

**NON-HUMAN ANIMALS HAVING A HEXANUCLEOTIDE REPEAT
EXPANSION IN A *C9ORF72* LOCUS**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/402,613, filed September 30, 2016, and U.S. Provisional Application No. 62/452,795, filed January 31, 2017, each of which is hereby incorporated herein in its entirety by reference.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0002] An official copy of the sequence listing is submitted concurrently with the specification electronically to the United States Patent and Trademark Office as the Receiving Office via EFS-Web as an ASCII formatted sequence listing with a file name of “2017-09-29-10267WO01-SEQ-LIST_ST25”, a creation date of September 29, 2017, and a size of about 94KB. The sequence listing contained in this ASCII formatted document is part of the specification and is herein incorporated by reference in its entirety.

BACKGROUND

[0003] Neurodegenerative diseases are major contributors to disability and disease. In particular, amyotrophic lateral sclerosis (ALS, also referred to as Lou Gehrig's disease) and frontotemporal dementia (FTD) are rare nervous system disorders characterized by progressive neuronal loss and/or death.

[0004] Although aging is viewed as the greatest risk factor for neurodegenerative disease, several genetic components have been discovered. For example, mutations in the copper-zinc superoxide dismutase (*SOD1*) gene have long been associated with ALS. Also, expanded hexanucleotide repeats of GGGGCC within a non-coding region of the

C9ORF72 gene have been linked to both ALS and FTD. Currently, there is no cure for either disease, although some treatments are able to prolong life by about 3-5 months..

[0005] While various laboratory animal models are extensively used in the development of most therapeutics, very few if any models exist that address neurodegenerative and inflammatory diseases in ways that provide for elucidation of the exact molecular mechanism by which identified genetic components cause disease, which elucidation in turn may uncover potential therapeutic modalities for not only ALS or other neurodegenerative diseases having a similar clinical presentation. Thus, the manner in which genetic mutations cause neurodegenerative disease remains largely unknown. Ideal animal models would contain the same genetic components and represent similar characteristics of human disease. Given the genetic differences between species, there is a high unmet need for the development of improved animal models that closely recapitulate human neurodegenerative and/or inflammatory disease. Of course, such improved animal models provide significant value in the development of effective therapeutic and/or prophylactic agents.

SUMMARY

[0006] The present invention encompasses the recognition that it is desirable to engineer non-human animals or non-human animal cells (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) to permit improved *in vivo* or *in vitro* systems for identifying and developing new therapeutics and, in some embodiments, therapeutic regimens, which can be used for the treatment of neurodegenerative diseases, disorders and conditions. In some embodiments, the *in vivo* or *in vitro* systems as described herein can be used for identifying and developing new therapeutics for treating diseases, disorders, and/or conditions associated with the *C9ORF72* locus, particularly a heterologous hexanucleotide repeat expansion sequence in the locus, such as, e.g., neurodegenerative disorders. Further, non-human animals or non-human animal cells (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein that comprise an insertion of a hexanucleotide repeat expansion sequence in a *C9ORF72* locus are desirable, for example, for use in identifying and developing therapeutics that target a GGGGCC hexanucleotide repeat (SEQ ID NO:1), products derived therefrom, e.g., sense or antisense RNA transcribed

therefrom, a RAN translation product and/or dipeptide repeat protein encoded by the hexanucleotide repeat, etc. In some embodiments, non-human animals and non-human animal cells (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) as described herein respectively provide improved *in vivo* and *in vitro* systems (or models) for neurodegenerative diseases, disorders and conditions (e.g., ALS and/or FTD).

[0007] A non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its genome a heterologous hexanucleotide repeat expansion sequence inserted into an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises at least one repeat of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its germline genome a heterologous hexanucleotide repeat expansion sequence inserted into an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises at least one repeat of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous hexanucleotide expansion sequence is a non-rodent (e.g., non-rat or non-mouse, e.g., a human) hexanucleotide expansion sequence that comprises at least one instance, e.g., repeat, of the hexanucleotide sequence set forth as SEQ ID NO:1. In some embodiments, the (human) heterologous hexanucleotide repeat expansion sequence comprises more than one, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the (human) heterologous hexanucleotide repeat expansion sequence comprises at least about three, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about five, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about ten, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about fifteen, preferably contiguous, repeats of the hexanucleotide

sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about twenty, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about thirty, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about forty, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about fifty, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about sixty, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about seventy, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about eighty, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about ninety, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the heterologous (human) hexanucleotide repeat expansion sequence comprises at least about one-hundred, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1. In some embodiments, the non-human animal comprises the heterologous (human) hexanucleotide repeat expansion sequence in its germline genome.

[0008] In some embodiments, the heterologous (e.g., non-rodent, non-rat, non-mouse and/or human) hexanucleotide repeat expansion sequence comprises heterologous (e.g., non-rodent, non-rat, non-mouse and/or human) sequences that flank the at least one, e.g., at least about three, at least about five, at least about ten, at least about fifteen, at least about twenty, at least about thirty, at least about forty, at least about fifty, at least about sixty, at least about seventy, at least about eighty, at least about ninety or at least about one-hundred, preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO:1. Accordingly, a heterologous (e.g., non-rodent, non-rat, non-mouse,

and/or human) hexanucleotide repeat expansion sequence may comprise from 5' to 3': a first heterologous hexanucleotide flanking sequence, one or more (preferably contiguous) instances of the hexanucleotide set forth as SEQ ID NO:1, and a second heterologous hexanucleotide flanking sequence. In some embodiments, a heterologous hexanucleotide repeat expansion sequence is identical to or substantially identical to a naturally occurring genomic sequence comprising a first heterologous hexanucleotide flanking sequence, one or more instances of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second heterologous hexanucleotide flanking sequence. Naturally occurring first and/or second heterologous hexanucleotide flanking sequences may each independently be, e.g., at least 4 base pairs in length, e.g., at least 10 base pairs in length, e.g., at least 20 base pairs in length etc.

[0009] In some embodiments, a heterologous human hexanucleotide expansion sequence spans (and optionally encompasses) all or portions of exons 1a and/or exon 1b of a human *C9orf72* gene. In some embodiments, a first heterologous hexanucleotide flanking sequence comprises all or part of the sequence of exon 1a of a human *C9orf72* gene (set forth as SEQ ID NO:34) and/or a second heterologous hexanucleotide flanking sequence comprises all or part of the sequence of exon 1b of a human *C9orf72* gene (set forth as SEQ ID NO:35). In some embodiments, a first heterologous hexanucleotide flanking sequence comprises the sequence set forth as SEQ ID NO:36, or a portion thereof, and/or a second heterologous hexanucleotide flanking sequence comprises the sequence set forth as SEQ ID NO:37, or a portion thereof.

[00010] An exemplary human hexanucleotide repeat expansion sequence is set forth as SEQ ID NO:2 (comprising from 5' to 3': a first heterologous hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36, 3 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second heterologous hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37). Another exemplary human hexanucleotide repeat expansion sequence is set forth as SEQ ID NO:3 (comprising from 5' to 3': a first heterologous hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36, 100 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second heterologous hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37). Accordingly, disclosed herein are non-human animals, e.g., rodents such as a rat or a mouse, whose genomes comprise in an endogenous *C9orf72* locus a sequence set forth as SEQ ID NO:2, a

variant of SEQ ID NO:2, a sequence set forth as SEQ ID NO:3, or a variant of SEQ ID NO:3.

[00011] In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) comprises in its genome a hexanucleotide repeat expansion sequence comprising a sequence that is a SEQ ID NO:2 variant, which comprises from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:34), one or two contiguous repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:35). In some embodiments, a non-human animal or a non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) as described herein comprises in its genome a hexanucleotide repeat expansion sequence comprising a sequence that is a SEQ ID NO:3 variant, which comprises from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:34), more than one and less than 100 contiguous repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:35). In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) comprises in its (germline) genome a hexanucleotide repeat expansion sequence comprising a sequence that is a SEQ ID NO:3 variant, comprises from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:34), 36 contiguous repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:35). In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) as described herein comprises in its genome a hexanucleotide repeat expansion sequence comprising a

sequence that is a SEQ ID NO:3 variant, which comprises from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:34), 92 contiguous repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:35).

[00012] In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) as disclosed herein is heterozygous or homozygous for a hexanucleotide repeat expansion sequence comprising a sequence that is a SEQ ID NO:2 variant, which comprises from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:34), one or two contiguous repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:35). In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) comprises in its (germline) genome a hexanucleotide repeat expansion sequence comprising a sequence that is a SEQ ID NO:3 variant, which comprises from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:34), more than one and less than 100 contiguous repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:35). In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) comprises in its (germline) genome a hexanucleotide repeat expansion sequence comprising a sequence that is a SEQ ID NO:3 variant, comprises from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:34), 36 contiguous repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37 (or

a portion thereof, e.g., a sequence set forth as SEQ ID NO:35). In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) comprises in its (germline) genome a hexanucleotide repeat expansion sequence comprising a sequence that is a SEQ ID NO:3 variant, which comprises from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:34), 92 contiguous repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37 (or a portion thereof, e.g., a sequence set forth as SEQ ID NO:35).

[00013] In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) comprises in its (germline) genome a replacement of 5' untranslated and/or non-coding endogenous non-human sequences of the endogenous *C9orf72* locus with the heterologous (human) hexanucleotide repeat expansion sequence. In some embodiments, the untranslated and/or non-coding sequence spanning between (and optionally encompassing at least a portion of) endogenous exon 1 (e.g., exon 1a and/or 1b) and the ATG start codon of the endogenous non-human *C9orf72* locus, or a portion thereof, is replaced with the heterologous hexanucleotide repeat expansion sequence. Additional sequences (e.g., recombinase recognition sequences, a drug resistance cassette, a reporter gene, etc.) linked to the heterologous (human) hexanucleotide expansion sequence, may also replace the untranslated and/or non-coding sequence spanning between (and optionally encompassing) endogenous exon 1 (e.g., exon 1a and/or exon 1b) and the ATG start codon of the endogenous non-human *C9orf72* locus, or a portion thereof.

[00014] Accordingly, in some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) as disclosed herein may comprise a heterozygous or homozygous replacement of an endogenous sequence that (1) starts from the 5' end, within, or the 3' end of an endogenous exon 1 and (2) ends 5' of the endogenous ATG start codon, or a portion thereof, with a heterologous hexanucleotide repeat expansion sequence, e.g., a hexanucleotide repeat expansion sequence comprising a least one repeat of the hexanucleotide sequence set forth as SEQ ID NO:1. In some

embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) as disclosed herein may comprise a heterozygous or homozygous replacement of an endogenous sequence that (i) starts from the 5' end of, within, or from the 3' end of an endogenous exon 1 and (ii) ends 5' of the endogenous ATG start codon, or a portion thereof, with a heterologous hexanucleotide repeat expansion comprising from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 34, at least one instance of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:35. In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) as disclosed herein may comprise a heterozygous or homozygous replacement of an endogenous sequence that (ii) starts from the 5' end of, within, or the 3' end of an endogenous exon 1 and (ii) ends 5' of the endogenous ATG start codon, or a portion thereof, with a heterologous hexanucleotide repeat expansion sequence comprising from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36, at least one instance of the hexanucleotide sequence set forth as SEQ ID NO:1, and a second human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37. In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) as disclosed herein may comprise a heterozygous or homozygous replacement of an endogenous sequence that (ii) starts from the 5' end of, within, or the 3' end of an endogenous exon 1 and (ii) ends 5' of the endogenous ATG start codon, or a portion thereof, with a heterologous hexanucleotide repeat expansion sequence comprising the sequence set forth as SEQ ID NO:2, a variant thereof, SEQ ID NO:3 or a variant thereof.

[00015] In some embodiments, a non-human animal or non-human animal cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its (germline) genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises one or more repeats of the hexanucleotide sequence set forth as SEQ ID NO:

1, and wherein the non-human animal or cell exhibits one or more of the following characteristics: (i) increased expression of *C9orf72* RNA sense and/or antisense transcripts compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by quantitative PCR (ii) an increased number of RNA foci comprising a *C9orf72* RNA sense and/or antisense transcript compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by fluorescence activated *in situ* hybridization, (iii) an increased level of dipeptide repeat proteins compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by immunofluorescence or (iv) any combination of (i) – (iii). In some embodiments, a non-human animal or cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its (germline) genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises three or more repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1, and wherein the non-human animal or cell exhibits one or more of the following characteristics: (i) increased expression of *C9orf72* RNA sense and/or antisense transcripts compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by quantitative PCR (ii) an increased number of RNA foci comprising a *C9orf72* RNA sense and/or antisense transcript compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by fluorescence activated *in situ* hybridization, (iii) an increased level of dipeptide repeat proteins compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by immunofluorescence or (iv) any combination of (i) – (iii). In some embodiments, a non-human animal or cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its (germline) genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises at least thirty repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1, and wherein the non-human animal or cell exhibits one or more of the following characteristics: (i) increased expression of *C9orf72* RNA sense and/or antisense transcripts compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by quantitative PCR (ii) an increased number of RNA foci comprising an increased number of RNA

foci comprising a *C9orf72* RNA sense and/or antisense transcript compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by fluorescence activated *in situ* hybridization, (iii) an increased level of dipeptide repeat proteins compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by immunofluorescence or (iv) any combination of (i) – (iii). In some embodiments, a non-human animal or cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its (germline) genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises ninety or more repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1, and wherein the non-human animal or cell exhibits one or more of the following characteristics: (i) increased expression of *C9orf72* RNA sense and/or antisense transcripts compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by quantitative PCR (ii) an increased number of RNA foci comprising a *C9orf72* RNA sense and/or antisense transcript compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by fluorescence activated *in situ* hybridization, (iii) an increased level of dipeptide repeat proteins compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by immunofluorescence or (iv) any combination of (i) – (iii). In some embodiments, a non-human animal or cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its (germline) genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises ninety-two repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1, and wherein the non-human animal or cell exhibits all of the following three characteristics: (i) increased expression of *C9orf72* RNA sense and/or antisense transcripts compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by quantitative PCR (ii) an increased number of RNA foci comprising an increased number of RNA foci comprising a *C9orf72* RNA sense and/or antisense transcript compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by fluorescence activated *in situ* hybridization, and (iii) an increased level of dipeptide repeat proteins compared to a control animal or cell comprising a

wildtype *C9orf72* locus, e.g., as evaluated by immunofluorescence. In some embodiments, a non-human animal or cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its (germline) genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises more than ninety repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1, and wherein the non-human animal or cell exhibits all of the following three characteristics: (i) increased expression of *C9orf72* RNA sense and/or antisense transcripts compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by quantitative PCR (ii) an increased number of RNA foci comprising an increased number of RNA foci comprising a *C9orf72* RNA sense and/or antisense transcript compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by fluorescence activated *in situ* hybridization, and (iii) an increased level of dipeptide repeat proteins compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by immunofluorescence. In some embodiments, a non-human animal or cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises at least 92 repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1, and wherein the non-human animal or cell exhibits all of the following three characteristics: (i) increased expression of *C9orf72* RNA sense and/or antisense transcripts compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by quantitative PCR (ii) an increased number of RNA foci comprising an increased number of RNA foci comprising a *C9orf72* RNA sense and/or antisense transcript compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by fluorescence activated *in situ* hybridization, and (iii) an increased level of dipeptide repeat proteins compared to a control animal or cell comprising a wildtype *C9orf72* locus, e.g., as evaluated by immunofluorescence.

[00016] In some embodiments, a non-human animal or cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its genome a heterologous hexanucleotide repeat

expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises a repeat of the hexanucleotide sequence set forth as SEQ ID NO: 1, and wherein one or more of the following characteristics of the non-human animal or cell is not significantly different compared to a control non-human animal or cell comprising a wildtype *C9orf72* locus: (i) the amount of *C9orf72* RNA sense and/or antisense transcripts compared, e.g., as evaluated by quantitative PCR (ii) the number of RNA foci comprising a *C9orf72* RNA sense and/or antisense transcript, e.g., as evaluated by fluorescence activated *in situ* hybridization, (iii) the level of dipeptide repeat proteins, e.g., as evaluated by immunofluorescence or (iv) any combination of (i) – (iii). In some embodiments, a non-human animal or cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises three repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1, and wherein one or more of the following characteristics of the non-human animal or cell is not significantly different compared to a control non-human animal or cell comprising a wildtype *C9orf72* locus: (i) the amount of *C9orf72* RNA sense and/or antisense transcripts compared, e.g., as evaluated by quantitative PCR (ii) the number of RNA foci comprising a *C9orf72* RNA sense and/or antisense transcript, e.g., as evaluated by fluorescence activated *in situ* hybridization, (iii) the level of dipeptide repeat proteins, e.g., as evaluated by immunofluorescence or (iv) any combination of (i) – (iii). In some embodiments, a non-human animal or cell (e.g., embryonic stem cell, embryonic stem cell derived-motor neuron, brain cell, neuronal cell, muscle cell, heart cell) described herein comprises in its genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises thirty repeats of the hexanucleotide sequence set forth as SEQ ID NO: 1, and wherein one or more of the following characteristics of the non-human animal or cell is not significantly different compared to a control non-human animal or cell comprising a wildtype *C9orf72* locus: (i) the amount of *C9orf72* RNA sense and/or antisense transcripts compared, e.g., as evaluated by quantitative PCR (ii) the number of RNA foci comprising a *C9orf72* RNA sense and/or antisense transcript, e.g., as evaluated by fluorescence activated *in situ* hybridization, (iii) the level of

dipeptide repeat proteins, e.g., as evaluated by immunofluorescence or (iv) any combination of (i) – (iii).

[00017] In some embodiments, a nucleic acid construct (or targeting construct, or targeting vector) as described herein is provided.

[00018] In some embodiments, a nucleic acid construct as described herein comprises, from 5' to 3', a 5' non-human targeting arm comprising a polynucleotide that is homologous to a 5' portion of a non-human (e.g., a rodent such as a mouse or a rat) *C9ORF72* locus, a heterologous hexanucleotide repeat expansion sequence comprising at least one of a hexanucleotide sequence set forth as SEQ ID NO:1, a first recombinase recognition site; a first promoter operably linked to a selectable marker, a second recombinase recognition site, and a 3' non-human targeting arm comprising a polynucleotide that is homologous to a 3' portion of a non-human (e.g., a rodent such as a mouse or a rat) *C9ORF72* locus. In some embodiments, the 5' portion of a non-human (e.g., a rodent such as a mouse or rat) *C9ORF72* locus includes a genomic sequence upstream of exon 1 of the non-human (e.g., rodent such as mouse or rat) *C9ORF72* gene.

[00019] In some embodiments, recombinase recognition sites include *loxP*, *lox511*, *lox2272*, *lox2372*, *lox66*, *lox71*, *loxM2*, *lox5171*, FRT, FRT11, FRT71, attp, att, FRT, rox, or a combination thereof. In some embodiments, a recombinase gene is included in the construct, e.g., under the control of an inducible promoter. The recombinase gene may be selected from the group consisting of Cre, Flp (e.g., Flpe, Flpo), and Dre. In some certain embodiments, first and second recombinase recognition sites are *lox* (e.g., *loxP*) sites, and a recombinase gene encodes a Cre recombinase.

[00020] In some embodiments, a first promoter is selected from the group consisting of protamine (Prot; e.g., Prot1 or Prot5), Blimp1, Blimp1 (1 kb fragment), Blimp1 (2 kb fragment), Gata6, Gata4, Igf2, Lhx2, Lhx5, hUB1, Em7 and Pax3. In some certain embodiments, a first promoter is a hUB1 promoter in combination with an Em7 promoter.

[00021] In some embodiments, a selectable marker is selected from group consisting of neomycin phosphotransferase (*neo^r*), hygromycin B phosphotransferase (*hyg^r*), puromycin-N-acetyltransferase (*puro^r*), blasticidin S deaminase (*bsr^r*), xanthine/guanine phosphoribosyl transferase (*gpt*), and Herpes simplex virus thymidine kinase (HSV-tk). In some certain embodiments, a selectable marker is *neo^r*.

[00022] In some embodiments, the nucleic acid construct comprises the sequence set forth as SEQ ID NO:8, which comprises from 5' to 3': a 5' non-human (mouse) targeting arm, a first human hexanucleotide flanking sequence comprising the sequence set forth as SEQ ID NO:36, three repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, a second human hexanucleotide flanking sequence comprising the sequence set forth as SEQ ID NO:37, a floxed drug resistance (neo^r) cassette and a 3' non-human (mouse) targeting arm. In some embodiments, the nucleic acid construct comprises the sequence set forth as SEQ ID NO:9, which comprises from 5' to 3': a 5' non-human (mouse) targeting arm, a first human hexanucleotide flanking sequence comprising the sequence set forth as SEQ ID NO:36, one-hundred repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, a second human hexanucleotide flanking sequence comprising the sequence set forth as SEQ ID NO:37, a floxed drug resistance (neo^r) cassette and a 3' non-human (mouse) targeting arm.

[00023] In some embodiments, a method of making a non-human animal or non-human animal cell is provided whose genome comprises an insertion of a heterologous hexanucleotide repeat expansion sequence into an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises at least one, e.g., at least about 3 repeats, e.g., at least about 30 repeats, e.g., at least about 90 repeats, of a hexanucleotide sequence set forth as SEQ ID NO:1, the method comprising (a) introducing a nucleic acid sequence, e.g., a nucleic acid construct as described herein (e.g., a nucleic acid construct comprising a sequence set forth as SEQ ID NO:8 or a nucleic acid construct comprising a sequence set forth as SEQ ID NO:9), into a non-human embryonic stem cell so that the heterologous hexanucleotide repeat expansion sequence is inserted into an endogenous *C9ORF72* locus, which nucleic acid comprises a polynucleotide that is homologous to the *C9ORF72* locus; (b) obtaining a genetically modified non-human embryonic stem cell from (a); and optionally, (c) creating a non-human animal using the genetically modified non-human embryonic stem cell of (b). In some embodiments, a method of making a non-human animal described herein further comprises a step of breeding a non-human animal generated in (c) so that a non-human animal homozygous for the insertion is created.

[00024] In some embodiments, a method for making a non-human animal whose genome comprises an insertion of a heterologous hexanucleotide repeat expansion sequence, which comprises at least one repeat of the hexanucleotide sequence set forth as

SEQ ID NO:1, in an endogenous *C9ORF72* locus is provided, the method comprising modifying the genome of a non-human animal so that it comprises an inserted heterologous hexanucleotide repeat expansion sequence in an endogenous *C9ORF72* locus, thereby making said non-human animal.

[00025] In some embodiments, a non-human animal is provided which is obtainable by, generated from, or produced from a method as described herein. In some embodiments, a non-human animal as disclosed herein is produced using a nucleic acid construct comprising a sequence set forth as SEQ ID NO:8. Such a non-human animal comprises a heterozygous or homozygous replacement of about 853 bp of an endogenous *C9orf72* locus starting from within endogenous exon 1 with a heterologous nucleotide sequence comprising from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36, one to three repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, a human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37, and a floxed drug resistance (*neo^r*) cassette, or upon excision of the *neo* gene, a lox recombination recognition sequence. In some embodiments, a non-human animal as disclosed herein is produced using a nucleic acid construct comprising a sequence set forth as SEQ ID NO:9. Such a non-human animal comprises a heterozygous or homozygous replacement of about 853 bp of an endogenous *C9orf72* locus starting from within endogenous exon 1 with a heterologous nucleotide sequence comprising from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36, one to one-hundred (e.g., 36 or 92) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, a human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37, and a floxed drug resistance (*neo^r*) cassette, or upon excision of the *neo* gene, a lox recombination recognition sequence. In some embodiments, a non-human animal comprises a heterologous nucleotide sequence set forth as SEQ ID NO:4 (8026), a heterologous nucleotide sequence set forth as SEQ ID NO:5 (8027), a heterologous nucleotide sequence set forth as SEQ ID NO:6 (8028), or a heterologous nucleotide sequence set forth as SEQ ID NO:7 (8029), wherein the heterologous nucleotide sequence optionally replaces about 853 bp of an untranslated and/or non-coding sequence of an endogenous *C9orf72* locus that starts within endogenous exon 1. In some embodiments, a non-human animal as disclosed herein is produced, e.g., by breeding an animal created using a nucleic acid construct comprising a sequence set forth as SEQ ID

NO:8 with an animal created using a nucleic acid construct comprising a sequence set forth as SEQ ID NO:9. Such animals may comprise both (1) a heterozygous replacement of about 853 bp of an endogenous *C9orf72* locus starting from within endogenous exon 1 with a heterologous nucleotide sequence comprising from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36, one to three repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, a human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37, and a floxed drug resistance (*neo^r*) cassette, or upon excision of the *neo* gene, a lox recombination recognition sequence and (2) a heterozygous replacement of about 853 bp of an endogenous *C9orf72* locus starting from within endogenous exon 1 with a heterologous nucleotide sequence comprising from 5' to 3': a first human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO: 36, one to one-hundred (e.g., 36 or 92) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, a human hexanucleotide flanking sequence comprising a sequence set forth as SEQ ID NO:37, and a floxed drug resistance (*neo^r*) cassette, or upon excision of the *neo* gene, a lox recombinase recognition sequence.

[00026] In some embodiments, an isolated non-human cell or tissue of a non-human animal as described herein, or as made by a method described herein, is provided. In some embodiments, an isolated cell or tissue comprises a *C9ORF72* locus as described herein. In some embodiments, a cell is a neuronal cell or a cell from a neuronal lineage. In some embodiments, an immortalized cell line is provided, which is made from an isolated cell of a non-human animal as described herein.

[00027] In some embodiments, a non-human embryonic stem cell is provided whose genome comprises a *C9ORF72* locus as described herein. In some embodiments, a non-human embryonic stem cell is a rodent embryonic stem cell. In some certain embodiments, a rodent embryonic stem cell is a mouse embryonic stem cell and is from a 129 strain, C57BL strain, or a mixture thereof. In some certain embodiments, a rodent embryonic stem cell is a mouse embryonic stem cell and is a mixture of 129 and C57BL strains.

[00028] Also described herein is a Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) system, or one or more components of a CRISPR/Cas system, which may be used to delete from a cell, e.g., an embryonic stem cell, a heterologous hexanucleotide repeat expansion sequence (or portion thereof)

inserted an endogenous *C9ORF72* locus as described herein. Such components include, for example, Cas proteins and/ or guide RNAs (gRNAs), which gRNA may include two separate RNA molecules; e.g., targeter-RNA (e.g., CRISPR RNAs (crRNA) and activator RNA (e.g., tracrRNAs); or a single-guide RNA (e.g., single-molecule gRNA (sgRNA)).

[00029] 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, or a type III system. Alternatively, a CRISPR/Cas system can be a type V system (e.g., subtype V-A or subtype V-B). A heterologous hexanucleotide repeat expansion sequence (or portion thereof) inserted an endogenous *C9ORF72* locus as described herein may be deleted by utilizing CRISPR complexes (comprising a guide RNA (gRNA) complexed with a Cas protein) for site-directed cleavage of nucleic acids.

