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Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

(54) **Title:** CRISPR-BASED THERAPEUTICS FOR C9ORF72 REPEAT EXPANSION DISEASE

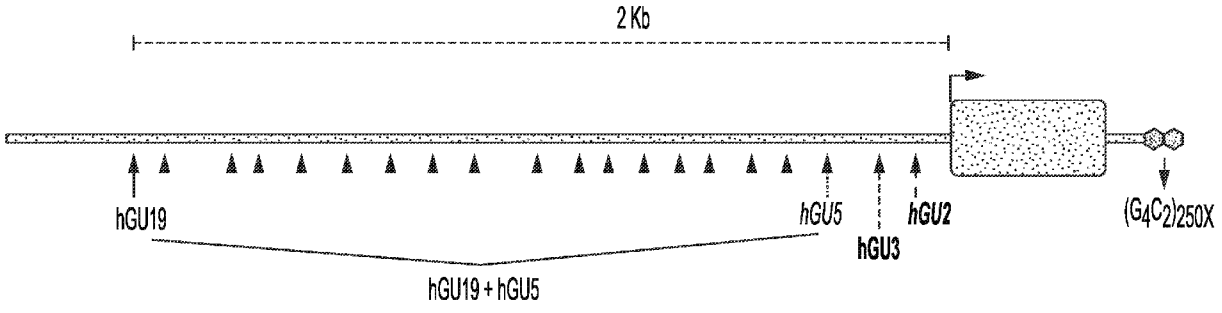

FIG. 15

(57) **Abstract:** Nuclease agents, including guide RNAs and CRISPR/Cas systems, targeting a *C9orf72* gene, lipid nanoparticles or viral vectors comprising such nuclease agents, and cells or animals comprising such nuclease agents are provided. Methods of modifying a *C9orf72* gene using the nuclease agents are also provided, as well as use of the nuclease agents in prophylactic and therapeutic applications for treatment and/or prevention of a *C9orf72* hexanucleotide repeat expansion associated disease and/or for ameliorating at least one symptom associated with such disease.

[Continued on next page]

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CRISPR-BASED THERAPEUTICS FOR C9ORF72 REPEAT EXPANSION DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of US Application No. 63/365,557, filed May 31, 2022, which is herein incorporated by reference in its entirety for all purposes.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS AN XML FILE VIA EFS WEB

[0002] The Sequence Listing written in file 057766-596445.xml is 179 kilobytes, was created on May 30, 2023, and is hereby incorporated by reference.

BACKGROUND

[0003] Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are progressive and fatal neurodegenerative diseases that cause motor neuron disease in the case of ALS and dementia in the case of FTLD. The most common cause of familial ALS is an expansion of a GGGGCC (G_4C_2) hexanucleotide repeat between two alternative 5' non-coding exons of the *C9orf72* gene. Normal individuals have between 3 and 35 G_4C_2 repeats, while ALS or FTLD patients harbor repeat numbers in the hundreds or thousands. The physiological function of the C9orf72 protein is not well understood, and no disease-causing mutations have been identified in its coding sequence. There are no effective cures currently available.

SUMMARY

[0004] Provided are nuclease agents, including guide RNAs and CRISPR/Cas systems, targeting a *C9orf72* gene, lipid nanoparticles or viral vectors comprising such nuclease agents, and cells or animals comprising such nuclease agents. Also provided are methods of modifying a *C9orf72* gene using the nuclease agents, as well as use of the nuclease agents in prophylactic and therapeutic applications for treatment and/or prevention of a *C9orf72* hexanucleotide repeat expansion associated disease and/or for ameliorating at least one symptom associated with such disease.

[0005] In one aspect, provided are methods of modifying a *C9orf72* gene in a cell. Some such methods comprise contacting the *C9orf72* gene with a first nuclease agent that targets a first

nuclease target sequence near the *C9orf72* exon 1A transcription start site, wherein the first nuclease agent cleaves the first nuclease target sequence to generate a targeted genetic modification in the *C9orf72* gene. Some such methods comprise contacting the *C9orf72* gene with a first nuclease agent that targets a first nuclease target sequence upstream of the *C9orf72* exon 1A transcription start site, wherein the first nuclease agent cleaves the first nuclease target sequence to generate a targeted genetic modification in the *C9orf72* gene. In some such methods, the *C9orf72* gene comprises a *C9orf72* hexanucleotide repeat expansion sequence between the first non-coding endogenous exon and exon 2 of the *C9orf72* gene, wherein the *C9orf72* hexanucleotide repeat expansion sequence has more than 30, more than 100, more than 200, more than 300, more than 400, or more than 500 repeats of the hexanucleotide sequence G_4C_2.

[0006] In some such methods, the first nuclease target sequence is within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. In some such methods, the first nuclease target sequence is within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[0007] In some such methods, the targeted genetic modification comprises a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. In some such methods, the targeted genetic modification comprises a deletion within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[0008] In some such methods, the targeted genetic modification comprises a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. In some such methods, the targeted genetic modification comprises a deletion within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site.

[0009] In some such methods, the targeted genetic modification comprises a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site. In some such methods, the targeted genetic modification comprises a deletion within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site.

[0010] In some such methods, the targeted genetic modification comprises a deletion of a

region of the *C9orf72* promoter. In some such methods, the deletion encompasses the *C9orf72* exon 1A transcription start site. In some such methods, the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In some such methods, the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A. In some such methods, the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such methods, the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts. In some such methods, the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such methods, the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. In some such methods, the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such methods, the targeted genetic modification reduces expression of polyGA dipeptide repeat proteins. In some such methods, the targeted genetic modification reduces expression of both polyGA and polyGP dipeptide repeat proteins.

[0011] In some such methods, the method comprises introducing the first nuclease agent or one or more nucleic acids encoding the first nuclease agent into the cell. In some such methods, the method further comprises contacting the *C9orf72* gene with a second nuclease agent that targets a second nuclease target sequence in the *C9orf72* gene, wherein the second nuclease agent cleaves the second nuclease target sequence. In some such methods, the targeted genetic modification comprises a deletion between the first nuclease target sequence and the second nuclease target sequence. In some such methods, the second nuclease target sequence is upstream of the *C9orf72* exon 1A transcription start site. In some such methods, the second nuclease target sequence is downstream of the *C9orf72* exon 1A transcription start site. In some such methods, the second nuclease target sequence is within 2500, within 2250, within 2000, within 1800, within 1600, within 1400, within 1200, within 1000, within 900, within 800, within 700, within 600, within 500, within 450, within 400, within 350, within 300, within 250, within

225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[0012] In some such methods, the method further comprises contacting the *C9orf72* gene with an exogenous donor nucleic acid for modification of the *C9orf72* gene. In some such methods, the exogenous donor nucleic acid comprises homology arms. In some such methods, the exogenous donor nucleic acid is between about 50 nucleotides to about 1 kb in length, optionally wherein the exogenous donor sequence is between about 80 nucleotides to about 200 nucleotides in length; and/or the exogenous donor nucleic acid is a single-stranded oligodeoxynucleotide.

[0013] In some such methods, the nuclease agent comprises: (a) a zinc finger nuclease (ZFN); (b) a transcription activator-like effector nuclease (TALEN); or (c) (i) a Cas protein; and (ii) a guide RNA, wherein the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence that is the nuclease target sequence, and wherein the guide RNA binds to the Cas protein and targets the Cas protein to the guide RNA target sequence. In some such methods, the nuclease agent comprises: (a) a Cas protein; and (b) a guide RNA, wherein the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence that is the nuclease target sequence, and wherein the guide RNA binds to the Cas protein and targets the Cas protein to the guide RNA target sequence. In some such methods, the method comprises introducing into the cell: (a) the Cas protein or a nucleic acid encoding the Cas protein; and (b) the guide RNA or one or more DNAs encoding the guide RNA. In some such methods, the guide RNA is a single guide RNA (sgRNA). In some such methods, the Cas protein is a Cas9 protein. In some such methods, the Cas9 protein is derived from a *Streptococcus pyogenes* Cas9 protein, a *Staphylococcus aureus* Cas9 protein, a *Campylobacter jejuni* Cas9 protein, a *Streptococcus thermophilus* Cas9 protein, or a *Neisseria meningitidis* Cas9 protein. In some such methods, the Cas protein is derived from a *Streptococcus pyogenes* Cas9 protein. In some such methods, the nucleic acid encoding the Cas protein is codon-optimized for expression in a mammalian cell or a human cell. In some such methods, the method comprises introducing the guide RNA in the form of RNA, optionally wherein the guide RNA comprises at least one modification. In some such methods, the at least one modification comprises a 2'-O-methyl-modified nucleotide and/or a phosphorothioate bond between nucleotides. In some such methods, the method comprises introducing the nucleic acid encoding the Cas protein, wherein the nucleic acid comprises an

mRNA encoding the Cas protein, optionally wherein the mRNA encoding the Cas protein comprises at least one modification. In some such methods, the method comprises introducing the nucleic acid encoding the Cas protein and the one or more DNAs encoding the guide RNA, wherein the nucleic acid encoding the Cas protein comprises DNA. In some such methods, the DNA encoding the Cas protein and the one or more DNAs encoding the guide RNA are in one or more vectors. In some such methods, the one or more vectors are one or more viral vectors. In some such methods, the one or more viral vectors are one or more adeno-associated virus (AAV) vectors. In some such methods, the Cas protein or the nucleic acid encoding the Cas protein and the guide RNA or the one or more DNAs encoding the guide RNA are associated with a lipid nanoparticle.

[0014] In some such methods, the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96. In some such methods, the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96. In some such methods, the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 33-71 or 34-71, optionally wherein guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 34-36, 53, 54, and 56. In some such methods, the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96. In some such methods, the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96. In some such methods, the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, and 56. In some such methods, the method further comprises contacting the *C9orf72* gene with a second nuclease agent that targets a second nuclease target sequence in the *C9orf72* gene, wherein the second nuclease agent cleaves the second nuclease target sequence,

wherein the second nuclease agent comprises the Cas protein and aa second guide RNA, wherein the second guide RNA comprises a second DNA-targeting segment that targets a second guide RNA target sequence that is the second nuclease target sequence, wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence, and wherein: (I) the second DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or (II) the second DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or (III) the second guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, 56, and 132.

[0015] In some such methods, the cell is a neuron, optionally wherein the neuron is a motor neuron. In some such methods, the cell is *in vitro* or *ex vivo*. In some such methods, the cell is in a subject *in vivo*, optionally wherein the subject is a human. In some such methods, the cell is a neuron in the brain of the subject. In some such methods, the subject has or is at risk for developing a *C9orf72* hexanucleotide repeat expansion associated disease. In some such methods, the *C9orf72* hexanucleotide repeat expansion associated disease is amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD). In some such methods, the first nuclease agent or one or more nucleic acids encoding the first nuclease agent are administered to the subject by intracerebroventricular injection, intracranial injection, or intrathecal injection. In some such methods, the cell is a mammalian cell, and the *C9orf72* gene is a mammalian *C9orf72* gene. In some such methods, the cell is a human cell. In some such methods, the cell is a mouse cell. In some such methods, the *C9orf72* gene comprises a human *C9orf72* promoter. In some such methods, the *C9orf72* gene is a human *C9orf72* gene or a humanized *C9orf72* gene.

[0016] In another aspect, provided are methods of modifying a *C9orf72* gene in a subject. Some such methods comprise administering to the subject a first nuclease agent or one or more nucleic acids encoding the first nuclease agent, wherein the first nuclease agent targets a first nuclease target sequence near the *C9orf72* exon 1A transcription start site, wherein the first nuclease agent cleaves the first nuclease target sequence to generate a targeted genetic modification in the *C9orf72* gene. Some such methods comprise administering to the subject a first nuclease agent or one or more nucleic acids encoding the first nuclease agent, wherein the first nuclease agent targets a first nuclease target sequence upstream of the *C9orf72* exon 1A

transcription start site, wherein the first nuclease agent cleaves the first nuclease target sequence to generate a targeted genetic modification in the *C9orf72* gene. In another aspect, provided are methods of preventing, treating, or ameliorating at least one symptom or indication of a *C9orf72* hexanucleotide repeat expansion associated disease. Some such methods comprise administering to a subject in need thereof a pharmaceutical composition comprising a therapeutically effective amount of a first nuclease agent or one or more nucleic acids encoding the first nuclease agent, wherein the first nuclease agent targets a first nuclease target sequence near the *C9orf72* exon 1A transcription start site, wherein the first nuclease agent cleaves the first nuclease target sequence to generate a targeted genetic modification in the *C9orf72* gene. Some such methods comprise administering to a subject in need thereof a pharmaceutical composition comprising a therapeutically effective amount of a first nuclease agent or one or more nucleic acids encoding the first nuclease agent, wherein the first nuclease agent targets a first nuclease target sequence upstream of the *C9orf72* exon 1A transcription start site, wherein the first nuclease agent cleaves the first nuclease target sequence to generate a targeted genetic modification in the *C9orf72* gene. In some such methods, the *C9orf72* hexanucleotide repeat expansion associated disease is amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD). In some such methods, the *C9orf72* gene comprises a *C9orf72* hexanucleotide repeat expansion sequence between the first non-coding endogenous exon and exon 2 of the *C9orf72* gene, wherein the *C9orf72* hexanucleotide repeat expansion sequence has more than 30, more than 100, more than 200, more than 300, more than 400, or more than 500 repeats of the hexanucleotide sequence G_4C_2. In some such methods, the first nuclease agent or one or more nucleic acids encoding the first nuclease agent are administered to the subject by intracerebroventricular injection, intracranial injection, or intrathecal injection.

[0017] In some such methods, the first nuclease target sequence is within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. In some such methods, the first nuclease target sequence is within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[0018] In some such methods, the targeted genetic modification comprises a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. In some such methods, the

targeted genetic modification comprises a deletion within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[0019] In some such methods, the targeted genetic modification comprises a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. In some such methods, the targeted genetic modification comprises a deletion within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site.

[0020] In some such methods, the targeted genetic modification comprises a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site. In some such methods, the targeted genetic modification comprises a deletion within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site.

[0021] In some such methods, the targeted genetic modification comprises a deletion of a region of the *C9orf72* promoter. In some such methods, the deletion encompasses the *C9orf72* exon 1A transcription start site. In some such methods, the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In some such methods, the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A. In some such methods, the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such methods, the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts. In some such methods, the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such methods, the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. In some such methods, the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such methods, the targeted genetic modification reduces expression of polyGA dipeptide repeat proteins. In some such methods, the targeted genetic modification reduces expression of both polyGA and polyGP dipeptide repeat

proteins.

[0022] In some such methods, the method further comprises administering to the subject a second nuclease agent or one or more nucleic acids encoding the second nuclease agent, wherein the second nuclease agent targets a second nuclease target sequence in the *C9orf72* gene, wherein the second nuclease agent cleaves the second nuclease target sequence. In some such methods, the targeted genetic modification comprises a deletion between the first nuclease target sequence and the second nuclease target sequence. In some such methods, the second nuclease target sequence is upstream of the *C9orf72* exon 1A transcription start site. In some such methods, the second nuclease target sequence is downstream of the *C9orf72* exon 1A transcription start site. In some such methods, the second nuclease target sequence is within 2500, within 2250, within 2000, within 1800, within 1600, within 1400, within 1200, within 1000, within 900, within 800, within 700, within 600, within 500, within 450, within 400, within 350, within 300, within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[0023] In some such methods, the method further comprises administering to the subject an exogenous donor nucleic acid for modification of the *C9orf72* gene. In some such methods, the exogenous donor nucleic acid comprises homology arms. In some such methods, (i) the exogenous donor nucleic acid is between about 50 nucleotides to about 1 kb in length, optionally wherein the exogenous donor sequence is between about 80 nucleotides to about 200 nucleotides in length; and/or (ii) the exogenous donor nucleic acid is a single-stranded oligodeoxynucleotide.

[0024] In some such methods, the nuclease agent comprises: (a) a zinc finger nuclease (ZFN); (b) a transcription activator-like effector nuclease (TALEN); or (c) (i) a Cas protein; and (ii) a guide RNA, wherein the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence that is the nuclease target sequence, and wherein the guide RNA binds to the Cas protein and targets the Cas protein to the guide RNA target sequence. In some such methods, the nuclease agent comprises: (a) a Cas protein; and (b) a guide RNA, wherein the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence that is the nuclease target sequence, and wherein the guide RNA binds to the Cas protein and targets the Cas protein to the guide RNA target sequence. In some such methods, the guide RNA is a single guide RNA (sgRNA). In some such methods, the Cas protein is a Cas9 protein. In some such methods, the Cas9 protein is derived from a *Streptococcus pyogenes* Cas9 protein, a

Staphylococcus aureus Cas9 protein, a *Campylobacter jejuni* Cas9 protein, a *Streptococcus thermophilus* Cas9 protein, or a *Neisseria meningitidis* Cas9 protein. In some such methods, the Cas protein is derived from a *Streptococcus pyogenes* Cas9 protein. In some such methods, the nucleic acid encoding the Cas protein is codon-optimized for expression in a mammalian cell or a human cell. In some such methods, the method comprises administering to the subject the guide RNA in the form of RNA, optionally wherein the guide RNA comprises at least one modification. In some such methods, the at least one modification comprises a 2'-O-methyl-modified nucleotide and/or a phosphorothioate bond between nucleotides. In some such methods, the method comprises administering to the subject the nucleic acid encoding the Cas protein, wherein the nucleic acid comprises an mRNA encoding the Cas protein, optionally wherein the mRNA encoding the Cas protein comprises at least one modification. In some such methods, the method comprises administering to the subject the nucleic acid encoding the Cas protein and the one or more DNAs encoding the guide RNA, wherein the nucleic acid encoding the Cas protein comprises DNA. In some such methods, the DNA encoding the Cas protein and the one or more DNAs encoding the guide RNA are in one or more vectors. In some such methods, the one or more vectors are one or more viral vectors. In some such methods, the one or more viral vectors are one or more adeno-associated virus (AAV) vectors. In some such methods, the Cas protein or the nucleic acid encoding the Cas protein and the guide RNA or the one or more DNAs encoding the guide RNA are associated with a lipid nanoparticle.

[0025] In some such methods, the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96. In some such methods, the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96. In some such methods, the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 33-71 or 34-71, optionally wherein guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS:

34-36, 53, 54, and 56. In some such methods, the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96. In some such methods, the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96. In some such methods, the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, and 56. In some such methods, the method further comprises contacting the *C9orf72* gene with a second nuclease agent that targets a second nuclease target sequence in the *C9orf72* gene, wherein the second nuclease agent cleaves the second nuclease target sequence, wherein the second nuclease agent comprises the Cas protein and aa second guide RNA, wherein the second guide RNA comprises a second DNA-targeting segment that targets a second guide RNA target sequence that is the second nuclease target sequence, wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence, and wherein: (I) the second DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or (II) the second DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or (III) the second guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, 56, and 132.

[0026] In some such methods, the targeted genetic modification in the *C9orf72* gene is generated in neurons in the subject, optionally wherein the neurons are motor neurons. In some such methods, the neurons are in the brain of the subject. In some such methods, the subject is a mammalian subject, and the *C9orf72* gene is a mammalian *C9orf72* gene. In some such methods, the subject is a human subject. In some such methods, the subject is a mouse subject. In some such methods, the *C9orf72* gene comprises a human *C9orf72* promoter. In some such methods, the *C9orf72* gene is a human *C9orf72* gene or a humanized *C9orf72* gene.

[0027] In another aspect, provided are CRISPR/Cas systems. Some such systems comprise a first guide RNA or one or more DNAs encoding the first guide RNA, wherein the first guide RNA comprises a DNA-targeting segment that targets a first guide RNA target sequence in a *C9orf72* gene, wherein the first guide RNA target sequence is near the *C9orf72* exon 1A transcription start site, and wherein the first guide RNA binds to a Cas protein and targets the

Cas protein to the first guide RNA target sequence. Some such systems comprise a first guide RNA or one or more DNAs encoding the first guide RNA, wherein the first guide RNA comprises a DNA-targeting segment that targets a first guide RNA target sequence in a *C9orf72* gene, wherein the first guide RNA target sequence is upstream of the *C9orf72* exon 1A transcription start site, and wherein the first guide RNA binds to a Cas protein and targets the Cas protein to the first guide RNA target sequence.

[0028] In some such systems, the first guide RNA target sequence is within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. In some such systems, the first guide RNA target sequence is within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[0029] In some such systems, cleavage of the first guide RNA target sequence by the Cas protein results in a deletion of a region of the *C9orf72* promoter. In some such systems, the deletion encompasses the *C9orf72* exon 1A transcription start site. In some such systems, cleavage of the first guide RNA target sequence by the Cas protein does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In some such systems, cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A. In some such systems, cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such systems, cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts. In some such systems, cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such systems, cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. In some such systems, cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such systems, cleavage of the first guide RNA target sequence by the Cas protein

reduces expression of polyGA dipeptide repeat proteins. In some such systems, cleavage of the first guide RNA target sequence by the Cas protein reduces expression of both polyGA and polyGP dipeptide repeat proteins.

[0030] Some such systems further comprise a second guide RNA or one or more DNAs encoding the second guide RNA, wherein the second guide RNA comprises a DNA-targeting segment that targets a second guide RNA target sequence in a *C9orf72* gene, and wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence. In some such systems, cleavage by the Cas protein at the first guide RNA target sequence and cleavage by the Cas protein at the second guide RNA target sequence results a deletion between the first guide RNA target sequence and the second guide RNA target sequence. In some such systems, the second guide RNA target sequence is upstream of the *C9orf72* exon 1A transcription start site. In some such systems, the second guide RNA target sequence is downstream of the *C9orf72* exon 1A transcription start site. In some such systems, the second guide RNA target sequence is within 2500, within 2250, within 2000, within 1800, within 1600, within 1400, within 1200, within 1000, within 900, within 800, within 700, within 600, within 500, within 450, within 400, within 350, within 300, within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[0031] Some such systems further comprise an exogenous donor nucleic acid for modification of the *C9orf72* gene. In some such systems, the exogenous donor nucleic acid comprises homology arms. In some such systems, (i) the exogenous donor nucleic acid is between about 50 nucleotides to about 1 kb in length, optionally wherein the exogenous donor sequence is between about 80 nucleotides to about 200 nucleotides in length; and/or (ii) the exogenous donor nucleic acid is a single-stranded oligodeoxynucleotide.

[0032] In some such systems, the first guide RNA is a single guide RNA (sgRNA). In some such systems, the CRISPR/Cas system comprises the first guide RNA in the form of RNA, optionally wherein the first guide RNA comprises at least one modification. In some such systems, the at least one modification comprises a 2'-O-methyl-modified nucleotide and/or a phosphorothioate bond between nucleotides.

[0033] In some such systems, the first DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ

ID NOS: 73-111 or 74-111, optionally wherein the first DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96. In some such systems, the first DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the first DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96. In some such systems, the first guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 33-71 or 34-71, optionally wherein first guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 34-36, 53, 54, and 56. In some such systems, the first DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96. In some such systems, the first DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96. In some such systems, the first guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, and 56. Some such systems further comprise a second guide RNA or one or more DNAs encoding the second guide RNA, wherein the second guide RNA comprises a DNA-targeting segment that targets a second guide RNA target sequence in a *C9orf72* gene, and wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence, and wherein: (I) the second DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or (II) the second DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or (III) the second guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, 56, and 132.

[0034] In some such systems, the Cas protein is a Cas9 protein. In some such systems, the Cas9 protein is derived from a *Streptococcus pyogenes* Cas9 protein, a *Staphylococcus aureus* Cas9 protein, a *Campylobacter jejuni* Cas9 protein, a *Streptococcus thermophilus* Cas9 protein, or a *Neisseria meningitidis* Cas9 protein. In some such systems, the Cas protein is derived from a

Streptococcus pyogenes Cas9 protein. Some such systems further comprise the Cas protein or a nucleic acid encoding the Cas protein. In some such systems, the nucleic acid encoding the Cas protein is codon-optimized for expression in a mammalian cell or a human cell. In some such systems, the CRISPR/Cas system comprises the nucleic acid encoding the Cas protein, wherein the nucleic acid comprises an mRNA encoding the Cas protein, optionally wherein the mRNA encoding the Cas protein comprises at least one modification. In some such systems, the CRISPR/Cas system comprises the nucleic acid encoding the Cas protein and the one or more DNAs encoding the first guide RNA, wherein the nucleic acid encoding the Cas protein comprises DNA. In some such systems, the DNA encoding the Cas protein and the one or more DNAs encoding the first guide RNA are in one or more vectors. In some such systems, the one or more vectors are one or more viral vectors. In some such systems, the one or more viral vectors are one or more adeno-associated virus (AAV) vectors. In some such systems, the Cas protein or the nucleic acid encoding the Cas protein and the first guide RNA or the one or more DNAs encoding the first guide RNA are associated with a lipid nanoparticle.

[0035] In some such systems, the *C9orf72* gene is a mammalian *C9orf72* gene. In some such systems, the *C9orf72* gene comprises a human *C9orf72* promoter. In some such systems, the *C9orf72* gene is a human *C9orf72* gene or a humanized *C9orf72* gene.

[0036] In another aspect, provided are pharmaceutical compositions comprising any of the above CRISPR/Cas systems and a pharmaceutically acceptable carrier.

[0037] In another aspect, provided are compositions comprising a guide RNA or one or more DNAs encoding the guide RNA. In some such compositions, the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence in a *C9orf72* gene, wherein the guide RNA target sequence is near the *C9orf72* exon 1A transcription start site, and wherein the guide RNA can bind to a Cas protein and target the Cas protein to the guide RNA target sequence. In some such compositions, the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence in a *C9orf72* gene, wherein the guide RNA target sequence is upstream of the *C9orf72* exon 1A transcription start site, and wherein the guide RNA can bind to a Cas protein and target the Cas protein to the guide RNA target sequence.

[0038] In some such compositions, the guide RNA target sequence is within 2500, within 2250, within 2000, within 1800, within 1600, within 1400, within 1200, within 1000, within 900, within 800, within 700, within 600, within 500, within 450, within 400, within 350, within 300,

within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. In some such compositions, the guide RNA target sequence is within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. In some such compositions, the guide RNA target sequence is within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[0039] In some such compositions, cleavage of the guide RNA target sequence by the Cas protein results in a deletion of a region of the *C9orf72* promoter. In some such compositions, the deletion encompasses the *C9orf72* exon 1A transcription start site. In some such compositions, cleavage of the guide RNA target sequence by the Cas protein does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In some such compositions, cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A. In some such compositions, cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such compositions, cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts. In some such compositions, cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such compositions, cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. In some such compositions, cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some such compositions, cleavage of the first guide RNA target sequence by the Cas protein reduces expression of polyGA dipeptide repeat proteins. In some such compositions, cleavage of the first guide RNA target sequence by the Cas protein reduces expression of both polyGA and polyGP dipeptide repeat proteins.

[0040] In some such compositions, the guide RNA is a single guide RNA (sgRNA). In some such compositions, the composition comprises the guide RNA in the form of RNA, optionally

wherein the guide RNA comprises at least one modification. In some such compositions, the at least one modification comprises a 2'-O-methyl-modified nucleotide and/or a phosphorothioate bond between nucleotides.

[0041] In some such compositions, the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96. In some such compositions, the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96. In some such compositions, the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 33-71 or 34-71, optionally wherein guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 34-36, 53, 54, and 56. In some such compositions, the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96. In some such compositions, the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96. In some such compositions, the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, and 56. In some such compositions, the composition further comprises a second guide RNA or one or more DNAs encoding the second guide RNA, wherein the second guide RNA comprises a DNA-targeting segment that targets a second guide RNA target sequence in a *C9orf72* gene, and wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence, and wherein: (I) the second DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or (II) the second DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or (III) the second guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20

contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, 56, and 132.

[0042] In some such compositions, the Cas protein is a Cas9 protein. In some such compositions, the Cas9 protein is derived from a *Streptococcus pyogenes* Cas9 protein, a *Staphylococcus aureus* Cas9 protein, a *Campylobacter jejuni* Cas9 protein, a *Streptococcus thermophilus* Cas9 protein, or a *Neisseria meningitidis* Cas9 protein. In some such compositions, the Cas protein is derived from a *Streptococcus pyogenes* Cas9 protein. In some such compositions, the one or more DNAs encoding the guide RNA are in one or more vectors. In some such compositions, the one or more vectors are one or more viral vectors. In some such compositions, the one or more viral vectors are one or more adeno-associated virus (AAV) vectors. In some such compositions, the guide RNA or the one or more DNAs encoding the guide RNA are associated with a lipid nanoparticle.

[0043] In some such compositions, the *C9orf72* gene is a mammalian *C9orf72* gene. In some such compositions, the *C9orf72* gene comprises a human *C9orf72* promoter. In some such compositions, the *C9orf72* gene is a human *C9orf72* gene or a humanized *C9orf72* gene.

[0044] In another aspect, provided are pharmaceutical compositions comprising any of the above compositions and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE FIGURES

[0045] **Figure 1** shows a schematic of RNA synthesis from the *C9orf72* gene in normal and disease states.

[0046] **Figure 2** shows a schematic of a precisely targeted humanized C9orf72 expansion allele that reproduces the molecular hallmarks of amyotrophic lateral sclerosis (ALS).

[0047] **Figure 3** shows a schematic of CRISPR/Cas9-mediated targeting of the potential promoter for exon 1a transcripts of *C9orf72*. Twenty gRNAs distributed across the ~2 kb region upstream of the *C9orf72* exon 1A transcription start site in 300X G_4C_2 repeat containing ES cells were tested.

[0048] **Figures 4A** and **4B** show bar graphs showing expression levels (as determined by the TAQMAN® quantitative reverse transcription-coupled PCR (RT-qPCR) assays shown in the depiction of the *C9orf72* locus at the top of each figure) of transcripts from the *C9orf72* locus (y-axis) that are exon 1A-exon 2 spliced transcripts (**Figure 4A**) or that are exon 1B-exon 2 spliced transcripts (**Figure 4B**) in mouse ES cells that comprise a humanized *C9orf72* locus comprising

300 repeats of the hexanucleotide sequence and a mouse promoter region following treatment with Cas9 and sequence-specific guide RNAs (gRNAs) targeting upstream of the transcription start site for exon 1A. Delta Ct values relative to Drosha are shown. **Figures 4A** and **4B** show that CRISPR/Cas9-mediated ablation of regions upstream of exon 1A reduced transcription from exon 1A (**Figure 4A**) while preserving transcripts initiating at exon 1B (**Figure 4B**) in 300X G_4C_2 repeat containing ES cells. In **Figure 4A**, the ES cell clones are sorted from highest to lowest expression of the exon 1A transcripts. In **Figure 4B**, the ES cell clones are sorted in the same order as in **Figure 4A**.

[0049] **Figure 5** shows a schematic of CRISPR/Cas9-mediated targeting of the potential promoter for exon 1A transcripts of *C9orf72* using a subset of the 20 gRNAs (mGU3, mGU4, mGU5, and mGU20).

[0050] **Figures 6A-6D** show bar graphs showing expression levels (as determined by the TAQMAN® quantitative reverse transcription-coupled PCR (RT-qPCR) assays shown in the depiction of the *C9orf72* locus at the top of each figure) of transcripts from the *C9orf72* locus (y-axis) that are exon 1A-exon 2 spliced transcripts (**Figure 6A**), that are exon 1B-exon 2 spliced transcripts (**Figure 6B**), that are exon 1A-intron-containing transcripts (**Figure 6C**), or that are intron-containing transcriptions (adjacent to G_4C_2 repeats) (**Figure 6D**) in mouse ES cells that comprise a humanized *C9orf72* locus comprising 300 repeats of the hexanucleotide sequence and a mouse promoter region following treatment with Cas9 and sequence-specific guide RNAs (gRNAs) targeting upstream of the transcription start site for exon 1a. Percent expression relative to control mouse ES cells comprising the humanized *C9orf72* locus comprising 300 repeats of the hexanucleotide sequence and the mouse promoter region is shown. **Figures 6A-6D** show that CRISPR/Cas9-mediated ablation of regions upstream of exon 1A using mGU3 reduced transcription from exon 1A (**Figures 6A, 6C,** and **6D**) while preserving transcripts initiating at exon 1B (**Figure 6B**) in 300X G_4C_2 repeat containing ES cells. The y-axis shows percent expression relative to untreated control 300X G_4C_2 repeat containing ES cells.

[0051] **Figures 7A-7D** show bar graphs showing expression levels (as determined by the TAQMAN® quantitative reverse transcription-coupled PCR (RT-qPCR) assays shown in the depiction of the *C9orf72* locus at the top of each figure) of transcripts from the *C9orf72* locus (y-axis) that are exon 1A-exon 2 spliced transcripts (**Figure 7A**), that are exon 1B-exon 2 spliced transcripts (**Figure 7B**), that are exon 1A-intron-containing transcripts (**Figure 7C**), or that are

intron-containing transcriptions (adjacent to G_4C_2 repeats) (**Figure 7D**) in mouse ES cells that comprise a humanized *C9orf72* locus comprising 300 repeats of the hexanucleotide sequence and a mouse promoter region following treatment with Cas9 and sequence-specific guide RNAs (gRNAs) targeting upstream of the transcription start site for exon 1A. Percent expression relative to control mouse ES cells comprising the humanized *C9orf72* locus comprising 300 repeats of the hexanucleotide sequence and the mouse promoter region is shown. **Figures 7A-7D** show that CRISPR/Cas9-mediated ablation of regions upstream of exon 1A using mGU3 + mGU5 reduced transcription from exon 1A (**Figures 7A, 7C,** and **7D**) while preserving transcripts initiating at exon 1B (**Figure 7B**) in 300X G_4C_2 repeat containing ES cells. The y-axis shows percent expression relative to untreated control 300X G_4C_2 repeat containing ES cells.

[0052] **Figure 8** shows the nature of deletions in CRISPR/Cas9-targeted regions in select ES cell clones that were targeted with mGU5 + mGU20, mGU3, mGU4, or mGU5, along with whether transcripts from exon 1A or exon 1B were reduced. Deletion mapping was performed in ES cells, and analysis of expression of transcriptions from exon 1A and exon 1B was done in ES-cell-derived motor neurons.

[0053] **Figure 9** shows accumulation of sense *C9orf72* transcripts (region E sense, 5' repeat sense, region G sense, region H sense, and region I sense) as assessed by NanoString NCOUNTER® technology in ES-cell-derived motor neurons.

[0054] **Figure 10** shows accumulation of antisense *C9orf72* transcripts (region A antisense, region E antisense, region G antisense, and 3' repeat antisense) as assessed by NanoString NCOUNTER® technology in ES-cell-derived motor neurons.

[0055] **Figure 11A** shows the nature of deletions in CRISPR/Cas9-targeted regions in select clones that were targeted with mGU5 + mGU20, mGU3, mGU4, or mGU5, along with whether sense or antisense transcripts were reduced. Deletion mapping was performed in ES cells, and analysis of expression of transcriptions from exon 1A and exon 1B or sense and antisense transcripts was done in ES-cell-derived motor neurons.

[0056] **Figure 11B** shows levels of dipeptide repeat proteins in ES-cell-derived motor neurons derived from select clones that were targeted with mGU5 + mGU20, mGU3, mGU4, or mGU5 as compared to 3X and 300X repeat untreated controls. Levels were assayed with antibodies against polyGlyAla (right) and polyGlyPro (left). Relative proteins levels for

polyGlyAla and polyGlyPro were quantitated and normalized to 300X repeat control samples (bottom).

[0057] **Figure 12** shows dot plots showing expression levels (as determined by the TAQMAN® quantitative reverse transcription-coupled PCR (RT-qPCR) assays) of transcripts from the *C9orf72* locus (y-axis) that are exon 1A-exon 2 spliced transcripts (left), that are transcripts containing the intron 1 region upstream of the hexanucleotide repeats (middle), or that are exon 1B-exon 2 spliced transcripts (right) in spinal cord and kidney samples from *Rosa26*Cas9/*C9orf72*G_4C_2*300x* heterozygous mice following injection of a dose of 7 x 10^{10} viral genomes of AAV-PHP.eB-U6-mGU3, mGU4, or mGU5 at postnatal day 0 by intracerebroventricular injection. Ct values are shown (lower Ct values = higher expression). Mice were sacrificed 28 days post-injection. The bar shows the median in each graph. Uninjected mice were used as a negative control.

[0058] **Figure 13** shows a schematic for generating a *C9orf72* allele with a humanized exon 1A promoter.

[0059] **Figure 14** shows bar graphs showing expression levels (as determined by the TAQMAN® quantitative reverse transcription-coupled PCR (RT-qPCR) assays shown in the depiction of the *C9orf72* locus at the top of each figure) of transcripts from the *C9orf72* locus (y-axis) that are exon 1A-exon 2 spliced transcripts, that are exon 1B-exon 2 spliced transcripts, that are exon 5-exon 6 spliced transcripts, that are transcripts containing exon 1A and intron 1 sequence, or that are transcripts containing intron 1 in mouse ES cells that comprise the allele with the humanized *C9orf72* promoter region and a humanized *C9orf72* locus comprising 3x or 250x repeats of the hexanucleotide sequence. Expression relative to 3x repeat control cells are shown. **Figure 14** shows that the allele with the humanized *C9orf72* promoter region reproduces the RNA expression pattern seen in repeat expansion alleles with the mouse promoter, with exon 1A and intron-containing transcripts being expressed at higher levels in 250x repeat mouse ES cells as compared to 3x repeat mouse ES cells, whereas exon 1B transcripts were unchanged.

[0060] **Figure 15** shows a schematic of CRISPR/Cas9-mediated targeting of the potential promoter for exon 1A transcripts of *C9orf72* in the allele with the humanized *C9orf72* promoter region. Nineteen gRNAs distributed across the ~2 kb human region upstream of the *C9orf72* exon 1A transcription start site in 250X G_4C_2 repeat containing ES cells were tested.

[0061] **Figure 16** shows bar graphs showing expression levels (as determined by the TAQMAN® quantitative reverse transcription-coupled PCR (RT-qPCR) assays shown in the depiction of the *C9orf72* locus at the top of each figure) of transcripts from the *C9orf72* locus (y-axis) that are exon 1A-exon 2 spliced transcripts (top) or that are exon 1B-exon 2 spliced transcripts (bottom) in mouse ES cells that comprise the allele with the humanized *C9orf72* promoter region and a humanized *C9orf72* locus comprising 250x repeats of the hexanucleotide sequence. Ct values are shown (lower Ct values = higher expression). **Figure 16** shows that, in various clones, CRISPR/Cas9-mediated ablation of regions upstream of exon 1A reduced transcription from exon 1A while preserving transcripts initiating at exon 1B in the allele with the humanized *C9orf72* promoter region.

[0062] **Figure 17** shows dot plots showing expression levels (as determined by the TAQMAN® quantitative reverse transcription-coupled PCR (RT-qPCR) assays) of transcripts from the *C9orf72* locus (y-axis) that are exon 1A-exon 2 spliced transcripts (left), that are transcripts containing the intron 1 region upstream of the hexanucleotide repeats (middle), or that are exon 1B-exon 2 spliced transcripts (right) in spinal cord and kidney samples from *Rosa26*Cas9/*C9orf72*G_4C_2*300x* heterozygous mice following injection of a dose of 7 x 10^{10} viral genomes of AAV-PHP.eB-U6-mGU3 and mGU5, mGU4 and mGU5, or mGU3 and mGU4 at postnatal day 0 by intracerebroventricular injection. RNA abundance normalized to PBS control is shown. The bar shows the median in each graph. PBS-injected mice were used as a negative control.

[0063] **Figure 18** shows the nature of CRISPR-induced mutations in CRISPR/Cas9-targeted regions in spinal cord and kidney samples from *Rosa26*Cas9/*C9orf72*G_4C_2*300x* heterozygous mice following injection of a dose of 7 x 10^{10} viral genomes of AAV-PHP.eB-U6-mGU3 and mGU5, mGU4 and mGU5, or mGU3 and mGU4 at postnatal day 0 by intracerebroventricular injection.

[0064] **Figure 19** shows dot plots showing expression levels (as determined by the TAQMAN® quantitative reverse transcription-coupled PCR (RT-qPCR) assays) of transcripts from the *C9orf72* locus (y-axis) that are exon 1A-exon 2 spliced transcripts (left), that are transcripts containing the intron 1 region upstream of the hexanucleotide repeats (middle), or that are exon 1B-exon 2 spliced transcripts (right) in spinal cord and kidney samples from *Rosa26*Cas9/*C9orf72*G_4C_2*300x* heterozygous mice following injection of a dose of 7 x 10^{10} viral

genomes of AAV-PHP.eB-U6-mGU3, mGU4, or GU5 at postnatal day 0 by intracerebroventricular injection. Delta Ct values are shown. The bar shows the median in each graph. PBS-injected mice were used as a negative control.

[0065] **Figure 20** shows the nature of CRISPR-induced mutations in CRISPR/Cas9-targeted regions in spinal cord and kidney samples from *Rosa26*Cas9/*C9orf72G$_4$C$_2$300x* heterozygous mice following injection of a dose of 7 x 10^{10} viral genomes of AAV-PHP.eB-U6-mGU3, mGU4, or GU5 at postnatal day 0 by intracerebroventricular injection.

[0066] **Figure 21** shows accumulation of antisense *C9orf72* transcripts (intron 1B antisense, 5' repeat antisense, region I antisense, and 3' repeat antisense) as assessed by NanoString NCOUNTER® technology in ES-cell-derived motor neurons.

[0067] **Figure 22** shows the nature of deletions in CRISPR/Cas9-targeted regions in select clones that were targeted with hGU3 and hGU21 (90099M clones) or hGU2 (90099G clones), along with whether sense or antisense transcripts were reduced. Deletion mapping was performed in ES cells and analysis of expression of transcriptions from exon 1A and exon 1B or sense and antisense transcripts was done in ES-cell-derived motor neurons.

DEFINITIONS

[0068] 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.

[0069] 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).

[0070] 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.

[0071] 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.

[0072] 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.

[0073] 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.

[0074] The term "isolated" with respect to cells, tissues, proteins, and nucleic acids includes cells, tissues, proteins, and nucleic acids that are relatively purified with respect to other bacterial, viral, cellular, or other components that may normally be present *in situ*, up to and including a substantially pure preparation of the cells, tissues, proteins, and nucleic acids. The term "isolated" also includes cells, tissues, proteins, and nucleic acids that have no naturally occurring counterpart, have been chemically synthesized and are thus substantially uncontaminated by other cells, tissues, proteins, and nucleic acids, or has been separated or purified from most other components (e.g., cellular components) with which they are naturally accompanied (e.g., other cellular proteins, polynucleotides, or cellular components).

[0075] 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).

[0076] The term "endogenous sequence" refers to a nucleic acid sequence that occurs naturally within a cell or subject. For example, an endogenous *C9orf72* sequence of a human refers to a native *C9orf72* sequence that naturally occurs at the *C9orf72* locus in the human.

[0077] "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.

[0078] 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.

[0079] "Codon optimization" (i.e., "codon optimized" sequences) 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 polypeptide of interest 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 Res.* 28(1):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).

[0080] 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 "*C9orf72* locus" may refer to the specific location of a *C9orf72* gene, *C9orf72* DNA sequence, or *C9orf72* position on a chromosome of the genome of an organism that has been identified as to where such a sequence resides. A "*C9orf72* locus" may comprise a regulatory element of a *C9orf72* gene, including, for example, an enhancer, a promoter, 5' and/or 3' untranslated region (UTR), or a combination thereof.

[0081] The term "gene" refers to DNA sequences in a chromosome that may contain, if naturally present, at least one coding and at least one non-coding region. The DNA sequence in a chromosome that codes for a product (e.g., but not limited to, an RNA product and/or a polypeptide product) can include 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). Additionally, other non-coding sequences including regulatory sequences (e.g., but not limited to, 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., but not

limited to, within 10 kb) or at distant sites, and they influence the level or rate of transcription and translation of the gene.

[0082] 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.

[0083] 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 human cell, a human liver cell, or a human liver hepatocyte). 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.

[0084] "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).

[0085] The methods and compositions provided herein employ a variety of different components. Some components throughout the description can have active variants and fragments. 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. 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.

[0086] 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).

[0087] 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).

[0088] "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).

[0089] "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.

[0090] 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.

[0091] 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 below.

[0092] 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

[0093] 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.

[0094] 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.

[0095] 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."

[0096] "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.

[0097] Designation of a range of values includes all integers within or defining the range, and all subranges defined by integers within the range. For example, 5-10 nucleotides is understood as 5, 6, 7, 8, 9, or 10 nucleotides, whereas 5-10% is understood to contain 5% and all possible values through 10%.

[0098] At least 17 nucleotides of a 20 nucleotide sequence is understood to include 17, 18, 19, or 20 nucleotides of the sequence provided, thereby providing a upper limit even if one is not specifically provided as it would be clearly understood. Similarly, up to 3 nucleotides would be understood to encompass 0, 1, 2, or 3 nucleotides, providing a lower limit even if one is not specifically provided. When "at least," "up to," or other similar language modifies a number, it can be understood to modify each number in the series.

[0099] As used herein, "no more than" or "less than" is understood as the value adjacent to the phrase and logical lower values or integers, as logical from context, to zero. For example, a

duplex region of "no more than 2 nucleotide base pairs" has a 2, 1, or 0 nucleotide base pairs. When "no more than" or "less than" is present before a series of numbers or a range, it is understood that each of the numbers in the series or range is modified.

[00100] As used herein, it is understood that when the maximum amount of a value is represented by 100% (e.g., 100% inhibition) that the value is limited by the method of detection. For example, 100% inhibition is understood as inhibition to a level below the level of detection of the assay.

[00101] Unless otherwise apparent from the context, the term "about" encompasses values ± 5% of a stated value. In certain embodiments, the term "about" is understood to encompass tolerated variation or error within the art, e.g., 2 standard deviations from the mean, or the sensitivity of the method used to take a measurement, or a percent of a value as tolerated in the art, e.g., with age. When "about" is present before the first value of a series, it can be understood to modify each value in the series.

[00102] 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").

[00103] The term "or" refers to any one member of a particular list and also includes any combination of members of that list.

[00104] 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.

[00105] Statistically significant means $p \leq 0.05$.

[00106] In the event of a conflict between a sequence in the application and an indicated accession number or position in an accession number, the sequence in the application predominates.

DETAILED DESCRIPTION

I. Overview

[00107] Sense and antisense repeat expansion *C9orf72* RNA detected as cytoplasmic and nuclear foci by fluorescence in situ hybridization (FISH) may sequester RNA binding proteins, leading to cellular toxicity. In addition, dipeptide repeat (DPR) proteins are proposed to be

produced from the *C9orf72* GGGGCC (G_4C_2) repeat expansion sense and antisense RNA by a non-canonical process that has been termed repeat associated non-AUG (RAN) translation, and there is strong evidence that DPR proteins are cytotoxic. DPR proteins can be translated from all sense and antisense reading frames. Sense DPR proteins include glycine-alanine, glycine-arginine, and glycine-proline DPR proteins. Antisense DPR proteins include proline-arginine, proline-alanine, and glycine-proline. Because G_4C_2 repeat-containing RNAs, either on their own or as templates for dipeptide repeat protein translation, appear to be pathogenic, a general therapeutic strategy is to either inhibit their synthesis or promote their destruction.

