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(71) Applicant: **REGENERON PHARMACEUTICALS, INC.** [US/US]; 777 Old Saw Mill River Road, Tarrytown, New York 10591 (US).

(72) Inventors: **HAINES, Jeffery**; c/o Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591 (US). **CROSBY, Keith**; c/o Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591 (US). **DRUMMOND-SAMUELSON, Meghan**; c/o Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, New Jersey 10591 (US). **FRENDEWEY, David**; c/o Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591 (US). **ZAMBROWICZ, Brian**; c/o Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591 (US). **MURPHY, Andrew J.**; c/o Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591 (US).

(74) Agent: **SCHILLING, Stephen H.** et al.; Alston & Bird LLP, Bank of America Plaza, 101 South Tryon Street, Suite 4000, Charlotte, North Carolina 28280-4000 (US).

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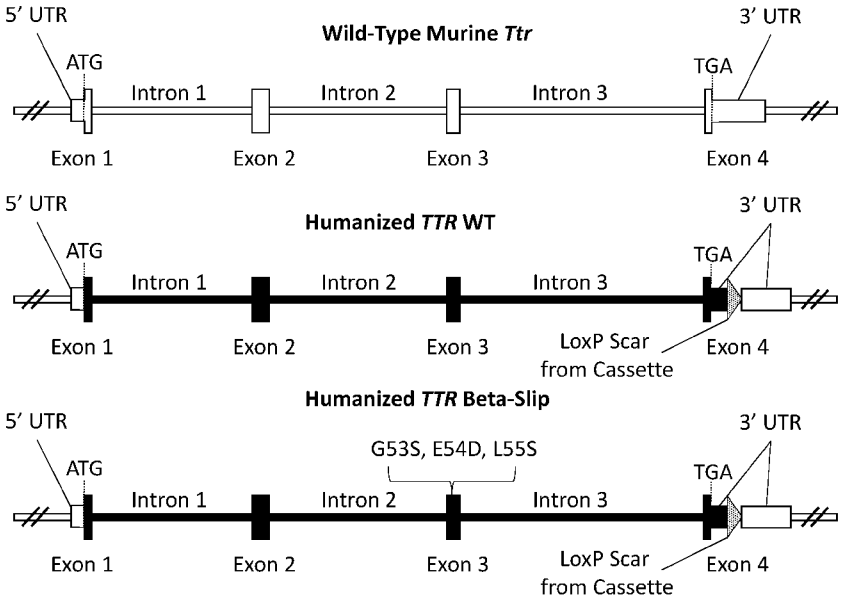


FIG. 2

(57) **Abstract:** Non-human animal cells and non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation and methods of using such non-human animal cells and non-human animals are provided. Non-human animal cells or non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation express a human transthyretin protein or a chimeric transthyretin protein, fragments of which are from human transthyretin. Methods are provided for using such non-human animals comprising a humanized *TTR* locus to assess *in vivo* efficacy of human-*TTR*-targeting reagents such as nuclease agents designed to target human *TTR*.

[Continued on next page]

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NON-HUMAN ANIMALS COMPRISING A HUMANIZED *TTR* LOCUS WITH A BETA-SLIP MUTATION AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of US Application No. 62/856,999, filed June 4, 2019, which is herein incorporated by reference in its entirety for all purposes.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS WEB

[0002] The Sequence Listing written in file 547028SEQLIST.txt is 124 kilobytes, was created on May 25, 2020, and is hereby incorporated by reference.

BACKGROUND

[0003] Transthyretin (TTR) is a protein found in the serum and cerebrospinal fluid that carries thyroid hormone and retinol-binding protein to retinol. The liver secretes TTR into the blood, while the choroid plexus secretes it into the cerebrospinal fluid. TTR is also produced in the retinal pigmented epithelium and secreted into the vitreous. Misfolded and aggregated TTR accumulates in multiple tissues and organs in the amyloid diseases senile systemic amyloidosis (SSA), familial amyloid polyneuropathy (FAP), and familial amyloid cardiomyopathy (FAC).

[0004] There remains a need for suitable non-human animals providing the true human target or a close approximation of the true human target of human-TTR-targeting reagents at the endogenous *Ttr* locus, thereby enabling testing of the efficacy and mode of action of such agents in live animals as well as pharmacokinetic and pharmacodynamics studies in a setting where the humanized protein and humanized gene are the only version of TTR present.

SUMMARY

[0005] Non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation are provided, as well as methods of using such non-human animals. Also provided are non-human animals, non-human animal cells, and non-human animal genomes comprising a humanized *TTR* locus comprising a beta-slip mutation are provided, as well as methods of making and using such non-human animals, non-human animal cells, and non-human animal

genomes. Also provided are humanized non-human animal *TTR* genes comprising a beta-slip mutation, nuclease agents and/or targeting vectors for use in humanizing a non-human animal *TTR* gene, and methods of making and using such humanized *TTR* genes.

[0006] In one aspect, provided are non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation. Also provided are non-human animals, non-human animal cells, and non-human animal genomes comprising in their genome a humanized *TTR* locus comprising a beta-slip mutation. Such non-human animals can comprise a genetically modified endogenous *Ttr* locus, wherein a region of the endogenous *Ttr* locus comprising both *Ttr* coding sequence and non-coding sequence has been deleted and replaced with an orthologous human *TTR* sequence comprising both *TTR* coding sequence and non-coding sequence, and wherein the genetically modified endogenous *Ttr* locus comprises a mutation that causes a shift in beta-strand D of the encoded transthyretin protein. Some such non-human animals, non-human animal cells, and non-human animal genomes can comprise a genetically modified endogenous *Ttr* locus, wherein a region of the endogenous *Ttr* locus comprising both *Ttr* coding sequence and non-coding sequence has been deleted and replaced with a corresponding human *TTR* sequence comprising both *TTR* coding sequence and non-coding sequence, and wherein the genetically modified endogenous *Ttr* locus comprises a mutation that causes a shift in beta-strand D of the encoded transthyretin protein. Optionally, the mutation causes a three-residue shift in beta strand D that places a residue corresponding to residue L58 in a human transthyretin protein at a position normally occupied by a residue corresponding to residue L55 in the human transthyretin protein when the encoded transthyretin protein is optimally aligned with the human transthyretin protein. Optionally, the mutation is a triple mutation corresponding to G53S/E54D/L55S in the human transthyretin protein when the encoded transthyretin protein is optimally aligned with the human transthyretin protein. Optionally, the triple mutation is in the orthologous human *TTR* sequence. Optionally, the triple mutation is in the corresponding human *TTR* sequence.

[0007] In some such non-human animals, the genetically modified endogenous *Ttr* locus comprises the endogenous *Ttr* promoter. In some such non-human animals, non-human animal cells, and non-human animal genomes the genetically modified endogenous *Ttr* locus comprises the endogenous *Ttr* promoter, wherein the human *TTR* sequence is operably linked to the endogenous *Ttr* promoter. Optionally, at least one intron and at least one exon of the endogenous *Ttr* locus have been deleted and replaced with the orthologous human *TTR* sequence.

Optionally, at least one intron and at least one exon of the endogenous *Ttr* locus have been deleted and replaced with the corresponding human *TTR* sequence.

[0008] In some such non-human animals, the entire *Ttr* coding sequence of the endogenous *Ttr* locus has been deleted and replaced with the orthologous human *TTR* sequence. Optionally, the region of the endogenous *Ttr* locus from the *Ttr* start codon to the *Ttr* stop codon has been deleted and replaced with the orthologous human *TTR* sequence. In some such non-human animals, non-human animal cells, and non-human animal genomes the entire *Ttr* coding sequence of the endogenous *Ttr* locus has been deleted and replaced with the corresponding human *TTR* sequence. Optionally, the region of the endogenous *Ttr* locus from the *Ttr* start codon to the *Ttr* stop codon has been deleted and replaced with the corresponding human *TTR* sequence.

[0009] In some such non-human animals, the genetically modified endogenous *Ttr* locus comprises a human *TTR* 3' untranslated region. In some such non-human animals, the endogenous *Ttr* 5' untranslated region has not been deleted and replaced with the orthologous human *TTR* sequence. In some such non-human animals, non-human animal cells, and non-human animal genomes the genetically modified endogenous *Ttr* locus comprises a human *TTR* 3' untranslated region. In some such non-human animals, non-human animal cells, and non-human animal genomes the endogenous *Ttr* 5' untranslated region has not been deleted and replaced with the corresponding human *TTR* sequence.

[0010] In some such non-human animals, the region of the endogenous *Ttr* locus from the *Ttr* start codon to the *Ttr* stop codon has been deleted and replaced with a human *TTR* sequence comprising the orthologous human *TTR* sequence and a human *TTR* 3' untranslated region, and the endogenous *Ttr* 5' untranslated region has not been deleted and replaced with the orthologous human *TTR* sequence, and the endogenous *Ttr* promoter has not been deleted and replaced with the orthologous human *TTR* sequence. In some such non-human animals, non-human animal cells, and non-human animal genomes the region of the endogenous *Ttr* locus from the *Ttr* start codon to the *Ttr* stop codon has been deleted and replaced with a human *TTR* sequence comprising the corresponding human *TTR* sequence and a human *TTR* 3' untranslated region, and the endogenous *Ttr* 5' untranslated region has not been deleted and replaced with the corresponding human *TTR* sequence, and the endogenous *Ttr* promoter has not been deleted and replaced with the corresponding human *TTR* sequence. Optionally, the human *TTR* sequence at

the genetically modified endogenous *Ttr* locus comprises, consists essentially of, or consists of a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 14. Optionally, the human *TTR* sequence at the genetically modified endogenous *Ttr* locus comprises, consists essentially of, or consists of a sequence at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to the sequence set forth in SEQ ID NO: 14. Optionally, the genetically modified endogenous *Ttr* locus encodes a protein comprising, consisting essentially of, or consisting of a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 9. Optionally, the genetically modified endogenous *Ttr* locus encodes a protein comprising, consisting essentially of, or consisting of a sequence at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to the sequence set forth in SEQ ID NO: 9. Optionally, the genetically modified endogenous *Ttr* locus comprises a coding sequence comprising, consisting essentially of, or consisting of a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 10. Optionally, the genetically modified endogenous *Ttr* locus comprises a coding sequence comprising, consisting essentially of, or consisting of a sequence at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to the sequence set forth in SEQ ID NO: 10. Optionally, the genetically modified endogenous *Ttr* locus comprises, consists essentially of, or consists of a sequence at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the sequence set forth in SEQ ID NO: 12 or 13. Optionally, the genetically modified endogenous *Ttr* locus comprises, consists essentially of, or consists of a sequence at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to the sequence set forth in SEQ ID NO: 12 or 13.

[0011] In some such non-human animals, the genetically modified endogenous *Ttr* locus encodes a transthyretin precursor protein comprising a signal peptide, and the region of the endogenous *Ttr* locus encoding the signal peptide has not been deleted and replaced with the orthologous human *TTR* sequence. Optionally, the first exon of the endogenous *Ttr* locus has not been deleted and replaced with the orthologous human *TTR* sequence. Optionally, the first exon and first intron of the endogenous *Ttr* locus have not been deleted and replaced with the orthologous human *TTR* sequence. Optionally, the region of the endogenous *Ttr* locus from the

start of the second *Ttr* exon to the *Ttr* stop codon has been deleted and replaced with the orthologous human *TTR* sequence. Optionally, the genetically modified endogenous *Ttr* locus comprises a human *TTR* 3' untranslated region. In some such non-human animals, non-human animal cells, and non-human animal genomes the genetically modified endogenous *Ttr* locus encodes a transthyretin precursor protein comprising a signal peptide, and the region of the endogenous *Ttr* locus encoding the signal peptide has not been deleted and replaced with the corresponding human *TTR* sequence. Optionally, the first exon of the endogenous *Ttr* locus has not been deleted and replaced with the corresponding human *TTR* sequence. Optionally, the first exon and first intron of the endogenous *Ttr* locus have not been deleted and replaced with the corresponding human *TTR* sequence. Optionally, the region of the endogenous *Ttr* locus from the start of the second *Ttr* exon to the *Ttr* stop codon has been deleted and replaced with the corresponding human *TTR* sequence. Optionally, the genetically modified endogenous *Ttr* locus comprises a human *TTR* 3' untranslated region.

[0012] In some such non-human animals, the region of the endogenous *Ttr* locus from the second *Ttr* exon to the *Ttr* stop codon has been deleted and replaced with a human *TTR* sequence comprising the orthologous human *TTR* sequence and a human *TTR* 3' untranslated region, and the endogenous *Ttr* 5' untranslated region has not been deleted and replaced with the orthologous human *TTR* sequence, and the endogenous *Ttr* promoter has not been deleted and replaced with the orthologous human *TTR* sequence. In some such non-human animals, non-human animal cells, and non-human animal genomes the region of the endogenous *Ttr* locus from the second *Ttr* exon to the *Ttr* stop codon has been deleted and replaced with a human *TTR* sequence comprising the corresponding human *TTR* sequence and a human *TTR* 3' untranslated region, and the endogenous *Ttr* 5' untranslated region has not been deleted and replaced with the corresponding human *TTR* sequence, and the endogenous *Ttr* promoter has not been deleted and replaced with the corresponding human *TTR* sequence.

[0013] In some such non-human animals, the genetically modified endogenous *Ttr* locus does not comprise a selection cassette or a reporter gene. In some such non-human animals, the genetically modified endogenous *Ttr* locus does comprise a selection cassette or a reporter gene. In some such non-human animals, the non-human animal is homozygous for the genetically modified endogenous *Ttr* locus. In some such non-human animals, the non-human animal is heterozygous for the genetically modified endogenous *Ttr* locus. In some such non-human

animals, non-human animal cells, and non-human animal genomes the genetically modified endogenous *Ttr* locus does not comprise a selection cassette or a reporter gene. In some such non-human animals, non-human animal cells, and non-human animal genomes the genetically modified endogenous *Ttr* locus does comprise a selection cassette or a reporter gene. In some such non-human animals, non-human animal cells, and non-human animal genomes the non-human animal is homozygous for the genetically modified endogenous *Ttr* locus. In some such non-human animals, non-human animal cells, and non-human animal genomes the non-human animal is heterozygous for the genetically modified endogenous *Ttr* locus. In some such non-human animals, the non-human animal comprises the genetically modified endogenous *Ttr* locus in its germline.

[0014] In some such non-human animals, the non-human animal is a mammal. In some such non-human animals, non-human animal cells, and non-human animal genomes the non-human animal is a mammal. Optionally, the mammal is a rodent. Optionally, the rodent is a rat or mouse. Optionally, the non-human animal is a mouse.

[0015] In some such non-human animals, the non-human animal is hyperactive relative to a control wild type non-human animal or a non-human animal comprising the genetically modified endogenous *Ttr* locus without the mutation. In some such non-human animals, non-human animal cells, and non-human animal genomes the non-human animal is hyperactive relative to a control wild type non-human animal or a non-human animal comprising the genetically modified endogenous *Ttr* locus without the mutation. Optionally, the hyperactivity is as measured by one or more or all of total distance, total activity, or total rearings in an open field test. In some such non-human animals, the non-human animal displays hindlimb dystonia. In some such non-human animals, non-human animal cells, and non-human animal genomes the non-human animal displays hindlimb dystonia. In some such non-human animals, wherein the non-human animal comprises amyloid deposits. In some such non-human animals, non-human animal cells, and non-human animal genomes wherein the non-human animal comprises amyloid deposits. Optionally, the non-human animal comprises amyloid deposits in the sciatic nerve. Optionally, the non-human animal develops amyloidosis by about two months of age.

[0016] In another aspect, provided are targeting vectors for generating a genetically modified endogenous *Ttr* locus in which a region of the endogenous *Ttr* locus comprising both *Ttr* coding sequence and non-coding sequence has been deleted and replaced with a corresponding human

TTR sequence comprising both *TTR* coding sequence and non-coding sequence, and wherein the genetically modified endogenous *Ttr* locus comprises a mutation that causes a shift in beta-strand D of the encoded transthyretin protein, wherein the targeting vector comprises an insert nucleic acid comprising the corresponding human *TTR* sequence flanked by a 5' homology arm targeting a 5' target sequence at the endogenous *Ttr* locus and a 3' homology arm targeting a 3' target sequence at the endogenous *Ttr* locus.

[0017] In another aspect, provided are genetically modified non-human animal *Ttr* genes in which a region of the endogenous *Ttr* gene comprising both *Ttr* coding sequence and non-coding sequence has been deleted and replaced with a corresponding human *TTR* sequence comprising both *TTR* coding sequence and non-coding sequence, and wherein the genetically modified non-human animal *Ttr* gene comprises a mutation that causes a shift in beta-strand D of the encoded transthyretin protein.

[0018] In another aspect, provided are methods of using the non-human animals comprising a humanized *TTR* locus to assess the activity of human-TTR-targeting reagents *in vivo*. Such methods can comprise: (a) administering the human-TTR-targeting reagent to any of the above non-human animals; and (b) assessing the activity of the human-TTR-targeting reagent in the non-human animal.

[0019] In some such methods, the introducing comprises adeno-associated virus (AAV)-mediated delivery, lipid nanoparticle (LNP)-mediated delivery, or hydrodynamic delivery (HDD). Optionally, the introducing comprises LNP-mediated delivery. Optionally, the introducing comprises AAV8-mediated delivery.

[0020] In some such methods, step (b) comprises isolating a liver from the non-human animal and assessing activity of the human-TTR-targeting reagent in the liver. Optionally, step (b) further comprises assessing activity of the human-TTR-targeting reagent in an organ or tissue other than the liver.

[0021] In some such methods, the assessing comprises assessing modification of the genetically modified *Ttr* locus. In some such methods, the assessing comprises assessing expression of a *Ttr* messenger RNA encoded by the genetically modified *Ttr* locus. In some such methods, the assessing comprises assessing expression of a TTR protein encoded by the genetically modified *Ttr* locus. Optionally, assessing expression of the TTR protein comprises measuring serum levels of the TTR protein in the non-human animal. Optionally, the activity is

assessed in the liver of the non-human animal.

[0022] In some such methods, the assessing comprises assessing hyperactivity. In some such methods, the assessing comprises assessing hindlimb dystonia. In some such methods, the assessing comprises assessing amyloid deposition. Optionally, the assessing comprises assessing amyloid deposition in the sciatic nerve. In some such methods, the assessing is in comparison to an untreated control non-human animal.

[0023] In some such methods, the human-TTR-targeting reagent comprises a nuclease agent designed to target a region of a human *TTR* gene. Optionally, the nuclease agent comprises a Cas protein and a guide RNA designed to target a guide RNA target sequence in the human *TTR* gene. Optionally, the Cas protein is a Cas9 protein. In some such methods, the human-TTR-targeting reagent comprises an exogenous donor nucleic acid, wherein the exogenous donor nucleic acid is designed to recombine with the human *TTR* gene. Optionally, the exogenous donor nucleic acid is a single-stranded oligodeoxynucleotide (ssODN). In some such methods, the human-TTR-targeting reagent comprises an antigen-binding protein. In some such methods, the human-TTR-targeting reagent comprises an RNAi agent or an antisense oligonucleotide.

[0024] In some such methods, assessing the activity of the human-TTR-targeting reagent in the non-human animal comprises assessing transthyretin activity. In some such methods, the assessing is in comparison to an untreated control non-human animal.

[0025] In another aspect, provided are methods of optimizing the activity of a human-TTR-targeting reagent *in vivo*. Such methods can comprise: (I) performing any of the above methods of assessing the activity of human-TTR-targeting reagents *in vivo* a first time in a first non-human animal; (II) changing a variable and performing the method of step (I) a second time with the changed variable in a second non-human animal; and (III) comparing the activity of the human-TTR-targeting reagent in step (I) with the activity of the human-TTR-targeting reagent in step (II), and selecting the method resulting in the higher efficacy, higher precision, higher consistency, or higher specificity. Such methods can comprise: (I) performing any of the above methods of assessing the activity of human-TTR-targeting reagents *in vivo* a first time in a first non-human animal comprising in its genome the genetically modified endogenous *Ttr* locus; (II) changing a variable and performing the method of step (I) a second time with the changed variable in a second non-human animal comprising in its genome the genetically modified endogenous *Ttr* locus; and (III) comparing the activity of the human-TTR-targeting reagent in

step (I) with the activity of the human-TTR-targeting reagent in step (II), and selecting the method resulting in the higher efficacy, higher precision, higher consistency, or higher specificity.

[0026] Optionally, the changed variable in step (II) is the delivery method of introducing the human-TTR-targeting reagent into the non-human animal. Optionally, the changed variable in step (II) is the route of administration of introducing the human-TTR-targeting reagent into the non-human animal. Optionally, the changed variable in step (II) is the concentration or amount of the human-TTR-targeting reagent introduced into the non-human animal. Optionally, the changed variable in step (II) is the form of the human-TTR-targeting reagent introduced into the non-human animal. Optionally, the changed variable in step (II) is the human-TTR-targeting reagent introduced into the non-human animal.

[0027] In another aspect, provided are methods of making the non-human animals comprising a humanized *TTR* locus. Such methods can comprise: (a) modifying the genome of a pluripotent non-human animal cell to comprise the genetically modified endogenous *Ttr* locus; (b) identifying or selecting the genetically modified pluripotent non-human animal cell comprising the genetically modified endogenous *Ttr* locus; (c) introducing the genetically modified pluripotent non-human animal cell into a non-human animal host embryo; and (d) gestating the non-human animal host embryo in a surrogate mother. Such methods can alternatively comprise: (a) modifying the genome of a non-human animal one-cell stage embryo to comprise the genetically modified endogenous *Ttr* locus; (b) selecting the genetically modified non-human animal one-cell stage embryo comprising the genetically modified endogenous *Ttr* locus; and (c) gestating the genetically modified non-human animal one-cell stage embryo in a surrogate mother.

BRIEF DESCRIPTION OF THE FIGURES

[0028] **Figure 1A** shows an alignment of mouse, human wild type, and human beta-slip transthyretin (TTR) precursor proteins (SEQ ID NOS: 5, 1, and 9, respectively). The signal peptide, T4 binding domain, phase 0 exon/intron boundaries, and phase 1/2 exon/intron boundaries are denoted, along with the beta-slip mutations.

[0029] **Figure 1B** shows an alignment of mouse, human wild type, and human beta-slip transthyretin (TTR) coding sequences (SEQ ID NOS: 8, 4, and 10, respectively). The signal

peptide, T4 binding domain, phase 0 exon/intron boundaries, and phase 1/2 exon/intron boundaries are denoted, along with the beta-slip mutations.

[0030] **Figure 2** shows schematics (not drawn to scale) of the wild-type murine *Ttr* locus, a wild type humanized mouse *Ttr* locus (wild type human *TTR*), and a mutant humanized mouse *Ttr* locus (beta-slip human *TTR*). Exons, introns, 5' untranslated regions (UTRs), 3' UTRs, start codons (ATG), stop codons (TGA), and loxP scars from selection cassettes are denoted. White boxes indicate murine sequence; black boxes indicate human sequence.

[0031] **Figure 3** shows a schematic (not drawn to scale) of the targeting to create the mutant (beta-slip) humanized mouse *Ttr* locus. The wild type mouse *Ttr* locus, the F0 allele of the mutant humanized mouse *Ttr* locus with the self-deleting neomycin (SDC-Puro) selection cassette (MAID 8530), and the F1 allele of the mutant humanized mouse *Ttr* locus with the loxP scar from removal of the SDC-Puro selection cassette (MAID 8531) are shown. White boxes indicate murine sequence; black boxes indicate human sequence.

[0032] **Figure 4** shows a schematic (not drawn to scale) of the strategy for screening of the targeted mouse *Ttr* locus, including loss-of-allele assays (7576mTU, 4552mTU, 9212mTU, 7655mTU, 9090mTM, 7576mTD, 9212mTD, and 7655mTD), gain of allele assays (7576hTU, 7655hTU, 7576hTD, Puro), retention assays (9204mretU, 9090retU, 9090retU2, 9090retU3, 9090retD, 9090retD2, 9090retD3, 9204mretD), and CRISPR assays designed to cover the region that is disrupted by the CRISPR guides (9090mTGU, mGU, 9090mTGD, and mGD). White boxes indicate murine sequence; black boxes indicate human sequence.

[0033] **Figures 5A-5D** show plasma hTTR levels (**Figure 5A**), serum total T4 levels (**Figure 5B**), serum free T4 levels (**Figure 5C**), and body temperature (**Figure 5D**) in two-month-old humanized *TTR* wild type mice, humanized *TTR* beta-slip mice, and F1H4 control mice. Dystonic mice are marked by the encircled red triangles. TTR and T4 levels were measured by ELISA.

[0034] **Figure 6A** shows a western blot following denaturing PAGE of plasma samples from two-month old humanized *TTR* beta-slip mice. F1H4 samples were used as a negative control, and recombinant WT human TTR was used as a positive control. Transferrin was used as a loading control.

[0035] **Figure 6B** shows a western blot following native PAGE of plasma samples from two-month old humanized *TTR* beta-slip mice and samples from two-month old humanized *TTR* wild

type mice. F1H4 samples were used as a negative control, and recombinant WT human TTR was used as a positive control.

[0036] **Figures 7A-7B** show the dystonic phenotype in humanized *TTR* beta-slip mice. **Figure 7A** show pictures of two-month old humanized *TTR* beta-slip mice, humanized *TTR* wild type mice, and control F1H4 mice after scruffing to assess the angle of their hindlimbs relative to the axis of their bodies. **Figure 7B** shows a quantification of the number of normal mice and the number of mice with a dystonic phenotype. Dystonic mice are marked by the encircled red dots.

[0037] **Figures 8A-8C** show various readouts from two-month old humanized *TTR* beta-slip mice, humanized *TTR* wild type mice, and control F1H4 mice in an open field behavioral test, including total distance (**Figure 8A**), total activity (**Figure 8B**), and rearings (**Figure 8C**).

[0038] **Figures 9A-9B** show body weight (**Figure 9A**) and grip strength (**Figure 9B**) in two-month old humanized *TTR* beta-slip mice, humanized *TTR* wild type mice, and control F1H4 mice. Dystonic mice are marked by the encircled red triangles and dots.

[0039] **Figures 10A-10B** show Congo Red staining in sciatic nerve samples (**Figure 10A**) and liver samples (**Figure 10B**) isolated from two-month old humanized *TTR* beta-slip mice, humanized *TTR* wild type mice, and control F1H4 mice. The top portions of each show the stained tissue imaged under white light. The bottom portions of each show the stained tissue illuminated using linear polarized light.

DEFINITIONS

[0040] The terms "protein," "polypeptide," and "peptide," used interchangeably herein, include polymeric forms of amino acids of any length, including coded and non-coded amino acids and chemically or biochemically modified or derivatized amino acids. The terms also include polymers that have been modified, such as polypeptides having modified peptide backbones. The term "domain" refers to any part of a protein or polypeptide having a particular function or structure.

[0041] Proteins are said to have an "N-terminus" and a "C-terminus." The term "N-terminus" relates to the start of a protein or polypeptide, terminated by an amino acid with a free amine group (-NH2). The term "C-terminus" relates to the end of an amino acid chain (protein or polypeptide), terminated by a free carboxyl group (-COOH).

[0042] The terms "nucleic acid" and "polynucleotide," used interchangeably herein, include polymeric forms of nucleotides of any length, including ribonucleotides, deoxyribonucleotides, or analogs or modified versions thereof. They include single-, double-, and multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, and polymers comprising purine bases, pyrimidine bases, or other natural, chemically modified, biochemically modified, non-natural, or derivatized nucleotide bases.

[0043] Nucleic acids are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. An end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring. An end of an oligonucleotide is referred to as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of another mononucleotide pentose ring. A nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements.

[0044] The term "genomically integrated" refers to a nucleic acid that has been introduced into a cell such that the nucleotide sequence integrates into the genome of the cell. Any protocol may be used for the stable incorporation of a nucleic acid into the genome of a cell.

[0045] The term "expression vector" or "expression construct" or "expression cassette" refers to a recombinant nucleic acid containing a desired coding sequence operably linked to appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host cell or organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, as well as other sequences. Eukaryotic cells are generally known to utilize promoters, enhancers, and termination and polyadenylation signals, although some elements may be deleted and other elements added without sacrificing the necessary expression.

[0046] The term "targeting vector" refers to a recombinant nucleic acid that can be introduced by homologous recombination, non-homologous-end-joining-mediated ligation, or any other means of recombination to a target position in the genome of a cell.

[0047] The term "viral vector" refers to a recombinant nucleic acid that includes at least one element of viral origin and includes elements sufficient for or permissive of packaging into a viral vector particle. The vector and/or particle can be utilized for the purpose of transferring DNA, RNA, or other nucleic acids into cells *in vitro*, *ex vivo*, or *in vivo*. Numerous forms of viral vectors are known.

[0048] The term "isolated" with respect to proteins, nucleic acids, and cells includes proteins, nucleic acids, and cells that are relatively purified with respect to other cellular or organism components that may normally be present *in situ*, up to and including a substantially pure preparation of the protein, nucleic acid, or cell. The term "isolated" also includes cells, proteins, and nucleic acids that have no naturally occurring counterpart or proteins or nucleic acids that have been chemically synthesized and are thus substantially uncontaminated by other proteins or nucleic acids. The term "isolated" also includes proteins, nucleic acids, or cells that have been separated or purified from most other cellular components or organism components with which they are naturally accompanied (e.g., other cellular proteins, nucleic acids, or cellular or extracellular components).

[0049] The term "wild type" includes entities having a structure and/or activity as found in a normal (as contrasted with mutant, diseased, altered, or so forth) state or context. Wild type genes and polypeptides often exist in multiple different forms (e.g., alleles).

[0050] The term "endogenous sequence" refers to a nucleic acid sequence that occurs naturally within a cell or non-human animal. For example, an endogenous *Ttr* sequence of a non-human animal refers to a native *Ttr* sequence that naturally occurs at the *Ttr* locus in the non-human animal.

[0051] "Exogenous" molecules or sequences include molecules or sequences that are not normally present in a cell in that form. Normal presence includes presence with respect to the particular developmental stage and environmental conditions of the cell. An exogenous molecule or sequence, for example, can include a mutated version of a corresponding endogenous sequence within the cell, such as a humanized version of the endogenous sequence, or can include a sequence corresponding to an endogenous sequence within the cell but in a different form (i.e., not within a chromosome). In contrast, endogenous molecules or sequences include molecules or sequences that are normally present in that form in a particular cell at a particular developmental stage under particular environmental conditions.

[0052] The term "heterologous" when used in the context of a nucleic acid or a protein indicates that the nucleic acid or protein comprises at least two segments that do not naturally occur together in the same molecule. For example, the term "heterologous," when used with reference to segments of a nucleic acid or segments of a protein, indicates that the nucleic acid or protein comprises two or more sub-sequences that are not found in the same relationship to each other (e.g., joined together) in nature. As one example, a "heterologous" region of a nucleic acid vector is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a nucleic acid vector could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Likewise, a "heterologous" region of a protein is a segment of amino acids within or attached to another peptide molecule that is not found in association with the other peptide molecule in nature (e.g., a fusion protein, or a protein with a tag). Similarly, a nucleic acid or protein can comprise a heterologous label or a heterologous secretion or localization sequence.

[0053] "Codon optimization" takes advantage of the degeneracy of codons, as exhibited by the multiplicity of three-base pair codon combinations that specify an amino acid, and generally includes a process of modifying a nucleic acid sequence for enhanced expression in particular host cells by replacing at least one codon of the native sequence with a codon that is more frequently or most frequently used in the genes of the host cell while maintaining the native amino acid sequence. For example, a nucleic acid encoding a Cas9 protein can be modified to substitute codons having a higher frequency of usage in a given prokaryotic or eukaryotic cell, including a bacterial cell, a yeast cell, a human cell, a non-human cell, a mammalian cell, a rodent cell, a mouse cell, a rat cell, a hamster cell, or any other host cell, as compared to the naturally occurring nucleic acid sequence. Codon usage tables are readily available, for example, at the "Codon Usage Database." These tables can be adapted in a number of ways. *See* Nakamura *et al.* (2000) *Nucleic Acids Research* 28:292, herein incorporated by reference in its entirety for all purposes. Computer algorithms for codon optimization of a particular sequence for expression in a particular host are also available (*see, e.g.*, Gene Forge).

[0054] The term "locus" refers to a specific location of a gene (or significant sequence), DNA sequence, polypeptide-encoding sequence, or position on a chromosome of the genome of an organism. For example, a "*Ttr* locus" may refer to the specific location of a *Ttr* gene, *Ttr*

DNA sequence, transthyretin-encoding sequence, or *Ttr* position on a chromosome of the genome of an organism that has been identified as to where such a sequence resides. A "*Ttr* locus" may comprise a regulatory element of a *Ttr* gene, including, for example, an enhancer, a promoter, 5' and/or 3' untranslated region (UTR), or a combination thereof.

[0055] The term "gene" refers to a DNA sequence in a chromosome that codes for a product (e.g., an RNA product and/or a polypeptide product) and includes the coding region interrupted with non-coding introns and sequence located adjacent to the coding region on both the 5' and 3' ends such that the gene corresponds to the full-length mRNA (including the 5' and 3' untranslated sequences). The term "gene" also includes other non-coding sequences including regulatory sequences (e.g., promoters, enhancers, and transcription factor binding sites), polyadenylation signals, internal ribosome entry sites, silencers, insulating sequence, and matrix attachment regions may be present in a gene. These sequences may be close to the coding region of the gene (e.g., within 10 kb) or at distant sites, and they influence the level or rate of transcription and translation of the gene.

[0056] The term "allele" refers to a variant form of a gene. Some genes have a variety of different forms, which are located at the same position, or genetic locus, on a chromosome. A diploid organism has two alleles at each genetic locus. Each pair of alleles represents the genotype of a specific genetic locus. Genotypes are described as homozygous if there are two identical alleles at a particular locus and as heterozygous if the two alleles differ.

[0057] The "coding region" or "coding sequence" of a gene consists of the portion of a gene's DNA or RNA, composed of exons, that codes for a protein. The region begins at the start codon on the 5' end and ends at the stop codon on the 3' end.

[0058] A "promoter" is a regulatory region of DNA usually comprising a TATA box capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular polynucleotide sequence. A promoter may additionally comprise other regions which influence the transcription initiation rate. The promoter sequences disclosed herein modulate transcription of an operably linked polynucleotide. A promoter can be active in one or more of the cell types disclosed herein (e.g., a eukaryotic cell, a non-human mammalian cell, a human cell, a rodent cell, a pluripotent cell, a one-cell stage embryo, a differentiated cell, or a combination thereof). A promoter can be, for example, a constitutively active promoter, a conditional promoter, an inducible promoter, a temporally restricted promoter (e.g., a

developmentally regulated promoter), or a spatially restricted promoter (e.g., a cell-specific or tissue-specific promoter). Examples of promoters can be found, for example, in WO 2013/176772, herein incorporated by reference in its entirety for all purposes.

[0059] "Operable linkage" or being "operably linked" includes juxtaposition of two or more components (e.g., a promoter and another sequence element) such that both components function normally and allow the possibility that at least one of the components can mediate a function that is exerted upon at least one of the other components. For example, a promoter can be operably linked to a coding sequence if the promoter controls the level of transcription of the coding sequence in response to the presence or absence of one or more transcriptional regulatory factors. Operable linkage can include such sequences being contiguous with each other or acting in trans (e.g., a regulatory sequence can act at a distance to control transcription of the coding sequence).

[0060] "Complementarity" of nucleic acids means that a nucleotide sequence in one strand of nucleic acid, due to orientation of its nucleobase groups, forms hydrogen bonds with another sequence on an opposing nucleic acid strand. The complementary bases in DNA are typically A with T and C with G. In RNA, they are typically C with G and U with A. Complementarity can be perfect or substantial/sufficient. Perfect complementarity between two nucleic acids means that the two nucleic acids can form a duplex in which every base in the duplex is bonded to a complementary base by Watson-Crick pairing. "Substantial" or "sufficient" complementary means that a sequence in one strand is not completely and/or perfectly complementary to a sequence in an opposing strand, but that sufficient bonding occurs between bases on the two strands to form a stable hybrid complex in set of hybridization conditions (e.g., salt concentration and temperature). Such conditions can be predicted by using the sequences and standard mathematical calculations to predict the Tm (melting temperature) of hybridized strands, or by empirical determination of Tm by using routine methods. Tm includes the temperature at which a population of hybridization complexes formed between two nucleic acid strands are 50% denatured (i.e., a population of double-stranded nucleic acid molecules becomes half dissociated into single strands). At a temperature below the Tm, formation of a hybridization complex is favored, whereas at a temperature above the Tm, melting or separation of the strands in the hybridization complex is favored. Tm may be estimated for a nucleic acid having a known G+C content in an aqueous 1 M NaCl solution by using, e.g., Tm=81.5+0.41(% G+C), although other known Tm computations consider nucleic acid structural characteristics.

[0061] "Hybridization condition" includes the cumulative environment in which one nucleic acid strand bonds to a second nucleic acid strand by complementary strand interactions and hydrogen bonding to produce a hybridization complex. Such conditions include the chemical components and their concentrations (e.g., salts, chelating agents, formamide) of an aqueous or organic solution containing the nucleic acids, and the temperature of the mixture. Other factors, such as the length of incubation time or reaction chamber dimensions may contribute to the environment. *See, e.g.*, Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, 2.sup.nd ed., pp. 1.90-1.91, 9.47-9.51, 1 1.47-11.57 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), herein incorporated by reference in its entirety for all purposes.

[0062] Hybridization requires that the two nucleic acids contain complementary sequences, although mismatches between bases are possible. The conditions appropriate for hybridization between two nucleic acids depend on the length of the nucleic acids and the degree of complementation, variables which are well known. The greater the degree of complementation between two nucleotide sequences, the greater the value of the melting temperature (Tm) for hybrids of nucleic acids having those sequences. For hybridizations between nucleic acids with short stretches of complementarity (e.g. complementarity over 35 or fewer, 30 or fewer, 25 or fewer, 22 or fewer, 20 or fewer, or 18 or fewer nucleotides) the position of mismatches becomes important (*see* Sambrook *et al.*, supra, 11.7-11.8). Typically, the length for a hybridizable nucleic acid is at least about 10 nucleotides. Illustrative minimum lengths for a hybridizable nucleic acid include at least about 15 nucleotides, at least about 20 nucleotides, at least about 22 nucleotides, at least about 25 nucleotides, and at least about 30 nucleotides. Furthermore, the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the region of complementation and the degree of complementation.

[0063] The sequence of polynucleotide need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. Moreover, a polynucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). A polynucleotide (e.g., gRNA) can comprise at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, or 100% sequence complementarity to a target region within the target nucleic acid sequence to which they are targeted. For example, a gRNA in which 18 of 20 nucleotides are complementary to a target region, and would therefore specifically hybridize, would represent 90% complementarity.

In this example, the remaining noncomplementary nucleotides may be clustered or interspersed with complementary nucleotides and need not be contiguous to each other or to complementary nucleotides.

[0064] Percent complementarity between particular stretches of nucleic acid sequences within nucleic acids can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs (Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410; Zhang and Madden (1997) *Genome Res.* 7:649-656, each of which is herein incorporated by reference in its entirety for all purposes) or by using the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wis.), using default settings, which uses the algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2:482-489, herein incorporated by reference in its entirety for all purposes.

[0065] The methods and compositions provided herein employ a variety of different components. Some components throughout the description can have active variants and fragments. Such components include, for example, Cas proteins, CRISPR RNAs, tracrRNAs, and guide RNAs. Biological activity for each of these components is described elsewhere herein. The term "functional" refers to the innate ability of a protein or nucleic acid (or a fragment or variant thereof) to exhibit a biological activity or function. Such biological activities or functions can include, for example, the ability of a Cas protein to bind to a guide RNA and to a target DNA sequence. The biological functions of functional fragments or variants may be the same or may in fact be changed (e.g., with respect to their specificity or selectivity or efficacy) in comparison to the original molecule, but with retention of the molecule's basic biological function.

[0066] The term "variant" refers to a nucleotide sequence differing from the sequence most prevalent in a population (e.g., by one nucleotide) or a protein sequence different from the sequence most prevalent in a population (e.g., by one amino acid).

[0067] The term "fragment" when referring to a protein means a protein that is shorter or has fewer amino acids than the full-length protein. The term "fragment" when referring to a nucleic acid means a nucleic acid that is shorter or has fewer nucleotides than the full-length nucleic acid. A fragment can be, for example, when referring to a protein fragment, an N-terminal fragment (i.e., removal of a portion of the C-terminal end of the protein), a C-terminal fragment (i.e., removal of a portion of the N-terminal end of the protein), or an internal fragment (i.e.,

removal of a portion of each of the N-terminal and C-terminal ends of the protein). A fragment can be, for example, when referring to a nucleic acid fragment, a 5' fragment (i.e., removal of a portion of the 3' end of the nucleic acid), a 3' fragment (i.e., removal of a portion of the 5' end of the nucleic acid), or an internal fragment (i.e., removal of a portion each of the 5' and 3' ends of the nucleic acid).