[00030] A CRISPR/Cas system as described herein may comprise a Cas protein (e.g., 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, Cu1966, and homologs or modified versions thereof) and/or one or more guide RNA (gRNA), which target(s) a gRNA recognition sequence. A CRISPR/Cas system as described herein may further comprise at least one expression construct, which comprises a nucleic acid encoding a Cas protein (e.g., which may be operably linked to a promoter) and/or DNA encoding a gRNA as described herein.

[00031] In some embodiments a gRNA recognition sequence, e.g., a target nucleic acid sequence to which a DNA-targeting segment of a gRNA will bind provided sufficient conditions for binding exist, is found in SEQ ID NO:45, or portion thereof. Site-specific binding and cleavage of SEQ ID NO:45 by Cas proteins can occur at locations determined by both (i) base-pairing complementarity between the gRNA and the target DNA and (ii) a short motif, called the protospacer adjacent motif (PAM), in the target DNA. The PAM can flank the guide RNA recognition sequence. Optionally, the guide RNA recognition sequence can be flanked on the 3' end by the PAM. Alternatively, the guide RNA recognition sequence can be flanked on the 5' end by the

PAM. 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. In some cases (e.g., when Cas9 from *S. pyogenes* or a closely related Cas9 is used), the PAM sequence of the non-complementary strand can be 5'-N₁GG-3', where N₁ is any DNA nucleotide and is immediately 3' of the guide RNA recognition sequence of the non-complementary strand of the target DNA. As such, the PAM sequence of the complementary strand would be 5'-CCN₂-3', where N₂ is any DNA nucleotide and is immediately 5' of the guide RNA recognition sequence of the complementary strand of the target DNA. In some such cases, N₁ and N₂ can be complementary and the N₁-N₂ base pair can be any base pair (e.g., N₁=C and N₂=G; N₁=G and N₂=C; N₁=A and N₂=T; or N₁=T, and N₂=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 some cases (e.g., for FnCpf1), the PAM sequence can be upstream of the 5' end and have the sequence 5'-TTN-3'. In some embodiments, a gRNA recognition sequence starts at position 190, 196, 274, 899, 905, 1006, or 1068 of SEQ ID NO:45.

[00032] As disclosed herein, guide RNAs may be provided in any form. In some embodiments, gRNA can be provided in the form of RNA, either as two molecules (a 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. In some embodiments, the DNA encoding the gRNA can encode a single RNA molecule (sgRNA) or separate RNA molecules (e.g., separate crRNA and tracrRNA) (wherein the separate RNA molecules may be provided as one DNA molecule, or as separate DNA molecules encoding the crRNA and tracrRNA, respectively).

[00033] In one embodiment, a CRISPR/Cas system as described herein comprises Cas9 protein or a protein derived from a Cas9 from a type II CRISPR/Cas system and/or at least one gRNA, wherein the at least one gRNA is encoded by DNA that encodes a crRNA and/or a tracrRNA. In some embodiments, a DNA encoding a crRNA comprises a sequence selected from the group consisting of AGTACTGTGAGAGCAAGTAG (R) (SEQ ID NO:38), GCTCTCACAGTACTCGCTGA (SEQ ID NO:39), CCGCAGCCTGTAGCAAGCTC (SEQ ID NO:40), CGGCCGCTAGCGCGATCGCG (SEQ ID NO:41), ACGCCCCGCGATCGCGCTAG (R) (SEQ ID NO:42),

TGGCGAGTGGGTGAGTGAGG (SEQ ID NO:43), GGAAGAGGCGCGGGTAGAAG (SEQ ID NO:44), GAGTACTGTGAGAGCAAGTAG (R) (SEQ ID NO:46), GCCGCAGCCTGTAGCAAGCTC (SEQ ID NO:47), GCGGCCGCTAGCGCGATCGCG (SEQ ID NO:48), GACGCCCCGCGATCGCGCTAG (R) (SEQ ID NO:49), and GTGGCGAGTGGGTGAGTGAGG (SEQ ID NO:50). In one embodiment, a CRISPR/Cas system described herein comprises a combination of at least seven crRNA encoding sequences, wherein each of the seven crRNA encoding sequences comprises a sequence set forth as SEQ ID NO: 38, 39, 40, 41, 42, 43 or 44. In one embodiment, a CRISPR/Cas 9 system described herein comprises a combination of at least seven distinct crRNA encoding sequences, wherein each of the seven crRNA encoding sequences comprises a sequence set forth as SEQ ID NO: 46, 39, 47, 48, 49, 50 or 44. In one embodiment, a CRISPR/Cas 9 system described herein comprises a combination of at least three distinct crRNA encoding sequences, each comprising a sequence set forth as SEQ ID NO: 40, 43 or 44. In one embodiment, a CRISPR/Cas 9 system described herein comprises a combination of at least three distinct crRNA encoding sequences, each comprising a sequence set forth as SEQ ID NO: 47, 50 or 44. In one embodiment, a CRISPR/Cas 9 system described herein comprises a combination of at least four distinct crRNA encoding sequences, each comprising a sequence set forth as SEQ ID NO: 38, 39, 41 or 42. In one embodiment, a CRISPR/Cas 9 system described herein comprises a combination of at least four distinct crRNA encoding sequences, each comprising a sequence set forth as SEQ ID NO: 46, 39, 48, or 49.

[00034] In some embodiments, a gRNA disclosed herein is encoded by DNA encoding a tracrRNA. In some embodiments, the tracrRNA encoding sequence comprises a sequence set forth as SEQ ID NO:63, 64 or 65. In some embodiments a gRNA as described herein comprises a crRNA and a tracrRNA. In some embodiments, a gRNA as disclosed herein comprises one or more crRNA (e.g., encoded by DNA comprising a sequence set forth as SEQ ID NO: 38, 39, 40, 41, 42, 43, 44, 46, 47, 48, 49 or 50) and a tracrRNA, e.g., a DNA comprising a sequence set forth as SEQ ID NO:63, 64 or 65. In some embodiments, the DNA encoding the gRNA can encode a single RNA molecule (sgRNA) or separate RNA molecules (e.g., separate crRNA and tracrRNA) (wherein the separate RNA molecules may be provided as one DNA molecule, or as separate DNA molecules encoding the crRNA and tracrRNA, respectively).

[00035] Targeted genetic modifications can be generated by contacting a cell with a Cas protein and one or more guide RNAs that hybridize to one or more guide RNA recognition sequences within a target genomic locus. At least one of the one or more guide RNAs can form a complex with and can guide the Cas protein to at least one of the one or more guide RNA recognition sequences, and the Cas protein can cleave the target genomic locus within at least one of the one or more guide RNA recognition sequences. 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). The end sequences generated by the double-strand break or the single-strand break can then undergo recombination.

[00036] In some embodiments, a non-human germ cell is provided whose genome comprises a *C9ORF72* locus as described herein. In some embodiments, a non-human germ cell is a rodent germ cell. In some certain embodiments, a rodent germ cell is a mouse germ cell and is from a 129 strain, C57BL strain, or a mixture thereof. In some certain embodiments, a rodent germ cell is a mouse germ cell and is a mixture of 129 and C57BL strains.

[00037] In some embodiments, the use of a non-human embryonic stem cell or germ cell as described herein is provided to make a genetically modified non-human animal. In some certain embodiments, a non-human embryonic stem cell or germ cell is a mouse embryonic stem cell or germ cell and is used to make a mouse comprising a *C9ORF72* locus as described herein. In some certain embodiments, a non-human embryonic stem cell or germ cell is a rat embryonic stem cell germ cell and is used to make a rat comprising a *C9ORF72* locus as described herein.

[00038] In some embodiments, a non-human embryo is provided comprising, made from, obtained from, or generated from a non-human embryonic stem cell comprising a *C9ORF72* locus as described herein. In some certain embodiments, a non-human embryo is a rodent embryo; in some embodiments, a mouse embryo; in some embodiments, a rat embryo.

[00039] In some embodiments, the use of a non-human embryo as described herein is provided to make a genetically modified non-human animal. In some certain embodiments, a non-human embryo is a mouse embryo and is used to make a mouse comprising a *C9ORF72* locus as described herein. In some certain embodiments, a non-human embryo is a rat embryo and is used to make a rat comprising a *C9ORF72* locus as described herein.

[00040] In some embodiments, a non-human animal model of amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD) is provided, which non-human animal has an endogenous *C9ORF72* locus comprising a heterologous hexanucleotide repeat expansion sequence as disclosed herein.

[00041] In some embodiments, a non-human animal model of amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD) is provided, which is obtained by an insertion of a heterologous hexanucleotide repeat expansion sequence in an endogenous *C9ORF72* locus.

[00042] In some embodiments, a method for identifying a therapeutic candidate for the treatment of a neurodegenerative disease, disorder or condition is provided, the method comprising (a) administering a candidate agent to a non-human animal or non-human animal cell (e.g., embryonic stem cell, an embryonic stem cell-derived motor neuron, a brain cell, a cortical cell, a neuronal cell, a muscle cell, a heart cell) whose genome comprises an endogenous *C9ORF72* locus modified as described herein; (b) performing one or more assays to determine if the candidate agent has a modulating effect on one or more signs, symptoms and/or conditions associated with the disease, disorder or condition (e.g., increased transcription of sense or antisense *C9orf72* RNA from the *C9orf72* locus, increased nuclear and/or cytoplasmic RNA foci comprising sense or antisense *C9orf72* RNA, increased RAN translation products (e.g., dipeptide repeat proteins); and (c) identifying the candidate agent that has a modulating effect on the one or more signs, symptoms and/or conditions associated with the disease, disorder or condition as the therapeutic candidate. In some embodiments, the disease or condition is selected from the group consisting of a neurodegenerative disease or condition. In some embodiments, the candidate agent is administered *in vivo* to a non-human animal as described herein, and one or more assays are performed on tissue comprising a brain cell, a cortical cell, a neuronal cell, a muscle cell, a heart cell, or a germ cell isolated from the non-human animal after administration. In some embodiments, the candidate agent is administered to a cell (e.g., an embryonic stem cell, an embryonic stem cell-derived motor neuron, a brain cell, a cortical cell, a neuronal cell, a muscle cell, a heart cell) comprising a hexanucleotide repeat expansion sequence at the *C9orf72* locus as described herein, and the assay performed, *in vitro*. In some embodiments, the assay is quantitative polymerase chain reaction (qPCR) to detect *C9orf72* gene products, e.g., sense and antisense *C9orf72* RNA. In some embodiments, qPCR may be performed with

a primer and/or probe having a nucleotide sequence set forth in SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, or any combination thereof. In some embodiments, the assay measures RNA foci comprising a *C9orf72* sense or antisense RNA transcript, e.g., an RNA transcript of a hexanucleotide repeat expansion sequence. In some embodiments, the assay that measures RNA foci comprising a *C9orf72* sense or antisense RNA transcript, e.g., an RNA transcript of a hexanucleotide repeat expansion sequence, using one or more probes having a nucleotide sequence as set forth in any one of SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, and/or SEQ ID NO:84. In some embodiments, the assay is measures RAN translation products, e.g., the assay is immunofluorescence and RAN translation products (e.g., dipeptide repeat proteins, e.g., polyGA dipeptide repeat proteins) are measured with an anti-polyGA antibody. In some embodiments, the assay is measures *C9orf72* protein levels.

[00043] In some embodiments, use of a non-human animal as described herein is provided in the manufacture of a medicament for the treatment of a neurodegenerative disease, disorder or condition.

[00044] In some embodiments, a neurodegenerative disease, disorder or condition is amyotrophic lateral sclerosis (ALS). In some embodiments, a neurodegenerative disease, disorder or condition is frontotemporal dementia (FTD).

[00045] In various embodiments, one or more phenotypes as described herein is or are as compared to a reference or control. In some embodiments, a reference or control includes a non-human animal having a modification as described herein, a modification that is different than a modification as described herein, or no modification (e.g., a wild type non-human animal). Non-human animals comprising a heterologous hexanucleotide repeat expansion sequence comprising a sequence set forth as SEQ ID NO:2, a variant thereof, SEQ ID NO: 4, a variant thereof, or SEQ ID NO:5, or a variant thereof, may exhibit a wildtype phenotype, e.g., may be used as a reference, or control, non-human animal in the methods described herein.

[00046] In various embodiments, a non-human animal is homozygous for the *C9orf72* locus described herein. In various embodiments, the non-human animal is heterozygous for the *C9orf72* locus described herein.

[00047] In various embodiments, a non-human animal described herein is a rodent; in some embodiments, a mouse; in some embodiments, a rat.

[00048] As used in this application, the terms "about" and "approximately" are used as equivalents. Any numerals used in this application with or without about/approximately are meant to cover any normal fluctuations appreciated by one of ordinary skill in the relevant art.

[00049] Other features, objects, and advantages of non-human animals, cells and methods provided herein are apparent in the detailed description of certain embodiments that follows. It should be understood, however, that the detailed description, while indicating certain embodiments, is given by way of illustration only, not limitation. Various changes and modifications within the scope of the invention will become apparent to those skilled in the art from the detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[00050] **Figure 1A** shows a schematic illustration, not to scale, of the three reported mouse *C9orf72* transcript isoforms (V1, V2 and V3) in the top box and a schematic illustration, not to scale, of a targeting strategy for insertion of one of two human heterologous hexanucleotide repeat expansion sequences spanning exons 1a and 1b of the human *C9orf72* gene and comprising 3 or 100 repeats into an endogenous mouse *C9orf72* locus. In Figure 1A, white filled boxes represent mouse exons, with white diagonally striped boxes representing non-coding mouse exons of the mouse *C9orf72* locus. Horizontally striped boxes are non-coding exons of a human *C9orf72* locus and the diamond represents the hexanucleotide repeat. A first targeting vector comprising a sequence set forth as SEQ ID NO:2 and a second targeting vector comprising a sequence set forth as SEQ ID NO:4 were generated. The first targeting vector includes from 5' to 3': a mouse homology arm 89 Kb upstream from RP23-434N2 of mouse the *3110043021Rik* gene and comprising SEQ ID NO:6, a human sequence set forth as SEQ ID NO:8 which spans non-coding exons 1a and 1b of human *C9orf72* and includes the intervening intron containing three repeats of the hexanucleotide sequence GGGGCC; a drug selection cassette that comprises a promoter from the human ubiquitin 1 gene (hUb1) and the bacterial *Em7* gene operably linked to a neomycin phosphotransferase resistance gene (neo-r) and is flanked by *loxP* sites), and a mouse homology arm 86 Kb downstream from RP23-434N2 of mouse the *3110043021Rik* gene and comprising SEQ

ID NO:7. The second targeting vector includes from 5' to 3': a mouse homology arm 89 Kb upstream from RP23-434N2 of mouse the *3110043021Rik* gene and comprising SEQ ID NO:6; a human sequence set forth as SEQ ID NO:9 which spans non-coding exons 1a and 1b of human *C9orf72* and includes the intervening intron containing 100 repeats of the hexanucleotide sequence GGGGCC; a drug selection cassette that comprises a promoter from the human ubiquitin 1 gene (hUb1) and the bacterial Em7 gene operably linked to a neomycin phosphotransferase resistance gene (neo-r) and is flanked by *loxP* sites); and a mouse homology arm 86 Kb downstream from RP23-434N2 of mouse the *3110043021Rik* gene and comprising SEQ ID NO:7. Upon homologous recombination with the first or second targeting vector, a mouse genomic region of about 853 bp, including a portion of exon 1 and part of intron 1 of mouse *3110043021Rik* is replaced with a sequence comprising the genomic sequence spanning exons 1a-1b of the human *C9orf72* non-coding sequence. The resulting modified mouse *C9orf72-HRE₃* loci before and after excision of the drug resistance cassette are depicted in **Figure 1B**. The resulting modified mouse *C9orf72-HRE₁₀₀* loci before and after excision of the drug resistance cassette are depicted in **Figure 1C**. In Figures 1B and 1C, murine non-coding regions are represented by diagonally striped boxes, human non-coding exons are represented by horizontally striped boxes, and mouse coding exons are represented by white boxes. Also shown in the top panels of Figures 1B and 1C is an approximate location of a probe (vertical white rectangle) used for Southern blot analysis (SEQ ID NO:29).

[00051] Shown in **Figure 2A** is the result of Southern blot analysis of genomic DNA isolated from control ES cell clones, ES cell clones targeted with a targeting vector comprising a heterologous repeat expansion sequence comprising three repeats of the hexanucleotide sequence (8026) and after excision of the drug cassette (8027 A-C4), or ES cell clones targeted with a targeting vector comprising a heterologous repeat expansion sequence comprising 100 repeats of the hexanucleotide sequence (8028) and after excision of the drug cassette (8029 A-A3, 8029 A-A6, 8029 B-A4, 8029 B-A10). **Figure 2B** shows the genotypic results of genotyping samples (n=6) including a control ES cell clone, the 8027 A-C4 clone, the 8029 A-A3 clone, the 8029 A-A6 clone, the 8029 B-A4 clone, the 8029 B-A10 clone, and controls (n=7) obtained from human samples containing three hexanucleotide repeat expansion sequences.

[00052] **Figure 3** shows a schematic illustration, not to scale, of the humanized *C9orf72-HRE*_x (where $x \geq 1$), the humanized region, and the wildtype (WT) *C9orf72* mouse loci. Also shown in Figure 3 are the approximate locations of 5'- and 3'- primers (white arrows) and probes (filled rectangles) used in the TAQMAN® qualitative PCR analyses A, B, G, H, and D described in Table 1 to quantify gene expression products from the modified *C9orf72-HRE* loci (A, B, G, H) or both the modified and wildtype *C9orf72* loci (D). In Figure 3, murine non-coding regions are represented by diagonally striped boxes, human non-coding exons are represented by horizontally striped boxes, and mouse coding exons are represented by white boxes. The sequences for the primers and probes depicted in Figure 3 and described in Table 1 are provided in Table 5.

Table 1

Analyses	Location of 5'-primer	Location of 3'-primer	Location of probe
A	Mouse exon 1a	Human exon 1a	Spans junction of mouse exon 1a and human exon 1a
B	Human exon 1a	Mouse exon 2	Human intron 2
G	Human Intron 2	Human Intron 2	Human Intron 2
H	Human Intron 2	Human Intron 2	Human Intron 2
D	Mouse Exon 5	Mouse Exon 6	Mouse Intron 6

[00053] **FIGURE 4** provides bar graphs showing expression levels (as determined by the TAQMAN® qualitative PCR assays A, B, G, and H depicted in Figure 3) of the *C9orf72* locus (y-axis) by embryonic stem cell derived motor neurons (ESMNs), total brain tissue, or parental embryonic stem (ES) cells that are heterozygous (Het) or homozygous (Homo) for a wildtype *C9orf72* locus (control) or a modified *C9orf72* locus comprising three (3x), thirty (30x) or ninety-two (92x) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 relative to ESMNs, brain, or parental ESCs, respectively, that are heterozygous for a modified *C9orf72* locus comprising three (3x) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1. All ESMNs and parental ES cells were heterozygous for the modified *C9orf72* loci, and all controls were homozygous for the wildtype *C9orf72* locus.

[00054] **FIGURES 5A-5C** provides bar graphs showing the differences in the count values (Δ ct; y-axis) of *C9orf72* gene products (detected by the TAQMAN® qualitative PCR assay A (**FIGURE 5A**), assay B (**FIGURE 5B**), or assay D (**FIGURE 5C**) as

depicted in Figure 3) by embryonic stem cell derived motor neurons (ESMNs), total mouse brain, or parental embryonic stem (ES) cells that are heterozygous (het) or homozygous (homo) for a wildtype *C9orf72* locus (Controls) or a modified *C9orf72* locus comprising three (3x), thirty (30x) or ninety-two (92x) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 and the count values of GAPDH gene products. All ESMNs and parental ES cells were heterozygous for the modified *C9orf72* loci, and all controls were homozygous for the wildtype *C9orf72* locus.

[00055] **FIGURE 6** provides bar graphs showing the differences in the count values (Δ ct; y-axis) of *C9orf72* gene products (detected by the TAQMAN® qualitative PCR assay B as depicted in Figure 3) in tissues isolated from the cortex, brainstem, remaining (rem) brain, spinal cord, muscle, liver, heart, or kidneys of mice heterozygous (het) or homozygous (homo) for a wildtype *C9orf72* locus (WT) or a modified *c9orf72* locus comprising three (3x) or ninety-two (92x) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 and the count values of β 2-microglobulin (B2M) gene products.

[00056] **FIGURE 7** shows Western blot images (top) from reducing SDS-PAGE analysis of lysates from embryonic stem cell –derived motor neurons (ESMNs) homozygous for a wildtype *C9orf72* locus (CTRL) or heterozygous for a modified *C9orf72* locus comprising three (G_4C_23x), thirty (G_4C_230x) or ninety-two (G_4C_292x) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, blotted with anti-*C9orf72* antibody (top) or anti-GAPDH antibody (bottom). Bar graphs (bottom panel) of the protein levels of *C9orf72* of these samples normalized to protein levels of *C9orf72* of ESMNs heterozygous for a modified *C9orf72* locus comprising three repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 are also provided, as are molecular weight markers.

[00057] **FIGURE 8** shows a Western blot image (top) from reducing SDS-PAGE analysis of lysates of from embryonic stem cell-derived motor neurons (ESMNs) heterozygous for a modified *C9orf72* locus comprising three (G_4C_23x) or ninety-two (G_4C_292x) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1. Lysates containing 0 μ g, 1.25 μ g, 2.5 μ g, 5 μ g, or 10 μ g total proteins are blotted with anti-*C9orf72* antibody (shown) or anti-GAPDH antibody (data not shown). Bar graphs (bottom) of the protein levels of *C9orf72* of these samples normalized to protein levels of GAPDH by these samples are also provided, as are molecular weight markers.

[00058] FIGURES 9A and 9B are images obtained from fluorescent *in situ* hybridization (FISH) of embryonic stem cell derived motor neurons (ESMNs) heterozygous for a *C9orf72* locus modified to comprise three (*C9orf72* G₄C₂ 3x), thirty (*C9orf72* G₄C₂ 30x) or ninety-two (*C9orf72* G₄C₂ 92x) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 stained with DNA (FIGURE 9A) or LNA (FIGURE 9B) probes, which images show the nuclear and cytoplasmic locations of sense (FIGURE 9A) or antisense (FIGURE 9B) transcripts of the hexanucleotide repeat sequence set forth in SEQ ID NO:1 in the ESMNs. Arrows point to exemplary stained RNA foci.

[00059] FIGURE 10 provides images obtained from immunofluorescence of embryonic stem cell derived motor neurons (ESMNs) heterozygous for a *C9orf72* locus modified to comprise three (*C9orf72* G₄C₂ 3x) or ninety-two (*C9orf72* G₄C₂ 92x) repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, which images show the nuclear locations of dipeptide repeat proteins (polyGA) translated (through RAN translation, a non-AUG mechanism) from transcripts of the hexanucleotide repeat sequence set forth in SEQ ID NO:1 in the ESMNs. Arrows point to exemplary stained polyGA dipeptide repeat proteins.

[00060] FIGURE 11 shows a schematic illustration, not to scale, of about 1300 bp of a mouse *C9ORF72* locus comprising a heterologous (human) hexanucleotide repeat expansion comprising about 92 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and which may be used as a reference sequence to generate a CRISPR/Cas system for the deletion of the expansion sequence. Also depicted in FIGURE 11 are the approximate locations of (1) the 92 repeats of the hexanucleotide sequence depicted by downward pointing arrows, (2) the starting positions (190, 196 and 274) of three sites upstream of the hexanucleotide repeat expansion sequence that may be targeted by gRNA respectively comprising the sequence set forth as SEQ ID NO:38, SEQ ID NO:39, and SEQ ID NO:40, (3) the starting positions (899, 905 1006 and 1068) of four sites downstream of the hexanucleotide repeat expansion sequence that may be targeted by gRNA respectively comprising the sequence set forth as SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43 and SEQ ID NO:44, and (4) the approximate locations for forward (F-) and reverse (R-) primers that may be used to confirm the deletion in selected cell clones. The nucleic acid sequence of the reference sequence depicted in FIGURE 11 is set forth as SEQ ID NO:45.

[00061] **FIGURE 12** shows an exemplary 10,718 bp expression construct that may be used in a CRISP/Cas system. The expression construct comprises a nucleic acid encoding a mouse Cas9 protein "mouse opt Cas9" fused with an N-terminal nuclear localization signal (NLS) and C-terminal nuclear localization signal, the expression of the fusion protein being under the control of a CAGG promoter. Upstream of the nucleic acid is a kozak sequence, and downstream of the nucleic acid is a bovine growth hormone polyadenylation (bGHpA) tail. Also shown as part of the expression construct are an EF1 promoter driving the expression of a nucleotide sequence encoding a green fluorescence protein (GFP) fused with a puromycin resistance gene operably linked to an SV40 polyadenylation (SV40 polyA) tail, an origin of replication site (pMB1), and a β lactamase gene providing ampicillin (Amp) resistance. The expression construct allows for the insertion of DNA encoding gRNA, e.g., a crRNA, between a U6 promoter and a termination signal. An expression construct has depicted in Figure 4 may further comprise, downstream of the U6 promoter and upstream a termination signal, a tracrRNA encoding sequence. Such tracrRNA encoding sequence is placed such that it may be operably linked to the, e.g., crRNA, upon its insertion. In some embodiments, a tracrRNA encoding sequence comprises

GTTGGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTAT
CAACTTGAAAAAGTGGCACCGAGTCGGTGC (SEQ ID NO: 63);

GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTT
GAAAAAGTGGCACCGAGTCGGTGC (SEQ ID NO:64);

GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATAAGGCTAGTCCG
TTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC (SEQ ID NO:65), or portions thereof.

DEFINITIONS

[00062] This invention is not limited to particular methods and experimental conditions described herein, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention is defined by the claims.

[00063] Unless defined otherwise, all terms and phrases used herein include the meanings that the terms and phrases have attained in the art, unless the contrary is clearly

indicated or clearly apparent from the context in which the term or phrase is used.

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, particular methods and materials are now described. All publications mentioned herein are hereby incorporated by reference.

[00064] *"Administration"* includes the administration of a composition to a subject or system (e.g., to a cell, organ, tissue, organism, or relevant component or set of components thereof). Those of ordinary skill will appreciate that route of administration may vary depending, for example, on the subject or system to which the composition is being administered, the nature of the composition, the purpose of the administration, etc. For example, in certain embodiments, administration to an animal subject (e.g., to a human or a rodent) may be bronchial (including by bronchial instillation), buccal, enteral, interdermal, intra-arterial, intradermal, intragastric, intramedullary, intramuscular, intranasal, intraperitoneal, intrathecal, intravenous, intraventricular, mucosal, nasal, oral, rectal, subcutaneous, sublingual, topical, tracheal (including by intratracheal instillation), transdermal, vaginal and/or vitreal. In some embodiments, administration may involve intermittent dosing. In some embodiments, administration may involve continuous dosing (e.g., perfusion) for at least a selected period of time.

[00065] *"Amelioration"* includes the prevention, reduction or palliation of a state, or improvement of the state of a subject. Amelioration includes, but does not require complete recovery or complete prevention of a disease, disorder or condition (e.g., radiation injury).

