

COMPOSITIONS AND METHODS FOR IN VIVO NUCLEASE-MEDIATED GENE
TARGETING FOR THE TREATMENT OF GENETIC DISORDERS

5 BACKGROUND OF THE INVENTION

Site-specific nucleases (such as CRISPR-Cas9 or meganucleases) generate double strand breaks (DSBs) in the chromosome, leading to DNA repair. In the presence of donor DNA, homology directed repair (HDR) occurs and replaces genetic information in the chromosome with new information from the donor gene.

10 Homology-directed repair (HDR) is a process where a DNA double-strand break (DSB) is repaired by homologous recombination using a DNA template. This template can come from within the cell during late S phase or G2 phase of the cell cycle, when sister chromatids are available prior to the completion of mitosis. Additionally, exogenous repair templates can be delivered into a cell, most often in the form of a synthetic, single-strand
15 DNA donor oligo or donor plasmid, to generate a precise change in the genome.

Safe harbor sites (SHS) are genomic loci where genes or other genetic elements can be safely inserted and expressed. These SHS are critical for effective human disease gene therapies; for investigating gene structure, function and regulation; and for cell marking and tracking.

20 What are needed are improved compositions and methods for gene editing.

SUMMARY OF THE INVENTION

Provided herein are compositions, methods, systems, and kits for gene editing, which allow knockdown or ablation of the native PCSK9 gene and insertion and/or expression of
25 an exogenous transgene in the PCSK9 gene locus.

In a first aspect, provided herein is a system for treating a genetic disorder. The system includes a gene editing component comprising an expression cassette comprising a nucleic acid sequence encoding a nuclease that targets the PCSK9 gene and regulatory sequences that direct expression of the nuclease in a target cell comprising the PCSK9 gene.
30 The system further includes a donor vector comprising a transgene cassette comprising a nucleic acid sequence encoding a transgene and regulatory sequences that direct expression of the transgene in the target cell, the donor vector further comprising homology-directed

recombination (HDR) arms 5' and 3' to the transgene cassette, wherein the transgene is not PCSK9. The nuclease targets the PCSK9 gene. In some embodiments, the nuclease targets PCSK9 exon 7. In some embodiments, the meganuclease is the ARCUS meganuclease.

In some embodiments, the gene editing component comprises a sequence that
5 encodes a Cas9. In certain embodiments, the gene editing vector further comprises sequence that encodes a sgRNA comprising at an least 20 nucleotide seed region, wherein the sgRNA specifically binds to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9.

In other embodiments, the donor vector further comprises sequences that encode a
10 sgRNA comprising an at least 20 nucleotide seed region, wherein the sgRNA specifically binds to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9.

In another aspect, provided herein is a system for treating a genetic disorder. The system includes a gene editing component comprising a nucleic acid sequence encoding a
15 nuclease that targets the PCSK9 gene. The system further includes a donor vector comprising a transgene cassette comprising a nucleic acid sequence encoding a transgene and regulatory sequences that direct expression of the transgene in the target cell, the donor vector further comprising homology-directed recombination (HDR) arms 5' and 3' to the transgene cassette, wherein the transgene is not PCSK9. The nuclease targets the PCSK9
20 gene. In certain embodiments, the gene editing component is provided in a lipid nanoparticle.

In some embodiments, the gene editing component comprises a sequence that encodes a Cas9. In certain embodiments, the gene editing vector further comprises a sequence that encodes a sgRNA comprising at an least 20 nucleotide seed region, wherein
25 the sgRNA specifically binds to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9.

In other embodiments, the donor vector further component comprises a sequence that encodes a sgRNA comprising an at least 20 nucleotide seed region, wherein the sgRNA specifically binds to a target site in the PCSK9 gene, said target site being 5' to a
30 protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9.

In certain embodiments, the transgene relates to a liver metabolic disorder. In certain embodiments, the transgene is OTC, PKU, CTLN1, or LDLR.

In certain embodiments, the vectors are adeno-associated viral (AAV) vectors, and the vectors comprise AAV 5' ITRs and AAV 3' ITRs.

5 In another embodiment, the dual vector system for treating a genetic disorder includes a gene editing AAV comprising an AAV capsid and a first vector genome comprising a 5' ITR, a sequence encoding a meganuclease that targets PCSK9 under control of regulatory sequences that direct expression of the meganuclease in a target cell comprising a PCSK9 gene, and a 3' ITR; and a donor AAV vector comprising an AAV
10 capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR, wherein the transgene does not encode PCSK9.

In another embodiment, the dual vector system for treating a genetic disorder
15 includes a gene editing AAV comprising an AAV capsid and a first vector genome comprising a 5' ITR, a 5' nuclear localization signal (NLS), a sequence encoding a Cas9 and regulatory sequences that direct expression of the saCas9 in a target cell comprising the PCSK9 gene, a 3' NLS, and a 3' ITR; and a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination
20 (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, a U6 promoter, a sgRNA comprising at least 20 nucleotides that specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9, and a 3' ITR, wherein the transgene does not encode PCSK9.

25 In yet another embodiment, the dual vector system for treating a genetic disorder includes a gene editing AAV vector comprising an AAV capsid and a first vector genome comprising a 5' ITR, a U6 promoter, a sgRNA comprising at least 20 nucleotides that specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9, a 5' nuclear localization
30 signal (NLS), a sequence encoding a Cas9 and regulatory sequences that direct expression of the Cas9 in a target cell comprising the PCSK9 gene, a 3' NLS, and a 3' ITR; and a donor

AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR.

5 In some embodiments, the gene editing AAV vector and the donor AAV vector have the same AAV capsid. In other embodiments, the gene editing AAV vector and the donor AAV vector have different AAV capsids. In some embodiments, the AAV capsid is selected from AAV8, AAV9, rh10, AAV6.2, AAV3B, hu37, rh79, and rh64.

In another aspect, a method of treating a disorder in humans by co-administering the dual vector system as described herein, is provided.

10 In another aspect, a method of treating a liver metabolic disorder in a subject is provided, the method includes co-administering to the subject having a liver metabolic disorder a gene editing AAV vector comprising a sequence encoding a nuclease and regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene; and a donor AAV vector comprising a transgene and regulatory sequences that
15 direct expression of the transgene in the target cell, the donor vector further comprising homology-directed recombination (HDR) arms 5' and 3' to the transgene cassette, wherein the transgene is not PCSK9. In certain embodiments, the liver metabolic disorder is ornithine transcarbamylase. In other embodiments, the familial hypercholesterolemia or phenylketonuria. In one embodiment, the subject is a neonate.

20 In another aspect, a system for treating genetic disorders is provided. The system includes a lipid nanoparticle (LNP) comprising a mRNA sequence encoding a nuclease that targets the PCSK9 gene; and a donor AAV vector comprising a transgene and regulatory sequences which direct its expression in the target cell, the donor vector further comprising a homology-directed recombination (HDR) arms 5' and 3' to the transgene, wherein the
25 transgene is not PCSK9. In some embodiments, the nuclease targets PCSK9 exon 7. In some embodiments, the meganuclease is the ARCUS meganuclease.

In other embodiments, the gene editing vector encodes a Cas9. In certain embodiments, the gene editing vector further encodes a sgRNA comprising at least 20 nucleotides, which specifically binds to a target site in the PCSK9 gene, said target site being
30 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9. In

some embodiments, where the system includes an LNP, the LNP comprises both the Cas9 coding sequence and gRNA.

5 In other embodiments, the donor vector further encodes a sgRNA comprising an at least 20 nucleotide seed region, wherein the sgRNA specifically binds to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9.

In yet another aspect, a dual vector system for treating a genetic disorder is provided. The system includes a gene editing vector comprising an expression cassette comprising a nucleic acid sequence encoding a nuclease and regulatory sequences that direct expression of
10 the nuclease in a target cell comprising a PCSK9 gene; and a donor vector comprising a nucleic acid sequence encoding an exogenous product for expression from the PCSK9 locus, wherein the inserted nucleic acid sequence does not encode PCSK9, wherein the system further comprises sequences that direct the nuclease to specifically targets the native PCSK9 gene locus; and wherein the native PCSK9 in the target cell is optionally ablated or reduced
15 post-dosing with the dual vector system.

In yet another aspect, a method of treating a patient is provided, using a system as described herein, wherein the patient's native PCSK9 expression levels are reduced and wherein the patient expresses the exogenous product.

20 In yet another aspect, an engineered coding sequence for ornithine transcarbamalase is provided. Vectors, expression cassettes, and recombinant viruses comprising the same are also included.

Other aspects and advantages of the invention will be apparent from the following detailed description of the invention.

25 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a schematic representation of the rhPCSK9 locus showing the donor splice site within exon 7, and a HDR donor vector comprising a donor template of interest, e.g., hFIX, hOTC.

30 FIG. 2 shows a timeline for a pilot study comprising an hFIX mini-gene knock-in in PCSK9 locus by ARCUS2 or SaCas9 in newborn NHPs.

FIGs. 3A to 3C show a schematic representation for a dual AAV vector system for SaCas9- or ARCUS-mediated gene correction. FIG. 3A shows a schematic representation for a dual AAVhu37 vector system for ARCUS2-mediated gene correction, wherein the AAVhu37-donor vector comprises an hOTC donor template sequence. FIG. 3B shows a schematic representation for a dual AAVhu37 vector system for SaCas9-mediated gene correction (trans; AAVhu37-SaCas9), wherein the expression cassettes for SaCas9 and sgRNA are in two separate vectors, and AAVhu37.sgRNA-donor vector comprises an hOTC donor template sequence and a U6.sgRNA cassette. FIG. 3C shows a schematic representation for a dual AAVhu37 vector system for SaCas9-mediated gene correction (cis; AAVhu37.PCSK9-sgRNA.SaCas9), wherein the expression cassettes for SaCas9 and sgRNA are in the same vector, and the hOTC donor vector is in a separate vector.

FIGs. 4A to 4H show an in vivo test of nuclease-mediated gene targeting in newborn NHPs. Animals were administered 1×10^{13} GC/kg of AAVhu37.ARCUS2.WPRE and 3×10^{13} GC/kg of AAVhu37.hFIXco-HDR or 1×10^{13} GC/kg of AAVhu37.SaCas9.WPRE and 3×10^{13} GC/kg of AAVhu37.hFIXco-HDR.U6.sgR or 1×10^{13} GC/kg of AAVhu37.GFP.WPRE and 3×10^{13} GC/kg of AAVhu37.hFIXco-HDR.U6.sgR, as shown in FIGs 4A, 4B and 5G. FIG. 4C shows hFIX levels at the indicated timepoints (plotted as ng/mL) in newborn NHPs. FIG. 4D shows PCSK9 levels at the indicated timepoints (plotted as percentage of baseline at day 0) in newborn NHPs. FIG. 4E shows ALT (Alanine Aminotransferase) levels at the indicated timepoints (plotted as U/L) in newborn NHPs. FIG. 4F shows anti-FIX IgG levels at the indicated timepoints (plotted as dilution factor, 1/dilution) in newborn NHPs. FIG. 4G shows PCSK9 levels at the indicated timepoints (plotted as ng/mL) in newborn NHPs. FIG. 4H shows weight as measured (plotted as g) in newborn NHPs.

FIGs. 5A to 5H show the results of the in vivo test described for FIG. 4, administered to 3-month-old infant NHPs. FIG. 5A shows hFIX levels at the indicated timepoints (plotted as ng/mL) in infant NHPs. FIG. 5B shows PCSK9 levels at the indicated timepoints (plotted as percentage of baseline at day 0) in infant NHPs. FIG. 5C shows ALT (Alanine Aminotransferase) levels at the indicated timepoints (plotted as U/L) in infant NHPs. FIG. 5D shows anti-FIX IgG levels at the indicated timepoints (plotted as dilution factor, 1/dilution) in infant NHPs. FIG. 5E shows PCSK9 levels at the indicated timepoints (plotted as ng/mL) in infant NHPs. FIG. 5F shows weight as measured at the indicated timepoints

(plotted as g) in infant NHPs. FIG. 5G is a summary table showing data from the experiment described in FIGs. 4A-5G. FIG. 5H shows a comparison of various data between newborn and infant NHPs tested.

FIGs. 6A to 6G show vector transduction (GC) and transgene expression in liver biopsies samples collected at various days post treatment in NHPs treated as described for FIGs 4A-4H. FIG. 6A shows vector transduction levels in liver biopsies samples, plotted as AAV genome copies (GC) per diploid cell. FIG. 6B shows relative expression of transgene RNA in liver biopsies samples. FIG. 6C shows dual in situ hybridization (ISH) using specific probes to detect FIX and ARCUS in liver biopsies. FIG. 6D shows digitized ISH images used for quantification of transduction percentage. FIG. 6E shows transduction efficiency of FIX transgene as quantified by ISH, and plotted as percent transduction.

FIGs. 7A to 7L show dual in situ hybridization (ISH) using specific probes to detect FIX and ARCUS in liver biopsies collected at 84 days post treatment in NHPs; showed at various magnification views (NHPs treated with AAVhu37.ARCUS2 and AAVhu37.Donor-HDR-hFIX). FIG. 7A shows ISH-detected ARCUS in liver biopsies, viewed at 4x magnification. FIG. 7B shows ISH-detected hFIX in liver biopsies, viewed at 4x magnification. FIG. 7C shows overlay image of ISH-detected ARCUS and hFIX, viewed at 4x magnification. FIG. 7D shows ISH-detected ARCUS and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 4x magnification. FIG. 7E shows ISH-detected ARCUS in liver biopsies, viewed at 10x magnification. FIG. 7F shows ISH-detected hFIX in liver biopsies, viewed at 10x magnification. FIG. 7G shows overlay image of ISH-detected ARCUS and hFIX, viewed at 10x magnification. FIG. 7H shows ISH-detected ARCUS and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 10x magnification. FIG. 7I shows ISH-detected ARCUS expression in liver biopsies, viewed at 20x magnification. FIG. 7J shows ISH-detected hFIX in liver biopsies, viewed at 20x magnification. FIG. 7K shows overlay image of ISH-detected ARCUS and hFIX, viewed at 20x magnification. FIG. 7L shows ISH-detected ARCUS and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 20x magnification.

FIGs. 8A to 8M show dual in situ hybridization (ISH) using specific probes to detect FIX and ARCUS in liver biopsies collected at 84 days post treatment in NHPs; showed at various magnification views (NHPs treated with AAVhu37.EGFP and AAVhu37.Donor-

HDR-hFIX.U6.sgR). FIG. 8A shows ISH-detected GFP-WRPE in liver biopsies, viewed at 4x magnification. FIG. 8B shows ISH-detected hFIX in liver biopsies, viewed at 4x magnification. FIG. 8C shows overlay image of ISH-detected GFP-WRPE and hFIX, viewed at 4x magnification. FIG. 8D shows ISH-detected GFP-WRPE and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 4x magnification. FIG. 8E shows ISH-detected GFP-WRPE in liver biopsies, viewed at 10x magnification. FIG. 8F shows ISH-detected hFIX in liver biopsies, viewed at 10x magnification. FIG. 8G shows overlay image of ISH-detected GFP-WRPE and hFIX, viewed at 10x magnification. FIG. 8H shows ISH-detected GFP-WRPE and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 10x magnification. FIG. 8I shows ISH-detected GFP-WRPE expression in liver biopsies, viewed at 20x magnification. FIG. 8J shows ISH-detected hFIX in liver biopsies, viewed at 20x magnification. FIG. 8K shows overlay image of ISH-detected GFP-WRPE and hFIX, viewed at 20x magnification. FIG. 8L shows ISH-detected GFP-WRPE and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 20x magnification. FIG. 8M shows ISH-detected GFP-WRPE and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 20x magnification in an untreated control.

FIG. 9 shows ARCUS-mediated on-target editing in NHP treated with AAVhu37.ARCUS2 and AAVhu37.Donor-HDR-hFIX. At 84 days post treatment, liver biopsies samples were collected, and percentage of total indels in the target region present in was calculated based on amplicon-seq.

FIGs. 10A and 10B show schematic representations of a PCSK9-hE7-KI Mouse Model. FIG. 10A shows schematic representation of the mouse *pcsk9* exon 7 which is replaced with human *pcsk9* exon 7 (hE7 contains ARCUS targeting sequence). Human PCSK9 exon 7 sequence is shown in SEQ ID NO: 44. FIG. 10B shows schematic representation of crossing PCSK9-hE7-KI mouse model with other disease mouse models, such as OTC *spf^{ash}*, the KI-*spf^{ash}* model. The *PCSK9-hE7-KI* knock-in mouse model was first generated by replacing a region including exon 7 of the murine *Pcsk9* gene with a region of human *PCSK9* gene containing exon 7. The *PCSK9-hE7-KI* mouse was then crossed with sparse fur ash (*spf^{ash}*) mouse, which exhibits a 20-fold reduction in OTC expression due to a G to A point mutation at the splice donor site at the end of exon 4 of the *Otc* gene. The mice from this cross were termed *PCSK9-hE7-KI.spf^{ash}* mice and were utilized as described

herein. *Abbreviations*: bp, base pairs; E6, exon 6; E7: exon 7; E8, exon 8; HDR, homology-dependent recombination; *PCSK9*, proprotein convertase subtilisin/kexin type 9 (gene, human); *Pcsk9*, proprotein convertase subtilisin/kexin type 9 (gene, mouse).

FIGs. 11A-11I show an in vivo test of nuclease-mediated gene targeting in newborn NHPs for vectors as shown in FIG. 11I. FIG. 11A is a chart showing experimental design of in vivo test of nuclease-mediated gene targeting in newborn NHPs for vectors as described in Example 3. Animals 21-111, 21-122, and 21-113 were AAV binding antibody (BAb) positive prior to dosing. Day 0 sample of 21-178 was collected post vector dosing which would interfere with the Bab assay. ^c: number of OT sites identified in independent ITRseq assay are listed. FIG. 11B shows PCSK9 levels shown as ng/mL (top row) or % of day 0 (bottom row) for the groups as shown in FIG. 11A. FIG. 11C shows ALT levels shown as U/L (top row) or AST shown as U/L (bottom row) for the groups, as shown in FIG. 11A. FIG. 11D shows transduction efficiency of OTC transgene as quantified by ISH or IF, and plotted as percent hepatocytes transduced. FIG. 11E shows body weight of mice. FIG. 11F shows vector GCs in liver by quantitative PCR analysis at day 84. FIG. 11G shows expression of hOTC and nuclease in macaque liver at day 84 measured by quantitative PCR on total RNA isolated from the liver biopsy samples followed by reverse transcription and presented as relative expression levels normalized by GAPDH levels. FIG. 11H shows Indel analysis on the *rhPCSK9*-targeted locus performed by amplicon-seq. FIG. 11I is a schematic of a timeline of an in vivo test of nuclease-mediated gene targeting in newborn NHPs including vectors tested for experiment described in Example 3.

FIG. 12 shows sequence alignment of 265 bp sequence that represents the human PCSK9 sequence of the pcsk9-hE7 knock-in allele, mouse PCSK9 (mPCSK9) and rhesus macaques PCSK9 (rhPCSK9). *Abbreviations*: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, genome copies; hOTC, human ornithine transcarbamylase; OT, off-target; PCR, polymerase chain reaction; *rhPCSK9*, proprotein convertase subtilisin/kexin type 9 (rhesus gene); RNA, ribonucleic acid.

FIG. 13 shows a schematic representation of donor constructs for a dual AAV vector system for ARCUS2-mediated gene correction, wherein the AAV-donor vector comprises an hOTC donor template sequence. The homology of the HDR arms in the constructs with the knock in mouse model (FIG. 10A-10B), NHP, and human target regions is shown.

FIG. 14A shows a timeline for a study comprising an hOTC mini-gene knock-in in PCSK9 locus by ARCUS2 performed in PCSK9-hE7-KI.spf-ash pups (partial OTC deficiency model), as described in Example 5. FIG. 14B shows the vectors and dosages each group will receive for the study of FIG. 14A.

5 FIGs. 14C-14I show results of a study of mice treated with vectors as shown in FIG. 7, or untreated (KI WT) and fed a high protein (HP) diet for 10 days. FIG. 14C shows probability of survival. FIG. 14D shows weight as a percentage of weight prior to introduction of the HP diet. FIG. 14E shows plasma NH₃ levels at day 10 of HP diet. FIG. 14F shows mPCSK9 protein levels at day 48. FIG. 14G shows indel % as measured by
10 amplicon-seq on day 59. FIG. 14H shows vector transduction levels in liver biopsy samples, plotted as AAV genome copies (GC) per diploid cell, measured on day 59. FIG. 14I shows OTC IF at 8 weeks.

FIG. 15 is schematic of the experimental design described in Example 10 to generate hLDLR mini gene knock-in in PCSK9 locus by SaCas9 in PCSK9-hE7-KI.lldlr-/ldlr-.apobec-
15 /apobec- Pups (hoFH model).

FIG. 16 is a schematic showing the vectors used in Example 10.

FIG. 17 shows the experimental design of Example 10.

FIG. 18A-18D shows the results of the experiment of Example 10. FIG. 18A shows serum LDL levels for shHDR + saCas9, mhHDR + saCas9, shHDR only and untreated mice.
20 FIG. 18B shows indel percentages for shHDR + saCas9, mhHDR + saCas9, shHDR only treated mice. FIG. 18C shows hLDLR genome copies per diploid genome as measured in liver at day 63. FIG. 18D shows serum LDL levels at day 63 for shHDR + saCas9, mhHDR + saCas9, shHDR only and untreated mice.

FIG. 19 shows immunohistochemistry data for liver samples taken at day 63 for mice
25 of Example 10.

DETAILED DESCRIPTION OF THE INVENTION

Provided herein are compositions, kits, and methods which provide stable, long term therapeutic effects to patients with certain genetic disorders, including liver metabolic
30 disorders. The compositions, kits, and methods utilize a nuclease that targets the PCSK9 locus of the target cell, and a donor vector provides a template which includes an exogenous

product for integration into, and expression from, the PCSK9 locus, wherein the inserted nucleic acid sequence does not encode PCSK9, and the expression of the endogenous PCSK9 is disrupted and expression levels are reduced.

5 PCSK9

Proprotein convertase subtilisin kexin 9 (PCSK9) is a serine protease that reduces both hepatic and extrahepatic low-density lipoprotein (LDL) receptor (LDLR; 606945) levels and increases plasma LDL cholesterol. PCSK9 is critical in the regulation of plasma cholesterol homeostasis. PCSK9 binds to the low-density lipid receptor family members low
10 density lipoprotein receptor (LDLR), very low-density lipoprotein receptor (VLDLR), apolipoprotein E receptor (LRP1/APOER) and apolipoprotein receptor 2 (LRP8/APOER2), and promotes their degradation in intracellular acidic compartments. Human PCSK9 has a protein sequence of NP_777596.2, as shown in SEQ ID NO: 23, with the coding sequence shown in SEQ ID NO: 22.

15 While the PCSK9 gene has been targeted for treatment of cholesterol related diseases, it is demonstrated herein that the PCSK9 gene locus is a safe harbor for gene targeting for insertion of other, non-PCSK9 transgenes. Thus, the compositions, kits, and methods provided herein utilize nucleases which target the PCSK9 gene locus, and insert a therapeutic transgene into the target PCSK9 locus, using a donor template.

20 The compositions, kits, and methods provided herein include a gene editing component (in some embodiments, a vector), and a donor vector which provides the therapeutic transgene to be expressed in the host cell.

GENE EDITING COMPONENT

25 The compositions, kits, and methods provided herein include a gene editing component that comprises a nuclease (or the coding sequence therefore) and sequences which direct the nuclease to specifically target the native PCSK9 gene locus on chromosome 1. As used herein, the “target PCSK9 locus” or “PCSK9 gene locus” is any site in the PCSK9 coding region where insertion of the heterologous transgene is desired. In certain
30 embodiments, the target PCSK9 locus is in Exon 7 of the PCSK9 coding sequence. FIG. 12 provides an alignment of the human (h), rhesus (rh), and mouse (m) PCSK9 exon 7 splice

sites which are exemplified herein using a SaCas9 and a meganuclease targeted to PCSK9 (referred to as ARCUS).