[0068] "Sequence identity" or "identity" in the context of two polynucleotides or polypeptide sequences refers to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins, residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known. Typically, this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

[0069] "Percentage of sequence identity" includes the value determined by comparing two optimally aligned sequences (greatest number of perfectly matched residues) over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. Unless otherwise

specified (e.g., the shorter sequence includes a linked heterologous sequence), the comparison window is the full length of the shorter of the two sequences being compared.

[0070] Unless otherwise stated, sequence identity/similarity values include the value obtained using GAP Version 10 using the following parameters: % identity and % similarity for a nucleotide sequence using GAP Weight of 50 and Length Weight of 3, and the nwsgapdna.cmp scoring matrix; % identity and % similarity for an amino acid sequence using GAP Weight of 8 and Length Weight of 2, and the BLOSUM62 scoring matrix; or any equivalent program thereof. "Equivalent program" includes any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

[0071] The term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine, or leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, or between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine, or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, or methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue. Typical amino acid categorizations are summarized in **Table 1** below.

[0072] Table 1. Amino Acid Categorizations.

Alanine	Ala	A	Nonpolar	Neutral	1.8
Arginine	Arg	R	Polar	Positive	-4.5
Asparagine	Asn	N	Polar	Neutral	-3.5
Aspartic acid	Asp	D	Polar	Negative	-3.5
Cysteine	Cys	C	Nonpolar	Neutral	2.5
Glutamic acid	Glu	E	Polar	Negative	-3.5
Glutamine	Gln	Q	Polar	Neutral	-3.5
Glycine	Gly	G	Nonpolar	Neutral	-0.4
Histidine	His	H	Polar	Positive	-3.2
Isoleucine	Ile	I	Nonpolar	Neutral	4.5
Leucine	Leu	L	Nonpolar	Neutral	3.8
Lysine	Lys	K	Polar	Positive	-3.9
Methionine	Met	M	Nonpolar	Neutral	1.9
Phenylalanine	Phe	F	Nonpolar	Neutral	2.8
Proline	Pro	P	Nonpolar	Neutral	-1.6
Serine	Ser	S	Polar	Neutral	-0.8
Threonine	Thr	T	Polar	Neutral	-0.7
Tryptophan	Trp	W	Nonpolar	Neutral	-0.9
Tyrosine	Tyr	Y	Polar	Neutral	-1.3
Valine	Val	V	Nonpolar	Neutral	4.2

[0073] A "homologous" sequence (e.g., nucleic acid sequence) includes a sequence that is either identical or substantially similar to a known reference sequence, such that it is, for example, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the known reference sequence. Homologous sequences can include, for example, orthologous sequence and paralogous sequences. Homologous genes, for example, typically descend from a common ancestral DNA sequence, either through a speciation event (orthologous genes) or a genetic duplication event (paralogous genes). "Orthologous" genes include genes in different species that evolved from a common ancestral gene by speciation. Orthologs typically retain the same function in the course of evolution. "Paralogous" genes include genes related by duplication within a genome. Paralogs can evolve new functions in the course of evolution.

[0074] The term "*in vitro*" includes artificial environments and to processes or reactions that occur within an artificial environment (e.g., a test tube or an isolated cell or cell line). The term "*in vivo*" includes natural environments (e.g., a cell or organism or body) and to processes or

reactions that occur within a natural environment. The term "*ex vivo*" includes cells that have been removed from the body of an individual and processes or reactions that occur within such cells.

[0075] The term "reporter gene" refers to a nucleic acid having a sequence encoding a gene product (typically an enzyme) that is easily and quantifiably assayed when a construct comprising the reporter gene sequence operably linked to an endogenous or heterologous promoter and/or enhancer element is introduced into cells containing (or which can be made to contain) the factors necessary for the activation of the promoter and/or enhancer elements. Examples of reporter genes include, but are not limited, to genes encoding beta-galactosidase (lacZ), the bacterial chloramphenicol acetyltransferase (cat) genes, firefly luciferase genes, genes encoding beta-glucuronidase (GUS), and genes encoding fluorescent proteins. A "reporter protein" refers to a protein encoded by a reporter gene.

[0076] The term "fluorescent reporter protein" as used herein means a reporter protein that is detectable based on fluorescence wherein the fluorescence may be either from the reporter protein directly, activity of the reporter protein on a fluorogenic substrate, or a protein with affinity for binding to a fluorescent tagged compound. Examples of fluorescent proteins include green fluorescent proteins (e.g., GFP, GFP-2, tagGFP, turboGFP, eGFP, Emerald, Azami Green, Monomeric Azami Green, CopGFP, AceGFP, and ZsGreenl), yellow fluorescent proteins (e.g., YFP, eYFP, Citrine, Venus, YPet, PhiYFP, and ZsYellowl), blue fluorescent proteins (e.g., BFP, eBFP, eBFP2, Azurite, mKalamal, GFPuv, Sapphire, and T-sapphire), cyan fluorescent proteins (e.g., CFP, eCFP, Cerulean, CyPet, AmCyanl, and Midoriishi-Cyan), red fluorescent proteins (e.g., RFP, mKate, mKate2, mPlum, DsRed monomer, mCherry, mRFP1, DsRed-Express, DsRed2, DsRed-Monomer, HcRed-Tandem, HcRedl, AsRed2, eqFP611, mRaspberry, mStrawberry, and Jred), orange fluorescent proteins (e.g., mOrange, mKO, Kusabira-Orange, Monomeric Kusabira-Orange, mTangerine, and tdTomato), and any other suitable fluorescent protein whose presence in cells can be detected by flow cytometry methods.

[0077] Repair in response to double-strand breaks (DSBs) occurs principally through two conserved DNA repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ). *See* Kasparek & Humphrey (2011) *Seminars in Cell & Dev. Biol.* 22:886-897, herein incorporated by reference in its entirety for all purposes. Likewise, repair of a target nucleic acid mediated by an exogenous donor nucleic acid can include any process of exchange

of genetic information between the two polynucleotides.

[0078] The term "recombination" includes any process of exchange of genetic information between two polynucleotides and can occur by any mechanism. Recombination can occur via homology directed repair (HDR) or homologous recombination (HR). HDR or HR includes a form of nucleic acid repair that can require nucleotide sequence homology, uses a "donor" molecule as a template for repair of a "target" molecule (i.e., the one that experienced the double-strand break), and leads to transfer of genetic information from the donor to target. Without wishing to be bound by any particular theory, such transfer can involve mismatch correction of heteroduplex DNA that forms between the broken target and the donor, and/or synthesis-dependent strand annealing, in which the donor is used to resynthesize genetic information that will become part of the target, and/or related processes. In some cases, the donor polynucleotide, a portion of the donor polynucleotide, a copy of the donor polynucleotide, or a portion of a copy of the donor polynucleotide integrates into the target DNA. *See* Wang *et al.* (2013) *Cell* 153:910-918; Mandalos *et al.* (2012) *PLOS ONE* 7:e45768:1-9; and Wang *et al.* (2013) *Nat Biotechnol.* 31:530-532, each of which is herein incorporated by reference in its entirety for all purposes.

[0079] Non-homologous end joining (NHEJ) includes the repair of double-strand breaks in a nucleic acid by direct ligation of the break ends to one another or to an exogenous sequence without the need for a homologous template. Ligation of non-contiguous sequences by NHEJ can often result in deletions, insertions, or translocations near the site of the double-strand break. For example, NHEJ can also result in the targeted integration of an exogenous donor nucleic acid through direct ligation of the break ends with the ends of the exogenous donor nucleic acid (i.e., NHEJ-based capture). Such NHEJ-mediated targeted integration can be preferred for insertion of an exogenous donor nucleic acid when homology directed repair (HDR) pathways are not readily usable (e.g., in non-dividing cells, primary cells, and cells which perform homology-based DNA repair poorly). In addition, in contrast to homology-directed repair, knowledge concerning large regions of sequence identity flanking the cleavage site is not needed, which can be beneficial when attempting targeted insertion into organisms that have genomes for which there is limited knowledge of the genomic sequence. The integration can proceed via ligation of blunt ends between the exogenous donor nucleic acid and the cleaved genomic sequence, or via ligation of sticky ends (i.e., having 5' or 3' overhangs) using an exogenous donor nucleic acid

that is flanked by overhangs that are compatible with those generated by a nuclease agent in the cleaved genomic sequence. *See, e.g.*, US 2011/020722, WO 2014/033644, WO 2014/089290, and Maresca *et al.* (2013) *Genome Res.* 23(3):539-546, each of which is herein incorporated by reference in its entirety for all purposes. If blunt ends are ligated, target and/or donor resection may be needed to generation regions of microhomology needed for fragment joining, which may create unwanted alterations in the target sequence.

[0080] The term "antigen-binding protein" includes any protein that binds to an antigen. Examples of antigen-binding proteins include an antibody, an antigen-binding fragment of an antibody, a multispecific antibody (e.g., a bi-specific antibody), an scFV, a bis-scFV, a diabody, a triabody, a tetrabody, a V-NAR, a VHH, a VL, a F(ab), a $F(ab)_2$, a DVD (dual variable domain antigen-binding protein), an SVD (single variable domain antigen-binding protein), a bispecific T-cell engager (BiTE), or a Davisbody (US Pat. No. 8,586,713, herein incorporated by reference herein in its entirety for all purposes).

[0081] The term "antigen" refers to a substance, whether an entire molecule or a domain within a molecule, which is capable of eliciting production of antibodies with binding specificity to that substance. The term antigen also includes substances, which in wild type host organisms would not elicit antibody production by virtue of self-recognition, but can elicit such a response in a host animal with appropriate genetic engineering to break immunological tolerance.

[0082] The term "epitope" refers to a site on an antigen to which an antigen-binding protein (e.g., antibody) binds. An epitope can be formed from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of one or more proteins. Epitopes formed from contiguous amino acids (also known as linear epitopes) are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding (also known as conformational epitopes) are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. *See, e.g.*, Epitope Mapping Protocols, in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed. (1996), herein incorporated by reference in its entirety for all purposes.

[0083] An antibody paratope as described herein generally comprises at a minimum a complementarity determining region (CDR) that specifically recognizes the heterologous epitope (e.g., a CDR3 region of a heavy and/or light chain variable domain).

[0084] The term "antibody" includes immunoglobulin molecules comprising four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable domain and a heavy chain constant region (C_H). The heavy chain constant region comprises three domains: C_H1, C_H2 and C_H3. Each light chain comprises a light chain variable domain and a light chain constant region (C_L). The heavy chain and light chain variable domains can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each heavy and light chain variable domain comprises three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 (heavy chain CDRs may be abbreviated as HCDR1, HCDR2 and HCDR3; light chain CDRs may be abbreviated as LCDR1, LCDR2 and LCDR3). The term "high affinity" antibody refers to an antibody that has a K_D with respect to its target epitope about of 10^{-9} M or lower (e.g., about 1×10^{-9} M, 1×10^{-10} M, 1×10^{-11} M, or about 1×10^{-12} M). In one embodiment, K_D is measured by surface plasmon resonance, e.g., BIACORE™; in another embodiment, K_D is measured by ELISA.

[0085] Specific binding of an antigen-binding protein to its target antigen includes binding with an affinity of at least 10^6, 10^7, 10^8, 10^9, or 10^{10} M^{-1}. Specific binding is detectably higher in magnitude and distinguishable from non-specific binding occurring to at least one unrelated target. Specific binding can be the result of formation of bonds between particular functional groups or particular spatial fit (e.g., lock and key type) whereas non-specific binding is usually the result of van der Waals forces. Specific binding does not however necessarily imply that an antigen-binding protein binds one and only one target.

[0086] The term "antisense RNA" refers to a single-stranded RNA that is complementary to a messenger RNA strand transcribed in a cell.

[0087] The term "small interfering RNA (siRNA)" refers to a typically double-stranded RNA molecule that induces the RNA interference (RNAi) pathway. These molecules can vary in length (generally between 18-30 base pairs) and contain varying degrees of complementarity to their target mRNA in the antisense strand. Some, but not all, siRNAs have unpaired

overhanging bases on the 5' or 3' end of the sense strand and/or the antisense strand. The term "siRNA" includes duplexes of two separate strands, as well as single strands that can form hairpin structures comprising a duplex region. The double-stranded structure can be, for example, less than 20, 25, 30, 35, 40, 45, or 50 nucleotides in length. For example, the double-stranded structure can be from about 21-23 nucleotides in length, from about 19-25 nucleotides in length, or from about 19-23 nucleotides in length.

[0088] The term "short hairpin RNA (shRNA)" refers to a single strand of RNA bases that self-hybridizes in a hairpin structure and can induce the RNA interference (RNAi) pathway upon processing. These molecules can vary in length (generally about 50-90 nucleotides in length, or in some cases up to greater than 250 nucleotides in length, e.g., for microRNA-adapted shRNA). shRNA molecules are processed within the cell to form siRNAs, which in turn can knock down gene expression. shRNAs can be incorporated into vectors. The term "shRNA" also refers to a DNA molecule from which a short, hairpin RNA molecule may be transcribed.

[0089] Compositions or methods "comprising" or "including" one or more recited elements may include other elements not specifically recited. For example, a composition that "comprises" or "includes" a protein may contain the protein alone or in combination with other ingredients. The transitional phrase "consisting essentially of" means that the scope of a claim is to be interpreted to encompass the specified elements recited in the claim and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. Thus, the term "consisting essentially of" when used in a claim of this invention is not intended to be interpreted to be equivalent to "comprising."

[0090] "Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur and that the description includes instances in which the event or circumstance occurs and instances in which the event or circumstance does not.

[0091] Designation of a range of values includes all integers within or defining the range, and all subranges defined by integers within the range.

[0092] Unless otherwise apparent from the context, the term "about" encompasses values within a standard margin of error of measurement (e.g., SEM) of a stated value.

[0093] The term "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

[0094] The term "or" refers to any one member of a particular list and also includes any combination of members of that list.

[0095] The singular forms of the articles "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a protein" or "at least one protein" can include a plurality of proteins, including mixtures thereof.

[0096] Statistically significant means $p \leq 0.05$.

DETAILED DESCRIPTION

I. Overview

[0097] Disclosed herein are non-human animal cells and non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation and methods of using such non-human animal cells and non-human animals. Also disclosed herein are methods of making such non-human animal cells and non-human animals. Also disclosed herein are non-human animal genomes comprising a humanized *TTR* locus comprising a beta-slip mutation and methods of using such non-human animal genomes. Also disclosed herein are humanized non-human animal *TTR* genes comprising a beta-slip mutation and nuclease agents and targeting vectors for use in humanizing a non-human animal *TTR* gene. Non-human animal cells or non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation express a human transthyretin protein or a chimeric transthyretin protein comprising one or more fragments of a human transthyretin protein. Non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation develop amyloidosis very early. For example, mice comprising a humanized *TTR* locus comprising a beta-slip mutation develop amyloidosis as early as about two months of age. This is the first reported *in vivo* model of TTR amyloidosis to develop amyloidosis so rapidly. Such non-human animal cells and non-human animals can be used to assess delivery or efficacy of human-TTR-targeting agents (e.g., CRISPR/Cas9 genome editing agents) *ex vivo* or *in vivo* and can be used in methods of optimizing the delivery of efficacy of such agents *ex vivo* or *in vivo*.

[0098] In some of the non-human animal cells and non-human animals disclosed herein, most or all of the non-human animal genomic DNA is replaced one-for-one with orthologous human genomic DNA. Compared to non-human animals with cDNA insertions, expression levels should be higher when the intron-exon structure and splicing machinery are maintained

because conserved regulator elements are more likely to be left intact, and spliced transcripts that undergo RNA processing are more stable than cDNAs. In contrast, insertion of human *TTR* cDNA (e.g., along with insertion of an artificial beta-globin intron in the 5' UTR) into a non-human animal *Ttr* locus would abolish conserved regulatory elements such as those contained within the first exon and intron of the non-human animal *Ttr*. Replacing the non-human animal genomic sequence with the orthologous human genomic sequence is more likely to result in faithful expression of the transgene from the endogenous *Ttr* locus. Similarly, transgenic non-human animals with transgenic insertion of human-TTR-coding sequences at a random genomic locus rather than the endogenous non-human-animal *Ttr* locus will not as accurately reflect the endogenous regulation of *Ttr* expression. A humanized *TTR* allele resulting from replacing most or all of the non-human animal genomic DNA one-for-one with orthologous human genomic DNA will provide the true human target or a close approximation of the true human target of human-TTR-targeting reagents (e.g., CRISPR/Cas9 reagents designed to target human *TTR*), thereby enabling testing of the efficacy and mode of action of such agents in live animals as well as pharmacokinetic and pharmacodynamics studies in a setting where the humanized protein and humanized gene are the only version of TTR present.

II. Non-Human Animals Comprising a Humanized TTR *Locus* Comprising a Beta-Slip Mutation

[0099] The cells and non-human animals disclosed herein comprise a humanized *TTR* locus comprising a beta-slip mutation. Cells or non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation express a human transthyretin protein or a partially humanized, chimeric transthyretin protein in which one or more fragments of the native transthyretin protein have been replaced with corresponding fragments from human transthyretin.

A. Transthyretin (TTR)

[00100] The cells and non-human animals described herein comprise a humanized *transthyretin* (*Ttr*) locus comprising a beta-slip mutation. Transthyretin (TTR) is a 127-amino acid, 55 kDa serum and cerebrospinal fluid transport protein primarily synthesized by the liver but also produced by the choroid plexus. It has also been referred to as prealbumin, thyroxine

binding prealbumin, ATTR, TBPA, CTS, CTS1, HEL111, HsT2651, and PALB. In its native state, TTR exists as a tetramer. In homozygotes, homo-tetramers comprise identical 127-amino-acid beta-sheet-rich subunits. In heterozygotes, TTR tetramers can be made up of variant and/or wild-type subunits, typically combined in a statistical fashion. TTR is responsible for carrying thyroxine (T4) and retinol-bound RBP (retinol-binding protein) in both the serum and the cerebrospinal fluid.

[00101] Unless otherwise apparent from context, reference to human transthyretin (TTR) or its fragments or domains includes the natural, wild type human amino acid sequences including isoforms and allelic variants thereof. Transthyretin precursor protein includes a signal sequence (typically 20 amino acids), whereas the mature transthyretin protein does not. Exemplary TTR polypeptide sequences are designated by Accession Numbers NP_000362.1 (NCBI) and P02766.1 (UniProt) (identical, each set forth SEQ ID NO: 1). Residues may be numbered according to UniProt Accession Number P02766.1, with the first amino acid of the mature protein (i.e., not including the 20 amino acid signal sequence) designated residue 1. In any other TTR protein, residues are numbered according to the corresponding residues in UniProt Accession Number P02766.1 on maximum alignment.

[00102] The human *TTR* gene is located on chromosome 18 and includes four exons and three introns. An exemplary human *TTR* gene is from residues 5001-12258 in the sequence designated by GenBank Accession Number NG_009490.1 (SEQ ID NO: 3). The four exons in SEQ ID NO: 3 include residues 1-205, 1130-1260, 3354-3489, and 6802-7258, respectively. The *TTR* coding sequence in SEQ ID NO: 3 includes residues 137-205, 1130-1260, 3354-3489, and 6802-6909. An exemplary human *TTR* mRNA is designated by NCBI Accession Number NM_000371.3 (SEQ ID NO: 2). An exemplary human *TTR* coding sequence encoding a TTR protein comprising the G53S/E54D/L55S beta-slip mutation is set forth in SEQ ID NO: 10. An transthyretin precursor protein comprising the G53S/E54D/L55S beta-slip mutation is set forth in SEQ ID NO: 9.

[00103] The mouse *Ttr* gene is located and chromosome 18 and also includes four exons and three introns. An exemplary mouse *Ttr* gene is from residues 20665250 to 20674326 the sequence designated by GenBank Accession Number NC_000084.6 (SEQ ID NO: 7). The four exons in SEQ ID NO: 7 include residues 1-258, 1207-1337, 4730-4865, and 8382-9077, respectively. The *Ttr* coding sequence in SEQ ID NO: 7 includes residues 190-258, 1207-1337,

4730-4865, and 8382-8489. An exemplary mouse TTR protein is designated by UniProt Accession Number P07309.1 or NCBI Accession Number NP_038725.1 (identical, each set forth SEQ ID NO: 5). An exemplary mouse *Ttr* mRNA is designated by NCBI Accession Number NM_013697.5 (SEQ ID NO: 6).

[00104] An exemplary rat TTR protein is designated by UniProt Accession Number P02767. An exemplary pig TTR protein is designated by UniProt Accession Number P50390. An exemplary chicken TTR protein is designated by UniProt Accession Number P27731. An exemplary cow TTR protein is designated by UniProt Accession Number O46375. An exemplary sheep TTR protein is designated by UniProt Accession Number P12303. An exemplary chimpanzee TTR protein designated by UniProt Accession Number Q5U7I5. An exemplary orangutan TTR protein is designated by UniProt Accession Number Q5NVS2. An exemplary rabbit TTR protein is designated by UniProt Accession Number P07489. An exemplary cynomolgus monkey (macaque) TTR protein is designated by UniProt Accession Number Q8HXW1.

[00105] Transthyretin (TTR) amyloidosis is a systemic disorder characterized by pathogenic, misfolded TTR and the extracellular deposition of amyloid fibrils composed of TTR. TTR amyloidosis is generally caused by destabilization of the native TTR tetramer form (due to environmental or genetic conditions), leading to dissociation, misfolding, and aggregation of TTR into amyloid fibrils that accumulate in various organs and tissues, causing progressive dysfunction. The dissociated monomers have a propensity to form misfolded protein aggregates and amyloid fibrils.

[00106] In humans, both wild-type TTR tetramers and mixed tetramers made up of mutant and wild-type subunits can dissociate, misfold, and aggregate, with the process of amyloidogenesis leading to the degeneration of post-mitotic tissue. Thus, TTR amyloidoses encompass diseases caused by pathogenic misfolded TTR resulting from mutations in TTR or resulting from non-mutated, misfolded TTR.

[00107] Senile systemic amyloidosis (SSA) and senile cardiac amyloidosis (SCA) are age-related types of amyloidosis that result from the deposition of wild-type TTR amyloid outside and within the cardiomyocytes of the heart. TTR amyloidosis is also the most common form of hereditary (familial) amyloidosis, which is caused by mutations that destabilize the TTR protein. TTR amyloidoses associated with point mutations in the TTR gene include familial amyloid

polyneuropathy (FAP), familial amyloid cardiomyopathy (FAC), and central nervous system selective amyloidosis (CNSA).

B. Humanized *TTR* Loci Comprising a Beta-Slip Mutation

[00108] The humanized *TTR* loci described herein comprise a beta-slip mutation. An example of a beta-slip mutation is one that describes a conformational change caused by the human transthyretin triple mutant G53S/E54D/L55S. The numbering of the residues here and below refers to numbering in the mature human transthyretin protein without the signal peptide (e.g., beginning at residue 21 of the transthyretin precursor protein, so these residues in the transthyretin precursor protein would be residues 73, 74, and 75, respectively). A three-residue shift in the beta strand D places L58 at the position normally occupied by L55. This results in structural consequences on the neighboring residues in the CD loop, beta strand D, and DE loop area comprising residues S50–G63, but leaves the position of the beta strand C intact. *See* Eneqvist et al. (2000) *Mol. Cell* 6:1207-1218, herein incorporated by reference in its entirety for all purposes. The G53S/E54D/L55S variant polymerizes spontaneously at physiological conditions and gives rise to high molecular weight aggregates showing all the characteristics of amyloid, except they are still soluble. The G53S/E54D/L55S variant binds thioflavin T and Congo red, has an elevated sensitivity to trypsin, and forms fibrillar structures, which produce a fiber diffraction pattern consistent with cross-beta structure. *See* Eneqvist et al. (2000) *Mol. Cell* 6:1207-1218, herein incorporated by reference in its entirety for all purposes.

[00109] Generally, a beta-slip mutation as referred to herein comprises a mutation that causes a shift in beta-strand D of a TTR protein. By beta-strand D is meant beta-strand D in the human TTR protein or a corresponding region of a non-human TTR protein when optimally aligned with the human TTR protein. For example, the mutation can cause a three-residue shift in beta strand D that places a residue corresponding to residue L58 in human TTR at a position normally occupied by a residue corresponding to residue L55 in human TTR when the mutated TTR protein is optimally aligned with the human TTR protein. More specifically, the beta-slip mutation can be a triple mutation corresponding to G53S/E54D/L55S in the human TTR protein when the mutated TTR protein is optimally aligned with the human TTR protein. A residue (e.g., nucleotide or amino acid) in an endogenous *Ttr* gene (or TTR protein) can be determined to correspond with a residue in the human *TTR* gene (or TTR protein) by optimally aligning the two

sequences for maximum correspondence over a specified comparison window (e.g., the *TTR* coding sequence), wherein the portion of the polynucleotide (or amino acid) sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences (*see, e.g.*, discussion elsewhere herein with regard to sequence identity and complementarity). Two residues correspond if they are located at the same position when optimally aligned.

[00110] A humanized beta-slip *TTR* locus disclosed herein can be a *Ttr* locus in which the entire *Ttr* gene is replaced with the corresponding orthologous human *TTR* sequence comprising a beta-slip mutation, or it can be a *Ttr* locus in which only a portion of the *Ttr* gene is replaced with the corresponding orthologous human *TTR* sequence (i.e., humanized). Alternatively, it can be a *Ttr* locus in which a portion of the *Ttr* gene is deleted and a portion of the corresponding orthologous human *TTR* sequence is inserted. If only a portion of the *Ttr* locus is humanized, the beta-slip mutation can be in the remaining endogenous *Ttr* sequence or in the inserted orthologous human *TTR* sequence. In some examples, the portion of the orthologous human *TTR* locus that is inserted comprises more of the human *TTR* locus than is deleted from the endogenous *Ttr* locus. A human *TTR* sequence corresponding to a particular segment of endogenous *Ttr* sequence refers to the region of human *TTR* that aligns with the particular segment of endogenous *Ttr* sequence when human *TTR* and the endogenous *Ttr* are optimally aligned (greatest number of perfectly matched residues). The corresponding orthologous human sequence can comprise, for example, complementary DNA (cDNA) or genomic DNA. Optionally, the corresponding orthologous human *TTR* sequence is modified to be codon-optimized based on codon usage in the non-human animal. Replaced or inserted (i.e., humanized) regions can include coding regions such as an exon, non-coding regions such as an intron, untranslated regions, or regulatory regions (e.g., a promoter, an enhancer, or a transcriptional repressor-binding element), or any combination thereof. A humanized *TTR* locus can also comprise human *TTR* sequence inserted into an endogenous *Ttr* locus without replacing the corresponding orthologous endogenous sequence. As one example, exons corresponding to 1, 2, 3, or all 4 exons (or all or portions of 1, 2, 3, or all 4 exons) of the human *TTR* gene can be humanized. In a specific example, exons corresponding to exons 2 and 3 and the coding regions of exons 1 and 4 (i.e., not including the 5' UTR and the 3' UTR) can be deleted from the

endogenous *TTR* locus, and a region of the human *TTR* gene including exons 2-4 and the coding region of exon 1 (i.e., not including the 5' UTR) of the human *TTR* gene can be inserted. In a specific example, exons corresponding to exons 2 and 3 and the coding regions of exons 1 and 4 (i.e., not including the 5' UTR and the 3' UTR) can be deleted from the endogenous *TTR* locus, and a region of the human *TTR* gene including exons 2 and 3 and the coding regions of exons 1 and 4 as well as all or part of the 3' UTR (i.e., not including the 5' UTR) of the human *TTR* gene can be inserted. Alternatively, a region of *TTR* encoding an epitope recognized by an anti-human-TTR antigen-binding protein or a region targeted by human-TTR-targeting reagent (e.g., a small molecule) can be humanized. Likewise, introns corresponding to 1, 2, or all 3 introns of the human *TTR* gene can be humanized or can remain endogenous. In one example, introns corresponding to all 3 introns of the human *TTR* gene can be humanized (e.g., deleted from the endogenous locus and replaced with the corresponding human introns).

[00111] A humanized *TTR* locus can be one in which a region of the endogenous *Ttr* locus has been deleted and replaced with an orthologous human *TTR* sequence (e.g., orthologous wild type human *TTR* sequence). As one example, the replaced region of the endogenous *Ttr* locus can comprise both a coding sequence (i.e., all or part of an exon) and a non-coding sequence (i.e., all or part of intron), such as at least one exon and at least one intron. For example, the replaced region can comprise at least one exon and at least one intron. The replaced region comprising both coding sequence and non-coding sequence can be a contiguous region of the endogenous *Ttr* locus, meaning there is no intervening sequence between the replaced coding sequence and the replaced non-coding sequence. For example, the replaced region can comprise at least one exon and at least one adjacent intron. The replaced region can comprise one exon, two exons, three exons, four exons, or all exons of the endogenous *Ttr* locus. The inserted human *TTR* sequence can comprise one exon, two exons, three exons, four exons, or all exons of a human *TTR* gene. Likewise, the replaced region can comprise one intron, two introns, three introns, or all introns of the endogenous *Ttr* locus. The inserted human *TTR* sequence can comprise one intron, two introns, three introns, or all introns of a human *TTR* gene. Optionally, one or more introns and/or one or more exons of the endogenous *Ttr* locus remain unmodified (i.e., not deleted and replaced). For example, the first exon of the endogenous *Ttr* locus can remain unmodified. Similarly, the first exon and the first intron of the endogenous *Ttr* locus can remain unmodified.

[00112] The transthyretin precursor protein encoded by the humanized *TTR* locus can retain the activity of the native transthyretin precursor protein and/or the human transthyretin precursor protein. For example, the transthyretin precursor protein encoded by the humanized *TTR* locus can retain the activity of a native transthyretin precursor protein comprising a beta-slip mutation and/or a human transthyretin precursor protein comprising the beta-slip mutation.

[00113] In one specific example, the entire coding sequence for the transthyretin precursor protein can be deleted and replaced with the orthologous human *TTR* sequence. For example, the region of the endogenous *Ttr* locus beginning at the start codon and ending at the stop codon can be deleted and replaced with the orthologous human *TTR* sequence.

[00114] Flanking untranslated regions including regulatory sequences can also be humanized. Alternatively, flanking untranslated regions including regulatory sequences can remain endogenous. The first exon of a *Ttr* locus typically include a 5' untranslated region upstream of the start codon. Likewise, the last exon of a *Ttr* locus typically includes a 3' untranslated region downstream of the stop codon. Regions upstream of the *Ttr* start codon and downstream of the *Ttr* stop codon can either be unmodified or can be deleted and replaced with the orthologous human *TTR* sequence. For example, the 5' untranslated region (UTR), the 3'UTR, or both the 5' UTR and the 3' UTR can be humanized, or the 5' UTR, the 3'UTR, or both the 5' UTR and the 3' UTR can remain endogenous. One or both of the human 5' and 3' UTRs can be inserted, and/or one or both of the endogenous 5' and 3' UTRs can be deleted. In one specific example, the 5' UTR remains endogenous. In another specific example, the 3' UTR is humanized, but the 5' UTR remains endogenous. In another specific example, the 5' UTR remains endogenous, and a human *TTR* 3' UTR is inserted into the endogenous *Ttr* locus. For example, the human *TTR* 3' UTR can replace the endogenous 3' UTR or can be inserted without replacing the endogenous 3' UTR (e.g., it can be inserted upstream of the endogenous 3' UTR). For example, the endogenous 5' UTR (or a portion thereof) and the endogenous 3' UTR (or a portion thereof) can remain at the humanized *TTR* locus, and the human 3' UTR (or a portion thereof) can be inserted upstream of the endogenous 3' UTR.

[00115] One or more regions of the endogenous *Ttr* locus encoding one or more domains of the transthyretin precursor protein can be humanized. Likewise, one or more regions of the endogenous *Ttr* locus encoding one or more domains of the transthyretin precursor protein can remain unmodified (i.e., not deleted and replaced). For example, transthyretin precursor proteins

typically have a signal peptide at the N-terminus. The signal peptide can be, for example, about 20 amino acids in length. The region of the endogenous *Ttr* locus encoding the signal peptide can remain unmodified (i.e., not deleted and replaced), or can be deleted and replaced with the orthologous human *TTR* sequence. Similarly, a region of the endogenous *Ttr* locus encoding an epitope recognized by an anti-human-TTR antigen-binding protein can be humanized.

[00116] Depending on the extent of replacement by orthologous sequences, regulatory sequences, such as a promoter, can be endogenous or supplied by the replacing human orthologous sequence. For example, the humanized *TTR* locus can include the endogenous non-human animal *Ttr* promoter. The coding sequence for the transthyretin precursor protein at the genetically modified endogenous *Ttr* locus can be operably linked to the endogenous *Ttr* promoter.

[00117] As a specific example, the humanized *TTR* locus comprising the beta-slip mutation can be one in which the region of the endogenous *Ttr* locus being deleted and replaced with the orthologous human *TTR* sequence comprises, consists essentially of, or consists of the region from the *Ttr* start codon to the stop codon. The human *TTR* sequence being inserted can further comprise a human *TTR* 3' UTR. For example, the human *TTR* sequence at the humanized *TTR* locus comprising the beta-slip mutation can comprise, consist essentially of, or consist of the region from the *TTR* start codon to the end of the 3' UTR. Optionally, the *Ttr* coding sequence in the modified endogenous *Ttr* locus is operably linked to the endogenous *Ttr* promoter. The human *TTR* sequence at the humanized *TTR* locus comprising the beta-slip mutation can comprise, consist essentially of, or consist of a sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 14. The human *TTR* sequence at the humanized *TTR* locus comprising the beta-slip mutation can comprise, consist essentially of, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 14. The humanized *TTR* locus comprising the beta-slip mutation can comprise, consist essentially of, or consist of a sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 12 or 13. The humanized *TTR* locus comprising the beta-slip mutation can comprise, consist essentially of, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 12 or 13. The

coding sequence (CDS) at the humanized *TTR* locus comprising the beta-slip mutation can comprise, consist essentially of, or consist of a sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 10 (or degenerates thereof that encode the same protein). The coding sequence (CDS) at the humanized *TTR* locus comprising the beta-slip mutation can comprise, consist essentially of, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 10 (or degenerates thereof that encode the same protein). The resulting human transthyretin precursor protein encoded by the humanized *TTR* locus comprising the beta-slip mutation can comprise, consist essentially of, or consist of a sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 9. The resulting human transthyretin precursor protein encoded by the humanized *TTR* locus comprising the beta-slip mutation can comprise, consist essentially of, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 9.

[00118] A control non-human animal comprising a humanized *TTR* wild type locus can also be generated. The wild type human *TTR* sequence at the humanized *TTR* locus can comprise, consist essentially of, or consist of a sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 17. The wild type human *TTR* sequence at the humanized *TTR* locus can comprise, consist essentially of, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 17. The humanized *TTR* wild type locus can comprise, consist essentially of, or consist of a sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 15 or 16. The humanized *TTR* wild type locus can comprise, consist essentially of, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 15 or 16. The coding sequence (CDS) at the humanized *TTR* wild type locus can comprise, consist essentially of, or consist of a sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 4 (or degenerates thereof that encode the same protein). The coding sequence (CDS) at the humanized *TTR* wild type locus can comprise, consist essentially

of, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 4 (or degenerates thereof that encode the same protein). The resulting human transthyretin precursor protein encoded by the humanized *TTR* wild type locus can comprise, consist essentially of, or consist of a sequence that is at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 1. The resulting human transthyretin precursor protein encoded by the humanized *TTR* wild type locus can comprise, consist essentially of, or consist of a sequence that is at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or about 100% identical to SEQ ID NO: 1.

[00119] As another specific example, the humanized *TTR* locus can be one in which the region of the endogenous *Ttr* locus being deleted and replaced with the orthologous human *TTR* sequence comprises, consists essentially of, or consists of the region from the start of the second *Ttr* exon to the stop codon. The human *TTR* sequence being inserted can further comprise a human *TTR* 3' UTR. For example, the human *TTR* sequence at the humanized *TTR* locus can comprise, consist essentially of, or consist of the region from the start of the second human *TTR* exon to the end of the 3' UTR. Optionally, the *Ttr* coding sequence in the modified endogenous *Ttr* locus is operably linked to the endogenous *Ttr* promoter.

[00120] TTR protein expressed from a humanized *TTR* locus can be an entirely human TTR protein or a chimeric endogenous/human TTR protein (e.g., if the non-human animal is a mouse, a chimeric mouse/human TTR protein). For example, the signal peptide of the transthyretin precursor protein can be endogenous, and the remainder of the protein can be human. Alternatively, the N-terminus of the transthyretin precursor protein can be endogenous, and the remainder of the protein can be human. For example, the N-terminal 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids can be endogenous, and the remainder can be human. In a specific example, the 23 amino acids at the N-terminus are endogenous, and the remainder of the protein is human.

[00121] Optionally, a humanized *TTR* locus can comprise other elements. Examples of such elements can include selection cassettes, reporter genes, recombinase recognition sites, or other elements. As one example, a humanized *TTR* locus can comprise a removable selection cassette (e.g., a self-deleting selection cassette) flanked by recombinase recognition sequences (e.g., loxP

sites). Alternatively, the humanized *TTR* locus can lack other elements (e.g., can lack a selection cassette and/or can lack a reporter gene). Examples of suitable reporter genes and reporter proteins are disclosed elsewhere herein. Examples of suitable selection markers include neomycin phosphotransferase (neo_r), hygromycin B phosphotransferase (hyg_r), puromycin-N-acetyltransferase ($puro_r$), blasticidin S deaminase (bsr_r), xanthine/guanine phosphoribosyl transferase (gpt), and herpes simplex virus thymidine kinase (HSV-k). Examples of recombinases include Cre, Flp, and Dre recombinases. One example of a Cre recombinase gene is Crei, in which two exons encoding the Cre recombinase are separated by an intron to prevent its expression in a prokaryotic cell. Such recombinases can further comprise a nuclear localization signal to facilitate localization to the nucleus (e.g., NLS-Crei). Recombinase recognition sites include nucleotide sequences that are recognized by a site-specific recombinase and can serve as a substrate for a recombination event. Examples of recombinase recognition sites include FRT, FRT11, FRT71, attp, att, rox, and lox sites such as loxP, lox511, lox2272, lox66, lox71, loxM2, and lox5171.

[00122] Other elements such as reporter genes or selection cassettes can be self-deleting cassettes flanked by recombinase recognition sites. *See, e.g.,* US 8,697,851 and US 2013/0312129, each of which is herein incorporated by reference in its entirety for all purposes. As an example, the self-deleting cassette can comprise a Crei gene (comprises two exons encoding a Cre recombinase, which are separated by an intron) operably linked to a mouse *Prm1* promoter and a neomycin resistance gene operably linked to a human ubiquitin promoter. By employing the *Prm1* promoter, the self-deleting cassette can be deleted specifically in male germ cells of F0 animals. The polynucleotide encoding the selection marker can be operably linked to a promoter active in a cell being targeted. Examples of promoters are described elsewhere herein. As another specific example, a self-deleting selection cassette can comprise a hygromycin resistance gene coding sequence operably linked to one or more promoters (e.g., both human ubiquitin and EM7 promoters) followed by a polyadenylation signal, followed by a Crei coding sequence operably linked to one or more promoters (e.g., an mPrm1 promoter), followed by another polyadenylation signal, wherein the entire cassette is flanked by loxP sites.

[00123] The humanized *TTR* locus can also be a conditional allele. For example, the conditional allele can be a multifunctional allele, as described in US 2011/0104799, herein incorporated by reference in its entirety for all purposes. For example, the conditional allele can

comprise: (a) an actuating sequence in sense orientation with respect to transcription of a target gene; (b) a drug selection cassette (DSC) in sense or antisense orientation; (c) a nucleotide sequence of interest (NSI) in antisense orientation; and (d) a conditional by inversion module (COIN, which utilizes an exon-splitting intron and an invertible gene-trap-like module) in reverse orientation. *See, e.g.*, US 2011/0104799. The conditional allele can further comprise recombinable units that recombine upon exposure to a first recombinase to form a conditional allele that (i) lacks the actuating sequence and the DSC; and (ii) contains the NSI in sense orientation and the COIN in antisense orientation. *See, e.g.*, US 2011/0104799.

C. Non-Human Cells and Non-Human Animals Comprising a Humanized *TTR* Locus Comprising a Beta-Slip Mutation

[00124] Non-human animal cells and non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation as described elsewhere herein are provided. Non-human animal genomes comprising a humanized *TTR* locus comprising a beta-slip mutation as described elsewhere herein are also provided. The genomes, cells, or non-human animals can be male or female. The cells or non-human animals can be heterozygous or homozygous for the humanized *TTR* locus comprising the beta-slip mutation. Likewise, the genomes can be heterozygous or homozygous for the humanized *TTR* locus comprising the beta-slip mutation. A diploid organism has two alleles at each genetic locus. Each pair of alleles represents the genotype of a specific genetic locus. Genotypes are described as homozygous if there are two identical alleles at a particular locus and as heterozygous if the two alleles differ. A non-human animal comprising a humanized *TTR* locus comprising a beta-slip mutation can comprise the humanized *TTR* locus in its germline.

