simply preserve editing activity while reducing immunogenicity. Instead, the mutations enhanced editing activity, rather than merely preserving it.

[0505] Notably, scaffold 320 showed a significant increase in potency over scaffold 235. Scaffold 320 includes mutations to only two regions of the scaffold; in the pseudoknot stem and

the extended stem (regions 1 and 4). Further, some combinations of mutations produced worse editing than scaffold 320. However, even the CpG-reduced scaffolds that performed worse than scaffold 320, such as scaffolds 331 and 334, performed similar to or better than scaffold 235.

[0506] Based on these results, without wishing to be bound by theory, it is believed that the boost in potency seen in many of the CpG-reduced and CpG-depleted scaffolds is likely caused by one of the mutations present in all CpG-reduced scaffolds (i.e., region 1 and/or 4). Since the mutation to region 4 is not present in the scaffolds with the extended stem loop replacement (i.e., the third mutation to region 3) and these scaffolds show a similar improvement in potency over 235 as 320 did, it is believed that the beneficial effect is likely caused by the mutation in region 1 (pseudoknot stem), which is present in all of the tested scaffolds. Further experiments will be performed to test the effect of the individual mutations in the pseudoknot stem (region 1) and the extended stem (region 4) separately.

[0507] Further, the N=1 data as presented in FIG. 28 indicate that all the new scaffolds carrying the mutation in region 2 (scaffold stem) edited at a slightly lower level than their respective counterparts without this mutation. This suggests that mutating this position in the scaffold stem may have a small deleterious effect on editing potency. This will be examined in additional experiments.

[0508] The results described here demonstrate that introducing mutations that reduced the CpG content of the DNA encoding the guide RNA scaffold resulted in improvements in gene editing relative to guide scaffold 235.

Example 13: Use of a catalytically-dead CasX repressor system fused with additional domains from DNMT3A and DNMT3L to induce durable silencing of the *B2M* locus

[0509] Experiments were performed to determine whether rationally-designed LTRP constructs, with three repressor domains composed of a transcriptional repressor domain, the catalytic domain from DNMT3A and the interaction domain from DNMT3L fused to catalytically-dead CasX (dCasX) 491, would induce durable long-term repression of the endogenous *B2M* locus *in vitro*. In addition, multiple configurations of the LTRP molecules, which contain varying placements of the epigenetic domains relative to dCasX, were designed to assess how their arrangement would affect the duration of silencing of the *B2M* locus, as well as the specificity of their on-target methylation activity.

Materials and Methods:

Generation of LTRP constructs and lentiviral plasmid cloning:

[0510] Lentiviral plasmid constructs coding for an LTRP molecule were built using standard molecular cloning techniques. These constructs comprised of sequences coding for catalytically-dead CasX protein 491 (dCasX491), a KRAB domain from ZNF10 or ZIM3, and the catalytic domain and interaction domain from DNMT3A (D3A) and DNMT3L (D3L) respectively. Briefly, constructs were ordered as oligonucleotides and assembled by overlap extension PCR followed by isothermal assembly. Amino acid sequences of these key LTRP elements are provided in Table 44. The resulting plasmids contained constructs positioned in varying configurations to generate an LTRP molecule. The protein sequences for the LTRP molecules are listed in Table 45, and the LTRP configurations are illustrated in FIG. 1. Sequences encoding the LTRP molecules also contained a 2x FLAG tag. Plasmids also harbored sequences encoding gRNA scaffold variant 174 having either a spacer targeting the endogenous *B2M* locus or a non-targeting control (spacer sequences listed in Table 46). These constructs were all cloned upstream of a P2A-puromycin element on the lentiviral plasmid. Cloned and sequence-validated constructs were midi-prepped and subjected to quality assessment prior to transfection in HEK293T cells.

Table 44: Sequences of LTRP components (e.g., additional domains fused to CasX) to generate LTRP variant plasmids (illustrated in FIG. 1)

Component	SEQ ID NO
ZNF10 KRAB domain	3239
ZIM3 KRAB domain	3240
DNMT3A catalytic domain	3241
DNMT3L interaction domain	127
dCasX491	4
Linker 1	123
Linker 2	122
Linker 3A	120
Linker 3B	
Linker 4	121
NLS A	30

Table 45: Protein sequences of LTRP molecules

LTRP ID	SEQ ID NO
1.A	3242
1.B	3243
2.A	3244
2.B	3245
3.A	3246
3.B	3247
4.A	3248
4.B	3249
5.A	3250
5.B	3251

Table 46: Sequences of spacers used in constructs

Spacer ID	Target gene	PAM	Sequence	SEQ ID NO
7.37	*B2M*	TTC	GGCCGAGAUGUCUCGCUCCG	3137
7.148	*B2M*	NGG	CGCGAGCACAGCUAAGGCCA	3101
0.0	Non-target	N/A	CGAGACGUAAUUACGUCUCG	3232

Transfection of HEK293T cells:

[0511] HEK293T cells were seeded at a density of 30,000 cells in each well of a 96-well plate. The next day, each well was transiently transfected using lipofectamine with 100 ng of LTRP variant plasmids, each containing a construct encoding for a differently configured LTRP protein (FIG. 1), with the gRNA having either non-targeting spacer 0.0 or targeting spacer 7.37 to the B2M locus. Specifically, for one experiment, HEK293T cells were transfected with plasmids encoding LTRP proteins #1-3, and in a second experiment, cells were lipofected with plasmids encoding LTRP protein #1, 4, and 5. In both experiments, LTRP molecules harbored a KRAB domain either from ZNF10 or ZIM3. Experimental controls included dCasX491 (with or without the ZNF10 repressor domain), catalytically-active CasX 491, and a catalytically-dead Cas9 fused to both the ZNF10-KRAB domain and DNMT3A/L domains, each with the same B2M-targeting or non-targeting gRNA. Each construct was tested in triplicate. 24 hours post-transfection, cells were selected with 1μg/mL puromycin for two days. Starting six days after transfection, cells were harvested for repression analysis every 2-3 days by analyzing B2M protein expression via

HLA immunostaining followed by flow cytometry. B2M expression was determined by using an antibody that would detect the B2M-dependent HLA protein expressed on the cell surface. HLA+ cells were measured using the Attune™ NxT flow cytometer. In addition, in a separate experiment, HEK293T cells transiently transfected with LTRP variant plasmids and the *B2M*-targeting gRNA or non-targeting gRNA were harvested at five days post-lipofection for genomic DNA (gDNA) extraction for bisulfite sequencing.

Bisulfite sequencing to assess LTRP specificity measured by off-target methylation levels at target locus:

[0512] To determine off-target methylation levels at the B2M locus, gDNA from harvested cells was extracted using the Zymo Quick-DNA Miniprep Plus kit following the manufacturer's instructions. The extracted gDNA was then subjected to bisulfite conversion using the EZ DNA Methylation™ Kit (Zymo) following the manufacturer's protocol, converting any non-methylated cytosine into uracil. The resulting bisulfite-treated DNA was subsequently sequenced using next-generation sequencing (NGS) to determine the levels of off-target methylation at the *B2M* and *VEGFA* loci.

NGS processing and analysis:

[0513] Target amplicons were amplified from 100 ng bisulfite-treated DNA via PCR with a set of primers specific to the bisulfite-converted target locations of interest (human *B2M* and *VEGFA* loci). These gene-specific primers contained an additional sequence at the 5′ end to introduce an Illumina™ adapter. Amplified DNA products were purified with the Cytiva Sera-Mag Select DNA cleanup kit. Quality and quantification of the amplicon were assessed using a Fragment Analyzer DNA Analysis kit (Agilent, dsDNA 35-1500 bp). Amplicons were sequenced on the Illumina™ Miseq™ according to the manufacturer's instructions. Raw fastq files from sequencing were processed using Bismark Bisulfite Read Mapper and Methylation caller. PCR amplification of the bisulfite-treated DNA would convert all uracil nucleotides into thymine, and sequencing of the PCR product would determine the rate of cytosine-to-thymine conversion as a readout of the level of potential off-target methylation at the *B2M* and *VEGFA* loci mediated by each LTRP molecule.

Results:

[0514] LTRP variant plasmids encoding for differently configured LTRP proteins (FIG. 1) were transiently transfected into HEK293T cells to determine whether the rationally-designed LTRP molecules could heritably silence gene expression of the target B2M locus *in vitro*. FIGS.

32A and 32B depict the results of a time-course experiment assessing B2M protein repression mediated by LTRP proteins #1-3, each of which harbored a KRAB domain from ZNF10 (FIG. 32A) or ZIM3 (FIG. 32B). Table 47 shows the average percentage of cells characterized as HLA-negative (indicative of depleted B2M expression) for each condition at 50 days post-transfection. The results illustrate that all LTRP molecules with a gRNA targeting the B2M locus were able to demonstrate sustained B2M repression for 50 days *in vitro*, although the potency of repression varied by the choice of KRAB domain and LTRP configuration. For instance, harboring a ZIM3-KRAB domain rendered the LTRP protein a more efficacious repressor than harboring a ZNF10-KRAB, and this effect was most prominently observed for LTRP #2 (compare FIG. 32A to FIG. 32B). Furthermore, positioning the DNMT3A/L domains at the N-terminus of dCasX491 (LTRP #1) resulted in more stable silencing of B2M expression compared to effects mediated by LTRPs with DNMT3A/L domains at the C-terminus of dCasX491 (LTRP #2 and #3; FIGS. 32A and 32B). These results also revealed that the relative positioning of the two types of repressor domains (i.e., dCasX491-KRAB-DNMT3A/L for LTRP #2 vs. dCasX491-DNMT3A/L-KRAB for LTRP #3) could also influence the overall potency of the LTRP molecule, despite both configurations being C-terminal fusions of dCasX491 (LTRP #2 and #3; FIGS. 32A and 32B).

[0515] In a second time-course experiment, durable B2M repression was assessed for LTRP proteins #1, #4, and #5, where both the DNMT3A/L and KRAB domains were positioned at the N-terminus of dCasX491 for LTRP #4 and #5 (FIG. 1). Table 48 shows the average percentage of HLA-negative cells for each condition at 73 days post-lipofection. As similarly seen in the first time-course, all LTRP conditions with a B2M-targeting gRNA maintained durable silencing of the B2M locus (FIGS. 33A and 33B; Table 48). In fact, the results in this experiment demonstrate that LTRP #5 was able to achieve and sustain the highest level of B2M repression compared to that achieved by LTRP #1 or LTRP #4 for 73 days *in vitro* (FIGS. 33A and 33B). Furthermore, LTRP #4 containing the ZIM3-KRAB also appeared to outperform its LTRP #1 counterpart (FIG. 33B). For both time-course experiments discussed above, CasX 491-mediated editing resulted in durable silencing of the B2M expression, while an XR construct fusing only the KRAB domain to dCasX491 (dCasX491-ZNF10) only resulted in transient B2M knockdown.

Table 47: Levels of B2M repression mediated by CasX and Cas9 molecules and LTRP constructs #1-3 quantified at 50 days post-transfection

Molecule	Spacer	% HLA-negative cells (mean)	Standard deviation
CasX 491	0.0	0.29	0.09
dCasX491	0.0	N/A	N/A
dCasX491-ZNF10	0.0	0.40	0.18
dCas9-ZNF10-D3A/L	0.0	1.05	0.63
LTRP1-ZNF10	0.0	0.99	0.35
LTRP2-ZNF10	0.0	0.61	0.11
LTRP3-ZNF10	0.0	0.79	0.29
LTRP1-ZIM3	0.0	0.99	0.22
LTRP2-ZIM3	0.0	0.78	0.27
LTRP3-ZIM3	0.0	0.71	0.53
CasX 491	7.37	76.57	11.03
dCasX491	7.37	0.49	0.10
dCasX491-ZNF10	7.148	0.89	0.19
dCas9-ZNF10-D3A/L	7.148	57.30	17.36
LTRP1-ZNF10 (LTRP #1.B)	7.37	69.97	7.89
LTRP2-ZNF10 (LTRP #2.B)	7.37	36.87	8.31
LTRP3-ZNF10 (LTRP #3.B)	7.37	17.07	3.50
LTRP1-ZIM3 (LTRP #1.A)	7.37	73.70	9.28
LTRP2-ZIM3 (LTRP #2.A)	7.37	58.83	0.87
LTRP3-ZIM3 (LTRP #3.A)	7.37	17.50	4.30

Table 48: Levels of B2M repression mediated by CasX and Cas9 molecules and LTRP constructs #1, #4, and #5 quantified at 73 days post-transfection

Molecule	Spacer	% HLA-negative cells (mean)	Standard deviation
CasX 491	0.0	0.71	0.05
dCasX491	0.0	N/A	N/A
dCasX491-ZNF10	0.0	0.76	0.12
dCas9-ZNF10-D3A/L	0.0	0.83	0.08
LTRP1-ZNF10	0.0	1.04	0.44

Molecule	Spacer	% HLA-negative cells (mean)	Standard deviation
LTRP4-ZNF10	0.0	1.17	0.52
LTRP5-ZNF10	0.0	1.94	1.27
LTRP1-ZIM3	0.0	1.83	0.76
LTRP4-ZIM3	0.0	N/A	N/A
LTRP5-ZIM3	0.0	1.15	0.26
CasX 491	7.37	73.30	8.43
dCasX491	7.37	0.83	0.16
dCasX491-ZNF10	7.148	1.37	0.37
dCas9-ZNF10-D3A/L	7.148	68.97	5.21
LTRP1-ZNF10 (LTRP #1.B)	7.37	48.27	3.66
LTRP4-ZNF10 (LTRP #4.B)	7.37	55.17	4.83
LTRP5-ZNF10 (LTRP #5.B)	7.37	60.77	8.12
LTRP1-ZIM3 (LTRP #1.A)	7.37	58.90	2.69
LTRP4-ZIM3 (LTRP #4.A)	7.37	69.00	6.58
LTRP5-ZIM3 (LTRP #5.A)	7.37	74.90	10.61

[0516] To evaluate the degree of off-target CpG methylation at the *B2M* locus mediated by the DNMT3A/L domains within the LTRP molecules, bisulfite sequencing was performed using genomic DNA extracted from HEK293T cells treated with LTRP proteins #1-3 containing the ZIM3-KRAB domain and harvested at five days post-lipofection. FIG. 34 illustrates the findings from bisulfite sequencing, specifically showing the distribution of the number of CpG sites around the transcription start site of the *B2M* locus that harbored a certain level of CpG methylation for each experimental condition. The results revealed that while LTRP #1 demonstrated the strongest on-target CpG-methylating activity (LTRP1-ZIM3 7.37), it induced the highest level of off-target CpG methylation (LTRP1-ZIM3 NT). LTRP #2 and LTRP #3 displayed weaker on-target CpG-methylating activity but relatively lower off-target methylation (FIG. 34). FIG. 35 is a scatterplot mapping the activity-specificity profiles for LTRP proteins #1-3 benchmarked against CasX 491 and dCas9-ZNF10-DNMT3A/L, where activity was measured as the average percentage of HLA-negative cells at day 21, and specificity was represented by the percentage of off-target CpG methylation at the *B2M* locus quantified at day 5.

[0517] The degree of off-target CpG methylation mediated by the DNMT3A/L domain was further evaluated by assessing the level of CpG methylation at a different locus, i.e., *VEGFA*, by performing bisulfite sequencing using the same extracted gDNA as was used previously for FIG. 34. The violin plot in FIG. 36 illustrates the bisulfite sequencing results showing the distribution of CpG sites with CpG methylation at the *VEGFA* locus in cells treated with LTRP proteins #1-3 containing the ZIM3-KRAB domain and a *B2M*-targeting gRNA. The findings further demonstrate that use of LTRP #1 resulted in the highest level of off-target CpG methylation, supporting the data shown earlier in FIG. 34. In comparison, use of either LTRP #2 or LTRP #3 resulted in substantially lower off-target methylation at the *-3* locus (FIG. 36).

[0518] The extent of off-target CpG methylation at the *VEGFA* locus for LTRP molecules #1, #4, and #5 was also analyzed. The plots in FIG. 37A-37B illustrate bisulfite sequencing results showing the distribution of CpG-methylated sites at the *VEGFA* locus in cells treated with LTRP #1, 4, and 5 containing a ZNF10 or ZIM3-KRAB domain and either a non-targeting gRNA (FIG. 37B) or a *B2M*-targeting gRNA (FIG. 37A). The data in FIG. 37B show that use of LTRP4-ZNF10, LTRP5-ZFN10, or LTRP5-ZIM3 resulted in markedly lower off-target CpG methylation at the *VEGFA* locus in comparison to use of LTRP1-ZNF10 or LTRP1-ZIM3. Similarly, the data in FIG. 37A show that use of LTRP #4 or LTRP #5 with either KRAB domain resulted in substantially lower levels of off-target CpG methylated sites compared to use with LTRP1-ZNF10. As exhibited in both FIG. 37A and 37B, the level of non-specific CpG methylation demonstrated by LTRP #1 is comparable to that achieved by the dCas9-ZNF10-DNMT3A/L benchmark.

[0519] FIG. 38 is a scatterplot mapping the activity-specificity profiles for LTRP molecules #1-5, containing either ZNF10- or ZIM3-KRAB domain, benchmarked against CasX 491 and dCas9-ZNF10-DNMT3A/L, where activity was measured as the average percentage of HLA-negative cells at day 21, and specificity was represented by the median percentage of off-target CpG methylation at the *VEGFA* locus detected at day 5. The data show that of the five LTRP molecules assessed, use of LTRP #5 resulted in the highest level of repressive activity, while use of LTRP #4 resulted in the strongest level of specificity.

[0520] The experiments demonstrate that the rationally-engineered LTRP molecules were able to transcriptionally and heritably repress the endogenous *B2M* locus, resulting in sustained depletion of the target protein. The findings also show that the choice of KRAB domain and

position and relative configuration of the DNMT3A/L domains could affect the overall potency and specificity of the LTRP molecule in durably silencing the target locus.

Example 14: Demonstration that inclusion of the ADD domain from DNMT3A enhances activity and specificity of LTRP molecules

[0521] In addition to its C-terminal methyltransferase domain, DNMT3A contains two N-terminal domains that regulate its function and recruitment to chromatin: the ADD domain and the PWWP domain. The PWWP domain reportedly interacts with methylated histone tails, including H3K36me3. The ADD domain is known to have two key functions: 1) it allosterically regulates the catalytic activity of DNMT3A by serving as a methyltransferase auto-inhibitory domain, and 2) it recognizes unmethylated H3K4 (H3K4me0). The interaction of the ADD domain with the H3K4me0 mark unveils the catalytic site of DNMT3A, thereby recruiting an active DNMT3A to chromatin to implement de novo methylation at these sites.

[0522] Given these functions of the ADD domain, experiments were performed to assess whether the incorporation of the ADD domain into the LTRP #5 construct, described previously in Example 13, would result in improved long-term repression of the target locus and reduced off-target methylation. The effect of incorporating the PWWP domain along with the ADD domain on LTRP activity and specificity was also assessed.

Materials and Methods:

Generation of LTRP constructs and plasmid cloning:

[0523] Plasmid constructs encoding for variants of the LTRP #5 construct with the ZIM3-KRAB domain (LTRP #5.A; see FIG. 1 for LTRP #5 configuration) were built using standard molecular cloning techniques. The resulting constructs comprised of sequences encoding for one of the following four alternative variations of LTRP5-ZIM3, where the additional DNMT3A domains were incorporated: 1) LTRP5-ZIM3 + ADD; 2) LTRP5-ZIM3 + ADD + PWWP; 3) LTRP5-ZIM3 + ADD without the DNMT3A catalytic domain; and 4) LTRP5-ZIM3 + ADD + PWWP without the DNMT3A catalytic domain. The sequences of key elements within the LTRP5-ZIM3 molecule and its variants are listed in Table 49, with the full-length protein sequence for each LTRP5-ZIM3 and its variants listed in Table 50. FIG. 45 is a schematic that illustrates the various LTRP #5 architectures assayed in this example. Sequences encoding the LTRP molecules also contained a 2x FLAG tag. Plasmids also harbored constructs encoding for

the gRNA scaffold variant 174 having either a spacer targeting the endogenous *B2M* locus or a non-targeting control (spacer sequences listed in Table 51).

Table 49: Sequences of LTRP components (e.g., additional domains fused to dCasX) to generate LTRP5 variant plasmids illustrated in FIG. 45

Component	Amino acid sequence SEQ ID NO
ZIM3 KRAB domain	3240
DNMT3A catalytic domain (CD)	126
DNMT3L interaction domain	127
dCasX491	4
Linker 1	123
Linker 2	122
Linker 3A'	124
Linker 3B	120
Linker 4	121
NLS A	30
NLS B	
DNMT3A ADD domain	125
DNMT3A PWWP domain	3252
Endogenous sequence between DNMT3A PWWP and ADD domains (endo)	3253

Table 50: Protein sequences of LTRP5 variants assayed in this example

LTRP ID	Amino acid sequence SEQ ID NO
LTRP5-ZIM3	3131
LTRP5-ZIM3 + ADD	3132
LTRP5-ZIM3 + ADD + PWWP	3254
LTRP5-ZIM3 + ADD - CD	3255
LTRP5-ZIM3 + ADD + PWWP - CD	3256

Table 51: Sequences of spacers used in constructs

Spacer ID	Target gene	Sequence	SEQ ID NO
0.0	Non-target	CGAGACGUAAUUACGUCUCG	3232
7.37	*B2M*	GGCCGAGAUGUCUCGCUCCG	3137
7.160	*B2M*	UAAACAUCACGAGACUCUAA	3113
7.165	*B2M*	UCCCUAUGUCCUUGCUGUUU	3114

Transfection of HEK293T cells:

[0524] Seeded HEK293T cells were transiently transfected with 100 ng of LTRP5 variant plasmids, each containing an LTRP:gRNA construct encoding for LTRP5-ZIM3 or one of its alternative variations (FIG. 45; Table 50 for sequences), with the gRNA having either non-targeting spacer 0.0 or a *B2M*-targeting spacer (Table 51 for spacer sequences). Spacers 7.160 and 7.165 have been shown to be repress the *B2M* locus when used with LTRPs, but not when used with dXRs made up of a dCasX fused to the ZIM3 KRAB domain (data not shown). Each construct was tested in triplicate. 24 hours post-transfection, cells were selected with 1μg/mL puromycin for three days. Cells were harvested for repression analysis at day 5, day 12, day 21, and day 51 post-transfection. Briefly, repression analysis was conducted by analyzing B2M protein expression via HLA immunostaining followed by flow cytometry, as described in Example 13. In addition, HEK293T cells transiently transfected with LTRP5 variant plasmids and a *B2M*-targeting gRNA or non-targeting gRNA were harvested at seven days post-transfection for gDNA extraction for bisulfite sequencing to assess off-target methylation at the *VEGFA* locus, which was performed as described in Example 13.

Results:

[0525] The effects of incorporating the ADD domain with or without the PWWP domain into the LTRP5 molecule on increasing long-term repression of the target *B2M* locus and reducing off-target methylation were assessed. Variations of the LTRP5-ZIM3 molecule were evaluated with either a *B2M*-targeting gRNA (with spacer 7.37 and LTRP-specific spacers 7.160 and 7.165) or a non-targeting gRNA, and the results are depicted in the plots in FIGS. 39-42. FIG. 39 shows that use of spacer 7.37 resulted in saturating levels of repression activity when paired with LTRP5-ZIM3, LTRP5-ZIM3 + ADD, and LTRP5-ZIM3 + ADD + PWWP, rendering it more challenging to assess activity differences among the LTRP5 variants. However, the differences in repression activity among the LTRP5 variants were more pronounced when using spacers 7.160 and 7.165 (FIGS. 40 and 41). The data demonstrate that incorporation of the ADD domain resulted in a significant increase in long-term repression when paired with the two LTRP-specific spacers compared to the repression levels achieved with the other LTRP5-ZIM3 molecules. Meanwhile, incorporation of both ADD and PWWP domains did not result in improved repression of the *B2M* locus, especially compared to the baseline LTRP5-ZIM3 molecule. As anticipated, the two LTRP5 variants without the DNMT3A catalytic domain exhibited poor long-term repression. Furthermore, the results depicted in FIG. 42 indicate that

addition of the ADD domain appeared to result in increased specificity, given the lower percentage of HLA-negative cells observed, relative to the baseline LTRP5-ZIM3 molecule.

[0526] Off-target CpG methylation at the *VEGFA* locus potentially mediated by the LTRP5 variants was assessed using bisulfite sequencing. FIG. 43 depicts the results from bisulfite sequencing, specifically showing the percentage of CpG methylation around the *VEGFA* locus. The results demonstrate that for all the *B2M*-targeting gRNAs, as well as the non-targeting gRNA, incorporation of the ADD domain into the LTRP5-ZIM3 molecule dramatically reduced the level of off-target methylation at the *VEGFA* locus (FIG. 43). FIG. 44 is a scatterplot mapping the activity-specificity profiles for the LTRP5-ZIM3 variants investigated in this example, where activity was measured as the average percentage of HLA-negative cells at day 21 when paired with spacer 7.160, and specificity was represented by the percentage of off-target CpG methylation at the *VEGFA* locus quantified at day 7 when paired with spacer 7.160. The scatterplot clearly shows that addition of the ADD domain significantly increased the activity of the LTRP5 molecule relative to the baseline ELX5 molecule without the ADD domain (FIG. 44).

[0527] The experiments demonstrate that inclusion of the DNMT3A ADD domain, but not inclusion of both the ADD and PWWP domains, improved repression activity and specificity of LTRP molecules. This enhancement of activity and specificity was observed with multiple gRNAs, demonstrating the significance of the incorporation of the ADD domain into LTRPs.

Example 15: Demonstration that inclusion of the ADD domain from DNMT3A into LTRPs enhances on-target activity and decreases off-target methylation

[0528] Experiments were performed to assess the effects of incorporating the ADD domain into LTRP molecules having configurations #1, #4, and #5, described previously in Example 13, on long-term repression of the target locus and off-target methylation.

Materials and Methods:

Generation of LTRP constructs and plasmid cloning:

[0529] Plasmid constructs encoding for LTRP molecules having configurations #1, #4, and #5 with the ZNF10-KRAB or ZIM3-KRAB domain and the DNMT3A ADD domain were built using standard molecular cloning techniques. Sequences of the resulting LTRP molecules are listed in Table 52, which also shows the abbreviated construct names for a particular LTRP molecule (e.g., LTRP #1.A, #1.B). FIG. 46 is a schematic that illustrates the general

architectures of LTRP molecules with the ADD domain incorporated for LTRP configuration #1, #4, and #5. Sequences encoding the LTRP molecules also contained a 2x FLAG tag. Plasmids also harbored sequences encoding gRNA scaffold 174 having either a spacer targeting the endogenous *B2M* locus or a non-targeting control (spacer sequences listed in Table 51).

Table 52: Protein sequences of the various LTRP #1, #4, and #5 variants assayed in this example

LTRP #	Domains	Amino acid sequence SEQ ID NO
LTRP #1	ZNF10-KRAB, DNMT3A ADD, DNMT3A CD, DNMT3L Interaction (LTRP #1.D)	3257
	ZIM3-KRAB, DNMT3A ADD, DNMT3A CD, DNMT3L Interaction (LTRP #1.C)	3258
	ZNF10-KRAB, DNMT3A CD, DNMT3L Interaction (LTRP #1.B)	3259
	ZIM3-KRAB, DNMT3A CD, DNMT3L Interaction (LTRP #1.A)	3066
LTRP #4	ZNF10-KRAB, DNMT3A ADD, DNMT3A CD, DNMT3L Interaction (LTRP #4.D)	3260
	ZIM3-KRAB, DNMT3A ADD, DNMT3A CD, DNMT3L Interaction (LTRP #4.C)	3261
	ZNF10-KRAB, DNMT3A CD, DNMT3L Interaction (LTRP #4.B)	3262
	ZIM3-KRAB, DNMT3A CD, DNMT3L Interaction (LTRP #4.A)	3263
LTRP #5	ZNF10-KRAB, DNMT3A ADD, DNMT3A CD, DNMT3L Interaction (LTRP #5.D)	3264
	ZIM3-KRAB, DNMT3A ADD, DNMT3A CD, DNMT3L Interaction (LTRP #5.C)	3132
	ZNF10-KRAB, DNMT3A CD, DNMT3L Interaction (LTRP #5.B)	3265
	ZIM3-KRAB, DNMT3A CD, DNMT3L Interaction (LTRP #5.A)	3131

Transfection of HEK293T cells:

[0530] Seeded HEK293T cells were transiently transfected with 100 ng of LTRP variant plasmids, each containing an LTRP:gRNA construct encoding for an LTRP molecule (Table 52;

FIG. 46), with the gRNA having either non-targeting spacer 0.0 or a *B2M*-targeting spacer (Table 51). Each construct was tested in triplicate. 24 hours post-transfection, cells were selected with 1μg/mL puromycin for 3 days. Cells were harvested for repression analysis at day 8, day 13, day 20, and day 27 post-transfection. Briefly, repression analysis was conducted by analyzing B2M protein expression via HLA immunostaining followed by flow cytometry, as described in Example 13. In addition, cells were also harvested on day 5 post-transfection for gDNA extraction for bisulfite sequencing to assess off-target methylation at the non-targeted *VEGFA* locus, which was performed using similar methods as described in Example 13.

Results:

[0531] The effects of incorporating the ADD domain into the LTRP molecules having configurations #1, #4, or #5 (see FIG. 2), with either a ZNF10 or ZIM-KRAB, on long-term repression of the *B2M* locus and off-target methylation were evaluated. LTRP molecules were tested with either a *B2M*-targeting gRNA or a non-targeting gRNA, and the results are depicted in the plots in FIGS. 47A-50B. The data demonstrate that incorporation of the ADD domain into the LTRP molecules clearly resulted in a substantial increase in B2M repression across all the time points for all LTRP configurations containing the ZIM3-KRAB when using spacer 7.160 (FIG. 47A), and similar findings were observed when using spacers 7.165 and 7.37 (data not shown). FIG. 47B shows the resulting B2M repression upon use of LTRP #5 containing either the ZNF10 or ZIM3-KRAB when paired with a gRNA with spacer 7.160; the data demonstrate that including the ADD domain increased durable B2M repression overall, with LTRP5-ZIM3 + ADD having a higher activity compared with that of LTRP5-ZNF10 + ADD. Similar time course findings were observed for LTRP #1 and LTRP #4 and the other two spacers (data not shown). FIG. 47C shows the resulting B2M repression upon use of LTRP #5 containing the ZIM3-KRAB when paired with any of the three *B2M*-targeting gRNAs, and the data demonstrate that inclusion of the ADD domain resulted in higher B2M repression overall. Similar time course findings were also observed for LTRP #1 and LTRP #4 (data not shown).

[0532] FIGS. 48A-48C shows the resulting B2M repression at the day 27 time point for all the LTRP configurations and gRNAs tested. The results show that the increase in B2M repression was more prominent with use of the sub-optimal spacers 7.160 and 7.165 compared to use of spacer 7.37. Furthermore, use of LTRP #1 and LTRP #5, which contained the DNMT3A and DNMT3L domains on the N-terminus of the molecule, resulted in the highest increase in B2M repression upon addition of the DNMT3A ADD domain (FIGS. 48A-48C). Use of LTRP #4,

which harbored the DNMT3A/3L domains 3' to the KRAB domain and 5' to the dCasX, resulted in lower activity gains, which may be attributable to a decreased ability of the ADD domain to interact with chromatin properly.

[0533] The specificity of LTRP molecules was determined by profiling the level of CpG methylation at the *VEGFA* gene, an off-target locus, using bisulfite sequencing, and the data are illustrated in FIGS. 49A-52B. The data demonstrate that inclusion of the DNMT3A ADD domain resulted in a substantial decrease in off-target methylation of the *VEGFA* locus across all conditions tested (FIGS. 49A-49C). Notably, the increased specificity mediated by the inclusion of the ADD domain was most prominent with the LTRP #1 and LTRP #5 configurations, both of which harbored the DNMT3A/3L domains on the N-terminal end of the molecule. Interestingly, LTRP molecules containing the ZIM3-KRAB domain led to stronger off-target methylation of the *VEGFA* locus. Furthermore, use of LTRP #4 and #5 configurations, even in the absence of an ADD domain, resulted in higher specificity compared to use of the LTRP #1 configuration. Compared to LTRP1-ZIM3 and LTRP4-ZIM3 configurations, inclusion of the ADD domain into LTRP5-ZIM3 resulted in the lowest off-target methylation.

[0534] FIGS. 50A-52B are a series of scatterplots mapping the activity-specificity profiles for the various LTRP molecules, where activity was measured as the average percentage of HLA-negative cells at day 27, and specificity was determined by the percentage of off-target CpG methylation at the *VEGFA* locus at day 5. The data demonstrate that across all three *B2M*-targeting spacers tested, inclusion of the ADD domain resulted in increased on-target B2M repression and decreased off-target methylation at the *VEGFA* locus. LTRP molecules having #1 and #5 configurations exhibited the greatest increases in activity and specificity at each spacer tested.

[0535] The results of the experiments discussed in this example support the findings in Example 14, in that the data demonstrate that inclusion of the DNMT3A ADD domain enhances both the strength of repression at early timepoints and the heritability of silencing across cell divisions, as well as decreases the off-target methylation incurred by the DNMT3A catalytic domain in the LTRP molecules. The data also confirm that different LTRP configurations have intrinsic differences in specificity, which can be exacerbated by use of a more potent KRAB domain. This decrease in specificity can be mitigated by inclusion of the DNMT3A ADD domain, which also can lead to greater on-target repression overall. The gains in repression activity are believed to be mediated by the function of the DNMT3A ADD domain to recognize

H3K4me0 and subsequent recruitment to chromatin. The gains in specificity are believed to be mediated via the function of the DNMT3A ADD domain to induce allosteric inhibition of the catalytic domain of DNMT3A in the absence of binding to H3K4me0. The results also highlight that positioning of the ADD domain in the different configurations tested is important to achieve the strongest gains in both specificity and activity of LTRP molecules.

Example 16: Demonstration of dXR effectiveness on HBEGF for high-throughput screening

[0536] Experiments were performed to determine the feasibility of using dXR constructs for high-throughput screening of molecules in mammalian cells.

Materials and Methods:

[0537] HEK293T cells were seeded in a 6-well plate at 300,000 cells/well and lipofected with 1 μg of plasmid encoding either a CasX molecule (491), a catalytically-dead CasX 491 with the ZNF10-KRAB repressor domain (dXR) and a guide scaffold 174 (SEQ ID NO: 1744) with a spacer targeting the *HBEGF* gene or a non-targeting spacer. Five combinations of CasX-based molecules and gRNAs with the indicated spacers (Table 53) were transfected into five separate wells. HBEGF is the receptor that mediates entry of diphtheria toxin that, when added to the cells, inhibits translation and leads to cell death. Targeting of the *HBEGF* gene with a CasX or dXR molecule and targeting gRNA should prevent toxin entry and allow survival of the cells, whereas cells treated with CasX and dXR molecules and a non-targeting gRNA should not survive. One day post-transfection, cells in each transfected well were split into 12 different wells in a 96-well plate and selected with puromycin. Over three days, cells were treated with six different concentrations of diphtheria toxin (0, 0.2, 2, 20, 200, and 2000 ng/mL), and biological duplicates were performed. After another two days, cells were split into fresh media, and total cell counts were measured on an ImageXpress Pico Automated Cell Imaging System.

Table 53: Sequences of spacers tested

Spacer ID	RNA sequence	SEQ ID NO	Molecule
34.19	ACUGGGAGGCUCAGCCCAUG	3266	CasX
34.21	UGUUCUGUCUUGAACUAGCU	3267	CasX
34.28	UGAGUGUCUUGUCUUGCUCA	3268	dXR
0.0	CGAGACGUAAUUACGUCUCG	3232	CasX & dXR

Results:

[0538] The results of the diphtheria toxin assay are illustrated in the plot in FIG. 53. dXR-mediated repression of the *HBEGF* gene resulted in survival of cells, but only at low doses of toxin (0.2 - 20 ng/mL). However, those same doses led to complete cell death in the control cells treated with non-targeting constructs. High doses (> 20 ng/mL) of toxin led to cell death in both the dXR and control samples, suggesting that the basal level of transcription permitted by dXR allows sufficient toxin to enter and trigger cell death. The results show that CasX-edited cells remained protected as editing of the locus leads to complete loss of functional protein. The non-targeting controls died at all doses, demonstrating the efficacy of the toxin when HBEGF is not repressed or edited.

[0539] The results show that dXR protects at low doses of toxin, demonstrating that this construct can be screened in a range of 0.2-20 ng/mL diphtheria toxin, with highest fold-enrichment between dXR and control observed at 0.2 ng/mL. Note that while CasX protects at all doses, repression by dXR still induced low basal expression of the target that led to toxicity of the cells at high doses of the toxin.

Example 17: Development of a selection scheme to identify improved repressor domains for inclusion in repressor fusion proteins To develop better LTRP fusion protein constructs, a library of transcriptional effector domains from many non-human species was tested in a selection assay. As KRAB domains are one of the largest and most rapidly-evolved domains in vertebrates, repressor domains from species not previously evaluated were anticipated to provide improved strength and permanence of repression.

Materials and Methods:

Identification of candidate repressor domains:

[0540] Homologs of KRAB domains were identified by downloading all sequences annotated with Prosite accession ps50805 (the accession number for KRAB domains). All domains were extended by 100 amino acids (with the annotation centered in the middle) to include potential unannotated functional sequences. In addition, HMMER, a tool to identify domains, was run on a set of high-quality primate annotations from recently completed alignments of long-read primate genome assemblies described (Warren, WC, et al. Sequence diversity analyses of an improved rhesus macaque genome enhance its biomedical utility. Science 370, Issue 6523, eabc6617in (2020); Fiddes, IT, et al. Comparative Annotation Toolkit (CAT)-simultaneous clade

and personal genome annotation. Genome Res. 28(7):1029 (2018); Mao, Y, et al. A high-quality bonobo genome refines the analysis of hominid evolution. Nature 594:77 (2021)), to identify domains in these assemblies, most of which were not present in UniProt. The search resulted in 32,120 unique sequences from 159 different organisms for testing for their potency in transcriptional repression. Additionally, 580 random amino acid sequence 80 residues in length were included in the library as negative controls, and 304 human KRAB domains were included based on work by Tycko, J. et al. (*Cell.* 2020 Dec 23;183(7):2020-2035).

Screening methods:

[0541] The domains described above were synthesized as DNA oligos, amplified, and cloned into a dCasX491 C-terminal GS linker lentiviral construct along with guide scaffold 174 (SEQ ID NO: 1744) with Spacer 34.28, to repress *HBEGF* and confer survival in a diphtheria toxin selection as described in Example 16, above. For each domain, the C-terminal GS linker was synonymously substituted to produce unique DNA barcodes that could be differentiated by NGS allowing internal technical replicates to be assessed in each pooled experiment. These plasmids were used to generate the lentiviral constructs of the library.

[0542] HEK293T cells were transduced, treated with 1 μg/mL puromycin to remove untransduced cells, and selection was carried out at 2 ng/mL diphtheria toxin for 48 hours. gDNA was extracted, amplified, and sequenced as described above. gDNA samples were also extracted, amplified, and sequenced from the cells before selection with diphtheria toxin, as a control. Two independent replicates were performed for the diphtheria toxin selection.