[00108] Transcription of the *C9orf72* gene initiates at two alternative non-coding exons: exon 1A (upstream) and exon 1B (downstream). The G_4C_2 repeat lies between exons 1A and 1B. Exons 1A and 1B can be spliced to exon 2, the first protein-coding exon, creating mRNAs with alternative 5'-untranslated regions. In healthy people with short G_4C_2 repeat expansions, transcription predominantly initiates at exon 1B; RNAs that include exon 1A are rare, and repeat-containing RNAs are undetectable. People suffering from C9orf72 ALS or FTLD accumulate transcripts in which exon 1A is spliced to exon 2, and both sense and antisense repeat-containing RNAs and the DPR proteins translated from them can be detected by *in situ* hybridization and immunohistochemistry. *See* **Figure 1**. These pathological findings suggest that the longer disease-associated G_4C_2 repeat expansions promote the use of the upstream exon 1A transcription initiation site, which is the only way that repeat-containing RNAs and their DPR proteins could be produced. It also follows that the production of antisense repeat-containing RNA, which depends on a long repeat expansion, is linked to increased use of the upstream transcription initiation site.

[00109] Thus, a possible therapeutic strategy for C9orf72 repeat-expansion disease would be to inhibit or abolish transcription that initiates upstream of the G_4C_2 repeat at exon 1A while retaining transcription that initiates at exon 1B downstream of the repeat, which will retain production of the mRNA for C9orf72 protein synthesis.

[00110] Disclosed herein are nuclease agents, including guide RNAs and CRISPR/Cas systems, targeting a *C9orf72* gene, lipid nanoparticles or viral vectors comprising such nuclease agents, and cells or animals comprising such nuclease agents. Methods of modifying a *C9orf72* gene using the nuclease agents are also provided, as well as use of the nuclease agents in prophylactic and therapeutic applications for treatment and/or prevention of a *C9orf72*

hexanucleotide repeat expansion associated disease and/or for ameliorating at least one symptom associated with such disease. The nuclease agents disclosed herein can target promoter elements upstream of exon 1A that control transcription of the *C9orf72* gene, selectively or preferentially reducing or abolishing transcripts that initiate at exon 1A while retaining transcripts that initiate at exon 1B.

[00111] The nuclease agents disclosed herein can, for example, reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1A. The nuclease agents disclosed herein can also, for example, reduce or abolish expression of *C9orf72* hexanucleotide-repeat-containing transcripts. The nuclease agents disclosed herein can also, for example, reduce or abolish expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts. The nuclease agents disclosed herein can also, for example, reduce or abolish expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts. The nuclease agents disclosed herein can also, for example, reduce or abolish expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. The nuclease agents disclosed herein can, for example, selectively or preferentially reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1A relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduce expression of transcripts that initiate at *C9orf72* exon 1A to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease agents disclosed herein can also, for example, selectively or preferentially reduce or abolish expression of *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduce expression of *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease agents disclosed herein can also, for example, selectively or preferentially reduce or abolish expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease agents disclosed herein can also, for example, selectively or preferentially reduce or abolish expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of

transcripts that initiate at *C9orf72* exon 1B). The nuclease agents disclosed herein can also, for example, selectively or preferentially reduce or abolish expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). With some nuclease agents disclosed herein, the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. With some nuclease agents disclosed herein, the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. With some nuclease agents disclosed herein, the targeted genetic modification reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. With some nuclease agents disclosed herein, the targeted genetic modification reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. With some nuclease agents disclosed herein, the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. With some nuclease agents disclosed herein, the targeted genetic modification reduces expression of polyGA dipeptide repeat proteins. With some nuclease agents disclosed herein, the targeted genetic modification reduces expression of polyGP dipeptide repeat proteins. With some nuclease agents disclosed herein, the targeted genetic modification reduces expression of both polyGA dipeptide repeat proteins and polyGP dipeptide repeat proteins.

II. Nuclease Agents, CRISPR/Cas Systems, and/or Exogenous Donor Nucleic Acids Targeting a C9orf72 *Gene*

[00112] The methods and compositions disclosed herein utilize nuclease agents such as Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems or components of such systems to modify a *C9orf72* gene within a cell, optionally

in combination with an exogenous donor nucleic acids. The *C9orf72* can be, for example, a human or mouse *C9orf72* or a humanized *C9orf72* gene. The cell can be, for example, within a subject, such as a human (e.g., a neuron).

A. *C9orf72*, Hexanucleotide Repeat Expansion Sequences, and Associated Diseases

[00113] Amyotrophic lateral sclerosis (ALS), also referred to as Lou Gehrig's disease, is the most frequent adult-onset paralytic disorder, characterized by the loss of upper and/or lower motor neurons. ALS occurs in as many as 20,000 individuals across the United States with about 5,000 new cases occurring each year. Frontotemporal dementia (FTD; also referred to as Pick's disease, frontotemporal lobar degeneration, or FTLD) is a group of disorders caused by progressive cell degeneration in the frontal or temporal lobes of the brain. FTD is reported to account for 10%-15% of all dementia cases. A hexanucleotide repeat expansion sequence between exons 1A and 1B, two non-coding exons of the human *C9orf72* gene, has been linked to both ALS and FTD. It is estimated that the G_4C_2 hexanucleotide repeat expansion accounts for about 50% of familial and many non-familial ALS cases. It is present in about 25% of familial FTD cases and about 8% of sporadic FTD cases.

[00114] Many pathological aspects related to the hexanucleotide repeat expansion sequence in *C9orf72* have been reported such as, for example, repeat-length-dependent formation of RNA foci, sequestration of specific RNA-binding proteins, and accumulation and aggregation of dipeptide repeat proteins (e.g., poly(glycine-alanine), poly(glycine-proline), poly(glycine-arginine), poly(alanine-proline), and poly(proline-arginine)) resulting from repeat-associated non-AUG (AUG) translation in their neurons.

[00115] Although C9orf72 has been reported to regulate endosomal trafficking, much of the cellular function of C9orf72 remains unknown. Indeed, *C9orf72* is a gene that encodes an uncharacterized protein with unknown function.

[00116] It is not known how the *C9orf72* hexanucleotide repeat expansion causes motor neuron disease and dementia, but two universal postmortem pathological findings in *C9orf72* ALS and FTD patients are associated with the repeat expansion: (1) sense and antisense repeat-containing RNA can be visualized as distinct foci in neurons and other cells; and (2) dipeptide repeat proteins—poly(glycine-alanine), poly(glycine-proline), poly(glycine-arginine), poly(alanine-proline), and poly(proline-arginine)—synthesized by repeat-associated non-AUG-

dependent translation from the sense and antisense repeat-containing RNAs can be detected in cells. One disease hypothesis proposes that the repeat-containing RNAs, visualized as foci, disrupt cellular RNA metabolism by sequestering RNA binding proteins. Another disease hypothesis posits that the dipeptide repeat proteins exert wide-spread toxic effects on RNA metabolism, proteostasis, and nucleocytoplasmic transport. If *C9orf72* repeat-containing RNA transcripts, either on their own or as templates for translation of dipeptide repeat proteins, promote pathogenesis in ALS and FTD, then a general therapeutic strategy would be to inhibit the synthesis of hexanucleotide repeat-containing RNA.

[00117] The *C9orf72* gene produces transcripts from two transcription initiation sites. The upstream site initiates transcription with alternative non-coding exon 1A, while the downstream site initiates transcription with alternative exon 1B. Both exons 1A and 1B can be spliced to exon 2, which contains the start of the protein-coding sequence. The pathogenic hexanucleotide repeat expansion is located between exons 1A and 1B. Therefore, transcription initiated from exon 1A can produce repeat-containing RNAs, while initiation from exon 1B cannot.

[00118] Mouse *C9orf72* transcript variants have been reported. *See, e.g.*, Koppers et al. (2015) *Ann. Neurol.* 78:426-438 and Atkinson et al. (2015) *Acta Neuropathologica Communications* 3:59, each of which is herein incorporated by reference in its entirety for all purposes. The genomic information for the three reported mouse *C9orf72* transcript variants is also available at the Ensembl web site under designations of ENSMUST00000108127 (V1), ENSMUST00000108126 (V2), and ENSMUST00000084724 (V3). Exemplary non-human (e.g., rodent) *C9orf72* mRNA and amino acid sequences are set forth in SEQ ID NOS: 22-25. The mRNA and amino acid sequences of mouse *C9orf72* can be found at GenBank accession numbers NM_001081343 and NP_001074812, respectively, and are hereby incorporated by reference in their entirety for all purposes. The sequences of NM_001081343.1 and NP_001074812.1 are set forth in SEQ ID NOS: 22 and 23, respectively. The mRNA and amino acid sequences of rat *C9orf72* can be found at GenBank accession numbers NM_001007702 and NP_001007703, respectively, and are hereby incorporated by reference in their entirety for all purposes. The sequences of NM_001007702.1 and NP_001007703.1 are set forth in SEQ ID NOS: 24 and 25, respectively.

[00119] Human *C9orf72* transcript variants are also known. One human *C9orf72* transcript variant lacks multiple exons in the central and 3' coding regions, and its 3' terminal exon extends

beyond a splice site that is used in variant 3 (see below), which results in a novel 3' untranslated region (UTR) as compared to variant 3. This variant encodes a significantly shorter polypeptide and its C-terminal amino acid is distinct as compared to that which is encoded by two other variants. The mRNA and amino acid sequences of this variant can be found at GenBank accession numbers NM_145005.6 and NP_659442.2, respectively, and are hereby incorporated by reference in their entirety for all purposes. The sequences of NM_145005.6 and NP_659442.2 are set forth in SEQ ID NO: 26 and SEQ ID NO: 27, respectively. A second human *C9orf72* transcript variant (2) differs in the 5' untranslated region (UTR) compared to variant 3. The mRNA and amino acid sequences of this variant can be found at GenBank accession numbers NM_018325.4 and NP_060795.1, respectively, and are hereby incorporated by reference in their entirety for all purposes. The sequences of NM_018325.4 and NP_060795.1 are set forth in SEQ ID NO: 28 and SEQ ID NO: 29, respectively. A third human *C9orf72* transcript variant (3) contains the longest sequence among three reported variants and encodes the longer isoform. The mRNA and amino acid sequences of this variant can be found at GenBank accession numbers NM_001256054.2 and NP_001242983.1, respectively, and are hereby incorporated by reference in their entirety for all purposes. The sequences of NM_001256054.2 and NP_001242983.1 are set forth in SEQ ID NO: 30 and SEQ ID NO: 31, respectively. Variants 2 and 3 encode the same protein.

B. Nuclease Agents and CRISPR/Cas Systems

[00120] The methods and compositions disclosed herein can utilize nuclease agents such as Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) systems, zinc finger nuclease (ZFN) systems, or Transcription Activator-Like Effector Nuclease (TALEN) systems or components of such systems to modify a target genomic locus in a *C9orf72* gene. Generally, the nuclease agents involve the use of engineered cleavage systems to induce a double strand break or a nick (i.e., a single strand break) in a nuclease target site. Cleavage or nicking can occur through the use of specific nucleases such as engineered ZFNs, TALENs, or CRISPR/Cas systems with an engineered guide RNA to guide specific cleavage or nicking of the nuclease target site. 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. The nuclease agent can be used to create a targeted genetic modification in the *C9orf72* gene. For

example, the targeted genetic modification can comprise a deletion of a region of the *C9orf72* promoter. In some embodiments, the deletion encompasses the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion of *C9orf72* exon 1A. In other embodiments, the targeted genetic modification does not result in deletion of the *C9orf72* hexanucleotide repeat expansion sequence. In one example, the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In another example, the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In another example, the targeted genetic modification reduces or abolishes expression of sense se *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In another example, the targeted genetic modification reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In another example, the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In another example, the targeted genetic modification reduces expression of polyGA dipeptide repeat proteins. In another example, the targeted genetic modification reduces expression of polyGP dipeptide repeat proteins. In another example, the targeted genetic modification reduces expression of both polyGA dipeptide repeat proteins and polyGP dipeptide repeat proteins.

[00121] In one example, the nuclease agent is a CRISPR/Cas system. In another example, the nuclease agent comprises one or more ZFNs. In yet another example, the nuclease agent comprises one or more TALENs. In a specific example, the CRISPR/Cas systems or components of such systems target a nuclease target sequence upstream of the *C9orf72* exon 1A transcription start site. In a more specific example, the CRISPR/Cas systems or components of such systems target within about 250, about 225, about 200, about 175, about 150, about 125, about 100, about 75, or about 50 nucleotides of the *C9orf72* exon 1A transcription start site. In a more specific

example, the CRISPR/Cas systems or components of such systems target within about 125, about 100, about 75, or about 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[00122] 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 system, or a type V system (e.g., subtype V-A or subtype V-B). The methods and compositions disclosed herein can employ CRISPR/Cas systems by utilizing CRISPR complexes (comprising a guide RNA (gRNA) complexed with a Cas protein) for site-directed binding or cleavage of nucleic acids. A CRISPR/Cas system targeting a *C9orf72* gene comprises a Cas protein (or a nucleic acid encoding the Cas protein) and one or more guide RNAs (or DNAs encoding the one or more guide RNAs), with each of the one or more guide RNAs targeting a different guide RNA target sequence in the target genomic locus.

[00123] 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.

(1) Target Genomic Loci

[00124] The nuclease agents and CRISPR/Cas systems described in the compositions and methods disclosed herein target a nuclease target sequence (e.g., a guide RNA target sequence) in a *C9orf72* gene. The *C9orf72* gene can be, for example, a mammalian *C9orf72* gene. In a specific example, the *C9orf72* gene comprises a human *C9orf72* promoter. In a specific example, the *C9orf72* gene is a human *C9orf72* gene. In another specific example, the *C9orf72* gene is a humanized *C9orf72* gene. For example, the *C9orf72* gene can be a non-human animal (e.g., non-human mammal, rodent, rat, or mouse) *C9orf72* gene in which a human hexanucleotide repeat expansion sequence and flanking human sequence is inserted at an endogenous *C9orf72* locus to replace the corresponding endogenous sequence. *See, e.g.*, US 2020-0196581 and WO

2020/131632, each of which is herein incorporated by reference in its entirety for all purposes.

[00125] Optionally, the *C9orf72* gene comprises a *C9orf72* hexanucleotide repeat expansion sequence between the first non-coding endogenous exon and exon 2 of the *C9orf72* gene. A *C9orf72* hexanucleotide repeat expansion sequence is generally a nucleotide sequence comprising at least two tandem repeats (i.e., contiguous repeats that are adjacent to each other without intervening sequence) of the hexanucleotide sequence G_4C_2. The hexanucleotide repeat expansion sequence can have any number of repeats. Optionally, the hexanucleotide repeat expansion sequence has more than about 30 repeats. In some embodiments, the hexanucleotide repeat expansion sequence has more than about 100 repeats, more than about 200 repeats, more than about 300 repeats, more than about 400 repeats, more than about 500 repeats, more than about 600 repeats, more than about 700 repeats, more than about 800 repeats, more than about 900 repeats, or more than about 1000 repeats.

[00126] The nuclease target site can be near (e.g., upstream or downstream) the *C9orf72* exon 1A transcription start site. The nuclease target site can be upstream of the *C9orf72* exon 1A transcription start site. Transcription of the *C9orf72* gene initiates at two alternative non-coding exons: exon 1A (upstream) and exon 1B (downstream). The G_4C_2 repeat lies between exons 1A and 1B. Exons 1A and 1B can be spliced to exon 2, the first protein-coding exon, creating mRNAs with alternative 5'-untranslated regions. In healthy people with short G_4C_2 repeat expansions, transcription predominantly initiates at exon 1B; RNAs that include exon 1A are rare, and repeat-containing RNAs are undetectable. People suffering from C9orf72 ALS or FTLD accumulate transcripts in which exon 1A is spliced to exon 2, and both sense and antisense repeat-containing RNAs and the DPR proteins translated from them can be detected by *in situ* hybridization and immunohistochemistry. In one example, the nuclease target sequence can be within about 2500, within about 2250, within about 2000, within about 1800, within about 1600, within about 1400, within about 1200, within about 1000, within about 900, within about 800, within about 700, within about 600, within about 500, within about 450, within about 400, within about 350, within about 300, within about 250, within about 225, within about 200, within about 175, within about 150, within about 125, within about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 nucleotides of the *C9orf72* exon 1A transcription start site. In one example, the nuclease target sequence can be within about 250, within about 225, within about 200, within about 175, within about 150, within about 125, within

about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 nucleotides of the *C9orf72* exon 1A transcription start site. In another example, the nuclease target sequence can be within about 125, within about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 of the *C9orf72* exon 1A transcription start site.

[00127] The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion that encompasses the *C9orf72* exon 1A transcription start site. Alternatively, the nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion or modification that does not encompass deletion or modification of the *C9orf72* exon 1A transcription start site. In other embodiments, the nuclease target sequence is positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In other embodiments, the nuclease target sequence is positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent does not result in deletion of *C9orf72* exon 1A. In other embodiments, the nuclease target sequence is positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent does not result in deletion of the *C9orf72* hexanucleotide repeat expansion sequence.

[00128] The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease

target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site.

[00129] The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of transcripts that initiate at *C9orf72* exon 1A to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of sense *C9orf72*

hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

[00130] In some of the compositions and methods disclosed herein, two or more nuclease agents or CRISPR/Cas systems are used to target two or more nuclease target sequences (e.g., guide RNA target sequences) in a *C9orf72* gene. For example, the first nuclease target sequence can be upstream of the *C9orf72* exon 1A transcription start site as described above. In some cases, the second nuclease target sequence is also upstream (i.e., 5') of the *C9orf72* exon 1A transcription start site. For example, the second nuclease target sequence can be within about 2500, within about 2250, within about 2000, within about 1800, within about 1600, within about 1400, within about 1200, within about 1000, within about 900, within about 800, within about 700, within about 600, within about 500, within about 450, within about 400, within about 350, within about 300, within about 250, within about 225, within about 200, within about 175, within about 150, within about 125, within about 100, within about 75, within about 50, within about

25, within about 20, or within about 10 nucleotides of the *C9orf72* exon 1A transcription start site. In some cases, the second nuclease target sequence is downstream (i.e., 3') of the *C9orf72* exon 1A transcription start site. For example, the second nuclease target sequence can be within about 2500, within about 2250, within about 2000, within about 1800, within about 1600, within about 1400, within about 1200, within about 1000, within about 900, within about 800, within about 700, within about 600, within about 500, within about 450, within about 400, within about 350, within about 300, within about 250, within about 225, within about 200, within about 175, within about 150, within about 125, within about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 nucleotides of the *C9orf72* exon 1A transcription start site. In one example, the second nuclease target sequence can be downstream (i.e., 3') of the *C9orf72* exon 1A transcription start site and within exon 1A. In another example, the second nuclease target sequence can be downstream (i.e., 3') of the *C9orf72* exon 1A transcription start site but not within exon 1A. In another example, the second nuclease target sequence can be downstream (i.e., 3') of the *C9orf72* exon 1A transcription start site but upstream (i.e., 5') of the *C9orf72* hexanucleotide repeat expansion sequence. In another example, the second nuclease target sequence can be downstream (i.e., 3') of *C9orf72* exon 1A but upstream (i.e., 5') of the *C9orf72* hexanucleotide repeat expansion sequence. In another example, the second nuclease target sequence can be downstream (i.e., 3') of the *C9orf72* hexanucleotide repeat expansion sequence but upstream of *C9orf72* exon 1B.

[00131] In one example, the first and second nuclease target sequences flank a region of the *C9orf72* promoter upstream of the *C9orf72* exon 1A transcription start site such that the region of the promoter is deleted. In another example, the first and second nuclease target sequences flank the *C9orf72* exon 1A transcription start site such that the *C9orf72* exon 1A transcription start site is deleted. In another example, the first and second nuclease target sequences flank *C9orf72* exon 1A such that *C9orf72* exon 1A is deleted. In another example, the first and second nuclease target sequences flank a region of the *C9orf72* gene comprising *C9orf72* exon 1A and the *C9orf72* hexanucleotide repeat expansion sequence such that the region of the *C9orf72* gene comprising *C9orf72* exon 1A and the *C9orf72* hexanucleotide repeat expansion sequence is deleted (e.g., but no portion of *C9orf72* exon 1B is deleted).

[00132] When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from

cleavage by the nuclease agents results in a deletion that encompasses the *C9orf72* exon 1A transcription start site. Alternatively, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents results in a deletion that does not encompass the *C9orf72* exon 1A transcription start site. In other embodiments, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In other embodiments, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents does not result in deletion of *C9orf72* exon 1A. In other embodiments, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents does not result in deletion of the *C9orf72* hexanucleotide repeat expansion sequence.

[00133] The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72*

exon 1A transcription start site.

[00134] When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of transcripts that initiate at *C9orf72* exon 1A to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic

modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces expression of polyGA dipeptide repeat proteins. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces expression of polyGP dipeptide repeat proteins. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces expression of both polyGA dipeptide repeat proteins and polyGP dipeptide repeat proteins.

(2) Cas Proteins

[00135] Cas proteins generally comprise at least one RNA recognition or binding domain that can interact with guide RNAs. 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.

[00136] 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.

[00137] 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) (e.g., assigned UniProt accession number Q99ZW2) is an exemplary Cas9 protein. An exemplary SpCas9 protein sequence is set forth in SEQ ID NO: 1 (encoded by the DNA sequence set forth in SEQ ID NO: 2). Smaller Cas9 proteins (e.g., Cas9 proteins whose coding sequences are compatible with the maximum AAV packaging capacity when combined with a guide RNA coding sequence and regulatory elements for the Cas9 and guide RNA, such as SaCas9 and CjCas9 and Nme2Cas9) are other exemplary Cas9 proteins. For example, Cas9 from *S. aureus* (SaCas9) (e.g., assigned UniProt accession number J7RUA5) is another exemplary Cas9 protein. Likewise, Cas9 from *Campylobacter jejuni* (CjCas9) (e.g., assigned UniProt accession number Q0P897) is another exemplary Cas9 protein. *See, e.g.*, Kim et al. (2017) *Nat. Commun.* 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. Cas9 from *Neisseria meningitidis* (Nme2Cas9) is another exemplary Cas9 protein. *See, e.g.*, Edraki et al. (2019) *Mol. Cell* 73(4):714-726, herein incorporated by reference in its entirety for all purposes. Cas9 proteins from *Streptococcus thermophilus* (e.g., *Streptococcus thermophilus* LMD-9 Cas9 encoded by the CRISPR1 locus (St1Cas9) or *Streptococcus thermophilus* Cas9 from the CRISPR3 locus (St3Cas9)) are other exemplary Cas9 proteins. Cas9 from *Francisella novicida* (FnCas9) or the RHA *Francisella novicida* Cas9 variant that recognizes an alternative PAM (E1369R/E1449H/R1556A substitutions) are other exemplary Cas9 proteins. These and other exemplary Cas9 proteins are reviewed, e.g., in Cebrian-Serrano and Davies (2017) *Mamm. Genome* 28(7):247-261, herein incorporated by reference in its entirety for all purposes. Examples of Cas9 coding sequences, Cas9 mRNAs, and Cas9 protein sequences are provided in WO 2013/176772, WO 2014/065596, WO 2016/106121, WO 2019/067910, WO 2020/082042, US 2020/0270617, WO 2020/082041, US 2020/0268906, WO 2020/082046, and US 2020/0289628, each of which is herein incorporated by reference in its entirety for all purposes. Specific examples of ORFs and Cas9

amino acid sequences are provided in Table 30 at paragraph [0449] WO 2019/067910, and specific examples of Cas9 mRNAs and ORFs are provided in paragraphs [0214]-[0234] of WO 2019/067910. *See also* WO 2020/082046 A2 (pp. 84-85) and Table 24 in WO 2020/069296, each of which is herein incorporated by reference in its entirety for all purposes.

[00138] Another example of a Cas protein is a Cpf1 (CRISPR from *Prevotella* and *Francisella* 1; Cas12a) 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.

[00139] Another example of a Cas protein is CasX (Cas12e). CasX is an RNA-guided DNA endonuclease that generates a staggered double-strand break in DNA. CasX is less than 1000 amino acids in size. Exemplary CasX proteins are from *Deltaproteobacteria* (DpbCasX or DpbCas12e) and *Planctomycetes* (PlmCasX or PlmCas12e). Like Cpf1, CasX uses a single RuvC active site for DNA cleavage. *See, e.g.*, Liu et al. (2019) *Nature* 566(7743):218-223, herein incorporated by reference in its entirety for all purposes.

[00140] Another example of a Cas protein is CasΦ (CasPhi or Cas12j), which is uniquely found in bacteriophages. CasΦ is less than 1000 amino acids in size (e.g., 700-800 amino acids). CasΦ cleavage generates staggered 5' overhangs. A single RuvC active site in CasΦ is capable of crRNA processing and DNA cutting. *See, e.g.*, Pausch et al. (2020) *Science* 369(6501):333-337, herein incorporated by reference in its entirety for all purposes.

[00141] 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.

[00142] 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. These and other modified Cas proteins are reviewed, e.g., in Cebrian-Serrano and Davies (2017) *Mamm. Genome* 28(7):247-261, herein incorporated by reference in its entirety for all purposes. Another example of a modified Cas9 protein is xCas9, which is a SpCas9 variant that can recognize an expanded range of PAM sequences. *See, e.g.*, Hu et al. (2018) *Nature* 556:57-63, herein incorporated by reference in its entirety for all purposes.

[00143] 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 of or a property of the Cas protein.

[00144] 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. Likewise, CasX and CasΦ generally comprise a single RuvC-like domain that cleaves both strands of a target DNA. 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(6096):816-821, herein incorporated by reference in its entirety for all purposes.

[00145] One or more 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 none of the nuclease domains is deleted or mutated in a Cas9 protein, the Cas9 protein will retain double-strand-break-inducing activity. 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 Res.* 39(21):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.

[00146] Examples of inactivating mutations in the catalytic domains of xCas9 are the same as those described above for SpCas9. 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) or a substitution at position D10 (e.g., D10A substitution) to generate a Cas nickase. *See, e.g.*,

WO 2016/106236, herein incorporated by reference in its entirety for all purposes. Examples of inactivating mutations in the catalytic domains of Nme2Cas9 are also known (e.g., D16A or H588A). Examples of inactivating mutations in the catalytic domains of St1Cas9 are also known (e.g., D9A, D598A, H599A, or N622A). Examples of inactivating mutations in the catalytic domains of St3Cas9 are also known (e.g., D10A or N870A). Examples of inactivating mutations in the catalytic domains of CjCas9 are also known (e.g., combination of D8A or H559A). Examples of inactivating mutations in the catalytic domains of FnCas9 and RHA FnCas9 are also known (e.g., N995A).

[00147] 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.

[00148] Examples of inactivating mutations in the catalytic domains of CasX proteins are also known. With reference to CasX proteins from *Deltaproteobacteria*, D672A, E769A, and D935A (individually or in combination) or corresponding positions in other CasX orthologs are inactivating. *See, e.g.*, Liu et al. (2019) *Nature* 566(7743):218-223, herein incorporated by reference in its entirety for all purposes.

[00149] Examples of inactivating mutations in the catalytic domains of CasΦ proteins are also known. For example, D371A and D394A, alone or in combination, are inactivating mutations. *See, e.g.*, Pausch et al. (2020) *Science* 369(6501):333-337, herein incorporated by reference in its entirety for all purposes.

[00150] 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. *See* WO 2014/089290, herein incorporated by reference in its entirety for all purposesCas 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.

[00151] 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(8):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.

[00152] A Cas protein may, for example, be fused with 1-10 NLSs (e.g., fused with 1-5 NLSs or fused with one NLS. Where one NLS is used, the NLS may be linked at the N-terminus or the C-terminus of the Cas protein sequence. It may also be inserted within the Cas protein sequence. Alternatively, the Cas protein may be fused with more than one NLS. For example, the Cas protein may be fused with 2, 3, 4, or 5 NLSs. In a specific example, the Cas protein may be fused with two NLSs. In certain circumstances, the two NLSs may be the same (e.g., two SV40 NLSs) or different. For example, the Cas protein can be fused to two SV40 NLS sequences linked at the carboxy terminus. Alternatively, the Cas protein may be fused with two NLSs, one linked at the N-terminus and one at the C-terminus. In other examples, the Cas protein may be fused with 3 NLSs or with no NLS. The NLS may be a monopartite sequence, such as, e.g., the SV40 NLS, PKKKRKV (SEQ ID NO: 3) or PKKKRRV (SEQ ID NO: 4). The NLS may be a bipartite sequence, such as the NLS of nucleoplasmin, KRPAATKKAGQAKKKK (SEQ ID NO: 5). In a specific example, a single PKKKRKV (SEQ ID NO: 3) NLS may be linked at the C-terminus of the Cas protein. One or more linkers are optionally included at the fusion site.

[00153] 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.

[00154] 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.

[00155] Cas proteins can also be tethered to 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 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 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 labeled nucleic acid. That is, the 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 labeled nucleic acid.

[00156] 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.

[00157] 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 a human cell, a human neuron, or a human motor neuron. 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. In certain embodiments, promotors are accepted by regulatory authorities for use in humans. In certain embodiments, promotors drive expression in a neuron.

[00158] Different promoters can be used to drive Cas expression or Cas9 expression. In some methods, small promoters are used so that the Cas or Cas9 coding sequence can fit into an AAV construct. For example, Cas or Cas9 and one or more gRNAs (e.g., 1 gRNA or 2 gRNAs or 3 gRNAs or 4 gRNAs) can be delivered via LNP-mediated delivery (e.g., in the form of RNA) or adeno-associated virus (AAV)-mediated delivery (e.g., AAV8-mediated delivery). For example, the nuclease agent can be CRISPR/Cas9, and a Cas9 mRNA and a gRNA (e.g., targeting a *C9orf72* gene (e.g., a human *C9orf72* gene) upstream of the exon 1A transcription start site) can be delivered via LNP-mediated delivery or AAV-mediated delivery. The Cas or Cas9 and the gRNA(s) can be delivered in a single AAV or via two separate AAVs. For example, a first AAV can carry a Cas or Cas9 expression cassette, and a second AAV can carry a gRNA expression cassette. Similarly, a first AAV can carry a Cas or Cas9 expression cassette, and a second AAV

can carry two or more gRNA expression cassettes. Alternatively, a single AAV can carry a Cas or Cas9 expression cassette (e.g., Cas or Cas9 coding sequence operably linked to a promoter) and a gRNA expression cassette (e.g., gRNA coding sequence operably linked to a promoter). Similarly, a single AAV can carry a Cas or Cas9 expression cassette (e.g., Cas or Cas9 coding sequence operably linked to a promoter) and two or more gRNA expression cassettes (e.g., gRNA coding sequences operably linked to promoters). Different promoters can be used to drive expression of the gRNA, such as a U6 promoter or the small tRNA Gln. Likewise, different promoters can be used to drive Cas9 expression. For example, small promoters are used so that the Cas9 coding sequence can fit into an AAV construct. Similarly, small Cas9 proteins (e.g., SaCas9 or CjCas9 are used to maximize the AAV packaging capacity).

[00159] 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. mRNA encoding Cas proteins can also be capped. Cas mRNAs can further comprise a poly-adenylated (poly-A or poly(A) or poly-adenine) tail. For example, a Cas mRNA can include a modification to one or more nucleosides within the mRNA, the Cas mRNA can be capped, and the Cas mRNA can comprise a poly(A) tail.

(3) Guide RNAs

[00160] 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" (also called "guide sequence") 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. A guide RNA can refer to either a CRISPR RNA (crRNA) or the combination of a crRNA and a trans-activating CRISPR RNA (tracrRNA). The crRNA and tracrRNA can be associated as a single RNA molecule (single

guide RNA or sgRNA) or in two separate RNA molecules (dual guide RNA or dgRNA). For Cas9, for example, a single-guide RNA can comprise a crRNA fused to a tracrRNA (e.g., via a linker). For Cpf1 and CasΦ, 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. In some of the methods and compositions disclosed herein, a gRNA is a *S. pyogenes* Cas9 gRNA or an equivalent thereof. In some of the methods and compositions disclosed herein, a gRNA is a *S. aureus* Cas9 gRNA or an equivalent thereof.

[00161] 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-activating 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 (e.g., for use with *S. pyogenes* Cas9), located downstream (3') of the DNA-targeting segment, comprises, consists essentially of, or consists of GUUUUAGAGCUAUGCU (SEQ ID NO: 6) or GUUUUAGAGCUAUGCUGUUUUG (SEQ ID NO: 7). Any of the DNA-targeting segments disclosed herein can be joined to the 5' end of SEQ ID NO: 6 or 7 to form a crRNA.

[00162] 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. Examples of tracrRNA sequences (e.g., for use with *S. pyogenes* Cas9) comprise, consist essentially of, or consist of any one of AGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACC GAGUCGGUGCUUU (SEQ ID NO: 8), AAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGG CACCGAGUCGGUGCUUUU (SEQ ID NO: 9), or GUUGGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCA ACUUGAAAAAGUGGCACCGAGUCGGUGC (SEQ ID NO: 10).

[00163] 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(6121):823-826; Jinek et al. (2012) *Science* 337(6096):816-821; Hwang et al. (2013) *Nat. Biotechnol.* 31(3):227-229; Jiang et al. (2013) *Nat. Biotechnol.* 31(3):233-239; and Cong et al. (2013) *Science* 339(6121):819-823, each of which is herein incorporated by reference in its entirety for all purposes.

[00164] 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.

[00165] The DNA-targeting segment can have, for example, a length of at least about 12, at least about 15, at least about 17, at least about 18, at least about 19, at least about 20, at least about 25, at least about 30, at least about 35, or at least about 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.

[00166] In one example, the DNA-targeting segment can be about 20 nucleotides in length. However, shorter and longer sequences can also be used for the targeting segment (e.g., 15-25 nucleotides in length, such as 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length). The degree of identity between the DNA-targeting segment and the corresponding guide RNA target sequence (or degree of complementarity between the DNA-targeting segment and the other strand of the guide RNA target sequence) can be, for example, about 75%, about 80%, about 85%, about 90%, about 95%, or 100%. The DNA-targeting segment and the corresponding guide RNA target sequence can contain one or more mismatches. For example, the DNA-targeting segment of the guide RNA and the corresponding guide RNA target sequence can contain 1-4, 1-3, 1-2, 1, 2, 3, or 4 mismatches (e.g., where the total length of the guide RNA target sequence is at least 17, at least 18, at least 19, or at least 20 or more nucleotides). For example, the DNA-targeting segment of the guide RNA and the corresponding guide RNA target sequence can contain 1-4, 1-3, 1-2, 1, 2, 3, or 4 mismatches where the total length of the guide RNA target sequence 20 nucleotides.

[00167] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 73-91 or 74-91. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 73-91 or 74-91. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at

least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 73-91 or 74-91. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 73-91 or 74-91. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 73-91 or 74-91. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 73-91 or 74-91. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 73-91 or 74-91. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 73-91 or 74-91.

[00168] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide

sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 74-76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 74-76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 74-76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 74-76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 74-76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 74-76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 74-76. Alternatively, a

guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 74-76.

[00169] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 74. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 74. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 74. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 74. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 74. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A

transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 74. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in SEQ ID NO: 74. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 74.

[00170] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 75. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 75. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 75. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the

sequence (DNA-targeting segment) set forth in SEQ ID NO: 75. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 75. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 75. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in SEQ ID NO: 75. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 75.

[00171] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth

in SEQ ID NO: 76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in SEQ ID NO: 76. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 76.

[00172] As one example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 72-73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 72-73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 72-73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 72-73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 72-73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 72-73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the

C9orf72 exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 72-73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 72-73.

[00173] As one example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 72. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 72. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 72. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 72. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%,

at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 72. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 72. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in SEQ ID NO: 72. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 72.

[00174] As one example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to

the sequence (DNA-targeting segment) set forth in SEQ ID NO: 73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in SEQ ID NO: 73. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 73.

[00175] In some cases, two guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 75.

Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 75. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 75. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 75. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 75. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 75. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 75. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences

upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 75.

[00176] In some cases, two guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the

C9orf72 exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 76.

[00177] In some cases, two guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72*

guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 76. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 76.

[00178] In some cases, two guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and

downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 72. Alternatively, guide RNAs targeting a *C9orf72*

gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 72.

[00179] In some cases, two guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences

(DNA-targeting segments) set forth in SEQ ID NOS: 75 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 72.

[00180] In some cases, two guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A

transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 72. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 72.

[00181] In some cases, two guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 73. Alternatively,

guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 74 and 73.

[00182] In some cases, two guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to

the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 75 and 73.

[00183] In some cases, two guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA

target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 73. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse

C9orf72 guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 76 and 73.

[00184] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 92-111. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 92-111. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 92-111. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 92-111. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 92-111. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can

comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 92-111. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 92-111. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 92-111.

[00185] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 93, 94, and 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 93, 94, and 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 93, 94, and 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-

targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 93, 94, and 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 93, 94, and 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 93, 94, and 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 93, 94, and 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in any one of SEQ ID NOS: 93, 94, and 96.

[00186] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 93. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-

targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 93. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 93. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 93. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 93. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 93. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in SEQ ID NO: 93. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at

least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 93.

[00187] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 94. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 94. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 94. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 94. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 94. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 94. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human

C9orf72 guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in SEQ ID NO: 94. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 94.

[00188] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least

90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in SEQ ID NO: 96. Alternatively, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 96.

[00189] As one example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment (i.e., guide sequence) comprising, consisting essentially of, or consisting of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 133. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 133. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%

identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 133. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to the sequence (DNA-targeting segment) set forth in SEQ ID NO: 133. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 133. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment that is at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 133. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequence (DNA-targeting segment) set forth in SEQ ID NO: 133. Alternatively, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can comprise a DNA-targeting segment comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence (DNA-targeting segment) set forth in SEQ ID NO: 133.

[00190] In some cases, two or more guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and

94. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 94. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 94. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 94. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 94. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 94. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 94. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences

upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 94.

[00191] In some cases, two or more guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the

C9orf72 exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 96.

[00192] In some cases, two or more guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72*

guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 96. Alternatively, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 96.

[00193] In some cases, two or more guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene

upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 133. Alternatively, guide RNAs targeting a *C9orf72*

gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 93 and 133.

[00194] In some cases, two or more guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences

(DNA-targeting segments) set forth in SEQ ID NOS: 94 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 94 and 133.

[00195] In some cases, two or more guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments (i.e., guide sequences) comprising, consisting essentially of, or consisting of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 96 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 96 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the

C9orf72 exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 96 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 96 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 96 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments that are at least 90% or at least 95% identical to at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 96 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of a sequence that differs by no more than 3, no more than 2, or no more than 1 nucleotide from the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 96 and 133. Alternatively, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can comprise DNA-targeting segments comprising, consisting essentially of, or consisting of sequences that differs by no more than 3, no more than 2, or no more than 1 nucleotide from at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequences (DNA-targeting segments) set forth in SEQ ID NOS: 96 and 133.

[00196] 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(7340):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.

[00197] 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%, 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).

[00198] 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.

[00199] 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 (e.g., for use with *S. pyogenes* Cas9) comprise, consist essentially of, or consist of:

GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU (version 1; SEQ ID NO: 11);

GUUGGAACCAUUCAAAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC (version 2; SEQ ID NO: 12);

GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC (version 3; SEQ ID NO: 13); and

GUUUAAGAGCUAUGCUGGAAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC (version 4; SEQ ID NO: 14);

GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU (version 5; SEQ ID NO: 15);

GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU (version 6; SEQ ID NO: 16);

GUUUAAGAGCUAUGCUGGAAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU (version 7; SEQ ID NO: 17); or

GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGGCACCGAGUCGGUGC (version 8; SEQ ID NO: 18). In some guide sgRNAs, the four terminal

U residues of version 6 are not present. In some sgRNAs, only 1, 2, or 3 of the four terminal U residues of version 6 are present. 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).

[00200] 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). That is, guide RNAs can include one or more modified nucleosides or nucleotides, or one or more non-naturally and/or naturally occurring components or configurations that are used instead of or in addition to the canonical A, G, C, and U residues. 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 transcriptional activators, transcriptional repressors, 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.

[00201] 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 (an exemplary backbone modification); (2) alteration or replacement of a constituent of the ribose sugar such as alteration or replacement of the 2' hydroxyl on the ribose sugar (an exemplary sugar modification); (3) replacement (e.g., wholesale replacement) of the phosphate moiety with dephospho linkers (an exemplary backbone modification); (4) modification or replacement of a naturally occurring nucleobase, including with a non-canonical nucleobase (an exemplary base modification); (5) replacement or modification of the ribose-phosphate backbone (an exemplary backbone modification); (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, cap, or linker (such 3' or 5' cap modifications may comprise a sugar and/or backbone modification); and (7) modification or replacement of the sugar (an exemplary sugar modification). 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. For example, Cas mRNAs can be modified by depletion of uridine using synonymous codons.

[00202] Chemical modifications such as those listed above can be combined to provide modified gRNAs and/or mRNAs comprising residues (nucleosides and nucleotides) that can have two, three, four, or more modifications. For example, a modified residue can have a modified sugar and a modified nucleobase. In one example, every base of a gRNA is modified (e.g., all bases have a modified phosphate group, such as a phosphorothioate group). For example, all or substantially all of the phosphate groups of a gRNA can be replaced with phosphorothioate groups. Alternatively or additionally, a modified gRNA can comprise at least one modified residue at or near the 5' end. Alternatively or additionally, a modified gRNA can comprise at least one modified residue at or near the 3' end.

[00203] Some gRNAs comprise one, two, three or more modified residues. For example, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, 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%, or 100% of the positions in a modified gRNA can be modified nucleosides or nucleotides.

[00204] 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. Some gRNAs described herein can contain one or more modified nucleosides or nucleotides to introduce stability toward intracellular or serum-based nucleases. Some modified gRNAs described herein can exhibit a reduced innate immune response when introduced into a population of cells.

[00205] In a dual guide RNA, each of the crRNA and the tracrRNA can contain modifications. Such modifications may be at one or both ends of the crRNA and/or tracrRNA. In a sgRNA, one or more residues at one or both ends of the sgRNA may be chemically modified, and/or internal nucleosides may be modified, and/or the entire sgRNA may be chemically modified. Some gRNAs comprise a 5' end modification. Some gRNAs comprise a 3' end modification. Some gRNAs comprise a 5' end modification and a 3' end modification.

[00206] The guide RNAs disclosed herein can comprise one of the modification patterns disclosed in WO 2018/107028 A1, herein incorporated by reference in its entirety for all purposes. The guide RNAs disclosed herein can also comprise one of the structures/modification patterns disclosed in US 2017/0114334, herein incorporated by reference in its entirety for all purposes. The guide RNAs disclosed herein can also comprise one of the structures/modification patterns disclosed in WO 2017/136794, WO 2017/004279, US 2018/0187186, or US 2019/0048338, each of which is herein incorporated by reference in its entirety for all purposes.

[00207] As one example, any of the guide RNAs described herein can comprise at least one modification. In one example, the at least one modification comprises a 2'-O-methyl (2'-O-Me) modified nucleotide, a phosphorothioate (PS) bond between nucleotides, a 2'-fluoro (2'-F) modified nucleotide, or a combination thereof. For example, the at least one modification can comprise a 2'-O-methyl (2'-O-Me) modified nucleotide. Alternatively or additionally, the at least one modification can comprise a phosphorothioate (PS) bond between nucleotides. Alternatively or additionally, the at least one modification can comprise a 2'-fluoro (2'-F) modified nucleotide. In one example, a guide RNA described herein comprises one or more 2'-O-methyl (2'-O-Me) modified nucleotides and one or more phosphorothioate (PS) bonds between nucleotides.

[00208] 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.

[00209] 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 a human cell, a human neuron, or a human motor neuron. 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.

[00210] 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.

[00211] Guide RNAs (or nucleic acids encoding guide RNAs) can be in compositions comprising one or more guide RNAs (e.g., 1, 2, 3, 4, or more guide RNAs) and a carrier increasing the stability of the guide RNA (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. Such compositions can further comprise a Cas protein, such as a Cas9 protein, or a nucleic acid encoding a Cas protein.

(4) Guide RNA Target Sequences

[00212] 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."

[00213] 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.

[00214] 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.

[00215] 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'. In

the case of DpbCasX, the PAM can have the sequence 5'-TTCN-3'. In the case of CasΦ, the PAM can have the sequence 5'-TBN-3', where B is G, T, or C.

[00216] 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: 19) or $N_{20}NGG$ (SEQ ID NO: 20). *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: 21) 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: 19-21, 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: 19-21.

[00217] 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.

[00218] The guide RNA target sequence can also be selected to minimize off-target modification or avoid off-target effects (e.g., by avoiding two or fewer mismatches to off-target genomic sequences).

[00219] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in any one of SEQ ID NOS: 33-51 or 34-51. As another example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in any one of SEQ ID NOS: 33-51 or 34-51.

[00220] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in any one of SEQ ID NOS: 34-36. As another example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in any one of SEQ ID NOS: 34-36.

[00221] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in SEQ ID NO: 34. As another example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in SEQ ID NO: 34.

[00222] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of

the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in SEQ ID NO: 35. As another example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in SEQ ID NO: 35.

[00223] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in SEQ ID NO: 36. As another example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in SEQ ID NO: 36.

[00224] As one example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in any one of SEQ ID NOS: 32-33. As another example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in any one of SEQ ID NOS: 32-33.

[00225] As one example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in SEQ ID NO: 32. As another example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in SEQ ID NO: 32.

[00226] As one example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in SEQ ID NO: 33. As another example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a mouse *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in SEQ ID NO: 33.

[00227] In some cases two or more guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 34 and 35. As another example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth SEQ ID NOS: 34 and 35.

[00228] As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 34 and 36. As another example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth SEQ ID NOS: 34 and 36.

[00229] As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 35 and 36. As another example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at

least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth SEQ ID NOS: 35 and 36.

[00230] As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 34 and 32. As another example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth SEQ ID NOS: 34 and 32.

[00231] As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 35 and 32. As another example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth SEQ ID NOS: 35 and 32.

[00232] As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 36 and 32. As another example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth SEQ ID NOS: 36 and 32.

[00233] As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 34 and 33. As another example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start

site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth SEQ ID NOS: 34 and 33.

[00234] As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 35 and 33. As another example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth SEQ ID NOS: 35 and 33.

[00235] As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 36 and 33. As another example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., mouse *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth SEQ ID NOS: 36 and 33.

[00236] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in any one of SEQ ID NOS: 52-71. As another example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in any one of SEQ ID NOS: 52-71.

[00237] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in any one of SEQ ID NOS: 53, 54, and 56. As another example, a guide RNA targeting a

C9orf72 gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in any one of SEQ ID NOS: 53, 54, and 56.

[00238] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in SEQ ID NO: 53. As another example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in SEQ ID NO: 53.

[00239] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in SEQ ID NO: 54. As another example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in SEQ ID NO: 54.

[00240] As one example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set forth in SEQ ID NO: 56. As another example, a guide RNA targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in SEQ ID NO: 56.

[00241] As one example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequence set

forth in SEQ ID NO: 132. As another example, a guide RNA targeting a *C9orf72* gene downstream of the *C9orf72* exon 1A transcription start site (e.g., a human *C9orf72* guide RNA target sequence downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequence set forth in SEQ ID NO: 132.

[00242] In some cases two or more guide RNAs are used. As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 53 and 54. As another example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequences set forth in SEQ ID NOS: 53 and 54.

[00243] As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 53 and 56. As another example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequences set forth in SEQ ID NOS: 53 and 56.

[00244] As one example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 54 and 56. As another example, guide RNAs targeting a *C9orf72* gene upstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequences set forth in SEQ ID NOS: 54 and 56.

[00245] As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences

upstream and downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 53 and 132. As another example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequences set forth in SEQ ID NOS: 53 and 132.