[00125] The non-human animal cells provided herein can be, for example, any non-human cell comprising a *Ttr* locus or a genomic locus homologous or orthologous to the human *TTR* locus. Likewise, the non-human animal genomes provided herein can be, for example, any non-human animal genome comprising a *Ttr* locus or a genomic locus homologous or orthologous to the human *TTR* locus. The cells can be eukaryotic cells, which include, for example, animal cells, mammalian cells, non-human mammalian cells, and human cells. The term "animal" includes mammals, fishes, and birds. Likewise, the genomes can be from eukaryotic cells. A mammalian cell can be, for example, a non-human mammalian cell, a rodent cell, a rat cell, a

mouse cell, or a hamster cell. Other non-human mammals include, for example, non-human primates, monkeys, apes, orangutans, cats, dogs, rabbits, horses, livestock (e.g., bovine species such as cows, steer, and so forth; ovine species such as sheep, goats, and so forth; and porcine species such as pigs and boars). Domesticated animals and agricultural animals are also included. The term "non-human" excludes humans.

[00126] The cells can also be any type of undifferentiated or differentiated state. For example, a cell can be a totipotent cell, a pluripotent cell (e.g., a human pluripotent cell or a non-human pluripotent cell such as a mouse embryonic stem (ES) cell or a rat ES cell), or a non-pluripotent cell. Totipotent cells include undifferentiated cells that can give rise to any cell type, and pluripotent cells include undifferentiated cells that possess the ability to develop into more than one differentiated cell types. Such pluripotent and/or totipotent cells can be, for example, ES cells or ES-like cells, such as an induced pluripotent stem (iPS) cells. ES cells include embryo-derived totipotent or pluripotent cells that are capable of contributing to any tissue of the developing embryo upon introduction into an embryo. ES cells can be derived from the inner cell mass of a blastocyst and are capable of differentiating into cells of any of the three vertebrate germ layers (endoderm, ectoderm, and mesoderm).

[00127] The cells provided herein can also be germ cells (e.g., sperm or oocytes). The cells can be mitotically competent cells or mitotically-inactive cells, meiotically competent cells or meiotically-inactive cells. Similarly, the cells can also be primary somatic cells or cells that are not a primary somatic cell. Somatic cells include any cell that is not a gamete, germ cell, gametocyte, or undifferentiated stem cell. For example, the cells can be liver cells, such as hepatoblasts or hepatocytes.

[00128] Suitable cells provided herein also include primary cells. Primary cells include cells or cultures of cells that have been isolated directly from an organism, organ, or tissue. Primary cells include cells that are neither transformed nor immortal. They include any cell obtained from an organism, organ, or tissue which was not previously passed in tissue culture or has been previously passed in tissue culture but is incapable of being indefinitely passed in tissue culture. Such cells can be isolated by conventional techniques and include, for example, hepatocytes.

[00129] Other suitable cells provided herein include immortalized cells. Immortalized cells include cells from a multicellular organism that would normally not proliferate indefinitely but, due to mutation or alteration, have evaded normal cellular senescence and instead can keep

undergoing division. Such mutations or alterations can occur naturally or be intentionally induced. A specific example of an immortalized cell line is the HepG2 human liver cancer cell line. Numerous types of immortalized cells are well known. Immortalized or primary cells include cells that are typically used for culturing or for expressing recombinant genes or proteins.

[00130] The cells provided herein also include one-cell stage embryos (i.e., fertilized oocytes or zygotes). Such one-cell stage embryos can be from any genetic background (e.g., BALB/c, C57BL/6, 129, or a combination thereof for mice), can be fresh or frozen, and can be derived from natural breeding or *in vitro* fertilization.

[00131] The cells provided herein can be normal, healthy cells, or can be diseased or mutant-bearing cells.

[00132] In a specific example, the non-human animal cells are embryonic stem (ES) cells or liver cells, such as mouse or rat ES cells or liver cells.

[00133] Non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation as described herein can be made by the methods described elsewhere herein. The term "animal" includes mammals, fishes, and birds. Non-human mammals include, for example, non-human primates, monkeys, apes, orangutans, cats, dogs, horses, rabbits, rodents (e.g., mice, rats, hamsters, and guinea pigs), and livestock (e.g., bovine species such as cows and steer; ovine species such as sheep and goats; and porcine species such as pigs and boars). Domesticated animals and agricultural animals are also included. The term "non-human animal" excludes humans. Preferred non-human animals include, for example, rodents, such as mice and rats.

[00134] The non-human animals can be from any genetic background. For example, suitable mice can be from a 129 strain, a C57BL/6 strain, a mix of 129 and C57BL/6, a BALB/c strain, or a Swiss Webster strain. Examples of 129 strains include 129P1, 129P2, 129P3, 129X1, 129S1 (e.g., 129S1/SV, 129S1/Svlm), 129S2, 129S4, 129S5, 129S9/SvEvH, 129S6 (129/SvEvTac), 129S7, 129S8, 129T1, and 129T2. *See, e.g.*, Festing *et al.* (1999) *Mammalian Genome* 10:836, herein incorporated by reference in its entirety for all purposes. Examples of C57BL strains include C57BL/A, C57BL/An, C57BL/GrFa, C57BL/Kal_wN, C57BL/6, C57BL/6J, C57BL/6ByJ, C57BL/6NJ, C57BL/10, C57BL/10ScSn, C57BL/10Cr, and C57BL/Ola. Suitable mice can also be from a mix of an aforementioned 129 strain and an aforementioned C57BL/6 strain (e.g., 50% 129 and 50% C57BL/6). Likewise, suitable mice can be from a mix of aforementioned 129 strains or a mix of aforementioned BL/6 strains (e.g., the 129S6

(129/SvEvTac) strain).

[00135] Similarly, rats can be from any rat strain, including, for example, an ACI rat strain, a Dark Agouti (DA) rat strain, a Wistar rat strain, a LEA rat strain, a Sprague Dawley (SD) rat strain, or a Fischer rat strain such as Fisher F344 or Fisher F6. Rats can also be obtained from a strain derived from a mix of two or more strains recited above. For example, a suitable rat can be from a DA strain or an ACI strain. The ACI rat strain is characterized as having black agouti, with white belly and feet and an *RT1*^{av1} haplotype. Such strains are available from a variety of sources including Harlan Laboratories. The Dark Agouti (DA) rat strain is characterized as having an agouti coat and an *RT1*^{av1} haplotype. Such rats are available from a variety of sources including Charles River and Harlan Laboratories. Some suitable rats can be from an inbred rat strain. *See, e.g.*, US 2014/0235933, herein incorporated by reference in its entirety for all purposes.

[00136] Non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation can have several phenotypes. As one example, such non-human animals can be hyperactive relative to control wild type non-human animals or control animals comprising a humanized *TTR* locus without the beta-slip mutation. Hyperactivity can be assessed by measuring one or more or all of total distance, total activity, and total rearings in an open field test as described in more detail in the working examples.

[00137] The non-human animals can also display dystonia or of a dystopic muscle phenotype. For example, the non-human animal can display hindlimb dystonia or a hindlimb dystonic phenotype (e.g., dystonic hindlimb retraction) as described in more detail in the working examples.

[00138] The non-human animals can also comprise aggregated forms of TTR and/or amyloid deposits (e.g., specifically TTR amyloid deposits). For example, the amyloid deposits can be in the sciatic nerve as shown in more detail in the working examples. However, the amyloid deposits can be in other organs and tissues as well.

[00139] In some non-human animals, any of these phenotypes (e.g., amyloid deposits) are apparent as early as about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months of age. For example, the phenotypes (e.g., amyloid deposits) can be apparent by about 2 months of age.

[00140] Non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation can express the humanized TTR protein at any level. For example, non-human animals

comprising a humanized *TTR* locus comprising a beta-slip mutation can express humanized TTR proteins at levels of at least about 0.1, at least about 0.2, or at least about 0.3 μg/mL in the serum. Alternatively, non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation can express humanized TTR protein at levels of at least about 0.5, at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 12, at least about 14, at least about 15, at least about 16, at least about 18, at least about 20, at least about 22, at least about 24, at least about 25, at least about 26, at least about 28, or at least about 30 μg/mL in the serum.

III. Methods of Using Non-Human Animals Comprising a Humanized TTR *Locus Comprising a Beta-Slip Mutation for Assessing Efficacy of Human-TTR-Targeting Reagents* In Vivo *or* Ex Vivo

[00141] Various methods are provided for using the non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation as described elsewhere herein for assessing or optimizing delivery or efficacy of human-TTR-targeting reagents (e.g., therapeutic molecules or complexes) *in vivo* or *ex vivo*. The non-human animals produce a humanized TTR protein comprising a beta-slip mutation that results in amyloid deposition and phenotypes reflecting the phenotypes of TTR amyloidosis in humans. These phenotypes and the amyloid deposition occur at a very early age. Because this is the first reported *in vivo* model of TTR amyloidosis to develop amyloidosis so rapidly, the non-human animals are a useful tool to study TTR amyloidosis. In addition, because the non-human animals comprise a humanized *TTR* locus, the non-human animals will more accurately reflect the efficacy of a human TTR-targeting reagent. Such non-human animals are particularly useful for testing genome-editing reagents designed to target the human *TTR* gene because the non-human animals disclosed herein comprise humanized endogenous *Ttr* loci rather than transgenic insertions of human *TTR* sequence at random genomic loci, and the humanized endogenous *Ttr* loci comprise orthologous human genomic *TTR* sequence from both coding and non-coding regions rather than an artificial cDNA sequence.

A. Methods of Testing Efficacy of Human-TTR-Targeting Reagents *In Vivo* or *Ex Vivo*

[00142] Various methods are provided for assessing delivery or efficacy of human-TTR-

targeting reagents *in vivo* using non-human animals comprising a humanized *TTR* locus comprising a beta-slip mutation as described elsewhere herein. Such methods can comprise: (a) introducing into the non-human animal a human-TTR-targeting reagent; and (b) assessing the activity of the human-TTR-targeting reagent.

[00143] The human-TTR-targeting reagent can be any biological or chemical agent that targets the human *TTR* locus (the human *TTR* gene), the human *TTR* mRNA, or the human transthyretin protein. Examples of human-TTR-targeting reagents are disclosed elsewhere herein. For example, the human-TTR-targeting reagent can be a TTR-targeting nucleic acid (e.g., CRISPR/Cas guide RNAs, short hairpin RNAs (shRNAs), or small interfering RNAs (siRNAs)) or nucleic acid encoding a TTR-targeting protein (e.g., a Cas proteins such as Cas9, a ZFN, or a TALEN). Alternatively, the human-TTR-targeting reagent can be a TTR-targeting antibody or antigen-binding protein, or any other large molecule or small molecule that targets human TTR.

[00144] Such human-TTR-targeting reagents can be administered by any delivery method (e.g., AAV, LNP, or HDD) as disclosed in more detail elsewhere herein and by any route of administration. Means of delivering therapeutic complexes and molecules and routes of administration are disclosed in more detail elsewhere herein. In particular methods, the reagents delivered via AAV-mediated delivery. For example, AAV8 can be used to target the liver. In other particular methods, the reagents are delivered by LNP-mediated delivery. In other particular methods, the reagents are delivered by hydrodynamic delivery (HDD). The dose can be any suitable dose. For example, in some methods in which the reagents (e.g., Cas9 mRNA and gRNA) are delivered by LNP-mediated delivery, the dose can be between about 0.01 and about 10 mg/kg, about 0.01 and about 5 mg/kg, between about 0.01 and about 4 mg/kg, between about 0.01 and about 3 mg/kg, between about 0.01 and about 2 mg/kg, between about 0.01 and about 1 mg/kg, between about 0.1 and about 10 mg/kg, between about 0.1 and about 6 mg/kg; between about 0.1 and about 5 mg/kg, between about 0.1 and about 4 mg/kg, between about 0.1 and about 3 mg/kg, between about 0.1 and about 2 mg/kg, between about 0.1 and about 1 mg/kg, between about 0.3 and about 10 mg/kg, between about 0.3 and about 6 mg/kg; between about 0.3 and about 5 mg/kg, between about 0.3 and about 4 mg/kg, between about 0.3 and about 3 mg/kg, between about 0.3 and about 2 mg/kg, between about 0.3 and about 1 mg/kg, about 0.1 mg/kg, about 0.3 mg/kg, about 1 mg/kg, about 2 mg/kg, or about 3 mg/kg. In a specific example, the

dose is between about 0.1 and about 6 mg/kg; between about 0.1 and about 3 mg/kg, or between about 0.1 and about 2 mg/kg. In a specific example, the human-TTR-targeting reagent is a genome editing reagent, the LNP dose is about 1 mg/kg, and the percent genome editing at the humanized *TTR* locus is between about 70% and about 80%. In another specific example, the human-TTR-targeting reagent is a genome editing reagent, the LNP dose is about 0.3 mg/kg, and the percent editing is between about 50% and about 80%. In another specific example, the human-TTR-targeting reagent is a genome editing reagent, the LNP dose is about 0.1 mg/kg, and the percent editing is between about 20% and about 80%. In another specific example, the LNP dose is about 1 mg/kg, and the serum TTR levels are reduced to between about 0% and about 10% or between about 0% and about 35% of control levels. In another specific example, the LNP dose is about 0.3 mg/kg, and the serum TTR levels are reduced to between about 0% and about 20% or about 0% and about 95% of control levels. In another specific example, the LNP dose is about 0.1 mg/kg, and the serum TTR levels are reduced to between about 0% and about 60% or about 0% and about 99% of control levels.

[00145] Methods for assessing activity of the human-TTR-targeting reagent are well-known and are provided elsewhere herein. Assessment of activity can be in any cell type, any tissue type, or any organ type as disclosed elsewhere herein. In some methods, assessment of activity is in liver cells. As one example, the assessing can comprise measuring non-homologous end joining (NHEJ) activity at the humanized *TTR* locus. This can comprise, for example, measuring the frequency of insertions or deletions within the humanized *TTR* locus. If the TTR-targeting reagent is a genome editing reagent (e.g., a nuclease agent), such methods can comprise assessing modification of the humanized *TTR* locus comprising the beta-slip mutation. For example, the assessing can comprise sequencing the humanized *TTR* locus in one or more cells isolated from the non-human animal (e.g., next-generation sequencing). Assessment can comprise isolating a target organ (e.g., liver) or tissue from the non-human animal and assessing modification of humanized *TTR* locus in the target organ or tissue. Assessment can also comprise assessing modification of humanized *TTR* locus in two or more different cell types within the target organ or tissue. Similarly, assessment can comprise isolating a non-target organ or tissue (e.g., two or more non-target organs or tissues) from the non-human animal and assessing modification of humanized *TTR* locus in the non-target organ or tissue.

[00146] Such methods can also comprise measuring expression levels of the mRNA produced

by the humanized *TTR* locus comprising the beta-slip mutation, or by measuring expression levels of the protein encoded by the humanized *TTR* locus comprising the beta-slip mutation. For example, protein levels can be measured in a particular cell, tissue, or organ type (e.g., liver), or secreted levels can be measured in the serum. Methods for assessing expression of *Ttr* mRNA or protein expressed from the humanized *TTR* locus are provided elsewhere herein and are well-known.

[00147] As one specific example, if the human-TTR-targeting reagent is a genome editing reagent (e.g., a nuclease agent), percent editing (e.g., total number of insertions or deletions observed over the total number of sequences read in the PCR reaction from a pool of lysed cells) at the humanized *TTR* locus can be assessed (e.g., in liver cells).

[00148] As one example, if the human-TTR-targeting reagent is a genome editing reagent (e.g., a nuclease agent), percent editing at the humanized *TTR* locus can be assessed (e.g., in liver cells). For example, the percent editing (e.g., total number of insertions or deletions observed over the total number of sequences read in the PCR reaction from a pool of lysed cells) can be at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99%, or, for example, between about 1% and about 99%, between about 10% and about 99%, between about 20% and about 99%, between about 30% and about 99%, between about 40% and about 99%, between about 50% and about 99%, between about 60% and about 99%, between about 1% and about 90%, between about 10% and about 90%, between about 20% and about 90%, between about 30% and about 90%, between about 40% and about 90%, between about 50% and about 90%, between about 60% and about 90%, between about 1% and about 80%, between about 10% and about 80%, between about 20% and about 80%, between about 30% and about 80%, between about 40% and about 80%, between about 50% and about 80%, or between about 60% and about 80%.

[00149] As another example, serum TTR levels can be assessed. For example, serum TTR levels can be reduced by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 65%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99%, or, for example, between about 1% and about 99%, between about 10% and about 99%, between about 20% and about 99%, between about 30% and about 99%, between about 40% and about 99%, between about

50% and about 99%, between about 60% and about 99%, between about 70% and about 99%, between about 80% and about 99%, between about 1% and about 90%, between about 10% and about 90%, between about 20% and about 90%, between about 30% and about 90%, between about 40% and about 90%, between about 50% and about 90%, between about 60% and about 90%, between about 70% and about 90%, or between about 80% and about 90%.

[00150] Such methods can also comprise assessing activity/hyperactivity of the non-human animals, such as in an open field test as described in more detail elsewhere herein. Such methods can also comprise assessing the presence of aggregated forms of TTR (e.g., by native PAGE and western blots) or assessing the presence of amyloid deposits as described in more detail elsewhere herein. Such methods can also comprise assessing whether the non-human animals display dystonia or dystonic phenotypes as described in more detail elsewhere herein.

[00151] The various methods provided above for assessing activity *in vivo* can also be used to assess the activity of human-TTR-targeting reagents *ex vivo* as described elsewhere herein.

[00152] In some methods, the human-TTR-targeting reagent is a nuclease agent, such as a CRISPR/Cas nuclease agent, that targets the human *TTR* gene. Such methods can comprise, for example: (a) introducing into the non-human animal a nuclease agent designed to cleave the human *TTR* gene (e.g., Cas protein such as Cas9 and a guide RNA designed to target a guide RNA target sequence in the human *TTR* gene); and (b) assessing modification of the humanized *TTR* locus comprising the beta-slip mutation.

[00153] In the case of a CRISPR/Cas nuclease, for example, modification of the humanized *TTR* locus comprising the beta-slip mutation will be induced when the guide RNA forms a complex with the Cas protein and directs the Cas protein to the humanized *TTR* locus, and the Cas/guide RNA complex cleaves the guide RNA target sequence, triggering repair by the cell (e.g., via non-homologous end joining (NHEJ) if no donor sequence is present).

[00154] Optionally, two or more guide RNAs can be introduced, each designed to target a different guide RNA target sequence within the human *TTR* gene. For example, two guide RNAs can be designed to excise a genomic sequence between the two guide RNA target sequences. Modification of the humanized *TTR* locus will be induced when the first guide RNA forms a complex with the Cas protein and directs the Cas protein to the humanized *TTR* locus, the second guide RNA forms a complex with the Cas protein and directs the Cas protein to the humanized *TTR* locus, the first Cas/guide RNA complex cleaves the first guide RNA target

sequence, and the second Cas/guide RNA complex cleaves the second guide RNA target sequence, resulting in excision of the intervening sequence.

[00155] Optionally, an exogenous donor nucleic acid capable of recombining with and modifying a human *TTR* gene is also introduced into the non-human animal. Optionally, the nuclease agent or Cas protein can be tethered to the exogenous donor nucleic acid as described elsewhere herein. Modification of the humanized *TTR* locus will be induced, for example, when the guide RNA forms a complex with the Cas protein and directs the Cas protein to the humanized *TTR* locus, the Cas/guide RNA complex cleaves the guide RNA target sequence, and the humanized *TTR* locus recombines with the exogenous donor nucleic acid to modify the humanized *TTR* locus. The humanized *TTR* locus can then be repaired with the exogenous donor nucleic acid, for example, via homology-directed repair (HDR) or via NHEJ-mediated insertion. Any type of exogenous donor nucleic acid can be used, examples of which are provided elsewhere herein.

B. Methods of Optimizing Delivery or Efficacy of Human-TTR-Targeting Reagent *In Vivo* or *Ex Vivo*

[00156] Various methods are provided for optimizing delivery of human-TTR-targeting reagents to a cell or non-human animal or optimizing the activity or efficacy of human-TTR-targeting reagents *in vivo*. Such methods can comprise, for example: (a) performing the method of testing the efficacy of a human-TTR-targeting reagent as described above a first time in a first non-human animal or first cell; (b) changing a variable and performing the method a second time in a second non-human animal (i.e., of the same species) or a second cell with the changed variable; and (c) comparing the activity of the human-TTR-targeting reagent in step (a) with the activity of the human-TTR-targeting reagent in step (b), and selecting the method resulting in the higher activity.

[00157] Methods of measuring delivery, efficacy, or activity of human-TTR-targeting reagents are disclosed elsewhere herein. For example, such methods can comprise measuring modification of the humanized *TTR* locus comprising the beta-slip mutation. More effective modification of the humanized *TTR* locus can mean different things depending on the desired effect within the non-human animal or cell. For example, more effective modification of the humanized *TTR* locus can mean one or more or all of higher levels of modification, higher

precision, higher consistency, or higher specificity. Higher levels of modification (i.e., higher efficacy) of the humanized *TTR* locus refers to a higher percentage of cells is targeted within a particular target cell type, within a particular target tissue, or within a particular target organ (e.g., liver). Higher precision refers to more precise modification of the humanized *TTR* locus (e.g., a higher percentage of targeted cells having the same modification or having the desired modification without extra unintended insertions and deletions (e.g., NHEJ indels)). Higher consistency refers to more consistent modification of the humanized *TTR* locus among different types of targeted cells, tissues, or organs if more than one type of cell, tissue, or organ is being targeted (e.g., modification of a greater number of cell types within the liver). If a particular organ is being targeted, higher consistency can also refer to more consistent modification throughout all locations within the organ (e.g., the liver). Higher specificity can refer to higher specificity with respect to the genomic locus or loci targeted, higher specificity with respect to the cell type targeted, higher specificity with respect to the tissue type targeted, or higher specificity with respect to the organ targeted. For example, increased genomic locus specificity refers to less modification of off-target genomic loci (e.g., a lower percentage of targeted cells having modifications at unintended, off-target genomic loci instead of or in addition to modification of the target genomic locus). Likewise, increased cell type, tissue, or organ type specificity refers to less modification of off-target cell types, tissue types, or organ types if a particular cell type, tissue type, or organ type is being targeted (e.g., when a particular organ is targeted (e.g., the liver), there is less modification of cells in organs or tissues that are not intended targets).

[00158] Alternatively, such methods can comprise measuring expression of *TTR* mRNA or TTR protein. In one example, a more effective human-TTR-targeting agent results in a greater decrease in *TTR* mRNA or TTR protein expression. Alternatively, such methods can comprise measuring TTR activity. In one example, a more effective human-TTR-targeting agent results in a greater decrease in TTR activity.

[00159] The variable that is changed can be any parameter. As one example, the changed variable can be the packaging or the delivery method by which the human-TTR-targeting reagent or reagents are introduced into the cell or non-human animal. Examples of delivery methods, such as LNP, HDD, and AAV, are disclosed elsewhere herein. For example, the changed variable can be the AAV serotype. Similarly, the administering can comprise LNP-mediated

delivery, and the changed variable can be the LNP formulation. As another example, the changed variable can be the route of administration for introduction of the human-TTR-targeting reagent or reagents into the cell or non-human animal. Examples of routes of administration, such as intravenous, intravitreal, intraparenchymal, and nasal instillation, are disclosed elsewhere herein.

[00160] As another example, the changed variable can be the concentration or amount of the human-TTR-targeting reagent or reagents introduced. As another example, the changed variable can be the concentration or the amount of one human-TTR-targeting reagent introduced (e.g., guide RNA, Cas protein, exogenous donor nucleic acid, RNAi agent, or ASO) relative to the concentration or the amount another human-TTR-targeting reagent introduced (e.g., guide RNA, Cas protein, exogenous donor nucleic acid, RNAi agent, or ASO).

[00161] As another example, the changed variable can be the timing of introducing the human-TTR-targeting reagent or reagents relative to the timing of assessing the activity or efficacy of the reagents. As another example, the changed variable can be the number of times or frequency with which the human-TTR-targeting reagent or reagents are introduced. As another example, the changed variable can be the timing of introduction of one human-TTR-targeting reagent introduced (e.g., guide RNA, Cas protein, exogenous donor nucleic acid, RNAi agent, or ASO) relative to the timing of introduction of another human-TTR-targeting reagent introduced (e.g., guide RNA, Cas protein, exogenous donor nucleic acid, RNAi agent, or ASO).

[00162] As another example, the changed variable can be the form in which the human-TTR-targeting reagent or reagents are introduced. For example, a guide RNA can be introduced in the form of DNA or in the form of RNA. A Cas protein (e.g., Cas9) can be introduced in the form of DNA, in the form of RNA, or in the form of a protein (e.g., complexed with a guide RNA). An exogenous donor nucleic acid can be DNA, RNA, single-stranded, double-stranded, linear, circular, and so forth. Similarly, each of the components can comprise various combinations of modifications for stability, to reduce off-target effects, to facilitate delivery, and so forth. Likewise, RNAi agents and ASOs, for example, can comprise various combinations of modifications for stability, to reduce off-target effects, to facilitate delivery, and so forth. As another example, the changed variable can be the human-TTR-targeting reagent or reagents that are introduced (e.g., introducing a different guide RNA with a different sequence, introducing a different Cas protein (e.g., introducing a different Cas protein with a different sequence, or a

nucleic acid with a different sequence but encoding the same Cas protein amino acid sequence), or introducing a different exogenous donor nucleic acid with a different sequence).

[00163] In a specific example, the human-TTR-targeting reagent comprises a Cas protein and a guide RNA designed to target a guide RNA target sequence in a human *TTR* gene. In such methods, the changed variable can be the guide RNA sequence and/or the guide RNA target sequence. Similarly, if the human-TTR-targeting reagent comprises an RNAi agent or an ASO, the changed variable can be introducing a different RNAi agent or ASO with a different sequence. In some such methods, the Cas protein and the guide RNA can each be administered in the form of RNA, and the changed variable can be the ratio of Cas mRNA to guide RNA (e.g., in an LNP formulation). In some such methods, the changed variable can be guide RNA modifications (e.g., a guide RNA with a modification is compared to a guide RNA without the modification).

C. Human-TTR-Targeting Reagents

[00164] A human-TTR-targeting reagent can be any reagent that targets a human *TTR* gene, a human *TTR* mRNA, or a human TTR protein. The human-TTR-targeting reagent can target any region of a human *TTR* gene, a human *TTR* mRNA, or a human TTR protein (i.e., not only the region comprising the beta-slip mutation but also any other region as well). For example, it can be a genome editing reagent such as a nuclease agent that cleaves a target sequence within the human *TTR* gene, it can be an antisense oligonucleotide targeting a human *TTR* mRNA, it can be an antigen-binding protein targeting an epitope of a human TTR protein, or it can be a small molecule targeting human TTR. Human-TTR-targeting reagents in the methods disclosed herein can be known human-TTR-targeting reagents, can be putative-TTR-targeting reagents (e.g., candidate reagents designed to target human TTR), or can be reagents being screened for human-TTR-targeting activity.

(1) Nuclease Agents Targeting Human TTR *Gene*

[00165] A human-TTR-targeting reagent can be a genome editing reagent such as a nuclease agent that cleaves a target sequence within the human *TTR* gene. A nuclease target sequence includes a DNA sequence at which a nick or double-strand break is induced by a nuclease agent. The target sequence for a nuclease agent can be endogenous (or native) to the cell or the target

sequence can be exogenous to the cell. A target sequence that is exogenous to the cell is not naturally occurring in the genome of the cell. The target sequence can also exogenous to the polynucleotides of interest that one desires to be positioned at the target locus. In some cases, the target sequence is present only once in the genome of the host cell.

[00166] The length of the target sequence can vary, and includes, for example, target sequences that are about 30-36 bp for a zinc finger nuclease (ZFN) pair (i.e., about 15-18 bp for each ZFN), about 36 bp for a Transcription Activator-Like Effector Nuclease (TALEN), or about 20 bp for a CRISPR/Cas9 guide RNA.

[00167] Any nuclease agent that induces a nick or double-strand break at a desired target sequence can be used in the methods and compositions disclosed herein. A naturally occurring or native nuclease agent can be employed so long as the nuclease agent induces a nick or double-strand break in a desired target sequence. Alternatively, a modified or engineered nuclease agent can be employed. An "engineered nuclease agent" includes a nuclease that is engineered (modified or derived) from its native form to specifically recognize and induce a nick or double-strand break in the desired target sequence. Thus, an engineered nuclease agent can be derived from a native, naturally occurring nuclease agent or it can be artificially created or synthesized. The engineered nuclease can induce a nick or double-strand break in a target sequence, for example, wherein the target sequence is not a sequence that would have been recognized by a native (non-engineered or non-modified) nuclease agent. The modification of the nuclease agent can be as little as one amino acid in a protein cleavage agent or one nucleotide in a nucleic acid cleavage agent. Producing a nick or double-strand break in a target sequence or other DNA can be referred to herein as "cutting" or "cleaving" the target sequence or other DNA.

[00168] Active variants and fragments of the exemplified target sequences are also provided. Such active variants can comprise at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the given target sequence, wherein the active variants retain biological activity and hence are capable of being recognized and cleaved by a nuclease agent in a sequence-specific manner. Assays to measure the double-strand break of a target sequence by a nuclease agent are well-known. *See, e.g.*, Frendewey *et al.* (2010) *Methods in Enzymology* 476:295-307, which is incorporated by reference herein in its entirety for all purposes.

[00169] The target sequence of the nuclease agent can be positioned anywhere in or near the *Ttr* locus. The target sequence can be located within a coding region of the *Ttr* gene, or within regulatory regions that influence the expression of the gene. A target sequence of the nuclease agent can be located in an intron, an exon, a promoter, an enhancer, a regulatory region, or any non-protein coding region.

[00170] One type of nuclease agent is a Transcription Activator-Like Effector Nuclease (TALEN). TAL effector nucleases are a class of sequence-specific nucleases that can be used to make double-strand breaks at specific target sequences in the genome of a prokaryotic or eukaryotic organism. TAL effector nucleases are created by fusing a native or engineered transcription activator-like (TAL) effector, or functional part thereof, to the catalytic domain of an endonuclease, such as, for example, *FokI*. The unique, modular TAL effector DNA binding domain allows for the design of proteins with potentially any given DNA recognition specificity. Thus, the DNA binding domains of the TAL effector nucleases can be engineered to recognize specific DNA target sites and thus, used to make double-strand breaks at desired target sequences. *See* WO 2010/079430; Morbitzer *et al.* (2010) *PNAS* 10.1073/pnas.1013133107; Scholze & Boch (2010) *Virulence* 1:428-432; Christian *et al. Genetics* (2010) 186:757-761; Li *et al.* (2010) *Nuc. Acids Res.* (2010) doi:10.1093/nar/gkq704; and Miller *et al.* (2011) *Nature Biotechnology* 29:143–148, each of which is herein incorporated by reference in its entirety.

[00171] Examples of suitable TAL nucleases, and methods for preparing suitable TAL nucleases, are disclosed, e.g., in US 2011/0239315 A1, US 2011/0269234 A1, US 2011/0145940 A1, US 2003/0232410 A1, US 2005/0208489 A1, US 2005/0026157 A1, US 2005/0064474 A1, US 2006/0188987 A1, and US 2006/0063231 A1, each of which is herein incorporated by reference in its entirety. In various embodiments, TAL effector nucleases are engineered that cut in or near a target nucleic acid sequence in, *e.g.*, a locus of interest or a genomic locus of interest, wherein the target nucleic acid sequence is at or near a sequence to be modified by a targeting vector. The TAL nucleases suitable for use with the various methods and compositions provided herein include those that are specifically designed to bind at or near target nucleic acid sequences to be modified by targeting vectors as described herein.

[00172] In some TALENs, each monomer of the TALEN comprises 33-35 TAL repeats that recognize a single base pair via two hypervariable residues. In some TALENs, the nuclease agent is a chimeric protein comprising a TAL-repeat-based DNA binding domain operably

linked to an independent nuclease such as a FokI endonuclease. For example, the nuclease agent can comprise a first TAL-repeat-based DNA binding domain and a second TAL-repeat-based DNA binding domain, wherein each of the first and the second TAL-repeat-based DNA binding domains is operably linked to a FokI nuclease, wherein the first and the second TAL-repeat-based DNA binding domain recognize two contiguous target DNA sequences in each strand of the target DNA sequence separated by a spacer sequence of varying length (12-20 bp), and wherein the FokI nuclease subunits dimerize to create an active nuclease that makes a double strand break at a target sequence.

[00173] The nuclease agent employed in the various methods and compositions disclosed herein can further comprise a zinc-finger nuclease (ZFN). In some ZFNs, each monomer of the ZFN comprises 3 or more zinc finger-based DNA binding domains, wherein each zinc finger-based DNA binding domain binds to a 3 bp subsite. In other ZFNs, the ZFN is a chimeric protein comprising a zinc finger-based DNA binding domain operably linked to an independent nuclease such as a FokI endonuclease. For example, the nuclease agent can comprise a first ZFN and a second ZFN, wherein each of the first ZFN and the second ZFN is operably linked to a FokI nuclease subunit, wherein the first and the second ZFN recognize two contiguous target DNA sequences in each strand of the target DNA sequence separated by about 5-7 bp spacer, and wherein the FokI nuclease subunits dimerize to create an active nuclease that makes a double strand break. *See, e.g.*, US20060246567; US20080182332; US20020081614; US20030021776; WO/2002/057308A2; US20130123484; US20100291048; WO/2011/017293A2; and Gaj *et al.* (2013) *Trends in Biotechnology*, 31(7):397-405, each of which is herein incorporated by reference.

[00174] Another type of nuclease agent is a meganuclease. Meganucleases have been classified into four families based on conserved sequence motifs, the families are the LAGLIDADG, GIY-YIG, H-N-H, and His-Cys box families. These motifs participate in the coordination of metal ions and hydrolysis of phosphodiester bonds. Meganucleases are notable for their long target sequences, and for tolerating some sequence polymorphisms in their DNA substrates. Meganuclease domains, structure and function are known, see for example, Guhan and Muniyappa (2003) *Crit Rev Biochem Mol Biol* 38:199-248; Lucas *et al.*, (2001) *Nucleic Acids Res* 29:960-9; Jurica and Stoddard, (1999) *Cell Mol Life Sci* 55:1304-26; Stoddard, (2006) *Q Rev Biophys* 38:49-95; and Moure *et al.*, (2002) *Nat Struct Biol* 9:764. In some examples, a

naturally occurring variant and/or engineered derivative meganuclease is used. Methods for modifying the kinetics, cofactor interactions, expression, optimal conditions, and/or target sequence specificity, and screening for activity are known. *See, e.g.*, Epinat *et al.*, (2003) *Nucleic Acids Res* 31:2952-62; Chevalier *et al.*, (2002) *Mol Cell* 10:895-905; Gimble *et al.*, (2003) *Mol Biol* 334:993-1008; Seligman *et al.*, (2002) *Nucleic Acids Res* 30:3870-9; Sussman *et al.*, (2004) *J Mol Biol* 342:31-41; Rosen *et al.*, (2006) *Nucleic Acids Res* 34:4791-800; Chames *et al.*, (2005) *Nucleic Acids Res* 33:e178; Smith *et al.*, (2006) *Nucleic Acids Res* 34:e149; Gruen *et al.*, (2002) *Nucleic Acids Res* 30:e29; Chen and Zhao, (2005) *Nucleic Acids Res* 33:e154; WO2005105989; WO2003078619; WO2006097854; WO2006097853; WO2006097784; and WO2004031346, each of which is herein incorporated by reference in its entirety.

[00175] Any meganuclease can be used, including, for example, I-SceI, I-SceII, I-SceIII, I-SceIV, I-SceV, I-SceVI, I-SceVII, I-CeuI, I-CeuAIIP, I-CreI, I-CrepsbIP, I-CrepsbIIP, I-CrepsbIIIP, I-CrepsbIVP, I-TliI, I-PpoI, PI-PspI, F-SceI, F-SceII, F-SuvI, F-TevI, F-TevII, I-AmaI, I-AniI, I-ChuI, I-CmoeI, I-CpaI, I-CpaII, I-CsmI, I-CvuI, I-CvuAIP, I-DdiI, I-DdiII, I-DirI, I-DmoI, I-HmuI, I-HmuII, I-HsNIP, I-LlaI, I-MsoI, I-NaaI, I-NanI, I-NcIIP, I-NgrIP, I-NitI, I-NjaI, I-Nsp236IP, I-PakI, I-PboIP, I-PcuIP, I-PcuAI, I-PcuVI, I-PgrIP, I-PobIP, I-PorI, I-PorIIP, I-PbpIP, I-SpBetaIP, I-ScaI, I-SexIP, I-SneIP, I-SpomI, I-SpomCP, I-SpomIP, I-SpomIIP, I-SquIP, I-Ssp6803I, I-SthPhiJP, I-SthPhiST3P, I-SthPhiSTe3bP, I-TdeIP, I-TevI, I-TevII, I-TevIII, I-UarAP, I-UarHGPAIP, I-UarHGPA13P, I-VinIP, I-ZbiIP, PI-MtuI, PI-MtuHIP PI-MtuHIIP, PI-PfuI, PI-PfuII, PI-PkoI, PI-PkoII, PI-Rma43812IP, PI-SpBetaIP, PI-SceI, PI-TfuI, PI-TfuII, PI-ThyI, PI-TliI, PI-TliII, or any active variants or fragments thereof.

[00176] Meganucleases can recognize, for example, double-stranded DNA sequences of 12 to 40 base pairs. In some cases, the meganuclease recognizes one perfectly matched target sequence in the genome.

[00177] Some meganucleases are homing nucleases. One type of homing nuclease is a LAGLIDADG family of homing nucleases including, for example, I-SceI, I-CreI, and I-Dmol.

[00178] Nuclease agents can further comprise CRISPR/Cas systems as described in more detail below.

[00179] Active variants and fragments of nuclease agents (i.e., an engineered nuclease agent) are also provided. Such active variants can comprise at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the native

nuclease agent, wherein the active variants retain the ability to cut at a desired target sequence and hence retain nick or double-strand-break-inducing activity. For example, any of the nuclease agents described herein can be modified from a native endonuclease sequence and designed to recognize and induce a nick or double-strand break at a target sequence that was not recognized by the native nuclease agent. Thus, some engineered nucleases have a specificity to induce a nick or double-strand break at a target sequence that is different from the corresponding native nuclease agent target sequence. Assays for nick or double-strand-break-inducing activity are known and generally measure the overall activity and specificity of the endonuclease on DNA substrates containing the target sequence.

[00180] The nuclease agent may be introduced into a cell or non-human animal by any known means. A polypeptide encoding the nuclease agent may be directly introduced into the cell or non-human animal. Alternatively, a polynucleotide encoding the nuclease agent can be introduced into the cell or non-human animal. When a polynucleotide encoding the nuclease agent is introduced, the nuclease agent can be transiently, conditionally, or constitutively expressed within the cell. The polynucleotide encoding the nuclease agent can be contained in an expression cassette and be operably linked to a conditional promoter, an inducible promoter, a constitutive promoter, or a tissue-specific promoter. Examples of promoters are discussed in further detail elsewhere herein. Alternatively, the nuclease agent can be introduced into the cell as an mRNA encoding the nuclease agent.

[00181] A polynucleotide encoding a nuclease agent can be stably integrated in the genome of a cell and operably linked to a promoter active in the cell. Alternatively, a polynucleotide encoding a nuclease agent can be in a targeting vector.

[00182] When the nuclease agent is provided to the cell through the introduction of a polynucleotide encoding the nuclease agent, such a polynucleotide encoding a nuclease agent can be modified to substitute codons having a higher frequency of usage in the cell of interest, as compared to the naturally occurring polynucleotide sequence encoding the nuclease agent. For example, the polynucleotide encoding the nuclease agent can be modified to substitute codons having a higher frequency of usage in a given eukaryotic cell of interest, including a human cell, a non-human cell, a mammalian cell, a rodent cell, a mouse cell, a rat cell or any other host cell of interest, as compared to the naturally occurring polynucleotide sequence.

(2) CRISPR/Cas Systems Targeting Human TTR *Gene*

[00183] A particular type of human-TTR-targeting reagent can be a CRISPR/Cas system that targets the human *TTR* gene. CRISPR/Cas systems include transcripts and other elements involved in the expression of, or directing the activity of, Cas genes. A CRISPR/Cas system can be, for example, a type I, a type II, a type III, or a type V system (e.g., subtype V-A or subtype V-B). CRISPR/Cas systems used in the compositions and methods disclosed herein can be non-naturally occurring. A "non-naturally occurring" system includes anything indicating the involvement of the hand of man, such as one or more components of the system being altered or mutated from their naturally occurring state, being at least substantially free from at least one other component with which they are naturally associated in nature, or being associated with at least one other component with which they are not naturally associated. For example, some CRISPR/Cas systems employ non-naturally occurring CRISPR complexes comprising a gRNA and a Cas protein that do not naturally occur together, employ a Cas protein that does not occur naturally, or employ a gRNA that does not occur naturally.

