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(54) Title: DMD REPORTER MODELS CONTAINING HUMANIZED DUSCHENE MUSCULAR DYSTROPHY MUTATIONS

(57) Abstract: CRISPR/Cas9-mediated genome editing holds clinical potential for treating genetic diseases, such as Duchenne muscular dystrophy (DMD), which is caused by mutations in the dystrophin gene. *In vivo* AAV-mediated delivery of gene-editing components machinery has been shown to successfully remove mutant sequence to generate an exon skipping in the cardiac and skeletal muscle cells of postnatal *mdx* mice, a model of DMD. Using different modes of AAV9 delivery, the restoration of dystrophin protein expression in cardiac and skeletal muscle of *mdx* mice was achieved. Here, a humanized mouse model for DMD is created to help test the efficacy of genome editing to cure DMD. Additionally, to facilitate the analysis of exon skipping strategies *in vivo* in a non-invasive way, a reporter luciferase knock-in version of the mouse model was prepared. These humanized mouse models provide the ability to study correcting of mutations responsible for DMD *in vivo*.

DESCRIPTION

DMD REPORTER MODELS CONTAINING HUMANIZED DUSCHENE MUSCULAR DYSTROPHY MUTATIONS

PRIORITY CLAIM

The present application claims benefit of priority to U.S. Provisional Application Serial No. 62/431,699, filed December 8, 2016, the entire contents of which are hereby incorporated by reference.

FEDERAL FUNDING SUPPORT CLAUSE

This invention was made with government support under grant no. U54 HD 087351 awarded by National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on December 7, 2017, is named UTFD_P3125WO.txt and is 186,485 bytes in size.

FIELD OF THE DISCLOSURE

The present disclosure relates to the fields of molecular biology, medicine and genetics. More particularly, the disclosure relates to the use of genome editing to create humanized animal models for different forms of Duchenne muscular dystrophy (DMD), each containing distinct DMD mutations.

BACKGROUND

Muscular dystrophies (MD) are a group of more than 30 genetic diseases characterized by progressive weakness and degeneration of the skeletal muscles that control movement. Duchenne muscular dystrophy (DMD) is one of the most severe forms of MD that affects approximately 1 in 5000 boys and is characterized by progressive muscle weakness and premature death. Cardiomyopathy and heart failure are common, incurable and lethal features of DMD. The disease is caused by mutations in the gene encoding dystrophin (*DMD*), a large

intracellular protein that links the dystroglycan complex at the cell surface with the underlying cytoskeleton, thereby maintaining integrity of the muscle cell membrane during contraction. Mutations in the dystrophin gene result in loss of expression of dystrophin causing muscle membrane fragility and progressive muscle wasting.

SUMMARY

Despite intense efforts to find cures through a variety of approaches, including myoblast transfer, viral delivery, and oligonucleotide-mediated exon skipping, there remains no cure for any type of muscular dystrophy. The present inventors recently used clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR/Cas9)-mediated genome editing to correct the dystrophin gene (DMD) mutation in postnatal *mdx* mice, a model for DMD. *In vivo* AAV-mediated delivery of gene-editing components successfully removed the mutant genomic sequence by exon skipping in the cardiac and skeletal muscle cells of *mdx* mice. Using different modes of AAV9 delivery, the inventors restored dystrophin protein expression in cardiac and skeletal muscle of *mdx* mice. The mdx mouse model and the correction exon 23 using AAV delivery of myoediting machinery has been useful to show proof-of concept of exon skipping approach using several cuts in genomic region encompassing the mutation *in vivo*. However, there is a lack of other models for the various known DMD mutations, and for new mutations that continue to be discovered.

In some embodiments, a composition comprises a sequence encoding a Cas9 polypeptide, a sequence encoding a first guide RNA (gRNA) targeting a first genomic target sequence, and a sequence encoding a second gRNA targeting a second genomic target sequence, wherein the first and second genomic target sequences each comprise an intronic sequence surrounding an exon of the murine dystrophin gene. In some embodiments, the exon comprises exon 50 of the murine dystrophin gene. In some embodiments, the sequence encoding a Cas9 polypeptide is isolated or derived from a sequence encoding a *S. aureus* Cas9 polypeptide. In some embodiments, at least one of the sequence encoding the Cas9 polypeptide, the sequence encoding the first gRNA, or the sequence encoding the second gRNA comprises an RNA sequence. In some embodiments, the RNA sequence comprises an mRNA sequence. In some embodiments, the RNA sequence comprises at least one chemically-modified nucleotide. In some embodiments, at least one of the sequence encoding the Cas9 polypeptide, the sequence

encoding the first gRNA, or the sequence encoding the second gRNA comprises a DNA sequence.

In some embodiments, a first vector comprises the sequence encoding the Cas9 polypeptide and a second vector comprises at least one of the sequence encoding the first gRNA or the sequence encoding the second gRNA. In some embodiments, the first vector or the sequence encoding the Cas9 polypeptide further comprises a first polyA sequence. In some embodiments, the second vector or the sequence encoding the first gRNA or the sequence encoding the second gRNA encodes a second polyA sequence. In some embodiments, the first vector or the sequence encoding the Cas9 polypeptide further comprises a first promoter sequence. In some embodiments, the second gRNA comprises a second promoter sequence. In some embodiments, the first promoter sequence and the second promoter sequence are identical. In some embodiments, the first promoter sequence and the second promoter sequence are not identical. In some embodiments, the first promoter sequence or the second promoter sequence comprises a CK8 promoter sequence. In some embodiments, the first promoter sequence or the second promoter sequence comprises a CK8e promoter sequence. In some embodiments, the first promoter sequence or the second promoter sequence comprises a constitutive promoter. In some embodiments, the first promoter sequence or the second promoter sequences comprises an inducible promoter.

In some embodiments, at least one of the first vector and the second vector is a non-viral vector. In some embodiments, the non-viral vector is a plasmid. In some embodiments, a liposome or nanoparticle comprises the non-viral vector. In some embodiments, at least one of the first vector and the second vector is a viral vector. In some embodiments, the viral vector is an adeno-associated viral (AAV) vector. The AAV vector may be replication-defective or conditionally replication defective. In some embodiments, the AAV vector is a recombinant AAV vector. In some embodiments, the AAV vector comprises a sequence isolated or derived from an AAV vector of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or any combination thereof.

In some embodiments, one vector comprises the sequence encoding the Cas9 polypeptide, the sequence encoding the first gRNA and the sequence encoding the second gRNA. In embodiments, the vector further comprises a polyA sequence. In embodiments, the vector further comprises a promoter sequence. In embodiments, the promoter sequence comprises a constitutive promoter. In embodiments, the promoter sequence comprises an

inducible promoter. In embodiments, the promoter sequence comprises a CK8 promoter sequence. In embodiments, the promoter sequence comprises a CK8e promoter sequence.

In embodiments, the composition comprises a sequence codon optimized for expression in a mammalian cell. In embodiments, the composition comprises a sequence codon optimized for expression in a human cell or a mouse cell. In some embodiments, the sequence encoding the Cas9 polypeptide is codon optimized for expression in human cells or mouse cells. In some embodiments, a composition of the disclosure further comprises a pharmaceutically carrier.

In some embodiments, a cell comprises a composition of the disclosure. In embodiments, the cell is a murine cell. In some embodiments, the cell is an oocyte. In embodiments, a composition may comprise the cell. In embodiments, a genetically engineered mouse may comprise the cell. In some embodiments, a method for creating a genetically engineered mouse comprises contacting the cell with a mouse.

In some embodiments, a genetically engineered mouse is provided, wherein the genome of the mouse comprises a deletion of exon 50 of the dystrophin gene resulting in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene. In some embodiments, the genetically engineered mouse further comprises a reporter gene located downstream of and in frame with exon 79 of the dystrophin gene, and upstream of a dystrophin 3'-UTR, wherein the reporter gene is expressed when exon 79 is translated in frame with exon 49. In some embodiments, the reporter gene is luciferase. In some embodiments, the genetically engineered mouse further comprises a protease coding sequence upstream of and in frame with the reporter gene, and downstream of and in frame with exon 79. In some embodiments, the protease is autocatalytic. In some embodiments, the protease is 2A protease.

In some embodiments, the genetically engineered mouse is heterozygous for a deletion. In some embodiments, the genetically engineered mouse is homozygous for a deletion. In some embodiments, the mouse exhibits increased creatine kinase levels compared to a wildtype mouse. In some embodiments, the mouse does not exhibit detectable dystrophin protein in heart or skeletal muscle.

In some embodiments, a method of producing a genetically engineered mouse comprises contacting a fertilized oocyte with CRISPR/Cas9 elements and two single guide RNA (sgRNA) targeting sequences flanking exon 50 of the dystrophin gene, thereby creating a modified oocyte, wherein deletion of exon 50 by CRISPR/Cas9 results in an out of frame

shift and a premature stop codon in exon 51 of the dystrophin gene; and transferring the modified oocyte into a recipient female. In some embodiments, the oocyte comprises a dystrophin gene having a reporter gene located downstream of and in frame with exon 79 of the dystrophin gene, and upstream of a dystrophin 3'-UTR, wherein the reporter gene is expressed when exon 79 is translated in frame with exon 49. In some embodiments, the reporter gene is luciferase. In some embodiments, the oocyte comprises a protease coding sequence upstream of and in frame with the reporter gene, and downstream of and in frame with exon 79. In embodiments, the protease is autocatalytic. In embodiments, the protease is 2A protease. In embodiments, the mouse is heterozygous for a deletion. In embodiments, the mouse is homozygous for a deletion. In embodiments, wherein the mouse exhibits increased creatine kinase levels compared to a wildtype mouse. In embodiments, the mouse does not exhibit detectable dystrophin protein in heart or skeletal muscle.

In some embodiments, an isolated cell is obtained from a genetically engineered mouse of the disclosure. In some embodiments, the cell comprises a reporter gene located downstream of and in frame with exon 79 of the dystrophin gene, and upstream of a dystrophin 3'-UTR, wherein the reporter gene is expressed when exon 79 is translated in frame with exon 49. In some embodiments, the reporter gene is luciferase. In some embodiments, the cell comprises a protease coding sequence upstream of and in frame with the reporter gene, and downstream of and in frame with exon 79. In some embodiments, the protease is autocatalytic. In some embodiments, the protease is 2A protease. In some embodiments, the cell is heterozygous for a deletion. In some embodiments, the cell is homozygous for a deletion.

In some embodiments, a genetically engineered mouse is produced by a method comprising the steps of contacting a fertilized oocyte with CRISPR/Cas9 elements and two single guide RNA (sgRNA) targeting sequences flanking exon 50 of the dystrophin gene, thereby creating a modified oocyte, wherein deletion of exon 50 by CRISPR/Cas9 results in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene; and transferring the modified oocyte into a recipient female.

In some embodiments, a method of screening a candidate substance for DMD exon-skipping activity comprises contacting a mouse according to any of claims 43, 46, 47, or 74 with the candidate substance; and assessing in frame transcription and/or translation of exon

79 of the dystrophin gene, wherein the presence of in frame transcription and/or translation of exon 79 indicates the candidate substance exhibits exon-skipping activity.

In some embodiments, a method of producing a genetically engineered mouse comprises contacting a fertilized oocyte with CRISPR/Cpf1 elements and two single guide RNA (sgRNA) targeting sequences flanking exon 50 of the dystrophin gene, thereby creating a modified oocyte, wherein deletion of exon 50 by CRISPR/Cpf1 results in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene; and transferring the modified oocyte into a recipient female.

In some embodiments, a genetically engineered mouse is produced by a method comprising the steps of contacting a fertilized oocyte with CRISPR/Cpf1 elements and two single guide RNA (sgRNA) targeting sequences flanking exon 50 of the dystrophin gene, thereby creating a modified oocyte, wherein deletion of exon 50 by CRISPR/Cpf1 results in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene; and transferring the modified oocyte into a recipient female.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

Other objects, features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the disclosure, are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure. The disclosure may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIGS. 1A-E. "Humanized"-ΔEx50 mouse model. (FIG. 1A) Outline of the CRISPR/Cas9 strategy used for generation of the mice. (FIG. 1B) RT-PCR analysis to validate the depletion of exon 50. (FIG. 1C) Sequence analysis of RT-PCR band to validate the depletion of exon and generation of an out of frame sequence (Nucleic Acid = tataaggaaa aaccaagcac tcagccagtg aagctgccag tcagactgtt actctagtga cac, SEQ ID NO: 805; Amino Acid = YKEKPSTQPVKLPVRL; SEQ ID NO: 806). (FIG. 1D) Serum creatine kinase (CK), a marker of muscle dystrophy that reflects muscle damage and membrane leakage was measured in wild type (WT), ΔEx50 and mdx mice. (FIG. 1E) Hematoxylin and eosin (H&E) and dystrophin staining of skeletal and cardiac muscle. Scale bar: 50 μm.

FIGS. 2A-B. Luciferase reporter mouse model. (FIG. 2A) Schematic of strategy for creation of dystrophin reporter mice. Dystrophin (Dmd) gene with exons is indicated in blue. Using CRISPR/Cas9 mutagenesis, the inventors inserted a Luciferase reporter with the protease 2A cleavage site at the 3' end of the dystrophin coding region. (FIG. 2B) Bioluminescence imaging of wild-type (WT) and *Dmd* knock-in luciferase reporter mice.

FIGS. 3A-D. Luciferase Dmd-mutant reporter mouse model. (FIG. 3A) Schematic outline of strategy for generating Δex50-luciferase reporter mice. (FIG. 3B) Genotyping results of ΔEx50-*Dmd*-KI-luciferase reporter mice. Schematic view of genotyping strategy forward (Fw) and reverse (Rv) primers. (FIG. 3C) Bioluminescence imaging of wild-type (WT), *Dmd* knock-in luciferase reporter and Δex50-Dmd knock-in luciferase reporter mice. (FIG. 3D) Western blot analysis of dystrophin (DMD), Luciferin and vinculin (VCL) expression in skeletal muscle and heart tissues.

FIGS. 4A-D. Strategy for CRISPR/Cas9-mediated genome editing in ΔEx50-KI-luciferase mice. (FIG. 4A) Scheme showing the CRISPR/Cas9-mediated genome editing approach to correct the reading frame in ΔEx50-KI-luciferase mice by skipping exon 51. Gray exons are out of frame. (FIG. 4B) Illustration of sgRNA binding position and sequence for sgRNA-ex51-SA. PAM sequence for sgRNA is indicated in red. Black arrow indicates the

cleavage site. (FIG. 4C) Genomic deep sequencing analysis of PCR amplicons generated across the exon 51 target site in 10T1/2 cells. Sequence of representative indels aligned with sgRNA sequence (indicated in blue) revealing insertions (highlighted in green) and deletions (highlighted in red). The line indicates the predicted exon splicing enhancers (ESEs) sequence located at the site of sgRNA. Black arrow indicates the cleavage site. (FIG. 4C) The muscle creatine kinase 8 (CK8e) promoter was used to express SpCas9. The U6, H1 and 7SK promoters for RNA polymerase III were used to express sgRNAs.

FIGS. 5A-D. *In Vivo* Investigation of Correction of dystrophin expression by intra-muscular injection of AAV9s. (FIG. 5A) TA muscles of ΔEx50-KI-luciferase mice were injected with AAV9s encoding sgRNA and Cas9. ΔEx50-KI-luciferase mice were analyzed weekly by bioluminescence. (FIG. 5B) Bioluminescence imaging of wild-type (WT), *Dmd* KI-luciferase reporter and ΔEx50-KI-luciferase reporter mice injected with AAV9s encoding sgRNA and Cas9 1 week and 3 weeks after injection. (FIG. 5C) Dystrophin immunohistochemistry of entire tibialis anterior muscle of wild-type (WT), *Dmd* KI- luciferase reporter and ΔEx50-KI-luciferase reporter mice injected with AAV9s encoding sgRNA and Cas9. (FIG. 5D) Dystrophin immunohistochemistry of tibialis anterior muscle of wild-type (WT), *Dmd* KI- luciferase reporter and ΔEx50-KI-luciferase reporter mice injected with AAV9s encoding sgRNA and Cas9.

DETAILED DESCRIPTION

DMD is a new mutation syndrome with more than 4,000 independent mutations that have been identified in humans (world-wide web at dmd.nl). The majority of patient's mutations carry deletions that cluster in a hotspot, and thus a therapeutic approach for skipping certain exon applies to large group of patients. The rationale of the exon skipping approach is based on the genetic difference between DMD and Becker muscular dystrophy (BMD) patients. In DMD patients, the reading frame of dystrophin mRNA is disrupted resulting in prematurely truncated, non-functional dystrophin proteins. BMD patients have mutations in the DMD gene that maintain the reading frame allowing the production of internally deleted, but partially functional dystrophins leading to much milder disease symptoms compared to DMD patients.

One the most common hot spots in DMD is the between exons 45 and 51, where skipping of exon 51 would apply to the largest group (i.e., 13-14% of DMD mutations). To further assess the efficiency and optimize CRISPR/Cas9-mediated exon skipping *in vivo*, a mimic of the human "hot spot" region was generated in a mouse model by deleting exon 50 using CRISPR/Cas9 system directed by two single guide RNAs (sgRNAs). The ΔEx50 mouse model exhibits dystrophic myofibers and increased serum creatine kinase level, thus providing a representative model of DMD. To accelerate the analysis of exon skipping strategies *in vivo* and in a non-invasive way, a reporter mouse was generated by insertion of a Luciferase expression cassette into the 3' end of the *Dmd* gene so that Luciferase would be translated in-frame with exon 79 of dystrophin. Then, the same 2 sgRNA were used to delete exon 50 in the *Dmd*-Luciferase line, generating a ΔEx50-*Dmd*-Luciferase mouse. Deletion of exon 50 in the *Dmd*-Luciferase line resulted in the decrease of bioluminescence signal in skeletal muscle and heart. These and other aspects of the disclosure are reproduced below.