Assessment of B2M repression:

[0543] Representative domains were cloned into a dCasX491 C-terminal GS linker lentiviral construct along with guide scaffold 316 (SEQ ID NO: 1746) with spacer 7.15 (GGAAUGCCCGCCAGCGCGAC; SEQ ID NO: 3110), targeting the *B2M* locus. Separately, representative domains were cloned into a dCasX491 C-terminal GS linker lentiviral construct along with guide scaffold 174 (SEQ ID NO: 1744) with spacer 7.37 (GGCCGAGAUGUCUCGCUCCG; SEQ ID NO: 3137), targeting the *B2M* locus. The lentiviral plasmid constructs encoding dXRs with various domains were generated using standard molecular cloning techniques. These constructs included sequences encoding dCasX491, and a KRAB domain from ZNF10, ZIM3, or one of the domains tested in the library. Cloned and sequence-validated constructs were midi-prepped and subjected to quality assessment prior to transfection in HEK293T cells.

[0544] HEK293T cells were seeded at a density of 30,000 cells in each well of a 96-well plate. The next day, each well was transiently transfected using lipofectamine with 100 ng of dXR plasmids, each containing a dXR construct with a different domain and a gRNA having a targeting spacer to the *B2M* locus. Experimental controls included dXR constructs with KRAB domains from ZNF10 or ZIM3, domains that were in the library but not among the top 95 or 1597 most effective repressors, or dCas9-ZNF10, each with a corresponding *B2M*-targeting gRNA. Each construct was tested in triplicate. 24 hours post-transfection, cells were selected with 1μg/mL puromycin for two days. Seven or ten days after transfection, cells were harvested for editing repression analysis by analyzing B2M protein expression via HLA immunostaining followed by flow cytometry. B2M expression was determined by using an antibody that would detect the B2M-dependent HLA protein expressed on the cell surface. HLA+ cells were measured using the Attune™ NxT flow cytometer.

Data analysis:

[0545] To understand the diversity of protein sequences in the tested library, an evolutionary scale modeling (ESM) transformer (ESM-1b) was applied to the initial library of 32,120 domain amino acid sequences to generate a high dimensional representation of the sequences (Rives, A. et al. *Proc Natl Acad Sci USA*. 2021 Apr 13;118(15)). Next, Uniform Manifold Approximation and Projection (UMAP) was applied to reduce the data set to a two-dimensional representation of the sequence diversity (McInnes, L., Healy, J., *ArXiv e-prints* 1802.03426, 2018). Using this technique, 75 clusters of domain sequences were identified.

[0546] Protein sequence motifs were generated using the STREME algorithm (Bailey, T., *Bioinformatics*. 2021 Mar 24;37(18):2834-2840) to identify motifs enriched in strong repressors.

Results:

[0547] Selections were performed to identify the domains out of a library of 32,120 unique sequences that were the most potent transcriptional repressors. The fold change in the abundance of each domain in the library before and after selection was calculated for each barcode-domain pair such that together the two independent replicates of the experiment represent 12 measurements of each domain's fitness.

[0548] FIG. 54 shows the range of $\log_2$(fold change) values for the entire library, the randomized sequences that served as negative controls, a positive control set of KRAB domains that were shown to have a $\log_2$(fold change) greater than 1 on day 5 of the HT-recruit experiment performed by Tycko et al. (*Cell*. 2020 Dec 23;183(7):2020-2035). As shown in FIG.

54, the diphtheria toxin selection successfully enriched for domains that were more potent transcriptional repressors. The negative control sequences were de-enriched from the library following selection.

[0549] To identify the domains that were reproducibly enriched in the post-selection library, a p-value threshold of less than 0.01 and a $\log_2$(fold change) threshold of greater than 2 was set. 1597 domains met these criteria. P-values were calculated via the MAGeCK algorithm which uses a permutation test and false discovery rate adjustment for multiple testing (Wei, L. et al. *Genome Biol.* 2014;15(12):554). The $\log_2$(fold change) values of these top 1597 repressor domains are shown in FIG. 54, and the amino acid sequences, p-values, and $\log_2$(fold change) values are provided in Table 54, below. In contrast, ZIM3 had a $\log_2$(fold change) of 1.7787, standard ZNF10 had a $\log_2$(fold change) of 1.3637, and an alternate ZNF10 corresponding to the ZNF10 KRAB domain used in Tycko, J. et al. (*Cell.* 2020 Dec 23;183(7):2020-2035) had a $\log_2$(fold change) of 1.6182. Therefore, the 1597 top repressor domains were substantially superior transcriptional repressors to ZNF10 and ZIM3. Many of these top repressor domains contained amino acids with residues that are predicted to stabilize interactions with the Trim28 protein when compared to ZIM3 and ZNF10 (Stoll, G.A. et al., *bioRxiv* 2022.03.17.484746).

[0550] To further narrow down the list of repressor domains while maintaining a breadth of amino acid sequence diversity, a set of 95 repressor domains was chosen from within the 1597 by selecting the most effective repressor from each cluster, as well as the top 25 best repressors of the 1597, as shown in Table 54.

Table 54: List of 1,597 repressor domain candidates identified from the high-throughput screen assessing dXR repression of the *HBEGF* gene and subsequent application of the following criteria: p-value < 0.01 and $\log_2$(fold change) > 2

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
Top 95 most effective repressor domains				
DOMAIN_7694	Columba livia	130	3.7111	1.13E-04
DOMAIN_10123	Rattus norvegicus	131	3.6356	8.11E-06
DOMAIN_15507	Cebus imitator	132	3.8531	1.53E-07
DOMAIN_17905	Chimp	133	2.5038	5.60E-04
DOMAIN_20505	Chlorocebus sabaeus	134	3.4989	2.91E-06
DOMAIN_26749	Ophiophagus hannah	135	5.4323	1.53E-07
DOMAIN_27604	Ailuropoda melanoleuca	136	2.8198	6.05E-05

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_29304	Peromyscus maniculatus bairdii	137	4.0496	1.53E-07
DOMAIN_30173	Phyllostomus discolor	138	2.2538	5.41E-04
DOMAIN_737	Bonobo	139	4.544	1.53E-07
DOMAIN_10331	Colobus angolensis palliatus	140	3.6796	1.53E-07
DOMAIN_10948	Colobus angolensis palliatus	141	3.2959	2.30E-06
DOMAIN_11029	Mandrillus leucophaeus	142	3.5748	1.53E-07
DOMAIN_17358	Bos indicus x Bos taurus	143	4.9878	1.53E-07
DOMAIN_17759	Felis catus	144	3.3159	1.38E-06
DOMAIN_18258	Physeter macrocephalus	145	3.75	3.42E-04
DOMAIN_19804	Callorhinus ursinus	146	3.8217	1.53E-07
DOMAIN_221	Bonobo	147	3.5533	3.06E-06
DOMAIN_881	Bonobo	148	4.3546	4.59E-07
DOMAIN_2380	Orangutan	149	3.2024	1.74E-04
DOMAIN_2942	Gibbon	150	3.3658	1.38E-06
DOMAIN_4687	Marmoset	151	5.2288	3.22E-06
DOMAIN_4806	Marmoset	152	3.3896	1.58E-04
DOMAIN_4968	Marmoset	153	3.0315	0.0022262
DOMAIN_5066	Marmoset	154	2.9062	0.0067409
DOMAIN_5290	Owl Monkey	155	3.0993	5.16E-05
DOMAIN_5463	Owl Monkey	156	3.2102	0.0022788
DOMAIN_6248	Saimiri boliviensis boliviensis	157	2.4415	0.0056883
DOMAIN_6445	Alligator sinensis	158	3.1151	4.51E-04
DOMAIN_6802	Pantherophis guttatus	159	3.0403	5.18E-04
DOMAIN_6807	Xenopus laevis	160	3.1615	5.16E-05
DOMAIN_7255	Microcaecilia unicolor	161	4.5265	1.38E-06
DOMAIN_8503	Mus caroli	162	2.8193	0.003503
DOMAIN_8790	Marmota monax	163	2.7436	2.06E-04
DOMAIN_8853	Mesocricetus auratus	164	4.6199	1.53E-07
DOMAIN_9114	Peromyscus maniculatus bairdii	165	2.2058	0.0048423
DOMAIN_9331	Peromyscus maniculatus bairdii	166	4.1063	4.59E-07
DOMAIN_9538	Mus musculus	167	3.5443	1.20E-04
DOMAIN_9960	Octodon degus	168	3.4751	1.07E-06
DOMAIN_10277	Dipodomys ordii	169	2.8257	4.16E-04
DOMAIN_10577	Colobus angolensis palliatus	170	4.1248	1.53E-07

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_11348	Chlorocebus sabaeus	171	3.3651	2.95E-05
DOMAIN_11386	Capra hircus	172	3.7637	4.75E-06
DOMAIN_11486	Bos mutus	173	4.8326	1.53E-07
DOMAIN_11683	Nomascus leucogenys	174	2.9249	0.0015672
DOMAIN_12292	Sus scrofa	175	4.3194	1.53E-07
DOMAIN_12452	Neophocaena asiaeorientalis asiaeorientalis	176	3.8774	5.05E-06
DOMAIN_12631	Macaca fascicularis	177	3.6926	1.53E-07
DOMAIN_13331	Macaca fascicularis	178	3.5154	2.15E-04
DOMAIN_13468	Phascolarctos cinereus	179	4.1548	1.38E-06
DOMAIN_13539	Gorilla	180	3.4924	1.79E-05
DOMAIN_14659	Acinonyx jubatus	181	4.0495	1.06E-05
DOMAIN_14755	Cebus imitator	182	3.1667	1.88E-04
DOMAIN_15126	Callithrix jacchus	183	2.9781	4.08E-04
DOMAIN_16444	Acinonyx jubatus	184	3.2246	2.30E-06
DOMAIN_16688	Lipotes vexillifer	185	3.5601	4.26E-05
DOMAIN_16806	Sapajus apella	186	3.9386	1.53E-07
DOMAIN_17317	Otolemur garnettii	187	3.4551	1.81E-04
DOMAIN_17432	Otolemur garnettii	188	3.11	1.36E-05
DOMAIN_18137	Monodelphis domestica	189	3.292	3.51E-05
DOMAIN_18216	Physeter macrocephalus	190	3.0602	9.40E-04
DOMAIN_18563	Owl Monkey	191	3.0406	0.0034849
DOMAIN_19229	Enhydra lutris kenyoni	192	4.0294	5.01E-05
DOMAIN_19460	Monodelphis domestica	193	3.995	1.97E-05
DOMAIN_19476	Owl Monkey	194	4.1343	1.53E-07
DOMAIN_19821	Rhinopithecus roxellana	195	3.583	1.53E-07
DOMAIN_19892	Ursus maritimus	196	3.1396	5.21E-04
DOMAIN_19896	Ovis aries	197	2.2228	1.58E-04
DOMAIN_19949	Callorhinus ursinus	198	3.2903	2.62E-04
DOMAIN_21247	Neovison vison	199	2.741	0.0043129
DOMAIN_21317	Pteropus vampyrus	200	4.0893	1.18E-05
DOMAIN_21336	Equus caballus	201	2.738	0.005135
DOMAIN_21603	Lipotes vexillifer	202	2.8535	4.35E-04
DOMAIN_21755	Equus caballus	203	3.1889	0.0028238
DOMAIN_22153	Zalophus californianus	204	3.6967	3.52E-06

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_22270	Bonobo	205	2.3813	0.0030391
DOMAIN_23394	Vicugna pacos	206	4.0769	3.06E-07
DOMAIN_23723	Carlito syrichta	207	3.5301	8.71E-05
DOMAIN_24125	Saimiri boliviensis boliviensis	208	3.9692	1.53E-07
DOMAIN_24458	Lynx pardinus	209	3.4012	9.66E-05
DOMAIN_24663	Myotis brandtii	210	2.9806	1.49E-04
DOMAIN_25289	Ursus maritimus	211	3.4113	7.70E-05
DOMAIN_25379	Sapajus apella	212	3.5892	1.53E-07
DOMAIN_25405	Desmodus rotundus	213	3.8846	3.20E-05
DOMAIN_26070	Geotrypetes seraphini	214	3.7958	1.53E-07
DOMAIN_26322	Geotrypetes seraphini	215	2.9265	7.13E-04
DOMAIN_26732	Meleagris gallopavo	216	2.7548	0.0057183
DOMAIN_27060	Gopherus agassizii	217	2.7943	0.0029172
DOMAIN_27385	Octodon degus	218	4.1339	2.77E-05
DOMAIN_27506	Bos mutus	219	3.8121	4.29E-06
DOMAIN_27811	Callithrix jacchus	220	2.9728	8.34E-05
DOMAIN_28640	Colinus virginianus	221	3.624	4.13E-06
DOMAIN_28803	Monodelphis domestica	222	3.0697	2.07E-05
DOMAIN_30661	Physeter macrocephalus	223	2.15	4.76E-05
DOMAIN_31643	Micrurus lemniscatus lemniscatus	224	3.8782	3.57E-04
Remaining repressor domains in the top 1597 most effective repressor domains				
DOMAIN_10870	Vicugna pacos	225	2.5964	0.004315
DOMAIN_10918	Odobenus rosmarus divergens	226	3.2079	9.21E-04
DOMAIN_92	Bonobo	227	2.1475	0.0021413
DOMAIN_98	Bonobo	228	2.7848	0.0055875
DOMAIN_134	Bonobo	229	2.9322	0.004676
DOMAIN_143	Bonobo	230	3.63	3.17E-05
DOMAIN_145	Bonobo	231	3.1497	4.09E-05
DOMAIN_214	Bonobo	232	2.1073	0.00941
DOMAIN_225	Bonobo	233	2.259	0.0013991
DOMAIN_226	Bonobo	234	3.0188	2.76E-04
DOMAIN_235	Bonobo	235	2.9615	0.0016622
DOMAIN_302	Bonobo	236	2.5092	0.0033327
DOMAIN_313	Bonobo	237	2.4558	0.0049862

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_344	Bonobo	238	2.4948	0.0087725
DOMAIN_362	Bonobo	239	3.6736	2.38E-04
DOMAIN_382	Bonobo	240	3.1625	0.0019781
DOMAIN_389	Bonobo	241	3.011	3.42E-04
DOMAIN_407	Bonobo	242	3.8312	1.59E-04
DOMAIN_418	Bonobo	243	3.2429	1.37E-04
DOMAIN_419	Bonobo	244	3.5913	5.13E-05
DOMAIN_421	Bonobo	245	3.2969	1.06E-05
DOMAIN_451	Bonobo	246	3.0774	0.0018269
DOMAIN_504	Bonobo	247	3.2187	4.17E-04
DOMAIN_516	Bonobo	248	2.0448	0.0018554
DOMAIN_621	Bonobo	249	2.1025	0.0034678
DOMAIN_623	Bonobo	250	3.3299	6.50E-04
DOMAIN_624	Bonobo	251	2.8281	0.0031625
DOMAIN_629	Bonobo	252	3.6318	1.09E-05
DOMAIN_668	Bonobo	253	2.9256	6.60E-04
DOMAIN_718	Bonobo	254	3.9	8.73E-06
DOMAIN_731	Bonobo	255	2.1318	0.0058273
DOMAIN_749	Bonobo	256	3.1162	0.0060655
DOMAIN_759	Bonobo	257	3.3019	0.0046077
DOMAIN_761	Bonobo	258	3.181	9.64E-04
DOMAIN_784	Bonobo	259	2.4886	0.0083818
DOMAIN_801	Bonobo	260	2.4863	0.0040602
DOMAIN_802	Bonobo	261	2.6563	5.66E-04
DOMAIN_811	Bonobo	262	2.4706	0.0035997
DOMAIN_812	Bonobo	263	2.8201	0.0013526
DOMAIN_888	Bonobo	264	2.8951	0.0033756
DOMAIN_893	Bonobo	265	2.7511	5.41E-04
DOMAIN_938	Bonobo	266	2.2926	0.0040367
DOMAIN_966	Chimp	267	3.3535	5.49E-04
DOMAIN_972	Chimp	268	3.7627	5.59E-05
DOMAIN_980	Chimp	269	2.9297	0.0011707
DOMAIN_987	Chimp	270	2.6881	5.48E-04
DOMAIN_999	Chimp	271	2.7361	0.0038248

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_1006	Chimp	272	3.2119	1.28E-04
DOMAIN_1079	Chimp	273	3.7915	3.90E-05
DOMAIN_1137	Chimp	274	3.1719	4.58E-04
DOMAIN_1153	Chimp	275	3.7928	5.16E-04
DOMAIN_1184	Chimp	276	3.2772	5.47E-04
DOMAIN_1237	Chimp	277	2.1795	0.0059151
DOMAIN_1242	Chimp	278	2.7144	0.0037672
DOMAIN_1247	Chimp	279	2.9622	4.18E-04
DOMAIN_1378	Gorilla	280	3.2279	0.0022191
DOMAIN_1381	Gorilla	281	4.1424	3.35E-05
DOMAIN_1382	Gorilla	282	3.0579	1.91E-04
DOMAIN_1457	Gorilla	283	2.6896	0.0026956
DOMAIN_1523	Gorilla	284	2.8607	0.0042127
DOMAIN_1539	Gorilla	285	2.9337	0.0028055
DOMAIN_1561	Gorilla	286	2.8783	0.0011557
DOMAIN_1565	Gorilla	287	2.771	3.04E-04
DOMAIN_1578	Gorilla	288	3.4875	5.97E-04
DOMAIN_1621	Gorilla	289	3.3004	1.20E-04
DOMAIN_1790	Gorilla	290	3.0669	0.0038707
DOMAIN_1816	Gorilla	291	3.108	0.0011178
DOMAIN_1818	Gorilla	292	3.2866	6.15E-04
DOMAIN_1822	Gorilla	293	2.4697	1.04E-04
DOMAIN_1870	Gorilla	294	2.215	0.0044522
DOMAIN_1875	Gorilla	295	2.5576	0.0043383
DOMAIN_1893	Gorilla	296	2.3898	0.0043422
DOMAIN_1946	Orangutan	297	3.1449	9.41E-04
DOMAIN_1952	Orangutan	298	3.0762	5.53E-04
DOMAIN_1964	Orangutan	299	2.3009	0.0099771
DOMAIN_1978	Orangutan	300	3.2215	0.0029968
DOMAIN_2014	Orangutan	301	2.7323	3.95E-04
DOMAIN_2034	Orangutan	302	3.7415	1.38E-06
DOMAIN_2119	Orangutan	303	2.2117	0.0054271
DOMAIN_2208	Orangutan	304	2.3044	0.009903
DOMAIN_2223	Orangutan	305	2.6106	0.0087315

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_2229	Orangutan	306	2.9337	0.0032308
DOMAIN_2245	Orangutan	307	3.2712	0.0012727
DOMAIN_2255	Orangutan	308	3.1952	0.002815
DOMAIN_2295	Orangutan	309	3.2816	6.61E-04
DOMAIN_2299	Orangutan	310	2.5125	0.0042678
DOMAIN_2376	Orangutan	311	2.1539	9.52E-04
DOMAIN_2391	Orangutan	312	2.4608	0.0045936
DOMAIN_2398	Orangutan	313	3.3125	3.44E-04
DOMAIN_2470	Orangutan	314	2.3815	0.0031273
DOMAIN_2499	Orangutan	315	3.114	0.0050479
DOMAIN_2563	Orangutan	316	2.8105	0.003781
DOMAIN_2576	Orangutan	317	3.1733	2.56E-04
DOMAIN_2590	Orangutan	318	2.8348	0.0091663
DOMAIN_2629	Orangutan	319	3.092	0.0015715
DOMAIN_2652	Orangutan	320	4.3981	4.59E-07
DOMAIN_2744	Gibbon	321	2.863	0.003897
DOMAIN_2754	Gibbon	322	3.7601	1.17E-04
DOMAIN_2786	Gibbon	323	2.5449	0.0037666
DOMAIN_2806	Gibbon	324	3.1649	0.0083733
DOMAIN_2808	Gibbon	325	2.6227	0.0079231
DOMAIN_2813	Gibbon	326	2.9522	4.12E-04
DOMAIN_2851	Gibbon	327	3.3945	3.80E-04
DOMAIN_2867	Gibbon	328	3.0591	4.79E-04
DOMAIN_2888	Gibbon	329	2.4267	0.0043214
DOMAIN_2891	Gibbon	330	2.7489	0.0082897
DOMAIN_2896	Gibbon	331	2.7253	0.0094587
DOMAIN_2904	Gibbon	332	2.8035	0.0019408
DOMAIN_2908	Gibbon	333	2.6452	0.0062379
DOMAIN_2943	Gibbon	334	2.9574	9.75E-04
DOMAIN_2962	Gibbon	335	2.1784	6.34E-04
DOMAIN_2992	Gibbon	336	2.6341	0.0045667
DOMAIN_2994	Gibbon	337	3.1921	0.0022412
DOMAIN_2997	Gibbon	338	2.9911	0.0016588
DOMAIN_3000	Gibbon	339	2.9522	5.36E-04

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_3062	Gibbon	340	2.6076	0.0035414
DOMAIN_3087	Gibbon	341	2.7999	5.44E-04
DOMAIN_3092	Gibbon	342	3.1954	2.80E-05
DOMAIN_3094	Gibbon	343	3.7195	2.83E-05
DOMAIN_3096	Gibbon	344	3.3962	2.16E-04
DOMAIN_3123	Gibbon	345	3.1293	1.88E-05
DOMAIN_3137	Gibbon	346	2.8303	0.0038836
DOMAIN_3300	Gibbon	347	3.0127	2.76E-04
DOMAIN_3328	Gibbon	348	2.3718	0.0015893
DOMAIN_3332	Gibbon	349	2.8786	0.0036582
DOMAIN_3335	Gibbon	350	4.0001	4.75E-06
DOMAIN_3336	Gibbon	351	3.5946	4.75E-06
DOMAIN_3337	Gibbon	352	2.9398	0.0053162
DOMAIN_3344	Gibbon	353	3.2218	4.60E-04
DOMAIN_3373	Gibbon	354	3.0768	0.0030033
DOMAIN_3434	Gibbon	355	2.4767	0.0035835
DOMAIN_3463	Gibbon	356	3.5462	5.96E-04
DOMAIN_3557	Rhesus	357	2.4416	0.0024889
DOMAIN_3575	Rhesus	358	3.7842	1.53E-07
DOMAIN_3585	Rhesus	359	2.4981	0.0036466
DOMAIN_3586	Rhesus	360	2.365	0.0033728
DOMAIN_3602	Rhesus	361	2.0444	0.0061662
DOMAIN_3661	Rhesus	362	2.4083	0.0088114
DOMAIN_3691	Rhesus	363	2.8393	0.0018244
DOMAIN_3759	Rhesus	364	2.5324	0.004454
DOMAIN_3760	Rhesus	365	2.7025	0.0017399
DOMAIN_3781	Rhesus	366	2.9317	0.0024892
DOMAIN_3782	Rhesus	367	2.3058	0.0048669
DOMAIN_3803	Rhesus	368	3.0165	0.0083941
DOMAIN_3832	Rhesus	369	2.7334	0.0026058
DOMAIN_4030	Rhesus	370	2.5274	0.0038526
DOMAIN_4036	Rhesus	371	2.7725	0.001577
DOMAIN_4046	Rhesus	372	2.7847	0.0088564
DOMAIN_4120	Rhesus	373	3.3237	4.55E-05

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_4121	Rhesus	374	3.3195	1.53E-07
DOMAIN_4126	Rhesus	375	3.529	1.65E-04
DOMAIN_4129	Rhesus	376	3.7382	9.33E-04
DOMAIN_4184	Rhesus	377	3.2397	9.40E-04
DOMAIN_4185	Rhesus	378	2.9116	0.0032623
DOMAIN_4199	Rhesus	379	2.6844	0.0058444
DOMAIN_4239	Rhesus	380	4.4187	9.19E-07
DOMAIN_4394	Marmoset	381	3.8103	4.09E-05
DOMAIN_4425	Marmoset	382	2.9741	0.0087646
DOMAIN_4461	Marmoset	383	3.0094	0.0076595
DOMAIN_4463	Marmoset	384	2.9717	0.008252
DOMAIN_4515	Marmoset	385	4.2166	1.21E-05
DOMAIN_4516	Marmoset	386	2.7603	0.0027577
DOMAIN_4534	Marmoset	387	2.6242	0.0034292
DOMAIN_4574	Marmoset	388	2.7135	9.16E-04
DOMAIN_4580	Marmoset	389	2.9618	3.22E-06
DOMAIN_4589	Marmoset	390	2.507	0.0070104
DOMAIN_4665	Marmoset	391	3.2985	0.0011116
DOMAIN_4705	Marmoset	392	3.5232	5.02E-04
DOMAIN_4722	Marmoset	393	4.8639	1.53E-07
DOMAIN_4748	Marmoset	394	3.0477	5.73E-04
DOMAIN_4749	Marmoset	395	3.5545	2.83E-05
DOMAIN_4751	Marmoset	396	3.238	4.91E-05
DOMAIN_4774	Marmoset	397	2.8894	0.0029528
DOMAIN_4823	Marmoset	398	2.7527	0.0083334
DOMAIN_4913	Marmoset	399	2.8878	0.0028098
DOMAIN_4921	Marmoset	400	3.5291	4.44E-06
DOMAIN_4922	Marmoset	401	4.0258	1.82E-05
DOMAIN_4978	Marmoset	402	2.7787	0.0025526
DOMAIN_5005	Marmoset	403	2.8406	0.00183
DOMAIN_5006	Marmoset	404	3.8614	1.38E-06
DOMAIN_5029	Marmoset	405	2.2642	0.0022609
DOMAIN_5031	Marmoset	406	2.8605	0.0025559
DOMAIN_5060	Marmoset	407	2.6043	8.74E-04

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_5096	Marmoset	408	2.456	0.008963
DOMAIN_5099	Marmoset	409	3.1407	0.0021138
DOMAIN_5102	Marmoset	410	2.7241	0.0024099
DOMAIN_5103	Marmoset	411	2.1016	0.0093552
DOMAIN_5125	Marmoset	412	2.911	0.0015369
DOMAIN_5188	OwlMonkey	413	2.1842	0.0046295
DOMAIN_5201	OwlMonkey	414	3.3658	1.53E-07
DOMAIN_5217	OwlMonkey	415	2.4689	0.0031316
DOMAIN_5235	OwlMonkey	416	3.437	4.62E-04
DOMAIN_5246	OwlMonkey	417	2.7473	0.0042075
DOMAIN_5248	OwlMonkey	418	4.1052	1.53E-07
DOMAIN_5267	OwlMonkey	419	3.1247	0.0016383
DOMAIN_5273	OwlMonkey	420	2.4023	0.0069063
DOMAIN_5299	OwlMonkey	421	2.7399	0.0093892
DOMAIN_5337	OwlMonkey	422	3.7616	4.52E-05
DOMAIN_5370	OwlMonkey	423	3.0452	0.0088803
DOMAIN_5440	OwlMonkey	424	2.7871	0.0048658
DOMAIN_5485	OwlMonkey	425	2.7826	0.0080202
DOMAIN_5489	OwlMonkey	426	2.6774	0.0021808
DOMAIN_5518	OwlMonkey	427	2.8542	0.0030235
DOMAIN_5527	OwlMonkey	428	3.1092	0.0016793
DOMAIN_5603	OwlMonkey	429	3.2806	0.0015418
DOMAIN_5716	OwlMonkey	430	3.0606	5.36E-04
DOMAIN_5742	Homo sapiens	431	2.8617	0.0029913
DOMAIN_5765	Rattus norvegicus	432	4.2973	1.53E-07
DOMAIN_5774	Homo sapiens	433	2.9608	3.75E-05
DOMAIN_5782	Homo sapiens	434	2.9086	4.56E-04
DOMAIN_5791	Homo sapiens	435	2.6823	0.0051494
DOMAIN_5792	Homo sapiens	436	3.0218	8.56E-04
DOMAIN_5806	Homo sapiens	437	2.866	0.0037801
DOMAIN_5822	Homo sapiens	438	2.9335	0.0074467
DOMAIN_5843	Homo sapiens	439	3.1821	2.83E-05
DOMAIN_5866	Homo sapiens	440	2.6362	0.0080677
DOMAIN_5883	Homo sapiens	441	3.0097	5.52E-04

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_5896	Bos taurus	442	2.9429	0.0023166
DOMAIN_5901	Homo sapiens	443	3.2935	0.0012981
DOMAIN_5914	Homo sapiens	444	2.5527	0.0029099
DOMAIN_5921	Homo sapiens	445	2.4715	0.00101
DOMAIN_5943	Mus musculus	446	2.501	0.0027917
DOMAIN_5946	Homo sapiens	447	3.2998	1.38E-06
DOMAIN_5968	Bos taurus	448	3.2856	3.86E-04
DOMAIN_5984	Homo sapiens	449	2.9852	2.37E-04
DOMAIN_5989	Mus musculus	450	3.6632	9.30E-04
DOMAIN_5994	Orangutan	451	2.9214	5.04E-04
DOMAIN_6038	Homo sapiens	452	3.3315	2.59E-04
DOMAIN_6053	Orangutan	453	3.2566	1.21E-04
DOMAIN_6063	Homo sapiens	454	3.5653	0.0019059
DOMAIN_6078	Homo sapiens	455	2.6246	0.0075453
DOMAIN_6134	Homo sapiens	456	2.7081	0.0034203
DOMAIN_6169	Homo sapiens	457	3.3909	1.68E-06
DOMAIN_6172	Homo sapiens	458	3.883	1.07E-06
DOMAIN_6249	Saimiri boliviensis boliviensis	459	3.5469	4.44E-06
DOMAIN_6293	Rattus norvegicus	460	2.6707	0.0034812
DOMAIN_6354	Terrapene carolina triunguis	461	2.4812	0.0095055
DOMAIN_6356	Terrapene carolina triunguis	462	2.9197	0.0031965
DOMAIN_6382	Gopherus agassizii	463	3.2875	1.66E-04
DOMAIN_6398	Gopherus agassizii	464	2.8238	0.0059966
DOMAIN_6410	Podarcis muralis	465	2.7633	0.0034243
DOMAIN_6433	Podarcis muralis	466	3.0313	1.16E-04
DOMAIN_6458	Gopherus agassizii	467	2.8973	0.0048435
DOMAIN_6472	Alligator sinensis	468	2.9259	0.0052565
DOMAIN_6482	Paroedura picta	469	3.3106	0.0019705
DOMAIN_6501	Paroedura picta	470	3.4172	0.0010204
DOMAIN_6539	Paroedura picta	471	3.2371	0.0025654
DOMAIN_6555	Paroedura picta	472	3.534	4.92E-04
DOMAIN_6577	Terrapene carolina triunguis	473	3.3168	3.95E-04
DOMAIN_6595	Terrapene carolina triunguis	474	2.2407	0.0027133
DOMAIN_6599	Terrapene carolina triunguis	475	3.3653	4.49E-05

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_6697	Podarcis muralis	476	2.6712	7.35E-04
DOMAIN_6737	Microcaecilia unicolor	477	2.4861	0.0065704
DOMAIN_6738	Microcaecilia unicolor	478	2.9275	7.79E-04
DOMAIN_6741	Microcaecilia unicolor	479	3.5726	2.50E-04
DOMAIN_6866	Alligator mississippiensis	480	3.5825	1.02E-04
DOMAIN_6936	Callipepla squamata	481	3.5294	9.07E-04
DOMAIN_6938	Alligator mississippiensis	482	2.6093	0.0020584
DOMAIN_6952	Alligator mississippiensis	483	2.3403	0.0084774
DOMAIN_6970	Phasianus colchicus	484	3.343	3.02E-04
DOMAIN_7000	Phasianus colchicus	485	2.8279	0.0039843
DOMAIN_7098	Microcaecilia unicolor	486	2.7074	0.0030553
DOMAIN_7109	Microcaecilia unicolor	487	2.9932	0.0077318
DOMAIN_7123	Microcaecilia unicolor	488	2.9074	0.0043723
DOMAIN_7166	Microcaecilia unicolor	489	3.1419	5.72E-04
DOMAIN_7183	Microcaecilia unicolor	490	2.4918	1.27E-04
DOMAIN_7184	Microcaecilia unicolor	491	2.2019	0.0099168
DOMAIN_7328	Terrapene carolina triunguis	492	3.1808	5.04E-05
DOMAIN_7353	Microcaecilia unicolor	493	2.6649	0.0042219
DOMAIN_7365	Microcaecilia unicolor	494	2.597	0.0042403
DOMAIN_7480	Gopherus agassizii	495	3.1707	5.44E-04
DOMAIN_7510	Gopherus agassizii	496	3.0452	6.73E-04
DOMAIN_7534	Gopherus agassizii	497	3.4086	2.50E-04
DOMAIN_7553	Gopherus agassizii	498	2.9036	0.0088341
DOMAIN_7605	Alligator sinensis	499	2.8444	0.0018789
DOMAIN_7607	Alligator sinensis	500	2.7102	0.0018612
DOMAIN_7641	Gallus gallus	501	3.6727	4.51E-04
DOMAIN_7653	Gallus gallus	502	3.3772	0.0028364
DOMAIN_7678	Chelonia mydas	503	2.7348	0.0039197
DOMAIN_7711	Columba livia	504	3.7965	1.67E-05
DOMAIN_7716	Pogona vitticeps	505	3.1171	0.0011931
DOMAIN_7745	Meleagris gallopavo	506	3.4946	0.0016126
DOMAIN_7750	Columba livia	507	2.8111	0.0012249
DOMAIN_7774	Pogona vitticeps	508	3.427	8.09E-04
DOMAIN_7796	Chelonia mydas	509	2.9513	1.04E-04

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_7813	Columba livia	510	3.4645	7.95E-04
DOMAIN_7824	Columba livia	511	2.9383	5.45E-04
DOMAIN_7850	Terrapene carolina triunguis	512	3.124	5.15E-04
DOMAIN_7895	Patagioenas fasciata monilis	513	3.2254	0.0013863
DOMAIN_7925	Gallus gallus	514	3.3919	0.0025195
DOMAIN_8012	Callipepla squamata	515	3.2046	0.0023734
DOMAIN_8013	Callipepla squamata	516	3.9783	2.13E-05
DOMAIN_8014	Callipepla squamata	517	3.7425	6.23E-05
DOMAIN_8036	Alligator mississippiensis	518	2.3504	0.0094483
DOMAIN_8041	Dipodomys ordii	519	3.6568	3.47E-04
DOMAIN_8054	Cavia porcellus	520	3.5889	4.15E-05
DOMAIN_8148	Cricetulus griseus	521	3.6904	4.82E-05
DOMAIN_8151	Cricetulus griseus	522	3.1527	0.0034782
DOMAIN_8154	Cricetulus griseus	523	2.8774	0.0027807
DOMAIN_8167	Mus musculus	524	3.9362	1.04E-04
DOMAIN_8179	Mesocricetus auratus	525	3.0623	0.0026242
DOMAIN_8182	Mus caroli	526	2.2411	0.0018051
DOMAIN_8216	Cricetulus griseus	527	3.1747	9.05E-05
DOMAIN_8226	Rattus norvegicus	528	2.4602	0.0090772
DOMAIN_8235	Mus caroli	529	2.8965	0.0012522
DOMAIN_8282	Peromyscus maniculatus bairdii	530	3.9882	1.07E-06
DOMAIN_8289	Peromyscus maniculatus bairdii	531	3.3026	2.94E-04
DOMAIN_8301	Mesocricetus auratus	532	3.1084	0.0017647
DOMAIN_8303	Ictidomys tridecemlineatus	533	3.6843	1.34E-04
DOMAIN_8305	Ictidomys tridecemlineatus	534	2.5554	0.0084633
DOMAIN_8308	Marmota monax	535	2.6564	3.69E-04
DOMAIN_8317	Mus caroli	536	3.3091	2.40E-05
DOMAIN_8340	Peromyscus maniculatus bairdii	537	2.2764	0.0086378
DOMAIN_8353	Peromyscus maniculatus bairdii	538	2.7989	4.14E-04
DOMAIN_8370	Cavia porcellus	539	3.5737	2.58E-04
DOMAIN_8412	Mus musculus	540	2.4486	0.0077639
DOMAIN_8418	Cricetulus griseus	541	2.4014	0.001307
DOMAIN_8424	Peromyscus maniculatus bairdii	542	2.7945	0.0019818
DOMAIN_8425	Peromyscus maniculatus bairdii	543	2.8391	0.004804

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_8460	Peromyscus maniculatus bairdii	544	3.1352	6.66E-05
DOMAIN_8467	Mesocricetus auratus	545	3.8156	7.15E-05
DOMAIN_8489	Mus caroli	546	2.8336	0.0042299
DOMAIN_8492	Mus musculus	547	3.3107	0.0032374
DOMAIN_8502	Cricetulus griseus	548	2.1429	4.22E-04
DOMAIN_8545	Rattus norvegicus	549	3.1044	0.0011282
DOMAIN_8546	Mus musculus	550	2.9439	0.0033958
DOMAIN_8547	Mus caroli	551	3.3997	0.0022286
DOMAIN_8549	Mus caroli	552	2.8508	0.0052033
DOMAIN_8555	Cricetulus griseus	553	3.2852	5.62E-05
DOMAIN_8618	Mesocricetus auratus	554	2.6363	0.008293
DOMAIN_8688	Mus musculus	555	2.4409	2.00E-04
DOMAIN_8689	Mus musculus	556	2.8548	6.62E-04
DOMAIN_8712	Mesocricetus auratus	557	2.7776	0.0028768
DOMAIN_8742	Peromyscus maniculatus bairdii	558	2.3354	0.002149
DOMAIN_8746	Mesocricetus auratus	559	3.317	1.64E-04
DOMAIN_8789	Marmota monax	560	3.1756	0.0021937
DOMAIN_8793	Mus caroli	561	2.6774	9.60E-05
DOMAIN_8816	Peromyscus maniculatus bairdii	562	2.4156	2.32E-04
DOMAIN_8830	Cavia porcellus	563	3.0644	0.0025588
DOMAIN_8839	Peromyscus maniculatus bairdii	564	3.0637	0.0036542
DOMAIN_8844	Peromyscus maniculatus bairdii	565	4.1629	7.81E-06
DOMAIN_8850	Peromyscus maniculatus bairdii	566	2.695	0.0040575
DOMAIN_8862	Marmota monax	567	2.3521	0.0061537
DOMAIN_8881	Cricetulus griseus	568	3.743	1.49E-05
DOMAIN_8886	Cricetulus griseus	569	3.5727	1.94E-05
DOMAIN_8899	Mesocricetus auratus	570	3.2182	9.45E-05
DOMAIN_8931	Cricetulus griseus	571	2.9497	8.73E-04
DOMAIN_8936	Cricetulus griseus	572	4.3486	1.07E-06
DOMAIN_8953	Mus caroli	573	2.5941	0.0032969
DOMAIN_8982	Mesocricetus auratus	574	3.1585	3.54E-05
DOMAIN_8989	Marmota monax	575	2.2309	0.0094553
DOMAIN_9012	Mus musculus	576	2.3905	0.0070058
DOMAIN_9042	Mus caroli	577	2.5894	0.0033885