[00184] ***Cas Proteins and Polynucleotides Encoding Cas Proteins.*** Cas proteins generally comprise at least one RNA recognition or binding domain that can interact with guide RNAs (gRNAs, described in more detail below). Cas proteins can also comprise nuclease domains (e.g., DNase domains or RNase domains), DNA-binding domains, helicase domains, protein-protein interaction domains, dimerization domains, and other domains. Some such domains (e.g., DNase domains) can be from a native Cas protein. Other such domains can be added to make a modified Cas protein. A nuclease domain possesses catalytic activity for nucleic acid cleavage, which includes the breakage of the covalent bonds of a nucleic acid molecule. Cleavage can produce blunt ends or staggered ends, and it can be single-stranded or double-stranded. For example, a wild type Cas9 protein will typically create a blunt cleavage product. Alternatively, a wild type Cpf1 protein (e.g., FnCpf1) can result in a cleavage product with a 5-nucleotide 5' overhang, with the cleavage occurring after the 18th base pair from the PAM sequence on the non-targeted strand and after the 23rd base on the targeted strand. A Cas protein can have full cleavage activity to create a double-strand break at a target genomic locus (e.g., a double-strand break with blunt ends), or it can be a nickase that creates a single-strand break at a target genomic locus.

[00185] Examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas5e (CasD), Cas6, Cas6e, Cas6f, Cas7, Cas8a1, Cas8a2, Cas8b, Cas8c, Cas9 (Csn1 or Csx12), Cas10, Cas10d, CasF, CasG, CasH, Csy1, Csy2, Csy3, Cse1 (CasA), Cse2 (CasB), Cse3 (CasE), Cse4 (CasC), Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, and Cu1966, and homologs or modified versions thereof.

[00186] An exemplary Cas protein is a Cas9 protein or a protein derived from a Cas9 protein. Cas9 proteins are from a type II CRISPR/Cas system and typically share four key motifs with a conserved architecture. Motifs 1, 2, and 4 are RuvC-like motifs, and motif 3 is an HNH motif. Exemplary Cas9 proteins are from *Streptococcus pyogenes*, *Streptococcus thermophilus*, *Streptococcus sp.*, *Staphylococcus aureus*, *Nocardiopsis dassonvillei*, *Streptomyces pristinaespiralis*, *Streptomyces viridochromogenes*, *Streptomyces viridochromogenes*, *Streptosporangium roseum*, *Streptosporangium roseum*, *Alicyclobacillus acidocaldarius*, *Bacillus pseudomycoides*, *Bacillus selenitireducens*, *Exiguobacterium sibiricum*, *Lactobacillus delbrueckii*, *Lactobacillus salivarius*, *Microscilla marina*, *Burkholderiales bacterium*, *Polaromonas naphthalenivorans*, *Polaromonas sp.*, *Crocosphaera watsonii*, *Cyanothece sp.*, *Microcystis aeruginosa*, *Synechococcus sp.*, *Acetohalobium arabaticum*, *Ammonifex degensii*, *Caldicelulosiruptor becscii*, *Candidatus Desulforudis*, *Clostridium botulinum*, *Clostridium difficile*, *Finegoldia magna*, *Natranaerobius thermophilus*, *Pelotomaculum thermopropionicum*, *Acidithiobacillus caldus*, *Acidithiobacillus ferrooxidans*, *Allochromatium vinosum*, *Marinobacter sp.*, *Nitrosococcus halophilus*, *Nitrosococcus watsoni*, *Pseudoalteromonas haloplanktis*, *Ktedonobacter racemifer*, *Methanohalobium evestigatum*, *Anabaena variabilis*, *Nodularia spumigena*, *Nostoc sp.*, *Arthrospira maxima*, *Arthrospira platensis*, *Arthrospira sp.*, *Lyngbya sp.*, *Microcoleus chthonoplastes*, *Oscillatoria sp.*, *Petrotoga mobilis*, *Thermosipho africanus*, *Acaryochloris marina*, *Neisseria meningitidis*, or *Campylobacter jejuni*. Additional examples of the Cas9 family members are described in WO 2014/131833, herein incorporated by reference in its entirety for all purposes. Cas9 from *S. pyogenes* (SpCas9) (assigned SwissProt accession number Q99ZW2) is an exemplary Cas9 protein. Cas9 from *S. aureus* (SaCas9) (assigned UniProt accession number J7RUA5) is another exemplary Cas9 protein. Cas9 from *Campylobacter jejuni* (CjCas9) (assigned UniProt accession number Q0P897) is another exemplary Cas9 protein. *See, e.g.*, Kim *et al.* (2017) *Nat. Comm.* 8:14500, herein incorporated

by reference in its entirety for all purposes. SaCas9 is smaller than SpCas9, and CjCas9 is smaller than both SaCas9 and SpCas9.

[00187] Another example of a Cas protein is a Cpf1 (CRISPR from *Prevotella* and *Francisella* 1) protein. Cpf1 is a large protein (about 1300 amino acids) that contains a RuvC-like nuclease domain homologous to the corresponding domain of Cas9 along with a counterpart to the characteristic arginine-rich cluster of Cas9. However, Cpf1 lacks the HNH nuclease domain that is present in Cas9 proteins, and the RuvC-like domain is contiguous in the Cpf1 sequence, in contrast to Cas9 where it contains long inserts including the HNH domain. *See, e.g.*, Zetsche *et al.* (2015) *Cell* 163(3):759-771, herein incorporated by reference in its entirety for all purposes. Exemplary Cpf1 proteins are from *Francisella tularensis 1*, *Francisella tularensis subsp. novicida*, *Prevotella albensis*, *Lachnospiraceae bacterium MC2017 1*, *Butyrivibrio proteoclasticus*, *Peregrinibacteria bacterium GW2011_GWA2_33_10*, *Parcubacteria bacterium GW2011_GWC2_44_17*, *Smithella sp. SCADC*, *Acidaminococcus sp. BV3L6*, *Lachnospiraceae bacterium MA2020*, *Candidatus Methanoplasma termitum*, *Eubacterium eligens*, *Moraxella bovoculi 237*, *Leptospira inadai*, *Lachnospiraceae bacterium ND2006*, *Porphyromonas crevioricanis 3*, *Prevotella disiens*, and *Porphyromonas macacae*. Cpf1 from *Francisella novicida* U112 (FnCpf1; assigned UniProt accession number A0Q7Q2) is an exemplary Cpf1 protein.

[00188] Cas proteins can be wild type proteins (i.e., those that occur in nature), modified Cas proteins (i.e., Cas protein variants), or fragments of wild type or modified Cas proteins. Cas proteins can also be active variants or fragments with respect to catalytic activity of wild type or modified Cas proteins. Active variants or fragments with respect to catalytic activity can comprise at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the wild type or modified Cas protein or a portion thereof, wherein the active variants retain the ability to cut at a desired cleavage site and hence retain nick-inducing or double-strand-break-inducing activity. Assays for nick-inducing or double-strand-break-inducing activity are known and generally measure the overall activity and specificity of the Cas protein on DNA substrates containing the cleavage site.

[00189] Cas proteins can be modified to increase or decrease one or more of nucleic acid binding affinity, nucleic acid binding specificity, and enzymatic activity. Cas proteins can also be modified to change any other activity or property of the protein, such as stability. For

example, one or more nuclease domains of the Cas protein can be modified, deleted, or inactivated, or a Cas protein can be truncated to remove domains that are not essential for the function of the protein or to optimize (e.g., enhance or reduce) the activity or a property of the Cas protein.

[00190] One example of a modified Cas protein is the modified SpCas9-HF1 protein, which is a high-fidelity variant of *Streptococcus pyogenes* Cas9 harboring alterations (N497A/R661A/Q695A/Q926A) designed to reduce non-specific DNA contacts. *See, e.g.*, Kleinstiver *et al.* (2016) *Nature* 529(7587):490-495, herein incorporated by reference in its entirety for all purposes. Another example of a modified Cas protein is the modified eSpCas9 variant (K848A/K1003A/R1060A) designed to reduce off-target effects. *See, e.g.*, Slaymaker *et al.* (2016) *Science* 351(6268):84-88, herein incorporated by reference in its entirety for all purposes. Other SpCas9 variants include K855A and K810A/K1003A/R1060A.

[00191] Cas proteins can comprise at least one nuclease domain, such as a DNase domain. For example, a wild type Cpf1 protein generally comprises a RuvC-like domain that cleaves both strands of target DNA, perhaps in a dimeric configuration. Cas proteins can also comprise at least two nuclease domains, such as DNase domains. For example, a wild type Cas9 protein generally comprises a RuvC-like nuclease domain and an HNH-like nuclease domain. The RuvC and HNH domains can each cut a different strand of double-stranded DNA to make a double-stranded break in the DNA. *See, e.g.*, Jinek *et al.* (2012) *Science* 337:816-821, herein incorporated by reference in its entirety for all purposes.

[00192] One or more or all of the nuclease domains can be deleted or mutated so that they are no longer functional or have reduced nuclease activity. For example, if one of the nuclease domains is deleted or mutated in a Cas9 protein, the resulting Cas9 protein can be referred to as a nickase and can generate a single-strand break within a double-stranded target DNA but not a double-strand break (i.e., it can cleave the complementary strand or the non-complementary strand, but not both). If both of the nuclease domains are deleted or mutated, the resulting Cas protein (e.g., Cas9) will have a reduced ability to cleave both strands of a double-stranded DNA (e.g., a nuclease-null or nuclease-inactive Cas protein, or a catalytically dead Cas protein (dCas)). An example of a mutation that converts Cas9 into a nickase is a D10A (aspartate to alanine at position 10 of Cas9) mutation in the RuvC domain of Cas9 from *S. pyogenes*. Likewise, H939A (histidine to alanine at amino acid position 839), H840A (histidine to alanine

at amino acid position 840), or N863A (asparagine to alanine at amino acid position N863) in the HNH domain of Cas9 from *S. pyogenes* can convert the Cas9 into a nickase. Other examples of mutations that convert Cas9 into a nickase include the corresponding mutations to Cas9 from *S. thermophilus*. *See, e.g.*, Sapranauskas *et al.* (2011) *Nucleic Acids Research* 39:9275-9282 and WO 2013/141680, each of which is herein incorporated by reference in its entirety for all purposes. Such mutations can be generated using methods such as site-directed mutagenesis, PCR-mediated mutagenesis, or total gene synthesis. Examples of other mutations creating nickases can be found, for example, in WO 2013/176772 and WO 2013/142578, each of which is herein incorporated by reference in its entirety for all purposes. If all of the nuclease domains are deleted or mutated in a Cas protein (e.g., both of the nuclease domains are deleted or mutated in a Cas9 protein), the resulting Cas protein (e.g., Cas9) will have a reduced ability to cleave both strands of a double-stranded DNA (e.g., a nuclease-null or nuclease-inactive Cas protein). One specific example is a D10A/H840A *S. pyogenes* Cas9 double mutant or a corresponding double mutant in a Cas9 from another species when optimally aligned with *S. pyogenes* Cas9. Another specific example is a D10A/N863A *S. pyogenes* Cas9 double mutant or a corresponding double mutant in a Cas9 from another species when optimally aligned with *S. pyogenes* Cas9.

[00193] Examples of inactivating mutations in the catalytic domains of *Staphylococcus aureus* Cas9 proteins are also known. For example, the *Staphylococcus aureus* Cas9 enzyme (SaCas9) may comprise a substitution at position N580 (e.g., N580A substitution) and a substitution at position D10 (e.g., D10A substitution) to generate a nuclease-inactive Cas protein. *See, e.g.*, WO 2016/106236, herein incorporated by reference in its entirety for all purposes.

[00194] Examples of inactivating mutations in the catalytic domains of Cpf1 proteins are also known. With reference to Cpf1 proteins from *Francisella novicida* U112 (FnCpf1), *Acidaminococcus* sp. BV3L6 (AsCpf1), *Lachnospiraceae bacterium* ND2006 (LbCpf1), and *Moraxella bovoculi* 237 (MbCpf1 Cpf1), such mutations can include mutations at positions 908, 993, or 1263 of AsCpf1 or corresponding positions in Cpf1 orthologs, or positions 832, 925, 947, or 1180 of LbCpf1 or corresponding positions in Cpf1 orthologs. Such mutations can include, for example one or more of mutations D908A, E993A, and D1263A of AsCpf1 or corresponding mutations in Cpf1 orthologs, or D832A, E925A, D947A, and D1180A of LbCpf1 or corresponding mutations in Cpf1 orthologs. *See, e.g.*, US 2016/0208243, herein incorporated by reference in its entirety for all purposes.

[00195] Cas proteins can also be operably linked to heterologous polypeptides as fusion proteins. For example, a Cas protein can be fused to a cleavage domain or an epigenetic modification domain. *See* WO 2014/089290, herein incorporated by reference in its entirety for all purposes. Cas proteins can also be fused to a heterologous polypeptide providing increased or decreased stability. The fused domain or heterologous polypeptide can be located at the N-terminus, the C-terminus, or internally within the Cas protein.

[00196] As one example, a Cas protein can be fused to one or more heterologous polypeptides that provide for subcellular localization. Such heterologous polypeptides can include, for example, one or more nuclear localization signals (NLS) such as the monopartite SV40 NLS and/or a bipartite alpha-importin NLS for targeting to the nucleus, a mitochondrial localization signal for targeting to the mitochondria, an ER retention signal, and the like. *See, e.g.*, Lange *et al.* (2007) *J. Biol. Chem.* 282:5101-5105, herein incorporated by reference in its entirety for all purposes. Such subcellular localization signals can be located at the N-terminus, the C-terminus, or anywhere within the Cas protein. An NLS can comprise a stretch of basic amino acids, and can be a monopartite sequence or a bipartite sequence. Optionally, a Cas protein can comprise two or more NLSs, including an NLS (e.g., an alpha-importin NLS or a monopartite NLS) at the N-terminus and an NLS (e.g., an SV40 NLS or a bipartite NLS) at the C-terminus. A Cas protein can also comprise two or more NLSs at the N-terminus and/or two or more NLSs at the C-terminus.

[00197] Cas proteins can also be operably linked to a cell-penetrating domain or protein transduction domain. For example, the cell-penetrating domain can be derived from the HIV-1 TAT protein, the TLM cell-penetrating motif from human hepatitis B virus, MPG, Pep-1, VP22, a cell penetrating peptide from Herpes simplex virus, or a polyarginine peptide sequence. *See, e.g.*, WO 2014/089290 and WO 2013/176772, each of which is herein incorporated by reference in its entirety for all purposes. The cell-penetrating domain can be located at the N-terminus, the C-terminus, or anywhere within the Cas protein.

[00198] Cas proteins can also be operably linked to a heterologous polypeptide for ease of tracking or purification, such as a fluorescent protein, a purification tag, or an epitope tag. Examples of fluorescent proteins include green fluorescent proteins (e.g., GFP, GFP-2, tagGFP, turboGFP, eGFP, Emerald, Azami Green, Monomeric Azami Green, CopGFP, AceGFP, ZsGreenl), yellow fluorescent proteins (e.g., YFP, eYFP, Citrine, Venus, YPet, PhiYFP,

ZsYellowl), blue fluorescent proteins (e.g., eBFP, eBFP2, Azurite, mKalamal, GFPuv, Sapphire, T-sapphire), cyan fluorescent proteins (e.g., eCFP, Cerulean, CyPet, AmCyanl, Midoriishi-Cyan), red fluorescent proteins (e.g., mKate, mKate2, mPlum, DsRed monomer, mCherry, mRFP1, DsRed-Express, DsRed2, DsRed-Monomer, HcRed-Tandem, HcRedl, AsRed2, eqFP611, mRaspberry, mStrawberry, Jred), orange fluorescent proteins (e.g., mOrange, mKO, Kusabira-Orange, Monomeric Kusabira-Orange, mTangerine, tdTomato), and any other suitable fluorescent protein. Examples of tags include glutathione-S-transferase (GST), chitin binding protein (CBP), maltose binding protein, thioredoxin (TRX), poly(NANP), tandem affinity purification (TAP) tag, myc, AcV5, AU1 , AU5, E, ECS, E2, FLAG, hemagglutinin (HA), nus, Softag 1, Softag 3, Strep, SBP, Glu-Glu, HSV, KT3, S, S1 , T7, V5, VSV-G, histidine (His), biotin carboxyl carrier protein (BCCP), and calmodulin.

[00199] Cas proteins can also be tethered to exogenous donor nucleic acids or labeled nucleic acids. Such tethering (i.e., physical linking) can be achieved through covalent interactions or noncovalent interactions, and the tethering can be direct (e.g., through direct fusion or chemical conjugation, which can be achieved by modification of cysteine or lysine residues on the protein or intein modification), or can be achieved through one or more intervening linkers or adapter molecules such as streptavidin or aptamers. *See, e.g.*, Pierce *et al.* (2005) *Mini Rev. Med. Chem.* 5(1):41-55; Duckworth *et al.* (2007) *Angew. Chem. Int. Ed. Engl.* 46(46):8819-8822; Schaeffer and Dixon (2009) *Australian J. Chem.* 62(10):1328-1332; Goodman *et al.* (2009) *Chembiochem.* 10(9):1551-1557; and Khatwani *et al.* (2012) *Bioorg. Med. Chem.* 20(14):4532-4539, each of which is herein incorporated by reference in its entirety for all purposes. Noncovalent strategies for synthesizing protein-nucleic acid conjugates include biotin-streptavidin and nickel-histidine methods. Covalent protein-nucleic acid conjugates can be synthesized by connecting appropriately functionalized nucleic acids and proteins using a wide variety of chemistries. Some of these chemistries involve direct attachment of the oligonucleotide to an amino acid residue on the protein surface (e.g., a lysine amine or a cysteine thiol), while other more complex schemes require post-translational modification of the protein or the involvement of a catalytic or reactive protein domain. Methods for covalent attachment of proteins to nucleic acids can include, for example, chemical cross-linking of oligonucleotides to protein lysine or cysteine residues, expressed protein-ligation, chemoenzymatic methods, and the use of photoaptamers. The exogenous donor nucleic acid or labeled nucleic acid can be tethered to the C-terminus, the

N-terminus, or to an internal region within the Cas protein. In one example, the exogenous donor nucleic acid or labeled nucleic acid is tethered to the C-terminus or the N-terminus of the Cas protein. Likewise, the Cas protein can be tethered to the 5' end, the 3' end, or to an internal region within the exogenous donor nucleic acid or labeled nucleic acid. That is, the exogenous donor nucleic acid or labeled nucleic acid can be tethered in any orientation and polarity. For example, the Cas protein can be tethered to the 5' end or the 3' end of the exogenous donor nucleic acid or labeled nucleic acid.

[00200] Cas proteins can be provided in any form. For example, a Cas protein can be provided in the form of a protein, such as a Cas protein complexed with a gRNA. Alternatively, a Cas protein can be provided in the form of a nucleic acid encoding the Cas protein, such as an RNA (e.g., messenger RNA (mRNA)) or DNA. Optionally, the nucleic acid encoding the Cas protein can be codon optimized for efficient translation into protein in a particular cell or organism. For example, the nucleic acid encoding the Cas protein can be modified to substitute codons having a higher frequency of usage in a bacterial cell, a yeast cell, a human cell, a non-human cell, a mammalian cell, a rodent cell, a mouse cell, a rat cell, or any other host cell of interest, as compared to the naturally occurring polynucleotide sequence. When a nucleic acid encoding the Cas protein is introduced into the cell, the Cas protein can be transiently, conditionally, or constitutively expressed in the cell.

[00201] Cas proteins provided as mRNAs can be modified for improved stability and/or immunogenicity properties. The modifications may be made to one or more nucleosides within the mRNA. Examples of chemical modifications to mRNA nucleobases include pseudouridine, 1-methyl-pseudouridine, and 5-methyl-cytidine. For example, capped and polyadenylated Cas mRNA containing N1-methyl pseudouridine can be used. Likewise, Cas mRNAs can be modified by depletion of uridine using synonymous codons.

[00202] Nucleic acids encoding Cas proteins can be stably integrated in the genome of a cell and operably linked to a promoter active in the cell. Alternatively, nucleic acids encoding Cas proteins can be operably linked to a promoter in an expression construct. Expression constructs include any nucleic acid constructs capable of directing expression of a gene or other nucleic acid sequence of interest (e.g., a Cas gene) and which can transfer such a nucleic acid sequence of interest to a target cell. For example, the nucleic acid encoding the Cas protein can be in a vector comprising a DNA encoding a gRNA. Alternatively, it can be in a vector or plasmid that

is separate from the vector comprising the DNA encoding the gRNA. Promoters that can be used in an expression construct include promoters active, for example, in one or more of a eukaryotic cell, a human cell, a non-human cell, a mammalian cell, a non-human mammalian cell, a rodent cell, a mouse cell, a rat cell, a pluripotent cell, an embryonic stem (ES) cell, an adult stem cell, a developmentally restricted progenitor cell, an induced pluripotent stem (iPS) cell, or a one-cell stage embryo. Such promoters can be, for example, conditional promoters, inducible promoters, constitutive promoters, or tissue-specific promoters. Optionally, the promoter can be a bidirectional promoter driving expression of both a Cas protein in one direction and a guide RNA in the other direction. Such bidirectional promoters can consist of (1) a complete, conventional, unidirectional Pol III promoter that contains 3 external control elements: a distal sequence element (DSE), a proximal sequence element (PSE), and a TATA box; and (2) a second basic Pol III promoter that includes a PSE and a TATA box fused to the 5′ terminus of the DSE in reverse orientation. For example, in the H1 promoter, the DSE is adjacent to the PSE and the TATA box, and the promoter can be rendered bidirectional by creating a hybrid promoter in which transcription in the reverse direction is controlled by appending a PSE and TATA box derived from the U6 promoter. *See, e.g.*, US 2016/0074535, herein incorporated by references in its entirety for all purposes. Use of a bidirectional promoter to express genes encoding a Cas protein and a guide RNA simultaneously allow for the generation of compact expression cassettes to facilitate delivery.

[00203] ***Guide RNAs.*** A "guide RNA" or "gRNA" is an RNA molecule that binds to a Cas protein (e.g., Cas9 protein) and targets the Cas protein to a specific location within a target DNA. Guide RNAs can comprise two segments: a "DNA-targeting segment" and a "protein-binding segment." "Segment" includes a section or region of a molecule, such as a contiguous stretch of nucleotides in an RNA. Some gRNAs, such as those for Cas9, can comprise two separate RNA molecules: an "activator-RNA" (e.g., tracrRNA) and a "targeter-RNA" (e.g., CRISPR RNA or crRNA). Other gRNAs are a single RNA molecule (single RNA polynucleotide), which can also be called a "single-molecule gRNA," a "single-guide RNA," or an "sgRNA." *See, e.g.*, WO 2013/176772, WO 2014/065596, WO 2014/089290, WO 2014/093622, WO 2014/099750, WO 2013/142578, and WO 2014/131833, each of which is herein incorporated by reference in its entirety for all purposes. For Cas9, for example, a single-guide RNA can comprise a crRNA fused to a tracrRNA (e.g., via a linker). For Cpf1, for example, only a crRNA is needed to

achieve binding to a target sequence. The terms "guide RNA" and "gRNA" include both double-molecule (i.e., modular) gRNAs and single-molecule gRNAs.

[00204] An exemplary two-molecule gRNA comprises a crRNA-like ("CRISPR RNA" or "targeter-RNA" or "crRNA" or "crRNA repeat") molecule and a corresponding tracrRNA-like ("trans-acting CRISPR RNA" or "activator-RNA" or "tracrRNA") molecule. A crRNA comprises both the DNA-targeting segment (single-stranded) of the gRNA and a stretch of nucleotides that forms one half of the dsRNA duplex of the protein-binding segment of the gRNA. An example of a crRNA tail, located downstream (3') of the DNA-targeting segment, comprises, consists essentially of, or consists of GUUUUAGAGCUAUGCU (SEQ ID NO: 84). Any of the DNA-targeting segments disclosed herein can be joined to the 5' end of SEQ ID NO: 84 to form a crRNA.

[00205] A corresponding tracrRNA (activator-RNA) comprises a stretch of nucleotides that forms the other half of the dsRNA duplex of the protein-binding segment of the gRNA. A stretch of nucleotides of a crRNA are complementary to and hybridize with a stretch of nucleotides of a tracrRNA to form the dsRNA duplex of the protein-binding domain of the gRNA. As such, each crRNA can be said to have a corresponding tracrRNA. An example of a tracrRNA sequence comprises, consists essentially of, or consists of AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACC GAGUCGGUGCUUU (SEQ ID NO: 85).

[00206] In systems in which both a crRNA and a tracrRNA are needed, the crRNA and the corresponding tracrRNA hybridize to form a gRNA. In systems in which only a crRNA is needed, the crRNA can be the gRNA. The crRNA additionally provides the single-stranded DNA-targeting segment that hybridizes to the complementary strand of a target DNA. If used for modification within a cell, the exact sequence of a given crRNA or tracrRNA molecule can be designed to be specific to the species in which the RNA molecules will be used. *See, e.g.*, Mali *et al.* (2013) *Science* 339:823-826; Jinek *et al.* (2012) *Science* 337:816-821; Hwang *et al.* (2013) *Nat. Biotechnol.* 31:227-229; Jiang *et al.* (2013) *Nat. Biotechnol.* 31:233-239; and Cong *et al.* (2013) *Science* 339:819-823, each of which is herein incorporated by reference in its entirety for all purposes.

[00207] The DNA-targeting segment (crRNA) of a given gRNA comprises a nucleotide sequence that is complementary to a sequence on the complementary strand of the target DNA,

as described in more detail below. The DNA-targeting segment of a gRNA interacts with the target DNA in a sequence-specific manner via hybridization (i.e., base pairing). As such, the nucleotide sequence of the DNA-targeting segment may vary and determines the location within the target DNA with which the gRNA and the target DNA will interact. The DNA-targeting segment of a subject gRNA can be modified to hybridize to any desired sequence within a target DNA. Naturally occurring crRNAs differ depending on the CRISPR/Cas system and organism but often contain a targeting segment of between 21 to 72 nucleotides length, flanked by two direct repeats (DR) of a length of between 21 to 46 nucleotides (*see, e.g.*, WO 2014/131833, herein incorporated by reference in its entirety for all purposes). In the case of *S. pyogenes*, the DRs are 36 nucleotides long and the targeting segment is 30 nucleotides long. The 3' located DR is complementary to and hybridizes with the corresponding tracrRNA, which in turn binds to the Cas protein.

[00208] The DNA-targeting segment can have, for example, a length of at least about 12, 15, 17, 18, 19, 20, 25, 30, 35, or 40 nucleotides. Such DNA-targeting segments can have, for example, a length from about 12 to about 100, from about 12 to about 80, from about 12 to about 50, from about 12 to about 40, from about 12 to about 30, from about 12 to about 25, or from about 12 to about 20 nucleotides. For example, the DNA targeting segment can be from about 15 to about 25 nucleotides (e.g., from about 17 to about 20 nucleotides, or about 17, 18, 19, or 20 nucleotides). *See, e.g.*, US 2016/0024523, herein incorporated by reference in its entirety for all purposes. For Cas9 from *S. pyogenes*, a typical DNA-targeting segment is between 16 and 20 nucleotides in length or between 17 and 20 nucleotides in length. For Cas9 from *S. aureus*, a typical DNA-targeting segment is between 21 and 23 nucleotides in length. For Cpf1, a typical DNA-targeting segment is at least 16 nucleotides in length or at least 18 nucleotides in length.

[00209] TracrRNAs can be in any form (e.g., full-length tracrRNAs or active partial tracrRNAs) and of varying lengths. They can include primary transcripts or processed forms. For example, tracrRNAs (as part of a single-guide RNA or as a separate molecule as part of a two-molecule gRNA) may comprise, consist essentially of, or consist of all or a portion of a wild type tracrRNA sequence (e.g., about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild type tracrRNA sequence). Examples of wild type tracrRNA sequences from *S. pyogenes* include 171-nucleotide, 89-nucleotide, 75-nucleotide, and 65-nucleotide versions. *See, e.g.*, Deltcheva *et al.* (2011) *Nature* 471:602-607; WO 2014/093661,

each of which is herein incorporated by reference in its entirety for all purposes. Examples of tracrRNAs within single-guide RNAs (sgRNAs) include the tracrRNA segments found within +48, +54, +67, and +85 versions of sgRNAs, where "+n" indicates that up to the +n nucleotide of wild type tracrRNA is included in the sgRNA. *See* US 8,697,359, herein incorporated by reference in its entirety for all purposes.

[00210] The percent complementarity between the DNA-targeting segment of the guide RNA and the complementary strand of the target DNA can be at least 60% (e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100%). The percent complementarity between the DNA-targeting segment and the complementary strand of the target DNA can be at least 60% over about 20 contiguous nucleotides. As an example, the percent complementarity between the DNA-targeting segment and the complementary strand of the target DNA can be 100% over the 14 contiguous nucleotides at the 5' end of the complementary strand of the target DNA and as low as 0% over the remainder. In such a case, the DNA-targeting segment can be considered to be 14 nucleotides in length. As another example, the percent complementarity between the DNA-targeting segment and the complementary strand of the target DNA can be 100% over the seven contiguous nucleotides at the 5' end of the complementary strand of the target DNA and as low as 0% over the remainder. In such a case, the DNA-targeting segment can be considered to be 7 nucleotides in length. In some guide RNAs, at least 17 nucleotides within the DNA-targeting segment are complementary to the complementary strand of the target DNA. For example, the DNA-targeting segment can be 20 nucleotides in length and can comprise 1, 2, or 3 mismatches with the complementary strand of the target DNA. In one example, the mismatches are not adjacent to the region of the complementary strand corresponding to the protospacer adjacent motif (PAM) sequence (i.e., the reverse complement of the PAM sequence) (e.g., the mismatches are in the 5' end of the DNA-targeting segment of the guide RNA, or the mismatches are at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19 base pairs away from the region of the complementary strand corresponding to the PAM sequence).

[00211] The protein-binding segment of a gRNA can comprise two stretches of nucleotides that are complementary to one another. The complementary nucleotides of the protein-binding segment hybridize to form a double-stranded RNA duplex (dsRNA). The protein-binding

segment of a subject gRNA interacts with a Cas protein, and the gRNA directs the bound Cas protein to a specific nucleotide sequence within target DNA via the DNA-targeting segment.

[00212] Single-guide RNAs can comprise a DNA-targeting segment and a scaffold sequence (i.e., the protein-binding or Cas-binding sequence of the guide RNA). For example, such guide RNAs can have a 5' DNA-targeting segment joined to a 3' scaffold sequence. Exemplary scaffold sequences comprise, consist essentially of, or consist of: GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU (version 1; SEQ ID NO: 86); GUUGGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC (version 2; SEQ ID NO: 87); GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC (version 3; SEQ ID NO: 88); and GUUUAAGAGCUAUGCUGGAAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC (version 4; SEQ ID NO: 89). Guide RNAs targeting any of the guide RNA target sequences disclosed herein can include, for example, a DNA-targeting segment on the 5' end of the guide RNA fused to any of the exemplary guide RNA scaffold sequences on the 3' end of the guide RNA. That is, any of the DNA-targeting segments disclosed herein can be joined to the 5' end of any one of the above scaffold sequences to form a single guide RNA (chimeric guide RNA).

[00213] Guide RNAs can include modifications or sequences that provide for additional desirable features (e.g., modified or regulated stability; subcellular targeting; tracking with a fluorescent label; a binding site for a protein or protein complex; and the like). Examples of such modifications include, for example, a 5' cap (e.g., a 7-methylguanylate cap (m7G)); a 3' polyadenylated tail (i.e., a 3' poly(A) tail); a riboswitch sequence (e.g., to allow for regulated stability and/or regulated accessibility by proteins and/or protein complexes); a stability control sequence; a sequence that forms a dsRNA duplex (i.e., a hairpin); a modification or sequence that targets the RNA to a subcellular location (e.g., nucleus, mitochondria, chloroplasts, and the like); a modification or sequence that provides for tracking (e.g., direct conjugation to a fluorescent molecule, conjugation to a moiety that facilitates fluorescent detection, a sequence that allows for fluorescent detection, and so forth); a modification or sequence that provides a binding site for proteins (e.g., proteins that act on DNA, including DNA methyltransferases,

DNA demethylases, histone acetyltransferases, histone deacetylases, and the like); and combinations thereof. Other examples of modifications include engineered stem loop duplex structures, engineered bulge regions, engineered hairpins 3' of the stem loop duplex structure, or any combination thereof. *See, e.g.*, US 2015/0376586, herein incorporated by reference in its entirety for all purposes. A bulge can be an unpaired region of nucleotides within the duplex made up of the crRNA-like region and the minimum tracrRNA-like region. A bulge can comprise, on one side of the duplex, an unpaired 5′-XXXY-3′ where X is any purine and Y can be a nucleotide that can form a wobble pair with a nucleotide on the opposite strand, and an unpaired nucleotide region on the other side of the duplex.

[00214] Unmodified nucleic acids can be prone to degradation. Exogenous nucleic acids can also induce an innate immune response. Modifications can help introduce stability and reduce immunogenicity. Guide RNAs can comprise modified nucleosides and modified nucleotides including, for example, one or more of the following: (1) alteration or replacement of one or both of the non-linking phosphate oxygens and/or of one or more of the linking phosphate oxygens in the phosphodiester backbone linkage; (2) alteration or replacement of a constituent of the ribose sugar such as alteration or replacement of the 2' hydroxyl on the ribose sugar; (3) replacement of the phosphate moiety with dephospho linkers; (4) modification or replacement of a naturally occurring nucleobase; (5) replacement or modification of the ribose-phosphate backbone; (6) modification of the 3' end or 5' end of the oligonucleotide (e.g., removal, modification or replacement of a terminal phosphate group or conjugation of a moiety); and (7) modification of the sugar. Other possible guide RNA modifications include modifications of or replacement of uracils or poly-uracil tracts. *See, e.g.*, WO 2015/048577 and US 2016/0237455, each of which is herein incorporated by reference in its entirety for all purposes. Similar modifications can be made to Cas-encoding nucleic acids, such as Cas mRNAs.

[00215] As one example, nucleotides at the 5' or 3' end of a guide RNA can include phosphorothioate linkages (e.g., the bases can have a modified phosphate group that is a phosphorothioate group). For example, a guide RNA can include phosphorothioate linkages between the 2, 3, or 4 terminal nucleotides at the 5' or 3' end of the guide RNA. As another example, nucleotides at the 5' and/or 3' end of a guide RNA can have 2'-O-methyl modifications. For example, a guide RNA can include 2'-O-methyl modifications at the 2, 3, or 4 terminal nucleotides at the 5' and/or 3' end of the guide RNA (e.g., the 5' end). *See, e.g.*, WO

2017/173054 A1 and Finn et al. (2018) *Cell Reports* 22:1-9, each of which is herein incorporated by reference in its entirety for all purposes. In one specific example, the guide RNA comprises 2'-O-methyl analogs and 3' phosphorothioate internucleotide linkages at the first three 5' and 3' terminal RNA residues. In another specific example, the guide RNA is modified such that all 2'OH groups that do not interact with the Cas9 protein are replaced with 2'-O-methyl analogs, and the tail region of the guide RNA, which has minimal interaction with Cas9, is modified with 5' and 3' phosphorothioate internucleotide linkages. *See, e.g.*, Yin et al. (2017) *Nat. Biotech.* 35(12):1179-1187, herein incorporated by reference in its entirety for all purposes. Other examples of modified guide RNAs are provided, e.g., in WO 2018/107028 A1, herein incorporated by reference in its entirety for all purposes.

[00216] Guide RNAs can be provided in any form. For example, the gRNA can be provided in the form of RNA, either as two molecules (separate crRNA and tracrRNA) or as one molecule (sgRNA), and optionally in the form of a complex with a Cas protein. The gRNA can also be provided in the form of DNA encoding the gRNA. The DNA encoding the gRNA can encode a single RNA molecule (sgRNA) or separate RNA molecules (e.g., separate crRNA and tracrRNA). In the latter case, the DNA encoding the gRNA can be provided as one DNA molecule or as separate DNA molecules encoding the crRNA and tracrRNA, respectively.

[00217] When a gRNA is provided in the form of DNA, the gRNA can be transiently, conditionally, or constitutively expressed in the cell. DNAs encoding gRNAs can be stably integrated into the genome of the cell and operably linked to a promoter active in the cell. Alternatively, DNAs encoding gRNAs can be operably linked to a promoter in an expression construct. For example, the DNA encoding the gRNA can be in a vector comprising a heterologous nucleic acid, such as a nucleic acid encoding a Cas protein. Alternatively, it can be in a vector or a plasmid that is separate from the vector comprising the nucleic acid encoding the Cas protein. Promoters that can be used in such expression constructs include promoters active, for example, in one or more of a eukaryotic cell, a human cell, a non-human cell, a mammalian cell, a non-human mammalian cell, a rodent cell, a mouse cell, a rat cell, a hamster cell, a rabbit cell, a pluripotent cell, an embryonic stem (ES) cell, an adult stem cell, a developmentally restricted progenitor cell, an induced pluripotent stem (iPS) cell, or a one-cell stage embryo. Such promoters can be, for example, conditional promoters, inducible promoters, constitutive promoters, or tissue-specific promoters. Such promoters can also be, for example, bidirectional

promoters. Specific examples of suitable promoters include an RNA polymerase III promoter, such as a human U6 promoter, a rat U6 polymerase III promoter, or a mouse U6 polymerase III promoter.

[00218] Alternatively, gRNAs can be prepared by various other methods. For example, gRNAs can be prepared by *in vitro* transcription using, for example, T7 RNA polymerase (*see, e.g.*, WO 2014/089290 and WO 2014/065596, each of which is herein incorporated by reference in its entirety for all purposes). Guide RNAs can also be a synthetically produced molecule prepared by chemical synthesis.

[00219] ***Guide RNA Target Sequences.*** Target DNAs for guide RNAs include nucleic acid sequences present in a DNA to which a DNA-targeting segment of a gRNA will bind, provided sufficient conditions for binding exist. Suitable DNA/RNA binding conditions include physiological conditions normally present in a cell. Other suitable DNA/RNA binding conditions (e.g., conditions in a cell-free system) are known in the art (*see, e.g.*, Molecular Cloning: A Laboratory Manual, 3rd Ed. (Sambrook *et al.*, Harbor Laboratory Press 2001), herein incorporated by reference in its entirety for all purposes). The strand of the target DNA that is complementary to and hybridizes with the gRNA can be called the "complementary strand," and the strand of the target DNA that is complementary to the "complementary strand" (and is therefore not complementary to the Cas protein or gRNA) can be called "noncomplementary strand" or "template strand."

[00220] The target DNA includes both the sequence on the complementary strand to which the guide RNA hybridizes and the corresponding sequence on the non-complementary strand (e.g., adjacent to the protospacer adjacent motif (PAM)). The term "guide RNA target sequence" as used herein refers specifically to the sequence on the non-complementary strand corresponding to (i.e., the reverse complement of) the sequence to which the guide RNA hybridizes on the complementary strand. That is, the guide RNA target sequence refers to the sequence on the non-complementary strand adjacent to the PAM (e.g., upstream or 5' of the PAM in the case of Cas9). A guide RNA target sequence is equivalent to the DNA-targeting segment of a guide RNA, but with thymines instead of uracils. As one example, a guide RNA target sequence for an SpCas9 enzyme can refer to the sequence upstream of the 5'-NGG-3' PAM on the non-complementary strand. A guide RNA is designed to have complementarity to the complementary strand of a target DNA, where hybridization between the DNA-targeting

segment of the guide RNA and the complementary strand of the target DNA promotes the formation of a CRISPR complex. Full complementarity is not necessarily required, provided that there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex. If a guide RNA is referred to herein as targeting a guide RNA target sequence, what is meant is that the guide RNA hybridizes to the complementary strand sequence of the target DNA that is the reverse complement of the guide RNA target sequence on the non-complementary strand.

[00221] A target DNA or guide RNA target sequence can comprise any polynucleotide, and can be located, for example, in the nucleus or cytoplasm of a cell or within an organelle of a cell, such as a mitochondrion or chloroplast. A target DNA or guide RNA target sequence can be any nucleic acid sequence endogenous or exogenous to a cell. The guide RNA target sequence can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory sequence) or can include both.