I. Duchenne Muscular Dystrophy

A. Background

Duchenne muscular dystrophy (DMD) is a recessive X-linked form of muscular dystrophy, affecting around 1 in 5000 boys, which results in muscle degeneration and premature death. The disorder is caused by a mutation in the gene dystrophin, (see GenBank Accession No. NC_000023.11), located on the human X chromosome, which codes for the protein dystrophin (GenBank Accession No. AAA53189; SEQ ID NO. 383), the sequence of which is reproduced below:

1 mlwweevedc yeredvqkkt ftkwvnaqfs kfgkqhienl fsdlqdgrrl ldllegltgq
61 klpkekgstr vhalnnvnka lrvlqnnnvd lvnigstdiv dgnhkltlgl iwniilhwqv
121 knvmknimag lqqtnsekil lswvrqstrn ypqvnvinft tswsdglaln alihshrpdl
181 fdwnsvvcqq satqrlehaf niaryqlgie klldpedvdt typdkksilm yitslfqvlp
241 qqvsieaiqe vemlprppkv tkeehfqlhh qmhysqqitv slaqgyerts spkprfksya
301 ytqaayvtts dptrspfpsq hleapedksf gsslmesevn ldryqtalee vlswllsaed
361 tlqaqgeisn dvevvkdqfh thegymmdlt ahqgrvgnil qlgskligtg klsedeetev
421 qeqmnllnsr weclrvasme kqsnlhrvlm dlqnqklkel ndwltkteer trkmeeeplg
481 pdledlkrqv qqhkvlqedl eqeqvrvnsl thmvvvvdes sgdhataale eqlkvlgdrw
541 anicrwtedr wvllqdillk wqrlteeqcl fsawlseked avnkihttgf kdqnemlssl
601 qklavlkadl ekkkqsmgkl yslkqdllst lknksvtqkt eawldnfarc wdnlvqklek
661 staqisqavt ttqpsltqtt vmetvttvtt reqilvkhaq eelpppppqk krqitvdsei
721 rkrldvdite lhswitrsea vlqspefaif rkegnfsdlk ekvnaierek aekfrklqda
781 srsaqalveq mvnegvnads ikqaseqlns rwiefcqlls erlnwleyqn niiafynqlq
841 qleqmtttae nwlkiqpttp septaiksql kickdevnrl sglqpqierl kiqsialkek
901 gqgpmfldad fvaftnhfkq vfsdvqarek elqtifdtlp pmryqetmsa irtwvqqset
961 klsipqlsvt dyeimeqrlg elqalqsslq eqqsglyyls ttvkemskka pseisrkyqs
1021 efeeiegrwk klssqlvehc qkleeqmnkl rkiqnhiqtl kkwmaevdvf lkeewpalgd
1081 seilkkqlkq crllvsdiqt iqpslnsvne ggqkikneae pefasrlete lkelntqwdh
1141 mcqqvyarke alkgglektv slqkdlsemh ewmtqaeeey lerdfeyktp delqkaveem
1201 krakeeaqqk eakvklltes vnsviaqapp vaqealkkel etlttnyqwl ctrlngkckt
1261 leevwacwhe llsylekank wlnevefklk ttenipggae eisevldsle nlmrhsednp
1321 nqirilaqtl tdggvmdeli neeletfnsr wrelheeavr rqklleqsiq saqetekslh
1381 liqesltfid kqlaayiadk vdaaqmpqea qkiqsdltsh eisleemkkh nqgkeaaqrv
1441 lsqidvaqkk lqdvsmkfrl fqkpanfelr lqeskmilde vkmhlpalet ksveqevvqs
1501 qlnhcvnlyk slsevkseve mviktgrqiv qkkqtenpke ldervtalkl hynelgakvt
1561 erkqqlekcl klsrkmrkem nvltewlaat dmeltkrsav egmpsnldse vawgkatqke
1621 iekqkvhlks itevgealkt vlgkketlve dklsllnsnw iavtsraeew lnllleyqkh
1681 metfdqnvdh itkwiiqadt lldesekkkp qqkedvlkrl kaelndirpk vdstrdqaan

1741 lmanrgdhcr klvepqisel nhrfaaishr iktgkasipl keleqfnsdi qkllepleae
1801 iqqgvnlkee dfnkdmnedn egtvkellqr gdnlqqritd erkreeikik qqllqtkhna
1861 lkdlrsqrrk kaleishqwy qykrqaddll kclddiekkl aslpeprder kikeidrelq
1921 kkkeelnavr rqaeglsedg aamaveptqi qlskrwreie skfaqfrrln faqihtvree
1981 tmmvmtedmp leisyvpsty lteithvsqa lleveqllna pdlcakdfed lfkqeeslkn
2041 ikdslqqssg ridiihskkt aalqsatpve rvklqealsq ldfqwekvnk mykdrqgrfd
2101 rsvekwrrfh ydikifnqwl teaeqflrkt qipenwehak ykwylkelqd gigqrqtvvr
2161 tlnatgeeii qqssktdasi lqeklgslnl rwqevckqls drkkrleeqk nilsefqrdl
2221 nefvlwleea dniasiplep gkeqqlkekl eqvkllveel plrqgilkql netggpvlvs
2281 apispeeqdk lenklkqtnl qwikvsralp ekqgeieaqi kdlgqlekkl edleeqlnhl
2341 llwlspirnq leiynqpnqe gpfdvqetei avqakqpdve eilskgqhly kekpatqpvk
2401 rkledlssew kavnrllqel rakqpdlapg lttigasptq tvtlvtqpvv tketaiskle
2461 mpsslmlevp aladfnrawt eltdwlslld qviksqrvmv gdledinemi ikqkatmqdl
2521 eqrrpqleel itaaqnlknk tsnqeartii tdrieriqnq wdevqehlqn rrqqlnemlk
2581 dstqwleake eaeqvlgqar akleswkegp ytvdaiqkki tetkqlakdl rqwqtnvdva
2641 ndlalkllrd ysaddtrkvh miteninasw rsihkrvser eaaleethrl lqqfpldlek
2701 flawlteaet tanvlqdatr kerlledskg vkelmkqwqd lqgeieahtd vyhnldensq
2761 kilrslegsd davllqrrld nmnfkwselr kkslnirshl eassdqwkrl hlslqellvw
2821 lqlkddelsr qapiggdfpa vqkqndvhra fkrelktkep vimstletvr iflteqpleg
2881 leklyqepre lppeeraqnv trllrkqaee vnteweklnl hsadwqrkid etlerlqelq
2941 eatdeldlkl rqaevikgsw qpvgdllids lqdhlekvka lrgeiaplke nvshvndlar
3001 qlttlgiqls pynlstledl ntrwkllqva vedrvrqlhe ahrdfgpasq hflstsvqgp
3061 weraispnkv pyyinhetqt tcwdhpkmte lyqsladlnn vrfsayrtam klrrlqkalc
3121 ldllslsaac daldqhnlkq ndqpmdilqi inclttiydr leqehnnlvn vplcvdmcln
3181 wllnvydtgr tgrirvlsfk tgiislckah ledkyrylfk qvasstgfcd qrrlglllhd
3241 siqiprqlge vasfggsnie psvrscfqfa nnkpeieaal fldwmrlepq smvwlpvlhr
3301 vaaaetakhq akcnickecp iigfryrslk hfnydicqsc ffsgrvakgh kmhypmveyc
3361 tpttsgedvr dfakvlknkf rtkryfakhp rmgylpvqtv legdnmetpv tlinfwpvds
3421 apasspqlsh ddthsriehy asrlaemens ngsylndsis pnesiddehl liqhycqsln

3481 qdsplsqprs paqilisles eergeleril adleeenrnl qaeydrlkqq hehkglsplp

3541 sppemmptsp qsprdaelia eakllrqhkg rlearmqile dhnkqlesql hrlrqlleqp

3601 qaeakvngtt vsspstslqr sdssqpmllr vvgsqtsdsm geedllsppq dtstgleevm

3661 eqlnnsfpss rgrntpgkpm redtm

In humans, dystrophin mRNA contains 79 exons. Dystrophin mRNA is known to be alternatively spliced, resulting in various isoforms. Exemplary dystrophin isoforms are listed in Table 1.

Table 1: Dystrophin isoforms

Sequence Name	**Nucleic Acid Accession No.**	**Nucleic Acid SEQ ID NO:**	**Protein Accession No.**	**Protein SEQ ID NO:**	**Description**
DMD Genomic Sequence	NC_000023.11 (positions 31119219 to 33339609)	None	None	None	Sequence from Human X Chromosome (at positions Xp21.2 to p21.1) from Assembly GRCh38.p7 (GCF_000001405.33)
Dystrophin Dp427c isoform	NM_000109.3	384	NP_000100.2	385	Transcript Variant: transcript Dp427c is expressed predominantly in neurons of the cortex and the CA regions of the hippocampus. It uses a unique promoter/exon 1 located about 130 kb upstream of the Dp427m transcript promoter. The transcript includes the common exon 2 of transcript Dp427m and has a similar length of 14 kb. The Dp427c isoform contains a unique N-

Sequence Name	Nucleic Acid Accession No.	Nucleic Acid SEQ ID NO:	Protein Accession No.	Protein SEQ ID NO:	Description
					terminal MED sequence, instead of the MLWWEEVEDCY sequence of isoform Dp427m. The remainder of isoform Dp427c is identical to isoform Dp427m.
Dystrophin Dp427m isoform	NM_004006.2	386	NP_003997.1	387	Transcript Variant: transcript Dp427m encodes the main dystrophin protein found in muscle. As a result of alternative promoter use, exon 1 encodes a unique N-terminal MLWWEEVEDCY aa sequence.
Dystrophin Dp427p1 isoform	NM_004009.3	388	NP_004000.1	389	Transcript Variant: transcript Dp427p1 initiates from a unique promoter/exon 1 located in what corresponds to the first intron of transcript Dp427m. The transcript adds the common exon 2 of Dp427m and has a similar length (14 kb). The Dp427p1 isoform replaces the MLWWEEVEDCY - start of Dp427m with a unique N-terminal MSEVSSD aa sequence.
Dystrophin Dp260-1 isoform	NM_004011.3	390	NP_004002.2	391	Transcript Variant: transcript Dp260-1 uses exons 30-79,

Sequence Name	Nucleic Acid Accession No.	Nucleic Acid SEQ ID NO:	Protein Accession No.	Protein SEQ ID NO:	Description
					and originates from a promoter/exon 1 sequence located in intron 29 of the dystrophin gene. As a result, Dp260-1 contains a 95 bp exon 1 encoding a unique N-terminal 16 aa MTEIILLIFFPAYFL N-sequence that replaces amino acids 1-1357 of the full-length dystrophin product (Dp427m isoform).
Dystrophin Dp260-2 isoform	NM_004012.3	392	NP_004003.1	393	Transcript Variant: transcript Dp260-2 uses exons 30-79, starting from a promoter/exon 1 sequence located in intron 29 of the dystrophin gene that is alternatively spliced and lacks N-terminal amino acids 1-1357 of the full length dystrophin (Dp427m isoform). The Dp260-2 transcript encodes a unique N-terminal MSARKLRNLSYK K sequence.
Dystrophin Dp140 isoform	NM_004013.2	394	NP_004004.1	395	Transcript Variant: Dp140 transcripts use exons 45-79, starting at a promoter/exon 1 located in intron 44. Dp140 transcripts

Sequence Name	Nucleic Acid Accession No.	Nucleic Acid SEQ ID NO:	Protein Accession No.	Protein SEQ ID NO:	Description
					have a long (1 kb) 5' UTR since translation is initiated in exon 51 (corresponding to aa 2461 of dystrophin). In addition to the alternative promoter and exon 1, differential splicing of exons 71-74 and 78 produces at least five Dp140 isoforms. Of these, this transcript (Dp140) contains all of the exons.
Dystrophin Dp116 isoform	NM_004014.2	396	NP_004005.1	397	Transcript Variant: transcript Dp116 uses exons 56-79, starting from a promoter/exon 1 within intron 55. As a result, the Dp116 isoform contains a unique N-terminal MLHRKTYHVK aa sequence, instead of aa 1-2739 of dystrophin. Differential splicing produces several Dp116-subtypes. The Dp116 isoform is also known as S-dystrophin or apo-dystrophin-2.
Dystrophin Dp71 isoform	NM_004015.2	398	NP_004006.1	399	Transcript Variant: Dp71 transcripts use exons 63-79 with a novel 80- to 100-nt exon containing an ATG start site for a

Sequence Name	Nucleic Acid Accession No.	Nucleic Acid SEQ ID NO:	Protein Accession No.	Protein SEQ ID NO:	Description
					new coding sequence of 17 nt. The short coding sequence is in-frame with the consecutive dystrophin sequence from exon 63. Differential splicing of exons 71 and 78 produces at least four Dp71 isoforms. Of these, this transcript (Dp71) includes both exons 71 and 78.
Dystrophin Dp71b isoform	NM_004016.2	400	NP_004007.1	401	Transcript Variant: Dp71 transcripts use exons 63-79 with a novel 80- to 100-nt exon containing an ATG start site for a new coding sequence of 17 nt. The short coding sequence is in-frame with the consecutive dystrophin sequence from exon 63. Differential splicing of exons 71 and 78 produces at least four Dp71 isoforms. Of these, this transcript (Dp71b) lacks exon 78 and encodes a protein with a different C-terminus than Dp71 and Dp71a isoforms.
Dystrophin Dp71a isoform	NM_004017.2	402	NP_004008.1	403	Transcript Variant: Dp71 transcripts use exons 63-79 with a novel 80- to 100-nt exon containing an

Sequence Name	Nucleic Acid Accession No.	Nucleic Acid SEQ ID NO:	Protein Accession No.	Protein SEQ ID NO:	Description
					ATG start site for a new coding sequence of 17 nt. The short coding sequence is in-frame with the consecutive dystrophin sequence from exon 63. Differential splicing of exons 71 and 78 produces at least four Dp71 isoforms. Of these, this transcript (Dp71a) lacks exon 71.
Dystrophin Dp71ab isoform	NM_004018.2	404	NP_004009.1	405	Transcript Variant: Dp71 transcripts use exons 63-79 with a novel 80- to 100-nt exon containing an ATG start site for a new coding sequence of 17 nt. The short coding sequence is in-frame with the consecutive dystrophin sequence from exon 63. Differential splicing of exons 71 and 78 produces at least four Dp71 isoforms. Of these, this transcript (Dp71ab) lacks both exons 71 and 78 and encodes a protein with a C-terminus like isoform Dp71b.
Dystrophin Dp40 isoform	NM_004019.2	406	NP_004010.1	407	Transcript Variant: transcript Dp40 uses exons 63-70. The 5' UTR and encoded first 7 aa are identical

Sequence Name	Nucleic Acid Accession No.	Nucleic Acid SEQ ID NO:	Protein Accession No.	Protein SEQ ID NO:	Description
					to that in transcript Dp71, but the stop codon lies at the splice junction of the exon/intron 70. The 3' UTR includes nt from intron 70 which includes an alternative polyadenylation site. The Dp40 isoform lacks the normal C-terminal end of full-length dystrophin (aa 3409-3685).
Dystrophin Dp140c isoform	NM_004020.3	408	NP_004011.2	409	Transcript Variant: Dp140 transcripts use exons 45-79, starting at a promoter/exon 1 located in intron 44. Dp140 transcripts have a long (1 kb) 5' UTR since translation is initiated in exon 51 (corresponding to aa 2461 of dystrophin). In addition to the alternative promoter and exon 1, differential splicing of exons 71-74 and 78 produces at least five Dp140 isoforms. Of these, this transcript (Dp140c) lacks exons 71-74.
Dystrophin Dp140b isoform	NM_004021.2	410	NP_004012.1	411	Transcript Variant: Dp140 transcripts use exons 45-79, starting at a promoter/exon 1

Sequence Name	Nucleic Acid Accession No.	Nucleic Acid SEQ ID NO:	Protein Accession No.	Protein SEQ ID NO:	Description
					located in intron 44. Dp140 transcripts have a long (1 kb) 5' UTR since translation is initiated in exon 51 (corresponding to aa 2461 of dystrophin). In addition to the alternative promoter and exon 1, differential splicing of exons 71-74 and 78 produces at least five Dp140 isoforms. Of these, this transcript (Dp140b) lacks exon 78 and encodes a protein with a unique C-terminus.
Dystrophin Dp140ab isoform	NM_004022.2	412	NP_004013.1	413	Transcript Variant: Dp140 transcripts use exons 45-79, starting at a promoter/exon 1 located in intron 44. Dp140 transcripts have a long (1 kb) 5' UTR since translation is initiated in exon 51 (corresponding to aa 2461 of dystrophin). In addition to the alternative promoter and exon 1, differential splicing of exons 71-74 and 78 produces at least five Dp140 isoforms. Of these, this transcript (Dp140ab)

Sequence Name	Nucleic Acid Accession No.	Nucleic Acid SEQ ID NO:	Protein Accession No.	Protein SEQ ID NO:	Description
					lacks exons 71 and 78 and encodes a protein with a unique C-terminus.
Dystrophin Dp140bc isoform	NM_004023.2	414	NP_004014.1	415	Transcript Variant: Dp140 transcripts use exons 45-79, starting at a promoter/exon 1 located in intron 44. Dp140 transcripts have a long (1 kb) 5' UTR since translation is initiated in exon 51 (corresponding to aa 2461 of dystrophin). In addition to the alternative promoter and exon 1, differential splicing of exons 71-74 and 78 produces at least five Dp140 isoforms. Of these, this transcript (Dp140bc) lacks exons 71-74 and 78 and encodes a protein with a unique C-terminus.
Dystrophin isoform X2	XM_006724469.3	416	XP_006724532.1	417	
Dystrophin isoform X5	XM_011545467.1	418	XP_011543769.1	419	
Dystrophin isoform X6	XM_006724473.2	420	XP_006724536.1	421	
Dystrophin isoform X8	XM_006724475.2	422	XP_006724538.1	423	

Sequence Name	Nucleic Acid Accession No.	Nucleic Acid SEQ ID NO:	Protein Accession No.	Protein SEQ ID NO:	Description
Dystrophin isoform X4	XM_017029328.1	424	XP_016884817.1	425	
Dystrophin isoform X1	XM_006724468.2	426	XP_006724531.1	427	
Dystrophin isoform X13	XM_017029331.1	428	XP_016884820.1	429	
Dystrophin isoform X3	XM_006724470.3	430	XP_006724533.1	431	
Dystrophin isoform X7	XM_006724474.3	432	XP_006724537.1	433	
Dystrophin isoform X9	XM_011545468.2	434	XP_011543770.1	435	
Dystrophin isoform X11	XM_017029330.1	436	XP_016884819.1	437	
Dystrophin isoform X10	XM_017029329.1	438	XP_016884818.1	439	
Dystrophin isoform X12	XM_011545469.1	440	XP_011543771.1	441	

The murine dystrophin protein has the following amino acid sequence (Uniprot Accession No. P11531, SEQ. ID. NO. 786):

1 MWWVDCYRDV KKTTKWNASK GKHDNSDDGK RDGTGKKKGS TRVHANNVNK ARVKNNVDVN

61 GSTDVDGNHK TGWNHWVKNV MKTMAGTNSK SWVRSTRNYV NVNTSSWSDG ANAHSHRDDW

121 NSVVSHSATR HANAKCGKDD VATTYDKKSM YTSVVSAVMR TSSKVTRHHH MHYSTVSAGY

181 TSSSKRKSYA TAAYVATSDS TSYSHARDKS DSSMTVNDSY TAVSWSADTR AGSNDVVKHA

241 HGMMDTSHGV GNVGSVGKGK SDAVMNNSRW CRVASMKSKH KVMDNKKDDW TKTRTKKMGD

301 DKCVHKVDVR VNSTHMVVVV DSSGDHATAA KVGDRWANCR WTDRWVDKWH TCSTWSKDAM

361 KNTSGKDNMM SSHKSTKDKK KTMKSSNDSA KNKSVTKMWM NARWDNTKKS SASAVTTTST

421 TTVMTVTMVT TRMVKHAKKR TVDSRKRDVD THSWTRSAVS SAVYRKGNSD KVNAARKAKR

481 KDASRSAAVM ANGVNASRAS NSRWTCSRVN WYTNTYNMTT TANKTSTTST AKSKCKDVNR

541 SAKSKKGGMD ADVATNHNHD GVRAKKTDTM RYTMSSRTWS SKSVYSVTYM RGKASSKNGN

601 YSDTVKMAKK ASCKYSGHWK KSSVSCKHMN KRKNHKTKWM AVDVKWAGDA KKKCRVGDTS

661 NSVNGGKKSA ASRTRNTWDH CRVYTRKAKA GDKTVSKDSM HWMTAYRDYK TDTAVMKRAK

721 AKTKVKTTVN SVAHASAAKK TTTNYWCTRN GKCKTVWACW HSYKANKWNV KKTMNVAGTV

781 SNMHHSNNRA TTDGGVMDNT NSRWRHAVRK KSSAKSHSDK AAYTDKVDAA MAKSDTSHSM

841 KKHNGKDANR VSDVAKKDVS MKRKANRSKM DVKMHATKSV VSSHCVNYKS SVKSVMVKTG

901 RVKKTNKDRV TAKHYNGAKV TRKKCKSRKM RKMNVTWAAT DTTKRSAVGM SNDSVAWGKA

961 TKKKAHKSVT GSKMVGKKTV DKSNSNWAVT SRVWNYKHMT DNTKWHADDS KKKKDKRKAM

1021 NDMRKVDSTR DAAKMANRGD HCRKVVSNRR AASHRKTGKA SKNSDKAGVN KDNKDMSDNG

1081 TVNRGDNRTD RKRKKTKHNA KDRSRRKKAS HWYYKRADDK CDKKASRDRK KDRKKKNAVR

1141 RAGSNGAAMA VTSKRWRSNA RRNAHTHTMV VTTDMDVSYV STYTSHASVD HNTCAKDDKS

1201 KNKDNSGRDH KKKTAASATS MKVKVAVAMD GKHRMYKRGR DRSVKWRHHY DMKVNWNVKK

1261 TNNWHAKYKW YKDGGRAVVR TNATGSSKTD VNKGSSRWHD CKARRKRKNV SRDNVWADNA

1321 TGDKVKARGK NTGGAVVSAR DKKKKTNWKV SRAKGVHKDR DHWSRNYNSA GDKVTVHGKA

1381 DVRSKGHYKK STVKRKDRSW AVNHRRTKDR AGSTTGASAS TVTVTSVVTK TVSKMSSVAA

1441 DNRAWTTDWS DRVKSRVMVG DDNMKKATDR RTAANKNKTS NARTTDRRWD VNRRNMKDST

1501 WAKAVGVRGK DSWKGHTVDA KKTTKAKDRR SVDVANDAKR DYSADDTRKV HMTNNTSWGN

1561 HKRVSAATHR DKSWTATTAN VDASRKKDSR GVRMKWDGTH TDYHNDNGKR SGSDARRDNM

1621 NKWSKKSNRS HASSDWKRHS VWKDDSRAGG DAVKNDHRAK RKTKVMSTTV RTGKYRRANV

1681 TRRKAVNAWD KNRSADWRKD ARAADDKRAV KGSWVGDDSD HKVKARGAKN VNRVNDAHTT

1741 GSYNSTDNTR WRVAVDRVRH AHRDGASHST SVGWRASNKV YYNHTTTCWD HKMTYSADNN

1801 VRSAYRTAMK RRKACDSSAA CDADHNKNDM DNCTTYDRHN NVNVCVDMCN WNVYDTGRTG

1861 RRVSKTGSCK AHDKYRYKVA SSTGCDRRGH DSRGVASGGS NSVRSCANNK AADWMRSMVW

1921 VHRVAAATAK HAKCNCKCGR YRSKHNYDCS CSGRVAKGHK MHYMVYCTTT SGDVRDAKVK

1981 NKRTKRYAKH RMGYVTVGDN MTVTNWVDSA ASSSHDDTHS RHYASRAMNS NGSYNDSSNS

2041 DDHHYCSNDS SRSASSRGRA DNRNAYDRKH HKGSSMMTSS RDAAAKRHKG RARMDHNKSH

2101 RRAAKVNGTT VSSSTSRSDS SMRVVGSTSS MGDSDTSTGV MNNSSSRGRN AGKMRDTM

Dystrophin is an important component within muscle tissue that provides structural stability to the dystroglycan complex (DGC) of the cell membrane. While both sexes can carry the mutation, females are rarely affected with the skeletal muscle form of the disease.