[00066] *"Approximately"*, as applied to one or more values of interest, includes to a value that is similar to a stated reference value. In certain embodiments, the term *"approximately"* or *"about"* refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[00067] *"Biologically active"* includes a characteristic of any agent that has activity in a biological system, *in vitro* or *in vivo* (e.g., in an organism). For instance, an agent that, when present in an organism, has a biological effect within that organism is considered to be biologically active. In particular embodiments, where a protein or

polypeptide is biologically active, a portion of that protein or polypeptide that shares at least one biological activity of the protein or polypeptide is typically referred to as a "*biologically active*" portion.

[00068] "*Comparable*" includes two or more agents, entities, situations, sets of conditions, etc. that may not be identical to one another but that are sufficiently similar to permit comparison there between so that conclusions may reasonably be drawn based on differences or similarities observed. Those of ordinary skill in the art will understand, in context, what degree of identity is required in any given circumstance for two or more such agents, entities, situations, sets of conditions, etc. to be considered comparable.

[00069] "*Conservative*", when describing a conservative amino acid substitution, includes substitution of an amino acid residue by another amino acid residue having a side chain R group with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of interest of a protein, for example, the ability of a receptor to bind to a ligand. Examples of groups of amino acids that have side chains with similar chemical properties include: aliphatic side chains such as glycine, alanine, valine, leucine, and isoleucine; aliphatic-hydroxyl side chains such as serine and threonine; amide-containing side chains such as asparagine and glutamine; aromatic side chains such as phenylalanine, tyrosine, and tryptophan; basic side chains such as lysine, arginine, and histidine; acidic side chains such as aspartic acid and glutamic acid; and sulfur-containing side chains such as cysteine and methionine. Conservative amino acids substitution groups include, for example, valine/leucine/isoleucine, phenylalanine/tyrosine, lysine/arginine, alanine/valine, glutamate/aspartate, and asparagine/glutamine. In some embodiments, a conservative amino acid substitution can be a substitution of any native residue in a protein with alanine, as used in, for example, alanine scanning mutagenesis. In some embodiments, a conservative substitution is made that has a positive value in the PAM250 log-likelihood matrix disclosed in Gonnet, G.H. et al., 1992, Science 256:1443-1445. In some embodiments, a substitution is a moderately conservative substitution wherein the substitution has a nonnegative value in the PAM250 log-likelihood matrix.

[00070] "*Control*" includes the art-understood meaning of a "*control*" being a standard against which results are compared. Typically, controls are used to augment integrity in experiments by isolating variables in order to make a conclusion about such

variables. In some embodiments, a control is a reaction or assay that is performed simultaneously with a test reaction or assay to provide a comparator. A "*control*" also includes a "*control animal*." A "*control animal*" may have a modification as described herein, a modification that is different as described herein, or no modification (i.e., a wild type animal). In one experiment, a "*test*" (i.e., a variable being tested) is applied. In a second experiment, the "*control*," the variable being tested is not applied. In some embodiments, a control is a historical control (i.e., of a test or assay performed previously, or an amount or result that is previously known). In some embodiments, a control is or comprises a printed or otherwise saved record. A control may be a positive control or a negative control.

[00071] "*Disruption*" includes the result of a homologous recombination event with a DNA molecule (e.g., with an endogenous homologous sequence such as a gene or gene locus). In some embodiments, a disruption may achieve or represent an insertion, deletion, substitution, replacement, missense mutation, or a frame-shift of a DNA sequence(s), or any combination thereof. Insertions may include the insertion of entire genes or fragments of genes, e.g., exons, which may be of an origin other than the endogenous sequence (e.g., a heterologous sequence). In some embodiments, a disruption may increase expression and/or activity of a gene or gene product (e.g., of a protein encoded by a gene). In some embodiments, a disruption may decrease expression and/or activity of a gene or gene product. In some embodiments, a disruption may alter sequence of a gene or an encoded gene product (e.g., an encoded protein). In some embodiments, a disruption may truncate or fragment a gene or an encoded gene product (e.g., an encoded protein). In some embodiments, a disruption may extend a gene or an encoded gene product. In some such embodiments, a disruption may achieve assembly of a fusion protein. In some embodiments, a disruption may affect level, but not activity, of a gene or gene product. In some embodiments, a disruption may affect activity, but not level, of a gene or gene product. In some embodiments, a disruption may have no significant effect on level of a gene or gene product. In some embodiments, a disruption may have no significant effect on activity of a gene or gene product. In some embodiments, a disruption may have no significant effect on either level or activity of a gene or gene product.

[00072] "*Determining*", "*measuring*", "*evaluating*", "*assessing*", "*assaying*" and "*analyzing*" includes any form of measurement, and include determining if an element

is present or not. These terms include both quantitative and/or qualitative determinations. Assaying may be relative or absolute. "*Assaying for the presence of*" can be determining the amount of something present and/or determining whether or not it is present or absent.

[00073] "*Endogenous locus*" or "*endogenous gene*" includes a genetic locus found in a parent or reference organism prior to introduction of a disruption, deletion, replacement, alteration, or modification as described herein. In some embodiments, the endogenous locus has a sequence found in nature. In some embodiments, the endogenous locus is a wild type locus. In some embodiments, the reference organism is a wild type organism. In some embodiments, the reference organism is an engineered organism. In some embodiments, the reference organism is a laboratory-bred organism (whether wild type or engineered).

[00074] "*Endogenous promoter*" includes a promoter that is naturally associated, e.g., in a wild type organism, with an endogenous gene.

[00075] "*Gene*" includes a DNA sequence in a chromosome that codes for a product (e.g., an RNA product and/or a polypeptide product). In some embodiments, a gene includes coding sequence (i.e., sequence that encodes a particular product). In some embodiments, a gene includes non-coding sequence. In some particular embodiments, a gene may include both coding (e.g., exonic) and non-coding (e.g., intronic) sequence. In some embodiments, a gene may include one or more regulatory sequences (e.g., promoters, enhancers, *etc.*) and/or intron sequences that, for example, may control or impact one or more aspects of gene expression (e.g., cell-type-specific expression, inducible expression, *etc.*). For the purpose of clarity we note that, as used in the present application, the term "*gene*" generally refers to a portion of a nucleic acid that encodes a polypeptide; the term may optionally encompass regulatory sequences, as will be clear from context to those of ordinary skill in the art. This definition is not intended to exclude application of the term "*gene*" to non-protein-coding expression units but rather to clarify that, in most cases, the term as used in this document refers to a polypeptide-coding nucleic acid.

[00076] "*Heterologous*" includes an agent or entity from a different source. For example, when used in reference to a polypeptide, nucleic acid sequence, gene, or gene product present in a particular cell or organism, the term clarifies that the relevant polypeptide, nucleic acid sequence, gene, or gene product: 1) was engineered by the

hand of man; 2) was introduced into the cell or organism (or a precursor thereof) through the hand of man (e.g., via genetic engineering); and/or 3) is not naturally produced by or present in the relevant cell or organism (e.g., the relevant cell type or organism type).

"*Heterologous*" also includes a polypeptide, nucleic acid sequence, gene or gene product that is normally present in a particular native cell or organism, but has been modified, for example, by mutation or placement under the control of non-naturally associated and, in some embodiments, non-endogenous regulatory elements (e.g., a promoter).

[00077] "*Host cell*" includes a cell into which a nucleic acid or protein has been introduced. Persons of skill upon reading this disclosure will understand that such terms refer not only to the particular subject cell, but also is used to refer to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the phrase "*host cell*". In some embodiments, a host cell is or comprises a prokaryotic or eukaryotic cell. In general, a host cell is any cell that is suitable for receiving and/or producing a heterologous nucleic acid or protein, regardless of the Kingdom of life to which the cell is designated.

Exemplary cells include those of prokaryotes and eukaryotes (single-cell or multiple-cell), bacterial cells (e.g., strains of *Escherichia coli*, *Bacillus* spp., *Streptomyces* spp., etc.), mycobacteria cells, fungal cells, yeast cells (e.g., *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Pichia methanolica*, etc.), plant cells, insect cells (e.g., SF-9, SF-21, baculovirus-infected insect cells, *Trichoplusia ni*, etc.), non-human animal cells, human cells, or cell fusions such as, for example, hybridomas or quadromas. In some embodiments, the cell is a human, monkey, ape, hamster, rat, or mouse cell. In some embodiments, the cell is eukaryotic and is selected from the following cells: CHO (e.g., CHO K1, DXB-11 CHO, Veggie-CHO), COS (e.g., COS-7), retinal cell, Vero, CV1, kidney (e.g., HEK293, 293 EBNA, MSR 293, MDCK, HaK, BHK), HeLa, HepG2, WI38, MRC 5, Colo205, HB 8065, HL-60, (e.g., BHK21), Jurkat, Daudi, A431 (epidermal), CV-1, U937, 3T3, L cell, C127 cell, SP2/0, NS-0, MMT 060562, Sertoli cell, BRL 3A cell, HT1080 cell, myeloma cell, tumor cell, and a cell line derived from an aforementioned cell. In some embodiments, the cell comprises one or more viral genes, e.g., a retinal cell that expresses a viral gene (e.g., a PER.C6® cell). In some embodiments, a host cell is or comprises an isolated cell. In some embodiments, a host cell is part of a tissue. In some embodiments, a host cell is part of an organism.

[00078] *"Identity"*, in connection with a comparison of sequences, includes identity as determined by a number of different algorithms known in the art that can be used to measure nucleotide and/or amino acid sequence identity. In some embodiments, identities as described herein are determined using a ClustalW v. 1.83 (slow) alignment employing an open gap penalty of 10.0, an extend gap penalty of 0.1, and using a Gonnet similarity matrix (MACVECTOR™ 10.0.2, MacVector Inc., 2008).

[00079] *"Improve"*, *"increase"*, *"eliminate"*, or *"reduce"* includes indicated values that are relative to a baseline measurement, such as a measurement in the same individual (or animal) prior to initiation of a treatment described herein, or a measurement in a control individual (or animal) or multiple control individuals (or animals) in the absence of the treatment described herein.

[00080] *"Isolated"* includes a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) designed, produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. In some embodiments, a substance is *"pure"* if it is substantially free of other components. In some embodiments, as will be understood by those skilled in the art, a substance may still be considered *"isolated"* or even *"pure"*, after having been combined with certain other components such as, for example, one or more carriers or excipients (e.g., buffer, solvent, water, etc.); in such embodiments, percent isolation or purity of the substance is calculated without including such carriers or excipients. To give but one example, in some embodiments, a biological polymer such as a polypeptide or polynucleotide that occurs in nature is considered to be *"isolated"* when: a) by virtue of its origin or source of derivation is not associated with some or all of the components that accompany it in its native state in nature; b) it is substantially free of other polypeptides or nucleic acids of the same species from the species that produces it in nature; or c) is expressed by or is otherwise in association with components from a cell

or other expression system that is not of the species that produces it in nature. Thus, for instance, in some embodiments, a polypeptide that is chemically synthesized or is synthesized in a cellular system different from that which produces it in nature is considered to be an "*isolated*" polypeptide. Alternatively or additionally, in some embodiments, a polypeptide that has been subjected to one or more purification techniques may be considered to be an "*isolated*" polypeptide to the extent that it has been separated from other components: a) with which it is associated in nature; and/or b) with which it was associated when initially produced.

[00081] "*Locus*" or "*Loci*" includes a specific location(s) 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, *C9ORF72*-encoding 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, but not limited to, an enhancer, a promoter, 5' and/or 3' UTR, or a combination thereof. Those of ordinary skill in the art will appreciate that chromosomes may, in some embodiments, contain hundreds or even thousands of genes and demonstrate physical co-localization of similar genetic loci when comparing between different species. Such genetic loci can be described as having shared synteny.

[00082] "*Non-human animal*" includes any vertebrate organism that is not a human. In some embodiments, a non-human animal is a cyclostome, a bony fish, a cartilaginous fish (e.g., a shark or a ray), an amphibian, a reptile, a mammal, and a bird. In some embodiments, a non-human mammal is a primate, a goat, a sheep, a pig, a dog, a cow, or a rodent. In some embodiments, a non-human animal is a rodent such as a rat or a mouse.

[00083] "*Nucleic acid*" includes any compound and/or substance that is or can be incorporated into an oligonucleotide chain. In some embodiments, a "*nucleic acid*" is a compound and/or substance that is or can be incorporated into an oligonucleotide chain via a phosphodiester linkage. As will be clear from context, in some embodiments, "*nucleic acid*" refers to individual nucleic acid residues (e.g., nucleotides and/or nucleosides); in some embodiments, "*nucleic acid*" refers to an oligonucleotide chain comprising individual nucleic acid residues. In some embodiments, a "*nucleic acid*" is

or comprises RNA; in some embodiments, a "*nucleic acid*" is or comprises DNA. In some embodiments, a "*nucleic acid*" is, comprises, or consists of one or more natural nucleic acid residues. In some embodiments, a "*nucleic acid*" is, comprises, or consists of one or more nucleic acid analogs. In some embodiments, a nucleic acid analog differs from a "*nucleic acid*" in that it does not utilize a phosphodiester backbone. For example, in some embodiments, a "*nucleic acid*" is, comprises, or consists of one or more "*peptide nucleic acids*", which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention. Alternatively or additionally, in some embodiments, a "*nucleic acid*" has one or more phosphorothioate and/or 5'-N-phosphoramidite linkages rather than phosphodiester bonds. In some embodiments, a "*nucleic acid*" is, comprises, or consists of one or more natural nucleosides (e.g., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine). In some embodiments, a "*nucleic acid*" is, comprises, or consists of one or more nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyladenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, 2-thiocytidine, methylated bases, intercalated bases, and combinations thereof). In some embodiments, a "*nucleic acid*" comprises one or more modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose) as compared with those in natural nucleic acids. In some embodiments, a "*nucleic acid*" has a nucleotide sequence that encodes a functional gene product such as an RNA or protein. In some embodiments, a "*nucleic acid*" includes one or more introns. In some embodiments, a "*nucleic acid*" includes one or more exons. In some embodiments, a "*nucleic acid*" is prepared by one or more of isolation from a natural source, enzymatic synthesis by polymerization based on a complementary template (*in vivo* or *in vitro*), reproduction in a recombinant cell or system, and chemical synthesis. In some embodiments, a "*nucleic acid*" is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 or more residues long. In some embodiments, a "*nucleic acid*" is

single stranded; in some embodiments, a "*nucleic acid*" is double stranded. In some embodiments, a "*nucleic acid*" has a nucleotide sequence comprising at least one element that encodes, or is the complement of a sequence that encodes, a polypeptide. In some embodiments, a "*nucleic acid*" has enzymatic activity.

[00084] "*Operably linked*" includes a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "*operably linked*" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. "*Operably linked*" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest. The term "*expression control sequence*" includes polynucleotide sequences, which are necessary to affect the expression and processing of coding sequences to which they are ligated. "*Expression control sequences*" include: appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. The nature of such control sequences differs depending upon the host organism. For example, in prokaryotes, such control sequences generally include promoter, ribosomal binding site and transcription termination sequence, while in eukaryotes typically such control sequences include promoters and transcription termination sequence. The term "*control sequences*" is intended to include components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

[00085] "*Phenotype*" includes a trait, or to a class or set of traits displayed by a cell or organism. In some embodiments, a particular phenotype may correlate with a particular allele or genotype. In some embodiments, a phenotype may be discrete; in some embodiments, a phenotype may be continuous.

[00086] "*Physiological conditions*" includes its art-understood meaning referencing conditions under which cells or organisms live and/or reproduce. In some embodiments, the term includes conditions of the external or internal milieu that may occur in nature for an organism or cell system. In some embodiments, physiological conditions are

those conditions present within the body of a human or non-human animal, especially those conditions present at and/or within a surgical site. Physiological conditions typically include, e.g., a temperature range of 20 – 40°C, atmospheric pressure of 1, pH of 6 – 8, glucose concentration of 1 – 20 mM, oxygen concentration at atmospheric levels, and gravity as it is encountered on earth. In some embodiments, conditions in a laboratory are manipulated and/or maintained at physiologic conditions. In some embodiments, physiological conditions are encountered in an organism.

[00087] *"Polypeptide"* includes any polymeric chain of amino acids. In some embodiments, a polypeptide has an amino acid sequence that occurs in nature. In some embodiments, a polypeptide has an amino acid sequence that does not occur in nature. In some embodiments, a polypeptide has an amino acid sequence that contains portions that occur in nature separately from one another (i.e., from two or more different organisms, for example, human and non-human portions). In some embodiments, a polypeptide has an amino acid sequence that is engineered in that it is designed and/or produced through action of the hand of man.

[00088] *"Prevent"* or *"prevention"* in connection with the occurrence of a disease, disorder, and/or condition, includes reducing the risk of developing the disease, disorder and/or condition and/or to delaying onset of one or more characteristics or symptoms of the disease, disorder or condition. Prevention may be considered complete when onset of a disease, disorder or condition has been delayed for a predefined period of time.

[00089] *"Reference"* includes a standard or control agent, animal, cohort, individual, population, sample, sequence or value against which an agent, animal, cohort, individual, population, sample, sequence or value of interest is compared. In some embodiments, a reference agent, animal, cohort, individual, population, sample, sequence or value is tested and/or determined substantially simultaneously with the testing or determination of the agent, animal, cohort, individual, population, sample, sequence or value of interest. In some embodiments, a reference agent, animal, cohort, individual, population, sample, sequence or value is a historical reference, optionally embodied in a tangible medium. In some embodiments, a reference may refer to a control. A *"reference"* also includes a *"reference animal"*. A *"reference animal"* may have a modification as described herein, a modification that is different as described herein or no modification (i.e., a wild type animal). Typically, as would be understood by those skilled in the art, a reference agent, animal, cohort, individual, population, sample, sequence or value is

determined or characterized under conditions comparable to those utilized to determine or characterize the agent, animal (e.g., a mammal), cohort, individual, population, sample, sequence or value of interest.

[00090] *"Response"* includes any beneficial alteration in a subject's condition that occurs as a result of or correlates with treatment. Such alteration may include stabilization of the condition (e.g., prevention of deterioration that would have taken place in the absence of the treatment), amelioration of symptoms of the condition, and/or improvement in the prospects for cure of the condition, etc. It may refer to a subject's response or to a neuron's response. Neuron or subject response may be measured according to a wide variety of criteria, including clinical criteria and objective criteria. Examination of the motor system of a subject may include examination of one or more of strength, tendon reflexes, superficial reflexes, muscle bulk, coordination, muscle tone, abnormal movements, station and gait. Techniques for assessing response include, but are not limited to, clinical examination, stretch flex (myotatic reflex), Hoffmann's reflex, and/or pressure tests. Methods and guidelines for assessing response to treatment are discussed in Brodal, A.: Neurological Anatomy in Relation to Clinical Medicine, ed. 2, New York, Oxford University Press, 1969; Medical Council of the U.K.: Aids to the Examination of the Peripheral Nervous System, Palo Alto, Calif., Pendragon House, 1978; Monrad-Krohn, G.H., Refsum, S.: The Clinical Examination of the Nervous System, ed. 12, London, H.K. Lewis & Co., 1964; and Wolf, J.K.: Segmental Neurology, A Guide to the Examination and Interpretation of Sensory and Motor Function, Baltimore, University Park Press, 1981. The exact response criteria can be selected in any appropriate manner, provided that when comparing groups of neurons and/or patients, the groups to be compared are assessed based on the same or comparable criteria for determining response rate. One of ordinary skill in the art will be able to select appropriate criteria.

[00091] *"Risk"*, as will be understood from context, of a disease, disorder, and/or condition comprises likelihood that a particular individual will develop a disease, disorder, and/or condition (e.g., a radiation injury). In some embodiments, risk is expressed as a percentage. In some embodiments, risk is from 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 and up to 100%. In some embodiments, risk is expressed as a risk relative to a risk associated with a reference sample or group of reference samples. In some embodiments, a reference sample or group of reference

samples have a known risk of a disease, disorder, condition and/or event (e.g., a radiation injury). In some embodiments a reference sample or group of reference samples are from individuals comparable to a particular individual. In some embodiments, relative risk is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more.

[00092] *"Substantially"* includes the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term *"substantially"* is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[00093] *"Substantial homology"* includes a comparison between amino acid or nucleic acid sequences. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be *"substantially homologous"* if they contain homologous residues in corresponding positions. Homologous residues may be identical residues. Alternatively, homologous residues may be non-identical residues with appropriately similar structural and/or functional characteristics. For example, as is well known by those of ordinary skill in the art, certain amino acids are typically classified as *"hydrophobic"* or *"hydrophilic"* amino acids, and/or as having *"polar"* or *"non-polar"* side chains. Substitution of one amino acid for another of the same type may often be considered a *"homologous"* substitution. Typical amino acid categorizations are summarized below.

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

Ambiguous Amino Acids	3-Letter	1-Letter
Asparagine or aspartic acid	Asx	B
Glutamine or glutamic acid	Glx	Z
Leucine or Isoleucine	Xle	J
Unspecified or unknown amino acid	Xaa	X

[00094] As is well known in this art, amino acid or nucleic acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST, and PSI-BLAST for amino acid sequences. Exemplary such programs are described in Altschul, S. F. et al., 1990, *J. Mol. Biol.*, 215(3): 403-410; Altschul, S. F. et al., 1997, *Methods in Enzymology*; Altschul, S. F. et al., 1997, *Nucleic Acids Res.*, 25:3389-3402; Baxevanis, A.D., and B. F. F. Ouellette (eds.) *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins*, Wiley, 1998; and Misener et al. (eds.) *Bioinformatics Methods and Protocols (Methods in Molecular Biology, Vol. 132)*, Humana Press, 1998. In addition to identifying homologous sequences, the programs mentioned above typically provide an indication of the degree of homology. In some embodiments, two sequences are considered to be substantially homologous if at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of their corresponding residues are homologous over a relevant stretch of residues. In some embodiments, the relevant stretch is a complete sequence. In some embodiments, the relevant stretch is at least 9, 10, 11, 12, 13, 14, 15, 16, 17 or more residues. In some embodiments, the relevant stretch includes contiguous residues along a complete sequence. In some embodiments, the relevant stretch includes

discontinuous residues along a complete sequence, for example, noncontiguous residues brought together by the folded conformation of a polypeptide or a portion thereof. In some embodiments, the relevant stretch is at least 10, 15, 20, 25, 30, 35, 40, 45, 50, or more residues.

[00095] *"Substantial identity"* includes a comparison between amino acid or nucleic acid sequences. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be *"substantially identical"* if they contain identical residues in corresponding positions. As is well known in this art, amino acid or nucleic acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST, and PSI-BLAST for amino acid sequences. Exemplary such programs are described in Altschul, S.F. et al., 1990, J. Mol. Biol., 215(3): 403-410; Altschul, S.F. et al., 1997, Methods in Enzymology; Altschul, S.F. et al., 1997, Nucleic Acids Res., 25:3389-3402; Baxevanis, A.D., and B.F.F. Ouellette (eds.) Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins, Wiley, 1998; and Misener et al. (eds.) Bioinformatics Methods and Protocols (Methods in Molecular Biology, Vol. 132), Humana Press, 1998. In addition to identifying identical sequences, the programs mentioned above typically provide an indication of the degree of identity. In some embodiments, two sequences are considered to be substantially identical if at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of their corresponding residues are identical over a relevant stretch of residues. In some embodiments, the relevant stretch is a complete sequence. In some embodiments, the relevant stretch is at least 10, 15, 20, 25, 30, 35, 40, 45, 50, or more residues.

[00096] *"Targeting vector," "targeting construct" or "nucleic acid construct"* includes a polynucleotide molecule that comprises a targeting region. A targeting region comprises a sequence that is identical or substantially identical to a sequence in a target cell, tissue or animal and provides for integration of the targeting construct into a position within the genome of the cell, tissue or animal via homologous recombination. Targeting regions that target using site-specific recombinase recognition sites (e.g., *loxP* or Frt sites) are also included. In some embodiments, a targeting construct as described herein further comprises a nucleic acid sequence or gene (e.g., a reporter gene or homologous or heterologous gene) of particular interest, a selectable marker, control and

or regulatory sequences, and other nucleic acid sequences that encodes a recombinase or recombinogenic protein. In some embodiments, a targeting construct may comprise a gene of interest in whole or in part, wherein the gene of interest encodes a polypeptide, in whole or in part, that has a similar function as a protein encoded by an endogenous sequence. In some embodiments, a targeting construct may comprises a humanized gene of interest, in whole or in part, wherein the humanized gene of interest encodes a polypeptide, in whole or in part, that has a similar function as a polypeptide encoded by an endogenous sequence. In some embodiments, a targeting construct may comprise a reporter gene, in whole or in part, wherein the reporter gene encodes a polypeptide that is easily identified and/or measured using techniques known in the art.

[00097] *"Transgenic animal", "transgenic non-human animal" or "Tg⁺"* includes any non-naturally occurring non-human animal in which one or more of the cells of the non-human animal contain heterologous nucleic acid and/or gene encoding a polypeptide of interest, in whole or in part. In some embodiments, a heterologous nucleic acid and/or gene is introduced into the cell, directly or indirectly by introduction into a precursor cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classic breeding techniques, but rather is directed to introduction of recombinant DNA molecule(s). This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. The term *"Tg⁺"* includes animals that are heterozygous or homozygous for a heterologous nucleic acid and/or gene, and/or animals that have single or multi-copies of a heterologous nucleic acid and/or gene.

[00098] *"Treatment", "Treat" or "Treating"* includes any administration of a substance (e.g., a therapeutic candidate) that partially or completely alleviates, ameliorates, relieves, inhibits, delays onset of, reduces severity of, and/or reduces incidence of one or more symptoms, features, and/or causes of a particular disease, disorder, and/or condition. In some embodiments, such treatment may be administered to a subject who does not exhibit signs of the relevant disease, disorder and/or condition and/or of a subject who exhibits only early signs of the disease, disorder, and/or condition. Alternatively or additionally, in some embodiments, treatment may be administered to a subject who exhibits one or more established signs of the relevant disease, disorder and/or condition. In some embodiments, treatment may be of a subject who has been diagnosed as suffering from the relevant disease, disorder, and/or

condition. In some embodiments, treatment may be of a subject known to have one or more susceptibility factors that are statistically correlated with increased risk of development of the relevant disease, disorder, and/or condition.