[00222] Site-specific binding and cleavage of a target DNA by a Cas protein can occur at locations determined by both (i) base-pairing complementarity between the guide RNA and the complementary strand of the target DNA and (ii) a short motif, called the protospacer adjacent motif (PAM), in the non-complementary strand of the target DNA. The PAM can flank the guide RNA target sequence. Optionally, the guide RNA target sequence can be flanked on the 3' end by the PAM (e.g., for Cas9). Alternatively, the guide RNA target sequence can be flanked on the 5' end by the PAM (e.g., for Cpf1). For example, the cleavage site of Cas proteins can be about 1 to about 10 or about 2 to about 5 base pairs (e.g., 3 base pairs) upstream or downstream of the PAM sequence (e.g., within the guide RNA target sequence). In the case of SpCas9, the PAM sequence (i.e., on the non-complementary strand) can be 5'-N_1GG-3', where N_1 is any DNA nucleotide, and where the PAM is immediately 3' of the guide RNA target sequence on the non-complementary strand of the target DNA. As such, the sequence corresponding to the PAM on the complementary strand (i.e., the reverse complement) would be 5'-CCN_2-3', where N_2 is any DNA nucleotide and is immediately 5' of the sequence to which the DNA-targeting segment of the guide RNA hybridizes on the complementary strand of the target DNA. In some such cases, N_1 and N_2 can be complementary and the N_1- N_2 base pair can be any base pair (e.g., N_1=C and N_2=G; N_1=G and N_2=C; N_1=A and N_2=T; or N_1=T, and N_2=A). In the case of Cas9 from *S. aureus*, the PAM can be NNGRRT or NNGRR, where N can A, G, C, or T, and R can be

G or A. In the case of Cas9 from *C. jejuni*, the PAM can be, for example, NNNNACAC or NNNNRYAC, where N can be A, G, C, or T, and R can be G or A. In some cases (e.g., for FnCpf1), the PAM sequence can be upstream of the 5' end and have the sequence 5'-TTN-3'.

[00223] An example of a guide RNA target sequence is a 20-nucleotide DNA sequence immediately preceding an NGG motif recognized by an SpCas9 protein. For example, two examples of guide RNA target sequences plus PAMs are $GN_{19}NGG$ (SEQ ID NO: 90) or $N_{20}NGG$ (SEQ ID NO: 91). *See, e.g.*, WO 2014/165825, herein incorporated by reference in its entirety for all purposes. The guanine at the 5' end can facilitate transcription by RNA polymerase in cells. Other examples of guide RNA target sequences plus PAMs can include two guanine nucleotides at the 5' end (e.g., $GGN_{20}NGG$; SEQ ID NO: 92) to facilitate efficient transcription by T7 polymerase *in vitro*. *See, e.g.*, WO 2014/065596, herein incorporated by reference in its entirety for all purposes. Other guide RNA target sequences plus PAMs can have between 4-22 nucleotides in length of SEQ ID NOS: 90–92, including the 5' G or GG and the 3' GG or NGG. Yet other guide RNA target sequences plus PAMs can have between 14 and 20 nucleotides in length of SEQ ID NOS: 90–92.

[00224] Formation of a CRISPR complex hybridized to a target DNA can result in cleavage of one or both strands of the target DNA within or near the region corresponding to the guide RNA target sequence (i.e., the guide RNA target sequence on the non-complementary strand of the target DNA and the reverse complement on the complementary strand to which the guide RNA hybridizes). For example, the cleavage site can be within the guide RNA target sequence (e.g., at a defined location relative to the PAM sequence). The "cleavage site" includes the position of a target DNA at which a Cas protein produces a single-strand break or a double-strand break. The cleavage site can be on only one strand (e.g., when a nickase is used) or on both strands of a double-stranded DNA. Cleavage sites can be at the same position on both strands (producing blunt ends; e.g. Cas9)) or can be at different sites on each strand (producing staggered ends (i.e., overhangs); e.g., Cpf1). Staggered ends can be produced, for example, by using two Cas proteins, each of which produces a single-strand break at a different cleavage site on a different strand, thereby producing a double-strand break. For example, a first nickase can create a single-strand break on the first strand of double-stranded DNA (dsDNA), and a second nickase can create a single-strand break on the second strand of dsDNA such that overhanging sequences are created. In some cases, the guide RNA target sequence or cleavage site of the nickase on the

first strand is separated from the guide RNA target sequence or cleavage site of the nickase on the second strand by at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 75, 100, 250, 500, or 1,000 base pairs.

(3) Exogenous Donor Nucleic Acids Targeting Human TTR *Gene*

[00225] The methods and compositions disclosed herein can utilize exogenous donor nucleic acids to modify the humanized *TTR* locus comprising the beta-slip mutation following cleavage of the humanized *TTR* locus with a nuclease agent. In such methods, the nuclease agent protein cleaves the humanized *TTR* locus to create a single-strand break (nick) or double-strand break, and the exogenous donor nucleic acid recombines the humanized *TTR* locus via non-homologous end joining (NHEJ)-mediated ligation or through a homology-directed repair event. Optionally, repair with the exogenous donor nucleic acid removes or disrupts the nuclease target sequence so that alleles that have been targeted cannot be re-targeted by the nuclease agent.

[00226] Exogenous donor nucleic acids can comprise deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), they can be single-stranded or double-stranded, and they can be in linear or circular form. For example, an exogenous donor nucleic acid can be a single-stranded oligodeoxynucleotide (ssODN). *See, e.g.*, Yoshimi *et al.* (2016) *Nat. Commun.* 7:10431, herein incorporated by reference in its entirety for all purposes. An exemplary exogenous donor nucleic acid is between about 50 nucleotides to about 5 kb in length, is between about 50 nucleotides to about 3 kb in length, or is between about 50 to about 1,000 nucleotides in length. Other exemplary exogenous donor nucleic acids are between about 40 to about 200 nucleotides in length. For example, an exogenous donor nucleic acid can be between about 50-60, 60-70, 70-80, 80-90, 90-100, 100-110, 110-120, 120-130, 130-140, 140-150, 150-160, 160-170, 170-180, 180-190, or 190-200 nucleotides in length. Alternatively, an exogenous donor nucleic acid can be between about 50-100, 100-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800, 800-900, or 900-1000 nucleotides in length. Alternatively, an exogenous donor nucleic acid can be between about 1-1.5, 1.5-2, 2-2.5, 2.5-3, 3-3.5, 3.5-4, 4-4.5, or 4.5-5 kb in length. Alternatively, an exogenous donor nucleic acid can be, for example, no more than 5 kb, 4.5 kb, 4 kb, 3.5 kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb, 900 nucleotides, 800 nucleotides, 700 nucleotides, 600 nucleotides, 500 nucleotides, 400 nucleotides, 300 nucleotides, 200 nucleotides, 100

nucleotides, or 50 nucleotides in length. Exogenous donor nucleic acids (e.g., targeting vectors) can also be longer.

[00227] In one example, an exogenous donor nucleic acid is an ssODN that is between about 80 nucleotides and about 200 nucleotides in length. In another example, an exogenous donor nucleic acids is an ssODN that is between about 80 nucleotides and about 3 kb in length. Such an ssODN can have homology arms, for example, that are each between about 40 nucleotides and about 60 nucleotides in length. Such an ssODN can also have homology arms, for example, that are each between about 30 nucleotides and 100 nucleotides in length. The homology arms can be symmetrical (e.g., each 40 nucleotides or each 60 nucleotides in length), or they can be asymmetrical (e.g., one homology arm that is 36 nucleotides in length, and one homology arm that is 91 nucleotides in length).

[00228] Exogenous donor nucleic acids can include modifications or sequences that provide for additional desirable features (e.g., modified or regulated stability; tracking or detecting with a fluorescent label; a binding site for a protein or protein complex; and so forth). Exogenous donor nucleic acids can comprise one or more fluorescent labels, purification tags, epitope tags, or a combination thereof. For example, an exogenous donor nucleic acid can comprise one or more fluorescent labels (e.g., fluorescent proteins or other fluorophores or dyes), such as at least 1, at least 2, at least 3, at least 4, or at least 5 fluorescent labels. Exemplary fluorescent labels include fluorophores such as fluorescein (e.g., 6-carboxyfluorescein (6-FAM)), Texas Red, HEX, Cy3, Cy5, Cy5.5, Pacific Blue, 5-(and-6)-carboxytetramethylrhodamine (TAMRA), and Cy7. A wide range of fluorescent dyes are available commercially for labeling oligonucleotides (e.g., from Integrated DNA Technologies). Such fluorescent labels (e.g., internal fluorescent labels) can be used, for example, to detect an exogenous donor nucleic acid that has been directly integrated into a cleaved target nucleic acid having protruding ends compatible with the ends of the exogenous donor nucleic acid. The label or tag can be at the 5' end, the 3' end, or internally within the exogenous donor nucleic acid. For example, an exogenous donor nucleic acid can be conjugated at 5' end with the IR700 fluorophore from Integrated DNA Technologies (5'IRDYE®700).

[00229] Exogenous donor nucleic acids can also comprise nucleic acid inserts including segments of DNA to be integrated at the humanized *TTR* locus comprising the beta-slip mutation. Integration of a nucleic acid insert at a humanized *TTR* locus can result in addition of

a nucleic acid sequence of interest to the humanized *TTR* locus, deletion of a nucleic acid sequence of interest at the humanized *TTR* locus, or replacement of a nucleic acid sequence of interest at the humanized *TTR* locus (i.e., deletion and insertion). Some exogenous donor nucleic acids are designed for insertion of a nucleic acid insert at the humanized *TTR* locus without any corresponding deletion at the humanized *TTR* locus. Other exogenous donor nucleic acids are designed to delete a nucleic acid sequence of interest at the humanized *TTR* locus without any corresponding insertion of a nucleic acid insert. Yet other exogenous donor nucleic acids are designed to delete a nucleic acid sequence of interest at the humanized *TTR* locus and replace it with a nucleic acid insert.

[00230] The nucleic acid insert or the corresponding nucleic acid at the humanized *TTR* locus being deleted and/or replaced can be various lengths. An exemplary nucleic acid insert or corresponding nucleic acid at the humanized *TTR* locus being deleted and/or replaced is between about 1 nucleotide to about 5 kb in length or is between about 1 nucleotide to about 1,000 nucleotides in length. For example, a nucleic acid insert or a corresponding nucleic acid at the humanized *TTR* locus being deleted and/or replaced can be between about 1-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-110, 110-120, 120-130, 130-140, 140-150, 150-160, 160-170, 170-180, 180-190, or 190-120 nucleotides in length. Likewise, a nucleic acid insert or a corresponding nucleic acid at the humanized *TTR* locus being deleted and/or replaced can be between 1-100, 100-200, 200-300, 300-400, 400-500, 500-600, 600-700, 700-800, 800-900, or 900-1000 nucleotides in length. Likewise, a nucleic acid insert or a corresponding nucleic acid at the humanized *TTR* locus being deleted and/or replaced can be between about 1-1.5, 1.5-2, 2-2.5, 2.5-3, 3-3.5, 3.5-4, 4-4.5, or 4.5-5 kb in length or longer.

[00231] The nucleic acid insert can comprise a sequence that is homologous or orthologous to all or part of sequence targeted for replacement. For example, the nucleic acid insert can comprise a sequence that comprises one or more point mutations (e.g., 1, 2, 3, 4, 5, or more) compared with a sequence targeted for replacement at the humanized *TTR* locus. Optionally, such point mutations can result in a conservative amino acid substitution (e.g., substitution of aspartic acid [Asp, D] with glutamic acid [Glu, E]) in the encoded polypeptide.

[00232] ***Donor Nucleic Acids for Non-Homologous-End-Joining-Mediated Insertion.*** Some exogenous donor nucleic acids have short single-stranded regions at the 5' end and/or the 3' end that are complementary to one or more overhangs created by nuclease-mediated cleavage

at the humanized *TTR* locus comprising the beta-slip mutation. These overhangs can also be referred to as 5' and 3' homology arms. For example, some exogenous donor nucleic acids have short single-stranded regions at the 5' end and/or the 3' end that are complementary to one or more overhangs created by nuclease-mediated cleavage at 5' and/or 3' target sequences at the humanized *TTR* locus. Some such exogenous donor nucleic acids have a complementary region only at the 5' end or only at the 3' end. For example, some such exogenous donor nucleic acids have a complementary region only at the 5' end complementary to an overhang created at a 5' target sequence at the humanized *TTR* locus or only at the 3' end complementary to an overhang created at a 3' target sequence at the humanized *TTR* locus. Other such exogenous donor nucleic acids have complementary regions at both the 5' and 3' ends. For example, other such exogenous donor nucleic acids have complementary regions at both the 5' and 3' ends e.g., complementary to first and second overhangs, respectively, generated by nuclease-mediated cleavage at the humanized *TTR* locus. For example, if the exogenous donor nucleic acid is double-stranded, the single-stranded complementary regions can extend from the 5' end of the top strand of the donor nucleic acid and the 5' end of the bottom strand of the donor nucleic acid, creating 5' overhangs on each end. Alternatively, the single-stranded complementary region can extend from the 3' end of the top strand of the donor nucleic acid and from the 3' end of the bottom strand of the template, creating 3' overhangs.

[00233] The complementary regions can be of any length sufficient to promote ligation between the exogenous donor nucleic acid and the target nucleic acid. Exemplary complementary regions are between about 1 to about 5 nucleotides in length, between about 1 to about 25 nucleotides in length, or between about 5 to about 150 nucleotides in length. For example, a complementary region can be at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. Alternatively, the complementary region can be about 5-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-110, 110-120, 120-130, 130-140, or 140-150 nucleotides in length, or longer.

[00234] Such complementary regions can be complementary to overhangs created by two pairs of nickases. Two double-strand breaks with staggered ends can be created by using first and second nickases that cleave opposite strands of DNA to create a first double-strand break, and third and fourth nickases that cleave opposite strands of DNA to create a second double-strand break. For example, a Cas protein can be used to nick first, second, third, and fourth

guide RNA target sequences corresponding with first, second, third, and fourth guide RNAs. The first and second guide RNA target sequences can be positioned to create a first cleavage site such that the nicks created by the first and second nickases on the first and second strands of DNA create a double-strand break (i.e., the first cleavage site comprises the nicks within the first and second guide RNA target sequences). Likewise, the third and fourth guide RNA target sequences can be positioned to create a second cleavage site such that the nicks created by the third and fourth nickases on the first and second strands of DNA create a double-strand break (i.e., the second cleavage site comprises the nicks within the third and fourth guide RNA target sequences). Preferably, the nicks within the first and second guide RNA target sequences and/or the third and fourth guide RNA target sequences can be off-set nicks that create overhangs. The offset window can be, for example, at least about 5 bp, 10 bp, 20 bp, 30 bp, 40 bp, 50 bp, 60 bp, 70 bp, 80 bp, 90 bp, 100 bp or more. *See* Ran *et al.* (2013) *Cell* 154:1380-1389; Mali *et al.* (2013) *Nat. Biotech.*31:833-838; and Shen *et al.* (2014) *Nat. Methods* 11:399-404, each of which is herein incorporated by reference in its entirety for all purposes. In such cases, a double-stranded exogenous donor nucleic acid can be designed with single-stranded complementary regions that are complementary to the overhangs created by the nicks within the first and second guide RNA target sequences and by the nicks within the third and fourth guide RNA target sequences. Such an exogenous donor nucleic acid can then be inserted by non-homologous-end-joining-mediated ligation.

[00235] ***Donor Nucleic Acids for Insertion by Homology-Directed Repair.*** Some exogenous donor nucleic acids comprise homology arms. If the exogenous donor nucleic acid also comprises a nucleic acid insert, the homology arms can flank the nucleic acid insert. For ease of reference, the homology arms are referred to herein as 5' and 3' (i.e., upstream and downstream) homology arms. This terminology relates to the relative position of the homology arms to the nucleic acid insert within the exogenous donor nucleic acid. The 5' and 3' homology arms correspond to regions within the humanized *TTR* locus, which are referred to herein as "5' target sequence" and "3' target sequence," respectively.

[00236] A homology arm and a target sequence "correspond" or are "corresponding" to one another when the two regions share a sufficient level of sequence identity to one another to act as substrates for a homologous recombination reaction. The term "homology" includes DNA sequences that are either identical or share sequence identity to a corresponding sequence. The

sequence identity between a given target sequence and the corresponding homology arm found in the exogenous donor nucleic acid can be any degree of sequence identity that allows for homologous recombination to occur. For example, the amount of sequence identity shared by the homology arm of the exogenous donor nucleic acid (or a fragment thereof) and the target sequence (or a fragment thereof) can be at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity, such that the sequences undergo homologous recombination. Moreover, a corresponding region of homology between the homology arm and the corresponding target sequence can be of any length that is sufficient to promote homologous recombination. Exemplary homology arms are between about 25 nucleotides to about 2.5 kb in length, are between about 25 nucleotides to about 1.5 kb in length, or are between about 25 to about 500 nucleotides in length. For example, a given homology arm (or each of the homology arms) and/or corresponding target sequence can comprise corresponding regions of homology that are between about 25-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400-450, or 450-500 nucleotides in length, such that the homology arms have sufficient homology to undergo homologous recombination with the corresponding target sequences within the target nucleic acid. Alternatively, a given homology arm (or each homology arm) and/or corresponding target sequence can comprise corresponding regions of homology that are between about 0.5 kb to about 1 kb, about 1 kb to about 1.5 kb, about 1.5 kb to about 2 kb, or about 2 kb to about 2.5 kb in length. For example, the homology arms can each be about 750 nucleotides in length. The homology arms can be symmetrical (each about the same size in length), or they can be asymmetrical (one longer than the other).

[00237] When a nuclease agent is used in combination with an exogenous donor nucleic acid, the 5' and 3' target sequences are preferably located in sufficient proximity to the nuclease cleavage site (e.g., within sufficient proximity to a the nuclease target sequence) so as to promote the occurrence of a homologous recombination event between the target sequences and the homology arms upon a single-strand break (nick) or double-strand break at the nuclease cleavage site. The term "nuclease cleavage site" includes a DNA sequence at which a nick or double-strand break is created by a nuclease agent (e.g., a Cas9 protein complexed with a guide RNA). The target sequences within the targeted locus that correspond to the 5' and 3' homology arms of the exogenous donor nucleic acid are "located in sufficient proximity" to a nuclease cleavage site

if the distance is such as to promote the occurrence of a homologous recombination event between the 5' and 3' target sequences and the homology arms upon a single-strand break or double-strand break at the nuclease cleavage site. Thus, the target sequences corresponding to the 5' and/or 3' homology arms of the exogenous donor nucleic acid can be, for example, within at least 1 nucleotide of a given nuclease cleavage site or within at least 10 nucleotides to about 1,000 nucleotides of a given nuclease cleavage site. As an example, the nuclease cleavage site can be immediately adjacent to at least one or both of the target sequences.

[00238] The spatial relationship of the target sequences that correspond to the homology arms of the exogenous donor nucleic acid and the nuclease cleavage site can vary. For example, target sequences can be located 5' to the nuclease cleavage site, target sequences can be located 3' to the nuclease cleavage site, or the target sequences can flank the nuclease cleavage site.

(4) Other Human-TTR-Targeting Reagents

[00239] The activity of any other known or putative human-TTR-targeting reagent can also be assessed using the non-human animals disclosed herein. Similarly, any other molecule can be screened for human-TTR-targeting activity using the non-human animals disclosed herein.

[00240] Other human-TTR-targeting reagents can include RNAi agents. An "RNAi agent" is a composition that comprises a small double-stranded RNA or RNA-like (e.g., chemically modified RNA) oligonucleotide molecule capable of facilitating degradation or inhibition of translation of a target RNA, such as messenger RNA (mRNA), in a sequence-specific manner. The oligonucleotide in the RNAi agent is a polymer of linked nucleosides, each of which can be independently modified or unmodified. RNAi agents operate through the RNA interference mechanism (i.e., inducing RNA interference through interaction with the RNA interference pathway machinery (RNA-induced silencing complex or RISC) of mammalian cells). While it is believed that RNAi agents, as that term is used herein, operate primarily through the RNA interference mechanism, the disclosed RNAi agents are not bound by or limited to any particular pathway or mechanism of action. RNAi agents disclosed herein comprise a sense strand and an antisense strand, and include, but are not limited to, short interfering RNAs (siRNAs), double-stranded RNAs (dsRNA), micro RNAs (miRNAs), short hairpin RNAs (shRNA), and dicer substrates. The antisense strand of the RNAi agents described herein is at least partially

complementary to a sequence (i.e., a succession or order of nucleobases or nucleotides, described with a succession of letters using standard nomenclature) in the target RNA.

[00241] Other human-TTR-targeting reagents can include antisense oligonucleotides (ASOs). Single-stranded ASOs and RNA interference (RNAi) share a fundamental principle in that an oligonucleotide binds a target RNA through Watson-Crick base pairing. Without wishing to be bound by theory, during RNAi, a small RNA duplex (RNAi agent) associates with the RNA-induced silencing complex (RISC), one strand (the passenger strand) is lost, and the remaining strand (the guide strand) cooperates with RISC to bind complementary RNA. Argonaute 2 (Ago2), the catalytic component of the RISC, then cleaves the target RNA. The guide strand is always associated with either the complementary sense strand or a protein (RISC). In contrast, an ASO must survive and function as a single strand. ASOs bind to the target RNA and block ribosomes or other factors, such as splicing factors, from binding the RNA or recruit proteins such as nucleases. Different modifications and target regions are chosen for ASOs based on the desired mechanism of action. A gapmer is an ASO oligonucleotide containing 2–5 chemically modified nucleotides (e.g. LNA or 2'-MOE) on each terminus flanking a central 8–10 base gap of DNA. After binding the target RNA, the DNA-RNA hybrid acts substrate for RNase H.

[00242] Other human-TTR-targeting reagents include antibodies or antigen-binding proteins designed to specifically bind a human TTR epitope. The term "antigen-binding protein" includes any protein that binds to an antigen. Examples of antigen-binding proteins include an antibody, an antigen-binding fragment of an antibody, a multispecific antibody (e.g., a bi-specific antibody), an scFV, a bis-scFV, a diabody, a triabody, a tetrabody, a V-NAR, a VHH, a VL, a F(ab), a $F(ab)_2$, a DVD (dual variable domain antigen-binding protein), an SVD (single variable domain antigen-binding protein), a bispecific T-cell engager (BiTE), or a Davisbody (US Pat. No. 8,586,713, herein incorporated by reference herein in its entirety for all purposes).

[00243] Other human-TTR-targeting reagents include small-molecule reagents. One example of such a small-molecule reagent is tafamidis, which functions by kinetic stabilization of the correctly folded tetrameric form of the transthyretin (TTR) protein. *See, e.g.*, Hammarstrom et al. (2003) *Science* 299:713-716, herein incorporated by reference in its entirety for all purposes.

D. Administering Human-TTR-Targeting Reagents to Non-Human Animals or Cells

[00244] The methods disclosed herein can comprise introducing into a non-human animal or cell various molecules (e.g., human-TTR-targeting reagents such as therapeutic molecules or complexes), including nucleic acids, proteins, nucleic-acid-protein complexes, protein complexes, or small molecules. "Introducing" includes presenting to the cell or non-human animal the molecule (e.g., nucleic acid or protein) in such a manner that it gains access to the interior of the cell or to the interior of cells within the non-human animal. The introducing can be accomplished by any means, and two or more of the components (e.g., two of the components, or all of the components) can be introduced into the cell or non-human animal simultaneously or sequentially in any combination. For example, a Cas protein can be introduced into a cell or non-human animal before introduction of a guide RNA, or it can be introduced following introduction of the guide RNA. As another example, an exogenous donor nucleic acid can be introduced prior to the introduction of a Cas protein and a guide RNA, or it can be introduced following introduction of the Cas protein and the guide RNA (e.g., the exogenous donor nucleic acid can be administered about 1, 2, 3, 4, 8, 12, 24, 36, 48, or 72 hours before or after introduction of the Cas protein and the guide RNA). *See, e.g.,* US 2015/0240263 and US 2015/0110762, each of which is herein incorporated by reference in its entirety for all purposes. In addition, two or more of the components can be introduced into the cell or non-human animal by the same delivery method or different delivery methods. Similarly, two or more of the components can be introduced into a non-human animal by the same route of administration or different routes of administration.

[00245] In some methods, components of a CRISPR/Cas system are introduced into a non-human animal or cell. A guide RNA can be introduced into a non-human animal or cell in the form of an RNA (e.g., *in vitro* transcribed RNA) or in the form of a DNA encoding the guide RNA. When introduced in the form of a DNA, the DNA encoding a guide RNA can be operably linked to a promoter active in a cell in the non-human animal. For example, a guide RNA may be delivered via AAV and expressed *in vivo* under a U6 promoter. Such DNAs can be in one or more expression constructs. For example, such expression constructs can be components of a single nucleic acid molecule. Alternatively, they can be separated in any combination among two or more nucleic acid molecules (i.e., DNAs encoding one or more CRISPR RNAs and

DNAs encoding one or more tracrRNAs can be components of a separate nucleic acid molecules).

[00246] Likewise, Cas proteins can be provided in any form. For example, a Cas protein can be provided in the form of a protein, such as a Cas protein complexed with a gRNA. Alternatively, a Cas protein can be provided in the form of a nucleic acid encoding the Cas protein, such as an RNA (e.g., messenger RNA (mRNA)) or DNA. Optionally, the nucleic acid encoding the Cas protein can be codon optimized for efficient translation into protein in a particular cell or organism. For example, the nucleic acid encoding the Cas protein can be modified to substitute codons having a higher frequency of usage in a mammalian cell, a rodent cell, a mouse cell, a rat cell, or any other host cell of interest, as compared to the naturally occurring polynucleotide sequence. When a nucleic acid encoding the Cas protein is introduced into a non-human animal, the Cas protein can be transiently, conditionally, or constitutively expressed in a cell in the non-human animal.

[00247] Nucleic acids encoding Cas proteins or guide RNAs can be operably linked to a promoter in an expression construct. Expression constructs include any nucleic acid constructs capable of directing expression of a gene or other nucleic acid sequence of interest (e.g., a Cas gene) and which can transfer such a nucleic acid sequence of interest to a target cell. For example, the nucleic acid encoding the Cas protein can be in a vector comprising a DNA encoding one or more gRNAs. Alternatively, it can be in a vector or plasmid that is separate from the vector comprising the DNA encoding one or more gRNAs. Suitable promoters that can be used in an expression construct include promoters active, for example, in one or more of a eukaryotic cell, a human cell, a non-human cell, a mammalian cell, a non-human mammalian cell, a rodent cell, a mouse cell, a rat cell, a hamster cell, a rabbit cell, a pluripotent cell, an embryonic stem (ES) cell, an adult stem cell, a developmentally restricted progenitor cell, an induced pluripotent stem (iPS) cell, or a one-cell stage embryo. Such promoters can be, for example, conditional promoters, inducible promoters, constitutive promoters, or tissue-specific promoters. Optionally, the promoter can be a bidirectional promoter driving expression of both a Cas protein in one direction and a guide RNA in the other direction. Such bidirectional promoters can consist of (1) a complete, conventional, unidirectional Pol III promoter that contains 3 external control elements: a distal sequence element (DSE), a proximal sequence element (PSE), and a TATA box; and (2) a second basic Pol III promoter that includes a PSE and

a TATA box fused to the 5′ terminus of the DSE in reverse orientation. For example, in the H1 promoter, the DSE is adjacent to the PSE and the TATA box, and the promoter can be rendered bidirectional by creating a hybrid promoter in which transcription in the reverse direction is controlled by appending a PSE and TATA box derived from the U6 promoter. *See, e.g.*, US 2016/0074535, herein incorporated by references in its entirety for all purposes. Use of a bidirectional promoter to express genes encoding a Cas protein and a guide RNA simultaneously allows for the generation of compact expression cassettes to facilitate delivery.

[00248] Molecules (e.g., Cas proteins or guide RNAs or RNAi agents or ASOs) introduced into the non-human animal or cell can be provided in compositions comprising a carrier increasing the stability of the introduced molecules (e.g., prolonging the period under given conditions of storage (e.g., -20°C, 4°C, or ambient temperature) for which degradation products remain below a threshold, such below 0.5% by weight of the starting nucleic acid or protein; or increasing the stability in vivo). Non-limiting examples of such carriers include poly(lactic acid) (PLA) microspheres, poly(D,L-lactic-coglycolic-acid) (PLGA) microspheres, liposomes, micelles, inverse micelles, lipid cochleates, and lipid microtubules.

[00249] Various methods and compositions are provided herein to allow for introduction of a molecule (e.g., nucleic acid or protein) into a cell or non-human animal. Methods for introducing molecules into various cell types are known and include, for example, stable transfection methods, transient transfection methods, and virus-mediated methods.

[00250] Transfection protocols as well as protocols for introducing nucleic acid sequences into cells may vary. Non-limiting transfection methods include chemical-based transfection methods using liposomes; nanoparticles; calcium phosphate (Graham *et al.* (1973) *Virology* 52 (2): 456–67, Bacchetti *et al.* (1977) *Proc. Natl. Acad. Sci. USA* 74 (4): 1590–4, and Kriegler, M (1991). Transfer and Expression: A Laboratory Manual. New York: W. H. Freeman and Company. pp. 96–97); dendrimers; or cationic polymers such as DEAE-dextran or polyethylenimine. Non-chemical methods include electroporation, sonoporation, and optical transfection. Particle-based transfection includes the use of a gene gun, or magnet-assisted transfection (Bertram (2006) *Current Pharmaceutical Biotechnology* 7, 277–28). Viral methods can also be used for transfection.

[00251] Introduction of molecules (e.g., nucleic acids or proteins) into a cell can also be mediated by electroporation, by intracytoplasmic injection, by viral infection, by adenovirus, by

adeno-associated virus, by lentivirus, by retrovirus, by transfection, by lipid-mediated transfection, or by nucleofection. Nucleofection is an improved electroporation technology that enables nucleic acid substrates to be delivered not only to the cytoplasm but also through the nuclear membrane and into the nucleus. In addition, use of nucleofection in the methods disclosed herein typically requires much fewer cells than regular electroporation (e.g., only about 2 million compared with 7 million by regular electroporation). In one example, nucleofection is performed using the LONZA® NUCLEOFECTOR™ system.

[00252] Introduction of molecules (e.g., nucleic acids or proteins) into a cell (e.g., a zygote) can also be accomplished by microinjection. In zygotes (i.e., one-cell stage embryos), microinjection can be into the maternal and/or paternal pronucleus or into the cytoplasm. If the microinjection is into only one pronucleus, the paternal pronucleus is preferable due to its larger size. Microinjection of an mRNA is preferably into the cytoplasm (e.g., to deliver mRNA directly to the translation machinery), while microinjection of a Cas protein or a polynucleotide encoding a Cas protein or encoding an RNA is preferable into the nucleus/pronucleus. Alternatively, microinjection can be carried out by injection into both the nucleus/pronucleus and the cytoplasm: a needle can first be introduced into the nucleus/pronucleus and a first amount can be injected, and while removing the needle from the one-cell stage embryo a second amount can be injected into the cytoplasm. If a Cas protein is injected into the cytoplasm, the Cas protein preferably comprises a nuclear localization signal to ensure delivery to the nucleus/pronucleus. Methods for carrying out microinjection are well known. *See, e.g.*, Nagy *et al.* (Nagy A, Gertsenstein M, Vintersten K, Behringer R., 2003, Manipulating the Mouse Embryo. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press); *see also* Meyer *et al.* (2010) *Proc. Natl. Acad. Sci. USA* 107:15022-15026 and Meyer *et al.* (2012) *Proc. Natl. Acad. Sci. USA* 109:9354-9359.

[00253] Other methods for introducing molecules (e.g., nucleic acid or proteins) into a cell or non-human animal can include, for example, vector delivery, particle-mediated delivery, exosome-mediated delivery, lipid-nanoparticle-mediated delivery, cell-penetrating-peptide-mediated delivery, or implantable-device-mediated delivery. As specific examples, a nucleic acid or protein can be introduced into a cell or non-human animal in a carrier such as a poly(lactic acid) (PLA) microsphere, a poly(D,L-lactic-coglycolic-acid) (PLGA) microsphere, a liposome, a micelle, an inverse micelle, a lipid cochleate, or a lipid microtubule. Some specific

examples of delivery to a non-human animal include hydrodynamic delivery, virus-mediated delivery (e.g., adeno-associated virus (AAV)-mediated delivery), and lipid-nanoparticle-mediated delivery.

[00254] Introduction of nucleic acids and proteins into cells or non-human animals can be accomplished by hydrodynamic delivery (HDD). For gene delivery to parenchymal cells, only essential DNA sequences need to be injected via a selected blood vessel, eliminating safety concerns associated with current viral and synthetic vectors. When injected into the bloodstream, DNA is capable of reaching cells in the different tissues accessible to the blood. Hydrodynamic delivery employs the force generated by the rapid injection of a large volume of solution into the incompressible blood in the circulation to overcome the physical barriers of endothelium and cell membranes that prevent large and membrane-impermeable compounds from entering parenchymal cells. In addition to the delivery of DNA, this method is useful for the efficient intracellular delivery of RNA, proteins, and other small compounds *in vivo*. *See, e.g.*, Bonamassa *et al.* (2011) *Pharm. Res.* 28(4):694-701, herein incorporated by reference in its entirety for all purposes.

[00255] Introduction of nucleic acids can also be accomplished by virus-mediated delivery, such as AAV-mediated delivery or lentivirus-mediated delivery. Other exemplary viruses/viral vectors include retroviruses, adenoviruses, vaccinia viruses, poxviruses, and herpes simplex viruses. The viruses can infect dividing cells, non-dividing cells, or both dividing and non-dividing cells. The viruses can integrate into the host genome or alternatively do not integrate into the host genome. Such viruses can also be engineered to have reduced immunity. The viruses can be replication-competent or can be replication-defective (e.g., defective in one or more genes necessary for additional rounds of virion replication and/or packaging). Viruses can cause transient expression, long-lasting expression (e.g., at least 1 week, 2 weeks, 1 month, 2 months, or 3 months), or permanent expression (e.g., of Cas9 and/or gRNA). Exemplary viral titers (e.g., AAV titers) include 10^{12}, 10^{13}, 10^{14}, 10^{15}, and 10^{16} vector genomes/mL.

[00256] The ssDNA AAV genome consists of two open reading frames, Rep and Cap, flanked by two inverted terminal repeats that allow for synthesis of the complementary DNA strand. When constructing an AAV transfer plasmid, the transgene is placed between the two ITRs, and Rep and Cap can be supplied *in trans*. In addition to Rep and Cap, AAV can require a helper plasmid containing genes from adenovirus. These genes (E4, E2a, and VA) mediate AAV

replication. For example, the transfer plasmid, Rep/Cap, and the helper plasmid can be transfected into HEK293 cells containing the adenovirus gene E1+ to produce infectious AAV particles. Alternatively, the Rep, Cap, and adenovirus helper genes may be combined into a single plasmid. Similar packaging cells and methods can be used for other viruses, such as retroviruses.

[00257] Multiple serotypes of AAV have been identified. These serotypes differ in the types of cells they infect (i.e., their tropism), allowing preferential transduction of specific cell types. Serotypes for CNS tissue include AAV1, AAV2, AAV4, AAV5, AAV8, and AAV9. Serotypes for heart tissue include AAV1, AAV8, and AAV9. Serotypes for kidney tissue include AAV2. Serotypes for lung tissue include AAV4, AAV5, AAV6, and AAV9. Serotypes for pancreas tissue include AAV8. Serotypes for photoreceptor cells include AAV2, AAV5, and AAV8. Serotypes for retinal pigment epithelium tissue include AAV1, AAV2, AAV4, AAV5, and AAV8. Serotypes for skeletal muscle tissue include AAV1, AAV6, AAV7, AAV8, and AAV9. Serotypes for liver tissue include AAV7, AAV8, and AAV9, and particularly AAV8.

[00258] Tropism can be further refined through pseudotyping, which is the mixing of a capsid and a genome from different viral serotypes. For example AAV2/5 indicates a virus containing the genome of serotype 2 packaged in the capsid from serotype 5. Use of pseudotyped viruses can improve transduction efficiency, as well as alter tropism. Hybrid capsids derived from different serotypes can also be used to alter viral tropism. For example, AAV-DJ contains a hybrid capsid from eight serotypes and displays high infectivity across a broad range of cell types *in vivo*. AAV-DJ8 is another example that displays the properties of AAV-DJ but with enhanced brain uptake. AAV serotypes can also be modified through mutations. Examples of mutational modifications of AAV2 include Y444F, Y500F, Y730F, and S662V. Examples of mutational modifications of AAV3 include Y705F, Y731F, and T492V. Examples of mutational modifications of AAV6 include S663V and T492V. Other pseudotyped/modified AAV variants include AAV2/1, AAV2/6, AAV2/7, AAV2/8, AAV2/9, AAV2.5, AAV8.2, and AAV/SASTG.

[00259] To accelerate transgene expression, self-complementary AAV (scAAV) variants can be used. Because AAV depends on the cell's DNA replication machinery to synthesize the complementary strand of the AAV's single-stranded DNA genome, transgene expression may be delayed. To address this delay, scAAV containing complementary sequences that are capable of spontaneously annealing upon infection can be used, eliminating the requirement for host cell

DNA synthesis. However, single-stranded AAV (ssAAV) vectors can also be used.

[00260] To increase packaging capacity, longer transgenes may be split between two AAV transfer plasmids, the first with a 3' splice donor and the second with a 5' splice acceptor. Upon co-infection of a cell, these viruses form concatemers, are spliced together, and the full-length transgene can be expressed. Although this allows for longer transgene expression, expression is less efficient. Similar methods for increasing capacity utilize homologous recombination. For example, a transgene can be divided between two transfer plasmids but with substantial sequence overlap such that co-expression induces homologous recombination and expression of the full-length transgene.

[00261] Introduction of nucleic acids and proteins can also be accomplished by lipid nanoparticle (LNP)-mediated delivery. For example, LNP-mediated delivery can be used to deliver a combination of Cas mRNA and guide RNA or a combination of Cas protein and guide RNA. Delivery through such methods results in transient Cas expression, and the biodegradable lipids improve clearance, improve tolerability, and decrease immunogenicity. Lipid formulations can protect biological molecules from degradation while improving their cellular uptake. Lipid nanoparticles are particles comprising a plurality of lipid molecules physically associated with each other by intermolecular forces. These include microspheres (including unilamellar and multilamellar vesicles, e.g., liposomes), a dispersed phase in an emulsion, micelles, or an internal phase in a suspension. Such lipid nanoparticles can be used to encapsulate one or more nucleic acids or proteins for delivery. Formulations which contain cationic lipids are useful for delivering polyanions such as nucleic acids. Other lipids that can be included are neutral lipids (i.e., uncharged or zwitterionic lipids), anionic lipids, helper lipids that enhance transfection, and stealth lipids that increase the length of time for which nanoparticles can exist *in vivo*. Examples of suitable cationic lipids, neutral lipids, anionic lipids, helper lipids, and stealth lipids can be found in WO 2016/010840 A1 and WO 2017/173054 A1, each of which is herein incorporated by reference in its entirety for all purposes. An exemplary lipid nanoparticle can comprise a cationic lipid and one or more other components. In one example, the other component can comprise a helper lipid such as cholesterol. In another example, the other components can comprise a helper lipid such as cholesterol and a neutral lipid such as DSPC. In another example, the other components can comprise a helper lipid such as cholesterol, an optional neutral lipid such as DSPC, and a stealth lipid such as S010, S024, S027,

S031, or S033.

[00262] The LNP may contain one or more or all of the following: (i) a lipid for encapsulation and for endosomal escape; (ii) a neutral lipid for stabilization; (iii) a helper lipid for stabilization; and (iv) a stealth lipid. *See, e.g.*, Finn et al. (2018) *Cell Reports* 22:1-9 and WO 2017/173054 A1, each of which is herein incorporated by reference in its entirety for all purposes. In certain LNPs, the cargo can include a guide RNA or a nucleic acid encoding a guide RNA. In certain LNPs, the cargo can include an mRNA encoding a Cas nuclease, such as Cas9, and a guide RNA or a nucleic acid encoding a guide RNA.

[00263] The lipid for encapsulation and endosomal escape can be a cationic lipid. The lipid can also be a biodegradable lipid, such as a biodegradable ionizable lipid. One example of a suitable lipid is Lipid A or LP01, which is (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate. *See, e.g.*, Finn et al. (2018) *Cell Reports* 22:1-9 and WO 2017/173054 A1, each of which is herein incorporated by reference in its entirety for all purposes. Another example of a suitable lipid is Lipid B, which is ((5-((dimethylamino)methyl)-1,3-phenylene)bis(oxy))bis(octane-8,1-diyl)bis(decanoate), also called ((5-((dimethylamino)methyl)-1,3-phenylene)bis(oxy))bis(octane-8,1-diyl)bis(decanoate). Another example of a suitable lipid is Lipid C, which is 2-((4-(((3-(dimethylamino)propoxy)carbonyl)oxy)hexadecanoyl)oxy)propane-1,3-diyl(9Z,9'Z,12Z,12'Z)-bis(octadeca-9,12-dienoate). Another example of a suitable lipid is Lipid D, which is 3-(((3-(dimethylamino)propoxy)carbonyl)oxy)-13-(octanoyloxy)tridecyl 3-octylundecanoate. Other suitable lipids include heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (also known as Dlin-MC3-DMA (MC3))).