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_9060	Cricetulus griseus	578	2.5974	0.0027286
DOMAIN_9119	Mesocricetus auratus	579	2.2985	0.0052412
DOMAIN_9141	Mus caroli	580	3.035	2.62E-05
DOMAIN_9159	Dipodomys ordii	581	3.0141	0.0023052
DOMAIN_9174	Peromyscus maniculatus bairdii	582	2.5194	0.0035749
DOMAIN_9175	Peromyscus maniculatus bairdii	583	2.4231	0.0042293
DOMAIN_9189	Heterocephalus glaber	584	3.3801	1.76E-04
DOMAIN_9192	Mus caroli	585	2.7981	0.008526
DOMAIN_9217	Mesocricetus auratus	586	3.8919	5.43E-05
DOMAIN_9235	Mus musculus	587	2.7307	0.0035899
DOMAIN_9250	Marmota monax	588	3.466	0.0012007
DOMAIN_9265	Mus musculus	589	2.1221	0.0021172
DOMAIN_9290	Peromyscus maniculatus bairdii	590	4.256	1.07E-06
DOMAIN_9303	Marmota monax	591	2.5344	0.0051732
DOMAIN_9313	Mus musculus	592	2.7692	0.0061916
DOMAIN_9324	Peromyscus maniculatus bairdii	593	3.1782	0.0020198
DOMAIN_9329	Peromyscus maniculatus bairdii	594	4.263	7.81E-06
DOMAIN_9332	Peromyscus maniculatus bairdii	595	3.9002	1.38E-06
DOMAIN_9356	Ictidomys tridecemlineatus	596	2.9297	0.0037302
DOMAIN_9389	Marmota monax	597	3.1785	2.65E-05
DOMAIN_9424	Dipodomys ordii	598	3.771	1.53E-07
DOMAIN_9435	Fukomys damarensis	599	3.1672	3.01E-04
DOMAIN_9446	Marmota monax	600	2.8722	3.80E-04
DOMAIN_9489	Dipodomys ordii	601	3.0215	0.0074336
DOMAIN_9503	Ictidomys tridecemlineatus	602	2.9864	0.0021536
DOMAIN_9526	Mesocricetus auratus	603	2.9435	0.0042492
DOMAIN_9530	Mesocricetus auratus	604	2.7003	0.0026178
DOMAIN_9541	Dipodomys ordii	605	2.8442	0.0028404
DOMAIN_9542	Octodon degus	606	2.6734	0.0036809
DOMAIN_9544	Octodon degus	607	2.9143	0.0054966
DOMAIN_9559	Mus caroli	608	3.327	0.001653
DOMAIN_9563	Mus musculus	609	3.7261	3.81E-05
DOMAIN_9576	Octodon degus	610	2.1952	0.0094564
DOMAIN_9617	Mesocricetus auratus	611	2.4034	0.0040152

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_9643	Dipodomys ordii	612	3.4306	0.0023603
DOMAIN_9697	Octodon degus	613	2.7566	0.0063579
DOMAIN_9704	Dipodomys ordii	614	3.1674	0.0013462
DOMAIN_9706	Octodon degus	615	2.821	0.0041809
DOMAIN_9713	Cricetulus griseus	616	3.0323	0.002243
DOMAIN_9716	Mus caroli	617	2.9009	0.0040762
DOMAIN_9723	Mus caroli	618	2.1903	0.0058971
DOMAIN_9725	Mus caroli	619	2.9654	0.0028095
DOMAIN_9776	Marmota monax	620	2.6258	0.0084697
DOMAIN_9787	Mus caroli	621	3.2962	8.37E-05
DOMAIN_9789	Mus musculus	622	2.5801	0.0012534
DOMAIN_9822	Ictidomys tridecemlineatus	623	2.9382	0.0065879
DOMAIN_9824	Heterocephalus glaber	624	3.1306	8.34E-05
DOMAIN_9827	Mus caroli	625	2.1904	0.0077554
DOMAIN_9843	Mus musculus	626	2.3385	0.0035982
DOMAIN_9846	Cricetulus griseus	627	2.7865	0.0025033
DOMAIN_9857	Mesocricetus auratus	628	3.3666	8.92E-04
DOMAIN_9858	Mesocricetus auratus	629	3.0047	1.33E-04
DOMAIN_9878	Marmota monax	630	3.7349	2.61E-04
DOMAIN_9891	Mus caroli	631	2.8116	3.13E-04
DOMAIN_9915	Mus caroli	632	3.4011	3.45E-04
DOMAIN_9962	Rattus norvegicus	633	2.7249	0.004063
DOMAIN_9993	Rattus norvegicus	634	2.7601	0.0035973
DOMAIN_10018	Octodon degus	635	3.3372	4.27E-04
DOMAIN_10041	Mus caroli	636	2.8662	0.0062437
DOMAIN_10044	Mus musculus	637	2.826	0.0043095
DOMAIN_10050	Octodon degus	638	3.3147	0.0020066
DOMAIN_10057	Mus musculus	639	2.2961	0.0026799
DOMAIN_10091	Fukomys damarensis	640	2.1679	4.36E-04
DOMAIN_10127	Peromyscus maniculatus bairdii	641	3.6912	3.83E-06
DOMAIN_10160	Ictidomys tridecemlineatus	642	2.9333	4.23E-04
DOMAIN_10184	Mus caroli	643	4.2854	1.53E-07
DOMAIN_10241	Octodon degus	644	3.5766	8.19E-05
DOMAIN_10257	Octodon degus	645	3.1757	5.20E-04

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_10294	Mus musculus	646	2.689	0.0067073
DOMAIN_10334	Mustela putorius furo	647	3.3529	5.07E-05
DOMAIN_10351	Delphinapterus leucas	648	3.3309	3.78E-04
DOMAIN_10359	Delphinapterus leucas	649	2.9199	0.0036842
DOMAIN_10381	Vicugna pacos	650	2.215	0.0057838
DOMAIN_10386	Odobenus rosmarus divergens	651	2.8337	0.0028753
DOMAIN_10403	Vicugna pacos	652	3.3993	0.0016441
DOMAIN_10420	Odobenus rosmarus divergens	653	3.7185	1.01E-04
DOMAIN_10425	Delphinapterus leucas	654	2.8616	0.0041775
DOMAIN_10427	Carlito syrichta	655	2.3719	0.0078328
DOMAIN_10491	Vicugna pacos	656	3.7199	0.0012761
DOMAIN_10495	Delphinapterus leucas	657	3.4705	5.27E-04
DOMAIN_10526	Delphinapterus leucas	658	2.4499	0.0033355
DOMAIN_10573	Cervus elaphus hippelaphus	659	2.4077	5.02E-04
DOMAIN_10612	Vicugna pacos	660	2.4997	0.0035134
DOMAIN_10613	Odobenus rosmarus divergens	661	2.9148	5.62E-05
DOMAIN_10623	Carlito syrichta	662	3.2233	0.0018333
DOMAIN_10646	Delphinapterus leucas	663	2.9354	0.0036496
DOMAIN_10647	Delphinapterus leucas	664	2.9514	7.60E-04
DOMAIN_10675	Ornithorhynchus anatinus	665	3.2777	5.13E-05
DOMAIN_10684	Odobenus rosmarus divergens	666	4.531	1.64E-05
DOMAIN_10704	Colobus angolensis palliatus	667	3.1582	0.004292
DOMAIN_10705	Colobus angolensis palliatus	668	3.6392	4.09E-05
DOMAIN_10733	Odobenus rosmarus divergens	669	3.315	0.0028523
DOMAIN_10762	Erinaceus europaeus	670	3.9254	4.55E-05
DOMAIN_10763	Mustela putorius furo	671	2.5924	0.0073193
DOMAIN_10765	Mustela putorius furo	672	2.5661	0.0076445
DOMAIN_10807	Erinaceus europaeus	673	3.5237	1.54E-04
DOMAIN_10882	Vicugna pacos	674	3.6289	2.93E-04
DOMAIN_10902	Vicugna pacos	675	3.1052	0.0096752
DOMAIN_10917	Odobenus rosmarus divergens	676	3.7871	1.53E-07
DOMAIN_10943	Cervus elaphus hippelaphus	677	2.5554	0.0037715
DOMAIN_10974	Chelonia mydas	678	2.6444	0.0091318
DOMAIN_11006	Loxodonta africana	679	2.6669	6.71E-04

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_11024	Suricata suricatta	680	3.2397	2.77E-04
DOMAIN_11031	Mandrillus leucophaeus	681	2.5516	0.005857
DOMAIN_11034	Mandrillus leucophaeus	682	2.2541	0.0042161
DOMAIN_11040	Sus scrofa	683	3.5161	3.39E-04
DOMAIN_11049	Neophocaena asiaeorientalis asiaeorientalis	684	2.7072	0.0015299
DOMAIN_11053	Nomascus leucogenys	685	3.677	4.44E-06
DOMAIN_11069	Capra hircus	686	3.2745	0.0036948
DOMAIN_11071	Chrysochloris asiatica	687	3.1268	0.0012421
DOMAIN_11097	Mandrillus leucophaeus	688	3.239	0.0011508
DOMAIN_11110	Sus scrofa	689	3.6632	4.76E-04
DOMAIN_11129	Nomascus leucogenys	690	2.3864	1.88E-04
DOMAIN_11130	Nomascus leucogenys	691	2.3487	6.64E-04
DOMAIN_11132	Bos indicus	692	3.5671	3.08E-05
DOMAIN_11157	Suricata suricatta	693	3.6671	8.22E-05
DOMAIN_11158	Chrysochloris asiatica	694	2.6889	0.0035388
DOMAIN_11162	Mandrillus leucophaeus	695	3.2804	2.65E-04
DOMAIN_11178	Sus scrofa	696	2.4845	0.0043413
DOMAIN_11192	Neophocaena asiaeorientalis asiaeorientalis	697	2.8798	2.10E-04
DOMAIN_11202	Nomascus leucogenys	698	3.5851	4.18E-05
DOMAIN_11204	Nomascus leucogenys	699	3.5793	5.22E-05
DOMAIN_11225	Capra hircus	700	3.606	0.0011566
DOMAIN_11227	Capra hircus	701	2.7556	0.0032733
DOMAIN_11264	Sus scrofa	702	3.5019	5.64E-04
DOMAIN_11265	Sus scrofa	703	4.2521	1.53E-07
DOMAIN_11282	Suricata suricatta	704	3.536	1.53E-07
DOMAIN_11289	Suricata suricatta	705	2.69	2.48E-04
DOMAIN_11291	Suricata suricatta	706	4.0373	4.59E-07
DOMAIN_11307	Mandrillus leucophaeus	707	3.6383	1.07E-06
DOMAIN_11312	Sus scrofa	708	3.8532	9.26E-05
DOMAIN_11314	Sus scrofa	709	2.9575	0.0015357
DOMAIN_11321	Nomascus leucogenys	710	2.9718	0.0086853
DOMAIN_11331	Capra hircus	711	3.0611	4.37E-04
DOMAIN_11332	Capra hircus	712	3.0468	2.19E-04
DOMAIN_11356	Sus scrofa	713	2.6549	0.0027629

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_11359	Sus scrofa	714	3.1036	0.0092232
DOMAIN_11381	Nomascus leucogenys	715	3.1705	4.83E-04
DOMAIN_11393	Suricata suricatta	716	3.4256	1.65E-04
DOMAIN_11401	Suricata suricatta	717	2.6345	0.0077459
DOMAIN_11403	Suricata suricatta	718	3.4222	2.27E-04
DOMAIN_11413	Sus scrofa	719	2.1814	0.0084919
DOMAIN_11433	Neophocaena asiaeorientalis asiaeorientalis	720	3.3986	1.91E-05
DOMAIN_11446	Nomascus leucogenys	721	2.6971	3.26E-04
DOMAIN_11461	Equus caballus	722	2.508	0.0090515
DOMAIN_11466	Suricata suricatta	723	3.4716	0.0027896
DOMAIN_11470	Mandrillus leucophaeus	724	3.1038	0.0012895
DOMAIN_11502	Trichechus manatus latirostris	725	3.601	4.21E-05
DOMAIN_11505	Trichechus manatus latirostris	726	3.0969	9.19E-07
DOMAIN_11534	Sus scrofa	727	3.8118	1.91E-05
DOMAIN_11554	Nomascus leucogenys	728	3.0498	4.11E-04
DOMAIN_11567	Zalophus californianus	729	3.4239	0.0010611
DOMAIN_11581	Equus caballus	730	3.1882	4.10E-04
DOMAIN_11612	Loxodonta africana	731	3.3006	0.0040119
DOMAIN_11621	Chrysochloris asiatica	732	3.2074	5.42E-04
DOMAIN_11643	Nomascus leucogenys	733	2.3544	0.0020207
DOMAIN_11662	Capra hircus	734	3.7889	2.36E-04
DOMAIN_11672	Suricata suricatta	735	3.318	0.0022931
DOMAIN_11701	Capra hircus	736	2.5282	0.0084694
DOMAIN_11726	Sus scrofa	737	3.4183	1.09E-05
DOMAIN_11749	Chlorocebus sabaeus	738	3.2721	0.0023817
DOMAIN_11753	Mandrillus leucophaeus	739	2.6119	0.0062269
DOMAIN_11760	Neophocaena asiaeorientalis asiaeorientalis	740	2.8102	0.0039794
DOMAIN_11796	Sus scrofa	741	2.2811	0.0010219
DOMAIN_11813	Canis lupus familiaris	742	3.5195	7.62E-04
DOMAIN_11825	Mandrillus leucophaeus	743	3.9893	1.53E-07
DOMAIN_11851	Nomascus leucogenys	744	3.0241	1.32E-04
DOMAIN_11858	Canis lupus familiaris	745	3.6419	1.53E-07
DOMAIN_11862	Canis lupus familiaris	746	2.8817	0.0032412
DOMAIN_11865	Muntiacus muntjak	747	3.0474	0.0026931

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_11868	Mandrillus leucophaeus	748	3.5158	4.44E-06
DOMAIN_11908	Canis lupus familiaris	749	2.894	0.0035529
DOMAIN_11923	Sus scrofa	750	3.2271	0.0018734
DOMAIN_11925	Mandrillus leucophaeus	751	3.5582	3.04E-04
DOMAIN_11928	Neophocaena asiaeorientalis asiaeorientalis	752	3.751	7.59E-04
DOMAIN_11933	Neophocaena asiaeorientalis asiaeorientalis	753	4.1135	1.52E-05
DOMAIN_11944	Bos indicus	754	3.2762	0.0022727
DOMAIN_11950	Canis lupus familiaris	755	4.3869	2.91E-06
DOMAIN_11988	Muntiacus muntjak	756	3.5916	3.83E-06
DOMAIN_11996	Canis lupus familiaris	757	3.0831	0.0015161
DOMAIN_11999	Canis lupus familiaris	758	3.7891	5.04E-05
DOMAIN_12001	Mandrillus leucophaeus	759	2.4384	0.0057376
DOMAIN_12021	Canis lupus familiaris	760	2.4637	0.0018489
DOMAIN_12051	Muntiacus muntjak	761	2.7925	0.0039375
DOMAIN_12057	Muntiacus muntjak	762	2.0631	0.0086017
DOMAIN_12079	Muntiacus muntjak	763	2.4029	0.0095567
DOMAIN_12092	Bos mutus	764	3.1752	1.82E-05
DOMAIN_12114	Neophocaena asiaeorientalis asiaeorientalis	765	3.3227	6.62E-04
DOMAIN_12133	Canis lupus familiaris	766	3.0204	0.0034751
DOMAIN_12139	Canis lupus familiaris	767	2.8097	0.0066678
DOMAIN_12147	Neophocaena asiaeorientalis asiaeorientalis	768	2.6974	9.14E-04
DOMAIN_12158	Nomascus leucogenys	769	3.0332	0.006631
DOMAIN_12187	Canis lupus familiaris	770	3.6477	5.13E-05
DOMAIN_12191	Muntiacus muntjak	771	3.6138	8.18E-04
DOMAIN_12195	Canis lupus familiaris	772	2.9023	1.11E-04
DOMAIN_12206	Bos mutus	773	2.9101	5.13E-04
DOMAIN_12210	Bos indicus	774	3.6136	0.0018284
DOMAIN_12214	Muntiacus muntjak	775	2.613	9.76E-04
DOMAIN_12231	Nomascus leucogenys	776	2.6703	0.00421
DOMAIN_12261	Neophocaena asiaeorientalis asiaeorientalis	777	2.7989	0.0029785
DOMAIN_12285	Gorilla	778	2.2573	0.0091023
DOMAIN_12313	Bos indicus	779	2.6903	0.0012684
DOMAIN_12320	Muntiacus muntjak	780	2.5075	0.0023021

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_12365	Nomascus leucogenys	781	3.5626	7.78E-04
DOMAIN_12395	Ailuropoda melanoleuca	782	3.1504	3.56E-04
DOMAIN_12459	Bos indicus	783	4.0425	3.06E-06
DOMAIN_12463	Ailuropoda melanoleuca	784	3.2567	0.009339
DOMAIN_12467	Gorilla	785	2.9575	4.85E-04
DOMAIN_12498	Muntiacus muntjak	786	2.8947	0.0075569
DOMAIN_12499	Muntiacus muntjak	787	2.2932	0.0064341
DOMAIN_12508	Gorilla	788	3.0173	0.0024497
DOMAIN_12511	Gorilla	789	3.0694	0.0023557
DOMAIN_12517	Lynx canadensis	790	2.6983	0.0017522
DOMAIN_12544	Gorilla	791	3.306	4.83E-04
DOMAIN_12550	Ailuropoda melanoleuca	792	3.0229	2.37E-04
DOMAIN_12576	Gorilla	793	3.04	0.0044151
DOMAIN_12590	Bos indicus	794	2.5531	0.0020023
DOMAIN_12591	Bos indicus	795	3.4169	0.0011553
DOMAIN_12598	Muntiacus muntjak	796	3.3709	4.18E-05
DOMAIN_12599	Muntiacus muntjak	797	2.2098	0.007064
DOMAIN_12630	Macaca fascicularis	798	3.6424	4.03E-05
DOMAIN_12646	Myotis lucifugus	799	3.487	0.0014708
DOMAIN_12686	Phascolarctos cinereus	800	2.76	0.0032103
DOMAIN_12698	Phascolarctos cinereus	801	2.8029	0.0066675
DOMAIN_12704	Myotis lucifugus	802	2.9127	0.0034078
DOMAIN_12712	Puma concolor	803	2.1195	0.008023
DOMAIN_12728	Lynx canadensis	804	3.1999	9.49E-04
DOMAIN_12734	Phyllostomus discolor	805	3.5207	1.38E-06
DOMAIN_12755	Oryctolagus cuniculus	806	2.8082	0.0061475
DOMAIN_12764	Desmodus rotundus	807	3.9505	1.53E-07
DOMAIN_12769	Macaca fascicularis	808	2.0555	0.0080928
DOMAIN_12777	Phascolarctos cinereus	809	2.1778	0.0057731
DOMAIN_12780	Phascolarctos cinereus	810	3.2671	1.01E-04
DOMAIN_12801	Sapajus apella	811	2.0238	0.006988
DOMAIN_12811	Macaca fascicularis	812	2.4278	0.0068959
DOMAIN_12815	Macaca fascicularis	813	2.7296	0.0029445
DOMAIN_12818	Macaca fascicularis	814	3.6211	9.69E-05

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_12829	Phascolarctos cinereus	815	3.3994	3.20E-04
DOMAIN_12831	Phascolarctos cinereus	816	2.9845	0.0029084
DOMAIN_12839	Oryctolagus cuniculus	817	3.4039	3.03E-04
DOMAIN_12849	Muntiacus muntjak	818	4.1042	1.53E-07
DOMAIN_12896	Macaca fascicularis	819	2.0413	0.0010397
DOMAIN_12901	Macaca fascicularis	820	3.5686	4.75E-06
DOMAIN_12902	Macaca fascicularis	821	3.3489	0.0016432
DOMAIN_12912	Puma concolor	822	2.7422	4.78E-04
DOMAIN_12941	Phyllostomus discolor	823	2.4012	0.0062382
DOMAIN_12985	Phascolarctos cinereus	824	3.7331	3.05E-05
DOMAIN_13004	Macaca fascicularis	825	3.2216	1.37E-04
DOMAIN_13022	Phascolarctos cinereus	826	3.0468	0.003082
DOMAIN_13029	Myotis lucifugus	827	3.1708	3.58E-04
DOMAIN_13062	Ursus maritimus	828	2.9752	2.10E-04
DOMAIN_13068	Ailuropoda melanoleuca	829	3.6132	2.43E-05
DOMAIN_13089	Sapajus apella	830	2.8761	0.0065934
DOMAIN_13111	Ailuropoda melanoleuca	831	2.6151	0.0090675
DOMAIN_13121	Macaca fascicularis	832	3.353	3.98E-04
DOMAIN_13125	Macaca fascicularis	833	3.2101	3.31E-04
DOMAIN_13171	Phascolarctos cinereus	834	3.0052	0.0061932
DOMAIN_13193	Sapajus apella	835	3.8948	1.53E-07
DOMAIN_13227	Oryctolagus cuniculus	836	2.3234	0.0034855
DOMAIN_13269	Desmodus rotundus	837	2.7236	0.0010081
DOMAIN_13277	Macaca fascicularis	838	2.9151	4.66E-04
DOMAIN_13282	Phascolarctos cinereus	839	3.5504	8.75E-04
DOMAIN_13284	Phascolarctos cinereus	840	3.0903	0.0057642
DOMAIN_13293	Myotis lucifugus	841	2.5884	6.56E-04
DOMAIN_13325	Macaca fascicularis	842	2.4051	0.0085787
DOMAIN_13332	Phascolarctos cinereus	843	2.685	0.0052498
DOMAIN_13333	Phascolarctos cinereus	844	2.9787	0.0079948
DOMAIN_13339	Puma concolor	845	3.2731	5.64E-04
DOMAIN_13346	Oryctolagus cuniculus	846	2.9551	0.0031649
DOMAIN_13363	Phyllostomus discolor	847	2.2178	0.0041619
DOMAIN_13364	Macaca fascicularis	848	3.5606	2.40E-05

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_13379	Phascolarctos cinereus	849	3.2967	0.0018734
DOMAIN_13380	Myotis lucifugus	850	3.6615	1.09E-05
DOMAIN_13387	Sapajus apella	851	2.8731	0.001777
DOMAIN_13417	Ailuropoda melanoleuca	852	3.7056	1.17E-04
DOMAIN_13439	Sapajus apella	853	2.5091	0.0050786
DOMAIN_13470	Phascolarctos cinereus	854	3.7598	2.40E-05
DOMAIN_13486	Puma concolor	855	3.4895	7.93E-04
DOMAIN_13501	Macaca fascicularis	856	2.8162	0.0083892
DOMAIN_13509	Phascolarctos cinereus	857	2.8053	0.00351
DOMAIN_13516	Phascolarctos cinereus	858	2.4421	0.0034809
DOMAIN_13536	Gorilla	859	3.3269	0.0064418
DOMAIN_13537	Ailuropoda melanoleuca	860	3.3265	8.83E-05
DOMAIN_13562	Phascolarctos cinereus	861	3.7608	4.71E-04
DOMAIN_13565	Phascolarctos cinereus	862	2.994	0.0032926
DOMAIN_13574	Puma concolor	863	3.1114	6.89E-04
DOMAIN_13591	Lynx canadensis	864	3.215	5.12E-04
DOMAIN_13601	Macaca fascicularis	865	2.4865	0.0065955
DOMAIN_13609	Phascolarctos cinereus	866	3.1787	0.002393
DOMAIN_13610	Phascolarctos cinereus	867	3.1925	0.0018707
DOMAIN_13644	Phascolarctos cinereus	868	3.2677	0.001927
DOMAIN_13648	Oryctolagus cuniculus	869	3.1393	0.0014022
DOMAIN_13650	Ailuropoda melanoleuca	870	3.8556	4.44E-06
DOMAIN_13664	Macaca fascicularis	871	2.7443	0.002582
DOMAIN_13670	Phascolarctos cinereus	872	3.154	4.21E-04
DOMAIN_13690	Sapajus apella	873	3.2587	0.0017001
DOMAIN_13691	Sapajus apella	874	2.6205	0.0033052
DOMAIN_13703	Lynx canadensis	875	3.7947	2.43E-05
DOMAIN_13705	Phyllostomus discolor	876	2.496	0.009207
DOMAIN_13722	Phascolarctos cinereus	877	2.4814	0.0058557
DOMAIN_13723	Phascolarctos cinereus	878	2.9677	0.0026349
DOMAIN_13733	Sapajus apella	879	3.3285	1.82E-04
DOMAIN_13783	Macaca fascicularis	880	2.5821	0.0093056
DOMAIN_13805	Lynx canadensis	881	3.1769	0.0088613
DOMAIN_13823	Macaca fascicularis	882	4.219	1.53E-07

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_13830	Phascolarctos cinereus	883	2.6435	0.0033465
DOMAIN_13832	Phascolarctos cinereus	884	2.9705	0.0077505
DOMAIN_13843	Phascolarctos cinereus	885	3.6119	1.81E-04
DOMAIN_13851	Canis lupus familiaris	886	2.6472	0.0033845
DOMAIN_13859	Macaca fascicularis	887	2.2006	0.0086366
DOMAIN_13878	Ailuropoda melanoleuca	888	4.3232	4.75E-06
DOMAIN_13880	Lynx canadensis	889	3.0991	0.0013743
DOMAIN_13907	Phascolarctos cinereus	890	2.4263	0.0084749
DOMAIN_13910	Bos mutus	891	2.9556	0.0048664
DOMAIN_13915	Muntiacus muntjak	892	2.8554	0.0080147
DOMAIN_13958	Phascolarctos cinereus	893	3.2926	9.78E-05
DOMAIN_13970	Lynx canadensis	894	2.89	0.0058701
DOMAIN_13979	Macaca fascicularis	895	2.6188	0.0016793
DOMAIN_13981	Phascolarctos cinereus	896	2.8041	0.0024451
DOMAIN_13984	Phascolarctos cinereus	897	2.8513	0.0029797
DOMAIN_13987	Myotis lucifugus	898	3.0633	4.59E-04
DOMAIN_13997	Puma concolor	899	2.984	2.51E-04
DOMAIN_14009	Ailuropoda melanoleuca	900	2.9207	5.05E-05
DOMAIN_14013	Ailuropoda melanoleuca	901	2.4619	0.0082352
DOMAIN_14031	Phyllostomus discolor	902	3.0963	0.0045422
DOMAIN_14040	Phascolarctos cinereus	903	3.0933	0.0065673
DOMAIN_14041	Phascolarctos cinereus	904	2.9069	0.0077333
DOMAIN_14049	Phascolarctos cinereus	905	2.7761	0.0052936
DOMAIN_14069	Lynx canadensis	906	2.9182	0.0020008
DOMAIN_14082	Phyllostomus discolor	907	3.2495	2.19E-04
DOMAIN_14083	Phyllostomus discolor	908	2.7465	0.0042213
DOMAIN_14108	Canis lupus familiaris	909	3.0621	0.004127
DOMAIN_14129	Lynx canadensis	910	2.8195	0.0026925
DOMAIN_14135	Bos mutus	911	2.426	0.0033513
DOMAIN_14147	Canis lupus familiaris	912	3.3683	2.59E-04
DOMAIN_14153	Muntiacus muntjak	913	2.883	0.0011637
DOMAIN_14197	Muntiacus muntjak	914	2.9589	0.0041555
DOMAIN_14219	Ailuropoda melanoleuca	915	2.6653	0.0035657
DOMAIN_14226	Lynx canadensis	916	3.1176	0.0020645

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_14228	Lynx canadensis	917	3.3445	7.54E-04
DOMAIN_14256	Lynx canadensis	918	2.4946	0.0066852
DOMAIN_14287	Bos indicus	919	3.6232	1.66E-04
DOMAIN_14295	Muntiacus muntjak	920	3.4018	7.22E-04
DOMAIN_14322	Desmodus rotundus	921	3.3716	1.94E-04
DOMAIN_14337	Muntiacus muntjak	922	3.2753	2.86E-05
DOMAIN_14338	Ailuropoda melanoleuca	923	3.1071	0.0022421
DOMAIN_14358	Lynx canadensis	924	2.7094	8.85E-04
DOMAIN_14365	Desmodus rotundus	925	3.0706	1.39E-04
DOMAIN_14373	Macaca fascicularis	926	2.5861	0.0069375
DOMAIN_14382	Phascolarctos cinereus	927	4.0523	7.52E-05
DOMAIN_14444	Phyllostomus discolor	928	2.4641	0.0037357
DOMAIN_14487	Ailuropoda melanoleuca	929	2.7981	0.0050538
DOMAIN_14526	Ailuropoda melanoleuca	930	3.2232	0.003818
DOMAIN_14532	Lynx canadensis	931	3.2071	2.43E-04
DOMAIN_14534	Lynx canadensis	932	2.8122	0.0039834
DOMAIN_14546	Muntiacus muntjak	933	3.5039	5.01E-05
DOMAIN_14551	Ailuropoda melanoleuca	934	3.6894	2.30E-06
DOMAIN_14557	Lynx canadensis	935	2.9876	2.85E-04
DOMAIN_14574	Gorilla	936	3.3356	7.83E-04
DOMAIN_14576	Ailuropoda melanoleuca	937	3.2158	0.0028459
DOMAIN_14602	Gorilla	938	3.2145	0.0037718
DOMAIN_14627	Acinonyx jubatus	939	2.9501	0.0033732
DOMAIN_14639	Rhesus	940	2.7046	0.0033915
DOMAIN_14714	Odocoileus virginianus texanus	941	3.2752	2.48E-04
DOMAIN_14746	Odocoileus virginianus texanus	942	2.605	0.0084645
DOMAIN_14773	Sapajus apella	943	3.5997	1.45E-05
DOMAIN_14794	Acinonyx jubatus	944	3.4295	4.09E-04
DOMAIN_14795	Rhinopithecus roxellana	945	2.8119	0.0024062
DOMAIN_14800	Rhinopithecus roxellana	946	2.274	0.0012494
DOMAIN_14815	Cebus imitator	947	3.3826	0.0075808
DOMAIN_14820	Callithrix jacchus	948	2.8836	0.0021743
DOMAIN_14829	Rhinopithecus roxellana	949	2.7188	4.08E-04
DOMAIN_14845	Cebus imitator	950	2.7224	0.0041993

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_14849	Cebus imitator	951	2.3659	0.0093133
DOMAIN_14862	Callithrix jacchus	952	2.8116	0.0079314
DOMAIN_14864	Rhesus	953	3.3492	2.46E-04
DOMAIN_14885	Cebus imitator	954	3.5373	4.09E-05
DOMAIN_14901	Bos taurus	955	2.9774	0.0085175
DOMAIN_14905	Rhinopithecus roxellana	956	3.372	0.0034794
DOMAIN_14928	Callithrix jacchus	957	3.1547	2.58E-04
DOMAIN_14939	Callorhinus ursinus	958	2.3884	0.0071338
DOMAIN_14946	Acinonyx jubatus	959	3.2842	7.46E-04
DOMAIN_14948	Acinonyx jubatus	960	3.3727	1.73E-04
DOMAIN_14974	Sapajus apella	961	2.9963	0.0091608
DOMAIN_14977	Sapajus apella	962	3.0085	5.11E-04
DOMAIN_14978	Acinonyx jubatus	963	3.0358	0.0017363
DOMAIN_14983	Rhinopithecus roxellana	964	3.704	1.53E-07
DOMAIN_14994	Bison bison bison	965	2.4997	0.0054874
DOMAIN_14995	Cebus imitator	966	3.5057	4.13E-06
DOMAIN_15042	Ovis aries	967	3.0774	0.0045881
DOMAIN_15070	Callithrix jacchus	968	4.0108	2.60E-04
DOMAIN_15083	Ovis aries	969	2.7541	7.89E-04
DOMAIN_15086	Ovis aries	970	3.5994	2.56E-04
DOMAIN_15089	Vulpes vulpes	971	2.3585	0.0076298
DOMAIN_15102	Acinonyx jubatus	972	3.0929	0.0033921
DOMAIN_15103	Bison bison bison	973	2.652	0.0021839
DOMAIN_15119	Callithrix jacchus	974	3.3838	2.60E-06
DOMAIN_15137	Ovis aries	975	2.7071	0.0022528
DOMAIN_15138	Vulpes vulpes	976	3.1771	6.85E-04
DOMAIN_15159	Ovis aries	977	3.2135	0.0012084
DOMAIN_15171	Vulpes vulpes	978	3.2837	2.40E-05
DOMAIN_15174	Vulpes vulpes	979	3.1387	0.0033116
DOMAIN_15184	Acinonyx jubatus	980	3.0092	0.0021588
DOMAIN_15197	Acinonyx jubatus	981	3.0957	0.0012736
DOMAIN_15227	Rhinopithecus roxellana	982	3.5532	4.75E-06
DOMAIN_15233	Rhinopithecus roxellana	983	2.788	0.0046622
DOMAIN_15234	Acinonyx jubatus	984	3.546	0.0019916

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_15241	Odocoileus virginianus texanus	985	3.3955	3.85E-04
DOMAIN_15251	Callithrix jacchus	986	2.2209	9.47E-04
DOMAIN_15254	Callithrix jacchus	987	3.5159	2.32E-04
DOMAIN_15267	Ovis aries	988	2.8528	0.0020149
DOMAIN_15269	Ovis aries	989	2.0839	0.0057336
DOMAIN_15278	Callithrix jacchus	990	3.2523	0.0089241
DOMAIN_15279	Callithrix jacchus	991	3.8574	6.87E-05
DOMAIN_15352	Cebus imitator	992	3.0832	0.0079363
DOMAIN_15354	Tursiops truncatus	993	3.5099	5.16E-05
DOMAIN_15356	Acinonyx jubatus	994	3.5466	0.0019099
DOMAIN_15360	Neophocaena asiaeorientalis asiaeorientalis	995	3.2575	3.95E-04
DOMAIN_15363	Orangutan	996	4.3121	1.53E-07
DOMAIN_15391	Leptonychotes weddellii	997	3.9053	1.53E-07
DOMAIN_15406	Chimp	998	3.4616	1.53E-07
DOMAIN_15419	Rhinopithecus roxellana	999	2.6943	0.0012439
DOMAIN_15426	Odocoileus virginianus texanus	1000	2.9673	0.0024959
DOMAIN_15447	Rhinopithecus roxellana	1001	3.1112	0.0031907
DOMAIN_15451	Bison bison bison	1002	3.2905	0.0024601
DOMAIN_15527	Balaenoptera acutorostrata scammoni	1003	3.0354	0.0023685
DOMAIN_15536	Cebus imitator	1004	2.4515	0.0048713
DOMAIN_15540	Callithrix jacchus	1005	3.124	0.0020464
DOMAIN_15575	Callithrix jacchus	1006	2.594	0.0095671
DOMAIN_15577	Callithrix jacchus	1007	2.4456	0.0010642
DOMAIN_15581	Callorhinus ursinus	1008	3.2465	0.0031873
DOMAIN_15586	Callorhinus ursinus	1009	2.6157	0.002815
DOMAIN_15603	Cebus imitator	1010	3.5111	0.0027084
DOMAIN_15605	Cebus imitator	1011	3.8196	2.50E-04
DOMAIN_15634	Delphinapterus leucas	1012	3.3574	0.0025587
DOMAIN_15636	Chimp	1013	2.2086	0.0062339
DOMAIN_15638	Sapajus apella	1014	3.4277	1.53E-07
DOMAIN_15669	Callorhinus ursinus	1015	2.8865	0.0027889
DOMAIN_15687	Cebus imitator	1016	2.5362	0.0063187
DOMAIN_15688	Cebus imitator	1017	3.2098	6.98E-04
DOMAIN_15693	Rhesus	1018	3.8571	9.95E-06

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_15699	Bos taurus	1019	3.5255	7.09E-04
DOMAIN_15753	Ovis aries	1020	3.1699	0.0035272
DOMAIN_15759	Ovis aries	1021	3.1884	0.0011061
DOMAIN_15764	Otolemur garnettii	1022	3.107	3.97E-04
DOMAIN_15800	Otolemur garnettii	1023	3.4462	1.80E-04
DOMAIN_15814	Rhesus	1024	3.9503	3.26E-05
DOMAIN_15823	Ovis aries	1025	2.8458	0.0034405
DOMAIN_15834	Otolemur garnettii	1026	3.7629	1.30E-05
DOMAIN_15839	Callithrix jacchus	1027	2.3399	0.0090833
DOMAIN_15863	Vulpes vulpes	1028	2.7434	0.0042734
DOMAIN_15931	Ovis aries	1029	3.0861	0.0028731
DOMAIN_15940	Enhydra lutris kenyoni	1030	2.8684	0.007571
DOMAIN_15956	Bos taurus	1031	3.2271	4.36E-04
DOMAIN_15972	Enhydra lutris kenyoni	1032	2.3299	1.05E-04
DOMAIN_16009	Zalophus californianus	1033	3.2738	0.0020718
DOMAIN_16011	Delphinapterus leucas	1034	4.3363	1.53E-07
DOMAIN_16017	Ovis aries	1035	2.6715	0.0041604
DOMAIN_16023	Rhinopithecus bieti	1036	2.2831	0.0064672
DOMAIN_16050	Ovis aries	1037	2.7105	0.0086883
DOMAIN_16063	Rhesus	1038	2.1603	0.0054023
DOMAIN_16084	Enhydra lutris kenyoni	1039	3.0131	0.0022672
DOMAIN_16115	Bos taurus	1040	2.9023	0.0027605
DOMAIN_16123	Ovis aries	1041	2.3799	0.0079176
DOMAIN_16147	Orangutan	1042	2.5699	4.83E-04
DOMAIN_16184	Ovis aries	1043	3.7743	4.44E-06
DOMAIN_16188	Otolemur garnettii	1044	2.5145	0.0014414
DOMAIN_16238	Orangutan	1045	3.8734	2.87E-04
DOMAIN_16246	Rhesus	1046	2.3971	3.89E-04
DOMAIN_16253	Ovis aries	1047	4.488	1.53E-07
DOMAIN_16266	Otolemur garnettii	1048	3.075	0.0019834
DOMAIN_16274	Otolemur garnettii	1049	2.7655	0.0014904
DOMAIN_16312	Vicugna pacos	1050	2.4302	0.0024702
DOMAIN_16323	Trichechus manatus latirostris	1051	4.0053	2.15E-04
DOMAIN_16340	Ovis aries	1052	2.778	0.0034068