Mutations vary in nature and frequency. Large genetic deletions are found in about 60-70% of cases, large duplications are found in about 10% of cases, and point mutants or other small changes account for about 15-30% of cases. Bladen *et al.* (2015), who examined some 7000 mutations, catalogued a total of 5,682 large mutations (80% of total mutations), of which 4,894 (86%) were deletions (1 exon or larger) and 784 (14%) were duplications (1 exon or

larger). There were 1,445 small mutations (smaller than 1 exon, 20% of all mutations), of which 358 (25%) were small deletions and 132 (9%) small insertions, while 199 (14%) affected the splice sites. Point mutations totaled 756 (52% of small mutations) with 726 (50%) nonsense mutations and 30 (2%) missense mutations. Finally, 22 (0.3%) mid-intronic mutations were observed. In addition, mutations were identified within the database that would potentially benefit from novel genetic therapies for DMD including stop codon read-through therapies (10% of total mutations) and exon skipping therapy (80% of deletions and 55% of total mutations).

B. Symptoms

Symptoms usually appear in boys between the ages of 2 and 3 and may be visible in early infancy. Even though symptoms do not appear until early infancy, laboratory testing can identify children who carry the active mutation at birth. Progressive proximal muscle weakness of the legs and pelvis associated with loss of muscle mass is observed first. Eventually this weakness spreads to the arms, neck, and other areas. Early signs may include pseudohypertrophy (enlargement of calf and deltoid muscles), low endurance, and difficulties in standing unaided or inability to ascend staircases. As the condition progresses, muscle tissue experiences wasting and is eventually replaced by fat and fibrotic tissue (fibrosis). By age 10, braces may be required to aid in walking but most patients are wheelchair dependent by age 12. Later symptoms may include abnormal bone development that lead to skeletal deformities, including curvature of the spine. Due to progressive deterioration of muscle, loss of movement occurs, eventually leading to paralysis. Intellectual impairment may or may not be present but if present, does not progressively worsen as the child ages. The average life expectancy for males afflicted with DMD is around 25.

The main symptom of Duchenne muscular dystrophy, a progressive neuromuscular disorder, is muscle weakness associated with muscle wasting with the voluntary muscles being first affected, especially those of the hips, pelvic area, thighs, shoulders, and calves. Muscle weakness also occurs later, in the arms, neck, and other areas. Calves are often enlarged. Symptoms usually appear before age 6 and may appear in early infancy. Other physical symptoms are:

- Awkward manner of walking, stepping, or running – (patients tend to walk on their forefeet, because of an increased calf muscle tone. Also, toe walking is a compensatory adaptation to knee extensor weakness.)

- Frequent falls
- Fatigue
- Difficulty with motor skills (running, hopping, jumping)
- Lumbar hyperlordosis, possibly leading to shortening of the hip-flexor muscles. This has an effect on overall posture and a manner of walking, stepping, or running.
- Muscle contractures of Achilles tendon and hamstrings impair functionality because the muscle fibers shorten and fibrose in connective tissue
- Progressive difficulty walking
- Muscle fiber deformities
- Pseudohypertrophy (enlarging) of tongue and calf muscles. The muscle tissue is eventually replaced by fat and connective tissue, hence the term pseudohypertrophy.
- Higher risk of neurobehavioral disorders (*e.g.*, ADHD), learning disorders (dyslexia), and non-progressive weaknesses in specific cognitive skills (in particular short-term verbal memory), which are believed to be the result of absent or dysfunctional dystrophin in the brain.
- Eventual loss of ability to walk (usually by the age of 12)
- Skeletal deformities (including scoliosis in some cases)
- Trouble getting up from lying or sitting position

The condition can often be observed clinically from the moment the patient takes his first steps, and the ability to walk usually completely disintegrates between the time the patient is 9 to 12 years of age. Most men affected with DMD become essentially "paralyzed from the neck down" by the age of 21. Muscle wasting begins in the legs and pelvis, then progresses to the muscles of the shoulders and neck, followed by loss of arm muscles and respiratory muscles. Calf muscle enlargement (pseudohypertrophy) is quite obvious. Cardiomyopathy particularly (dilated cardiomyopathy) is common, but the development of congestive heart failure or arrhythmia (irregular heartbeat) is only occasional.

A positive Gowers' sign reflects the more severe impairment of the lower extremities muscles. The child helps himself to get up with upper extremities: first by rising to stand on his arms and knees, and then "walking" his hands up his legs to stand upright. Affected children usually tire more easily and have less overall strength than their peers. Creatine kinase (CPK-MM) levels in the bloodstream are extremely high. An electromyography (EMG) shows that weakness is caused by destruction of muscle tissue rather than by damage to nerves. Genetic

testing can reveal genetic errors in the Xp21 gene. A muscle biopsy (immunohistochemistry or immunoblotting) or genetic test (blood test) confirms the absence of dystrophin, although improvements in genetic testing often make this unnecessary.

Other symptoms include:

- Abnormal heart muscle (cardiomyopathy)
- Congestive heart failure or irregular heart rhythm (arrhythmia)
- Deformities of the chest and back (scoliosis)
- Enlarged muscles of the calves, buttocks, and shoulders (around age 4 or 5). These muscles are eventually replaced by fat and connective tissue (pseudohypertrophy).
- Loss of muscle mass (atrophy)
- Muscle contractures in the heels, legs
- Muscle deformities
- Respiratory disorders, including pneumonia and swallowing with food or fluid passing into the lungs (in late stages of the disease)

C. Causes

Duchenne muscular dystrophy (DMD) is caused by a mutation of the dystrophin gene at locus Xp21, located on the short arm of the X chromosome. Dystrophin is responsible for connecting the cytoskeleton of each muscle fiber to the underlying basal lamina (extracellular matrix), through a protein complex containing many subunits. The absence of dystrophin permits excess calcium to penetrate the sarcolemma (the cell membrane). Alterations in calcium and signaling pathways cause water to enter into the mitochondria, which then burst.

In skeletal muscle dystrophy, mitochondrial dysfunction gives rise to an amplification of stress-induced cytosolic calcium signals and an amplification of stress-induced reactive-oxygen species (ROS) production. In a complex cascading process that involves several pathways and is not clearly understood, increased oxidative stress within the cell damages the sarcolemma and eventually results in the death of the cell. Muscle fibers undergo necrosis and are ultimately replaced with adipose and connective tissue.

DMD is inherited in an X-linked recessive pattern. Females will typically be carriers for the disease while males will be affected. Typically, a female carrier will be unaware they carry a mutation until they have an affected son. The son of a carrier mother has a 50% chance of inheriting the defective gene from his mother. The daughter of a carrier mother has a 50%

chance of being a carrier and a 50% chance of having two normal copies of the gene. In all cases, an unaffected father will either pass a normal Y to his son or a normal X to his daughter. Female carriers of an X-linked recessive condition, such as DMD, can show symptoms depending on their pattern of X-inactivation.

Exon deletions preceding exon 51 of the human *DMD* gene, which disrupt the open reading frame (ORF) by juxtaposing out of frame exons, represent the most common type of human DMD mutation. Skipping of exon 51 can, in principle, restore the DMD ORF in 13% of DMD patients with exon deletions.

Duchenne muscular dystrophy has an incidence of 1 in 5000 male infants. Mutations within the dystrophin gene can either be inherited or occur spontaneously during germline transmission. A table of exemplary but non-limiting mutations and corresponding models are set forth below:

Deletion, small insertion and nonsense mutations	**Name of Mouse Model**
Exon 44	ΔEx44
Exon 52	ΔEx52
Exon 43	ΔEx43

D. Diagnosis

Genetic counseling is advised for people with a family history of the disorder. Duchenne muscular dystrophy can be detected with about 95% accuracy by genetic studies performed during pregnancy.

DNA test. The muscle-specific isoform of the dystrophin gene is composed of 79 exons, and DNA testing and analysis can usually identify the specific type of mutation of the exon or exons that are affected. DNA testing confirms the diagnosis in most cases.

Muscle biopsy. If DNA testing fails to find the mutation, a muscle biopsy test may be performed. A small sample of muscle tissue is extracted (usually with a scalpel instead of a needle) and a dye is applied that reveals the presence of dystrophin. Complete absence of the protein indicates the condition.

Over the past several years DNA tests have been developed that detect more of the many mutations that cause the condition, and muscle biopsy is not required as often to confirm the presence of Duchenne's.

Prenatal tests. DMD is carried by an X-linked recessive gene. Males have only one X chromosome, so one copy of the mutated gene will cause DMD. Fathers cannot pass X-linked traits on to their sons, so the mutation is transmitted by the mother.

If the mother is a carrier, and therefore one of her two X chromosomes has a DMD mutation, there is a 50% chance that a female child will inherit that mutation as one of her two X chromosomes, and be a carrier. There is a 50% chance that a male child will inherit that mutation as his one X chromosome, and therefore have DMD.

Prenatal tests can tell whether an unborn child has the most common mutations. There are many mutations responsible for DMD, and some have not been identified, so genetic testing only works when family members with DMD have a mutation that has been identified.

Prior to invasive testing, determination of the fetal sex is important; while males are sometimes affected by this X-linked disease, female DMD is extremely rare. This can be achieved by ultrasound scan at 16 weeks or more recently by free fetal DNA testing. Chorion villus sampling (CVS) can be done at 11–14 weeks, and has a 1% risk of miscarriage. Amniocentesis can be done after 15 weeks, and has a 0.5% risk of miscarriage. Fetal blood sampling can be done at about 18 weeks. Another option in the case of unclear genetic test results is fetal muscle biopsy.

E. Treatment

There is no current cure for DMD, and an ongoing medical need has been recognized by regulatory authorities. Phase 1-2a trials with exon skipping treatment for certain mutations have halted decline and produced small clinical improvements in walking. Treatment is generally aimed at controlling the onset of symptoms to maximize the quality of life, and include the following:

- Corticosteroids such as prednisolone and deflazacort increase energy and strength and defer severity of some symptoms.
- Randomized control trials have shown that beta-2-agonists increase muscle strength but do not modify disease progression. Follow-up time for most RCTs on beta2-agonists is only around 12 months and hence results cannot be extrapolated beyond that time frame.
- Mild, non-jarring physical activity such as swimming is encouraged. Inactivity (such as bed rest) can worsen the muscle disease.
- Physical therapy is helpful to maintain muscle strength, flexibility, and function.

- Orthopedic appliances (such as braces and wheelchairs) may improve mobility and the ability for self-care. Form-fitting removable leg braces that hold the ankle in place during sleep can defer the onset of contractures.
- Appropriate respiratory support as the disease progresses is important.

Comprehensive multi-disciplinary care standards/guidelines for DMD have been developed by the Centers for Disease Control and Prevention (CDC), and are available at www.treat-nmd.eu/dmd/care/diagnosis-management-DMD.

DMD generally progresses through five stages, as outlined in Bushby *et al.*, Lancet Neurol., 9(1): 77-93 (2010) and Bushby *et al.*, Lancet Neurol., 9(2): 177-198 (2010), incorporated by reference in their entireties. During the presymptomatic stage, patients typically show developmental delay, but no gait disturbance. During the early ambulatory stage, patients typically show the Gowers' sign, waddling gait, and toe walking. During the late ambulatory stage, patients typically exhibit an increasingly labored gait and begin to lose the ability to climb stairs and rise from the floor. During the early non-ambulatory stage, patients are typically able to self-propel for some time, are able to maintain posture, and may develop scoliosis. During the late non-ambulatory stage, upper limb function and postural maintenance is increasingly limited.

In some embodiments, treatment is initiated in the presymptomatic stage of the disease. In some embodiments, treatment is initiated in the early ambulatory stage. In some embodiments, treatment is initiated in the late ambulatory stage. In embodiments, treatment is initiated during the early non-ambulatory stage. In embodiments, treatment is initiated during the late non-ambulatory stage.

1. Physical Therapy

Physical therapists are concerned with enabling patients to reach their maximum physical potential. Their aim is to:

- minimize the development of contractures and deformity by developing a program of stretches and exercises where appropriate
- anticipate and minimize other secondary complications of a physical nature by recommending bracing and durable medical equipment
- monitor respiratory function and advise on techniques to assist with breathing exercises and methods of clearing secretions

2. Respiration Assistance

Modern "volume ventilators/respirators," which deliver an adjustable volume (amount) of air to the person with each breath, are valuable in the treatment of people with muscular dystrophy related respiratory problems. The ventilator may require an invasive endotracheal or tracheotomy tube through which air is directly delivered, but, for some people non-invasive delivery through a face mask or mouthpiece is sufficient. Positive airway pressure machines, particularly bi-level ones, are sometimes used in this latter way. The respiratory equipment may easily fit on a ventilator tray on the bottom or back of a power wheelchair with an external battery for portability.

Ventilator treatment may start in the mid to late teens when the respiratory muscles can begin to collapse. If the vital capacity has dropped below 40% of normal, a volume ventilator/respirator may be used during sleeping hours, a time when the person is most likely to be under ventilating ("hypoventilating"). Hypoventilation during sleep is determined by a thorough history of sleep disorder with an oximetry study and a capillary blood gas (See Pulmonary Function Testing). A cough assist device can help with excess mucus in lungs by hyperinflation of the lungs with positive air pressure, then negative pressure to get the mucus up. If the vital capacity continues to decline to less than 30 percent of normal, a volume ventilator/respirator may also be needed during the day for more assistance. The person gradually will increase the amount of time using the ventilator/respirator during the day as needed.

F. Prognosis

Duchenne muscular dystrophy is a progressive disease which eventually affects all voluntary muscles and involves the heart and breathing muscles in later stages. The life expectancy is currently estimated to be around 25, but this varies from patient to patient. Recent advancements in medicine are extending the lives of those afflicted. The *Muscular Dystrophy Campaign*, which is a leading UK charity focusing on all muscle disease, states that "with high standards of medical care young men with Duchenne muscular dystrophy are often living well into their 30s."

In rare cases, persons with DMD have been seen to survive into the forties or early fifties, with the use of proper positioning in wheelchairs and beds, ventilator support (via tracheostomy or mouthpiece), airway clearance, and heart medications, if required. Early

planning of the required supports for later-life care has shown greater longevity in people living with DMD.

Curiously, in the mdx mouse model of Duchenne muscular dystrophy, the lack of dystrophin is associated with increased calcium levels and skeletal muscle myonecrosis. The intrinsic laryngeal muscles (ILM) are protected and do not undergo myonecrosis. ILM have a calcium regulation system profile suggestive of a better ability to handle calcium changes in comparison to other muscles, and this may provide a mechanistic insight for their unique pathophysiological properties. The ILM may facilitate the development of novel strategies for the prevention and treatment of muscle wasting in a variety of clinical scenarios.

II. CRISPR Systems

A. CRISPRs

CRISPRs (clustered regularly interspaced short palindromic repeats) are DNA loci containing short repetitions of base sequences. Each repetition is followed by short segments of "spacer DNA" from previous exposures to a virus. CRISPRs are found in approximately 40% of sequenced eubacteria genomes and 90% of sequenced archaea. CRISPRs are often associated with cas genes that code for proteins related to CRISPRs. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as plasmids and phages and provides a form of acquired immunity. CRISPR spacers recognize and silence these exogenous genetic elements like RNAi in eukaryotic organisms.

CRISPR repeats range in size from 24 to 48 base pairs. They usually show some dyad symmetry, implying the formation of a secondary structure such as a hairpin, but are not truly palindromic. Repeats are separated by spacers of similar length. Some CRISPR spacer sequences exactly match sequences from plasmids and phages, although some spacers match the prokaryote's genome (self-targeting spacers). New spacers can be added rapidly in response to phage infection.

B. Cas Nucleases

CRISPR-associated (*cas*) genes are often associated with CRISPR repeat-spacer arrays. As of 2013, more than forty different Cas protein families had been described. Of these protein

families, Cas1 appears to be ubiquitous among different CRISPR/Cas systems. Particular combinations of *cas* genes and repeat structures have been used to define 8 CRISPR subtypes (Ecoli, Ypest, Nmeni, Dvulg, Tneap, Hmari, Apern, and Mtube), some of which are associated with an additional gene module encoding repeat-associated mysterious proteins (RAMPs). More than one CRISPR subtype may occur in a single genome. The sporadic distribution of the CRISPR/Cas subtypes suggests that the system is subject to horizontal gene transfer during microbial evolution.

Exogenous DNA is apparently processed by proteins encoded by Cas genes into small elements (~30 base pairs in length), which are then somehow inserted into the CRISPR locus near the leader sequence. RNAs from the CRISPR loci are constitutively expressed and are processed by Cas proteins to small RNAs composed of individual, exogenously-derived sequence elements with a flanking repeat sequence. The RNAs guide other Cas proteins to silence exogenous genetic elements at the RNA or DNA level. Evidence suggests functional diversity among CRISPR subtypes. The Cse (Cas subtype *Ecoli*) proteins (called CasA-E in *E. coli*) form a functional complex, Cascade, that processes CRISPR RNA transcripts into spacer-repeat units that Cascade retains. In other prokaryotes, Cas6 processes the CRISPR transcripts. Interestingly, CRISPR-based phage inactivation in *E. coli* requires Cascade and Cas3, but not Cas1 and Cas2. The Cmr (Cas RAMP module) proteins found in *Pyrococcus furiosus* and other prokaryotes form a functional complex with small CRISPR RNAs that recognizes and cleaves complementary target RNAs. RNA-guided CRISPR enzymes are classified as type V restriction enzymes.

Cas9 is a nuclease, an enzyme specialized for cutting DNA, with two active cutting sites, one for each strand of the double helix. The team demonstrated that they could disable one or both sites while preserving Cas9's ability to locate its target DNA. tracrRNA and spacer RNA can be combined into a "single-guide RNA" molecule that, mixed with Cas9, can find and cut the correct DNA targets. and Such synthetic guide RNAs are able to be used for gene editing.

Cas9 proteins are highly enriched in pathogenic and commensal bacteria. CRISPR/Cas-mediated gene regulation may contribute to the regulation of endogenous bacterial genes, particularly during bacterial interaction with eukaryotic hosts. For example, Cas protein Cas9 of *Francisella novicida* uses a unique, small, CRISPR/Cas-associated RNA (scaRNA) to repress an endogenous transcript encoding a bacterial lipoprotein that is critical for *F. novicida*

to dampen host response and promote virulence. Wang *et al.* (2013) showed that coinjection of Cas9 mRNA and sgRNAs into the germline (zygotes) generated nice with mutations. Delivery of Cas9 DNA sequences also is contemplated.