[00264] Some such lipids suitable for use in the LNPs described herein are biodegradable *in vivo*. For example, LNPs comprising such a lipid include those where at least 75% of the lipid is cleared from the plasma within 8, 10, 12, 24, or 48 hours, or 3, 4, 5, 6, 7, or 10 days. As another example, at least 50% of the LNP is cleared from the plasma within 8, 10, 12, 24, or 48 hours, or 3, 4, 5, 6, 7, or 10 days.

[00265] Such lipids may be ionizable depending upon the pH of the medium they are in. For example, in a slightly acidic medium, the lipids may be protonated and thus bear a positive

charge. Conversely, in a slightly basic medium, such as, for example, blood where pH is approximately 7.35, the lipids may not be protonated and thus bear no charge. In some embodiments, the lipids may be protonated at a pH of at least about 9, 9.5, or 10. The ability of such a lipid to bear a charge is related to its intrinsic pKa. For example, the lipid may, independently, have a pKa in the range of from about 5.8 to about 6.2.

[00266] Neutral lipids function to stabilize and improve processing of the LNPs. Examples of suitable neutral lipids include a variety of neutral, uncharged or zwitterionic lipids. Examples of neutral phospholipids suitable for use in the present disclosure include, but are not limited to, 5-heptadecylbenzene-1,3-diol (resorcinol), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), phosphocholine (DOPC), dimyristoylphosphatidylcholine (DMPC), phosphatidylcholine (PLPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DAPC), phosphatidylethanolamine (PE), egg phosphatidylcholine (EPC), dilauryloylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), 1-myristoyl-2-palmitoyl phosphatidylcholine (MPPC), 1-palmitoyl-2-myristoyl phosphatidylcholine (PMPC), 1-palmitoyl-2-stearoyl phosphatidylcholine (PSPC), 1,2-diarachidoyl-sn-glycero-3-phosphocholine (DBPC), 1-stearoyl-2-palmitoyl phosphatidylcholine (SPPC), 1,2-dieicosenoyl-sn-glycero-3-phosphocholine (DEPC), palmitoyloleoyl phosphatidylcholine (POPC), lysophosphatidyl choline, dioleoyl phosphatidylethanolamine (DOPE), dilinoleoylphosphatidylcholine distearoylphosphatidylethanolamine (DSPE), dimyristoyl phosphatidylethanolamine (DMPE), dipalmitoyl phosphatidylethanolamine (DPPE), palmitoyloleoyl phosphatidylethanolamine (POPE), lysophosphatidylethanolamine, and combinations thereof. For example, the neutral phospholipid may be selected from the group consisting of distearoylphosphatidylcholine (DSPC) and dimyristoyl phosphatidyl ethanolamine (DMPE).

[00267] Helper lipids include lipids that enhance transfection. The mechanism by which the helper lipid enhances transfection can include enhancing particle stability. In certain cases, the helper lipid can enhance membrane fusogenicity. Helper lipids include steroids, sterols, and alkyl resorcinols. Examples of suitable helper lipids suitable include cholesterol, 5-heptadecylresorcinol, and cholesterol hemisuccinate. In one example, the helper lipid may be cholesterol or cholesterol hemisuccinate.

[00268] Stealth lipids include lipids that alter the length of time the nanoparticles can exist in

vivo. Stealth lipids may assist in the formulation process by, for example, reducing particle aggregation and controlling particle size. Stealth lipids may modulate pharmacokinetic properties of the LNP. Suitable stealth lipids include lipids having a hydrophilic head group linked to a lipid moiety.

[00269] The hydrophilic head group of stealth lipid can comprise, for example, a polymer moiety selected from polymers based on PEG (sometimes referred to as poly(ethylene oxide)), poly(oxazoline), poly(vinyl alcohol), poly(glycerol), poly(N- vinylpyrrolidone), polyaminoacids, and poly N-(2-hydroxypropyl)methacrylamide. The term PEG means any polyethylene glycol or other polyalkylene ether polymer. In certain LNP formulations, the PEG, is a PEG-2K, also termed PEG 2000, which has an average molecular weight of about 2,000 daltons. *See, e.g.,* WO 2017/173054 A1, herein incorporated by reference in its entirety for all purposes.

[00270] The lipid moiety of the stealth lipid may be derived, for example, from diacylglycerol or diacylglycamide, including those comprising a dialkylglycerol or dialkylglycamide group having alkyl chain length independently comprising from about C4 to about C40 saturated or unsaturated carbon atoms, wherein the chain may comprise one or more functional groups such as, for example, an amide or ester. The dialkylglycerol or dialkylglycamide group can further comprise one or more substituted alkyl groups.

[00271] As one example, the stealth lipid may be selected from PEG-dilauroylglycerol, PEG-dimyristoylglycerol (PEG-DMG), PEG-dipalmitoylglycerol, PEG-distearoylglycerol (PEG-DSPE), PEG-dilaurylglycamide, PEG- dimyristylglycamide, PEG-dipalmitoylglycamide, and PEG-distearoylglycamide, PEG- cholesterol (l-[8'-(Cholest-5-en-3[beta]-oxy)carboxamido-3',6'-dioxaoctanyl]carbamoyl-[omega]-methyl-poly(ethylene glycol), PEG-DMB (3,4-ditetradecoxylbenzyl-[omega]-methyl-poly(ethylene glycol)ether), 1,2-dimyristoyl-sn- glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2k- DMG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2k-DSPE), 1,2-distearoyl-sn-glycerol, methoxypoly ethylene glycol (PEG2k-DSG), poly(ethylene glycol)-2000-dimethacrylate (PEG2k-DMA), and 1,2- distearyloxypropyl-3-amine-N-[methoxy(polyethylene glycol)-2000] (PEG2k-DSA). In one particular example, the stealth lipid may be PEG2k-DMG.

[00272] The LNPs can comprise different respective molar ratios of the component lipids in the formulation. The mol-% of the CCD lipid may be, for example, from about 30 mol-% to

about 60 mol-%, from about 35 mol-% to about 55 mol-%, from about 40 mol-% to about 50 mol-%, from about 42 mol-% to about 47 mol-%, or about 45%. The mol-% of the helper lipid may be, for example, from about 30 mol-% to about 60 mol-%, from about 35 mol-% to about 55 mol-%, from about 40 mol-% to about 50 mol-%, from about 41 mol-% to about 46 mol-%, or about 44 mol-%. The mol-% of the neutral lipid may be, for example, from about 1 mol-% to about 20 mol-%, from about 5 mol-% to about 15 mol-%, from about 7 mol-% to about 12 mol-%, or about 9 mol-%. The mol-% of the stealth lipid may be, for example, from about 1 mol-% to about 10 mol-%, from about 1 mol-% to about 5 mol-%, from about 1 mol-% to about 3 mol-%, about 2 mol-%, or about 1 mol-%.

[00273] The LNPs can have different ratios between the positively charged amine groups of the biodegradable lipid (N) and the negatively charged phosphate groups (P) of the nucleic acid to be encapsulated. This may be mathematically represented by the equation N/P. For example, the N/P ratio may be from about 0.5 to about 100, from about 1 to about 50, from about 1 to about 25, from about 1 to about 10, from about 1 to about 7, from about 3 to about 5, from about 4 to about 5, about 4, about 4.5, or about 5. The N/P ratio can also be from about 4 to about 7 or from about 4.5 to about 6. In specific examples, the N/P ratio can be 4.5 or can be 6.

[00274] In some LNPs, the cargo can comprise Cas mRNA and gRNA. The Cas mRNA and gRNAs can be in different ratios. For example, the LNP formulation can include a ratio of Cas mRNA to gRNA nucleic acid ranging from about 25:1 to about 1:25, ranging from about 10:1 to about 1:10, ranging from about 5:1 to about 1:5, or about 1:1. Alternatively, the LNP formulation can include a ratio of Cas mRNA to gRNA nucleic acid from about 1:1 to about 1:5, or about 10:1. Alternatively, the LNP formulation can include a ratio of Cas mRNA to gRNA nucleic acid of about 1:10, 25:1, 10:1, 5:1, 3:1, 1:1, 1:3, 1:5, 1:10, or 1:25. Alternatively, the LNP formulation can include a ratio of Cas mRNA to gRNA nucleic acid of from about 1:1 to about 1:2. In specific examples, the ratio of Cas mRNA to gRNA can be about 1:1 or about 1:2.

[00275] In some LNPs, the cargo can comprise exogenous donor nucleic acid and gRNA. The exogenous donor nucleic acid and gRNAs can be in different ratios. For example, the LNP formulation can include a ratio of exogenous donor nucleic acid to gRNA nucleic acid ranging from about 25:1 to about 1:25, ranging from about 10:1 to about 1:10, ranging from about 5:1 to about 1:5, or about 1:1. Alternatively, the LNP formulation can include a ratio of exogenous donor nucleic acid to gRNA nucleic acid from about 1:1 to about 1:5, about 5:1 to about 1:1,

about 10:1, or about 1:10. Alternatively, the LNP formulation can include a ratio of exogenous donor nucleic acid to gRNA nucleic acid of about 1:10, 25:1, 10:1, 5:1, 3:1, 1:1, 1:3, 1:5, 1:10, or 1:25.

[00276] A specific example of a suitable LNP has a nitrogen-to-phosphate (N/P) ratio of 4.5 and contains biodegradable cationic lipid, cholesterol, DSPC, and PEG2k-DMG in a 45:44:9:2 molar ratio. The biodegradable cationic lipid can be (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate. *See, e.g.*, Finn et al. (2018) *Cell Reports* 22:1-9, herein incorporated by reference in its entirety for all purposes. The Cas9 mRNA can be in a 1:1 ratio by weight to the guide RNA. Another specific example of a suitable LNP contains Dlin-MC3-DMA (MC3), cholesterol, DSPC, and PEG-DMG in a 50:38.5:10:1.5 molar ratio.

[00277] Another specific example of a suitable LNP has a nitrogen-to-phosphate (N/P) ratio of 6 and contains biodegradable cationic lipid, cholesterol, DSPC, and PEG2k-DMG in a 50:38:9:3 molar ratio. The biodegradable cationic lipid can be (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate. The Cas9 mRNA can be in a 1:2 ratio by weight to the guide RNA.

[00278] The mode of delivery can be selected to decrease immunogenicity. For example, a Cas protein and a gRNA may be delivered by different modes (e.g., bi-modal delivery). These different modes may confer different pharmacodynamics or pharmacokinetic properties on the subject delivered molecule (e.g., Cas or nucleic acid encoding, gRNA or nucleic acid encoding, or exogenous donor nucleic acid/repair template). For example, the different modes can result in different tissue distribution, different half-life, or different temporal distribution. Some modes of delivery (e.g., delivery of a nucleic acid vector that persists in a cell by autonomous replication or genomic integration) result in more persistent expression and presence of the molecule, whereas other modes of delivery are transient and less persistent (e.g., delivery of an RNA or a protein). Delivery of Cas proteins in a more transient manner, for example as mRNA or protein, can ensure that the Cas/gRNA complex is only present and active for a short period of time and

can reduce immunogenicity caused by peptides from the bacterially-derived Cas enzyme being displayed on the surface of the cell by MHC molecules. Such transient delivery can also reduce the possibility of off-target modifications.

[00279] Administration *in vivo* can be by any suitable route including, for example, parenteral, intravenous, oral, subcutaneous, intra-arterial, intracranial, intrathecal, intraperitoneal, topical, intranasal, or intramuscular. Systemic modes of administration include, for example, oral and parenteral routes. Examples of parenteral routes include intravenous, intraarterial, intraosseous, intramuscular, intradermal, subcutaneous, intranasal, and intraperitoneal routes. A specific example is intravenous infusion. Nasal instillation and intravitreal injection are other specific examples. Local modes of administration include, for example, intrathecal, intracerebroventricular, intraparenchymal (e.g., localized intraparenchymal delivery to the striatum (e.g., into the caudate or into the putamen), cerebral cortex, precentral gyrus, hippocampus (e.g., into the dentate gyrus or CA3 region), temporal cortex, amygdala, frontal cortex, thalamus, cerebellum, medulla, hypothalamus, tectum, tegmentum, or substantia nigra), intraocular, intraorbital, subconjuctival, intravitreal, subretinal, and transscleral routes. Significantly smaller amounts of the components (compared with systemic approaches) may exert an effect when administered locally (for example, intraparenchymal or intravitreal) compared to when administered systemically (for example, intravenously). Local modes of administration may also reduce or eliminate the incidence of potentially toxic side effects that may occur when therapeutically effective amounts of a component are administered systemically.

[00280] Administration *in vivo* can be by any suitable route including, for example, parenteral, intravenous, oral, subcutaneous, intra-arterial, intracranial, intrathecal, intraperitoneal, topical, intranasal, or intramuscular. A specific example is intravenous infusion. Compositions comprising the guide RNAs and/or Cas proteins (or nucleic acids encoding the guide RNAs and/or Cas proteins) can be formulated using one or more physiologically and pharmaceutically acceptable carriers, diluents, excipients or auxiliaries. The formulation can depend on the route of administration chosen. The term "pharmaceutically acceptable" means that the carrier, diluent, excipient, or auxiliary is compatible with the other ingredients of the formulation and not substantially deleterious to the recipient thereof.

[00281] The frequency of administration and the number of dosages can depend on the half-

life of the exogenous donor nucleic acids, guide RNAs, or Cas proteins (or nucleic acids encoding the guide RNAs or Cas proteins) and the route of administration among other factors. The introduction of nucleic acids or proteins into the cell or non-human animal can be performed one time or multiple times over a period of time. For example, the introduction can be performed at least two times over a period of time, at least three times over a period of time, at least four times over a period of time, at least five times over a period of time, at least six times over a period of time, at least seven times over a period of time, at least eight times over a period of time, at least nine times over a period of times, at least ten times over a period of time, at least eleven times, at least twelve times over a period of time, at least thirteen times over a period of time, at least fourteen times over a period of time, at least fifteen times over a period of time, at least sixteen times over a period of time, at least seventeen times over a period of time, at least eighteen times over a period of time, at least nineteen times over a period of time, or at least twenty times over a period of time.

E. Measuring Delivery, Activity, or Efficacy of Human-TTR-Targeting Reagents *In Vivo* or *Ex Vivo*

[00282] The methods disclosed herein can further comprise detecting or measuring activity of human-TTR-targeting reagents. For example, if the human-TTR-targeting reagent is a genome editing reagent (e.g., CRISPR/Cas designed to target the human *TTR* locus), the measuring can comprise assessing the humanized *TTR* locus comprising the beta-slip mutation for modifications.

[00283] Various methods can be used to identify cells having a targeted genetic modification. The screening can comprise a quantitative assay for assessing modification of allele (MOA) of a parental chromosome. *See, e.g.*, US 2004/0018626; US 2014/0178879; US 2016/0145646; WO 2016/081923; and Frendewey et al. (2010) *Methods Enzymol.* 476:295-307, each of which is herein incorporated by reference in its entirety for all purposes. For example, the quantitative assay can be carried out via a quantitative PCR, such as a real-time PCR (qPCR). The real-time PCR can utilize a first primer set that recognizes the target locus and a second primer set that recognizes a non-targeted reference locus. The primer set can comprise a fluorescent probe that recognizes the amplified sequence. Other examples of suitable quantitative assays include fluorescence-mediated in situ hybridization (FISH), comparative genomic hybridization,

isothermic DNA amplification, quantitative hybridization to an immobilized probe(s), INVADER® Probes, TAQMAN® Molecular Beacon probes, or ECLIPSE™ probe technology (*see, e.g.*, US 2005/0144655, herein incorporated by reference in its entirety for all purposes).

[00284] Next-generation sequencing (NGS) can also be used for screening. Next-generation sequencing can also be referred to as "NGS" or "massively parallel sequencing" or "high throughput sequencing." NGS can be used as a screening tool in addition to the MOA assays to define the exact nature of the targeted genetic modification and whether it is consistent across cell types or tissue types or organ types.

[00285] Assessing modification of the humanized *TTR* locus comprising the beta-slip mutation in a non-human animal can be in any cell type from any tissue or organ. For example, the assessment can be in multiple cell types from the same tissue or organ or in cells from multiple locations within the tissue or organ. This can provide information about which cell types within a target tissue or organ are being targeted or which sections of a tissue or organ are being reached by the human-TTR-targeting reagent. As another example, the assessment can be in multiple types of tissue or in multiple organs. In methods in which a particular tissue, organ, or cell type is being targeted, this can provide information about how effectively that tissue or organ is being targeted and whether there are off-target effects in other tissues or organs.

[00286] One example of an assay that can be used are the RNASCOPE™ and BASESCOPE™ RNA *in situ* hybridization (ISH) assays, which are methods that can quantify cell-specific edited transcripts, including single nucleotide changes, in the context of intact fixed tissue. The BASESCOPE™ RNA ISH assay can complement NGS and qPCR in characterization of gene editing. Whereas NGS/qPCR can provide quantitative average values of wild type and edited sequences, they provide no information on heterogeneity or percentage of edited cells within a tissue. The BASESCOPE™ ISH assay can provide a landscape view of an entire tissue and quantification of wild type versus edited transcripts with single-cell resolution, where the actual number of cells within the target tissue containing the edited mRNA transcript can be quantified. The BASESCOPE™ assay achieves single-molecule RNA detection using paired oligo ("ZZ") probes to amplify signal without non-specific background. However, the BASESCOPE™ probe design and signal amplification system enables single-molecule RNA detection with a ZZ probe, and it can differentially detect single nucleotide edits and mutations in intact fixed tissue.

[00287] If the reagent is designed to inactivate the humanized *TTR* locus comprising the beta-slip mutation, affect expression of the humanized *TTR* locus, prevent translation of the humanized *TTR* mRNA, or clear the humanized TTR protein, the measuring can comprise assessing humanized *TTR* mRNA or protein expression. This measuring can be within the liver or particular cell types or regions within the liver, or it can involve measuring serum levels of secreted humanized TTR protein.

[00288] Production and secretion of the humanized TTR protein comprising the beta-slip mutation can be assessed by any known means. For example, expression can be assessed by measuring levels of the encoded mRNA in the liver of the non-human animal or levels of the encoded protein in the liver of the non-human animal using known assays. Secretion of the humanized TTR protein can be assessed by measuring or plasma levels or serum levels of the encoded humanized TTR protein in the non-human animal using known assays. For example, the measuring can be to determine if the human-TTR-targeting reagent reduces TTR levels in the non-human animal.

[00289] The measuring can also comprise assessing aggregation of the humanized TTR protein. For example, native PAGE and western blots can be used to assess the presence of aggregated humanized TTR protein (e.g., higher molecular weight forms of humanized TTR protein) and whether the human-TTR-targeting reagent prevents aggregation, reduces aggregation, disrupts aggregation, or increases clearance of aggregated forms of humanized TTR protein.

[00290] The measuring can also comprise assessing amyloid deposition or the presence of amyloid deposits (amyloidosis). For example, the measuring can be to determine whether the human-TTR-targeting reagent prevents, reduces, disrupts, or clears amyloid deposits. As on example, Congo Red is a widely used stain to detect amyloidosis. Tissues can be imaged under white light which reveals overall tissue architecture and a characteristic red stain. When Congo Red stained tissue is illuminated using linear polarized light, only the dye which is bound to amyloids will refract the polarized light (e.g., amyloid bound CongoRed dye will become birefringent), which is viewed as a light green/white color. The presence of these greenish-white deposits is indicative of amyloid deposition. Such assays can be used to assess whether the human-TTR-targeting reagent prevents or reduces or disrupts or clears amyloid deposits. The assessment can be in any tissue or organ in which TTR amyloid deposition occurs. As one non-

limiting example, the assessment can be in the sciatic nerve.

[00291] The measuring can also comprise assessing activity or hyperactivity levels in the non-human animal. For example, the measuring can be to determine if the human-TTR-targeting reagent prevents or reduces hyperactivity in the non-human animal. Hyperactivity can be assessed by measuring one or more or all of total distance, total activity, and total rearings in an open field test. The open field test gives an overall measure of mouse locomotion and hyperactivity. Three of the readouts from the open field test are the total distance traveled, the total activity, and the total number of rearings. The open field is a behavioral test used to measure the general motor health and activity of mice during a 60-minute period. The mouse is tracked for the total distance it travels inside an enclosed, square apparatus during a 60-minute time period. Total activity is measured by the number of times a mouse in the apparatus interrupts the path of infrared breams in the X and Y planes. Rearings are a measure of how many times a mouse will stand on its hindlimbs to explore the walls of the apparatus. A greater rearings value is indicative of less anxious and hyperactive mice. Rearings are measured by infrared beam breaks in the Z-plane (beams placed at a height above that of a quadrupedal mouse).

[00292] The assessment can also comprise assessing the presence of dystonia or dystonic phenotypes. For example, the assessment can comprise assessing whether the non-human animal displays hindlimb dystonia or a hindlimb dystonic phenotype (e.g., dystonic hindlimb retraction) as described in the working examples. For example, the measuring can be to determine whether a human-TTR-targeting reagent ameliorates or prevents such phenotypes.

[00293] The assessment of any of these phenotypes can be at any age of non-human animal, such as at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months of age. In a specific example, the non-human animal can be at least about 2 months of age.

[00294] The assessment of any of these phenotypes can be done in comparison to a control non-human animal. One example of a control non-human animal is a corresponding wild type animal (e.g., of the same species). For example, the control non-human animal can be a wild type littermate. Another example of a control non-human animal is a corresponding non-human animal comprising a humanized *TTR* locus without the beta-slip mutation (e.g., the humanized *TTR* locus is identical except for the absence of the beta-slip mutation). The control non-human animals can be, for example, the same age as the test non-human animal and/or the same sex as

the test non-human animal. The assessment of any of these phenotypes can also be done in comparison to a control non-human animal that is identical to the test non-human animal except not treated with the human-TTR-targeting reagent.

[00295] The assessment of any of these phenotypes can be in a single non-human animal and assessing changes in that non-human animal. Alternatively, the assessment can be in a population of non-human animals and comparing, for example, the percentage of non-human animals having a particular phenotype. As one example, the assessment can comprise assessing the percentage of non-human animals having amyloid deposits or having a dystonic phenotype in a test population (treated with the human-TTR-targeting reagent) compared to a control population (not treated with the human-TTR-targeting reagent).

IV. Methods of Making Non-Human Animals Comprising a Humanized TTR *Locus* Comprising a Beta-Slip Mutation

[00296] Various methods are provided for making a non-human animal comprising a humanized *TTR* locus comprising a beta-slip mutation as disclosed elsewhere herein. Any convenient method or protocol for producing a genetically modified organism is suitable for producing such a genetically modified non-human animal. *See, e.g.*, Cho *et al.* (2009) *Current Protocols in Cell Biology* 42:19.11:19.11.1–19.11.22 and Gama Sosa *et al.* (2010) *Brain Struct. Funct.* 214(2-3):91-109, each of which is herein incorporated by reference in its entirety for all purposes. Such genetically modified non-human animals can be generated, for example, through gene knock-in at a targeted *Ttr* locus.

[00297] For example, the method of producing a non-human animal comprising a humanized *TTR* locus comprising a beta-slip mutation can comprise: (1) modifying the genome of a pluripotent cell to comprise the humanized *TTR* locus comprising the beta-slip mutation; (2) identifying or selecting the genetically modified pluripotent cell comprising the humanized *TTR* locus comprising the beta-slip mutation; (3) introducing the genetically modified pluripotent cell into a non-human animal host embryo; and (4) implanting and gestating the host embryo in a surrogate mother. Alternatively, the method of producing a non-human animal comprising a humanized *TTR* locus comprising a beta-slip mutation can comprise: (1) modifying the genome of a pluripotent cell to comprise the humanized *TTR* locus comprising the beta-slip mutation; (2) identifying or selecting the genetically modified pluripotent cell comprising the humanized *TTR*

locus comprising the beta-slip mutation; (3) introducing the genetically modified pluripotent cell into a non-human animal host embryo; and (4) gestating the host embryo in a surrogate mother. Optionally, the host embryo comprising modified pluripotent cell (e.g., a non-human ES cell) can be incubated until the blastocyst stage before being implanted into and gestated in the surrogate mother to produce an F0 non-human animal. The surrogate mother can then produce an F0 generation non-human animal comprising the humanized *TTR* locus comprising the beta-slip mutation.

[00298] The methods can further comprise identifying a cell or animal having a modified target genomic locus (i.e., a humanized *TTR* locus comprising the beta-slip mutation). Various methods can be used to identify cells and animals having a targeted genetic modification.

[00299] The screening step can comprise, for example, a quantitative assay for assessing modification of allele (MOA) of a parental chromosome. For example, the quantitative assay can be carried out via a quantitative PCR, such as a real-time PCR (qPCR). The real-time PCR can utilize a first primer set that recognizes the target locus and a second primer set that recognizes a non-targeted reference locus. The primer set can comprise a fluorescent probe that recognizes the amplified sequence.

[00300] Other examples of suitable quantitative assays include fluorescence-mediated in situ hybridization (FISH), comparative genomic hybridization, isothermic DNA amplification, quantitative hybridization to an immobilized probe(s), INVADER® Probes, TAQMAN® Molecular Beacon probes, or ECLIPSE™ probe technology (*see, e.g.*, US 2005/0144655, incorporated herein by reference in its entirety for all purposes).

[00301] An example of a suitable pluripotent cell is an embryonic stem (ES) cell (e.g., a mouse ES cell or a rat ES cell). The modified pluripotent cell can be generated, for example, through recombination by (a) introducing into the cell one or more targeting vectors comprising an insert nucleic acid flanked by 5' and 3' homology arms corresponding to 5' and 3' target sites, wherein the insert nucleic acid comprises a humanized *TTR* locus comprising the beta-slip mutation; and (b) identifying at least one cell comprising in its genome the insert nucleic acid integrated at the endogenous *Ttr* locus. Alternatively, the modified pluripotent cell can be generated by (a) introducing into the cell: (i) a nuclease agent, wherein the nuclease agent induces a nick or double-strand break at a target sequence within the endogenous *Ttr* locus; and (ii) one or more targeting vectors comprising an insert nucleic acid flanked by 5' and 3'

homology arms corresponding to 5' and 3' target sites located in sufficient proximity to the nuclease target sequence, wherein the insert nucleic acid comprises the humanized *TTR* locus comprising the beta-slip mutation; and (c) identifying at least one cell comprising a modification (e.g., integration of the insert nucleic acid) at the endogenous *Ttr* locus. Any nuclease agent that induces a nick or double-strand break into a desired target sequence can be used. Examples of suitable nucleases include a Transcription Activator-Like Effector Nuclease (TALEN), a zinc-finger nuclease (ZFN), a meganuclease, and Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems or components of such systems (e.g., CRISPR/Cas9). *See, e.g.*, US 2013/0309670 and US 2015/0159175, each of which is herein incorporated by reference in its entirety for all purposes.

[00302] The donor cell can be introduced into a host embryo at any stage, such as the blastocyst stage or the pre-morula stage (i.e., the 4 cell stage or the 8 cell stage). Progeny that are capable of transmitting the genetic modification though the germline are generated. *See, e.g.*, US Patent No. 7,294,754, herein incorporated by reference in its entirety for all purposes.

[00303] Alternatively, the method of producing the non-human animals described elsewhere herein can comprise: (1) modifying the genome of a one-cell stage embryo to comprise the humanized *TTR* locus comprising the beta-slip mutation using the methods described above for modifying pluripotent cells; (2) selecting the genetically modified embryo; and (3) implanting and gestating the genetically modified embryo into a surrogate mother. Alternatively, the method of producing the non-human animals described elsewhere herein can comprise: (1) modifying the genome of a one-cell stage embryo to comprise the humanized *TTR* locus comprising the beta-slip mutation using the methods described above for modifying pluripotent cells; (2) selecting the genetically modified embryo; and (3) gestating the genetically modified embryo into a surrogate mother. Progeny that are capable of transmitting the genetic modification though the germline are generated.

[00304] Nuclear transfer techniques can also be used to generate the non-human mammalian animals. Briefly, methods for nuclear transfer can include the steps of: (1) enucleating an oocyte or providing an enucleated oocyte; (2) isolating or providing a donor cell or nucleus to be combined with the enucleated oocyte; (3) inserting the cell or nucleus into the enucleated oocyte to form a reconstituted cell; (4) implanting the reconstituted cell into the womb of an animal to form an embryo; and (5) allowing the embryo to develop. In such methods, oocytes are

generally retrieved from deceased animals, although they may be isolated also from either oviducts and/or ovaries of live animals. Oocytes can be matured in a variety of well-known media prior to enucleation. Enucleation of the oocyte can be performed in a number of well-known manners. Insertion of the donor cell or nucleus into the enucleated oocyte to form a reconstituted cell can be by microinjection of a donor cell under the zona pellucida prior to fusion. Fusion may be induced by application of a DC electrical pulse across the contact/fusion plane (electrofusion), by exposure of the cells to fusion-promoting chemicals, such as polyethylene glycol, or by way of an inactivated virus, such as the Sendai virus. A reconstituted cell can be activated by electrical and/or non-electrical means before, during, and/or after fusion of the nuclear donor and recipient oocyte. Activation methods include electric pulses, chemically induced shock, penetration by sperm, increasing levels of divalent cations in the oocyte, and reducing phosphorylation of cellular proteins (as by way of kinase inhibitors) in the oocyte. The activated reconstituted cells, or embryos, can be cultured in well-known media and then transferred to the womb of an animal. *See, e.g.*, US 2008/0092249, WO 1999/005266, US 2004/0177390, WO 2008/017234, and US Patent No. 7,612,250, each of which is herein incorporated by reference in its entirety for all purposes.

[00305] The various methods provided herein allow for the generation of a genetically modified non-human F0 animal wherein the cells of the genetically modified F0 animal comprise the humanized *TTR* locus comprising the beta-slip mutation. Depending on the method used to generate the F0 animal, the number of cells within the F0 animal that have the humanized *TTR* locus comprising the beta-slip mutation will vary. The introduction of the donor ES cells into a pre-morula stage embryo from a corresponding organism (e.g., an 8-cell stage mouse embryo) via for example, the VELOCIMOUSE® method allows for a greater percentage of the cell population of the F0 animal to comprise cells having the nucleotide sequence of interest comprising the targeted genetic modification. For example, at least 50%, 60%, 65%, 70%, 75%, 85%, 86%, 87%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the cellular contribution of the non-human F0 animal can comprise a cell population having the targeted modification.

[00306] The cells of the genetically modified F0 animal can be heterozygous for the humanized *TTR* locus comprising the beta-slip mutation or can be homozygous for the humanized *TTR* locus comprising the beta-slip mutation.

[00307] All patent filings, websites, other publications, accession numbers and the like cited above or below are incorporated by reference in their entirety for all purposes to the same extent as if each individual item were specifically and individually indicated to be so incorporated by reference. If different versions of a sequence are associated with an accession number at different times, the version associated with the accession number at the effective filing date of this application is meant. The effective filing date means the earlier of the actual filing date or filing date of a priority application referring to the accession number if applicable. Likewise, if different versions of a publication, website or the like are published at different times, the version most recently published at the effective filing date of the application is meant unless otherwise indicated. Any feature, step, element, embodiment, or aspect of the invention can be used in combination with any other unless specifically indicated otherwise. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

BRIEF DESCRIPTION OF THE SEQUENCES

[00308] The nucleotide and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three-letter code for amino acids. The nucleotide sequences follow the standard convention of beginning at the 5' end of the sequence and proceeding forward (i.e., from left to right in each line) to the 3' end. Only one strand of each nucleotide sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand. When a nucleotide sequence encoding an amino acid sequence is provided, it is understood that codon degenerate variants thereof that encode the same amino acid sequence are also provided. The amino acid sequences follow the standard convention of beginning at the amino terminus of the sequence and proceeding forward (i.e., from left to right in each line) to the carboxy terminus.

[00309] Table 2. Description of Sequences.

SEQ ID NO	Type	Description
1	Protein	Human TTR Protein NP_000362.1 and P02766.1
2	DNA	Human *TTR* mRNA NM_000371.3
3	DNA	Human *TTR* Gene NG_009490.1
4	DNA	Human *TTR* CDS
5	Protein	Mouse TTR protein P07309.1 and NP_038725.1

SEQ ID NO	Type	Description
6	DNA	Mouse *Ttr* mRNA NM_013697.5
7	DNA	Mouse *Ttr* gene NC_000084.6
8	DNA	Mouse *TTR* CDS
9	DNA	Human TTR Beta-Slip Protein
10	DNA	Human TTR Beta-Slip CDS
11	DNA	Mouse *Ttr* Locus – Start Codon to Stop Codon
12	DNA	Expected Beta-Slip TTR Humanization – F0, with SDC Puro cassette
13	DNA	Expected Beta-Slip TTR Humanization – F1, Cassette-Deleted
14	DNA	Human *TTR* Sequence Inserted in Humanized Beta-Slip *TTR*
15	DNA	Expected WT TTR Humanization – F0, with SDC Neo cassette
16	DNA	Expected WT TTR Humanization – F1, Cassette-Deleted
17	DNA	Human *TTR* Sequence Inserted in Humanized WT *TTR*
18	DNA	9090retU3 – F Primer
19	DNA	9090retU2 – F Primer
20	DNA	9090retU – F Primer
21	DNA	9090mTGU – F Primer
22	DNA	7576mTU – F Primer
23	DNA	9090mTM – F Primer
24	DNA	7576mTD – F Primer
25	DNA	9090mTGD – F Primer
26	DNA	9090retD – F Primer
27	DNA	9090retD2 – F Primer
28	DNA	9090retD3 – F Primer
29	DNA	7576hTU – F Primer
30	DNA	7576hTD – F Primer
31	DNA	Puro – F Primer
32	DNA	7655hTU – F Primer
33	DNA	9212mTU – F Primer
34	DNA	9212mTGD – F Primer
35	DNA	7655mTU – F Primer
36	DNA	7655mTD – F Primer
37	DNA	9204mretD – F Primer
38	DNA	9204mretU – F Primer
39	DNA	4552mTU – F Primer
40	DNA	9090retU3 – R Primer
41	DNA	9090retU2 – R Primer
42	DNA	9090retU – R Primer
43	DNA	9090mTGU – R Primer
44	DNA	7576mTU – R Primer
45	DNA	9090mTM – R Primer
46	DNA	7576mTD – R Primer
47	DNA	9090mTGD – R Primer
48	DNA	9090retD – R Primer
49	DNA	9090retD2 – R Primer
50	DNA	9090retD3 – R Primer
51	DNA	7576hTU – R Primer
52	DNA	7576hTD – R Primer
53	DNA	Puro – R Primer
54	DNA	7655hTU – R Primer
55	DNA	9212mTU – R Primer
56	DNA	9212mTGD – R Primer
57	DNA	7655mTU – R Primer

SEQ ID NO	Type	Description
58	DNA	7655mTD – R Primer
59	DNA	9204mretD – R Primer
60	DNA	9204mretU – R Primer
61	DNA	4552mTU – R Primer
62	DNA	9090retU3 – Probe
63	DNA	9090retU2 – Probe
64	DNA	9090retU – Probe
65	DNA	9090mTGU – Probe
66	DNA	7576mTU – Probe
67	DNA	9090mTM – Probe
68	DNA	7576mTD – Probe
69	DNA	9090mTGD – Probe
70	DNA	9090retD – Probe
71	DNA	9090retD2 – Probe
72	DNA	9090retD3 – Probe
73	DNA	7576hTU – Probe
74	DNA	7576hTD – Probe
75	DNA	Puro – Probe
76	DNA	7655hTU – Probe
77	DNA	9212mTU – Probe
78	DNA	9212mTGD – Probe
79	DNA	7655mTU – Probe
80	DNA	7655mTD – Probe
81	DNA	9204mretD – Probe
82	DNA	9204mretU – Probe
83	DNA	4552mTU – Probe
84	RNA	crRNA tail
85	RNA	tracrRNA
86	RNA	Generic Guide RNA Scaffold v1
87	RNA	Generic Guide RNA Scaffold v2
88	RNA	Generic Guide RNA Scaffold v3
89	RNA	Generic Guide RNA Scaffold v4
90	DNA	Generic Guide RNA Recognition Sequence plus PAM v1
91	DNA	Generic Guide RNA Recognition Sequence plus PAM v2
92	DNA	Generic Guide RNA Recognition Sequence plus PAM v3

EXAMPLES

Example 1. Generation of Mice Comprising a Humanized *TTR* Beta-Slip Locus

[00310] A humanized *Ttr* allele made was a complete deletion of the mouse transthyretin coding sequence and its replacement with the orthologous part of the human *TTR* gene. The orthologous part of the human *TTR* gene encoded three point mutations (G53S, E54D, and L55S, referred to collectively as TTR beta-slip) that shift the position of a beta-sheet involved in intermolecular interactions of the TTR complex and renders the TTR particularly aggregate-prone. The altered interactions the TTR complex that is formed by the beta-slip mutations results in the formation of protofibrils and amyloids (*see, e.g.*, Eneqvist et al. (2000) *Mol. Cell*

6(5):1207-1218, herein incorporated by reference in its entirety for all purposes), which have been reported to be toxic to the IMR-32 cell line (*see, e.g.*, Andersson et al. (2013) *PLoS One* 8(2):e55766, herein incorporated by reference in its entirety for all purposes). These mutations, however, have never been modeled *in vivo*.

[00311] A large targeting vector comprising a 5' homology arm including 33.7 kb of sequence upstream from the mouse *Ttr* start codon and 34.5 kb of the sequence downstream of the mouse *Ttr* stop codon was generated to replace the approximately 8.3 kb region from the mouse *Ttr* start codon to the mouse *Ttr* stop codon with the approximately 7.1 kb orthologous human *TTR* sequence from the human *TTR* start codon to the end of the last human *TTR* exon (exon 4, including the human 3' UTR) and a self-deleting puromycin selection cassette (SDC Puro) flanked by loxP sites. *See* **Figure 3**. The SDC Puro cassette includes the following components from 5' to 3': loxP site, mouse protamine (*Prm1*) promoter, Crei (Cre coding sequence optimized to include intron), polyA, human ubiquitin promoter, puromycin-N-acetyltransferase ($puro_r$) coding sequence, polyA, loxP. To generate the humanized allele, CRISPR/Cas9 components were introduced into F1H4 mouse embryonic stem cells together with the large targeting vector. Loss-of-allele assays, gain-of-allele assays, retention assays, and CRISPR assays using primers and probes set forth in **Figure 4** and in **Table 3** were performed to confirm the humanization of the mouse *Ttr* allele. Loss-of-allele, gain-of-allele assays, and retention assays are described, for example, in US 2014/0178879; US 2016/0145646; WO 2016/081923; and Frendewey *et al.* (2010) *Methods Enzymol.* 476:295-307, each of which is herein incorporated by reference in its entirety for all purposes. CRISPR assays are TAQMAN® assays designed to cover the region that is disrupted by the CRISPR gRNAs. When a CRISPR gRNA cuts and creates an indel (insertion or deletion), the TAQMAN® assay will fail to amplify and thus reports CRISPR cleavage. Versions with the SDC Puro cassette and after excision of the SDC Puro cassette are shown in **Figure 3**. F0 mice were then generated using the VELOCIMOUSE® method. *See, e.g.*, US 7,576,259; US 7,659,442; US 7,294,754; US 2008/007800; and Poueymirou et al. (2007) *Nature Biotech.* 25(1):91-99, each of which is herein incorporated by reference in its entirety for all purposes.

[00312] F0 generation mice (50% C57BL/6NTac and 50% 129S6/SvEvTac) were generated from multiple humanized ES cell clones. The sequence for the expected humanized *TTR* beta-slip locus in the F0 generation mice is set forth in SEQ ID NO: 12 and includes the SDC Puro

cassette (referred to as MAID8530). F1 and F2 generation mice (75% C57BL/6NTac and 25% 129S6/SvEvTac) were then generated by breeding. The sequence for the expected humanized *TTR* beta-slip locus in the F1 and F2 generation mice is set forth in SEQ ID NO: 13 and does not include the SDC Puro cassette (referred to as MAID8531). All humanized *TTR* beta-slip mice that were characterized in the experiments below were $TTR^{8531/8531}$ (cassette removed in both humanized *TTR* beta-slip alleles).

[00313] Corresponding humanized *TTR* wild type mice were made in the same manner except with a SDC Neo cassette. The sequence for the expected humanized mouse *Ttr* wild type locus in the F0 generation mice is set forth in SEQ ID NO: 15 and includes the SDC Neo cassette (referred to as 7576). F1 and F2 generation mice (75% C57BL/6NTac and 25% 129S6/SvEvTac) were then generated by breeding. The sequence for the expected humanized mouse *Ttr* wild type locus in the F1 and F2 generation mice is set forth in SEQ ID NO: 16 and does not include the SDC Neo cassette (referred to as MAID7577). All humanized *TTR* wild type mice that were characterized in the experiments below were $TTR^{7577/7577}$ (cassette removed in both humanized *TTR* wild type alleles).