[00099] *"Variant"* includes an entity that shows significant structural identity with a reference entity, but differs structurally from the reference entity in the presence or level of one or more chemical moieties as compared with the reference entity. In many embodiments, a *"variant"* also differs functionally from its reference entity. In general, whether a particular entity is properly considered to be a *"variant"* of a reference entity is based on its degree of structural identity with the reference entity. As will be appreciated by those skilled in the art, any biological or chemical reference entity has certain characteristic structural elements. A *"variant"*, by definition, is a distinct chemical entity that shares one or more such characteristic structural elements. To give but a few examples, a small molecule may have a characteristic core structural element (e.g., a macrocycle core) and/or one or more characteristic pendent moieties so that a variant of the small molecule is one that shares the core structural element and the characteristic pendent moieties but differs in other pendent moieties and/or in types of bonds present (single vs. double, E vs. Z, etc.) within the core, a polypeptide may have a characteristic sequence element comprised of a plurality of amino acids having designated positions relative to one another in linear or three-dimensional space and/or contributing to a particular biological function, a nucleic acid may have a characteristic sequence element comprised of a plurality of nucleotide residues having designated positions relative to one another in linear or three-dimensional space. For example, a *"variant polypeptide"* may differ from a reference polypeptide as a result of one or more differences in amino acid sequence and/or one or more differences in chemical moieties (e.g., carbohydrates, lipids, etc.) covalently attached to the polypeptide backbone. In some embodiments, a *"variant polypeptide"* shows an overall sequence identity with a reference polypeptide that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 99%. Alternatively or additionally, in some embodiments, a *"variant polypeptide"* does not share at least one characteristic sequence element with a reference polypeptide. In some embodiments, the reference polypeptide has one or more biological activities. In some embodiments, a *"variant polypeptide"* shares one or more of the biological activities of the reference polypeptide. In some embodiments, a *"variant polypeptide"* lacks one or more of the biological activities of the reference polypeptide. In some

embodiments, a "*variant polypeptide*" shows a reduced level of one or more biological activities as compared with the reference polypeptide. In many embodiments, a polypeptide of interest is considered to be a "*variant*" of a parent or reference polypeptide if the polypeptide of interest has an amino acid sequence that is identical to that of the parent but for a small number of sequence alterations at particular positions. Typically, fewer than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, or 2% of the residues in the variant are substituted as compared with the parent. In some embodiments, a "*variant*" has 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 substituted residue(s) as compared with a parent. Often, a "*variant*" has a very small number (e.g., fewer than 5, 4, 3, 2, or 1) number of substituted functional residues (i.e., residues that participate in a particular biological activity). Furthermore, a "*variant*" typically has not more than 5, 4, 3, 2, or 1 additions or deletions, and often has no additions or deletions, as compared with the parent. Moreover, any additions or deletions are typically fewer than about 25, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 10, about 9, about 8, about 7, about 6, and commonly are fewer than about 5, about 4, about 3, or about 2 residues. In some embodiments, the parent or reference polypeptide is one found in nature. As will be understood by those of ordinary skill in the art, a plurality of variants of a particular polypeptide of interest may commonly be found in nature, particularly when the polypeptide of interest is an infectious agent polypeptide. In some embodiments, a non-human animal will comprise a variant of a nucleic acid construct used for targeted insertion of a heterologous hexanucleotide expansion sequence. As non-limiting examples, such nucleic acid constructs may comprise a 5' first heterologous hexanucleotide flanking sequence, n repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, a 3' second heterologous hexanucleotide flanking sequence, and optionally, a drug resistance reporter gene preferably flanked by recombinase recognition sequences. As shown in Example 1, an animal resulting from the targeted insertion may comprise in an endogenous locus a variant of the nucleic acid construct, e.g., wherein the variant comprises less than n repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 and/or lacks the drug resistance gene, *see, e.g.*, FIGURES 1B and 1C. Accordingly, a variant of a sequence included herein includes sequences essentially identical to the reference parent sequence, but lacking one or more repeats and/or drug resistance gene(s).

[000100] "**Vector**" includes a nucleic acid molecule capable of transporting another nucleic acid to which it is associated. In some embodiment, vectors are capable of extra-chromosomal replication and/or expression of nucleic acids to which they are linked in a host cell such as a eukaryotic and/or prokaryotic cell. Vectors capable of directing the expression of operably linked genes are referred to herein as "*expression vectors*."

[000101] "**Wild type**" includes an entity having a structure and/or activity as found in nature in a "*normal*" (as contrasted with mutant, diseased, altered, etc.) state or context. Those of ordinary skill in the art will appreciate that wild type genes and polypeptides often exist in multiple different forms (e.g., alleles).

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[000102] Non-human animals are provided having an insertion of a heterologous hexanucleotide repeat expansion sequence in an endogenous *C9ORF72* locus. In some embodiments, non-human animals described herein are heterozygous for a modified *C9ORF72* locus as described herein. In some embodiments, non-human animals as described herein comprise a first modified *C9orf72* locus and a second modified *C9orf72* locus, wherein the first and second loci are different. In some embodiments, non-human animals described herein are homozygous for a modified *C9ORF72* locus as described herein. In some embodiments, non-human animals described herein develop ALS- and/or FTD-like disease due to the presence of the heterologous hexanucleotide repeat expansion sequence.

[000103] Various aspects of the invention are described in detail in the following sections. The use of sections is not meant to limit the invention. Each section can apply to any aspect of the invention. In this application, the use of "or" means "and/or" unless stated otherwise.

C9ORF72

[000104] 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), originally referred to as Pick's disease after physician Arnold Pick, is a group of disorders caused by progressive cell degeneration in the frontal or temporal lobes of the brain. FTD is reported to count for 10-15% of all dementia cases. A hexanucleotide

repeat expansion sequence between (and optionally spanning) exons 1a and 1b, two non-coding exons, of the human *C9ORF72* gene have been linked to both ALS and FTD (DeJesus-Hernandez, M. et al., 2011, *Neuron* 72:245-256; Renton, A.E. et al., 2011, *Neuron* 72:257-268; Majounie, E. et al., 2012, *Lancet Neurol.* 11:323-330; Waite, A.J. et al., 2014, *Neurobiol. Aging* 35:1779.e5-1779.e13). It is estimated that the GGGGCC (SEQ ID NO:1) 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.

[000105] 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., reviewed in Stepto, A. et al., 2014, *Acta Neuropathol.* 127:377-389; see also Almeida, S. et al., 2013, *Acta Neuropathol.* 126:385-399; Bieniek, K.F. et al., 2014, *JAMA Neurol.* 71(6): 775–781; van Blitterswijk, M. et al., 2014, *Mol. Neurodegen.* 9:38, 10 pages). Knock-in mice that have been generated to contain a heterologous hexanucleotide repeat expansion sequence comprising 66 repeats of the hexanucleotide sequence (GGGGCC; SEQ ID NO:1) exhibit RNA foci and dipeptide protein aggregates in their neurons. These mice showed cortical neuron loss and exhibited behavior and motor deficits at 6 months of age (Chew, J. et al., 2015, *Science* May 14. Pii:aaa9344). However, the mechanism through which such repeat expansions cause disease, whether through a loss- or gain-of-function of toxicity, remains unclear. Additionally, the contribution of a lower number of repeats in the hexanucleotide repeat expansion sequence to ALS/FTD is also unknown.

[000106] Although *C9ORF72* has been reported to regulate endosomal trafficking (Farg, M.A. et al., 2014, *Human Mol. Gen.* 23(13):3579-3595), much of the cellular function of *C9ORF72* remains unknown. Indeed, *C9ORF72* is a gene that encodes an uncharacterized protein with unknown function. Despite the lack of understanding surrounding *C9ORF72*, several animal models, including engineered cell lines, for ALS and/or FTD have been developed (Roberson, E.D., 2012, *Ann. Neurol.* 72(6):837-849; Panda, S.K. et al., 2013, *Genetics* 195:703-715; Suzuki, N. et al., 2013, *Nature Neurosci.* 16(12):1725-1728; Xu, Z. et al., 2013, *Proc. Nat. Acad. Sci. U.S.A.* 110(19):7778-7783; Hukema, R.K. et al., 2014, *Acta Neuropathol. Comm.* 2:166, 4 pages). Another report using a transgenic mouse strain containing a heterologous hexanucleotide repeat

expansion sequence comprising 80 GGGGCC repeats operably linked with a fluorescent reporter and controlled by a tetracycline responsive element without any surrounding *C9orf72* sequences demonstrated neuronal cytoplasmic inclusions similar to those seen in ALS-FTD patients, which suggests that expanded repeats of the hexanucleotide GGGGCC sequence itself may be responsible for disease (Hukema, R.K. et al., 2014, *Acta Neuropathol. Comm.* 2:166, 4 pages). These mice have been useful to establish an initial *C9orf72* expression profile in cells of the CNS and provide some understanding of the mechanism of action associated with the repeat expansion; however, construct design can influence the phenotype of the resulting transgenic animal (see, e.g., Muller, U., 1999, *Mech. Develop.* 81:3-21). For example, a transgenic mouse strain containing an inducible GGGGCC repeat (Hukema, 2014, *supra*) was designed without human flanking sequence presumably due to the fact that such surrounding sequence was thought to affect translation of repeat sequences. Thus, such *in vivo* systems exploiting C9ORF72-mediated biology for therapeutic applications are incomplete.

C9ORF72 and Hexanucleotide Repeat Expansion Sequences

[000107] Mouse *C9ORF72* transcript variants have been reported in the art (e.g., Koppers et al., *Ann Neurol* (2015); 78: 426-438; Atkinson et al., *Acta Neuropathologica Communications* (2015) 3: 59), and are also depicted in Figure 1A. 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 Table 2. For mRNA sequences, bold font contained within parentheses indicates coding sequence and consecutive exons, where indicated, are separated by alternating lower and upper case letters. For amino acid sequences, mature polypeptide sequences, where indicated, are in bold font.

[000108] Human *C9ORF72* transcript variants are known in the art. 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. The sequences of NM_145005.6 and NP_659442.2 are respectively set forth as SEQ ID NO:10 and SEQ ID NO:11. 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. The sequences of NM_018325.4 and NP_060795.1 are respectively set forth as SEQ ID NO:12 and SEQ ID NO:13. 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. The sequences of NM_001256054.2 and NP_001242983.1 are respectively set forth as SEQ ID NO:14 and SEQ ID NO:15. Variants 2 and 3 encode the same protein.

[000109] A hexanucleotide repeat expansion sequence is generally a nucleotide sequence comprising at least one instance, e.g., one repeat, of the hexanucleotide sequence GGGGCC set forth as SEQ ID NO:1. For purposes of insertion into an endogenous non-human *C9orf72* locus, a heterologous hexanucleotide repeat expansion sequence comprises at least one instance (repeat) and preferably more than one instance (repeat) of the hexanucleotide sequence set forth as SEQ ID NO:1 and may be identical to, or substantially identical to a genomic nucleic acid sequence spanning (and optionally including) non-coding exons 1a and 1b of a human 'chromosome 9 open reading frame 72' (*C9orf72*), or a portion thereof. Non-limiting examples of heterologous hexanucleotide expansion sequences include the sequences set forth as SEQ ID NO:1, SEQ ID NO:2 (comprising three repeats of the GGGGCC hexanucleotide sequence) and SEQ ID NO:3 (comprising 100 repeats of the GGGGCC hexanucleotide sequence).

TABLE 2

***Mus musculus* *C9orf72* mRNA (NM_001081343; SEQ ID NO:16)**

gtgtccggggcgggcggtcccgggcgggcgggcgggcgagcgggctgcggtgcggtccctgcgccggcggtgaaggcgc
agcagcgcgagtgCTATTGCAAGCGTTCGGATAATGTGAGACCTGGAATGCAGTG

AGACCTGGGATGCAGGG(ATGTCTGACTATCTGCCCCCACCATCTCCTGCT
GTTGCCAAGACAGAGATTGCTTTAAGTGGTGAATCACCCCTGTTGGCGG
CTACCTTTGCTTACTGGGATAATATTCTTGGTCCTAGAGTAAGGCATATT
TGGGCTCCAAAGACAGACCAAGTGCTTCTCAGTGATGGAGAAATAACTT
TTCTTGCCAACCACACTCTAAATGGAGAAATCTTCGAAATGCAGAGAG
TGGGGCTATAGATGTAAAAATTTTTTGTCTTATCTGAAAAAGGGGTAAATTA
TTGTTTCATTAATCTTCGACGGAAACTGGAATGGAGATCGGAGCACTTA
TGGACTATCAATTATACTGCCGCAGACAGAGCTGAGCTTCTACCTCCCA
CTTCACAGAGTGTGTGTTGACAGGCTAACACACATTATTCGAAAAGGAA
GAATATGGATGCATAAGgaaagacaagaaaatgtccagaaaatgtcttgaaggcacagagaggat
ggaagatcagGGTCAGAGTATCATTTCCCATGCTTACTGGGGAAGTCATTCTGT
TAATGGAGCTGCTTGCATCTATGAAATCCCACAGTGTTCTGAAGACAT
TGATatagctgatacagtgctcaatgatgatgacattgggtgacagctgtcacgaaggctttcttctcaaTGCCAT
CAGCTCACACCTGCAGACCTGTGGCTGTTCCGTTGTAGTTGGCAGCAGT
GCAGAGAAAGTAAATAAGatagtaagaacgctgtgcttttctgacaccagcagagaggaaatgctc
caggetgtgtgaagcagaatcgtctttaaagtacgaatcgggactcttgtgcaaggcttgetaaagGATGCAAC
AGGCAGTTTTTGTCTACCTTCCGGCAAGTTATGTATGCCCCGTACCCC
ACCACGCACATTGATGTGGATGTCAACACTGTCAAGCAGATGCCACCGT
GTCATGAACATATTTATAATCAACGCAGATACATGAGGTCAGAGCTGAC
AGCCTTCTGGAGGGCAACTTCAGAAGAGGACATGGCGCAGGACACCAT
CATCTACACAGATGAGAGCTTCACTCCTGATTTgaatatttccaagatgtcttacacaga
gacactctagtgaaagccttctggatcagGTCTTCCATTTGAAGCCTGGCCTGTCTCTCA
GGAGTACTTTCCTTGACAGTTCTCCTCATTCTTCACAGAAAAGCCTTG
ACACTAATCAAGTACATCGAGGATGATACgcagaaggggaaaaagcccttaagtctcttc
ggaacctgaagatagactcttgatttaacagcagagggcgatcttaacataataatggctctagctgagaaaattaagcc
aggcctacactctttcatcttgggagacctttctacactagtgtaagaacgtgatgttctaatgacctttga)ccgtgt
ggtttgctgtgtctcttccacagtcacacctgctgttacagtgctcagcagtggtgggcacatcttctcccgagtcctgct
gcaggacagggtagactacactgtcagtagaagctgtacctgatgtcaggtgcatcggtacagtgatgactcttctagaata
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ttattgaagcatgtgtaaacactgttatatatctttctcctagatggggaatttgaataaaatacctttgaaattctgtgt

Mus musculus C9orf72 amino acid (NP_001074812; SEQ ID NO:17)

MSTICPPPSPAVAKTEIALSGESPLLAATFAYWDNILGPRVRHIWAPKTDQV
LLSDGEITFLANHTLNGEILRNAESGAIDVKFFVLSEKGVIIIVSLIFDGNWNG
DRSTYGLSIILPQTELSFYLP LHRVCVDRLTHIIRKGRIVMHKERQENVQKI
VLEGTERMEDQGQSIIPMLTGEVIPVMELLASMKSHSVPEDIDIADTVLNDD
DIGDSCHEGFLNAISSHLQTCGCSVVVGSSAEKVNKIVRTLCLFLTPAERK
CSRLCEAESSFKYESGLFVQGLLKDATGSFVLPFRQVMYAPYPTTHIDVDVN
TVKQMPPCHEHIYNQRRYMRSELTAFWRATSEEDMAQDTHIYTDESFTPD
NIFQDVLHRDRTLKAFDLQVFHLKPGLSLRSTFLAQFLILHRKALTLIKIE
DDTQKGKKPFKSLRNLKIDLDLTAEGDLNIIMALAEKIKPGLHSFIFGRPFYTSVQ
ERDVLMTF

Rattus norvegicus C9orf72 mRNA (NM_001007702; SEQ ID NO:18)

CGTTTGTAGTGTGAGCCATCCCAATTGCCTGTTCTTCTCTGTGGGAGTGGTG
TCTAGACAGTCCAGGCAGGGTATGCTAGGCAGGTGCGTTTTGGTTGCCTCAG
ATCGCAACTTGACTCCATAACGGTGACCAAAGACAAAAGAAGGAAACCAGA
TTAAAAAGAACCGGACACAGACCCCTGCAGAACTCTGGAGCGGCCGTGGTTG
GGGGCGGGGCTACGACGGGGCGGACTCGGGGGCGTGGGAGGGCGGGGCCG
GGGCGGGGCGGAGCCGGCTGCGGTTGCGGTCCCTGCGCCGGCGGTGAAG
GCGCAGCGGCGGCGAGTGGCTATTGCAAGCGTTTGGATAATGTGAGACCTGG
GATGCAGGG(ATGTGCGACTATCTGCCCCCACCATCTCCTGCTGTTGCCA
AGACAGAGATTGCTTTAAGTGGTGAATCACCCCTTGTGGCGGCTACCTT
TGCTTACTGGGATAATATTCTTGGTCCTAGAGTAAGGCACATTTGGGCT
CCAAAGACAGACCAAGTACTCCTCAGTGATGGAGAAATCACTTTTCTTG
CCAACCACACTCTGAATGGAGAAATTCTTCGGAATGCGGAGAGTGGGGC
AATAGATGTAAAGTTTTTTGTCTTATCTGAAAAGGGCGTCATTATTGTTT
CATTAACTTCGACGGGAACTGGAACGGAGATCGGAGCACTTACGGACT
ATCAATTATACTGCCGCAGACGGAGCTGAGTTTCTACCTCCCACTGCAC
AGAGTGTGTGTTGACAGGCTAACGCACATCATTCGAAAAGGAAGGATAT
GGATGCACAAGGAAAGACAAGAAAATGTCCAGAAAATTGTCTTGGAAGG
CACCGAGAGGATGGAAGATCAGGGTCAGAGTATCATCCCTATGCTTACT
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TACTACCTTTCCGGCAAGTTATGTATGCCCCCTATCCCACCACACACATC
GATGTGGATGTCAAACTGTCAAGCAGATGCCACCGTGTGATGAACATA

TTTATAATCAACGCAGATACATGAGGTCAGAGCTGACAGCCTTCTGGAG
GGCAACTTCAGAAGAGGACATGGCTCAGGACACCATCATCTACACAGAT
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CACTCTAGTGAAAGCCTTTCTGGATCAGGTCTTCCATTTGAAGCCTGGC
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CCTCCAGGGTCCTGCCGCAGGACACGTTACACTACACTTGTCAGTAGAGGTC
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TTGCCAAGTTTTAAGGTTAATTTTCATTATTTTTTGAGCCAAATTAAAATGTGC
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CTGGACTTTGGTGTTCTCCACGTTCCCTGCATCCTAAGGACAGCAGGAGAAC
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AAGCTCCGTGCTACAGCAGTGTAGGGTATACACAAACACAAGTAAGTGTGTTT
ATTTAAACTGTGGACTTAGCATAAAAAGGGAGACTATATTTATTTTTTACA
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AACATTCTATTCTGAAAGGTCACAGTGGTTTTGACATGACACATCAGAACAA
CGCACACTGTCCATGATGGCTTATGAACTCCAAGTCACTCCATCATGGTAAA
TGGGTAGATCCCTCCTTCTAGTGTGCCACACCATTGCTTCCCACAGTAGAATC
TTATTTAAGTGCTAAGTGTGCTCTGCTGGTTTACTCTGTTGTTTTAGAGAAT
GTAAGTTGTATAGTGAATAAGTTATTGAAGCATGTGTAAACACTGTTATACA
TCTTTTCTCCTAGATGGGGAATTTGGAATAAAATACCTTTAAATTCAAAAA
AAAAAAAAAAAAAAAAAAAA

Rattus norvegicus C9orf72 amino acid (NP_001007703; SEQ ID NO:19)

MSTICPPPSPAVAKTEIALSGESPLLAATFAYWDNILGPRVRHIWAPKTDQVLLS
 DGEITFLANHTLNGEILRNAESGAIDVKFFVLSEKGVIIIVSLIFDGNWNGDRSTYG
 LSILPQTELSFYLPVLCVDRDLTHIRKGRWIMHKERQENVQKIVLEGTERMED
 QGQSIIPMLTGEVIPVMELLASMRSHSVPELDIADTVLNDDDIGDSCHEGFLN
 AISSHLQTCGCSVVVGSSAEKVNKIVRTLCLFLTPAERKCSRLCEAESSFKYESGL
 FVQGLLKDATGSFVLPFRQVMYAPYPTTHIDVDVNTVKQMPPCHEHIYNQRRY
 MRSELTAFWRATSEEDMAQDTIIYTDESFTPLNIFQDVLHRDTLVKAFLDQVFN
 LKPGLSLRSTFLAQFLILHRKALTLIKIEDDTQKGKKPFKSLRNLKIDLDLTAE
 GDLNIIMALAEKIKPGLHSFIFGRPFYTSVQERDVLMTF

***C9ORF72 Targeting Vectors and Production of Non-Human Animals Having a
 Heterologous Hexanucleotide Repeat Expansion Sequence Inserted in a C9ORF72
 Locus***

[000110] Provided herein are targeting vectors or targeting constructs for the production of non-human animals having a heterologous hexanucleotide expansion sequence inserted into an endogenous *C9ORF72* locus as described herein.

A. Large Targeting Vectors

[000111] In cells other than one-cell stage embryos, a targeting vector that is a “large targeting vector” or “LTVEC” can be used, 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).

[000112] A targeting vector includes homology arms. If the targeting vector 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 repair template. The 5' and 3' homology arms correspond to regions within the genomic region of interest, which are referred to herein as "5' target sequence" and "3' target sequence," respectively.

[000113] 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 repair template 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 repair template (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. 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. The homology arms can be symmetrical (each about the same size in length), or they can be asymmetrical (one longer than the other).

[000114] The homology arms can correspond to a locus that is native to a cell (e.g., the targeted locus). Alternatively, for example, they can correspond to a region of a heterologous or exogenous segment of DNA that was integrated into the genome of the cell, including, for example, transgenes, expression cassettes, or heterologous or exogenous regions of DNA. Alternatively, the homology arms of the targeting vector can correspond to a region of a yeast artificial chromosome (YAC), a bacterial artificial chromosome (BAC), a human artificial chromosome, or any other engineered region contained in an appropriate host cell. Still further, the homology arms of the targeting vector can correspond to or be derived from a region of a BAC library, a cosmid library, or a P1 phage library, or can be derived from synthetic DNA.

[000115] Examples of LTVECs include vectors derived from a bacterial artificial chromosome (BAC), a human artificial chromosome, or a yeast artificial chromosome (YAC). Non-limiting examples of LTVECs and methods for making them are described,

e.g., in US Patent Nos. 6,586,251; 6,596,541; and 7,105,348; and in WO 2002/036789, each of which is herein incorporated by reference in its entirety for all purposes.

LTVECs can be in linear form or in circular form.

[000116] LTVECs can be of any length and are typically at least 10 kb in length. For example, an LTVEC can be from about 50 kb to about 500 kb, from about 50 kb to about 75 kb, from about 75 kb to about 100 kb, from about 100 kb to about 125 kb, from about 125 kb to about 150 kb, from about 150 kb to about 175 kb, from about 175 kb to about 200 kb, from about 200 kb to about 225 kb, from about 225 kb to about 250 kb, from about 250 kb to about 275 kb, from about 275 kb to about 300 kb, from about 300 kb to about 325 kb, from about 325 kb to about 350 kb, from about 350 kb to about 375 kb, from about 375 kb to about 400 kb, from about 400 kb to about 425 kb, from about 425 kb to about 450 kb, from about 450 kb to about 475 kb, or from about 475 kb to about 500 kb. Alternatively, an LTVEC can be at least 10 kb, at least 15 kb, at least 20 kb, at least 30 kb, at least 40 kb, at least 50 kb, at least 60 kb, at least 70 kb, at least 80 kb, at least 90 kb, at least 100 kb, at least 150 kb, at least 200 kb, at least 250 kb, at least 300 kb, at least 350 kb, at least 400 kb, at least 450 kb, or at least 500 kb or greater. The size of an LTVEC can be too large to enable screening of targeting events by conventional assays, e.g., southern blotting and long-range (e.g., 1 kb to 5 kb) PCR.

[000117] The sum total of the 5' homology arm and the 3' homology arm in an LTVEC is typically at least 10 kb. As an example, the 5' homology arm can range from about 5 kb to about 150 kb and/or the 3' homology arm can range from about 5 kb to about 150 kb. Each homology arm can be, for example, from about 5 kb to about 10 kb, from about 10 kb to about 20 kb, from about 20 kb to about 30 kb, from about 30 kb to about 40 kb, from about 40 kb to about 50 kb, from about 50 kb to about 60 kb, from about 60 kb to about 70 kb, from about 70 kb to about 80 kb, from about 80 kb to about 90 kb, from about 90 kb to about 100 kb, from about 100 kb to about 110 kb, from about 110 kb to about 120 kb, from about 120 kb to about 130 kb, from about 130 kb to about 140 kb, from about 140 kb to about 150 kb, from about 150 kb to about 160 kb, from about 160 kb to about 170 kb, from about 170 kb to about 180 kb, from about 180 kb to about 190 kb, or from about 190 kb to about 200 kb. The sum total of the 5' and 3' homology arms can be, for example, from about 10 kb to about 20 kb, from about 20 kb to about 30 kb, from about 30 kb to about 40 kb, from about 40 kb to about 50 kb, from about 50 kb to about 60 kb, from about 60 kb to about 70 kb, from about 70 kb to about 80 kb, from

about 80 kb to about 90 kb, from about 90 kb to about 100 kb, from about 100 kb to about 110 kb, from about 110 kb to about 120 kb, from about 120 kb to about 130 kb, from about 130 kb to about 140 kb, from about 140 kb to about 150 kb, from about 150 kb to about 160 kb, from about 160 kb to about 170 kb, from about 170 kb to about 180 kb, from about 180 kb to about 190 kb, from about 190 kb to about 200 kb, from about 200 kb to about 250 kb, from about 250 kb to about 300 kb, from about 300 kb to about 350 kb, or from about 350 kb to about 400 kb. Alternatively, each homology arm can be at least 5 kb, at least 10 kb, at least 15 kb, at least 20 kb, at least 30 kb, at least 40 kb, at least 50 kb, at least 60 kb, at least 70 kb, at least 80 kb, at least 90 kb, at least 100 kb, at least 110 kb, at least 120 kb, at least 130 kb, at least 140 kb, at least 150 kb, at least 160 kb, at least 170 kb, at least 180 kb, at least 190 kb, or at least 200 kb. Likewise, the sum total of the 5' and 3' homology arms can be at least 10 kb, at least 15 kb, at least 20 kb, at least 30 kb, at least 40 kb, at least 50 kb, at least 60 kb, at least 70 kb, at least 80 kb, at least 90 kb, at least 100 kb, at least 110 kb, at least 120 kb, at least 130 kb, at least 140 kb, at least 150 kb, at least 160 kb, at least 170 kb, at least 180 kb, at least 190 kb, at least 200 kb, at least 250 kb, at least 300 kb, at least 350 kb, or at least 400 kb.

[000118] LTVECs can comprise nucleic acid inserts having nucleic acid sequences larger than those typically used by other approaches intended to perform homologous recombination in cells. For example, an LTVEC can comprise a nucleic acid insert ranging from about 1 kb to about 5 kb, from about 5 kb to about 10 kb, from about 10 kb to about 20 kb, from about 20 kb to about 40 kb, from about 40 kb to about 60 kb, from about 60 kb to about 80 kb, from about 80 kb to about 100 kb, from about 100 kb to about 150 kb, from about 150 kb to about 200 kb, from about 200 kb to about 250 kb, from about 250 kb to about 300 kb, from about 300 kb to about 350 kb, from about 350 kb to about 400 kb, from about 400 kb to about 450 kb, from about 450 kb to about 500 kb, or greater. Alternatively, the nucleic acid insert can be at least 1 kb, at least 5 kb, at least 10 kb, at least 20 kb, at least 30 kb, at least 40 kb, at least 60 kb, at least 80 kb, at least 100 kb, at least 150 kb, at least 200 kb, at least 250 kb, at least 300 kb, at least 350 kb, at least 400 kb, at least 450 kb, or at least 500 kb.