The systems CRISPR/Cas are separated into three classes. Class 1 uses several Cas proteins together with the CRISPR RNAs (crRNA) to build a functional endonuclease. Class 2 CRISPR systems use a single Cas protein with a crRNA. Cpf1 has been recently identified as a Class II, Type V CRISPR/Cas systems containing a 1,300 amino acid protein. See also U.S. Patent Publication 2014/0068797, which is incorporated by reference in its entirety.

In some embodiments, the compositions of the disclosure include a small version of a Cas9 from the bacterium *Staphylococcus aureus* (UniProt Accession No. J7RUA5). The small version of the Cas9 provides advantages over wild type or full length Cas9. In some embodiments the Cas9 is a spCas9 (AddGene).

C. **Cpf1 Nucleases**

Clustered Regularly Interspaced Short Palindromic Repeats from *Prevotella* and *Francisella* 1 or CRISPR/Cpf1 is a DNA-editing technology which shares some similarities with the CRISPR/Cas9 system. Cpf1 is an RNA-guided endonuclease of a class II CRISPR/Cas system. This acquired immune mechanism is found in *Prevotella* and *Francisella* bacteria. It prevents genetic damage from viruses. Cpf1 genes are associated with the CRISPR locus, coding for an endonuclease that use a guide RNA to find and cleave viral DNA. Cpf1 is a smaller and simpler endonuclease than Cas9, overcoming some of the CRISPR/Cas9 system limitations.

Cpf1 appears in many bacterial species. The ultimate Cpf1 endonuclease that was developed into a tool for genome editing was taken from one of the first 16 species known to harbor it.

In embodiments, the Cpf1 is a Cpf1 enzyme from *Acidaminococcus* (species BV3L6, UniProt Accession No. U2UMQ6; SEQ ID NO. 442), having the sequence set forth below:

1 mtqfegftnl yqvsktlrfe lipqgktlkh iqeqgfieed karndhykel kpiidriykt
61 yadqclqlvq ldwenlsaai dsyrkektee trnalieeqa tyrnaihdyf igrtdnltda
121 inkrhaeiyk glfkaelfng kvlkqlgtvt ttehenallr sfdkfttyfs gfyenrknvf
181 saedistaip hrivqdnfpk fkenchiftr litavpslre hfenvkkaig ifvstsieev
241 fsfpfynqll tqtqidlynq llggisreag tekikglnev lnlaiqknde tahiiaslph

301 rfiplfkqil sdrntlsfil eefksdeevi qsfckyktll rnenvletae alfnelnsid

361 lthifishkk letissalcd hwdtlrnaly erriseltgk itksakekvq rslkhedinl

421 qeiisaagke lseafkqkts eilshahaal dqplpttlkk qeekeilksq ldsllglyhl

481 ldwfavdesn evdpefsarl tgiklemeps lsfynkarny atkkpysvek fklnfqmptl

541 asgwdvnkek nngailfvkn glyylgimpk qkgrykalsf eptektsegf dkmyydyfpd

601 aakmipkcst qlkavtahfq thttpillsn nfiepleitk eiydlnnpek epkkfqtaya

661 kktgdqkgyr ealckwidft rdflskytkt tsidlsslrp ssqykdlgey yaelnpllyh

721 isfqriaeke imdavetgkl ylfqiynkdf akghhgkpnl htlywtglfs penlaktsik

781 lngqaelfyr pksrmkrmah rlgekmlnkk lkdqktpipd tlyqelydyv nhrlshdlsd

841 earallpnvi tkevsheiik drrftsdkff fhvpitlnyq aanspskfnq rvnaylkehp

901 etpiigidrg ernliyitvi dstgkileqr slntiqqfdy qkkldnreke rvaarqawsv

961 vgtikdlkqg ylsqviheiv dlmihyqavv vlenlnfgfk skrtgiaeka vyqqfekmli

1021 dklnclvlkd ypaekvggvl npyqltdqft sfakmgtqsg flfyvpapyt skidpltgfv

1081 dpfvwktikn hesrkhfleg fdflhydvkt gdfilhfkmn rnlsfqrglp gfmpawdivf

1141 eknetqfdak gtpfiagkri vpvienhrft gryrdlypan elialleekg ivfrdgsnil

1201 pkllenddsh aidtmvalir svlqmrnsna atgedyinsp vrdlngvcfd srfqnpewpm

1261 dadangayhi alkgqlllnh lkeskdlklq ngisnqdwla yiqelrn

In some embodiments, the Cpf1 is a Cpf1 enzyme from *Lachnospiraceae* (species ND2006, UniProt Accession No. A0A182DWE3; SEQ ID NO. 443), having the sequence set forth below:

1 AASKLEKFTN CYSLSKTLRF KAIPVGKTQE NIDNKRLLVE DEKRAEDYKG VKKLLDRYYL

61 SFINDVLHSI KLKNLNNYIS LFRKKTRTEK ENKELENLEI NLRKEIAKAF KGAAGYKSLF

121 KKDIIETILP EAADDKDEIA LVNSFNGFTT AFTGFFDNRE NMFSEEAKST SIAFRCINEN

181 LTRYISNMDI FEKVDAIFDK HEVQEIKEKI LNSDYDVEDF FEGEFFNFVL TQEGIDVYNA

241 IIGGFVTESG EKIKGLNEYI NLYNAKTKQA LPKFKPLYKQ VLSDRESLSF YGEGYTSDEE

301 VLEVFRNTLN KNSEIFSSIK KLEKLFKNFD EYSSAGIFVK NGPAISTISK DIFGEWNLIR

361 DKWNAEYDDI HLKKKAVVTE KYEDDRRKSF KKIGSFSLEQ LQEYADADLS VVEKLKEIII

421 QKVDEIYKVY GSSEKLFDAD FVLEKSLKKN DAVVAIMKDL LDSVKSFENY IKAFFGEGKE

481 TNRDESFYGD FVLAYDILLK VDHIYDAIRN YVTQKPYSKD KFKLYFQNPQ FMGGWDKDKE

541 TDYRATILRY GSKYYLAIMD KKYAKCLQKI DKDDVNGNYE KINYKLLPGP NKMLPKVFFS

601 KKWMAYYNPS EDIQKIYKNG TFKKGDMFNL NDCHKLIDFF KDSISRYPKW SNAYDFNFSE

661 TEKYKDIAGF YREVEEQGYK VSFESASKKE VDKLVEEGKL YMFQIYNKDF SDKSHGTPNL

721 HTMYFKLLFD ENNHGQIRLS GGAELFMRRA SLKKEELVVH PANSPIANKN PDNPKKTTTL

781 SYDVYKDKRF SEDQYELHIP IAINKCPKNI FKINTEVRVL LKHDDNPYVI GIDRGERNLL

841 YIVVVDGKGN IVEQYSLNEI INNFNGIRIK TDYHSLLDKK EKERFEARQN WTSIENIKEL

901 KAGYISQVVH KICELVEKYD AVIALEDLNS GFKNSRVKVE KQVYQKFEKM LIDKLNYMVD

961 KKSNPCATGG ALKGYQITNK FESFKSMSTQ NGFIFYIPAW LTSKIDPSTG FVNLLKTKYT

1021 SIADSKKFIS SFDRIMYVPE EDLFEFALDY KNFSRTDADY IKKWKLYSYG NRIRIFAAAK

1081 KNNVFAWEEV CLTSAYKELF NKYGINYQQG DIRALLCEQS DKAFYSSFMA LMSLMLQMRN

1141 SITGRTDVDF LISPVKNSDG IFYDSRNYEA QENAILPKNA DANGAYNIAR KVLWAIGQFK

1201 KAEDEKLDKV KIAISNKEWL EYAQTSVK

In some embodiments, the Cpf1 is codon optimized for expression in mammalian cells. In some embodiments, the Cpf1 is codon optimized for expression in human cells or mouse cells.

The Cpf1 locus contains a mixed alpha/beta domain, a RuvC-I followed by a helical region, a RuvC-II and a zinc finger-like domain. The Cpf1 protein has a RuvC-like endonuclease domain that is similar to the RuvC domain of Cas9. Furthermore, Cpf1 does not have a HNH endonuclease domain, and the N-terminal of Cpf1 does not have the alpha-helical recognition lobe of Cas9.

Cpf1 CRISPR-Cas domain architecture shows that Cpf1 is functionally unique, being classified as Class 2, type V CRISPR system. The Cpf1 loci encode Cas1, Cas2 and Cas4 proteins more similar to types I and III than from type II systems. Database searches suggest the abundance of Cpf1-family proteins in many bacterial species.

Functional Cpf1 does not require a tracrRNA. Therefore, functional Cpf1 gRNAs of the disclosure may comprise or consist of a crRNA. This benefits genome editing because Cpf1 is not only a smaller nuclease than Cas9, but also it has a smaller sgRNA molecule (approximately half as many nucleotides as Cas9).

The Cpf1-gRNA (e.g. Cpf1-crRNA) complex cleaves target DNA or RNA by identification of a protospacer adjacent motif 5'-YTN-3' (where "Y" is a pyrimidine and "N" is any nucleobase) or 5'-TTN-3', in contrast to the G-rich PAM targeted by Cas9. After identification of PAM, Cpf1 introduces a sticky-end-like DNA double- stranded break of 4 or 5 nucleotides overhang.

The CRISPR/Cpf1 system comprises or consists of a Cpf1 enzyme and a guide RNA that finds and positions the complex at the correct spot on the double helix to cleave target DNA. In its native bacterial hosts, CRISPR/Cpf1 systems activity has three stages:

Adaptation, during which Cas1 and Cas2 proteins facilitate the adaptation of small fragments of DNA into the CRISPR array;

Formation of crRNAs: processing of pre-cr-RNAs producing of mature crRNAs to guide the Cas protein; and

Interference, in which the Cpf1 is bound to a crRNA to form a binary complex to identify and cleave a target DNA sequence.

This system has been modified to utilize non-naturally occurring crRNAs, which guide Cpf1 to a desired target sequence in a non-bacterial cell, such as a mammalian cell.

D. gRNA

As an RNA guided protein, Cas9 requires a short RNA to direct the recognition of DNA targets. Though Cas9 preferentially interrogates DNA sequences containing a PAM sequence

NGG it can bind here without a protospacer target. However, the Cas9-gRNA complex requires a close match to the gRNA to create a double strand break. CRISPR sequences in bacteria are expressed in multiple RNAs and then processed to create guide strands for RNA. Because Eukaryotic systems lack some of the proteins required to process CRISPR RNAs the synthetic construct gRNA was created to combine the essential pieces of RNA for Cas9 targeting into a single RNA expressed with the RNA polymerase type III promoter U6. Synthetic gRNAs are slightly over 100bp at the minimum length and contain a portion which targets the 20 protospacer nucleotides immediately preceding the PAM sequence NGG; gRNAs do not contain a PAM sequence.

In some embodiments, the gRNA targets a site within a wildtype dystrophin gene. In some embodiments, the gRNA targets a site within a mutant dystrophin gene. In some embodiments, the gRNA targets a dystrophin intron. In some embodiments, the gRNA targets a dystrophin exon. In some embodiments, the gRNA targets a site in a dystrophin exon that is expressed and is present in one or more of the dystrophin isoforms shown in Table 1. In embodiments, the gRNA targets a dystrophin splice site. In some embodiments, the gRNA targets a splice donor site on the dystrophin gene. In embodiments, the gRNA targets a splice acceptor site on the dystrophin gene.

In embodiments, the guide RNA targets a mutant DMD exon. In some embodiments, the mutant exon is exon 23 or 51. In some embodiments, the guide RNA targets at least one of exons 1, 23, 41, 44, 46, 47, 48, 49, 50, 51, 52, 53, 54, or 55 of the dystrophin gene. In embodiments, the guide RNA targets at least one of introns 44, 45, 50, 51, 52, 53, 54, or 55 of the dystrophin gene. In preferred embodiments, the guide RNAs are designed to induce skipping of exon 51 or exon 23. In embodiments, the gRNA is targeted to a splice acceptor site of exon 51 or exon 23.

Suitable gRNAs for use in various compositions and methods disclosed herein are provided as SEQ ID NOs. 448-770. (Table E). In preferred embodiments, the gRNA is selected from any one of SEQ ID No. 448 to SEQ ID No. 770.

In some embodiments, gRNAs of the disclosure comprise a sequence that is complementary to a target sequence within a coding sequence or a non-coding sequence corresponding to the *DMD* gene, and, therefore, hybridize to the target sequence. In some embodiments, gRNAs for Cpf1 comprise a single crRNA containing a direct repeat scaffold

sequence followed by 24 nucleotides of guide sequence. In some embodiments, a "guide" sequence of the crRNA comprises a sequence of the gRNA that is complementary to a target sequence. In some embodiments, crRNA of the disclosure comprises a sequence of the gRNA that is not complementary to a target sequence. "Scaffold" sequences of the disclosure link the gRNA to the Cpf1 polypeptide. "Scaffold" sequences of the disclosure are not equivalent to a tracrRNA sequence of a gRNA-Cas9 construct.

E. **Cas9 *versus* Cpf1**

Cas9 requires two RNA molecules to cut DNA while Cpf1 needs one. The proteins also cut DNA at different places, offering researchers more options when selecting an editing site. Cas9 cuts both strands in a DNA molecule at the same position, leaving behind 'blunt' ends. Cpf1 leaves one strand longer than the other, creating 'sticky' ends that are easier to work with. Cpf1 appears to be more able to insert new sequences at the cut site, compared to Cas9. Although the CRISPR/Cas9 system can efficiently disable genes, it is challenging to insert genes or generate a knock-in. Cpf1 lacks tracrRNA, utilizes a T-rich PAM and cleaves DNA via a staggered DNA DSB.

In summary, important differences between Cpf1 and Cas9 systems are that Cpf1 recognizes different PAMs, enabling new targeting possibilities, creates 4-5 nt long sticky ends, instead of blunt ends produced by Cas9, enhancing the efficiency of genetic insertions and specificity during NHEJ or HDR, and cuts target DNA further away from PAM, further away from the Cas9 cutting site, enabling new possibilities for cleaving the DNA.

Feature	**Cas9**	**Cpf1**
Structure	Two RNA required (Or 1 fusion transcript (crRNA+tracrRNA=gRNA)	One RNA required
Cutting mechanism	Blunt end cuts	Staggered end cuts
Cutting site	Proximal to recognition site	Distal from recognition site
Target sites	G-rich PAM	T-rich PAM
Cell type	Fast growing cells, including cancer cells	Non-dividing cells, including nerve cells

F. CRISPR/Cpf1-mediated gene editing

The first step in editing the DMD gene using CRISPR/Cpf1 is to identify the genomic target sequence. The genomic target for the gRNAs of the disclosure can be any ~24 nucleotide DNA sequence within the dystrophin gene, provided that the sequence is unique compared to the rest of the genome. In some embodiments, the genomic target sequence corresponds to a sequence within exon 51, exon 45, exon 44, exon 53, exon 46, exon 52, exon 50, exon 43, exon 6, exon 7, exon 8, and/or exon 55 of the human dystrophin gene. In some embodiments, the genomic target sequence is a 5' or 3' splice site of exon 51, exon 45, exon 44, exon 53, exon 46, exon 52, exon 50, exon 43, exon 6, exon 7, exon 8, and/or exon 55 of the human dystrophin gene. In some embodiments, the genomic target sequence corresponds to a sequence within an intron immediately upstream or downstream of exon 51, exon 45, exon 44, exon 53, exon 46, exon 52, exon 50, exon 43, exon 6, exon 7, exon 8, and/or exon 55 of the human dystrophin gene. Exemplary genomic target sequences can be found in Table D.

The next step in editing the DMD gene using CRISPR/Cpf1 is to identify all Protospacer Adjacent Motif (PAM) sequences within the genetic region to be targeted. Cpf1 utilizes a T-rich PAM sequence (TTTN, wherein N is any nucleotide). The target sequence must be immediately upstream of a PAM. Once all possible PAM sequences and putative target sites have been identified, the next step is to choose which site is likely to result in the most efficient on-target cleavage. The gRNA targeting sequence needs to match the target sequence, and the gRNA targeting sequence must not match additional sites within the genome. In preferred embodiments, the gRNA targeting sequence has perfect homology to the target with no homology elsewhere in the genome. In some embodiments, a given gRNA targeting sequence will have additional sites throughout the genome where partial homology exists. These sites are called "off-targets" and should be considered when designing a gRNA. In general, off-target sites are not cleaved as efficiently when mismatches occur near the PAM sequence, so gRNAs with no homology or those with mismatches close to the PAM sequence will have the highest specificity. In addition to "off-target activity", factors that maximize cleavage of the desired target sequence ("on-target activity") must be considered. It is known to those of skill in the art that two gRNA targeting sequences, each having 100% homology to the target DNA may not result in equivalent cleavage efficiency. In fact, cleavage efficiency may increase or decrease depending upon the specific nucleotides within

the selected target sequence. Close examination of predicted on-target and off-target activity of each potential gRNA targeting sequence is necessary to design the best gRNA. Several gRNA design programs have been developed that are capable of locating potential PAM and target sequences and ranking the associated gRNAs based on their predicted on-target and off-target activity (e.g. CRISPRdirect, available at www.crispr.dbcls.jp).

The next step is to synthesize and clone desired gRNAs. Targeting oligos can be synthesized, annealed, and inserted into plasmids containing the gRNA scaffold using standard restriction-ligation cloning. However, the exact cloning strategy will depend on the gRNA vector that is chosen. The gRNAs for Cpf1 are notably simpler than the gRNAs for Cas9, and only consist of a single crRNA containing direct repeat scaffold sequence followed by ~24 nucleotides of guide sequence. Cpf1 requires a minimum of 16 nucleotides of guide sequence to achieve detectable DNA cleavage, and a minimum of 18 nucleotides of guide sequence to achieve efficient DNA cleavage in vitro. In some embodiments, 20-24 nucleotides of guide sequence is used. The seed region of the Cpf1 gRNA is generally within the first 5 nucleotides on the 5' end of the guide sequence. Cpf1 makes a staggered cut in the target genomic DNA. In AsCpf1 and LbCpf1, the cut occurs 19 bp after the PAM on the targeted (+) strand, and 23 bp on the other strand.

Each gRNA should then be validated in one or more target cell lines. For example, after the CRISPR and gRNA are delivered to the cell, the genomic target region may be amplified using PCR and sequenced according to methods known to those of skill in the art.

In some embodiments, gene editing may be performed in vitro or ex vivo. In some embodiments, cells are contacted in vitro or ex vivo with a Cpf1 and a gRNA that targets a dystrophin splice site. In some embodiments, the cells are contacted with one or more nucleic acids encoding the Cpf1 and the guide RNA. In some embodiments, the one or more nucleic acids are introduced into the cells using, for example, lipofection or electroporation. Gene editing may also be performed in zygotes. In embodiments, zygotes may be injected with one or more nucleic acids encoding Cpf1 and a gRNA that targets a dystrophin splice site. The zygotes may subsequently be injected into a host.

In embodiments, the Cpf1 is provided on a vector. In embodiments, the vector contains a Cpf1 sequence derived from a *Lachnospiraceae* bacterium. See, for example, Uniprot Accession No. A0A182DWE3; SEQ ID NO. 443. In embodiments, the vector

contains a Cpf1 sequence derived from an *Acidaminococcus* bacterium. See, for example, Uniprot Accession No. U2UMQ6; SEQ ID NO. 442. In some embodiments, the Cpf1 sequence is codon optimized for expression in human cells or mouse cells. In some embodiments, the vector further contains a sequence encoding a fluorescent protein, such as GFP, which allows Cpf1-expressing cells to be sorted using fluorescence activated cell sorting (FACS). In some embodiments, the vector is a viral vector such as an adeno-associated viral vector.

In embodiments, the gRNA is provided on a vector. In some embodiments, the vector is a viral vector such as an adeno-associated viral vector. In embodiments, the Cpf1 and the guide RNA are provided on the same vector. In embodiments, the Cpf1 and the guide RNA are provided on different vectors.

In some embodiments, the cells are additionally contacted with a single-stranded DMD oligonucleotide to effect homology directed repair. In some embodiments, small INDELs restore the protein reading frame of dystrophin ("reframing" strategy). When the reframing strategy is used, the cells may be contacted with a single gRNA. In embodiments, a splice donor or splice acceptor site is disrupted, which results in exon skipping and restoration of the protein reading frame ("exon skipping" strategy). When the exon skipping strategy is used, the cells may be contacted with two or more gRNAs.