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_16372	Odocoileus virginianus texanus	1053	4.2664	1.53E-07
DOMAIN_16378	Callithrix jacchus	1054	2.9868	0.0037718
DOMAIN_16399	Rhinopithecus roxellana	1055	4.0639	1.53E-07
DOMAIN_16408	Cebus imitator	1056	2.0194	0.009233
DOMAIN_16461	Cebus imitator	1057	3.1155	0.0020676
DOMAIN_16471	Acinonyx jubatus	1058	3.3465	0.0021006
DOMAIN_16478	Rhinopithecus roxellana	1059	2.8285	0.0023275
DOMAIN_16516	Rhesus	1060	3.8473	1.94E-05
DOMAIN_16517	Callithrix jacchus	1061	3.3189	2.60E-06
DOMAIN_16534	Acinonyx jubatus	1062	2.7531	0.0057425
DOMAIN_16556	Rhinopithecus roxellana	1063	2.4217	0.0084734
DOMAIN_16566	Odocoileus virginianus texanus	1064	3.3903	7.82E-04
DOMAIN_16576	Chimp	1065	2.6949	0.0021998
DOMAIN_16597	Cebus imitator	1066	2.9869	0.0023416
DOMAIN_16611	Papio anubis	1067	3.4786	1.53E-07
DOMAIN_16618	Ursus maritimus	1068	3.1184	0.0015351
DOMAIN_16629	Cebus imitator	1069	3.7569	1.57E-04
DOMAIN_16630	Cebus imitator	1070	3.2435	1.36E-04
DOMAIN_16638	Macaca nemestrina	1071	3.3871	0.0011337
DOMAIN_16648	Physeter macrocephalus	1072	3.629	1.88E-05
DOMAIN_16651	Delphinapterus leucas	1073	2.0926	0.0074143
DOMAIN_16659	Leptonychotes weddellii	1074	3.8913	2.37E-05
DOMAIN_16664	Leptonychotes weddellii	1075	3.4502	1.76E-05
DOMAIN_16673	Phascolarctos cinereus	1076	3.0938	0.0039727
DOMAIN_16677	Orangutan	1077	3.1577	0.0023254
DOMAIN_16694	Callorhinus ursinus	1078	2.0979	0.0094743
DOMAIN_16695	Callorhinus ursinus	1079	3.965	3.06E-07
DOMAIN_16696	Tursiops truncatus	1080	3.0806	0.002705
DOMAIN_16703	Phascolarctos cinereus	1081	3.3969	2.19E-04
DOMAIN_16731	Ursus arctos horribilis	1082	2.849	1.30E-05
DOMAIN_16734	Leptonychotes weddellii	1083	3.4791	2.57E-04
DOMAIN_16738	Chimp	1084	3.5957	8.11E-06
DOMAIN_16744	Enhydra lutris kenyoni	1085	3.637	6.38E-05
DOMAIN_16763	Monodelphis domestica	1086	2.9244	0.0053031

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_16771	Saimiri boliviensis boliviensis	1087	3.3025	0.0027295
DOMAIN_16773	Balaenoptera acutorostrata scammoni	1088	4.5309	1.38E-06
DOMAIN_16776	Callorhinus ursinus	1089	3.0877	0.0024757
DOMAIN_16809	Delphinapterus leucas	1090	2.4357	0.0068567
DOMAIN_16811	Balaenoptera acutorostrata scammoni	1091	3.5141	3.08E-04
DOMAIN_16856	Ursus maritimus	1092	2.7613	0.0040844
DOMAIN_16865	Papio anubis	1093	3.9619	1.53E-07
DOMAIN_16876	Callorhinus ursinus	1094	3.2183	4.66E-04
DOMAIN_16877	Rhinolophus ferrumequinum	1095	3.3745	3.78E-05
DOMAIN_16936	Rhinopithecus roxellana	1096	2.9808	0.0044295
DOMAIN_16953	Callorhinus ursinus	1097	3.3286	1.62E-04
DOMAIN_16973	Delphinapterus leucas	1098	3.0187	0.0041062
DOMAIN_16994	Odocoileus virginianus texanus	1099	3.0575	0.0025431
DOMAIN_17001	Rhinolophus ferrumequinum	1100	3.045	0.003661
DOMAIN_17023	Sapajus apella	1101	2.5472	0.0041588
DOMAIN_17027	Balaenoptera acutorostrata scammoni	1102	3.131	0.0028042
DOMAIN_17041	Rhinopithecus roxellana	1103	2.7589	0.0074146
DOMAIN_17062	Rhinopithecus roxellana	1104	3.2594	7.33E-05
DOMAIN_17105	Rhesus	1105	2.637	0.0054256
DOMAIN_17108	Phyllostomus discolor	1106	2.4499	0.0018315
DOMAIN_17134	Panthera pardus	1107	3.2502	0.0016926
DOMAIN_17139	Ursus arctos horribilis	1108	4.0326	2.13E-05
DOMAIN_17153	Ursus arctos horribilis	1109	2.1759	0.0043459
DOMAIN_17167	Ursus maritimus	1110	4.1644	1.52E-05
DOMAIN_17177	Physeter macrocephalus	1111	3.2446	0.002928
DOMAIN_17180	Zalophus californianus	1112	2.945	0.0082198
DOMAIN_17195	Ursus maritimus	1113	3.0566	0.0037464
DOMAIN_17202	Ursus arctos horribilis	1114	2.6589	0.0072284
DOMAIN_17206	Pteropus vampyrus	1115	3.7092	5.05E-06
DOMAIN_17234	Delphinapterus leucas	1116	2.0152	0.0059669
DOMAIN_17236	Rhinolophus ferrumequinum	1117	2.7166	0.0039056
DOMAIN_17241	Muntiacus muntjak	1118	2.2217	0.003544
DOMAIN_17264	Vicugna pacos	1119	3.0866	0.0021294
DOMAIN_17278	Tursiops truncatus	1120	3.4898	4.12E-05

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_17279	Bison bison bison	1121	3.591	8.11E-06
DOMAIN_17333	Camelus dromedarius	1122	2.8765	0.003642
DOMAIN_17340	Leptonychotes weddellii	1123	3.1536	5.34E-05
DOMAIN_17382	Leptonychotes weddellii	1124	3.075	0.0035284
DOMAIN_17383	Leptonychotes weddellii	1125	2.953	0.0032519
DOMAIN_17412	Ovis aries	1126	4.9319	1.53E-07
DOMAIN_17421	Vulpes vulpes	1127	3.3129	2.83E-05
DOMAIN_17474	Monodelphis domestica	1128	2.683	0.0036059
DOMAIN_17483	Cercocebus atys	1129	3.5742	3.44E-05
DOMAIN_17495	Neomonachus schauinslandi	1130	3.1828	5.59E-05
DOMAIN_17497	Monodelphis domestica	1131	2.8088	5.07E-05
DOMAIN_17509	Physeter macrocephalus	1132	3.438	8.07E-04
DOMAIN_17516	Monodelphis domestica	1133	3.1523	4.18E-04
DOMAIN_17525	Myotis davidii	1134	3.4986	7.28E-04
DOMAIN_17534	Cercocebus atys	1135	2.9374	0.0033612
DOMAIN_17547	Neomonachus schauinslandi	1136	3.2455	5.64E-04
DOMAIN_17548	Neomonachus schauinslandi	1137	2.8002	5.08E-04
DOMAIN_17574	Cercocebus atys	1138	3.4893	2.80E-05
DOMAIN_17632	Monodelphis domestica	1139	3.3689	2.06E-04
DOMAIN_17658	Monodelphis domestica	1140	3.8781	1.99E-06
DOMAIN_17662	Monodelphis domestica	1141	2.7612	0.0040459
DOMAIN_17666	Monodelphis domestica	1142	2.6895	0.002059
DOMAIN_17671	Monodelphis domestica	1143	3.0937	0.008519
DOMAIN_17689	Cercocebus atys	1144	3.6469	1.53E-07
DOMAIN_17704	Neomonachus schauinslandi	1145	3.1047	0.0028404
DOMAIN_17714	Monodelphis domestica	1146	2.2724	0.0043612
DOMAIN_17717	Physeter macrocephalus	1147	2.9442	7.54E-04
DOMAIN_17748	Leptonychotes weddellii	1148	3.0918	2.44E-04
DOMAIN_17752	Leptonychotes weddellii	1149	3.2541	4.59E-04
DOMAIN_17775	Camelus dromedarius	1150	2.6595	0.0033885
DOMAIN_17798	Orangutan	1151	3.3458	5.16E-05
DOMAIN_17801	Orangutan	1152	2.9733	0.0022819
DOMAIN_17871	Leptonychotes weddellii	1153	3.1894	1.49E-05
DOMAIN_17873	Leptonychotes weddellii	1154	3.4076	3.00E-04

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_17890	Cercocebus atys	1155	4.2356	2.80E-05
DOMAIN_17898	Enhydra lutris kenyoni	1156	3.2117	0.0034476
DOMAIN_17903	Orangutan	1157	2.4683	0.0030976
DOMAIN_17925	Otolemur garnettii	1158	2.7982	0.0042639
DOMAIN_18048	OwlMonkey	1159	2.5186	0.0087422
DOMAIN_18083	Papio anubis	1160	2.9283	4.79E-04
DOMAIN_18100	Neomonachus schauinslandi	1161	2.3606	0.0061598
DOMAIN_18103	Monodelphis domestica	1162	2.7334	0.0056181
DOMAIN_18136	Monodelphis domestica	1163	2.7288	6.75E-04
DOMAIN_18155	Sarcophilus harrisii	1164	2.7528	0.0052222
DOMAIN_18161	Cercocebus atys	1165	2.6663	0.0060803
DOMAIN_18181	Physeter macrocephalus	1166	4.696	4.59E-07
DOMAIN_18203	Monodelphis domestica	1167	3.7912	4.81E-04
DOMAIN_18206	Monodelphis domestica	1168	2.3929	0.0046062
DOMAIN_18214	Physeter macrocephalus	1169	2.6389	0.0094737
DOMAIN_18227	OwlMonkey	1170	3.5267	5.66E-06
DOMAIN_18241	Leptonychotes weddellii	1171	3.8187	9.60E-05
DOMAIN_18243	Felis catus	1172	3.5331	6.96E-04
DOMAIN_18244	Leptonychotes weddellii	1173	3.1726	0.0050817
DOMAIN_18272	Neomonachus schauinslandi	1174	2.9141	0.0085916
DOMAIN_18303	Monodelphis domestica	1175	2.9174	0.0018489
DOMAIN_18312	Monodelphis domestica	1176	2.8473	8.20E-04
DOMAIN_18323	Monodelphis domestica	1177	2.3956	0.0040336
DOMAIN_18325	Monodelphis domestica	1178	2.7636	0.0038297
DOMAIN_18332	Monodelphis domestica	1179	3.4328	4.68E-04
DOMAIN_18345	Monodelphis domestica	1180	3.349	4.43E-04
DOMAIN_18356	Monodelphis domestica	1181	3.1967	4.67E-04
DOMAIN_18385	Neomonachus schauinslandi	1182	2.1472	0.0044932
DOMAIN_18415	Neomonachus schauinslandi	1183	2.9768	4.55E-04
DOMAIN_18424	Physeter macrocephalus	1184	3.7744	3.31E-04
DOMAIN_18426	Physeter macrocephalus	1185	2.8011	0.0079672
DOMAIN_18428	Physeter macrocephalus	1186	2.5903	0.0095383
DOMAIN_18433	OwlMonkey	1187	3.4614	0.0022427
DOMAIN_18441	Felis catus	1188	3.7534	1.77E-04

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_18458	Monodelphis domestica	1189	3.1061	0.0018603
DOMAIN_18459	Monodelphis domestica	1190	3.1352	2.38E-04
DOMAIN_18483	Monodelphis domestica	1191	2.8259	5.19E-04
DOMAIN_18485	Monodelphis domestica	1192	2.8817	0.0011922
DOMAIN_18498	OwlMonkey	1193	2.7354	0.0021141
DOMAIN_18502	Myotis davidii	1194	3.4127	1.93E-04
DOMAIN_18504	Cercocebus atys	1195	3.2213	5.38E-04
DOMAIN_18536	Camelus dromedarius	1196	3.2028	0.0011217
DOMAIN_18580	Cercocebus atys	1197	4.4477	3.22E-06
DOMAIN_18589	Neomonachus schauinslandi	1198	3.039	0.0025063
DOMAIN_18594	Monodelphis domestica	1199	3.2119	0.0036607
DOMAIN_18618	Physeter macrocephalus	1200	2.6489	0.0072165
DOMAIN_18646	Monodelphis domestica	1201	2.4678	0.007646
DOMAIN_18670	Neomonachus schauinslandi	1202	3.1792	3.80E-04
DOMAIN_18677	Monodelphis domestica	1203	2.2686	0.0068996
DOMAIN_18693	Camelus dromedarius	1204	3.0179	0.0013759
DOMAIN_18698	Felis catus	1205	3.3067	0.0093304
DOMAIN_18711	Vulpes vulpes	1206	2.2749	0.0063986
DOMAIN_18724	Chimp	1207	3.2062	5.16E-04
DOMAIN_18726	Myotis davidii	1208	2.9362	0.0025771
DOMAIN_18734	Monodelphis domestica	1209	2.8813	0.0092612
DOMAIN_18752	Monodelphis domestica	1210	3.5544	4.85E-05
DOMAIN_18753	Monodelphis domestica	1211	2.6101	3.54E-04
DOMAIN_18760	Chimp	1212	3.1806	7.49E-05
DOMAIN_18785	Leptonychotes weddellii	1213	2.9139	0.0019203
DOMAIN_18817	Monodelphis domestica	1214	2.2496	0.0091589
DOMAIN_18830	Monodelphis domestica	1215	3.2719	0.0032764
DOMAIN_18835	Camelus dromedarius	1216	2.4878	8.56E-05
DOMAIN_18873	Camelus dromedarius	1217	3.262	0.0049846
DOMAIN_18891	Orangutan	1218	3.6429	1.38E-06
DOMAIN_18923	Callithrix jacchus	1219	2.2053	0.0054504
DOMAIN_18935	Ovis aries	1220	3.4507	3.14E-05
DOMAIN_18947	Enhydra lutris kenyoni	1221	3.3167	7.58E-04
DOMAIN_18971	Enhydra lutris kenyoni	1222	3.3941	5.05E-06

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_18977	Orangutan	1223	3.6262	9.03E-06
DOMAIN_18979	Orangutan	1224	2.0034	0.0071822
DOMAIN_19005	Enhydra lutris kenyoni	1225	3.4092	4.57E-04
DOMAIN_19028	Orangutan	1226	2.3618	0.0022277
DOMAIN_19056	Bos indicus x Bos taurus	1227	3.0542	0.001874
DOMAIN_19072	Vulpes vulpes	1228	2.8133	0.0016331
DOMAIN_19079	Otolemur garnettii	1229	4.0159	4.88E-05
DOMAIN_19125	Otolemur garnettii	1230	2.9892	8.36E-04
DOMAIN_19207	Enhydra lutris kenyoni	1231	2.655	0.0091617
DOMAIN_19220	Camelus dromedarius	1232	3.1947	0.0088687
DOMAIN_19221	Camelus dromedarius	1233	3.1733	4.21E-04
DOMAIN_19299	Myotis davidii	1234	2.8882	0.0043533
DOMAIN_19351	Orangutan	1235	3.1988	2.17E-04
DOMAIN_19385	Monodelphis domestica	1236	2.9198	0.008105
DOMAIN_19387	Monodelphis domestica	1237	3.4706	1.85E-04
DOMAIN_19388	Physeter macrocephalus	1238	3.2831	7.71E-04
DOMAIN_19404	Monodelphis domestica	1239	2.0125	0.0031965
DOMAIN_19423	Monodelphis domestica	1240	3.49	0.002544
DOMAIN_19424	Monodelphis domestica	1241	2.5838	0.0041846
DOMAIN_19437	OwlMonkey	1242	2.826	0.001773
DOMAIN_19445	Monodelphis domestica	1243	2.1105	0.0078325
DOMAIN_19447	Monodelphis domestica	1244	3.4492	1.40E-04
DOMAIN_19487	Monodelphis domestica	1245	3.4312	6.00E-04
DOMAIN_19497	Monodelphis domestica	1246	3.466	2.80E-05
DOMAIN_19517	Monodelphis domestica	1247	3.3361	1.04E-04
DOMAIN_19533	Papio anubis	1248	2.5831	4.67E-04
DOMAIN_19563	Papio anubis	1249	2.5522	0.0089134
DOMAIN_19580	Monodelphis domestica	1250	3.5716	3.29E-05
DOMAIN_19585	Monodelphis domestica	1251	3.0031	0.0032403
DOMAIN_19596	Monodelphis domestica	1252	3.8583	8.18E-05
DOMAIN_19597	Monodelphis domestica	1253	3.5081	4.46E-04
DOMAIN_19600	Monodelphis domestica	1254	2.5854	0.0042185
DOMAIN_19602	Physeter macrocephalus	1255	2.7219	0.0058524
DOMAIN_19611	Lipotes vexillifer	1256	3.3901	4.24E-04

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_19629	Monodelphis domestica	1257	3.0535	0.0017954
DOMAIN_19699	Otolemur garnettii	1258	2.8474	3.15E-04
DOMAIN_19708	Bos indicus x Bos taurus	1259	3.6339	8.02E-04
DOMAIN_19713	Chimp	1260	3.845	2.95E-05
DOMAIN_19721	Otolemur garnettii	1261	2.6913	0.0089069
DOMAIN_19776	Enhydra lutris kenyoni	1262	2.617	0.0093497
DOMAIN_19777	Orangutan	1263	3.2427	0.0075444
DOMAIN_19780	Orangutan	1264	3.0867	1.72E-04
DOMAIN_19786	Chimp	1265	2.9155	5.94E-04
DOMAIN_19788	Enhydra lutris kenyoni	1266	3.3393	4.71E-04
DOMAIN_19800	Zalophus californianus	1267	2.368	0.009162
DOMAIN_19805	Rhinolophus ferrumequinum	1268	2.6527	0.0030997
DOMAIN_19818	Rhinopithecus roxellana	1269	2.3477	0.0022161
DOMAIN_19883	Zalophus californianus	1270	3.5504	3.42E-04
DOMAIN_19886	Panthera pardus	1271	2.8642	4.04E-05
DOMAIN_19889	Vicugna pacos	1272	3.1963	4.15E-05
DOMAIN_19891	Zalophus californianus	1273	3.2135	0.0010023
DOMAIN_19921	Callorhinus ursinus	1274	2.0083	0.0055679
DOMAIN_19944	Zalophus californianus	1275	3.8559	8.71E-05
DOMAIN_19947	Bonobo	1276	2.2608	0.00818
DOMAIN_19967	Tursiops truncatus	1277	2.9548	0.0027997
DOMAIN_19968	Tursiops truncatus	1278	2.8089	0.004093
DOMAIN_19990	Panthera pardus	1279	3.5329	0.0018768
DOMAIN_19993	Tursiops truncatus	1280	3.4227	0.0047476
DOMAIN_20012	Leptonychotes weddellii	1281	3.8253	3.41E-05
DOMAIN_20023	Physeter macrocephalus	1282	3.6893	5.78E-04
DOMAIN_20025	Carlito syrichta	1283	2.2451	0.002157
DOMAIN_20030	Tursiops truncatus	1284	4.1273	3.22E-06
DOMAIN_20089	Panthera pardus	1285	4.2275	8.99E-05
DOMAIN_20095	Phascolarctos cinereus	1286	3.7141	1.55E-05
DOMAIN_20115	Physeter macrocephalus	1287	3.1154	0.0030089
DOMAIN_20134	Acinonyx jubatus	1288	3.2457	3.20E-04
DOMAIN_20136	Sus scrofa	1289	3.3856	2.94E-04
DOMAIN_20147	Odocoileus virginianus texanus	1290	3.7467	1.53E-07

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_20171	Trichechus manatus latirostris	1291	3.951	1.03E-05
DOMAIN_20208	Pteropus vampyrus	1292	2.4805	0.0041634
DOMAIN_20249	Vicugna pacos	1293	2.7041	0.0043741
DOMAIN_20250	Phascolarctos cinereus	1294	3.5525	1.37E-04
DOMAIN_20287	Cercocebus atys	1295	3.4486	5.29E-04
DOMAIN_20318	Callithrix jacchus	1296	3.5311	3.52E-06
DOMAIN_20332	Callithrix jacchus	1297	3.2855	0.0011689
DOMAIN_20336	Panthera pardus	1298	2.3293	0.0076785
DOMAIN_20345	Cebus imitator	1299	3.8132	1.53E-07
DOMAIN_20352	Vicugna pacos	1300	2.9839	9.79E-04
DOMAIN_20359	Pteropus vampyrus	1301	3.9594	4.06E-05
DOMAIN_20371	Ursus arctos horribilis	1302	2.8418	0.0061393
DOMAIN_20381	Saimiri boliviensis boliviensis	1303	2.0412	0.0013486
DOMAIN_20398	Physeter macrocephalus	1304	3.1266	0.0039215
DOMAIN_20436	Sus scrofa	1305	2.724	0.0058616
DOMAIN_20455	Nomascus leucogenys	1306	3.112	2.94E-04
DOMAIN_20462	Trichechus manatus latirostris	1307	5.4429	1.53E-07
DOMAIN_20469	Equus caballus	1308	2.7506	0.0077201
DOMAIN_20487	Mandrillus leucophaeus	1309	2.8325	0.0020982
DOMAIN_20524	Nomascus leucogenys	1310	3.2893	0.0024993
DOMAIN_20537	Chlorocebus sabaeus	1311	3.2762	0.0027249
DOMAIN_20540	Mandrillus leucophaeus	1312	2.8477	0.0021931
DOMAIN_20545	Sus scrofa	1313	2.711	0.0086718
DOMAIN_20561	Chrysochloris asiatica	1314	3.8309	3.52E-05
DOMAIN_20565	Suricata suricatta	1315	3.148	2.90E-04
DOMAIN_20601	Sus scrofa	1316	2.9097	0.0037911
DOMAIN_20652	Neophocaena asiaeorientalis asiaeorientalis	1317	2.7283	0.0038931
DOMAIN_20667	Suricata suricatta	1318	3.7485	1.38E-06
DOMAIN_20674	Mandrillus leucophaeus	1319	3.3115	1.53E-07
DOMAIN_20716	Suricata suricatta	1320	3.6174	3.02E-05
DOMAIN_20729	Mandrillus leucophaeus	1321	2.5535	0.0090894
DOMAIN_20746	Chrysochloris asiatica	1322	3.4727	4.79E-04
DOMAIN_20767	Sus scrofa	1323	3.1224	3.16E-04
DOMAIN_20835	Suricata suricatta	1324	3.0025	0.0031432

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_20915	Mandrillus leucophaeus	1325	2.4373	0.0054586
DOMAIN_20998	Bonobo	1326	2.6659	0.0044767
DOMAIN_21010	Equus caballus	1327	2.2253	0.0040982
DOMAIN_21023	Sarcophilus harrisii	1328	3.1196	0.0023342
DOMAIN_21067	Zalophus californianus	1329	3.0246	0.0010917
DOMAIN_21082	Loxodonta africana	1330	3.2032	0.0040056
DOMAIN_21086	Pteropus vampyrus	1331	2.1339	0.0079029
DOMAIN_21095	Trichechus manatus latirostris	1332	2.5003	0.0091721
DOMAIN_21110	Neovison vison	1333	2.499	0.0065113
DOMAIN_21123	Callorhinus ursinus	1334	3.237	4.13E-04
DOMAIN_21133	Suricata suricatta	1335	3.1021	4.18E-04
DOMAIN_21161	Sarcophilus harrisii	1336	3.2208	5.87E-04
DOMAIN_21162	Sarcophilus harrisii	1337	2.885	6.85E-04
DOMAIN_21175	Callorhinus ursinus	1338	3.3334	2.29E-04
DOMAIN_21197	Tursiops truncatus	1339	2.214	0.0073288
DOMAIN_21226	Sarcophilus harrisii	1340	2.6942	0.0033484
DOMAIN_21260	Pteropus vampyrus	1341	3.1806	0.0039855
DOMAIN_21276	Mandrillus leucophaeus	1342	3.0178	0.0029699
DOMAIN_21277	OwlMonkey	1343	2.7115	0.0075352
DOMAIN_21312	Lipotes vexillifer	1344	3.5287	4.75E-06
DOMAIN_21333	Zalophus californianus	1345	3.5801	3.57E-05
DOMAIN_21334	Equus caballus	1346	2.9508	8.67E-04
DOMAIN_21335	Equus caballus	1347	2.518	0.0034809
DOMAIN_21367	Equus caballus	1348	2.9921	0.0091001
DOMAIN_21369	Equus caballus	1349	2.7947	0.0011824
DOMAIN_21371	Physeter macrocephalus	1350	3.8804	4.44E-06
DOMAIN_21421	Pteropus vampyrus	1351	2.7713	7.52E-05
DOMAIN_21481	Bonobo	1352	2.7056	0.0012415
DOMAIN_21494	Tursiops truncatus	1353	3.783	1.36E-04
DOMAIN_21583	Sarcophilus harrisii	1354	3.1529	0.0026931
DOMAIN_21588	Callorhinus ursinus	1355	3.4914	5.39E-04
DOMAIN_21612	OwlMonkey	1356	3.2931	4.09E-05
DOMAIN_21626	Monodelphis domestica	1357	3.5419	1.57E-04
DOMAIN_21632	Monodelphis domestica	1358	2.6551	0.0071923

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_21658	Monodelphis domestica	1359	3.1325	2.50E-04
DOMAIN_21786	Trichechus manatus latirostris	1360	3.2249	2.76E-04
DOMAIN_21822	Equus caballus	1361	3.5647	3.22E-06
DOMAIN_21823	Equus caballus	1362	3.2474	0.0072446
DOMAIN_21844	OwlMonkey	1363	3.467	4.44E-06
DOMAIN_21862	Chlorocebus sabaeus	1364	2.3797	0.0032299
DOMAIN_21889	Equus caballus	1365	3.6563	4.18E-04
DOMAIN_21896	Lipotes vexillifer	1366	2.8718	0.0093653
DOMAIN_21900	Equus caballus	1367	2.7606	0.0041711
DOMAIN_21909	Suricata suricatta	1368	3.2301	3.40E-04
DOMAIN_21928	Callorhinus ursinus	1369	3.758	1.67E-05
DOMAIN_21947	Trichechus manatus latirostris	1370	3.1204	0.003623
DOMAIN_21951	Equus caballus	1371	2.8972	3.24E-04
DOMAIN_21985	Suricata suricatta	1372	3.6273	1.99E-06
DOMAIN_21988	Sarcophilus harrisii	1373	3.3393	0.0011817
DOMAIN_21993	Lipotes vexillifer	1374	2.5494	0.0039206
DOMAIN_22022	Tursiops truncatus	1375	3.9558	4.44E-06
DOMAIN_22079	Trichechus manatus latirostris	1376	3.4511	6.43E-04
DOMAIN_22117	Sarcophilus harrisii	1377	2.5969	0.0040801
DOMAIN_22143	Pteropus vampyrus	1378	2.6595	9.36E-04
DOMAIN_22151	Trichechus manatus latirostris	1379	3.1615	5.26E-04
DOMAIN_22158	Lipotes vexillifer	1380	2.0562	0.0010562
DOMAIN_22166	Trichechus manatus latirostris	1381	4.2024	2.53E-05
DOMAIN_22192	Trichechus manatus latirostris	1382	2.8134	0.0083622
DOMAIN_22220	Bonobo	1383	2.8922	0.0013379
DOMAIN_22268	Lipotes vexillifer	1384	2.6534	0.0053876
DOMAIN_22278	Pteropus vampyrus	1385	3.3575	0.0037798
DOMAIN_22280	Pteropus vampyrus	1386	3.1521	0.0017347
DOMAIN_22285	Trichechus manatus latirostris	1387	3.0261	6.83E-04
DOMAIN_22297	Sarcophilus harrisii	1388	2.4261	0.0066953
DOMAIN_22311	Monodelphis domestica	1389	2.9903	0.0017115
DOMAIN_22322	Tursiops truncatus	1390	3.4452	3.85E-04
DOMAIN_22366	OwlMonkey	1391	4.848	3.06E-07
DOMAIN_22375	Tursiops truncatus	1392	2.5484	0.0090894

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_22381	Tursiops truncatus	1393	3.8641	2.63E-04
DOMAIN_22383	Pteropus vampyrus	1394	3.4752	2.48E-04
DOMAIN_22407	OwlMonkey	1395	2.5308	0.0081831
DOMAIN_22425	OwlMonkey	1396	3.0333	0.0032208
DOMAIN_22430	Callorhinus ursinus	1397	2.982	0.0064761
DOMAIN_22454	Monodelphis domestica	1398	2.6042	0.0022491
DOMAIN_22458	Monodelphis domestica	1399	3.0003	0.0025373
DOMAIN_22459	Monodelphis domestica	1400	2.9261	0.0013171
DOMAIN_22462	Monodelphis domestica	1401	3.5597	2.34E-05
DOMAIN_22471	Papio anubis	1402	3.6293	1.68E-06
DOMAIN_22479	OwlMonkey	1403	3.9668	4.18E-05
DOMAIN_22483	OwlMonkey	1404	2.1702	0.0013107
DOMAIN_22495	Callorhinus ursinus	1405	2.2623	0.0043918
DOMAIN_22512	OwlMonkey	1406	2.93	0.003255
DOMAIN_22518	Lipotes vexillifer	1407	2.8869	0.0024472
DOMAIN_22520	Callorhinus ursinus	1408	3.3586	2.83E-05
DOMAIN_22527	Tursiops truncatus	1409	2.989	9.71E-04
DOMAIN_22566	Papio anubis	1410	3.5278	6.63E-05
DOMAIN_22586	Nomascus leucogenys	1411	2.1811	0.0021723
DOMAIN_22615	Homo sapiens	1412	3.0957	4.43E-04
DOMAIN_22654	Ursus arctos horribilis	1413	3.248	5.59E-05
DOMAIN_22667	Saimiri boliviensis boliviensis	1414	3.4947	0.0037256
DOMAIN_22669	Balaenoptera acutorostrata scammoni	1415	3.583	4.34E-04
DOMAIN_22692	Propithecus coquereli	1416	3.2791	3.52E-04
DOMAIN_22710	Propithecus coquereli	1417	3.4387	0.0032081
DOMAIN_22740	Panthera pardus	1418	2.692	0.0027611
DOMAIN_22742	Panthera pardus	1419	2.9133	0.0027938
DOMAIN_22768	Ursus maritimus	1420	4.0609	7.81E-06
DOMAIN_22771	Ursus americanus	1421	3.3498	2.83E-05
DOMAIN_22776	Propithecus coquereli	1422	2.7757	2.88E-04
DOMAIN_22778	Saimiri boliviensis boliviensis	1423	3.1251	4.93E-04
DOMAIN_22782	Vombatus ursinus	1424	3.1663	4.24E-04
DOMAIN_22917	Cervus elaphus hippelaphus	1425	3.8061	2.77E-05
DOMAIN_22919	Colobus angolensis palliatus	1426	2.8609	0.003796

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_22928	Tupaia chinensis	1427	3.0141	0.0015348
DOMAIN_22937	Ursus arctos horribilis	1428	3.0779	0.0032951
DOMAIN_22939	Muntiacus reevesi	1429	3.6187	1.78E-04
DOMAIN_22944	Muntiacus reevesi	1430	3.3908	5.28E-04
DOMAIN_23007	Lynx pardinus	1431	3.7329	1.09E-04
DOMAIN_23009	Saimiri boliviensis boliviensis	1432	3.1269	0.0062706
DOMAIN_23011	Cervus elaphus hippelaphus	1433	3.6236	3.51E-05
DOMAIN_23012	Cervus elaphus hippelaphus	1434	3.6131	2.50E-04
DOMAIN_23013	Cervus elaphus hippelaphus	1435	3.4615	4.85E-04
DOMAIN_23018	Colobus angolensis palliatus	1436	3.4177	2.30E-04
DOMAIN_23039	Saimiri boliviensis boliviensis	1437	2.8829	5.70E-04
DOMAIN_23040	Saimiri boliviensis boliviensis	1438	2.5742	0.0056531
DOMAIN_23041	Vombatus ursinus	1439	3.6194	1.92E-04
DOMAIN_23050	Balaenoptera acutorostrata scammoni	1440	2.9754	0.003318
DOMAIN_23082	Mustela putorius furo	1441	3.9481	5.17E-05
DOMAIN_23093	Propithecus coquereli	1442	3.2165	5.48E-04
DOMAIN_23109	Mustela putorius furo	1443	2.9639	0.0019589
DOMAIN_23113	Camelus ferus	1444	3.4612	3.52E-04
DOMAIN_23136	Vicugna pacos	1445	3.285	2.16E-04
DOMAIN_23181	Colobus angolensis palliatus	1446	2.7665	0.0021609
DOMAIN_23196	Odobenus rosmarus divergens	1447	4.3363	3.22E-06
DOMAIN_23200	Ursus americanus	1448	3.755	1.84E-06
DOMAIN_23215	Vombatus ursinus	1449	3.0212	0.0035725
DOMAIN_23217	Vombatus ursinus	1450	4.1674	2.76E-06
DOMAIN_23239	Vicugna pacos	1451	3.0945	0.0090937
DOMAIN_23250	Delphinapterus leucas	1452	2.71	3.14E-04
DOMAIN_23260	Tupaia chinensis	1453	2.7567	0.0029622
DOMAIN_23281	Colobus angolensis palliatus	1454	2.5048	0.0036625
DOMAIN_23286	Mustela putorius furo	1455	3.3651	1.66E-04
DOMAIN_23301	Gulo gulo	1456	2.6839	0.0035226
DOMAIN_23323	Erinaceus europaeus	1457	3.2619	0.0031362
DOMAIN_23331	Carlito syrichta	1458	2.8995	5.23E-04
DOMAIN_23336	Carlito syrichta	1459	2.239	0.0065533
DOMAIN_23341	Carlito syrichta	1460	2.656	0.0058992

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_23375	Vicugna pacos	1461	3.266	7.64E-04
DOMAIN_23378	Odobenus rosmarus divergens	1462	3.0623	0.0016508
DOMAIN_23419	Gulo gulo	1463	3.5213	7.41E-04
DOMAIN_23453	Carlito syrichta	1464	2.2331	0.006161
DOMAIN_23454	Carlito syrichta	1465	3.0632	7.96E-04
DOMAIN_23458	Vicugna pacos	1466	2.4232	0.0045857
DOMAIN_23480	Odobenus rosmarus divergens	1467	3.4432	3.38E-04
DOMAIN_23494	Mustela putorius furo	1468	3.847	5.05E-06
DOMAIN_23508	Mustela putorius furo	1469	2.3582	0.0047712
DOMAIN_23513	Tupaia chinensis	1470	3.2927	5.31E-05
DOMAIN_23514	Odobenus rosmarus divergens	1471	3.0166	4.77E-04
DOMAIN_23561	Colobus angolensis palliatus	1472	3.2392	0.0021906
DOMAIN_23574	Gulo gulo	1473	2.939	0.0083249
DOMAIN_23575	Erinaceus europaeus	1474	3.4624	0.001589
DOMAIN_23576	Erinaceus europaeus	1475	3.8014	2.89E-05
DOMAIN_23590	Odobenus rosmarus divergens	1476	2.8653	0.0052881
DOMAIN_23604	Vicugna pacos	1477	2.6984	0.0046123
DOMAIN_23641	Carlito syrichta	1478	2.6942	0.0081075
DOMAIN_23642	Delphinapterus leucas	1479	3.8829	2.28E-04
DOMAIN_23654	Carlito syrichta	1480	2.337	0.0083622
DOMAIN_23679	Tupaia chinensis	1481	3.7951	5.10E-05
DOMAIN_23680	Vicugna pacos	1482	2.712	0.0034785
DOMAIN_23709	Carlito syrichta	1483	4.545	1.53E-07
DOMAIN_23711	Gulo gulo	1484	2.658	0.0016432
DOMAIN_23721	Carlito syrichta	1485	2.972	0.0022972
DOMAIN_23731	Colobus angolensis palliatus	1486	3.1609	2.35E-04
DOMAIN_23745	Myotis brandtii	1487	3.4544	3.54E-04
DOMAIN_23793	Odobenus rosmarus divergens	1488	2.7573	0.0081197
DOMAIN_23804	Colobus angolensis palliatus	1489	2.3403	0.0086366
DOMAIN_23827	Odobenus rosmarus divergens	1490	2.3013	0.009767
DOMAIN_23854	Gulo gulo	1491	3.838	7.18E-05
DOMAIN_23856	Erinaceus europaeus	1492	3.1072	0.0035694
DOMAIN_23863	Mustela putorius furo	1493	2.8758	0.0085493
DOMAIN_23885	Colobus angolensis palliatus	1494	3.033	0.0034316

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_23895	Mustela putorius furo	1495	2.6148	0.003318
DOMAIN_23898	Mustela putorius furo	1496	2.7383	0.0035921
DOMAIN_23916	Odobenus rosmarus divergens	1497	3.3232	1.63E-04
DOMAIN_23931	Gulo gulo	1498	3.8077	1.49E-05
DOMAIN_23940	Homo sapiens	1499	2.5087	0.0010424
DOMAIN_23953	Muntiacus reevesi	1500	2.4156	0.0075055
DOMAIN_23979	Balaenoptera acutorostrata scammoni	1501	4.0461	5.77E-05
DOMAIN_24020	Rhinolophus ferrumequinum	1502	3.1125	1.66E-04
DOMAIN_24028	Ursus arctos horribilis	1503	3.8797	1.53E-07
DOMAIN_24035	Propithecus coquereli	1504	3.2225	0.0017975
DOMAIN_24042	Propithecus coquereli	1505	3.3038	4.75E-06
DOMAIN_24083	Myotis brandtii	1506	3.9804	2.77E-05
DOMAIN_24113	Propithecus coquereli	1507	3.3264	2.89E-04
DOMAIN_24152	Vombatus ursinus	1508	3.3664	0.0022672
DOMAIN_24204	Propithecus coquereli	1509	3.0779	4.60E-04
DOMAIN_24212	Pteropus alecto	1510	2.498	0.0034998
DOMAIN_24230	Muntiacus reevesi	1511	3.1832	1.53E-07
DOMAIN_24256	Ursus arctos horribilis	1512	2.7933	0.0018808
DOMAIN_24282	Muntiacus reevesi	1513	2.694	0.0052575
DOMAIN_24306	Propithecus coquereli	1514	3.2084	0.0023952
DOMAIN_24317	Myotis brandtii	1515	3.9767	3.17E-05
DOMAIN_24379	Macaca nemestrina	1516	2.4643	0.0086804
DOMAIN_24393	Propithecus coquereli	1517	3.8008	2.45E-06
DOMAIN_24446	Propithecus coquereli	1518	3.6312	7.27E-05
DOMAIN_24463	Balaenoptera acutorostrata scammoni	1519	2.5362	0.007147
DOMAIN_24496	Ursus americanus	1520	3.6403	4.24E-04
DOMAIN_24515	Balaenoptera acutorostrata scammoni	1521	3.7358	5.28E-05
DOMAIN_24518	Balaenoptera acutorostrata scammoni	1522	3.4135	3.05E-05
DOMAIN_24546	Ursus americanus	1523	3.4262	8.42E-06
DOMAIN_24570	Saimiri boliviensis boliviensis	1524	3.6773	1.45E-05
DOMAIN_24571	Balaenoptera acutorostrata scammoni	1525	2.6912	0.0038376
DOMAIN_24600	Ursus americanus	1526	3.156	0.0012483
DOMAIN_24614	Cervus elaphus hippelaphus	1527	2.6295	0.0046463