Described herein are compositions, particularly nucleases, which are useful targeting a gene for the insertion of a transgene, for example, nucleases that are specific for PCSK9. In certain embodiments, the nuclease is naturally occurring. In other embodiments, the nuclease is non-naturally occurring, i.e., engineered in the DNA-binding domain and/or cleavage domain. For example, the DNA-binding domain of a naturally-occurring nuclease may be altered to bind to a selected target site (e.g., a meganuclease that has been engineered to bind to site different than the cognate binding site). In other embodiments, the nuclease comprises heterologous DNA-binding and cleavage domains (e.g., zinc finger nucleases; TAL-effector nucleases; meganuclease DNA-binding domains with heterologous cleavage domains).

In certain embodiments, the nuclease is a meganuclease that targets PCSK9. Meganucleases are endodeoxyribonucleases characterized by a large recognition site (double-stranded DNA sequences of 12 to 40 base pairs), for example, I-SceI. When combined with a nuclease, DNA can be cut at a specific location. The restriction enzymes can be introduced into cells, for use in gene editing or for genome editing *in situ*. In certain embodiments, the nuclease is a member of the LAGLIDADG (SEQ ID NO: 31) family of homing endonucleases. In certain embodiments, the nuclease is a member of the I-CreI family of homing endonucleases which recognizes and cuts a 22 base pair recognition sequence SEQ ID NO: 32 - CAAAACGTCGTGAGACAGTTTG. See, e.g., WO 2009/059195. Methods for rationally-designing mono-LAGLIDADG (SEQ ID NO: 32) homing endonucleases were described which are capable of comprehensively redesigning I-CreI and other homing endonucleases to target widely-divergent DNA sites, including sites in mammalian, yeast, plant, bacterial, and viral genomes (WO 2007/047859). In one embodiment, the nuclease is encoded by the sequence shown in SEQ ID NO: 19, nt 330 to 1424, or a sequence sharing at least 95%, 98%, or 99% identity thereto. In one embodiment, the nuclease protein sequence is the sequence shown in SEQ ID NO: 20, or a sequence sharing at least 95%, 98%, or 99% identity thereto. Such nuclease is sometimes referred to herein as the ARCUS nuclease. The term "homing endonuclease" is synonymous with the term "meganuclease." See, WO 2018/195449, describing certain PCSK9 meganucleases, which is incorporated herein in its entirety.

Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. Zinc finger domains can be engineered to target specific desired DNA sequences and this enables zinc-finger nucleases to target unique sequences within complex genomes. By taking advantage of endogenous
5 DNA repair machinery, these reagents can be used to precisely alter the genomes of higher organisms and serve as a prominent tool in the field of genome editing. Transcription activator-like effector nucleases (TALEN) are restriction enzymes that can be engineered to cut specific sequences of DNA. They are made by fusing a TAL effector DNA-binding domain to a DNA cleavage domain (a nuclease which cuts DNA strands). In another
10 embodiment, the coding sequence encodes a zinc finger nuclease or a transcription activator-like (TAL) effector nuclease (TALEN).

In certain embodiments, the nuclease is a CRISPR-associated nuclease (Cas), optionally, Cas9. "Cas9" (CRISPR associated protein 9) refers to family of RNA-guided DNA endonucleases which is characterized by two signature nuclease domains, RuvC
15 (cleaves non-coding strand) and HNH (coding strand). Suitable bacterial sources of Cas9 include *Staphylococcus aureus* (SaCas9), *Streptococcus pyogenes* (SpCas9), and *Neisseria meningitidis* [KM Estelt *et al*, Nat Meth, 10: 1116-1121 (2013)]. The wild-type coding sequences may be utilized in the constructs described herein. Alternatively, the bacterial codons are optimized for expression in humans, e.g., using any of a variety of known human
20 codon optimizing algorithms. Alternatively, these sequences may be produced synthetically, either in full or in part. Other endonucleases with similar properties may optionally be substituted. See, e.g., the public CRISPR database (db) accessible at <http://crispr.u-psud.fr/crispr>.

In certain embodiments, the compositions, kits, and methods the nuclease coding
25 sequence is comprised in a gene editing vector. The gene editing vector includes an expression cassette comprising a nucleic acid sequence encoding a nuclease and regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene.

A "vector" as used herein is a biological or chemical moiety comprising a nucleic acid sequence which can be introduced into an appropriate host cell for replication or expression
30 of said nucleic acid sequence. Common vectors include non-viral vectors and viral vectors. As used herein, a non-viral system might be selected from nanoparticles, electroporation

systems and novel biomaterials, naked DNA, phage, transposon, plasmids, cosmids (Phillip McClean, www.ndsu.edu/pubweb/~mcclean/plsc731/cloning/cloning4.htm) and artificial chromosomes (Gong, Shiaoqing, et al. "A gene expression atlas of the central nervous system based on bacterial artificial chromosomes." *Nature* 425.6961 (2003): 917-925).

5 As used herein, an "expression cassette" refers to a nucleic acid molecule which comprises a biologically useful nucleic acid sequence (e.g., a gene cDNA encoding a protein, enzyme or other useful gene product, mRNA, etc.) and regulatory sequences operably linked thereto which direct or modulate transcription, translation, and/or expression of the nucleic acid sequence and its gene product. As used herein, "operably linked" sequences include
10 both regulatory sequences that are contiguous with the nucleic acid sequence and regulatory sequences that act in trans or at a distance to control the sequence. Such regulatory sequences typically include, e.g., one or more of a promoter, an enhancer, an intron, a Kozak sequence, a polyadenylation sequence, and a TATA signal. The expression cassette may contain regulatory sequences upstream (5' to) of the gene sequence, e.g., one or more of a
15 promoter, an enhancer, an intron, etc., and one or more of an enhancer, or regulatory sequences downstream (3' to) a gene sequence, e.g., 3' untranslated region comprising a polyadenylation site, among other elements. In other embodiments, the term "transgene" refers to one or more DNA sequences from an exogenous source which are inserted into a target cell. Typically, such an expression cassette for generating a viral vector contains the
20 coding sequence for the gene product described herein flanked by packaging signals of the viral genome and other expression control sequences such as those described herein. In certain embodiments, a vector genome may contain two or more expression cassettes.

 In addition to the coding sequence for the nuclease, in certain embodiments the gene editing vector includes regulatory sequences which direct expression of the nuclease in a
25 host cell. In certain embodiments, the regulatory elements include a promoter. In certain embodiments, where the system is designed for treatment of metabolic disorders characterized by a mutation or phenotype in hepatocytes, the gene editing vector may be designed such that the nuclease is expressed under the control of a liver-specific promoter. An illustrative plasmid and vector described herein uses the liver-specific promoter thyroxine binding globulin (TBG), which is characterized by the sequence of SEQ ID NO: 41. In other
30 embodiments a shortened version of TBG, a variant termed herein TBG-S1, which is

characterized by the sequence of SEQ ID NO: 11, is useful. In another embodiment, the hybrid liver promoter (HLP) having the sequence of SEQ ID NO: 12 is utilized.

In some embodiments it is desirable to utilize a promoter having low-transcriptional activity, or weak promoter. In one embodiment, the promoter is a weakened version of the liver-specific thyroxin binding globulin (TBG) promoter. In one embodiment, the weak promoter is truncated at the 5' or 3' end of the native promoter, or TBG-S1 sequence. In another embodiment, the promoter retains only the 3' terminal 113 nt from the TBG-S1 promoter and is termed F113 (also called TBG-S1-F113) (SEQ ID NO: 19, nt 206 to 318). US Provisional Patent Application Nos. 63/016,145, filed April 27, 2020, 63/033,738, filed June 2, 2020, and 63/089,796, filed October 9, 2020, PCT/US21/29386 and PCT/US21/29403, both filed April 27, 2021, each entitled COMPOSITIONS AND METHODS FOR REDUCING NUCLEASE EXPRESSION AND OFF-TARGET ACTIVITY USING A PROMOTER WITH LOW TRANSCRIPTIONAL ACTIVITY are incorporated herein by reference in their entirety.

Alternatively, other liver-specific promoters may be used such as alpha 1 anti-trypsin (A1AT), human albumin (Miyatake *et al.*, J. Virol., 71:5124-32 (1997)), and hepatitis B virus core promoter (Sandig *et al.*, Gene Ther., 3:1002-9 (1996), TTR minimal enhancer/promoter, alpha-antitrypsin promoter, LSP (845 nt). See, *e.g.*, The Liver Specific Gene Promoter Database, Cold Spring Harbor, <http://rulai.schl.edu/LSPD>. Alternatively, other tissue specific promoters, such as muscle-specific promoters, such as the muscle creatine kinase (MCK) promoter, or muscle hybrid (MH) promoter, may be used. Alternatively, other promoters, such as constitutive promoters (CMV, CBG, CB7, etc.), regulatable (inducible) promoters [see, *e.g.*, WO 2011/126808 and WO 2013/049493, incorporated by reference herein], or a promoter responsive to physiologic cues may be utilized in the vectors described herein. Optionally, if a regulatable system is selected, a third vector may be required in order to provide the regulatory function.

In addition to a promoter, the gene editing cassette, expression cassette and/or vector may contain one or more appropriate "regulatory elements" or "regulatory sequences", which comprise but are not limited to an enhancer; transcription factor; transcription terminator; efficient RNA processing signals such as splicing and polyadenylation signals (polyA); sequences that stabilize cytoplasmic mRNA, for example Woodchuck Hepatitis

Virus (WHP) Posttranscriptional Regulatory Element (WPRE); sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product.

Examples of suitable polyA sequences include, *e.g.*, SV40, bovine growth hormone (bGH), and TK polyA. Examples of suitable enhancers include, *e.g.*, the alpha fetoprotein enhancer, the TTR minimal promoter/enhancer, LSP (TH-binding globulin promoter/alpha1-microglobulin/bikunin enhancer), amongst others. These control sequences or the regulatory sequences are operably linked to the nucleic acid coding sequence or transgene coding sequence.

10 In certain embodiments, the gene editing vector includes a TBG promoter, one or more alpha mic/bik enhancer(s), coding sequence for the ARCUS meganuclease, optionally a WPRE, and a polyA. In certain embodiments, the expression cassette includes nt 211 to nt 2964 of SEQ ID NO: 42.

In some embodiments, the gene editing component further includes sequences which
 15 direct the nuclease to a target site in the PCSK9 target locus. In certain embodiments, such as a meganuclease specific for PCSK9, no further sequences are required to direct the nuclease to the target site. However, in the case, for example, of Cas9, an additional sequence, called a “single guide RNA” or “sgRNA” is provided, which is specific for the target sequence. The sgRNA may be provided on the same vector (*cis*) or a different vector from (*trans*) as the
 20 Cas9. As used herein, the sgRNA has at least a 20-base sequence (or about 24 - 28 bases, sometimes called the seed region) for specific DNA binding (i.e., homologous to the target DNA), in combination with the gRNA scaffold. Transcription of sgRNAs should start precisely at its 5' end. When targeting the template DNA strand, the base-pairing region of the sgRNA has the same sequence identity as the transcribed sequence. When targeting the
 25 non-template DNA strand, the base-pairing region of the sgRNA is the reverse-complement of the transcribed sequence. Optionally, the gene editing vector may contain more than one sgRNA. The sgRNA is 5' to a protospacer-adjacent motif (PAM) which is specifically recognized by the Cas9 (or Cpf1) enzyme. Typically, the sgRNA is “immediately” 5' to the PAM sequence, *i.e.*, there are no spacer or intervening sequences. In one embodiment, the
 30 sgRNA “seed” coding sequence is AAGTTGGTCCCCAAAGTCCC (SEQ ID NO: 8), which

is useful for targeting exon 7 of human and macaque PCSK9 by SaCas9. However, other sgRNAs can be designed by the person of skill in the art.

In certain embodiments, the sgRNA includes at least 20 nucleotides and specifically binds to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9. The seed region in some
5 embodiments shares 100% complementarity with the target site in the PCSK9 gene. In other embodiments, the seed region contains 1, 2, 3, 4, or 5 mismatches as compared to the target site.

The sgRNA is under control of an RNA polymerase promoter and/or terminator. In
10 certain embodiments, the RNA polymerase promoter is a Pol III promoter such as the U6 promoter. In another embodiment, the promoter is the H1 promoter. The sequence for an exemplary U6 promoter can be found in SEQ ID NO: 10. In other embodiments, the sgRNA and RNA polymerase promoter are located in the donor vector.

In other embodiments, for example, wherein the nuclease is a Cas9, the gene
15 editing component further includes one or more nuclear localization signal (NLSs). In one embodiment, the NLSs flank the coding sequence for the Cas9. In certain embodiments, the NLS has the sequence of nt 4241 to 4288 of SEQ ID NO: 5. See, e.g., Lu et al. Types of nuclear localization signals and mechanisms of protein import into the nucleus, Cell Commun Signal (May 2021) 19:60, which is incorporated herein by reference.

20 In certain embodiments, the nuclease coding sequence is provided as messenger RNA (mRNA). An mRNA may include a 5' untranslated region, a 3' untranslated region, and/or a coding or translating sequence. In certain embodiments, the coding sequence for a Cas9 is provided as mRNA.

An mRNA may be a naturally or non-naturally occurring mRNA. An mRNA may
25 include one or more modified nucleobases, nucleosides, or nucleotides. In some embodiments, the mRNA in the compositions of the invention comprise at least one modification which confers increased or enhanced stability to the nucleic acid, including, for example, improved resistance to nuclease digestion in vivo. An mRNA may include any number of base pairs, including tens, hundreds, or thousands of base pairs. Any number (e.g.,
30 all, some, or none) of nucleobases, nucleosides, or nucleotides may be an analog of a canonical species, substituted, modified, or otherwise non-naturally occurring. In certain

embodiments, all of a particular nucleobase type may be modified. For example, all cytosine in an mRNA may be 5-methylcytosine. As used herein, the terms “modification” and “modified” as such terms relate to the nucleic acids provided herein, include at least one alteration which preferably enhances stability and renders the mRNA more stable (e.g., resistant to nuclease digestion) than the wild-type or naturally occurring version of the mRNA. As used herein, the terms “stable” and “stability” as such terms relate to the nucleic acids of the present invention, and particularly with respect to the mRNA, refer to increased or enhanced resistance to degradation by, for example nucleases (i.e., endonucleases or exonucleases) which are normally capable of degrading such mRNA. Increased stability can include, for example, less sensitivity to hydrolysis or other destruction by endogenous enzymes (e.g., endonucleases or exonucleases) or conditions within the target cell or tissue, thereby increasing or enhancing the residence of such mRNA in the target cell, tissue, subject and/or cytoplasm. The stabilized mRNA molecules provided herein demonstrate longer half-lives relative to their naturally occurring, unmodified counterparts (e.g. the wild-type version of the mRNA). Also contemplated by the terms “modification” and “modified” as such terms related to the mRNA of the present invention are alterations which improve or enhance translation of mRNA nucleic acids, including for example, the inclusion of sequences which function in the initiation of protein translation (e.g., the Kozak consensus sequence).

In some embodiments, the mRNA described herein have undergone a chemical or biological modification to render them more stable. Exemplary modifications to an mRNA include the depletion of a base (e.g., by deletion or by the substitution of one nucleotide for another) or modification of a base, for example, the chemical modification of a base. The phrase “chemical modifications” as used herein, includes modifications which introduce chemistries which differ from those seen in naturally occurring mRNA, for example, covalent modifications such as the introduction of modified nucleotides, (e.g., nucleotide analogs, or the inclusion of pendant groups which are not naturally found in such mRNA molecules).

In some embodiments, the number of C and/or U residues in an mRNA sequence is reduced. In another embodiment, the number of C and/or U residues is reduced by substitution of one codon encoding a particular amino acid for another codon encoding the

same or a related amino acid. Contemplated modifications to the mRNA nucleic acids of the present invention also include the incorporation of pseudouridines pseudouridine (ψ) or 5-methylcytosine (m5C). Substitutions and modifications to the mRNA of the present invention may be performed by methods readily known to one of ordinary skill in the art.

5 In certain embodiments, the mRNA includes a 5' cap structure, a chain terminating nucleotide, a stem loop, and/or a polyadenylation signal. A cap structure or cap species is a compound including two nucleoside moieties joined by a linker and may be selected from a naturally occurring cap, a non-naturally occurring cap or cap analog, or an anti-reverse cap analog. An mRNA may instead or additionally include a chain terminating nucleoside.

10 In certain embodiments, the mRNA includes a stem loop, such as a histone stem loop. A stem loop may include 1, 2, 3, 4, 5, 6, 7, 8, or more nucleotide base pairs. A stem loop may be located in any region of an mRNA. For example, a stem loop may be located in, before, or after an untranslated region (a 5' untranslated region or a 3' untranslated region), a coding region, or a polyA sequence or tail.

15 In certain embodiments, the mRNA includes a polyA sequence. A polyA sequence may be comprised entirely or mostly of adenine nucleotides or analogs or derivatives thereof. In certain embodiments, the polyA sequence is a tail located adjacent to a 3' untranslated region of an mRNA.

20 An mRNA may encode any polypeptide of interest, e.g., a nuclease, including any naturally or non-naturally occurring or otherwise modified polypeptide. A polypeptide encoded by an mRNA may be of any size and may have any secondary structure or activity. In some embodiments, a polypeptide encoded by an mRNA may have a therapeutic effect when expressed in a cell.

25 DONOR VECTOR

 The compositions, kits, and methods include a donor vector, which provides the coding sequence for the therapeutic transgene. In certain embodiments, the donor vector contains an expression cassette comprising a nucleic acid sequence encoding a transgene, and regulatory sequences that direct expression of the transgene in the target cell. In certain
30 embodiments, the transgene encodes a protein that is aberrantly expressed in a liver metabolic disorder or other genetic disorder. The transgene encodes a protein other than

PCSK9. Such proteins include, but are not limited to OTC, low density lipoprotein receptor (LDLR), Factor IX such as a sequence shown in SEQ ID NO: 55 or 56, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith, and. Factor VIII such as a sequence shown in SEQ ID NO: 53 or 54, or
 5 sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith.

Further illustrative genes which may be delivered via the donor vector include, without limitation, glucose-6-phosphatase, associated with glycogen storage disease or deficiency type 1A (GSD1), phosphoenolpyruvate-carboxykinase (PEPCK), associated with
 10 PEPCK deficiency; cyclin-dependent kinase-like 5 (CDKL5), also known as serine/threonine kinase 9 (STK9) associated with seizures and severe neurodevelopmental impairment; galactose-1 phosphate uridyl transferase, associated with galactosemia such as a sequence shown in SEQ ID NO: 63 or 64, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith; phenylalanine
 15 hydroxylase (PAH), associated with phenylketonuria (PKU); gene products associated with Primary Hyperoxaluria Type 1 including Hydroxyacid Oxidase 1 (GO/HAO1) such as a sequence shown in SEQ ID NO: 49 or 50, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith and AGXT such as a sequence shown in SEQ ID NO: 47 or 48, or sequence sharing at least 70%, 75%,
 20 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith, branched chain alpha-ketoacid dehydrogenase, including BCKDH, BCKDH-E2, BAKDH-E1a, and BAKDH-E1b, associated with Maple syrup urine disease; fumarylacetoacetate hydrolase, associated with tyrosinemia type 1; methylmalonyl-CoA mutase, associated with methylmalonic acidemia; medium chain acyl CoA dehydrogenase, associated with medium
 25 chain acetyl CoA deficiency; ornithine transcarbamylase (OTC), associated with ornithine transcarbamylase deficiency; argininosuccinic acid synthetase (ASS1) such as a sequence shown in SEQ ID NO: 69 or 70, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith, associated with citrullinemia; lecithin-cholesterol acyltransferase (LCAT) deficiency; amethylmalonic
 30 acidemia (MMA); NPC1 associated with Niemann-Pick disease, type C1); propionic academia (PA); low density lipoprotein receptor (LDLR) protein, associated with familial

- hypercholesterolemia (FH) such as a sequence shown in SEQ ID NO: 73 or 74, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith, LDLR variant, such as those described in WO 2015/164778, or having a sequence shown in SEQ ID NO: or , or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith; ApoE and ApoC proteins, associated with dementia; lipoprotein lipase (LPL) (Lipoprotein Lipase Deficiency) such as a sequence shown in SEQ ID NO: 67 or 68, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith, UDP-glucouronosyltransferase, associated with Crigler-Najjar disease; adenosine deaminase, associated with severe combined immunodeficiency disease; hypoxanthine guanine phosphoribosyl transferase, associated with Gout and Lesch-Nyan syndrome; biotinidase, associated with biotinidase deficiency; alpha-galactosidase A (a-Gal A) associated with Fabry disease such as a sequence shown in SEQ ID NO: 75 or 76, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith; beta-galactosidase (GLB1) associated with GM1 gangliosidosis; ATP7B associated with Wilson's Disease; beta-glucocerebrosidase, associated with Gaucher disease type 2 and 3 such as a sequence shown in SEQ ID NO: 51 or 52, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith; peroxisome membrane protein 70 kDa, associated with Zellweger syndrome; arylsulfatase A (ARSA) associated with metachromatic leukodystrophy, galactocerebrosidase (*GALC*) enzyme associated with Krabbe disease, alpha-glucosidase (GAA) associated with Pompe disease such as a sequence shown in SEQ ID NO: 79 or 80, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith; sphingomyelinase (SMPD1) gene associated with Nieman Pick disease type A; carnosinase (CNI); hypoxanthine-guanine phosphoribosyltransferase (HGPRT); erythropoietin (EPO); Carbamyl Phosphate Synthetase (CPS1), N-Acetylglutamate Synthetase (NAGS); Argininosuccinate Lyase (ASL) (Argininosuccinic Aciduria) such as a sequence shown in SEQ ID NO: 57 or 58, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith; and Arginase (AG); argininosuccinate synthase associated with adult onset type II citrullinemia (CTLN2) (WO 2018/144709, which is incorporated herein

by reference); carbamoyl-phosphate synthase 1 (CPS1) associated with urea cycle disorders; survival motor neuron (SMN) protein, associated with spinal muscular atrophy; ceramidase associated with Farber lipogranulomatosis; b-hexosaminidase associated with GM2 gangliosidosis and Tay-Sachs and Sandhoff diseases; aspartylglucosaminidase associated with aspartyl-glucosaminuria; α -fucosidase associated with fucosidosis; α -mannosidase associated with alpha-mannosidosis; porphobilinogen deaminase, associated with acute intermittent porphyria (AIP); alpha-1 antitrypsin for treatment of alpha-1 antitrypsin deficiency (emphysema) such as a sequence shown in SEQ ID NO: 77 or 78, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith; erythropoietin for treatment of anemia due to thalassemia or to renal failure; vascular endothelial growth factor, angiopoietin-1, and fibroblast growth factor for the treatment of ischemic diseases; thrombomodulin and tissue factor pathway inhibitor for the treatment of occluded blood vessels as seen in, for example, atherosclerosis, thrombosis, or embolisms; aromatic amino acid decarboxylase (AADC), and tyrosine hydroxylase (TH) for the treatment of Parkinson's disease; the beta adrenergic receptor, anti-sense to, or a mutant form of, phospholamban, the sarco(endo)plasmic reticulum adenosine triphosphatase-2 (SERCA2), and the cardiac adenylyl cyclase for the treatment of congestive heart failure; a tumor suppressor gene such as p53 for the treatment of various cancers; a cytokine such as one of the various interleukins for the treatment of inflammatory and immune disorders and cancers; dystrophin or minidystrophin and utrophin or miniutrophin for the treatment of muscular dystrophies; and, insulin or GLP-1 for the treatment of diabetes.