[00246] As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 54 and 132. As another example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequences set forth in SEQ ID NOS: 54 and 132.

[00247] As one example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target the guide RNA target sequences set forth in SEQ ID NOS: 56 and 132. As another example, guide RNAs targeting a *C9orf72* gene upstream and downstream of the *C9orf72* exon 1A transcription start site (e.g., human *C9orf72* guide RNA target sequences upstream and downstream of the *C9orf72* exon 1A transcription start site) can target at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the guide RNA target sequences set forth in SEQ ID NOS: 56 and 132.

(5) Lipid Nanoparticles Comprising Nuclease Agents

[00248] Lipid nanoparticles comprising the nuclease agents (e.g., CRISPR/Cas systems) are also provided. The lipid nanoparticles can alternatively or additionally comprise an exogenous donor nucleic acid as disclosed herein. For example, the lipid nanoparticles can comprise a nuclease agent (e.g., CRISPR/Cas system), can comprise an exogenous donor nucleic acid, or can comprise both a nuclease agent (e.g., a CRISPR/Cas system) and an exogenous donor nucleic acid. Regarding CRISPR/Cas systems, the lipid nanoparticles can comprise the Cas protein in any form (e.g., protein, DNA, or mRNA) and/or can comprise the guide RNA(s) in

any form (e.g., DNA or RNA). In one example, the lipid nanoparticles comprise the Cas protein in the form of mRNA (e.g., a modified RNA as described herein) and the guide RNA(s) in the form of RNA (e.g., a modified guide RNA as disclosed herein). As another example, the lipid nanoparticles can comprise the Cas protein in the form of protein and the guide RNA(s) in the form of RNA). In a specific example, the guide RNA and the Cas protein are each introduced in the form of RNA via LNP-mediated delivery in the same LNP. As discussed in more detail elsewhere herein, one or more of the RNAs can be modified. Delivery through such methods can result in transient Cas expression and/or transient presence of the guide RNA, 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*. *See, e.g.*, 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.

[00249] In some LNPs, the cargo can comprise Cas mRNA (e.g., Cas9 mRNA) and gRNA. The Cas mRNA and gRNAs can be in different ratios. In some LNPs, the cargo can comprise an exogenous donor nucleic acid and a nuclease agent (e.g., CRISPR/Cas system). The exogenous donor nucleic acid and nuclease agent components can be in different ratios.

[00250] Examples of suitable LNPs can be found, e.g., in WO 2019/067992, WO 2020/082042, US 2020/0270617, WO 2020/082041, US 2020/0268906, WO 2020/082046 (*see, e.g.*, pp. 85-86), and US 2020/0289628, each of which is herein incorporated by reference in its entirety for all purposes. A specific example of using LNPs to deliver to the brain is disclosed in Nabhan et al. (2016) *Sci. Rep.* 6:20019, herein incorporated by reference in its entirety for all purposes.

(6) Vectors Comprising Nuclease Agents

[00251] The nuclease agents disclosed herein (e.g., ZFN, TALEN, or CRISPR/Cas) can be provided in a vector for expression. A vector can comprise additional sequences such as, for example, replication origins, promoters, and genes encoding antibiotic resistance.

[00252] Some vectors may be circular. Alternatively, the vector may be linear. The vector can be in the packaged for delivered via a lipid nanoparticle, liposome, non-lipid nanoparticle, or viral capsid. Non-limiting exemplary vectors include plasmids, phagemids, cosmids, artificial chromosomes, minichromosomes, transposons, viral vectors, and expression vectors.

[00253] Introduction of nucleic acids can also be accomplished by virus-mediated delivery, such as AAV-mediated delivery or lentivirus-mediated delivery. The vectors can be, for example, viral vectors such as adeno-associated virus (AAV) vectors. The AAV may be any suitable serotype and may be a single-stranded AAV (ssAAV) or a self-complementary AAV (scAAV). Other exemplary viruses/viral vectors include retroviruses, lentiviruses, 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). Viral vectors may be genetically modified from their wild type counterparts. For example, the viral vector may comprise an insertion, deletion, or substitution of one or more nucleotides to facilitate cloning or such that one or more properties of the vector is changed. Such properties may include packaging capacity, transduction efficiency, immunogenicity, genome integration, replication, transcription, and translation. In some examples, a portion of the viral genome may be deleted such that the virus is capable of packaging exogenous sequences having a larger size. In some examples, the viral vector may have an enhanced transduction efficiency. In some examples, the immune response induced by the virus in a host may be reduced. In some examples, viral genes (such as integrase) that promote integration of the viral sequence into a host genome may be mutated such that the virus becomes non-integrating. In some examples, the viral vector may be replication defective. In some examples, the viral vector may comprise exogenous transcriptional or translational control

sequences to drive expression of coding sequences on the vector. In some examples, the virus may be helper-dependent. For example, the virus may need one or more helper components to supply viral components (such as viral proteins) required to amplify and package the vectors into viral particles. In such a case, one or more helper components, including one or more vectors encoding the viral components, may be introduced into a host cell or population of host cells along with the vector system described herein. In other examples, the virus may be helper-free. For example, the virus may be capable of amplifying and packaging the vectors without a helper virus. In some examples, the vector system described herein may also encode the viral components required for virus amplification and packaging.

[00254] Exemplary viral titers (e.g., AAV titers) include about 10^{12} to about 10^{16} vg/mL. Other exemplary viral titers (e.g., AAV titers) include about 10^{12} to about 10^{16} vg/kg of body weight.

[00255] Adeno-associated viruses (AAVs) are endemic in multiple species including human and non-human primates (NHPs). At least 12 natural serotypes and hundreds of natural variants have been isolated and characterized to date. *See, e.g.*, Li et al. (2020) *Nat. Rev. Genet.* 21:255-272, herein incorporated by reference in its entirety for all purposes. AAV particles are naturally composed of a non-enveloped icosahedral protein capsid containing a single-stranded DNA (ssDNA) genome. The DNA genome is flanked by two inverted terminal repeats (ITRs) which serve as the viral origins of replication and packaging signals. The *rep* gene encodes four proteins required for viral replication and packaging whilst the *cap* gene encodes the three structural capsid subunits which dictate the AAV serotype, and the Assembly Activating Protein (AAP) which promotes virion assembly in some serotypes.

[00256] Recombinant AAV (rAAV) is currently one of the most commonly used viral vectors used in gene therapy to treat human diseases by delivering therapeutic transgenes to target cells *in vivo*. rAAV vectors are composed of icosahedral capsids similar to natural AAVs, but rAAV virions do not encapsidate AAV protein-coding or AAV replicating sequences. These viral vectors are non-replicating. The only viral sequences required in rAAV vectors are the two ITRs, which are needed to guide genome replication and packaging during manufacturing of the rAAV vector. rAAV genomes are devoid of AAV *rep* and *cap* genes, rendering them non-replicating *in vivo*. rAAV vectors are produced by expressing *rep* and *cap* genes along with additional viral helper proteins *in trans*, in combination with the intended transgene cassette flanked by AAV

ITRs.

[00257] In rAAV genomes, a gene expression cassette can be placed between ITR sequences. Typically, rAAV genome cassettes comprise of a promoter to drive expression of a transgene, followed by a polyadenylation sequence. The ITRs flanking a rAAV expression cassette are usually derived from AAV2, the first serotype to be isolated and converted into a recombinant viral vector. Since then, most rAAV production methods rely on AAV2 *Rep*-based packaging systems. *See, e.g.*, Colella et al. (2017) *Mol. Ther. Methods Clin. Dev.* 8:87-104, herein incorporated by reference in its entirety for all purposes.

[00258] The specific serotype of a recombinant AAV vector influences its *in vivo* tropism to specific tissues. AAV capsid proteins are responsible for mediating attachment and entry into target cells, followed by endosomal escape and trafficking to the nucleus. Thus, the choice of serotype when developing a rAAV vector will influence what cell types and tissues the vector is most likely to bind to and transduce when injected *in vivo*.

[00259] Once in the nucleus, the ssDNA genome is released from the virion and a complementary DNA strand is synthesized to generate a double-stranded DNA (dsDNA) molecule. Double-stranded AAV genomes naturally circularize via their ITRs and become episomes which will persist extrachromosomally in the nucleus. Therefore, for episomal gene therapy programs, rAAV-delivered rAAV episomes provide long-term, promoter-driven gene expression in non-dividing cells. However, this rAAV-delivered episomal DNA is diluted out as cells divide. In contrast, the gene therapy described herein is based on gene insertion to allow long-term gene expression.

[00260] 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.

[00261] 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. The term AAV includes, for example, AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAVrh.64R1, AAVhu.37, AAVrh.8, AAVrh.32.33, AAV8, AAV9, AAV-DJ, AAV2/8, AAVrh10, AAVLK03, AV10, AAV11, AAV12, rh10, and hybrids thereof, avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, non-primate AAV, and ovine AAV. The genomic sequences of various serotypes of AAV, as well as the sequences of the native terminal repeats (TRs), Rep proteins, and capsid subunits are known in the art. Such sequences may be found in the literature or in public databases such as GenBank. A "AAV vector" as used herein refers to an AAV vector comprising a heterologous sequence not of AAV origin (*i.e.*, a nucleic acid sequence heterologous to AAV), typically comprising a sequence encoding an exogenous polypeptide of interest. The construct may comprise an AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAVrh.64R1, AAVhu.37, AAVrh.8, AAVrh.32.33, AAV8, AAV9, AAV-DJ, AAV2/8, AAVrh10, AAVLK03, AV10, AAV11, AAV12, rh10, and hybrids thereof, avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, non-primate AAV, and ovine AAV capsid sequence. In general, the heterologous nucleic acid sequence (the transgene) is flanked by at least one, and generally by two, AAV inverted terminal repeat sequences (ITRs). An AAV vector may either be single-stranded (ssAAV) or self-complementary (scAAV). Serotypes for CNS tissue include AAV1, AAV2, AAV4, AAV5, AAV8, and AAV9. Selectivity of AAV serotypes for gene delivery in neurons is discussed, for example, in Hammond et al. (2017) *PLoS One* 12(12):e0188830, herein incorporated by reference in its entirety for all purposes. In a specific example, an AAV-PHP.eB vector is used. The AAV-PHP.eB vector shows high ability to cross the blood-brain barrier, increasing its CNS transduction efficiency. In another specific example, an AAV9 vector is used.

[00262] 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.

[00263] 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.

[00264] 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.

[00265] In certain AAVs, the cargo can include nucleic acids encoding one or more guide RNAs (e.g., DNA encoding a guide RNA, or DNA encoding two or more guide RNAs). In certain AAVs, the cargo can include a nucleic acid (e.g., DNA) encoding a Cas nuclease, such as Cas9, and DNA encoding one or more guide RNAs (e.g., DNA encoding a guide RNA, or DNA encoding two or more guide RNAs). In certain AAVs, the cargo can include an exogenous donor nucleic acid as described herein. In certain AAVs, the cargo can include a nucleic acid (e.g., DNA) encoding a Cas nuclease, such as Cas9, a DNA encoding a guide RNA (or multiple guide RNAs), and an exogenous donor nucleic acid.

[00266] For example, Cas or Cas9 and one or more gRNAs (e.g., 1 gRNA or 2 gRNAs or 3 gRNAs or 4 gRNAs) can be delivered via LNP-mediated delivery (e.g., in the form of RNA) or adeno-associated virus (AAV)-mediated delivery. For example, a Cas9 mRNA and a gRNA can be delivered via LNP-mediated delivery, or DNA encoding Cas9 and DNA encoding a gRNA

can be delivered via AAV-mediated delivery. The Cas or Cas9 and the gRNA(s) can be delivered in a single AAV or via two separate AAVs. For example, a first AAV can carry a Cas or Cas9 expression cassette, and a second AAV can carry a gRNA expression cassette. Similarly, a first AAV can carry a Cas or Cas9 expression cassette, and a second AAV can carry two or more gRNA expression cassettes. Alternatively, a single AAV can carry a Cas or Cas9 expression cassette (e.g., Cas or Cas9 coding sequence operably linked to a promoter) and a gRNA expression cassette (e.g., gRNA coding sequence operably linked to a promoter). Similarly, a single AAV can carry a Cas or Cas9 expression cassette (e.g., Cas or Cas9 coding sequence operably linked to a promoter) and two or more gRNA expression cassettes (e.g., gRNA coding sequences operably linked to promoters). Different promoters can be used to drive expression of the gRNA, such as a U6 promoter or the small tRNA Gln. Likewise, different promoters can be used to drive Cas9 expression. For example, small promoters are used so that the Cas9 coding sequence can fit into an AAV construct. Similarly, small Cas9 proteins (e.g., SaCas9 or CjCas9 are used to maximize the AAV packaging capacity).

C. Exogenous Donor Nucleic Acids

[00267] The methods and compositions disclosed herein can utilize exogenous donor nucleic acids to modify the *C9orf72* gene (e.g., *C9orf72* promoter) following cleavage of the *C9orf72* gene (e.g., *C9orf72* promoter) with a nuclease agent or independent of cleavage of the *C9orf72* gene (e.g., *C9orf72* promoter) with a nuclease agent. In such methods using a nuclease agent, the nuclease agent protein cleaves the *C9orf72* gene (e.g., *C9orf72* promoter) to create a single-strand break (nick) or double-strand break, and the exogenous donor nucleic acid recombines the *C9orf72* gene (e.g., *C9orf72* promoter) 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.

[00268] The exogenous donor nucleic acid can target any sequence in the *C9orf72* gene (e.g., *C9orf72* promoter). Some exogenous donor nucleic acids comprise homology arms. Other exogenous donor nucleic acids do not comprise homology arms. The exogenous donor nucleic acids can be capable of insertion into a *C9orf72* gene (e.g., *C9orf72* promoter) by homology-directed repair, and/or they can be capable of insertion into a *C9orf72* gene (e.g., *C9orf72*

promoter) by non-homologous end joining. In one example, the exogenous donor nucleic acid (e.g., a targeting vector) can target upstream of the *C9orf72* exon 1A transcription start site. For example, the exogenous donor nucleic acid can target within about 2500, within about 2250, within about 2000, within about 1800, within about 1600, within about 1400, within about 1200, within about 1000, within about 900, within about 800, within about 700, within about 600, within about 500, within about 450, within about 400, within about 350, within about 300, within about 250, within about 225, within about 200, within about 175, within about 150, within about 125, within about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 nucleotides of the *C9orf72* exon 1A transcription start site. For example, the exogenous donor nucleic acid can target within about 250, about 225, about 200, about 175, about 150, about 125, about 100, about 75, or about 50 nucleotides of the *C9orf72* exon 1A transcription start site. For example, the exogenous donor nucleic acid can target within about 125, about 100, about 75, or about 50 nucleotides of the *C9orf72* exon 1A transcription start site.

[00269] 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. Exogenous donor nucleic acids can be naked nucleic acids or can be delivered by viruses, such as AAV. In a specific example, the exogenous donor nucleic acid can be delivered via AAV and can be capable of insertion into a *C9orf72* gene by non-homologous end joining (e.g., the exogenous donor nucleic acid can be one that does not comprise homology arms).

[00270] 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.

[00271] 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).

[00272] 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).

[00273] Exogenous donor nucleic acids can also comprise nucleic acid inserts including segments of DNA to be integrated in the *C9orf72* gene. Integration of a nucleic acid insert in the *C9orf72* gene can result in addition of a nucleic acid sequence of interest to the *C9orf72* gene, deletion of a nucleic acid sequence of interest in the *C9orf72* gene, or replacement of a nucleic acid sequence of interest in the *C9orf72* gene (i.e., deletion and insertion). Some exogenous donor nucleic acids are designed for insertion of a nucleic acid insert in the *C9orf72* gene without any corresponding deletion in the *C9orf72* gene. Other exogenous donor nucleic acids are designed to delete a nucleic acid sequence of interest in the *C9orf72* gene 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 in the *C9orf72* gene and replace it with a nucleic acid insert.

[00274] The nucleic acid insert or the corresponding nucleic acid in the *C9orf72* gene being deleted and/or replaced can be various lengths. An exemplary nucleic acid insert or corresponding nucleic acid in the *C9orf72* gene 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 in the *C9orf72* gene 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 in the *C9orf72* gene 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 in the *C9orf72* gene 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.

[00275] 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 in the *C9orf72* gene. 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.

[00276] ***Donor Nucleic Acids for Non-Homologous-End-Joining-Mediated Insertion.*** Some exogenous donor nucleic acids are capable of insertion into a *C9orf72* gene by non-homologous end joining. In some cases, such exogenous donor nucleic acids do not comprise homology arms. For example, such exogenous donor nucleic acids can be inserted into a blunt end double-strand break following cleavage with a nuclease agent. In a specific example, the exogenous donor nucleic acid can be delivered via AAV and can be capable of insertion into a *C9orf72* gene by non-homologous end joining (e.g., the exogenous donor nucleic acid can be one that does not comprise homology arms). In a specific example, the exogenous donor nucleic acid can be inserted via homology-independent targeted integration. For example, the insert sequence in the exogenous donor nucleic acid to be inserted into a *C9orf72* gene can be flanked on each side by a target site for a nuclease agent (e.g., the same target site as in the *C9orf72* gene, and the same nuclease agent being used to cleave the target site in the *C9orf72* gene). The nuclease agent can then cleave the target sites flanking the insert sequence. In a specific example, the exogenous donor nucleic acid is delivered AAV-mediated delivery, and cleavage of the target sites flanking the insert sequence can remove the inverted terminal repeats (ITRs) of the AAV. In some methods, the target site in the *C9orf72* gene (e.g., a gRNA target sequence including the flanking protospacer adjacent motif) is no longer present if the insert sequence is inserted into the *C9orf72* gene in the correct orientation but it is reformed if the insert sequence is inserted into the *C9orf72* gene in the opposite orientation. This can help ensure that the insert sequence is inserted in the correct orientation for expression.

[00277] Other 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 in the *C9orf72* gene. 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 in the *C9orf72* gene. 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 in the *C9orf72* gene or only at the 3' end complementary to an overhang created at a 3' target sequence in the *C9orf72* gene. 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 in the *C9orf72* gene. 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.

[00278] 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.

[00279] 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). Optionally, 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.

[00280] ***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 *C9orf72* gene, which are referred to herein as "5' target sequence" and "3' target sequence," respectively.

[00281] 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).

[00282] When a nuclease agent is used in combination with an exogenous donor nucleic acid, the 5' and 3' target sequences are optionally located in sufficient proximity to the nuclease cleavage site (e.g., within sufficient proximity to 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.

[00283] 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.

III. Methods for Modifying a C9orf72 *Gene in a Cell or a Subject and Methods of Preventing, Treating, or Ameliorating at Least one Symptom or Indication of a* C9orf72 *Hexanucleotide Repeat Expansion Associated Disease*

[00284] Also disclosed herein are methods of modifying a *C9orf72* gene using the nuclease agents, CRISPR/Cas systems, or exogenous donor nucleic acids described herein, as well as use of the CRISPR/Cas systems in prophylactic and therapeutic applications for treatment and/or prevention of *C9orf72* hexanucleotide repeat expansion associated disease and/or for ameliorating at least one symptom associated with such disease. Such methods can, for example, reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1A. Such methods can also, for example, reduce or abolish expression of *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can also, for example, reduce or abolish expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can also, for example, reduce or abolish expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can also, for example, reduce or abolish expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can, for example, selectively or preferentially reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1A relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduce expression of transcripts that initiate at *C9orf72* exon 1A to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduce expression of *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or

abolish expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). In some methods, the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces expression of polyGA dipeptide repeat proteins. In some methods, the targeted genetic modification reduces expression of polyGP dipeptide repeat proteins. In some methods, the targeted genetic modification reduces expression of both polyGA dipeptide repeat proteins and polyGP dipeptide repeat proteins.

A. Methods of Modifying a *C9orf72* Gene in a Cell or a Subject

[00285] Various methods are provided for modifying a *C9orf72* gene using the nuclease agents or CRISPR/Cas systems (e.g., Cas protein or a nucleic acid encoding and one or more *C9orf72*-targeting gRNAs or DNAs encoding) described elsewhere herein. Optionally, an exogenous donor nucleic acid (e.g., targeting vector) targeting the *C9orf72* gene can be used

together with the nuclease agents or CRISPR/Cas reagents. Alternatively, the nuclease agents or CRISPR/Cas systems can be used without any exogenous donor nucleic acid.

[00286] The *C9orf72* gene can be in an animal or cell, and the methods can occur *in vitro*, *ex vivo*, or *in vivo*. The *C9orf72* gene can be in the genome of the cell, and can be endogenous to the cell or can be not endogenous to the cell. Animals include mammals, fishes, and birds. A mammal can be, for example, a non-human mammal, a human, a rodent, a rat, a mouse, or a hamster. In one example, the animal is a human. Other non-human mammals include, for example, non-human primates (e.g., cynomolgus), monkeys, apes, cats, dogs, rabbits, horses, bulls, deer, bison, 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). Birds include, for example, chickens, turkeys, ostrich, geese, ducks, and so forth. Domesticated animals and agricultural animals are also included. The animals in the methods disclosed herein can be humans or they can be non-human animals. In a specific example, the *C9orf72* gene is in a human or a human cell. The term "non-human" excludes humans. Particular examples of non-human animals include rodents, such as mice and rats, or non-human primates, such as cynomolgus.

[00287] Cells used in the methods can be from any type of animal, and they can be any type of undifferentiated or differentiated state. The cells can be *in vitro*, *ex vivo*, or *in vivo*. For example, a cell can be a non-human 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. In some embodiments, a human cell is a not a totipotent cell. In some embodiments, a human cell is not a pluripotent cell.

[00288] The cells provided herein can also be germ cells (e.g., sperm or oocytes) or non-human germ cells. 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, kidney cells, hematopoietic cells, endothelial cells, epithelial cells, fibroblasts, mesenchymal cells, keratinocytes, blood cells, melanocytes, monocytes, mononuclear cells,

monocytic precursors, B cells, erythroid-megakaryocytic cells, eosinophils, macrophages, T cells, islet beta cells, exocrine cells, pancreatic progenitors, endocrine progenitors, adipocytes, preadipocytes, neurons, glial cells, neural stem cells, neurons, hepatoblasts, hepatocytes, cardiomyocytes, skeletal myoblasts, smooth muscle cells, ductal cells, acinar cells, alpha cells, beta cells, delta cells, PP cells, cholangiocytes, white or brown adipocytes, or ocular cells (e.g., trabecular meshwork cells, retinal pigment epithelial cells, retinal microvascular endothelial cells, retinal pericyte cells, conjunctival epithelial cells, conjunctival fibroblasts, iris pigment epithelial cells, keratocytes, lens epithelial cells, non-pigment ciliary epithelial cells, ocular choroid fibroblasts, photoreceptor cells, ganglion cells, bipolar cells, horizontal cells, or amacrine cells). For example, the cells can be neurons, such as motor neurons. The cells provided herein can be normal, healthy cells, or can be diseased or mutant-bearing cells such as cells comprising a hexanucleotide repeat expansion at the *C9orf72* locus.

[00289] 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) *Mamm. Genome* 10(8):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).

[00290] 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*^{avl} haplotype. Such rats are available from a variety of sources including Charles River and Harlan Laboratories. In some cases, 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.

[00291] The nuclease agents or CRISPR/Cas reagents (and optionally exogenous donor nucleic acid) can be introduced into a cell or an animal in any form and by any means as described elsewhere herein, and all or some can be introduced simultaneously or sequentially in any combination as described elsewhere herein.

[00292] In some methods, one nuclease agent (e.g., one guide RNA) is used. In other methods, one or more additional nuclease agents that target one or more additional nuclease target sequences (e.g., one or more additional guide RNAs that target additional guide RNA target sequences) within the *C9orf72* gene can be used. By using one or more additional nuclease agents or guide RNAs (e.g., a second nuclease agent that targets a second nuclease target sequence or a second guide RNA that targets a second guide RNA target sequence), cleavage by the nuclease agent (e.g., Cas protein) can create two or more double-strand breaks or two or more single-strand breaks (e.g., if the nuclease agent or Cas protein is a nickase). In some methods, two or more nuclease agents (e.g., guide RNAs) targeting the *C9orf72* gene can be used (e.g., two nuclease agents or two guide RNAs can be used).

[00293] Optionally, one or more exogenous donor sequences which recombine with a target genomic locus in the *C9orf72* gene can be used together with the nuclease agent or CRISPR/Cas system to generate a targeted genetic modification. Examples and variations of exogenous donor sequences that can be used in the methods are disclosed elsewhere herein.

[00294] Targeted genetic modifications in a *C9orf72* gene in a genome can be generated by contacting the *C9orf72* gene with a nuclease agent targeting a nuclease target sequence in the *C9orf72* gene such that the nuclease agent cleaves (e.g., creates a double-strand break) in the nuclease target sequence (e.g., a complex comprising a Cas protein (e.g., a Cas9 protein) and a guide RNA targeting a guide RNA target sequence in the *C9orf72* gene, such that Cas protein creates one or more nicks or double-strand breaks at the guide RNA target sequence). Optionally, the *C9orf72* gene)can be further contacted with a second nuclease agent targeting a second nuclease target sequence in the *C9orf72* gene in the *C9orf72* gene such that the nuclease agent cleaves (e.g., creates a double-strand break) in the second nuclease target sequence (e.g., a

complex comprising a Cas protein (e.g., a Cas9 protein) and a guide RNA targeting a guide RNA target sequence in the *C9orf72* gene, such that Cas protein creates one or more nicks or double-strand breaks at the guide RNA target sequence). Optionally, the *C9orf72* gene can be further contacted with one or more exogenous donor nucleic acids. For example, targeted genetic modifications to a *C9orf72* gene can be generated by introducing into a cell or an animal a nuclease agent targeting a nuclease target sequence in the *C9orf72* gene (or one or more nucleic acids encoding the nuclease agent) such that the nuclease agent cleaves (e.g., creates a double-strand break) in the nuclease target sequence (e.g., a complex comprising a Cas protein (e.g., a Cas9 protein) and a guide RNA targeting a guide RNA target sequence in the *C9orf72* gene, such that Cas protein creates one or more nicks or double-strand breaks at the guide RNA target sequence). Optionally, a second nuclease agent targeting a second nuclease target sequence in the *C9orf72* gene can be introduced into the cell or the animal such that the nuclease agent cleaves (e.g., creates a double-strand break) in the second nuclease target sequence (e.g., a complex comprising a Cas protein (e.g., a Cas9 protein) and a guide RNA targeting a guide RNA target sequence in the *C9orf72* gene, such that Cas protein creates one or more nicks or double-strand breaks at the guide RNA target sequence). Optionally, one or more exogenous donor nucleic acids can also be introduced into the cell. The Cas protein forms a different complex with each guide RNA, and the Cas protein cleaves the guide RNA target sequence. An exogenous donor nucleic acid, if used, can recombine with the target genomic locus. Cleavage by the Cas protein can create a double-strand break or a single-strand break (e.g., if the Cas protein is a nickase). Examples and variations of nuclease agents and examples and variations of Cas proteins and guide RNAs that can be used in the methods are described elsewhere herein.

[00295] A guide RNA can be introduced into an animal or cell, for example, in the form of an RNA (e.g., *in vitro* transcribed RNA, such as the modified guide RNAs disclosed herein) 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 the cell or in a cell in the 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).

[00296] Likewise, Cas proteins can be introduced into an animal or cell 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)), such as a modified mRNA as disclosed herein, 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 human 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 cell or an animal, the Cas protein can be transiently, conditionally, or constitutively expressed in the cell or in a cell in the animal.

[00297] In one example, the Cas protein is introduced in the form of an mRNA (e.g., a modified mRNA as disclosed herein), and the guide RNA is introduced in the form of RNA such as a modified gRNA as disclosed herein (e.g., together within the same lipid nanoparticle).

[00298] Guide RNAs can be modified as disclosed elsewhere herein. Likewise, Cas mRNAs can be modified as disclosed elsewhere herein.

[00299] The nuclease target site can be near (e.g., upstream or downstream) the *C9orf72* exon 1A transcription start site. The nuclease target site can be upstream of the *C9orf72* exon 1A transcription start site. Transcription of the *C9orf72* gene initiates at two alternative non-coding exons: exon 1A (upstream) and exon 1B (downstream). The G_4C_2 repeat lies between exons 1A and 1B. Exons 1A and 1B can be spliced to exon 2, the first protein-coding exon, creating mRNAs with alternative 5'-untranslated regions. In healthy people with short G_4C_2 repeat expansions, transcription predominantly initiates at exon 1B; RNAs that include exon 1A are rare, and repeat-containing RNAs are undetectable. People suffering from C9orf72 ALS or FTLD accumulate transcripts in which exon 1A is spliced to exon 2, and both sense and antisense repeat-containing RNAs and the DPR proteins translated from them can be detected by *in situ* hybridization and immunohistochemistry. In one example, the nuclease target sequence can be within about 2500, within about 2250, within about 2000, within about 1800, within about 1600, within about 1400, within about 1200, within about 1000, within about 900, within about

800, within about 700, within about 600, within about 500, within about 450, within about 400, within about 350, within about 300, within about 250, within about 225, within about 200, within about 175, within about 150, within about 125, within about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 nucleotides of the *C9orf72* exon 1A transcription start site. In one example, the nuclease target sequence can be within about 250, within about 225, within about 200, within about 175, within about 150, within about 125, within about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 nucleotides of the *C9orf72* exon 1A transcription start site. In another example, the nuclease target sequence can be within about 125, within about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 of the *C9orf72* exon 1A transcription start site.

[00300] The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site.

[00301] The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion that encompasses the *C9orf72* exon 1A transcription start site. Alternatively, the nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion or modification that does not encompass deletion or modification of the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion of *C9orf72* exon 1A. In other embodiments, the targeted genetic modification does not result in deletion of the *C9orf72* hexanucleotide repeat expansion sequence.

[00302] The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of transcripts that initiate at *C9orf72* exon 1A to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts to a

greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

[00303] In some of the compositions and methods disclosed herein, two or more nuclease agents or CRISPR/Cas systems are used to target two or more nuclease target sequences (e.g., guide RNA target sequences) in a *C9orf72* gene. For example, the first nuclease target sequence can be upstream of the *C9orf72* exon 1A transcription start site as described above. In some cases, the second nuclease target sequence is also upstream (i.e., 5') of the *C9orf72* exon 1A transcription start site. For example, the second nuclease target sequence can be within about 2500, within about 2250, within about 2000, within about 1800, within about 1600, within about 1400, within about 1200, within about 1000, within about 900, within about 800, within about

700, within about 600, within about 500, within about 450, within about 400, within about 350, within about 300, within about 250, within about 225, within about 200, within about 175, within about 150, within about 125, within about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 nucleotides of the *C9orf72* exon 1A transcription start site. In some cases, the second nuclease target sequence is downstream (i.e., 3') of the *C9orf72* exon 1A transcription start site. For example, the second nuclease target sequence can be within about 2500, within about 2250, within about 2000, within about 1800, within about 1600, within about 1400, within about 1200, within about 1000, within about 900, within about 800, within about 700, within about 600, within about 500, within about 450, within about 400, within about 350, within about 300, within about 250, within about 225, within about 200, within about 175, within about 150, within about 125, within about 100, within about 75, within about 50, within about 25, within about 20, or within about 10 nucleotides of the *C9orf72* exon 1A transcription start site. In one example, the second nuclease target sequence can be downstream (i.e., 3') of the *C9orf72* exon 1A transcription start site and within exon 1A. In another example, the second nuclease target sequence can be downstream (i.e., 3') of the *C9orf72* exon 1A transcription start site but not within exon 1A. In another example, the second nuclease target sequence can be downstream (i.e., 3') of the *C9orf72* exon 1A transcription start site but upstream (i.e., 5') of the *C9orf72* hexanucleotide repeat expansion sequence. In another example, the second nuclease target sequence can be downstream (i.e., 3') of *C9orf72* exon 1A but upstream (i.e., 5') of the *C9orf72* hexanucleotide repeat expansion sequence. In another example, the second nuclease target sequence can be downstream (i.e., 3') of the *C9orf72* hexanucleotide repeat expansion sequence but upstream of *C9orf72* exon 1B.

[00304] In one example, the first and second nuclease target sequences flank a region of the *C9orf72* promoter upstream of the *C9orf72* exon 1A transcription start site such that the region of the promoter is deleted. In another example, the first and second nuclease target sequences flank the *C9orf72* exon 1A transcription start site such that the *C9orf72* exon 1A transcription start site is deleted. In another example, the first and second nuclease target sequences flank *C9orf72* exon 1A such that *C9orf72* exon 1A is deleted. In another example, the first and second nuclease target sequences flank a region of the *C9orf72* gene comprising *C9orf72* exon 1A and the *C9orf72* hexanucleotide repeat expansion sequence such that the region of the *C9orf72* gene comprising *C9orf72* exon 1A and the *C9orf72* hexanucleotide repeat expansion sequence is

deleted (e.g., but no portion of *C9orf72* exon 1B is deleted).

[00305] When two or more nuclease agents or CRISPR/Cas systems are used, the two nuclease target sequences, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents results in a deletion that encompasses the *C9orf72* exon 1A transcription start site. Alternatively, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents results in a deletion that does not encompass the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion of *C9orf72* exon 1A. In other embodiments, the targeted genetic modification does not result in deletion of the *C9orf72* hexanucleotide repeat expansion sequence.

[00306] The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72*

exon 1A transcription start site.

[00307] When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of transcripts that initiate at *C9orf72* exon 1A to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic

modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

[00308] Nuclease target sequences (e.g., guide RNA target sequences) can also be selected to minimize off-target modification or avoid off-target effects (e.g., by avoiding two or fewer mismatches to off-target genomic sequences).

[00309] In methods in which one or more exogenous donor nucleic acids are used to modify the *C9orf72* gene following cleavage by the nuclease agent (e.g., Cas protein), the nuclease agent can cleave the target genomic locus to create a single-strand break (nick) or double-strand break, and the cleaved or nicked locus can be repaired by the exogenous donor nucleic acid via non-homologous end joining (NHEJ)-mediated insertion or homology-directed repair. Optionally, repair with the exogenous donor nucleic acid removes or disrupts the nuclease target sequence(s) so that alleles that have been targeted cannot be re-targeted by the nuclease agent(s).

[00310] The exogenous donor nucleic acid can target any sequence in *C9orf72* gene. Some exogenous donor nucleic acids comprise homology arms. Other exogenous donor nucleic acids

do not comprise homology arms. The exogenous donor nucleic acids can be capable of insertion into a *C9orf72* gene by homology-directed repair, and/or they can be capable of insertion into a *C9orf72* gene by non-homologous end joining.

[00311] 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. Exogenous donor nucleic acids can be naked nucleic acids or can be delivered by viruses, such as AAV. In a specific example, the exogenous donor nucleic acid can be delivered via AAV and can be capable of insertion into a the *C9orf72* gene by non-homologous end joining (e.g., the exogenous donor nucleic acid can be one that does not comprise homology arms).

[00312] An exemplary exogenous donor nucleic acid is between about 50 nucleotides to about 5 kb in length or between about 50 nucleotides to about 3 kb in length. Alternatively, an exogenous donor nucleic acid can be between about 1 kb to about 1.5 kb, about 1.5 kb to about 2 kb, about 2 kb to about 2.5 kb, about 2.5 kb to about 3 kb, about 3 kb to about 3.5 kb, about 3.5 kb to about 4 kb, about 4 kb to about 4.5 kb, or about 4.5 kb to about 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, or 2.5 kb in length.

[00313] In one example, an exogenous donor nucleic acid is an ssODN that is between about 80 nucleotides and about 3 kb in length. Such an ssODN can have homology arms or short single-stranded regions at the 5' end and/or the 3' end that are complementary to one or more overhangs created by Cas-agent-mediated cleavage at the target genomic locus, for example, that are each between about 40 nucleotides and about 60 nucleotides in length. Such an ssODN can also have homology arms or complementary regions, for example, that are each between about 30 nucleotides and 100 nucleotides in length. The homology arms or complementary regions can be symmetrical (e.g., each 40 nucleotides or each 60 nucleotides in length), or they can be asymmetrical (e.g., one homology arm or complementary region that is 36 nucleotides in length, and one homology arm or complementary region that is 91 nucleotides in length).

[00314] 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).

[00315] The exogenous donor nucleic acids disclosed herein also comprise nucleic acid inserts including segments of DNA to be integrated at target genomic loci. Integration of a nucleic acid insert at a target genomic locus can result in addition of a nucleic acid sequence of interest to the target genomic locus or replacement of a nucleic acid sequence of interest at the target genomic locus (i.e., deletion and insertion). Some exogenous donor nucleic acids are designed for deletion of a nucleic acid sequence at a target genomic locus without any corresponding insertion at the target genomic locus. Some exogenous donor nucleic acids are designed for insertion of a nucleic acid insert at a target genomic locus without any corresponding deletion at the target genomic locus. Other exogenous donor nucleic acids are designed to delete a nucleic acid sequence of interest at a target genomic locus and replace it with a nucleic acid insert.

[00316] The nucleic acid insert or the corresponding nucleic acid at the target genomic locus being deleted and/or replaced can be various lengths. An exemplary nucleic acid insert or corresponding nucleic acid at the target genomic 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 3 kb nucleotides in length. For example, a nucleic acid insert or a corresponding nucleic acid at the

target genomic locus being deleted and/or replaced can be between about 1 to about 100, about 100 to about 200, about 200 to about 300, about 300 to about 400, about 400 to about 500, about 500 to about 600, about 600 to about 700, about 700 to about 800, about 800 to about 900, or about 900 to about 1,000 nucleotides in length. Likewise, a nucleic acid insert or a corresponding nucleic acid at the target genomic locus being deleted and/or replaced can be between about 1 kb to about 1.5 kb, about 1.5 kb to about 2 kb, about 2 kb to about 2.5 kb, about 2.5 kb to about 3 kb, about 3 kb to about 3.5 kb, about 3.5 kb to about 4 kb, about 4 kb to about 4.5 kb, about 4.5 kb to about 5 kb in length, or longer.

[00317] The nucleic acid insert or the corresponding nucleic acid at the target genomic locus being deleted and/or replaced can be a coding region such as an exon; a non-coding region such as an intron, an untranslated region, or a regulatory region (e.g., a promoter or an enhancer); or any combination thereof.

[00318] The nucleic acid insert can also comprise a conditional allele. 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.

[00319] Nucleic acid inserts can also comprise a polynucleotide encoding a selection marker. Alternatively, the nucleic acid inserts can lack a polynucleotide encoding a selection marker. The selection marker can be contained in a selection cassette. Optionally, the selection cassette can be a self-deleting cassette. *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. Exemplary 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), or herpes simplex virus thymidine kinase (HSV-k), or a combination thereof. 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.

[00320] The nucleic acid insert can also comprise a reporter gene. Exemplary reporter genes include those encoding luciferase, β-galactosidase, green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), enhanced yellow fluorescent protein (eYFP), blue fluorescent protein (BFP), enhanced blue fluorescent protein (eBFP), DsRed, ZsGreen, MmGFP, mPlum, mCherry, tdTomato, mStrawberry, J-Red, mOrange, mKO, mCitrine, Venus, YPet, Emerald, CyPet, Cerulean, T-Sapphire, and alkaline phosphatase. Such reporter genes can be operably linked to a promoter active in a cell being targeted. Examples of promoters are described elsewhere herein.

[00321] The nucleic acid insert can also comprise one or more expression cassettes or deletion cassettes. A given cassette can comprise one or more of a nucleotide sequence of interest, a polynucleotide encoding a selection marker, and a reporter gene, along with various regulatory components that influence expression. Examples of selectable markers and reporter genes that can be included are discussed in detail elsewhere herein.

[00322] The nucleic acid insert can comprise a nucleic acid flanked with site-specific recombination target sequences. Alternatively, the nucleic acid insert can comprise one or more site-specific recombination target sequences. Although the entire nucleic acid insert can be flanked by such site-specific recombination target sequences, any region or individual polynucleotide of interest within the nucleic acid insert can also be flanked by such sites. Site-specific recombination target sequences, which can flank the nucleic acid insert or any polynucleotide of interest in the nucleic acid insert can include, for example, loxP, lox511, lox2272, lox66, lox71, loxM2, lox5171, FRT, FRT11, FRT71, attp, att, FRT, rox, or a combination thereof. In one example, the site-specific recombination sites flank a polynucleotide encoding a selection marker and/or a reporter gene contained within the nucleic acid insert. Following integration of the nucleic acid insert at a targeted locus, the sequences between the

site-specific recombination sites can be removed. Optionally, two exogenous donor nucleic acids can be used, each with a nucleic acid insert comprising a site-specific recombination site. The exogenous donor nucleic acids can be targeted to 5' and 3' regions flanking a nucleic acid of interest. Following integration of the two nucleic acid inserts into the target genomic locus, the nucleic acid of interest between the two inserted site-specific recombination sites can be removed.

[00323] Nucleic acid inserts can also comprise one or more restriction sites for restriction endonucleases (i.e., restriction enzymes), which include Type I, Type II, Type III, and Type IV endonucleases. Type I and Type III restriction endonucleases recognize specific recognition sites, but typically cleave at a variable position from the nuclease binding site, which can be hundreds of base pairs away from the cleavage site (recognition site). In Type II systems the restriction activity is independent of any methylase activity, and cleavage typically occurs at specific sites within or near to the binding site. Most Type II enzymes cut palindromic sequences, however Type IIa enzymes recognize non-palindromic recognition sites and cleave outside of the recognition site, Type IIb enzymes cut sequences twice with both sites outside of the recognition site, and Type IIs enzymes recognize an asymmetric recognition site and cleave on one side and at a defined distance of about 1-20 nucleotides from the recognition site. Type IV restriction enzymes target methylated DNA. Restriction enzymes are further described and classified, for example in the REBASE database (webpage at rebase.neb.com; Roberts et al., (2003) *Nucleic Acids Res.* 31:418-420; Roberts et al., (2003) *Nucleic Acids Res.* 31:1805-1812; and Belfort et al. (2002) in Mobile DNA II, pp. 761-783, Eds. Craigie et al., (ASM Press, Washington, DC)).

[00324] Some exogenous donor nucleic acids are capable of insertion into a *C9orf72* gene by non-homologous end joining. In some cases, such exogenous donor nucleic acids do not comprise homology arms. For example, such exogenous donor nucleic acids can be inserted into a blunt end double-strand break following cleavage with a nuclease agent. In a specific example, the exogenous donor nucleic acid can be delivered via AAV and can be capable of insertion into a *C9orf72* gene by non-homologous end joining (e.g., the exogenous donor nucleic acid can be one that does not comprise homology arms).

[00325] In a specific example, the exogenous donor nucleic acid can be inserted via homology-independent targeted integration. For example, the nucleic acid insert in the

exogenous donor nucleic acid is flanked on each side by a guide RNA target sequence (e.g., the same target site as in the *C9orf72* gene, and the CRISPR/Cas reagent (Cas protein and guide RNA) being used to cleave the target site in the *C9orf72* gene). The Cas protein can then cleave the target sites flanking the nucleic acid insert. In a specific example, the exogenous donor nucleic acid is delivered AAV-mediated delivery, and cleavage of the target sites flanking the nucleic acid insert can remove the inverted terminal repeats (ITRs) of the AAV. In some methods, the target site in the *C9orf72* gene (e.g., a guide RNA target sequence including the flanking protospacer adjacent motif) is no longer present if the nucleic acid insert is inserted into the *C9orf72* gene in the correct orientation but it is reformed if the nucleic acid insert is inserted into the *C9orf72* gene in the opposite orientation.

[00326] Other 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 *C9orf72* gene. 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 *C9orf72* gene. 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 *C9orf72* gene or only at the 3' end complementary to an overhang created at a 3' target sequence at the *C9orf72* gene. 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 *C9orf72* gene. 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.

[00327] 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 to about 10, about 10 to about 20, about 20 to about 30, about 30 to about 40, about 40 to about 50, about 50 to about 60, about 60 to about 70, about 70 to about 80, about 80 to about 90, about 90 to about 100, about 100 to about 110, about 110 to about 120, about 120 to about 130, about 130 to about 140, about 140 to about 150 nucleotides in length, or longer.

[00328] 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). 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. Biotechnol.* 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.

[00329] 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 target genomic locus in the *C9orf72* gene, which are referred to herein as "5' target sequence" and "3' target sequence," respectively.

[00330] 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 to about 30, about 30 to about 40, about 40 to about 50, about 50 to about 60, about 60 to about 70, about 70 to about 80, about 80 to about 90, about 90 to about 100, about 100 to about 150, about 150 to about 200, about 200 to about 250, about 250 to about 300, about 300 to about 350, about 350 to about 400, about 400 to about 450, or about 450 to about 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).

[00331] In some methods, the exogenous donor nucleic acid can be a "large targeting vector" or "LTVEC," which includes targeting vectors that comprise homology arms that correspond to and are derived from nucleic acid sequences larger than those typically used by other approaches intended to perform homologous recombination in cells. LTVECs also include targeting vectors comprising nucleic acid inserts having nucleic acid sequences larger than those typically used by other approaches intended to perform homologous recombination in cells. For example, LTVECs make possible the modification of large loci that cannot be accommodated by traditional plasmid-based targeting vectors because of their size limitations. For example, the targeted locus can be (i.e., the 5' and 3' homology arms can correspond to) a locus of the cell that is not targetable using a conventional method or that can be targeted only incorrectly or only with significantly low efficiency in the absence of a nick or double-strand break induced by a nuclease agent (e.g., a Cas protein). LTVECs can be of any length and are typically at least 10 kb in length. The sum total of the 5' homology arm and the 3' homology arm in an LTVEC is typically at least 10 kb. Generation and use of large targeting vectors (LTVECs) derived from bacterial artificial chromosome (BAC) DNA through bacterial homologous recombination (BHR) reactions using VELOCIGENE® genetic engineering technology is described, e.g., in US 6,586,251 and Valenzuela et al. (2003) *Nat. Biotechnol.* 21(6):652-659, each of which is herein incorporated by reference in its entirety for all purposes. Generation of LTVECs through *in vitro* assembly methods is described, e.g., in US 2015/0376628 and WO 2015/200334, each of which is herein incorporated by reference in its entirety for all purposes. CRISPR/Cas-assisted LTVEC targeting is described, e.g., in US 9,546,384; US 2015-0159174; US 9,228,208; US 2015-0159175; US 2016-0060657; US 10,208,317; US 2017-0067078; US 10,711,280; US 2019-0112619; and WO 2015/088643, each of which is herein incorporated by reference in its entirety for all purposes.

[00332] When a nuclease agent is used in combination with an exogenous donor nucleic acid, the 5' and 3' target sequences can be located in sufficient proximity to the nuclease agent cleavage site (e.g., within sufficient proximity to a guide RNA 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 agent site. The term "nuclease agent cleavage site" includes a DNA sequence at which a nick or double-strand break is created by a nuclease agent (e.g., Cas 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 agent 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 agent 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 agent cleavage site or within at least 10 nucleotides to about 1,000 nucleotides of a given nuclease agent cleavage site. As an example, the nuclease agent cleavage site can be immediately adjacent to at least one or both of the target sequences.

[00333] The spatial relationship of the target sequences that correspond to the homology arms of the exogenous donor nucleic acid and the nuclease agent cleavage site can vary. For example, target sequences can be located 5' to the nuclease agent cleavage site, target sequences can be located 3' to the nuclease agent cleavage site, or the target sequences can flank the nuclease agent cleavage site.