[00314] A comparison of the mouse transthyretin precursor protein, human wild type transthyretin precursor protein, and human beta-slip transthyretin precursor protein sequences is shown in **Figure 1A**. A comparison of the mouse transthyretin, human wild type transthyretin, and human beta-slip transthyretin coding sequences is shown in **Figure 1B**. A schematic showing the wild type mouse *Ttr* locus, the final wild type humanized mouse *Ttr* locus (with the SDC Neo cassette deleted) and the final beta-slip humanized mouse *Ttr* locus (with the SDC Puro cassette deleted) is shown in **Figure 2**. The endogenous mouse *Ttr* locus sequence from the start codon to the stop codon is provided in SEQ ID NO: 11. The coding sequence for the endogenous mouse *Ttr* locus is provided in SEQ ID NO: 8. The transthyretin precursor protein encoded by the endogenous mouse *Ttr* locus is provided in SEQ ID NO: 5. Sequences for the expected wild type humanized mouse *Ttr* locus with the SDC Neo cassette and without the SDC Neo cassette are set forth in SEQ ID NOS: 15 and 16, respectively. The expected coding sequence (CDS) of the wild type humanized mouse *Ttr* locus is set forth in SEQ ID NO: 4. The expected transthyretin precursor protein encoded by the wild type humanized mouse *Ttr* locus is set forth in SEQ ID NO: 1. Sequences for the expected beta-slip humanized mouse *Ttr* locus with the SDC Puro cassette and without the SDC Puro cassette are set forth in SEQ ID NOS: 12

and 13, respectively. The expected coding sequence (CDS) of the beta-slip humanized mouse *Ttr* locus is set forth in SEQ ID NO: 10. The expected transthyretin precursor protein encoded by the beta slip humanized mouse *Ttr* locus is set forth in SEQ ID NO: 9. These alleles provides a true human target of human *TTR* therapeutics, thereby enabling testing of the efficacy and mode of action of therapeutics in live animals as well as pharmacokinetic and pharmacodynamics studies in a setting where the mutated human protein is the only version of TTR present.

[00315] Table 3. Primers and Probes for Loss-of-Allele Assays, Gain-of-Allele Assays, and Retention Assays.

Assay	Forward Primer	Reverse Primer	Probe
9090retU3	CACAGACAATCAGACGTACCAGTA (SEQ ID NO: 18)	GGGACATCTCGGTTTCCTGACTT (SEQ ID NO: 40)	TCATGTAATCTGGCTTCAGAGTGGGA (SEQ ID NO: 62)
9090retU2	CCAGCTTTGCCAGTTTACGA (SEQ ID NO: 19)	TCCACACTACTGAACTCCACAA (SEQ ID NO: 41)	TGGGAGGCAATTCTTAGTTTCAATGGA (SEQ ID NO: 63)
9090retU	TTGGACGGTTGCCCTCTT (SEQ ID NO: 20)	CGGAACACTCGCTCTACGAAA (SEQ ID NO: 42)	TCCCAAAGGTGTCTGTCTGCACA (SEQ ID NO: 64)
9090mTGU	GATGGCTTCCCTTCGACTCTTC (SEQ ID NO: 21)	GGGCCAGCTTCAGACACA (SEQ ID NO: 43)	CTCCTTTGCCTCGCTGGACTGG (SEQ ID NO: 65)
7576mTU	CACTGACATTTCTCTTGTCTCCTCT (SEQ ID NO: 22)	CCCAGGGTGCTGGAGAATCCAA (SEQ ID NO: 44)	CGGACAGCATCCAGGACTT (SEQ ID NO: 66)
9090mTM	GGGCTCACCACAGATGAGAAG (SEQ ID NO: 23)	GCCAAGTGTCTTCCAGTACGAT (SEQ ID NO: 45)	AGAAGGAGTGTACAGAGTAGAACTGGACA (SEQ ID NO: 67)
7576mTD	CACTGTTCGCCACAGGTCTT (SEQ ID NO: 24)	GTTCCCTTTCTTGGGTTCAGA (SEQ ID NO: 46)	TGTTTGTGGGTGTCAGTGTTTCTACTC (SEQ ID NO: 68)
9090mTGD	GCTCAGCCCATACTCCTACA (SEQ ID NO: 25)	GATGCTACTGCTTTGGCAAGATC (SEQ ID NO: 47)	CACCACGGCTGTCGTCAGCAA (SEQ ID NO: 69)
9090retD	GCCCAGGAGGACCAGGAT (SEQ ID NO: 26)	CCTGAGCTGCTAACACGGTT (SEQ ID NO: 48)	CTTGCCAAAGCAGTAGCATCCCA (SEQ ID NO: 70)
9090retD2	GGCAACTTGCTTGAGGAAGA (SEQ ID NO: 27)	AGCTACAGACCATGCTTAGTGTA (SEQ ID NO: 49)	AGGTCAGAAAGCAGAGTGGACCA (SEQ ID NO: 71)
9090retD3	GCAGCAACCCAGCTTCACTT (SEQ ID NO: 28)	TGCCAGTTTAGGAGGAATATGTTC (SEQ ID NO: 50)	CCCAGGCAATTCCTACCTTCCCA (SEQ ID NO: 72)
7576hTU	ACTGAGCTGGGACTTGAAC (SEQ ID NO: 29)	CTGAGGAAACAGAGGTACCAGATAT (SEQ ID NO: 51)	TCTGAGCATTCTACCTCATTGCTTTGGT (SEQ ID NO: 73)

Assay	Forward Primer	Reverse Primer	Probe
7576hTD	TGCCTCACTCTGAGAACCA (SEQ ID NO: 30)	AGTCACACAGTTCTGTCAAATCAG (SEQ ID NO: 52)	AGGCTGTCCCAGCACCTGAGTCG (SEQ ID NO: 74)
Puro	CGCAACCTCCCCTTCTACG (SEQ ID NO: 31)	GTCCTTCGGGCACCTCG (SEQ ID NO: 53)	CGGCTCGGCTTCACCGTCACC (SEQ ID NO: 75)
7655hTU	GGCCGTGCATGTGTTCAG (SEQ ID NO: 32)	TCCTGTGGGAGGGTTCTTTG (SEQ ID NO: 54)	AAGGCTGCTGATGACACCTGGGA (SEQ ID NO: 76)
9212mTU	GGTTCCCATTTGCTCTTATTCGT (SEQ ID NO: 33)	CCCTCTCTCTGAGCCCTCTA (SEQ ID NO: 55)	AGATTCAGACACACACAACTTACCAGC (SEQ ID NO: 77)
9212mTGD	CCCACACTGCAGAAGGAAACTTG (SEQ ID NO: 34)	GCTGCCTAAGTCTTTGGAGCT (SEQ ID NO: 56)	AGACCTGCAATTCTCTAAGAGCTCCACA (SEQ ID NO: 78)
7655mTU	GGTTCCCATTTGCTCTTATTCGT (SEQ ID NO: 35)	CCCTCTCTCTGAGCCCTCTA (SEQ ID NO: 57)	AGATTCAGACACACACAACTTACCAGC (SEQ ID NO: 79)
7655mTD	CCAGCTTAGCATCCTGTGAACA (SEQ ID NO: 36)	GAGAGGAGAGACAGCTAGTTCTAAC (SEQ ID NO: 58)	TTGTCTGCAGCTCCTACCTCTGGG (SEQ ID NO: 80)
9204mretD	GGCAACTTGCTTGAGGAAGA (SEQ ID NO: 37)	AGCTACAGACCATGCTTAGTGTA (SEQ ID NO: 59)	AGGTCAGAAAGCAGAGTGGACCA (SEQ ID NO: 81)
9204mretU	TGTGGAGTTCAGTAGTGTGGAG (SEQ ID NO: 38)	GCCCTCTTCATACAGGAATCAC (SEQ ID NO: 60)	TTGACATGTGTGGGTGAGAGATTTTACTG (SEQ ID NO: 82)
4552mTU	CACTGACATTTCTCTTGTCTCCTCT (SEQ ID NO: 39)	CGGACAGCATCCAGGACTT (SEQ ID NO: 61)	CCCAGGGTGCTGGAGAATCCAA (SEQ ID NO: 83)

Example 2. Characterization of Mice Comprising a Humanized *TTR* Beta-Slip Locus.

[00316] A humanized *TTR* beta-slip mouse colony was established, and F2 cohorts were characterized at two months of age. Human TTR was measured in serum and detected at 0.3 μg/mL, which is substantially lower than the levels typically detected in the serum of the control wild type mice (approximately 1000 μg/mL of mouse TTR) or that circulating in humans (approximately 200 μg/mL). It is also lower than the levels seen in the humanized *TTR* wild type mice. *See* **Figure 5A** and **Table 4**.

[00317] Table 4. Plasma TTR levels in, Serum T4 Levels in, and Body Temperature of Humanized *TTR* Beta-Slip Mice, Humanized TTR Wild Type Mice, and Control F1H4 Mice.

Strain	hTTR, µg/mL (SD)	mTTR, µg/mL (SD)	Total T4, ng/mL (SD)	Free T4, ng/mL (SD)	Body Temp, °C (SD)
F1H4	N.D.	~800	158.6 (20.85)	423.1 (6.93)	36.95 (0.55)
WT $hTTR^{7577/7577}$	52.07 (10.67)	N.D.	66.88 (18)	286.8 (51.19)	36.7 (0.44)
β-slip $hTTR^{8531/8531}$	0.291 (0.083)	N.D.	51.19 (34.09)	250.9 (36.76)	36.79 (0.84)
Human Serum	234.5 (n.a.)	N.D.	64.71 (n.a.)	423.1 (n.a.)	N.D.

[00318] When the blood of humanized *TTR* beta-slip mice was analyzed on native PAGE, followed by western blot with an anti-human-specific antibody, TTR was observed as expected. *See* **Figure 6A**. Human mature WT TTR (pI: 5.31; MW: 13.76 kDa) and human mature beta-slip TTR (pI: 5.30 / MW: 13.75 kDa) have nearly identical molecular weights. When the blood of humanized *TTR* beta-slip mice was analyzed on native PAGE, followed by western blot with an anti-human-specific antibody, high molecular weight TTR species were observed in the serum of the humanized *TTR* beta-slip mice but not the corresponding humanized *TTR* wild type mice. *See* **Figure 6B**.

[00319] Despite having low levels of circulating TTR, the humanized *TTR* beta-slip mice had phenotypic differences when tested in behavioral assays. *See* **Figures 8A-8C**. One assay is the open field test, which gives an overall measure of mouse locomotion and hyperactivity. Three of the readouts from the open field test are the total distance traveled, the total activity, and the total number of rearings, measures which were each significantly increased in the humanized *TTR* beta-slip mice when compared to humanized *TTR* WT mice or littermate (F1H4) controls. *See* **Figures 8A**, **8B**, and **8C**, respectively. *See also* **Tables 5**, **6**, and **7**, respectively. The open field is a behavioral test used to measure the general motor health and activity of mice during a 60-minute period. The mice were tracked using computer software (Kinder Scientific MotorMonitor software, Kinder Scientific, Poway, CA) for the total distance they traveled inside an enclosed, square apparatus during a 60-minute time period. Total activity was measured by the number of times a mouse in the apparatus interrupts the path of infrared breams in the X and Y planes (i.e., "beam breaks"). Rearings were a measure of how many times a mouse stood on its hindlimbs (i.e., "rear") to explore the walls of the apparatus. A greater rearings value is

indicative of less anxious and more active mice. Rearings were measured by infrared beam breaks in the Z-plane (beams placed at a height above that of a quadrupedal mouse).

[00320] Table 5. Total Distance in Open Field Test of Humanized *TTR* Beta-Slip Mice, Humanized TTR Wild Type Mice, and Control F1H4 Mice.

	F1H4, cm (SD)	**WT hTTR$^{7577/7577}$** cm (SD)	**β-slip hTTR$^{8531/8531}$** cm (SD)
Total distance	10240 (3399)	9863 (4134)	16052 (2146)**

** = $p < 0.01$ vs. F1H4 and vs. WT hTTR$^{7577/7577}$ (ANOVA, Tukey)

[00321] Table 6. Total Activity of Humanized *TTR* Beta-Slip Mice, Humanized TTR Wild Type Mice, and Control F1H4 Mice.

	F1H4 n (SD)	**WT hTTR$^{7577/7577}$** n (SD)	**β-slip hTTR$^{8531/8531}$** n (SD)
Total Activity (Beam Breaks)	4217 (1306)	3470 (1856)	6717 (872.2)

[00322] Table 7. Total Rearings of Humanized *TTR* Beta-Slip Mice, Humanized TTR Wild Type Mice, and Control F1H4 Mice.

	F1H4 n (SD)	**WT hTTR$^{7577/7577}$** n (SD)	**β-slip hTTR$^{8531/8531}$** n (SD)
Rearings	174.2 (77.7)	153.3 (121.2)	497.8 (125.2)***

*** = $p < 0.001$ vs. F1H4 and vs. WT hTTR$^{7577/7577}$ (ANOVA, Tukey)

[00323] Increased hyperactivity has been reported in TTR-null mice (*see, e.g.*, Sousa et al. (2004) *J. Neurochem.* 88(5):1052-1058, herein incorporated by reference in its entirety for all purposes), suggesting TTR beta-slip may act as a loss-of-function mutant, despite being detectable in serum. Additional correlates of the expressed TTR beta-slip protein being non-functional is the decrease in serum total T4 and free T4, which were associated with a decreased body temperature, which suggests TTR beta-slip mice may be hypothyroid due to the loss of function of TTR. *See* **Figures 5B**, **5C**, and **5D**, respectively.

[00324] At least two possible explanations may account for the low level of circulating human TTR in the humanized *TTR* beta-slip mice. One possible explanation is poor or inefficient secretion of beta-slip TTR from liver hepatocytes. A second possible explanation is that the circulating human beta-slip TTR may be rapidly deposited onto peripheral organs/tissues.

[00325] The possibility of tissue deposition of human beta-slip TTR is supported by our observation that some mice had weak muscle tone in their hindlimbs, muscles which are highly innervated by the sciatic nerve. *See* **Figures 7A-7B**. This observation of humanized *TTR* beta-slip mice having altered muscle tone in hindlimbs (i.e., "dystonic") is an observation not previously reported in the literature for TTR null mice or transgenic mice expressing human forms of TTR. This phenotype was evaluated by scruffing the mouse and assessing the angle of its hindlimbs relative to the axis of the body. Normally, mice have a wide angle between the body axis and the hindlimb angle (*see* F1H4 and TTR WT humanization pictures in **Figure 7A**). In contrast, the beta-slip mice did not have their hindlimbs extended from their body (*see* TTR β-slip humanization pictures in **Figure 7A**). The significance of this is that it could be a proxy measure for sciatic nerve dysfunction because the sciatic nerve controls hindlimb function. The sciatic nerve is one of the major sites of amyloid deposition in human TTR diseases (e.g., FAP). Thus, the dystonic phenotype could be an indicator for abnormal sciatic nerve function.

[00326] Grip strength was then measured. Grip strength was measured by allowing the mouse to grab a bar connected to a grip strength meter, then manually pulling the mouse away from bar at a constant rate. The apparatus measures the maximal force at which the mouse pulls the bar before losing grip and releasing it. The forelimbs and hindlimbs were tested separately and graphed as the average units of force from three attempts per mouse. Grip strength did not correlate with the dystonic phenotype and was similar in humanized *TTR* beta-slip mice, humanized *TTR* wild type mice, and control (F1H4) mice. *See* **Figure 9B** and **Table 8**. Likewise, body weight was similar in humanized *TTR* beta-slip mice, humanized *TTR* wild type mice, and control (F1H4) mice. *See* **Figure 9A** and **Table 9**.

[00327] Table 8. Grip Strength of Humanized *TTR* Beta-Slip Mice, Humanized TTR Wild Type Mice, and Control F1H4 Mice.

	Forelimb, N (SD)	**Hindlimb, N (SD)**
F1H4	1.109 (0.137)	0.3115 (0.092)
WT $hTTR^{7577/7577}$	0.961 (0.155)	0.3029 (0.096)
β-slip $hTTR^{8531/8531}$	1.141 (0.185)	0.3107 (0.083)

[00328] Table 9. Body Weight of Humanized *TTR* Beta-Slip Mice, Humanized TTR Wild Type Mice, and Control F1H4 Mice.

	Males, g (SD)	Females, g (SD)
F1H4	27.12 (2.31)	20.51 (2.12)
WT $hTTR^{7577/7577}$	24.77 (3.16)	20.22 (1.67)
β-slip $hTTR^{8531/8531}$	25.38 (1.26)	20.69 (0.99)

[00329] Post-mortem histopathological analysis with an amyloid-specific dye, Congo Red, showed birefringent-positive amyloid deposits on the sciatic nerve of humanized *TTR* beta-slip mice and not on the sciatic nerves of F1H4 (littermate controls) or humanized *TTR* wild type mice. *See* **Figures 10A** and **10B**, respectively. Two-month old beta-slip mice were euthanized using CO_2 and transcardially perfused with phosphate buffer (20 mL) followed by perfusion with 20 mL of 4% paraformaldehyde (PFA). The liver and sciatic nerve were removed and post-fixed in 4% PFA for 2 days at 4°C, followed by a cryopreservation step by soaking the tissue in 30% (w/v) sucrose in phosphate buffer overnight. The next day, tissues were immersed and frozen in OCT ("optimal cutting temperature"). Sections were cut on a cryostat at 10 micron and mounted onto frosted microscope slides. The slides were stained with Congo Red solution according to the manufacturer's protocol (Sigma, cat no: HT60-1KT). Congo Red is a widely used stain to detect amyloidosis. Slides were imaged under white light which revealed overall tissue architecture and a characteristic red stain. *See* top panels in **Figures 10A** and **10B**. When the Congo Red stained tissue was illuminated using linear polarized light, only the dye which was bound to amyloids refracted the polarized light (e.g., amyloid bound CongoRed dye will become birefringent), which was viewed as a light green/white color. *See* bottom panels in **Figures 10A** and **10B**. The presence of these greenish-white deposits was indicative of amyloid deposition. We observed birefringence (i.e., the presence of amyloid deposits) in the sciatic nerve, which could explain the dystonic phenotype of mice. *See* bottom right panel (TTR β-slip humanization) in **Figure 10A**. As expected, we did not observe amyloids in the liver. *See* bottom right panel (TTR β-slip humanization) in **Figure 10B**.

[00330] This is the first reported *in vivo* model to develop amyloidosis so rapidly, with amyloid deposits observed in two-month-old beta-slip mice. Experiments are then done to co-stain with an anti-TTR antibody to co-label the birefringent deposits and confirm that they are caused by TTR deposition. Additional experiments are done to analyze whether there are deposits in other organs and tissues.

We claim:

1. A non-human animal comprising in its genome a genetically modified endogenous *Ttr* locus, wherein a region of the endogenous *Ttr* locus comprising both *Ttr* coding sequence and non-coding sequence has been deleted and replaced with a corresponding human *TTR* sequence comprising both *TTR* coding sequence and non-coding sequence, and wherein the genetically modified endogenous *Ttr* locus comprises a mutation that causes a shift in beta-strand D of the encoded transthyretin protein.

2. The non-human animal of claim 1, wherein the mutation causes a three-residue shift in beta strand D that places a residue corresponding to residue L58 in a human transthyretin protein at a position normally occupied by a residue corresponding to residue L55 in the human transthyretin protein when the encoded transthyretin protein is optimally aligned with the human transthyretin protein.

3. The non-human animal of claim 2, wherein the mutation is a triple mutation corresponding to G53S/E54D/L55S in the human transthyretin protein when the encoded transthyretin protein is optimally aligned with the human transthyretin protein.

4. The non-human animal of claim 3, wherein the triple mutation is in the corresponding human *TTR* sequence.

5. The non-human animal of any preceding claim, wherein the genetically modified endogenous *Ttr* locus comprises the endogenous *Ttr* promoter, wherein the human *TTR* sequence is operably linked to the endogenous *Ttr* promoter.

6. The non-human animal of any preceding claim, wherein at least one intron and at least one exon of the endogenous *Ttr* locus have been deleted and replaced with the corresponding human *TTR* sequence.

7. The non-human animal of any preceding claim, wherein the entire *Ttr* coding sequence of the endogenous *Ttr* locus has been deleted and replaced with the corresponding human *TTR* sequence.

8. The non-human animal of claim 7, wherein the region of the endogenous *Ttr* locus from the *Ttr* start codon to the *Ttr* stop codon has been deleted and replaced with the corresponding human *TTR* sequence.

9. The non-human animal of any preceding claim, wherein the genetically modified endogenous *Ttr* locus comprises a human *TTR* 3' untranslated region.

10. The non-human animal of any preceding claim, wherein the endogenous *Ttr* 5' untranslated region has not been deleted and replaced with the corresponding human *TTR* sequence.

11. The non-human animal of any preceding claim, wherein the region of the endogenous *Ttr* locus from the *Ttr* start codon to the *Ttr* stop codon has been deleted and replaced with a human *TTR* sequence comprising the corresponding human *TTR* sequence and a human *TTR* 3' untranslated region, and

wherein the endogenous *Ttr* 5' untranslated region has not been deleted and replaced with the corresponding human *TTR* sequence, and

wherein the endogenous *Ttr* promoter has not been deleted and replaced with the corresponding human *TTR* sequence.

12. The non-human animal of claim 11, wherein:

(i) the human *TTR* sequence at the genetically modified endogenous *Ttr* locus comprises a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the sequence set forth in SEQ ID NO: 14; or

(ii) the genetically modified endogenous *Ttr* locus encodes a protein comprising a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the sequence set forth in SEQ ID NO: 9;

(iii) the genetically modified endogenous *Ttr* locus comprises a coding sequence comprising a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the sequence set forth in SEQ ID NO: 10; or

(iv) the genetically modified endogenous *Ttr* locus comprises a sequence at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the sequence set forth in SEQ ID NO: 12 or 13.

13. The non-human animal of any one of claims 1-6, wherein the genetically modified endogenous *Ttr* locus encodes a transthyretin precursor protein comprising a signal peptide, and the region of the endogenous *Ttr* locus encoding the signal peptide has not been deleted and replaced with the corresponding human *TTR* sequence.

14. The non-human animal of claim 13, wherein the first exon of the endogenous *Ttr* locus has not been deleted and replaced with the corresponding human *TTR* sequence.

15. The non-human animal of claim 14, wherein the first exon and first intron of the endogenous *Ttr* locus have not been deleted and replaced with the corresponding human *TTR* sequence.

16. The non-human animal of any one of claims 13-15, wherein the region of the endogenous *Ttr* locus from the start of the second *Ttr* exon to the *Ttr* stop codon has been deleted and replaced with the corresponding human *TTR* sequence.

17. The non-human animal of any one of claims 13-16, wherein the genetically modified endogenous *Ttr* locus comprises a human *TTR* 3' untranslated region.

18. The non-human animal of any one of claims 13-17, wherein the region of the endogenous *Ttr* locus from the second *Ttr* exon to the *Ttr* stop codon has been deleted and replaced with a human *TTR* sequence comprising the corresponding human *TTR* sequence and a human *TTR* 3' untranslated region, and

wherein the endogenous *Ttr* 5' untranslated region has not been deleted and replaced with the corresponding human *TTR* sequence, and

wherein the endogenous *Ttr* promoter has not been deleted and replaced with the corresponding human *TTR* sequence.

19. The non-human animal of any preceding claim, wherein the genetically modified endogenous *Ttr* locus does not comprise a selection cassette or a reporter gene.

20. The non-human animal of any preceding claim, wherein the non-human animal is homozygous for the genetically modified endogenous *Ttr* locus.

21. The non-human animal of any preceding claim, wherein the non-human animal is a mammal.

22. The non-human animal of claim 21, wherein the non-human animal is a rat or mouse.

23. The non-human animal of claim 22, wherein the non-human animal is a mouse.

24. The non-human animal of any preceding claim, wherein the non-human animal comprises the genetically modified endogenous *Ttr* locus in its germline.

25. The non-human animal of any preceding claim, wherein the non-human animal is hyperactive relative to a control wild type non-human animal or a non-human animal comprising the genetically modified endogenous *Ttr* locus without the mutation.

26. The non-human animal of claim 25, wherein the hyperactivity is as measured by one or more or all of total distance, total activity, or total rearings in an open field test.

27. The non-human animal of any preceding claim, wherein the non-human animal displays hindlimb dystonia.

28. The non-human animal of any preceding claim, wherein the non-human animal comprises amyloid deposits.

29. The non-human animal of claim 28, wherein the non-human animal comprises amyloid deposits in the sciatic nerve.

30. The non-human animal of claim 28 or 29, wherein the non-human animal develops amyloidosis by about two months of age.

31. A non-human animal cell comprising in its genome a genetically modified endogenous *Ttr* locus, wherein a region of the endogenous *Ttr* locus comprising both *Ttr* coding sequence and non-coding sequence has been deleted and replaced with a corresponding human

TTR sequence comprising both *TTR* coding sequence and non-coding sequence, and wherein the genetically modified endogenous *Ttr* locus comprises a mutation that causes a shift in beta-strand D of the encoded transthyretin protein.

32. A non-human animal genome comprising a genetically modified endogenous *Ttr* locus, wherein a region of the endogenous *Ttr* locus comprising both *Ttr* coding sequence and non-coding sequence has been deleted and replaced with a corresponding human *TTR* sequence comprising both *TTR* coding sequence and non-coding sequence, and wherein the genetically modified endogenous *Ttr* locus comprises a mutation that causes a shift in beta-strand D of the encoded transthyretin protein.

33. A targeting vector for generating a genetically modified endogenous *Ttr* locus in which a region of the endogenous *Ttr* locus comprising both *Ttr* coding sequence and non-coding sequence has been deleted and replaced with a corresponding human *TTR* sequence comprising both *TTR* coding sequence and non-coding sequence, and wherein the genetically modified endogenous *Ttr* locus comprises a mutation that causes a shift in beta-strand D of the encoded transthyretin protein, wherein the targeting vector comprises an insert nucleic acid comprising the corresponding human *TTR* sequence flanked by a 5' homology arm targeting a 5' target sequence at the endogenous *Ttr* locus and a 3' homology arm targeting a 3' target sequence at the endogenous *Ttr* locus.

34. A genetically modified non-human animal *Ttr* gene in which a region of the endogenous *Ttr* gene comprising both *Ttr* coding sequence and non-coding sequence has been deleted and replaced with a corresponding human *TTR* sequence comprising both *TTR* coding sequence and non-coding sequence, and wherein the genetically modified non-human animal *Ttr* gene comprises a mutation that causes a shift in beta-strand D of the encoded transthyretin protein.

35. A method of assessing the activity of a human-TTR-targeting reagent *in vivo*, comprising:

(a) administering the human-TTR-targeting reagent to the non-human animal of any one of claims 1-30; and

(b) assessing the activity of the human-TTR-targeting reagent in the non-human animal.

36. The method of claim 35, wherein the introducing comprises adeno-associated virus (AAV)-mediated delivery, lipid nanoparticle (LNP)-mediated delivery, or hydrodynamic delivery (HDD).

37. The method of claim 36, wherein the introducing comprises LNP-mediated delivery.

38. The method of claim 36, wherein the introducing comprises AAV8-mediated delivery.

39. The method of any one of claims 35-38, wherein step (b) comprises isolating a liver from the non-human animal and assessing activity of the human-TTR-targeting reagent in the liver.

40. The method of claim 39, wherein step (b) further comprises assessing activity of the human-TTR-targeting reagent in an organ or tissue other than the liver.

41. The method of any one of claims 35-40, wherein the assessing comprises assessing modification of the genetically modified *Ttr* locus.

42. The method of any one of claims 35-41, wherein the assessing comprises assessing expression of a *Ttr* messenger RNA encoded by the genetically modified *Ttr* locus.

43. The method of any one of claims 35-42, wherein the assessing comprises assessing expression of a TTR protein encoded by the genetically modified *Ttr* locus.

44. The method of claim 43, wherein assessing expression of the TTR protein comprises measuring serum levels of the TTR protein in the non-human animal.

45. The method of any one of claims 41-43, wherein the activity is assessed in the liver of the non-human animal.

46. The method of any one of claims 35-45, wherein the assessing comprises assessing hyperactivity.

47. The method of any one of claims 35-46, wherein the assessing comprises assessing hindlimb dystonia.

48. The method of any one of claims 35-47, wherein the assessing comprises assessing amyloid deposition.

49. The method of claim 48, wherein the assessing comprises assessing amyloid deposition in the sciatic nerve.

50. The method of any one of claims 35-49, wherein the assessing is in comparison to an untreated control non-human animal.

51. The method of any one of claims 35-50, wherein the human-TTR-targeting reagent comprises a nuclease agent designed to target a region of a human *TTR* gene.

52. The method of claim 51, wherein the nuclease agent comprises a Cas protein and a guide RNA designed to target a guide RNA target sequence in the human *TTR* gene.

53. The method of claim 52, wherein the Cas protein is a Cas9 protein.

54. The method of any one of claims 35-53, wherein the human-TTR-targeting reagent comprises an exogenous donor nucleic acid, wherein the exogenous donor nucleic acid is designed to recombine with the human *TTR* gene.

55. The method of claim 54, wherein the exogenous donor nucleic acid is a single-stranded oligodeoxynucleotide (ssODN).

56. The method of any one of claims 35-55, wherein the human-TTR-targeting reagent comprises an antigen-binding protein.

57. The method of any one of claims 35-56, wherein the human-TTR-targeting reagent comprises an RNAi agent or an antisense oligonucleotide.

58. A method of optimizing the activity of a human-TTR-targeting reagent *in vivo*, comprising:

(I) performing the method of any one of claims 35-57 a first time in a first non-human animal comprising in its genome the genetically modified endogenous *Ttr* locus;

(II) changing a variable and performing the method of step (I) a second time with the changed variable in a second non-human animal comprising in its genome the genetically modified endogenous *Ttr* locus; and

(III) comparing the activity of the human-TTR-targeting reagent in step (I) with the activity of the human-TTR-targeting reagent in step (II), and selecting the method resulting in the higher efficacy, higher precision, higher consistency, or higher specificity.

59. The method of claim 58, wherein the changed variable in step (II) is the delivery method of introducing the human-TTR-targeting reagent into the non-human animal.

60. The method of claim 58, wherein the changed variable in step (II) is the route of administration of introducing the human-TTR-targeting reagent into the non-human animal.

61. The method of claim 58, wherein the changed variable in step (II) is the concentration or amount of the human-TTR-targeting reagent introduced into the non-human animal.

62. The method of claim 58, wherein the changed variable in step (II) is the form of the human-TTR-targeting reagent introduced into the non-human animal.

63. The method of claim 58, wherein the changed variable in step (II) is the human-TTR-targeting reagent introduced into the non-human animal.

64. A method of making the non-human animal of any one of claims 1-30, comprising:

(I) (a) modifying the genome of a pluripotent non-human animal cell to comprise the genetically modified endogenous *Ttr* locus;

(b) identifying or selecting the genetically modified pluripotent non-human animal cell comprising the genetically modified endogenous *Ttr* locus;

(c) introducing the genetically modified pluripotent non-human animal cell into a non-human animal host embryo; and

(d) gestating the non-human animal host embryo in a surrogate mother; or

(II) (a) modifying the genome of a non-human animal one-cell stage embryo to comprise the genetically modified endogenous *Ttr* locus;

(b) selecting the genetically modified non-human animal one-cell stage embryo comprising the genetically modified endogenous *Ttr* locus; and

(c) gestating the genetically modified non-human animal one-cell stage embryo in a surrogate mother.

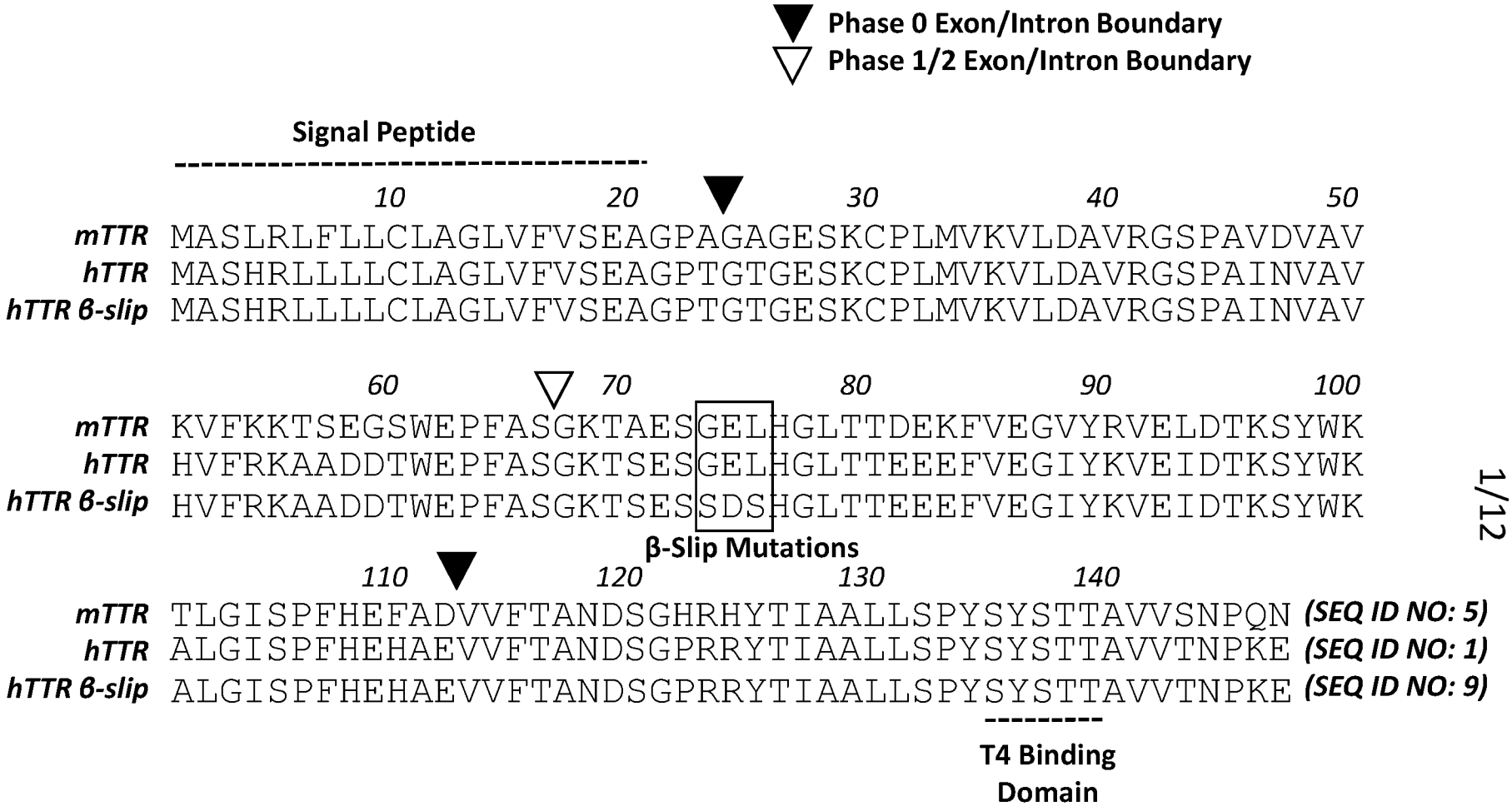

Phase 0 Exon/Intron Boundary
Phase 1/2 Exon/Intron Boundary
Signal Peptide
10 20 30 40 50
mTTR MASLRLFLLCLAGLVFVSEAGPAGAGESKCPLMVKVLDAVRGSPAVDVAV
hTTR MASHRLLLLCLAGLVFVSEAGPTGTGESKCPLMVKVLDAVRGSPAINVAV
hTTR β-slip MASHRLLLLCLAGLVFVSEAGPTGTGESKCPLMVKVLDAVRGSPAINVAV
60 70 80 90 100
mTTR KVFKKTSEGSWEPFASGKTAESGELHGLTTDEKFVEGVYRVELDTKSYWK
hTTR HVFRKAADDTWEPFASGKTSESGELHGLTTEEEFVEGIYKVEIDTKSYWK
hTTR β-slip HVFRKAADDTWEPFASGKTSESSDSHGLTTEEEFVEGIYKVEIDTKSYWK
β-Slip Mutations
110 120 130 140
mTTR TLGISPFHEFADVVFTANDSGHRHYTIAALLSPYSYSTTAVVSNPQN (SEQ ID NO: 5)
hTTR ALGISPFHEHAEVVFTANDSGPRRYTIAALLSPYSYSTTAVVTNPKE (SEQ ID NO: 1)
hTTR β-slip ALGISPFHEHAEVVFTANDSGPRRYTIAALLSPYSYSTTAVVTNPKE (SEQ ID NO: 9)
T4 Binding Domain

FIG. 1A

```
                10        20        30        40        50        60        70        80        90       100
mTTR            ATGGCTTCCCTTCGACTCTTCCTCCTTTGCCTCGCTGGACTGGTATTTGTGTCTGAAGCTGGCCCCGCGGGTGCTGGAGAATCCAAATGTCCTCTGATGG
hTTR            ATGGCTTCTCATCGTCTGCTCCTCCTCTGCCTTGCTGGACTGGTATTTGTGTCTGAGGCTGGCCCTACGGGCACCGGTGAATCCAAGTGTCCTCTGATGG
hTTR β-slip     ATGGCTTCTCATCGTCTGCTCCTCCTCTGCCTTGCTGGACTGGTATTTGTGTCTGAGGCTGGCCCTACGGGCACCGGTGAATCCAAGTGTCCTCTGATGG

               110       120       130       140       150       160       170       180       190       200
mTTR            TCAAAGTCCTGGATGCTGTCCGAGGCAGCCCTGCTGTAGACGTGGCTGTAAAAGTGTTCAAAAAGACCTCTGAGGGATCCTGGGAGCCCTTTGCCTCTGG
hTTR            TCAAAGTTCTAGATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTGCTGATGACACCTGGGAGCCATTTGCCTCTGG
hTTR β-slip     TCAAAGTTCTAGATGCTGTCCGAGGCAGTCCTGCCATCAATGTGGCCGTGCATGTGTTCAGAAAGGCTGCTGATGACACCTGGGAGCCATTTGCCTCTGG

               210       220       230       240       250       260       270       280       290       300
mTTR            GAAGACCGCGGAGTCTGGAGAGCTGCACGGGCTCACCACAGATGAGAAGTTTGTAGAAGGAGTGTACAGAGTAGAACTGGACACCAAATCGTACTGGAAG
hTTR            GAAAACCAGTGAGTCTGGAGAGCTGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATAGACACCAAATCTTACTGGAAG
hTTR β-slip     GAAAACCAGTGAGTCTAGCGATTCGCATGGGCTCACAACTGAGGAGGAATTTGTAGAAGGGATATACAAAGTGGAAATAGACACCAAATCTTACTGGAAG

               310       320       330       340       350       360       370       380       390       400
mTTR            ACACTTGGCATTTCCCCGTTCCATGAATTCGCGGATGTGGTTTTCACAGCCAACGACTCTGGCCATCGCCACTACACCATCGCAGCCCTGCTCAGCCCAT
hTTR            GCACTTGGCATCTCCCCATTCCATGAGCATGCAGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCTGCTGAGCCCCT
hTTR β-slip     GCACTTGGCATCTCCCCATTCCATGAGCATGCAGAGGTGGTATTCACAGCCAACGACTCCGGCCCCCGCCGCTACACCATTGCCGCCCTGCTGAGCCCCT

               410       420       430       440
mTTR            ACTCCTACAGCACCACGGCTGTCGTCAGCAACCCCCAGAATTGA (SEQ ID NO: 8)
hTTR            ACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATGA (SEQ ID NO: 4)
hTTR β-slip     ACTCCTATTCCACCACGGCTGTCGTCACCAATCCCAAGGAATGA (SEQ ID NO: 10)
```