Examples of suitable transgenes for delivery include, e.g., those associated with familial hypercholesterolemia (e.g., VLDLr, LDLr, ApoE, see, e.g., WO 2020/132155, WO 2018/152485, WO 2017/100682, which are incorporated herein by reference), muscular dystrophy, cystic fibrosis, and rare or orphan diseases. Examples of such rare disease may include spinal muscular atrophy (SMA), Huntingdon's Disease, Rett Syndrome (e.g., methyl-CpG-binding protein 2 (MeCP2); UniProtKB – P51608), Amyotrophic Lateral Sclerosis (ALS), Duchenne Type Muscular dystrophy, Friedrichs Ataxia (e.g., frataxin), progranulin (PRGN) (associated with non-Alzheimer's cerebral degenerations, including, frontotemporal dementia (FTD), progressive non-fluent aphasia (PNFA) and semantic

dementia), among others. Other useful gene products include, carbamoyl synthetase I, ornithine transcarbamylase (OTC), arginosuccinate synthetase, arginosuccinate lyase (ASL) for treatment of arginosuccinate lyase deficiency, arginase, fumarylacetate hydrolase, phenylalanine hydroxylase, alpha-1 antitrypsin, rhesus alpha- fetoprotein (AFP), rhesus

5 chorionic gonadotrophin (CG), glucose-6-phosphatase such as a sequence shown in SEQ ID NO: 59 or 60, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith, plasma protease C1 inhibitor (SERPING1) associated with hereditary angioedema such as a sequence shown in SEQ ID

10 NO: 61 or 62, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith, porphobilinogen deaminase, cystathione beta-synthase associated with homocystinuria such as a sequence shown in SEQ ID NO: 65 or 66, or sequence sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith, branched chain ketoacid decarboxylase, albumin, isovaleryl-coA dehydrogenase, propionyl CoA carboxylase, methyl malonyl CoA

15 mutase, glutaryl CoA dehydrogenase, insulin, beta-glucosidase, pyruvate carboxylate, hepatic phosphorylase, phosphorylase kinase, glycine decarboxylase, H-protein, T-protein, a cystic fibrosis transmembrane regulator (CFTR) sequence, and a dystrophin gene product [e.g., a mini- or micro-dystrophin]. Still other useful gene products include enzymes such as may be useful in enzyme replacement therapy, which is useful in a variety of conditions

20 resulting from deficient activity of enzyme. For example, enzymes that contain mannose-6-phosphate may be utilized in therapies for lysosomal storage diseases (e.g., a suitable gene includes that encoding β -glucuronidase (GUSB)). Examples of suitable transgene for delivery may include human frataxin delivered in an AAV vector as described, e.g., PCT/US20/66167, December 18, 2020, US Provisional Patent Application No. 62/950,834,

25 filed December 19, 2019, and US Provisional Application No. 63/136,059 filed on January 11, 2021 which are incorporated herein by reference. Another example of suitable transgene for delivery may include human acid- α -glucosidase (GAA) delivered in an AAV vector as described, e.g., PCT/US20/30493, April 30, 2020, now published as WO2020/223362A1, PCT/US20/30484, April 20, 2020, now published as WO 2020/223356 A1, US Provisional

30 Patent Application No. 62/840,911, filed April 30, 2019, US Provisional Application No. 62.913,401, filed October 10, 2019, US Provisional Patent Application No. 63/024,941, filed

May 14, 2020, and US Provisional Patent Application No. 63/109,677, filed November 4, 2020 which are incorporated herein by reference. Also, another example of suitable transgene for delivery may include human α -L-iduronidase (IDUA) delivered in an AAV vector as described, e.g., PCT/US2014/025509, March 13, 2014, now published as WO 2014/151341, and US Provisional Patent Application No. 61/788,724, filed March 15, 2013 which are incorporated herein by reference.

Other useful therapeutic products include those expressed in muscle, including heart muscle. Other useful therapeutic products encoded by the transgene include hormones and growth and differentiation factors including, without limitation, insulin, glucagon, glucagon-like peptide 1 (GLP-1), growth hormone (GH), parathyroid hormone (PTH), growth hormone releasing factor (GRF), follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), vascular endothelial growth factor (VEGF), angiopoietins, angiostatin, granulocyte colony stimulating factor (GCSF), erythropoietin (EPO), connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF), transforming growth factor α (TGF α), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), any one of the transforming growth factor β superfamily, including TGF β , activins, inhibins, or any of the bone morphogenic proteins (BMP) BMPs 1-15, any one of the heregulin/neuregulin/ARIA/neu differentiation factor (NDF) family of growth factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins NT-3 and NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), neurturin, agrin, any one of the family of semaphorins/collapsins, netrin-1 and netrin-2, hepatocyte growth factor (HGF), ephrins, noggin, sonic hedgehog and tyrosine hydroxylase. Other transgenes useful herein include those for treating mucopolysaccharidosis type I-VII (IDUA, IDS, GNA, HGSNAT, NAGLU, SGSH, GALNS, GLB1, ARSB, GUSB). Exemplary sequences for useful for treating MPSI can be found in WO 2019/010335, which is incorporated herein by reference. Exemplary sequences for useful for treating MPSII can be found in WO 2019/060662, which is incorporated herein by reference. Exemplary sequences for useful for treating MPSIIIa can be found in WO 2019/108857, which is incorporated herein by reference. Exemplary sequences for useful for

treating MPSIIIb can be found in WO 2019/108856 which is incorporated herein by reference.

In some embodiments, the transgene cassette includes a promoter, the transgene coding sequence, and a poly A sequence. In some embodiments, the promoter is a liver-specific promoter, such as the TBG promoter, TBG-S1 promoter, HLP promoter, or others described herein. In other embodiments, a transgene is provided without a promoter, and is inserted in the genome downstream of the native PCSK9 promoter.

The transgene cassette, expression cassette and/or vector (editing or donor) may contain one or more appropriate “regulatory elements” or “regulatory sequences”, which comprise but are not limited to an enhancer; transcription factor; transcription terminator; efficient RNA processing signals such as splicing and polyadenylation signals (polyA); sequences that stabilize cytoplasmic mRNA, for example Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE); sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. Examples of suitable polyA sequences include, *e.g.*, SV40, bovine growth hormone (bGH), and TK polyA. Examples of suitable enhancers include, *e.g.*, the alpha fetoprotein enhancer, the TTR minimal promoter/enhancer, LSP (TH-binding globulin promoter/alpha1-microglobulin/bikunin enhancer), amongst others. These control sequences or the regulatory sequences are operably linked to the nucleic acid coding sequences or transgene coding sequence.

In addition to the transgene cassette, in certain embodiments, the donor vector also includes homology-directed recombination (HDR) arms 5' and 3' to the transgene cassette, to facilitate homology directed recombination of the transgene into the endogenous genome. The homology arms are directed to the target PCSK9 locus and can be of varying length. In some embodiments, the HDR arms are each from about 100bp to about 1000bp in length. In other embodiments, the HDR arms are each from about 130bp to about 500bp. In other embodiments, the HDR arms are each from about 100bp to about 300bp. In other embodiments, the HDR arms are each from about 100bp to about 400bp. In other embodiments, the HDR arms are each from about 250bp to about 500bp. In other embodiments, the HDR arms are each from about 300bp to about 500bp. In certain

embodiments, the HDR arms are each about 100bp, 125bp, 150bp, 175bp, 200bp, 225bp, 250bp, 275bp, 300bp, 325bp, 350bp, 375bp, 400bp, 425bp, 450bp, 475bp, or 500bp. In one embodiment, the HDR arm is 130bp. In another embodiment, the HDR arm is 137bp. In other embodiments, the HDR arms are about 130bp to 140bp. In another embodiment, the

5 HDR arms are about 500bp. In another embodiment, the HDR arms are absent. The HDR arms ideally share a high level of complementarity with the target PCSK9 locus, although it need not be 100% complementarity. In some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more mismatches are permitted in each HDR arm. Suitable HDR arm sequences, for targeting PCSK9 exon 7 are shown in SEQ ID Nos: 24-29. In one

10 embodiment, the HDR arm sequences are selected from SEQ ID Nos: 24-29.

Also provided herein, are compositions, kits, and methods for nuclease-mediated, site-specific integration of an OTC transgene cassette in a PCSK9 safe harbor in the genome that provides long-term therapeutic benefits to patients with OTC deficiency. An engineered coding sequence for OTC, referred to herein as hOTCco2, and shown in SEQ ID NO: 17 is

15 provided. Nucleic acids having the sequence of SEQ ID NO: 17 or sequences sharing at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.9% identity are provided. In one embodiment, the nucleic acid shares less than 80%, less than 79%, less than 78%, less than 77%, less than 76%, less than 75%, less than 74%, less than 73%, less than 72%, less than 71%, or less than 70% identity with the native OTC coding

20 sequence which is shown in SEQ ID NO: 30.

Other sequences useful for the treatment of OTC are described in WO 2015/138348 and WO 2015/138357 which is incorporated herein by reference. Illustrative sequences useful for the treatment of PKU are described in WO 2018/126112, which is incorporated herein by reference. Other sequences are shown in SEQ ID NO: 71 or 72, or sequence

25 sharing at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity therewith.

VIRAL AND NON-VIRAL VECTORS

The (gene editing and donor) expression cassettes or coding sequences described

30 herein, may be engineered into any suitable genetic element for delivery to a target cell, e.g., a liver cell, such as a vector. A “vector” as used herein is a biological or chemical moiety

comprising a nucleic acid sequence which can be introduced into an appropriate host cell for replication or expression of said nucleic acid sequence. Common vectors include non-viral vectors and viral vectors. As used herein, a non-viral system might be selected from nanoparticles, electroporation systems and novel biomaterials, naked DNA, phage, transposon, plasmids, cosmids (Phillip McClean, www.ndsu.edu/pubweb/~mcclean/plsc731/cloning/cloning4.htm) and artificial chromosomes (Gong, Shiao-ching, et al. "A gene expression atlas of the central nervous system based on bacterial artificial chromosomes." *Nature* 425.6961 (2003): 917-925). In one embodiment, a nucleic acid is delivered via non-viral vector or lipid nanoparticle, as described herein or known in the art.

10 In certain embodiments, the gene editing component is encapsulated in a lipid nanoparticle (LNP). See, for example, Conway et al, Non-viral Delivery of Zinc Finger Nuclease mRNA Enables Highly Efficient In Vivo Genome Editing of Multiple Therapeutic Gene Targets, *Molecular Therapy*, 27(4):866-877 (April 2019), which is incorporated herein by reference). As used herein, the phrase "lipid nanoparticle" refers to a transfer vehicle
15 comprising one or more lipids (e.g., cationic lipids, non-cationic lipids, and PEG-modified lipids). Preferably, the lipid nanoparticles are formulated to deliver one or more mRNA to one or more target cells (e.g., liver and/or muscle). Examples of suitable lipids include, for example, the phosphatidyl compounds (e.g., phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebroside, and
20 gangliosides). Also contemplated is the use of polymers as transfer vehicles, whether alone or in combination with other transfer vehicles. Suitable polymers may include, for example, polyacrylates, polyalkylcyanoacrylates, polylactide, polylactide-polyglycolide copolymers, polycaprolactones, dextran, albumin, gelatin, alginate, collagen, chitosan, cyclodextrins, dendrimers and polyethylenimine. In one embodiment, the transfer vehicle is selected based
25 upon its ability to facilitate the transfection of a mRNA to a target cell. Useful lipid nanoparticles for mRNA comprise a cationic lipid to encapsulate and/or enhance the delivery of mRNA into the target cell that will act as a depot for protein production. As used herein, the phrase "cationic lipid" refers to any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH. The contemplated lipid nanoparticles may
30 be prepared by including multi-component lipid mixtures of varying ratios employing one or more cationic lipids, non-cationic lipids and PEG-modified lipids. Several cationic lipids

have been described in the literature, many of which are commercially available. See, e.g., WO2014/089486, US 2018/0353616A1, and US 8,853,377B2, which are incorporated by reference. In certain embodiments, LNP formulation is performed using routine procedures comprising cholesterol, ionizable lipid, helper lipid, PEG-lipid and polymer forming a lipid bilayer around the encapsulated nucleic acids (Kowalski et al., 2019, Mol. Ther. 27(4):710-728). In some embodiments, LNP comprises a cationic lipid (i.e. N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)) with helper lipid DOPE. In some embodiments, LNP comprises an ionizable lipid Dlin-MC3-DMA ionizable lipids, or diketopiperazine-based ionizable lipids (cKK-E12). In some embodiments, polymer comprises a polyethyleneimine (PEI), or a poly(β -amino)esters (PBAEs). See, e.g., WO2014/089486, US 2018/0353616A1, US2013/0037977A1, WO2015/074085A1, US9670152B2, and US 8,853,377B2, which are incorporated by reference. In certain embodiments, wherein the gene editing component includes a Cas9 mRNA, the LNP also includes a gRNA.

Certain LNPs useful herein include those that are described in WO 2021/077066 and WO 2021/055892, each of which is incorporated herein by reference in its entirety. Useful LNPs include those that show enhanced delivery to the liver. LNP formulations may be varied to enhance liver delivery. For example, the type and ionizable lipid:mRNA ratio, the mRNA:sgRNA ratio, molar ratio of ionizable lipid, phospholipid, cholesterol, and PEG-lipid, etc. may be varied. In one embodiment, the LNP is one described by Kauffman, K. J.; Dorkin, J. R.; Yang, J. H.; Heartlein, M. W.; DeRosa, F.; Mir, F. F.; Fenton, O. S.; Anderson, D. G., Optimization of lipid nanoparticle formulations for mRNA delivery in vivo with fractional factorial and definitive screening designs. Nano letters 2015, 15 (11), 7300-7306, which is incorporated herein by reference. In certain embodiments, the LNPs are designed with ionizable lipid: mRNA weight ratios varying between 5:1 to 25:1. In certain embodiments, the ionizable lipid: mRNA weight ratio is 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 12.5:1, 15:1, 20:1, or 25:1. In certain embodiments, the mRNA:sgRNA weight ratio is 1:1, 1:2, 2:1, 1:4, 1:5, 5:1, 4:1, 3:1, or 2:1.

Other LNPs have been described and are useful herein. See, e.g., WO 2016/118724, US 10,413,618B2, US 10,723,692B2, and US8754062B2, each of which is incorporated herein by reference.

Certain examples herein illustrate use of AAV vectors containing the gene editing component (nuclease) coding sequences and transgene coding sequences in AAV vector genomes. However, the use of constructs described herein is not limited to AAV constructs and can be used for other vectors. In certain embodiments, the vector genome may be
5 packaged into a different vector (e.g., a recombinant bocavirus). In certain embodiments, the expression cassette may be packaged into a different viral vector, into a non-viral vector, and/or into a different delivery system. In certain embodiments, the gene editing component is provided in an LNP.

“Plasmid” or “plasmid vector” generally is designated herein by a lowercase p
10 preceded and/or followed by a vector name. Plasmids, other cloning and expression vectors, properties thereof, and constructing/manipulating methods thereof that can be used in accordance with the present invention are readily apparent to those of skill in the art. In one embodiment, the nucleic acid sequence as described herein or the expression cassette as described herein are engineered into a suitable genetic element (a vector) useful for
15 generating viral vectors and/or for delivery to a host cell, e.g., naked DNA, phage, transposon, cosmid, episome, etc., which transfers the nuclease sequences carried thereon. The selected vector may be delivered by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. The methods used to make such constructs are
20 known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY.

In certain embodiments, the expression cassette is located in a vector genome for packaging into a viral capsid. For example, for an AAV vector genome, the components of
25 the expression cassette are flanked at the extreme 5' end and the extreme 3' end by AAV inverted terminal repeat sequences. For example, a 5' AAV ITR, expression cassette, 3' AAV ITR. In other embodiments, a self-complementary AAV may be selected. In other embodiments, retroviral system, lentivirus vector system, or an adenoviral system may be used.

30

AAV VECTORS

In certain embodiments, the gene editing vector and/or the donor vector is provided as a recombinant AAV. A “recombinant AAV” or “rAAV” is a DNase-resistant viral particle containing two elements, an AAV capsid and a vector genome containing at least 5 non-AAV coding sequence packaged within the AAV capsid. Unless otherwise specified, this term may be used interchangeably with the phrase “rAAV vector” or “AAV vector”. The rAAV is a “replication-defective virus” or “viral vector”, as it lacks any functional AAV rep gene or functional AAV cap gene and cannot generate progeny. In certain embodiments, the only AAV sequences are the AAV inverted terminal repeat sequences (ITRs), typically 10 located at the extreme 5’ and 3’ ends of the vector genome in order to allow the gene and regulatory sequences located between the ITRs to be packaged within the AAV capsid.

The source of the AAV capsid may be one of any of the dozens of naturally occurring and available adeno-associated viruses, as well as engineered AAVs. The source of the AAV capsid for the gene editing vector and/or the donor vector is, in one embodiment, 15 the same. In another embodiment, the source of the AAV capsid for the gene editing vector and/or the donor vector is different. An adeno-associated virus (AAV) viral vector is an AAV DNase-resistant particle having an AAV protein capsid into which is packaged nucleic acid sequences for delivery to target cells. An AAV capsid is composed of 60 capsid (cap) protein subunits, VP1, VP2, and VP3, that are arranged in an icosahedral symmetry in a ratio 20 of approximately 1:1:10 to 1:1:20, depending upon the selected AAV. Various AAVs may be selected as sources for capsids of AAV viral vectors as identified above. See, e.g., US Published Patent Application No. 2007-0036760-A1; US Published Patent Application No. 2009-0197338-A1; EP 1310571. See also, WO 2003/042397 (AAV7 and other simian AAV), US Patent 7790449 and US Patent 7282199 (AAV8), WO 2005/033321 and US 25 7,906,111 (AAV9), and WO 2006/110689, WO 2003/042397 (rh.10) and WO 2018/160582 (AAVhu68). These documents also describe other AAV which may be selected for generating AAV and are incorporated by reference.

Unless otherwise specified, the AAV capsid, ITRs, and other selected AAV components described herein, may be readily selected from among any AAV, including, 30 without limitation, the AAVs commonly identified as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV8bp, AAV7M8, AAVAnc80, AAVrh10, AAVrh79, and

AAVPHP.B and variants of any of the known or mentioned AAVs or AAVs yet to be discovered or variants or mixtures thereof. See, e.g., WO 2005/033321, which is incorporated herein by reference. In one embodiment, the AAV capsid is an AAV1 capsid or variant thereof, AAV8 capsid or variant thereof, an AAV9 capsid or variant thereof, an AAVhu.68 capsid or variant thereof, an AAVrh.10 capsid or variant thereof, an AAVrh64R1 capsid or variant thereof, an AAVhu.37 capsid or variant thereof, or an AAV3B or variant thereof. In one aspect, the capsid is an AAVhu.37 capsid. See, also WO 2019/168961 and WO 2019/169004, which are incorporated by reference herein in their entirety. In other embodiments, the AAV capsid is an AAVrh79 capsid or variant thereof. In other
10 embodiments, the AAV capsid is an AAVrh.90 or variant thereof.

 In certain embodiments, the rAAV comprises an AAVhu37 capsid. An AAVhu37 capsid comprises: a heterogeneous population of vp1 proteins which are the product of a nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 38, a heterogeneous population of vp2 proteins which are the product of a nucleic acid sequence
15 encoding the amino acid sequence of at least about amino acids 138 to 738 of SEQ ID NO: 38, and a heterogeneous population of vp3 proteins which are the product of a nucleic acid sequence encoding at least amino acids 204 to 738 of SEQ ID NO: 38 wherein: the vp1, vp2 and vp3 proteins contain subpopulations with amino acid modifications comprising at least two highly deamidated asparagines (N) in asparagine - glycine pairs in SEQ ID NO: 38 and
20 optionally further comprising subpopulations comprising other deamidated amino acids, wherein the deamidation results in an amino acid change. AAVhu37 is characterized by having highly deamidated residues, e.g., at positions N57, N263, N385, and/or N514 based on the numbering of the AAVhu37 VP1 (SEQ ID NO: 38).

 Deamidation has been observed in other residues, as shown in the table below, and
25 in, e.g., WO 2019/168961, published September 6, 2019, which is incorporated herein by reference. In certain embodiments, an AAVhu37 capsid is modified in one or more of the following positions, in the ranges provided below, as determined using mass spectrometry with a trypsin enzyme. In certain embodiments, one or more of the following positions, or the glycine following the N is modified as described herein. For example, in certain
30 embodiments, a G may be modified to an S or an A, e.g., at position 58, 264, 386, or 515. In one embodiment, the AAVhu37 capsid is modified at position N57/G58 to N57Q or G58A to

afford a capsid with reduced deamidation at this position. In another embodiment, N57/G58 is altered to NS57/58 or NA57/58. However, in certain embodiments, an increase in deamidation is observed when NG is altered to NS or NA. In certain embodiments, an N of an NG pair is modified to a Q while retaining the G. In certain embodiments, both amino acids of an NG pair are modified. In certain embodiments, N385Q results in significant reduction of deamidation in that location. In certain embodiments, N499Q results in significant increase of deamidation in that location.

In certain embodiments, AAVhu37 may have these or other residues deamidated, e.g., typically at less than 10% and/or may have other modifications, including methylations (e.g., ~R487) (typically less than 5%, more typically less than 1% at a given residue), isomerization (e.g., at D97) (typically less than 5%, more typically less than 1% at a given residue), phosphorylation (e.g., where present, in the range of about 10 to about 60%, or about 10 to about 30%, or about 20 to about 60%) (e.g., at one or more of S149, ~S153, ~S474, ~T570, ~S665), or oxidation (e.g. at one or more of W248, W307, W307, M405, M437, M473, W480, W480, W505, M526, M544, M561, W621, M637, and/or W697). Optionally the W may oxidize to kynurenine.

TABLE A	
AAVhu37 Deamidation based on VP1 numbering	% Deamidation
N57+Deamidation	65-90, 70-95, 80-95, 75 - 100, 80-100, or 90-100
N94+Deamidation	5 - 15, about 10
~N254+Deamidation	10 - 20
~N263+Deamidation	75 - 100
~N305+Deamidation	1 - 5

TABLE A	
AAVhu37 Deamidation based on VP1 numbering	% Deamidation
~N385+Deamidation	65-90, 70-95, 80-95, 75 - 100, 80-100, or 90-100
~N410+Deamidation	1 - 25,
N479+Deamidation	1 - 5, 1-3
~N514+Deamidation	65-90, 70-95, 80-95, 75 - 100, 80-100, or 90-100
~Q601+Deamidation	0-1
N653+Deamidation	0 -2

Still other positions may have such these or other modifications (e.g., acetylation or further deamidations). In certain embodiments, the nucleic acid sequence encoding the AAVhu37 vp1 capsid protein is provided in SEQ ID NO: 37. In other embodiments, a

5 nucleic acid sequence of 70% to 99.9% identity to SEQ ID NO: 37 may be selected to express the AAVhu37 capsid proteins. In certain other embodiments, the nucleic acid sequence is at least about 75% identical, at least 80% identical, at least 85%, at least 90%, at least 95%, at least 97% identical, or at least 99% identical to SEQ ID NO: 37. However,

10 other nucleic acid sequences which encode the amino acid sequence of SEQ ID NO: 38 may be selected for use in producing rAAVhu37 capsids. In certain embodiments, the nucleic acid sequence has the nucleic acid sequence of SEQ ID NO: 37 or a sequence at least 70% to at least 99% identical, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, identical to SEQ ID NO: 37 which encodes SEQ ID NO: 38. In certain embodiments, the nucleic acid sequence has the nucleic acid sequence of SEQ ID

- NO: 37 or a sequence at least 70% to 99%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, identical to about nt 412 to about nt 2214 of SEQ ID NO: 37 which encodes the vp2 capsid protein (about aa 138 to 738) of SEQ ID NO: 38. In certain embodiments, the nucleic acid sequence has the nucleic acid sequence of about
- 5 nt 610 to about nt 2214 of SEQ ID NO: 37 or a sequence at least 70% to 99%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, identical to nt SEQ ID NO: 37 which encodes the vp3 capsid protein (about aa 204 to 738) of SEQ ID NO: 38. See, EP 2 345 731 B1 and SEQ ID NO: 88 therein, which are incorporated by reference.
- 10 In certain embodiments, the rAAV comprises an AAV8 capsid. An AAV8 capsid comprises: a heterogeneous population of VP isoforms which are deamidated as defined in the following table, based on the total amount of VP proteins in the capsid, as determined using mass spectrometry. Suitable modifications include those described in the paragraph above labelled modulation of deamidation, which is incorporated herein. In certain
- 15 embodiments, the AAV capsid is modified at one or more of the following position, in the ranges provided below, as determined using mass spectrometry. In certain embodiments, one or more of the following positions, or the glycine following the N is modified as described herein. In certain embodiments, an artificial NG is introduced into a different position than one of the positions identified below. In certain embodiments, one or more of the following
- 20 positions, or the glycine following the N is modified as described herein. For example, in certain embodiments, a G may be modified to an S or an A, e.g., at position 58, 67, 95, 216, 264, 386, 411, 460, 500, 515, or 541. Significant reduction in deamidation is observed when NG57/58 is altered to NS 57/58 or NA57/58. However, in certain embodiments, an increase in deamidation is observed when NG is altered to NS or NA. In certain embodiments, an N
- 25 of an NG pair is modified to a Q while retaining the G. In certain embodiments, both amino acids of an NG pair are modified. In certain embodiments, N385Q results in significant reduction of deamidation in that location. In certain embodiments, N499Q results in significant increase of deamidation in that location. In certain embodiments, an NG mutation is made at the pair located at N263 (e.g., to N263A). In certain embodiments, an NG
- 30 mutation is made at the pair located at N514 (e.g., to N514A). In certain embodiments, an NG mutation is made at the pair located at N540 (e.g., N540A). In certain embodiments,

- AAV mutants containing multiple mutations and at least one of the mutations at these positions are engineered. In certain embodiments, no mutation is made at position N57. In certain embodiments, no mutation is made at position N94. In certain embodiments, no mutation is made at position N305. In certain embodiments, no mutation is made at position
- 5 G386. In certain embodiments, no mutation is made at position Q467. In certain embodiments, no mutation is made at position N479. In certain embodiments, no mutation is made at position N653. In certain embodiments, the capsid is modified to reduce “N” or “Q” at positions other than then “NG” pairs. Residue numbers are based on the published AAV8 sequence, reproduced in SEQ ID NO: 36.