B. Construction of Large Targeting Vectors

[000119] Many of the techniques used to construct targeting vectors described herein are standard molecular biology techniques well known to the skilled artisan (*see, e.g.*, Sambrook, J., E. F. Fritsch and T. Maniatis. *Molecular Cloning: A Laboratory Manual*,

Second Edition, Vols. 1, 2, and 3, 1989; Current Protocols in, Molecular Biology, Eds. Ausubel et al., Greene Publ. Assoc., Wiley Interscience, NY). Any methods known in the art for constructing large targeting vectors can be used.

[000120] In one example, the method for constructing a large targeting vector (LTVEC) comprises: (a) obtaining a large genomic DNA clone containing the gene/genes or chromosomal locus/loci of interest; and (b) appending homology boxes 1 and 2 to a modification cassette to generate the LTVEC. Optionally, such methods can further comprise verifying that each LTVEC has been engineered correctly. Optionally, such methods can further comprise purification, preparation, and linearization of LTVEC DNA for introduction into eukaryotic cells. Such methods are further described in US 2004/0018626, US 2013/0309670, and WO 2013/163394, each of which is herein incorporated by reference in its entirety for all purposes.

[000121] Genes or loci of interest can be selected based on specific criteria, such as detailed structural or functional data, or they can be selected in the absence of such detailed information as potential genes or gene fragments become predicted through the efforts of the various genome sequencing projects. It is not necessary to know the complete sequence and gene structure of a gene or locus of interest to produce LTVECs. The only sequence information that is required is approximately 80-100 nucleotides so as to obtain the genomic clone of interest as well as to generate the homology boxes used in making the LTVEC and to make probes for use in quantitative modification-of-allele (MOA) assays.

[000122] Once a gene or locus of interest has been selected, a large genomic clone containing this gene or locus can be obtained. This clone can be obtained in any one of several ways including, but not limited to, screening suitable DNA libraries (e.g., BAC, PAC, YAC, or cosmid) by standard hybridization or PCR techniques, or by any other methods familiar to the skilled artisan.

[000123] Homology boxes mark the sites of bacterial homologous recombination that are used to generate LTVECs from large cloned genomic fragments. Homology boxes are short segments of DNA, generally double-stranded and at least 40 nucleotides in length, that are homologous to regions within the large cloned genomic fragment flanking the region to be modified. The homology boxes are appended to the modification cassette so that following homologous recombination in bacteria, the modification cassette replaces the region to be modified. The technique of creating a

targeting vector using bacterial homologous recombination can be performed in a variety of systems (*see, e.g.*, Yang et al. (1997) *Nat. Biotechnol.* 15:859-865, Muyrers et al. (1999) *Nucleic Acids Res.* 27:1555-1557; Angrand et al. (1999) *Nucleic Acids Res.* 27:e16; Narayanan et al. (1999) *Gene Ther.* 6:442-447; Yu, et al. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97:5978-5983, each of which is herein incorporated by reference in its entirety for all purposes). One example of such a technology is ET cloning (*see, e.g.*, Zhang et al. (1998) *Nat. Genet.* 20:123-128; Narayanan et al. (1999) *Gene Ther.* 6:442-447, each of which is herein incorporated by reference in its entirety for all purposes) and variations of this technology (*see, e.g.*, Yu et al. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97:5978-5983, herein incorporated by reference in its entirety for all purposes). ET refers to the recE and recT proteins that carry out the homologous recombination reaction. RecE is an exonuclease that trims one strand of linear double-stranded DNA 5' to 3', thus leaving behind a linear double-stranded fragment with a 3' single-stranded overhang. This single-stranded overhang is coated by recT protein, which has single-stranded DNA (ssDNA) binding activity. ET cloning is performed using *E. coli* that transiently express the *E. coli* gene products of recE and recT and the bacteriophage lambda (λ) protein λ gam. The λ gam protein protects the donor DNA fragment from degradation by the recBC exonuclease system and it is preferred for efficient ET cloning in recBC⁺ hosts such as the frequently used *E. coli* strain DH10b.

[000124] LTVECs can also be generated by DNA assembly methods, such as *in vitro* DNA assembly methods including Gibson DNA assembly or modifications of Gibson DNA assembly. *See, e.g.*, US 2015/0376628, US 2016/0115486, WO 2015/200334, and US 2010/0035768, each of which is incorporated by reference in its entirety for all purposes.

[000125] Traditional methods of assembling nucleic acids employ time consuming steps of conventional enzymatic digestion with restriction enzymes, cloning of the nucleic acids, and ligating nucleic acids together. These methods are made more difficult when large fragments or vectors are being assembled together. However, the malleable target specificity of nucleases (*e.g.*, guide RNAs and Cas9 nucleases) can be taken advantage of to convert nucleic acids into a form suitable for use in rapid assembly reactions. *See, e.g.*, US 2015/0376628, US 2016/0115486, and WO 2015/200334, each of which is incorporated by reference in its entirety for all purposes.

[000126] Any DNA molecules of interest having overlapping sequences can be assembled by such methods, including DNAs which are naturally occurring, cloned DNA molecules, synthetically generated DNAs, and so forth. Assembling two nucleic acids includes any method of joining strands of two nucleic acids. For example, assembly includes joining digested nucleic acids such that strands from each nucleic acid anneal to the other and extension, in which each strand serves as a template for extension of the other.

[000127] Any *in vitro* or *in vivo* DNA assembly methods or rapid combinatorial methods can be used to assemble the nucleic acids. For example, a first and a second nucleic acid having overlapping ends can be combined with a ligase, exonuclease, DNA polymerase, and nucleotides and incubated at a constant temperature, such as at 50 °C. Specifically, a T5 exonuclease could be used to remove nucleotides from the 5' ends of dsDNA producing complementary overhangs. The complementary single-stranded DNA overhangs can then be annealed, DNA polymerase used for gap filling, and *Taq* DNA ligase used to seal the resulting nicks at 50 °C. Thus, two nucleic acids sharing overlapping end sequences can be joined into a covalently sealed molecule in a one-step isothermal reaction. *See, e.g.,* Gibson et al. (2009) *Nature Methods* 6(5): 343-345, herein incorporated by reference in its entirety for all purposes.

[000128] Site-directed nuclease agents (e.g., guide RNA-directed Cas proteins) allow rapid and efficient combination of nucleic acids by selecting and manipulating the end sequences generated by their endonuclease activity. For example, DNA assembly methods can combine a first polynucleotide with a nuclease agent (e.g., a gRNA-Cas complex) specific for a desired target site and an exonuclease. The target site can be chosen such that when the nuclease cleaves the nucleic acid, the resulting ends created by the cleavage have regions complementary to the ends of a second nucleic acid to be assembled with the first nucleic acid (e.g., overlapping ends). These complementary ends can then be assembled to yield a single assembled nucleic acid. Because the nuclease agent (e.g., gRNA-Cas complex) is specific for an individual target site, the method allows for modification of nucleic acids in a precise site-directed manner. By selecting a nuclease agent (e.g., a gRNA-Cas complex) specific for a target site such that, on cleavage, produces end sequences complementary to those of a second nucleic acid, isothermal assembly can be used to assemble the resulting digested nucleic acid. Thus, by selecting nucleic acids and nuclease agents (e.g., gRNA-Cas complexes) that result in

overlapping end sequences, nucleic acids can be assembled by rapid combinatorial methods to produce the final assembled nucleic acid in a fast and efficient manner. Alternatively, nucleic acids not having complementary ends can be assembled with joiner oligos designed to have complementary ends to each nucleic acid. By using the joiner oligos, two or more nucleic acids can be seamlessly assembled, thereby reducing unnecessary sequences in the resulting assembled nucleic acid.

[000129] Verification that the LTVEC has been engineered correctly can then be undertaken. For example, diagnostic PCR can be used to verify the novel junctions created by the introduction of the donor fragment into the gene or chromosomal locus of interest. Alternatively or additionally, diagnostic restriction enzyme digestion can be done to make sure that only the desired modifications have been introduced into the LTVEC during the bacterial homologous recombination process. Alternatively or additionally, direct sequencing of the LTVEC can be done, particularly the regions spanning the site of the modification to verify the novel junctions created by the introduction of the donor fragment into the gene or chromosomal locus of interest.

[000130] After any purification and further preparation of the LTVEC DNA for introduction into eukaryotic cells, the LTVEC is preferably linearized in a manner that leaves the modified endogenous gene or chromosomal locus DNA flanked with long homology arms. This can be accomplished by linearizing the LTVEC, preferably in the vector backbone, with any suitable restriction enzyme that digests only rarely. Examples of suitable restriction enzymes include NotI, Pad, SfiI, SrfI, SwaI, FseI, and so forth. The choice of restriction enzyme may be determined experimentally (i.e., by testing several different candidate rare cutters) or, if the sequence of the LTVEC is known, by analyzing the sequence and choosing a suitable restriction enzyme based on the analysis.

C. *C9orf72-HRE* nucleic acid constructs

[000131] DNA sequences can be used to prepare LTVECs for knock-in animals (e.g., an *C9ORF72-HRE*). Typically, a polynucleotide molecule (e.g., an insert nucleic acid) comprising a hexanucleotide expansion sequence and/or a selectable marker is inserted into a vector, preferably a DNA vector, in order to replicate the polynucleotide molecule in a suitable host cell.

[000132] A polynucleotide molecule (or insert nucleic acid) comprises a segment of DNA that one desires to integrate into a target locus. In some embodiments, an insert nucleic acid comprises one or more polynucleotides of interest. In some embodiments,

an insert nucleic acid comprises one or more expression cassettes. In some certain embodiments, an expression cassette comprises a polynucleotide of interest, a polynucleotide encoding a selection marker and/or a reporter gene along with, in some certain embodiments, various regulatory components that influence expression. Virtually any polynucleotide of interest may be contained within an insert nucleic acid and thereby integrated at a target genomic locus. Methods disclosed herein, provide for at least 1, 2, 3, 4, 5, 6 or more polynucleotides of interest to be integrated into a targeted *C9ORF72* genomic locus.

[000133] In some embodiments, a polynucleotide of interest contained in an insert nucleic acid encodes a reporter. In some embodiments, a polynucleotide of interest encodes a selectable marker.

[000134] In some embodiments, a polynucleotide of interest is flanked by or comprises site-specific recombination sites (e.g., *loxP*, *Frt*, etc.). In some certain embodiments, site-specific recombination sites flank a DNA segment that encodes a reporter and/or a DNA segment that encodes a selectable marker. Exemplary polynucleotides of interest, including selection markers and reporter genes that can be included within insert nucleic acids are described herein.

[000135] Various methods employed in preparation of plasmids, DNA constructs and/or targeting vectors and transformation of host organisms are known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning: A Laboratory Manual*, 2nd Ed., ed. by Sambrook, J. et al., Cold Spring Harbor Laboratory Press: 1989.

[000136] As described above, exemplary non-human (e.g., rodent) *C9ORF72* nucleic acid and amino acid sequences for use in constructing targeting vectors for knock-in animals are provided in Table 2. Other non-human *C9ORF72* sequences can also be found in the GenBank database. *C9ORF72* targeting vectors as disclosed herein comprise a heterologous hexanucleotide repeat expansion sequence, and optionally one or more sequences encoding a reporter gene and/or a selectable marker, flanked by sequences that are identical or substantially homologous to flanking sequences of a target region (also referred to as "homology arms") for insertion into the genome of a transgenic non-human animal.

[000137] To give but one example, an insertion start point may be set upstream (5'), within, or downstream (3') of a first exon, e.g., a first non-coding exon, to allow an insert

nucleic acid to be operably linked to an endogenous regulatory sequence (e.g., a promoter). A targeting strategy for making a targeted insertion of a heterologous hexanucleotide repeat expansion sequence is provided in Figure 1B and Figure 1C. The drug selection cassette is flanked by *loxP* (LP) recombinase recognition sites that enable Cre-mediated excision of the drug selection cassette. This allows for, among other things, excision of the selection cassette. Thus, prior to phenotypic analysis the drug selection cassette may be removed leaving only the heterologous hexanucleotide repeat expansion sequence, and in some embodiments, one copy of the recombinase recognition site.

[000138] Disclosed herein are nucleic acid constructs useful for the modified mouse *C9orf72* alleles depicted in Figures 1B and 1C, wherein the nucleic acid constructs comprise the sequences set forth in SEQ ID NO:8 and SEQ ID NO:9. SEQ ID NO:8 comprises from 5' to 3': a 5' homology arm (SEQ ID NO:20), a 962 human bp sequence spanning and including part of exon 1a and all of exon 1b of a human *C9orf72* gene (SEQ ID NO:2), a floxed neomycin resistance cassette containing the neomycin resistance gene under the control of a human ubiquitin 1 and/or Em7 promoter (SEQ ID NO:21), and a 3' homology arm (SEQ ID NO:22). SEQ ID NO:9 comprises from 5' to 3': a 5' homology arm (SEQ ID NO:23), a 1261 human bp sequence spanning and including part exon 1a and all of exon 1b of a human *C9orf72* gene (SEQ ID NO:3), a floxed neomycin resistance cassette containing the neomycin resistance gene under the control of a human ubiquitin 1 and/or Em7 promoter (SEQ ID NO:24), and a 3' homology arm (SEQ ID NO:25).

[000139] As described herein, insertion of heterologous hexanucleotide repeat expansion sequence into an endogenous *C9orf72* locus can comprise a replacement of or an insertion/addition to the *C9orf72* locus or a portion thereof with an insert nucleic acid. In some embodiments, an insert nucleic acid comprises a reporter gene. In some certain embodiments, a reporter gene is positioned in operable linkage with an endogenous *C9orf72* promoter. Such a modification allows for the expression of a reporter gene driven by an endogenous *C9orf72* promoter. Alternatively, a reporter gene is not placed in operable linkage with an endogenous *C9orf72* promoter.

[000140] A variety of reporter genes (or detectable moieties) can be used in targeting vectors described herein. Exemplary reporter genes include, for example, β -galactosidase (encoded *lacZ* gene), Green Fluorescent Protein (GFP), enhanced Green

Fluorescent Protein (eGFP), MmGFP, blue fluorescent protein (BFP), enhanced blue fluorescent protein (eBFP), mPlum, mCherry, tdTomato, mStrawberry, J-Red, DsRed, mOrange, mKO, mCitrine, Venus, YPet, yellow fluorescent protein (YFP), enhanced yellow fluorescent protein (eYFP), Emerald, CyPet, cyan fluorescent protein (CFP), Cerulean, T-Sapphire, luciferase, alkaline phosphatase, or a combination thereof. The methods described herein demonstrate the construction of targeting vectors that employ the use of a *lacZ* reporter gene that encodes β -galactosidase, however, persons of skill upon reading this disclosure will understand that non-human animals described herein can be generated in the absence of a reporter gene or with any reporter gene known in the art.

[000141] Where appropriate, the coding region of the genetic material or polynucleotide sequence(s) encoding a reporter polypeptide, in whole or in part, may be modified to include codons that are optimized for expression in the non-human animal (e.g., see U.S. Patent No.'s 5,670,356 and 5,874,304). Codon optimized sequences are synthetic sequences, and preferably encode the identical polypeptide (or a biologically active fragment of a full length polypeptide which has substantially the same activity as the full length polypeptide) encoded by the non-codon optimized parent polynucleotide. In some embodiments, the coding region of the genetic material encoding a reporter polypeptide (e.g. *lacZ*), in whole or in part, may include an altered sequence to optimize codon usage for a particular cell type (e.g., a rodent cell). For example, the codons of the reporter gene to be inserted into the genome of a non-human animal (e.g., a rodent) may be optimized for expression in a cell of the non-human animal. Such a sequence may be described as a codon-optimized sequence.

[000142] Compositions and methods for making non-human animals that comprises an insertion of heterologous hexanucleotide repeat expansion sequence disruption in an endogenous *C9ORF72* locus as described herein are provided, including compositions and methods for making non-human animals that express the heterologous hexanucleotide repeat expansion sequence, e.g., from a *C9ORF72* promoter, e.g., an endogenous mouse promoter, and a *C9ORF72* regulatory sequence, e.g., a human regulatory, e.g., found in exons 1a and 1b. In some embodiments, compositions and methods for making non-human animals that express a heterologous hexanucleotide repeat expansion sequence from an endogenous promoter and an endogenous regulatory sequence are also provided. Methods include inserting a targeting vector, as described

herein, encoding a heterologous hexanucleotide repeat expansion sequence into the genome of a non-human animal so that a non-coding sequence of a *C9ORF72* locus is deleted, in whole or in part. In some embodiments, a non-human animal described herein comprises an endogenous *C9ORF72* locus that comprises a targeting vector as described herein.

[000143] Targeting vectors described herein may be introduced into ES cells and screened for ES clones harboring a disruption in a *C9orf72* locus as described in Frendewey, D., et al., 2010, Methods Enzymol. 476:295-307. A variety of host embryos can be employed in the methods and compositions disclosed herein. For example, the pluripotent and/or totipotent cells having the targeted genetic modification can be introduced into a pre-morula stage embryo (e.g., an 8-cell stage embryo) from a corresponding organism. See, e.g., US 7,576,259, US 7,659,442, US 7,294,754, and US 2008/0078000 A1, all of which are incorporated by reference herein in their entireties. In other cases, the donor ES cells may be implanted into a host embryo at the 2-cell stage, 4-cell stage, 8-cell stage, 16-cell stage, 32-cell stage, or 64-cell stage. The host embryo can also be a blastocyst or can be a pre-blastocyst embryo, a pre-morula stage embryo, a morula stage embryo, an uncompacted morula stage embryo, or a compacted morula stage embryo.

[000144] In some embodiments, the VELOCIMOUSE® method (Poueymirou, W.T. et al., 2007, Nat. Biotechnol. 25:91-99) may be applied to inject positive ES cells into an 8-cell embryo to generate fully ES cell-derived F0 generation heterozygous mice ready for *lacZ* expression profiling or breeding to homozygosity. Exemplary methods for generating non-human animals having a disruption in a *C9orf72* locus are provided in Example 1.

[000145] Methods for generating transgenic non-human animals, including knockouts and knock-ins, are well known in the art (see, e.g., Gene Targeting: A Practical Approach, Joyner, ed., Oxford University Press, Inc. (2000)). For example, generation of transgenic rodents may optionally involve disruption of the genetic loci of an endogenous rodent gene and introduction of a reporter gene into the rodent genome, in some embodiments, at the same location as the endogenous rodent gene.

[000146] A schematic illustration (not to scale) of the genomic organization of a mouse *C9orf72* is provided in Figure 1A (top box). An exemplary targeting strategy for replacement of a non-coding sequence of an endogenous murine *C9orf72* locus with a

heterologous hexanucleotide repeat expansion sequence is also provided in Figure 1A (bottom box). As illustrated, genomic DNA spanning between exon 1 and the ATG start codon, or a portion thereof, is replaced with a heterologous hexanucleotide repeat expansion sequence and a drug selection cassette flanked by site-specific recombinase recognition sites. The targeting vector used in this strategy may optionally include a recombinase-encoding sequence that is operably linked to a promoter that is developmentally regulated such that the recombinase is expressed in undifferentiated cells. Exemplary developmentally regulated promoters that can be included in targeting vectors described herein are provided in Table 3. Additional suitable promoters that can be used in targeting vectors described herein include those described in U.S. Patent No.'s 8,697,851, 8,518,392 and 8,354,389; all of which are herein incorporated by reference). Upon homologous recombination, the non-coding sequence, e.g., approximately 800-1000 bp spanning from exon 1 (or within exon 1) to exon 2, of the endogenous murine *C9orf72* locus is replaced by the sequence contained in the targeting vector. The drug selection cassette may be removed, e.g., optionally in a development-dependent manner such that progeny derived from mice whose germ line cells containing a disruption in a *C9orf72* locus described above will shed the selectable marker from differentiated cells during development (see U.S. Patent No.'s 8,697,851, 8,518,392 and 8,354,389, all of which are herein incorporated by reference).

TABLE 3

Prot promoter (SEQ ID NO:26)

CCAGTAGCAGCACCCACGTCCACCTTCTGTCTAGTAATGTCCAACACCTCCCT
 CAGTCCAAACACTGCTCTGCATCCATGTGGCTCCCATTTATACCTGAAGCACT
 TGATGGGGCCTCAATGTTTTACTAGAGCCACCCCCCTGCAACTCTGAGACC
 CTCTGGATTTGTCTGTCAGTGCCTCACTGGGGCGTTGGATAATTTCTTAAAAG
 GTCAAGTTCCCTCAGCAGCATTCTCTGAGCAGTCTGAAGATGTGTGCTTTTCA
 CAGTTCAAATCCATGTGGCTGTTTACCCACCTGCCTGGCCTTGGGTTATCTA
 TCAGGACCTAGCCTAGAAGCAGGTGTGTGGCACTTAACACCTAAGCTGAGTG
 ACTAACTGAACACTCAAGTGGATGCCATCTTTGTCACTTCTTGACTGTGACAC
 AAGCAACTCCTGATGCCAAAGCCCTGCCCACCCCTCTCATGCCCATATTTGG
 ACATGGTACAGGTCCTCACTGGCCATGGTCTGTGAGGTCCTGGTCCTCTTTGA
 CTTCATAATTCCTAGGGGCCACTAGTATCTATAAGAGGAAGAGGGTGCTGGC
 TCCCAGGCCACAGCCCACAAAATTCCACCTGCTCACAGGTTGGCTGGCTCGA
 CCCAGGTGGTGTCCCCTGCTCTGAGCCAGCTCCCGGCCAAGCCAGCACC

Blimp1 promoter 1kb (SEQ ID NO:27)

TGCCATCATCACAGGATGTCCTTCCTTCTCCAGAAGACAGACTGGGGCTGAA

GGAAAAGCCGGCCAGGCTCAGAACGAGCCCCACTAATTACTGCCTCCAACA
GCTTTTCCACTCACTGCCCCCAGCCCAACATCCCCCTTTTAACTGGGAAGCATT
CCTACTCTCCATTGTACGCACACGCTCGGAAGCCTGGCTGTGGGTTTGGGCA
TGAGAGGCAGGGACAACAAAACCAGTATATATGATTATAACTTTTTCTGTT
TCCCTATTTCCAAATGGTCGAAAGGAGGAAGTTAGGTCTACCTAAGCTGAAT
GTATTCAAGTTAGCAGGAGAAATGAAATCCTATACGTTTAATACTAGAGGAGA
ACCGCCTTAGAATATTTATTTTCATTGGCAATGACTCCAGGACTACACAGCGA
AATTGTATTGCATGTGCTGCCAAAATACTTTAGCTCTTTTCCTTCGAAGTACGT
CGGATCCTGTAATTGAGACACCGAGTTTAGGTGACTAGGGTTTTCTTTTGAG
GAGGAGTCCCCACCCCGCCCCGCTCTGCCGCGACAGGAAGCTAGCGATCCG
GAGGACTTAGAATACAATCGTAGTGTGGGTAAACATGGAGGGCAAGCGCCT
GCAAAGGGGAAGTAAGAAGATTCCCAGTCCTTGTGAAATCCATTTGCAAACA
GAGGAAGCTGCCGCGGGTCGCAGTCGGTGGGGGGAAGCCCTGAACCCACG
CTGCACGGCTGGGCTGGCCAGGTGCGGCCACGCCCCCATCGCGGCGGCTGGT
AGGAGTGAATCAGACCGTCAGTATTGGTAAAGAAGTCTGCGGCAGGGCAGG
GAGGGGGAAGAGTAGTCAGTCGCTCGCTCACTCGCTCGCTCGCACAGACACT
GCTGCAGTGACACTCGGCCCTCCAGTGTGCGGGAGACGCAAGAGCAGCGCG
CAGCACCTGTCCGCCCGGAGCGAGCCCGGCCCGCGGCCGTAGAAAAGGAGG
GACCGCCGAGGTGCGCGTCAGTACTGCTCAGCCCGGCAGGGACGCGGGAGG
ATGTGGACTGGGTGGAC

Blimp1 promoter 2kb (SEQ ID NO:28)

GTGGTGCTGACTCAGCATCGGTTAATAAAACCCTCTGCAGGAGGCTGGATTTT
TTTTGTTTAAATTATCACTTGGACCTTTCTGAGAACTCTTAAGAATTGTTTCATT
GGGTTTTTTTTGTTTTGTTTTGGTTTTGGTTTTTTTTTTTTTTTTTTTTTTTT
TTTGGTTTTTTGGAGACAGGGTTTCTCTGTATATAGCCCTGGCACAAGAGCAA
GCTAACAGCCTGTTTCTTCTTGGTGCTAGCGCCCCCTCTGGCAGAAAAATGAA
ATAACAGGTGGACCTACAACCCCCCCCCCCCCCCCCAGTGATTCTACTCTTG
TCCCCGGTATAAATTTGATTGTTCCGAACCTACATAAATTGTAGAAGGATTTTT
TAGATGCACATATCATTTTTCTGTGATACCTTCCACACACCCCTCCCCCCCCAAA
AAAAATTTTTCTGGGAAAGTTTCTTGAAAGGAAAACAGAAGAACAAGCCTGTC
TTTATGATTGAGTTGGGCTTTTGTGTTTGCTGTGTTTCATTTCTTCCTGTAAACA
AATACTCAAATGTCCACTTCATTGTATGACTAAGTTGGTATCATTAGGTTGGG
TCTGGGTGTGTGAATGTGGGTGTGGATCTGGATGTGGGTGGGTGTGTATGCC
CCGTGTGTTTGAATACTAGAAAAGATAACCATCGTAAACTTTTGGGAGAG
ATGATTTTTTAAAAATGGGGGTGGGGGTGAGGGGAACCTGCGATGAGGCAAG
CAAGATAAGGGGAAGACTTGAGTTTCTGTGATCTAAAAAGTCGCTGTGATGG
GATGCTGGCTATAAATGGGCCCTTAGCAGCATTGTTTCTGTGAATTGGAGGA
TCCCTGCTGAAGGCAAAAGACCATTGAAGGAAGTACCGCATCTGGTTTGTGTT
TGTAATGAGAAGCAGGAATGCAAGGTCCACGCTCTTAATAATAAACAAACA
GGACATTGTATGCCATCATCACAGGATGTCCTTCCTTCTCCAGAAGACAGAC
TGGGGCTGAAGGAAAAGCCGGCCAGGCTCAGAACGAGCCCCACTAATTACT
GCCTCCAACAGCTTTCCACTCACTGCCCCCAGCCCAACATCCCCCTTTTAACT
GGGAAGCATTCTACTCTCATTGTACGCACACGCTCGGAAGCCTGGCTGTG
GGTTTGGGCATGAGAGGCAGGGACAACAAAACCAGTATATATGATTATAAC
TTTTTCTGTTTCCCTATTTCCAAATGGTCGAAAGGAGGAAGTTAGGTCTACC
TAAGCTGAATGTATTAGTTAGCAGGAGAAATGAAATCCTATACGTTTAAATA
CTAGAGGAGAACCGCCTTAGAATATTTATTTTCATTGGCAATGACTCCAGGAC
TACACAGCGAAATTGTATTGCATGTGCTGCCAAAATACTTTAGCTCTTTCTT

CGAAGTACGTCGGATCCTGTAATTGAGACACCGAGTTTAGGTGACTAGGGTT
 TTCTTTTGAGGAGGAGTCCCCACCCCGCCCGCTCTGCCGCGACAGGAAGC
 TAGCGATCCGGAGGACTTAGAATACAATCGTAGTGTGGGTAAACATGGAGG
 GCAAGCGCCTGCAAAGGGAAGTAAGAAGATTCCCAGTCCTTGTTGAAATCCA
 TTTGCAAACAGAGGAAGCTGCCGCGGGTCGCAGTCGGTGGGGGAAGCCCT
 GAACCCACGCTGCACGGCTGGGCTGGCCAGGTGCGGCCACGCCCCATCGC
 GGCGGCTGGTAGGAGTGAATCAGACCGTCAGTATTGGTAAAGAAGTCTGCG
 GCAGGGCAGGGAGGGGGAAGAGTAGTCAGTCGCTCGCTCACTCGCTCGCTC
 GCACAGACACTGCTGCAGTGACACTCGGCCCTCCAGTGTCGCGGAGACGCAA
 GAGCAGCGCGCAGCACCTGTCCGCCCGGAGCGAGCCCGGCCCGCGGCCGTA
 GAAAAGGAGGGACCGCCGAGGTGCGCGTCAGTACTGCTCAGCCCGGCAGGG
 ACGCGGGAGGATGTGGACTGGGTGGAC

D. Introduction of LTVEC into Cells

[000147] LTVEC DNA can be introduced into eukaryotic cells using any standard methodology. “Introducing” includes presenting to the cell the nucleic acid in such a manner that the sequence gains access to the interior of the cell. The introducing can be accomplished by any means.