[00334] Repair in response to cleavage of the *C9orf72* gene by a nuclease agent in the methods described herein can occur through any repair pathway. For example, repair can occur via homologous recombination (HR) or non-homologous end joining (NHEJ). 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) *Semin. Cell Dev. Biol.* 22(8):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.

[00335] 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.

[00336] 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.

[00337] Various types of targeted genetic modifications in a *C9orf72* gene can be introduced using the methods described herein. Such targeted modifications can include, for example, additions of one or more nucleotides, deletions of one or more nucleotides, substitutions of one or more nucleotides, a point mutation, or a combination thereof. For example, at least 1, 2, 3, 4, 5, 7, 8, 9, 10 or more nucleotides can be changed (e.g., deleted, inserted, or substituted) to form the targeted genomic modification. The deletions, insertions, or substitutions can be of any size, as disclosed elsewhere herein. *See, e.g.*, 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.

[00338] The nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion that encompasses the *C9orf72* exon 1A transcription start site. Alternatively, the nuclease target sequence can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion or modification that does not encompass deletion or modification of the *C9orf72* exon 1A transcription start site.

[00339] In one example, the first and second nuclease target sequences flank a region of the *C9orf72* promoter upstream of the *C9orf72* exon 1A transcription start site such that the region of the promoter is deleted. In another example, the first and second nuclease target sequences flank the *C9orf72* exon 1A transcription start site such that the *C9orf72* exon 1A transcription start site is deleted. In another example, the first and second nuclease target sequences flank *C9orf72* exon 1A such that *C9orf72* exon 1A is deleted. In another example, the first and second nuclease target sequences flank a region of the *C9orf72* gene comprising *C9orf72* exon 1A and the *C9orf72* hexanucleotide repeat expansion sequence such that the region of the *C9orf72* gene comprising *C9orf72* exon 1A and the *C9orf72* hexanucleotide repeat expansion sequence is deleted (e.g., but no portion of *C9orf72* exon 1B is deleted).

[00340] When two or more nuclease agents or CRISPR/Cas systems are used, the two

nuclease target sequences, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents results in a deletion that encompasses the *C9orf72* exon 1A transcription start site. Alternatively, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents results in a deletion that does not encompass the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion of *C9orf72* exon 1A. In other embodiments, the targeted genetic modification does not result in deletion of the *C9orf72* hexanucleotide repeat expansion sequence.

[00341] The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides upstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site. The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent results in a deletion within 125, within 100, within 75, or within 50 nucleotides downstream of the *C9orf72* exon 1A transcription start site.

[00342] When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease

target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of transcripts that initiate at *C9orf72* exon 1A to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to

expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. When two or more nuclease agents or CRISPR/Cas systems are used, the nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agent selectively reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduces expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than it reduces expression of transcripts that initiate at *C9orf72* exon 1B). The nuclease target sequences can be positioned such that a targeted genetic modification resulting from cleavage by the nuclease agents reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

[00343] In a specific example, a targeted modification can comprise a deletion between first and second nuclease target sequences or nuclease agent cleavage sites. If an exogenous donor sequence (e.g., repair template or targeting vector) is used, the modification can comprise a deletion between first and second nuclease target sequences or nuclease agent cleavage sites as well as an insertion of a nucleic acid insert between the 5' and 3' target sequences.

[00344] Alternatively, if an exogenous donor sequence is used in combination with a nuclease agent, the modification can comprise a deletion between the 5' and 3' target sequences as well as an insertion of a nucleic acid insert between the 5' and 3' target sequences in the pair of first and second homologous chromosomes, thereby resulting in a homozygous modified genome. Alternatively, if the exogenous donor sequence comprises 5' and 3' homology arms with no nucleic acid insert, the modification can comprise a deletion between the 5' and 3' target sequences.

[00345] The deletion between the first and second nuclease target sequences or nuclease agent cleavage sites or the deletion between the 5' and 3' target sequences can be a precise deletion

wherein the deleted nucleic acid consists of only the nucleic acid sequence between the first and second nuclease cleavage sites or only the nucleic acid sequence between the 5' and 3' target sequences such that there are no additional deletions or insertions at the modified genomic target locus. The deletion between the first and second nuclease target sequences or nuclease agent cleavage sites can also be an imprecise deletion extending beyond the first and second nuclease cleavage sites, consistent with imprecise repair by non-homologous end joining (NHEJ), resulting in additional deletions and/or insertions at the modified genomic locus. For example, the deletion can extend about 1 bp, about 2 bp, about 3 bp, about 4 bp, about 5 bp, about 10 bp, about 20 bp, about 30 bp, about 40 bp, about 50 bp, about 100 bp, about 200 bp, about 300 bp, about 400 bp, about 500 bp, or more beyond the first and second Cas protein cleavage sites. Likewise, the modified genomic locus can comprise additional insertions consistent with imprecise repair by NHEJ, such as insertions of about 1 bp, about 2 bp, about 3 bp, about 4 bp, about 5 bp, about 10 bp, about 20 bp, about 30 bp, about 40 bp, about 50 bp, about 100 bp, about 200 bp, about 300 bp, about 400 bp, about 500 bp, or more.

[00346] The targeted genetic modification can be, for example, a biallelic modification or a monoallelic modification. Biallelic modifications include events in which the same modification is made to the same locus on corresponding homologous chromosomes (e.g., in a diploid cell), or in which different modifications are made to the same locus on corresponding homologous chromosomes. In some methods, the targeted genetic modification is a monoallelic modification. A monoallelic modification includes events in which a modification is made to only one allele (i.e., a modification to the *C9orf72* gene in only one of the two homologous chromosomes). Homologous chromosomes include chromosomes that have the same genes at the same loci but possibly different alleles (e.g., chromosomes that are paired during meiosis). The term allele includes any of one or more alternative forms of a genetic sequence. In a diploid cell or organism, the two alleles of a given sequence typically occupy corresponding loci on a pair of homologous chromosomes.

[00347] A monoallelic mutation can result in a cell that is heterozygous for the targeted *C9orf72* modification. Heterozygosity includes situation in which only one allele of the *C9orf72* gene (i.e., corresponding alleles on both homologous chromosomes) have the targeted modification.

[00348] A biallelic modification can result in homozygosity for a targeted modification.

Homozygosity includes situations in which both alleles of the *C9orf72* gene (i.e., corresponding alleles on both homologous chromosomes) have the targeted modification. Alternatively, a biallelic modification can result in compound heterozygosity (e.g., hemizygosity) for the targeted modification. Compound heterozygosity includes situations in which both alleles of the target locus (i.e., the alleles on both homologous chromosomes) have been modified, but they have been modified in different ways (e.g., a targeted modification in one allele and inactivation or disruption of the other allele). For example, in the allele without the targeted modification, a double-strand break created by the nuclease agent may have been repaired by non-homologous end joining (NHEJ)-mediated DNA repair, which generates a mutant allele comprising an insertion or a deletion of a nucleic acid sequence and thereby causes disruption of that genomic locus. For example, a biallelic modification can result in compound heterozygosity if the cell has one allele with the targeted modification and another allele that is not capable of being expressed. Compound heterozygosity includes hemizygosity. Hemizygosity includes situations in which only one allele (i.e., an allele on one of two homologous chromosomes) of the target locus is present. For example, a biallelic modification can result in hemizygosity for a targeted modification if the targeted modification occurs in one allele with a corresponding loss or deletion of the other allele.

[00349] Methods for measuring expression of transcripts that initiate at *C9orf72* exon 1A, expression of transcripts that initiate at *C9orf72* exon 1B, and expression of sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts are known and are described elsewhere herein. Assessment of expression (or measuring RNA foci and dipeptide repeats as described elsewhere herein) can be in any cell type (e.g., neurons, such as motor neurons).

[00350] In some methods, the resulting percent expression from the *C9orf72* exon 1A transcription start site (i.e., expression of transcripts comprising exon 1A) in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00351] In some methods, the percent decrease in expression from the *C9orf72* exon 1A transcription start site in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00352] In some methods, the resulting percent expression of *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00353] In some methods, the percent decrease in expression of *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00354] In some methods, the resulting percent expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than

about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00355] In some methods, the percent decrease in expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00356] In some methods, the resulting percent expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00357] In some methods, the percent decrease in expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00358] In some methods, the resulting percent expression of both sense and antisense

C9orf72 hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00359] In some methods, the percent decrease in expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00360] In some methods, the percent decrease in expression of polyGA dipeptide repeat proteins in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00361] In some methods, the percent decrease in expression of polyGP dipeptide repeat proteins in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease

can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00362] In some methods, the percent decrease in expression of polyGA and polyGP dipeptide repeat proteins in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00363] Such methods can, for example, reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1A. Such methods can also, for example, reduce or abolish expression of *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can also, for example, reduce or abolish expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can also, for example, reduce or abolish expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can also, for example, reduce or abolish expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can, for example, selectively or preferentially reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1A relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduce expression of transcripts that initiate at *C9orf72* exon 1A to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduce expression of *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of antisense

C9orf72 hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). In other embodiments, the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion of *C9orf72* exon 1A. In other embodiments, the targeted genetic modification does not result in deletion of the *C9orf72* hexanucleotide repeat expansion sequence. In some methods, the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

[00364] Some methods result in a decrease in sense and/or antisense repeat-containing RNA foci in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*). Some methods result in a decrease in dipeptide repeat proteins (e.g., poly(glycine-alanine), poly(glycine-proline), poly(glycine-arginine), poly(alanine-proline), and/or poly(proline-arginine)) synthesized by

repeat-associated non-AUG-dependent translation from the sense and antisense repeat-containing RNAs in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*).

[00365] In the methods described herein, introduction or administration of the nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid) can result in a durable effect in editing of the *C9orf72* gene, and/or in reducing or abolishing expression of transcripts that initiate at *C9orf72* exon 1A, and/or in reducing or abolishing expression of *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. For example, the durable effect can extend at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, or at least 15 weeks, or it can extend at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 18, at least 24, at least 30, or at least 36 months, or it can extend at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 years. In one example, a single dose of the nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid) can result in a durable effect in editing of the *C9orf72* gene, and/or in reducing or abolishing expression of transcripts that initiate at *C9orf72* exon 1A, and/or in reducing or abolishing expression of *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts.

B. Prophylactic or Therapeutic Applications

[00366] The nuclease agents and CRISPR/Cas systems (and optionally exogenous donor nucleic acids) disclosed herein for targeting a *C9orf72* gene and the methods of modifying a *C9orf72* gene are useful for the treatment and/or prevention of a *C9orf72* hexanucleotide repeat expansion associated disease and/or for ameliorating at least one symptom or indication

associated with such disease. In a particular example, the disease is amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD). ALS, also referred to as Lou Gehrig's disease, is the most frequent adult-onset paralytic disorder, characterized by the loss of upper and/or lower motor neurons. ALS occurs in as many as 20,000 individuals across the United States with about 5,000 new cases occurring each year. Frontotemporal dementia (also referred to as Pick's disease, frontotemporal lobar degeneration, or FTLD) is a group of disorders caused by progressive cell degeneration in the frontal or temporal lobes of the brain. FTD is reported to account for 10%-15% of all dementia cases. A hexanucleotide repeat expansion sequence between exon 1A and 1B, two non-coding exons of the human *C9ORF72* gene, has been linked to both ALS and FTD. It is estimated that the G_4C_2 hexanucleotide repeat expansion accounts for about 50% of familial and many non-familial ALS cases. It is present in about 25% of familial FTD cases and about 8% of sporadic FTD cases.

[00367] Many signs or symptoms related to the hexanucleotide repeat expansion sequence in *C9ORF72* have been reported such as, for example, repeat-length-dependent formation of RNA foci, sequestration of specific RNA-binding proteins, and accumulation and aggregation of dipeptide repeat proteins (e.g., poly(glycine-alanine), poly(glycine-proline), poly(glycine-arginine), poly(alanine-proline), or poly(proline-arginine) dipeptide repeat proteins) resulting from repeat-associated non-AUG (AUG) translation.

[00368] It is not known how the *C9orf72* hexanucleotide repeat expansion causes motor neuron disease and dementia, but two universal postmortem pathological findings in *C9orf72* ALS and FTD patients are associated with the repeat expansion: (1) sense and antisense repeat-containing RNA can be visualized as distinct foci in neurons and other cells; and (2) dipeptide repeat proteins—poly(glycine-alanine), poly(glycine-proline), poly(glycine-arginine), poly(alanine-proline), and poly(proline-arginine)—synthesized by repeat-associated non-AUG-dependent translation from the sense and antisense repeat-containing RNAs can be detected in cells. One disease hypothesis proposes that the repeat-containing RNAs, visualized as foci, disrupt cellular RNA metabolism by sequestering RNA binding proteins. Another disease hypothesis posits that the dipeptide repeat proteins exert wide-spread toxic effects on RNA metabolism, proteostasis, and nucleocytoplasmic transport.

[00369] Another symptom or indication is the expression of repeat-containing RNAs from the *C9orf72* gene. The *C9orf72* gene produces transcripts from two transcription initiation sites. The

upstream site initiates transcription with alternative non-coding exon 1A, while the downstream site initiates transcription with alternative exon 1B. Both exons 1A and 1B can be spliced to exon 2, which contains the start of the protein-coding sequence. The pathogenic hexanucleotide repeat expansion is located between exons 1A and 1B. Therefore, transcription initiated from exon 1A can produce repeat-containing RNAs, while initiation from exon 1B cannot.

[00370] In some methods, a nuclease agent or CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene may be administered at a therapeutic dose to a subject with a *C9orf72* hexanucleotide repeat expansion associated disease. Such methods can comprise administering to a subject a therapeutically effective amount of the nuclease agent or CRISPR/Cas system (and optionally an exogenous donor nucleic acid) to the subject. A nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene can also be used for the preparation of a pharmaceutical composition or medicament for treating patients suffering from a *C9orf72* hexanucleotide repeat expansion associated disease. Therapeutic or pharmaceutical compositions comprising a nuclease agent or CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene can be administered with suitable carriers, excipients, and other agents that are incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. *See also* Powell et al. "Compendium of excipients for parenteral formulations" PDA (1998) *J. Pharm. Sci. Technol.* 52:238-311.

[00371] Likewise, the methods for modifying or a *C9orf72* gene disclosed herein can be used for treating a *C9orf72* hexanucleotide repeat expansion associated disease. Such therapeutic methods can comprise administering a therapeutically effective amount of a pharmaceutical composition comprising a nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene to the subject in need thereof. The *C9orf72* hexanucleotide repeat expansion associated disease treated can be any disease or condition associated with *C9orf72* hexanucleotide repeat expansion. Some such methods prevent, treat, or ameliorate at least one symptom of a *C9orf72* hexanucleotide repeat expansion associated disease (described above), the method comprising administering a therapeutically

effective amount of a nuclease agent or CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene to a subject in need thereof. In some methods, the nuclease agent or CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene may be administered prophylactically or therapeutically to a subject having or at risk of having a *C9orf72* hexanucleotide repeat expansion associated disease. The nuclease agent or CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene may be administered via intracerebroventricular injection, intracranial injection, intrathecal injection, or by any other suitable means.

[00372] A therapeutically effective amount is an amount that produces the desired effect for which it is administered. The exact amount will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. *See, e.g.*, Lloyd (1999) The Art, Science and Technology of Pharmaceutical Compounding.

[00373] A subject can be an animal, optionally a mammal, optionally a human, in need of amelioration, prevention, and/or treatment of a *C9orf72* hexanucleotide repeat expansion associated disease. The term includes human subjects who have or are at risk of having such a disease.

[00374] The terms treat, treating, or treatment refer to the reduction or amelioration of the severity of at least one symptom or indication of a *C9orf72* hexanucleotide repeat expansion associated disease due to the administration of a therapeutic agent such as a nuclease agent or a CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene to a subject in need thereof. The terms include inhibition of progression of disease or of worsening of a symptom/indication. The terms also include positive prognosis of disease (i.e., the subject may be free of disease or may have reduced disease upon administration of a therapeutic agent such as a nuclease agent or a CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene). The therapeutic agent may be administered at a therapeutic dose to the subject. The terms prevent, preventing, or prevention refer to inhibition of manifestation of a *C9orf72* hexanucleotide repeat expansion associated disease or any symptoms or indications of such a disease upon administration of a nuclease agent or a CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene.

[00375] In some methods, a single dose of a nuclease agent or a CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene may be administered to a subject in need thereof. In other methods, multiple doses of a nuclease agent or a CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene may be administered to a subject over a defined time course. Such methods can comprise sequentially administering to a subject multiple doses of a nuclease agent or a CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene. Sequentially administering means that each dose of the nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid) is administered to the subject at a different point in time, such as on different days separated by a predetermined interval (e.g., hours, days, weeks, or months). Some methods comprise sequentially administering to the patient a single initial dose of a nuclease agent or a CRISPR/Cas system (and optionally an exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene, followed by one or more secondary doses of the nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid), and optionally followed by one or more tertiary doses.

[00376] Initial dose, secondary doses, and tertiary doses refer to the temporal sequence of administration of the nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene. Thus, the initial dose is the dose which is administered at the beginning of the treatment regimen (also referred to as the baseline dose), the secondary doses are the doses which are administered after the initial dose, and the tertiary doses are the doses which are administered after the secondary doses. The initial, secondary, and tertiary doses may all contain the same amount of the nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid), but generally may differ from one another in terms of frequency of administration. In some methods, however, the amount of the nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid)contained in the initial, secondary, and/or tertiary doses varies from one another (e.g., adjusted up or down as appropriate) during the course of treatment. In some methods, two or more (e.g., 2, 3, 4, or 5) doses are administered at the beginning of the treatment regimen as loading doses followed by subsequent doses that are administered on a less frequent basis (e.g., maintenance doses).

[00377] Such methods may comprise administering to a patient any number of secondary and/or tertiary doses of a nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid) disclosed herein for targeting a *C9orf72* gene. In one example, only a single secondary dose is administered to the subject. In another example, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, or more) secondary doses are administered to the subject. Likewise, in another example, only a single tertiary dose is administered to the subject. In other examples, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, or more) tertiary doses are administered to the subject.

[00378] The frequency at which the secondary and/or tertiary doses are administered to a subject can vary over the course of the treatment regimen. The frequency of administration may also be adjusted during the course of treatment by a physician depending on the needs of the individual subject following clinical examination.

[00379] In some methods, the resulting percent expression from the *C9orf72* exon 1A transcription start site in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00380] In some methods, the percent decrease in expression from the *C9orf72* exon 1A transcription start site in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00381] In some methods, the resulting percent expression of *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be

less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00382] In some methods, the percent decrease in expression of *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00383] In some methods, the resulting percent expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00384] In some methods, the percent decrease in expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other

suitable time.

[00385] In some methods, the resulting percent expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00386] In some methods, the percent decrease in expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00387] In some methods, the resulting percent expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be less than about 90%, less than about 85%, less than about 80%, less than about 75%, less than about 70%, less than about 65%, less than about 60%, less than about 55%, less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, or less than about 25%. The resulting percent expression can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00388] In some methods, the percent decrease in expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in*

vitro, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00389] In some methods, the percent decrease in expression of polyGA dipeptide repeat proteins in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00390] In some methods, the percent decrease in expression of polyGP dipeptide repeat proteins in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00391] In some methods, the percent decrease in expression of polyGA and polyGP dipeptide repeat proteins in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*) can be at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, or at least about 75%. The percent decrease can be, e.g., at 1 week post-administration, 2 weeks post-administration, 3 weeks post-administration, or 4 weeks post-administration, or any other suitable time.

[00392] Such methods can, for example, reduce or abolish expression of transcripts that

initiate at *C9orf72* exon 1A. Such methods can also, for example, reduce or abolish expression of *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can also, for example, reduce or abolish expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can also, for example, reduce or abolish expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can also, for example, reduce or abolish expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. Such methods can, for example, selectively or preferentially reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1A relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduce expression of transcripts that initiate at *C9orf72* exon 1A to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (i.e., reduce expression of *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). Such methods can also, for example, selectively or preferentially reduce or abolish expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts relative to the effect on expression of transcripts that initiate at *C9orf72* exon 1B (e.g., reduce expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts to a greater extent than reducing expression of transcripts that initiate at *C9orf72* exon 1B). In other embodiments, the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site. In other embodiments, the targeted genetic modification does not result in deletion of *C9orf72* exon 1A.

In other embodiments, the targeted genetic modification does not result in deletion of the *C9orf72* hexanucleotide repeat expansion sequence. In some methods, the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B. In some methods, the targeted genetic modification reduces expression of polyGA dipeptide repeat proteins. In some methods, the targeted genetic modification reduces expression of polyGP dipeptide repeat proteins. In some methods, the targeted genetic modification reduces expression of both polyGA dipeptide repeat proteins and polyGP dipeptide repeat proteins.

[00393] Some methods result in a decrease in sense and/or antisense repeat-containing RNA foci in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*). Some methods result in a decrease in dipeptide repeat proteins (e.g., poly(glycine-alanine), poly(glycine-proline), poly(glycine-arginine), poly(alanine-proline), and/or poly(proline-arginine)) synthesized by repeat-associated non-AUG-dependent translation from the sense and antisense repeat-containing RNAs in treated cells (e.g., neurons, such as motor neurons) as compared to control untreated cells or as compared to pre-administration (*in vitro*, *ex vivo*, or *in vivo*).

[00394] Methods for measuring expression of transcripts that initiate at *C9orf72* exon 1A, expression of transcripts that initiate at *C9orf72* exon 1B, and expression of sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts are known and are described elsewhere herein. Assessment of expression (or measuring RNA foci and dipeptide repeats as described

elsewhere herein) can be in any cell type (e.g., neurons, such as motor neurons).

[00395] In the methods described herein, introduction or administration of the nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid) can result in a durable effect in editing of the *C9orf72* gene, and/or in reducing or abolishing expression of transcripts that initiate at *C9orf72* exon 1A, and/or in reducing or abolishing expression of *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts. For example, the durable effect can extend at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, or at least 15 weeks, or it can extend at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 18, at least 24, at least 30, or at least 36 months, or it can extend at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 years. In one example, a single dose of the nuclease agent or CRISPR/Cas system (and optionally exogenous donor nucleic acid) can result in a durable effect in editing of the *C9orf72* gene, and/or in reducing or abolishing expression of transcripts that initiate at *C9orf72* exon 1A, and/or in reducing or abolishing expression of *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of sense *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of antisense *C9orf72* hexanucleotide-repeat-containing transcripts, and/or in reducing or abolishing expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts.

[00396] The prophylactic or therapeutic methods described herein can be combined with other therapeutic or prophylactic treatments for *C9orf72* hexanucleotide repeat expansion associated diseases.

C. Administering Nuclease Agents and/or Exogenous Donor Nucleic Acids to Animals or Cells

[00397] The methods disclosed herein can comprise introducing into an animal (e.g., mammal, such as a human) or cell various nuclease agents (e.g., CRISPR/Cas reagents) targeting

C9orf72 (and optionally exogenous donor nucleic acids targeting *C9orf72*), including in the form of nucleic acids (e.g., DNA or RNA), proteins, or nucleic-acid-protein complexes. "Introducing" includes presenting to the cell or animal the molecule(s) (e.g., nucleic acid(s) or protein(s)) in such a manner that it gains access to the interior of the cell or to the interior of cells within the 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 animal simultaneously or sequentially in any combination. For example, a Cas protein can be introduced into a cell or 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 nuclease agent (e.g., a Cas protein and a guide RNA), or it can be introduced following introduction of the nuclease agent (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 nuclease agent). *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 animal by the same delivery method or different delivery methods. Similarly, two or more of the components can be introduced into an animal by the same route of administration or different routes of administration.

[00398] A nuclease agent can be introduced into an animal or cell or one or more nucleic acids encoding the nuclease agent can be introduced into the cell. A guide RNA can be introduced into an animal or cell, for example, in the form of an RNA (e.g., *in vitro* transcribed RNA) or in the form of a DNA encoding the guide RNA. Guide RNAs can be modified as disclosed elsewhere herein. When introduced in the form of a DNA, the DNA encoding a guide RNA can be operably linked to a promoter active in the cell or in a cell in the 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).

[00399] 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. Cas RNAs can be modified as disclosed elsewhere herein. 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 human 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 cell or an animal, the Cas protein can be transiently, conditionally, or constitutively expressed in the cell or in a cell in the animal.

[00400] 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. For example, a suitable promoter can be active in a neuron, such as a motor neuron. 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.

[00401] Molecules (e.g., Cas proteins or guide RNAs) introduced into the 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.

[00402] Various methods and compositions are provided herein to allow for introduction of molecule (e.g., a nucleic acid or protein) into a cell or 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.

[00403] Transfection protocols as well as protocols for introducing molecules 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. U.S.A.* 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.

[00404] Introduction of 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.

[00405] 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 optionally 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 optionally 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 optionally 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. U.S.A.* 107:15022-15026 and Meyer et al. (2012) *Proc. Natl. Acad. Sci. U.S.A.* 109:9354-9359, each of which is herein incorporated by reference in its entirety for all purposes.

[00406] Other methods for introducing molecules (e.g., nucleic acid or proteins) into a cell or 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 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 an animal include hydrodynamic delivery, virus-mediated delivery (e.g., adeno-associated virus (AAV)-mediated

delivery), and lipid-nanoparticle-mediated delivery.

[00407] Introduction of nucleic acids can also be accomplished by virus-mediated delivery, such as AAV-mediated delivery or lentivirus-mediated delivery. The vectors can be, for example, viral vectors such as adeno-associated virus (AAV) vectors. The AAV may be any suitable serotype and may be a single-stranded AAV (ssAAV) or a self-complementary AAV (scAAV). Other exemplary viruses/viral vectors include retroviruses, lentiviruses, 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). Viral vectors may be genetically modified from their wild type counterparts. For example, the viral vector may comprise an insertion, deletion, or substitution of one or more nucleotides to facilitate cloning or such that one or more properties of the vector is changed. Such properties may include packaging capacity, transduction efficiency, immunogenicity, genome integration, replication, transcription, and translation. In some examples, a portion of the viral genome may be deleted such that the virus is capable of packaging exogenous sequences having a larger size. In some examples, the viral vector may have an enhanced transduction efficiency. In some examples, the immune response induced by the virus in a host may be reduced. In some examples, viral genes (such as integrase) that promote integration of the viral sequence into a host genome may be mutated such that the virus becomes non-integrating. In some examples, the viral vector may be replication defective. In some examples, the viral vector may comprise exogenous transcriptional or translational control sequences to drive expression of coding sequences on the vector. In some examples, the virus may be helper-dependent. For example, the virus may need one or more helper components to supply viral components (such as viral proteins) required to amplify and package the vectors into viral particles. In such a case, one or more helper components, including one or more vectors encoding the viral components, may be introduced into a host cell or population of host cells along with the vector system described herein. In other examples, the virus may be helper-free. For example, the virus may be capable of amplifying and packaging the vectors without a helper virus. In some examples, the vector system described herein may also encode the viral

components required for virus amplification and packaging.

[00408] Exemplary viral titers (e.g., AAV titers) include about 10^{12} to about 10^{16} vg/mL. Other exemplary viral titers (e.g., AAV titers) include about 10^{12} to about 10^{16} vg/kg of body weight.

[00409] Adeno-associated viruses (AAVs) are endemic in multiple species including human and non-human primates (NHPs). At least 12 natural serotypes and hundreds of natural variants have been isolated and characterized to date. *See, e.g.*, Li et al. (2020) *Nat. Rev. Genet.* 21:255-272, herein incorporated by reference in its entirety for all purposes. AAV particles are naturally composed of a non-enveloped icosahedral protein capsid containing a single-stranded DNA (ssDNA) genome. The DNA genome is flanked by two inverted terminal repeats (ITRs) which serve as the viral origins of replication and packaging signals. The *rep* gene encodes four proteins required for viral replication and packaging whilst the *cap* gene encodes the three structural capsid subunits which dictate the AAV serotype, and the Assembly Activating Protein (AAP) which promotes virion assembly in some serotypes.

[00410] Recombinant AAV (rAAV) is currently one of the most commonly used viral vectors used in gene therapy to treat human diseases by delivering therapeutic transgenes to target cells *in vivo*. rAAV vectors are composed of icosahedral capsids similar to natural AAVs, but rAAV virions do not encapsidate AAV protein-coding or AAV replicating sequences. These viral vectors are non-replicating. The only viral sequences required in rAAV vectors are the two ITRs, which are needed to guide genome replication and packaging during manufacturing of the rAAV vector. rAAV genomes are devoid of AAV *rep* and *cap* genes, rendering them non-replicating *in vivo*. rAAV vectors are produced by expressing *rep* and *cap* genes along with additional viral helper proteins *in trans*, in combination with the intended transgene cassette flanked by AAV ITRs.

[00411] In rAAV genomes, a gene expression cassette can be placed between ITR sequences. Typically, rAAV genome cassettes comprise of a promoter to drive expression of a transgene, followed by a polyadenylation sequence. The ITRs flanking a rAAV expression cassette are usually derived from AAV2, the first serotype to be isolated and converted into a recombinant viral vector. Since then, most rAAV production methods rely on AAV2 *Rep*-based packaging systems. *See, e.g.*, Colella et al. (2017) *Mol. Ther. Methods Clin. Dev.* 8:87-104, herein incorporated by reference in its entirety for all purposes.

[00412] The specific serotype of a recombinant AAV vector influences its *in vivo* tropism to specific tissues. AAV capsid proteins are responsible for mediating attachment and entry into target cells, followed by endosomal escape and trafficking to the nucleus. Thus, the choice of serotype when developing a rAAV vector will influence what cell types and tissues the vector is most likely to bind to and transduce when injected *in vivo*.

[00413] Once in the nucleus, the ssDNA genome is released from the virion and a complementary DNA strand is synthesized to generate a double-stranded DNA (dsDNA) molecule. Double-stranded AAV genomes naturally circularize via their ITRs and become episomes which will persist extrachromosomally in the nucleus. Therefore, for episomal gene therapy programs, rAAV-delivered rAAV episomes provide long-term, promoter-driven gene expression in non-dividing cells. However, this rAAV-delivered episomal DNA is diluted out as cells divide. In contrast, the gene therapy described herein is based on gene insertion to allow long-term gene expression.

[00414] 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.

[00415] 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. The term AAV includes, for example, AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAVrh.64R1, AAVhu.37, AAVrh.8, AAVrh.32.33, AAV8, AAV9, AAV-DJ, AAV2/8, AAVrh10, AAVLK03, AV10, AAV11, AAV12, rh10, and hybrids thereof, avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, non-primate AAV, and ovine AAV. The genomic sequences of various serotypes of AAV, as well as the sequences of the native terminal repeats (TRs), Rep proteins, and capsid subunits are known in the art. Such

sequences may be found in the literature or in public databases such as GenBank. A "AAV vector" as used herein refers to an AAV vector comprising a heterologous sequence not of AAV origin (*i.e.*, a nucleic acid sequence heterologous to AAV), typically comprising a sequence encoding an exogenous polypeptide of interest. The construct may comprise an AAV1, AAV2, AAV3, AAV3B, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAVrh.64R1, AAVhu.37, AAVrh.8, AAVrh.32.33, AAV8, AAV9, AAV-DJ, AAV2/8, AAVrh10, AAVLK03, AV10, AAV11, AAV12, rh10, and hybrids thereof, avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, non-primate AAV, and ovine AAV capsid sequence. In general, the heterologous nucleic acid sequence (the transgene) is flanked by at least one, and generally by two, AAV inverted terminal repeat sequences (ITRs). An AAV vector may either be single-stranded (ssAAV) or self-complementary (scAAV). Serotypes for CNS tissue include AAV1, AAV2, AAV4, AAV5, AAV8, and AAV9. Selectivity of AAV serotypes for gene delivery in neurons is discussed, for example, in Hammond et al. (2017) *PLoS One* 12(12):e0188830, herein incorporated by reference in its entirety for all purposes. In a specific example, an AAV-PHP.eB vector is used. The AAV-PHP.eB vector shows high ability to cross the blood-brain barrier, increasing its CNS transduction efficiency. In another specific example, an AAV9 vector is used.

[00416] 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.

[00417] 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.

[00418] 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.

[00419] In certain AAVs, the cargo can include nucleic acids encoding one or more guide RNAs (e.g., DNA encoding a guide RNA, or DNA encoding two or more guide RNAs). In certain AAVs, the cargo can include a nucleic acid (e.g., DNA) encoding a Cas nuclease, such as Cas9, and DNA encoding one or more guide RNAs (e.g., DNA encoding a guide RNA, or DNA encoding two or more guide RNAs). In certain AAVs, the cargo can include an exogenous donor nucleic acid as described herein. In certain AAVs, the cargo can include a nucleic acid (e.g., DNA) encoding a Cas nuclease, such as Cas9, a DNA encoding a guide RNA (or multiple guide RNAs), and an exogenous donor nucleic acid.

[00420] For example, Cas or Cas9 and one or more gRNAs (e.g., 1 gRNA or 2 gRNAs or 3 gRNAs or 4 gRNAs) can be delivered via LNP-mediated delivery (e.g., in the form of RNA) or adeno-associated virus (AAV)-mediated delivery. For example, a Cas9 mRNA and a gRNA can be delivered via LNP-mediated delivery, or DNA encoding Cas9 and DNA encoding a gRNA can be delivered via AAV-mediated delivery. The Cas or Cas9 and the gRNA(s) can be delivered in a single AAV or via two separate AAVs. For example, a first AAV can carry a Cas or Cas9 expression cassette, and a second AAV can carry a gRNA expression cassette. Similarly, a first AAV can carry a Cas or Cas9 expression cassette, and a second AAV can carry two or more gRNA expression cassettes. Alternatively, a single AAV can carry a Cas or Cas9 expression cassette (e.g., Cas or Cas9 coding sequence operably linked to a promoter) and a gRNA expression cassette (e.g., gRNA coding sequence operably linked to a promoter). Similarly, a single AAV can carry a Cas or Cas9 expression cassette (e.g., Cas or Cas9 coding sequence

operably linked to a promoter) and two or more gRNA expression cassettes (e.g., gRNA coding sequences operably linked to promoters). Different promoters can be used to drive expression of the gRNA, such as a U6 promoter or the small tRNA Gln. Likewise, different promoters can be used to drive Cas9 expression. For example, small promoters are used so that the Cas9 coding sequence can fit into an AAV construct. Similarly, small Cas9 proteins (e.g., SaCas9 or CjCas9 are used to maximize the AAV packaging capacity).

[00421] Introduction of nucleic acids and proteins can also be accomplished by lipid nanoparticle (LNP)-mediated delivery. The lipid nanoparticles can comprise the nuclease agents (e.g., CRISPR/Cas systems) disclosed herein. The lipid nanoparticles can alternatively or additionally comprise an exogenous donor nucleic acid as disclosed herein. For example, the lipid nanoparticles can comprise a nuclease agent (e.g., CRISPR/Cas system), can comprise an exogenous donor nucleic acid, or can comprise both a nuclease agent (e.g., a CRISPR/Cas system) and an exogenous donor nucleic acid. Regarding CRISPR/Cas systems, the lipid nanoparticles can comprise the Cas protein in any form (e.g., protein, DNA, or mRNA) and/or can comprise the guide RNA(s) in any form (e.g., DNA or RNA). In one example, the lipid nanoparticles comprise the Cas protein in the form of mRNA (e.g., a modified RNA as described herein) and the guide RNA(s) in the form of RNA (e.g., a modified guide RNA as disclosed herein). As another example, the lipid nanoparticles can comprise the Cas protein in the form of protein and the guide RNA(s) in the form of RNA). In a specific example, the guide RNA and the Cas protein are each introduced in the form of RNA via LNP-mediated delivery in the same LNP. As discussed in more detail elsewhere herein, one or more of the RNAs can be modified. Delivery through such methods can result in transient Cas expression and/or transient presence of the guide RNA, 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*. *See, e.g.*, 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.

[00422] In some LNPs, the cargo can comprise Cas mRNA (e.g., Cas9 mRNA) and gRNA. The Cas mRNA and gRNAs can be in different ratios. In some LNPs, the cargo can comprise an exogenous donor nucleic acid and a nuclease agent (e.g., CRISPR/Cas system). The exogenous donor nucleic acid and nuclease agent components can be in different ratios.

[00423] Examples of suitable LNPs can be found, e.g., in WO 2019/067992, WO 2020/082042, US 2020/0270617, WO 2020/082041, US 2020/0268906, WO 2020/082046 (*see, e.g.*, pp. 85-86), and US 2020/0289628, each of which is herein incorporated by reference in its entirety for all purposes. A specific example of using LNPs to deliver to the brain is disclosed in Nabhan et al. (2016) *Sci. Rep.* 6:20019, herein incorporated by reference in its entirety for all purposes.

[00424] Exemplary dosing of LNPs includes about 0.1, about 0.25, about 0.3, about 0.5, about 1, about 2, about 3, about 4, about 5, about 6, about 8, or about 10 mg/kg body weight (mpk) or about 0.1 to about 10.

[00425] 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.

[00426] 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. For example, administration may be directly to the brain of a subject or to neurons in the brain of a subject. In a specific example, administration to a subject is by intrathecal injection or by intracranial injection (e.g., stereotactic surgery for injection in the hippocampus and other brain regions, or intracerebroventricular injection). In a specific example, administration to a subject is by intracerebroventricular injection. In another specific example, administration to a subject is by intracranial injection. In another specific example, administration to a subject is by intrathecal injection.

[00427] In some methods, administration is by a means such that the reagent being introduced reaches neurons or the nervous system. This can be achieved, for example, by peripheral delivery or by direct delivery to the nervous system. *See, e.g.,* Evers et al. (2015) *Adv. Drug Deliv. Res.* 87:90-103, herein incorporated by reference in its entirety for all purposes.

[00428] For reagents to reach the nervous system, they first have to cross the vascular barrier, made up of the blood brain barrier or the blood-spinal cord barrier. One mechanism that can be used to cross the vascular barrier is receptor-mediated endocytosis. Another mechanism that can

be used is cell-penetrating peptide (CPP)-based delivery systems. Different CPPs use distinct cellular translocation pathways, which depend on cell types and cargos. Another delivery mechanism that can be used is exosomes, which are extracellular vesicles known to mediate communication between cells through transfer of proteins and nucleic acids. For example, IV injection of exosomes transduced with short viral peptides derived from rabies virus glycoprotein (RVG) can result in crossing of the blood brain barrier and delivery to the brain.

[00429] Techniques are also available that bypass the vascular barriers through direct infusion into the cerebrospinal fluid. For example, reagents can be infused intracerebroventricularly (ICV), after which the reagents would have to pass the ependymal cell layer that lines the ventricular system to enter the parenchyma. Intrathecal (IT) delivery means delivery of the reagents into the subarachnoid space of the spinal cord. From here, reagents will have to pass the pia mater to enter the parenchyma. Reagents can be delivered ICT or IT through an outlet catheter that is connected to an implanted reservoir. Drugs can be injected into the reservoir and delivered directly to the CSF. Intranasal administration is an alternative route of delivery that can be used.

[00430] The frequency of administration and the number of dosages can depend on the half-life of the nuclease agents (e.g., guide RNAs, Cas proteins (or nucleic acids encoding the guide RNAs or Cas proteins)) or exogenous donor nucleic acids and the route of administration among other factors. The introduction of nucleic acids or proteins into the cell or animal can be performed one time or multiple times over a period of time. For example, the introduction can be performed only once over a period of time, 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.

IV. Cells or Animals

[00431] Cells or subjects (e.g., animals) comprising the nuclease agents or nucleic acids encoding nuclease agents, exogenous donor nucleic acids, nucleic acid constructs, expression constructs, vectors, or lipid nanoparticles disclosed herein or that have been modified to comprise a targeted genetic modification in a *C9orf72* gene as disclosed herein are also provided. Cells or subject (e.g., animals) comprising a humanized *C9orf72* locus (e.g., humanized promoter, exon 1A, and repeat-containing intron) as disclosed herein are also provided.

[00432] The cells or subjects can be, for example, mammalian, non-human mammalian, and human. A mammal can be, for example, a non-human mammal, a human, a rodent, a rat, a mouse, or a hamster. Other non-human mammals include, for example, non-human primates, monkeys, apes, cats, dogs, rabbits, horses, bulls, deer, bison, 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). The term "non-human" excludes humans.

[00433] The cells can be isolated cells (e.g., *in vitro*) or can be *in vivo* within a subject (e.g., animal or mammal). Cells can also be any type of undifferentiated or differentiated state. In one example, the cells are neurons.

[00434] The cells provided herein can be normal, healthy cells, or can be diseased cells comprising expanded hexanucleotide repeats at the *C9orf72* locus as described elsewhere herein.

[00435] In one example, the cell is a human cell, a rodent cell, a mouse cell, or a rat cell such as a human neuron, a rodent neuron, a mouse neuron, or a rat number. In a specific example, the cell is a human neuron. In a specific example, the cell is *in vivo* in a subject (e.g., a neuron in the brain of a subject).

[00436] 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

[00437] 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.

[00438] Table 2. Description of Sequences.

SEQ ID NO	Type	Description
1	Protein	Cas9 Protein
2	DNA	Cas9 CDS
3	Protein	SV40 NLS v1
4	Protein	SV40 NLS v2
5	Protein	Nucleoplasmin NLS
6	RNA	crRNA Tail v1
7	RNA	crRNA Tail v2
8	RNA	TracrRNA v1
9	RNA	TracrRNA v2
10	RNA	TracrRNA v3
11	RNA	gRNA Scaffold v1
12	RNA	gRNA Scaffold v2
13	RNA	gRNA Scaffold v3
14	RNA	gRNA Scaffold v4
15	RNA	gRNA Scaffold v5
16	RNA	gRNA Scaffold v6
17	RNA	gRNA Scaffold v7
18	RNA	gRNA Scaffold v8
19	DNA	Guide RNA Target Sequence Plus PAM v1
20	DNA	Guide RNA Target Sequence Plus PAM v2
21	DNA	Guide RNA Target Sequence Plus PAM v3

SEQ ID NO	Type	Description
22	DNA	*Mus musculus C9orf72* mRNA (NM_001081343.1)
23	Protein	*Mus musculus* C9orf72 Amino Acid (NP_001074812.1)
24	DNA	*Rattus norvegicus C9orf72* mRNA (NM_001007702.1)
25	Protein	*Rattus norvegicus* C9orf72 Amino Acid (NP_001007703.1)
26	DNA	*Homo sapiens C9orf72* mRNA (NM_145005.6)
27	Protein	*Homo sapiens* C9orf72 Amino Acid (NP_659442.2)
28	DNA	*Homo sapiens C9orf72* mRNA (NM_018325.4)
29	Protein	*Homo sapiens* C9orf72 Amino Acid (NP_060795.1)
30	DNA	*Homo sapiens C9orf72* mRNA (NM_001256054.2)
31	Protein	*Homo sapiens* C9orf72 Amino Acid (NP_001242983.1)
32-51	DNA	Mouse C9orf72 gRNA Target Sequences
52-71	DNA	Human C9orf72 gRNA Target Sequences
72-91	RNA	Mouse C9orf72 gRNA DNA-Targeting Segments
92-111	RNA	Human C9orf72 gRNA DNA-Targeting Segments
112-130	DNA	Primer and Probes
131	DNA	Alt-R sgRNA Target Sequence
132	DNA	Human C9orf72 gRNA Target Sequence hGU21
133	RNA	Human C9orf72 gRNA DNA-Targeting Segment hGU21

EXAMPLES

Example 1. Dissecting the mechanisms of pathogenic gene expression for *C9orf72* repeat expansion alleles

[00439] Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are progressive and fatal neurodegenerative diseases that cause motor neuron disease in the case of ALS and dementia in the case of FTLD. The most common cause of familial ALS is an expansion of a GGGGCC (G_4C_2) hexanucleotide repeat between two alternative 5' non-coding exons of the *C9orf72* gene. Normal individuals have between 3 and 35 G_4C_2 repeats, while ALS or FTLD patients harbor repeat numbers in the hundreds or thousands. The physiological function of the C9orf72 protein is not well understood, and no disease-causing mutations have been identified in its coding sequence. The G_4C_2 repeats are transcribed in a bidirectional manner, resulting in both G_4C_2 (sense with respect to the *C9orf72* mRNA) and G_2C_4 (antisense) repeat-containing transcripts, which are translated into six different dipeptide repeat (DPR) proteins by a non-canonical mechanism termed Repeat Associated Non-AUG (RAN) translation. Both the repeat-containing RNAs and the DPR proteins derived from them have been implicated as pathogenic agents in C9orf72 ALS and FTLD. Although the exact mechanisms by which repeat-containing RNA and DPR proteins promote the disease state are not known, it appears clear that synthesis of RNA from the repeat expansion is a pre-requisite for pathogenesis.

[00440] Transcription of the *C9orf72* gene initiates at two alternative non-coding exons: exon 1A (upstream) and exon 1B (downstream). The G_4C_2 repeat lies between exons 1A and 1B. Exons 1A and 1B can be spliced to exon 2, the first protein-coding exon, creating mRNAs with alternative 5'-untranslated regions. In healthy people with short G_4C_2 repeat expansions, transcription predominantly initiates at exon 1B; RNAs that include exon 1A are rare, and repeat-containing RNAs are undetectable. People suffering from C9orf72 ALS or FTLD accumulate transcripts in which exon 1A is spliced to exon 2, and both sense and antisense repeat-containing RNAs and the DPR proteins translated from them can be detected by *in situ* hybridization and immunohistochemistry. *See* **Figure 1**. These pathological findings suggest that the longer disease-associated G_4C_2 repeat expansions promote the use of the upstream exon 1A transcription initiation site, which is the only way that repeat-containing RNAs and their DPR proteins could be produced. It also follows that the production of antisense repeat-containing RNA, which depends on a long repeat expansion, is somehow linked to increased use of the upstream transcription initiation site.

[00441] Thus, a possible therapeutic strategy for C9orf72 repeat-expansion disease would be to inhibit or abolish transcription that initiates upstream of the G_4C_2 repeat at exon 1A while retaining transcription that initiates at exon 1B downstream of the repeat, which will retain production of the mRNA for C9orf72 protein synthesis. Under the assumption that there are promoter elements upstream of exon 1A that control transcription of the *C9orf72* gene, we designed experiments to determine if transcripts that initiate at exon 1A could selectively be abolished while retaining transcripts that initiate at exon 1B.

[00442] To test this, we used mouse embryonic stem (ES) cells in which we placed a fragment from the human *C9orf72* gene, including part of exon 1A, the intron sequence between 1A and 1B, including 300 G_4C_2 repeats (300X), all of exon 1B and part of the downstream intron, precisely at its homologous position in one allele of the mouse *C9orf72* gene. *See* **Figure 2**. *See also* US 2020-0196581 and WO 2020/131632, each of which is herein incorporated by reference in its entirety for all purposes. As a reliable model of C9orf72 repeat expansion disease, the 300X humanized allele reproduces the molecular hallmarks of disease: a switch in transcription initiation from exon 1B to exon 1A; accumulation of sense and antisense repeat-containing RNA; production of poly-Glycine-Alanine and poly-Glycine-Proline DPR proteins; accumulation of intron-containing sense RNAs; and accumulation of antisense RNAs that initiate

approximately 170 bp downstream of exon 1B and extend through the G_4C_2 repeat and into flanking sequences upstream of exon 1A.