10

TABLE B	
AAV8 Modification Based on VP1 numbering	%
N35+Deamidation	1
N57+Deamidation	65-90, 70-95, 80-95, 75 - 100, 80-100, or 90-100
N66+Deamidation	0-10
N94+Deamidation	1-15
N113+Deamidation	0-10
~Q166+Deamidation	0-10
~N173+Deamidation	0-10
N254/N255+Deamidation	5-45
N263+Deamidation	65-90, 70-95, 80-95, 75 - 100, 80-100, or 90-100
~N304+Deamidation	0-10
~N305+Deamidation	10-40
N320+Deamidation	0-10
~Q322+Deamidation	0-10
N385+Deamidation	65-90, 70-95, 80-95, 75 - 100, 80-100, or 90-100

TABLE B	
AAV8 Modification Based on VP1 numbering	%
N410+Deamidation	15-70
~Q431+Deamidation	0-10
N438+Deamidation	0-10
~N459+Deamidation	0-10
~Q467+Deamidation	0-10
~N479+Deamidation	0-10
N498/N499+Deamidation	0-10
N502+Deamidation	0-10
N514+Deamidation	65-90, 70-95, 80-95, 75 - 100, 80-100, or 90-100
N517+Deamidation	15 - 40
N540+Deamidation	65-90, 70-95, 80-95, 75 - 100, 80-100, or 90-100
~N554+Deamidation	0-10
~Q589+Deamidation	0-10
~N590+Deamidation	0-10
~N599+Deamidation	35 - 75
~Q601+Deamidation	45-75
~Q610+Deamidation	0-10
Q617+Deamidation	0-10
N630+Deamidation	5-30
Q648+Deamidation	0-10
N653+Deamidation	0-10
N665+Deamidation	5 - 30
N670+Deamidation	0-10
N693+Deamidation	0-10
~N706+Deamidation	0-10

TABLE B	
AAV8 Modification Based on VP1 numbering	%
N718+Deamidation	0-10
N737+Deamidation	0-10

- In certain embodiments, the rAAV comprises a AAVrh79 capsid, as described in WO 2019/169004, published September 6, 2019, which is incorporated herein by reference. In one embodiment, an AAVrh79 capsid comprises a heterogeneous population of AAVrh79
- 5 vp1 proteins, AAVrh79 vp2 proteins, and AAVrh79 vp3 proteins. In one embodiment, the AAVrh79 capsid is produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of 1 to 738 of SEQ ID NO: 34. Optionally, sequences co-expressing the vp3 protein from a nucleic acid sequence excluding the vp1-unique region (about aa 1 to 137) or the vp2-unique region (about aa 1 to 203), vp1 proteins produced from
- 10 SEQ ID NO: 33, or vp1 proteins produced from a nucleic acid sequence at least 70% identical to SEQ ID NO: 33 which encodes the predicted amino acid sequence of 1 to 738 of SEQ ID NO: 34. In other embodiments, the AAVrh79 vp2 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of at least about amino acids 138 to 738 of SEQ ID NO: 34, vp2 proteins produced from a sequence
- 15 comprising at least nucleotides 412 to 2214 of SEQ ID NO: 33, or vp2 proteins produced from a nucleic acid sequence at least 70% identical to at least nucleotides 412 to 2214 of SEQ ID NO: 33 which encodes the predicted amino acid sequence of at least about amino acids 138 to 738 of SEQ ID NO: 34, AAVrh79 vp3 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of at least about
- 20 amino acids 204 to 738 of SEQ ID NO: 34, vp3 proteins produced from a sequence comprising at least nucleotides 610 to 2214 of SEQ ID NO: 33, or vp3 proteins produced from a nucleic acid sequence at least 70% identical to at least nucleotides 610 to 2214 of SEQ ID NO: 33 which encodes the predicted amino acid sequence of at least about amino acids 204 to 738 of SEQ ID NO: 34.
- 25 In certain embodiments, an AAVrh79 capsid comprises: a heterogeneous population of vp1 proteins which are the product of a nucleic acid sequence encoding the amino acid

sequence of SEQ ID NO: 34, a heterogeneous population of vp2 proteins which are the product of a nucleic acid sequence encoding the amino acid sequence of at least about amino acids 138 to 738 of SEQ ID NO: 34, and a heterogeneous population of vp3 proteins which are the product of a nucleic acid sequence encoding at least amino acids 204 to 738 of SEQ ID NO: 34.

The AAVrh79 vp1, vp2 and vp3 proteins contain subpopulations with amino acid modifications comprising at least two highly deamidated asparagines (N) in asparagine - glycine pairs in SEQ ID NO: 34 and optionally further comprising subpopulations comprising other deamidated amino acids, wherein the deamidation results in an amino acid change. High levels of deamidation at N-G pairs N57, N263, N385 and/or N514 are observed, relative to the number of SEQ ID NO: 34. Deamidation has been observed in other residues, as shown in the table below and in the examples. In certain embodiments, AAVrh79 may have other residues deamidated, e.g., typically at less than 10% and/or may have other modifications, including methylations (e.g., ~R487) (typically less than 5%, more typically less than 1% at a given residue), isomerization (e.g., at D97) (typically less than 5%, more typically less than 1% at a given residue), phosphorylation (e.g., where present, in the range of about 10 to about 60%, or about 10 to about 30%, or about 20 to about 60%) (e.g., at one or more of S149, ~S153, ~S474, ~T570, ~S665), or oxidation (e.g., at one or more of W248, W307, W307, M405, M437, M473, W480, W480, W505, M526, M544, M561, W621, M637, and/or W697). Optionally the W may oxidize to kynurenine.

TABLE C	
AAVrh79 Deamidation based on VP1 numbering	% Deamidation
N57+Deamidation	65-90, 70-95, 80-95, 75 - 100, 80-100, or 90-100
N94+Deamidation	5 - 15, about 10

TABLE C	
AAVrh79 Deamidation based on VP1 numbering	% Deamidation
~N254+Deamidation	10 - 20
~N263+Deamidation	75 - 100
~N305+Deamidation	1 - 5
~N385+Deamidation	65-90, 70-95, 80- 95, 75 - 100, 80- 100, or 90-100
~N410+Deamidation	1 - 25,
N479+Deamidation	1 - 5, 1-3
~N514+Deamidation	65-90, 70-95, 80- 95, 75 - 100, 80- 100, or 90-100
~Q601+Deamidation	0-1
N653+Deamidation	0 - 2

In certain embodiments, an AAVrh79 capsid is modified in one or more of the positions identified in the preceding table, in the ranges provided below, as determined using mass spectrometry with a trypsin enzyme. In certain embodiments, one or more of the following positions, or the glycine following the N is modified as described herein. Residue numbers are based on the AAVrh79 sequence provided herein. See, SEQ ID NO: 34.

In certain embodiments, the nucleic acid sequence encoding the AAVrh79 vp1 capsid protein is provided in SEQ ID NO: 33. In other embodiments, a nucleic acid sequence of 70% to 99.9% identity to SEQ ID NO: 33 may be selected to express the AAVrh79 capsid proteins. In certain other embodiments, the nucleic acid sequence is at least about 75%

identical, at least 80% identical, at least 85%, at least 90%, at least 95%, at least 97% identical, at least 99% or at least 99.9% identical to SEQ ID NO: 33. However, other nucleic acid sequences which encode the amino acid sequence of SEQ ID NO: 34 may be selected for use in producing rAAV capsids. In certain embodiments, the nucleic acid sequence has the nucleic acid sequence of SEQ ID NO: 33 or a sequence at least 70% to 99% identical, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, identical to SEQ ID NO: 33 which encodes SEQ ID NO: 34. In certain embodiments, the nucleic acid sequence has the nucleic acid sequence of SEQ ID NO: 33 or a sequence at least 70% to 99%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, identical to about nt 412 to about nt 2214 of SEQ ID NO: 33 which encodes the vp2 capsid protein (about aa 138 to 738) of SEQ ID NO: 34. In certain embodiments, the nucleic acid sequence has the nucleic acid sequence of about nt 610 to about nt 2214 of SEQ ID NO: 33 or a sequence at least 70% to 99%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, identical to nt SEQ ID NO: 33 which encodes the vp3 capsid protein (about aa 204 to 738) of SEQ ID NO: 34.

The invention also encompasses nucleic acid sequences encoding mutant AAVrh79, in which one or more residues has been altered in order to decrease deamidation, or other modifications which are identified herein. Such nucleic acid sequences can be used in production of mutant rAAVrh79 capsids.

In certain embodiments, the rAAV comprises a AAVrh.90 capsid, as described in WO 2020/223232, published November 5, 2020, which is incorporated herein by reference. In a further aspect, a recombinant adeno-associated virus (rAAV) is provided which comprises: (A) an AAVrh.90 capsid comprising one or more of: (1) AAVrh.90 capsid proteins comprising: a heterogeneous population of AAVrh.90 vp1 proteins selected from: vp1 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of 1 to 738 of SEQ ID NO: 40, vp1 proteins produced from SEQ ID NO: 39, or vp1 proteins produced from a nucleic acid sequence at least 70% identical to SEQ ID NO: 39 which encodes the predicted amino acid sequence of 1 to 738 of SEQ ID NO: 40, a heterogeneous population of AAVrh.90 vp2 proteins selected from: vp2 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of at least about amino acids 138 to 738 of SEQ ID NO: 40, vp2

- proteins produced from a sequence comprising at least nucleotides 412 to 2214 of SEQ ID NO: 39, or vp2 proteins produced from a nucleic acid sequence at least 70% identical to at least nucleotides 412 to 2214 of SEQ ID NO: 39 which encodes the predicted amino acid sequence of at least about amino acids 138 to 738 of SEQ ID NO: 40, a heterogeneous
- 5 population of AAVrh.90 vp3 proteins selected from: vp3 proteins produced by expression from a nucleic acid sequence which encodes the predicted amino acid sequence of at least about amino acids 204 to 738 of SEQ ID NO: 40, vp3 proteins produced from a sequence comprising at least nucleotides 610 to 2214 of SEQ ID NO: 39, or vp3 proteins produced from a nucleic acid sequence at least 70% identical to at least nucleotides 610 to 2214 of
- 10 SEQ ID NO: 39 which encodes the predicted amino acid sequence of at least about amino acids 204 to 738 of SEQ ID NO: 40; and/or (2) a heterogeneous population of vp1 proteins which are the product of a nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 40, a heterogeneous population of vp2 proteins which are the product of a nucleic acid sequence encoding the amino acid sequence of at least about amino acids 138 to 738 of
- 15 SEQ ID NO: 40, and a heterogeneous population of vp3 proteins which are the product of a nucleic acid sequence encoding at least amino acids 204 to 738 of SEQ ID NO: 40, wherein: the vp1, vp2 and vp3 proteins contain subpopulations with amino acid modifications comprising at least two highly deamidated asparagines (N) in asparagine - glycine pairs in SEQ ID NO: 40 and optionally further comprising subpopulations comprising other
- 20 deamidated amino acids, wherein the deamidation results in an amino acid change; and (B) a vector genome in the AAVrh.90 capsid, the vector genome comprising a nucleic acid molecule comprising AAV inverted terminal repeat sequences and a non-AAV nucleic acid sequence encoding a product operably linked to sequences which direct expression of the product in a host cell.
- 25 In certain embodiments, the AAVrh.90 vp1, vp2 and vp3 proteins contain subpopulations with amino acid modifications comprising at least two highly deamidated asparagines (N) in asparagine - glycine pairs in SEQ ID NO: 40 and optionally further comprising subpopulations comprising other deamidated amino acids, wherein the deamidation results in an amino acid change. High levels of deamidation at N-G pairs N57, ~N263, ~N385, and/or ~N514 are observed, relative to the number of SEQ ID NO: 40.
- 30 Deamidation has been observed in other residues as shown in the table below. In certain

- embodiments, AAVrh.90 may have other residues deamidated (e.g., ~N305, ~N499, and/or ~N599, typically at less than 20%) and/or may have other modifications, including phosphorylation (e.g., where present, in the range of about 2 to about 30%, or about 2 to about 20%, or about 2 to about 10%) (e.g., at S149), or oxidation (e.g., at one or more of ~W23, ~M204, ~M212, W248, W282, M405, M473, W480, W505, M526, ~N544, M561, and/or ~M607). Optionally the W may oxidize to kynurenine.

TABLE D	
AAVrh.90 Deamidation based on VP1 numbering	% Deamidation
N57+Deamidation	65-90, 70-95, 80-95, 75-100, 80-100, or 90-100
N94+Deamidation	2-15 or 2-5
~N263+Deamidation	65-90, 70-95, 80-95, 75-100, 80-100, or 90-100
~N305+Deamidation	5-30, 5-20, or 10-20
~N385+Deamidation	65-90, 70-95, 80-95, 75-100, 80-100, or 90-100
~N499+Deamidation	2-15, 2-10, or 5-10
~N514+Deamidation	65-90, 70-95, 80-95, 75-100, 80-100, or 90-100
~N599+Deamidation	2- 15, 2-10, or 5-10

- In certain embodiments, an AAVrh.90 capsid is modified in one or more of the positions identified in the preceding table, in the ranges provided, as determined using mass spectrometry with a trypsin enzyme. In certain embodiments, one or more of the positions, or the glycine following the N is modified as described herein. Residue numbers are based on the AAVrh.90 sequence provided herein. See, SEQ ID NO: 40.

- In certain embodiments, an AAVrh.90 capsid comprises: a heterogeneous population of vp1 proteins which are the product of a nucleic acid sequence encoding the amino acid sequence of SEQ ID NO: 40, a heterogeneous population of vp2 proteins which are the product of a nucleic acid sequence encoding the amino acid sequence of at least about amino acids 138 to 738 of SEQ ID NO: 40, and a heterogeneous population of vp3 proteins which

are the product of a nucleic acid sequence encoding at least amino acids 204 to 738 of SEQ ID NO: 40.

In certain embodiments, the parvovirus vector capsids are selected for liver-tropism and the patient being treated has a liver metabolic disorder. In certain embodiments, the
5 parvovirus vector capsids are selected for cardiac-tropism and the patient being treated has a cardiac disorder. In certain embodiments, the parvovirus vector capsids are selected for tropism for cells in the skeletal muscle and the patient being treated has a muscular disorder.

As used herein, a “vector genome” refers to the nucleic acid sequence packaged inside the rAAV capsid which forms a viral particle. Such a nucleic acid sequence contains
10 AAV inverted terminal repeat sequences (ITRs). In the examples herein, a vector genome contains, at a minimum, from 5' to 3', an AAV 5' ITR, expression cassette containing the transgene or coding sequence(s) operably linked to regulatory sequences directing expression thereof, and an AAV 3' ITR. The ITRs are the genetic elements responsible for the replication and packaging of the genome during vector production and are the only viral
15 cis elements required to generate rAAV. In one embodiment, the ITRs are from an AAV different than that supplying a capsid. In a preferred embodiment, the ITR sequences from AAV2, or the deleted version thereof (Δ ITR), which may be used for convenience. However, ITRs from other AAV sources may be selected. Where the source of the ITRs is from AAV2 and the AAV capsid is from another AAV source, the resulting vector may be termed
20 pseudotyped. Typically, AAV vector genome comprises an AAV 5' ITR, the nucleic acid sequences encoding the gene product(s) and any regulatory sequences, and an AAV 3' ITR. However, other configurations of these elements may be suitable. In one embodiment, a self-complementary AAV is provided. A shortened version of the 5' ITR, termed Δ ITR, has been described in which the D-sequence and terminal resolution site (trs) are deleted. In certain
25 embodiments, the vector genome includes a shortened AAV2 ITR of 130 base pairs, wherein the external “a” element is deleted. The shortened ITR is reverted back to the wild-type length of 145 base pairs during vector DNA amplification using the internal A element as a template. In other embodiments, the full-length AAV 5' and 3' ITRs are used. In other embodiments, a full-length or engineered ITR may be selected. ITRs from AAV2, a different
30 source AAV than the capsid, or other than full-length ITRs may be selected. The ITRs are from the same AAV source as the AAV which provides the rep function during production

or a transcomplementing AAV. Further, other ITRs may be used. Examples of suitable ITR sequences are shown in the sequence listing, e.g., SEQ ID NO: 42, nt 1 to 130 and 3052 to 3181. Further, the vector genome contains regulatory sequences that direct modulate expression of the gene products (e.g., directly or indirectly by modulating transcription and/or translation). Suitable components of a vector genome are discussed in more detail herein.

In certain embodiments, the gene editing vector genome includes a TBG promoter, one or more alpha mic/bik enhancer(s), coding sequence for the ARCUS meganuclease, optionally a WPRE, and a polyA. In certain embodiments, the expression cassette includes nt 211 to nt 2964 of SEQ ID NO: 42, flanked by 5' and 3' ITRs.

For use in producing an AAV viral vector (e.g., a recombinant (r) AAV), the expression cassettes can be carried on any suitable vector, e.g., a plasmid, which is delivered to a packaging host cell. The plasmids useful in this invention may be engineered such that they are suitable for replication and packaging *in vitro* in prokaryotic cells, insect cells, mammalian cells, among others. Suitable transfection techniques and packaging host cells are known and/or can be readily designed by one of skill in the art.

Methods for generating and isolating AAVs suitable for use as vectors are known in the art. See generally, e.g., Grieger & Samulski, 2005, "Adeno-associated virus as a gene therapy vector: Vector development, production and clinical applications," *Adv. Biochem. Engin/Biotechnol.* 99: 119-145; Buning *et al.*, 2008, "Recent developments in adeno-associated virus vector technology," *J. Gene Med.* 10:717-733; and the references cited below, each of which is incorporated herein by reference in its entirety. For packaging a transgene into virions, the ITRs are the only AAV components required in *cis* in the same construct as the nucleic acid molecule containing the expression cassettes. The cap and rep genes can be supplied in *trans*.

The term "AAV intermediate" or "AAV vector intermediate" refers to an assembled rAAV capsid which lacks the desired genomic sequences packaged therein. These may also be termed an "empty" capsid. Such a capsid may contain no detectable genomic sequences of an expression cassette, or only partially packaged genomic sequences which are insufficient to achieve expression of the gene product. These empty capsids are non-functional to transfer the gene of interest to a host cell.

The recombinant adeno-associated virus (AAV) described herein may be generated using techniques which are known. *See, e.g.*, WO 2003/042397; WO 2005/033321, WO 2006/110689; US 7588772 B2. Such a method involves culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid protein; a functional rep gene; an

5 expression cassette composed of, at a minimum, AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the expression cassette into the AAV capsid protein. Methods of generating the capsid, coding sequences therefor, and methods for production of rAAV viral vectors have been described. *See, e.g.*, Gao, et al, Proc. Natl. Acad. Sci. U.S.A. 100 (10), 6081-6086 (2003) and US 2013/0045186A1.

10 In one embodiment, a production cell culture useful for producing a recombinant AAV is provided. Such a cell culture contains a nucleic acid which expresses the AAV capsid protein in the host cell; a nucleic acid molecule suitable for packaging into the AAV capsid, e.g., a vector genome which contains AAV ITRs and a non-AAV nucleic acid sequence encoding a gene product operably linked to sequences which direct expression of

15 the product in a host cell; and sufficient AAV rep functions and adenovirus helper functions to permit packaging of the nucleic acid molecule into the recombinant AAV capsid. In one embodiment, the cell culture is composed of mammalian cells (e.g., human embryonic kidney 293 cells, among others) or insect cells (e.g., baculovirus).

Optionally the rep functions are provided by an AAV other than the AAV providing the capsid. For example, the rep may be, but is not limited to, AAV1 rep protein, AAV2 rep protein, AAV3 rep protein, AAV4 rep protein, AAV5 rep protein, AAV6 rep protein, AAV7 rep protein, AAV8 rep protein; or rep 78, rep 68, rep 52, rep 40, rep68/78 and rep40/52; or a fragment thereof; or another source. Optionally, the rep and cap sequences are on the same genetic element in the cell culture. There may be a spacer between the rep sequence and cap

25 gene. Any of these AAV or mutant AAV capsid sequences may be under the control of exogenous regulatory control sequences which direct expression thereof in a host cell.

In one embodiment, cells are manufactured in a suitable cell culture (e.g., HEK 293) cells. Methods for manufacturing the gene therapy vectors described herein include methods well known in the art such as generation of plasmid DNA used for production of the gene

30 therapy vectors, generation of the vectors, and purification of the vectors. In some embodiments, the gene therapy vector is an AAV vector and the plasmids generated are an

AAV cis-plasmid encoding the AAV genome and the gene of interest, an AAV trans-plasmid containing AAV rep and cap genes, and an adenovirus helper plasmid. The vector generation process can include method steps such as initiation of cell culture, passage of cells, seeding of cells, transfection of cells with the plasmid DNA, post-transfection medium
5 exchange to serum free medium, and the harvest of vector-containing cells and culture media. The harvested vector-containing cells and culture media are referred to herein as crude cell harvest. In yet another system, the gene therapy vectors are introduced into insect cells by infection with baculovirus-based vectors. For reviews on these production systems, see generally, e.g., Zhang et al., 2009, "Adenovirus-adenovirus hybrid for large-
10 scale recombinant adeno-associated virus production," Human Gene Therapy 20:922-929, the contents of each of which is incorporated herein by reference in its entirety. Methods of making and using these and other AAV production systems are also described in the following U.S. patents, the contents of each of which is incorporated herein by reference in its entirety: 5,139,941; 5,741,683; 6,057,152; 6,204,059; 6,268,213; 6,491,907; 6,660,514;
15 6,951,753; 7,094,604; 7,172,893; 7,201,898; 7,229,823; and 7,439,065.

The crude cell harvest may thereafter be subject method steps such as concentration of the vector harvest, diafiltration of the vector harvest, microfluidization of the vector harvest, nuclease digestion of the vector harvest, filtration of microfluidized intermediate, crude purification by chromatography, crude purification by ultracentrifugation, buffer
20 exchange by tangential flow filtration, and/or formulation and filtration to prepare bulk vector.

A two-step affinity chromatography purification at high salt concentration followed anion exchange resin chromatography are used to purify the vector drug product and to remove empty capsids. These methods are described in more detail in International
25 Patent Publication No. WO 2017/160360, which is incorporated by reference herein. Purification methods for AAV8, International Patent Publication No. WO 2017/100676, and rh10, International Patent Publication No. WO 2017/100704, and for AAV1, International Patent Publication No. WO 2017/100674 are all incorporated by reference herein.

To calculate empty and full particle content, VP3 band volumes for a selected
30 sample (e.g., in examples herein an iodixanol gradient-purified preparation where # of GC = # of particles) are plotted against GC particles loaded. The resulting linear equation ($y =$

mx+c) is used to calculate the number of particles in the band volumes of the test article peaks. The number of particles (pt) per 20 μ L loaded is then multiplied by 50 to give particles (pt) /mL. Pt/mL divided by GC/mL gives the ratio of particles to genome copies (pt/GC). Pt/mL–GC/mL gives empty pt/mL. Empty pt/mL divided by pt/mL and x 100 gives the percentage of empty particles.