[000148] The methods provided herein do not depend on a particular method for introducing a nucleic acid into the cell, only that the nucleic acid gains access to the interior of a least one cell. Methods for introducing nucleic acids into various cell types are known in the art and include, for example, stable transfection methods, transient transfection methods, and virus-mediated methods.

[000149] Transfection protocols as well as protocols for introducing nucleic acids into cells may vary. Non-limiting transfection methods include chemical-based transfection methods using liposomes; nanoparticles; calcium phosphate (*see, e.g.,* Graham et al. (1973) *Virology* 52 (2): 456–67, Bacchetti et al. (1977) *Proc. Natl. Acad. Sci. USA* 74 (4): 1590–4, and Kriegler, M (1991). *Transfer and Expression: A Laboratory Manual*. New York: W. H. Freeman and Company. pp. 96–97, each of which is herein incorporated by reference in its entirety); dendrimers; or cationic polymers such as DEAE-dextran or polyethylenimine. Non-chemical methods include electroporation, Sono-poration, and optical transfection. Particle-based transfection includes the use of a gene gun, or magnet-assisted transfection (*see, e.g.,* Bertram (2006) *Current Pharmaceutical Biotechnology* 7, 277–285, herein incorporated by reference in its entirety). Viral methods can also be used for transfection.

[000150] Introduction of nucleic acids 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.

[000151] Introduction of nucleic acids into a cell (e.g., a one-cell stage embryo) can also be accomplished by microinjection. In one-cell stage embryos, microinjection can be into the maternal and/or paternal pronucleus or into the cytoplasm. If the microinjection is into only one pronucleus, the paternal pronucleus is preferable due to its larger size. Microinjection of an mRNA is preferably into the cytoplasm (e.g., to deliver mRNA directly to the translation machinery), while microinjection of a protein or a DNA encoding a Cas protein is preferably 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 nuclease agent protein is injected into the cytoplasm, the protein preferably comprises a nuclear localization signal to ensure delivery to the nucleus/pronucleus. Methods for carrying out microinjection are well known. *See, e.g.,* Nagy et al., 2003, *Manipulating the Mouse Embryo*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press); Meyer et al. (2010) *Proc. Natl. Acad. Sci. U.S.A.* 107:15022-15026, and Meyer et al. (2012) *Proc. Natl. Acad. Sci. USA* 109:9354-9359, each of which is herein incorporated by reference in its entirety.

[000152] Other methods for introducing nucleic acid or proteins into a cell 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.

[000153] The introduction of nucleic acids into the cell can be performed one time or multiple times over a period of time. For example, the introduction can be performed at least two times over a period of time, at least three times over a period of time, at least four times over a period of time, at least five times over a period of time, at least six

times over a period of time, at least seven times over a period of time, at least eight times over a period of time, at least nine times over a period of times, at least ten times over a period of time, at least eleven times, at least twelve times over a period of time, at least thirteen times over a period of time, at least fourteen times over a period of time, at least fifteen times over a period of time, at least sixteen times over a period of time, at least seventeen times over a period of time, at least eighteen times over a period of time, at least nineteen times over a period of time, or at least twenty times over a period of time.

E. Screening for and Identifying Cells with Targeted Genetic Modifications

[000154] Cells in which the LTVEC has been introduced successfully can be selected by exposure to selection agents, depending on whether a selectable marker gene that has been engineered into the LTVEC. As a non-limiting example, if the selectable marker is the neomycin phosphotransferase (neo) gene (*see, e.g., Beck et al. (1982) Gene 19:327-336, herein incorporated by reference in its entirety for all purposes*), then cells that have taken up the LTVEC can be selected in G418-containing media; cells that do not have the LTVEC will die whereas cells that have taken up the LTVEC will survive (*see, e.g., Santerre, et al. (1984) Gene 30:147-156, herein incorporated by reference in its entirety for all purposes*). Such selection markers can, for example, impart resistance to an antibiotic such as G418, hygromycin, blasticidin, neomycin, or puromycin. Such selection markers include neomycin phosphotransferase (neo^r), hygromycin B phosphotransferase (hyg^r), puromycin-N-acetyltransferase (puro^r), and blasticidin S deaminase (bsr^r). In still other embodiments, the selection marker is operably linked to an inducible promoter and the expression of the selection marker is toxic to the cell. Non-limiting examples of such selection markers include xanthine/guanine phosphoribosyl transferase (gpt), hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or herpes simplex virus thymidine kinase (HSV-TK).

[000155] The methods disclosed herein can further comprise identifying a cell having a modified genome. Various methods can be used to identify cells having a targeted genetic modification, such as a deletion or an insertion. Such methods can comprise identifying one cell having the targeted genetic modification at a target locus.

[000156] Conventional assays for screening for targeted modifications, such as long-range PCR, Sanger sequencing, or Southern blotting, link the inserted targeting vector to the targeted locus. For example, for a long-range PCR assay, one primer can recognize a sequence within the inserted DNA while the other recognizes a genomic region of

interest sequence beyond the ends of the targeting vector's homology arms. Because of their large homology arm sizes, however, LTVECs do not permit screening by such conventional assays. To screen LTVEC targeting, modification-of-allele (MOA) assays including loss-of-allele (LOA) and gain-of-allele (GOA) assays can be used (*see, e.g.*, US 2014/0178879 and Frendewey *et al.* (2010) *Methods Enzymol.* 476:295-307, each of which is herein incorporated by reference in its entirety for all purposes). The loss-of-allele (LOA) assay inverts the conventional screening logic and quantifies the number of copies of the native locus to which the mutation was directed. In a correctly targeted cell clone, the LOA assay detects one of the two native alleles (for genes not on the X or Y chromosome), the other allele being disrupted by the targeted modification. The same principle can be applied in reverse as a gain-of-allele (GOA) assay to quantify the copy number of the inserted targeting vector. For example, the combined use of GOA and LOA assays will reveal a correctly targeted heterozygous clone as having lost one copy of the native target gene and gained one copy of the drug resistance gene or other inserted marker.

[000157] As an example, quantitative polymerase chain reaction (qPCR) can be used as the method of allele quantification, but any method that can reliably distinguish the difference between zero, one, and two copies of the target gene or between zero, one, and two copies of the nucleic acid insert can be used to develop a MOA assay. For example, TAQMAN[®] can be used to quantify the number of copies of a DNA template in a genomic DNA sample, especially by comparison to a reference gene (*see, e.g.*, US 6,596,541, herein incorporated by reference in its entirety for all purposes). The reference gene is quantitated in the same genomic DNA as the target gene(s) or locus(loci). Therefore, two TAQMAN[®] amplifications (each with its respective probe) are performed. One TAQMAN[®] probe determines the "Ct" (Threshold Cycle) of the reference gene, while the other probe determines the Ct of the region of the targeted gene(s) or locus(loci) which is replaced by successful targeting (*i.e.*, a LOA assay). The Ct is a quantity that reflects the amount of starting DNA for each of the TAQMAN[®] probes, *i.e.* a less abundant sequence requires more cycles of PCR to reach the threshold cycle. Decreasing by half the number of copies of the template sequence for a TAQMAN[®] reaction will result in an increase of about one Ct unit. TAQMAN[®] reactions in cells where one allele of the target gene(s) or locus(loci) has been replaced by homologous recombination will result in an increase of one Ct for the target

TAQMAN[®] reaction without an increase in the Ct for the reference gene when compared to DNA from non-targeted cells. For a GOA assay, another TAQMAN[®] probe can be used to determine the Ct of the nucleic acid insert that is replacing the targeted gene(s) or locus(loci) by successful targeting.

[000158] The screening step can also comprise arm-specific assays, which are assays used to distinguish between correct targeted insertions of a nucleic acid insert into a target genomic locus from random transgenic insertions of the nucleic acid insert into genomic locations outside of the target genomic locus. Arm-specific assays determine copy numbers of a DNA template in LTVEC homology arms. *See, e.g.,* US 2016/0177339, WO 2016/100819, US 2016/0145646, and WO 2016/081923, each of which is herein incorporated by reference in its entirety for all purposes. It can be useful augment standard LOA and GOA assays to verify correct targeting by LTVECs. For example, LOA and GOA assays alone may not distinguish correctly targeted cell clones from clones in which a deletion of the target genomic locus coincides with random integration of a LTVEC elsewhere in the genome. Because the selection pressure in the targeted cell is based on the selection cassette, random transgenic integration of the LTVEC elsewhere in the genome will generally include the selection cassette and adjacent regions of the LTVEC but may exclude more distal regions of the LTVEC. For example, if a portion of an LTVEC is randomly integrated into the genome, and the LTVEC comprises a nucleic acid insert of around 5 kb or more in length with a selection cassette adjacent to the 3' homology arm, in some cases the 3' homology arm but not the 5' homology arm will be transgenically integrated with the selection cassette. Alternatively, if the selection cassette adjacent to the 5' homology arm, in some cases the 5' homology arm but not the 3' homology arm will be transgenically integrated with the selection cassette. As an example, if LOA and GOA assays are used to assess targeted integration of the LTVEC, and the GOA assay utilizes probes against the selection cassette or any other unique (non-arm) region of the LTVEC, a heterozygous deletion at the target genomic locus combined with a random transgenic integration of the LTVEC will give the same readout as a heterozygous targeted integration of the LTVEC at the target genomic locus. To verify correct targeting by the LTVEC, arm-specific assays can be used in conjunction with LOA and/or GOA assays.

[000159] Other examples of suitable quantitative assays include fluorescence-mediated in situ hybridization (FISH), comparative genomic hybridization, isothermic DNA

amplification, quantitative hybridization to an immobilized probe(s), INVADER[®] Probes, TAQMAN[®] Molecular Beacon probes, or ECLIPSE[™] probe technology (*see, e.g.,* US 2005/0144655, herein incorporated by reference in its entirety for all purposes). **[000160]** Next generation sequencing (NGS) can also be used for screening, particularly in one-cell stage embryos that have been modified. Next-generation sequencing can also be referred to as “NGS” or “massively parallel sequencing” or “high throughput sequencing.” Such NGS can be used as a screening tool in addition to the MOA assays and retention assays to define the exact nature of the targeted genetic modification and to detect mosaicism. Mosaicism refers to the presence of two or more populations of cells with different genotypes in one individual who has developed from a single fertilized egg (i.e., zygote). In the methods disclosed herein, it is not necessary to screen for targeted clones using selection markers. For example, the MOA and NGS assays described herein can be relied on without using selection cassettes.

F. Methods of Making Genetically Modified Non-Human Animals

[000161] Genetically modified non-human animals can be generated employing the various methods disclosed herein. Any convenient method or protocol for producing a genetically modified organism, including the methods described herein, is suitable for producing such a genetically modified non-human animal. Such methods starting with genetically modifying a pluripotent cell such as an embryonic stem (ES) cell generally comprise: (1) modifying the genome of a pluripotent cell that is not a one-cell stage embryo using the methods described herein; (2) identifying or selecting the genetically modified pluripotent cell; (3) introducing the genetically modified pluripotent cell into a host embryo; and (4) implanting and gestating the host embryo comprising the genetically modified pluripotent cell in a surrogate mother. The surrogate mother can then produce F0 generation non-human animals comprising the targeted genetic modification and capable of transmitting the targeted genetic modification through the germline. Animals bearing the genetically modified genomic locus can be identified via a modification of allele (MOA) assay as described herein. The donor cell can be introduced into a host embryo at any stage, such as the blastocyst stage or the pre-morula stage (i.e., the 4 cell stage or the 8 cell stage). Progeny that are capable of transmitting the genetic modification through the germline are generated. The pluripotent cell can be, for example, an ES cell (e.g., a rodent ES cell, a mouse ES cell, or a rat ES cell) as

discussed elsewhere herein. *See, e.g.*, US Patent No. 7,294,754, herein incorporated by reference in its entirety for all purposes.

[000162] Alternatively, such methods starting with genetically modifying a one-cell stage embryo generally comprise: (1) modifying the genome of a one-cell stage embryo using the methods described herein; (2) identifying or selecting the genetically modified embryo; and (3) implanting and gestating the genetically modified embryo in a surrogate mother. The surrogate mother can then produce F0 generation non-human animals comprising the targeted genetic modification and capable of transmitting the targeted genetic modification through the germline. Animals bearing the genetically modified genomic locus can be identified via a modification of allele (MOA) assay as described herein.

[000163] Nuclear transfer techniques can also be used to generate the non-human mammalian animals. Briefly, methods for nuclear transfer can include the steps of: (1) enucleating an oocyte or providing an enucleated oocyte; (2) isolating or providing a donor cell or nucleus to be combined with the enucleated oocyte; (3) inserting the cell or nucleus into the enucleated oocyte to form a reconstituted cell; (4) implanting the reconstituted cell into the womb of a non-human animal to form an embryo; and (5) allowing the embryo to develop. In such methods, oocytes are generally retrieved from deceased animals, although they may be isolated also from either oviducts and/or ovaries of live animals. Oocytes can be matured in a variety of media known to those of ordinary skill in the art prior to enucleation. Enucleation of the oocyte can be performed in a number of manners well known to those of ordinary skill in the art. Insertion of the donor cell or nucleus into the enucleated oocyte to form a reconstituted cell can be by microinjection of a donor cell under the zona pellucida prior to fusion. Fusion may be induced by application of a DC electrical pulse across the contact/fusion plane (electrofusion), by exposure of the cells to fusion-promoting chemicals, such as polyethylene glycol, or by way of an inactivated virus, such as the Sendai virus. A reconstituted cell can be activated by electrical and/or non-electrical means before, during, and/or after fusion of the nuclear donor and recipient oocyte. Activation methods include electric pulses, chemically induced shock, penetration by sperm, increasing levels of divalent cations in the oocyte, and reducing phosphorylation of cellular proteins (as by way of kinase inhibitors) in the oocyte. The activated reconstituted cells, or embryos, can be cultured in medium well known to those of ordinary skill in the art and

then transferred to the womb of an animal. *See, e.g.*, US 2008/0092249, WO 1999/005266, US 2004/0177390, WO 2008/017234, and US Patent No. 7,612,250, each of which is herein incorporated by reference in its entirety for all purposes.

[000164] The various methods provided herein allow for the generation of a genetically modified non-human F0 animal wherein the cells of the genetically modified F0 animal that comprise the targeted genetic modification. It is recognized that depending on the method used to generate the F0 animal, the number of cells within the F0 animal that have the targeted genetic modification will vary. The introduction of the donor ES cells into a pre-morula stage embryo from a corresponding organism (e.g., an 8-cell stage mouse embryo) via, for example, the VELOCIMOUSE[®] method allows for a greater percentage of the cell population of the F0 animal to comprise cells having the targeted genetic modification. *See, e.g.*, US 2014/0331340, US 2008/0078001, US 2008/0028479, US 2006/0085866, and WO 2006/044962, each of which is herein incorporated by reference in its entirety for all purposes. For example, at least 50%, 60%, 65%, 70%, 75%, 85%, 86%, 87%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the cellular contribution of the non-human F0 animal can comprise a cell population having the targeted genetic modification. In addition, at least one or more of the germ cells of the F0 animal can have the targeted genetic modification.

[000165] A genetically modified founder non-human animal can be identified based upon the absence of endogenous genomic *C9ORF72* sequences in its genome that are replaced with the heterologous hexanucleotide repeat expansion sequence and/or the presence (and/or expression) of the heterologous hexanucleotide repeat expansion sequence, drug resistance gene and/or reporter in tissues or cells of the non-human animal. A transgenic founder non-human animal can then be used to breed additional non-human animals carrying the heterologous hexanucleotide repeat expansion sequence thereby creating a series of non-human animals each carrying one or more copies of a *C9ORF72* locus as described herein.

[000166] Transgenic non-human animals may also be produced to contain selected systems that allow for regulated or directed expression of the transgene. Exemplary systems include the Cre/*loxP* recombinase system of bacteriophage P1 (see, e.g., Lakso, M. et al., 1992, Proc. Natl. Acad. Sci. USA 89:6232-6236) and the FLP/Frt recombinase system of *S. cerevisiae* (O'Gorman, S. et al, 1991, Science 251:1351-1355). Such

animals can be provided through the construction of "*double*" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding the heterologous hexanucleotide repeat expansion sequence and the other containing a transgene encoding a recombinase (e.g., a Cre recombinase).

[000167] Although embodiments employing an insertion of a heterologous hexanucleotide repeat expansion sequence in an endogenous *C9ORF72* locus in a mouse are extensively discussed herein, other non-human animals that comprise a disruption in a *C9ORF72* locus are also provided. Such non-human animals include any of those which can be genetically modified to replace a non-coding sequence of a *C9ORF72* locus as disclosed herein, including, e.g., mammals, e.g., mouse, rat, rabbit, pig, bovine (e.g., cow, bull, buffalo), deer, sheep, goat, chicken, cat, dog, ferret, primate (e.g., marmoset, rhesus monkey), etc. For example, for those non-human animals for which suitable genetically modifiable ES cells are not readily available, other methods are employed to make a non-human animal comprising the genetic modification. Such methods include, e.g., modifying a non-ES cell genome (e.g., a fibroblast or an induced pluripotent cell) and employing somatic cell nuclear transfer (SCNT) to transfer the genetically modified genome to a suitable cell, e.g., an enucleated oocyte, and gestating the modified cell (e.g., the modified oocyte) in a non-human animal under suitable conditions to form an embryo.

[000168] Briefly, methods for nuclear transfer include steps of: (1) enucleating an oocyte; (2) isolating a donor cell or nucleus to be combined with the enucleated oocyte; (3) inserting the cell or nucleus into the enucleated oocyte to form a reconstituted cell; (4) implanting the reconstituted cell into the womb of an animal to form an embryo; and (5) allowing the embryo to develop. In such methods oocytes are generally retrieved from deceased animals, although they may be isolated also from either oviducts and/or ovaries of live animals. Oocytes may be matured in a variety of medium known to persons of skill in the art prior to enucleation. Enucleation of the oocyte can be performed in a variety of ways known to persons of skill in the art. Insertion of a donor cell or nucleus into an enucleated oocyte to form a reconstituted cell is typically achieved by microinjection of a donor cell under the zona pellucida prior to fusion. Fusion may be induced by application of a DC electrical pulse across the contact/fusion plane (electrofusion), by exposure of the cells to fusion-promoting chemicals, such as polyethylene glycol, or by way of an inactivated virus, such as the Sendai virus. A

reconstituted cell is typically activated by electrical and/or non-electrical means before, during, and/or after fusion of the nuclear donor and recipient oocyte. Activation methods include electric pulses, chemically induced shock, penetration by sperm, increasing levels of divalent cations in the oocyte, and reducing phosphorylation of cellular proteins (as by way of kinase inhibitors) in the oocyte. The activated reconstituted cells, or embryos, are typically cultured in medium known to persons of skill in the art and then transferred to the womb of an animal. See, e.g., U.S. Patent Application Publication No. 2008-0092249 A1, WO 1999/005266 A2, U.S. Patent Application Publication No. 2004-0177390 A1, WO 2008/017234 A1, and U.S. Patent No. 7,612,250, each of which is herein incorporated by reference.

[000169] Methods for modifying a non-human animal genome (e.g., a pig, cow, rodent, chicken, etc.) include, e.g., employing a zinc finger nuclease (ZFN) or a transcription activator-like effector nuclease (TALEN) to modify a genome to include an insertion of a heterologous hexanucleotide repeat expansion sequence in a *C9ORF72* locus as described herein.

[000170] In some embodiments, a non-human animal described herein is a mammal. In some embodiments, a non-human animal described herein is a small mammal, e.g., of the superfamily Dipodoidea or Muroidea. In some embodiments, a genetically modified animal described herein is a rodent. In some embodiments, a rodent described herein is selected from a mouse, a rat, and a hamster. In some embodiments, a rodent described herein is selected from the superfamily Muroidea. In some embodiments, a genetically modified animal described herein is from a family selected from Calomyscidae (e.g., mouse-like hamsters), Cricetidae (e.g., hamster, New World rats and mice, voles), Muridae (true mice and rats, gerbils, spiny mice, crested rats), Nesomyidae (climbing mice, rock mice, with-tailed rats, Malagasy rats and mice), Platacanthomyidae (e.g., spiny dormice), and Spalacidae (e.g., mole rates, bamboo rats, and zokors). In some certain embodiments, a genetically modified rodent described herein is selected from a true mouse or rat (family Muridae), a gerbil, a spiny mouse, and a crested rat. In some certain embodiments, a genetically modified mouse described herein is from a member of the family Muridae. In some embodiment, a non-human animal described herein is a rodent. In some certain embodiments, a rodent described herein is selected from a mouse and a rat. In some embodiments, a non-human animal described herein is a mouse.

[000171] In some embodiments, a non-human animal described herein is a rodent that is a mouse of a C57BL strain selected from C57BL/A, C57BL/An, C57BL/GrFa, C57BL/KaLwN, C57BL/6, C57BL/6J, C57BL/6ByJ, C57BL/6NJ, C57BL/10, C57BL/10ScSn, C57BL/10Cr, and C57BL/Ola. In some certain embodiments, a mouse described herein is a 129 strain selected from the group consisting of a strain that is 129P1, 129P2, 129P3, 129X1, 129S1 (e.g., 129S1/SV, 129S1/SvIm), 129S2, 129S4, 129S5, 129S9/SvEvH, 129/SvJae, 129S6 (129/SvEvTac), 129S7, 129S8, 129T1, 129T2 (see, e.g., Festing et al., 1999, *Mammalian Genome* 10:836; Auerbach, W. et al., 2000, *Biotechniques* 29(5):1024-1028, 1030, 1032). In some certain embodiments, a genetically modified mouse described herein is a mix of an aforementioned 129 strain and an aforementioned C57BL/6 strain. In some certain embodiments, a mouse described herein is a mix of aforementioned 129 strains, or a mix of aforementioned BL/6 strains. In some certain embodiments, a 129 strain of the mix as described herein is a 129S6 (129/SvEvTac) strain. In some embodiments, a mouse described herein is a BALB strain, e.g., BALB/c strain. In some embodiments, a mouse described herein is a mix of a BALB strain and another aforementioned strain.

[000172] In some embodiments, a non-human animal described herein is a rat. In some certain embodiments, a rat described herein is selected from a Wistar rat, an LEA strain, a Sprague Dawley strain, a Fischer strain, F344, F6, and Dark Agouti. In some certain embodiments, a rat strain as described herein is a mix of two or more strains selected from the group consisting of Wistar, LEA, Sprague Dawley, Fischer, F344, F6, and Dark Agouti.

[000173] A rat pluripotent and/or totipotent cell 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. Rat pluripotent and/or totipotent cells can also be obtained from a strain derived from a mix of two or more strains recited above. For example, the rat pluripotent and/or totipotent cell 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. An example of a rat ES cell line from an ACI rat is an ACI.G1 rat ES cell. The Dark Agouti (DA) rat strain is characterized as having an agouti coat and an *RT1^{av1}* haplotype. Such rats are available from a variety of sources including Charles River and

Harlan Laboratories. Examples of a rat ES cell line from a DA rat are the DA.2B rat ES cell line and the DA.2C rat ES cell line. In some cases, the rat pluripotent and/or totipotent cells are from an inbred rat strain. See, e.g., U.S. 2014/0235933 A1, filed on February 20, 2014, and herein incorporated by reference in its entirety.

[000174] Non-human animals are provided that comprise an insertion of a heterologous hexanucleotide repeat expansion sequence in an endogenous *C9ORF72* locus. In some embodiments, insertion of a heterologous hexanucleotide repeat expansion sequence is not pathogenic. In some embodiments, insertion of a heterologous hexanucleotide repeat expansion sequence results in one or more phenotypes as described herein, e.g., a phenotype associated with ALS and/or FTD. Insertion of a heterologous hexanucleotide repeat expansion sequence may be measured directly, e.g., by determining the approximate number of instance, e.g., repeats, of the hexanucleotide sequence set forth as SEQ ID NO:1 in the heterologous hexanucleotide repeat expansion sequence, e.g., by Southern Blot or polymerase chain reaction genotyping reactions.

Methods Employing Non-human Animals Having An Insertion of a Heterologous Hexanucleotide Repeat Expansion Sequence in an Endogenous C9ORF72 Locus

[000175] Non-human animals as described herein provide improved animal models for neurodegenerative diseases, disorders and conditions. In particular, non-human animals as described herein provide improved animal models that translate to human diseases such as, for example, ALS and/or FTD, characterized by upper motor neuron symptoms and/or non-motor neuron loss.

[000176] Non-human animals as described herein provide an improved *in vivo* system and source of biological materials (e.g., cells) that comprise and/or express the inserted pathogenic heterologous hexanucleotide repeat expansion sequence in an endogenous *C9ORF72* locus that are useful for a variety of assays. In various embodiments, non-human animals described herein may be used to develop therapeutics that treat, prevent and/or inhibit one or more symptoms associated with expression and/or activity of a pathogenic heterologous hexanucleotide repeat expansion. In various embodiments, non-human animals described herein are used to identify, screen and/or develop candidate therapeutics (e.g., antibodies, gRNAs (comprising CRISPR RNA and tracrRNA) and siRNA, etc.) that bind a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation.