[00443] Using these mouse ES cells, we used the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 targeted genome modification technology to create mutations in the promoter of the *C9orf72* gene. To screen for mutations with the desired therapeutic properties, we introduced a collection of 20 CRISPR guide RNA (gRNA)/Cas9 ribonucleoprotein complexes that target the mouse *C9orf72* genomic locus in a region spanning 2 kb upstream of exon 1A into the mouse embryonic stem (ES) cells in which we placed a fragment from the human *C9orf72* gene, including part of exon 1A, the intron sequence between 1A and 1B, including 300 G_4C_2 repeats (300X), all of exon 1B and part of the downstream intron, precisely at its homologous position in one allele of the mouse *C9orf72* gene. *See* **Figure 3**. A total of 384 ES cell clones were picked, plated, and screened for *C9orf72* mRNA, exon 1A and exon 1B transcription, and intron-containing transcripts. The results of the CRISPR/Cas9 screen revealed small deletion mutations within a few hundred base pairs of the transcription initiation site for exon 1Athat were able to block or substantially reduce sense transcripts that initiated at exon 1A while sparing transcripts that initiated at exon 1B. *See* **Figures 4A** and **4B**, respectively. As shown below, the same mutations also had the unexpected property of blocking or substantially reducing the accumulation of antisense RNA that extended from its mapped initiation site through the repeat region in the antisense direction. Mutations with these properties had no discernable effect on the abundance of the *C9orf72* mRNA.

[00444] A *C9orf72* promoter ablation experiment was then done using a subset of the gRNAs: mGU3 (54 bp from exon 1A transcription start site), mGU4 (118 bp from exon 1A transcription start site), mGU5 (215 bp from exon 1A transcription start site), and mGU20 (~2 kb from exon 1A transcription start site). *See* **Figure 5**. Clones targeted with mGU3 had perturbed transcription from exon 1A (**Figure 6A, 6C,** and **6D**), while sparing transcripts that initiated at exon 1B (**Figure 6B**), whereas transcripts that initiated at either exon 1A or exon 1B were generally spared in clones targeted with mGU5. Likewise, clones targeted with the combination of mGU3 and mGU5 had perturbed transcription from exon 1A (**Figure 7A, 7C,** and **7D**), while sparing transcripts that initiated at exon 1B (**Figure 7B**), whereas transcripts that initiated at either exon 1A or exon 1B were generally spared in clones targeted with the combination of

mGU5 and mGU20. Repeat stability in the CRISPR/Cas9-targeted clones was confirmed using the AmplideX PCR/CE C9orf72 Kit (Asuragen) (data not shown).

[00445] To gain further insight into the selective abolishment of transcripts that initiate at exon 1A while retaining transcripts that initiate at exon 1B, PCR and Sanger sequencing were used to reveal the nature of deletions in the CRISPR/Cas9-targeted regions. As shown in **Figure 8**, deletions that extended into the region between 0-215 bp upstream of the exon 1A transcription start site perturbed transcription from exon 1A without affecting transcription from exon 1B, whereas deletions between 215 bp to ~2 kb upstream of the exon 1A transcription start site had minimal effect on transcription from exon 1A or exon 1B.

[00446] Using the same ES cell clones from **Figure 8**, NanoString NCOUNTER® was used to further assess whether accumulation of sense transcripts (**Figure 9**) or antisense transcripts (**Figure 10**) was perturbed in motor neurons derived from the ES cells. Surprisingly, a subset of clones that harbored deletions upstream of exon 1A had perturbed accumulation of both sense and antisense transcripts. As shown in **Figure 11A**, deletions that encompassed the exon 1A transcription start site reduced both sense and antisense transcripts. As shown in **Figure 11B**, levels of poly(GlyAla) dipeptide repeat proteins were reduced in clones with reduced sense transcripts, and levels of poly(GlyPro) dipeptide repeat proteins were reduced in clones with reduced sense and antisense transcripts. These results support the expectation that a sense RNA serves as the template for translation of poly(GlyAla), while poly(GlyPro) is synthesized from both sense and antisense RNA templates. The stronger inhibition of poly(GlyPro) synthesis achieved in clones with reduced sense and antisense transcripts indicates that the majority of this DPR protein is produced from antisense transcripts in the 300X model. These results showed that CRISPR gRNAs that can direct Cas9 to induce mutations in the promoter region of a *C9orf72* gene can reduce or abolish synthesis of both sense and antisense pathogenic repeat-containing RNAs while sparing the mRNA for the C9orf72 protein. It is surprising that a single deletion mutation in the promoter region upstream of a *C9orf72* exon 1A would affect only upstream transcription initiation from exon 1A while having little or no effect on transcription that initiates from exon 1B. It is also surprising that small deletion mutations in the upstream flanking region of the *C9orf72* gene would reduce or abolish antisense transcription that initiates in the opposite direction approximately 2.4 kb distal from the site of the mutation.

[00447] Having demonstrated the principle that CRISPR/Cas9 can be used to achieve a therapeutic effect in C9orf72 ALS and FTLD using mouse ES cells, we next sought to reproduce the preferential ablation of exon 1A transcripts in mice. C9orf72 $G_4C_2$300x heterozygous mice (described in US 2020-0196581 and WO 2020/131632, each of which is herein incorporated by reference in its entirety for all purposes) were crossed with Cas9-ready mice (described in US 2019-0032155 and WO 2019/028032, each of which is herein incorporated by reference in its entirety for all purposes). A dose of 7 x 10^{10} viral genomes of AAV-PHP.eB-U6-mGU3, mGU4, or mGU5 were injected into the *Rosa26*Cas9/*C9orf72*G_4C_2*300x* heterozygous mice at postnatal day 0 by intracerebroventricular injection. Mice were sacrificed 28 days post-injection. As shown in **Figure 12**, delivery of the gRNAs to the CNS of the Cas9-ready G_4C_2 repeat expansion mice suggested that there is reduced transcription from exon 1A that is accompanied by a similar reduction in intron-containing transcripts, whereas transcription from exon 1B is unaffected.

[00448] Having demonstrated the principle that CRISPR/Cas9 can be used to achieve a therapeutic effect in C9orf72 ALS and FTLD in mouse ES cells and mice, we generated an extended mouse humanized *C9orf72* repeat expansion model in which the humanization extends to include approximately 3 kb of flanking sequence upstream of exon 1A in order to repeat the CRISPR/Cas9 screening using gRNAs targeting the human *C9orf72* promoter region. A schematic for generating the *C9orf72* allele with the humanized exon 1A promoter is shown in **Figure 13**.

[00449] To validate the allele with the humanized *C9orf72* promoter, we assessed the expression of transcripts in mouse ES cells having the allele with the humanized *C9orf72* promoter and either 3x repeats or 250x repeats through use of TAQMAN® qualitative PCR assays spanning different sections of the *C9orf72* pre-mRNA. **Figure 14** shows that the allele with the humanized *C9orf72* promoter region reproduces the RNA expression pattern seen in repeat expansion alleles with the mouse promoter, with exon 1A and intron-containing transcripts being expressed at higher levels in 250x repeat mouse ES cells as compared to 3x repeat mouse ES cells, and exon 1B transcripts being expressed at similar levels in 250x repeat mouse ES cells as compared to 3x repeat mouse ES cells.

[00450] To screen for mutations with the desired therapeutic properties, we introduced a collection of 19 CRISPR guide RNA (gRNA)/Cas9 ribonucleoprotein complexes that target the human *C9orf72* promoter region in a region spanning ~2 kb upstream of exon 1A into the mouse

embryonic stem (ES) cells having the allele with the humanized *C9orf72* promoter. *See* **Figure 15**. ES cell clones were picked, plated, and screened for exon 1A and exon 1B transcription. The results of the CRISPR/Cas9 screen revealed small deletion mutations upstream of the transcription initiation site for exon 1A that were able to block or substantially reduce sense transcripts that initiated at exon 1A while sparing transcripts that initiated at exon 1B. *See* **Figure 16**.

[00451] A *C9orf72* promoter ablation experiment was then done in ESMNs using a subset of the gRNAs: hGU5, hGU3, hGU2, and hGU21. CRISPR-induced mutations upstream of E1A in the human promoter were shown to be able to abolish sense and antisense transcripts across the G_4C_2 repeats. *See* **Figures 21-22**. For 90099M clones, a combination of hGU3 and hGU21 was used. For 90099G clones, hGU2 was used.

Materials and Methods

[00452] **ES cell culture, gRNA electroporations into ES cells and isolation of modified ES cell clones**. ES cells were grown on cell culture plates coated with gelatin (0.1% in water, Stem Cell Technologies) containing a monolayer of irradiated primary mouse embryonic fibroblasts (Produced in-house). Dulbecco's Minimal Essential Medium (DMEM, Gibco) with high glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, penicillin and streptomycin (50 µg/mL, Gibco), 15% FBS (Gemini Bio), 1200 U/mL LIF (Life Technologies) was used to grow and maintain ES cells at 37°C, 5% CO_2, and atmospheric O_2 level and passaged as needed. Electroporation of gRNAs into ES cells was done with nucleofector 4D (Lonza) with the CP-105 program as per manufacturer instructions. Electroporation conditions for each type of experiment was as follows.

[00453] **20 gRNAs (all together) directed against mouse promoter (Figures 3, 4A, and 4B)**. For introducing all 20 gRNAs directed against the mouse promoter region and SpCas9 into mouse ES cells (**Figures 3, 4A**, and **4B**), four complexes were made with each complex containing 5 gRNAs (each gRNA was used at 62.5 pmoles, total volume=5 µL) along with 2.5 µL of SpCas9 (TrueCut Cas9 (Thermo Fisher Cat# A36499) 78.125 pmoles) for a total volume of 7.5 µL. Once the complexes were made, they were mixed together (7.5 µL X 4 = 30 µL). This was then mixed with 70 µL of P3 buffer (Lonza). This 100 µL of RNP complexes+ P3 buffer was used to resuspend an ES cell pellet containing 2 million ES cells (This was an ES cell clone

that harbored 300 G_4C_2 repeats). Electroporation was done with nucleofector 4D (Lonza) with the CP-105 program. Subsequently, 60,000 cells from this EP suspension was plated onto 1 well of a 6-well plate (pre-plated with feeders). Three days later, the cells were trypsinized to single cells and plated at 2000 cells per 10 cm dish to form colonies. Seven days later, colonies were picked and transferred onto individual wells of 96 well plates. Three days later, all wells of the 96 well plates was trypsinized and split into 3 separate 96-well plate, one for RNA analysis, one for DNA analysis and one plate frozen to be retrieved later after the RNA and DNA analysis was complete.

[00454] Individual and dual gRNAs against mouse promoter (Figures 5, 6A, 6B, 7A, 7B, and 8). For introducing one or two gRNAs directed against the mouse promoter region and SpCas9 into mouse ES cells (**Figures 5, 6A, 6B, 7A, 7B**, and **8**), complexes were made with each complex containing 1 or 2 gRNAs (each gRNA was used at 125 pmoles, total volume = 2 or 4 µL) along with 1 or 2 µL of SpCas9 (TrueCut Cas9 31.25 or 62.5 pmoles) for a total volume of 3-6 µL. This was mixed with 94-96 µL of P3 buffer (Lonza). This 100 µL of RNP complexes + P3 buffer was used to resuspend an ES cell pellet containing 2 million ES cells (This was an ES cell clone that harbored 300 G_4C_2 repeats). Electroporation was done with nucleofector 4D (Lonza) with the CP-105 program. Subsequently, 60,000 cells from this EP suspension was plated onto 1 well of a 6-well plate (pre-plated with feeders). Three days later, the cells were trypsinized to single cells and plated at 2000 cells per 10 cm dish to form colonies. Seven days later, colonies were picked and transferred onto individual wells of 96 well plates. Three days later, all wells of the 96 well plates was trypsinized and split into 3 separate 96-well plate, one for RNA analysis, one for DNA analysis and one plate frozen to be retrieved later after the RNA and DNA analysis was complete.

[00455] Table 3. Guide RNAs targeting mouse *C9orf72* promoter or near E1A Start Site.

gRNA	Guide RNA Target Sequence (SEQ ID NO)	PAM	Location	Strand	Distance from E1A Start Site (UCSC annotated)
mGU	CTTTCCTTCTAGGTGGAAAG (SEQ ID NO: 32)	TGG	chr4:35,173,321-35,173,343		(-)49 bp
mGU2	CGTCTCTGCTTGACGCGCCG (SEQ ID NO: 33)	CGG	chr4:35,173,396-35,173,418	Minus	*43 bp
mGU3	AAGCGCCAACCCGGAACTTA (SEQ ID NO: 34)	CGG	chr4:35,173,434-35,173,456		64 bp
mGU4	AGCCCATGACAGCCTCCGCC (SEQ ID NO: 35)	TGG	chr4:35,173,498-35,173,520		128 bp
mGU5	GTAACCTAGATAGATAACGC (SEQ ID NO: 36)	AGG	chr4:35,173,595-35,173,617		225 bp

gRNA	Guide RNA Target Sequence (SEQ ID NO)	PAM	Location	Strand	Distance from E1A Start Site (UCSC annotated)
mGU6	ACGGCATTTAACCGTGTCAT (SEQ ID NO: 37)	AGG	chr4:35,173,724-35,173,746		354 bp
mGU7	AACACGGTGACTACGCACAG (SEQ ID NO: 38)	AGG	chr4:35,173,804-35,173,826		434 bp
mGU8	AATCAATAAATCAGACGTCA (SEQ ID NO: 39)	TGG	chr4:35,173,895-35,173,917		525 bp
mGU9	AGTTCTATTTTTACCGAAGG (SEQ ID NO: 40)	TGG	chr4:35,174,042-35,174,064		672 bp
mGU10	TCTTACAGTGTTTTGTACCG (SEQ ID NO: 41)	TGG	chr4:35,174,148-35,174,170	Minus	795 bp
mGU11	AAATAGCATAGGACCATGTT (SEQ ID NO: 42)	TGG	chr4:35,174,237-35,174,259	Minus	884 bp
mGU12	GTCAGCTAATCCACAACACG (SEQ ID NO: 43)	AGG	chr4:35,174,337-35,174,359	Minus	984 bp
mGU13	ATCAATTACATTGATAGCTC (SEQ ID NO: 44)	TGG	chr4:35,174,482-35,174,504		1112 bp
mGU14	TGTTAGTTTGACTCACAGAC (SEQ ID NO: 45)	AGG	chr4:35,174,590-35,174,612		1220 bp
mGU15	GTCTCTAGGTAAAATTTTGA (SEQ ID NO: 46)	AGG	chr4:35,174,678-35,174,700		1308 bp
mGU16	CTAAAATATTGTATGACCAC (SEQ ID NO: 47)	AGG	chr4:35,174,909-35,174,931	Minus	1556 bp
mGU17	TGGAACAATTACTTAGGCAT (SEQ ID NO: 48)	AGG	chr4:35,175,002-35,175,024	Minus	1649 bp
mGU18	TAATTGTTGTATTGCCTGAA (SEQ ID NO: 49)	AGG	chr4:35,175,107-35,175,129	Minus	1754 bp
mGU19	AATCTACCTCAAATAACTCC (SEQ ID NO: 50)	AGG	chr4:35,175,255-35,175,277	Minus	1902 bp
mGU20	GGAGTAAACCAGTACGTGGT (SEQ ID NO: 51)	AGG	chr4:35,175,353-35,175,375		1983 bp

*Depending on the annotation used, the guide RNA target sequence is considered upstream or downstream of the E1A start site.

[00456] Individual and dual gRNAs against human promoter (Figures 15 and 16). For introducing one or two gRNAs directed against the human promoter region and SpCas9 into mouse ES cells (**Figures 15** and **16**), complexes were made with each complex containing 1 or 2 gRNAs (each gRNA was used at 125 pmoles, total volume= 2 or 4 µL) along with 1 or 2 µL of SpCas9 (TrueCut Cas9 31.25 or 62.5 pmoles) for a total volume of 3-6 µL. This was mixed with 94-96 µL of P3 buffer (Lonza). This 100 µL of RNP complexes + P3 buffer was used to resuspend an ES cell pellet containing 2 million ES cells (This was an ES cell clone that harbored 300 G_4C_2 repeats). Electroporation was done with nucleofector 4D (Lonza) with the CP-105 program. Subsequently, 60,000 cells from this EP suspension was plated onto 1 well of a 6-well plate (pre-plated with feeders). Three days later, the cells were trypsinized to single cells and plated at 2000 cells per 10 cm dish to form colonies. Seven days later, colonies were picked and transferred onto individual wells of 96 well plates. Three days later, all wells of the 96 well

plates was trypsinized and split into 3 separate 96-well plate, one for RNA analysis, one for DNA analysis and one plate frozen to be retrieved later after the RNA and DNA analysis was complete.

[00457] Table 4. Guide RNAs targeting human *C9orf72* promoter or near E1A Start Site.

Name	Guide RNA Target Sequence (SEQ ID NO)	PAM	Location	Strand	Distance from E1A Start Site
hGU21	AAACAGACAGACGTAACCTA (SEQ ID NO: 132)	CGG		plus	(-) 10 bp
hGU	TGCGATGACGTTTTCTCACG (SEQ ID NO: 52)	AGG	chr9:27573881-27573903	minus	36 bp
hGU2	CTCACGAGGCTAGCGAAATG (SEQ ID NO: 53)	GGG	chr9:27573895-27573917	minus	50 bp
hGU3	GCGCCAACGCTCCTCCAGAG (SEQ ID NO: 54)	CGG	chr9:27573952-27573974		90 bp
hGU4	TGTGCGAACCTTAATAGGGG (SEQ ID NO: 55)	AGG	chr9:27573982-27574004	minus	137 bp
hGU5	GGCGCTCAACACATAATTGG (SEQ ID NO: 56)	TGG	chr9:27574076-27574098	minus	231 bp
hGU6	ACCCTGGTAGGGGACAGCTC (SEQ ID NO: 57)	CGG	chr9:27574156-27574178	minus	311 bp
hGU7	CAAGCATGATTTTCACACTG (SEQ ID NO: 58)	GGG	chr9:27574250-27574272	minus	405 bp
hGU8	CCTGCAGACCAAAAGACGCA (SEQ ID NO: 59)	AGG	chr9:27574378-27574400		516 bp
hGU9	TTTGATCTGCAAGTGTGAGA (SEQ ID NO: 60)	TGG	chr9:27574486-27574508	minus	641 bp
hGU10	AACGTTTTAATCATTCACCG (SEQ ID NO: 61)	AGG	chr9:27574562-27574584	minus	717 bp
hGU11	TTCACAAAGCATAGAACAGT (SEQ ID NO: 62)	AGG	chr9:27574654-27574676		792 bp
hGU12	ATACCCAACTAAAATAGTGG (SEQ ID NO: 63)	GGG	chr9:27574708-27574730	minus	863 bp
hGU13	ACTTGGAGAGAATTACACTG (SEQ ID NO: 64)	TGG	chr9:27574770-27574792		908 bp
hGU14	TTGGCACTATTAAGGATCTG (SEQ ID NO: 65)	AGG	chr9:27574913-27574935		1051 bp
hGU15	GGGCATACATGTAGATCAGA (SEQ ID NO: 66)	TGG	chr9:27575021-27575043		1159 bp
hGU16	AGTTGTACTTCACTTATACG (SEQ ID NO: 67)	TGG	chr9:27575159-27575181	minus	1314 bp
hGU17	CAGAGTAGACCCTTGGTTGG (SEQ ID NO: 68)	GGG	chr9:27575210-27575232	minus	1365 bp
hGU18	GGGAGACCCCAGACTTCACA (SEQ ID NO: 69)	TGG	chr9:27575494-27575516	minus	1649 bp
hGU19	TGGCTTTCAGACTTGCAATG (SEQ ID NO: 70)	GGG	chr9:27576007-27576029		2145 bp
hGU20	TCTCCTTTGACTCACACCCA (SEQ ID NO: 71)	GGG	chr9:27576066-27576088	minus	2221 bp

[00458] ES cell-derived motor neuron (ESMN) differentiation (Figures 11A, 11B, 12, 21, and 22). To differentiate mouse ES cells to ES cell-derived motor neurons (ESMNs), we thawed mESCs on cell culture plates coated with gelatin (0.1% in water, Stem Cell Technologies) containing a monolayer of EMBRYOMAX™ primary mouse embryonic fibroblasts (Millipore

#PMEF-CF) and grew them for three days in mESC medium with daily media changes, after which cells were removed by treatment with trypsin and harvested by a 5 min centrifugation at 800 rpm. Cells were resuspended in neural induction media composed of 43.5% Advanced DMEM/F12K medium (Gibco), 43.5% neurobasal medium (Gibco), 10% Knockout™ serum replacement (Gibco), 1% each of L-glutamine (100X, Gibco), penicillin-streptomycin (100X, Gibco), and EMBRYOMAX™ 2-mercaptoethanol (100X, Sigma Aldrich #ES-007-E) and cultured for two days, after which embryoid bodies were switched to neural induction medium supplemented with all-*trans* retinoic acid (1 μM; Sigma) and mouse Smoothened agonist (SAG; 1 μM;) and cultured for another four days, after which the ESMNs were harvested for RNA extraction.

[00459] PCR for verifying nature of CRISPR/Cas9 induced mutations (Figures 8 and 11A). We used the Quick-DNA miniprep kit (Zymo) to purify genomic DNA from mouse ES cells containing G_4C_2 repeats (That were previously electroporated with specific gRNAs as described above) and analyzed the resulting genomic DNA by PCR (98 °C for 30 s, 5 minutes, 35 cycles of 98 °C for 10 s, 63°C for 20 s, 72°C for 3 min, and a final extension at 72°C for 2 min) using Q5 polymerase (NEB) with two primers that flank the region targeted by all indicated gRNAs. The forward primer is in the mouse region and the reverse primer is in the humanized region (forward, 5′-CCTGCAGCAATGCACATCAC-3′ (SEQ ID NO: 112); reverse, 5′-TACAGGCTGCGGTTGTTTC-3’ (SEQ ID NO: 113)).

[00460] Asuragen PCR for estimation of G_4C_2 repeat stability. We used the Quick-DNA miniprep kit (Zymo) to purify genomic DNA from mouse ES cells containing G_4C_2 repeats and analyzed it by the AMPLIDEX® PCR/CE C9orf72 Kit (Cat. # 49581, Asuragen). Briefly, we amplified gDNA by PCR (98°C for 5 minutes, 37 cycles of 97 °C for 35s, 62°C for 35s, 72°C for 3 min, and a final extension at 72°C for 10 min) with two primers that flank the repeat region (forward, 5′-CGCAGCCTGTAGCAAGCTCTGGAACTCAGGAGTCG-3′ (SEQ ID NO: 114); reverse, 5′ 6-FAM/TGCGCCTCCGCCGCCGCGGGCGCAGGCACCGCAACCGCA-3′ (SEQ ID NO: 115)). The PCR product thus generated was then analyzed by agarose gel electrophoresis.

[00461] Reverse transcription-quantitative PCR (RT-qPCR) (Figures 4A, 4B, 6A-6D, 7A-7D, 14 and 16). We purified RNA from mouse ES cells with the RNeasy plus mini kit (Qiagen, Cat No: 74136) as per the manufacturer’s instructions, followed by genomic DNA

removal with the Turbo DNA-free kit (ThermoFisher Scientific, # AM1907). We diluted the purified RNA samples to a concentration of 20 ng/μL and performed RT-qPCR in a one-step reaction with QuantiNova Probe RT-PCR kit (Qiagen) according to the manufacturer's recommendations. The RT-qPCR contained 2 μL of RNA and an 8 μL of a mixture containing RT-PCR Master mix, ROX dye, RT-mix, and custom-designed primer-probe mix. The custom TaqMan assays (*see* **Table 5**) were obtained from IDT and the reference assay for Drosha mRNA from ThermoFisher (cat. # Mm01310009_m1). We performed the qRT-PCR reactions in quadruplicate on a VIIA™ 7 Real-Time PCR Detection System (ThermoFisher) with the RT reaction at 45°C for 10 min followed by PCR cycling (95°C 10 min, 45 cycles of 95°C 5s, 60°C 30s) in an optical 384-well plate. We determined ΔCt values between the target RNA assays and the Drosha mRNA reference and calculated relative RNA abundance by the $2^{-\Delta\Delta Ct}$ method. A lower Ct and ΔCt value indicates higher amounts of target RNA detected.

[00462] Table 5. List of RT-qPCR TAQMAN® assays.

Assay Name	Primer/Probe	Sequence	SEQ ID NO
Exon 5 - Exon 6	Forward Primer	GCTGTCACGAAGGCTTTCTTC	116
	Reverse Primer	GCACTGCTGCCAACTACAAC	117
	Probe	TCAATGCCATCAGCTCACACCTGC	118
Exon 1A - Exon 2	Forward Primer	GAGCAGGTGTGGGTTTAGGA	119
	Reverse Primer	CCAGGTCTCACTGCATTCCA	120
	Probe	ATTGCAAGCGTTCGGATAATGTGAGA	121
Exon 1B - Exon 2	Forward Primer	GGTGGCGAGTGGCTATTG	122
	Reverse Primer	GATAGTCGACATCCCTGCATC	123
	Probe	AAGCGTTCGGATAATGTGAGACCTGG	124
Exon 1A-Intron	Forward Primer	AAGAGCAGGTGTGGGTTTAG	125
	Reverse Primer	CGGTTGTTTCCCTCCTTGT	126
	Probe	TCTCACAGTACTCGCTGAGGGTGA	127
Intron (Adjacent to G_4C_2 repeats)	Forward Primer	CCCACTACTTGCTCTCACAG	128
	Reverse Primer	TACAGGCTGCGGTTGTTT	129
	Probe	ACTCGCTGAGGGTGAACAAGAAA	130

[00463] NanoString NCOUNTER® technology. Nanostring technology was used to separately detect sense and antisense transcripts. NCOUNTER® specific probe sequences were custom designed and manufactured by NanoString and mixed to form a codeset. Each codeset also includes a number of housekeeping genes to correct for RNA input amount and/or quality differences. Protocol was followed according to standard NCOUNTER® instructions. Briefly, using the Nanostring NCOUNTER® Analysis System (Nanostring Technologies), gene expression analysis was conducted for each sample using a custom-designed codeset containing eight targets. Each reaction contained 400 ng of total RNA in a 7 μL volume, plus reporter and

capture probes. Analysis and normalization of the raw Nanostring data was conducted using nSolver Analysis Software v4.0 (Nanostring Technologies). Raw counts were normalized to internal levels of five reference genes: DROSHA, GUSB, HPRT, LMNA, and TBP.

[00464] Cloning *C9orf72* G_4C_2 constructs with human promoter (Figure 13). Previously, we generated a targeting vector that contains the mouse promoter region and either 3 or 250 G_4C_2 repeats. To swap the promoter with human sequence, we digested the 3X targeting vector using in vitro CRISPR/Cas9 reaction using recombinant Cas9 protein and an Alt-R sgRNA (IDT) corresponding to the sequence: GCATTTTTGATGAAGCAAGA (SEQ ID NO 131) and then using BbVCI restriction enzyme (NEB). A synthetic double stranded DNA fragment containing a ~20 bp mouse homology arm and a 3 kb human promoter region was generated (GBLOCK™, Integrated DNA Technologies, IDT) and cloned into the digested targeting vector described above using Nebuilder HiFi DNA assembly kit (NEB) as per the manufacturer's instructions. Once the targeting vector containing the human promoter and a 3X G_4C_2 repeat was generated, this vector was digested with BbVCI and PspXI restriction enzymes to remove the 3 G_4C_2 repeats. The original 250 G_4C_2 repeats, with the mouse promoter was also subjected to restriction digestion using BbVCI and PspXI restriction enzymes and a fragment containing the 250 G_4C_2 repeats was recovered. This fragment was ligated into the above mentioned 3 G_4C_2 repeat containing vector followed by transformation of ELECTROMAX™ DH10β competent cells (Invitrogen # 18290015) and culturing at 25°C to reduce recombination events that collapse the G_4C_2 repeat expansion. Correctly recombined bacterial clones were expanded in LB broth plus carbenicillin (100 µg/mL; Sigma Aldrich), and the plasmids were purified with the EndoFree Plasmid maxi-prep kit (Zymo Research).

[00465] Slot blot assays (Figure 11B). For slot blot assays, protein extracts from ES-cell-derived motor neurons were quantified using the RC DC protein assay (BioRad). Lysates containing 1.25 µg or 2.5 µg were immobilized onto nitrocellulose membranes with Bio-Slot 48-well microfiltration system (Bio-Rad) under vacuum. The membranes were washed in TBS-T and blotted with an antibody against poly(GP) (1:5,000, Novus biologicals) and poly GA (1:5000, Millipore). After the membrane was incubated with HRP conjugated secondary antibody, bands were visualized by the ECL plus Western Blotting Detection System (Pierce).

[00466] Generation of ES cells with CRISPR-induced deletions in the humanized region upstream of E1A. Cas9 RNP complexes are formed by combining 31.25 pmol of Cas9 protein

per 125 pmol of each sgRNA for 15 minutes at room temperature before mixing with Lonza P3 buffer. The ES cells used for this experiment contains a 250X G_4C_2 repeat expansion while also containing a humanization of a 3 kb region upstream of E1A. Cas9 RNPs are delivered into these ES cells using the Lonza 4D nucleofector instrument. Following nucleofection, cells are plated onto feeder-coated 6-well plates. After 3 days of recovery the cells are trypsinized and sparsely plated to produce single-cell derived colonies. Colonies are picked and expanded for screening 7 days later. Screening the clones is done by RT-qPCR as described below but with ES cell RNA.

[00467] RT-qPCR for *C9orf72* RNA transcripts in ESMNs (Figures 21 and 22). ESMNs were harvested and total RNA was isolated using RNeasy plus kit (Qiagen) followed by DNase treatment using the Turbo DNA free kit (Thermo Fisher Scientific). RT-qPCR analysis was performed in a one-step reaction with QuantiNova Probe RT-PCR kit (Qiagen) according to the manufacturer's recommendations. The RT-qPCR contained 2 μL of RNA and an 8 μL of a mixture containing RT-PCR Master mix, ROX dye, RT-mix, and custom-designed primer-probe mix. The custom TaqMan assays were obtained from IDT and the reference assay for Drosha mRNA from ThermoFisher (cat. # Mm01310009_m1). We performed the qRT-PCR reactions in quadruplicate on a ViiA™ 7 Real-Time PCR Detection System (ThermoFisher) with the RT reaction at 45°C for 10 min followed by PCR cycling (95°C 10 min, 45 cycles of 95°C 5 sec, 60°C 30s) in an optical 384-well plate. We determined ΔCt values between the target RNA assays and the Drosha mRNA reference.

Example 2. *C9orf72* promoter deletion using paired or single guide RNAs *in vivo*

Intracerebroventricular injection of paired gRNAs in neonatal mice

[00468] We next sought to reproduce the preferential ablation of exon 1A transcripts in mice. C9orf72 $G_4C_2$300x heterozygous mice (described in US 2020-0196581 and WO 2020/131632, each of which is herein incorporated by reference in its entirety for all purposes) were crossed with Cas9-ready mice (described in US 2019-0032155 and WO 2019/028032, each of which is herein incorporated by reference in its entirety for all purposes). A dose of 7 x 10^{10} viral genomes of AAV-PHP.eB-U6-mGU3 and mGU4, mGU3 and mGU5, or mGU4 and mGU5 were injected into the *Rosa26*Cas9/*C9orf72*G_4C_2*300x* heterozygous mice at postnatal day 0 by intracerebroventricular injection. PBS (no gRNA) was used as a negative control. Specifically, newborn pups (P0/P1) were anesthetized on ice for ~7-10 minutes until toe pinch reflexes were

diminished. Pups were placed under a dissecting microscope with an attached light source. Injections were done using a 10 µL Hamilton syringe (Hamilton cat# 7653-01) fitted with a custom removable needle (32 gauge, 12 degree point angle, 0.75 inches long). Pups were given a single injection into the left ventricle at a position approximately 2 mm laterally from the superior sagittal sinus and 2 mm rostrally from the transverse sinus to a depth of approximately 2 mm. Each injection contains 3.5-5.0E10 viral genomes in a volume of 5 µL diluted with PBS and containing FastGreen dye (0.03%) to visualize fluid distribution. After injection, pups were placed on a 37°C heating pad, monitored for full recovery, and then placed back in their home cages.

RT-qPCR for C9orf72 RNA transcripts

[00469] Mice were sacrificed 24-28 days post-injection, and different tissues were dissected and immediately placed in RNAlater and stored at -20°C until RNA isolation. Total RNA was isolated using Trizol reagent followed by DNase treatment. RT-qPCR analysis was performed using performed RT-qPCR in a one-step reaction with QuantiNova Probe RT-PCR kit (Qiagen) according to the manufacturer's recommendations. The RT-qPCR contained 2 µL of RNA and an 8 µL of a mixture containing RT-PCR Master mix, ROX dye, RT-mix, and custom-designed primer-probe mix. The custom TaqMan assays (*see* **Table 5**) were obtained from IDT and the reference assay for Drosha mRNA from ThermoFisher (cat. # Mm01310009_m1). We performed the qRT-PCR reactions in quadruplicate on a ViiA™ 7 Real-Time PCR Detection System (ThermoFisher) with the RT reaction at 45°C for 10 min followed by PCR cycling (95°C 10 min, 45 cycles of 95°C 5 sec, 60°C 30 s) in an optical 384-well plate. We determined ΔCt values between the target RNA assays and the Drosha mRNA reference. As shown in **Figure 17**, delivery of the gRNAs to the CNS of the Cas9-ready G_4C_2 repeat expansion mice resulted in reduced transcription from exon 1A that is accompanied by a similar reduction in intron-containing transcripts, whereas transcription from exon 1B is unaffected.

Amplicon sequencing for mapping CRISPR induced deletions

[00470] Tissue samples from the above experiment were harvested and genomic DNA (gDNA) was extracted using a proteinase K-based lysis buffer. Target specific oligos were designed (21-27 base pairs, bp) to generate a maximum amplicon size of 350 bp with primer

melting temperature (Tm) of 60-65°C degrees. Barcode adapter sequences were added to the target specific oligo and the full sequence was ordered from Integrated DNA Technologies (IDT). Polymerase Chain Reaction (PCR) was completed on each gDNA sample. Briefly, in each reaction, 4 nanograms (ng) of gDNA was combined with IDT oligos, Q5 polymerase (#M0491, New England Biolabs), 10 µM dNTPs, buffer, and water per manufacturer's specifications. Next, the amplification products were diluted 1:100 and used for the PCR barcoding reaction to create the final sequencing library. Each barcoding reaction contained a single amplified target with a forward and reverse primer containing a unique barcode and index. Each plate of PCRs was pooled in equal volumes and then purified in a single tube using AMPure XP reagent (#A63881, Beckmann-Coulter), per the manufacturer's instructions. Final library concentration was measured using the Qubit fluorometer (#Q32866, Invitrogen). Four nanomoles of the prepared library was loaded onto the Illumina MiSeq according to the manufacturer's instruction utilizing the 2x300 read kit (#MS-102-3003, Illumina).

[00471] Barcoded samples were de-multiplexed to individual reads (FASTQ format). Forward and reverse reads of each FASTQ file were then merged using PEAR (*see, e.g.*, Zhang et al. (2014) *Bioinformatics* 30(5):614-620, herein incorporated by reference in its entirety for all purposes). Merged reads were mapped to the *Mus musculus* genome version 9 (mm9) using Bowtie2 (*see, e.g.*, Langmead et al. (2012) *Nat. Methods* 9(4):357-359, herein incorporated by reference in its entirety for all purposes). Each sample was sequenced with a minimum of 20,000 merged reads across the expected guide cleavage location. Finally, characterization of barcoded samples was performed using a custom perl script. Briefly, all insertions, deletions, or base changes (INDEL) within a window of 20 bases upstream and downstream of the expected cut site were considered to be CRISPR induced modifications. The number of reads containing an INDEL was compared to the number of reads with wild type sequence to determine the percent editing per animal and tissue. As shown in **Figure 18**, PCR amplicon sequence revealed large deletions in ~30% of reads in the spinal cord of the mice targeted with the gRNAs.

Intracerebroventricular injection of single gRNAs in neonatal mice

[00472] We next sought to reproduce the preferential ablation of exon 1A transcripts in mice. C9orf72 $G_4C_2$300x heterozygous mice (described in US 2020-0196581 and WO 2020/131632, each of which is herein incorporated by reference in its entirety for all purposes) were crossed

with Cas9-ready mice (described in US 2019-0032155 and WO 2019/028032, each of which is herein incorporated by reference in its entirety for all purposes). A dose of 7 x 10^{10} viral genomes of AAV-PHP.eB-U6-mGU3, mGU4, or GU5 were injected into the *Rosa26*Cas9/*C9orf72*G_4C_2*300x* heterozygous mice at postnatal day 0 by intracerebroventricular injection. PBS (no gRNA) was used as a negative control. Specifically, newborn pups (P0/P1) were anesthetized on ice for ~7-10 minutes until toe pinch reflexes were diminished. Pups were placed under a dissecting microscope with an attached light source. Injections were done using a 10 µL Hamilton syringe (Hamilton cat# 7653-01) fitted with a custom removable needle (32 gauge, 12 degree point angle, 0.75 inches long). Pups were given a single injection into the left ventricle at a position approximately 2 mm laterally from the superior sagittal sinus and 2 mm rostrally from the transverse sinus to a depth of approximately 2 mm. Each injection contains 3.5-5.0E10 viral genomes in a volume of 5 µL diluted with PBS and containing FastGreen dye (0.03%) to visualize fluid distribution. After injection, pups were placed on a 37°C heating pad, monitored for full recovery, and then placed back in their home cages.

RT-qPCR for C9orf72 RNA transcripts

[00473] Mice were sacrificed 24-28 days post-injection, and different tissues were dissected and immediately placed in RNAlater and stored at -20°C until RNA isolation. Total RNA was isolated using Trizol reagent followed by DNase treatment. RT-qPCR analysis was performed using performed RT-qPCR in a one-step reaction with QuantiNova Probe RT-PCR kit (Qiagen) according to the manufacturer's recommendations. The RT-qPCR contained 2 µL of RNA and an 8 µL of a mixture containing RT-PCR Master mix, ROX dye, RT-mix, and custom-designed primer-probe mix. The custom TaqMan assays (*see* **Table 5**) were obtained from IDT and the reference assay for Drosha mRNA from ThermoFisher (cat. # Mm01310009_m1). We performed the qRT-PCR reactions in quadruplicate on a ViiA™ 7 Real-Time PCR Detection System (ThermoFisher) with the RT reaction at 45°C for 10 min followed by PCR cycling (95°C 10 min, 45 cycles of 95°C 5 sec, 60°C 30 s) in an optical 384-well plate. We determined ΔCt values between the target RNA assays and the Drosha mRNA reference. As shown in **Figure 19**, delivery of the gRNAs to the CNS of the Cas9-ready G_4C_2 repeat expansion mice resulted in reduced transcription from exon 1A that is accompanied by a similar reduction in intron-containing transcripts, whereas transcription from exon 1B is unaffected.

Amplicon sequencing for mapping CRISPR induced deletions

[00474] Tissue samples from the above experiment were harvested and genomic DNA (gDNA) was extracted using a proteinase K-based lysis buffer. Target specific oligos were designed (21-27 base pairs, bp) to generate a maximum amplicon size of 350 bp with primer melting temperature (Tm) of 60-65°C degrees. Barcode adapter sequences were added to the target specific oligo and the full sequence was ordered from Integrated DNA Technologies (IDT). Polymerase Chain Reaction (PCR) was completed on each gDNA sample. Briefly, in each reaction, 4 nanograms (ng) of gDNA was combined with IDT oligos, Q5 polymerase (#M0491, New England Biolabs), 10 μM dNTPs, buffer, and water per manufacturer's specifications. Next, the amplification products were diluted 1:100 and used for the PCR barcoding reaction to create the final sequencing library. Each barcoding reaction contained a single amplified target with a forward and reverse primer containing a unique barcode and index. Each plate of PCRs was pooled in equal volumes and then purified in a single tube using AMPure XP reagent (#A63881, Beckmann-Coulter), per the manufacturer's instructions. Final library concentration was measured using the Qubit fluorometer (#Q32866, Invitrogen). Four nanomoles of the prepared library was loaded onto the Illumina MiSeq according to the manufacturer's instruction utilizing the 2x300 read kit (#MS-102-3003, Illumina).

[00475] Barcoded samples were de-multiplexed to individual reads (FASTQ format). Forward and reverse reads of each FASTQ file were then merged using PEAR (*see, e.g.*, Zhang et al. (2014) *Bioinformatics* 30(5):614-620, herein incorporated by reference in its entirety for all purposes). Merged reads were mapped to the *Mus musculus* genome version 9 (mm9) using Bowtie2 (*see, e.g.*, Langmead et al. (2012) *Nat. Methods* 9(4):357-359, herein incorporated by reference in its entirety for all purposes). Each sample was sequenced with a minimum of 20,000 merged reads across the expected guide cleavage location. Finally, characterization of barcoded samples was performed using a custom perl script. Briefly, all insertions, deletions, or base changes (INDEL) within a window of 20 bases upstream and downstream of the expected cut site were considered to be CRISPR induced modifications. The number of reads containing an INDEL was compared to the number of reads with wild type sequence to determine the percent editing per animal and tissue. As shown in **Figure 20**, PCR amplicon sequence revealed gRNA-

specific indels induced in the spinal cord but not the kidneys of the mice targeted with the gRNAs.

We claim:

1. A method of modifying a *C9orf72* gene in a cell, comprising contacting the *C9orf72* gene with a first nuclease agent that targets a first nuclease target sequence near and optionally upstream of the *C9orf72* exon 1A transcription start site, wherein the first nuclease agent cleaves the first nuclease target sequence to generate a targeted genetic modification in the *C9orf72* gene.

2. The method of claim 1, wherein the *C9orf72* gene comprises a *C9orf72* hexanucleotide repeat expansion sequence between the first non-coding endogenous exon and exon 2 of the *C9orf72* gene, wherein the *C9orf72* hexanucleotide repeat expansion sequence has more than 30, more than 100, more than 200, more than 300, more than 400, or more than 500 repeats of the hexanucleotide sequence G_4C_2.

3. The method of claim 1 or 2, wherein the first nuclease target sequence is within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site, or wherein the targeted genetic modification comprises a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

4. The method of any preceding claim, wherein the first nuclease target sequence is within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site, or wherein the targeted genetic modification comprises a deletion within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

5. The method of any preceding claim, wherein the targeted genetic modification comprises a deletion of a region of the *C9orf72* promoter.

6. The method of claim 5, wherein the deletion encompasses the *C9orf72* exon 1A transcription start site.

7. The method of any one of claims 1-5, wherein the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site.

8. The method of any preceding claim, wherein the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A.

9. The method of any preceding claim, wherein the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

10. The method of any preceding claim, wherein the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts.

11. The method of any preceding claim, wherein the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

12. The method of any preceding claim, wherein the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts.

13. The method of any preceding claim, wherein the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

14. The method of any preceding claim, wherein the targeted genetic modification reduces expression of polyGA dipeptide repeat proteins.

15. The method of any preceding claim, wherein the targeted genetic modification reduces expression of both polyGA and polyGP dipeptide repeat proteins.

16. The method of any preceding claim, wherein the method comprises introducing the first nuclease agent or one or more nucleic acids encoding the first nuclease agent into the cell.

17. The method of any preceding claim, wherein the method further comprises contacting the *C9orf72* gene with a second nuclease agent that targets a second nuclease target sequence in the *C9orf72* gene, wherein the second nuclease agent cleaves the second nuclease target sequence.

18. The method of claim 17, wherein the targeted genetic modification comprises a deletion between the first nuclease target sequence and the second nuclease target sequence.

19. The method of claim 17 or 18, wherein the second nuclease target sequence is upstream of the *C9orf72* exon 1A transcription start site.

20. The method of claim 17 or 18, wherein the second nuclease target sequence is downstream of the *C9orf72* exon 1A transcription start site.

21. The method of claim 19 or 20, wherein the second nuclease target sequence is within 2500, within 2250, within 2000, within 1800, within 1600, within 1400, within 1200, within 1000, within 900, within 800, within 700, within 600, within 500, within 450, within 400, within 350, within 300, within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

22. The method of any preceding claim, wherein the method further comprises contacting the *C9orf72* gene with an exogenous donor nucleic acid for modification of the *C9orf72* gene.

23. The method of claim 22, wherein the exogenous donor nucleic acid comprises homology arms.

24. The method of claim 22 or 23, wherein:

(i) the exogenous donor nucleic acid is between about 50 nucleotides to about

1 kb in length, optionally wherein the exogenous donor sequence is between about 80 nucleotides to about 200 nucleotides in length; and/or

(ii) the exogenous donor nucleic acid is a single-stranded oligodeoxynucleotide.

25. The method of any preceding claim, wherein the nuclease agent comprises:

(a) a zinc finger nuclease (ZFN);

(b) a transcription activator-like effector nuclease (TALEN); or

(c) (i) a Cas protein; and

(ii) a guide RNA, wherein the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence that is the nuclease target sequence, and wherein the guide RNA binds to the Cas protein and targets the Cas protein to the guide RNA target sequence.

26. The method of any one of claims 1-24, wherein the nuclease agent comprises:

(a) a Cas protein; and

(b) a guide RNA, wherein the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence that is the nuclease target sequence, and wherein the guide RNA binds to the Cas protein and targets the Cas protein to the guide RNA target sequence.

27. The method of claim 26, wherein the method comprises introducing into the cell:

(a) the Cas protein or a nucleic acid encoding the Cas protein; and

(b) the guide RNA or one or more DNAs encoding the guide RNA.

28. The method of any one of claims 25-27, wherein the guide RNA is a single guide RNA (sgRNA).

29. The method of any one of claims 25-28, wherein the Cas protein is a Cas9 protein.

30. The method of claim 29, wherein the Cas9 protein is derived from a *Streptococcus pyogenes* Cas9 protein, a *Staphylococcus aureus* Cas9 protein, a *Campylobacter jejuni* Cas9 protein, a *Streptococcus thermophilus* Cas9 protein, or a *Neisseria meningitidis* Cas9 protein.

31. The method of claim 29, wherein the Cas protein is derived from a *Streptococcus pyogenes* Cas9 protein.

32. The method of any one of claims 25-31, wherein the nucleic acid encoding the Cas protein is codon-optimized for expression in a mammalian cell or a human cell.

33. The method of any one of claims 25-32, wherein the method comprises introducing the guide RNA in the form of RNA, optionally wherein the guide RNA comprises at least one modification.

34. The method of claim 33, wherein the at least one modification comprises a 2'-O-methyl-modified nucleotide and/or a phosphorothioate bond between nucleotides.

35. The method of any one of claims 25-34, wherein the method comprises introducing the nucleic acid encoding the Cas protein, wherein the nucleic acid comprises an mRNA encoding the Cas protein, optionally wherein the mRNA encoding the Cas protein comprises at least one modification.

36. The method of any one of claims 25-32, wherein the method comprises introducing the nucleic acid encoding the Cas protein and the one or more DNAs encoding the guide RNA, wherein the nucleic acid encoding the Cas protein comprises DNA.

37. The method of claim 36, wherein the DNA encoding the Cas protein and the one or more DNAs encoding the guide RNA are in one or more vectors.

38. The method of claim 37, wherein the one or more vectors are one or more viral vectors.

39. The method of claim 38, wherein the one or more viral vectors are one or more adeno-associated virus (AAV) vectors.

40. The method of any one of claims 25-39, wherein the Cas protein or the nucleic acid encoding the Cas protein and the guide RNA or the one or more DNAs encoding the guide RNA are associated with a lipid nanoparticle.

41. The method of any one of claims 25-40, wherein:

(I) the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96; and/or

(II) the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96; and/or

(III) the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 33-71 or 34-71, optionally wherein guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 34-36, 53, 54, and 56.