Generally, methods for assaying for empty capsids and AAV vector particles with packaged genomes have been known in the art. See, e.g., Grimm et al., *Gene Therapy* (1999) 6:1322-1330; Sommer et al., *Molec. Ther.* (2003) 7:122-128. To test for denatured capsid, the methods include subjecting the treated AAV stock to SDS-polyacrylamide gel electrophoresis, consisting of any gel capable of separating the three capsid proteins, for example, a gradient gel containing 3-8% Tris-acetate in the buffer, then running the gel until sample material is separated, and blotting the gel onto nylon or nitrocellulose membranes, preferably nylon. Anti-AAV capsid antibodies are then used as the primary antibodies that bind to denatured capsid proteins, preferably an anti-AAV capsid monoclonal antibody, most preferably the B1 anti-AAV-2 monoclonal antibody (Wobus et al., *J. Virol.* (2000) 74:9281-9293). A secondary antibody is then used, one that binds to the primary antibody and contains a means for detecting binding with the primary antibody, more preferably an anti-IgG antibody containing a detection molecule covalently bound to it, most preferably a sheep anti-mouse IgG antibody covalently linked to horseradish peroxidase. A method for detecting binding is used to semi-quantitatively determine binding between the primary and secondary antibodies, preferably a detection method capable of detecting radioactive isotope emissions, electromagnetic radiation, or colorimetric changes, most preferably a chemiluminescence detection kit. For example, for SDS-PAGE, samples from column fractions can be taken and heated in SDS-PAGE loading buffer containing reducing agent (e.g., DTT), and capsid proteins were resolved on pre-cast gradient polyacrylamide gels (e.g., Novex). Silver staining may be performed using SilverXpress (Invitrogen, CA) according to the manufacturer's instructions or other suitable staining method, i.e. SYPRO ruby or coomassie stains. In one embodiment, the concentration of AAV vector genomes (vg) in column fractions can be measured by quantitative real time PCR (Q-PCR). Samples are diluted and digested with DNase I (or another suitable nuclease) to remove exogenous DNA. After inactivation of the nuclease, the samples are further diluted and amplified using

primers and a TaqMan™ fluorogenic probe specific for the DNA sequence between the primers. The number of cycles required to reach a defined level of fluorescence (threshold cycle, Ct) is measured for each sample on an Applied Biosystems Prism 7700 Sequence Detection System. Plasmid DNA containing identical sequences to that contained in the AAV vector is employed to generate a standard curve in the Q-PCR reaction. The cycle threshold (Ct) values obtained from the samples are used to determine vector genome titer by normalizing it to the Ct value of the plasmid standard curve. End-point assays based on the digital PCR can also be used.

In one aspect, an optimized q-PCR method is used which utilizes a broad spectrum serine protease, e.g., proteinase K (such as is commercially available from Qiagen). More particularly, the optimized qPCR genome titer assay is similar to a standard assay, except that after the DNase I digestion, samples are diluted with proteinase K buffer and treated with proteinase K followed by heat inactivation. Suitably samples are diluted with proteinase K buffer in an amount equal to the sample size. The proteinase K buffer may be concentrated to 2-fold or higher. Typically, proteinase K treatment is about 0.2 mg/mL, but may be varied from 0.1 mg/mL to about 1 mg/mL. The treatment step is generally conducted at about 55 °C for about 15 minutes, but may be performed at a lower temperature (e.g., about 37 °C to about 50 °C) over a longer time period (e.g., about 20 minutes to about 30 minutes), or a higher temperature (e.g., up to about 60 °C) for a shorter time period (e.g., about 5 to 10 minutes). Similarly, heat inactivation is generally at about 95 °C for about 15 minutes, but the temperature may be lowered (e.g., about 70 to about 90 °C) and the time extended (e.g., about 20 minutes to about 30 minutes). Samples are then diluted (e.g., 1000 fold) and subjected to TaqMan analysis as described in the standard assay.

Additionally, or alternatively, droplet digital PCR (ddPCR) may be used. For example, methods for determining single-stranded and self-complementary AAV vector genome titers by ddPCR have been described. See, e.g., M. Lock et al, *Hum Gene Therapy Methods*, Hum Gene Ther Methods. 2014 Apr;25(2):115-25. doi: 10.1089/hgtb.2013.131. Epub 2014 Feb 14. The ddPCR method directly measures the concentration of encapsidated vector genomes. The sample is treated with DNase I to digest any non-encapsidated DNA present in the sample followed by treatment with Proteinase K to disrupt the capsid. The sample is then diluted to fit the assay range. The sample is mixed with ddPCR Supermix, and

detection is accomplished using sequence-specific primers targeting the Meganuclease specific to the *PCSK9* gene (M2PCSK9) in combination with a fluorescently-tagged probe hybridizing to this same region. Twenty microliters of ddPCR reaction mixture are processed in the Bio-Rad droplet generator, and the ddPCR reaction mixture is partitioned into $\geq 10,000$ droplets. After droplet generation, the ddPCR reaction mixture undergoes PCR amplification, and the amplified ddPCR reaction mixture is read using a Bio-Rad Droplet Reader.

The infectious unit (IU) assay may be used to determine the productive uptake and replication of rAAV vector in RC32 cells (rep2 expressing HeLa cells). A 96-well endpoint format has been employed similar to that previously published. Briefly, RC32 cells will be co-infected by serial dilutions of rAAV BDS and a uniform dilution of Ad5 with 12 replicates at each dilution of rAAV. Seventy-two hours after infection, the cells will be lysed, and qPCR will be performed to detect rAAV vector amplification over input. An endpoint dilution 50% tissue culture infectious dose (TCID₅₀) calculation (Spearman-Kärber) will be performed to determine a infectious titer expressed as IU/mL. Since “infectivity” values are dependent on each particle’s contact with cells, receptor binding, internalization, transport to the nucleus, and genome replication, they are influenced by assay geometry and the presence of appropriate receptors and post-binding pathways in the cell line used. Receptors and post-binding pathways are not usually maintained in immortalized cell lines, and thus infectivity assay titers are not an absolute measure of the number of “infectious” particles present. However, the ratio of encapsidated GC to “infectious units” (described as GC/IU ratio) can be used as a measure of product consistency from lot to lot.

In brief, the method for separating rAAV particles having packaged genomic sequences from genome-deficient AAV intermediates involves subjecting a suspension comprising recombinant AAV viral particles and AAV capsid intermediates to fast performance liquid chromatography, wherein the AAV viral particles and AAV intermediates are bound to a strong anion exchange resin equilibrated at a high pH, and subjected to a salt gradient while monitoring eluate for ultraviolet absorbance at about 260 and about 280. The pH may be adjusted depending upon the AAV selected. See, e.g., WO2017/160360 (AAV9), WO2017/100704 (AAVrh10), WO 2017/100676 (e.g., AAV8), and WO 2017/100674 (AAV1)] which are incorporated by reference herein. In this method,

the AAV full capsids are collected from a fraction which is eluted when the ratio of A260/A280 reaches an inflection point. In one example, for the Affinity Chromatography step, the diafiltered product may be applied to a Capture Select™ Poros- AAV2/9 affinity resin (Life Technologies) that efficiently captures the AAV2 serotype. Under these ionic conditions, a significant percentage of residual cellular DNA and proteins flow through the column, while AAV particles are efficiently captured.

DUAL VECTOR SYSTEM

In another aspect, a dual vector system for treating a genetic disorder is provided. The system includes (a) a gene editing component that includes a nucleic acid sequence encoding a nuclease that targets PCSK9 and, optionally, regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene; and (b) a donor vector comprising a nucleic acid sequence encoding an exogenous product for expression from the PCSK9 locus, wherein the inserted nucleic acid sequence does not encode PCSK9, and wherein the system further comprises sequences that direct the nuclease to specifically target the native PCSK9 gene locus. The system optionally comprises a component which allows the native PCSK9 in the target cell to be ablated or reduced post-dosing with the dual vector system, e.g., via use of an inducing agent with an inducible promoter. In one embodiment the gene editing component is comprised in a gene editing vector comprising an expression cassette comprising a nucleic acid sequence encoding a nuclease and regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene. The components of the dual vector are as those described herein.

While the system can be effective if the ratio of gene editing component to donor vector is about 1 to about 1, it is desirable for the donor template vector to be present in excess of the gene editing component. In one embodiment, the ratio of editing vector (a) to donor vector (b) is about 1:3 to about 1:100, or about 1:10. This ratio of gene editing enzyme (e.g., Cas9 or meganuclease) to donor template may be maintained even if the enzyme is additionally or alternatively supplied by a source other than the AAV vector.

In one embodiment, the dual vector system includes a gene editing AAV vector comprising an AAV capsid and a first vector genome comprising a 5' ITR, a sequence encoding a meganuclease that targets PCSK9 under control of regulatory sequences that

direct expression of the meganuclease in a target cell comprising a PCSK9 gene, and a 3' ITR; and (b) a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR.

In another embodiment, the dual vector system includes a gene editing AAV comprising an AAV capsid and a first vector genome comprising a 5' ITR, a 5' nuclear localization signal (NLS), a sequence encoding a Cas9 and regulatory sequences that direct expression of the SaCas9 in a target cell comprising the PCSK9 gene, a 3' NLS, and a 3' ITR; and a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, a U6 promoter, a sgRNA comprising at least 20 nucleotides that specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9, and a 3' ITR.

In another embodiment, the dual vector system includes a gene editing AAV vector comprising an AAV capsid and a first vector genome comprising a 5' ITR, a U6 promoter, a sgRNA comprising at least 20 nucleotides that specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9, a 5' nuclear localization signal (NLS), a sequence encoding a Cas9 and regulatory sequences that direct expression of the Cas9 in a target cell comprising the PCSK9 gene, a 3' NLS, and a 3' ITR; and a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR.

In certain embodiments of the systems described herein, the gene editing AAV vector the donor AAV vector have the same AAV capsid. In other embodiments, the gene editing AAV vector and the donor AAV vector have different AAV capsids. In some embodiments, the AAV capsid is selected from AAV8, AAV9, rh10, AAV6.2, AAV3B, hu37, rh79, and rh64.

In certain embodiments, the nuclease is a Cas9 nuclease, and the Cas9 is selected from *Staphylococcus aureus* or *Streptococcus pyogenes* Cas9.

In certain embodiments, the nuclease and/or transgene is under the control of a tissue-specific promoter. In certain embodiments, the nuclease and/or transgene is under the control of a constitutive promoter. In certain embodiments, the nuclease and/or transgene is under the control of an inducible promoter. In certain embodiments, the nuclease and/or transgene is under the control of a liver-specific promoter, optionally a human thyroxine-binding globulin (TBG) promoter, or hybrid liver promoter (HLP). In certain embodiments, the system further comprises an inducing agent.

In another embodiment, the system includes (a) a gene editing component that includes a nucleic acid sequence encoding a nuclease that targets PCSK9 and regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene encapsulated in a LNP; and (b) a donor vector comprising a nucleic acid sequence encoding an exogenous product for expression from the PCSK9 locus encapsulated in a LNP, wherein the inserted nucleic acid sequence does not encode PCSK9, and wherein the system further comprises sequences that direct the nuclease to specifically target the native PCSK9 gene locus. The system optionally comprises a component which allows the native PCSK9 in the target cell to be ablated or reduced post-dosing with the dual vector system, e.g., via use of an inducing agent with an inducible promoter.

In another embodiment, the system includes (a) a gene editing component that includes a nucleic acid sequence encoding a nuclease that targets PCSK9 and regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene wherein the gene editing component is provided via AAV vector; and (b) a donor vector comprising a nucleic acid sequence encoding an exogenous product for expression from the PCSK9 locus encapsulated in a LNP, wherein the inserted nucleic acid sequence does not encode PCSK9, and wherein the system further comprises sequences that direct the nuclease to specifically targets the native PCSK9 gene locus. The system optionally comprises a component which allows the native PCSK9 in the target cell to be ablated or reduced post-dosing with the dual vector system, e.g., via use of an inducing agent with an inducible promoter.

In another embodiment, the system includes (a) a gene editing component that includes a nucleic acid sequence encoding a nuclease that targets PCSK9 and regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene encapsulated in a LNP; and (b) a donor vector comprising a nucleic acid sequence encoding an exogenous product for expression from the PCSK9 locus, wherein the donor vector is an AAV vector, wherein the inserted nucleic acid sequence does not encode PCSK9, and wherein the system further comprises sequences that direct the nuclease to specifically targets the native PCSK9 gene locus. The system optionally comprises a component which allows the native PCSK9 in the target cell to be ablated or reduced post-dosing with the dual vector system, e.g., via use of an inducing agent with an inducible promoter.

In one embodiment, the dual vector system includes (a) a LNP comprising mRNA that encodes a meganuclease that targets PCSK9 under control of regulatory sequences that direct expression of the meganuclease in a target cell comprising a PCSK9 gene; and (b) a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR.

In another embodiment, the dual vector system includes (a) a LNP comprising a nucleic acid comprising a sequence encoding a Cas9 and a sgRNA comprising at least 20 nucleotides that specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9; and (b) a donor AAV vector comprising an AAV capsid and a vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR. The sequence encoding Cas9 is provided as mRNA.

In another embodiment, the dual vector system includes a gene editing AAV vector comprising an AAV capsid and a first vector genome comprising a 5' ITR, a U6 promoter, a sgRNA comprising at least 20 nucleotides that specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9, a 5' nuclear localization signal (NLS), a sequence encoding a Cas9 and regulatory sequences that direct expression of the Cas9 in a target cell comprising the

PCSK9 gene, a 3' NLS, and a 3' ITR; and a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR.

5

PHARMACEUTICAL COMPOSITIONS

In another aspect, a pharmaceutical composition is provided which contains a first rAAV stock comprising rAAV gene editing vectors comprising an expression cassette comprising a nucleic acid sequence encoding a nuclease that targets PCSK9 and regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene; and a second rAAV stock comprising rAAV donor vectors comprising a transgene cassette comprising a nucleic acid sequence encoding a transgene and regulatory sequences that direct expression of the transgene in the target cell. The pharmaceutical composition contains an optional carrier, excipient, and/or preservative. In some embodiments, the donor vector further includes homology-directed recombination (HDR) arms 5' and 3' to the transgene cassette. In one embodiment, the AAV capsid for the donor vector, gene editing vector, or both, is an AAVrh79 capsid. In another embodiment, the AAV capsid for the donor vector, gene editing vector, or both, is an AAVrh.90 capsid. In another embodiment, the AAV capsid for the donor vector, gene editing vector, or both, is an AAVhu.37 capsid. In one embodiment, the AAV capsid for the donor vector, gene editing vector, or both, is an AAV8 capsid. In one embodiment, the AAV capsid for the donor vector, gene editing vector, or both, is an AAVrh.91 capsid. In one embodiment, the AAV capsid for the donor vector, gene editing vector, or both, is an AAVhu.68 capsid.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a host. Delivery vehicles such as liposomes, nanocapsules, microparticles, microspheres, lipid particles,

vesicles, and the like, may be used for the introduction of the compositions of the present invention into suitable host cells. In particular, the rAAV vector delivered vector genomes may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

5 In one embodiment, a composition includes a final formulation suitable for delivery to a subject, e.g., is an aqueous liquid suspension buffered to a physiologically compatible pH and salt concentration. Optionally, one or more surfactants are present in the formulation. In another embodiment, the composition may be transported as a concentrate which is diluted for administration to a subject. In other embodiments, the composition may be
10 lyophilized and reconstituted at the time of administration.

 Methods and agents well known in the art for making formulations are described, for example, in "Remington's Pharmaceutical Sciences," Mack Publishing Company, Easton, Pa. Formulations may, for example, contain excipients, carriers, stabilizers, or diluents such as sterile water, saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable
15 origin, or hydrogenated naphthalenes, preservatives (such as octadecyldimethylbenzyl, ammonium chloride, hexamethonium chloride, benzalkonium chloride, benzethonium chloride, phenol, butyl or benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol), low molecular weight polypeptides, proteins such as serum albumin, gelatin, or immunoglobulins, hydrophilic
20 polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, histidine, arginine, and lysine, monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, and dextrans, chelating agents such as EDTA, sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™,
25 PLURONICS™ or polyethylene glycol (PEG).

 The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes,
30 albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in

macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

A suitable surfactant, or combination of surfactants, may be selected from among non-ionic surfactants that are nontoxic. In one embodiment, a difunctional block copolymer surfactant terminating in primary hydroxyl groups is selected, e.g., such as Pluronic® F68 [BASF], also known as Poloxamer 188, which has a neutral pH, has an average molecular weight of 8400. Other surfactants and other Poloxamers may be selected, i.e., nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)), SOLUTOL HS 15 (Macrogol-15 Hydroxystearate), LABRASOL (Polyoxy caprylic glyceride), polyoxy 10 oleyl ether, TWEEN (polyoxyethylene sorbitan fatty acid esters), ethanol and polyethylene glycol. In one embodiment, the formulation contains a poloxamer. These copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits: the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content. In one embodiment Poloxamer 188 is selected. The surfactant may be present in an amount up to about 0.0005 % to about 0.001% of the suspension.

The vectors are administered in sufficient amounts to transfect the cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse effects, or with medically acceptable physiological effects, which can be determined by those skilled in the medical arts. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to a desired organ (*e.g.*, the liver (optionally via the hepatic artery), lung, heart, eye, kidney,), oral, inhalation, intranasal, intrathecal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, and other parental routes of administration. Routes of administration may be combined, if desired.

Dosages of the viral vector depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. For example, a therapeutically effective human dosage of the viral vector is generally in the range of from about 25 to about 1000 microliters to about 100 mL of solution containing concentrations of from about 1×10^9 to 1×10^{16} genomes virus vector. The dosage is

adjusted to balance the therapeutic benefit against any side effects and such dosages may vary depending upon the therapeutic application for which the recombinant vector is employed. The levels of expression of the transgene product can be monitored to determine the frequency of dosage resulting in viral vectors, preferably AAV vectors containing the minigene. Optionally, dosage regimens similar to those described for therapeutic purposes may be utilized for immunization using the compositions of the invention.

The vector compositions can be formulated in dosage units to contain an amount of replication-defective virus that is in the range of about 1.0×10^9 GC to about 1.0×10^{16} GC (to treat an average subject of 70 kg in body weight) including all integers or fractional amounts within the range, and preferably 1.0×10^{12} GC to 1.0×10^{14} GC for a human patient. In one embodiment, the compositions are formulated to contain at least 1×10^9 , 2×10^9 , 3×10^9 , 4×10^9 , 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , or 9×10^9 GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{10} , 2×10^{10} , 3×10^{10} , 4×10^{10} , 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , or 9×10^{10} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{11} , 2×10^{11} , 3×10^{11} , 4×10^{11} , 5×10^{11} , 6×10^{11} , 7×10^{11} , 8×10^{11} , or 9×10^{11} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{12} , 2×10^{12} , 3×10^{12} , 4×10^{12} , 5×10^{12} , 6×10^{12} , 7×10^{12} , 8×10^{12} , or 9×10^{12} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{13} , 2×10^{13} , 3×10^{13} , 4×10^{13} , 5×10^{13} , 6×10^{13} , 7×10^{13} , 8×10^{13} , or 9×10^{13} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{14} , 2×10^{14} , 3×10^{14} , 4×10^{14} , 5×10^{14} , 6×10^{14} , 7×10^{14} , 8×10^{14} , or 9×10^{14} GC per dose including all integers or fractional amounts within the range. In another embodiment, the compositions are formulated to contain at least 1×10^{15} , 2×10^{15} , 3×10^{15} , 4×10^{15} , 5×10^{15} , 6×10^{15} , 7×10^{15} , 8×10^{15} , or 9×10^{15} GC per dose including all integers or fractional amounts within the range. In one embodiment, for human application the dose can range from 1×10^{10} to about 1×10^{12} GC per dose including all integers or fractional amounts within the range.

These above doses may be administered in a variety of volumes of carrier, excipient or buffer formulation, ranging from about 25 to about 1000 microliters, or higher volumes, including all numbers within the range, depending on the size of the area to be treated, the viral titer used, the route of administration, and the desired effect of the method.

5 Any suitable route of administration may be selected. Accordingly, pharmaceutical compositions may be formulated for any appropriate route of administration, for example, in the form of liquid solutions or suspensions (as, for example, for intravenous administration, for oral administration, etc.). Alternatively, pharmaceutical compositions may be in solid form (*e.g.*, in the form of tablets or capsules, for example for oral administration). In some
10 embodiments, pharmaceutical compositions may be in the form of powders, drops, aerosols, etc.

In one aspect, provided herein is a pharmaceutical composition comprising at least parvovirus vector comprising at least one gene editing vector and at least one donor vector as described herein in a formulation buffer. In certain embodiments, the pharmaceutical
15 composition comprises a combination of different vector populations. In one embodiment, provided is a pharmaceutical composition comprising a single rAAV population described herein in a formulation buffer. The methods provided herein provide for co-administration of two separate vector-containing suspensions.

20 METHODS

The compositions provided herein are useful for treatment of various genetic disorders, including liver metabolic disorders. In certain embodiments, the compositions are useful in treating ornithine transcarbamylase. In other embodiments, the compositions are
25 useful in treating familial hypercholesterolemia. In other embodiments, the compositions are useful in treating phenylketonuria.

Exemplary liver diseases or disorders that can be treated include, but are not limited to, hepatitis A, hepatitis B, hepatitis C, autoimmune hepatitis, primary biliary cholangitis, primary sclerosing cholangitis, hemochromatosis, Wilson's disease, alpha-1 antitrypsin
30 deficiency, liver cancer, bile duct cancer, liver adenoma, transthyretin (TTR), proprotein convertase subtilisin/kexin type 9 (PCSK9)-based diseases or disorders, or any combination thereof. Further disorders include glycogen storage disease or deficiency type 1A (GSD1),

PEPCK deficiency, CDKL5 deficiency, galactosemia, phenylketonuria (PKU), Primary Hyperoxaluria Type 1, Maple syrup urine disease, tyrosinemia type 1, methylmalonic acidemia, medium chain acetyl CoA deficiency, ornithine transcarbamylase deficiency, citrullinemia; lecithin-cholesterol acyltransferase (LCAT) deficiency, amethylmalonic acidemia (MMA), Niemann-Pick disease, propionic academia (PA); familial hypercholesterolemia (FH), dementia, Lipoprotein Lipase Deficiency, Crigler-Najjar disease, severe combined immunodeficiency disease, Gout and Lesch-Nyan syndrome, biotinidase deficiency, Fabry disease, GM1 gangliosidosis, Wilson's Disease, Gaucher disease type 2 and 3, Zellweger syndrome, metachromatic leukodystrophy, Krabbe disease, Pompe disease, Nieman Pick disease type A, Argininosuccinic Aciduria, adult onset type II citrullinemia, urea cycle disorders; Farber lipogranulomatosis, aspartyl-glucosaminuria, fucosidosis, alpha-mannosidosis, acute intermittent porphyria (AIP), alpha-1 antitrypsin deficiency (emphysema), anemia due to thalassemia or to renal failure, ischemic diseases, occluded blood vessels as seen in, for example, atherosclerosis, thrombosis, or embolisms, Parkinson's disease, congestive heart failure, muscular dystrophies, and diabetes.

In certain of the methods described herein, the native PCSK9 expression is reduced or ablated and a transgene is expressed from the insertion in the native PCSK9 locus. In another embodiment, the native PCSK9 expression is reduced or ablated, and the transgene is expressed exogenously, i.e., without being integrated into the subject's genome.

Provided herein is a method of treating a disorder in a human by co-administering the dual vector system as described herein.

In one embodiment, a method of treating a liver metabolic disorder in a subject is provided. The method includes co-administering to the subject having a liver metabolic disorder a gene editing AAV vector comprising a sequence encoding a nuclease that targets PCSK9 and regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene; and a donor AAV vector comprising a transgene and regulatory sequences that direct expression of the transgene in the target cell. In another embodiment, the method includes co-administering to the subject having a liver metabolic disorder an LNP comprising a sequence encoding a Cas9 nuclease and sgRNA that target PCSK9 in a target cell comprising a PCSK9 gene; and a donor AAV vector comprising a transgene and

regulatory sequences that direct expression of the transgene in the target cell. In one embodiment, the subject is a neonate.