FIG. 1B

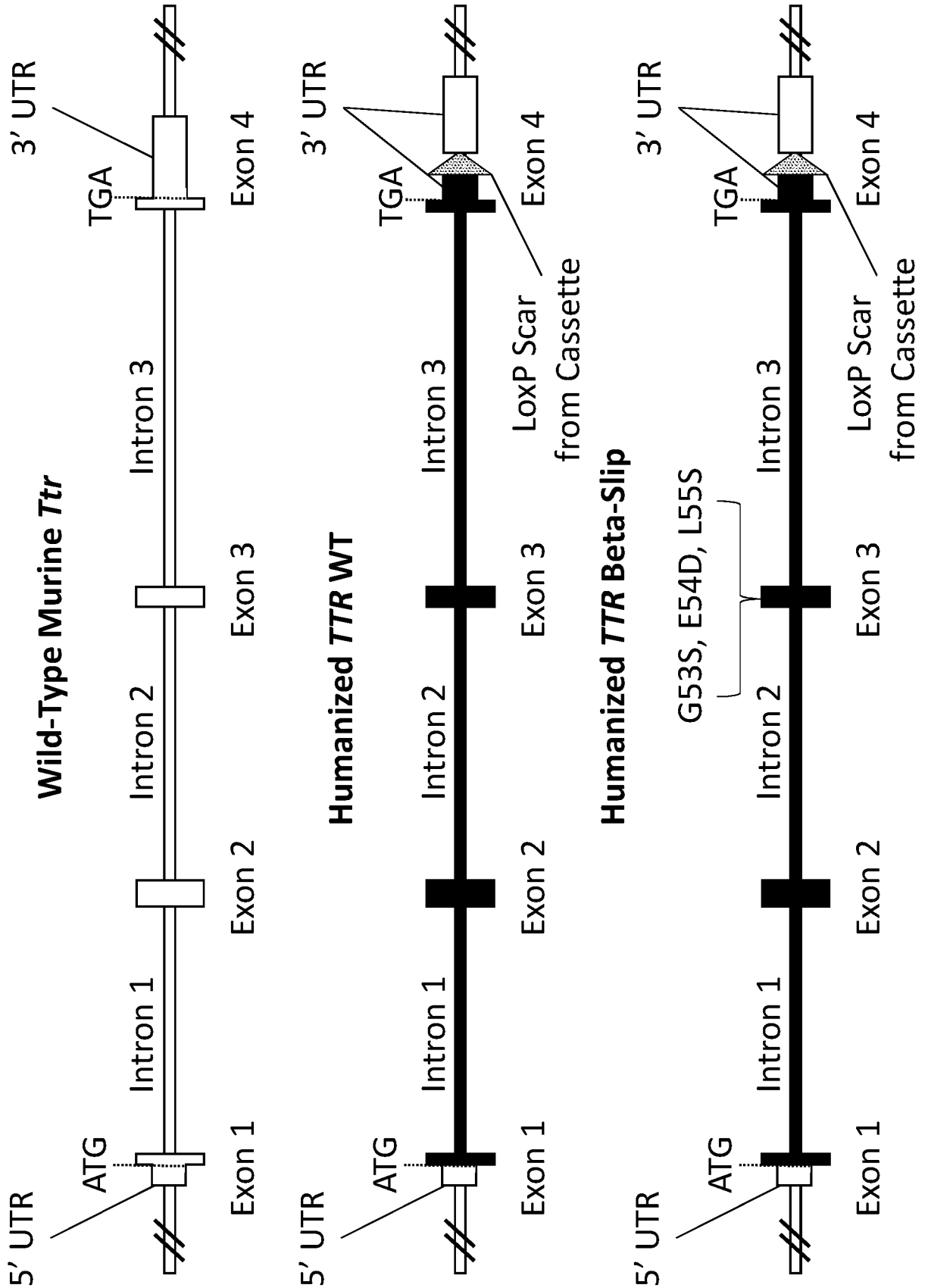

Wild-Type Murine *Ttr*
5' UTR
ATG
Exon 1
Intron 1
Exon 2
Intron 2
Exon 3
Intron 3
TGA
3' UTR
Exon 4
Humanized *TTR* WT
5' UTR
ATG
Exon 1
Intron 1
Exon 2
Intron 2
Exon 3
Intron 3
TGA
3' UTR
LoxP Scar
from Cassette
Exon 4
Humanized *TTR* Beta-Slip
G53S, E54D, L55S
5' UTR
ATG
Exon 1
Intron 1
Exon 2
Intron 2
Exon 3
Intron 3
TGA
3' UTR
LoxP Scar
from Cassette
Exon 4

FIG. 2

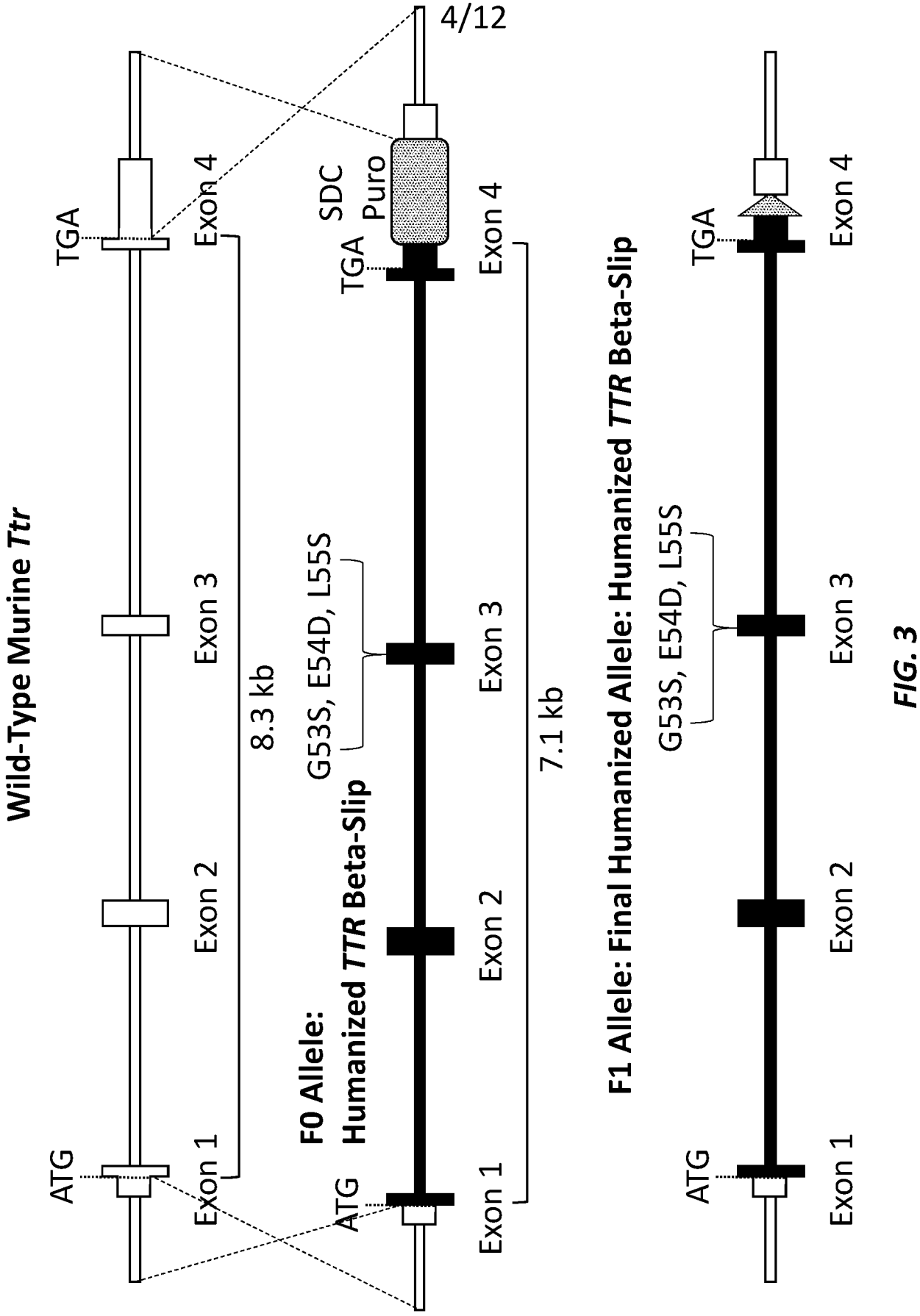

Wild-Type Murine Ttr
ATG
TGA
Exon 1
Exon 2
Exon 3
Exon 4
8.3 kb
F0 Allele:
Humanized TTR Beta-Slip
G53S, E54D, L55S
SDC
Puro
7.1 kb
F1 Allele: Final Humanized Allele: Humanized TTR Beta-Slip

FIG. 3

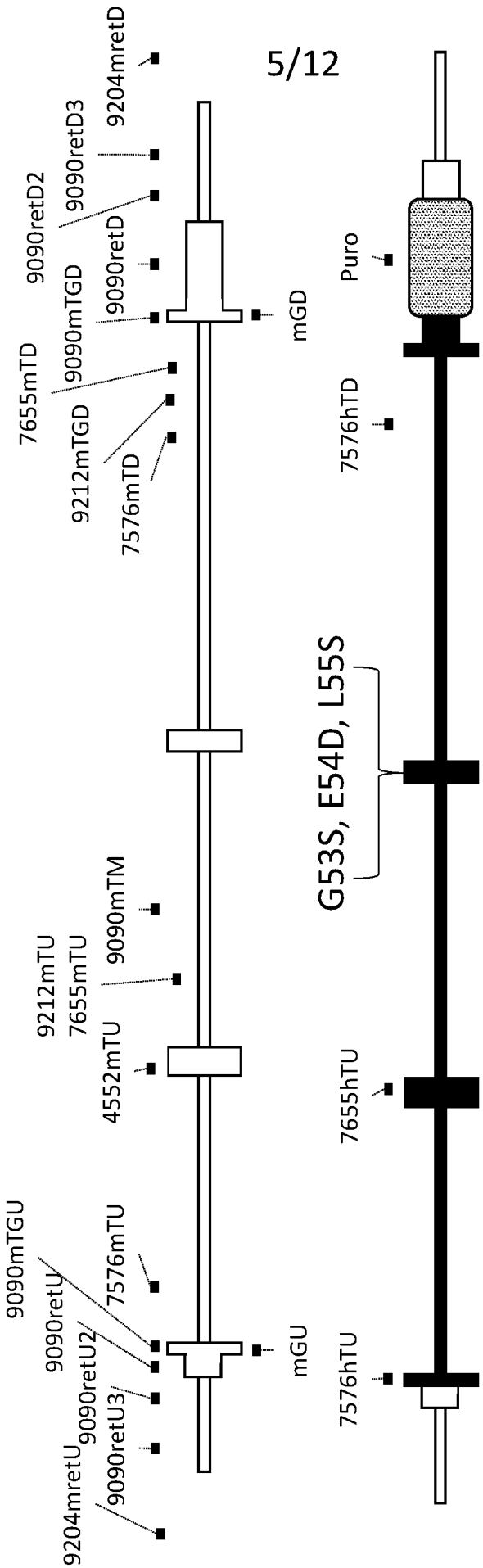

9204mretD
9090retD2
9090retD3
9090retD
9090mTGD
7655mTD
9212mTGD
7576mTD
mGD
Puro
7576hTD
G53S, E54D, L55S
9212mTU
7655mTU
9090mTM
4552mTU
7655hTU
9090mTGU
9090retU
9090retU2
9090retU3
9204mretU
7576mTU
mGU
7576hTU

FIG. 4

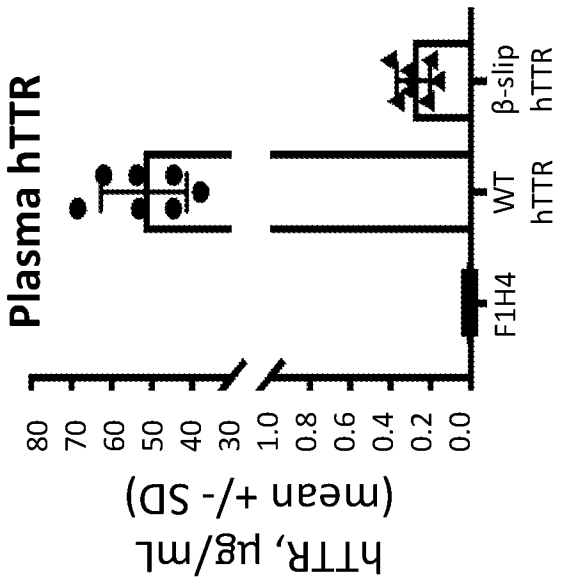


FIG. 5A

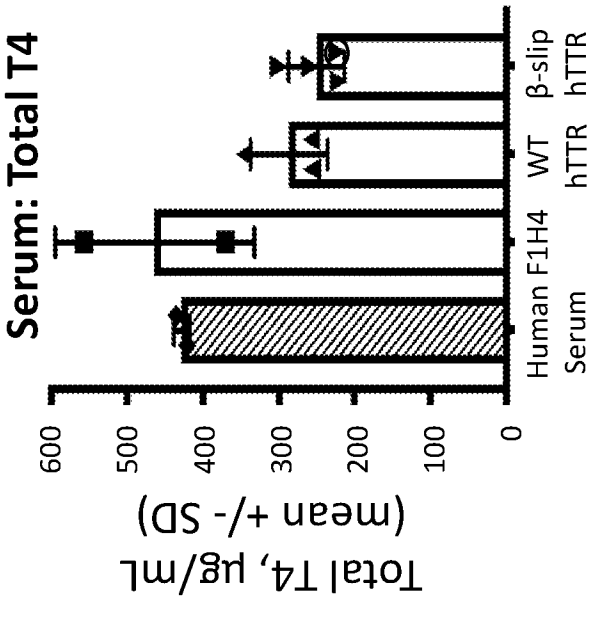


FIG. 5B

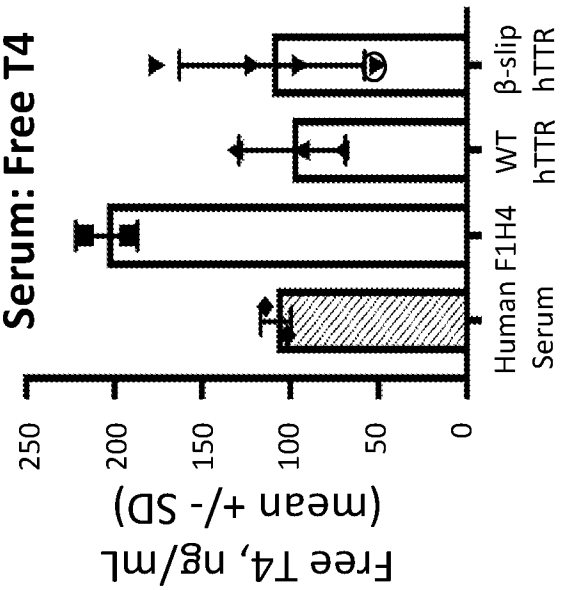


FIG. 5C

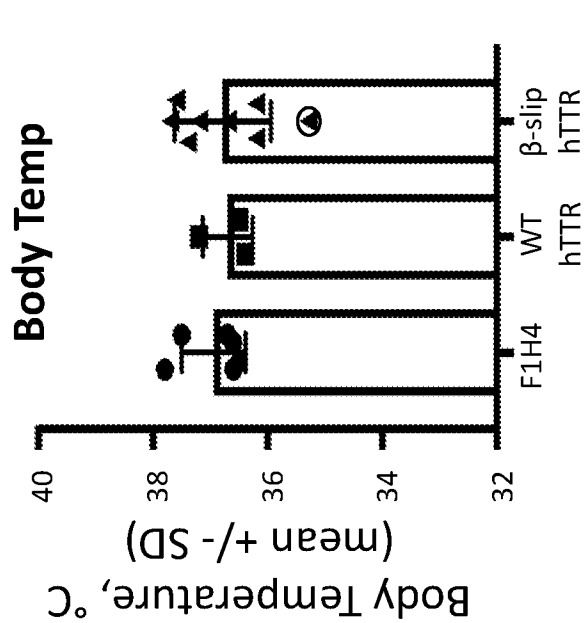


FIG. 5D

or = Dystonic Mouse

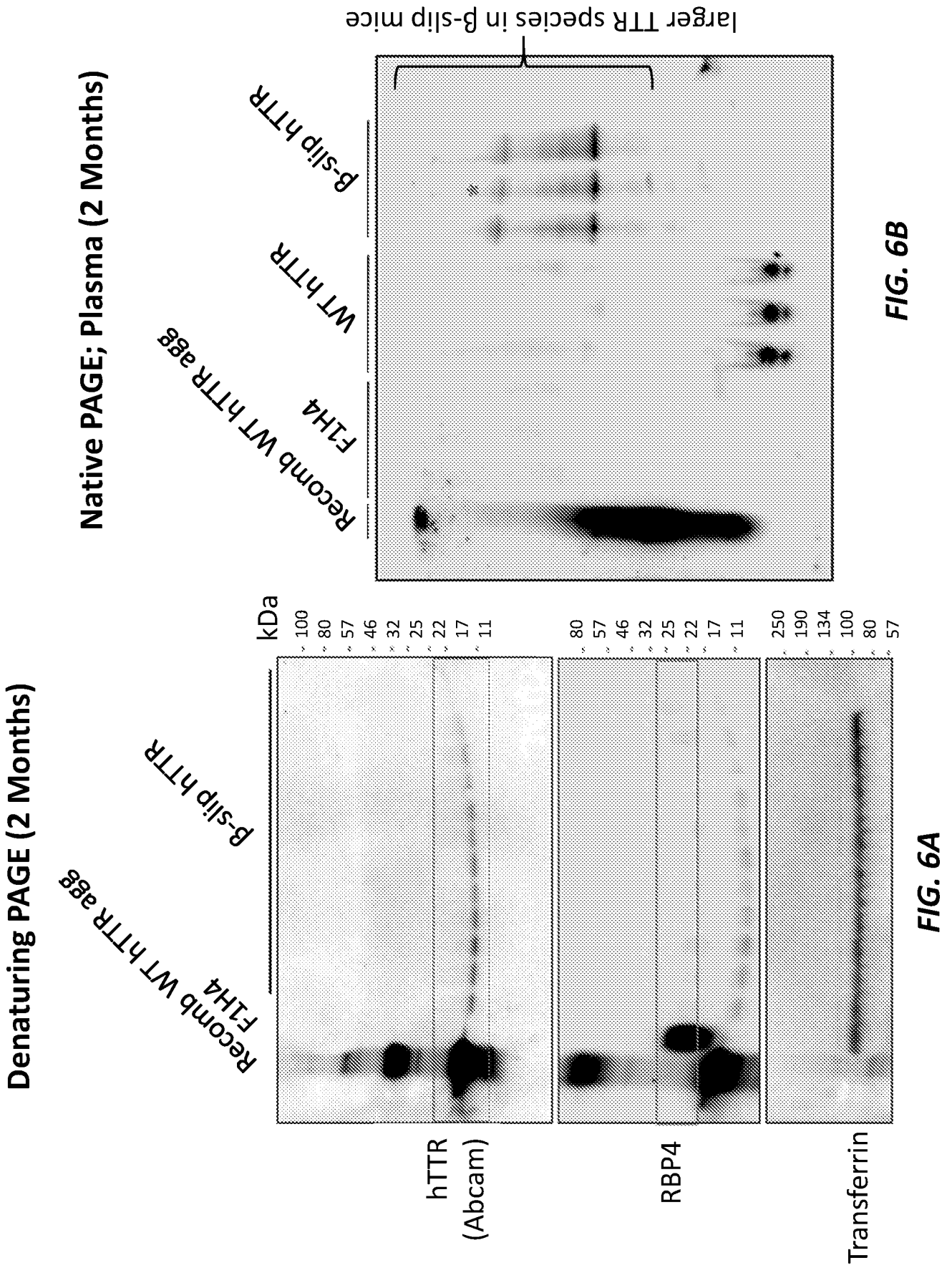

Denaturing PAGE (2 Months)
Recomb WT hTTR abb F1H4
β-slip hTTR
kDa
100
80
57
46
32
25
22
17
11
80
57
46
32
25
22
17
11
250
190
134
100
80
57
hTTR (Abcam)
RBP4
Transferrin
Native PAGE; Plasma (2 Months)
Recomb WT hTTR abb
F1H4
WT hTTR
β-slip hTTR
larger TTR species in β-slip mice

FIG. 6A

FIG. 6B

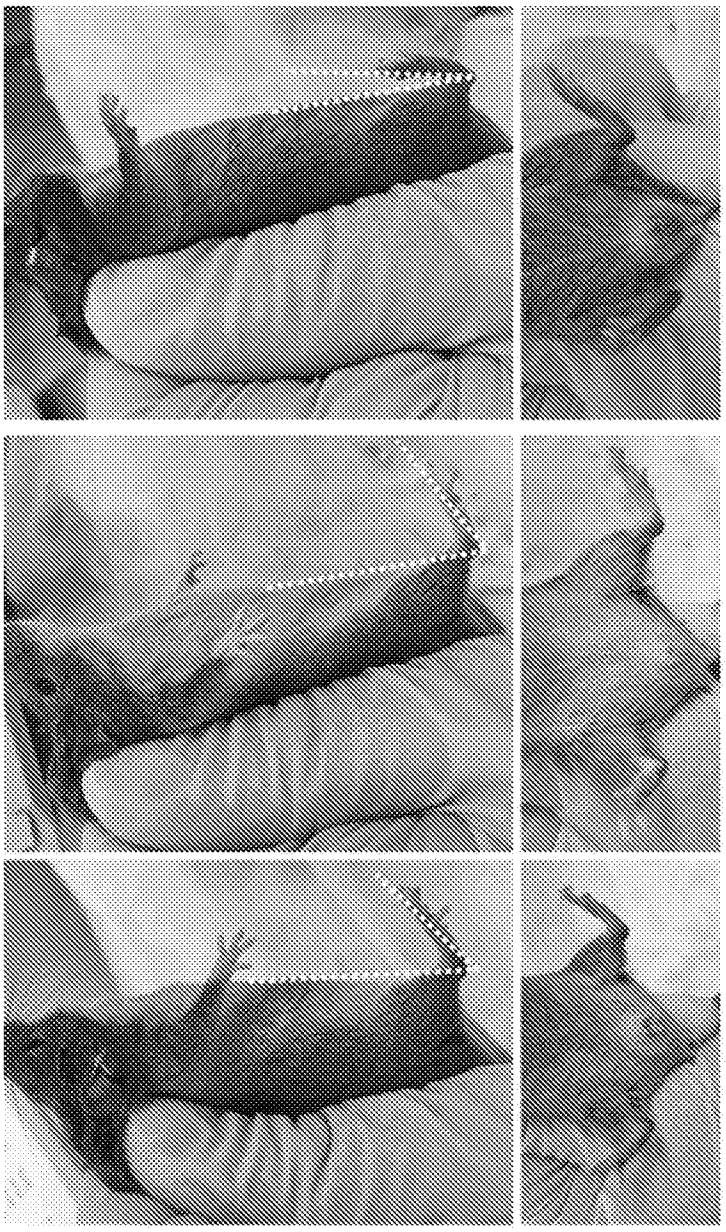

F1H4
TTR WT HumIn
TTR β-slip HumIn

FIG. 7A

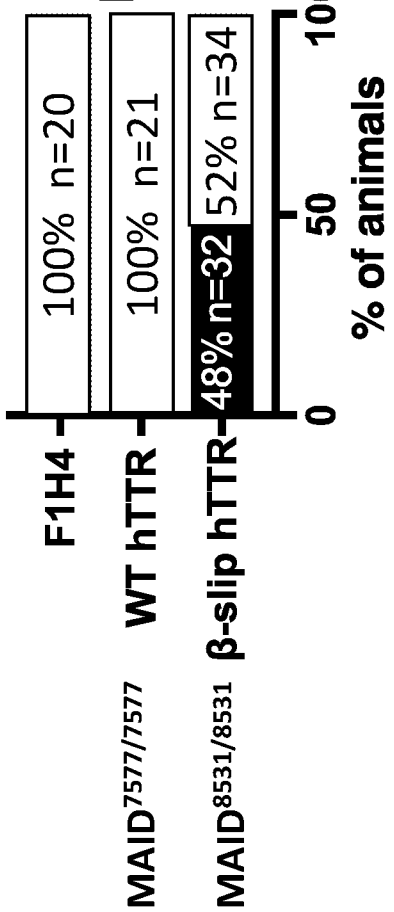

F1H4
WT hTTR
β-slip hTTR
MAID7577/7577
MAID8531/8531
100% n=20
100% n=21
48% n=32
52% n=34
Normal
"Dystonic"
0
50
100
% of animals

FIG. 7B

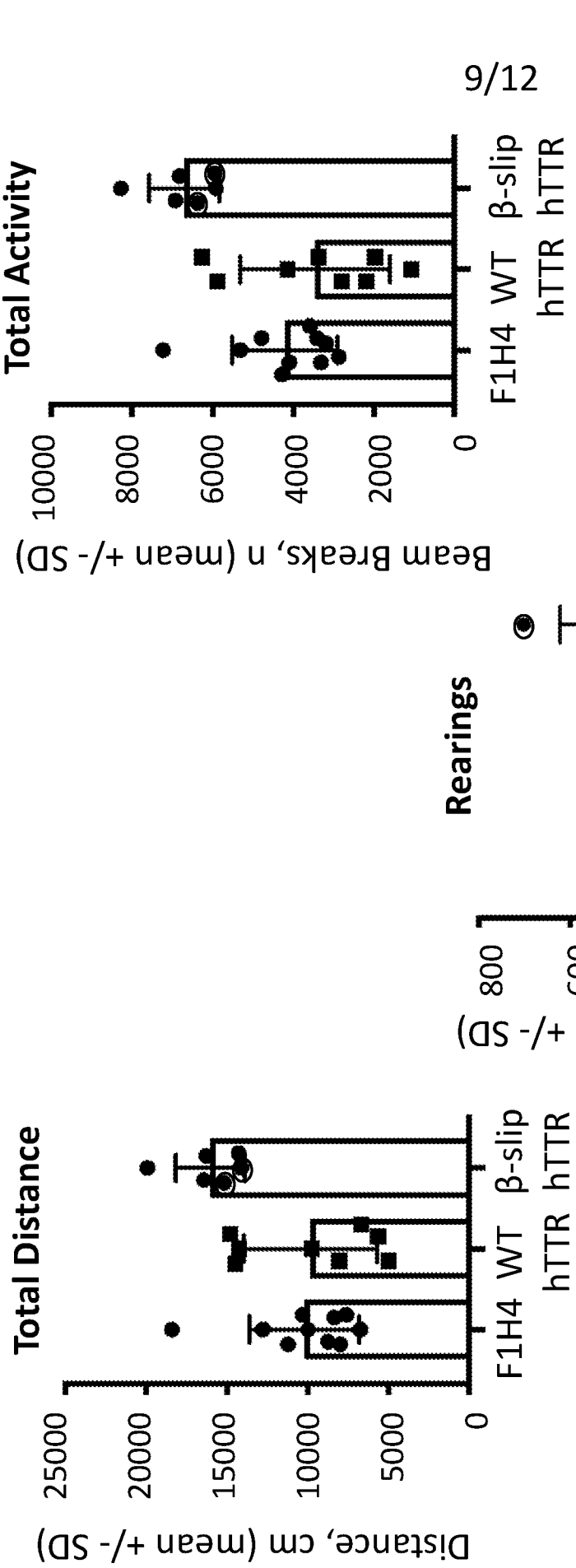


FIG. 8A

FIG. 8B

FIG. 8C

◭or◔ = Dystonic Mouse

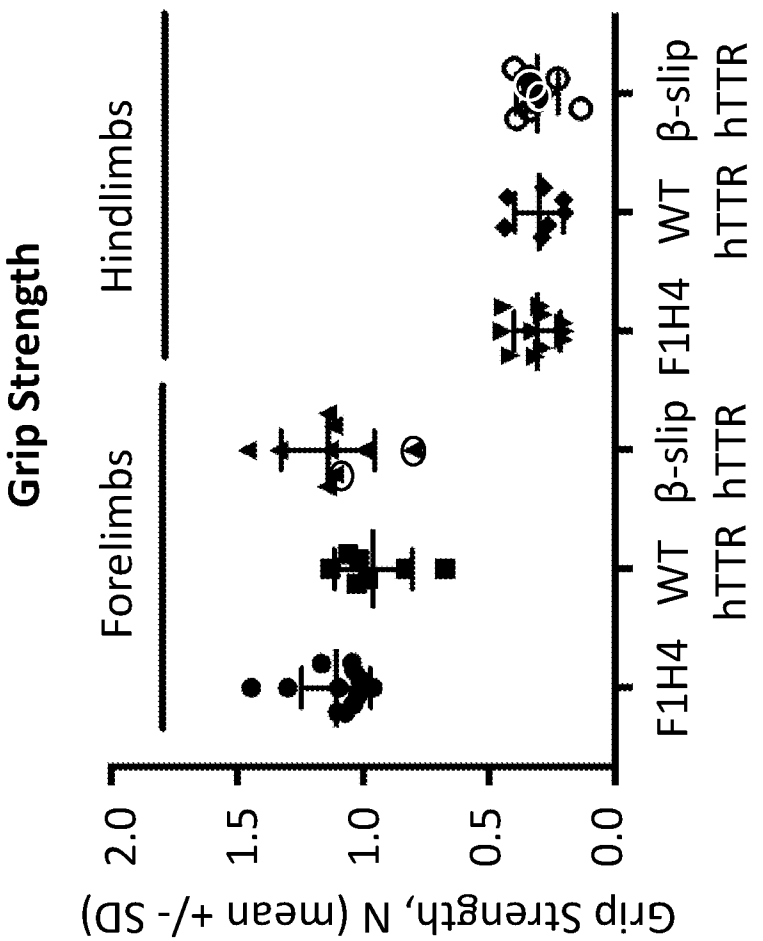

Grip Strength
Forelimbs
Hindlimbs
Grip Strength, N (mean +/- SD)
2.0
1.5
1.0
0.5
0.0
F1H4
WT hTTR
β-slip hTTR
F1H4
WT hTTR
β-slip hTTR
or = Dystonic Mouse

FIG. 9B

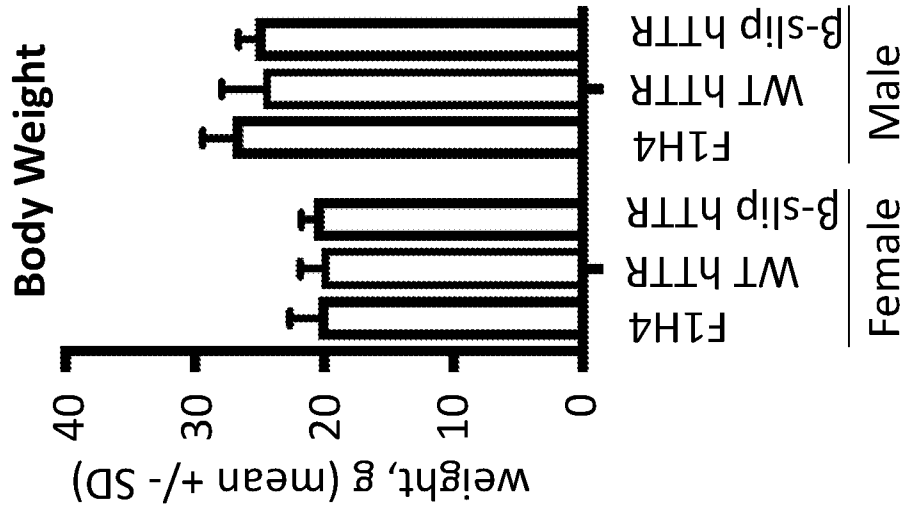

Body Weight
weight, g (mean +/- SD)
40
30
20
10
0
Female
Male
F1H4
WT hTTR
β-slip hTTR
F1H4
WT hTTR
β-slip hTTR

FIG. 9A

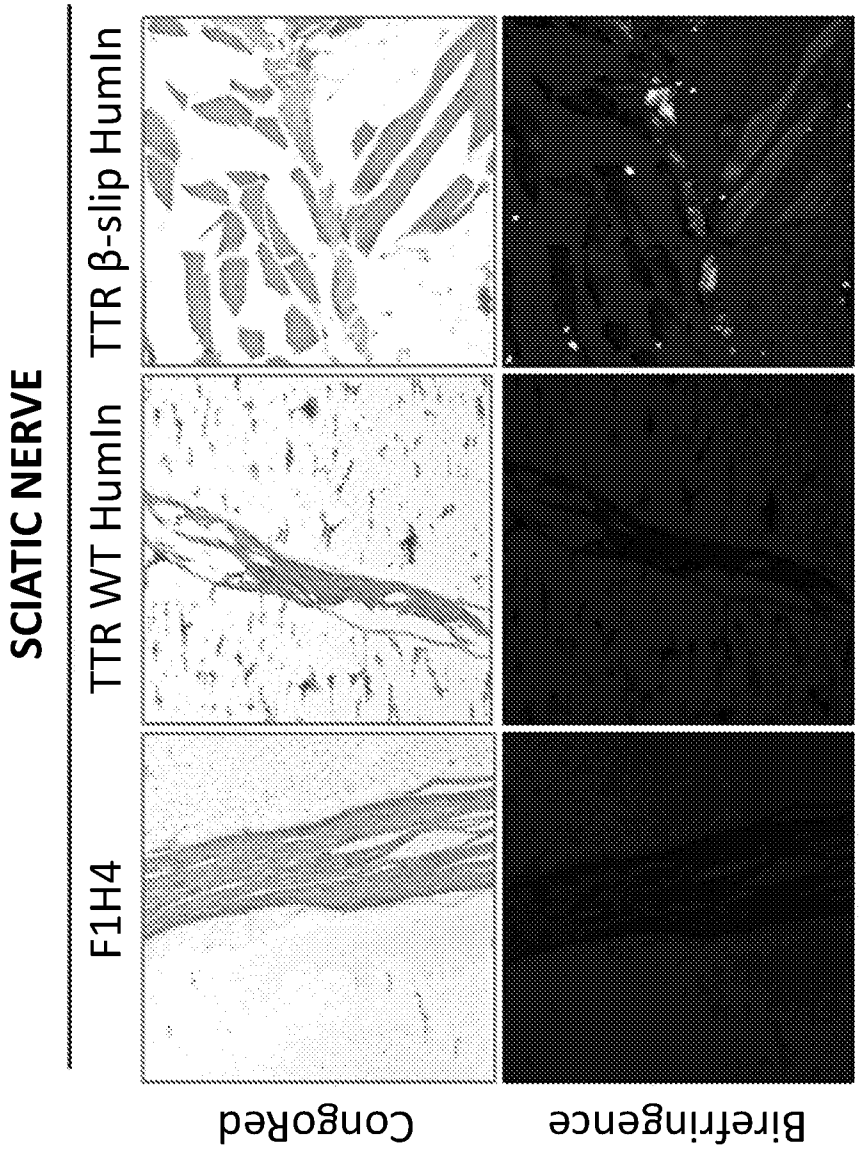

SCIATIC NERVE
F1H4
TTR WT HumIn
TTR β-slip HumIn
CongoRed
Birefringence

FIG. 10A

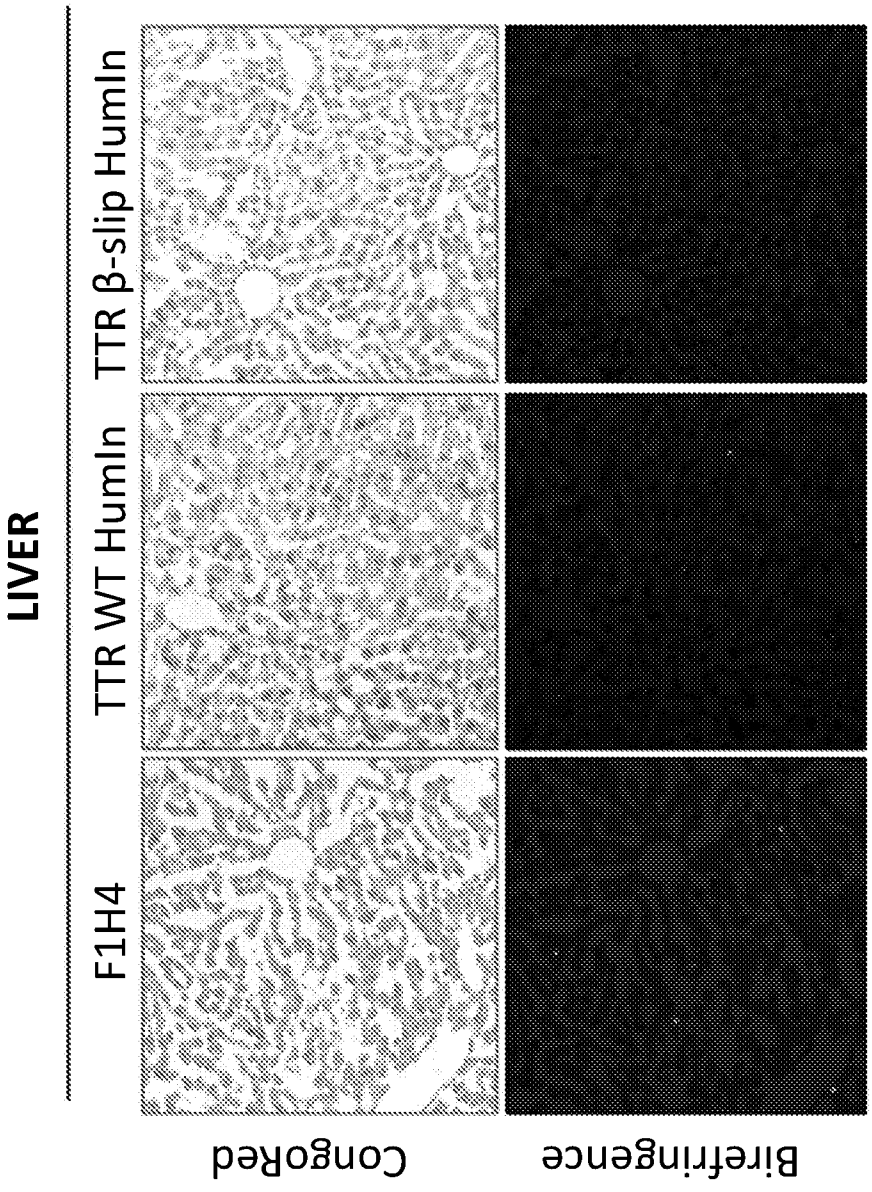

LIVER
F1H4
TTR WT HumIn
TTR β-slip HumIn
CongoRed
Birefringence

FIG. 10B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/035859

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01K67/027 C07K14/47
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	H.-W. KAN ET AL: "Sensory nerve degeneration in a mouse model mimicking early manifestations of familial amyloid polyneuropathy due to transthyretin Ala97Ser", NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY., vol. 44, no. 7, 25 March 2018 (2018-03-25), pages 673-686, XP055726084, GB ISSN: 0305-1846, DOI: 10.1111/nan.12477 abstract; p 674-675; fig 1 ----- -/--	1-64

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 September 2020	17/09/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brero, Alessandro

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI XIANGSHUN ET AL: "Amyloid deposition in a mouse model humanized at the transthyretin and retinol-binding protein 4 loci", LABORATORY INVESTIGATION, NATURE PUBLISHING GROUP, THE UNITED STATES AND CANADIAN ACADEMY OF PATHOLOGY, INC, vol. 98, no. 4, 12 January 2018 (2018-01-12), pages 512-524, XP036492977, ISSN: 0023-6837, DOI: 10.1038/S41374-017-0019-Y [retrieved on 2018-01-12] abstract; p 513-515; fig 1	1-64
A	T Eneqvist ET AL: "The beta-slip: a novel concept in transthyretin amyloidosis", Molecular cell, 1 November 2000 (2000-11-01), pages 1207-1218, XP055726071, United States Retrieved from the Internet: URL:https://reader.elsevier.com/reader/sd/pii/S1097276500001179?token=D82DA0E709CDB249DDD28BFFA88966F82D04F86D51263CE15E5A805056087C1484C643B4153B189A210680BF2584A262 cited in the application the whole document	1-64
A	MEI-HUI TENG ET AL: "Amyloid and Nonfibrillar Deposits in Mice Transgenic for Wild-Type Human Transthyretin: A Possible Model for Senile Systemic Amyloidosis", LABORATORY INVESTIGATION, vol. 81, no. 3, 1 March 2001 (2001-03-01), pages 385-396, XP055530441, The United States and Canadian Academy of Pathology, Inc. ISSN: 0023-6837, DOI: 10.1038/labinvest.3780246 abstract; p 386	1-64
A	WO 2019/067872 A1 (INTELLIA THERAPEUTICS INC [US]) 4 April 2019 (2019-04-04) the whole document	1-64
A	WO 2019/067875 A1 (REGENERON PHARMA [US]) 4 April 2019 (2019-04-04) the whole document	1-64

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RIDWAN BABATUNDE IBRAHIM ET AL: "Contributions of Animal Models to the Mechanisms and Therapies of Transthyretin Amyloidosis", FRONTIERS IN PHYSIOLOGY, vol. 10, 2 April 2019 (2019-04-02), XP055726074, DOI: 10.3389/fphys.2019.00338 the whole document	1-64
A	BUXBAUM ET AL: "Animal models of human amyloidoses: Are transgenic mice worth the time and trouble?", FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 583, no. 16, 20 August 2009 (2009-08-20), pages 2663-2673, XP026524939, ISSN: 0014-5793, DOI: 10.1016/J.FEBSLET.2009.07.031 [retrieved on 2009-07-20] the whole document	1-64

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2020/035859

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2019067872 A1	04-04-2019	AU 2018338787 A1	16-04-2020
		CA 3077251 A1	04-04-2019
		CN 111417728 A	14-07-2020
		CO 2020005116 A2	15-05-2020
		EP 3688161 A1	05-08-2020
		KR 20200058509 A	27-05-2020
		SG 11202002565Y A	29-04-2020
		TW 201932479 A	16-08-2019
		US 2020248180 A1	06-08-2020
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