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_24615	Colobus angolensis palliatus	1528	2.4075	0.0069247
DOMAIN_24653	Cervus elaphus hippelaphus	1529	2.9883	0.0083016
DOMAIN_24677	Lynx pardinus	1530	2.3115	0.0094713
DOMAIN_24719	Muntiacus reevesi	1531	2.7499	0.005142
DOMAIN_24725	Ursus arctos horribilis	1532	3.4496	4.09E-05
DOMAIN_24771	Myotis brandtii	1533	3.3701	0.0025351
DOMAIN_24786	Vombatus ursinus	1534	2.9237	0.0078001
DOMAIN_24788	Vombatus ursinus	1535	2.7694	0.0021557
DOMAIN_24838	Pteropus alecto	1536	2.3323	0.0042954
DOMAIN_24867	Nomascus leucogenys	1537	3.469	2.97E-04
DOMAIN_24903	Ailuropoda melanoleuca	1538	3.0377	0.0030054
DOMAIN_24939	Phascolarctos cinereus	1539	3.3066	6.08E-04
DOMAIN_24947	Ursus maritimus	1540	2.9491	0.0055208
DOMAIN_24975	Muntiacus muntjak	1541	3.2737	0.0069767
DOMAIN_24993	Oryctolagus cuniculus	1542	3.3817	5.00E-04
DOMAIN_25016	Oryctolagus cuniculus	1543	2.9822	0.0034776
DOMAIN_25052	Pteropus alecto	1544	2.3634	0.0072024
DOMAIN_25060	Ailuropoda melanoleuca	1545	3.6002	4.82E-04
DOMAIN_25063	Phascolarctos cinereus	1546	2.9436	0.0042752
DOMAIN_25070	Sapajus apella	1547	2.9649	0.0043634
DOMAIN_25091	Phascolarctos cinereus	1548	2.9006	0.0039332
DOMAIN_25094	Phascolarctos cinereus	1549	3.0413	0.0026876
DOMAIN_25106	Canis lupus familiaris	1550	2.8622	0.0075508
DOMAIN_25126	Puma concolor	1551	2.1478	0.005514
DOMAIN_25128	Sapajus apella	1552	2.588	0.0029475
DOMAIN_25131	Sapajus apella	1553	2.592	0.0051895
DOMAIN_25146	Macaca nemestrina	1554	3.629	1.68E-06
DOMAIN_25150	Muntiacus reevesi	1555	3.147	0.0018391
DOMAIN_25157	Myotis brandtii	1556	3.0902	0.0012442
DOMAIN_25194	Macaca nemestrina	1557	2.4613	0.003597
DOMAIN_25204	Panthera pardus	1558	2.7595	0.0027917
DOMAIN_25234	Saimiri boliviensis boliviensis	1559	2.743	0.0042296
DOMAIN_25235	Oryctolagus cuniculus	1560	3.6965	1.76E-05
DOMAIN_25334	Phascolarctos cinereus	1561	2.7501	0.0096299

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_25384	Rhinolophus ferrumequinum	1562	3.5139	8.10E-05
DOMAIN_25389	Ursus maritimus	1563	3.0814	6.54E-04
DOMAIN_25400	Lynx canadensis	1564	2.2285	3.10E-04
DOMAIN_25410	Puma concolor	1565	2.8699	0.0022843
DOMAIN_25443	Muntiacus reevesi	1566	3.2531	0.0016615
DOMAIN_25534	Ursus maritimus	1567	2.2698	0.0054246
DOMAIN_25554	Panthera pardus	1568	3.0101	0.003898
DOMAIN_25564	Muntiacus reevesi	1569	3.4378	6.04E-04
DOMAIN_25565	Muntiacus reevesi	1570	2.6133	0.0011572
DOMAIN_25623	Ursus maritimus	1571	3.4886	2.91E-06
DOMAIN_25628	Rhinopithecus bieti	1572	2.8332	0.0022213
DOMAIN_25649	Ursus arctos horribilis	1573	3.6884	5.62E-05
DOMAIN_25654	Pteropus alecto	1574	2.2996	0.0031144
DOMAIN_25671	Muntiacus reevesi	1575	3.5244	1.53E-07
DOMAIN_25682	Rhinopithecus bieti	1576	2.5621	0.002108
DOMAIN_25686	Panthera pardus	1577	2.8635	0.0031882
DOMAIN_25726	Pteropus alecto	1578	2.8203	0.0039506
DOMAIN_25741	Sapajus apella	1579	3.7244	1.32E-04
DOMAIN_25780	Rhinopithecus bieti	1580	2.8383	0.0018385
DOMAIN_25807	Puma concolor	1581	3.6511	0.0018679
DOMAIN_25842	Rhinolophus ferrumequinum	1582	3.0942	2.44E-04
DOMAIN_25844	Ursus maritimus	1583	2.5635	0.0037997
DOMAIN_25857	Balaenoptera acutorostrata scammoni	1584	2.898	0.0026959
DOMAIN_25865	Vombatus ursinus	1585	3.1027	0.0066133
DOMAIN_25869	Vombatus ursinus	1586	2.3538	0.006932
DOMAIN_25972	Geotrypetes seraphini	1587	3.2178	0.0036689
DOMAIN_25973	Geotrypetes seraphini	1588	2.7804	0.001766
DOMAIN_25996	Geotrypetes seraphini	1589	3.984	1.24E-05
DOMAIN_26010	Geotrypetes seraphini	1590	2.1911	0.008383
DOMAIN_26012	Geotrypetes seraphini	1591	2.3532	9.70E-04
DOMAIN_26044	Geotrypetes seraphini	1592	2.8874	0.0068616
DOMAIN_26103	Geotrypetes seraphini	1593	2.5308	0.0033422
DOMAIN_26127	Geotrypetes seraphini	1594	2.5183	0.00586
DOMAIN_26131	Geotrypetes seraphini	1595	2.4087	0.0068533

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_26134	Geotrypetes seraphini	1596	2.4433	0.0072939
DOMAIN_26163	Geotrypetes seraphini	1597	2.4527	0.0041806
DOMAIN_26177	Geotrypetes seraphini	1598	3.4467	1.27E-05
DOMAIN_26180	Geotrypetes seraphini	1599	3.4522	1.35E-04
DOMAIN_26194	Geotrypetes seraphini	1600	2.8857	0.0031518
DOMAIN_26211	Pelodiscus sinensis	1601	2.6058	0.0064871
DOMAIN_26233	Colinus virginianus	1602	3.6739	1.77E-04
DOMAIN_26236	Pelodiscus sinensis	1603	2.7094	0.003991
DOMAIN_26265	Geotrypetes seraphini	1604	2.5922	3.31E-04
DOMAIN_26268	Geotrypetes seraphini	1605	2.1404	0.0020397
DOMAIN_26292	Geotrypetes seraphini	1606	2.4722	0.0074388
DOMAIN_26299	Geotrypetes seraphini	1607	2.3704	0.0058481
DOMAIN_26305	Geotrypetes seraphini	1608	3.0107	0.0084216
DOMAIN_26306	Geotrypetes seraphini	1609	2.6178	0.0051922
DOMAIN_26335	Colinus virginianus	1610	4.0965	3.41E-04
DOMAIN_26340	Pelodiscus sinensis	1611	3.1704	0.003352
DOMAIN_26353	Pelodiscus sinensis	1612	3.5785	1.16E-04
DOMAIN_26373	Pseudonaja textilis	1613	3.3204	5.13E-04
DOMAIN_26407	Colinus virginianus	1614	2.9778	0.0049206
DOMAIN_26414	Pelodiscus sinensis	1615	2.9544	0.0089308
DOMAIN_26415	Pelodiscus sinensis	1616	2.5032	0.0035489
DOMAIN_26416	Pelodiscus sinensis	1617	3.6321	4.36E-05
DOMAIN_26417	Pelodiscus sinensis	1618	4.1057	4.46E-05
DOMAIN_26423	Pelodiscus sinensis	1619	3.0169	0.0025697
DOMAIN_26430	Pelodiscus sinensis	1620	2.6946	0.0051824
DOMAIN_26439	Pelodiscus sinensis	1621	3.2468	0.0010568
DOMAIN_26463	Pelodiscus sinensis	1622	2.8812	0.003427
DOMAIN_26469	Pelodiscus sinensis	1623	3.021	5.08E-04
DOMAIN_26496	Geotrypetes seraphini	1624	2.7991	0.0040994
DOMAIN_26501	Geotrypetes seraphini	1625	2.6513	0.0041882
DOMAIN_26518	Geotrypetes seraphini	1626	2.397	0.0087878
DOMAIN_26577	Geotrypetes seraphini	1627	2.4722	0.0035247
DOMAIN_26634	Gopherus agassizii	1628	2.8182	0.0079972
DOMAIN_26636	Gopherus agassizii	1629	2.6934	0.0090052

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_26660	Phasianus colchicus	1630	3.201	4.90E-04
DOMAIN_26679	Paroedura picta	1631	2.6033	0.001326
DOMAIN_26780	Meleagris gallopavo	1632	3.1696	0.0031591
DOMAIN_26783	Meleagris gallopavo	1633	3.2848	0.0020241
DOMAIN_26795	Meleagris gallopavo	1634	3.3538	0.001228
DOMAIN_26800	Meleagris gallopavo	1635	3.8197	1.62E-04
DOMAIN_26803	Aquila chrysaetos chrysaetos	1636	3.4265	0.001246
DOMAIN_26852	Mus musculus	1637	2.8783	0.0025253
DOMAIN_26853	Mus musculus	1638	3.6235	7.59E-04
DOMAIN_26886	Homo sapiens	1639	3.3209	0.0016312
DOMAIN_26925	Alligator sinensis	1640	3.2248	0.0036928
DOMAIN_26999	Xenopus laevis	1641	3.4317	4.75E-06
DOMAIN_27032	Alligator mississippiensis	1642	3.4805	0.0019423
DOMAIN_27285	Peromyscus maniculatus bairdii	1643	3.092	5.16E-04
DOMAIN_27498	Sus scrofa	1644	2.9278	0.0029754
DOMAIN_27521	Suricata suricatta	1645	2.7447	0.0010703
DOMAIN_27563	Muntiacus muntjak	1646	3.6292	6.63E-05
DOMAIN_27566	Muntiacus muntjak	1647	2.7825	0.0020795
DOMAIN_27579	Muntiacus muntjak	1648	3.8878	7.50E-06
DOMAIN_27581	Canis lupus familiaris	1649	2.4582	0.0090172
DOMAIN_27639	Macaca fascicularis	1650	2.452	0.0032574
DOMAIN_27642	Puma concolor	1651	2.8615	0.0015287
DOMAIN_27690	Myotis lucifugus	1652	3.1465	0.0012118
DOMAIN_27705	Phascolarctos cinereus	1653	2.5921	0.0030483
DOMAIN_27759	Bos taurus	1654	2.2124	0.0070756
DOMAIN_27767	Callithrix jacchus	1655	2.2153	0.0023952
DOMAIN_27777	Odocoileus virginianus texanus	1656	2.6766	0.0067364
DOMAIN_27784	Ovis aries	1657	2.1631	0.0040915
DOMAIN_27809	Cebus imitator	1658	2.8715	0.0025161
DOMAIN_27827	Vulpes vulpes	1659	3.1318	2.13E-05
DOMAIN_27833	Callithrix jacchus	1660	3.0164	4.27E-04
DOMAIN_27866	Orangutan	1661	2.9226	0.0029981
DOMAIN_27886	Bison bison bison	1662	2.735	0.0036356
DOMAIN_27902	Vulpes vulpes	1663	2.9068	0.0039341

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_27988	Camelus dromedarius	1664	2.5381	0.0015476
DOMAIN_28051	Neomonachus schauinslandi	1665	2.4353	0.0018581
DOMAIN_28071	Enhydra lutris kenyoni	1666	3.2938	2.61E-04
DOMAIN_28085	Enhydra lutris kenyoni	1667	2.2962	0.0029074
DOMAIN_28103	Physeter macrocephalus	1668	2.4116	0.009594
DOMAIN_28118	OwlMonkey	1669	3.1049	0.0027807
DOMAIN_28158	Odocoileus virginianus texanus	1670	3.0762	0.0016156
DOMAIN_28164	Callithrix jacchus	1671	2.7356	0.0064115
DOMAIN_28299	Capra hircus	1672	3.5584	6.41E-05
DOMAIN_28309	Pteropus vampyrus	1673	3.5338	3.28E-04
DOMAIN_28335	Bonobo	1674	3.3013	2.50E-04
DOMAIN_28341	Homo sapiens	1675	2.7008	5.14E-04
DOMAIN_28417	Gulo gulo	1676	2.5366	5.02E-04
DOMAIN_28421	Erinaceus europaeus	1677	3.0763	0.0038713
DOMAIN_28507	Muntiacus reevesi	1678	3.2874	8.76E-04
DOMAIN_28513	Propithecus coquereli	1679	2.3747	0.0050076
DOMAIN_28533	Propithecus coquereli	1680	2.7575	0.0031303
DOMAIN_28588	Rhinolophus ferrumequinum	1681	2.6131	0.0030648
DOMAIN_28619	Rhinolophus ferrumequinum	1682	2.6504	0.0027237
DOMAIN_28823	Microcaecilia unicolor	1683	2.331	0.0078575
DOMAIN_28845	Camelus ferus	1684	3.0175	0.0017733
DOMAIN_28929	Mus musculus	1685	3.1025	6.70E-04
DOMAIN_29066	Xenopus tropicalis	1686	2.6393	3.67E-04
DOMAIN_29164	Chelonia mydas	1687	2.1345	0.0029635
DOMAIN_29260	Peromyscus maniculatus bairdii	1688	2.5127	0.0074146
DOMAIN_29339	Mesocricetus auratus	1689	2.9581	0.0028165
DOMAIN_29377	Mesocricetus auratus	1690	2.672	0.0070692
DOMAIN_29426	Mus caroli	1691	2.0491	6.64E-04
DOMAIN_29434	Mus caroli	1692	2.2707	0.005184
DOMAIN_29467	Mus caroli	1693	3.4689	5.79E-05
DOMAIN_29471	Cricetulus griseus	1694	3.1911	4.18E-05
DOMAIN_29511	Peromyscus maniculatus bairdii	1695	3.4739	7.00E-05
DOMAIN_29614	Peromyscus maniculatus bairdii	1696	3.4528	1.82E-04
DOMAIN_29616	Mesocricetus auratus	1697	2.2807	0.0035376

Domain ID	Species	SEQ ID NO	Log2 (fold change)	P-value
DOMAIN_29765	Erinaceus europaeus	1698	3.3088	9.79E-04
DOMAIN_29900	Nomascus leucogenys	1699	2.1583	0.0098463
DOMAIN_30185	Rhinopithecus roxellana	1700	3.0766	5.83E-05
DOMAIN_30211	Bison bison bison	1701	2.3322	0.0023122
DOMAIN_30236	Callithrix jacchus	1702	2.7293	0.0021744
DOMAIN_30329	Rhesus	1703	2.1216	0.0099018
DOMAIN_30783	Chimp	1704	2.952	0.001698
DOMAIN_31235	Vicugna pacos	1705	2.2828	0.0067045
DOMAIN_31340	Homo sapiens	1706	2.8261	0.0021028
DOMAIN_31383	Propithecus coquereli	1707	2.1919	0.0087058
DOMAIN_31638	Balaenoptera acutorostrata scammoni	1708	2.0254	0.0036297
DOMAIN_31798	Notechis scutatus	1709	4.8007	7.82E-04
DOMAIN_31935	Rhinolophus ferrumequinum	1710	3.5544	0.0084786
DOMAIN_32127	Human	1711	3.7547	2.62E-05
DOMAIN_32145	Human	1712	3.1866	1.67E-05
DOMAIN_32146	Human	1713	2.7628	0.0016129
DOMAIN_32159	Human	1714	2.7874	0.0021753
DOMAIN_32215	Human	1715	3.2653	0.001461
DOMAIN_32223	Human	1716	2.8836	0.0068873
DOMAIN_32255	Human	1717	3.8237	1.39E-05
DOMAIN_32279	Human	1718	2.4917	0.0060199
DOMAIN_32286	Human	1719	2.8921	0.0070992
DOMAIN_32312	Human	1720	2.9151	0.0030308
DOMAIN_32321	Human	1721	3.0441	0.0040854
DOMAIN_32327	Human	1722	3.1024	0.0044212
DOMAIN_32334	Human	1723	2.8117	0.0015241
DOMAIN_32351	Human	1724	2.0727	0.0036362
DOMAIN_32386	Human	1725	3.5521	3.87E-04
DOMAIN_32390	Human	1726	3.757	4.30E-05

[0551] The repressor domain with the highest $\log_2$ (fold change) was derived from the king cobra, *Ophiophagus hannah* (DOMAIN_26749; SEQ ID NO: 135). Surprisingly, this sequence was highly divergent from human KRAB domains (with only 41% sequence identity) and was grouped in a sequence cluster of poor repressor domains.

[0552] To verify that the domains identified in the selection supported transcriptional repression in an independent assay, representative members of the top 95 and 1597 repressor domains were used to generate dXR constructs, and their ability to repress transcription of the *B2M* locus was tested. As shown in FIG. 55, seven days after transduction, dXRs with all but one of the representative top 95 or 1597 repressor domains tested repressed *B2M* to a greater extent than did the dXR with the ZNF10 KRAB domain. As shown in FIG. 56, ten days after transduction, the majority of the dXRs with representative top 95 or 1597 repressor domains tested repressed *B2M* to a greater extent than did ZNF10 or ZIM3. dXR repression of a target locus tends to deteriorate over time, and ten days following transduction is believed to be a relatively late timepoint for measuring dXR repression. Therefore, it is particularly notable that many of the dXR constructs with repressor domains in the top 95 and 1597 were able to repress *B2M* to a greater extent than dXR with KRAB domains derived from ZNF10 or ZIM3 as late as ten days following transduction.

[0553] To further understand the basis of the superior ability of the identified repressor domains to repress transcription, protein sequence motifs were identified from the top 1597 repressor domains using the STREME algorithm. Specifically, five motifs (motifs 1-5) were generated by comparing the amino acid sequences of the top 1597 repressor domains to a negative training set of 1506 repressor domains with p-values less than 0.01, and $\log_2$ (fold change) values less than 0. Logos of motifs 1-5 are provided in FIGS. 57A, 57B, 57C, 57D, and 57E. In addition, four motifs (motifs 6-9) were generated by comparing the top 1597 repressor domains to shuffled sequences derived from the 1597 repressor domain sequences. Logos of motifs 6-9 are provided in FIGS. 57F, 57G, 57H, and 57I.

[0554] Table 55, below, provides the p-value, E-value (a measure of statistical significance), and number and percentage of sequences matching the motif in the top 1597 repressor domains for each of the nine motifs, as calculated by STREME. Table 56 provides the sequences of each motif, showing the amino acid residues present at each position within the motifs (from N- to C-terminus).

Table 55: Characteristics of protein sequence motifs of top 1597 repressor domains

Motif ID	P-value	E-value	Number and percentage of sites matching motif in top 1597 repressor domains
Motifs generated compared to a negative training set			
1	3.7e-014	7.1e-013	1158 (72.5%)
2	3.4e-012	6.4e-011	978 (61.2%)

Motif ID	P-value	E-value	Number and percentage of sites matching motif in top 1597 repressor domains
3	7.5e-010	1.4e-008	1017 (63.7%)
4	7.0e-008	1.3e-006	987 (61.8%)
5	1.7e-007	3.3e-006	678 (42.5%)
Motifs generated compared to shuffled sequences			
6	1.2e-048	1.5e-047	1597 (100.0%)
7	1.2e-048	1.5e-047	1597 (100.0%)
8	1.3e-042	1.6e-041	1377 (86.2%)
9	2.1e-040	2.7e-039	1483 (92.9%)

Table 56: Sequences of protein sequence motifs of top 1597 repressor domains

Motif ID	Position in motif	Amino acid residues with >5% representation in motif
Motifs generated compared to a negative training set		
1	1	P
	2	A, D, E, N
	3	L, V
	4	I, V
	5	S, T, F
	6	H, K, L, Q, R, W
	7	L, M
	8	E
	9	G, K, Q, R
2	1	L, V
	2	A, G, L, T, V
	3	A, F, S
	4	L, V
	5	G
	6	C, F, H, I, L, Y
	7	A, C, P, Q, S
	8	A, F, G, I, S, V
	9	A, P, S, T
	10	K, R
3	1	Q
	2	K, R
	3	A, D, E, G, N, S, T
	4	L
	5	Y
	6	R
	7	D, E, S
	8	V
	9	M

Motif ID	Position in motif	Amino acid residues with >5% representation in motif
Motifs generated compared to shuffled sequences		
6	1	L
	2	Y
	3	K, R
	4	D, E
	5	V
	6	M
	7	L, Q, R
	8	E
	9	N, T
	10	F, Y
	11	A, E, G, Q, R, S
	12	H, L, N
	13	L, V
	14	A, G, I, L, T, V
	15	A, F, S
7	1	F
	2	A, E, G, K, R
	3	D
	4	V
	5	A, S, T
	6	I, V
	7	D, E, N, Y
	8	F
	9	S, T
	10	E, L, P, Q, R, W
	11	D, E
	12	E
	13	W

Motif ID	Position in motif	Amino acid residues with >5% representation in motif
	10	L, R
4	1	A, L, P, S
	2	L, V
	3	S, T
	4	F
	5	A, E, G, K, R
	6	D
	7	V
	8	A, T
	9	I, V
	10	D, E, N, Y
	11	F
	12	S, T
	13	E, P, Q, R, W
	14	E, N
	15	E, Q
5	1	E, G, R
	2	E, K
	3	A, D, E
	4	P
	5	C, W
	6	I, K, L, M, T, V
	7	I, L, P, V
	8	D, E, K, V
	9	E, G, K, P, R
	10	A, D, R, G, K, Q, V
	11	D, E, G, I, L, R, S, V

Motif ID	Position in motif	Amino acid residues with >5% representation in motif
	14	A, E, G, Q, R
8	1	K, R
	2	P
	3	A, D, E, N
	4	I, L, M, V
	5	I, V
	6	F, S, T
	7	H, K, L, Q, R, W
	8	L
	9	E
	10	K, Q, R
	11	E, G, R
	12	D, E, K
	13	A, D, E
	14	L, P
	15	C, W
9	1	C, H, L, Q, W
	2	L
	3	D, G, N, R, S
	4	L, P, S, T
	5	A, S, T
	6	Q
	7	K, R
	8	A, D, E, K, N, S, T

[0555] Notably, motifs 6 and 7 were present in 100% of the top 1597 repressor domains. Many of the highly conserved positions in motif 6 (e.g., amino acid residues L1, Y2, V5, M6, and E8) are known to form an interface with Trim28 (also known as Kap1), which is responsible for recruiting transcriptional repressive machinery to a locus. Similarly, residues in motif 7 (D3, V4, E11, E12) all contribute to Trim28 recruitment. It is believed that many of the amino acid residues identified as enriched in the top repressor domains strengthen Trim28 recruitment. Notably, some of these residues are absent in commonly used KRAB domains. Specifically, in the site in ZNF10 that matches motif 6, the residue at the first position is a valine instead of a leucine. In the site in ZIM3 that matches motif 7, the residue at position 11 is a glycine instead of a glutamic acid. Many of the other motifs described above that are not present in all KRAB

domains may represent additional and novel mechanisms of repression that are specific to sequence clusters of repressor domains with homology to KRAB domains.

[0556] Taken together, the experiments described herein have identified a suite of non-human transcriptional repressor domains that are effective for promoting transcriptional repression in the context of a dXR molecule. These domains repressed transcription to a greater extent than ZNF10 and ZIM3. Finally, protein sequence motifs were identified that are associated with the domains that were the strongest transcriptional repressors.

Example 18: Members of the top 95 repressor domains increase LTRP5 activity

[0557] As described in Example 17, non-human repressor domains were identified that resulted in enhanced repression in the context of dXR constructs. Here, experiments were performed to test whether the enhanced repressor domains identified in Example 17 were also superior transcriptional repressors in the context of LTRP5.

Materials and Methods:

[0558] Representative repressor domains identified in Example 17 and determined to be members of the top 95 performing repressors (Table 65) were cloned into an LTRP5 construct without the DNMT3A ADD domain (FIG. 1). The LTRP5 constructs were constructed as described in Example 13 (Table 45), except that an SV40 NLS was present downstream of the repressor domains. An LTRP5 molecule with a ZIM3 KRAB domain was used as a control. A separate plasmid was used to encode guide scaffold 316 (SEQ ID NO: 1746) with spacer 7.165 (UCCCUAUGUCCUUGCUGUUU; SEQ ID NO: 3114) targeting the *B2M* locus. Additional controls included a dXR molecule with a ZIM3 KRAB domain with a gRNA having scaffold 316 and spacer 7.165, and LTRP5 and dXR molecules with the ZIM3 KRAB and a non-targeting gRNA. Spacer 7.165 was chosen because it is known to be a relatively inefficient spacer, which would therefore increase the dynamic range of the assay for discerning differences between the various LTRP molecules tested.

[0559] HEK293T cells were transfected as described in Example 14, except that the cells were transfected with 50 ng each of a plasmid encoding the LTRP construct and a plasmid encoding the gRNA. Repression analysis was conducted by analyzing B2M protein expression via HLA immunostaining followed by flow cytometry as described in Example 13. 72 of the 95 top repressor domains were assessed together in one experiment where B2M expression was measured at 7, 14, 19, and 26 days after transfection, and the data shown in Tables 57-60. For

the remaining 24 repressor domains assessed in a second experiment, B2M expression was measured at 6, 13, 20, and 27 days after transfection, and the data are shown in Tables 61-64. A construct encoding catalytically-active CasX 491, paired with the *B2M*-targeting spacer 7.37 (SEQ ID NO: 3137) was also included as a control.

Results:

[0560] The results of the B2M assay are provided in Tables 57-64, below.

Table 57. Levels of B2M repression mediated by dXR and LTRP constructs with various repressor domains quantified at 7 days post-transfection.

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
dXR	ZIM3	NT	7.347	1.955	15
LTRP5	ZIM3	NT	5.619	2.520	15
dXR	ZIM3	7.165	6.334	1.335	14
LTRP5	DOMAIN_11029	7.165	33.467	1.290	3
LTRP5	DOMAIN_4968	7.165	30.133	2.804	3
LTRP5	DOMAIN_27811	7.165	22.633	0.643	3
LTRP5	DOMAIN_5066	7.165	37.833	1.026	3
LTRP5	DOMAIN_15126	7.165	30.333	0.306	3
LTRP5	DOMAIN_17358	7.165	27.767	3.062	3
LTRP5	DOMAIN_8503	7.165	29.700	0.889	3
LTRP5	DOMAIN_11486	7.165	36.667	1.320	3
LTRP5	DOMAIN_28803	7.165	30.367	0.902	3
LTRP5	DOMAIN_17317	7.165	25.933	0.586	3
LTRP5	DOMAIN_24125	7.165	32.667	1.290	3
LTRP5	DOMAIN_8853	7.165	48.100	4.458	3
LTRP5	DOMAIN_19949	7.165	31.967	2.511	3
LTRP5	DOMAIN_737	7.165	41.467	3.258	3
LTRP5	DOMAIN_16444	7.165	36.367	1.704	3
LTRP5	DOMAIN_11386	7.165	35.633	1.677	3
LTRP5	DOMAIN_27506	7.165	39.467	1.504	3
LTRP5	DOMAIN_10331	7.165	38.300	1.308	3
LTRP5	DOMAIN_13539	7.165	40.800	2.307	3
LTRP5	DOMAIN_2380	7.165	41.100	1.277	3
LTRP5	DOMAIN_18258	7.165	29.133	0.777	3
LTRP5	DOMAIN_23723	7.165	33.400	2.170	3
LTRP5	DOMAIN_16806	7.165	35.667	1.450	3
LTRP5	DOMAIN_18216	7.165	41.400	0.819	3
LTRP5	DOMAIN_17432	7.165	43.967	0.907	3
LTRP5	DOMAIN_4806	7.165	36.767	1.747	3

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_25379	7.165	46.467	3.868	3
LTRP5	DOMAIN_16643	7.165	41.133	1.206	3
LTRP5	DOMAIN_21603	7.165	34.367	0.404	3
LTRP5	DOMAIN_21247	7.165	37.867	2.219	3
LTRP5	DOMAIN_28640	7.165	39.900	1.277	3
LTRP5	ZIM3	7.165	31.940	1.637	15
LTRP5	DOMAIN_14659	7.165	37.933	1.767	3
LTRP5	DOMAIN_6248	7.165	36.000	2.352	3
LTRP5	DOMAIN_11348	7.165	38.433	1.286	3
LTRP5	DOMAIN_19229	7.165	33.967	0.321	3
LTRP5	DOMAIN_17759	7.165	43.233	0.924	3
LTRP5	DOMAIN_24663	7.165	39.433	6.048	3
LTRP5	DOMAIN_18137	7.165	44.033	0.404	3
LTRP5	DOMAIN_13331	7.165	38.700	2.163	3
LTRP5	DOMAIN_6807	7.165	39.500	0.436	3
LTRP5	DOMAIN_16688	7.165	41.800	0.265	3
LTRP5	DOMAIN_26322	7.165	42.767	4.661	3
LTRP5	DOMAIN_6802	7.165	45.767	1.888	3
LTRP5	DOMAIN_22270	7.165	32.500	1.100	3
LTRP5	DOMAIN_7255	7.165	43.567	0.451	3
LTRP5	DOMAIN_5463	7.165	32.533	0.404	3
LTRP5	DOMAIN_12631	7.165	40.300	0.624	3
LTRP5	DOMAIN_9960	7.165	43.967	2.363	3
LTRP5	DOMAIN_6445	7.165	45.667	2.730	3
LTRP5	DOMAIN_23394	7.165	40.733	2.285	3
LTRP5	DOMAIN_10948	7.165	42.733	0.924	3
LTRP5	DOMAIN_19804	7.165	42.067	1.914	3
LTRP5	DOMAIN_5290	7.165	43.400	1.136	3
LTRP5	DOMAIN_24458	7.165	43.567	1.332	3
LTRP5	DOMAIN_19896	7.165	43.600	0.624	3
LTRP5	DOMAIN_21755	7.165	38.667	1.498	3
LTRP5	DOMAIN_8790	7.165	34.900	0.608	3
LTRP5	DOMAIN_881	7.165	43.533	1.858	3
LTRP5	DOMAIN_14755	7.165	37.350	0.071	2
LTRP5	DOMAIN_20505	7.165	45.367	0.569	3
LTRP5	DOMAIN_9114	7.165	43.267	1.501	3
LTRP5	DOMAIN_13468	7.165	43.700	1.572	3
LTRP5	DOMAIN_11683	7.165	40.267	0.153	3
LTRP5	DOMAIN_22153	7.165	46.833	0.643	3
LTRP5	DOMAIN_25289	7.165	38.533	0.945	3
LTRP5	DOMAIN_17905	7.165	36.933	1.447	3

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_221	7.165	43.433	0.751	3
LTRP5	DOMAIN_7694	7.165	52.400	0.436	3
LTRP5	DOMAIN_15507	7.165	44.067	0.907	3
LTRP5	DOMAIN_29304	7.165	51.700	1.400	3
LTRP5	DOMAIN_10123	7.165	47.833	0.666	3
LTRP5	DOMAIN_30173	7.165	53.900	0.100	3

Table 58. Levels of B2M repression mediated by dXR and LTRP constructs with various repressor domains quantified at 14 days post-transfection.

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
dXR	ZIM3	NT	1.768	0.862	15
LTRP5	ZIM3	NT	2.902	0.966	15
dXR	ZIM3	7.165	3.397	3.091	15
LTRP5	DOMAIN_11029	7.165	14.667	1.620	3
LTRP5	DOMAIN_4968	7.165	18.933	1.557	3
LTRP5	DOMAIN_27811	7.165	15.100	0.854	3
LTRP5	DOMAIN_5066	7.165	21.133	1.286	3
LTRP5	DOMAIN_15126	7.165	18.767	2.060	3
LTRP5	DOMAIN_17358	7.165	16.667	1.815	3
LTRP5	DOMAIN_8503	7.165	17.367	1.582	3
LTRP5	DOMAIN_11486	7.165	21.567	2.401	3
LTRP5	DOMAIN_28803	7.165	20.533	0.666	3
LTRP5	DOMAIN_17317	7.165	18.833	1.358	3
LTRP5	DOMAIN_24125	7.165	23.767	0.321	3
LTRP5	DOMAIN_8853	7.165	25.250	0.212	2
LTRP5	DOMAIN_19949	7.165	23.633	1.767	3
LTRP5	DOMAIN_737	7.165	26.767	2.589	3
LTRP5	DOMAIN_16444	7.165	25.267	0.862	3
LTRP5	DOMAIN_11386	7.165	26.800	1.868	3
LTRP5	DOMAIN_27506	7.165	26.267	1.501	3
LTRP5	DOMAIN_10331	7.165	25.733	1.286	3
LTRP5	DOMAIN_13539	7.165	28.700	2.152	3
LTRP5	DOMAIN_2380	7.165	27.533	1.858	3
LTRP5	DOMAIN_18258	7.165	21.967	0.777	3
LTRP5	DOMAIN_23723	7.165	23.333	1.501	3
LTRP5	DOMAIN_16806	7.165	26.367	2.060	3
LTRP5	DOMAIN_18216	7.165	27.667	1.358	3
LTRP5	DOMAIN_17432	7.165	29.433	0.462	3
LTRP5	DOMAIN_4806	7.165	25.500	2.272	3

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_25379	7.165	29.450	2.051	2
LTRP5	DOMAIN_16643	7.165	26.333	1.890	3
LTRP5	DOMAIN_21603	7.165	31.367	4.219	3
LTRP5	DOMAIN_21247	7.165	29.467	0.651	3
LTRP5	DOMAIN_28640	7.165	28.133	1.350	3
LTRP5	ZIM3	7.165	25.287	1.914	15
LTRP5	DOMAIN_14659	7.165	28.167	2.676	3
LTRP5	DOMAIN_6248	7.165	25.433	0.115	3
LTRP5	DOMAIN_11348	7.165	29.433	2.501	3
LTRP5	DOMAIN_19229	7.165	25.367	1.656	3
LTRP5	DOMAIN_17759	7.165	29.867	1.250	3
LTRP5	DOMAIN_24663	7.165	30.500	1.323	3
LTRP5	DOMAIN_18137	7.165	28.967	0.839	3
LTRP5	DOMAIN_13331	7.165	29.333	1.701	3
LTRP5	DOMAIN_6807	7.165	28.133	0.208	3
LTRP5	DOMAIN_16688	7.165	29.667	0.153	3
LTRP5	DOMAIN_26322	7.165	30.033	8.565	3
LTRP5	DOMAIN_6802	7.165	30.900	1.670	3
LTRP5	DOMAIN_22270	7.165	26.967	2.967	3
LTRP5	DOMAIN_7255	7.165	31.500	2.536	3
LTRP5	DOMAIN_5463	7.165	24.167	1.358	3
LTRP5	DOMAIN_12631	7.165	31.967	2.108	3
LTRP5	DOMAIN_9960	7.165	31.900	1.493	3
LTRP5	DOMAIN_6445	7.165	34.533	1.484	3
LTRP5	DOMAIN_23394	7.165	25.767	0.666	3
LTRP5	DOMAIN_10948	7.165	34.267	1.750	3
LTRP5	DOMAIN_19804	7.165	32.567	2.212	3
LTRP5	DOMAIN_5290	7.165	34.967	2.810	3
LTRP5	DOMAIN_24458	7.165	30.433	1.762	3
LTRP5	DOMAIN_19896	7.165	32.200	0.954	3
LTRP5	DOMAIN_21755	7.165	27.867	3.630	3
LTRP5	DOMAIN_8790	7.165	25.033	0.058	3
LTRP5	DOMAIN_881	7.165	27.533	0.987	3
LTRP5	DOMAIN_14755	7.165	29.867	0.404	3
LTRP5	DOMAIN_20505	7.165	35.733	1.150	3
LTRP5	DOMAIN_9114	7.165	34.467	0.981	3
LTRP5	DOMAIN_13468	7.165	28.633	1.721	3
LTRP5	DOMAIN_11683	7.165	32.333	0.757	3
LTRP5	DOMAIN_22153	7.165	34.533	3.592	3
LTRP5	DOMAIN_25289	7.165	30.467	2.495	3
LTRP5	DOMAIN_17905	7.165	32.800	0.529	3

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_221	7.165	33.233	1.012	3
LTRP5	DOMAIN_7694	7.165	42.367	1.531	3
LTRP5	DOMAIN_15507	7.165	34.600	2.600	3
LTRP5	DOMAIN_29304	7.165	42.067	2.948	3
LTRP5	DOMAIN_10123	7.165	39.667	2.984	3
LTRP5	DOMAIN_30173	7.165	49.167	1.266	3

Table 59. Levels of B2M repression mediated by dXR and LTRP constructs with various repressor domains quantified at 19 days post-transfection.

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
dXR	ZIM3	NT	7.326	4.796	15
LTRP5	ZIM3	NT	6.060	4.855	15
dXR	ZIM3	7.165	10.038	10.782	15
LTRP5	DOMAIN_11029	7.165	10.257	1.970	3
LTRP5	DOMAIN_4968	7.165	14.333	2.579	3
LTRP5	DOMAIN_27811	7.165	13.200	1.212	3
LTRP5	DOMAIN_5066	7.165	15.800	0.781	3
LTRP5	DOMAIN_15126	7.165	17.133	1.097	3
LTRP5	DOMAIN_17358	7.165	22.700	2.364	3
LTRP5	DOMAIN_8503	7.165	12.267	2.285	3
LTRP5	DOMAIN_11486	7.165	16.600	2.138	3
LTRP5	DOMAIN_28803	7.165	15.200	0.300	3
LTRP5	DOMAIN_17317	7.165	17.267	1.026	3
LTRP5	DOMAIN_24125	7.165	19.700	1.652	3
LTRP5	DOMAIN_8853	7.165	21.333	3.842	3
LTRP5	DOMAIN_19949	7.165	16.600	1.082	3
LTRP5	DOMAIN_737	7.165	19.367	1.168	3
LTRP5	DOMAIN_16444	7.165	20.433	2.026	3
LTRP5	DOMAIN_11386	7.165	19.333	0.924	3
LTRP5	DOMAIN_27506	7.165	23.300	1.970	3
LTRP5	DOMAIN_10331	7.165	22.800	2.007	3
LTRP5	DOMAIN_13539	7.165	24.267	2.150	3
LTRP5	DOMAIN_2380	7.165	25.067	3.668	3
LTRP5	DOMAIN_18258	7.165	21.467	2.344	3
LTRP5	DOMAIN_23723	7.165	20.367	3.329	3
LTRP5	DOMAIN_16806	7.165	22.267	1.656	3
LTRP5	DOMAIN_18216	7.165	22.200	2.193	3
LTRP5	DOMAIN_17432	7.165	25.967	0.404	3
LTRP5	DOMAIN_4806	7.165	20.133	2.593	3

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_25379	7.165	26.400	4.583	3
LTRP5	DOMAIN_16643	7.165	24.733	2.205	3
LTRP5	DOMAIN_21603	7.165	24.500	5.186	3
LTRP5	DOMAIN_21247	7.165	23.667	1.002	3
LTRP5	DOMAIN_28640	7.165	26.767	3.880	3
LTRP5	ZIM3	7.165	22.520	3.682	15
LTRP5	DOMAIN_14659	7.165	26.067	3.386	3
LTRP5	DOMAIN_6248	7.165	26.833	4.140	3
LTRP5	DOMAIN_11348	7.165	24.400	2.476	3
LTRP5	DOMAIN_19229	7.165	23.133	1.858	3
LTRP5	DOMAIN_17759	7.165	27.667	0.902	3
LTRP5	DOMAIN_24663	7.165	26.667	5.493	3
LTRP5	DOMAIN_18137	7.165	23.967	1.206	3
LTRP5	DOMAIN_13331	7.165	23.367	1.626	3
LTRP5	DOMAIN_6807	7.165	23.700	0.265	3
LTRP5	DOMAIN_16688	7.165	26.367	1.930	3
LTRP5	DOMAIN_26322	7.165	25.367	8.700	3
LTRP5	DOMAIN_6802	7.165	45.967	33.520	3
LTRP5	DOMAIN_22270	7.165	21.133	0.709	3
LTRP5	DOMAIN_7255	7.165	30.267	2.103	3
LTRP5	DOMAIN_5463	7.165	18.033	1.893	3
LTRP5	DOMAIN_12631	7.165	29.100	2.516	3
LTRP5	DOMAIN_9960	7.165	29.067	3.134	3
LTRP5	DOMAIN_6445	7.165	31.267	2.040	3
LTRP5	DOMAIN_23394	7.165	25.267	2.957	3
LTRP5	DOMAIN_10948	7.165	29.400	1.473	3
LTRP5	DOMAIN_19804	7.165	26.400	1.442	3
LTRP5	DOMAIN_5290	7.165	29.133	1.021	3
LTRP5	DOMAIN_24458	7.165	25.600	2.500	3
LTRP5	DOMAIN_19896	7.165	28.600	1.997	3
LTRP5	DOMAIN_21755	7.165	30.233	1.185	3
LTRP5	DOMAIN_8790	7.165	20.733	0.723	3
LTRP5	DOMAIN_881	7.165	34.400	3.378	3
LTRP5	DOMAIN_14755	7.165	25.000	1.700	3
LTRP5	DOMAIN_20505	7.165	33.800	2.095	3
LTRP5	DOMAIN_9114	7.165	28.533	0.961	3
LTRP5	DOMAIN_13468	7.165	31.333	3.580	3
LTRP5	DOMAIN_11683	7.165	29.533	2.219	3
LTRP5	DOMAIN_22153	7.165	32.167	3.383	3
LTRP5	DOMAIN_25289	7.165	31.233	1.890	3
LTRP5	DOMAIN_17905	7.165	40.933	5.052	3

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_221	7.165	33.933	1.662	3
LTRP5	DOMAIN_7694	7.165	38.067	2.003	3
LTRP5	DOMAIN_15507	7.165	31.633	3.609	3
LTRP5	DOMAIN_29304	7.165	36.900	4.424	3
LTRP5	DOMAIN_10123	7.165	45.250	0.212	2
LTRP5	DOMAIN_30173	7.165	42.200	1.414	2

Table 60. Levels of B2M repression mediated by dXR and LTRP constructs with various repressor domains quantified at 26 days post-transfection.