In various embodiments, non-human animals described herein are used to screen and develop candidate therapeutics (e.g., antibodies, gRNAs (comprising CRISPR RNA and tracrRNA) and siRNA, etc.) that block activity of a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation. In various embodiments, non-human animals described herein are used to determine the binding profile of antagonists and/or agonists of a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof (transcript), e.g., resulting from RAN translation, of a non-human animal as described herein. In some embodiments, non-human animals described herein are used to determine the epitope or epitopes of one or more candidate therapeutic antibodies that bind a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation.

[000177] In various embodiments, non-human animals described herein are used to determine the pharmacokinetic profiles of a drug targeting a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation. In various embodiments, one or more non-human animals described herein and one or more control or reference non-human animals are each exposed to one or more candidate drugs targeting a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation, at various doses (e.g., 0.1 mg/kg, 0.2 mg/kg, 0.3 mg/kg, 0.4 mg/kg, 0.5 mg/kg, 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 7.5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 40 mg/kg, or 50 mg/kg or more). Candidate therapeutic antibodies may be dosed via any desired route of administration including parenteral and non-parenteral routes of administration. Parenteral routes include, e.g., intravenous, intraarterial, intraportal, intramuscular, subcutaneous, intraperitoneal, intraspinal, intrathecal, intracerebroventricular, intracranial, intrapleural or other routes of injection. Non-parenteral routes include, e.g., oral, nasal, transdermal, pulmonary, rectal, buccal, vaginal, ocular. Administration may also be by continuous infusion, local administration, sustained release from implants (gels, membranes or the like), and/or intravenous injection. Blood is isolated from non-human animals (humanized and control) at various time points (e.g., 0 hr, 6 hr, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, or up to 30 or more days). Various assays may be performed to determine the pharmacokinetic profiles of administered drugs

targeting a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation, using samples obtained from non-human animals as described herein including, but not limited to, total IgG, anti-therapeutic antibody response, agglutination, etc.

[000178] In various embodiments, non-human animals as described herein are used to measure the therapeutic effect of blocking, modulating, and/or inhibiting activity of a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from Repeat-associated non-AUG (RAN) translation, and the effect on gene expression as a result of cellular changes. In various embodiments, a non-human animal as described herein or cells isolated therefrom are exposed to a drug targeting a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation, of the non-human animal and, after a subsequent period of time, analyzed for effects on processes (or interactions) dependent on the pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation, for example, formation of RNA foci, protein aggregation from RAN translation products, motor neuron and/or non-motor neuron function, etc.

[000179] Cells from non-human animals as described herein can be isolated and used on an ad hoc basis, or can be maintained in culture for many generations. In various embodiments, cells from a non-human animal as described herein are immortalized (e.g., via use of a virus) and maintained in culture indefinitely (e.g., in serial cultures).

[000180] Non-human animals described herein provide an *in vivo* system for assessing the pharmacokinetic properties and/or efficacy of a drug (e.g., a drug targeting a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation). In various embodiments, a drug may be delivered or administered to one or more non-human animals, cells derived therefrom or having the same genetic modifications thereof, as described herein, followed by monitoring of, or performing one or more assays on, the non-human animals (or cells isolated therefrom) to determine the effect of the drug on the non-human animal. Pharmacokinetic properties include, but are not limited to, how an animal processes the drug into various metabolites (or detection of the presence or absence of one or more drug metabolites, including, but not limited to, toxic metabolites), drug half-life, circulating levels of drug after administration (e.g., serum concentration of drug), anti-

drug response (e.g., anti-drug antibodies), drug absorption and distribution, route of administration, routes of excretion and/or clearance of the drug. In some embodiments, pharmacokinetic and pharmacodynamic properties of drugs are monitored in or through the use of non-human animals described herein.

[000181] In some embodiments, performing an assay includes determining the effect on the phenotype and/or genotype of the non-human animal to which the drug is administered. In some embodiments, performing an assay includes determining lot-to-lot variability for a drug. In some embodiments, performing an assay includes determining the differences between the effects of a drug administered to a non-human animal described herein and a reference non-human animal. In various embodiments, reference non-human animals may have a modification as described herein, e.g., insertion of a non-pathogenic heterologous hexanucleotide repeat expansion sequence or no modification (i.e., a wild type non-human animal).

[000182] Exemplary parameters that may be measured in non-human animals (or in and/or using cells isolated therefrom) for assessing the pharmacokinetic properties of a drug include, but are not limited to, agglutination, autophagy, cell division, cell death, complement-mediated hemolysis, DNA integrity, drug-specific antibody titer, drug metabolism, gene expression arrays, metabolic activity, mitochondrial activity, oxidative stress, phagocytosis, protein biosynthesis, protein degradation, protein secretion, stress response, target tissue drug concentration, non-target tissue drug concentration, transcriptional activity, and the like. In various embodiments, non-human animals described herein are used to determine a pharmaceutically effective dose of a drug (e.g., a drug targeting a pathogenic heterologous hexanucleotide repeat expansion sequence or expression product thereof, e.g., resulting from RAN translation).

EXAMPLES

[000183] The following examples are provided so as to describe to those of ordinary skill in the art how to make and use methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Unless indicated otherwise, temperature is indicated in Celsius, and pressure is at or near atmospheric.

Example 1. Insertion of a heterologous hexanucleotide repeat expansion sequence in a non-human embryonic stem cell at an endogenous non-human C9ORF72 locus

[000184] This example illustrates a targeted insertion of a heterologous hexanucleotide repeat expansion sequence into an embryonic stem cell at a *C9orf72* locus of a non-human animal, particularly rodent. In particular, this example specifically describes the replacement of a part of a non-coding sequence of a mouse *C9orf72* locus with a heterologous human hexanucleotide repeat expansion sequence placed in operable linkage with an mouse *C9orf72* promoter and/or human regulatory elements, e.g., those that may be found in exons 1a and/or 1b of the human *C9orf72* gene. The *C9orf72-HRE* targeting vector for inserting a heterologous hexanucleotide repeat expansion sequence in an endogenous mouse *C9orf72* locus was made as previously described (see, e.g., U.S. Patent No. 6,586,251; Valenzuela et al., 2003, *Nature Biotech.* 21(6):652-659; and Adams, N.C. and N.W. Gale, in *Mammalian and Avian Transgenesis—New Approaches*, ed. Lois, S.P.a.C., Springer Verlag, Berlin Heidelberg, 2006). The resulting modified *C9orf72* locus is depicted in Figure 1A, bottom box.

[000185] Briefly, targeting vectors comprising a sequence set forth in SEQ ID NO:8 or SEQ ID NO:9 were generated using bacterial artificial chromosome (BAC) clones from a mouse RP23 BAC library (Adams, D.J. et al., 2005, *Genomics* 86:753-758) and introduced into F1 hybrid (129S6SvEvTac/C57BL6NTac) embryonic stem (ES) cells followed by culturing in selection medium containing G418. Drug-resistant colonies were picked 10 days after electroporation and screened for correct targeting as previously described (Valenzuela et al., *supra*; Friendewey, D. et al., 2010, *Methods Enzymol.* 476:295-307). Targeted ES cells are analyzed to determine the approximate size of hexanucleotide repeat expansions present in targeted mouse ES cell clones by Southern blot analysis and/or amplification of the *C9orf72-HRE* locus.

[000186] Specifically, Southern blot analysis was performed to determine the approximate size of hexanucleotide repeat expansions present in targeted C9ORF72 transgenic mouse ES cells. Genomic DNA was extracted from targeted mouse ES clones grown in single wells of a gelatin-coated 96 well plate. Once ES cell clones reached 100% confluence, cells were washed twice with 1xPBS and lysed overnight at 37°C in 50 uL of lysis buffer (1M Tris pH 8.5, 0.5M EDTA, 20% SDS, 5M NaCl, and 1 mg/mL proteinase K). DNA was precipitated with the addition of 125 uL of ice cold 200 proof ethanol to each well, followed by an overnight incubation at 4°C. Precipitated DNA was washed twice with 70% ethanol, air dried, and resuspended in 30 uL 0.5x TE pH 8.0.

[000187] Extracted genomic DNAs (gDNA) were digested with HindII and ScaI overnight at 37°C and size separated on a 1% agarose gel. Post-electrophoresis agarose gels were denatured (1M NaCl, 5% NaOH) and neutralized (1.5M NaCl, 0.5M Tris pH 7.5). Digested gDNAs were then transferred to Hybond-N membranes (Amersham) via overnight capillary transfer.

[000188] A probe corresponding to a 252 bp XmaI fragment (see Figure 2A) contained within the humanized targeting vector

(5'-

CCGGGGCGGGGCTGCGGTTGCGGTGCCTGCGCCCGCGGCGGCGGAGGCGCA
GGCGGTGGCGAGTGGGTGAGTGAGGAGGCGGCATCCTGGCGGGTGGCTGTT
TGGGGTTTCGGCTGCCGGAAGAGGCGCGGGTAGAAGCGGGGGCTCTCCTCA
GAGCTCGACGCATTTTTACTTTCCCTCTCATTTCTCTGACCGAAGCTGGGTGT
CGGGCTTTCGCCTCTAGCGACTGGTGGAAATGCCTGCATCCGGGCC-3'; SEQ
ID NO:29)

was labeled with ³²P using Prime-It II Random Primer Labeling Kit (Agilent).

Denatured probe was diluted in ExpressHyb Hybridization Solution (Takara) and incubated with prepared membranes overnight at 65°C. Autoradiography film was exposed to the probed blots for 72 hours.

[000189] As shown in **Figure 2B**, an ES cell clone (8027 A-C4) comprising an inserted non-pathogenic heterologous hexanucleotide repeat expansion sequence comprising three repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 is obtained after introduction of the *C9orf72-HRE-3* targeting vector comprising a sequence set forth as SEQ ID NO:4 and excision of the drug resistance cassette. After introduction of the *C9orf72-HRE-100* targeting vector comprising a sequence set forth as SEQ ID NO:6, at least two ES cell clones (8029 A-A3 and 8029 A-A6) comprising an inserted heterologous hexanucleotide repeat expansion sequence, which is a variant of the sequence set forth as SEQ ID NO:7 and comprises about 92 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, were obtained. Also at least two ES cell clones (8029 B-A6 and 8029 B-A4) comprising an inserted heterologous hexanucleotide repeat expansion sequence, which is a variant of the sequence set forth as SEQ ID NO:7 and comprises about 30 repeats of the hexanucleotide sequence set forth

as SEQ ID NO:1, were obtained after introduction of the *C9orf72-HRE-100* targeting vector (8028) and excision of the drug resistance cassette.

[000190] AmpliX PCR/CE C9ORF72 Kit (Asuragen) was also used according to manufacturer's instructions to confirm the number of instances of the hexanucleotide sequence set forth as SEQ ID NO:1 in heterologous hexanucleotide repeat expansion sequence inserted into the endogenous *C9orf72* ES cell clones described. Purified mESC genomic total DNA from a 3X repeat clone (8027 A-C4), 2 individual 92x repeat clones (8029 A-A3, 8029 A-A6), and 2 individual 30x repeat clones (8029 B-A4, 8029 B-A10) was used as input DNA. F1H4 mESC genomic total DNA served as negative control, and Coriell Cell Repository purified human blood cell genomic DNA from patients with known *C9ORF72* hexanucleotide expanded repeat alleles (samples ND11836 (HRE genotype: 8/expanded), ND14442 (2/expanded), ND6769 (13/44)) served as positive controls (Coriell Institute for Medical Research). PCR using the primers in Table 4 was performed on a ABI 9700 thermal cycler (Thermo Fisher). Amplicons were sized by capillary electrophoresis on a ABI 3500xL GeneScan using POP-7 polymer (Thermo Fisher) and NuSieve agarose gels (Lonza). 2-log DNA ladder (New England BioLabs) molecular weight marker was loaded on agarose gels for comparison, and bands were visualized with SYBR Gold Nucleic Acid Stain (Thermo Fisher).

Table 4

Primer name	Sequence (SEQ ID NO:)
2-Primer Fwd	TGCGCCTCCGCCGCCGCGGGCGCAGGCACCGCAACCGCA (SEQ ID NO:30)
2-Primer Rev	CGCAGCCTGTAGCAAGCTCTGGAAGTCAGGAGTCG (SEQ ID NO:31)
3-Primer Fwd	ATGCAGGCAATTCCACCAGTCGCTAGAGGCGAAAGC (SEQ ID NO:32)
3-Primer Rev	TAACCAGAAGAAAACAAGGAGGGAAACAACCGCAGCCTGT (SEQ ID NO:33)

[000191] **Figure 2B** confirms the presence of 3 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 within the heterologous hexanucleotide repeat expansion

sequence inserted into the endogenous *C9orf72* locus of mouse ES cell clone 8027 A-C4, about 30 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 within the heterologous hexanucleotide repeat expansion sequences inserted into the endogenous *C9orf72* locus of mouse ES cell clones 8029 B-A9 and 8029 B-A10, and about 92 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 within the heterologous hexanucleotide repeat expansion sequences at the endogenous *C9orf72* locus of mouse ES cell clones 8029 A-A3 and 8029 A-A6.

Example 2: Generation of embryonic stem cell derived motor neurons and non-human animals comprising a heterologous hexanucleotide repeat expansion sequence at an endogenous mouse *C9ORF72* locus

[000192] Embryonic Stem Cell Derived Motor Neurons

[000193] Parental embryonic stem cells (ESCs) homozygous for a wildtype *C9orf72* locus (control) or heterozygous for a *C9orf72* locus genetically modified with about 3 repeats (*C9orf72HRE₃^{+/-}*), 30 repeats (*C9orf72HRE₃₀^{+/-}*), or 92 repeats (*C9orf72HRE₉₂^{+/-}*) of the hexanucleotide sequence set forth as SEQ ID NO:1 were cultured in embryonic stem cell medium (ESM; DMEM + 15% Fetal bovine serum + Penicillin/Streptomycin + Glutamine + Non-essential amino acids + nucleosides + β -mercaptoethanol + Sodium pyruvate + LIF) for 2 days, during which the medium was changed daily. ES medium was replaced with 7 ml of ADFNK medium (Advanced DMEM/F12 + Neurobasal medium + 10% Knockout serum + Penicillin/Streptomycin + Glutamine + β -mercaptoethanol) 1 hour before trypsinization. ADFNK medium was aspirated and ESC were trypsinized with 0.05% trypsin-EDTA. Pelleted cells were resuspended in 12ml of ADFNK and grown for two days in suspension. Cells were cultured for a further 4 days in ADFNK supplemented with retinoic acid (RA) and smoothened agonist to obtain motor neurons (ESMNs). Dissociated motor neurons were plated and matured in embryonic stem cell-derived motor neuron medium (ESMN; Neurobasal medium + 2% Horse serum + B27 + Glutamine + Penicillin/Streptomycin + β -mercaptoethanol + 10ng/ml GDNF, BDNF, CNTF).

[000194] Non-human animals

[000195] The VELOCIMOUSE® method (DeChiara, T.M. et al., 2010, Methods Enzymol. 476:285-294; DeChiara, T.M., 2009, Methods Mol. Biol. 530:311-324;

Poueymirou et al., 2007, Nat. Biotechnol. 25:91-99) was used, in which targeted ES cells were injected into uncompacted 8-cell stage Swiss Webster embryos, to produce healthy fully ES cell-derived F0 generation mice heterozygous for the *C9orf72-HRE* (3x or 100x) insertion. F0 generation heterozygous male were crossed with C57Bl6/NTac females to generate F1 heterozygotes that were intercrossed to produce F2 generation *C9orf72-HRE*^{+/+}, *C9orf72-HRE*^{+/-} and wild type mice for molecular and phenotypic analyses.

Example 3. Analysis of motor neurons or brain tissues having a heterologous hexanucleotide repeat expansion sequence in an endogenous *C9orf72* locus

[000196] Recently, Liu et al. (2017) *Cell Chem. Biol.* 24:141-148 used quantitative polymerase chain reaction (qPCR) and digital droplet polymerase chain reaction (ddPCR) to quantify the copy number of sense and antisense RNA transcripts from the *C9orf72* locus expressed by human fibroblast cell lines, or human astrocytes and motor neurons derived from induced pluripotent stem cells (iPSCs), isolated from patients suffering from ALS. Liu et al. (2017), *supra*, detected significantly higher numbers of sense intronic, antisense, and sense *C9orf72* transcripts in patient-derived fibroblasts compared to fibroblasts derived from healthy patients. On average, three to four copies of *C9orf72* intronic and antisense transcripts, and about 15-20 copies of *C9orf72* sense mRNA transcripts, were detected per patient-derived fibroblast. Liu et al. (2017) *supra*. Liu et al. (2017) et al., *supra*, show that, in contrast, one or less intronic and antisense transcripts, and 5-10 copies of *C9orf72* sense mRNA transcripts, were detected in non-disease fibroblast cell lines. Similarly to the fibroblasts, expression of intronic, antisense, and sense *C9orf72* transcripts was higher in patient-derived astrocytes and neuronal cells compared to healthy-control derived astrocytes and neuronal cells. Liu et al. (2017) et al., *supra*. By calculating the percentage of cells that contain RNA foci, the average number of foci per cell, and the distribution of different numbers of foci among cells, and in determining the number of *C9orf72* transcripts in disease- or healthy-derived cells, Liu et al. (2017) et al., *supra*, suggested that the each foci seen in disease-derived cell is a single mutant *C9orf72* intronic or antisense transcript, and further, that small numbers of RNA molecules may have a sizable impact on disease.

[000197] In this example, the stability of the size of the hexanucleotide repeat in a breeding colony was confirmed in F2 animals using AmplideX PCR/CE *C9ORF72* Kit

(Asuragen) as described above (data not shown). Additionally, RNA transcripts in mouse embryonic stem cell derived motor neurons (ESMNs), brain tissues, and parental embryonic stem cells comprising a wildtype *C9orf72* locus (control) or a genetically modified *C9orf72* locus that comprises three, thirty, or ninety-two repeats of the hexanucleotide sequence set forth in SEQ ID NO:1 were examined. RNA foci and dipeptide repeat protein levels were also evaluated in ESMNs derived from parental embryonic stem cells comprising a wildtype *C9orf72* locus (control) or a genetically modified *C9orf72* locus that comprises three, thirty, or ninety-two repeats of the hexanucleotide sequence set forth in SEQ ID NO:1.

[000198] *Materials and Methods*

[000199] Quantitative polymerase chain reaction

[000200] Total RNA from each sample was extracted and reverse transcribed using primers that flank various regions, and probes that detect those regions of the modified *C9orf72-HRE* locus. Detectable regions include those that span the junction of mouse and human sequences, only human sequences, or only mouse sequences. QPCR of GAPDH or β 2-microglobulin was performed using probes and primers of readily available kits.

[000201] Specifically, RNA was isolated from embryonic stem cell-derived motor neurons (ESMN), parental embryonic stem (ES) cells, or total brains isolated from mice comprising a wildtype (WT) *C9orf72* locus (control) or a genetically modified *C9orf72* locus comprising 3, 30 or 92 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1

[000202] Total RNA was isolated using Direct-zol RNA Miniprep plus kit according to the manufacturer's protocol (Zymo Research). About 1 μ g total RNA was treated with DNase I (ThermoFisher) at 25°C for 15 min. EDTA was added and the mixture incubated at 65°C for 10 min. Reverse transcription (RT) reactions were performed with a Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (ThermoFisher). After DNase I treatment, 10 μ L of RT mixture containing RT buffer, random hexamer primers, dNTPs, Maxima H minus enzyme mix was added to make final volume of 20 μ L. The RT reaction mixture was incubated at 25°C for 10 min, then at 50°C for 15 min, and then 5 min at 85°C to inactivate the enzyme. The cDNA mix was diluted with water to make 100 μ L final volume.

[000203] After reverse transcription, the PCR reaction solution was reconstituted to a final volume of 8 μ L containing 3 μ L cDNA and 5 μ L of PCR mixture, probe and gene specific primers. Unless otherwise noted final primer and probe concentrations were 0.5 μ M and 0.25 μ M respectively. qPCR was performed on a ViiA™ 7 Real-Time PCR Detection System (ThermoFisher). PCR reactions were done in quadruplicates at 95 °C 10 min and 95 °C 3 s, 60 °C 30 s for 45 cycles in an optical 384-well plate. The sequences of the primers and probes and SEQ ID NO used in each analysis (A, B, F, G, H) are provided in Table 5.

Table 5

Analysis A	
Forward Primer	CATCCCAATTGCCCTTTCC (SEQ ID NO:66)
Reverse Primer	CCCACACCTGCTCTTGCTAGA (SEQ ID NO:67)
Probe	TCTAGGTGGAAAGTGGG (SEQ ID NO:68)
Analysis B	
Forward Primer	GAGCAGGTGTGGGTTTAGGA (SEQ ID NO:69)
Reverse Primer	CCAGGTCTCACTGCATTCCA (SEQ ID NO:70)
Probe	ATTGCAAGCGTTCGGATAATGTGAGA (SEQ ID NO:71)
Analysis D	
Forward Primer	GCTGTACGAAGGCTTTCTTC (SEQ ID NO:72)
Reverse Primer	GCACTGCTGCCAACTACAAC (SEQ ID NO:73)
Probe	TCAATGCCATCAGCTCACACCTGC (SEQ ID NO:74)
Analysis G	
Forward Primer	AAGAGGCGCGGGTAGAA (SEQ ID NO:75)
Reverse Primer	CAGCTTCGGTCAGAGAAATGAG (SEQ ID NO:76)
Probe	CTCTCCTCAGAGCTCGACGCATTT (SEQ ID NO:77)
Analysis H	
Forward Primer	CTGCACAATTTTCAGCCCAAG (SEQ ID NO:78)
Reverse Primer	CAGGTCATGTCCCACAGAAT (SEQ ID NO:79)
Probe	CATATGAGGGCAGCAATGCAAGTC (SEQ ID NO:80)

[000204] Western Blot Analysis

[000205] Differentiated embryoid bodies (EBs) were collected and homogenized in SDS sample buffer (2% SDS, 10% glycerol, 5% β mercaptoethanol, 60 mM TrisHCl, pH 6.8, bromophenol blue). Protein extracts were quantified using the RC DC protein assay (BioRad). Extracts (10 μ g) were run on a 4-20 % SDS-PAGE gel (ThermoFisher) and transferred onto nitrocellulose membrane using an iBLOT transfer unit (ThermoFisher). Immunoblots were probed with primary antibodies against C9orf72 and GAPDH (Millipore). Bound antibody was detected by incubation with secondary antibodies conjugated to horseradish peroxidase (Abcam) followed by chemiluminescence using a SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Signal was detected by autoradiography using Full Speed Blue sensitive medical X-Ray film (Ewen Parker XRay Corporation). Relative protein levels were calculated using ImageJ.

[000206] Fluorescent in situ hybridization (FISH) and immunofluorescence (IF) for the detection of RNA and translation products

[000207] Fluorescent *in situ* hybridization (FISH) and immunofluorescence were respectively used to determine the location of RNA transcribed from the hexanucleotide repeat sequence set forth as SEQ ID NO:1, as well as dipeptide repeat proteins translated therefrom, in embryonic stem cell-derived motor neurons (ESMNs) generated as described in Example 3. Briefly, ESMNs were grown in four-well chamber slide (Lab-Tek II chamber slide system, ThermoFisher Scientific) and fixed with 4% PFA (Electron Microscopy Sciences) in PBS. Cells were then permeabilized with diethyl pyrocarbonate (DEPC) PBS/0.2% Triton X-100 (Fisher Scientific, catalog #BP151) and washed with DEPC-PBS, blocked and stained with LNA or DNA oligonucleotides for the detection of RNA transcription products, or anti-polyGA antibody for the detection of RAN translation products, as described below. After staining, slides were subsequently incubated with an appropriate fluorescent dye, mounted with Fluoromount G (Southern Biotech) and visualized using confocal microscopy.

[000208] Detection of sense or antisense RNA transcription products

[000209] Slides were pre-hybridized with buffer consisting of 50% formamide (IBI Scientific, catalog #IB72020), DEPC 2 $\times$ SSC [300 mM sodium chloride, 30mM sodium citrate (pH 7.0)], 10% (w/v) dextran sulfate (Sigma-Aldrich, catalog #D8960), and DEPC 50 mM sodium phosphate (pH 7.0) for 30 min at 66°C (for LNA probes) or 55°C (for DNA probes). The hybridization buffer was then drained off, and 400 μ l of 40 nM LNA probe mix or 200ng/ml of DNA probe mix in hybridization buffer was added to

each of the slides and incubated in the dark for 3 hours at 66°C (for LNA probes) or 55°C (for DNA probes). Slides incubated with LNA probes were rinsed once in DEPC 2× SSC/0.1% Tween 20 (Fisher Scientific, catalog no. BP337) at room temperature and in DEPC 0.1× SSC three times at 65°C. Slides incubated with DNA probes washed three times with 40% formamide in 2× SSC and briefly washed one time in PBS. Slides were subsequently incubated with 1 µg/mL DAPI (Molecular Probes Inc.).

[000210] The sequences and SEQ ID NOs: of the LNA and DNA oligonucleotide probes used in this example, as well as the hybridization conditions of the probes, are provided in Table 6 below.

Table 6

Probe	Sequence (SEQ ID NO:)	Hybridization method
LNA sense G ₄ C ₂ RNA	TYE563-CCCCGGCCCCGGCCCC (SEQ ID NO:81)	66°C hybridization and washes in 0.1 X SSC
LNA antisense G ₄ C ₂ RNA	TYE563-GGGGCCGGGGCCGGGG GGCCCC (SEQ ID NO:82)	66°C hybridization and washes in 0.1 X SSC
DNA sense G ₄ C ₂ RNA	CCCCGGCCCCGGCCCCGG—Cy3 (SEQ ID NO:83)	55°C hybridization and washes in 2 X SSC
DNA antisense G ₄ C ₂ RNA	GGGGCCGGGGCCGGGGC—Cy3 (SEQ ID NO:84)	55°C hybridization and washes in 2 X SSC

[000211] Detection of Dipeptide Repeat Protein Products

[000212] After permeabilization, slides were blocked with 5% normal donkey serum diluted in Tris buffered saline (pH 7.4) with 0.2% Triton X100 (TBS-T). Slides were incubated overnight at 4°C with primary antibodies against poly-GA (Millipore) diluted in TBS-T with 5% normal donkey. After washing 3 time with TBS-T, slides were incubated with species specific secondary antibodies coupled to Alexa 488 or 555 (1:1000 in TBS-T, ThermoFisher) and DAPI (1µg/ml) (Molecular Probes Inc.) for 1hr at room temperature. After washing 3 times with TBS-T slides were mounted with Fluoromount G (Southern Biotech) and visualized using confocal microscopy.