Efficiency of in vitro or ex vivo Cpf1-mediated DNA cleavage may be assessed using techniques known to those of skill in the art, such as the T7 E1 assay. Restoration of DMD expression may be confirmed using techniques known to those of skill in the art, such as RT-PCR, western blotting, and immunocytochemistry.

In some embodiments, in vitro or ex vivo gene editing is performed in a muscle or satellite cell. In some embodiments, gene editing is performed in iPSC or iCM cells. In embodiments, the iPSC cells are differentiated after gene editing. For example, the iPSC cells may be differentiated into a muscle cell or a satellite cell after editing. In embodiments, the iPSC cells are differentiated into cardiac muscle cells, skeletal muscle cells, or smooth muscle cells. In embodiments, the iPSC cells are differentiated into cardiomyocytes. iPSC cells may be induced to differentiate according to methods known to those of skill in the art.

In some embodiments, contacting the cell with the Cpf1 and the gRNA restores dystrophin expression. In embodiments, cells which have been edited in vitro or ex vivo, or

cells derived therefrom, show levels of dystrophin protein that is comparable to wild type cells. In embodiments, the edited cells, or cells derived therefrom, express dystrophin at a level that is 50%, 60%, 70%, 80%, 90%, 95% or any percentage in between of wild type dystrophin expression levels. In embodiments, the cells which have been edited in vitro or ex vivo, or cells derived therefrom, have a mitochondrial number that is comparable to that of wild type cells. In embodiments the edited cells, or cells derived therefrom, have 50%, 60%, 70%, 80%, 90%, 95% or any percentage in between as many mitochondria as wild type cells. In embodiments, the edited cells, or cells derived therefrom, show an increase in oxygen consumption rate (OCR) compared to non-edited cells at baseline.

III. Nucleic Acid Delivery

As discussed above, in certain embodiments, expression cassettes are employed to express a transcription factor product, either for subsequent purification and delivery to a cell/subject, or for use directly in a genetic-based delivery approach. Provided herein are expression vectors which contain one or more nucleic acids encoding Cpf1 and at least one DMD guide RNA that targets a dystrophin splice site. In some embodiments, a nucleic acid encoding Cpf1 and a nucleic acid encoding at least one guide RNA are provided on the same vector. In further embodiments, a nucleic acid encoding Cpf1 and a nucleic acid encoding least one guide RNA are provided on separate vectors.

Expression requires that appropriate signals be provided in the vectors, and include various regulatory elements such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in cells. Elements designed to optimize messenger RNA stability and translatability in host cells also are defined. The conditions for the use of a number of dominant drug selection markers for establishing permanent, stable cell clones expressing the products are also provided, as is an element that links expression of the drug selection markers to expression of the polypeptide.

A. Regulatory Elements

Throughout this application, the term "expression cassette" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed and translated, *i.e.*, is under

the control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene. An "expression vector" is meant to include expression cassettes comprised in a genetic construct that is capable of replication, and thus including one or more of origins of replication, transcription termination signals, poly-A regions, selectable markers, and multipurpose cloning sites.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (*tk*) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

In some embodiments, the Cpf1 constructs of the disclosure are expressed by a muscle-cell specific promoter. This muscle-cell specific promoter may be constitutively active or may be an inducible promoter.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

In certain embodiments, viral promotes such as the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the gene product.

Enhancers are genetic elements that increase transcription from a promoter located at a distant position on the same molecule of DNA. Enhancers are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a gene of interest in an expression construct. Additionally, any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the gene. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

The promoter and/or enhancer may be, for example, immunoglobulin light chain, immunoglobulin heavy chain, T-cell receptor, HLA DQ a and/or DQ β, β-interferon, interleukin-2, interleukin-2 receptor, MHC class II 5, MHC class II HLA-Dra, β-Actin, muscle creatine kinase (MCK), prealbumin (transthyretin), elastase I, metallothionein (MTII),

collagenase, albumin, α-fetoprotein, t-globin, β-globin, c-fos, c-HA-*ras,* insulin, neural cell adhesion molecule (NCAM), α_1-antitrypain, H2B (TH2B) histone, mouse and/or type I collagen, glucose-regulated proteins (GRP94 and GRP78), rat growth hormone, human serum amyloid A (SAA), troponin I (TN I), platelet-derived growth factor (PDGF), duchenne muscular dystrophy, SV40, polyoma, retroviruses, papilloma virus, hepatitis B virus, human immunodeficiency virus, cytomegalovirus (CMV), and gibbon ape leukemia virus.

In some embodiments, inducible elements may be used. In some embodiments, the inducible element is, for example, MTII, MMTV (mouse mammary tumor virus), β-interferon, adenovirus 5 E2, collagenase, stromelysin, SV40, murine MX gene, GRP78 gene, α-2-macroglobulin, vimentin, MHC class I gene H-2κb, HSP70, proliferin, tumor necrosis factor, and/or thyroid stimulating hormone α gene. In some embodiments, the inducer is phorbol ester (TFA), heavy metals, glucocorticoids, poly(rI)x, poly(rc), ElA, phorbol ester (TPA), interferon, Newcastle Disease Virus, A23187, IL-6, serum, interferon, SV40 large T antigen, PMA, and/or thyroid hormone. Any of the inducible elements described herein may be used with any of the inducers described herein.

Of particular interest are muscle specific promoters. These include the myosin light chain-2 promoter, the α-actin promoter, the troponin 1 promoter; the Na^+/Ca^{2+} exchanger promoter, the dystrophin promoter, the α7 integrin promoter, the brain natriuretic peptide promoter and the αB-crystallin/small heat shock protein promoter, α-myosin heavy chain promoter and the ANF promoter. In some embodiments, the muscle specific promoter is the CK8 promoter, which has the following sequence (SEQ ID NO: 787):

1 CTAGACTAGC ATGCTGCCCA TGTAAGGAGG CAAGGCCTGG GGACACCCGA GATGCCTGGT

61 TATAATTAAC CCAGACATGT GGCTGCCCCC CCCCCCCCAA CACCTGCTGC CTCTAAAAAT

121 AACCCTGCAT GCCATGTTCC CGGCGAAGGG CCAGCTGTCC CCCGCCAGCT AGACTCAGCA

181 CTTAGTTTAG GAACCAGTGA GCAAGTCAGC CCTTGGGGCA GCCCATACAA GGCCATGGGG

241 CTGGGCAAGC TGCACGCCTG GGTCCGGGGT GGGCACGGTG CCCGGGCAAC GAGCTGAAAG

301 CTCATCTGCT CTCAGGGGCC CCTCCCTGGG GACAGCCCCT CCTGGCTAGT CACACCCTGT

361 AGGCTCCTCT ATATAACCCA GGGGCACAGG GGCTGCCCTC ATTCTACCAC CACCTCCACA

421 GCACAGACAG ACACTCAGGA GCCAGCCAGC

In some embodiments, the muscle-cell cell specific promoter is a variant of the CK8 promoter, called CK8e. The CK8e promoter has the following sequence (SEQ ID NO: 788):

1 TGCCCATGTA AGGAGGCAAG GCCTGGGGAC ACCCGAGATG CCTGGTTATA ATTAACCCAG

61 ACATGTGGCT GCCCCCCCCC CCCCAACACC TGCTGCCTCT AAAAATAACC CTGCATGCCA

121 TGTTCCCGGC GAAGGGCCAG CTGTCCCCCG CCAGCTAGAC TCAGCACTTA GTTTAGGAAC

181 CAGTGAGCAA GTCAGCCCTT GGGGCAGCCC ATACAAGGCC ATGGGGCTGG GCAAGCTGCA

241 CGCCTGGGTC CGGGGTGGGC ACGGTGCCCG GGCAACGAGC TGAAAGCTCA TCTGCTCTCA

301 GGGGCCCCTC CCTGGGGACA GCCCCTCCTG GCTAGTCACA CCCTGTAGGC TCCTCTATAT

361 AACCCAGGGG CACAGGGGCT GCCCTCATTC TACCACCACC TCCACAGCAC AGACAGACAC

421 TCAGGAGCCA GCCAGC

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. Any polyadenylation sequence may be employed, such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

B. 2A Peptide

The inventor utilizes the 2A-like self-cleaving domain from the insect virus *Thosea asigna* (TaV 2A peptide; SEQ ID NO. 444; EGRGSLLTCGDVEENPGP) (Chang *et al.*, 2009). These 2A-like domains have been shown to function across Eukaryotes and cause cleavage of amino acids to occur co-translationally within the 2A-like peptide domain. Therefore, inclusion of TaV 2A peptide allows the expression of multiple proteins from a single mRNA transcript. Importantly, the domain of TaV when tested in eukaryotic systems has shown greater than 99% cleavage activity. Other acceptable 2A-like peptides include, but are not limited to, equine rhinitis A virus (ERAV) 2A peptide (SEQ ID NO. 445; QCTNYALLKLAGDVESNPGP), porcine teschovirus-1 (PTV1) 2A peptide (SEQ ID NO. 446; ATNFSLLKQAGDVEENPGP)

and foot and mouth disease virus (FMDV) 2A peptide (SEQ ID NO. 447; PVKQLLNFDLLKLAGDVESNPGP) or modified versions thereof.

In some embodiments, the 2A peptide is used to express a reporter and a Cfp1 simultaneously. The reporter may be, for example, GFP.

Other self-cleaving peptides that may be used include, but are not limited to nuclear inclusion protein a (Nia) protease, a P1 protease, a 3C protease, a L protease, a 3C-like protease, or modified versions thereof.

C. Delivery of Expression Vectors

There are a number of ways in which expression vectors may be introduced into cells. In certain embodiments, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. They can accommodate only up to 8 kB of foreign genetic material but can be readily introduced in a variety of cell lines and laboratory animals.

One of the preferred methods for *in vivo* delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to express an antisense polynucleotide that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kB, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kB. In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells

regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off. The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5□-tripartite leader (TPL) sequence which makes them preferred mRNAs for translation.

In one system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins. Since the E3 region is dispensable from the adenovirus genome, the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions. In nature, adenovirus can package approximately 105% of the wild-type genome, providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than

80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.* (1995) disclosed improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

The adenoviruses of the disclosure are replication defective or at least conditionally replication defective. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present disclosure.

As stated above, the typical vector according to the present disclosure is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding the gene of interest at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors, as

described by Karlsson *et al.* (1986), or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9-10^{12} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression and vaccine development. Animal studies suggested that recombinant adenovirus could be used for gene therapy. Studies in administering recombinant adenovirus to different tissues include trachea instillation, muscle injection, peripheral intravenous injections and stereotactic inoculation into the brain.

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription. The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5□ and 3□ ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome.

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed. When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged

into viral particles, which are then secreted into the culture media. The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells.

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses may be used, in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor are used. The antibodies are coupled via the biotin components by using streptavidin. Using antibodies against major histocompatibility complex class I and class II antigens, it has been demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.,* 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present disclosure. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes. Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact-sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (see, for example, Markowitz *et al.,* 1988; Hersdorffer *et al.,* 1990).

Other viral vectors may be employed as expression constructs in the present disclosure. Vectors derived from viruses such as vaccinia virus, adeno-associated virus (AAV), and herpesviruses may be employed. They offer several attractive features for various mammalian cells.

In embodiments, the AAV vector is replication-defective or conditionally replication defective. In embodiments, the AAV vector is a recombinant AAV vector. In some embodiments, the AAV vector comprises a sequence isolated or derived from an AAV vector

of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or any combination thereof. In some embodiments, the AAV vector is not an AAV9 vector.

In some embodiments, a single viral vector is used to deliver a nucleic acid encoding Cpf1 and at least one gRNA to a cell. In some embodiments, Cpf1 is provided to a cell using a first viral vector and at least one gRNA is provided to the cell using a second viral vector. In order to effect expression of sense or antisense gene constructs, the expression construct must be delivered into a cell. The cell may be a muscle cell, a satellite cell, a mesangioblast, a bone marrow derived cell, a stromal cell or a mesenchymal stem cell. In embodiments, the cell is a cardiac muscle cell, a skeletal muscle cell, or a smooth muscle cell. In embodiments, the cell is a cell in the tibialis anterior, quadriceps, soleus, diaphragm or heart. In some embodiments, the cell is an induced pluripotent stem cell (iPSC) or inner cell mass cell (iCM). In further embodiments, the cell is a human iPSC or a human iCM. In some embodiments, human iPSCs or human iCMs of the disclosure may be derived from a cultured stem cell line, an adult stem cell, a placental stem cell, or from another source of adult or embryonic stem cells that does not require the destruction of a human embryo. Delivery to a cell may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo*, as in the treatment of certain disease states. One mechanism for delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present disclosure. These include calcium phosphate precipitation, DEAE-dextran, electroporation, direct microinjection, DNA-loaded liposomes and lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, and receptor-mediated transfection. Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression construct has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation).

In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In yet another embodiment, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of calcium phosphate precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of calcium phosphate-precipitated plasmids results in expression of the transfected genes. DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

In still another embodiment for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them. Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force. The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

In some embodiments, the expression construct is delivered directly to the liver, skin, and/or muscle tissue of a subject. This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ, *i.e.*, *ex vivo* treatment. Again, DNA encoding a particular gene may be delivered via this method and still be incorporated by the present disclosure.

In a further embodiment, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess

of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers. Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. A reagent known as Lipofectamine 2000™ is widely used and commercially available.

In certain embodiments, the liposome may be complexed with a hemagglutinating virus (HVJ), to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA. In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present disclosure. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Other expression constructs which can be employed to deliver a nucleic acid encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific.

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) and transferrin. A synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells.

IV. Methods of Making Transgenic Mice

A particular embodiment provides transgenic animals that contain mutations in the dystrophin gene. Also, transgenic animals may express a marker that reflects the production of mutant or normal dystrophin gene product.

In a general aspect, a transgenic animal is produced by the integration of a given construct into the genome in a manner that permits the expression of the transgene using methods discussed above. Methods for producing transgenic animals are generally described by Wagner and Hoppe (U.S. Pat. No. 4,873,191; incorporated herein by reference), and Brinster *et al.* (1985; incorporated herein by reference).

Typically, the construct is transferred by microinjection into a fertilized egg. The microinjected eggs are implanted into a host female, and the progeny are screened for the expression of the transgene. Transgenic animals may be produced from the fertilized eggs from a number of animals including, but not limited to reptiles, amphibians, birds, mammals, and fish.

DNA for microinjection can be prepared by any means known in the art. For example, DNA for microinjection can be cleaved with enzymes appropriate for removing the bacterial plasmid sequences, and the DNA fragments electrophoresed on 1% agarose gels in TBE buffer, using standard techniques. The DNA bands are visualized by staining with ethidium bromide, and the band containing the expression sequences is excised. The excised band is then placed in dialysis bags containing 0.3 M sodium acetate, pH 7.0. DNA is electroeluted into the dialysis bags, extracted with a 1:1 phenol:chloroform solution and precipitated by two volumes of ethanol. The DNA is redissolved in 1 ml of low salt buffer (0.2 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) and purified on an Elutip-D® column. The column is first primed with 3 ml of high salt buffer (1 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) followed by washing with 5 ml of low salt buffer. The DNA solutions are passed through the column three times to bind DNA to the column matrix. After one wash with 3 ml of low salt buffer, the DNA is eluted with 0.4 ml high salt buffer and precipitated by two volumes of ethanol. DNA concentrations are measured by absorption at 260 nm in a UV spectrophotometer. For microinjection, DNA concentrations are adjusted to 3 μg/ml in 5 mM Tris, pH 7.4 and 0.1 mM EDTA. Other methods for purification of DNA for microinjection known to those of skill in the art may be used.

In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO.sub.2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's

phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5% BSA (EBSS) in a 37.5.degree. C. incubator with a humidified atmosphere at 5% CO_2, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Randomly cycling adult female mice are paired with vasectomized males. C57BL/6 or Swiss mice or other comparable strains can be used for this purpose. Recipient females are mated at the same time as donor females. At the time of embryo transfer, the recipient females are anesthetized with an intraperitoneal injection of 0.015 ml of 2.5% avertin per gram of body weight. The oviducts are exposed by a single midline dorsal incision. An incision is then made through the body wall directly over the oviduct. The ovarian bursa is then torn with watchmakers forceps. Embryos to be transferred are placed in DPBS (Dulbecco's phosphate buffered saline) and in the tip of a transfer pipet (about 10 to 12 embryos). The pipet tip is inserted into the infundibulum and the embryos transferred. After the transfer, the incision is closed by two sutures.

VI. Mouse Models of DMD

Provided herein is a novel mouse model of DMD, and methods of making the same. The instant disclosure can be used to produce novel mouse models for various DMD mutations.

In some embodiments, the mice are generated using a CRISPR/Cas9 or a CRISPR/Cpf1 system. In embodiments, a single gRNA is used to delete or modify a target DNA sequence. In embodiments, two or more gRNAs are used to delete or modify a target DNA sequence. In some embodiments, the target DNA sequence is an intron. In some embodiments, the target DNA sequence is an exon. In embodiments, the target DNA is a splice donor or acceptor site.

In embodiments, the mouse may be generated by first contacting a fertilized oocyte with CRISPR/Cas9 elements and two single guide RNA (sgRNA) targeting sequences flanking an exon of murine dystrophin. In some embodiments, the exon is exon 50, and in some embodiments the targeting sequences are intronic regions surrounding exon 50. Contacting the fertilized oocyte with the CRISPR/Cas9 elements and the two sgRNAs leads to excision of the exon, thereby creating a modified oocyte. For example, deletion of exon 50 by CRISPR/Cas9 results in an out of frame shift and a premature stop codon in exon 51. The modified oocyte is then transferred into a recipient female.

In embodiments, the fertilized oocyte is derived from a wildtype mouse. In embodiments, the fertilized oocyte is derived from a mouse whose genome contains an exogenous reporter gene. In some embodiments, the exogenous reporter gene is luciferase. In some embodiments, the exogenous reporter gene is a fluorescent protein such as GFP. In some embodiments, a reporter gene expression cassette is inserted into the 3' end of the dystrophin gene, so that luciferase is translated in-frame with exon 79 of dystrophin. In some embodiments, a self-cleaving peptide such as protease 2A is engineered at a cleavage site between the dystrophin and the luciferase, so that the reporter will be released from the protein after translation.

In some embodiments, the genetically engineered mice described herein have a mutation in the region between exons 45 to 51 of the dystrophin gene. In embodiments, the genetically engineered mice have a deletion of exon 50 of the dystrophin gene resulting in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene. Deletions and mutations can be confirmed by methods known to those of skill in the art, such as DNA sequencing.

In some embodiments, the genetically engineered mice have a reporter gene. In some embodiments, the reporter gene is located downstream of and in frame with exon 79 of the dystrophin gene, and upstream of a dystrophin 3'-UTR, wherein the reporter gene is expressed when exon 79 is translated in frame with exon 49. In some embodiments, a protease 2A is engineered at a cleavage site between the proteins, which is auto-catalytically cleaved so that the reporter protein is released from dystrophin after translation. In some embodiments, the reporter gene is green fluorescent protein (GFP). In some embodiments, the reporter gene is luciferase.

In embodiments, the mice do not express the dystrophin protein in one or more tissues, for example in skeletal muscle and/or in the heart. In embodiments, the mice exhibit a significant increase of creatine kinase (CK) levels compared to wildtype mice. Elevated CK levels are a sign of muscle damage.

V. Pharmaceutical Compositions and Delivery Methods

For clinical applications, pharmaceutical compositions are prepared in a form appropriate for the intended application. Generally, thisentails preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

Appropriate salts and buffers are used to render drugs, proteins or delivery vectors stable and allow for uptake by target cells. Aqueous compositions of the present disclosure comprise an effective amount of the drug, vector or proteins, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes solvents, buffers, solutions, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like acceptable for use in formulating pharmaceuticals, such as pharmaceuticals suitable for administration to humans. The use of such media and agents for pharmaceutically active substances is well known in the art. Any conventional media or agent that is not incompatible with the active ingredients of the present disclosure, its use in therapeutic compositions may be used. Supplementary active ingredients also can be incorporated into the compositions, provided they do not inactivate the vectors or cells of the compositions.