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
dXR	ZIM3	NT	3.113	3.228	15
LTRP5	ZIM3	NT	4.327	3.294	15
dXR	ZIM3	7.165	6.341	4.981	15
LTRP5	DOMAIN_11029	7.165	8.727	0.401	3
LTRP5	DOMAIN_4968	7.165	9.963	0.616	3
LTRP5	DOMAIN_27811	7.165	10.480	1.753	3
LTRP5	DOMAIN_5066	7.165	11.633	1.790	3
LTRP5	DOMAIN_15126	7.165	11.897	2.133	3
LTRP5	DOMAIN_17358	7.165	12.700	2.022	3
LTRP5	DOMAIN_8503	7.165	13.000	1.480	3
LTRP5	DOMAIN_11486	7.165	13.300	2.762	3
LTRP5	DOMAIN_28803	7.165	13.433	1.626	3
LTRP5	DOMAIN_17317	7.165	14.333	2.346	3
LTRP5	DOMAIN_24125	7.165	14.967	0.961	3
LTRP5	DOMAIN_8853	7.165	15.333	1.531	3
LTRP5	DOMAIN_19949	7.165	15.600	1.082	3
LTRP5	DOMAIN_737	7.165	16.600	1.127	3
LTRP5	DOMAIN_16444	7.165	17.067	0.833	3
LTRP5	DOMAIN_11386	7.165	17.633	1.270	3
LTRP5	DOMAIN_27506	7.165	17.667	1.450	3
LTRP5	DOMAIN_10331	7.165	18.033	2.055	3
LTRP5	DOMAIN_13539	7.165	18.100	2.390	3
LTRP5	DOMAIN_2380	7.165	18.133	0.723	3
LTRP5	DOMAIN_18258	7.165	18.200	1.852	3
LTRP5	DOMAIN_23723	7.165	18.667	1.290	3
LTRP5	DOMAIN_16806	7.165	18.800	2.718	3
LTRP5	DOMAIN_18216	7.165	19.333	2.802	3
LTRP5	DOMAIN_17432	7.165	19.367	1.626	3
LTRP5	DOMAIN_4806	7.165	19.400	2.022	3

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_25379	7.165	19.667	5.994	3
LTRP5	DOMAIN_16643	7.165	19.833	1.550	3
LTRP5	DOMAIN_21603	7.165	20.033	3.482	3
LTRP5	DOMAIN_21247	7.165	20.067	0.473	3
LTRP5	DOMAIN_28640	7.165	20.500	2.587	3
LTRP5	ZIM3	7.165	20.607	4.413	15
LTRP5	DOMAIN_14659	7.165	20.633	2.371	3
LTRP5	DOMAIN_6248	7.165	20.867	1.193	3
LTRP5	DOMAIN_11348	7.165	21.367	3.811	3
LTRP5	DOMAIN_19229	7.165	21.533	1.266	3
LTRP5	DOMAIN_17759	7.165	21.567	0.833	3
LTRP5	DOMAIN_24663	7.165	21.633	1.701	3
LTRP5	DOMAIN_18137	7.165	21.833	1.097	3
LTRP5	DOMAIN_13331	7.165	21.900	1.153	3
LTRP5	DOMAIN_6807	7.165	21.900	1.735	3
LTRP5	DOMAIN_16688	7.165	22.200	2.265	3
LTRP5	DOMAIN_26322	7.165	22.233	11.832	3
LTRP5	DOMAIN_6802	7.165	22.433	1.150	3
LTRP5	DOMAIN_22270	7.165	22.533	2.084	3
LTRP5	DOMAIN_7255	7.165	22.867	4.271	3
LTRP5	DOMAIN_5463	7.165	22.900	2.516	3
LTRP5	DOMAIN_12631	7.165	23.433	2.641	3
LTRP5	DOMAIN_9960	7.165	23.500	3.996	3
LTRP5	DOMAIN_6445	7.165	23.633	3.308	3
LTRP5	DOMAIN_23394	7.165	23.900	1.127	3
LTRP5	DOMAIN_10948	7.165	23.900	2.166	3
LTRP5	DOMAIN_19804	7.165	24.133	1.966	3
LTRP5	DOMAIN_5290	7.165	24.233	2.139	3
LTRP5	DOMAIN_24458	7.165	24.367	1.531	3
LTRP5	DOMAIN_19896	7.165	24.633	1.361	3
LTRP5	DOMAIN_21755	7.165	25.333	1.097	3
LTRP5	DOMAIN_8790	7.165	25.567	1.320	3
LTRP5	DOMAIN_881	7.165	26.367	0.208	3
LTRP5	DOMAIN_14755	7.165	26.867	1.563	3
LTRP5	DOMAIN_20505	7.165	27.467	3.101	3
LTRP5	DOMAIN_9114	7.165	28.100	0.872	3
LTRP5	DOMAIN_13468	7.165	28.100	2.663	3
LTRP5	DOMAIN_11683	7.165	28.300	1.300	3
LTRP5	DOMAIN_22153	7.165	28.500	3.716	3
LTRP5	DOMAIN_25289	7.165	28.600	3.579	3
LTRP5	DOMAIN_17905	7.165	30.367	0.839	3

Repressor construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_221	7.165	31.433	3.707	3
LTRP5	DOMAIN_7694	7.165	32.833	2.517	3
LTRP5	DOMAIN_15507	7.165	32.933	3.011	3
LTRP5	DOMAIN_29304	7.165	32.933	4.409	3
LTRP5	DOMAIN_10123	7.165	35.500	4.814	3
LTRP5	DOMAIN_30173	7.165	41.967	2.318	3

Table 61. Levels of B2M repression mediated by dXR and LTRP constructs with various repressor domains quantified at 6 days post-transfection.

Construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
dXR	ZIM3	7.165	7.165	2.389	6
dXR	ZIM3	NT	3.625	0.408	6
LTRP5	ZIM3	NT	5.888	0.976	6
LTRP5	DOMAIN_31643	7.165	15.633	0.551	3
LTRP5	DOMAIN_19460	7.165	17.133	0.231	3
LTRP5	DOMAIN_26732	7.165	21.333	0.751	3
LTRP5	DOMAIN_18563	7.165	21.700	1.100	3
LTRP5	DOMAIN_19892	7.165	21.967	0.569	3
LTRP5	DOMAIN_21317	7.165	25.633	0.416	3
LTRP5	DOMAIN_9114	7.165	23.233	0.907	3
LTRP5	DOMAIN_10277	7.165	22.900	0.361	3
LTRP5	DOMAIN_27060	7.165	26.533	0.666	3
LTRP5	DOMAIN_12452	7.165	26.900	1.510	3
LTRP5	DOMAIN_21336	7.165	23.533	0.961	3
LTRP5	DOMAIN_30661	7.165	24.867	1.801	3
LTRP5	DOMAIN_12292	7.165	30.700	0.436	3
LTRP5	DOMAIN_8853	7.165	31.167	0.987	3
LTRP5	DOMAIN_9538	7.165	29.800	1.952	3
LTRP5	DOMAIN_19821	7.165	26.200	1.652	3
LTRP5	ZIM3	7.165	23.083	3.178	6
LTRP5	DOMAIN_26070	7.165	29.433	2.272	3
LTRP5	DOMAIN_19476	7.165	27.867	0.808	3
LTRP5	DOMAIN_4687	7.165	30.133	0.808	3
LTRP5	DOMAIN_25405	7.165	33.467	1.097	3
LTRP5	DOMAIN_10577	7.165	28.933	0.473	3
LTRP5	DOMAIN_2942	7.165	30.633	1.429	3
LTRP5	DOMAIN_27604	7.165	28.700	1.300	3
LTRP5	DOMAIN_7694	7.165	36.633	0.666	3
LTRP5	DOMAIN_29304	7.165	34.933	0.289	3

Construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_9331	7.165	37.733	0.321	3
LTRP5	DOMAIN_30173	7.165	33.900	0.794	3
LTRP5	DOMAIN_26749	7.165	40.467	0.945	3
LTRP5	DOMAIN_27385	7.165	35.933	0.473	3
CasX 491	N/A	7.37	76.167	0.808	3

Table 62. Levels of B2M repression mediated by dXR and LTRP constructs with various repressor domains quantified at 13 days post-transfection.

Construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
dXR	ZIM3	7.165	7.258	14.130	6
dXR	ZIM3	NT	1.218	0.144	6
LTRP5	ZIM3	NT	1.712	0.328	6
LTRP5	DOMAIN_31643	7.165	6.800	0.786	3
LTRP5	DOMAIN_19460	7.165	9.690	0.147	3
LTRP5	DOMAIN_26732	7.165	10.800	0.173	3
LTRP5	DOMAIN_18563	7.165	12.100	1.587	3
LTRP5	DOMAIN_19892	7.165	12.900	0.458	3
LTRP5	DOMAIN_21317	7.165	13.967	0.513	3
LTRP5	DOMAIN_9114	7.165	13.767	1.504	3
LTRP5	DOMAIN_10277	7.165	15.333	1.150	3
LTRP5	DOMAIN_27060	7.165	13.867	1.457	3
LTRP5	DOMAIN_12452	7.165	15.700	1.562	3
LTRP5	DOMAIN_21336	7.165	14.267	0.651	3
LTRP5	DOMAIN_30661	7.165	14.933	1.537	3
LTRP5	DOMAIN_12292	7.165	17.967	0.666	3
LTRP5	DOMAIN_8853	7.165	18.600	0.361	3
LTRP5	DOMAIN_9538	7.165	18.733	1.102	3
LTRP5	DOMAIN_19821	7.165	15.867	2.237	3
LTRP5	ZIM3	7.165	18.267	3.578	6
LTRP5	DOMAIN_26070	7.165	18.233	1.960	3
LTRP5	DOMAIN_19476	7.165	20.100	1.323	3
LTRP5	DOMAIN_4687	7.165	19.767	0.961	3
LTRP5	DOMAIN_25405	7.165	22.100	1.609	3
LTRP5	DOMAIN_10577	7.165	21.133	1.266	3
LTRP5	DOMAIN_2942	7.165	21.633	2.101	3
LTRP5	DOMAIN_27604	7.165	24.700	2.364	3
LTRP5	DOMAIN_7694	7.165	26.900	0.436	3
LTRP5	DOMAIN_29304	7.165	25.367	0.462	3
LTRP5	DOMAIN_9331	7.165	27.133	0.681	3

Construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_30173	7.165	25.400	0.656	3
LTRP5	DOMAIN_26749	7.165	27.700	0.608	3
LTRP5	DOMAIN_27385	7.165	27.733	0.808	3
CasX 491	N/A	7.37	81.167	0.945	3

Table 63. Levels of B2M repression mediated by dXR and LTRP constructs with various repressor domains quantified at 20 days post-transfection.

Construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
dXR	ZIM3	7.165	1.120	0.345	6
dXR	ZIM3	NT	1.425	1.093	6
LTRP5	ZIM3	NT	2.412	2.055	6
LTRP5	DOMAIN_31643	7.165	6.147	1.909	3
LTRP5	DOMAIN_19460	7.165	8.290	0.460	3
LTRP5	DOMAIN_26732	7.165	7.950	1.171	3
LTRP5	DOMAIN_18563	7.165	10.163	1.163	3
LTRP5	DOMAIN_19892	7.165	11.800	1.249	3
LTRP5	DOMAIN_21317	7.165	12.833	2.581	3
LTRP5	DOMAIN_9114	7.165	12.467	0.681	3
LTRP5	DOMAIN_10277	7.165	13.633	0.635	3
LTRP5	DOMAIN_27060	7.165	12.400	1.375	3
LTRP5	DOMAIN_12452	7.165	13.233	0.451	3
LTRP5	DOMAIN_21336	7.165	12.733	0.503	3
LTRP5	DOMAIN_30661	7.165	13.633	0.379	3
LTRP5	DOMAIN_12292	7.165	14.300	0.100	3
LTRP5	DOMAIN_8853	7.165	16.200	2.524	3
LTRP5	DOMAIN_9538	7.165	16.200	1.100	3
LTRP5	DOMAIN_19821	7.165	14.233	1.790	3
LTRP5	ZIM3	7.165	17.033	4.457	6
LTRP5	DOMAIN_26070	7.165	16.467	1.401	3
LTRP5	DOMAIN_19476	7.165	17.133	0.569	3
LTRP5	DOMAIN_4687	7.165	16.700	0.755	3
LTRP5	DOMAIN_25405	7.165	19.300	1.114	3
LTRP5	DOMAIN_10577	7.165	18.167	0.551	3
LTRP5	DOMAIN_2942	7.165	18.567	2.001	3
LTRP5	DOMAIN_27604	7.165	21.533	1.850	3
LTRP5	DOMAIN_7694	7.165	22.467	0.709	3
LTRP5	DOMAIN_29304	7.165	22.033	1.365	3
LTRP5	DOMAIN_9331	7.165	22.800	1.510	3
LTRP5	DOMAIN_30173	7.165	24.800	3.936	3

Construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
LTRP5	DOMAIN_26749	7.165	24.633	1.290	3
LTRP5	DOMAIN_27385	7.165	23.633	1.935	3
CasX 491	N/A	7.37	83.633	3.443	3

Table 64. Levels of B2M repression mediated by dXR and LTRP constructs with various repressor domains quantified at 27 days post-transfection.

Construct	Repressor domain	Spacer	Mean % HLA-negative cells	Standard deviation	Sample size
dXR	ZIM3	7.165	1.845	1.522	6
dXR	ZIM3	NT	2.463	2.385	6
LTRP5	ZIM3	NT	3.022	2.959	6
LTRP5	DOMAIN_31643	7.165	3.687	0.562	3
LTRP5	DOMAIN_19460	7.165	5.853	0.416	3
LTRP5	DOMAIN_26732	7.165	6.647	0.985	3
LTRP5	DOMAIN_18563	7.165	7.660	0.939	3
LTRP5	DOMAIN_19892	7.165	8.920	0.596	3
LTRP5	DOMAIN_21317	7.165	9.640	0.487	3
LTRP5	DOMAIN_9114	7.165	10.167	2.191	3
LTRP5	DOMAIN_10277	7.165	10.237	0.446	3
LTRP5	DOMAIN_27060	7.165	10.377	1.046	3
LTRP5	DOMAIN_12452	7.165	10.473	0.654	3
LTRP5	DOMAIN_21336	7.165	10.767	0.404	3
LTRP5	DOMAIN_30661	7.165	11.133	1.790	3
LTRP5	DOMAIN_12292	7.165	11.833	0.252	3
LTRP5	DOMAIN_8853	7.165	11.967	1.401	3
LTRP5	DOMAIN_9538	7.165	12.100	1.000	3
LTRP5	DOMAIN_19821	7.165	12.200	1.868	3
LTRP5	ZIM3	7.165	12.483	2.585	6
LTRP5	DOMAIN_26070	7.165	13.533	2.401	3
LTRP5	DOMAIN_19476	7.165	13.900	1.153	3
LTRP5	DOMAIN_4687	7.165	13.900	1.970	3
LTRP5	DOMAIN_25405	7.165	14.467	1.387	3
LTRP5	DOMAIN_10577	7.165	15.233	1.301	3
LTRP5	DOMAIN_2942	7.165	15.433	2.255	3
LTRP5	DOMAIN_27604	7.165	18.600	2.307	3
LTRP5	DOMAIN_7694	7.165	19.067	1.021	3
LTRP5	DOMAIN_29304	7.165	19.167	1.935	3
LTRP5	DOMAIN_9331	7.165	19.333	0.808	3
LTRP5	DOMAIN_30173	7.165	20.133	0.764	3
LTRP5	DOMAIN_26749	7.165	20.467	0.493	3

LTRP5	DOMAIN_27385	7.165	21.000	1.411	3
CasX 491	N/A	7.37	80.933	0.451	3

[0561] As shown in Tables 57-64, treatment with most LTRP5 constructs containing an enhanced repressor domain, paired with a gRNA containing spacer 7.165, resulted in higher levels of B2M repression in comparison to the level of repression achieved with use of an LTRP5-ZIM3 construct. The data from these experiments were used to identify the top 9 candidate repressor domains using the following criteria. First, demonstration of at least a 20% increase in mean repressive activity as measured by B2M knockdown over the mean repressive activity exhibited by the LTRP5-ZIM3 control at all four timepoints in the experiments above, which resulted in the selection of three repressor domains. To identify more candidate repressor domains, domains that exhibited at least a 20% increase in mean activity over that exhibited by the LTRP5-ZIM3 control in at least two timepoints, prioritizing later timepoints; and, third, selecting the best performing domains from each of the 75 clusters (discussed in Example 17) to maintain diversity of amino acid sequences or eliminating domains with >90% sequence identity to previously identified human repressor domains (Tycko, J. et al., High-Throughput Discovery and Characterization of Human Transcriptional Effectors *Cell.* 183(7):2020-2035 (2020)). The nine most effective repressor domains identified were DOMAIN_7694, DOMAIN_10123, DOMAIN_15507, DOMAIN_17905, DOMAIN_20505, DOMAIN_26749, DOMAIN_27604, DOMAIN_29304, and DOMAIN_30173, and their sequences are listed in Table 65.

Table 65. List of top 9 and top 95 most effective repressor domains.

Domain ID	Description	SEQ ID NO
Top 9 repressor domains		
DOMAIN_7694	Columba livia repressor domain	130
DOMAIN_10123	Rattus norvegicus repressor domain	131
DOMAIN_15507	Cebus imitator repressor domain	132
DOMAIN_17905	Chimp repressor domain	133
DOMAIN_20505	Chlorocebus sabaeus repressor domain	134
DOMAIN_26749	Ophiophagus hannah repressor domain	135
DOMAIN_27604	Ailuropoda melanoleuca repressor domain	136

Domain ID	Description	SEQ ID NO
DOMAIN_29304	Peromyscus maniculatus bairdii repressor domain	137
DOMAIN_30173	Phyllostomus discolor repressor domain	138
Remaining repressor domains in the top 95 repressor domains		
DOMAIN_737	Bonobo repressor domain	139
DOMAIN_10331	Colobus angolensis palliatus repressor domain	140
DOMAIN_10948	Colobus angolensis palliatus repressor domain	141
DOMAIN_11029	Mandrillus leucophaeus repressor domain	142
DOMAIN_17358	Bos indicus x Bos taurus repressor domain	143
DOMAIN_17759	Felis catus repressor domain	144
DOMAIN_18258	Physeter macrocephalus repressor domain	145
DOMAIN_19804	Callorhinus ursinus repressor domain	146
DOMAIN_221	Bonobo repressor domain	147
DOMAIN_881	Bonobo repressor domain	148
DOMAIN_2380	Orangutan repressor domain	149
DOMAIN_2942	Gibbon repressor domain	150
DOMAIN_4687	Marmoset repressor domain	151
DOMAIN_4806	Marmoset repressor domain	152
DOMAIN_4968	Marmoset repressor domain	153
DOMAIN_5066	Marmoset repressor domain	154
DOMAIN_5290	Owl Monkey repressor domain	155
DOMAIN_5463	Owl Monkey repressor domain	156
DOMAIN_6248	Saimiri boliviensis boliviensis repressor domain	157
DOMAIN_6445	Alligator sinensis repressor domain	158
DOMAIN_6802	Pantherophis guttatus repressor domain	159
DOMAIN_6807	Xenopus laevis repressor domain	160
DOMAIN_7255	Microcaecilia unicolor repressor domain	161
DOMAIN_8503	Mus caroli repressor domain	162
DOMAIN_8790	Marmota monax repressor domain	163
DOMAIN_8853	Mesocricetus auratus repressor domain	164
DOMAIN_9114	Peromyscus maniculatus bairdii repressor domain	165
DOMAIN_9331	Peromyscus maniculatus bairdii repressor domain	166
DOMAIN_9538	Mus musculus repressor domain	167
DOMAIN_9960	Octodon degus repressor domain	168
DOMAIN_10277	Dipodomys ordii repressor domain	169

Domain ID	Description	SEQ ID NO
DOMAIN_10577	Colobus angolensis palliatus repressor domain	170
DOMAIN_11348	Chlorocebus sabaeus repressor domain	171
DOMAIN_11386	Capra hircus repressor domain	172
DOMAIN_11486	Bos mutus repressor domain	173
DOMAIN_11683	Nomascus leucogenys repressor domain	174
DOMAIN_12292	Sus scrofa repressor domain	175
DOMAIN_12452	Neophocaena asiaeorientalis asiaeorientalis repressor domain	176
DOMAIN_12631	Macaca fascicularis repressor domain	177
DOMAIN_13331	Macaca fascicularis repressor domain	178
DOMAIN_13468	Phascolarctos cinereus repressor domain	179
DOMAIN_13539	Gorilla repressor domain	180
DOMAIN_14659	Acinonyx jubatus repressor domain	181
DOMAIN_14755	Cebus imitator repressor domain	182
DOMAIN_15126	Callithrix jacchus repressor domain	183
DOMAIN_16444	Acinonyx jubatus repressor domain	184
DOMAIN_16688	Lipotes vexillifer repressor domain	185
DOMAIN_16806	Sapajus apella repressor domain	186
DOMAIN_17317	Otolemur garnettii repressor domain	187
DOMAIN_17432	Otolemur garnettii repressor domain	188
DOMAIN_18137	Monodelphis domestica repressor domain	189
DOMAIN_18216	Physeter macrocephalus repressor domain	190
DOMAIN_18563	OwlMonkey repressor domain	191

Domain ID	Description	SEQ ID NO
DOMAIN_19229	Enhydra lutris kenyoni repressor domain	192
DOMAIN_19460	Monodelphis domestica repressor domain	193
DOMAIN_19476	OwlMonkey repressor domain	194
DOMAIN_19821	Rhinopithecus roxellana repressor domain	195
DOMAIN_19892	Ursus maritimus repressor domain	196
DOMAIN_19896	Ovis aries repressor domain	197
DOMAIN_19949	Callorhinus ursinus repressor domain	198
DOMAIN_21247	Neovison vison repressor domain	199
DOMAIN_21317	Pteropus vampyrus repressor domain	200
DOMAIN_21336	Equus caballus repressor domain	201
DOMAIN_21603	Lipotes vexillifer repressor domain	202
DOMAIN_21755	Equus caballus repressor domain	203
DOMAIN_22153	Zalophus californianus repressor domain	204
DOMAIN_22270	Bonobo repressor domain	205
DOMAIN_23394	Vicugna pacos repressor domain	206
DOMAIN_23723	Carlito syrichta repressor domain	207
DOMAIN_24125	Saimiri boliviensis boliviensis repressor domain	208
DOMAIN_24458	Lynx pardinus repressor domain	209
DOMAIN_24663	Myotis brandtii repressor domain	210
DOMAIN_25289	Ursus maritimus repressor domain	211
DOMAIN_25379	Sapajus apella repressor domain	212
DOMAIN_25405	Desmodus rotundus repressor domain	213

Domain ID	Description	SEQ ID NO
DOMAIN_26070	Geotrypetes seraphini repressor domain	214
DOMAIN_26322	Geotrypetes seraphini repressor domain	215
DOMAIN_26732	Meleagris gallopavo repressor domain	216
DOMAIN_27060	Gopherus agassizii repressor domain	217
DOMAIN_27385	Octodon degus repressor domain	218
DOMAIN_27506	Bos mutus repressor domain	219
DOMAIN_27811	Callithrix jacchus repressor domain	220
DOMAIN_28640	Colinus virginianus repressor domain	221
DOMAIN_28803	Monodelphis domestica repressor domain	222
DOMAIN_30661	Physeter macrocephalus repressor domain	223
DOMAIN_31643	Micrurus lemniscatus lemniscatus repressor domain	224

[0562] Accordingly, the experiments described herein demonstrate that the use of the enhanced repressor domains identified in Example 17 resulted in improved levels of transcriptional repression in the context of an LTRP construct.

Example 19: Identification of alternative consensus protein sequence motifs of enhanced repressor domains

[0563] In the previous Example 17, nine protein sequence motifs (FIGS. 57A-57I) were generated for the top 1597 enhanced repressor domains using the following methods: 1) comparing the amino acid sequences of the top 1597 repressor domains to a negative training set of 1506 repressor domains with p-values less than 0.01 and $\log_2$(fold change) values less than 0, and 2), and comparing the amino acid sequences of the top 1597 domains to shuffled sequences derived from the 1597 sequences. In this example, five more alternative consensus protein sequence motifs were generated by comparing the amino acid sequences of the top 1597 repressors domains to a negative training set containing the amino acid sequences of human ZIM3 (SEQ ID NO: 128) and ZNF10 KRAB domains (SEQ ID NO: 129). Logos of these

resulting five motifs are provided in FIGS. 58A-58E. Table 66, below, provides the unadjusted p-value and number and percentage of sequences matching the motif in the top 1597 novel repressor domains for each of the five alternative motifs, as calculated by STREME. Table 67 provides the sequences of each motif, showing the amino acid residues present at each position within the motifs (from N- to C-terminus).

Table 66: Characteristics of alternative consensus protein sequence motifs of top 1597 enhanced repressor domains generated when compared to a negative training set containing ZIM3 and ZNF10.

Alternative Motif ID	Unadjusted P-value	Number and percentage of sites matching motif in 1597 enhanced domains
1	1.7E-001	1432 (89.7%)
2	2.1E-001	1236 (77.4%)
3	2.7E-001	1058 (66.2%)
4	4.2E-001	1554 (97.3%)
5	4.3E-001	679 (42.5%)

Table 67: Characteristics of alternative consensus protein sequence motifs of 1597 enhanced repressor domains.

Motif ID	Position in motif	Amino acid residues with >5% representation in motif
1	1	D
	2	V
	3	A
	4	V
	5	Y
	6	F
	7	S
	8	P
	9	E
	10	E
	11	W
	12	G
	13	C
	14	L
2	1	A, D, G, N, R, S
	2	P, S, T
	3	A, S, T
	4	Q
	5	K, R
	6	A, D, K, N, S, T
	7	L
	8	Y
3	1	A, P, S

Motif ID	Position in motif	Amino acid residues with >5% representation in motif
	2	K
	3	P
	4	A, D, E
	5	L, M, V
	6	I, V
	7	F, S, T
	8	H, K L, Q, R, W
4	1	L
	2	E
	3	E, K, Q, R
	4	E, G, R
	5	A, D, E, K
	6	A, D, E
	7	L, P
	8	C, W
5	1	D, E
	2	V
	3	M
	4	L
	5	E
	6	N, T
	7	Y
	8	A, E, G, Q, R, S
	9	H, N
	10	L, M, V
	11	A, L, V
	12	S
	13	L, V
	14	A, G, V
	15	C, F, L

[0564] The methods used as described in this example resulted in the generation of alternative motifs associated with the 1597 repressor domains that were identified as the strongest transcriptional repressors in Example 17. Notably, alternative motifs 1, 2, 3, and 5 were not found in either ZIM3 or ZNF10, and instead were uniquely found in the majority of the 1597 top repressor domains. Furthermore, every amino acid position of alternative motif 1 appeared to be highly conserved and was found in nearly 90% of the 1597 domains; this sequence is believed to be important in mediating the recruitment of Trim28 and downstream factors involved in transcriptional and epigenetic repression. As for the other alternative consensus motifs identified, these motifs may represent additional and novel mechanisms of repression that are specific to certain clusters of repressor domains.

Example 20: Demonstration that silencing of a target locus mediated by LTRP molecules is reversible using a DNMT1 inhibitor

[0565] Experiments were performed to demonstrate that durable repression of a target locus mediated by LTRP molecules is reversible, such that treatment with a DNMT1 inhibitor would remove methyl marks to reactivate expression of the target gene.

Materials and Methods:

[0566] LTRP #5 containing the ZIM3-KRAB domain, which was generated as described in Example 13, and CasX variant 491 were used in this experiment. A *B2M*-targeting gRNA with scaffold 174 containing spacer 7.37 (SEQ ID NO: 3137) or a non-targeting gRNA containing spacer 0.0 (SEQ ID NO: 3232) were used in this experiment.

Transfection of HEK293T cells:

[0567] HEK293T cells were transfected with 100 ng of a plasmid containing a construct encoding for either CasX 491 or LTRP #5 containing the ZIM3-KRAB domain with a B2M-targeting gRNA or non-targeting gRNA and cultured for 58 days. These transfected HEK293T cells were subsequently re-seeded at ~30,000 cells well of a 96-well plate and were treated with 5-aza-2′-deoxycytidine (5-azadC), a DNMT1 inhibitor, at concentrations ranging from 0μM to 20μM. Six days post-treatment with 5-azadC, cells were harvested for B2M silencing analysis at day 5, day 12, and day 21 post-transfection. Briefly, repression analysis was conducted by analyzing B2M protein expression via HLA immunostaining followed by flow cytometry, as described in Example 13. Treatments for each dose of 5-azadC for each experimental condition were performed in triplicates.

Results:

[0568] The plot in FIG. 59 shows the percentage of transfected HEK293T cells treated with the indicated concentrations of 5-azadC that expressed the B2M protein. The data demonstrate that 5-azadC treatment of cells transfected with a plasmid encoding LTRP5-ZIM3 with the *B2M*-targeting gRNA resulted in a reactivation of the *B2M* gene (FIG. 59). Specifically, ~75% of treated cells exhibited B2M expression with 20μM 5-azadC, compared to the 25% of cells with B2M expression at 0μM concentration (FIG. 59). Furthermore, 5-azadC treatment of cells transfected with a plasmid encoding CasX 491 with the B2M-targeting gRNA did not exhibit reactivation of the *B2M* gene. FIG. 60 is a plot that juxtaposes B2M repression activity with gene reactivation upon 5-azadC treatment. The data show B2M repression post-transfection with

either CasX 491 or LTRP5-ZIM3 with the *B2M*-targeting gRNA, resulting in ~75% repression of B2M expression by day 58; however, B2M expression is increased upon 5-azadC treatment (FIG. 60). As anticipated, 5-azadC treatment of cells transfected with either CasX 491 or LTRP5-ZIM3 with the non-targeting gRNA did not demonstrate repression or reactivation (FIGS. 59-60).

[0569] The experiments demonstrate reversibility of LTRP-mediated repression of a target locus. By using a DNMT1 inhibitor to remove methyl marks implemented by LTRP molecules, the silenced target gene was reactivated to induce expression of the target protein.

Example 21: Exemplary sequences of LTRP fusion proteins

[0570] Table 68 provides exemplary full-length LTRP fusion proteins in configurations 1, 4, or 5 with the ADD domain (FIG. 2), with human ZIM3 or ZNF10 KRAB domains or one of the top nine most effective repressor domains: DOMAIN_7694, DOMAIN_10123, DOMAIN_15507, DOMAIN_17905, DOMAIN_20505, DOMAIN_26749, DOMAIN_27604, DOMAIN_29304, and DOMAIN_30173. In Table 68, the components are listed in order from N- to C-terminus.

Table 68: Exemplary protein sequences of LTRP fusion proteins

LTRP #	Components	Domains	Amino acid sequence SEQ ID NO
LTRP1 with ADD domain	START codon + NLS + buffer sequence		3269
	START codon + DNMT3A ADD domain		3270
	DNMT3A catalytic domain		126
	Linker (L2)		122
	DNMT3L interaction domain		127
	Linker (L1)		123
	Linker (L3A) + buffer		3271
	dCasX491		4
	Buffer + linker (L3B)		3272
	Repressor domain 1	Human ZIM3	3240
		Human ZNF10	3239
		Columba livia repressor domain (DOMAIN_7694)	130
		Rattus norvegicus repressor domain (DOMAIN_10123)	131
		Cebus imitator repressor domain (DOMAIN_15507)	132

LTRP #	Components	Domains	Amino acid sequence SEQ ID NO
		Chimpanzee repressor domain (DOMAIN_17905)	133
		Chlorocebus sabaeus repressor domain (DOMAIN_20505)	134
		Ophiophagus hannah repressor domain (DOMAIN_26749)	135
		Ailuropoda melanoleuca repressor domain (DOMAIN_27604)	136
		Peromyscus maniculatus bairdii repressor domain (DOMAIN_29304)	137
		Phyllostomus discolor repressor domain (DOMAIN_30173)	138
	Buffer + NLS		3273
LTRP4 with ADD domain	START codon + NLS + buffer sequence		3269
	Repressor domain 1	Human ZIM3	3240
		Human ZNF10	3239
		Columba livia repressor domain (DOMAIN_7694)	130
		Rattus norvegicus repressor domain (DOMAIN_10123)	131
		Cebus imitator repressor domain (DOMAIN_15507)	132
		Chimpanzee repressor domain (DOMAIN_17905)	133
		Chlorocebus sabaeus repressor domain (DOMAIN_20505)	134
		Ophiophagus hannah repressor domain (DOMAIN_26749)	135
		Ailuropoda melanoleuca repressor domain (DOMAIN_27604)	136
		Peromyscus maniculatus bairdii repressor domain (DOMAIN_29304)	137
		Phyllostomus discolor repressor domain (DOMAIN_30173)	138
	Linker (L3A) + buffer		3271
	START codon + DNMT3A ADD domain		3270
	DNMT3A catalytic domain		126

LTRP #	Components	Domains	Amino acid sequence SEQ ID NO
	Linker (L2)		122
	DNMT3L interaction domain		127
	Linker (L1)		123
	dCasX491		4
	Buffer + linker (L3B)		3272
	NLS		30
LTRP5 with ADD domain	START codon + NLS + buffer sequence		3269
	START codon + DNMT3A ADD domain		3270
	DNMT3A catalytic domain		126
	Linker (L2)		122
	DNMT3L interaction domain		127
	Linker (L3A)		124
	Repressor domain 1	Human ZIM3	3240
		Human ZNF10	3239
		Columba livia repressor domain (DOMAIN_7694)	130
		Rattus norvegicus repressor domain (DOMAIN_10123)	131
		Cebus imitator repressor domain (DOMAIN_15507)	132
		Chimpanzee repressor domain (DOMAIN_17905)	133
		Chlorocebus sabaeus repressor domain (DOMAIN_20505)	134
		Ophiophagus hannah repressor domain (DOMAIN_26749)	135
		Ailuropoda melanoleuca repressor domain (DOMAIN_27604)	136
		Peromyscus maniculatus bairdii repressor domain (DOMAIN_29304)	137
		Phyllostomus discolor repressor domain (DOMAIN_30173)	138
	Linker (L1)		123
	dCasX491		4
	Buffer + linker (L3B)		3272
	NLS		30

[0571] Table 69 provides exemplary amino acid sequences of components of LTRP constructs. In Table 69, the protein domains are shown without starting methionines.

Table 69: Exemplary protein sequences of components of LTRP constructs.

Component	Amino acid sequence SEQ ID NO
DNMT3A catalytic domain (CD)	126
DNMT3L interaction domain	127
dCasX491	4
Linker 1 (L1)	123
Linker 2 (L2)	122
Linker 3A (L3A)	124
Linker 3B (L3B)	120
NLS	30
DNMT3A ADD domain	125

Example 22: A preliminary evaluation of genome-wide transcriptomic effects of using an LTRP molecule and a *PCSK9*-targeting gRNA to repress the *PCSK9* locus to decrease PCSK9 secretion in vitro

[0572] Experiments were performed to determine the effects of using an LTRP molecule with a targeting gRNA to repress the *PCSK9* locus and reduce PCSK9 secretion levels in human cells. Specifically, genome-wide transcriptomic effects were evaluated to determine the extent of off-target effects of treating the cells with LTRP5-ADD-ZIM3 and select *PCSK9*-targeting spacers.

Materials and Methods:

Transfection of human Huh7 cells:

[0573] mRNA encoding the following molecules were generated by IVT using similar methods as described in Example 2: 1) a catalytically-active CasX 676 (as described in Example 5), 2) dXR1 (as described in Example 2), and 3) LTRP5-ADD-ZIM3 (as described in Example 2). The DNA and mRNA sequences for CasX 676 are shown in Tables 21 and 22; the DNA and mRNA sequences for dXR1 are shown in Tables 11 and 12; the DNA and mRNA sequences for LTRP5-ADD-ZIM3 are shown in Tables 18 and 19.

[0574] gRNAs containing select NHP-conserved spacers targeting the *PCSK9* locus were designed using gRNA scaffold 316 and chemically synthesized with the v1 modification profile (as described in Example 7). These NHP-conserved spacers, which were selected given their efficacy in sustaining PCSK9 repression through at least day 36 as demonstrated in Example 3, were TG-06-154, TG-06-167, TG-06-133, TG-06-146, and TG-06-352, TG-06-138 (see Table 17 for SEQ ID NOS) was used to pair with dXR1 as well as LTRP5-ADD-ZIM3, and spacer

TG-06-001 (also known as spacer 6.1; SEQ ID NO: 1834) was used to pair with CasX 676. A non-targeting spacer was used as an experimental control.