[000213] Results

[000214] As shown in **Figures 4, 5 and 6**, ESMNs, total brain and neuronal tissues from mice comprising the hexanucleotide repeat expansion sequence set forth as SEQ ID

NO:1 at the *C9orf72* locus showed increased expression of the *C9orf72* mRNA transcripts. Such increase appears to be correlated with the number of the hexanucleotide repeats present between exons 1a and 1b of the *C9orf72* locus. **Figure 6** also shows that, similar to the neuronal tissues isolated from the mice comprising 3 or 92 repeats of the heterologous hexanucleotide sequence set forth as SEQ ID NO:1 at the endogenous *C9orf72* locus and ESMNs comprising the same, *C9orf72* expression was also enhanced in non-neuronal tissues, e.g., muscle and heart, in mice comprising 3 or 92 repeats of the heterologous hexanucleotide sequence set forth as SEQ ID NO:1 at the endogenous *C9orf72* locus. Furthermore, the enhancement was specific for the humanized *C9orf72* allele; no enhanced expression of the mouse *C9orf72* allele, which does not contain the repeat sequence, was seen in heterozygous mice (data not shown).

[000215] Preliminary calculations indicate that ESMNs or brain cells with thirty or ninety-two repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 have approximately 17 copies of a *C9orf72* mRNA per cell, consistent with the findings of Liu et al. (2017) *supra*. An increased number of repeats of a hexanucleotide sequence set forth in SEQ ID NO:1 is also directly correlated with an increase in *C9orf72* protein levels, (**Figures 7 and 8**), nuclear and cytoplasmic accumulation of sense and antisense *C9orf72* RNA foci (**Figures 9A and 9B**), and dipeptide repeat proteins (**Figure 10**). The data shown herein indicate that increased number of repeats of a hexanucleotide sequence set forth in SEQ ID NO:1 at the *C9orf72* locus results in cells exhibiting a molecular phenotype (e.g., increased transcription, accumulation of RNA foci, and/or increased dipeptide repeat proteins) similar to human cells isolated from patients diagnosed with ALS, and supports the use of the non-human animals disclosed herein as a disease model for neurodegenerative disease.

Example 4. Behavioral analysis of non-human animals having a heterologous hexanucleotide repeat expansion sequence in an endogenous *C9orf72* locus

[000216] This example describes behavioral analysis of non-human animals (e.g., rodents) described herein for ALS-like symptoms such as, for example, decreased body weight and/or significant motor abnormalities resulting from an insertion of a heterologous hexanucleotide repeat expansion sequence in an endogenous rodent (e.g., mouse) *C9orf72* locus as described in Example 1.

[000217] Phenotypic studies of mice having an a pathogenic heterologous hexanucleotide repeat expansion sequence inserted into an endogenous *C9orf72* locus as described above, and/or control mice, e.g., wildtype mice or mice having an a non-pathogenic heterologous hexanucleotide repeat expansion sequence inserted into an endogenous *C9orf72* locus as described above, is performed at 8, 18, 37 (female) and 57-60 weeks (male). Body weight is measured on a bi-weekly basis, and body composition is analyzed by μ CT scan (Dynamic 60). Standard 24 scan is used to visualize mass of the cervical region of the spine. All animal procedures were conducted in compliance with protocols approved by the Regeneron Pharmaceuticals Institutional Animal Care and Use Committee.

[000218] Assessment of overall motor function is performed using blinded subjective scoring assays. Analysis of motor impairment is conducted using rotarod, open field locomotor, and catwalk testing. Motor impairment score is measure using the system developed by the ALS Therapy Development Institute (ALSTDI, Gill A. et al., 2009, PLoS One 4:e6489). During catwalk testing, subjects walk across an illuminated glass platform while a video camera records from below. Gait related parameters such as stride pattern, individual paw swing speed, stance duration, and pressure are reported for each animal. This test is used to phenotype mice and evaluate novel chemical entities for their effect on motor performance. CatWalk XT is a system for quantitative assessment of footfalls and gait in rats and mice. It is used to evaluate the locomotor ability of rodents in almost any kind of experimental model of central nervous, peripheral nervous, muscular, or skeletal abnormality.

[000219] CatWalk Gait Analysis: Animals are placed at the beginning of the runway of Noldus CatWalk XT 10, with the open end in front of them. Mice spontaneously run to the end of the runway to attempt to escape. The camera records and the software of the system measures the footprints. The footprints are analyzed for abnormalities in paw placement.

[000220] Open Field Test: Mice are placed in the Kinder Scientific open field system and evaluated for 60 minutes. The apparatus uses infrared beams and computer software to calculate fine movements, X+Y ambulation, distance traveled, number of rearing events, time spent rearing, and immobility time.

[000221] Rotorod: The rotorod test (IITC Life Science, Woodland Hills, CA) measures the latency for a mouse to fall from a rotating beam. The rotorod is set to the

experimental regime that starts at 1 rpm and accelerates up to 15 rpm over 180 seconds. Then, the animals' latency to fall following the incremental regime is recorded. The average and maximum of the three longest durations of time that the animals stay on the beam without falling off are used to evaluate falling latency. Animals that manage to stay on the beam longer than 180 seconds are deemed to be asymptomatic.

[000222] Upper motor neuron impairment presents as spasticity (i.e., rigidity), increased reflexes, tremor, bradykinesia, and Babinski signs. Lower motor neuron impairment presents as muscle weakness, wasting, claspings, curling and dragging of feet, and fasciculations. Bulbar impairment presents as difficulty swallowing, slurring and tongue fasciculations. Overall motor function is also assessed starting at 32 weeks up to 60 weeks of age as percent of living animals at a given week. Mice are weighed weekly and assessment of overall motor function is performed using blinded subjective scoring assays (as described above). Weekly or bi-monthly clinical neurological exams are performed on the two groups of mice looking at their motor impairment, tremor and rigidity of their hind limb muscles. For motor impairment, a blinded neurological scoring scale from of zero (no symptoms) to four (mouse cannot right themselves within 30 seconds of being placed on their side) is used as shown in Table 7.

TABLE 7

ALS-TDI neurological scoring system

Score of 0:	Full extension of hind legs away from lateral midline when mouse is suspended by its tail, and mouse can hold this for two seconds, suspended two to three times.
Score of 1:	Collapse or partial collapse of leg extension towards lateral midline (weakness) or trembling of hind legs during tail suspension.
Score of 2:	Toes curl under at least twice during walking of 12 inches, or any part of foot is dragging along cage bottom/table.
Score of 3:	Rigid paralysis or minimal joint movement, foot not being used for generating forward motion.
Score of 4:	Mouse cannot right itself within 30 seconds after being placed on either side.

[000223] For tremor and rigidity, a scoring system with a scale from zero (no symptoms) to three (severe) is used. Table 8 sets forth the scoring methodology related to motor impairment, tremor and rigidity of animals during testing.

TABLE 8				
	0	1	2	3
Motor impairment	no phenotype	clasping	clasping & dragging	Paralysis
Tremor	none	mild	moderate	Severe
Rigidity	none	mild	moderate	Severe

[000224] In another experiment mice are examined using a grip strength test. Briefly, the grip strength measures the neuromuscular function as maximal muscle strength of forelimbs, and is assessed by the grasping applied by a mouse on a grid that is connected to a sensor. All grip strength values obtained are normalized against mouse body weight.

[000225] In another experiment, the lumbar portion of spinal cords from control mice and mice comprising a pathogenic heterologous hexanucleotide repeat expansion sequence inserted into an endogenous *C9orf72* locus at around 60 weeks old are collected for histopathological analysis. The total number of motor neurons in the spinal cords, and mean cell body area of motor neurons are observed in both test and control cohorts.

[000226] The thermal nociception of control mice, and test mice comprising an insertion of a pathogenic heterologous hexanucleotide repeat expansion sequence at 20 weeks of age is tested by placing animals on a metal surface maintained at 48°C, 52°C or 55°C (IITC, Woodland Hills, CA). Latency to respond, defined as the time elapsed until the animal licked or flicked a hind paw, to the heat stimulus is measured. Mice remain on the plate until they performed either of two nocifensive behaviors: hindpaw licking or hindpaw shaking.

Example 5. Deletion of a heterologous hexanucleotide repeat expansion sequence from an endogenous non-human C9ORF72 locus in a non-human embryonic stem cell using a CRISPR/Cas9 system

[000227] Potential guide RNA (gRNA) sequences for a reference hexanucleotide repeat expansion sequence (comprising at least one, at least about three, at least about five, at least about fifteen, at least about twenty, at least about thirty, at least about forty, at least about fifty, at least about 60 at least about 70, at least about 80, or at least about 90,

preferably contiguous, repeats of the hexanucleotide sequence set forth as SEQ ID NO:1) are analyzed and scored. DNA encoding potentially effective gRNA (e.g., crRNA and/or tracrRNA) is synthesized and placed into an expression construct, which may also comprise a nucleic acid encoding a Cas protein. *See, e.g., FIGURE 12.* ES cells comprising the reference hexanucleotide repeat expansion sequence are transfected with the expression construct (s) comprising the DNA encoding the gRNA and/or Cas protein, and a drug resistance gene. Drug –resistant clones are obtained by serial dilution, expanded for analysis and frozen. DNA from each drug-resistant ES cell clone is isolated and analyzed by PCR and visualization on an agarose gel. PCR products of a correct size are extracted and further sequenced to confirm deletion of the targeted hexanucleotide repeat expansion sequence.

[000228] **FIGURE 11** provides a not to scale depiction of a non-limiting exemplary reference hexanucleotide repeat expansion sequence, e.g., as found in 8029 A-A6 ES cells generated in Example 1, e.g., having a sequence as set forth as SEQ ID NO:45, and the positions of which that were more likely to be successfully targeted by gRNA. The DNA sequences encoding crRNA that target the positions depicted in **FIGURE 11**, an exemplary sequence for which is provided as SEQ ID NO:45, and the SEQ ID NO: of each are provided in Table 9. Notably, the sequences set forth as SEQ ID NOs:46-50 contain an initial guanine not found in the reference hexanucleotide repeat expansion sequence set forth as SEQ ID NO:45 for optimal expression with a U6 promoter.

Table 9 Designed gRNA sequences

Position in SEQ ID NO:45	crRNA encoding sequence (SEQ ID NO:)
190	GCTACTTGCTCTCACAGTACT (SEQ ID NO:46)
196	GCTCTCACAGTACTCGCTGA (SEQ ID NO:39)
274	GCCGCAGCCTGTAGCAAGCTC (SEQ ID NO:47)
899	GCGGCCGCTAGCGCGATCGCG (SEQ ID NO:48)
905	GCTAGCGCGATCGCGGGGCG (SEQ ID NO:49)

1006	GTGGCGAGTGGGTGAGTGAGG (SEQ ID NO:50)
1068	GGAAGAGGCGCGGGTAGAAG (SEQ ID NO:44)

[000229] DNA encoding the crRNA as set forth in Table 9 were made (Integrated DNA Technologies) and inserted into an expression construct in operable linkage with DNA encoding tracrRNA (e.g., DNA comprising the sequence set forth as SEQ ID NO:63). Successful ligation of the crRNA encoding sequences, was confirmed by polymerase chain reaction with the vector screening primers set forth in Table 10, and the sequences of the gRNA (crRNA and tracrRNA) encoding sequences were confirmed with sequence analysis using the vector sequencing primers, also set forth in Table 10. Expression constructs comprising the correct gRNA encoding sequences under the control of a U6 promoter, a nucleic acid encoding a cas9 protein, and a puromycin resistance gene, **Figure 12**, were amplified and purified.

Table 10

Vector Screening forward primer Position 190 gRNA	ACACCGCTCTCACAGTACTCGCTGAG (SEQ ID NO:51)
Vector Screening forward primer Position 196 gRNA	ACACCGCCGCGAGCCTGTAGCAAGCTCG (SEQ ID NO:52)
Vector Screening forward primer Position 274 gRNA	ACACCGAGTACTGTGAGAGCAAGTAGG (SEQ ID NO:53)
Vector Screening forward primer Position 899 gRNA	ACACCGACGCCCCGCGATCGCGCTAGG (SEQ ID NO:54)
Vector Screening forward primer Position 905 gRNA	ACACCGCGGCCGCTAGCGCGATCGCGG (SEQ ID NO:55)
Vector Screening forward primer Position 1006 gRNA	ACACCGTGGCGAGTGGGTGAGTGAGGG (SEQ ID NO:56)
Vector Screening forward primer Position 1068 gRNA	ACACCGGAAGAGGCGCGGGTAGAAGG (SEQ ID NO:57)
Vector Screening reverse primer All gRNA	GACGCGTTAATGCCAACTTT (SEQ ID NO:58)
Vector sequencing forward primer	GAGGGCCTATTTCCTATGAT (SEQ ID NO:59)
Vector sequencing reverse primer	GACGCGTTAATGCCAACTTT (SEQ ID NO:60)
Clone screening forward primer	GAACCTACGGAGTCCCACGA (SEQ ID NO:61)
Clone screening reverse primer	GGAGACAGCTCGGGTACTGA (SEQ ID NO:62)

[000230] 8029 A-A6 clones as obtained in Example 1 and comprising a hexanucleotide repeat expansion sequence comprising about 92 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 (e.g., a reference sequence set forth as SEQ ID NO:45) were transfected with different combinations of the crRNA set forth in Table 9 (plus tracrRNA sequence), a puromycin resistance gene, and a CRISPR/Cas9 endonuclease gene. In one combination, ES cells were transfected with a CRISPR/Cas9 system targeting sequences starting at positions 190, 196, 274, 899, 905, 1006, and 1068 of SEQ ID NO:45 (e.g., the expression construct(s) comprising a nucleic acid encoding cas9 protein and/or gRNA inserts having the sequences set forth as SEQ ID NOs: 39, 44 and 46-50. In a second combination, ES cells were transfected a CRISPR/Cas9 system targeting positions 196, 1006 and 1067 of SEQ ID NO: 45 (e.g., the expression construct(s) comprising a nucleic acid encoding cas9 protein and/or DNA encoding gRNA inserts comprising the sequence set forth as SEQ ID NOs: 39, 50 and 44, respectively). In a third combination, ES cells were transfected with gRNA inserts targeting positions 196, 272 and 1005 and 1067 of SEQ ID NO:45 (e.g., the expression construct(s) comprising a nucleic acid encoding cas9 protein and/or gRNA inserts comprising a sequence set forth as SEQ ID NO: 39, 47, 50 and 44, respectively).

[000231] Puromycin-resistant ES clones were obtained by serial dilution, cultured in media (500 ml KO DMEM media, 95ml Heat Inactivated FBS, 12mL L-Glutamine, 6mL Penn-Step, 6mL Non-Essential Amino Acids, 1.2mL B-mercaptoethonal), expanded for analysis, and frozen. DNA from each clone was isolated using the DNAase Blood and Tissue Kit according to the manufacturer's protocol (Qiagen) and analyzed by PCR using the clone screening forward and reverse screening primers set forth in Table 10. PCR products were visualized by agarose gel electrophoresis, and PCR products of a correct size were extracted and further sequenced to confirm deletion of the targeted hexanucleotide repeat expansion sequence.

[000232] Of one-hundred sixty (160) clones, one hundred clones were tested and eleven (11) demonstrated a deletion of the hexanucleotide repeat expansion sequence, e.g. as demonstrated an amplified PCR product between 300 and 700 base pairs (data not shown). Sequence analysis confirmed deletion of the hexanucleotide repeat expansion sequence (data not shown). Of the three combinations tested, a CRISPR/Cas system targeting the combination of positions 196, 1005 and 1067 of SEQ ID NO: 45 proved

most efficient in deleting the hexanucleotide repeat expansion sequence; this combination resulted in ten of the eleven positive clones. A CRISPR/Cas system targeting the combination of positions 196, 272, 1005 and 1067 of SEQ ID NO: 45 provided one clone.

EQUIVALENTS

[000233] Having thus described several aspects of at least one embodiment of this invention, it is to be appreciated by those skilled in the art that various alterations, modifications, and improvements will readily occur to those skilled in the art. Such alterations, modifications, and improvements are intended to be part of this disclosure, and are intended to be within the spirit and scope of the invention. Accordingly, the foregoing description and drawing are by way of example only and the invention is described in detail by the claims that follow.

[000234] Use of ordinal terms such as "first," "second," "third," etc., in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed, but are used merely as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term) to distinguish the claim elements.

[000235] The articles "a" and "an" in the specification and in the claims, unless clearly indicated to the contrary, should be understood to include the plural referents. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or the entire group members are present in, employed in, or otherwise relevant to a given product or process. Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the listed claims is introduced into another claim dependent on the same base claim (or, as

relevant, any other claim) unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise. Where elements are presented as lists, (e.g., in Markush group or similar format) it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, etc. For purposes of simplicity those embodiments have not in every case been specifically set forth in so many words herein. It should also be understood that any embodiment or aspect of the invention can be explicitly excluded from the claims, regardless of whether the specific exclusion is recited in the specification.

[000236] Those skilled in the art will appreciate typical standards of deviation or error attributable to values obtained in assays or other processes described herein.

[000237] The publications, websites and other reference materials referenced herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference.

We claim:

1. A non-human animal or non-human animal cell comprising in its genome a heterologous hexanucleotide repeat expansion sequence inserted at an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises at least one repeat of the hexanucleotide sequence set forth as SEQ ID NO: 1.
2. The non-human animal or non-human animal cell of claim 1, wherein the heterologous hexanucleotide repeat expansion sequence comprises at least at thirty repeats of the hexanucleotide sequence set forth as SEQ ID NO:1.
3. The non-human animal or non-human animal cell of claim 1 or claim 2, wherein the hexanucleotide repeat expansion comprises at least 90 repeats of the hexanucleotide sequence set forth as SEQ ID NO:1.
4. The non-human animal or non-human animal cell of any one of claims 1-3, wherein the non-human animal or non-human animal cell exhibits (i) increased expression of *C9orf72* transcripts compared to a control animal comprising a wildtype *C9orf72* locus, (ii) an increased number of RNA foci compared to a control animal comprising a wildtype *C9orf72* locus, (iii) an increased level of dipeptide repeat proteins compared to a control animal comprising a wildtype *C9orf72* locus, or (iv) any combination of (i) – (iii).
5. The non-human animal or non-human animal cell of any of the preceding claims, wherein the heterologous hexanucleotide repeat expansion sequence is located between the first non-coding endogenous exon and exon 2 of the endogenous *C9orf72* locus.
6. The non-human animal or non-human animal cell of claim 5, wherein the endogenous *C9orf72* locus comprises a nucleotide sequence set forth as SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or variants thereof.

7. The non-human animal or non-human animal cell of any one of the preceding claims, wherein the non-human animal is a rodent or the non-human animal cell is a rodent cell.
8. The non-human animal or non-human animal cell of claim 7, wherein the rodent is a rat or a mouse, or wherein the non-human animal cell is a rat cell or a mouse cell.
9. The non-human animal or non-human animal cell of claim 8, wherein the non-human animal is a mouse or the non-human animal cell is a mouse cell, wherein the heterologous hexanucleotide repeat expansion sequence comprises ninety-two repeats of the hexanucleotide sequence set forth as SEQ ID NO:1, and wherein the mouse or mouse cell exhibits all of the following three characteristics (i) increased expression of *C9orf72* transcripts compared to a control animal comprising a wildtype *C9orf72* locus, (ii) an increased number of RNA foci compared to a control animal comprising a wildtype *C9orf72* locus, and (iii) an increased level of dipeptide repeat proteins compared to a control animal comprising a wildtype *C9orf72* locus.
10. The non-human animal or non-human animal cell of any of the preceding claims, wherein the non-human animal is homozygous for the heterologous hexanucleotide repeat expansion sequence.
11. The non-human animal or non-human animal cell of any one of claims 1-9, wherein the non-human animal is heterozygous for the heterologous hexanucleotide repeat expansion sequence.
12. The non-human animal or non-human animal cell of claim 11, wherein the heterologous hexanucleotide repeat expansion sequence is a first heterologous hexanucleotide expansion sequence, wherein the non-human animal or non-human animal cell is also heterozygous for a second heterologous hexanucleotide repeat expansion sequence, and wherein the first expansion sequence has a different number of repeats than the second heterologous hexanucleotide repeat expansion sequence.

13. The non-human animal or non-human animal cell of claim 12, wherein the first heterologous repeat expansion sequence comprises one to three repeats of the hexanucleotide sequence set forth as SEQ ID NO:1 and the second heterologous repeat expansion sequence comprises four to one hundred repeats of the hexanucleotide sequence set forth as SEQ ID NO:1.
14. The non-human animal or non-human animal cell of any one of the preceding claims, wherein the non-human animal cell is an embryonic stem cell, an embryonic stem cell-derived motor neuron, a brain cell, a cortical cell, a neuronal cell, a muscle cell, a heart cell, or a germ cell.
15. An immortalized cell line or motor neuron derived from the embryonic stem cell of claim 14.
16. A non-human animal embryonic stem cell whose genome comprises a heterologous hexanucleotide repeat expansion sequence in an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises at least one repeat of the hexanucleotide sequence set forth as SEQ ID NO: 1.
17. A non-human animal embryo generated from the embryonic stem cell of claim 16.
18. A method of making a non-human animal whose genome comprises a hexanucleotide sequence, the method comprising
modifying the genome of the non-human animal so that it comprises a heterologous hexanucleotide repeat expansion sequence in an endogenous *C9orf72* locus, wherein the heterologous hexanucleotide repeat expansion sequence comprises at least one repeat of the hexanucleotide sequence set forth as SEQ ID NO: 1.
19. The method of claim 18, wherein the modifying is achieved by a process comprising
(a) introducing into a non-human embryonic stem cell a nucleic acid construct comprising an insert nucleic acid, wherein the insert nucleic acid comprises from 5' to 3'

- (i) a 5' homology arm that is homologous to a 5' target sequence of the *C9orf72* locus,
- (ii) a heterologous hexanucleotide repeat expansion sequence, and (iii) a 3' homology arm that is homologous to a 5' target sequence of the *C9orf72* locus;
- (b) obtaining a genetically modified rodent embryonic stem cell from (a); and
- (c) creating a rodent using the genetically modified rodent embryonic stem cell of (b).

20. The method of claim 19, wherein the insert nucleic acid further comprises between the 5' and 3' homology arms one or more genes encoding one or more selection markers.

21. The method of claim 19 or 20, wherein the insert nucleic acid further comprises one or more site-specific recombination sites between the 5' and 3' homology arms.

22. The method of claim 21, wherein the insert nucleic acid further comprises between the 5' and 3' homology arms a recombinase gene and a selection marker gene flanked by the recombinase recognition sites, which recombinase recognition sites are oriented to direct an excision.

23. The method of any one of claims 19-22, wherein the 5' homology arm is identical or substantially identical to exon 1 of the endogenous *C9orf72* locus, or a portion thereof.

24. The method of claim 23, wherein the 5' homology arm comprises the nucleotide sequence set forth as SEQ ID NO:20 or SEQ ID NO:23.

25. The method of any one of claims 19-24, wherein the 3' homology arm is identical or substantially identical to at least a portion of intron 1 of the endogenous *C9orf72* locus.

26. The method of claim 25, wherein the 3' homology arm comprises the nucleotide sequence set forth as SEQ ID NO:22 or SEQ ID NO:25.

27. The method of any one of claims 18-26, wherein the insert nucleic acid comprises a nucleic acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4.
28. The method of any one of claims 18-26, wherein the insert nucleic acid comprises a nucleic acid sequence set forth in SEQ ID NO:3 or SEQ ID NO:6.
29. The method of any one of claims 18-28, further comprising a step of breeding the rodent generated in (c) so that a rodent homozygous for the insertion is created.
30. The method of any one of claims 18-29, wherein the non-human animal is a rodent.
31. The method of claim 30, wherein the rodent is a rat or a mouse.
32. A non-human animal obtainable by the method of any one of claims 18-31.
33. A method of identifying a therapeutic candidate for the treatment of a disease or condition associated with the presence of a hexanucleotide repeat expansion sequence, the method comprising
- (a) administering a candidate agent to a non-human animal or a non-human animal cell comprising a *C9orf72* locus genetically modified to comprise a hexanucleotide repeat expansion sequence comprising at least one repeat of the hexanucleotide sequence set forth as SEQ ID NO:1;
 - (b) performing one or more assays to determine if the candidate agent has an effect on one or more signs, symptoms and/or conditions associated with the disease or condition; and
 - (c) identifying the candidate agent that has an effect on the one or more signs, symptoms and/or conditions associated with the disease or condition as the therapeutic candidate.
34. The method of claim 33, wherein the candidate agent is administered *in vivo* to a non-human animal, and optionally, the assay is performed *in vitro* on tissue isolated from the non-human animal after administration of the candidate agent.

35. The method of claim 33, wherein the candidate agent is administered *in vitro* to a non-human embryonic stem cell-derived motor neuron.
36. The method of any one of claims 33-35, wherein the assay is quantitative polymerase chain reaction (qPCR) to detect C9orf72 gene products.
37. The method of claim 36, wherein qPCR is performed with a primer and/or probe having a nucleotide sequence set forth in SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, or any combination thereof.
38. The method of any one of claims 33-35, wherein the assay is measures RNA foci comprising a C9orf72 sense or antisense RNA transcripts.
39. The method of claim 38, wherein the assay is fluorescent in situ hybridization using one or more probes having a nucleotide sequence as set forth in any one of SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, and/or SEQ ID NO:84.
40. The method of any one of claims 33-35, wherein the assay is measures polyGA dipeptide repeat proteins.
41. A host cell comprising a heterologous hexanucleotide repeat expansion sequence.
42. The host cell of claim 41, wherein the host cell is a bacterial cell.
43. A CRISPR/Cas system comprising a Cas protein and/or one or more gRNA, wherein the one or more gRNA is encoded by a DNA comprising a sequence selected from the group consisting of SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49; SEQ ID NO:50, and a combination thereof.

44. The CRISPR/Cas system of claim 43, wherein the one or more gRNA comprises a first, second and third gRNA, wherein the first gRNA is encoded by a DNA comprising the sequence set forth as SEQ ID NO: 39, wherein the second gRNA is encoded by a DNA comprising the sequence set forth as SEQ ID NO: 44, and wherein the third gRNA is encoded by a DNA comprising the sequence set forth as SEQ ID NO: 50.

45. The CRISPR/Cas system of claim 44, further comprising a fourth gRNA encoded by a DNA comprising the sequence set forth as SEQ ID NO: 47.

46. The CRISPR/Cas system of claim 45, further comprising a fifth, sixth, and seventh gRNA, wherein the fifth gRNA is encoded by a DNA comprising the sequence set forth as SEQ ID NO: 46, the sixth gRNA is encoded by a DNA comprising the sequence set forth as SEQ ID NO: 48, and the seventh gRNA is encoded by a DNA comprising the sequence set forth as SEQ ID NO: 49.

47. The CRISPR/Cas system of any one of claims 42-46, wherein the gRNA comprises a tracrRNA encoding by a DNA comprising a sequence set forth as SEQ ID NO:63, 64 or 65.

48. The CRISPR/Cas system of claim 47, wherein the tracrRNA is encoded by a DNA comprising the sequence set forth as SEQ ID NO:63.

49. The CRISPR/Cas system of claim 47, wherein the tracrRNA is encoded by a DNA comprising the sequence set forth as SEQ ID NO:64.

50. The CRISPR/Cas system of claim 47, wherein the tracrRNA is encoded by a DNA comprising the sequence set forth as SEQ ID NO:65.

51. The CRISPR/Cas system of any one of claims 43-50, further comprising an expression construct, wherein the expression construct comprises a nucleic acid encoding the Cas protein and/or DNA encoding the at least one gRNA, and wherein the expression construct optionally further comprises a drug resistance gene.