In some embodiments, the active compositions of the present disclosure include classic pharmaceutical preparations. Administration of these compositions according to the present disclosure may be via any common route so long as the target tissue is available via that route, but generally including systemic administration. This includes oral, nasal, or buccal. Alternatively, administration may be by intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection, or by direct injection into muscle tissue. Such compositions are normally administered as pharmaceutically acceptable compositions, as described *supra*.

The active compounds may also be administered parenterally or intraperitoneally. By way of illustration, solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as

hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations generally contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include, for example, sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Generally, these preparations are sterile and fluid to the extent that easy injectability exists. Preparations should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Appropriate solvents or dispersion media may contain, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions may be prepared by incorporating the active compounds in an appropriate amount into a solvent along with any other ingredients (for example as enumerated above) as desired, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the desired other ingredients, *e.g.*, as enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation include vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient(s) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

In some embodiments, the compositions of the present disclosure are formulated in a neutral or salt form. Pharmaceutically-acceptable salts include, for example, acid addition salts (formed with the free amino groups of the protein) derived from inorganic acids (*e.g.*,

hydrochloric or phosphoric acids, or from organic acids (*e.g.*, acetic, oxalic, tartaric, mandelic, and the like)). Salts formed with the free carboxyl groups of the protein can also be derived from inorganic bases (*e.g.*, sodium, potassium, ammonium, calcium, or ferric hydroxides) or from organic bases (*e.g.*, isopropylamine, trimethylamine, histidine, procaine and the like.

Upon formulation, solutions are preferably administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations may easily be administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution generally is suitably buffered and the liquid diluent first rendered isotonic for example with sufficient saline or glucose. Such aqueous solutions may be used, for example, for intravenous, intramuscular, subcutaneous and intraperitoneal administration. Preferably, sterile aqueous media are employed as is known to those of skill in the art, particularly in light of the present disclosure. By way of illustration, a single dose may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

In some embodiments, the Cpf1 and gRNAs described herein may be delivered to the patient using adoptive cell transfer (ACT). In adoptive cell transfer, one or more expression constructs are provided ex vivo to cells which have originated from the patient (autologous) or from one or more individual(s) other than the patient (allogeneic). The cells are subsequently introduced or reintroduced into the patient. Thus, in some embodiments, one or more nucleic acids encoding Cpf1 and a guide RNA that targets a dystrophin splice site are provided to a cell ex vivo before the cell is introduced or reintroduced to a patient.

The following tables provide exemplary primer and genomic targeting sequences for use in connection with the compositions and methods disclosed herein.

TABLE C – PRIMER SEQUENCES

	Primer Name		Primer Sequence
Cloning primers for pCpf1-2A-GFP	AgeI-nLbCpf1-F1	F	tttttttcaggttGGaccggtgccaccATGAGCAAGCTGGA (SEQ ID NO: 794)
	nLbCpf1-R1	R	TGGGGTTATAGTAGGCCATCCACTTC (SEQ ID NO: 795)
	nLbCpf1-F2	F	GATGGCCTACTATAACCCCAGCG (SEQ ID NO: 796)
	nLbCpf1-R2	R	GGCATAGTCGGGGACATCATATG (SEQ ID NO: 797)
	AgeI-nAsCpf1-F1	F	tttttttcaggttGGaccggtgccaccATGACACAGTTCGAG (SEQ ID NO: 798)
	nAsCpf1-R1	R	TCCTTCTCAGGATTGTTCAGGTCGTA (SEQ ID NO: 799)
	nAsCpf1-F2	F	CTGAACAATCCTGAGAAGGAGCC (SEQ ID NO: 800)
	nAsCpf1-R2	R	GGCATAGTCGGGGACATCATATG (SEQ ID NO: 801)
	nCpf1-2A-GFP-F	F	ATGATGTCCCCGACTATGCCgaattcGGCAGTGGAGAGGG (SEQ ID NO: 802)
	nCpf1-2A-GFP-R	R	AGCGAGCTCTAGttagaattcCTTGTACAG (SEQ ID NO: 803)
In vitro transcription of	T7-Scaffold-F	F	CACCAGCGCTGCTTAATACGACTCACTATAGGGAAAT (SEQ ID NO: 804)
	T7-Scaffold-R	R	AGTAGCGCTTCTAGACCCTCACTTCCTACTCAG (SEQ ID NO: 18)

LbCpf1 mRNA	T7-nLb-F1	F	AGAAGAAATATAAGACTCGAGgccaccATGAGCAAGCTGGAGAAGTTTAC (SEQ ID NO: 19)
	T7-nLb-R1	R	TGGGGTTATAGTAGGCCATCC (SEQ ID NO: 20)
	T7-nLB-NLS-F2	F	GATGGCCTACTATAACCCCAGCG (SEQ ID NO: 10)
	T7-nLB-NLS-R2	R	CCCGCAGAAGGCAGCGTCGACTTAGGCATAGTCGGGGACATCATATG (SEQ ID NO: 21)
	T7-nAs-F1	F	AGAAGAAATATAAGACTCGAGgccaccATGACACAGTTCGAGGGCTTTAC (SEQ ID NO: 22)
	T7-nAs-R1	R	TCCTTCTCAGGATTGTTCAGGTCGTA (SEQ ID NO: 13)
	T7-nAs-NLS-F2	F	CTGAACAATCCTGAGAAGGAGCC (SEQ ID NO: 14)
	T7-nAs-NLS-R2	R	CCCGCAGAAGGCAGCGTCGACTTAGGCATAGTCGGGGACATCATATG (SEQ ID NO: 21)
Human *DMD* Exon 51 gRNA	nLb-DMD-E51-g1-Top	F	CACCGTAATTTCTACTAAGTGTAGATgCTCCTACTCAGACTGTTACTCTGTTTTTTT (SEQ ID NO: 23)
	nLb-DMD-E51-g1-Bot	R	AAACAAAAAAAACAGAGTAACAGTCTGAGTAGGAGcATCTACACTTAGTAGAAATTAC (SEQ ID NO: 24)
	nLb-DMD-E51-g2-Top	F	CACCGTAATTTCTACTAAGTGTAGATtaccatgtattgctaaacaaagtaTTTTTTT (SEQ ID NO: 25)
	nLb-DMD-E51-g2-Bot	R	AAACAAAAAAAAtactttgttagcaatacatggtaATCTACACTTAGTAGAAATTAC (SEQ ID NO: 26)
	nLb-DMD-E51-g3-Top	F	CACCGTAATTTCTACTAAGTGTAGATattgaagagtaacaatttgagccaTTTTTTT (SEQ ID NO: 27)

	nLb-DMD-E51-g3-Bot	R	AAACAAAAAAAAtggctcaaattgttacttcttcaatATCTACACTTAGTAGAAATTAC (SEQ ID NO: 28)
	nAs-DMD-E51-g1-Top	F	CACCGTAATTTCTACTCTTGTAGATgCTCCTACTCAGACTGTTACTCTGTTTTTTTT (SEQ ID NO: 29)
	nAs-DMD-E51-g1-Bot	R	AAACAAAAAAAACAGAGTAACAGTCTGAGTAGGAGcATCTACAAGAGTAGAAATTAC(SEQ ID NO: 30)
Human *DMD* Exon 51 T7E1	DMD-E51-T7E1-F1	F	Ttcctggcaaggtctga (SEQ ID NO: 31)
	DMD-E51-T7E1-R1	R	ATCCTCAAGGTCACCCACC(SEQ ID NO: 32)
Human cardiomyocytes RT-PCR	Riken51-RT-PCR-F1	F	CCCAGAAGAGCAAGATAAACTTGAA (SEQ ID NO: 789)
	Riken51-RT-PCR-R1	R	CTCTGTTCCAAATCCTGCATTGT (SEQ ID NO: 33)
Human cardiomyocytes mtDNA copy number qPCR	hmtND1-qF1	F	CGCCACATCTACCATCACCCTC (SEQ ID NO: 790)
	hmtND1-qR1	R	CGGCTAGGCTAGAGGTGGCTA (SEQ ID NO: 791)
	hLPL-qF1	F	GAGTATGCAGAAGCCCCGAGTC (SEQ ID NO: 792)
	hLPL-qR1	R	TCAACATGCCCAACTGGTTTCTGG (SEQ ID NO: 793)

	Primer Name		Primer Sequence
Mouse *Dmd* Exon 23 gRNA genomic target sequence	nLb-dmd-E23-g1-Top	F	CACCGTAATTTCTACTAAGTGTAGATaggctctgcaaagttctTTGAAAGTTTTTTT (SEQ ID NO: 34)
	nLb-dmd-E23-g1-Bot	R	AAACAAAAAAACTTTCAAgaacttgcagaggcctATCTACACTTAGTAGAAATTAC (SEQ ID NO: 35)
	nLb-dmd-E23-g2-Top	F	CACCGTAATTTCTACTAAGTGTAGATAAAGAGCAACAAAATGGCttcaacTTTTTTT (SEQ ID NO: 36)
	nLb-dmd-E23-g2-Bot	R	AAACAAAAAAAAgttgaaGCCATTTTGTTGCTCTTTATCTACACTTAGTAGAAATTAC (SEQ ID NO: 37)
	nLb-mdmd-E23-g2-Top	F	CACCGTAATTTCTACTAAGTGTAGATAAAGAGCAATTAAAATGGCttcaacTTTTTTT (SEQ ID NO: 38)
	nLb-mdmd-E23-g2-Bot	R	AAACAAAAAAAAgttgaaGCCATTTTATTGCTCTTTATCTACACTTAGTAGAAATTAC (SEQ ID NO: 39)
	nLb-dmd-E23-g3-Top	F	CACCGTAATTTCTACTAAGTGTAGATAAAGAACTTTGCAGAGCctcaaaaTTTTTTT (SEQ ID NO: 40)
	nLb-dmd-E23-g3-Bot	R	AAACAAAAAAAAttttgagGCTCTGCAAAGTTCTTTATCTACACTTAGTAGAAATTAC (SEQ ID NO: 41)
	nLb-dmd-I22-g1-Top	F	CACCGTAATTTCTACTAAGTGTAGATctgaatatctatgcattaataactTTTTTTT (SEQ ID NO: 42)
	nLb-dmd-I22-g1-Bot	R	AAACAAAAAAAAagtttattaatgcatagatattcagATCTACACTTAGTAGAAATTAC (SEQ ID NO: 43)

	nLb-dmd-I22-g2-Top	F	CACCGTAATTTCTACTAAGTGTAGATtattatattacagggcatattataTTTTTTT (SEQ ID NO: 44)
	nLb-dmd-I22-g2-Bot	R	AAACAAAAAAAAtataaatatgccctgtaatataataATCTACACTTAGTAGAAATTAC (SEQ ID NO: 45)
	nLb-dmd-I23-g3-Top	F	CACCGTAATTTCTACTAAGTGTAGATAGgtaagccgaggtttggcctttaTTTTTTT (SEQ ID NO: 46)
	nLb-dmd-I23-g3-Bot	R	AAACAAAAAAAAtaaaggccaaacctcggcttacCTATCTACACTTAGTAGAAATTAC (SEQ ID NO: 47)
	nLb-dmd-I23-g4-Top	F	CACCGTAATTTCTACTAAGTGTAGATcccagagtccttcaaagatattgaTTTTTTT (SEQ ID NO: 48)
	nLb-dmd-I23-g4-Bot	R	AAACAAAAAAAAtcaatatctttgaaggactctgggATCTACACTTAGTAGAAATTAC (SEQ ID NO: 49)
In vitro transcription of LbCpf1 gRNA genomic target sequence	T7-Lb-dmd-E23-uF	F	GAATTGTAATACGACTCACTATAGGGTAATTTCTACTAAGTGTAGAT (SEQ ID NO: 50)
	T7-Lb-dmd-E23-g1-R	R	CTTTCAAgaacttgcagagcctATCTACACTTAGTAGAAATTA (SEQ ID NO:51)
	T7-Lb-dmd-E23-mg2-R	R	GttgaaGCCATTTTATTGCTCTTTATCTACACTTAGTAGAAATTA (SEQ ID NO:52)
	T7-Lb-dmd-E23-g3-R	R	ttttgagGCTCTGCAAAGTTCTTTATCTACACTTAGTAGAAATTA (SEQ ID NO:53)

	T7-Lb-dmd-I22-g2-R	R	tataatatgccctgtaatataataATCTACACTTAGTAGAAATTACCCTATAGTGAG (SEQ ID NO: 54)
	T7-Lb-dmd-I22-g4-R	R	tcaatatcttgaaggactctgggATCTACACTTAGTAGAAATTACCCTATAGTGAG (SEQ ID NO: 55)
Mouse *Dmd* Exon 23 T7E1	Dmd-E23-T7E1-F729	F	Gagaaacttctgtgatgtgaggacata (SEQ ID NO: 56)
	Dmd-E23-T7E1-R1	R	CAAACCTCGGCTTACCTGAAAT (SEQ ID NO: 57)
	Dmd-E23-T7E1-R729	R	Caatatctttgaaggactctgggtaaa (SEQ ID NO: 58)
	Dmd-E23-T7E1-R3	R	Aattaatagaagtcaatgtagggaagg (SEQ ID NO: 59)

TABLE D – Genomic Target Sequences

Targeted gRNA Exon	Guide #	Strand	Genomic Target Sequence*	PAM	SEQ ID NO.
Human-Exon 51	4	1	tctttttcttctttttccttttt	tttt	60
Human-Exon 51	5	1	cttttcttctttttccttttG	tttt	61
Human-Exon 51	6	1	tttttcttctttttccttttGC	tttc	62
Human-Exon 51	7	1	tcttctttttccttttGCAAAA	tttt	63
Human-Exon 51	8	1	cttctttttccttttGCAAAAA	tttt	64
Human-Exon 51	9	1	ttctttttccttttGCAAAAAC	tttc	65
Human-Exon 51	10	1	ttcctttttGCAAAAACCCAAAAT	tttt	66
Human-Exon 51	11	1	tcctttttGCAAAAACCCAAAATA	tttt	67
Human-Exon 51	12	1	cctttttGCAAAAACCCAAAATAT	tttt	68
Human-Exon 51	13	1	cttttGCAAAAACCCAAAATATT	tttc	69
Human-Exon 51	14	1	tGCAAAAACCCAAAATATTTTAGC	tttt	70
Human-Exon 51	15	1	GCAAAAACCCAAAATATTTTAGCT	tttt	71
Human-Exon 51	16	1	CAAAAACCCAAAATATTTTAGCTC	tttG	72
Human-Exon 51	17	1	AGCTCCTACTCAGACTGTTACTCT	TTTT	73
Human-Exon 51	18	1	GCTCCTACTCAGACTGTTACTCTG	TTTA	74
Human-Exon 51	19	-1	CTTAGTAACCACAGGTTGTGTCAC	TTTC	75
Human-Exon 51	20	-1	GAGATGGCAGTTTCCTTAGTAACC	TTTG	76
Human-Exon 51	21	-1	TAGTTTGGAGATGGCAGTTTCCTT	TTTC	77
Human-Exon 51	22	-1	TTCTCATACCTTCTGCTTGATGAT	TTTT	78
Human-Exon 51	23	-1	TCATTTTTTCTCATACCTTCTGCT	TTTA	79
Human-Exon 51	24	-1	ATCATTTTTTCTCATACCTTCTGC	TTTT	80
Human-Exon 51	25	-1	AAGAAAAACTTCTGCCAACTTTTA	TTTA	81
Human-Exon 51	26	-1	AAAGAAAAACTTCTGCCAACTTTT	TTTT	82
Human-Exon 51	27	1	TCTTTAAAATGAAGATTTTCCACC	TTTT	83
Human-Exon 51	28	1	CTTTAAAATGAAGATTTTCCACCA	TTTT	84

Human-Exon 51	29	1	TTTAAAATGAAGATTTTCCACCAA	TTTC	85
Human-Exon 51	30	1	AAATGAAGATTTTCCACCAATCAC	TTTA	86
Human-Exon 51	31	1	CCACCAATCACTTTACTCTCCTAG	TTTT	87
Human-Exon 51	32	1	CACCAATCACTTTACTCTCCTAGA	TTTC	88
Human-Exon 51	33	1	CTCTCCTAGACCATTTCCCACCAG	TTTA	89
Human-Exon 45	1	-1	agaaaagattaaacagtgtgctac	tttg	90
Human-Exon 45	2	-1	tttgagaaaagattaaacagtgtg	TTTa	91
Human-Exon 45	3	-1	atttgagaaaagattaaacagtgt	TTTT	92
Human-Exon 45	4	-1	Tatttgagaaaagattaaacagtg	TTTT	93
Human-Exon 45	5	1	atcttttctcaaatAAAAAGACAT	ttta	94
Human-Exon 45	6	1	ctcaaatAAAAAGACATGGGGCTT	tttt	95
Human-Exon 45	7	1	tcaaatAAAAAGACATGGGGCTTC	tttc	96
Human-Exon 45	8	1	TGTTTTGCCTTTTTGGTATCTTAC	TTTT	97
Human-Exon 45	9	1	GTTTTGCCTTTTTGGTATCTTACA	TTTT	98
Human-Exon 45	10	1	TTTTGCCTTTTTGGTATCTTACAG	TTTG	99
Human-Exon 45	11	1	GCCTTTTTGGTATCTTACAGGAAC	TTTT	100
Human-Exon 45	12	1	CCTTTTTGGTATCTTACAGGAACT	TTTG	101
Human-Exon 45	13	1	TGGTATCTTACAGGAACTCCAGGA	TTTT	102
Human-Exon 45	14	1	GGTATCTTACAGGAACTCCAGGAT	TTTT	103
Human-Exon 45	15	-1	AGGATTGCTGAATTATTTCTTCCC	TTTG	104
Human-Exon 45	16	-1	GAGGATTGCTGAATTATTTCTTCC	TTTT	105
Human-Exon 45	17	-1	TGAGGATTGCTGAATTATTTCTTC	TTTT	106
Human-Exon 45	18	-1	CTGTAGAATACTGGCATCTGTTTT	TTTC	107
Human-Exon 45	19	-1	CCTGTAGAATACTGGCATCTGTTT	TTTT	108
Human-Exon 45	20	-1	TCCTGTAGAATACTGGCATCTGTT	TTTT	109
Human-Exon 45	21	-1	CAGACCTCCTGCCACCGCAGATTC	TTTG	110
Human-Exon 45	22	-1	TGTCTGACAGCTGTTTGCAGACCT	TTTC	111
Human-Exon 45	23	-1	CTGTCTGACAGCTGTTTGCAGACC	TTTT	112

Human-Exon 45	24	-1	TCTGTCTGACAGCTGTTTGCAGAC	TTTT	113
Human-Exon 45	25	-1	TTCTGTCTGACAGCTGTTTGCAGA	TTTT	114
Human-Exon 45	26	-1	ATTCCTATTAGATCTGTCGCCCTA	TTTC	115
Human-Exon 45	27	-1	CATTCCTATTAGATCTGTCGCCCT	TTTT	116
Human-Exon 45	28	1	AGCAGACTTTTTAAGCTTTCTTTA	TTTT	117
Human-Exon 45	29	1	GCAGACTTTTTAAGCTTTCTTTAG	TTTA	118
Human-Exon 45	30	1	TAAGCTTTCTTTAGAAGAATATTT	TTTT	119
Human-Exon 45	31	1	AAGCTTTCTTTAGAAGAATATTTC	TTTT	120
Human-Exon 45	32	1	AGCTTTCTTTAGAAGAATATTTCA	TTTA	121
Human-Exon 45	33	1	TTTAGAAGAATATTTCATGAGAGA	TTTC	122
Human-Exon 45	34	1	GAAGAATATTTCATGAGAGATTAT	TTTA	123
Human-Exon 44	1	1	TCAGTATAACCAAAAAATATACGC	TTTG	124
Human-Exon 44	2	1	acataatccatctatttttcttga	tttt	125
Human-Exon 44	3	1	cataatccatctatttttcttgat	ttta	126
Human-Exon 44	4	1	tcttgatccatatgctttACCTG	tttt	127
Human-Exon 44	5	1	cttgatccatatgctttACCTGC	tttt	128
Human-Exon 44	6	1	ttgatccatatgctttACCTGCA	tttc	129
Human-Exon 44	7	-1	TCAACAGATCTGTCAAATCGCCTG	TTTC	130
Human-Exon 44	8	1	ACCTGCAGGCGATTTGACAGATCT	tttt	131
Human-Exon 44	9	1	CCTGCAGGCGATTTGACAGATCTG	tttA	132
Human-Exon 44	10	1	ACAGATCTGTTGAGAAATGGCGGC	TTTG	133
Human-Exon 44	11	-1	TATCATAATGAAAACGCCGCCATT	TTTA	134
Human-Exon 44	12	1	CATTATGATATAAAGATATTTAAT	TTTT	135
Human-Exon 44	13	-1	TATTTAGCATGTTCCCAATTCTCA	TTTG	136
Human-Exon 44	14	-1	GAAAAAACAAATCAAAGACTTACC	TTTC	137
Human-Exon 44	15	1	ATTTGTTTTTTCGAAATTGTATTT	TTTG	138
Human-Exon 44	16	1	TTTTTTCGAAATTGTATTTATCTT	TTTG	139
Human-Exon 44	17	1	TTCGAAATTGTATTTATCTTCAGC	TTTT	140