42. The method of any one of claims 25-41, wherein:

(I) the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96; and/or

(II) the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96; and/or

(III) the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, and 56.

43. The method of claim 42, wherein the method further comprises contacting the *C9orf72* gene with a second nuclease agent that targets a second nuclease target sequence in

the *C9orf72* gene, wherein the second nuclease agent cleaves the second nuclease target sequence,

wherein the second nuclease agent comprises the Cas protein and aa second guide RNA, wherein the second guide RNA comprises a second DNA-targeting segment that targets a second guide RNA target sequence that is the second nuclease target sequence, wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence, and wherein:

(I) the second DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or

(II) the second DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or

(III) the second guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, 56, and 132.

44. The method of any one of claims 1-43, wherein the cell is a neuron, optionally wherein the neuron is a motor neuron.

45. The method of any one of claims 1-44, wherein the cell is *in vitro* or *ex vivo*.

46. The method of any one of claims 1-44, wherein the cell is in a subject *in vivo*, optionally wherein the subject is a human.

47. The method of claim 46, wherein the cell is a neuron in the brain of the subject.

48. The method of claim 46 or 47, wherein the subject has or is at risk for developing a *C9orf72* hexanucleotide repeat expansion associated disease.

49. The method of claim 48, wherein the *C9orf72* hexanucleotide repeat expansion associated disease is amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD).

50. The method of any one of claims 46-49, wherein the first nuclease agent or one or more nucleic acids encoding the first nuclease agent are administered to the subject by intracerebroventricular injection, intracranial injection, or intrathecal injection.

51. The method of any one of claims 1-50, wherein the cell is a mammalian cell, and the *C9orf72* gene is a mammalian *C9orf72* gene.

52. The method of any one of claims 1-51, wherein the cell is a human cell.

53. The method of any one of claims 1-51, wherein the cell is a mouse cell.

54. The method of any one of claims 1-53, wherein the *C9orf72* gene comprises a human *C9orf72* promoter.

55. The method of any one of claims 1-54, wherein the *C9orf72* gene is a human *C9orf72* gene or a humanized *C9orf72* gene.

56. A method of modifying a *C9orf72* gene in a subject, comprising administering to the subject a first nuclease agent or one or more nucleic acids encoding the first nuclease agent, wherein the first nuclease agent targets a first nuclease target sequence near and optionally upstream of the *C9orf72* exon 1A transcription start site, wherein the first nuclease agent cleaves the first nuclease target sequence to generate a targeted genetic modification in the *C9orf72* gene.

57. A method of preventing, treating, or ameliorating at least one symptom or indication of a *C9orf72* hexanucleotide repeat expansion associated disease, comprising administering to a subject in need thereof a pharmaceutical composition comprising a therapeutically effective amount of a first nuclease agent or one or more nucleic acids encoding the first nuclease agent, wherein the first nuclease agent targets a first nuclease target sequence near and optionally upstream of the *C9orf72* exon 1A transcription start site, wherein the first nuclease agent cleaves the first nuclease target sequence to generate a targeted genetic modification in the *C9orf72* gene.

58. The method of claim 57, wherein the *C9orf72* hexanucleotide repeat expansion associated disease is amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD).

59. The method of any one of claims 56-58, wherein the *C9orf72* gene comprises a *C9orf72* hexanucleotide repeat expansion sequence between the first non-coding endogenous exon and exon 2 of the *C9orf72* gene, wherein the *C9orf72* hexanucleotide repeat expansion sequence has more than 30, more than 100, more than 200, more than 300, more than 400, or more than 500 repeats of the hexanucleotide sequence G_4C_2.

60. The method of any one of claims 56-59, wherein the first nuclease agent or one or more nucleic acids encoding the first nuclease agent are administered to the subject by intracerebroventricular injection, intracranial injection, or intrathecal injection.

61. The method of any one of claims 56-60, wherein the first nuclease target sequence is within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site, or wherein the targeted genetic modification comprises a deletion within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

62. The method of any one of claims 56-61, wherein the first nuclease target sequence is within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site, or wherein the targeted genetic modification comprises a deletion within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

63. The method of any one of claims 56-62, wherein the targeted genetic modification comprises a deletion of a region of the *C9orf72* promoter.

64. The method of claim 63, wherein the deletion encompasses the *C9orf72* exon 1A transcription start site.

65. The method of any one of claims 56-63, wherein the targeted genetic modification does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site.

66. The method of any one of claims 56-65, wherein the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A.

67. The method of any one of claims 56-66, wherein the targeted genetic modification reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

68. The method of any one of claims 56-67, wherein the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts.

69. The method of any one of claims 56-68, wherein the targeted genetic modification reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

70. The method of any one of claims 56-69, wherein the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts.

71. The method of any one of claims 56-70, wherein the targeted genetic modification reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

72. The method of any one of claims 56-71, wherein the targeted genetic modification reduces expression of polyGA dipeptide repeat proteins.

73. The method of any one of claims 56-72, wherein the targeted genetic modification reduces expression of both polyGA and polyGP dipeptide repeat proteins.

74. The method of any one of claims 56-73, wherein the method further comprises administering to the subject a second nuclease agent or one or more nucleic acids encoding the second nuclease agent, wherein the second nuclease agent targets a second nuclease target sequence in the *C9orf72* gene, wherein the second nuclease agent cleaves the second nuclease target sequence.

75. The method of claim 74, wherein the targeted genetic modification comprises a deletion between the first nuclease target sequence and the second nuclease target sequence.

76. The method of claim 74 or 75, wherein the second nuclease target sequence is upstream of the *C9orf72* exon 1A transcription start site.

77. The method of claim 74 or 75, wherein the second nuclease target sequence is downstream of the *C9orf72* exon 1A transcription start site.

78. The method of claim 76 or 77, wherein the second nuclease target sequence is within 2500, within 2250, within 2000, within 1800, within 1600, within 1400, within 1200, within 1000, within 900, within 800, within 700, within 600, within 500, within 450, within 400, within 350, within 300, within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

79. The method of any one of claims 56-78, wherein the method further comprises administering to the subject an exogenous donor nucleic acid for modification of the *C9orf72* gene.

80. The method of claim 79, wherein the exogenous donor nucleic acid comprises homology arms.

81. The method of claim 79 or 80, wherein:

(i) the exogenous donor nucleic acid is between about 50 nucleotides to about 1 kb in length, optionally wherein the exogenous donor sequence is between about 80 nucleotides to about 200 nucleotides in length; and/or

(ii) the exogenous donor nucleic acid is a single-stranded oligodeoxynucleotide.

82. The method of any one of claims 56-81, wherein the nuclease agent comprises:

(a) a zinc finger nuclease (ZFN);

(b) a transcription activator-like effector nuclease (TALEN); or

(c) (i) a Cas protein; and

(ii) a guide RNA, wherein the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence that is the nuclease target sequence, and wherein the guide RNA binds to the Cas protein and targets the Cas protein to the guide RNA target sequence.

83. The method of any one of claims 56-81, wherein the nuclease agent comprises:

(a) a Cas protein; and

(b) a guide RNA, wherein the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence that is the nuclease target sequence, and wherein the guide RNA binds to the Cas protein and targets the Cas protein to the guide RNA target sequence.

84. The method of any one of claims 82-83, wherein the guide RNA is a single guide RNA (sgRNA).

85. The method of any one of claims 82-84, wherein the Cas protein is a Cas9 protein.

86. The method of claim 85, wherein the Cas9 protein is derived from a *Streptococcus pyogenes* Cas9 protein, a *Staphylococcus aureus* Cas9 protein, a *Campylobacter jejuni* Cas9 protein, a *Streptococcus thermophilus* Cas9 protein, or a *Neisseria meningitidis* Cas9 protein.

87. The method of claim 85, wherein the Cas protein is derived from a *Streptococcus pyogenes* Cas9 protein.

88. The method of any one of claims 82-87, wherein the nucleic acid encoding the Cas protein is codon-optimized for expression in a mammalian cell or a human cell.

89. The method of any one of claims 82-88, wherein the method comprises administering to the subject the guide RNA in the form of RNA, optionally wherein the guide RNA comprises at least one modification.

90. The method of claim 89, wherein the at least one modification comprises a 2'-O-methyl-modified nucleotide and/or a phosphorothioate bond between nucleotides.

91. The method of any one of claims 82-90, wherein the method comprises administering to the subject the nucleic acid encoding the Cas protein, wherein the nucleic acid comprises an mRNA encoding the Cas protein, optionally wherein the mRNA encoding the Cas protein comprises at least one modification.

92. The method of any one of claims 82-88, wherein the method comprises administering to the subject the nucleic acid encoding the Cas protein and the one or more DNAs encoding the guide RNA, wherein the nucleic acid encoding the Cas protein comprises DNA.

93. The method of claim 92, wherein the DNA encoding the Cas protein and the one or more DNAs encoding the guide RNA are in one or more vectors.

94. The method of claim 93, wherein the one or more vectors are one or more viral vectors.

95. The method of claim 94, wherein the one or more viral vectors are one or more adeno-associated virus (AAV) vectors.

96. The method of any one of claims 82-95, wherein the Cas protein or the nucleic acid encoding the Cas protein and the guide RNA or the one or more DNAs encoding the guide RNA are associated with a lipid nanoparticle.

97. The method of any one of claims 82-96, wherein:

(I) the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 73-

111 or 74-111, optionally wherein the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96; and/or

(II) the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96; and/or

(III) the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 33-71 or 34-71, optionally wherein guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 34-36, 53, 54, and 56.

98. The method of any one of claims 82-97, wherein:

(I) the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96; and/or

(II) the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96; and/or

(III) the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, and 56.

99. The method of claim 98, wherein the method further comprises contacting the *C9orf72* gene with a second nuclease agent that targets a second nuclease target sequence in the *C9orf72* gene, wherein the second nuclease agent cleaves the second nuclease target sequence,

wherein the second nuclease agent comprises the Cas protein and aa second guide RNA, wherein the second guide RNA comprises a second DNA-targeting segment that targets a second guide RNA target sequence that is the second nuclease target sequence, wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence, and wherein:

(I) the second DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or

(II) the second DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or

(III) the second guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, 56, and 132.

100. The method of any one of claims 56-99, wherein the targeted genetic modification in the *C9orf72* gene is generated in neurons in the subject, optionally wherein the neurons are motor neurons.

101. The method of claim 100, wherein the neurons are in the brain of the subject.

102. The method of any one of claims 56-101, wherein the subject is a mammalian subject, and the *C9orf72* gene is a mammalian *C9orf72* gene.

103. The method of any one of claims 56-102, wherein the subject is a human subject.

104. The method of any one of claims 56-102, wherein the subject is a mouse subject.

105. The method of any one of claims 56-104, wherein the *C9orf72* gene comprises a human *C9orf72* promoter.

106. The method of any one of claims 56-105, wherein the *C9orf72* gene is a human *C9orf72* gene or a humanized *C9orf72* gene.

107. A CRISPR/Cas system comprising a first guide RNA or one or more DNAs encoding the first guide RNA, wherein the first guide RNA comprises a DNA-targeting segment that targets a first guide RNA target sequence in a *C9orf72* gene, wherein the first guide RNA target sequence is near and optionally upstream of the *C9orf72* exon 1A transcription start

site, and wherein the first guide RNA binds to a Cas protein and targets the Cas protein to the first guide RNA target sequence.

108. The CRISPR/Cas system of claim 107, wherein the first guide RNA target sequence is within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

109. The CRISPR/Cas system of claim 107 or 108, wherein the first guide RNA target sequence is within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

110. The CRISPR/Cas system of any one of claims 107-109, wherein cleavage of the first guide RNA target sequence by the Cas protein results in a deletion of a region of the *C9orf72* promoter.

111. The CRISPR/Cas system of claim 110, wherein the deletion encompasses the *C9orf72* exon 1A transcription start site.

112. The CRISPR/Cas system of any one of claims 107-110, wherein cleavage of the first guide RNA target sequence by the Cas protein does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site.

113. The CRISPR/Cas system of any one of claims 107-112, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A.

114. The CRISPR/Cas system of any one of claims 107-113, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

115. The CRISPR/Cas system of any one of claims 107-114, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts.

116. The CRISPR/Cas system of any one of claims 107-115, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

117. The CRISPR/Cas system of any one of claims 107-116, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts.

118. The CRISPR/Cas system of any one of claims 107-117, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

119. The CRISPR/Cas system of any one of claims 107-118, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces expression of polyGA dipeptide repeat proteins.

120. The CRISPR/Cas system of any one of claims 107-119, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces expression of both polyGA and polyGP dipeptide repeat proteins.

121. The CRISPR/Cas system of any one of claims 107-120, further comprising a second guide RNA or one or more DNAs encoding the second guide RNA, wherein the second guide RNA comprises a DNA-targeting segment that targets a second guide RNA target sequence in a *C9orf72* gene, and wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence.

122. The CRISPR/Cas system of claim 121, wherein cleavage by the Cas protein at the first guide RNA target sequence and cleavage by the Cas protein at the second guide RNA target sequence results a deletion between the first guide RNA target sequence and the second guide RNA target sequence.

123. The CRISPR/Cas system of claim 121 or 122, wherein the second guide RNA target sequence is upstream of the *C9orf72* exon 1A transcription start site.

124. The CRISPR/Cas system of claim 121 or 122, wherein the second guide RNA target sequence is downstream of the *C9orf72* exon 1A transcription start site.

125. The CRISPR/Cas system of claim 123 or 124, wherein the second guide RNA target sequence is within 2500, within 2250, within 2000, within 1800, within 1600, within 1400, within 1200, within 1000, within 900, within 800, within 700, within 600, within 500, within 450, within 400, within 350, within 300, within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

126. The CRISPR/Cas system of any one of claims 107-125, further comprising an exogenous donor nucleic acid for modification of the *C9orf72* gene.

127. The CRISPR/Cas system of claim 126, wherein the exogenous donor nucleic acid comprises homology arms.

128. The CRISPR/Cas system of claim 126 or 127, wherein:

(i) the exogenous donor nucleic acid is between about 50 nucleotides to about 1 kb in length, optionally wherein the exogenous donor sequence is between about 80 nucleotides to about 200 nucleotides in length; and/or

(ii) the exogenous donor nucleic acid is a single-stranded oligodeoxynucleotide.

129. The CRISPR/Cas system of any one of claims 107-128, wherein the first guide RNA is a single guide RNA (sgRNA).

130. The CRISPR/Cas system of any one of claims 107-129, wherein the CRISPR/Cas system comprises the first guide RNA in the form of RNA, optionally wherein the first guide RNA comprises at least one modification.

131. The CRISPR/Cas system of claim 130, wherein the at least one modification comprises a 2'-O-methyl-modified nucleotide and/or a phosphorothioate bond between nucleotides.

132. The CRISPR/Cas system of any one of claims 107-131, wherein:

(I) the first DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the first DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96; and/or

(II) the first DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the first DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96; and/or

(III) the first guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 33-71 or 34-71, optionally wherein first guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 34-36, 53, 54, and 56.

133. The CRISPR/Cas system of any one of claims 107-132, wherein:

(I) the first DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96; and/or

(II) the first DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96; and/or

(III) the first guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, and 56.

134. The CRISPR/Cas system of claim 133, further comprising a second guide RNA or one or more DNAs encoding the second guide RNA, wherein the second guide RNA

comprises a DNA-targeting segment that targets a second guide RNA target sequence in a *C9orf72* gene, and wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence, and wherein:

(I) the second DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or

(II) the second DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or

(III) the second guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, 56, and 132.

135. The CRISPR/Cas system of any one of claims 107-134, wherein the Cas protein is a Cas9 protein.

136. The CRISPR/Cas system of claim 135, wherein the Cas9 protein is derived from a *Streptococcus pyogenes* Cas9 protein, a *Staphylococcus aureus* Cas9 protein, a *Campylobacter jejuni* Cas9 protein, a *Streptococcus thermophilus* Cas9 protein, or a *Neisseria meningitidis* Cas9 protein.

137. The CRISPR/Cas system of claim 135, wherein the Cas protein is derived from a *Streptococcus pyogenes* Cas9 protein.

138. The CRISPR/Cas system of any one of claims 107-137, further comprising the Cas protein or a nucleic acid encoding the Cas protein.

139. The CRISPR/Cas system of claim 138, wherein the nucleic acid encoding the Cas protein is codon-optimized for expression in a mammalian cell or a human cell.

140. The CRISPR/Cas system of claim 138 or 139, wherein the CRISPR/Cas system comprises the nucleic acid encoding the Cas protein, wherein the nucleic acid comprises an mRNA encoding the Cas protein, optionally wherein the mRNA encoding the Cas protein comprises at least one modification.

141. The CRISPR/Cas system of any one of claims 138 or 139, wherein the CRISPR/Cas system comprises the nucleic acid encoding the Cas protein and the one or more DNAs encoding the first guide RNA, wherein the nucleic acid encoding the Cas protein comprises DNA.

142. The CRISPR/Cas system of claim 141, wherein the DNA encoding the Cas protein and the one or more DNAs encoding the first guide RNA are in one or more vectors.

143. The CRISPR/Cas system of claim 142, wherein the one or more vectors are one or more viral vectors.

144. The CRISPR/Cas system of claim 143, wherein the one or more viral vectors are one or more adeno-associated virus (AAV) vectors.

145. The CRISPR/Cas system of any one of claims 138-144, wherein the Cas protein or the nucleic acid encoding the Cas protein and the first guide RNA or the one or more DNAs encoding the first guide RNA are associated with a lipid nanoparticle.

146. The CRISPR/Cas system of any one of claims 107-145, wherein the *C9orf72* gene is a mammalian *C9orf72* gene.

147. The CRISPR/Cas system of any one of claims 107-146, wherein the *C9orf72* gene comprises a human *C9orf72* promoter.

148. The CRISPR/Cas system of any one of claims 107-147, wherein the *C9orf72* gene is a human *C9orf72* gene or a humanized *C9orf72* gene.

149. A pharmaceutical composition comprising the CRISPR/Cas system of any one of claims 107-148 and a pharmaceutically acceptable carrier.

150. A composition comprising a guide RNA or one or more DNAs encoding the guide RNA, wherein the guide RNA comprises a DNA-targeting segment that targets a guide RNA target sequence in a *C9orf72* gene, wherein the guide RNA target sequence is near and optionally upstream of the *C9orf72* exon 1A transcription start site, and wherein the guide RNA can bind to a Cas protein and target the Cas protein to the guide RNA target sequence.

151. The composition of claim 150, wherein the guide RNA target sequence is within 2500, within 2250, within 2000, within 1800, within 1600, within 1400, within 1200, within 1000, within 900, within 800, within 700, within 600, within 500, within 450, within 400, within 350, within 300, within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

152. The composition of claim 150 or 151, wherein the guide RNA target sequence is within 250, within 225, within 200, within 175, within 150, within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

153. The composition of any one of claims 150-152, wherein the guide RNA target sequence is within 125, within 100, within 75, or within 50 nucleotides of the *C9orf72* exon 1A transcription start site.

154. The composition of any one of claims 150-153, wherein cleavage of the guide RNA target sequence by the Cas protein results in a deletion of a region of the *C9orf72* promoter.

155. The composition of claim 154, wherein the deletion encompasses the *C9orf72* exon 1A transcription start site.

156. The composition of any one of claims 150-154, wherein cleavage of the guide RNA target sequence by the Cas protein does not result in deletion or disruption of the *C9orf72* exon 1A transcription start site.

157. The composition of any one of claims 150-156, wherein cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A.

158. The composition of any one of claims 150-157, wherein cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of transcripts that initiate at *C9orf72* exon 1A but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

159. The composition of any one of claims 150-158, wherein cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts.

160. The composition of any one of claims 150-159, wherein cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

161. The composition of any one of claims 150-160, wherein cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts.

162. The composition of any one of claims 150-161, wherein cleavage of the guide RNA target sequence by the Cas protein reduces or abolishes expression of both sense and antisense *C9orf72* hexanucleotide-repeat-containing transcripts but does not reduce or abolish expression of transcripts that initiate at *C9orf72* exon 1B.

163. The composition of any one of claims 150-162, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces expression of polyGA dipeptide repeat proteins.

164. The composition of any one of claims 150-163, wherein cleavage of the first guide RNA target sequence by the Cas protein reduces expression of both polyGA and polyGP dipeptide repeat proteins.

165. The composition of any one of claims 150-164, wherein the guide RNA is a single guide RNA (sgRNA).

166. The composition of any one of claims 150-165, wherein the composition comprises the guide RNA in the form of RNA, optionally wherein the guide RNA comprises at least one modification.

167. The composition of claim 166, wherein the at least one modification comprises a 2'-O-methyl-modified nucleotide and/or a phosphorothioate bond between nucleotides.

168. The composition of any one of claims 150-167, wherein:

(I) the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96; and/or

(II) the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 73-111 or 74-111, optionally wherein the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 74-76, 93, 94, and 96; and/or

(III) the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 33-71 or 34-71, optionally wherein guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 34-36, 53, 54, and 56.

169. The composition of any one of claims 150-168, wherein:

(I) the DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96; and/or

(II) the DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, and 96; and/or

(III) the guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, and 56.

170. The composition of claim 169, further comprising a second guide RNA or one or more DNAs encoding the second guide RNA, wherein the second guide RNA comprises a

DNA-targeting segment that targets a second guide RNA target sequence in a *C9orf72* gene, and wherein the second guide RNA binds to the Cas protein and targets the Cas protein to the second guide RNA target sequence, and wherein:

(I) the second DNA-targeting segment comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or

(II) the second DNA-targeting segment is at least 90% or at least 95% identical to the sequence set forth in any one of SEQ ID NOS: 93, 94, 96, and 133; and/or

(III) the second guide RNA target sequence comprises at least 17, at least 18, at least 19, or at least 20 contiguous nucleotides of the sequence set forth in any one of SEQ ID NOS: 53, 54, 56, and 132.

171. The composition of any one of claims 150-170, wherein the Cas protein is a Cas9 protein.

172. The composition of claim 171, wherein the Cas9 protein is derived from a *Streptococcus pyogenes* Cas9 protein, a *Staphylococcus aureus* Cas9 protein, a *Campylobacter jejuni* Cas9 protein, a *Streptococcus thermophilus* Cas9 protein, or a *Neisseria meningitidis* Cas9 protein.

173. The composition of claim 171, wherein the Cas protein is derived from a *Streptococcus pyogenes* Cas9 protein.

174. The composition of any one of claims 150-173, wherein the one or more DNAs encoding the guide RNA are in one or more vectors.

175. The composition of claim 174, wherein the one or more vectors are one or more viral vectors.

176. The composition of claim 175, wherein the one or more viral vectors are one or more adeno-associated virus (AAV) vectors.

177. The composition of any one of claims 150-176, wherein the guide RNA or the one or more DNAs encoding the guide RNA are associated with a lipid nanoparticle.

178. The composition of any one of claims 150-177, wherein the *C9orf72* gene is a mammalian *C9orf72* gene.

179. The composition of any one of claims 150-178, wherein the *C9orf72* gene comprises a human *C9orf72* promoter.

180. The composition of any one of claims 150-179, wherein the *C9orf72* gene is a human *C9orf72* gene or a humanized *C9orf72* gene.

181. A pharmaceutical composition comprising the composition of any one of claims 150-180 and a pharmaceutically acceptable carrier.

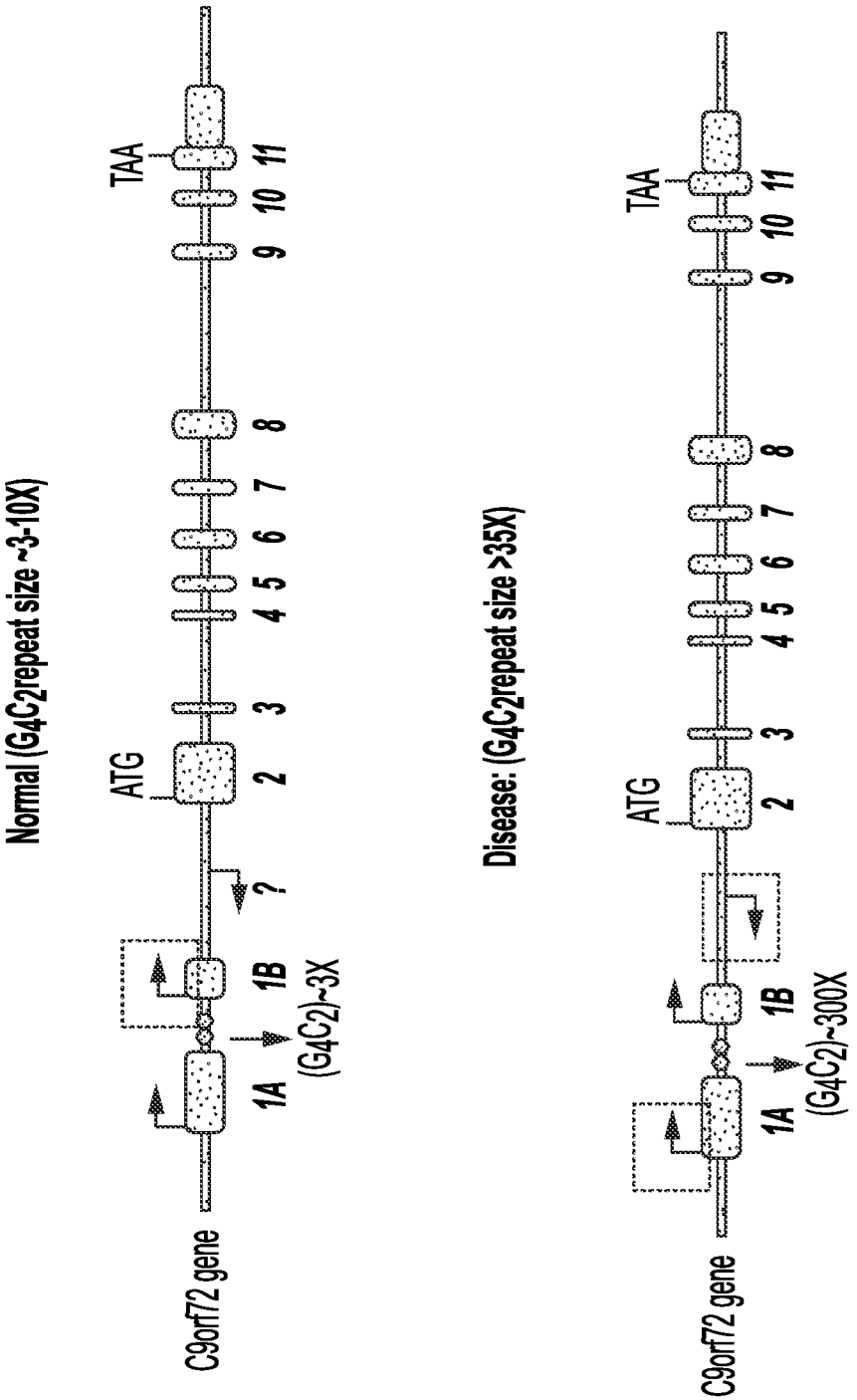
Normal (G4C2repeat size ~3-10X)
C9orf72 gene
ATG
TAA
1A
1B
(G4C2)~3X
2
3
4
5
6
7
8
9
10
11
?
Disease: (G4C2repeat size >35X)
C9orf72 gene
ATG
TAA
1A
1B
(G4C2)~300X
2
3
4
5
6
7
8
9
10
11

FIG. 1

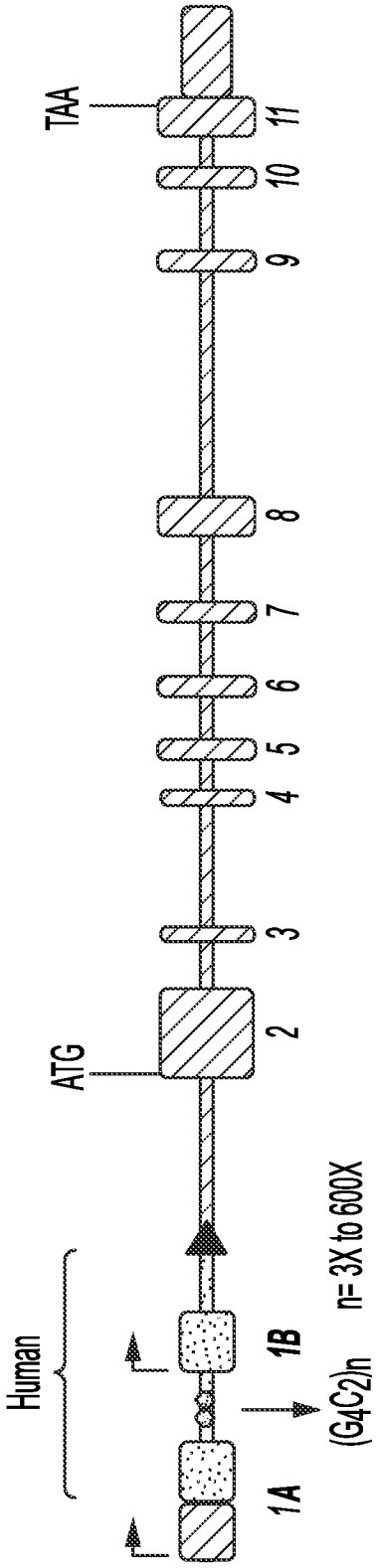
TAA
ATG
Human
1A
1B
(G4C2)n
n= 3X to 600X
2
3
4
5
6
7
8
9
10
11

FIG. 2

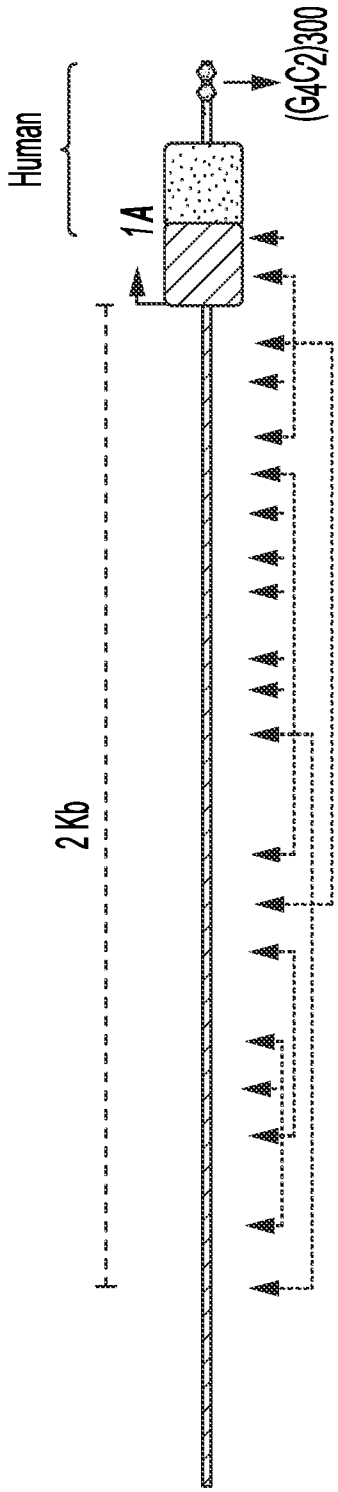
Human
1A
2 Kb
(G4C2)300

FIG. 3

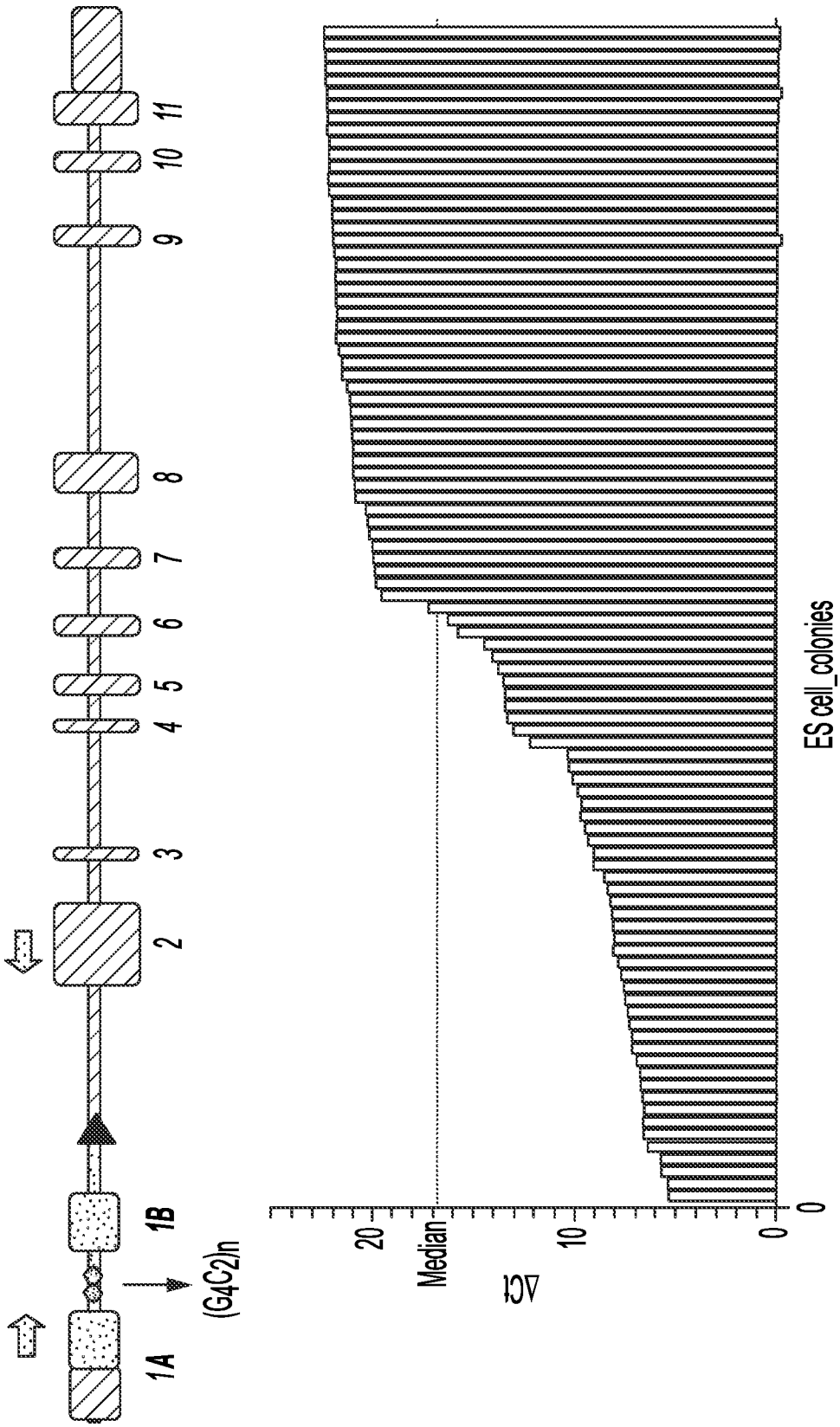
1A
(G4C2)n
1B
2
3
4
5
6
7
8
9
10
11
ΔCt
20
10
0
Median
0
ES cell_colonies

FIG. 4A

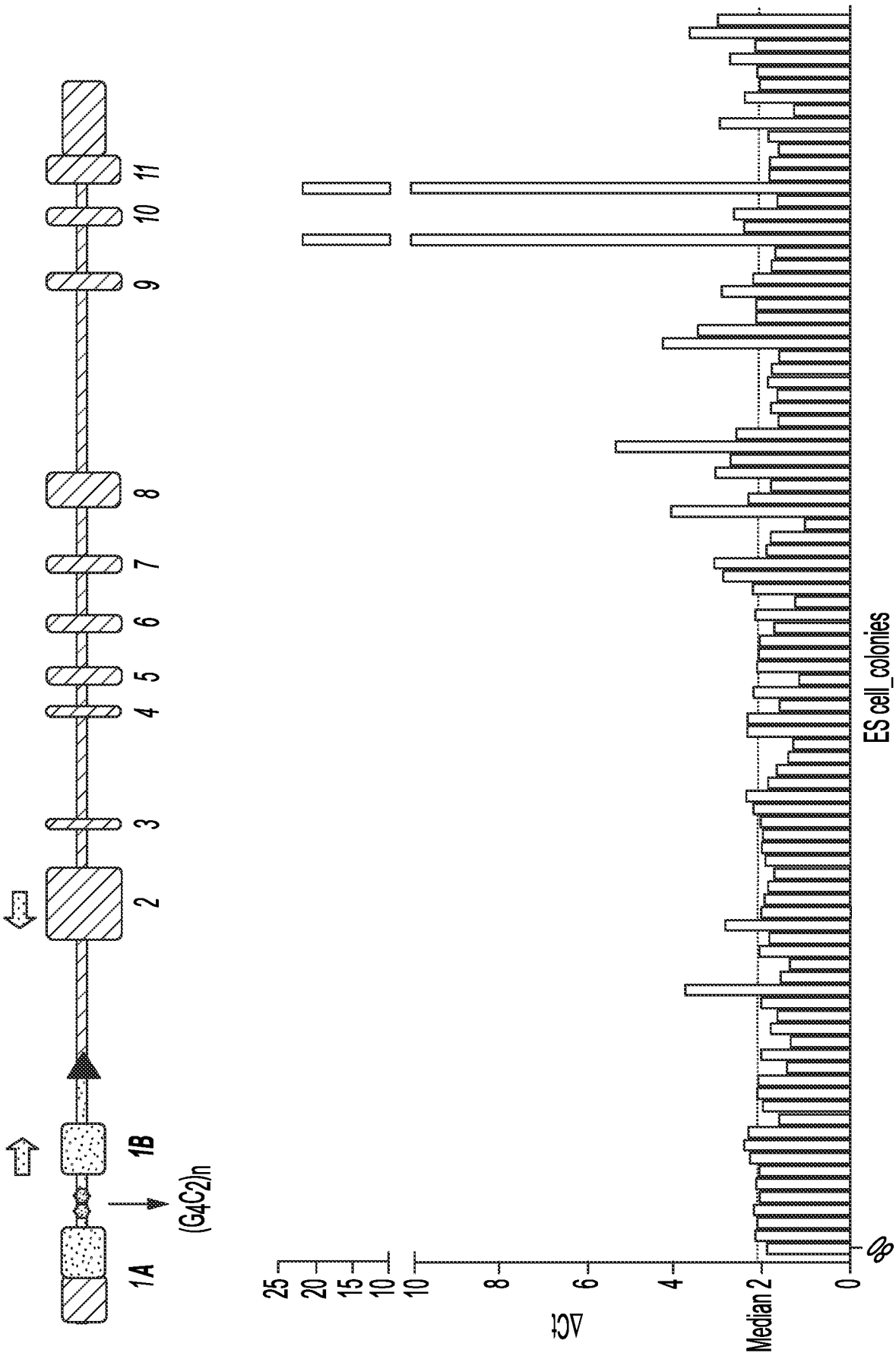
1A
1B
(G4C2)n
2
3
4
5
6
7
8
9
10
11
ΔCt
25
20
15
10
8
6
4
Median 2
0
08
ES cell_colonies

FIG. 4B

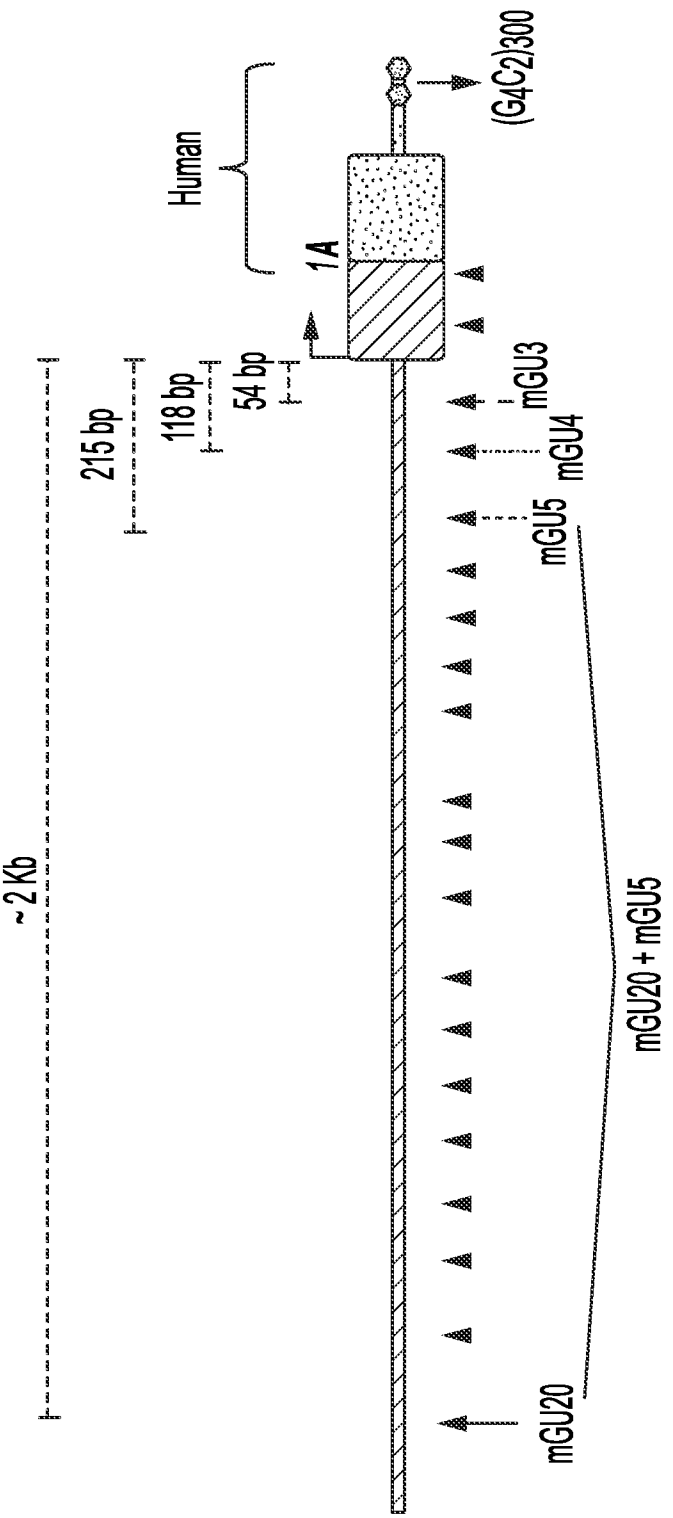
(G4C2)300
Human
1A
54 bp
118 bp
215 bp
~2 Kb
mGU3
mGU4
mGU5
mGU20 + mGU5
mGU20