[0575] Seeded Huh7 cells were transfected with mRNA encoding a catalytically-active CasX 676, dXR1, or LTRP5-ADD-ZIM3 and a gRNA with scaffold 316 and a spacer targeting the *PCSK9* locus. Cells were harvested at 6 and 26 days post-transfection and subsequently lysed and stored in DNA/RNA Shield (Zymo Research). As an additional control, untreated, naïve cells were also harvested. The collected samples were subjected to gDNA/RNA extraction using the Quick-DNA/RNA Miniprep Plus kit (Zymo Research). RNA samples were used for total RNA sequencing (RNA-seq), which was performed by a third-party. Raw FASTQ files were received and processed with FASTQC, and adapters were trimmed using Trim Galore. Transcript expression was then quantified against the hg38 genome using Salmon to generate normalized counts for each gene (transcripts per million, or TPM). Differential expression analysis was performed using DESeq2. Genes with fewer than 10 counts in all samples were omitted from analysis, and all pairwise comparisons (e.g., differential gene expression analyses between untreated and treated conditions at the two individual timepoints) were done with significance thresholds of $|\log2FC| > 2$ and adjusted p-value < 0.001.

Results:

[0576] Huh7 cells were transfected with mRNA encoding CasX 676, dXR1 or LTRP5-ADD-ZIM3 with a *PCSK9*-targeting gRNA and harvested at the 6 and 26 days post-transfection for RNA-seq analysis. Quantification of normalized *PCSK9* read counts was determined for each experimental condition, and log2 fold changes in normalized *PCSK9* transcript counts comparing each experimental condition and the untreated, naïve condition were calculated (Table 70). RNA-seq analyses revealed that use of spacers TG-06-154, TG-06-167, and TG-06-133, when paired with LTRP5-ADD-ZIM3, durably repress *PCSK9* expression through day 26 (Table 70). Furthermore, while use of the non-targeting spacer with LTRP5-ADD-ZIM3 appeared to reduce *PCSK9* expression, this level of repression was not as high compared to the repression levels achieved with spacers TG-06-154, TG-06-167, and TG-06-133. As anticipated, use of spacer TG-06-138 with dXR1 resulted in transient silencing of the *PCSK9* locus, although unexpectedly, *PCSK9* downregulation was attenuated by day 26 when using spacer 6.1 paired with CasX 676 (Table 70).

Table 70: Log2FC of normalized PCSK9 read counts for each indicated experimental condition compared to untreated, naïve condition.

Molecule	Spacer	Log2FC of PCSK9 read counts – Day 6	Log2FC of PCSK9 read counts – Day 26
CasX 676	NT	0.022	-0.001
CasX 676	6.1	-1.826	-0.818
dXR1	NT	0.016	0.068
dXR1	TG-06-138	-4.291	-0.266
LTRP5-ADD-ZIM3	NT	-0.700	-1.094
LTRP5-ADD-ZIM3	TG-06-138	-4.254	-1.861
LTRP5-ADD-ZIM3	TG-06-154	-5.202	-4.283
LTRP5-ADD-ZIM3	TG-06-167	-3.404	-2.856
LTRP5-ADD-ZIM3	TG-06-133	-3.342	-2.773
LTRP5-ADD-ZIM3	TG-06-146	-3.709	-1.755
LTRP5-ADD-ZIM3	TG-06-352	-3.330	-1.981

[0577] Differential gene expression analyses were performed by comparing each experimental condition to the untreated, naïve control to determine the number of upregulated and downregulated genes detected at 6 days and 26 days after treatment. Quantification of differentially expressed genes is shown in Table 71. Representative volcano plots illustrating the differential gene expression analyses for LTRP5-ADD-ZIM3 with the non-targeting spacer, LTRP5-ADD-ZIM3 with spacer TG-06-154, and LTRP5-ADD-ZIM3 with spacer TG-06-133 are shown in FIGS. 65A-65B, 66A-66B, and 67A-67B respectively. TG-06-154, TG-06-133, and TG-06-167 were selected based on their ability to repress *PCSK9* expression through day 26. The data demonstrate that of these three *PCSK9*-targeting spacers, when assessed with LTRP5-ADD-ZIM3, use of spacer TG-06-133 resulted in the lowest number of differentially regulated off-target genes. Meanwhile, use of spacer TG-06-154 or TG-06-167 resulted in a higher number of differentially regulated off-target genes, especially at the later timepoint of 26 days post-transfection (Table 71). Furthermore, transient repression using dXR1 and spacer TG-06-138 resulted in minimal transcriptomic changes by day 26.

Table 71: Number of differentially regulated off-target genes for each treatment condition compared to untreated, naïve control at the two indicated timepoints. Statistical significance thresholds applied were |log2FC| > 2 and adjusted p-value < 0.001.

Molecule	Spacer	# of differentially expressed genes (off-target) - Day 6		# of differentially expressed genes (off-target) - Day 26	
		Downregulated	Upregulated	Downregulated	Upregulated

CasX 676	NT	0	0	0	0
CasX 676	6.1	0	0	0	0
dXR1	NT	0	0	0	0
dXR1	TG-06-138	3	14	0	1
LTRP5-ADD-ZIM3	NT	0	0	9	35
LTRP5-ADD-ZIM3	TG-06-138	2	0	0	0
LTRP5-ADD-ZIM3	TG-06-154	2	0	6	10
LTRP5-ADD-ZIM3	TG-06-167	2	0	18	21
LTRP5-ADD-ZIM3	TG-06-133	2	0	0	0
LTRP5-ADD-ZIM3	TG-06-146	1	7	0	0
LTRP5-ADD-ZIM3	TG-06-352	2	1	0	0

[0578] These experiments show a preliminary analysis of the genome-wide transcriptomic effects of using an LTRP molecule and a PCSK9-targeting gRNA to repress the *PCSK9* locus in human cells. The data from these experiments show that analyzing these genome-wide transcriptomic effects would help with the identification of candidate spacers for targeting the *PCSK9* locus.

Example 23: Assessment of *PCSK9* spacers in achieving in human hepatocyte cells when paired with an LTRP5-ADD molecule

[0579] Experiments were performed to carry out a comprehensive evaluation of *PCSK9*-targeting spacers, with the TTC recognition motif, when paired with an LTRP molecule in configuration #5 containing the ADD domain (LTRP5; diagrammed in FIG. 2). Briefly, *in vitro* experiments were conducted to assess spacers that induce durable repression of the human *PCSK9* locus, resulting in substantial reduction in PCSK9 secretion.

Materials and Methods:

[0580] A computational screen was performed as described in Example 3 that resulted in the identification of 69 TTC spacers for subsequent experimental assessment. Of these 69 TTC spacers, 61 spacers were subjected to further in vitro screening using a cell-based assay as described in the ensuing methods. In addition to the 61 spacers, four more spacers were identified in an independent computational screen using methods similar to those described in Example 3, with the addition of one criterion: i.e., TTC spacers that overlapped with a SNP

having a minor allele frequency (MAF) of >0.05 were excluded. Therefore, a total of 65 spacers were subjected to an in vitro experiment using a cell-based assay described in the methods that follow. The 61 out of 65 *PCSK9*-targeting spacers tested in this experiment are listed in Table 72, with the corresponding SEQ ID NOS listed in Table 17. The four additional spacers that were independently identified are shown in Table 73.

Table 72: List of 61 out of 65 *PCSK9*-targeting spacers assessed in this example.

Spacer ID
TG-06-342
TG-06-117
TG-06-118
TG-06-119
TG-06-120
TG-06-122
TG-06-123
TG-06-121
TG-06-124
TG-06-125
TG-06-127
TG-06-128
TG-06-131
TG-06-132
TG-06-135
TG-06-133
TG-06-134
TG-06-138
TG-06-139
TG-06-140
TG-06-141

Spacer ID
TG-06-142
TG-06-144
TG-06-143
TG-06-146
TG-06-145
TG-06-147
TG-06-149
TG-06-150
TG-06-151
TG-06-152
TG-06-153
TG-06-154
TG-06-155
TG-06-157
TG-06-159
TG-06-158
TG-06-160
TG-06-161
TG-06-004
TG-06-001
TG-06-005

Spacer ID
TG-06-002
TG-06-343
TG-06-167
TG-06-168
TG-06-169
TG-06-170
TG-06-344
TG-06-345
TG-06-346
TG-06-347
TG-06-348
TG-06-171
TG-06-349
TG-06-249
TG-06-250
TG-06-251
TG-06-350
TG-06-351
TG-06-172

Table 73: RNA sequences of the four additional TTC spacers targeting the human *PCSK9* locus.

Spacer ID	Spacer RNA sequence	SEQ ID NO:
TG-06-126	CCUUUUCAUCCUCCUGCCUG	2672
TG-06-129	UUUUACACACCAUGUUCAAG	2675
TG-06-148	GCCGGGCCCACCUUUUCAGU	2694
TG-06-1046	UCUCUUACAUGGGGGGAAAC	2714

Assessment of PCSK9 secretion levels for the 65 PCSK9-targeting spacers:

[0581] mRNA encoding the LTRP5-ADD-ZIM3 molecule was generated by IVT using methods similar to those described in Example 2. The DNA and mRNA sequences for LTRP5-ADD-ZIM3 are shown in Tables 18 and 19.

[0582] gRNAs containing each of the 65 *PCSK9*-targeting spacers (Tables 72-73) were designed using gRNA scaffold 316 and chemically synthesized with the v1 modification profile (as described in Example 7). Furthermore, a non-targeting gRNA was used as non-targeting control.

[0583] To assess PCSK9 secretion, seeded Huh7 cells were co-transfected with mRNA encoding for LTRP5-ADD-ZIM3 and a gRNA with scaffold 316 and spacer targeting the *PCSK9* locus using lipofectamine 3000. Two doses of total RNA input at a 2:1 mass ratio of mRNA to gRNA were used for screening: 50ng mRNA:25ng gRNA and 25ng mRNA:12.5ng gRNA. Media supernatant was harvested at 6 days post-transfection to assess level of PCSK9 secretion by ELISA (Table 74). Levels of PCSK9 secretion were normalized to total cell count. As an additional control, PCSK9 secretion was also measured in the media supernatant harvested from cells transfected with mRNA encoding for mScarlet.

[0584] Media supernatant is further sampled at 13, 20, and 27 days post-transfection, and levels of PCSK9 secretion are measured by ELISA as described above.

Results:

[0585] PCSK9 secretion levels for Huh7 cells transfected with mRNA encoding LTRP5-ADD-ZIM3 with a *PCSK9*-targeting gRNA at the 6-day timepoint are shown in Table 74. Specifically, Table 74 provides the level of secreted PCSK9 (ng/mL) in cells transfected with gRNAs with each of the spacers, the percent reduction in PCSK9 secretion relative to the control transfected with a non-targeting gRNA, and the distance in basepairs of each targeting sequence to the *PCSK9* transcription start site (TSS). The average percent reduction in PCSK9 secretion with each dose for each spacer is also provided, and spacers in constructs that resulted in greater than 50% reduction in PCSK9 secretion averaged from the two doses are bolded in the table.

Table 74: Results of ELISA assay evaluating the functional effects of 65 PCSK9-targeting spacers on PCSK9 secretion levels when paired with LTRP5-ADD-ZIM3*

Spacer ID	Distance to TSS (bp)	50ng mRNA: 25ng gRNA		25ng mRNA: 12.5ng gRNA		Average percent reduction in PCSK9 secretion
		Secreted PCSK9 (ng/mL)	Percent reduction in PCSK9 secretion	Secreted PCSK9 (ng/mL)	Percent reduction in PCSK9 secretion	
TG-06-342	-1086	51.55	37.34	75.23	5.6	15.87
TG-06-117	-917	68.98	16.16	98.05	37.62	10.73
TG-06-118	-860	35.5	56.86	54.78	23.11	39.99
TG-06-119	-858	70.37	14.47	84.54	18.66	2.1
TG-06-120	**-843**	**21.43**	**73.95**	**47.97**	**32.67**	**53.31**
TG-06-122	-800	79.36	3.54	86.06	20.79	8.63
TG-06-123	-789	30.49	62.95	51.32	27.97	45.46
TG-06-121	**-787**	**28.33**	**65.57**	**37.13**	**47.89**	**56.73**
TG-06-124	**-786**	**16.54**	**79.9**	**30.59**	**57.06**	**68.48**
TG-06-125	**-767**	**24.93**	**69.7**	**46.11**	**35.28**	**52.49**
TG-06-126	**-707**	**23.48**	**71.46**	**44.82**	**37.09**	**54.27**
TG-06-127	**-699**	**22.57**	**72.56**	**49.1**	**31.08**	**51.82**
TG-06-128	-664	42.21	48.7	62.29	12.57	30.63
TG-06-129	-646	59.93	27.16	90.82	27.47	0.16
TG-06-131	-644	28.4	65.48	57.89	18.74	42.11
TG-06-132	-621	56.68	31.11	75.01	5.29	12.91
TG-06-135	**-601**	**25.66**	**68.82**	**44.59**	**37.42**	**53.12**
TG-06-133	**-596**	**29.45**	**64.21**	**42.13**	**40.86**	**52.53**
TG-06-134	-589	43.79	46.77	57.45	19.37	33.07
TG-06-138	**-527**	**18.99**	**76.92**	**30.87**	**56.67**	**66.79**
TG-06-139	**-463**	**15.03**	**81.73**	**25.45**	**64.28**	**73.01**
TG-06-140	-408	61.68	25.04	59.94	15.87	20.45
TG-06-141	**-383**	**13.86**	**83.16**	**12.58**	**82.34**	**82.75**
TG-06-142	**-379**	**30.87**	**62.48**	**27.27**	**61.73**	**62.1**
TG-06-144	**-351**	**23.42**	**71.53**	**29**	**59.29**	**65.41**
TG-06-143	**-337**	**38.69**	**52.97**	**22.33**	**68.66**	**60.81**
TG-06-146	**-316**	**40.84**	**50.36**	**20.34**	**71.45**	**60.9**
TG-06-145	**-299**	**15.8**	**80.8**	**18.54**	**73.98**	**77.39**
TG-06-147	-291	53.6	34.85	37.43	47.46	41.15
TG-06-148	-273	71.46	13.15	44.77	37.16	25.15
TG-06-149	**-177**	**21.18**	**74.26**	**38.33**	**46.21**	**60.23**

Spacer ID	Distance to TSS (bp)	50ng mRNA: 25ng gRNA		25ng mRNA: 12.5ng gRNA		Average percent reduction in PCSK9 secretion
		Secreted PCSK9 (ng/mL)	Percent reduction in PCSK9 secretion	Secreted PCSK9 (ng/mL)	Percent reduction in PCSK9 secretion	
TG-06-150	**-148**	**18.33**	**77.73**	**39.59**	**44.42**	**61.08**
TG-06-151	-145	53.44	35.05	66.42	6.77	20.91
TG-06-152	-126	36.95	55.09	57.43	19.39	37.24
TG-06-153	-76	73.23	10.99	86.58	21.52	5.27
TG-06-154	**-13**	**6.87**	**91.65**	**24.48**	**65.64**	**78.64**
TG-06-155	18	29.45	64.21	56.36	20.89	42.55
TG-06-157	**70**	**12.32**	**85.03**	**32.4**	**54.53**	**69.78**
TG-06-159	169	53.15	35.4	80.84	13.47	10.97
TG-06-158	**175**	**9.39**	**88.59**	**31.72**	**55.48**	**72.03**
TG-06-160	**182**	**20.63**	**74.93**	**48.67**	**31.69**	**53.31**
TG-06-161	204	29.55	64.08	53.16	25.38	44.73
TG-06-004	448	54.83	33.35	73.05	2.54	15.41
TG-06-001	**451**	**18.21**	**77.86**	**32.86**	**53.88**	**65.87**
TG-06-005	464	67.08	18.47	76.37	7.19	5.64
TG-06-002	503	81.95	0.4	83.15	16.71	8.16
TG-06-1046	592	78.9	4.1	76.27	7.05	1.48
TG-06-343	598	62.8	23.67	75.55	6.04	8.82
TG-06-167	751	39.55	51.93	66.61	6.51	29.22
TG-06-168	**768**	**19.1**	**76.78**	**50.96**	**28.47**	**52.62**
TG-06-169	781	66.39	19.3	62.55	12.2	15.75
TG-06-170	786	64.19	21.98	72.96	2.41	9.79
TG-06-344	851	82.29	0.01	76.43	7.28	3.64
TG-06-345	859	81.22	1.28	85.68	20.26	9.49
TG-06-346	861	77.02	6.39	81.63	14.58	4.09
TG-06-347	869	26.55	67.73	51.83	27.24	47.49
TG-06-348	**878**	**23.14**	**71.88**	**35.55**	**50.1**	**60.99**
TG-06-171	889	52.41	36.3	55.95	21.46	28.88
TG-06-349	908	91.34	11.02	87.55	22.89	16.95
TG-06-249	949	87.36	6.18	82.36	15.61	10.89
TG-06-250	959	86.92	5.64	80.42	12.87	9.26
TG-06-251	985	36.8	55.28	44.62	37.37	46.32

Spacer ID	Distance to TSS (bp)	50ng mRNA: 25ng gRNA		25ng mRNA: 12.5ng gRNA		Average percent reduction in PCSK9 secretion
		Secreted PCSK9 (ng/mL)	Percent reduction in PCSK9 secretion	Secreted PCSK9 (ng/mL)	Percent reduction in PCSK9 secretion	
TG-06-350	1063	71.25	13.4	67.87	4.74	9.07
TG-06-351	1074	56.34	31.52	62.93	11.67	21.6
TG-06-172	1097	44.44	45.99	59.31	16.75	31.37
Non-targeting	-	82.28	0	71.24	0	0
mScarlet mRNA	-	84.85	3.12	-	-	-

*Data are shown rounded to the nearest hundredth.

[0586] The data presented in Table 74 demonstrate that constructs with most of the tested spacers produced decreased levels of secreted PCSK9 at 6 days post-transfection. The spacers were complementary to the *PCSK9* locus in a region ranging from 1086 basepairs upstream to 1097 basepairs downstream of the TSS, and effective spacers were found throughout the tested region, with many of the most effective spacers clustering between the TSS and approximately 500 basepairs upstream of the TSS.

[0587] The construct with the TG-06-141 spacer was the most effective overall at the 6-day timepoint, with an 82.75% average reduction in secreted PCSK9 levels. Constructs with spacers TG-06-154, TG-06-145, TG-06-139, TG-06-158, TG-06-157, TG-06-124, TG-06-138, TG-06-001 and TG-06-144 were also highly effective, with each producing greater than a 65% average reduction in secreted PCSK9 levels.

[0588] 13, 20, and 27 days post-transfection timepoints are further assessed in order to identify spacers that support PCSK9 repression and a reduction of secreted PCSK9 levels over longer time periods.

[0589] These results demonstrate that delivery of mRNA encoding an LTRP molecule with the ADD domain with the appropriate targeting gRNA can result in repression of the *PCSK9* locus to reduce PCSK9 secretion in a cell-based assay.

Example 24: Additional assessment of the effects of using CpG-reduced or depleted gRNA scaffolds on CasX-mediated editing activity

[0590] As discussed in Example 12, above, unmethylated CpG motifs act as PAMPs that potently trigger undesired immune activation. Therefore, nucleotide substitutions to replace native CpG motifs in the AAV constructs, including constructs encoding for guide scaffold variants 235 and 316, were designed and generated. Here, experiments were performed to evaluate further the effects of using these resulting CpG-reduced or depleted gRNA scaffolds on CasX-mediated editing activity.

Materials and Methods:

[0591] The CpG-reduced or depleted scaffolds 320-341 were evaluated in three in vitro experiments described below; the sequences of scaffolds 320-341 are listed in Table 42. In addition, two newly engineered gRNA scaffolds, scaffold 382 and 392 (sequences listed in Table 75), were also assessed. As benchmark comparisons, scaffolds 174, 235, and 316 (sequences listed in Table 9 and Table 75) were also included for evaluation.

Table 75: Sequences of additional gRNA scaffolds tested in this example

Scaffold ID	DNA sequence	DNA SEQ ID NO:	RNA sequence	RNA SEQ ID NO:
Scaffold 382	ACTGGCGCTTCTATCTGATTACTCT GAGCCGCCATCACCAGCGACTATGT CGTAGTGGGTAAAGCTCCCTCTTCG GAGGGAGCATCAGAG	3448	ACUGGCGCUUCUAUCUGAUUACUCU GAGCCGCCAUCACCAGCGACUAUGU CGUAGUGGGUAAAGCUCCCUCUUCG GAGGGAGCAUCAGAG	3451
Scaffold 392	ACTGGGCCTTCTATCTGATTACTCT GAGGCCCATCACCAGCGACTATGTC GTAGTGGGTAAAGCCGCTTACGGAC TTCGGTCCGTAAGAGGCATCAGAG	3449	ACUGGGCCUUCUAUCUGAUUACUCU GAGGCCCAUCACCAGCGACUAUGUC GUAGUGGGUAAAGCCGCUUACGGAC UUCGGUCCGUAAGAGGCAUCAGAG	3452
Scaffold 174	ACTGGCGCTTTTATCTGATTACTTT GAGAGCCATCACCAGCGACTATGTC GTAGTGGGTAAAGCTCCCTCTTCGG AGGGAGCATCAAAG	3450	ACUGGCGCUUUUAUCUGAUUACUUU GAGAGCCAUCACCAGCGACUAUGUC GUAGUGGGUAAAGCUCCCUCUUCGG AGGGAGCAUCAAAG	1744

[0592] AAV constructs were designed and generated as previously described in Example 12. The CpG-reduced or depleted gRNA scaffolds were tested in two different AAV backbones.

Specifically, for the experiment involving lipofection of HEK293 cells as described below, scaffolds 235 and 320-341 were tested in AAV vectors that were CpG-depleted, with the exception of AAV2 ITRs, as previously described in Example 12. Briefly, the CpG-depleted AAV backbone construct encoded for CpG-depleted versions of the following elements: U1A promoter, CasX 491, bGH poly(A) signal sequence, and U6 promoter. For the experiment involving AAV transduction of human induced neurons (iNs) and HEK293 cells as described below, scaffolds 174, 235, 316, 320-341, 382, and 392 (see Tables 9, 42 and 75 for sequences) were tested in an AAV backbone that was not CpG-depleted (see Table 76 for sequences). Furthermore, spacer 7.37 targeting the *B2M* locus was used in two experiments described below involving HEK293 cells: lipofection and AAV transduction. Spacer 31.63 targeting the *AAVS1* locus was used in an experiment described below involving human iNs. Table 77 below lists the AAV constructs that were tested in the context of a non-CpG-depleted AAV vector and the experimental conditions in which these constructs were assessed.

Table 76: Sequences encoding for a base AAV plasmid into which gRNA scaffolds in Table 75 were cloned

Component Name	DNA sequence	SEQ ID NO
5' ITR	CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCG GGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCG AGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCC T	3233
buffer sequence	GCGGCCTCTAGACTCGAGGCGTT	3453
U1A promoter	AATGGAGGCGGTACTATGTAGATGAGAATTCAGGAGCAAACTG GGAAAAGCAACTGCTTCCAAATATTTGTGATTTTTACAGTGTA GTTTTGGAAAAACTCTTAGCCTACCAATTCTTCTAAGTGTTTT AAAATGTGGGAGCCAGTACACATGAAGTTATAGAGTGTTTTAA TGAGGCTTAAATATTTACCGTAACTATGAAATGCTACGCATAT CATGCTGTTCAGGCTCCGTGGCCACGCAACTCATACT	3454
buffer sequence	CTCTGGCTAACTACCGGT	3455
Kozak	GCCACC	N.D.
start codon + c-MYC NLS	ATGGCCCCAGCGGCCAAACGGGTGAAGCTGGAC	3456
linker	TCTAGA	N.D.
CasX 515	CAAGAGATCAAGAGAATCAACAAGATCAGAAGGAGACTGGTCA AGGACAGCAACACAAAGAAGGCCGGCAAGACAGGCCCCATGAA AACCCTGCTCGTCAGAGTGATGACCCCTGACCTGAGAGAGCGG CTGGAAAACCTGAGAAAGAAGCCCGAGAACATCCCTCAGCCTA	3457

	TCAGCAACACCAGCAGGGCCAACCTGAACAAGCTGCTGACCGA CTACACCGAGATGAAGAAAGCCATCCTGCACGTGTACTGGGAA GAGTTCCAGAAAGACCCCGTGGGCCTGATGAGCAGAGTTGCTC AGCCTGCCAGCAAGAAGATCGACCAGAACAAGCTGAAGCCCGA GATGGACGAGAAGGGCAATCTGACCACAGCCGGCTTTGCCTGC TCTCAGTGTGGCCAGCCTCTGTTCGTGTACAAGCTGGAACAGG TGTCCGAGAAAGGCAAGGCCTACACCAACTACTTCGGCAGATG TAACGTGGCCGAGCACGAGAAGCTGATTCTGCTGGCCCAGCTG AAACCTGAGAAGGACTCTGATGAGGCCGTGACCTACAGCCTGG GCAAGTTTGGACAGAGAGCCCTGGACTTCTACAGCATCCACGT GACCAAAGAAAGCACACACCCCGTGAAGCCCCTGGCTCAGATC GCCGGCAATAGATACGCCTCTGGACCTGTGGGCAAAGCCCTGT CCGATGCCTGCATGGGAACAATCGCCAGCTTCCTGAGCAAGTA CCAGGACATCATCATCGAGCACCAGAAGGTGGTCAAGGGCAAC CAGAAGAGACTGGAAAGCCTGAGGGAGCTGGCCGGCAAAGAGA ACCTGGAATACCCCAGCGTGACCCTGCCTCCTCAGCCTCACAC AAAAGAAGGCGTGGACGCCTACAACGAAGTGATCGCCAGAGTG AGAATGTGGGTCAACCTGAACCTGTGGCAGAAGCTGAAACTGT CCAGGGACGACGCCAAGCCTCTGCTGAGACTGAAGGGCTTCCC TAGCTTCCCTCTGGTGGAAAGACAGGCCAATGAAGTGGATTGG TGGGACATGGTCTGCAACGTGAAGAAGCTGATCAACGAGAAGA AAGAGGATGGCAAGGTTTTCTGGCAGAACCTGGCCGGCTACAA GAGACAAGAAGCCCTGAGGCCTTACCTGAGCAGCGAAGAGGAC CGGAAGAAGGGCAAGAAGTTCGCCAGATACCAGCTGGGCGACC TGCTGCTGCACCTGGAAAAGAAGCACGGCGAGGACTGGGGCAA AGTGTACGATGAGGCCTGGGAGAGAATCGACAAGAAGGTGGAA GGCCTGAGCAAGCACATTAAGCTGGAAGAGGAAAGAAGGAGCG AGGACGCCCAATCTAAAGCCGCTCTGACCGATTGGCTGAGAGC CAAGGCCAGCTTTGTGATCGAGGGCCTGAAAGAGGCCGACAAG GACGAGTTCTGCAGATGCGAGCTGAAGCTGCAGAAGTGGTACG GCGATCTGAGAGGCAAGCCCTTCGCCATTGAGGCCGAGAACAG CATCCTGGACATCAGCGGCTTCAGCAAGCAGTACAACTGCGCC TTCATTTGGCAGAAAGACGGCGTCAAGAAACTGAACCTGTACC TGATCATCAATTACTTCAAAGGCGGCAAGCTGCGGTTCAAGAA GATCAAACCCGAGGCCTTCGAGGCTAACAGATTCTACACCGTG ATCAACAAAAAGTCCGGCGAGATCGTGCCCATGGAAGTGAACT TCAACTTCGACGACCCCAACCTGATTATCCTGCCTCTGGCCTT CGGCAAGAGACAGGGCAGAGAGTTCATCTGGAACGATCTGCTG AGCCTGGAAACCGGCTCTCTGAAGCTGGCCAATGGCAGAGTGA TCGAGAAAACCCTGTACAACAGGAGAACCAGACAGGACGAGCC TGCTCTGTTTGTGGCCCTGACCTTCGAGAGAAGAGAGGTGCTG GACAGCAGCAACATCAAGCCCATGAACCTGATCGGCGTGGACC GGGGCGAGAATATCCCTGCTGTGATCGCCCTGACAGACCCTGA AGGATGCCCACTGAGCAGATTCAAGGACTCCCTGGGCAACCCT ACACACATCCTGAGAATCGGCGAGAGCTACAAAGAGAAGCAGA GGACAATCCAGGCCAAGAAAGAGGTGGAACAGAGAAGAGCCGG CGGATACTCTAGGAAGTACGCCAGCAAGGCCAAGAATCTGGCC GACGACATGGTCCGAAACACCGCCAGAGATCTGCTGTACTACG CCGTGACACAGGACGCCATGCTGATCTTCGAGAATCTGAGCAG AGGCTTCGGCCGGCAGGGCAAGAGAACCTTTATGGCCGAGAGG CAGTACACCAGAATGGAAGATTGGCTCACAGCTAAACTGGCCT ACGAGGGACTGCCCAGCAAGACCTACCTGTCCAAAACACTGGC CCAGTATACCTCCAAGACCTGCAGCAATTGCGGCTTCACCATC ACCAGCGCCGACTACGACAGAGTGCTGGAAAAGCTCAAGAAAA CCGCCACCGGCTGGATGACCACCATCAACGGCAAAGAGCTGAA GGTTGAGGGCCAGATCACCTACTACAACAGGTACAAGAGGCAG AACGTCGTGAAGGATCTGAGCGTGGAACTGGACAGACTGAGCG AAGAGAGCGTGAACAACGACATCAGCAGCTGGACAAAGGGCAG ATCAGGCGAGGCTCTGAGCCTGCTGAAGAAGAGGTTTAGCCAC	

	AGACCTGTGCAAGAGAAGTTCGTGTGCCTGAACTGCGGCTTCG AGACACACGCCGATGAACAGGCTGCCCTGAACATTGCCAGAAG CTGGCTGTTCCTGAGAAGCCAAGAGTACAAGAAGTACCAGACC AACAAGACCACCGGCAACACCGACAAGAGGGCCTTTGTGGAAA CCTGGCAGAGCTTCTACAGAAAAAAGCTGAAAGAAGTCTGGAA GCCCGCCGTG	
linker	GGATCC	N.D.
c-MYC NLS	CCAGCCGCGAAGCGAGTGAAACTGGAC	3458
stop codon	TAA	N.D.
buffer sequence	GAATTCCTAGAGCTCGCTGATCAGCCTCGA	3459
bGH poly(A) signal sequence	CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCC CGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTT TCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGT GTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGG GGAGGATTGGGAAGAGAATAGCAGGCATGCTGGGGA	3460
buffer sequence	GGTACCGT	N.D.
U6 promoter	GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGAT ACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACTGTAAA CACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAA TTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGG ACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTT GGCTTTATATATCTTGTGGAAAGGAC	3461
buffer sequence	GAAACACC	N.D.
Scaffold variants	*See sequences listed in Tables 9, 42, and 75*	*See sequences listed in Tables 9, 42, and 75*
B2M spacer (spacer 7.37)	GGCCGAGATGTCTCGCTCCG	3137
AAVS1 spacer (spacer 31.63)	CAAGAGGAGAAGCAGTTTGG	3462
Non-targeting spacer (spacer 0.0)	CGAGACGTAATTACGTCTCG	3232
buffer sequence	TTTTTTTTGGCGGCCGC	3463
3' ITR	AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGC TCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGC CCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCA GCTGCCTGCAGG	3238

Table 77: List of AAV constructs and scaffold variants tested in a non-CpG-depleted AAV vector (see Table 76 for sequences) and the experimental conditions in which these constructs were assessed

AAV construct ID	Scaffold variant	Spacer	Experimental conditions
262	235	31.63	AAV transduction in iNs

263	328	31.63	AAV transduction in iNs
264	329	31.63	AAV transduction in iNs
265	382	31.63	AAV transduction in iNs
266	174	31.63	AAV transduction in iNs
267	335	31.63	AAV transduction in iNs
268	325	31.63	AAV transduction in iNs
269	330	31.63	AAV transduction in iNs
270	327	31.63	AAV transduction in iNs
271	334	31.63	AAV transduction in iNs
272	339	31.63	AAV transduction in iNs
273	337	31.63	AAV transduction in iNs
274	235	Non-targeting	AAV transduction in iNs
275	331	7.37	AAV transduction in HEK293s
276	335	7.37	AAV transduction in HEK293s
277	316	7.37	AAV transduction in HEK293s
278	392	7.37	AAV transduction in HEK293s
279	325	7.37	AAV transduction in HEK293s
280	334	7.37	AAV transduction in HEK293s
281	324	7.37	AAV transduction in HEK293s
282	336	7.37	AAV transduction in HEK293s
283	330	7.37	AAV transduction in HEK293s
284	320	7.37	AAV transduction in HEK293s
285	332	7.37	AAV transduction in HEK293s
286	321	7.37	AAV transduction in HEK293s
287	339	7.37	AAV transduction in HEK293s
288	235	7.37	AAV transduction in HEK293s
289	235	Non-targeting	AAV transduction in HEK293s

[0593] AAV production was performed using methods described in Example 12. For the experiment involving lipofection of HEK293 cells as described below, AAV titering was performed following methods described in Example 12. For the two experiments involving AAV transduction of human iNs or HEK293 cells as described below, AAV titering was performed by ddPCR.

Cell-based assays evaluating the effects of using CpG-depleted or reduced gRNA scaffolds on editing activity:

[0594] In one experiment, ~20,000 HEK293 cells per well were seeded in 96-well plates 24 hours prior to transfection. Seeded cells were then transfected with CpG-depleted AAV plasmids containing various versions of the guide scaffold (scaffolds 320-341). 5 days post transfection, cells were harvested for B2M protein expression analysis via HLA immunostaining following by flow cytometry. A CpG-depleted AAV plasmid with scaffold variant 235 served as an experimental control. An AAV plasmid with a CMV promoter driving mCherry expression was used as a transfection control, and a ~41% transfection rate was observed. The results from this experiment are shown in FIG. 68.

[0595] In a second experiment, ~20,000 induced neuron (iN) cells per well were seeded on Matrigel-coated 96-well plates 7 days prior to transduction. AAVs expressing the CasX:gRNA system, containing various versions of the guide scaffold (AAV construct ID #262-274; see Table 77), were diluted in neuronal plating media and added to cells 7 days post-plating. Cells were transduced at three MOIs (3E4, 1E4 or 3E3 vg/cell). 7 days post-transduction, cells were gDNA extraction for editing analysis at the *AAVS1* locus using NGS. The results from this experiment are shown in FIGS. 69A-69C.

[0596] In a third experiment, ~10,000 HEK293 cells per well were seeded in 96-well plates 24 hours prior to transfection. Seeded cells were then transduced with AAVs expressing the CasX:gRNA system, containing various versions of the guide scaffold (AAV construct ID #275-289; see Table 77). Cells were transduced at three MOIs (1E4, 3E3, or 1E3 vg/cell). 5 days post-transduction, cells were harvested for B2M protein expression analysis via HLA immunostaining following by flow cytometry. The results from this experiment are shown in FIGS. 70A-70C.

Results:

[0597] Experiments were performed to evaluate further the effects of using CpG-reduced or depleted gRNA scaffolds on CasX-mediated editing activity. In the first experiment (N=1), HEK293 cells were lipofected with CpG-depleted AAV plasmids containing various versions of the gRNA scaffold (scaffolds 320-341, see Table 42 for sequences). B2M protein expression was subsequently analyzed, and the results of the assay are shown in FIG. 68. The data demonstrate that use of scaffolds 320-341 did not improve editing activity at the target *B2M* locus, since use of these scaffolds produced a lower percentage of cells with $B2M^-$ relative to the level achieved when using an AAV construct containing scaffold 235. These results do not recapitulate the results described in Example 12 (see FIGS. 28-31).

[0598] In the second experiment (N=1), human iNs were transduced with AAV particles expressing the CasX:gRNA system, containing various versions of the guide scaffold (AAV construct ID #262-274). Editing at the AAVS1 locus was analyzed, and the results of the assay are shown in FIGS. 69A-69C. The data demonstrate that of the scaffold variants tested, use of scaffold variant 329 and 382 appeared to improve editing at the *AAVS1* locus when compared to use of scaffold 235, especially at MOI of 1E4 and 3E3 vg/cell. Furthermore, the effects on editing activity were observed in a dose-dependent manner.

[0599] In the third experiment (N=1), HEK293 cells were transduced with AAV particles expressing the CasX:gRNA system, containing various versions of the guide scaffold (AAV construct ID #275-289). B2M protein expression was subsequently analyzed, and the results of the assay are shown in FIGS. 70A-70C. The data demonstrate that of the scaffold variants tested, use of scaffolds 316, 392 and 332 appeared to improve editing at the *B2M* locus when compared to use of scaffold 235 overall. Specifically, at the higher MOI of 1E4 and 3E3 vg/cell, slightly improved editing was observed with use of scaffolds 316, 392, and 332 (FIGS. 70A-70B), while a stronger editing improvement was observed at the lower MOI of 1E3 vg/cell (FIG. 70C). Notably, scaffold 332 and 392 both include CG > GC mutations in the pseudoknot stem (region 1; FIGS. 27A-27B), effectively reducing the overall number of CpGs when compared to scaffold 235, thereby potentially contributing to the increase in editing activity. Furthermore, scaffolds 316 and 332 both have a truncated extended stem when compared to scaffold 235, removing the bubble and the CG dinucleotide (region 3; FIGS. 27A-27B), thereby also potentially contributing to the observed increase in editing activity. Further experiments are performed, especially at lower MOIs, to unravel the intricacies of the effects of individual CpG mutations on editing potency.

[0600] The results from the experiments described here demonstrate that use of guide scaffolds with different levels of CpG depletion can result in varying levels of editing mediated by the CasX:gRNA system, and that the resulting editing levels can vary by method of delivery (e.g., plasmid transfection vs. AAV transduction).

287014549

CLAIMS

What is claimed is:

1. A system comprising a repressor fusion protein and a guide ribonucleic acid (gRNA), wherein the repressor fusion protein comprises:

(a) a catalytically-dead CRISPR protein;

(b) a repressor domain (RD1);

(c) a DNA methyltransferase (DNMT) 3A ATRX-DNMT3-DNMT3L domain (ADD);

(d) a DNMT3A catalytic domain (DNMT3A); and

(e) a DNMT3L interaction domain (DNMT3L);

wherein the gRNA comprises a targeting sequence complementary to a *proprotein convertase subtilisin/kexin Type 9* (*PCSK9*) gene target nucleic acid sequence, and

wherein the repressor fusion protein is capable of forming a ribonucleoprotein (RNP) with the gRNA.

2. The system of claim 1, wherein the RNP is capable of binding to the *PCSK9* gene target nucleic acid sequence.

3. The system of claim 1 or claim 2, wherein the RNP is capable of repressing transcription of the *PCSK9* gene upon binding to the *PCSK9* gene target nucleic acid sequence.

4. The system of any one of claims 1-3, wherein the ADD is fused to the N-terminus of the DNMT3A.

5. The system of any one of claims 1-4, wherein the repressor fusion protein comprises, from N- to C-terminus:

(a) the RD1;

(b) the ADD;

(c) the DNMT3A;

(d) the DNMT3L; and

(e) the catalytically-dead CRISPR protein.

6. The system of any one of claims 1-4, wherein the repressor fusion protein comprises, from N- to C-terminus:

(a) the ADD;

(b) the DNMT3A;

(c) the DNMT3L;

(d) the RD1; and

(e) the catalytically-dead CRISPR protein.