In certain embodiments, the gene editing AAV vector and the donor vector of are delivered essentially simultaneously via the same route. In other embodiments, the gene editing vector is delivered first. In other embodiments, the donor vector is delivered first.

In one embodiment, the dosage of an rAAV is about 1×10^9 GC to about 1×10^{15} genome copies (GC) per dose (to treat an average subject of 70 kg in body weight), and preferably 1.0×10^{12} GC to 2.0×10^{15} GC for a human patient. In another embodiment, the dose is less than about 1×10^{14} GC/kg body weight of the subject. In certain embodiments, the dose administered to a patient is at least about 1.0×10^9 GC/kg, about 1.5×10^9 GC/kg, about 2.0×10^9 GC/kg, about 2.5×10^9 GC/kg, about 3.0×10^9 GC/kg, about 3.5×10^9 GC/kg, about 4.0×10^9 GC/kg, about 4.5×10^9 GC/kg, about 5.0×10^9 GC/kg, about 5.5×10^9 GC/kg, about 6.0×10^9 GC/kg, about 6.5×10^9 GC/kg, about 7.0×10^9 GC/kg, about 7.5×10^9 GC/kg, about 8.0×10^9 GC/kg, about 8.5×10^9 GC/kg, about 9.0×10^9 GC/kg, about 9.5×10^9 GC/kg, about 1.0×10^{10} GC/kg, about 1.5×10^{10} GC/kg, about 2.0×10^{10} GC/kg, about 2.5×10^{10} GC/kg, about 3.0×10^{10} GC/kg, about 3.5×10^{10} GC/kg, about 4.0×10^{10} GC/kg, about 4.5×10^{10} GC/kg, about 5.0×10^{10} GC/kg, about 5.5×10^{10} GC/kg, about 6.0×10^{10} GC/kg, about 6.5×10^{10} GC/kg, about 7.0×10^{10} GC/kg, about 7.5×10^{10} GC/kg, about 8.0×10^{10} GC/kg, about 8.5×10^{10} GC/kg, about 9.0×10^{10} GC/kg, about 9.5×10^{10} GC/kg, about 1.0×10^{11} GC/kg, about 1.5×10^{11} GC/kg, about 2.0×10^{11} GC/kg, about 2.5×10^{11} GC/kg, about 3.0×10^{11} GC/kg, about 3.5×10^{11} GC/kg, about 4.0×10^{11} GC/kg, about 4.5×10^{11} GC/kg, about 5.0×10^{11} GC/kg, about 5.5×10^{11} GC/kg, about 6.0×10^{11} GC/kg, about 6.5×10^{11} GC/kg, about 7.0×10^{11} GC/kg, about 7.5×10^{11} GC/kg, about 8.0×10^{11} GC/kg, about 8.5×10^{11} GC/kg, about 9.0×10^{11} GC/kg, about 9.5×10^{11} GC/kg, about 1.0×10^{12} GC/kg, about 1.5×10^{12} GC/kg, about 2.0×10^{12} GC/kg, about 2.5×10^{12} GC/kg, about 3.0×10^{12} GC/kg, about 3.5×10^{12} GC/kg, about 4.0×10^{12} GC/kg, about 4.5×10^{12} GC/kg, about 5.0×10^{12} GC/kg, about 5.5×10^{12} GC/kg, about 6.0×10^{12} GC/kg, about 6.5×10^{12} GC/kg, about 7.0×10^{12} GC/kg, about 7.5×10^{12} GC/kg, about 8.0×10^{12} GC/kg, about 8.5×10^{12} GC/kg, about 9.0×10^{12} GC/kg, about 9.5×10^{12} GC/kg, about 1.0×10^{13} GC/kg, about 1.5×10^{13} GC/kg, about 2.0×10^{13} GC/kg, about 2.5×10^{13} GC/kg, about 3.0×10^{13} GC/kg, about 3.5×10^{13} GC/kg, about 4.0×10^{13} GC/kg, about 4.5×10^{13} GC/kg.

GC/kg , about 5.0×10^{13} GC/kg , about 5.5×10^{13} GC/kg , about 6.0×10^{13} GC/kg , about 6.5×10^{13} GC/kg , about 7.0×10^{13} GC/kg , about 7.5×10^{13} GC/kg , about 8.0×10^{13} GC/kg , about 8.5×10^{13} GC/kg , about 9.0×10^{13} GC/kg , about 9.5×10^{13} GC/kg , or about 1.0×10^{14} GC/kg body weight or the subject.

5 Other examples of suitable diseases which may be treated using the compositions described herein are familial hypercholesterolemia, muscular dystrophy, cystic fibrosis, and rare or orphan diseases. Examples of such rare diseases may include spinal muscular atrophy, Huntingdon's Disease, Rett Syndrome, Amyotrophic Lateral Sclerosis (ALS), Duchenne Type Muscular dystrophy, Friedrichs Ataxia, spinocerebellar ataxia type 2
10 (SCA2)/ALS, progranulin (PRGN) (associated with non-Alzheimer's cerebral degenerations, including, frontotemporal dementia (FTD), progressive non-fluent aphasia (PNFA) and semantic dementia), among others. See, e.g., www.orpha.net/consor/cgi-bin/Disease_Search_List.php; rarediseases.info.nih.gov/diseases. Other diseases indicted by the transgenes described herein may also be treated using the methods described herein.

15 The vectors are administered in sufficient amounts to transfect the cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse effects, or with medically acceptable physiological effects, which can be determined by those skilled in the medical arts. Desirable routes of administration include direct delivery to a desired organ (e.g., the liver (optionally via the hepatic artery), lung,
20 heart, eye, kidney), oral, inhalation, intranasal, intratracheal, intrathecal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, and other parental routes of administration. Routes of administration may be combined, if desired.

The system described herein may be therapeutically useful if a sufficient amount of functional enzyme or protein is generated to improve the patient's condition. In certain
25 embodiments, gene expression levels as low as 5% of healthy patients will provide sufficient therapeutic effect for the patient to be treatable to non-gene therapy approaches. In other embodiments, gene expression levels are at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%,
30 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%,

76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, to up to 100% of the normal range (levels) observed in humans (or other veterinary subject). For example, by “functional enzyme”, is meant a gene which encodes the wild-type enzyme (*e.g.*, OTCase) which provides at least

5 about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%,

10 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or about the same, or greater than 100% of the biological activity level of the wild-type enzyme, or a natural variant or polymorph thereof which is not associated with disease. More particularly, as heterozygous patients may have as low an enzyme functional level as about 50% or lower, effective treatment may not require replacement of enzyme activity to levels

15 within the range of “normal” or non-deficient patients. Similarly, patients having no detectable amounts of enzyme may be rescued by delivering enzyme function to less than 100% activity levels, and may optionally be subject to further treatment subsequently. In certain embodiments, where gene function is being delivered by the donor template, patients may express higher levels than found in “normal”, healthy subjects. In still other

20 embodiments, where reduction in gene expression is desired, as much as a 20% reduction to a 50% reduction, or up to about 100% reduction, may provide desired benefits. As described herein, the therapy described herein may be used in conjunction with other treatments, *i.e.*, the standard of care for the subject’s (patient’s) diagnosis.

In one embodiment, the method further comprises administering an

25 immunosuppressive co-therapy to the subject. Such immunosuppressive co-therapy may be started prior to delivery of an rAAV or a composition as disclosed, *e.g.*, if undesirably high neutralizing antibody levels to the AAV capsid are detected. In certain embodiments, co-therapy may also be started prior to delivery of the rAAV as a precautionary measure. In certain embodiments, immunosuppressive co-therapy is started following delivery of the

30 rAAV, *e.g.*, if an undesirable immune response is observed following treatment.

Immunosuppressants for such co-therapy include, but are not limited to, a glucocorticoid, steroids, antimetabolites, T-cell inhibitors, a macrolide (*e.g.*, a rapamycin or rapalog), and cytostatic agents including an alkylating agent, an anti-metabolite, a cytotoxic antibiotic, an antibody, or an agent active on immunophilin. The immune suppressant may include prednisolone, a nitrogen mustard, nitrosourea, platinum compound, methotrexate, azathioprine, mercaptopurine, fluorouracil, dactinomycin, an anthracycline, mitomycin C, bleomycin, mithramycin, IL-2 receptor- (CD25-) or CD3-directed antibodies, anti-IL-2 antibodies, ciclosporin, tacrolimus, sirolimus, IFN- β , IFN- γ , an opioid, or TNF- α (tumor necrosis factor-alpha) binding agent. In certain embodiments, the immunosuppressive therapy may be started 0, 1, 2, 7, or more days prior to the rAAV administration, or 0, 1, 2, 3, 7, or more days post the rAAV administration. Such therapy may involve a single drug (*e.g.*, prednisolone) or co-administration of two or more drugs, the (*e.g.*, prednisolone, micophenolate mofetil (MMF) and/or sirolimus (*i.e.*, rapamycin)) on the same day. One or more of these drugs may be continued after gene therapy administration, at the same dose or an adjusted dose. Such therapy may be for about 1 week (7 days), two weeks, three weeks, about 60 days, or longer, as needed. In certain embodiments, a tacrolimus-free regimen is selected.

In another embodiment, the method includes co-treatment with a standard OTC therapy. Treatment of OTC deficiency is largely focused on dietary management of blood ammonia levels to avoid hyperammonemia or to remove excess ammonia from the blood during hyperammonemic episodes (NORD, 2021). Individuals with OTC deficiency follow dietary restrictions limiting their protein intake to control blood ammonia levels. Dietary restrictions must be carefully balanced in infants, who need to consume enough protein to ensure proper growth while avoiding excess protein intake that could trigger a hyperammonemic episode (Berry and Steiner, 2001). As such, infants are placed on high calorie, low protein diets supplemented by essential amino acids. In hyperammonemic episodes, all protein may be removed from a patient's diet for 24 hours (NORD, 2021).

There are several medications designed to stimulate the removal of nitrogen from the blood stream. Sodium phenylbutyrate (Buphenyl) is approved by the United States Food and Drug Administration (FDA) for the treatment of chronic hyperammonemia in patients with OTC deficiency. Once metabolized, Buphenyl is converted to phenylacetate, which

conjugates with glutamine to form phenylacetylglutamine, which is excreted by the kidney, providing an alternative pathway for nitrogen excretion. Glycerol phenylbutyrate (Ravicti) is also FDA-approved for the treatment of chronic hyperammonemia in patients with urea cycle disorders. Like Buphenyl, Ravicti is converted to phenylacetate and follows the same mechanism for excreting nitrogen (Lichter-Konecki et al., 1993; Gordon, 2003; Magellan, 2021). Finally, Ammonul (sodium phenylacetate and sodium benzoate), is approved by the FDA as an adjunctive therapy for the treatment of acute hyperammonemia in patients with urea cycle disorders. The sodium phenylacetate component of Ammonul follows the same mechanism for nitrogen excretion as the phenylacetate metabolite created by Buphenyl and Ravicti. The sodium benzoate component of Ammonul conjugates with glycine to form hippuric acid, which is excreted by the kidneys and removing nitrogen through this process. Sodium benzoate is also given as an oral preparation for long-term maintenance of OTC deficiency and is often preferred over Buphenyl and Ravicti since it thought to have fewer side effects (Lichter-Konecki et al., 1993).

In one aspect, a method is provided for treating a patient having ornithine transcarbamylase (OTC) deficiency, using a nuclease expression cassette comprising a meganuclease coding sequence that recognizes a site within the human PCSK9 gene, under the control of a promoter as described herein. The method further includes administration of an expression cassette carrying the OTC transgene of SEQ ID NO: 17, or a sequence sharing at least 90% identity therewith, as described herein. Such expression cassettes may be delivered via a viral or non-viral vector. In certain embodiments, the expression cassettes may be delivered using an LNP. The native human OTC coding sequence is shown in SEQ ID NO: 30. SEQ ID NO: 17 and SEQ ID NO: 30 share about 75.89% identity.

In another aspect, a method is provided for treating a patient having ornithine transcarbamylase (OTC) deficiency, using a nuclease expression cassette comprising a sgRNA and Cas9 coding sequence that recognizes a site within the human PCSK9 gene. The method further includes administration of an expression cassette carrying the OTC transgene of SEQ ID NO: 17, or a sequence sharing at least 90% identity therewith, as described herein. Such expression cassettes may be delivered via a viral or non-viral vector. In certain embodiments, the expression cassettes may be delivered using an LNP.

A variety of assays exist for measuring OTC expression and activity levels in vitro. See, e.g., X Ye, *et al*, 1996 Prolonged metabolic correction in adult ornithine transcarbamylase-deficient mice with adenoviral vectors. J Biol Chem 271:3639–3646) or in vivo. For example, OTC enzyme activity can be measured using a liquid chromatography

5 mass spectrometry stable isotope dilution method to detect the formation of citrulline normalized to [1,2,3,4,5-13C5] citrulline (98% 13C). The method is adapted from a previously developed assay for detection of N-acetylglutamate synthase activity [Morizono H, *et al*, Mammalian N-acetylglutamate synthase. Mol Genet Metab. 2004;81(Suppl 1):S4–11.]. Slivers of fresh frozen liver are weighed and briefly homogenized in buffer containing

10 10 mM HEPES, 0.5 % Triton X-100, 2.0 mM EDTA and 0.5 mM DTT. Volume of homogenization buffer is adjusted to obtain 50 mg/ml tissue. Enzyme activity is measured using 250 µg liver tissue in 50 mM Tris-acetate, 4 mM ornithine, 5 mM carbamyl phosphate, pH 8.3. Enzyme activity is initiated with the addition of freshly prepared 50 mM carbamyl phosphate dissolved in 50 mM Tris-acetate pH 8.3, allowed to proceed for 5 minutes at 25

15 °C and quenched by addition of an equal volume of 5 mM13C5-citrulline in 30%TCA. Debris is separated by 5 minutes of microcentrifugation, and the supernatants are transferred to vials for mass spectroscopy. Ten µL of sample are injected into an Agilent 1100 series LC-MS under isocratic conditions with a mobile phase of 93% solvent A (1 ml trifluoroacetic acid in 1 L water):7% solvent B (1ml trifluoroacetic acid in 1L of 1:9

20 water/acetonitrile). Peaks corresponding to citrulline [176.1 mass:charge ratio (m/z)] and 13C5-citrulline (181.1 m/z) are quantitated, and their ratios are compared to ratios obtained for a standard curve of citrulline run with each assay. Samples are normalized to either total liver tissue or to protein concentration determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Other assays, which do not require liver biopsy, may also be used. One

25 such assay is a plasma amino acid assays in which the ratio of glutamine and citrulline is assessed and if glutamine is high (>800 microliters/liter) and citrulline low (e.g., single digits), a urea cycle defect is suspected. Plasma ammonia levels can be measured and a concentration of about 100 micromoles per liter is indicative of OTCD. Blood gases can be assessed if a patient is hyperventilating; respiratory alkalosis is frequent in OTCD. Orotic

30 acid in urine, e.g., greater than about 20 micromoles per millimole creatine is indicative of OTCD, as is elevated urinary orotate after allopurinol challenge test. Diagnostic criteria for

OTCD have been set forth in Tuchman *et al*, 2008, Urea Cycle Disorders Consortium (UCDC) of the Rare Disease Clinical Research Network (RDCRN). Tuchman M, *et al*, Consortium of the Rare Diseases Clinical Research Network. Cross-sectional multicenter study of patients with urea cycle disorders in the United States. *Mol Genet Metab*.

- 5 2008;94:397–402, which is incorporated by reference herein. See, also, <http://www.ncbi.nlm.nih.gov/books/NBK154378/>, which provides a discussion of the present standard of care for OTCD.

In certain embodiments, a nuclease expression cassette, non-viral vector, viral vector (e.g., rAAV), or any of the same in a pharmaceutical composition, as described herein is
10 administrable for gene editing in a patient. In certain embodiments, the method is useful for non-embryonic gene editing. In certain embodiments, the patient is an infant (e.g., birth to about 9 months). In certain embodiments, the patient is older than an infant, e.g., 12 months or older.

As used herein, “a,” “an,” or “the” can mean one or more than one. For example, “a”
15 cell can mean a single cell or a multiplicity of cells.

As used herein, the term “specificity” means the ability of a nuclease to recognize and cleave double-stranded DNA molecules only at a particular sequence of base pairs referred to as the recognition sequence, or only at a particular set of recognition sequences. The set of recognition sequences will share certain conserved positions or sequence motifs,
20 but may be degenerate at one or more positions. A highly-specific nuclease is capable of cleaving only one or a very few recognition sequences. Specificity can be determined by any method known in the art.

The abbreviation “sc” refers to self-complementary. “Self-complementary AAV” refers a construct in which a coding region carried by a recombinant AAV nucleic acid sequence has been designed to form an intra-molecular double-stranded DNA template. Upon infection, rather than waiting for cell mediated synthesis of the second strand, the two complementary halves of scAAV will associate to form one double stranded DNA (dsDNA) unit that is ready for immediate replication and transcription. *See, e.g., D M McCarty et al*, “Self-complementary recombinant adeno-associated virus (scAAV) vectors promote
25 efficient transduction independently of DNA synthesis”, *Gene Therapy*, (August 2001), Vol 8, Number 16, Pages 1248-1254. Self-complementary AAVs are described in, *e.g., U.S.*

Patent Nos. 6,596,535; 7,125,717; and 7,456,683, each of which is incorporated herein by reference in its entirety.

As used herein, the term “operably linked” refers to both expression control sequences that are contiguous with the gene of interest and expression control sequences that
5 act in trans or at a distance to control the gene of interest.

The term “exogenous” as used to describe a nucleic acid sequence or protein means that the nucleic acid or protein does not naturally occur in the position in which it exists in a chromosome, or host cell. An exogenous nucleic acid sequence also refers to a sequence derived from and inserted into the same expression cassette or host cell, but which is present
10 in a non-natural state, e.g., a different copy number, or under the control of different regulatory elements.

The term “heterologous” when used with reference to a protein or a nucleic acid indicates that the protein or the nucleic acid comprises two or more sequences or subsequences which are not found in the same relationship to each other in nature. For
15 instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid. For example, in one embodiment, the nucleic acid has a promoter from one gene arranged to direct the expression of a coding sequence from a different gene.

As used herein, the term “host cell” may refer to the packaging cell line in which a
20 vector (e.g., a recombinant AAV) is produced from a production plasmid. In the alternative, the term “host cell” may refer to any target cell in which expression of the transgene is desired. Thus, a “host cell,” refers to a prokaryotic or eukaryotic cell that contains a exogenous or heterologous nucleic acid sequence that has been introduced into the cell by any means, e.g., electroporation, calcium phosphate precipitation, microinjection,
25 transformation, viral infection, transfection, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. In certain embodiments herein, the term “host cell” refers to cultures of cells of various mammalian species for in vitro assessment of the compositions described herein. In other embodiments herein, the term “host cell” refers to the cells employed to generate and package the viral
30 vector or recombinant virus. Still in other embodiment, the term “host cell” is intended to

reference the target cells of the subject being treated in vivo for the diseases or conditions as described herein. In certain embodiments, the term “host cell” is a liver cell or hepatocyte.

A “subject” is a mammal, *e.g.*, a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, or non-human primate, such as a monkey, chimpanzee, baboon or gorilla. A patient
5 refers to a human. A veterinary subject refers to a non-human mammal. In certain embodiments, the subject does not have a defect in their PCSK9 gene.

A “replication-defective virus” or “viral vector” refers to a synthetic or artificial viral particle in which an expression cassette containing a gene of interest is packaged in a viral capsid or envelope, where any viral genomic sequences also packaged within the viral capsid
10 or envelope are replication-deficient; *i.e.*, they cannot generate progeny virions but retain the ability to infect target cells. In one embodiment, the genome of the viral vector does not include genes encoding the enzymes required to replicate (the genome can be engineered to be “gutless” - containing only the gene of interest flanked by the signals required for amplification and packaging of the artificial genome), but these genes may be supplied
15 during production. Therefore, it is deemed safe for use in gene therapy since replication and infection by progeny virions cannot occur except in the presence of the viral enzyme required for replication.

The terms “sequence identity” “percent sequence identity” or “percent identical” in the context of nucleic acid sequences refers to the residues in the two sequences which are
20 the same when aligned for maximum correspondence. The length of sequence identity comparison may be over the full-length of the genome, the full-length of a gene coding sequence, or a fragment of at least about 500 to 5000 nucleotides, is desired. However, identity among smaller fragments, *e.g.* of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more
25 nucleotides, may also be desired. Similarly, “percent sequence identity” may be readily determined for amino acid sequences, over the full-length of a protein, or a fragment thereof. Suitably, a fragment is at least about 8 amino acids in length and may be up to about 700 amino acids. Examples of suitable fragments are described herein.

The term “substantial homology” or “substantial similarity,” when referring to amino
30 acids or fragments thereof, indicates that, when optimally aligned with appropriate amino acid insertions or deletions with another amino acid (or its complementary strand), there is

amino acid sequence identity in at least about 95 to 99% of the aligned sequences.

Preferably, the homology is over full-length sequence, or a protein thereof, *e.g.*, a cap protein, a rep protein, or a fragment thereof which is at least 8 amino acids, or more desirably, at least 15 amino acids in length. Examples of suitable fragments are described
5 herein.

By the term “highly conserved” is meant at least 80% identity, preferably at least 90% identity, and more preferably, over 97% identity. Identity is readily determined by one of skill in the art by resort to algorithms and computer programs known by those of skill in the art.

10 Generally, when referring to “identity”, “homology”, or “similarity” between two different adeno-associated viruses, “identity”, “homology” or “similarity” is determined in reference to “aligned” sequences. “Aligned” sequences or “alignments” refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence. In the
15 examples, AAV alignments are performed using the published AAV9 sequences as a reference point. Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Examples of such programs include, “Clustal Omega”, “Clustal W”, “CAP Sequence Assembly”, “MAP”, and “MEME”, which are accessible through Web Servers on the internet. Other sources for such
20 programs are known to those of skill in the art. Alternatively, Vector NTI utilities are also used. There are also a number of algorithms known in the art that can be used to measure nucleotide sequence identity, including those contained in the programs described above. As another example, polynucleotide sequences can be compared using Fasta™, a program in GCG Version 6.1. Fasta™ provides alignments and percent sequence identity of the regions
25 of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid sequences can be determined using Fasta™ with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) as provided in GCG Version 6.1, herein incorporated by reference. Multiple sequence alignment programs are also available for amino acid sequences, *e.g.*, the “Clustal Omega”, “Clustal X”, “MAP”,
30 “PIMA”, “MSA”, “BLOCKMAKER”, “MEME”, and “Match-Box” programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter

these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. *See, e.g.,* J. D. Thomson et al, Nucl. Acids. Res., “A comprehensive comparison of multiple sequence alignments”, 27(13):2682-2690 (1999).

5 As used herein, the term “about” refers to a variant of $\pm 10\%$ from the reference integer and values therebetween. For example, “about” 40 base pairs, includes ± 4 (i.e., 36 – 44, which includes the integers 36, 37, 38, 39, 40, 41, 42, 43, 44). For other values, particularly when reference is to a percentage (e.g., 90% identity, about 10% variance, or about 36% mismatches), the term “about” is inclusive of all values within the range
10 including both the integer and fractions.

Throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have
15 specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the
20 range.

As used throughout this specification and the claims, the terms “comprising”, “containing”, “including”, and its variants are inclusive of other components, elements, integers, steps and the like. Conversely, the term “consisting” and its variants are exclusive of other components, elements, integers, steps and the like.

25 Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art and by reference to published texts, which provide one skilled in the art with a general guide to many of the terms used in the present application.