FIG. 5

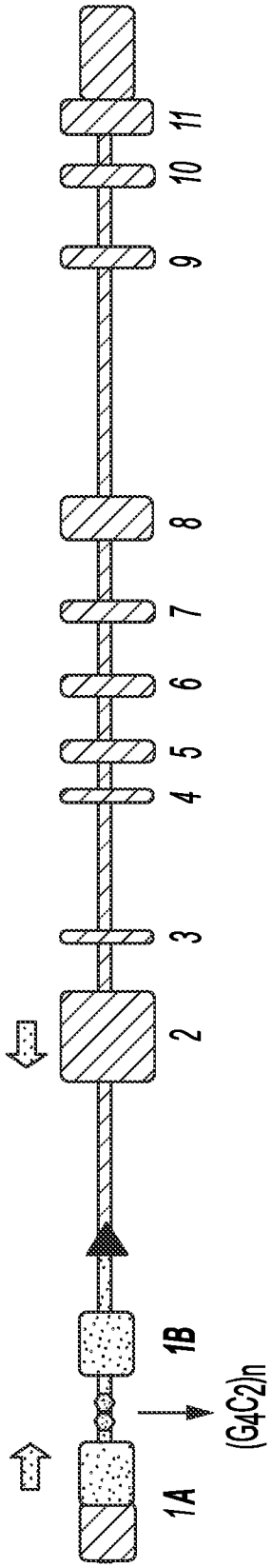

FIG. 6A

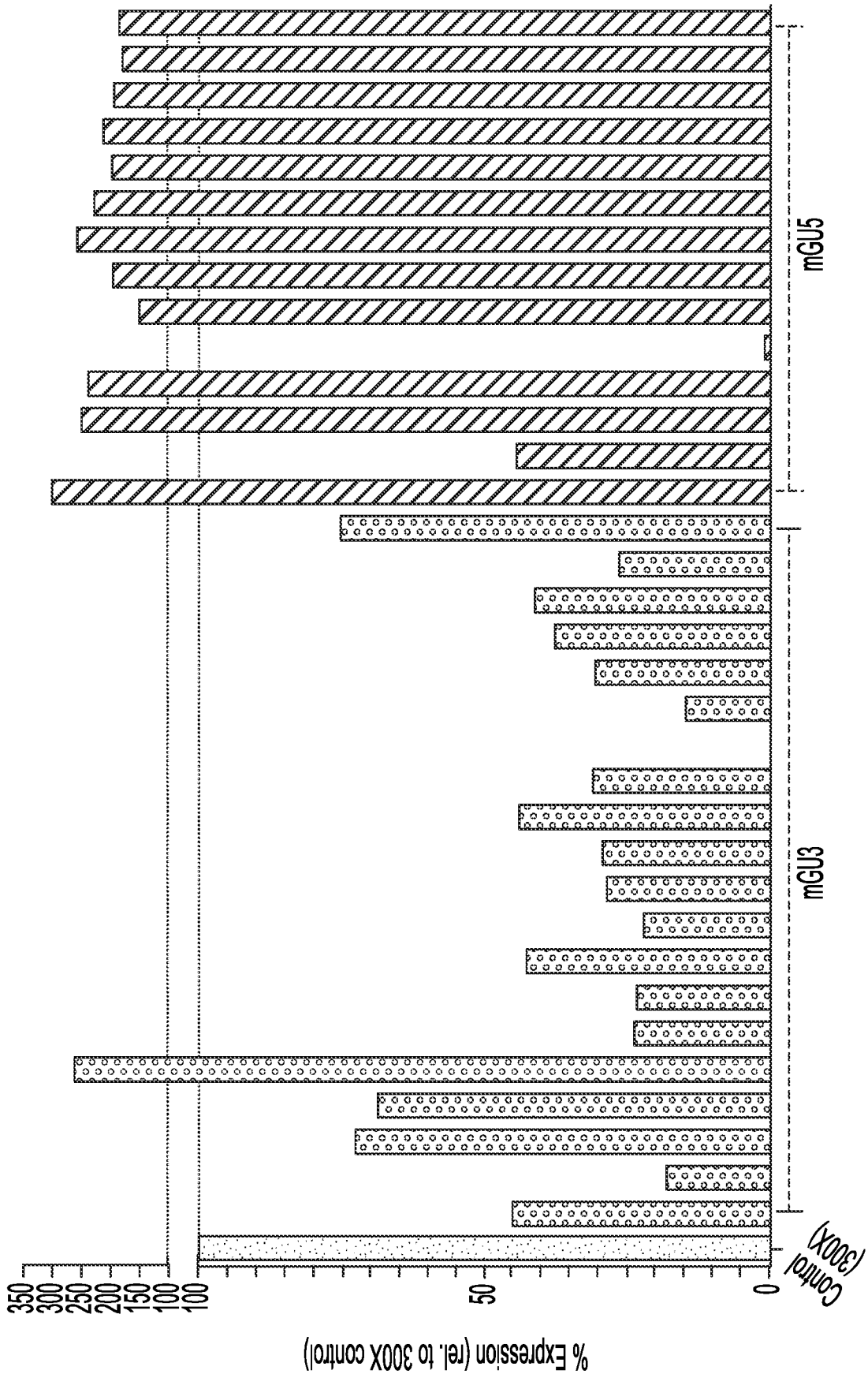

FIG. 6A
CONTINUED

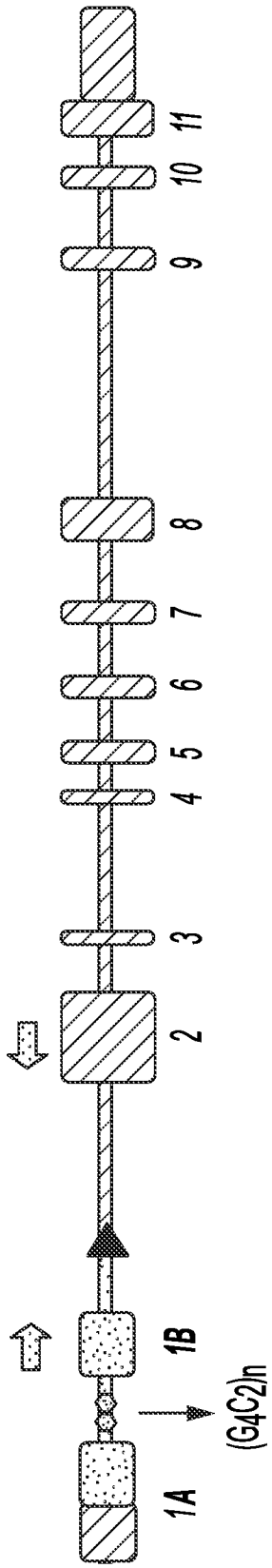

FIG. 6B

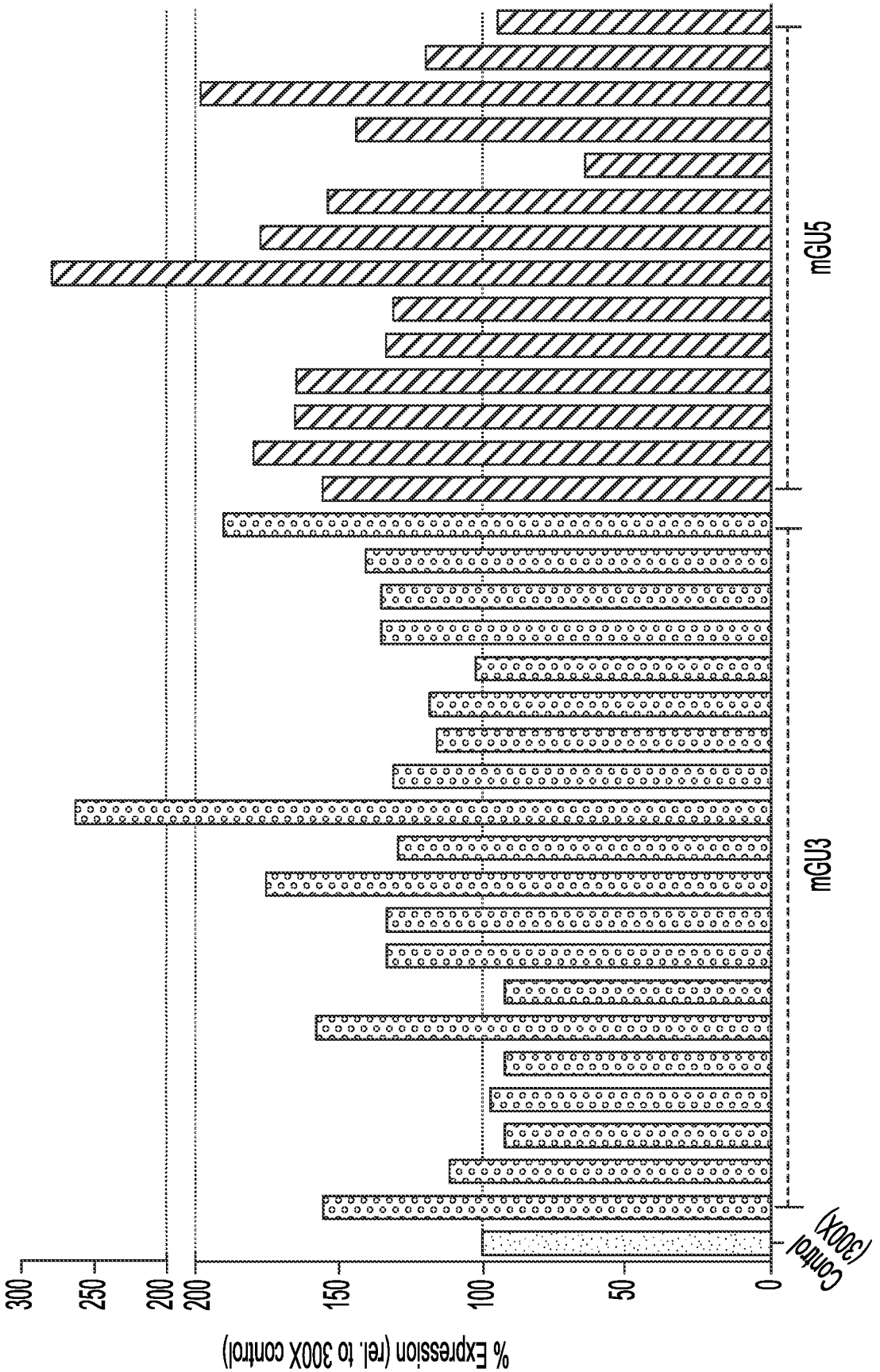

FIG. 6B
CONTINUED

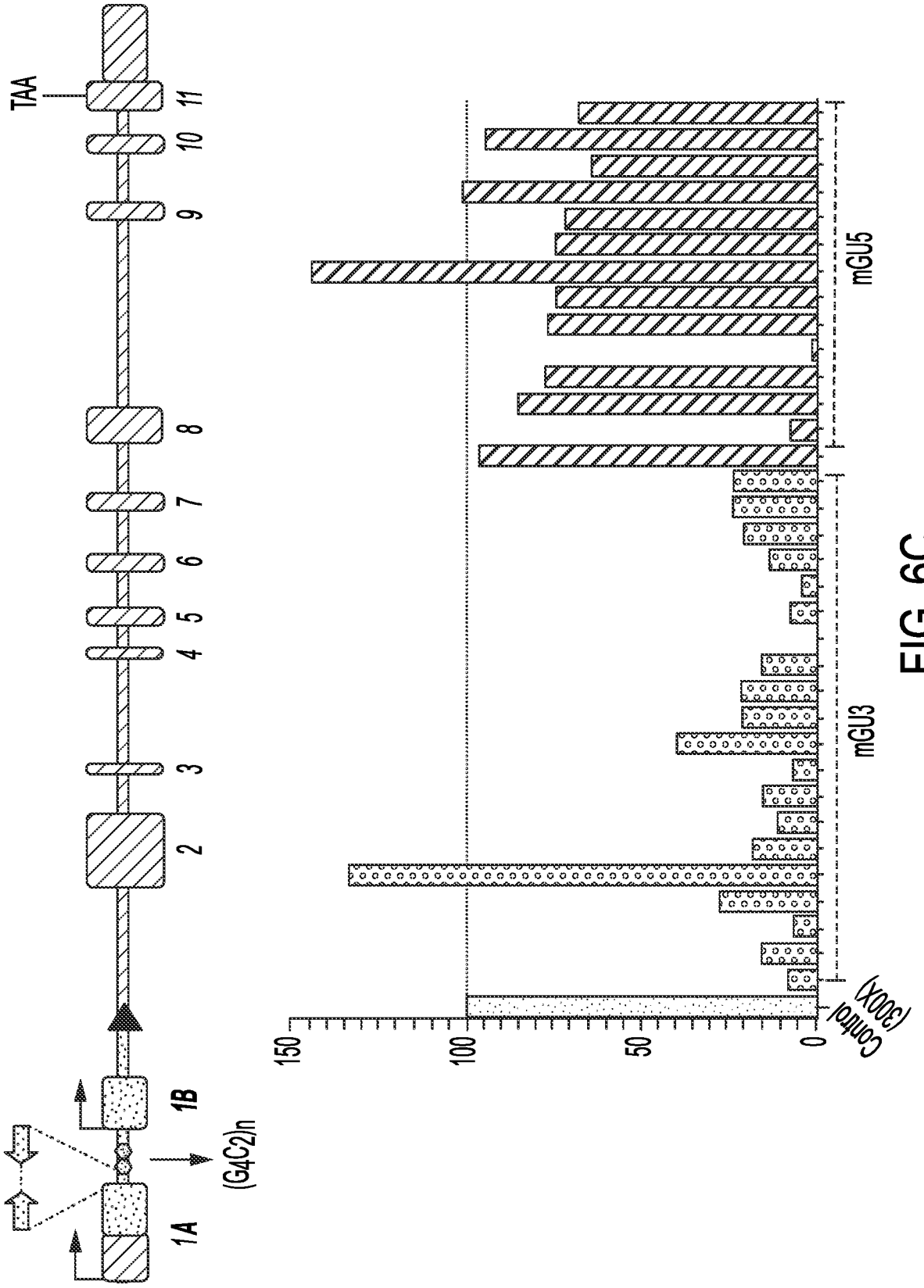
TAA
11
10
9
8
7
6
5
4
3
2
1B
1A
(G4C2)n
150
100
50
0
Control (300X)
mGU3
mGU5

FIG. 6C

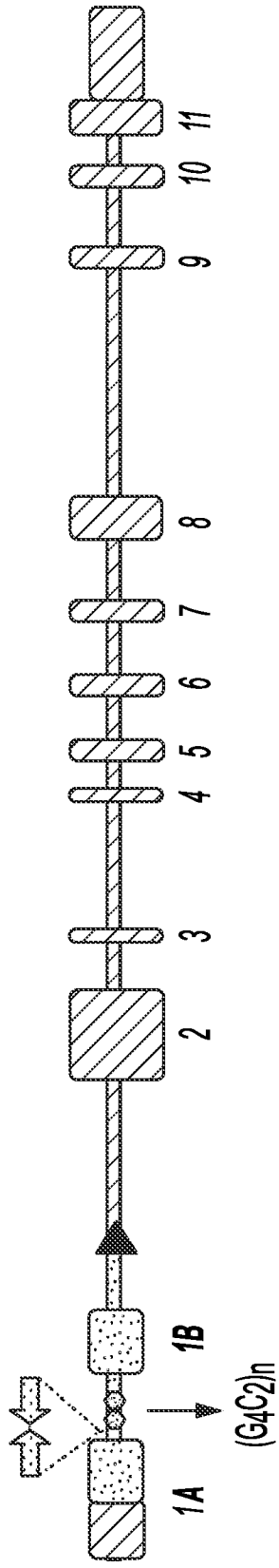

FIG. 6D

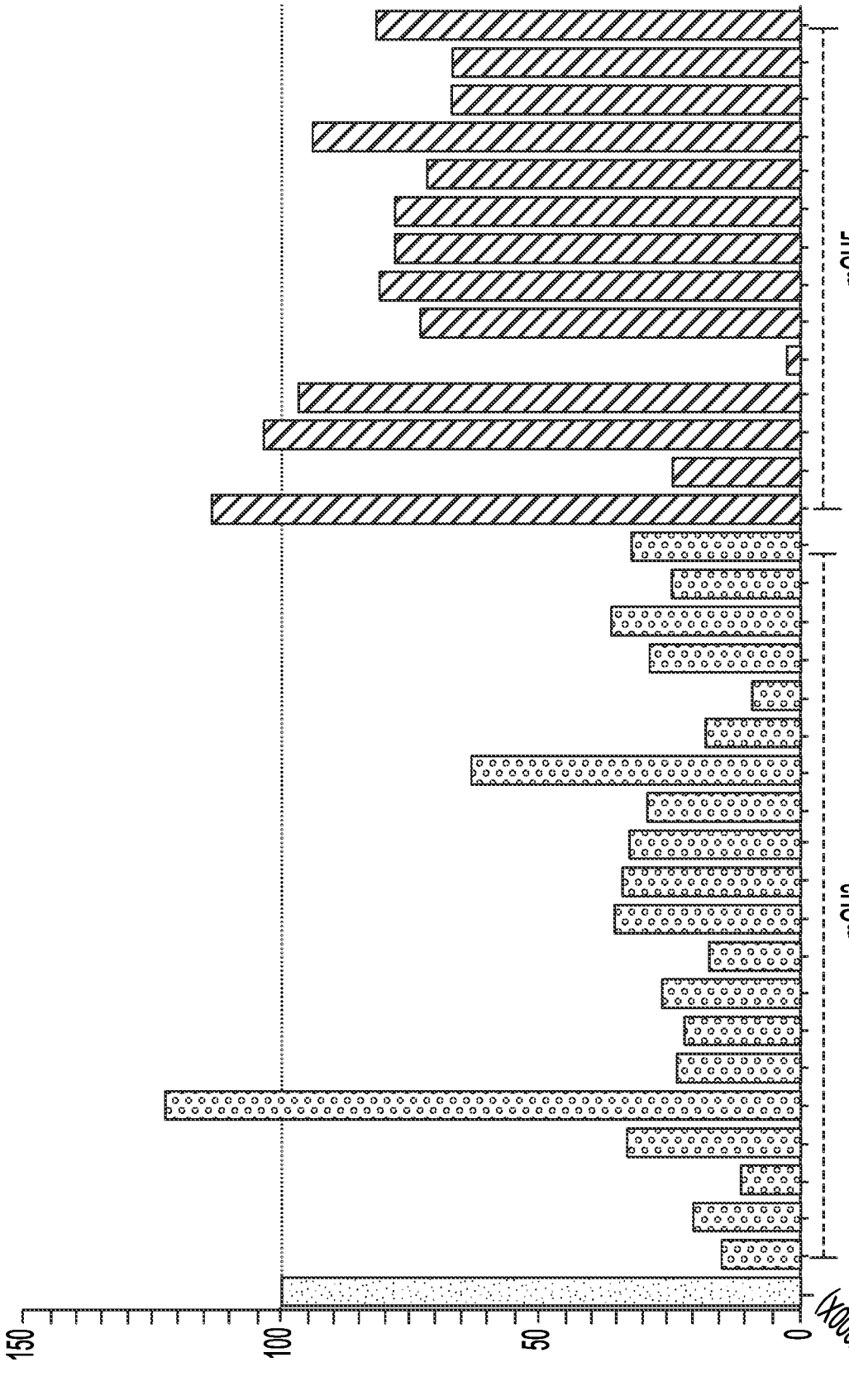

FIG. 6D
CONTINUED

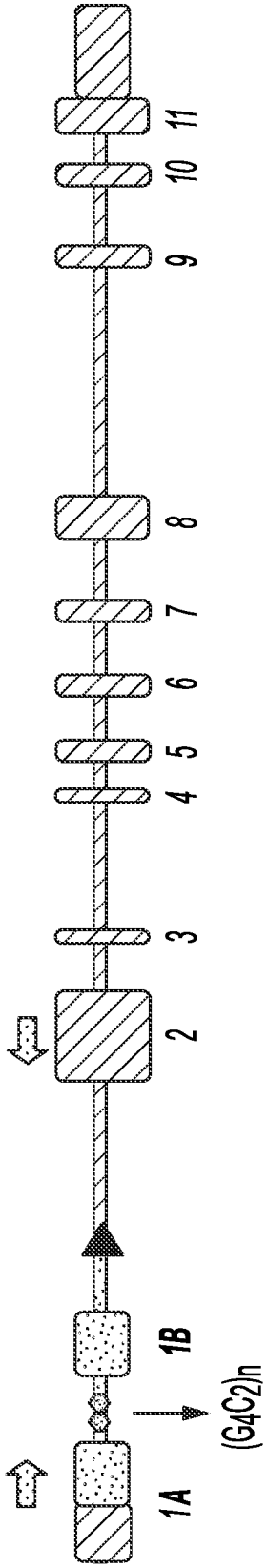

FIG. 7A

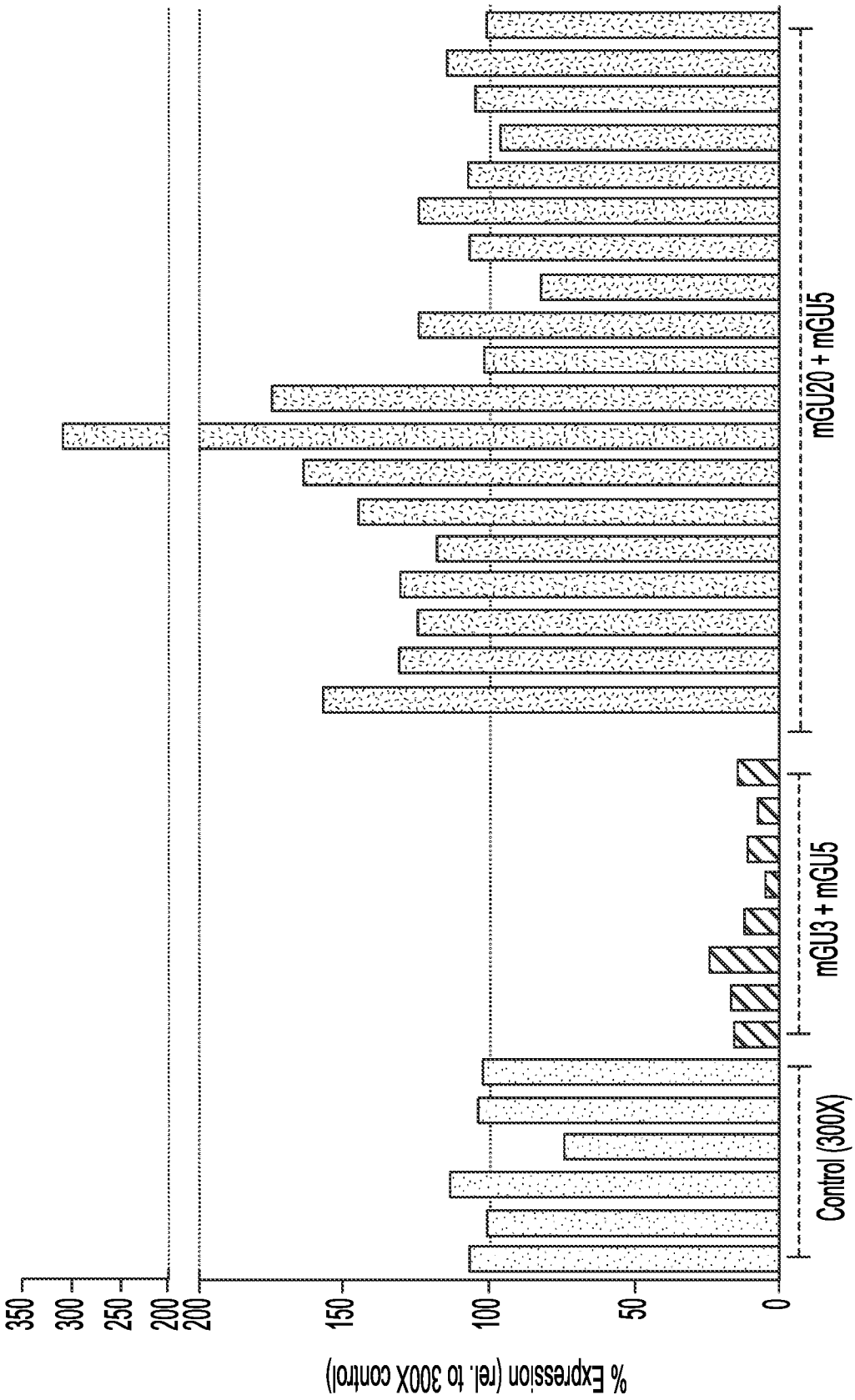

FIG. 7A
CONTINUED

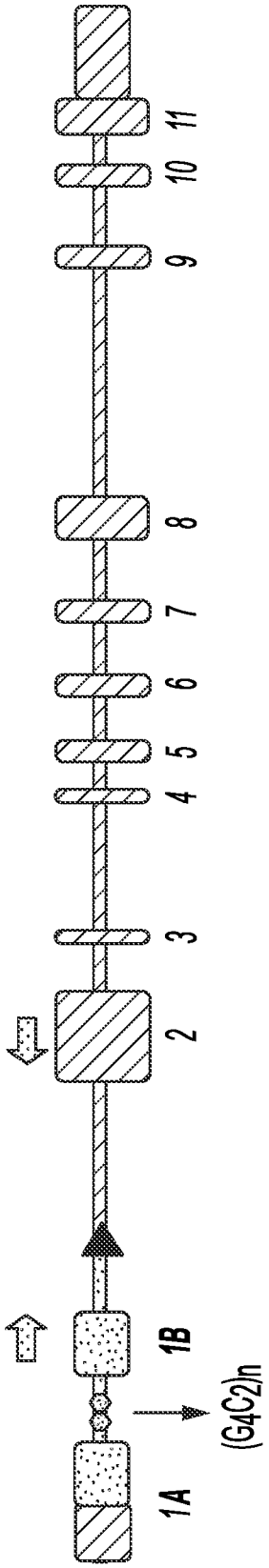

FIG. 7B

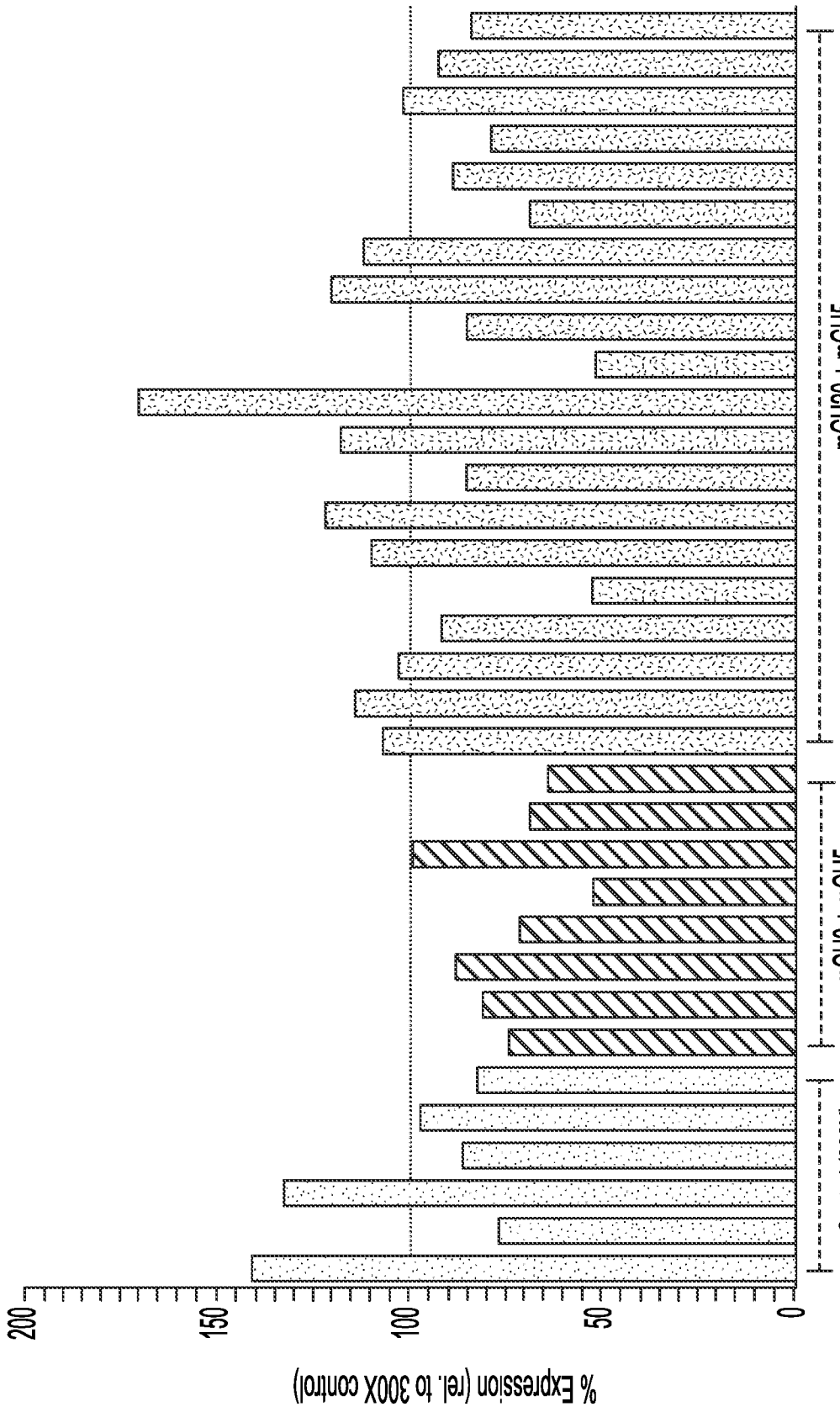

FIG. 7B
CONTINUED

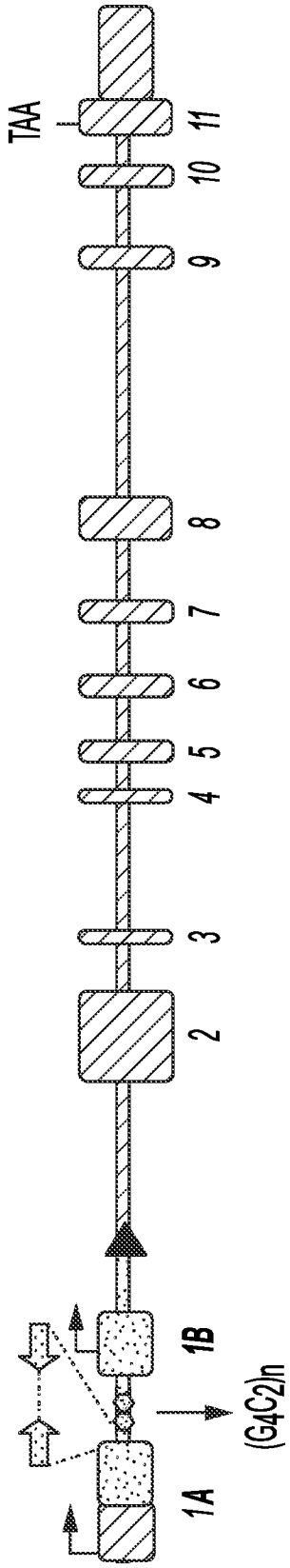

FIG. 7C

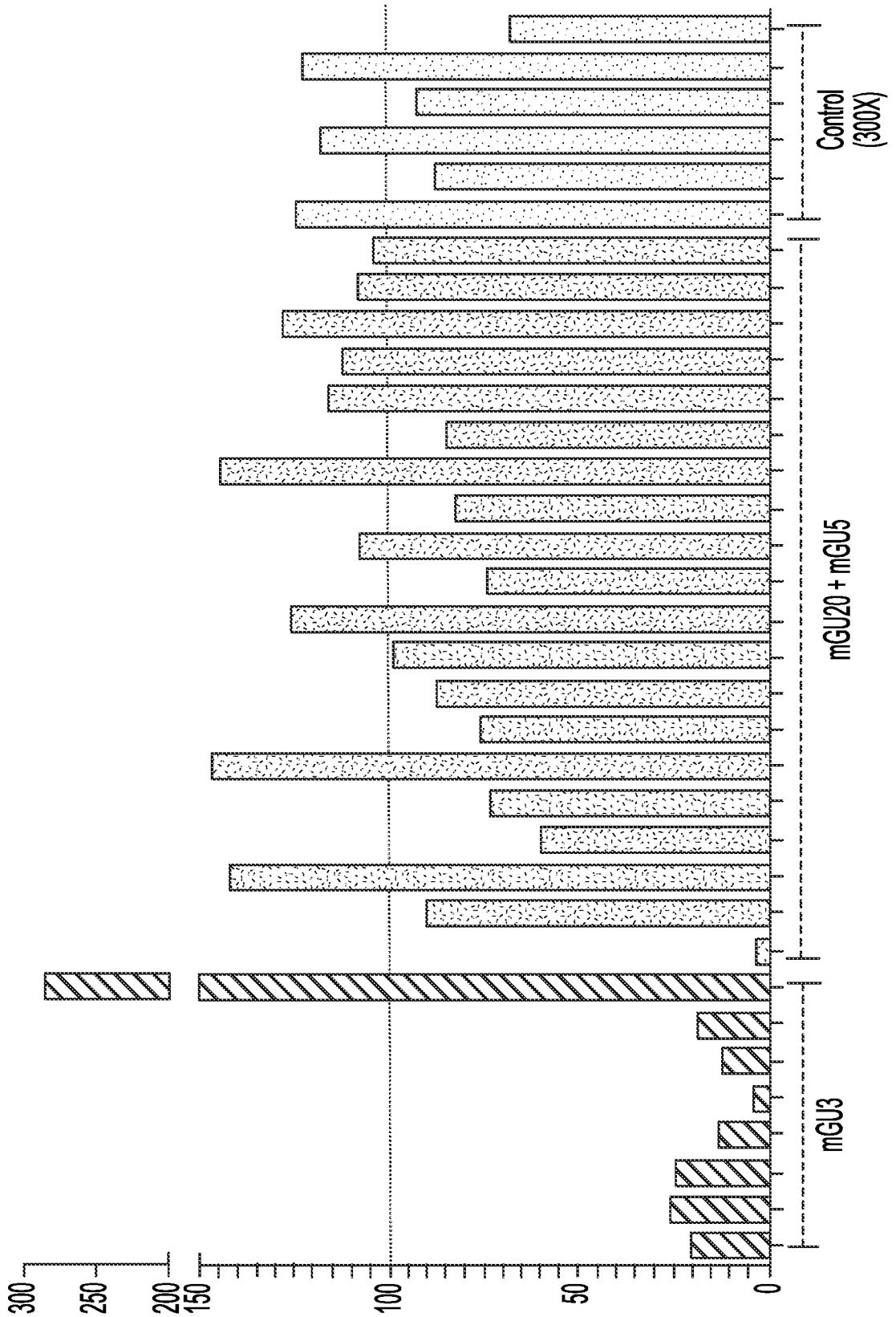

FIG. 7C
CONTINUED

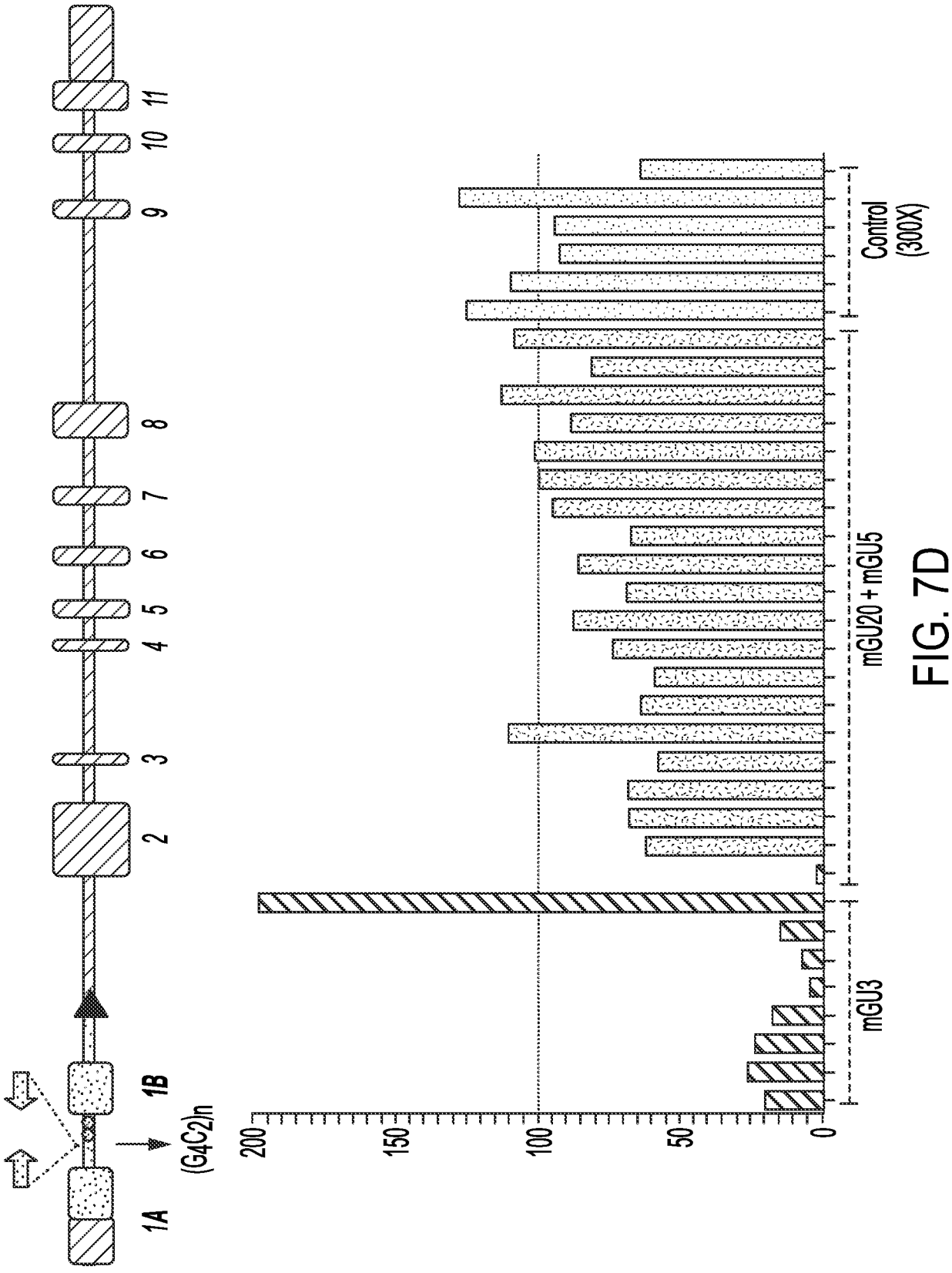
1A
1B
(G4C2)n
2
3
4
5
6
7
8
9
10
11
200
150
100
50
0
mGU3
mGU20 + mGU5
Control
(300X)

FIG. 7D

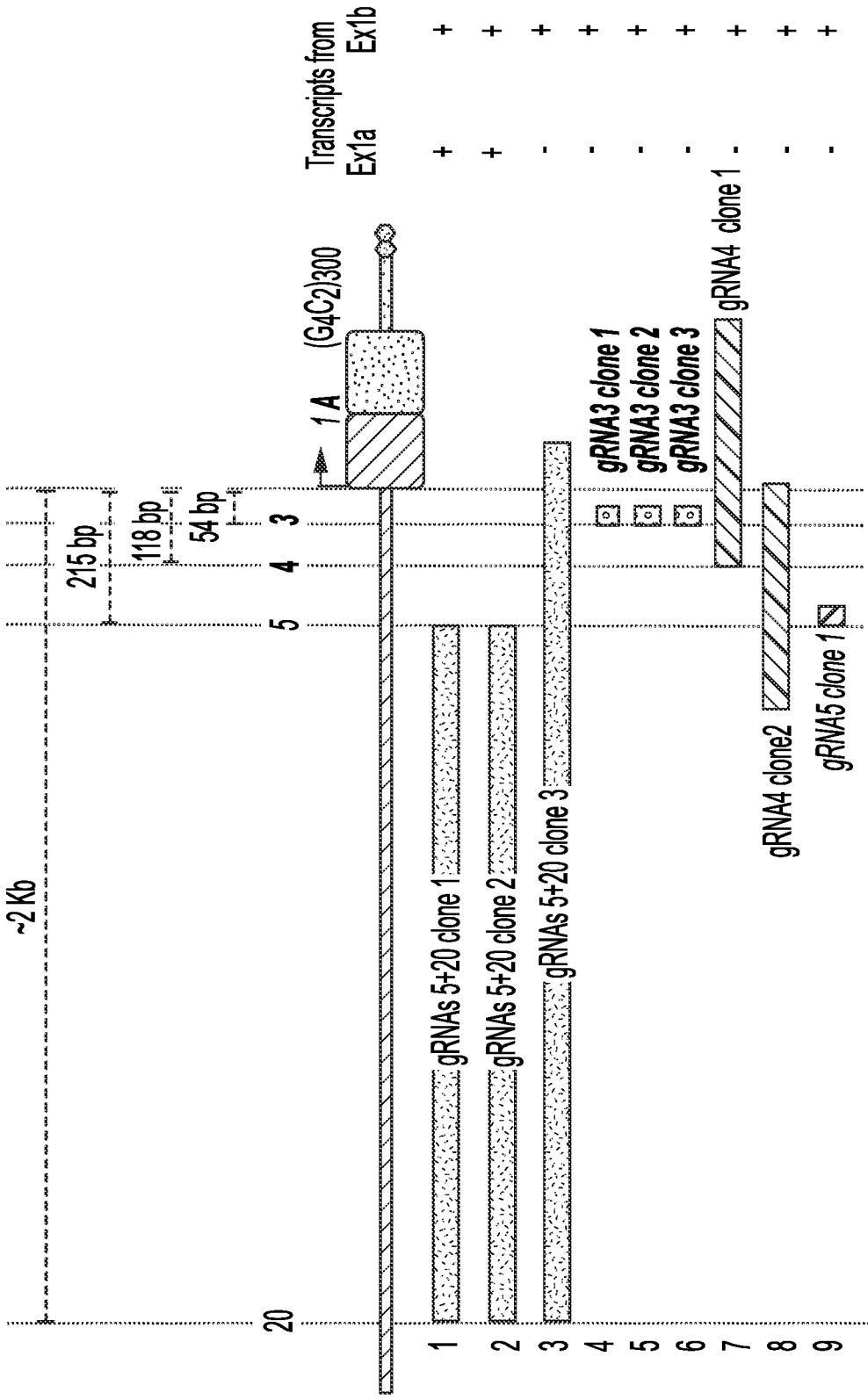
Transcripts from
Ex1a
Ex1b
(G4C2)300
1 A
gRNA3 clone 1
gRNA3 clone 2
gRNA3 clone 3
gRNA4 clone 1
gRNA4 clone2
gRNA5 clone 1
gRNAs 5+20 clone 1
gRNAs 5+20 clone 2
gRNAs 5+20 clone 3
~2 Kb
215 bp
118 bp
54 bp
20
5
4
3
1
2
3
4
5
6
7
8
9

FIG. 8

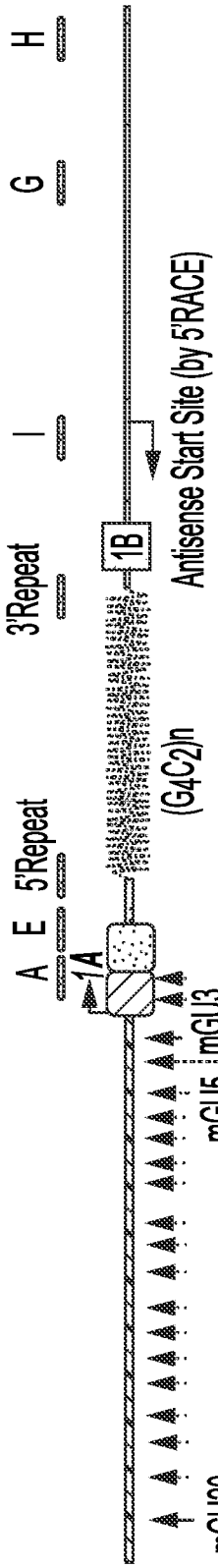
A
E
5'Repeat
1A
(G4C2)n
3'Repeat
I
G
H
1B
Antisense Start Site (by 5'RACE)
mGU3
mGU5
mGU4
mGU20 + mGU5
mGU20

FIG. 9

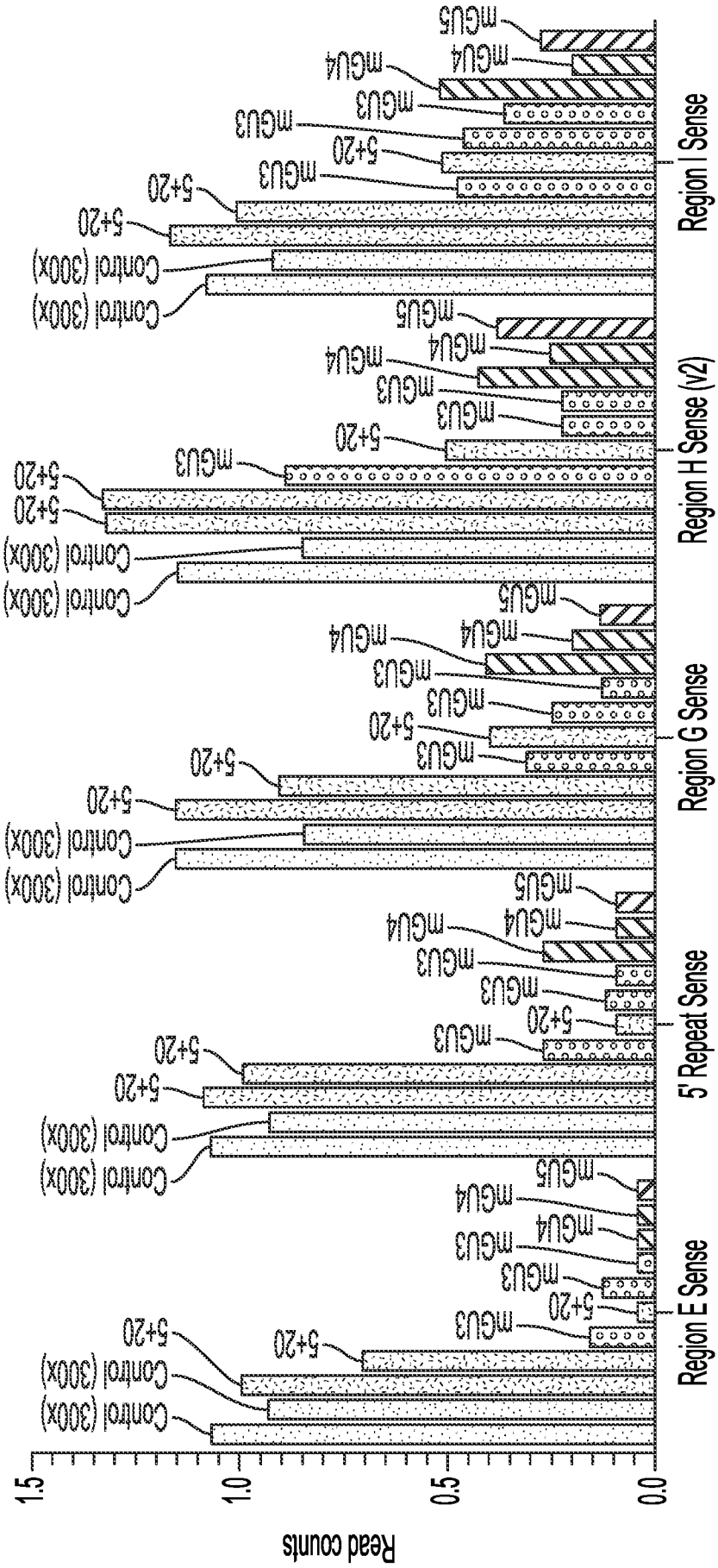

FIG. 9
CONTINUED


A E 5'Repeat
3'Repeat I G H
1A
1B
(G4C2)n
Antisense Start Site (by 5'RACE)
mGU20
mGU20 + mGU5
mGU5
mGU3
mGU4

FIG. 10

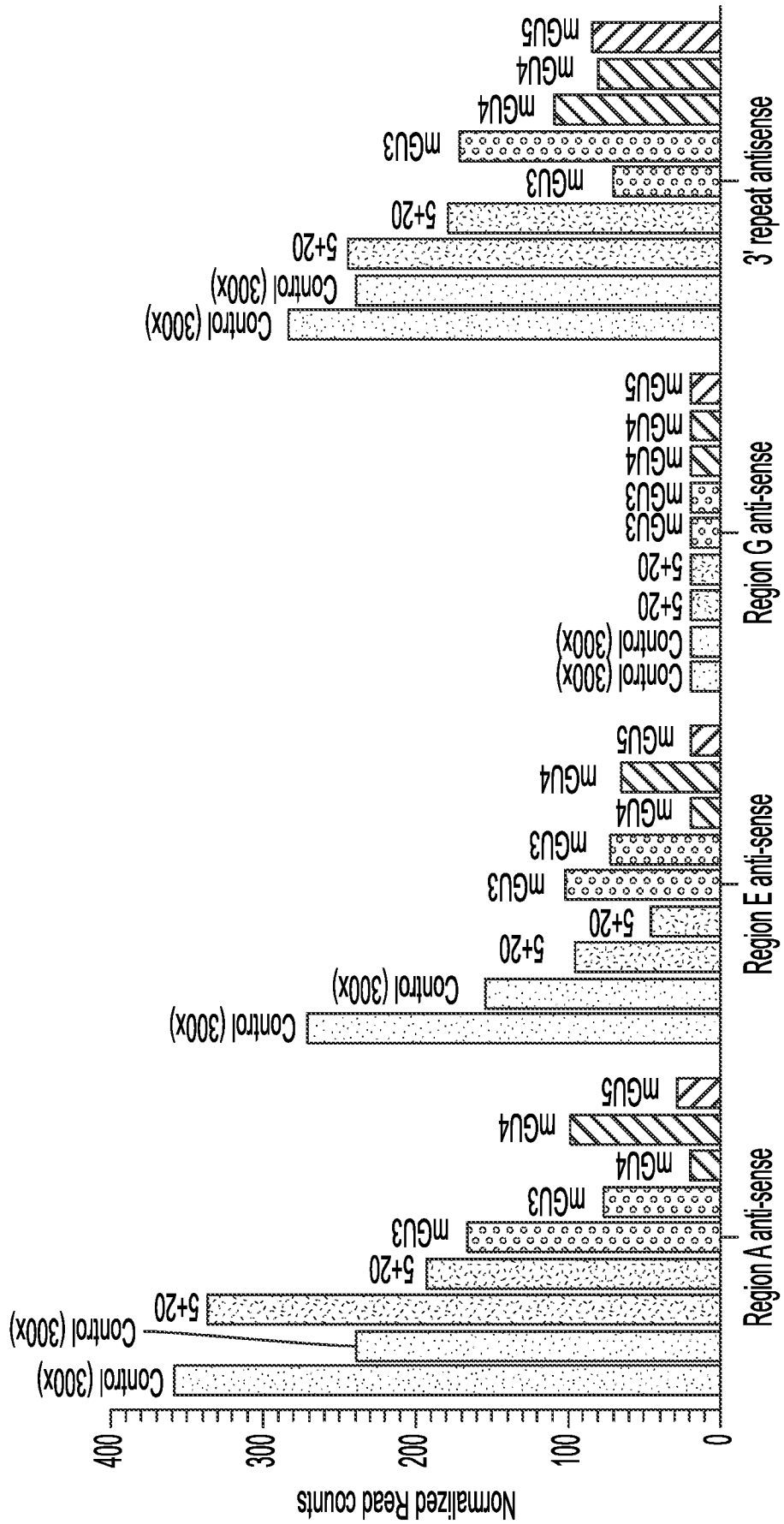

FIG. 10
CONTINUED

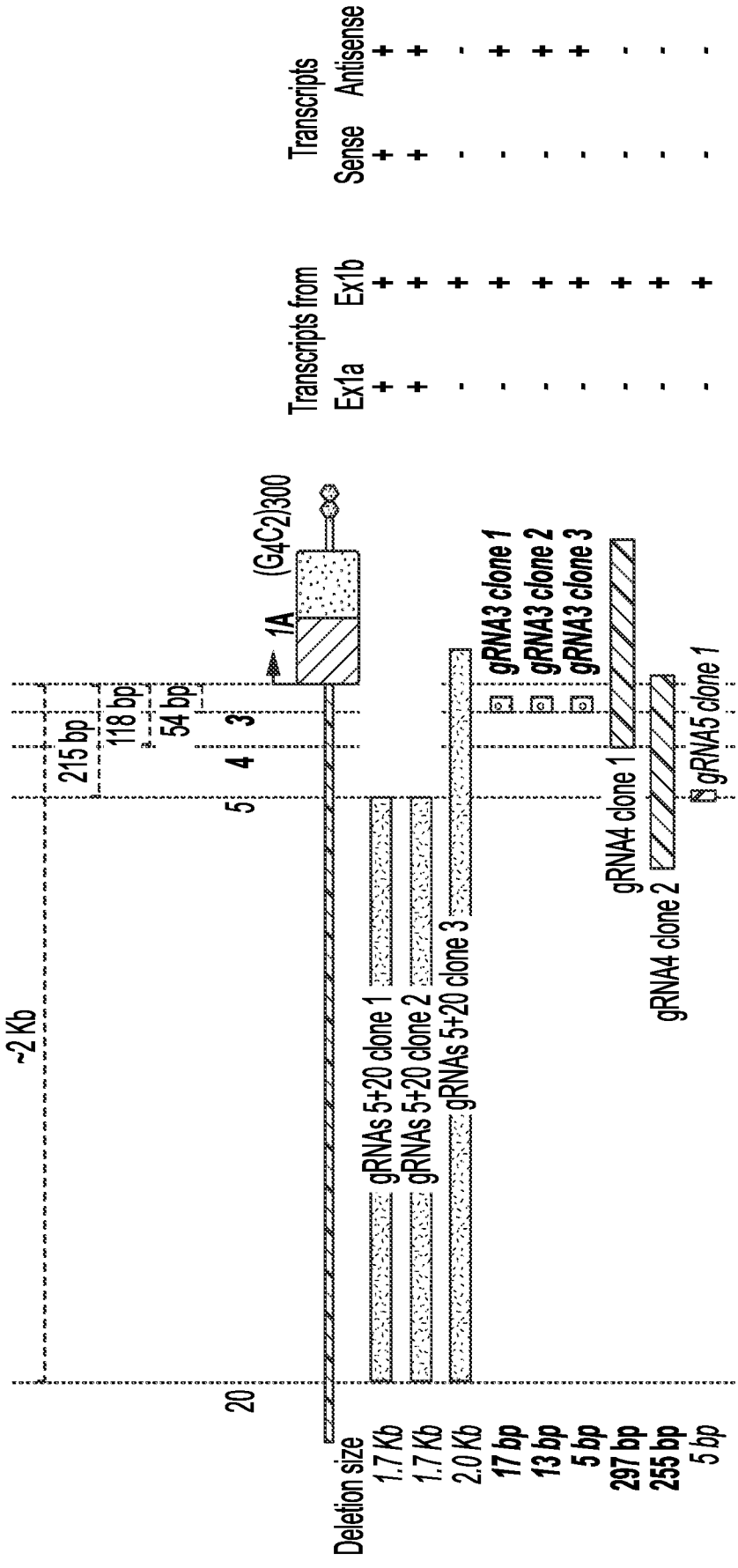
Transcripts
Sense
Antisense
Transcripts from
Ex1a
Ex1b
(G4C2)300
1A
215 bp
118 bp
54 bp
5
4
3
~2 Kb
20
gRNAs 5+20 clone 1
gRNAs 5+20 clone 2
gRNAs 5+20 clone 3
gRNA3 clone 1
gRNA3 clone 2
gRNA3 clone 3
gRNA4 clone 1
gRNA4 clone 2
gRNA5 clone 1
Deletion size
1.7 Kb
1.7 Kb
2.0 Kb
17 bp
13 bp
5 bp
297 bp
255 bp
5 bp