Human-Exon 44	18	1	TCGAAATTGTATTTATCTTCAGCA	TTTT	141
Human-Exon 44	19	1	CGAAATTGTATTTATCTTCAGCAC	TTTT	142
Human-Exon 44	20	1	GAAATTGTATTTATCTTCAGCACA	TTTC	143
Human-Exon 44	21	-1	AGAAGTTAAAGAGTCCAGATGTGC	TTTA	144
Human-Exon 44	22	1	TCTTCAGCACATCTGGACTCTTTA	TTTA	145
Human-Exon 44	23	-1	CATCACCCTTCAGAACCTGATCTT	TTTC	146
Human-Exon 44	24	1	ACTTCTTAAAGATCAGGTTCTGAA	TTTA	147
Human-Exon 44	25	1	GACTGTTGTTGTCATCATTATATT	TTTT	148
Human-Exon 44	26	1	ACTGTTGTTGTCATCATTATATTA	TTTG	149
Human-Exon 53	1	-1	AACTAGAATAAAAGGAAAAATAAA	TTTC	150
Human-Exon 53	2	1	CTACTATATATTTATTTTTCCTTT	TTTA	151
Human-Exon 53	3	1	TTTTTCCTTTTATTCTAGTTGAAA	TTTA	152
Human-Exon 53	4	1	TCCTTTTATTCTAGTTGAAAGAAT	TTTT	153
Human-Exon 53	5	1	CCTTTTATTCTAGTTGAAAGAATT	TTTT	154
Human-Exon 53	6	1	CTTTTATTCTAGTTGAAAGAATTC	TTTC	155
Human-Exon 53	7	1	ATTCTAGTTGAAAGAATTCAGAAT	TTTT	156
Human-Exon 53	8	1	TTCTAGTTGAAAGAATTCAGAATC	TTTA	157
Human-Exon 53	9	-1	ATTCAACTGTTGCCTCCGGTTCTG	TTTC	158
Human-Exon 53	10	-1	ACATTTCATTCAACTGTTGCCTCC	TTTA	159
Human-Exon 53	11	-1	CTTTTGGATTGCATCTACTGTATA	TTTT	160
Human-Exon 53	12	-1	TGTGATTTTCTTTTGGATTGCATC	TTTC	161
Human-Exon 53	13	-1	ATACTAACCTTGGTTTCTGTGATT	TTTG	162
Human-Exon 53	14	-1	AAAAGGTATCTTTGATACTAACCT	TTTA	163
Human-Exon 53	15	-1	AAAAAGGTATCTTTGATACTAACC	TTTT	164
Human-Exon 53	16	-1	TTTTAAAAAGGTATCTTTGATACT	TTTA	165
Human-Exon 53	17	-1	ATTTTAAAAAGGTATCTTTGATAC	TTTT	166
Human-Exon 46	1	-1	TTAATGCAAACTGGGACACAAACA	TTTG	167
Human-Exon 46	2	1	TAAATTGCCATGTTTGTGTCCCAG	TTTT	168

Human-Exon 46	3	1	AAATTGCCATGTTTGTGTCCCAGT	TTTT	169
Human-Exon 46	4	1	AATTGCCATGTTTGTGTCCCAGTT	TTTA	170
Human-Exon 46	5	1	TGTCCCAGTTTGCATTAACAAATA	TTTG	171
Human-Exon 46	6	-1	CAACATAGTTCTCAAACTATTTGT	tttC	172
Human-Exon 46	7	-1	CCAACATAGTTCTCAAACTATTTG	tttt	173
Human-Exon 46	8	-1	tCCAACATAGTTCTCAAACTATTT	tttt	174
Human-Exon 46	9	-1	tttCCAACATAGTTCTCAAACTAT	tttt	175
Human-Exon 46	10	-1	ttttCCAACATAGTTCTCAAACTA	tttt	176
Human-Exon 46	11	-1	tttttCCAACATAGTTCTCAAACT	tttt	177
Human-Exon 46	12	1	CATTAACAAATAGTTTGAGAACTA	TTTG	178
Human-Exon 46	13	1	AGAACTATGTTGGaaaaaaaaaTA	TTTG	179
Human-Exon 46	14	-1	GTTCTTCTAGCCTGGAGAAAGAAG	TTTT	180
Human-Exon 46	15	1	ATTCTTCTTTCTCCAGGCTAGAAG	TTTT	181
Human-Exon 46	16	1	TTCTTCTTTCTCCAGGCTAGAAGA	TTTA	182
Human-Exon 46	17	1	TCCAGGCTAGAAGAACAAAAGAAT	TTTC	183
Human-Exon 46	18	-1	AAATTCTGACAAGATATTCTTTTG	TTTG	184
Human-Exon 46	19	-1	CTTTTAGTTGCTGCTCTTTTCCAG	TTTT	185
Human-Exon 46	20	-1	AGAAAATAAAATTACCTTGACTTG	TTTG	186
Human-Exon 46	21	-1	TGCAAGCAGGCCCTGGGGGATTTG	TTTA	187
Human-Exon 46	22	1	ATTTTCTCAAATCCCCCAGGGCCT	TTTT	188
Human-Exon 46	23	1	TTTTCTCAAATCCCCCAGGGCCTG	TTTA	189
Human-Exon 46	24	1	CTCAAATCCCCCAGGGCCTGCTTG	TTTT	190
Human-Exon 46	25	1	TCAAATCCCCCAGGGCCTGCTTGC	TTTC	191
Human-Exon 46	26	1	TTAATTCAATCATTGGTTTTCTGC	TTTT	192
Human-Exon 46	27	1	TAATTCAATCATTGGTTTTCTGCC	TTTT	193
Human-Exon 46	28	1	AATTCAATCATTGGTTTTCTGCCC	TTTT	194
Human-Exon 46	29	1	ATTCAATCATTGGTTTTCTGCCCA	TTTA	195
Human-Exon 46	30	-1	GCAAGGAACTATGAATAACCTAAT	TTTA	196

Human-Exon 46	31	1	CTGCCCATTAGGTTATTCATAGTT	TTTT	197
Human-Exon 46	32	1	TGCCCATTAGGTTATTCATAGTTC	TTTC	198
Human-Exon 52	1	-1	TAGAAAACAATTTAACAGGAAATA	TTTA	199
Human-Exon 52	2	1	CTGTTAAATTGTTTTCTATAAACC	TTTC	200
Human-Exon 52	3	-1	GAAATAAAAAAGATGTTACTGTAT	TTTA	201
Human-Exon 52	4	-1	AGAAATAAAAAAGATGTTACTGTA	TTTT	202
Human-Exon 52	5	1	CTATAAACCCTTATACAGTAACAT	TTTT	203
Human-Exon 52	6	1	TATAAACCCTTATACAGTAACATC	TTTC	204
Human-Exon 52	7	1	TTATTTCTAAAAGTGTTTTGGCTG	TTTT	205
Human-Exon 52	8	1	TATTTCTAAAAGTGTTTTGGCTGG	TTTT	206
Human-Exon 52	9	1	ATTTCTAAAAGTGTTTTGGCTGGT	TTTT	207
Human-Exon 52	10	1	TTTCTAAAAGTGTTTTGGCTGGTC	TTTA	208
Human-Exon 52	11	1	TAAAAGTGTTTTGGCTGGTCTCAC	TTTC	209
Human-Exon 52	12	-1	CATAATACAAAGTAAAGTACAATT	TTTA	210
Human-Exon 52	13	-1	ACATAATACAAAGTAAAGTACAAT	TTTT	211
Human-Exon 52	14	1	GGCTGGTCTCACAATTGTACTTTA	TTTT	212
Human-Exon 52	15	1	GCTGGTCTCACAATTGTACTTTAC	TTTG	213
Human-Exon 52	16	1	CTTTGTATTATGTAAAAGGAATAC	TTTA	214
Human-Exon 52	17	1	TATTATGTAAAAGGAATACACAAC	TTTG	215
Human-Exon 52	18	1	TTCTTACAGGCAACAATGCAGGAT	TTTG	216
Human-Exon 52	19	1	GAACAGAGGCGTCCCCAGTTGGAA	TTTG	217
Human-Exon 52	20	-1	GGCAGCGGTAATGAGTTCTTCCAA	TTTG	218
Human-Exon 52	21	-1	TCAAATTTTGGGCAGCGGTAATGA	TTTT	219
Human-Exon 52	22	1	AAAAACAAGACCAGCAATCAAGAG	TTTG	220
Human-Exon 52	23	-1	TGTGTCCCATGCTTGTTAAAAAAC	TTTG	221
Human-Exon 52	24	1	TTAACAAGCATGGGACACACAAAG	TTTT	222
Human-Exon 52	25	1	TAACAAGCATGGGACACACAAAGC	TTTT	223
Human-Exon 52	26	1	AACAAGCATGGGACACACAAAGCA	TTTT	224

Human-Exon 52	27	1	ACAAGCATGGGACACACAAAGCAA	TTTA	225
Human-Exon 52	28	-1	TTGAAACTTGTCATGCATCTTGCT	TTTA	226
Human-Exon 52	29	-1	ATTGAAACTTGTCATGCATCTTGC	TTTT	227
Human-Exon 52	30	-1	TATTGAAACTTGTCATGCATCTTG	TTTT	228
Human-Exon 52	31	1	AATAAAAACTTAAGTTCATATATC	TTTC	229
Human-Exon 50	1	-1	GTGAATATATTATTGGATTTCTAT	TTTG	230
Human-Exon 50	2	-1	AAGATAATTCATGAACATCTTAAT	TTTG	231
Human-Exon 50	3	-1	ACAGAAAAGCATACACATTACTTA	TTTA	232
Human-Exon 50	4	1	CTGTTAAAGAGGAAGTTAGAAGAT	TTTT	233
Human-Exon 50	5	1	TGTTAAAGAGGAAGTTAGAAGATC	TTTC	234
Human-Exon 50	6	-1	CCGCCTTCCACTCAGAGCTCAGAT	TTTA	235
Human-Exon 50	7	-1	CCCTCAGCTCTTGAAGTAAACGGT	TTTG	236
Human-Exon 50	8	1	CTTCAAGAGCTGAGGGCAAAGCAG	TTTA	237
Human-Exon 50	9	-1	AACAAATAGCTAGAGCCAAAGAGA	TTTG	238
Human-Exon 50	10	-1	GAACAAATAGCTAGAGCCAAAGAG	TTTT	239
Human-Exon 50	11	1	GCTCTAGCTATTTGTTCAAAAGTG	TTTG	240
Human-Exon 50	12	1	TTCAAAAGTGCAACTATGAAGTGA	TTTG	241
Human-Exon 50	13	-1	TCTCTCACCCAGTCATCACTTCAT	TTTC	242
Human-Exon 50	14	-1	CTCTCTCACCCAGTCATCACTTCA	TTTT	243
Human-Exon 43	1	1	tatatatatatatTTTTCTCTT	TTTG	244
Human-Exon 43	2	1	TCTCTTTCTATAGACAGCTAATTC	tTTT	245
Human-Exon 43	3	1	CTCTTTCTATAGACAGCTAATTCA	TTTT	246
Human-Exon 43	4	-1	AAACAGTAAAAAAATGAATTAGCT	TTTA	247
Human-Exon 43	5	1	TCTTTCTATAGACAGCTAATTCAT	TTTC	248
Human-Exon 43	6	-1	AAAACAGTAAAAAAATGAATTAGC	TTTT	249
Human-Exon 43	7	1	TATAGACAGCTAATTCATTTTTTT	TTTC	250
Human-Exon 43	8	-1	TATTCTGTAATATAAAAATTTTAA	TTTA	251
Human-Exon 43	9	-1	ATATTCTGTAATATAAAAATTTTA	TTTT	252

Human-Exon 43	10	1	TTTACTGTTTTAAAATTTTTATAT	TTTT	253
Human-Exon 43	11	1	TTACTGTTTTAAAATTTTTATATT	TTTT	254
Human-Exon 43	12	1	TACTGTTTTAAAATTTTTATATTA	TTTT	255
Human-Exon 43	13	1	ACTGTTTTAAAATTTTTATATTAC	TTTT	256
Human-Exon 43	14	1	CTGTTTTAAAATTTTTATATTACA	TTTA	257
Human-Exon 43	15	1	AAAATTTTTATATTACAGAATATA	TTTT	258
Human-Exon 43	16	1	AAATTTTTATATTACAGAATATAA	TTTA	259
Human-Exon 43	17	-1	TTGTAGACTATCTTTTATATTCTG	TTTG	260
Human-Exon 43	18	1	TATATTACAGAATATAAAAGATAG	TTTT	261
Human-Exon 43	19	1	ATATTACAGAATATAAAAGATAGT	TTTT	262
Human-Exon 43	20	1	TATTACAGAATATAAAAGATAGTC	TTTA	263
Human-Exon 43	21	-1	CAATGCTGCTGTCTTCTTGCTATG	TTTG	264
Human-Exon 43	22	1	CAATGGGAAAAAGTTAACAAAATG	TTTC	265
Human-Exon 43	23	-1	TGCAAGTATCAAGAAAAATATATG	TTTC	266
Human-Exon 43	24	1	TCTTGATACTTGCAGAAATGATTT	TTTT	267
Human-Exon 43	25	1	CTTGATACTTGCAGAAATGATTTG	TTTT	268
Human-Exon 43	26	1	TTGATACTTGCAGAAATGATTTGT	TTTC	269
Human-Exon 43	27	1	TTTTCAGGGAACTGTAGAATTTAT	TTTG	270
Human-Exon 43	28	-1	CATGGAGGGTACTGAAATAAATTC	TTTC	271
Human-Exon 43	29	-1	CCATGGAGGGTACTGAAATAAATT	TTTT	272
Human-Exon 43	30	1	CAGGGAACTGTAGAATTTATTTCA	TTTT	273
Human-Exon 43	31	-1	TCCATGGAGGGTACTGAAATAAAT	TTTT	274
Human-Exon 43	32	1	AGGGAACTGTAGAATTTATTTCAG	TTTC	275
Human-Exon 43	33	-1	TTCCATGGAGGGTACTGAAATAAA	TTTT	276
Human-Exon 43	34	-1	CCTGTCTTTTTTCCATGGAGGGTA	TTTC	277
Human-Exon 43	35	-1	CCCTGTCTTTTTTCCATGGAGGGT	TTTT	278
Human-Exon 43	36	-1	TCCCTGTCTTTTTTCCATGGAGGG	TTTT	279
Human-Exon 43	37	1	TTTCAGTACCCTCCATGGAAAAAA	TTTA	280

Human-Exon 43	38	1	AGTACCCTCCATGGAAAAAAGACA	TTTC	281
Human-Exon 6	1	1	AGTTTGCATGGTTCTTGCTCAAGG	TTTA	282
Human-Exon 6	2	-1	ATAAGAAAATGCATTCCTTGAGCA	TTTC	283
Human-Exon 6	3	-1	CATAAGAAAATGCATTCCTTGAGC	TTTT	284
Human-Exon 6	4	1	CATGGTTCTTGCTCAAGGAATGCA	TTTG	285
Human-Exon 6	5	-1	ACCTACATGTGGAAATAAATTTTC	TTTG	286
Human-Exon 6	6	-1	GACCTACATGTGGAAATAAATTTT	TTTT	287
Human-Exon 6	7	-1	TGACCTACATGTGGAAATAAATTT	TTTT	288
Human-Exon 6	8	1	CTTATGAAAATTTATTTCCACATG	TTTT	289
Human-Exon 6	9	1	TTATGAAAATTTATTTCCACATGT	TTTC	290
Human-Exon 6	10	-1	ATTACATTTTTGACCTACATGTGG	TTTC	291
Human-Exon 6	11	-1	CATTACATTTTTGACCTACATGTG	TTTT	292
Human-Exon 6	12	-1	TCATTACATTTTTGACCTACATGT	TTTT	293
Human-Exon 6	13	1	TTTCCACATGTAGGTCAAAAATGT	TTTA	294
Human-Exon 6	14	1	CACATGTAGGTCAAAAATGTAATG	TTTC	295
Human-Exon 6	15	-1	TTGCAATCCAGCCATGATATTTTT	TTTG	296
Human-Exon 6	16	-1	ACTGTTGGTTTGTTGCAATCCAGC	TTTC	297
Human-Exon 6	17	-1	CACTGTTGGTTTGTTGCAATCCAG	TTTT	298
Human-Exon 6	18	1	AATGCTCTCATCCATAGTCATAGG	TTTG	299
Human-Exon 6	19	-1	ATGTCTCAGTAATCTTCTTACCTA	TTTA	300
Human-Exon 6	20	-1	CAAGTTATTTAATGTCTCAGTAAT	TTTA	301
Human-Exon 6	21	-1	ACAAGTTATTTAATGTCTCAGTAA	TTTT	302
Human-Exon 6	22	1	GACTCTGATGACATATTTTTCCCC	TTTA	303
Human-Exon 6	23	1	TCCCCAGTATGGTTCCAGATCATG	TTTT	304
Human-Exon 6	24	1	CCCCAGTATGGTTCCAGATCATGT	TTTT	305
Human-Exon 6	25	1	CCCAGTATGGTTCCAGATCATGTC	TTTC	306
Human-Exon 7	1	1	TATTTGTCTTtgtgtatgtgtgta	TTTA	307
Human-Exon 7	2	1	TCTTtgtgtatgtgtgtatgtgta	TTTG	308

Human-Exon 7	3	1	tgtatgtgtgtatgtgtatgtgtt	TTtg	309
Human-Exon 7	4	1	AGGCCAGACCTATTTGACTGGAAT	ttTT	310
Human-Exon 7	5	1	GGCCAGACCTATTTGACTGGAATA	tTTA	311
Human-Exon 7	6	1	ACTGGAATAGTGTGGTTTGCCAGC	TTTG	312
Human-Exon 7	7	1	CCAGCAGTCAGCCACACAACGACT	TTTG	313
Human-Exon 7	8	-1	TCTATGCCTAATTGATATCTGGCG	TTTC	314
Human-Exon 7	9	-1	CCAACCTTCAGGATCGAGTAGTTT	TTTA	315
Human-Exon 7	10	1	TGGACTACCACTGCTTTTAGTATG	TTTC	316
Human-Exon 7	11	1	AGTATGGTAGAGTTTAATGTTTTC	TTTT	317
Human-Exon 7	12	1	GTATGGTAGAGTTTAATGTTTTCA	TTTA	318
Human-Exon 8	1	-1	AGACTCTAAAAGGATAATGAACAA	TTTG	319
Human-Exon 8	2	1	ACTTTGATTTGTTCATTATCCTTT	TTTA	320
Human-Exon 8	3	-1	TATATTTGAGACTCTAAAAGGATA	TTTC	321
Human-Exon 8	4	1	ATTTGTTCATTATCCTTTTAGAGT	TTTG	322
Human-Exon 8	5	-1	GTTTCTATATTTGAGACTCTAAAA	TTTG	323
Human-Exon 8	6	-1	GGTTTCTATATTTGAGACTCTAAA	TTTT	324
Human-Exon 8	7	-1	TGGTTTCTATATTTGAGACTCTAA	TTTT	325
Human-Exon 8	8	1	TTCATTATCCTTTTAGAGTCTCAA	TTTG	326
Human-Exon 8	9	1	AGAGTCTCAAATATAGAAACCAAA	TTTT	327
Human-Exon 8	10	1	GAGTCTCAAATATAGAAACCAAAA	TTTA	328
Human-Exon 8	11	-1	CACTTCCTGGATGGCTTCAATGCT	TTTC	329
Human-Exon 8	12	1	GCCTCAACAAGTGAGCATTGAAGC	TTTT	330
Human-Exon 8	13	1	CCTCAACAAGTGAGCATTGAAGCC	TTTG	331
Human-Exon 8	14	-1	GGTGGCCTTGGCAACATTTCCACT	TTTA	332
Human-Exon 8	15	-1	GTCACTTTAGGTGGCCTTGGCAAC	TTTA	333
Human-Exon 8	16	-1	ATGATGTAACTGAAAATGTTCTTC	TTTG	334
Human-Exon 8	17	-1	CCTGTTGAGAATAGTGCATTTGAT	TTTA	335
Human-Exon 8	18	1	CAGTTACATCATCAAATGCACTAT	TTTT	336