7. The system of any one of claims 1-6, wherein the catalytically-dead CRISPR protein is a catalytically-dead Class 1 CRISPR protein or a catalytically-dead Class 2 CRISPR protein.

8. The system of claim 7, wherein the catalytically-dead Class 2 CRISPR protein is a catalytically-dead Type II protein, a catalytically-dead Type V protein, or a catalytically-dead Type VI protein.

9. The system of claim 8, wherein the catalytically-dead Type II protein is a catalytically-dead Cas9 protein.

10. The system of claim 8, wherein the catalytically-dead Type V protein is selected from the group consisting of a catalytically-dead Cas12a (Cpf1), a catalytically-dead Cas12b (C2c1), a catalytically-dead Cas12c (C2c3), a catalytically-dead Cas12d (CasY), a catalytically-dead Cas12e (CasX), a catalytically-dead Cas12f, a catalytically-dead Cas12g, a catalytically-dead Cas12h, a catalytically-dead Cas12i, a catalytically-dead Cas12j, a catalytically-dead Cas12k, a catalytically-dead Cas14, and a catalytically-dead CasΦ.

11. The system of claim 10, wherein the catalytically-dead Type V protein is a catalytically-dead CasX (dCasX).

12. The system of claim 11, wherein the dCasX comprises a sequence selected from the group consisting of SEQ ID NOS: 4-29, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

13. The system of claim 11, wherein the dCasX comprises a sequence of SEQ ID NOS: 3281-3441, or 3444-3446, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto, and wherein the sequence comprises a RuvC domain comprising one or more mutations that inactivates the cleavage activity of the RuvC domain.

14. The system of claim 13, wherein the one or more mutations comprise D659A, E756A and/or D922A substitutions corresponding to a sequence of SEQ ID NO: 2.

15. The system of claim 11, wherein the dCasX comprises a sequence of SEQ ID NO: 4.

16. The system of any one of claims 1-15, wherein the RD1 comprises an amino acid sequence motif selected from the group consisting of:

(a) $PX_1X_2X_3X_4X_5X_6EX_7$, wherein

i) X_1 is A, D, E, or N,

ii) X_2 is L or V,

iii) X_3 is I or V,

iv) X_4 is S, T, or F,

v) X_5 is H, K, L, Q, R or W,

vi) X_6 is L or M, and

vii) X_7 is G, K, Q, or R;

(b) $X_1X_2X_3X_4GX_5X_6X_7X_8X_9$, wherein

i) X_1 is L or V,

ii) X_2 is A, G, L, T or V,

iii) X_3 is A, F, or S,

iv) X_4 is L or V,

v) X_5 is C, F, H, I, L or Y,

vi) X_6 is A, C, P, Q, or S,

vii) X_7 is A, F, G, I, S, or V,

viii) X_8 is A, P, S, or T, and

ix) X_9 is K or R;

(c) $QX_1X_2LYRX_3VMX_4$ (SEQ ID NO: 1727), wherein

i) X_1 is K or R,

ii) X_2 is A, D, E, G, N, S, or T,

iii) X_3 is D, E, or S, and

iv) X_4 is L or R;

(d) $X_1X_2X_3FX_4DVX_5X_6X_7FX_8X_9X_{10}X_{11}$ (SEQ ID NO: 1728), wherein

i) X_1 is A, L, P, or S,

ii) X_2 is L or V,

iii) X_3 is S or T,

iv) X_4 is A, E, G, K, or R,

v) X_5 is A or T,

vi) X_6 is I or V,

vii) X_7 is D, E, N, or Y,

viii) X_8 is S or T,

ix) X_9 is E, P, Q, R, or W,

x) X_{10} is E or N, and

xi) X_{11} is E or Q;

(e) $X_1X_2X_3PX_4X_5X_6X_7X_8X_9X_{10}$, wherein

i) X_1 is E, G, or R,

ii) X_2 is E or K,

iii) X_3 is A, D, or E,

iv) X_4 is C or W,

v) X_5 is I, K, L, M, T, or V,

vi) X_6 is I, L, P, or V,

vii) X_7 is D, E, K, or V,

viii) X_8 is E, G, K, P, or R,

ix) X_9 is A, D, R, G, K, Q, or V, and

x) X_{10} is D, E, G, I, L, R, S, or V;

(f) $LYX_1X_2VMX_3EX_4X_5X_6X_7X_8X_9X_{10}$ (SEQ ID NO: 1729), wherein

i) X_1 is K or R,

ii) X_2 is D or E,

iii) X_3 is L, Q, or R,

iv) X_4 is N or T,

v) X_5 is F or Y,

vi) X_6 is A, E, G, Q, R, or S,

vii) X_7 is H, L, or N,

viii) X_8 is L or V,

ix) X_9 is A, G, I, L, T, or V, and

x) X_{10} is A, F, or S,

(g) $FX_1DVX_2X_3X_4FX_5X_6X_7EWX_8$ (SEQ ID NO: 1730), wherein

i) X_1 is A, E, G, K, or R,

ii) X_2 is A, S, or T,

iii) X_3 is I or V,

iv) X_4 is D, E, N, or Y,

v) X_5 is S or T,

vi) X_6 is E, L, P, Q, R, or W,

vii) X_7 is D or E, and

viii) X_8 is A, E, G, Q, or R;

(h) $X_1PX_2X_3X_4X_5X_6LEX_7X_8X_9X_{10}X_{11}X_{12}$, wherein

i) X_1 is K or R,

ii) X_2 is A, D, E, or N,

iii) X_3 is I, L, M, or V,

iv) X_4 is I or V,

v) X_5 is F, S, or T,

vi) X_6 is H, K, L, Q, R, or W,

vii) X_7 is K, Q, or R,

viii) X_8 is E, G, or R,

ix) X_9 is D, E, or K,

x) X_{10} is A, D, or E,

xi) X_{11} is L or P, and

xii) X_{12} is C or W; and

(i) $X_1LX_2X_3X_4QX_5X_6$, wherein

i) X_1 is C, H, L, Q, or W,

ii) X_2 is D, G, N, R, or S,

iii) X_3 is L, P, S, or T,

iv) X_4 is A, S, or T,

v) X_5 is K or R, and

vi) X_6 is A, D, E, K, N, S, or T.

17. The system of claim 16, wherein the RD1 comprises a first and a second amino acid sequence motif wherein:

(a) the first amino acid sequence motif comprises $LYX_1X_2VMX_3EX_4X_5X_6X_7X_8X_9X_{10}$ (SEQ ID NO: 1729), wherein

i) X_1 is K or R,

ii) X_2 is D or E,

iii) X_3 is L, Q, or R,

iv) X_4 is N or T,

v) X_5 is F or Y,

vi) X_6 is A, E, G, Q, R, or S,

vii) X_7 is H, L, or N,

viii) X_8 is L or V,

ix) X_9 is A, G, I, L, T, or V, and

x) X_{10} is A, F, or S; and

(b) the second amino acid sequence motif comprises $FX_1DVX_2X_3X_4FX_5X_6X_7EWX_8$ (SEQ ID NO: 1730), wherein

i) X_1 is A, E, G, K, or R,

ii) X_2 is A, S, or T,

iii) X_3 is I or V,

iv) X_4 is D, E, N, or Y,

v) X_5 is S or T,

vi) X_6 is E, L, P, Q, R, or W,

vii) X_7 is D or E, and

viii) X_8 is A, E, G, Q, or R.

18. The system of any one of claims 1-15, wherein the RD1 comprises an amino acid sequence motif selected from the group consisting of:

(a) DVAVYFSPEEWGCL (SEQ ID NO: 2945);

(b) $X_1X_2X_3QX_4X_5LY$, wherein

i) X_1 is A, D, G, N, R, or S,

ii) X_2 is P, S, or T,

iii) X_3 is A, S, or T,

iv) X_4 is K or R, and

v) X_5 is A, D, K, N, S, or T;

(c) $X_1KPX_2X_3X_4X_5X_6$, wherein

i) X_1 is A, P, or S,

ii) X_2 is A, D, or E,

iii) X_3 is L, M, or V,

iv) X_4 is I or V,

v) X_5 is F, S, or T, and

vi) X_6 is H, K, L, Q, R, or W;

(d) $LEX_1X_2X_3X_4X_5X_6$, wherein

i) X_1 is E, K, Q or R,

ii) X_2 is E, G, or R,

iii) X_3 is A, D, E, or K,

iv) X_4 is A, D, or E,

v) X_5 is L or P, and

vi) X_6 is C or W; and

(e) $X_1VMLEX_2YX_3X_4X_5X_6SX_7X_8X_9$ (SEQ ID NO: 2946), wherein

i) X_1 is D or E,

ii) X_2 is N or T,

iii) X_3 is A, E, G, Q, R, or S,

iv) X_4 is H or N,

v) X_5 is L, M, or V,

vi) X_6 is A, L, or V,

vii) X_7 is L or V,

viii) X_8 is A, G, or V, and

ix) X_9 is C, F, or L.

19. The system of any one of claims 1-18, wherein the RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 130-1726, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

20. The system of any one of claims 1-18, wherein the RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 130-224, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

21. The system of any one of claims 1-18, wherein the RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 130-138, or sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

22. The system of any one of claims 1-18, wherein the RD1 comprises the sequence of SEQ ID NO: 135, or sequence having at least about 70%, at least about 80%, at least about 85%, at

least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

23. The system of any one of claims 1-18, wherein the RD1 comprises the sequence of SEQ ID NO: 131, or sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

24. The system of any one of claims 1-15, wherein the RD1 comprises a Krüppel-associated box (KRAB) domain.

25. The system of claim 24, wherein the KRAB domain is a ZIM3 KRAB domain or a ZNF10 KRAB domain.

26. The system of claim 25, wherein the ZIM3 KRAB domain comprises a sequence of SEQ ID NO: 128, or sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

27. The system of claim 25, wherein the ZNF10 KRAB domain comprises a sequence of SEQ ID NO: 129, or sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

28. The system of any one of claims 1-27, wherein the ADD comprises a sequence of SEQ ID NO: 125, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

29. The system of any one of claims 1-28, wherein the DNMT3A comprises a sequence of SEQ ID NO: 126, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

30. The system of any one of claims 1-29, wherein the DNMT3L comprises a sequence of SEQ ID NO: 127, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

31. The system of any one of claims 6-30, wherein

(a) the RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 128, 129, 131, and 135, or sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto; and

(b) the catalytically-dead CRISPR protein is a dCasX.

32. The system of any one of claims 1-31, wherein the repressor fusion protein comprises one or more linker peptides.

33. The system of claim 32, wherein at least one of the one or more linker peptides comprises a sequence selected from the group consisting of SEQ ID NOS: 98-124.

34. The system of claim 32, wherein at least one of the one or more linker peptides comprises a sequence selected from the group consisting of SEQ ID NOS: 120 and 122-124.

35. The system of any one of claims 1-34, wherein the repressor fusion protein comprises one or more nuclear localization signals (NLS).

36. The system of claim 35, wherein at least one of the one or more NLS comprises a sequence selected from the group consisting of SEQ ID NOS: 30-97.

37. The system of claim 35, wherein at least one of the one or more NLS is a simian virus 40 (SV40) NLS.

38. The system of claim 37, wherein the SV40 NLS comprises a sequence of SEQ ID NO: 30.

39. The system of any one of claims 6-38, wherein the repressor fusion protein comprises, from N- to C-terminus,

(a) a first NLS;

(b) the ADD;

(c) the DNMT3A;

(d) a first peptide linker;

(e) the DNMT3L;

(f) a second peptide linker;

(g) the RD1;

(h) a third peptide linker;

(i) the catalytically-dead CRISPR protein;

(j) a fourth peptide linker; and

(k) a second NLS.

40. The system of claim 39, wherein the first linker comprises a sequence of SEQ ID NO: 122, the second and the fourth linker comprise a sequence of SEQ ID NO: 124, and the third linker comprises a sequence of SEQ ID NO: 123.

41. The system of any one of claims 1-40, wherein the *PCSK9* gene target nucleic acid sequence is:

(a) within 1 kilobase (kb) of a transcription start site (TSS) in the *PCSK9* gene;

(b) within 1 kb of an enhancer of the *PCSK9* gene.

(c) within the 3' untranslated region of the *PCSK9* gene;

(d) within the 3' untranslated region of the *PCSK9* gene; or

(e) within an exon of the *PCSK9* gene.

42. The system of claim 41, wherein the *PCSK9* gene target nucleic acid sequence is within exon 1 of the *PCSK9* gene.

43. The system of any one of claims 1-42, wherein the targeting sequence of the gRNA comprises a sequence selected from the group consisting of SEQ ID NOS: 1824-2944, or a sequence having at least about 65%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95% identity thereto.

44. The system of any one of claims 1-42, wherein the targeting sequence of the gRNA comprises a sequence selected from the group consisting of SEQ ID NOS: 1824-2944.

45. The system of any one of claims 1-42, wherein the targeting sequence of the gRNA comprises a sequence of SEQ ID NOS: 1824-2944 with 1, 2, 3, 4, or 5 nucleotide(s) removed from the 3' end of the sequence.

46. The system of any one of claims 1-42, wherein the targeting sequence of the gRNA comprises a sequence selected from the group consisting of SEQ ID NOS: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714, or a sequence having at least about 65%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95% identity thereto.

47. The system of any one of claims 1-42, wherein the targeting sequence of the gRNA comprises a sequence selected from the group consisting of SEQ ID NOS: 1824-1890, 1910, 1925, 2672, 2675, 2694, and 2714.

48. The system of any one of claims 1-47, wherein the gRNA is a single-molecule gRNA (sgRNA) and comprises a scaffold stem loop comprising the sequence of

CCAGCGACUAUGUCGUAGUGG (SEQ ID NO: 1822), or a sequence with at least 1, 2, 3, 4 or 5 mismatches thereto.

49. The system of any one of claims 1-48, wherein the gRNA comprises a scaffold comprising a sequence selected from the group consisting of SEQ ID NOS: 1744-1821, 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.

50. The system of claim 49, wherein the gRNA comprises a scaffold comprising a sequence selected from the group consisting of SEQ ID NOS: 1744-1821.

51. The system of any one of claims 1-49, wherein the gRNA is a chimeric gRNA.

52. The system of claim 51, wherein the gRNA comprises a scaffold sequence comprising SEQ ID NO: 1746, or a sequence having 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.

53. The system of claim 52, wherein the gRNA comprises a scaffold sequence comprising SEQ ID NO: 1746.

54. The system of any one of claims 1-53, wherein the gRNA is chemically modified.

55. The system of claim 54, wherein the chemical modification to the gRNA comprises an addition of a 2'O-methyl group to one or more nucleotides of the gRNA.

56. The system of claim 54 or claim 55, wherein the chemical modification to the gRNA comprises a substitution of a phosphorothioate bond between two or more nucleosides of the gRNA.

57. The system of any one of claims 54-56, wherein the chemically modified gRNA comprises a sequence selected from the group consisting of SEQ ID NOS: 2968-2976, or a sequence having 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.

58. The system of claim 57, wherein the chemically modified gRNA comprises a sequence of SEQ ID NO: 2968, or a sequence having 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.

59. The system of claim 57 or claim 58, wherein the chemically modified gRNA sequence has a substitution of a 20 nucleotide targeting sequence complementary to a target nucleic acid of the *PCSK9* gene for the 20 nucleotides at the 3' end of the gRNA.

60. A system comprising a repressor fusion protein comprising:

(a) a DNA-binding protein capable of binding to a *PCSK9* gene target nucleic acid sequence;

(b) a DNA methyltransferase (DNMT) 3A ATRX-DNMT3-DNMT3L domain (ADD); and

(c) a DNMT3A catalytic domain (DNMT3A).

61. The system of claim 60, wherein the ADD is fused to the N-terminus of the DNMT3A.

62. The system of claim 60 or claim 61, wherein the repressor fusion protein comprises:

(a) a repressor domain (RD1);

(b) a DNMT3L interaction domain (DNMT3L); and

(c) one or more linker peptides.

63. The system of claim 62, wherein the repressor fusion protein comprises, from N- to C-terminus:

(a) the RD1;

(b) the ADD;

(c) the DNMT3A;

(d) the DNMT3L; and

(e) the DNA-binding protein.

64. The system of claim 62, wherein the repressor fusion protein comprises, from N- to C-terminus:

(a) the ADD;

(b) the DNMT3A;

(c) the DNMT3L;

(d) the RD1; and

(e) the DNA-binding protein.

65. The system of any one of claims 62-64, wherein the one or more linker sequences are selected from the group consisting of SEQ ID NOS: 122-124 and link any two components selected from the RD1, the ADD, the DNMT3A, the DNMT3L and the DNA binding protein.

66. The system of any one of claims 60-64, wherein the DNA-binding protein comprises a zinc finger (ZF) domain.

67. The system of any one of claims 60-64, wherein the DNA-binding protein comprises a transcription-activator-like effector (TALE) domain.

68. The system of any one of claims 60-64, wherein the DNA-binding protein comprises a catalytically-dead Class 1 CRISPR protein or a catalytically-dead Class 2 CRISPR protein.

69. The system of claim 68, comprising a guide ribonucleic acid (gRNA), wherein the gRNA comprises a targeting sequence complementary to a *PCSK9* gene target nucleic acid sequence.

70. The system of claim 69, wherein the gRNA is capable of forming a ribonucleoprotein (RNP) complex with the repressor fusion protein.

71. The system of claim 70, wherein the RNP is capable of binding to the *PCSK9* gene target nucleic acid sequence.

72. The system of any one of claims 60-71, wherein the repressor fusion protein is capable of repressing transcription of the *PCSK9* gene upon binding to the *PCSK9* gene target nucleic acid sequence.

73. The system of any one of claims 68-72, wherein the catalytically-dead Class 2 CRISPR protein is a catalytically-dead Class 2, Type II protein, a catalytically-dead Class 2, Type V protein, or a catalytically-dead Class 2, Type VI protein.

74. The system of claim 73, wherein the catalytically-dead Class 2, Type II protein is a catalytically-dead Cas9 protein.

75. The system of claim 73, wherein the catalytically-dead Class2, Type V protein is a catalytically-dead Cas12a (Cpf1), a catalytically-dead Cas12b (C2c1), a catalytically-dead Cas12c (C2c3), a catalytically-dead Cas12d (CasY), a catalytically-dead Cas12e (CasX), a catalytically-dead Cas12f, a catalytically-dead Cas12g, a catalytically-dead Cas12h, a catalytically-dead Cas12i, a catalytically-dead Cas12j, a catalytically-dead Cas12k, a catalytically-dead Cas14, or a catalytically-dead CasΦ.

76. The system of claim 73, wherein the catalytically-dead Class 2, Type V protein is a catalytically-dead CasX variant (dCasX).

77. The system of claim 76, wherein the dCasX comprises a sequence selected from the group consisting of SEQ ID NOS: 4-29, or a sequence having at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%

sequence identity thereto, wherein the repressor fusion protein comprising the dCasX retains the ability to form an RNP with a gRNA.

78. The system of claim 76, wherein the dCasX comprises a sequence of SEQ ID NOS: 3281-3441, or 3444-3446, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto, and wherein, wherein the sequence comprises a RuvC domain comprising one or more mutations that inactivates the cleavage activity of the RuvC domainc.

79. The system of claim 78, wherein the one or more mutations comprise D659A, E756A and/or D922A substitutions corresponding to a sequence of SEQ ID NO: 2.

80. The system of claim 76, wherein the dCasX comprises the sequence of SEQ ID NO: 4.

81. The system of any one of claims 62-80, wherein the RD1 comprises an amino acid sequence motif selected from the group consisting of:

(a) $PX_1X_2X_3X_4X_5X_6EX_7$, wherein

i) X_1 is A, D, E, or N,

ii) X_2 is L or V,

iii) X_3 is I or V,

iv) X_4 is S, T, or F,

v) X_5 is H, K, L, Q, R or W,

vi) X_6 is L or M, and

vii) X_7 is G, K, Q, or R;

(b) $X_1X_2X_3X_4GX_5X_6X_7X_8X_9$, wherein

i) X_1 is L or V,

ii) X_2 is A, G, L, T or V,

iii) X_3 is A, F, or S,

iv) X_4 is L or V,

v) X_5 is C, F, H, I, L or Y,

vi) X_6 is A, C, P, Q, or S,

vii) X_7 is A, F, G, I, S, or V,

viii) X_8 is A, P, S, or T, and

ix) X_9 is K or R;

(c) $QX_1X_2LYRX_3VMX_4$ (SEQ ID NO: 1727), wherein

i) X_1 is K or R,

ii) X_2 is A, D, E, G, N, S, or T,

iii) X_3 is D, E, or S, and

iv) X_4 is L or R;

(d) $X_1X_2X_3FX_4DVX_5X_6X_7FX_8X_9X_{10}X_{11}$ (SEQ ID NO: 1728), wherein

i) X_1 is A, L, P, or S,

ii) X_2 is L or V,

iii) X_3 is S or T,

iv) X_4 is A, E, G, K, or R,

v) X_5 is A or T,

vi) X_6 is I or V,

vii) X_7 is D, E, N, or Y,

viii) X_8 is S or T,

ix) X_9 is E, P, Q, R, or W,

x) X_{10} is E or N, and

xi) X_{11} is E or Q;

(e) $X_1X_2X_3PX_4X_5X_6X_7X_8X_9X_{10}$, wherein

i) X_1 is E, G, or R,

ii) X_2 is E or K,

iii) X_3 is A, D, or E,

iv) X_4 is C or W,

v) X_5 is I, K, L, M, T, or V,

vi) X_6 is I, L, P, or V,

vii) X_7 is D, E, K, or V,

viii) X_8 is E, G, K, P, or R,

ix) X_9 is A, D, R, G, K, Q, or V, and

x) X_{10} is D, E, G, I, L, R, S, or V;

(f) $LYX_1X_2VMX_3EX_4X_5X_6X_7X_8X_9X_{10}$ (SEQ ID NO: 1729), wherein

i) X_1 is K or R,

ii) X_2 is D or E,

iii) X_3 is L, Q, or R,

iv) X_4 is N or T,

v) X_5 is F or Y,

vi) X_6 is A, E, G, Q, R, or S,

vii) X_7 is H, L, or N,

viii) X_8 is L or V,

ix) X_9 is A, G, I, L, T, or V, and

x) X_{10} is A, F, or S,

(g) $FX_1DVX_2X_3X_4FX_5X_6X_7EWX_8$ (SEQ ID NO: 1730), wherein

i) X_1 is A, E, G, K, or R,

ii) X_2 is A, S, or T,

iii) X_3 is I or V,

iv) X_4 is D, E, N, or Y,

v) X_5 is S or T,

vi) X_6 is E, L, P, Q, R, or W,

vii) X_7 is D or E, and

viii) X_8 is A, E, G, Q, or R;

(h) $X_1PX_2X_3X_4X_5X_6LEX_7X_8X_9X_{10}X_{11}X_{12}$, wherein

i) X_1 is K or R,

ii) X_2 is A, D, E, or N,

iii) X_3 is I, L, M, or V,

iv) X_4 is I or V,

v) X_5 is F, S, or T,

vi) X_6 is H, K, L, Q, R, or W,

vii) X_7 is K, Q, or R,

viii) X_8 is E, G, or R,

ix) X_9 is D, E, or K,

x) X_{10} is A, D, or E,

xi) X_{11} is L or P, and

xii) X_{12} is C or W; and

(i) $X_1LX_2X_3X_4QX_5X_6$, wherein

i) X_1 is C, H, L, Q, or W,

ii) X_2 is D, G, N, R, or S,

iii) X_3 is L, P, S, or T,

iv) X_4 is A, S, or T,

v) X_5 is K or R, and

vi) X_6 is A, D, E, K, N, S, or T.

82. The system of claim 81, wherein the RD1 comprises a first and a second amino acid sequence motif wherein:

(a) the first amino acid sequence motif comprises $LYX_1X_2VMX_3EX_4X_5X_6X_7X_8X_9X_{10}$ (SEQ ID NO: 1729), wherein

i) X_1 is K or R,

ii) X_2 is D or E,

iii) X_3 is L, Q, or R,

iv) X_4 is N or T,

v) X_5 is F or Y,

vi) X_6 is A, E, G, Q, R, or S,

vii) X_7 is H, L, or N,

viii) X_8 is L or V,

ix) X_9 is A, G, I, L, T, or V, and

x) X_{10} is A, F, or S; and

(b) the second amino acid sequence motif comprises $FX_1DVX_2X_3X_4FX_5X_6X_7EWX_8$ (SEQ ID NO: 1730), wherein

i) X_1 is A, E, G, K, or R,

ii) X_2 is A, S, or T,

iii) X_3 is I or V,

iv) X_4 is D, E, N, or Y,

v) X_5 is S or T,

vi) X_6 is E, L, P, Q, R, or W,

vii) X_7 is D or E, and

viii) X_8 is A, E, G, Q, or R.

83. The system of any one of claims 62-80, wherein the RD1 comprises an amino acid sequence motif selected from the group consisting of:

(a) DVAVYFSPEEWGCL (SEQ ID NO: 2945);

(b) $X_1X_2X_3QX_4X_5LY$, wherein

i) X_1 is A, D, G, N, R, or S,

ii) X_2 is P, S, or T,

iii) X_3 is A, S, or T,

iv) X_4 is K or R, and

v) X_5 is A, D, K, N, S, or T;

(c) $X_1KPX_2X_3X_4X_5X_6$, wherein

i) X_1 is A, P, or S,

ii) X_2 is A, D, or E,

iii) X_3 is L, M, or V,

iv) X_4 is I or V,

v) X_5 is F, S, or T, and

vi) X_6 is H, K, L, Q, R, or W;

(d) $LEX_1X_2X_3X_4X_5X_6$, wherein

i) X_1 is E, K, Q or R,

ii) X_2 is E, G, or R,

iii) X_3 is A, D, E, or K,

iv) X_4 is A, D, or E,

v) X_5 is L or P, and

vi) X_6 is C or W; and

(e) $X_1VMLEX_2YX_3X_4X_5X_6SX_7X_8X_9$ (SEQ ID NO: 2946), wherein

i) X_1 is D or E,

ii) X_2 is N or T,

iii) X_3 is A, E, G, Q, R, or S,

iv) X_4 is H or N,

v) X_5 is L, M, or V,

vi) X_6 is A, L, or V,

vii) X_7 is L or V,

viii) X_8 is A, G, or V, and

ix) X_9 is C, F, or L.

84. The system of any one of claims 81-83, wherein the RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 130-1726, or a sequence having at least about 70%,

at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

85. The system of any one of claims 81-83, wherein the RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 130-224, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

86. The system of any one of claims 81-83, wherein the RD1 comprises a sequence selected from the group consisting of SEQ ID NOS: 130-138, or sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

87. The system of any one of claims 81-83, wherein the RD1 comprises the sequence of SEQ ID NO: 135, or sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

88. The system of any one of claims 81-83, wherein the RD1 comprises the sequence of SEQ ID NO: 131, or sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

89. The system of any one of claims 60-88, wherein the ADD comprises the sequence of SEQ ID NO: 125, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

90. The system of any one of claims 60-89, wherein the DNMT3A comprises the sequence of SEQ ID NO: 126, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

91. The system of any one of claims 60-90, wherein the DNMT3L comprises the sequence of SEQ ID NO: 127, or a sequence having at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% sequence identity thereto.

92. A nucleic acid comprising a sequence that encodes the gRNA of any one of claims 1-59.

93. A nucleic acid comprising a sequence that encodes the repressor fusion protein of any one of claims 1-91.

94. The nucleic acid of claim 93, wherein the sequencing encoding the repressor fusion protein is codon-optimized.

95. The nucleic acid of claim 94, wherein the sequencing encoding the repressor fusion protein is codon-optimized for expression in a human cell.

96. The nucleic acid of any one of claims 93-95, wherein the sequence encoding the repressor fusion protein comprises one or more sequences selected from the group consisting of SEQ ID NO: 3105, 3116-3124, and 3127-3128, 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.

97. The nucleic acid of any one of claims 93-96, wherein the nucleic acid is a messenger RNA (mRNA).

98. The nucleic acid of claim 97, wherein at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99%, or 100% of uridine nucleosides of the mRNA sequence are replaced with N1-methylpseudouridine.

99. The nucleic acid of claim 97 or claim 98, comprising a 5' untranslated region (UTR).

100. The nucleic acid of any one of claims 97-99, comprising a 3' untranslated region (UTR).

101. A lipid nanoparticle (LNP) comprising the nucleic acid of any one of claims 97-100.

102. A lipid nanoparticle (LNP) comprising the gRNA of any one of claims 1-59.

103. A lipid nanoparticle (LNP) comprising

(a) the gRNA of any one of claims 1-59;

(b) the nucleic acid of any one of claims 93-97; or

(c) a combination of (a) and (b).

104. The LNP of any one of claims 101-103, wherein the LNP comprises one or more components selected from the group consisting of an ionizable lipid, a helper phospholipid, a polyethylene glycol (PEG)-modified lipid, and cholesterol or a derivative thereof.

105. The LNP of claim 104, wherein the LNP comprises an ionizable lipid, a helper phospholipid, a polyethylene glycol (PEG)-modified lipid, and cholesterol or a derivative thereof.

106. The LNP of any one of claims 101-105, wherein the LNP comprises a cationic lipid comprising a pKa of 5 to 8.

107. A vector comprising the nucleic acid of any one of claims 92-97.

108. The vector of claim 107, wherein the vector is selected from the group consisting of a retroviral vector, a lentiviral vector, an adenoviral vector, an adeno-associated viral (AAV) vector, a herpes simplex virus (HSV) vector, a plasmid, a minicircle, a nanoplasmid, a DNA vector, and an RNA vector.

109. A host cell comprising the vector of claim 107 or claim 108.

110. The host cell of claim 109, wherein the host cell is selected from the group consisting of a Baby Hamster Kidney fibroblast (BHK), a human embryonic kidney 293 (HEK293) cell, a human embryonic kidney 293T (HEK293T) cell, a NS0 cell, a SP2/0 cell, a YO myeloma cell, a P3X63 mouse myeloma cell, a PER cell, a PER.C6 cell, a hybridoma cell, a NIH3T3 cell, a CV-1 (simian) in Origin with SV40 genetic material (COS) cell, a HeLa cell, a Chinese hamster ovary (CHO) cell, and a yeast cell.

111. A pharmaceutical composition comprising:

(a) the system of any one of claims 1-91;

(b) the nucleic acid of any one of claims 92-100;

(c) the LNP of any one of claims 101-106; or

(d) the vector of claim 107 or claim 108,

and one or more pharmaceutically suitable excipients.

112. A pharmaceutical composition comprising a plurality of the lipid nanoparticles of any one of claims 101-106, and a pharmaceutically acceptable carrier or diluent.

113. The pharmaceutical composition of claim 112, wherein an average diameter of lipid nanoparticles in the plurality is between about 20 nm and about 200 nm.

114. The pharmaceutical composition of any one of claims 111-113, wherein the pharmaceutical composition is formulated for a route of administration selected from the group consisting of intravenous, intraarterial, intraportal vein injection, intraperitoneal, intramuscular, intracerebroventricular, intracisternal, intrathecal, intracranial, intralumbar, intraocular, subcutaneous, and oral routes.

115. A method of repressing transcription of a *PCSK9* gene in a population of cells, the method comprising introducing into cells of the population:

(a) the system of any one of claims 1-91;

(b) the nucleic acid of any one of claims 92-100;

(c) the LNP of any one of claims 101-106;

(d) the vector of claim 107 or claim 108;

(e) the pharmaceutical composition of any one of claims 111-114; or

(f) combinations of two or more of (a)-(d),

wherein transcription of the *PCSK9* gene is repressed by the repressor fusion protein.

116. The method of claim 115, wherein transcription of the *PCSK9* gene of at least about 1%, at least about 2%, at least about 3%, at least about 4%, at least about 5%, at least about 6%, at least about 7%, at least about 8%, at least about 9%, or at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60% or more of the cells of the population is repressed.

117. The method of claim 115 or claim 116, wherein transcription of the *PCSK9* gene is repressed in the cells of the population such that expression of PCSK9 protein is decreased by at least about 10%, at least about 20%, at least about 30%, at least about 40%, 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 at least 99%, in comparison to a cell where transcription of the *PCSK9* gene has not been repressed.

118. The method of any one of claims 115-117, wherein transcription of the *PCSK9* gene of the cells of the population is repressed such that at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% of the cells of the population do not express a detectable level of PCSK9 protein.

119. The method of any one of claims 115-117, wherein repression of transcription of the *PCSK9* gene of the cells of the population is heritable.

120. The method of claim 119, wherein the repression of the PCKS9 gene persists for at least 1, 2, 3, 4, 5, or 6 or more cell divisions.

121. The method of any one of claims 115-120, wherein repression of transcription of the *PCSK9* gene of the cells of the population is sustained for at least about 8 hours, at least about 1 day, at least about 7 days, at least 2 weeks, at least about 3 weeks, at least about 1 month, or at least about 2 months, when assayed in an in vitro assay.

122. The method of any one of claims 115-121, wherein the cells are eukaryotic.

123. The method of claim 122, wherein the eukaryotic cells are selected from the group consisting of rodent cells, mouse cells, rat cells, and non-human primate cells.

124. The method of claim 122, wherein the eukaryotic cells are human cells.

125. The method of any one of claims 115-124, wherein the eukaryotic cells are selected from the group consisting of hepatocytes, cells of the intestine, cells of the kidney, cells of the central nervous system, smooth muscle cells, macrophages, cells of the retina, and arterial endothelial cells.

126. The method of any one of claims 115-124, wherein the eukaryotic cells are selected from the group consisting of embryonic stem cells, induced pluripotent stem cells, germ cells, fibroblasts, oligodendrocytes, glial cells, hematopoietic stem cells, neuron progenitor cells, neurons, astrocytes, muscle cells, bone cells, hepatocytes, pancreatic cells, retinal cells, cancer cells, T-cells, B-cells, NK cells, fetal cardiomyocytes, myofibroblasts, mesenchymal stem cells, autotransplanted expanded cardiomyocytes, adipocytes, totipotent cells, pluripotent cells, blood stem cells, myoblasts, bone marrow cells, mesenchymal cells, parenchymal cells, epithelial cells, an endothelial cells, mesothelial cells, fibroblasts, osteoblasts, chondrocytes, hematopoietic stem cells, bone-marrow derived progenitor cells, myocardial cells, skeletal cells, fetal cells, undifferentiated cells, multi-potent progenitor cells, unipotent progenitor cells, monocytes, cardiac myoblast, skeletal myoblast, macrophage, capillary endothelial cells, xenogeneic cells, allogenic cells, or post-natal stem cells.

127. The method of any one of claims 115-126, wherein the repression of transcription of the *PCSK9* gene of the population of cells occurs *in vitro* or *ex vivo*.

128. The method of any one of claims 115-126, wherein the repression of transcription of the *PCSK9* gene of the population of cells occurs *in vivo* in a subject.

129. The method of claim 128, wherein the subject is selected from the group consisting of a rodent, a mouse, a rat, and a non-human primate.

130. The method of claim 128, wherein the subject is a human.

131. The method of any one of claims 115-130, wherein the repression is stable through one or more cell divisions.

132. The method of any one of claims 115-131, wherein the repression is reversible.

133. The method of claim 132, wherein the repression is reversible by introducing into cells of the population a DNA methyltransferase inhibitor.

134. The method of claim 133, wherein the DNA methyltransferase inhibitor is a cytidine analog.

135. The method of claim 133 or claim 134, wherein the DNA methyltransferase inhibitor is selected from the group consisting of azacytidine, decitabine, clofarabine, and zebularine.

136. A method of treating a *PCSK9*-related disease or disorder in a subject in need thereof, comprising contacting cells of the subject with a therapeutically effective dose of:

(a) the system of any one of claims 1-91;

(b) the nucleic acid of any one of claims 92-100;

(c) the LNP of any one of claims 101-106;

(d) the vector of claim 107 or claim 108;

(e) the pharmaceutical composition of any one of claims 111-114; or

(f) combinations of two or more of (a)-(d),

wherein transcription of the *PCSK9* gene is repressed by the repressor fusion protein in the contacted cells.

137. The method of claim 136, wherein the cells are contacted with a therapeutically effective dose of the LNP.

138. The method of claim 137, wherein the LNP is administered by a route of administration selected from the group consisting of intravenous, intraarterial, intraportal vein injection, intraperitoneal, intramuscular, intracerebroventricular, intracisternal, intrathecal, intracranial, intralumbar, intraocular, subcutaneous, and oral routes.

139. The method of any one of claims 136-138, wherein the subject is pretreated with a therapeutic agent that increases hepatic LDL receptor (LDLR) expression.

140. The method of claim 139, wherein the therapeutic agent is selected from the group consisting of evolocumab, inclisiran, alirocumab, and MK-0616.

141. The method of any one of claims 136-140, wherein the *PCSK9*-related disease is autosomal dominant hypercholesterolemia (ADH), hypercholesterolemia, elevated total cholesterol levels, hyperlipidemia, elevated low-density lipoprotein (LDL) levels, elevated LDL-cholesterol levels, reduced high-density lipoprotein levels, liver steatosis, coronary heart disease, ischemia, stroke, peripheral vascular disease, thrombosis, type 2 diabetes, high elevated blood pressure, atherosclerosis, obesity, aortic stenosis, elevated PCSK9 levels, or a combination thereof.

142. The method of any one of claims 136-141, wherein the method results in improvement in at least one clinically-relevant endpoint selected from the group consisting of change from baseline in LDL-cholesterol, decrease in plaque atheroma volume, reduction in in coronary plaque, reduction in atherosclerotic cardiovascular disease (ASCVD), cardiovascular death, nonfatal myocardial infarction, ischemic stroke, nonfatal stroke, coronary revascularization, unstable angina, and visual acuity.

143. The method of any one of claims 136-141, wherein the method results in improvement in at least two clinically-relevant endpoints selected from the group consisting of change from baseline in LDL-cholesterol, decrease in plaque atheroma volume, reduction in in coronary plaque, reduction in atherosclerotic cardiovascular disease (ASCVD), cardiovascular death, nonfatal myocardial infarction, ischemic stroke, nonfatal stroke, coronary revascularization, unstable angina and visual acuity.

144. The system of any one of claims 1-91, the nucleic acid of any one of claims 92-100, the LNP of any one of claims 101-106, the vector of claim 107 or claim 108, the pharmaceutical composition of any one of claims 111-114, or combinations thereof, for use in the manufacture of a medicament for the treatment of a *PCSK9*-related disease.

145. The system of any one of claims 1-91, the nucleic acid of any one of claims 92-100, the LNP of any one of claims 101-106, the vector of claim 107 or claim 108, the pharmaceutical composition of any one of claims 111-114, or combinations thereof, for use in repressing an *PCSK9* target nucleic acid sequence in a population of cells, or treating an *PCSK9*-related disease.

146. A kit comprising the system of any one of claims 1-91, the nucleic acid of any one of claims 92-100, the LNP of any one of claims 101-106, the vector of claim 107 or claim 108, the pharmaceutical composition of any one of claims 111-114, or combinations thereof, and a suitable container.

147. The kit of claim 146, comprising a buffer, an excipient, a nuclease inhibitor, a protease inhibitor, a liposome, a therapeutic agent, a label, a label visualization reagent, instructions for use, or any combination of the foregoing.