30

EXAMPLES

Ornithine transcarbamylase (OTC) deficiency is an X-linked urea cycle disorder associated with high mortality. Although a promising treatment for late-onset OTC deficiency, adeno-associated virus (AAV) neonatal gene therapy would only provide short-term therapeutic effects as the non-integrated genome gets lost during hepatocyte proliferation. Nuclease-mediated, site-specific integration of an OTC mini gene cassette in a safe harbor in the genome would provide long-term therapeutic benefits to patients with OTC deficiency. One of the safe harbors for gene targeting is the PCSK9 gene, such as the exon 7 region. The nucleases could be an engineered meganuclease targeting PCSK9 (ARCUS2) or CRISPR/Cas9 with specific sgRNA targeting PCSK9. The donor vector contains a mini gene including a liver-specific promoter such as a TBG promoter, a codon optimized hOTC coding sequence, and a poly A sequence. Both the nuclease and the donor template could be delivered by AAV vectors (dual AAV vector system). Demonstrated persistent transgene expression and efficient gene targeting in 12% of hepatocytes at 12 weeks after a single intravenous injection of the dual AAV vectors in a newborn nonhuman primate (NHP). The mini gene in the donor vector is flanked with homolog-directed recombination (HDR) arms.

First time demonstration in NHPs of in vivo nuclease-mediated gene targeting to the PCSK9 locus to express a therapeutic protein following a single injection of the dual AAV vectors as newborns or infants. Composition of the donor vector containing the OTC mini gene for gene targeting to the human/NHP PCSK9 locus has not been tested in the clinics for the treatment of OTC deficiency. We will test the hOTC donor vectors in newborn NHPs for gene targeting efficiency and in newborn transgenic OTC deficient mice for efficacy.

Many metabolic diseases require early intervention and therapy; however, AAV-mediated neonatal gene therapy is unstable due to fast liver proliferation in the neonatal stage and non-integrative nature of the AAV vector. Targeted integration of a therapeutic mini gene cassette in a safe harbor would persistently express the therapeutic gene on the genome level and the therapeutic effects would be maintained through cell division. For many metabolic diseases such as OTC deficiency, sufficient transduction efficiency in liver needs to be achieved for clinical benefits.

We describe a genome editing approach for the treatment of ornithine transcarbamylase deficiency (OTCD) which can cause lethal episodes of hyperammonemia in infancy. The goal of genome editing is for the therapeutic effect to be durable and achieved in all OTCD patients independent of their mutation. We propose to accomplish this

5 by treating surviving newborns with two AAV vectors: one to deliver a nuclease to create a double stranded break in a safe-harbor site and the second to deliver an OTC minigene for knock-in into this site. Our assumption is that dividing hepatocytes of the newborn liver will be conducive to efficient knock-in of the OTC gene and will eliminate, through dilution, the non-integrated input vector genomes. We decided to use the PCSK9 gene as a safe-harbor

10 site and a meganuclease, called ARCUS to target it, based on our previous work in adult macaques which showed safe, efficient and stable reductions of PCSK9 following AAV delivery ARCUS. Our initial studies of genome editing for OTCD were performed in an OTC deficient mouse rendered susceptible to the PCSK9 ARCUS nuclease through germ line modification of exon 7 of the endogenous PCSK9 gene. Injection of the two vectors into

15 newborn mice resulted in efficient knock-in of the human OTC minigene and protection to lethal hyperammonemia when challenged with a high protein diet. In preparation for clinical studies, we evaluated key safety and efficacy parameters in newborn and infant macaques. A total of 24 animals were treated with AAV vectors with analyses to include examination of liver biopsies at 3 and 12 months. In these studies we evaluated the impact of the following

20 parameters on editing efficiency and toxicity: transgene (human factor IX and human OTC), promoters driving ARCUS, Clade E capsids, length of donor flanking the transgene and age of the macaque at time of dosing. We report here preliminary data of 16/24 animals that includes, at a minimum, 3 month biopsy results. We found the injection of AAV vectors was quite safe with no evidence of transaminase elevations or liver histopathology in any

25 ARCUS treated animals. The key measure of efficacy in the primate model is transduction efficiency measured by in situ hybridization and immunostaining to detect cells expressing the human OTC mRNA and protein, respectively. The highest and most consistent results were obtained with vectors using a novel clade E capsid driving ARCUS with a TBG promoter in the first vector and using 500 bp flanking homology arms on the donor vector.

30 With this combination we achieved $10.0 \pm 6.4\%$ (N=6) transduction which is higher than the threshold we believe can provide substantial benefit to patients which is ~5% OTC

expressing cells. Preliminary data suggests that the level of editing is stable over one year and that efficient targeted insertion can be achieved when injected into macaques up to 3 months of age. Molecular analyses of the PCSK9 target locus suggested the vast majority of the knocked-in of vector genome were through non-homologous end joining (NHEJ) rather than homology-directed repair (HDR). In summary, the substantial unmet need of the neonatal form of OTCD warrants consideration of experimental therapies such as genome editing such as that described in this report.

EXAMPLE 1 – MATERIALS AND METHODS

10 Materials and Methods

AAV vectors were constructed according to previously established procedures and manufacturer's instructions. The AAVhu37 capsid was used for the experiments as described herein, where indicated.

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

EXAMPLE 2 – PILOT STUDY: HFIX MINI GENE KNOCK-IN IN PCSK9 LOCUS BY ARCUS2 OR SACAS9 IN NEWBORN NHPS

In this study, we evaluated on-target (PSK9) SaCas9- or ARCUS- mediated gene editing and hFIX or OTC mini-gene knock in efficiency in newborn non-human primates (NHPS). FIG. 1 shows a schematic representation of the rhPCSK9 locus showing the donor splice site within exon 7, and a HDR donor vector comprising donor template of interest, e.g., hFIX, hOTC. Furthermore, FIGs. 3A to 3C show a schematic representation for a dual AAV vector system for SaCas9- or ARCUS-mediated gene correction. FIG. 3A shows a schematic representation for a dual AAVhu37 vector system for ARCUS2-mediated gene correction, wherein the AAVhu37-donor vector comprises an hOTC donor template sequence. FIG. 3B shows a schematic representation for a dual AAVhu37 vector system for Sa-Cas9-mediated gene correction (trans; AAVhu37-SaCas9), wherein the AAV.hu37.shRNA-donor vector comprises an hOTC donor template sequence. FIG. 3C shows a schematic representation for a dual AAVhu37 vector system for Sa-Cas9-mediated

gene correction (cis; AAVhu37.PCSK9-sgRN.SaCas9), wherein the AAV.hu37-donor vector comprises an hOTC donor template sequence.

The above described dual AAVhu37 vectors comprising gene editing nuclease and donor templates, were used in newborn NHP to examine the hFIX mini-gene knock-in in PCSK9 locus as mediated by either SaCas9 or ARCUS2. Gene editing AAVhu37 vector was delivered at a dose of 1×10^{13} GC/kg, and donor template AAVhu37 vector was delivered at 3×10^{13} GC/kg. Overall, there were three treatment NHP groups: 1) AAVhu37.EGFP and AAVhu37.Donor-HDR-hFIX.U6.sgR; 2) AAVhu37.ARCUS2 and AAVhu37.Donor-HDR-hFIX; 3) AAVhu37.SaCas9 and AAVhu37.Donor-HDR-hFIX.U6.sgR. FIG. 2 shows a timeline for a pilot study comprising an hFIX mini-gene knock-in in PCSK9 locus by ARCUS2 or SaCas9 in newborn NHPs. In this study, NHPs were injected at day 0, blood samples were collected at every 2-4 weeks (to examine serum chemistry, hFIX expression in plasma, PCSK9 levels in serum, LDL levels and neutralizing antibodies (NAb) levels), first liver biopsy was performed at day 84 (to examine vector genome levels, gene expression levels, on- an off-target editing, and histology).

An in vivo test of nuclease-mediated gene targeting was performed in newborn and infant NHPs. Animals were administered with 1×10^{13} GC/kg of AAVhu37.ARCUS2.WPRE and 3×10^{13} GC/kg of AAVhu37.hFIXco-HDR or 1×10^{13} GC/kg of AAVhu37.SaCas9.WPRE and 3×10^{13} GC/kg of AAVhu37.hFIXco-HDR.U6.sgR or 1×10^{13} GC/kg of AAVhu37.GFP.WPRE and 3×10^{13} GC/kg of AAVhu37.hFIXco-HDR.U6.sgR, as shown in FIGs 4A, 4B, and 5G. FIG. 4C shows hFIX levels at the indicated timepoints from day 0 to 13 months post treatment (plotted as ng/mL). FIG. 4D shows PCSK9 levels at the indicated timepoints from day 0 to 12 months post treatment (plotted as percentage of baseline at day 0). FIG. 4E shows ALT (Alanine Aminotransferase) levels at the indicated timepoints from day 0 to day 196 post treatment (plotted as U/L). FIG. 4F shows anti-FIX IgG levels at the indicated timepoints from day 0 to day 196 post treatment (plotted as dilution factor, 1/dilution). FIG. 4G shows PCSK9 levels at the indicated timepoints from day 0 to day 196 post treatment (plotted as ng/mL). FIG. 4H shows weight as measured at the indicated timepoints from day 0 to day 196 post treatment (plotted as g). FIG. 5A shows hFIX levels at the indicated timepoints (plotted as ng/mL) in infant NHPs. FIG. 5B shows PCSK9 levels at the indicated timepoints (plotted as percentage of baseline at day 0) in infant NHPs. FIG. 5C

shows ALT (Alanine Aminotransferase) levels at the indicated timepoints (plotted as U/L) in infant NHPs. FIG. 5D shows anti-FIX IgG levels at the indicated timepoints (plotted as dilution factor, 1/dilution) in infant NHPs. FIG. 5E shows PCSK9 levels at the indicated timepoints (plotted as ng/mL) in infant NHPs. FIG. 5F shows weight as measured at the indicated timepoints (plotted as g) in infant NHPs. FIG. 5G is a summary table showing data from the experiment described in FIGs. 4A-5G. FIG. 5H shows a comparison of various data between newborn and infant NHPs tested.

FIGs. 6A to 6E show vector transduction (GC) and transgene expression in liver biopsies samples collected at days shown post treatment in the NHPs. 6A shows vector transduction levels in liver biopsies samples, plotted as AAV genome copies (GC) per diploid cell. FIG. 6B shows relative expression of transgene RNA in liver biopsies samples. FIG. 6C shows dual in situ hybridization (ISH) using specific probes to detect FIX and ARCUS in liver biopsies. FIG. 6D shows digitized ISH images used for quantification of transduction. FIG. 6E shows transduction efficiency of FIX transgene as quantified by ISH, and plotted as percent transduction. FIG. 6F shows genome copies in liver. Higher FIXco GC was observed in the infant (3x) than the newborn at both time points. Reduced FIXco GC in the 2nd biopsy (1.5 – 2x) than in the 1st biopsy. FIG. 6G shows transgene mRNA in liver biopsy. FIXco mRNA was stable between 3m and 1y in the infant, while FIXco mRNA was reduced by 3-fold between 3m and 1y in the newborn-treated animal. FIG. 6H shows the results of molecular analysis on liver samples. Indels as measured by amplicon-seq and off targets as measured by ITR-seq are shown. In addition, the results of nanopore long-read seq is shown for animal 20-196 at day 366. 0.6% of reads showed incorporation of the HDR on both sides. FIG. 6I is a summary table of the data described in FIGs. 6A-6H.

FIGs. 7A to 7L show dual in situ hybridization (ISH) using specific probes to detect FIX and ARCUS in liver biopsies collected at 84 days post treatment in NHPs; showed at various magnification views (NHPs treated with AAVhu37.ARCUS2 and AAVhu37.Donor-HDR-hFIX). FIG. 7A shows ISH-detected ARCUS in liver biopsies, viewed at 4x magnification. FIG. 7B shows ISH-detected hFIX in liver biopsies, viewed at 4x magnification. FIG. 7C shows overlay image of ISH-detected ARCUS and hFIX, viewed at 4x magnification. FIG. 7D shows ISH-detected ARCUS and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 4x magnification. FIG. 7E shows ISH-detected

- ARCUS in liver biopsies, viewed at 10x magnification. FIG. 7F shows ISH-detected hFIX in liver biopsies, viewed at 10x magnification. FIG. 7G shows overlay image of ISH-detected ARCUS and hFIX, viewed at 10x magnification. FIG. 7H shows ISH-detected ARCUS and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 10x magnification.
- 5 FIG. 7I shows ISH-detected ARCUS expression in liver biopsies, viewed at 20x magnification. FIG. 7J shows ISH-detected hFIX in liver biopsies, viewed at 20x magnification. FIG. 7K shows overlay image of ISH-detected ARCUS and hFIX, viewed at 20x magnification. FIG. 7L shows ISH-detected ARCUS and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 20x magnification. Summary of vector
- 10 transduction (GC/diploid genome) is shown in table 1 below.

Table 1.

	GC/diploid genome
hFIX	0.63
ARCUS	0.13
Ratio (FIX/ARCUS)	4.8
Ratio of vector dose (FIX/ARCUS)	3.0

- FIGs. 8A to 8M show dual in situ hybridization (ISH) using specific probes to detect FIX and ARCUS in liver biopsies collected at 84 days post treatment in NHPs; showed at various magnification views (NHPs treated with AAVhu37.EGFP and AAVhu37.Donor-
- 15 HDR-hFIX.U6.sgR). FIG. 8A shows ISH-detected GFP-WRPE in liver biopsies, viewed at 4x magnification. FIG. 8B shows ISH-detected hFIX in liver biopsies, viewed at 4x magnification. FIG. 8C shows overlay image of ISH-detected GFP-WRPE and hFIX, viewed at 4x magnification. FIG. 8D shows ISH-detected GFP-WRPE and hFIX as an overlaid
- 20 image with DAPI (staining for nuclei), viewed at 4x magnification. FIG. 8E shows ISH-detected GFP-WRPE in liver biopsies, viewed at 10x magnification. FIG. 8F shows ISH-detected hFIX in liver biopsies, viewed at 10x magnification. FIG. 8G shows overlay image of ISH-detected GFP-WRPE and hFIX, viewed at 10x magnification. FIG. 8H shows ISH-

detected GFP-WRPE and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 10x magnification. FIG. 8I shows ISH-detected GFP-WRPE expression in liver biopsies, viewed at 20x magnification. FIG. 8J shows ISH-detected hFIX in liver biopsies, viewed at 20x magnification. FIG. 8K shows overlay image of ISH-detected GFP-WRPE and hFIX, viewed at 20x magnification. FIG. 8L shows ISH-detected GFP-WRPE and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 20x magnification. FIG. 8M shows ISH-detected GFP-WRPE and hFIX as an overlaid image with DAPI (staining for nuclei), viewed at 20x magnification in an untreated control. Summary of vector transduction (GC/diploid genome) is shown in table 2 below.

Table 2.

	GC/diploid genome
hFIX	0.017
ARCUS	0.006
Ratio (FIX/ARCUS)	2.7
Ratio of vector dose (FIX/ARCUS)	3.0

FIG. 9 shows ARCUS-mediated on-target editing in NHP treated with AAVhu37.ARCUS2 and AAVhu37.Donor-HDR-hFIX. At 84 days post treatment, liver biopsies samples were collected, and percentage of total indels in the target region present in was calculated. Furthermore, ARCUS-mediated on-target editing in NHP treated with AAVhu37.ARCUS2 and AAVhu37.Donor-HDR-hFIX. At 84 days post treatment, liver biopsies samples were collected, and frequencies of total indels in the target region present in was calculated, plotted as frequency of unique UMI OT reads relative to the target. Summary of indel as quantified by amplicon sequencing is shown in Table 3 below.

Table 3.

ID	20-196 (NB, d84)	20-196 (NB, d366)	AH0120 (3m, d98)	RA3567 (ctl)
Ins %	1.1	1.3	1.2	0.00
Del %	9.2	10.9	13.2	0.04
Total Indel %	10.2	12.1	14.4	0.04
HDR% (LMU- seq)	2.8	2.0	1.2	

EXAMPLE 3 - ARCUS2-MEDIATED HOTC GENE TARGETING IN NEWBORN NHPS

Newborn (1–16 days old) or infant (3–4 months old) rhesus macaques were used in non-GLP-compliant POC pharmacology studies. The M2PCSK9 meganuclease targets a 22-bp sequence present in the human and rhesus macaque *PCSK9* gene. Thus, rhesus macaques can be used to evaluate on-target editing (pharmacology) and safety/toxicology. Furthermore, newborn and infant rhesus macaques have similar anatomical and physiological features as human infants and will allow for the use of the intended clinical ROA (IV). It is anticipated that the similarity in anatomy and ROA will result in representative vector distribution and transduction profiles, which will enable more accurate assessment of the pharmacology and toxicity of the test article, including on- and off-target editing, and clinical pathology, which is not possible in newborn mice.

In this study newborn NHPs were administered ARCUS2 nuclease vectors, and donor vectors having HDR arms of varying length – 500bp arm or short HDR arm. Vector schematic is shown in FIG. 11J. FIG. 12A is a summary table showing data from the experiment. All 14 newborn macaques tolerated vector infusions well (i.e., no apparent clinical sequelae) and gained weight over time (FIG. 11I). Liver enzyme levels were within

the normal range except for transient and modest elevation of ALT levels in some animals on Day 14 (FIG. 11C).

Analysis on the Day 0 plasma samples collected from the newborns prior to dosing showed 3 animals (21-111, 21-113, 21-122) had high levels (≥ 400) of binding antibodies to AAVrh79 (FIG. 11A). These pre-existing anti-AAVrh79 antibodies would block AAV gene transfer.

PCSK9 levels were followed in all newborn animals including the donor-only control animals over time. PCSK9 levels on Day 0 varied between the newborns (FIG. 11B). Nine animals showed a trend of reduced PCSK9 levels post vector administration including one donor-only control animal, while the remaining five animals showed persistent or transient elevation of PCSK9 levels post dosing FIG. 11B).

On Day 84, a liver biopsy via laparotomy was performed. Transduction efficiencies of hOTC in liver were evaluated by dual ISH with hOTC- and M2PCSK9-specific probes to detect transgene mRNA, and by OTC immunofluorescence to detect human OTC protein, followed by quantification on scanned slides (FIG. 11D). The three animals (21-111, 21-113, and 21-122) with pre-existing anti-AAVrh79 binding antibodies at the time of dosing did show any OTC-positive hepatocytes by both methods. The two donor-only control animals showed low level ($\leq 1\%$) of hOTC transduction. The highest transduction efficiencies (11.9 and 18.6% by OTC immunofluorescence) were detected in the two animals that received a co-administration of AAVrh79.TBG.PI.M2PCSK9.WPRE.bGH and AAVrh79.rhHDR.TBG.hOTCco.bGH donor vectors (G6). Positive hOTC-expressing hepatocytes were also found to be present in clusters. These levels are above the threshold for substantially benefitting patients, which is $\sim 5\%$ OTC-expressing cells.

Molecular analysis on the Day 84 liver biopsy samples from each animal were performed to measure transgene copy numbers per diploid genome, mRNA expression levels, on-target editing, and off-target editing (FIG. 11F). Consistent with the transduction efficiency analyses, the two animals (21-157 and 21-175) in Group 6 had the highest hOTC vector GC (FIG. 11F), hOTC mRNA (FIG. 12I), and on-target indel% (FIG. 11H). The M2PCSK9 vector GC in animals were 2-fold to 7-fold lower than the hOTC vector GC, while M2PCSK9 mRNA levels were 23-fold and 765-fold lower than the hOTC mRNA levels (FIG. 11F and 11G).

Off-target activity evaluated by ITR-seq identified 2 to 40 potential off-targets in the Day 84 liver biopsy samples in this study. Some off-target sites were detected in multiple animals, including the hFIX infant and hFIX newborn animals in Study 2 and Study 3, respectively. Off-target editing will be further characterized by amplicon-seq on the potential off-target sites.

In summary, we identified an M2PCSK9 vector and hOTCco donor vector combination that when co-administered into newborn macaques could achieve 12–18.6% transduction efficiency in liver at 3 months post dosing, both higher than the threshold for substantially benefitting patients, which is ~5% OTC-expressing hepatocytes. Animals in this study are being followed for long-term efficiency and safety evaluation. We will perform a second liver biopsy at 1 year post dosing to evaluate the stability of hOTC transduction, histopathology in liver, and on- and off-targeting in liver.

EXAMPLE 4 - PCSK9-HE7-KI MOUSE MODEL

Since the M2PCSK9 targeting sequence in human and macaque *PCSK9* gene is not conserved with the murine *Pcsk9* gene, we cannot use M2PCSK9 for genome editing in the genomic locus in mouse. Therefore, we commissioned The Jackson Laboratory to generate a knock-in mouse model that replaces a region including the exon 7 of the murine *Pcsk9* gene with a region of human *PCSK9* gene containing exon 7, named *PCSK9-hE7-KI* mouse (FIG. 10A-10C). This model can be used for evaluation of *in vivo* genome editing and gene targeting efficiency. We then crossed *PCSK9-hE7-KI* mouse with sparse fur ash (*spf^{ash}*) mouse. *spf^{ash}* mice have a G to A point mutation at the splice donor site at the end of exon 4 of the *Otc* gene, which leads to abnormal splicing of *Otc* mRNA and a 20-fold reduction in both OTC mRNA and protein expression (Hodges and Rosenberg, 1989). Affected animals have 5-10% residual OTC activity and can survive on a chow diet, but they develop hyperammonia that can be lethal when on a high-protein diet (Yang et al., 2016).

The *PCSK9-hE7-KI.spf^{ash}* mouse model can be used for evaluation of the efficacy of *in vivo* gene targeting of human *OTC* and demonstration of correlations of targeting efficiency and efficacy. However, due to the small size of neonatal mice, evaluation of blood clinical pathology and clinical efficacy of gene targeting can only be performed after mice are weaned, once they have reached sufficient body weight, and as a terminal procedure.

FIG. 12 shows sequence alignment of 265 bp sequence represents the human PCSK9 sequence of the PCSK9-hE7 knock-in allele, mouse PCSK9 (mPCSK9) and rhesus macaques PCSK9 (rhPCSK9). There are 6 mismatches between human and rhesus sequences in this 265 bp region. Rodent and primate sequences diverge beyond this window due to insertion of various LINE and LTR. A 2 amino acid difference in exon 7 between human and mouse is present. The hE7-KI mouse is expressing normal levels of mPCSK9 by as measured ELISA.

EXAMPLE 5 - IN VIVO OTC GENE TARGETING TO THE *PCSK9* LOCUS IN PCSK9-HE7-KI.SPF^{ASH} PUPS

This ongoing non-GLP-compliant pharmacology study aims to assess whether M2PCSK9 meganuclease-mediated knock-in of the human *OTC* gene in newborn PCSK9-hE7-KI.SPF^{ASH} mice can achieve therapeutic human OTC expression in the target tissue for treatment of OTC deficiency (liver) following a single co-administration of an M2PCSK9 nuclease-expressing vector and a human *OTC* donor vector via the intended clinical ROA (IV). A schematic of the experimental design is shown in FIG. 14A, with dosage groups shown in FIG. 14B.

On Day 0, newborn (PND 1–2) male *PCSK9-hE7-KI.SPF^{ASH}* mice were IV co-administered an AAVrh79 vector expressing M2PCSK9 meganuclease (AAVrh79.TBG.PI.M2PCSK9.WPRE.bGH) at a dose of 1.0×10^{13} GC/kg in combination with one of three different AAVrh79 hOTCco donor vectors at a dose of 3.0×10^{13} GC/kg. The M2PCSK9 meganuclease-expressing vector evaluated in this study (AAVrh79.TBG.PI.M2PCSK9.WPRE.bGH) was identical to the lead clinical candidate, while each hOTCco donor vector was identical to the lead clinical candidate except for the HDR arms. Specifically, while the clinical candidate includes a long version of the human HDR sequence (AAVrh79.hHDR.TBG.hOTCco.bGH), the hOTCco donor vectors assessed in this study included a mouse-human hybrid HDR sequence (AAVrh79.mhHDR.TBG.hOTCco.bGH), a shorter version of the human HDR sequence (AAVrh79.shHDR.TBG.hOTCco.bGH), or no HDR sequence (AAVrh79.TBG.hOTCco.bGH). FIG. 13 shows a comparison of the homology of the HDR arms with human, knock in mouse and NHP sequences. As a negative control, additional

age-matched *PCSK9-hE7-KI.spf^{ash}* mice were administered an AAVrh79 vector expressing no meganuclease (AAVrh79.TBG.PI.EGFP.WPRE.bGH) in combination with AAVrh79.mhHDR.TBG.hOTCco.bGH.