FIG. 11A

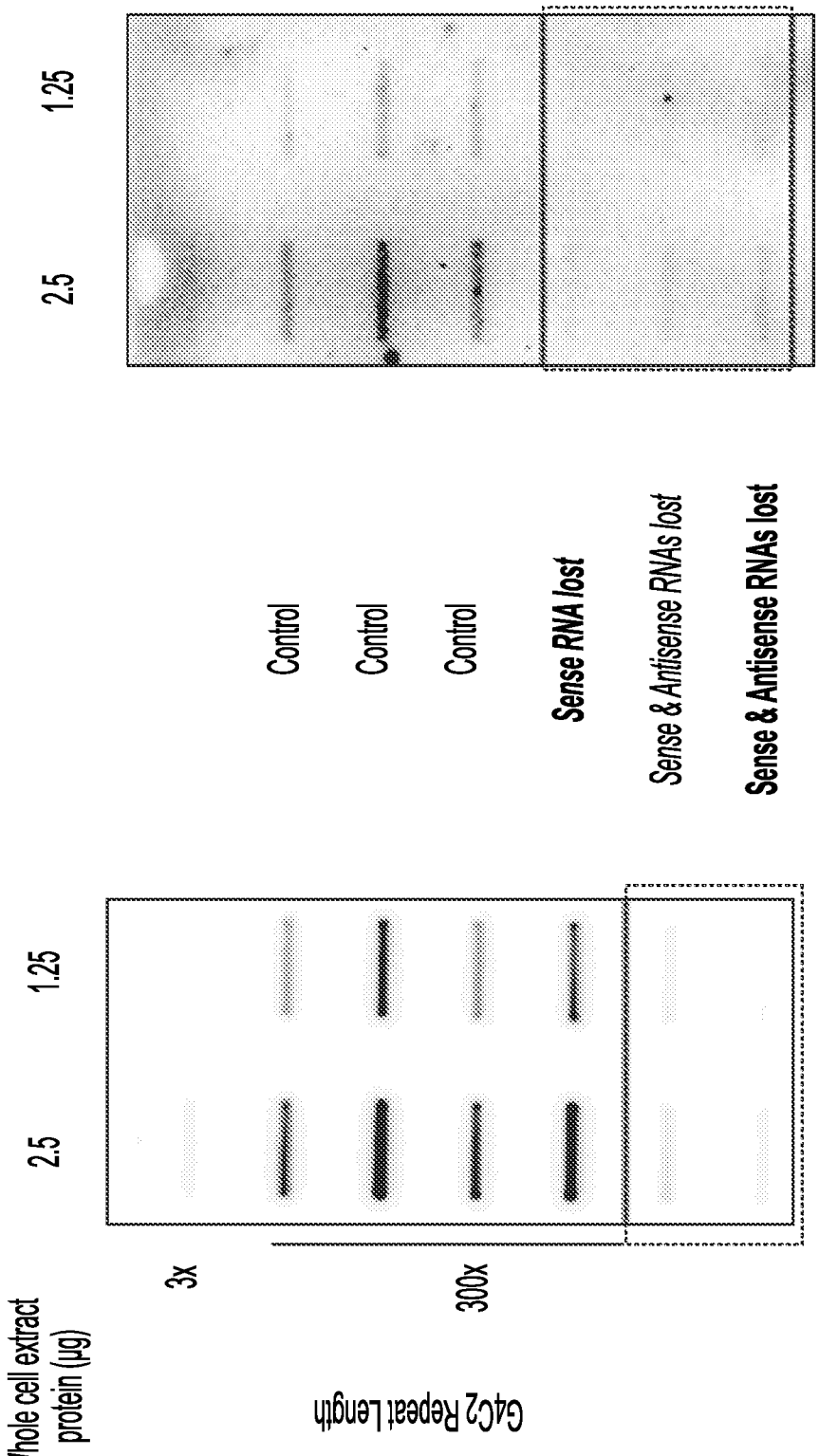
Western slot blot ES cell-derived motor neurons
Whole cell extract protein (µg)
2.5
1.25
2.5
1.25
3x
300x
G4C2 Repeat Length
Control
Control
Control
Sense RNA lost
Sense & Antisense RNAs lost
Sense & Antisense RNAs lost
anti-polyGlyPro antibody (sense & antisense)
anti-polyGlyAla antibody (sense)

FIG. 11B

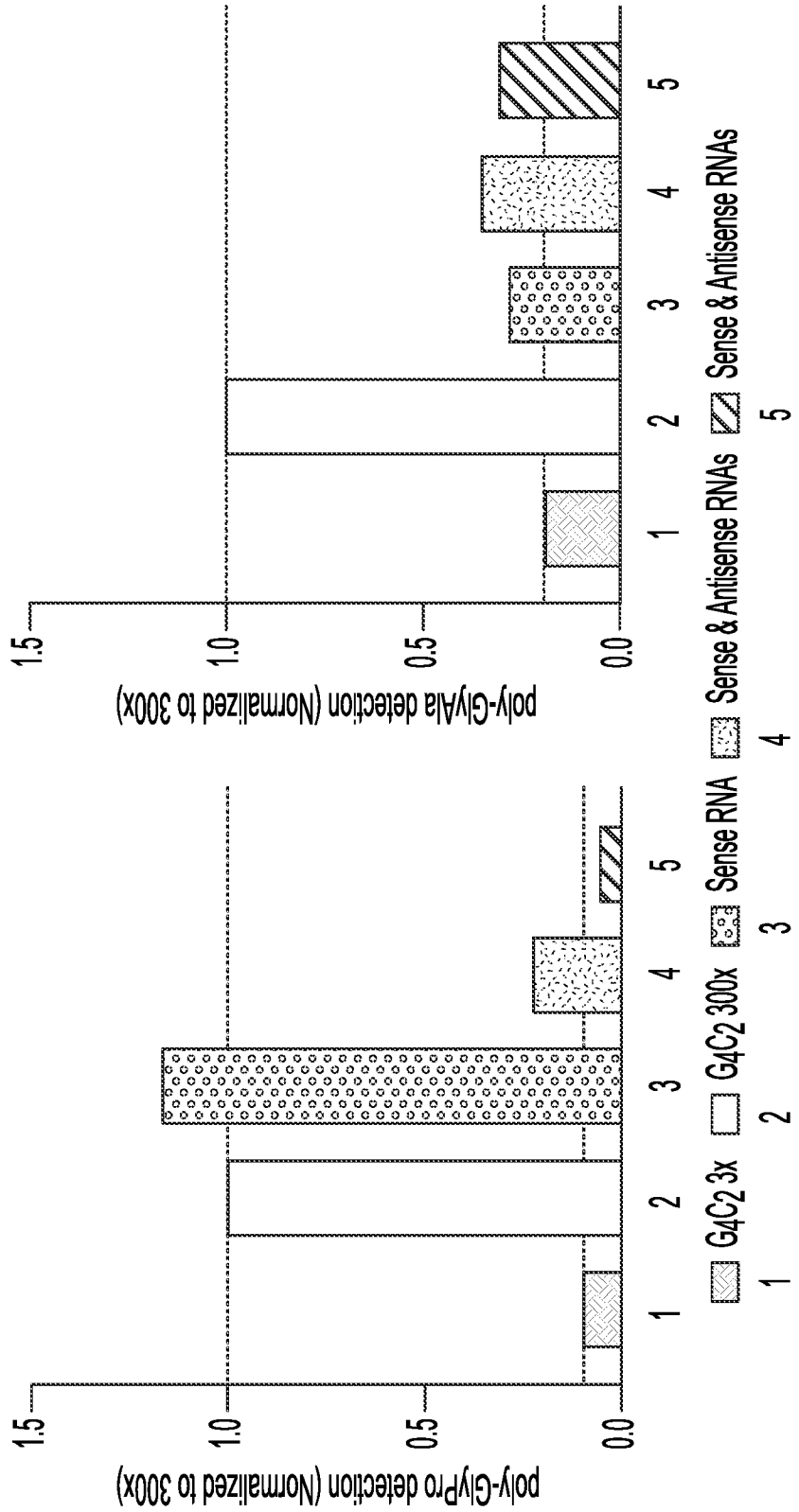

FIG. 11B
CONTINUED

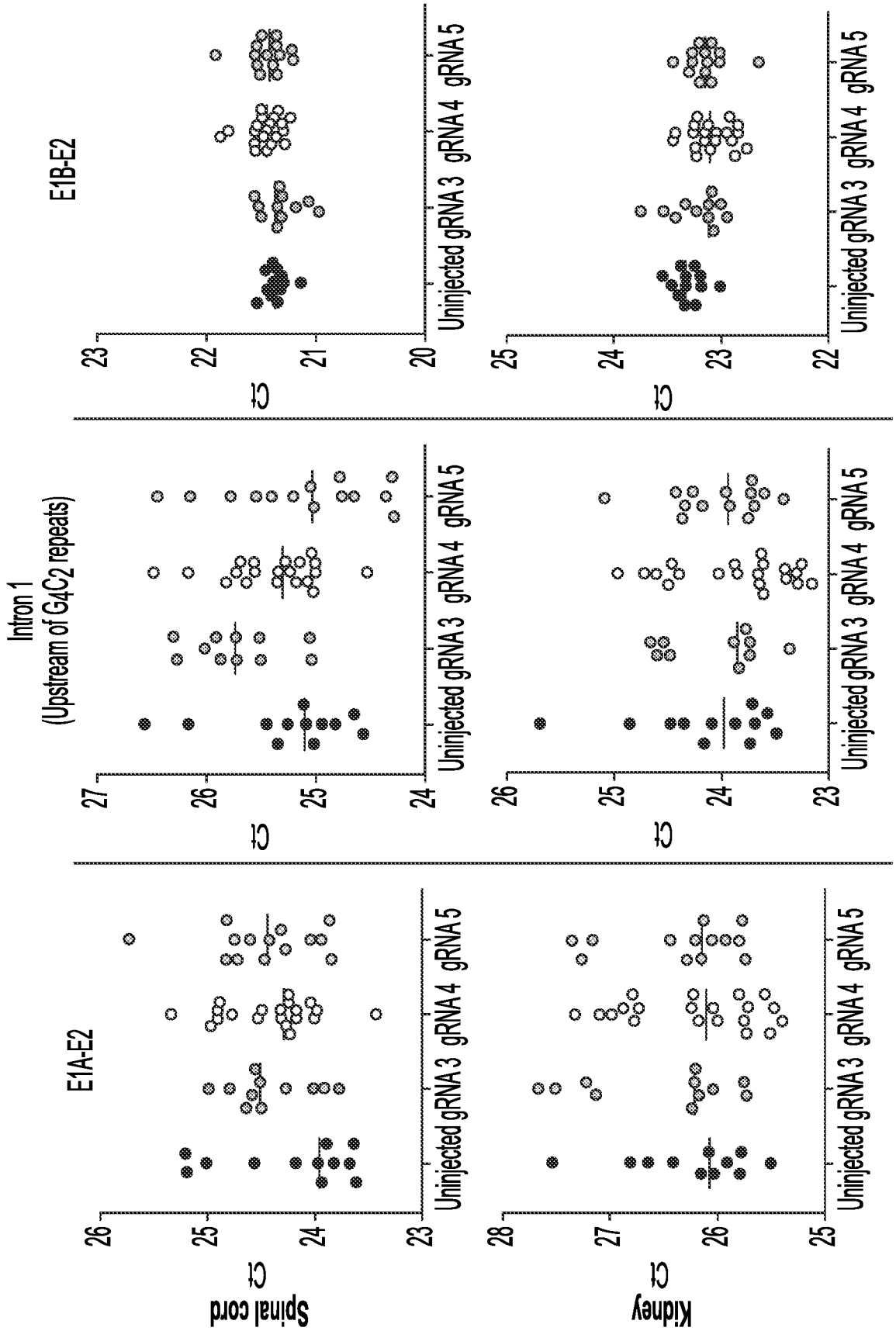
Spinal cord
Kidney
E1A-E2
E1B-E2
Intron 1
(Upstream of G4C2 repeats)
Ct
Uninjected gRNA 3 gRNA 4 gRNA 5

FIG. 12

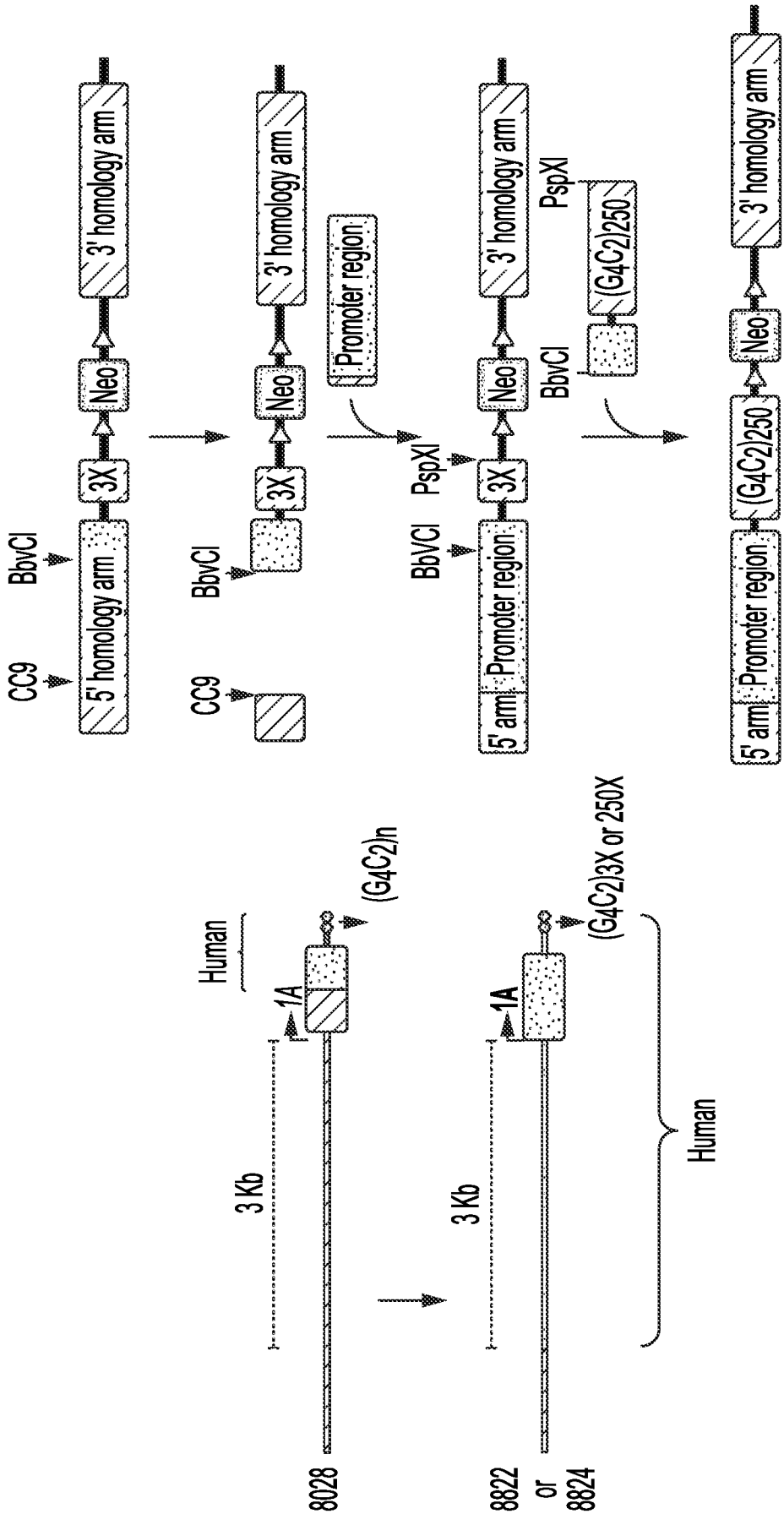
CC9
BbvCI
5' homology arm
3X
Neo
3' homology arm
Promoter region
PspXI
(G4C2)250
5' arm
Human
1A
(G4C2)n
(G4C2)3X or 250X
3 Kb
8028
8822
or
8824

FIG. 13

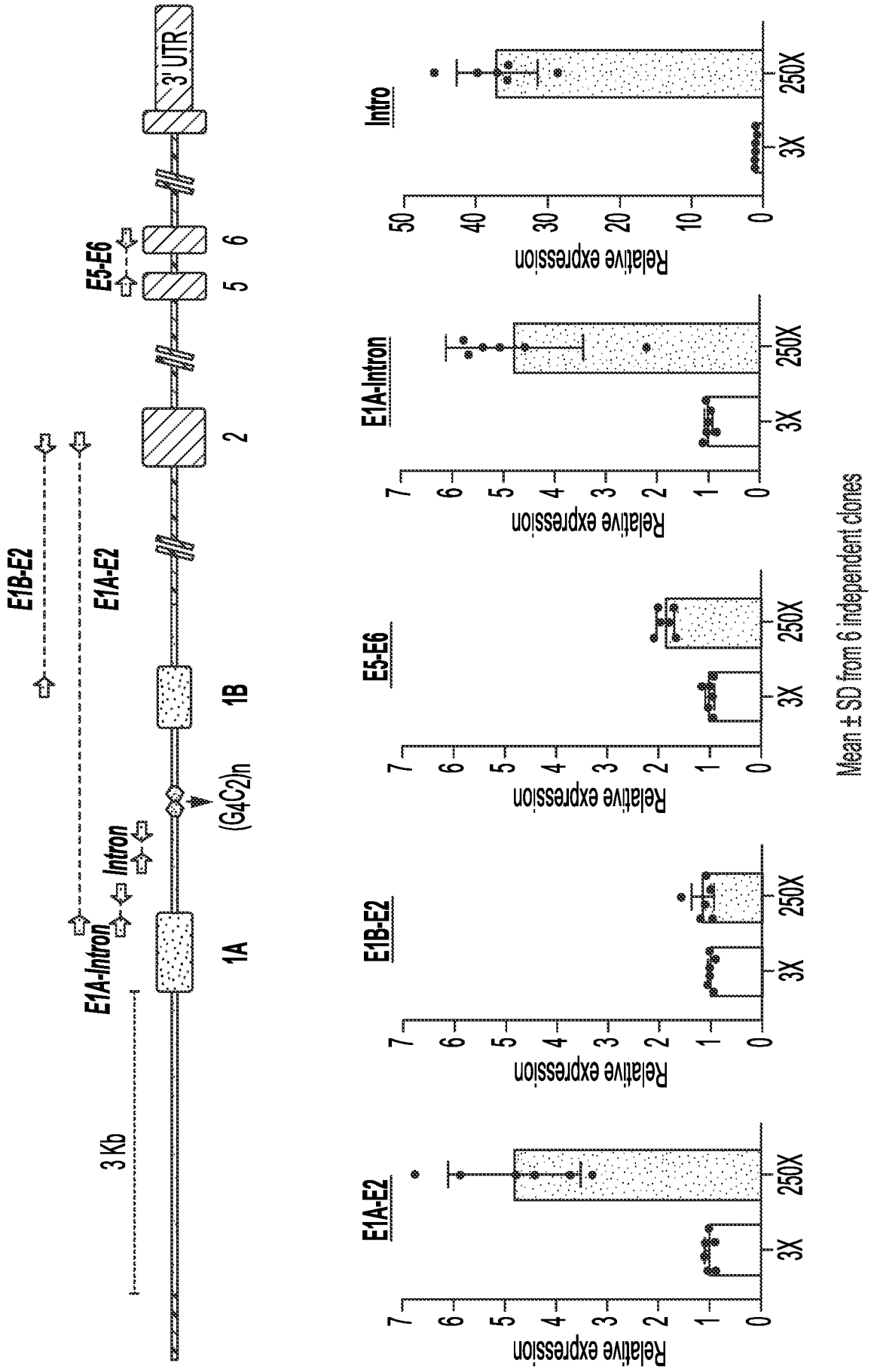
3 Kb
E1A-Intron
Intron
E1A-E2
E1B-E2
E5-E6
3' UTR
1A
1B
2
5
6
(G4C2)n
E1A-E2
E1B-E2
E5-E6
E1A-Intron
Intro
Relative expression
3X
250X
Mean ± SD from 6 independent clones

FIG. 14

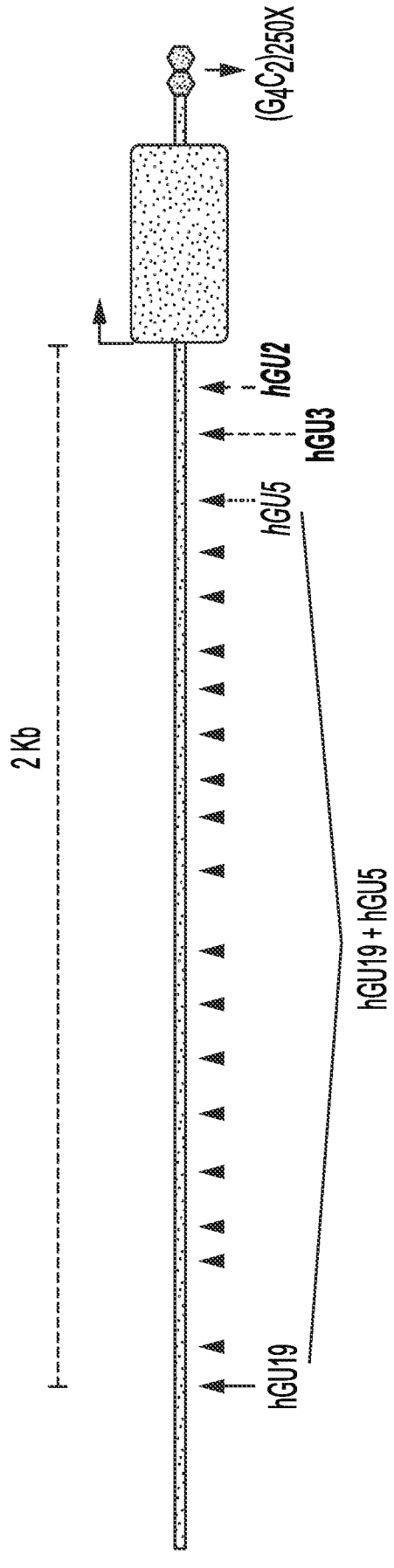

FIG. 15

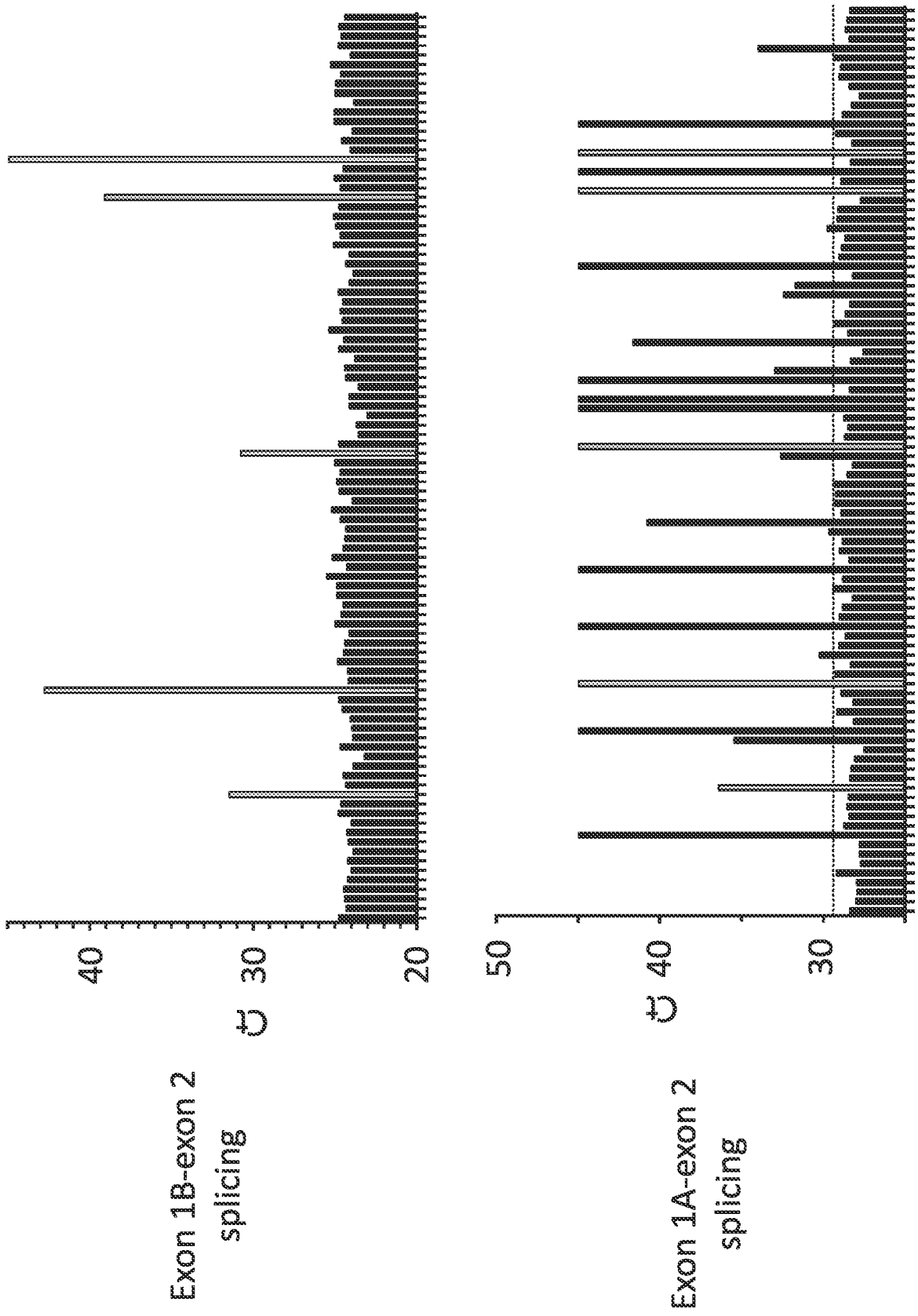
Exon 1B-exon 2 splicing
Ct
40
30
20
Exon 1A-exon 2 splicing
Ct
50
40
30

FIG. 16

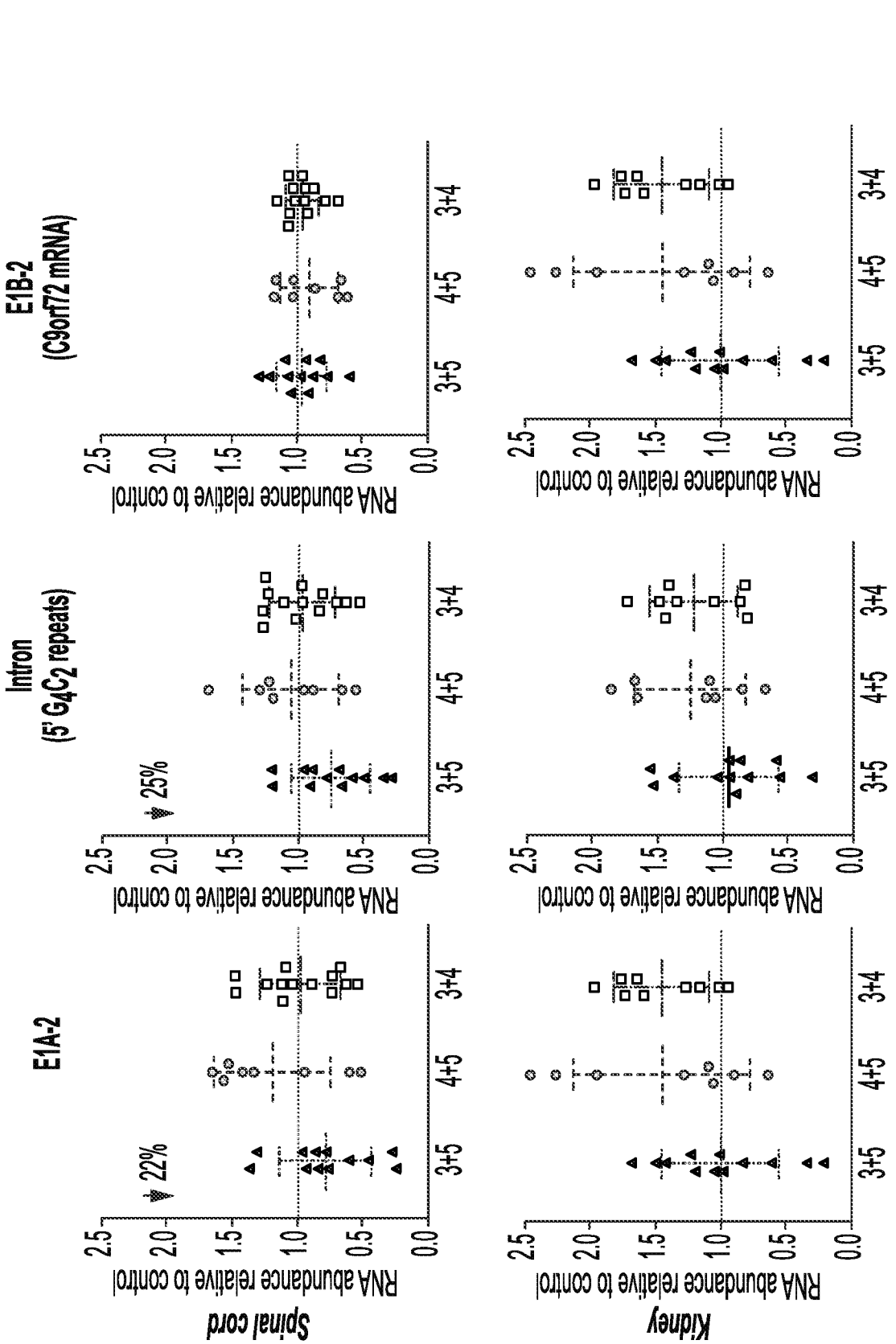
E1A-2
Intron
(5' G4C2 repeats)
E1B-2
(C9orf72 mRNA)
Spinal cord
Kidney
RNA abundance relative to control
22%
25%
3+5
4+5
3+4
gRNA 3 & 5
gRNA 4 & 5
gRNA 3 & 4

FIG. 17

gRNA	gRNA3 + gRNA5		gRNA4 + gRNA5		gRNA3 + gRNA4	
	Tail	Spinal cord	Tail	Spinal cord	Tail	Spinal cord
% of alleles with no deletion	95.2	56.9	96.8	67.6	97.8	75
% of alleles with deletion between the Cas9 cut sites	0.7	30.3	0	18.4	0	9.8
% of alleles with other mutations	0.2	2.7	0	4.4	0	7.5
% not categorized	3.9	10	3.2	9.6	2.2	7.7

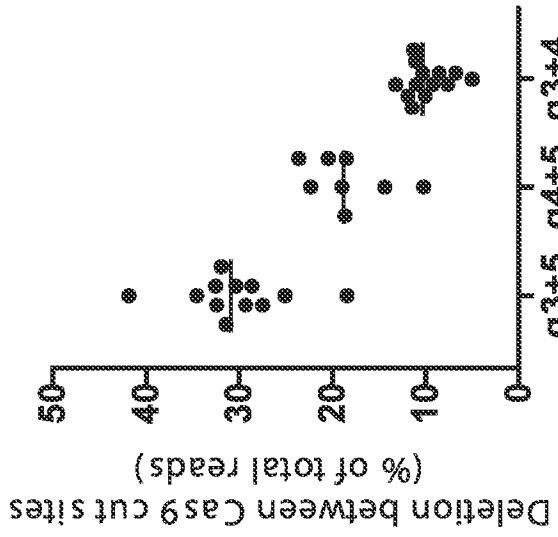

FIG. 18

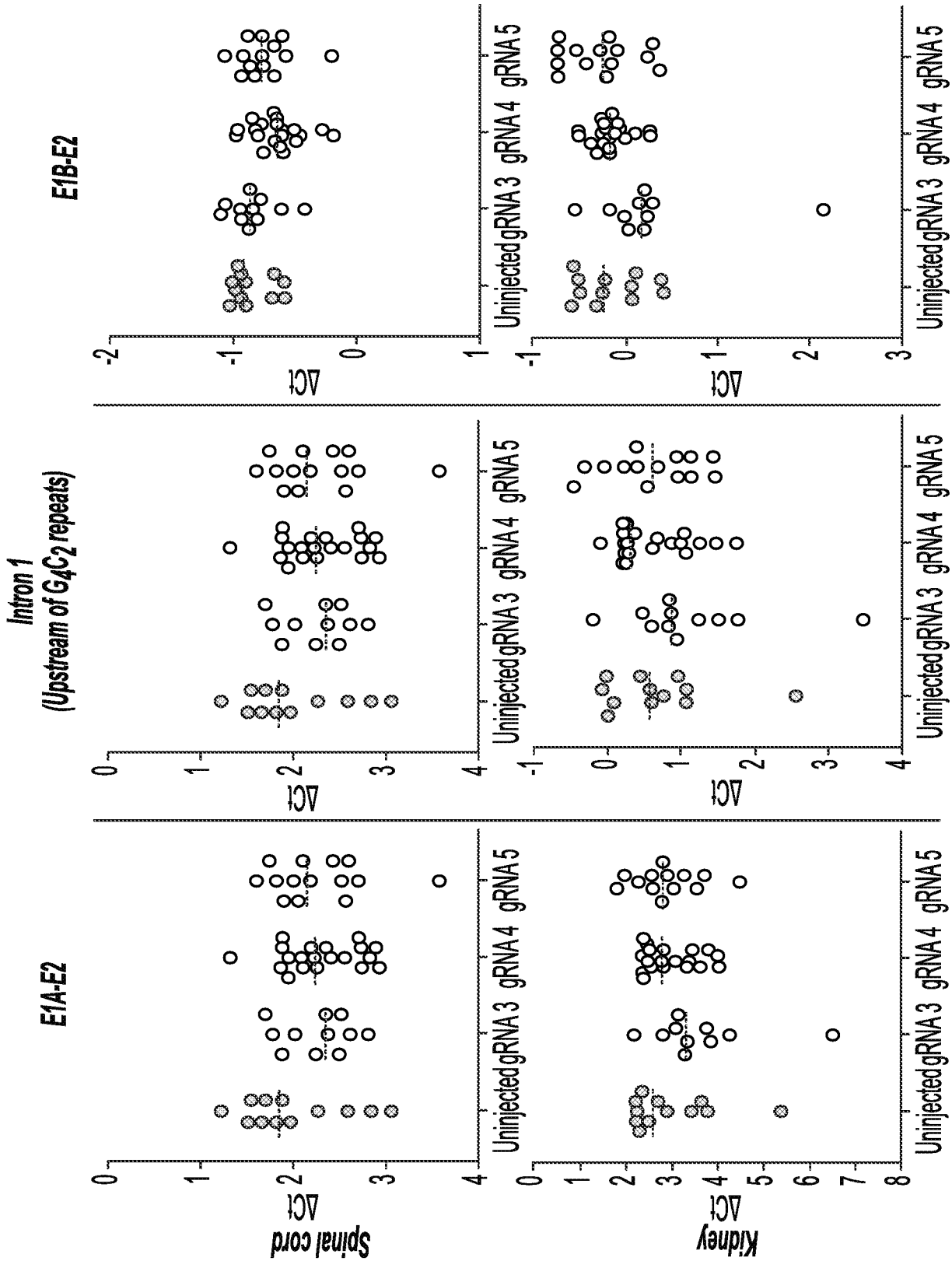
E1A-E2
Intron 1
(Upstream of G4C2 repeats)
E1B-E2
Spinal cord
Kidney
ΔCt
Uninjected
gRNA 3
gRNA 4
gRNA 5

FIG. 19

gRNA	Percentage Indels in spinal cord	Percentage Indels in kidney
No gRNA	0	0
gRNA3	5.72	1.49
gRNA4	21.13	6.08
gRNA5	25.70	4.39

FIG. 20

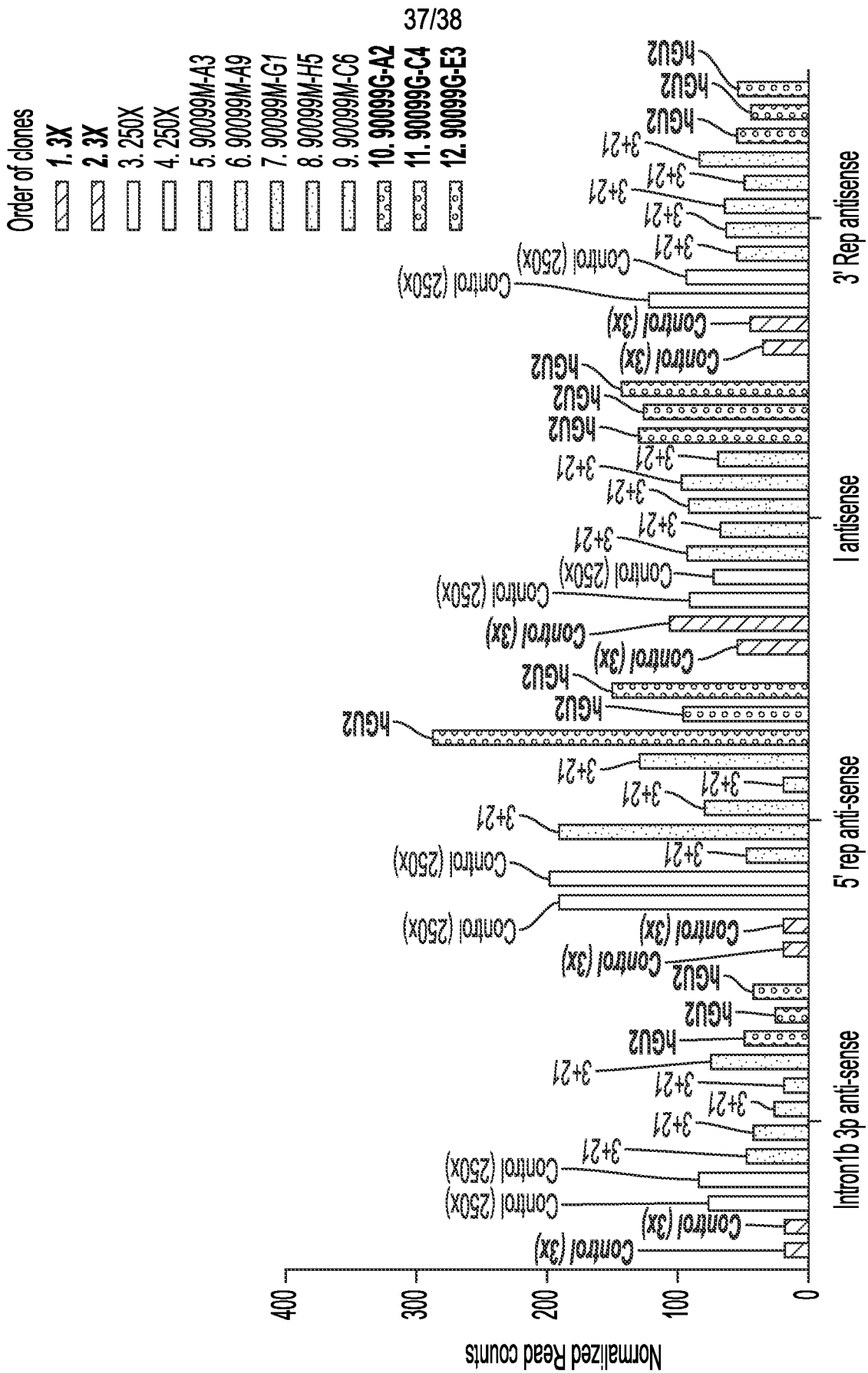
Order of clones
1. 3X
2. 3X
3. 250X
4. 250X
5. 90099M-A3
6. 90099M-A9
7. 90099M-G1
8. 90099M-H5
9. 90099M-C6
10. 90099G-A2
11. 90099G-C4
12. 90099G-E3
Normalized Read counts
0
100
200
300
400
Intron1b 3p anti-sense
5' rep anti-sense
I antisense
3' Rep antisense
Control (3x)
Control (250x)
3+21
hGUS

FIG. 21

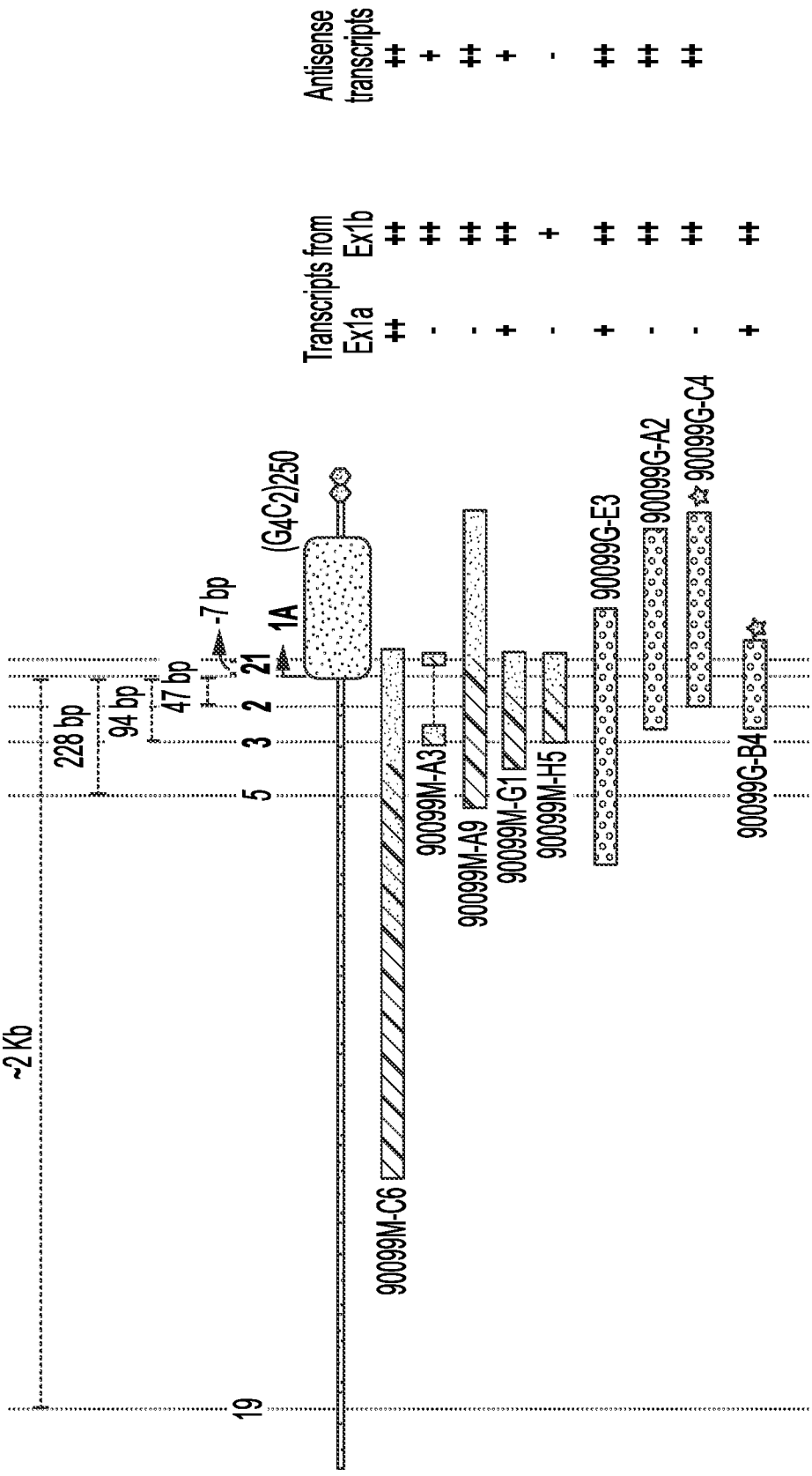
Antisense transcripts
Transcripts from Ex1a Ex1b
(G4C2)250
1A
21
-7 bp
47 bp
94 bp
228 bp
~2 Kb
5
3
2
19
90099M-C6
90099M-A3
90099M-A9
90099M-G1
90099M-H5
90099G-E3
90099G-A2
90099G-C4
90099G-B4

FIG. 22