In-life evaluations include viability monitoring performed daily, body weight measurements, assessment of plasma PCSK9, and plasma NH₃ and urine orotic acid levels following the high protein diet challenge, and a partial hepatectomy at Day 120 to evaluate the stability of human *OTC* transduction following two-third partial hepatectomy. On Days 49 and 170, a subgroup from each cohort is challenged with a 10-day high protein diet followed by necropsy at the end of the challenge. At necropsy, livers are collected to evaluate the knock-in of the human *OTC* gene, including assessment of human *OTC* mRNA expression (*in situ* hybridization), OTC protein expression (immunostaining), and OTC enzyme activity assessed by staining and/or an enzyme activity assay. Liver DNA is isolated to assess on-target editing (amplicon-seq, Oxford nanopore long-read sequencing) and evaluate vector genome copies.

Preliminary results show mice dosed with vectors having the mhHDR arms show survival equivalent to wild type mice, with shHDR-treated mice achieving 80% viability after the 10-day high protein diet challenge (FIG. 14C). All treated mice maintained weight better than KI-spf-ash untreated mice (FIG. 14D). Plasma ammonia levels of mhHDR -treated mice were markedly reduced as compared to untreated mice (FIG. 14E).

mPCSK9 levels were measured at date 48 and all treated mice showed a reduction (FIG. 14F). Indel percentage was fairly consistent across HDR types (FIG. 14G). hOTC levels were increased in mice treated with shHDR and mhHDR (FIG. 14H).

EXAMPLE 6 - IN VIVO OTC GENE TARGETING TO THE *PCSK9* LOCUS IN NEWBORN RHESUS MACAQUES

This ongoing non-GLP-compliant pharmacology study aims to assess whether M2PCSK9 meganuclease-mediated knock-in of the human *OTC* gene in newborn rhesus macaques can achieve therapeutic human OTC expression in the target tissue for treatment of OTC deficiency (liver) following a single co-administration of an M2PCSK9 meganuclease-expressing vector and a human *OTC* donor vector via the intended clinical ROA (IV).

On Day 0, newborn (1 to 16-day-old) rhesus macaques were IV co-administered one of two different vectors expressing M2PCSK9 meganuclease at a dose of 1.0×10^{13} GC/kg in combination with one of two different AAV hOTCco donor vectors at a dose of 3.0×10^{13} GC/kg. A non-nuclease group that only received the AAV hOTCco donor vector at a dose of 3.0×10^{13} GC/kg was included as donor-only controls.

For the AAV vectors targeting the *PCSK9* gene, we compared two AAV vector constructs that express the M2PCSK9 in liver. AAV.TBG.PI.M2PCSK9.WPRE.bGH contains the full-length TBG promoter and two copies of enhancer elements and WPRE expresses higher levels of nuclease than AAV.TBG-S1-F113.PI.M2PCSK9.bGH, which contains a short and weak promoter. For the hOTC donor vectors, we compared two AAV.hOTCco donor vectors which differ in the length of the homology arm flanking the hOTCco transgene cassette.

NHPs were IV-administered two vectors on Day 0 and are being monitored daily for viability. In-life evaluations include measurement of body weights, clinical pathology of the blood, and gene editing analysis of plasma. Two laparotomy procedures are planned to isolate liver tissue for analysis of genome editing efficiency, vector genome copy, transgene expression, histopathology, immunostaining, and RNA ISH staining. NHPs will be followed long-term and will be necropsied (date to be determined), at which time, tissues from the liver and other major organs will be collected for evaluation of genome editing efficiency, vector genome copy, transgene expression, histopathology, immunostaining, and RNA ISH staining.

EXAMPLE 7 - ASSESSING THE EFFICACY AND DETERMINING THE RATIO OF VECTORS IN PCSK9-HE7-KI.SPF^{ASH} PUPS

This planned non-GLP-compliant pharmacology study aims to assess the ratio of vector components required to achieve the highest efficacy of M2PCSK9 meganuclease-mediated knock-in of the human *OTC* gene in newborn PCSK9-hE7-KI.*spf^{ash}* mice for treatment of OTC deficiency (liver) following a single co-administration of an M2PCSK9 nuclease-expressing vector and a human *OTC* donor vector via the intended clinical ROA (IV).

On Day 0, newborn (PND 1–2) male PCSK9-hE7-KI.*spf^{ash}* mice will be IV co-administered an AAVrh79 vector expressing M2PCSK9 meganuclease (AAVrh79.TBG.PI.M2PCSK9.WPRE.bGH) at one of three doses in combination with one of three doses of the hOTCco donor vector including a mouse-human hybrid HDR sequence (AAVrh79.mhHDR.TBG.hOTCco.bGH). The M2PCSK9 meganuclease-expressing vector evaluated in this study (AAVrh79.TBG.PI.M2PCSK9.WPRE.bH) is identical to the clinical candidate, while the hOTCco donor vector is identical to the clinical candidate except for the HDR arms. Specifically, while the clinical candidate includes a long version of the human HDR sequence (AAVrh79.hHDR.TBG.hOTCco.bGH), the hOTCco donor vectors assessed in this study included a mouse-human hybrid HDR sequence (AAVrh79.mhHDR.TBG.hOTCco.bGH).

The mouse-human hybrid HDR sequence within the donor vector (AAVrh79.mhHDR.TBG.hOTCco.bGH) was selected for this study to enable evaluation of the pharmacology of this approach where the donor sequence is directly homologous to the sequence in the PCSK9-hE7-KI.*spf^{ash}* mice.

In-life evaluations include viability monitoring performed daily, body weight measurements, assessment of plasma NH₃ and urine orotic acid levels following the high protein diet challenge. On Day 81, mice will be challenged with a 10-day high protein diet followed by necropsy at the end of the challenge. At necropsy, livers will be collected to evaluate the knock-in of the human *OTC* gene, including assessment of human *OTC* mRNA expression (*in situ* hybridization), OTC protein expression (immunostaining), and OTC enzyme activity assessed by staining and/or an enzyme activity assay. Liver DNA will also be isolated to assess on-target editing (amplicon-seq) and evaluate vector genome copies.

EXAMPLE 8 - ASSESSING THE EFFICACY AND DETERMINING THE MINIMUM EFFECTIVE DOSE IN PCSK9-HE7-KI.SPF^{ASH} PUPS

This planned GLP-compliant pharmacology study aims to evaluate the efficacy and determine the MED of IV-administered AAV in the newborn PCSK9-hE7-KI.*spf^{ash}* mouse model. The AAVrh79 vector expressing M2PCSK9 meganuclease (AAVrh79.TBG.PI.M2PCSK9.WPRE.bGH) will be the toxicological vector lot that will be manufactured for the planned GLP-compliant toxicology study. Instead of utilizing the test

article, which includes a long version of the human HDR sequence (AAVrh79.hHDR.TBG.hOTCco.bGH), this study will utilize the hOTCco donor vector that includes the mouse-human hybrid HDR sequence (AAVrh79.mhHDR.TBG.hOTCco.bGH). This vector will be manufactured in a comparable method to that of the toxicological vector lot of the clinical candidate.

We have chosen to use the mouse-human hybrid HDR sequence within the donor vector (AAVrh79.mhHDR.TBG.hOTCco.bGH) for this study to enable us to effectively study the pharmacology of this approach where the donor sequence is directly homologous to the sequence in the PCSK9-hE7-KI.*spf^{ash}* mice.

10 This study will evaluate N=60 neonatal (PND 1–2) newborn PCSK9-hE7-KI.*spf^{ash}* mice and N=15 age-matched male PCSK9-hE7-KI.WT (wild type) as controls. The study will include one necropsy time point (90 days). For efficacy evaluation, mice will be challenged by a 10-day course of a high protein diet from Day 81 to Day 90. Survival, body conditions, and biomarker changes will be evaluated. Three dose levels of AAV will be
15 evaluated using IV administration. The dose levels will be selected based on the range of doses evaluated in previous nonclinical studies. The dose levels evaluated will bracket the anticipated clinical doses.

20 In-life assessments will include daily viability checks, monitoring for survival, body weight measurements, assessment of serum PCSK9 levels, plasma NH₃ and urine orotic acid levels following high protein diet challenge. Necropsies will be performed on Day 90. At necropsy, blood will be collected for CBC/differentials and serum clinical chemistry analysis. A list of tissues will be collected for histopathological evaluation. Liver will be collected to evaluate the knock-in of the human *OTC* gene, including assessment of human *OTC* mRNA expression (*in situ* hybridization), OTC protein expression (immunostaining),
25 and OTC enzyme activity assessed by staining and/or an enzyme activity assay. Liver DNA will also be isolated to assess on-target editing (amplicon-seq) and evaluate vector genome copies.

30 The MED will be determined based upon analysis of survival following the high protein diet, plasma NH₃ levels at the end of the high protein diet challenge human *OTC* mRNA and protein expression, OTC enzyme activity, and on-target editing of AAV-treated

newborn PCSK9-hE7-KI.*spf^{ash}* compared to vehicle-treated newborn PCSK9-hE7-KI.*spf^{ash}* control mice.

EXAMPLE 9 – TOXICOLOGY STUDY IN PCSK9-HE7-KI.SPF^{ASH} PUPS

5 A 6-month GLP-compliant safety study will be conducted in newborn (PND 1–2) PCSK9-hE7-KI.*spf^{ash}* mice to investigate the safety, tolerability, pharmacology, and pharmacokinetics of the test article following IV co-administration. Interim analyses, including on-target editing, off-target editing, transgene expression, and histopathological analyses, will be performed on Day 60 and Day 180 as these time points will allow sufficient
10 time for the nuclease-dependent gene insertion to have reached stable plateau levels following administration. Newborn PCSK9-hE7-KI.*spf^{ash}* mice will receive one of three dose levels of the test article (1.0×10^{12} GC/kg nuclease vector and 3.0×10^{12} GC/kg donor vector, 3.3×10^{12} GC/kg nuclease vector and 1.0×10^{13} GC/kg donor vector, or 1.0×10^{13} GC/kg nuclease vector and 3.0×10^{13} GC/kg; N=20 per dose) or vehicle (phosphate-buffered
15 saline [PBS]; N=20). After the test article or vehicle administration, in-life evaluations will include clinical observations to monitor daily for signs of distress and abnormal behavior, body weight measurements, and blood clinical serum chemistry (specifically ALT, AST, and total bilirubin).

 On Day 60 after test article administration, cohorts 1, 3, 5, and 7 of will be
20 euthanized, and a histopathological analysis will be performed on a comprehensive list of tissues, including, but not limited to, brain, spinal cord, heart, liver, spleen, kidney, lungs, reproductive organs, adrenal glands, and lymph nodes. Organs will be weighed as appropriate.

 Liver samples will be collected and analyzed for on-target editing by amplicon-seq
25 and AMP-seq, off-target editing by ITR-seq and amplicon-seq, vector biodistribution, and transgene expression. In liver samples, biodistribution will be evaluated by PCR and meganuclease RNA expression will be analyzed by RT-PCR will be performed. Highly perfused organs will be analyzed for meganuclease RNA and tissues with detectable expression of meganuclease RNA will be evaluated for on-target editing by amplicon-seq.
30 Tissues with detectable on-target editing will be further evaluated for off-target editing.

For vector biodistribution, qPCR detection specific to the transgenes of the dual vectors, M2PCSK9 and hOTCco, will be developed. The efficiency, linearity, precision, reproducibility and limit of detection of the assays will be assessed using the AAV cis plasmids as surrogates of target sequence. The lower limit of quantification (LLOQ) of the assays will be determined prior to the assay on test tissues or excreta initiated. A qualification plan will be implemented to bridge the transgene specific assays to the qualification studies conducted previously. The matrix tested will include intended target, liver for biodistribution. The matrix effect will be further evaluated based on the recovery of spiked target controls from all samples tested in the course of biodistribution studies as well as the data subtracted from the qualification studies conducted previously.

EXAMPLE 10 –HDLR MINI GENE KNOCK-IN IN PCSK9 LOCUS BY SACAS9 IN PCSK9-HE7-KI.LDLR/LDLR⁻.APOBEC⁻/APOBEC⁻ PUPS (HOFH MODEL)

This study aims to assess whether Cas9-mediated knock-in of the human *LDLR* gene in newborn *PCSK9-hE7-KI.ldr/ldr.apobec/apobec* mice can achieve therapeutic human LDLR expression in the target tissue for treatment of familial hypercholesterolemia (liver) following a single co-administration of a SaCas9 nuclease-expressing vector and a human *LDLR* donor vector via the intended clinical ROA (IV). A mouse model was generated using the experimental design in FIG. 15. In the mouse model, mouse PCSK9 exon 7 is replaced with human PCSK9 exon 7, that contains the SaCas9 targeting sequences.

On Day 0, newborn *PCSK9-hE7-KI.ldr/ldr.apobec/apobec* mice were IV co-administered an AAVrh79 vector expressing Cas9 (AAVrh79.U6.sgR3.PSCK9.APB2.HLP.SaCas9.bGH) at a dose of 1.0×10^{13} GC/kg in combination with one of two different AAVrh79 hLDLR donor vectors at a dose of 3.0×10^{13} GC/kg. A schematic showing the vectors used is shown in FIG. 16. Specifically, one of the donor vectors assessed in this study included a mouse-human hybrid HDR sequence (AAVrh79.mhHDR.hLDLR011) and the other included a shorter version of the human HDR sequence (AAVrh79.shHDR.hLDLR011). As a negative control, additional age-matched *PCSK9-hE7-KI.sp^{fash}* mice were administered an AAVrh79 vector expressing no saCas9 in combination with AAVrh79.shHDR.hLDLR011.

In-life evaluations include viability monitoring performed daily, and assessment of serum LDL-c levels at days 42, 63, 90, 120, and 150. A partial hepatectomy at Day 63 to evaluate the stability of human *LDLR* transduction, and necropsy at date 150. At necropsy, livers are collected to evaluate the knock-in of the human *LDLR* gene, including assessment of human *LDLR* mRNA expression (*in situ* hybridization), LDLR protein expression (immunostaining). Liver DNA is isolated to assess on-target editing (amplicon-seq, Oxford nanopore long-read sequencing) and evaluate vector genome copies. Experimental design is shown in FIG. 17.

Preliminary results show mice dosed with saCas9 and donor vectors had significantly reduced serum LDL levels. There was no change of LDL following 2/3 partial hepatectomy indicating stable integration (FIG. 18A). Indels were consistent using mhHDR and shHDR donor vectors (FIG. 18B). At day 63, shHDR-treated mice showed slightly higher hLDLR levels (FIG. 18C), while serum LDL levels were similar for mhHDR and shHDR (with saCas9) vectors (FIG. 18D).

FIG. 19 shows immunohistochemistry evaluation of hLDLR expression in day 63 liver following partial hepatectomy.

All documents cited in this specification are incorporated herein by reference, as are sequences and text of the Sequence Listing filed herewith are incorporated by reference. US Provisional Patent Application Nos. 63/180,603 filed April 27, 2021, 63/242,474, filed September 9, 2021, 63/244,205, filed September 14, 2021, 63/301,933, filed January 21, 2022, 63/331,385, filed April 15, 2022 are each incorporated by reference in their entirety. While the invention has been described with reference to particular embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

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42	<221> CDS
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WHAT IS CLAIMED IS:

1. A system for treating a genetic disorder, the system comprising:
 - (a) a gene editing vector comprising a nucleic acid sequence encoding a nuclease that targets the PCSK9 gene; and
 - (b) a donor vector comprising a transgene cassette comprising a nucleic acid sequence encoding a transgene and regulatory sequences that direct expression of the transgene in the target cell, the donor vector further comprising homology-directed recombination (HDR) arms 5' and 3' to the transgene cassette, wherein the transgene is not PCSK9.
2. The system according to claim 1, further comprising regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene
3. The system according to claim 1 or claim 2, wherein the nuclease targets PCSK9 exon 7.
4. The system according to any one of claims 1 to 3, wherein the nuclease is a meganuclease specific for PCSK9.
5. The system according to claim 4, wherein the meganuclease is the ARCUS meganuclease.
6. The system according to claim 1 or 2, wherein the gene editing vector comprises a sequence that encodes a Cas9 flanked by nuclear localization signals.
7. The system according to claim 6, wherein the gene editing vector further comprises an sgRNA comprising at least 20 nucleotides, which specifically binds to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9.

8. The system according to claim 6, wherein the donor vector further comprises an sgRNA comprising an at least 20 nucleotide seed region, wherein the sgRNA specifically binds to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9.
9. The system according to any one of claims 6 to 8, further comprising an RNA polymerase promoter.
10. The system according to claim 9, wherein the RNA polymerase promoter is the U6 promoter.
11. The system according to claim 10, wherein the U6 promoter is located 5' of the sgRNA.
12. The system according to any one of claims 7 to 11, wherein the seed region is 100% complementary to the target site sequence.
13. The system according to any one of claims 7 to 11, wherein the seed region is less than 100% complementary to the target site sequence.
14. The system according to any one of claims 1 to 13, wherein the transgene is OTC, PKU, CTLN1, or LDLR.
15. The system according to any one of claims 1 to 14, wherein at least one of the donor vector and gene editing vector is an adeno-associated viral (AAV) vector, and the AAV vector comprises AAV 5' ITRs and AAV 3' ITRs.
16. The system according to claim 15, wherein the ratio of gene editing AAV vector of (a) to donor AAV vector of (b) is such that donor AAV vector of (b) is in excess of gene editing vector of (a).

17. A system for treating a genetic disorder, the system comprising:

(a) a gene editing AAV comprising an AAV capsid and a first vector genome comprising a 5' ITR, a sequence encoding a meganuclease that targets PCSK9 under control of regulatory sequences that direct expression of the meganuclease in a target cell comprising a PCSK9 gene, and a 3' ITR; and

(b) a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR.

18. A system for treating a genetic disorder, the system comprising:

(a) a gene editing AAV comprising an AAV capsid and a first vector genome comprising a 5' ITR, a 5' nuclear localization signal (NLS), a sequence encoding a Cas9 and regulatory sequences that direct expression of the saCas9 in a target cell comprising the PCSK9 gene, a 3' NLS, and a 3' ITR; and

(b) a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, a U6 promoter, a sgRNA comprising at least 20 nucleotides that specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9, and a 3' ITR.

19. A system for treating a genetic disorder, the system comprising:

(a) a gene editing AAV vector comprising an AAV capsid and a first vector genome comprising a 5' ITR, a U6 promoter, a sgRNA comprising at least 20 nucleotides that specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9, a 5' nuclear localization signal (NLS), a sequence encoding a Cas9 and regulatory sequences that direct expression of the Cas9 in a target cell comprising the PCSK9 gene, a 3' NLS, and a 3' ITR; and

(b) a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and

regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR.

20. A system for treating a genetic disorder, the system comprising:
- (a) a gene editing vector comprising:
 - (i) a lipid nanoparticle;
 - (ii) a sgRNA comprising at least 20 nucleotides that specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by a Cas9;
 - (iii) an mRNA comprising a 5' nuclear localization signal (NLS), a sequence encoding the Cas9, a 3' NLS; and
 - (b) a donor AAV vector comprising an AAV capsid and a second vector genome comprising: a 5' ITR, a 5' homology directed recombination (HDR) arm, a transgene and regulatory sequences that direct expression of the transgene in the target cell, a 3' HDR arm, and a 3' ITR.
21. The system according to any one of claims 17 to 19, wherein the gene editing AAV vector of (a) and the donor AAV vector of (b) have the same AAV capsid.
22. The system according to claim 21, wherein the AAV capsid is selected from AAV8, AAV9, rh10, AAV6.2, AAV3B, hu37, rh79, and rh64.
23. The system according to any one of claims 6 to 18, or 18 to 22, wherein Cas9 is selected from *Staphylococcus aureus* or *Streptococcus pyogenes* Cas9.
24. The system according to any one of claims 2 to 19, wherein the nuclease is under the control of a tissue-specific promoter.
25. The system according to any one of claims 2 to 19, wherein the nuclease is under the control of a constitutive promoter.

26. The system according to claim 24, wherein the nuclease is under the control of a liver-specific promoter, optionally a human thyroxin-binding globulin (TBG) promoter, or hybrid liver promoter (HLP).
27. A method of treating a disorder in humans by co-administering the system according to any one of claims 1 to 26.
28. A method of treating a liver metabolic disorder in a neonate subject, the method comprising: co-administering to the subject having a liver metabolic disorder:
- (a) a gene editing AAV vector comprising a sequence encoding a nuclease and regulatory sequences that direct expression of the nuclease in a target cell comprising a PCSK9 gene; and
 - (b) a donor AAV vector comprising a transgene and regulatory sequences that direct expression of the transgene in the target cell, the donor vector further comprising homology-directed recombination (HDR) arms 5' and 3' to the transgene cassette.
29. The method according to claim 28, wherein the gene editing AAV vector of (a) and the donor vector of (b) are delivered essentially simultaneously via the same route.
30. The method according to claim 28 or claim 29, wherein the gene editing AAV vector of (a) is suspended in a vehicle for injection at a concentration of about 2×10^{11} GC/mL to about 2×10^{12} GC/mL.
31. The method according to claim 28 or claim 29, wherein the AAV targeting vector of (a) is suspended in a vehicle for injection at a concentration of about 2×10^{12} GC/mL to about 1×10^{13} GC/mL.
32. The method according to any one of claims 28 to 31, wherein the liver metabolic disorder is ornithine transcarbamylase.

33. The method according to any one of claims 28 to 31, wherein the liver metabolic disorder is OTC, FH, citrullinemia type I (CTLN1) or phenylketonuria.
34. A system for treating genetic disorders, the system comprising:
(a) a lipid nanoparticle (LNP) comprising a mRNA sequence encoding a nuclease;
and
(b) a donor AAV vector comprising a transgene and regulatory sequences which direct its expression in the target cell, the donor vector further comprising a homology-directed recombination (HDR) arms 5' and 3' to the transgene.
35. The system according to claim 34, wherein the nuclease targets the PCSK9 gene.
36. The system according to claim 34, wherein the nuclease targets PCSK9 exon 7.
37. The system according to claim 34, wherein the nuclease is a meganuclease specific for PCSK9.
38. The system according to claim 37, wherein the meganuclease is the ARCUS meganuclease.
39. The system according to claim 34, wherein the nuclease is a Cas9 nuclease and wherein said LNP comprises an sgRNA.
40. The system according to claim 39, wherein the Cas9 nuclease is flanked by nuclear localization signals.
41. The system according to claim 39 or 40, wherein the sgRNA comprises at least 20 nucleotides which specifically bind to a target site in the PCSK9 gene, said target site being 5' to a protospacer-adjacent motif (PAM) that is specifically recognized by the Cas9.

42. The system according to any one of claims 34 to 41, further comprising an RNA polymerase promoter.
43. The system according to claim 43, wherein the RNA polymerase promoter is the U6 promoter.
44. The system according to claim 46, wherein the U6 promoter is located 5' of the sgRNA.
45. The system according to any one of claims 34 to 44, wherein the sgRNA is 100% complementary to the target site sequence.
46. The system according to any one of claims 34 to 44, wherein the sgRNA is less than 100% complementary to the target site sequence.
47. The system according to any one of claims 34 to 46, wherein the transgene is a liver-expressed gene.
48. The system according to any one of claims 34 to 47, wherein the transgene is selected from OTC, PKU, CTLN1 and FH.
49. A system for treating a genetic disorder, the system comprising:
(a) a gene editing vector comprising a nucleic acid sequence encoding a nuclease;
and
(b) a donor vector comprising a nucleic acid sequence encoding an exogenous product for expression from the PCSK9 locus, wherein the inserted nucleic acid sequence does not encode PCSK9,
wherein the system further comprises sequences that direct the nuclease to specifically targets the native PCSK9 gene locus; and
wherein the native PCSK9 in the target cell is optionally ablated or reduced post-dosing with the dual vector system.

50. A method of treating a patient using the system of claim 49, wherein the patient's native PCSK9 expression levels are reduced and wherein the patient expresses the exogenous product.
51. An expression cassette comprising the engineered coding sequence of SEQ ID NO: 17, or a sequence sharing at least 90% identity therewith.
52. The expression cassette according to claim 51, further comprising AAV 5' and 3' ITRs.
53. An AAV vector comprising the expression cassette according to claim 51 or 52.