Human-Exon 8	19	1	AGTTACATCATCAAATGCACTATT	TTTC	337
Human-Exon 8	20	-1	CACACTTTACCTGTTGAGAATAGT	TTTA	338
Human-Exon 8	21	1	CTGTTTTATATGCATTTTTAGGTA	TTTT	339
Human-Exon 8	22	1	TGTTTTATATGCATTTTTAGGTAT	TTTC	340
Human-Exon 8	23	1	ATATGCATTTTTAGGTATTACGTG	TTTT	341
Human-Exon 8	24	1	TATGCATTTTTAGGTATTACGTGC	TTTA	342
Human-Exon 8	25	1	TAGGTATTACGTGCACatatatat	TTTT	343
Human-Exon 8	26	1	AGGTATTACGTGCACatatatata	TTTT	344
Human-Exon 8	27	1	GGTATTACGTGCACatatatatat	TTTA	345
Human-Exon 55	1	-1	AGCAACAACTATAATATTGTGCAG	TTTA	346
Human-Exon 55	2	1	GTTCCTCCATCTTTCTCTTTTTAT	TTTA	347
Human-Exon 55	3	1	TCTTTTTATGGAGTTCACTAGGTG	TTTC	348
Human-Exon 55	4	1	TATGGAGTTCACTAGGTGCACCAT	TTTT	349
Human-Exon 55	5	1	ATGGAGTTCACTAGGTGCACCATT	TTTT	350
Human-Exon 55	6	1	TGGAGTTCACTAGGTGCACCATTC	TTTA	351
Human-Exon 55	7	1	ATAATTGCATCTGAACATTTGGTC	TTTA	352
Human-Exon 55	8	1	GTCCTTTGCAGGGTGAGTGAGCGA	TTTG	353
Human-Exon 55	9	-1	TTCCAAAGCAGCCTCTCGCTCACT	TTTC	354
Human-Exon 55	10	1	CAGGGTGAGTGAGCGAGAGGCTGC	TTTG	355
Human-Exon 55	11	1	GAAGAAACTCATAGATTACTGCAA	TTTG	356
Human-Exon 55	12	-1	CAGGTCCAGGGGGAACTGTTGCAG	TTTC	357
Human-Exon 55	13	-1	CCAGGTCCAGGGGGAACTGTTGCA	TTTT	358
Human-Exon 55	14	-1	AGCTTCTGTAAGCCAGGCAAGAAA	TTTC	359
Human-Exon 55	15	1	TTGCCTGGCTTACAGAAGCTGAAA	TTTC	360
Human-Exon 55	16	-1	CTTACGGGTAGCATCCTGTAGGAC	TTTC	361
Human-Exon 55	17	-1	CTCCCTTGGAGTCTTCTAGGAGCC	TTTA	362
Human-Exon 55	18	-1	ACTCCCTTGGAGTCTTCTAGGAGC	TTTT	363
Human-Exon 55	19	-1	ATCAGCTCTTTTACTCCCTTGGAG	TTTC	364

Human-Exon 55	20	1	CGCTTTAGCACTCTTGTGGATCCA	TTTC	365
Human-Exon 55	21	1	GCACTCTTGTGGATCCAATTGAAC	TTTA	366
Human-Exon 55	22	-1	TCCCTGGCTTGTCAGTTACAAGTA	TTTG	367
Human-Exon 55	23	-1	GTCCCTGGCTTGTCAGTTACAAGT	TTTT	368
Human-Exon 55	24	-1	TTTTGTCCCTGGCTTGTCAGTTAC	TTTG	369
Human-Exon 55	25	-1	GTTTTGTCCCTGGCTTGTCAGTTA	TTTT	370
Human-Exon 55	26	1	TACTTGTAACTGACAAGCCAGGGA	TTTG	371
Human-G1-exon51		1	gCTCCTACTCAGACTGTTACTCTG	TTTA	372
Human-G2-exon51		1	taccatgtattgctaaacaaagta	TTTC	373
Human-G3-exon51		-1	attgaagagtaacaatttgagcca	TTTA	374
mouse-Exon23-G1		1	aggctctgcaaagttctTTGAAAG	TTTG	375
mouse-Exon23-G2		1	AAAGAGCAACAAAATGGCttcaac	TTTG	376
mouse-Exon23-G3		1	AAAGAGCAATAAAATGGCttcaac	TTTG	377
mouse-Exon23-G4		-1	AAAGAACTTTGCAGAGCctcaaaa	TTTC	378
mouse-Exon23-G5		-1	ctgaatatctatgcattaataact	TTTA	379
mouse-Exon23-G6		-1	tattatattacagggcatattata	TTTC	380
mouse-Exon23-G7		1	Aggtaagccgaggtttggccttta	TTTC	381
mouse-Exon23-G8		1	cccagagtccttcaaagatattga	TTTA	382

* In this table, upper case letters represent nucleotides that align to the exon sequence of the gene. Lower case letters represent nucleotides that align to the intron sequence of the gene.

TABLE E – gRNA sequences

Targeted gRNA Exon	Guide #	Strand	gRNA sequence*	PAM	SEQ ID NO.
Human-Exon 51	4	1	aaaaaggaaaaaagaagaaaaaga	tttt	448
Human-Exon 51	5	1	Caaaaaggaaaaaagaagaaaaag	tttt	449
Human-Exon 51	6	1	GCaaaaaggaaaaaagaagaaaaa	tttc	450
Human-Exon 51	7	1	UUUUGCaaaaaggaaaaaagaaga	tttt	451
Human-Exon 51	8	1	UUUUUGCaaaaaggaaaaaagaag	tttt	452
Human-Exon 51	9	1	GUUUUUGCaaaaaggaaaaaagaa	tttc	453
Human-Exon 51	10	1	AUUUUGGGUUUUUGCaaaaaggaa	tttt	454
Human-Exon 51	11	1	UAUUUUGGGUUUUUGCaaaaagga	tttt	455
Human-Exon 51	12	1	AUAUUUUGGGUUUUUGCaaaaagg	tttt	456
Human-Exon 51	13	1	AAUAUUUUGGGUUUUUGCaaaaag	tttc	457
Human-Exon 51	14	1	GCUAAAAUAUUUUGGGUUUUUGCa	tttt	458
Human-Exon 51	15	1	AGCUAAAAUAUUUUGGGUUUUUGC	tttt	459
Human-Exon 51	16	1	GAGCUAAAAUAUUUUGGGUUUUUG	tttG	460
Human-Exon 51	17	1	AGAGUAACAGUCUGAGUAGGAGCU	TTTT	461
Human-Exon 51	18	1	CAGAGUAACAGUCUGAGUAGGAGC	TTTA	462
Human-Exon 51	19	-1	GUGACACAACCUGUGGUUACUAAG	TTTC	463
Human-Exon 51	20	-1	GGUUACUAAGGAAACUGCCAUCU	TTTG	464
Human-Exon 51	21	-1	AAGGAAACUGCCAUCUCCAAACUA	TTTC	465
Human-Exon 51	22	-1	AUCAUCAAGCAGAAGGUAUGAGAA	TTTT	466
Human-Exon 51	23	-1	AGCAGAAGGUAUGAGAAAAAAUGA	TTTA	467
Human-Exon 51	24	-1	GCAGAAGGUAUGAGAAAAAAUGAU	TTTT	468
Human-Exon 51	25	-1	UAAAAGUUGGCAGAAGUUUUUCUU	TTTA	469

Human-Exon 51	26	-1	AAAAGUUGGCAGAAGUUUUUCUUU	TTTT	470
Human-Exon 51	27	1	GGUGGAAAAUCUUCAUUUUAAAGA	TTTT	471
Human-Exon 51	28	1	UGGUGGAAAAUCUUCAUUUUAAAG	TTTT	472
Human-Exon 51	29	1	UUGGUGGAAAAUCUUCAUUUUAAA	TTTC	473
Human-Exon 51	30	1	GUGAUUGGUGGAAAAUCUUCAUUU	TTTA	474
Human-Exon 51	31	1	CUAGGAGAGUAAAGUGAUUGGUGG	TTTT	475
Human-Exon 51	32	1	UCUAGGAGAGUAAAGUGAUUGGUG	TTTC	476
Human-Exon 51	33	1	CUGGUGGGAAAUGGUCUAGGAGA	TTTA	477
Human-Exon 45	1	-1	guagcacacuguuuaaucuuuucu	tttg	478
Human-Exon 45	2	-1	cacacuguuuaaucuuuucucaaa	TTTa	479
Human-Exon 45	3	-1	acacuguuuaaucuuuucucaaau	TTTT	480
Human-Exon 45	4	-1	cacuguuuaaucuuuucucaaauA	TTTT	481
Human-Exon 45	5	1	AUGUCUUUUUauuugagaaaagau	ttta	482
Human-Exon 45	6	1	AAGCCCCAUGUCUUUUUauuugag	tttt	483
Human-Exon 45	7	1	GAAGCCCCAUGUCUUUUUauuuga	tttc	484
Human-Exon 45	8	1	GUAAGAUACCAAAAAGGCAAAACA	TTTT	485
Human-Exon 45	9	1	UGUAAGAUACCAAAAAGGCAAAAC	TTTT	486
Human-Exon 45	10	1	CUGUAAGAUACCAAAAAGGCAAAA	TTTG	487
Human-Exon 45	11	1	GUUCCUGUAAGAUACCAAAAAGGC	TTTT	488
Human-Exon 45	12	1	AGUUCCUGUAAGAUACCAAAAAGG	TTTG	489
Human-Exon 45	13	1	UCCUGGAGUUCCUGUAAGAUACCA	TTTT	490
Human-Exon 45	14	1	AUCCUGGAGUUCCUGUAAGAUACC	TTTT	491
Human-Exon 45	15	-1	GGGAAGAAAUAAUUCAGCAAUCCU	TTTG	492
Human-Exon 45	16	-1	GGAAGAAAUAAUUCAGCAAUCCUC	TTTT	493
Human-Exon 45	17	-1	GAAGAAAUAAUUCAGCAAUCCUCA	TTTT	494
Human-Exon 45	18	-1	AAAACAGAUGCCAGUAUUCUACAG	TTTC	495
Human-Exon 45	19	-1	AAACAGAUGCCAGUAUUCUACAGG	TTTT	496
Human-Exon 45	20	-1	AACAGAUGCCAGUAUUCUACAGGA	TTTT	497

Human-Exon 45	21	-1	GAAUCUGCGGUGGCAGGAGGUCUG	TTTG	498
Human-Exon 45	22	-1	AGGUCUGCAAACAGCUGUCAGACA	TTTC	499
Human-Exon 45	23	-1	GGUCUGCAAACAGCUGUCAGACAG	TTTT	500
Human-Exon 45	24	-1	GUCUGCAAACAGCUGUCAGACAGA	TTTT	501
Human-Exon 45	25	-1	UCUGCAAACAGCUGUCAGACAGAA	TTTT	502
Human-Exon 45	26	-1	UAGGGCGACAGAUCUAAUAGGAAU	TTTC	503
Human-Exon 45	27	-1	AGGGCGACAGAUCUAAUAGGAAUG	TTTT	504
Human-Exon 45	28	1	UAAAGAAAGCUUAAAAAGUCUGCU	TTTT	505
Human-Exon 45	29	1	CUAAAGAAAGCUUAAAAAGUCUGC	TTTA	506
Human-Exon 45	30	1	AAAUAUUCUUCUAAAGAAAGCUUA	TTTT	507
Human-Exon 45	31	1	GAAAUAUUCUUCUAAAGAAAGCUU	TTTT	508
Human-Exon 45	32	1	UGAAAUAUUCUUCUAAAGAAAGCU	TTTA	509
Human-Exon 45	33	1	UCUCUCAUGAAAUAUUCUUCUAAA	TTTC	510
Human-Exon 45	34	1	AUAAUCUCUCAUGAAAUAUUCUUC	TTTA	511
Human-Exon 44	1	1	GCGUAUAUUUUUUGGUUAUACUGA	TTTG	512
Human-Exon 44	2	1	ucaagaaaaauagauggauuaugu	tttt	513
Human-Exon 44	3	1	aucaagaaaaauagauggauuaug	ttta	514
Human-Exon 44	4	1	CAGGUaaaagcauauggaucaaga	tttt	515
Human-Exon 44	5	1	GCAGGUaaaagcauauggaucaag	tttt	516
Human-Exon 44	6	1	UGCAGGUaaaagcauauggaucaa	tttc	517
Human-Exon 44	7	-1	CAGGCGAUUUGACAGAUCUGUUGA	TTTC	518
Human-Exon 44	8	1	AGAUCUGUCAAAUCGCCUGCAGGU	tttt	519
Human-Exon 44	9	1	CAGAUCUGUCAAAUCGCCUGCAGG	tttA	520
Human-Exon 44	10	1	GCCGCCAUUUCUCAACAGAUCUGU	TTTG	521
Human-Exon 44	11	-1	AAUGGCGGCGUUUUCAUUAUGAUA	TTTA	522
Human-Exon 44	12	1	AUUAAAUAUCUUUAUAUCAUAAUG	TTTT	523
Human-Exon 44	13	-1	UGAGAAUUGGGAACAUGCUAAAUA	TTTG	524
Human-Exon 44	14	-1	GGUAAGUCUUUGAUUUGUUUUUUC	TTTC	525

Human-Exon 44	15	1	AAAUACAAUUUCGAAAAAACAAAU	TTTG	526
Human-Exon 44	16	1	AAGAUAAAUACAAUUUCGAAAAAA	TTTG	527
Human-Exon 44	17	1	GCUGAAGAUAAAUACAAUUUCGAA	TTTT	528
Human-Exon 44	18	1	UGCUGAAGAUAAAUACAAUUUCGA	TTTT	529
Human-Exon 44	19	1	GUGCUGAAGAUAAAUACAAUUUCG	TTTT	530
Human-Exon 44	20	1	UGUGCUGAAGAUAAAUACAAUUUC	TTTC	531
Human-Exon 44	21	-1	GCACAUCUGGACUCUUUAACUUCU	TTTA	532
Human-Exon 44	22	1	UAAAGAGUCCAGAUGUGCUGAAGA	TTTA	533
Human-Exon 44	23	-1	AAGAUCAGGUUCUGAAGGGUGAUG	TTTC	534
Human-Exon 44	24	1	UUCAGAACCUGAUCUUUAAGAAGU	TTTA	535
Human-Exon 44	25	1	AAUAUAAUGAUGACAACAACAGUC	TTTT	536
Human-Exon 44	26	1	UAAUAUAAUGAUGACAACAACAGU	TTTG	537
Human-Exon 53	1	-1	UUUAUUUUUCCUUUUAUUCUAGUU	TTTC	538
Human-Exon 53	2	1	AAAGGAAAAAUAAAUAUAUAGUAG	TTTA	539
Human-Exon 53	3	1	UUUCAACUAGAAUAAAAGGAAAAA	TTTA	540
Human-Exon 53	4	1	AUUCUUUCAACUAGAAUAAAAGGA	TTTT	541
Human-Exon 53	5	1	AAUUCUUUCAACUAGAAUAAAAGG	TTTT	542
Human-Exon 53	6	1	GAAUUCUUUCAACUAGAAUAAAAG	TTTC	543
Human-Exon 53	7	1	AUUCUGAAUUCUUUCAACUAGAAU	TTTT	544
Human-Exon 53	8	1	GAUUCUGAAUUCUUUCAACUAGAA	TTTA	545
Human-Exon 53	9	-1	CAGAACCGGAGGCAACAGUUGAAU	TTTC	546
Human-Exon 53	10	-1	GGAGGCAACAGUUGAAUGAAAUGU	TTTA	547
Human-Exon 53	11	-1	UAUACAGUAGAUGCAAUCCAAAAG	TTTT	548
Human-Exon 53	12	-1	GAUGCAAUCCAAAAGAAAAUCACA	TTTC	549
Human-Exon 53	13	-1	AAUCACAGAAACCAAGGUUAGUAU	TTTG	550
Human-Exon 53	14	-1	AGGUUAGUAUCAAAGAUACCUUU	TTTA	551
Human-Exon 53	15	-1	GGUUAGUAUCAAAGAUACCUUUUU	TTTT	552
Human-Exon 53	16	-1	AGUAUCAAAGAUACCUUUUUAAAA	TTTA	553

Human-Exon 53	17	-1	GUAUCAAAGAUACCUUUUUAAAAU	TTTT	554
Human-Exon 46	1	-1	UGUUUGUGUCCCAGUUUGCAUUAA	TTTG	555
Human-Exon 46	2	1	CUGGGACACAAACAUGGCAAUUUA	TTTT	556
Human-Exon 46	3	1	ACUGGGACACAAACAUGGCAAUUU	TTTT	557
Human-Exon 46	4	1	AACUGGGACACAAACAUGGCAAUU	TTTA	558
Human-Exon 46	5	1	UAUUUGUUAAUGCAAACUGGGACA	TTTG	559
Human-Exon 46	6	-1	ACAAAUAGUUUGAGAACUAUGUUG	tttC	560
Human-Exon 46	7	-1	CAAAUAGUUUGAGAACUAUGUUGG	tttt	561
Human-Exon 46	8	-1	AAAUAGUUUGAGAACUAUGUUGGa	tttt	562
Human-Exon 46	9	-1	AUAGUUUGAGAACUAUGUUGGaaa	tttt	563
Human-Exon 46	10	-1	UAGUUUGAGAACUAUGUUGGaaaa	tttt	564
Human-Exon 46	11	-1	AGUUUGAGAACUAUGUUGGaaaaa	tttt	565
Human-Exon 46	12	1	UAGUUCUCAAACUAUUUGUUAAUG	TTTG	566
Human-Exon 46	13	1	UAuuuuuuuuuCCAACAUAGUUCU	TTTG	567
Human-Exon 46	14	-1	CUUCUUUCUCCAGGCUAGAAGAAC	TTTT	568
Human-Exon 46	15	1	CUUCUAGCCUGGAGAAAGAAGAAU	TTTT	569
Human-Exon 46	16	1	UCUUCUAGCCUGGAGAAAGAAGAA	TTTA	570
Human-Exon 46	17	1	AUUCUUUUGUUCUUCUAGCCUGGA	TTTC	571
Human-Exon 46	18	-1	CAAAAGAAUAUCUUGUCAGAAUUU	TTTG	572
Human-Exon 46	19	-1	CUGGAAAAGAGCAGCAACUAAAAG	TTTT	573
Human-Exon 46	20	-1	CAAGUCAAGGUAAUUUUAUUUUCU	TTTG	574
Human-Exon 46	21	-1	CAAAUCCCCCAGGGCCUGCUUGCA	TTTA	575
Human-Exon 46	22	1	AGGCCCUGGGGGAUUUGAGAAAAU	TTTT	576
Human-Exon 46	23	1	CAGGCCCUGGGGGAUUUGAGAAAA	TTTA	577
Human-Exon 46	24	1	CAAGCAGGCCCUGGGGGAUUUGAG	TTTT	578
Human-Exon 46	25	1	GCAAGCAGGCCCUGGGGGAUUUGA	TTTC	579
Human-Exon 46	26	1	GCAGAAAACCAAUGAUUGAAUUAA	TTTT	580
Human-Exon 46	27	1	GGCAGAAAACCAAUGAUUGAAUUA	TTTT	581

Human-Exon 46	28	1	GGGCAGAAAACCAAUGAUUGAAUU	TTTT	582
Human-Exon 46	29	1	UGGGCAGAAAACCAAUGAUUGAAU	TTTA	583
Human-Exon 46	30	-1	AUUAGGUUAUUCAUAGUUCCUUGC	TTTA	584
Human-Exon 46	31	1	AACUAUGAAUAACCUAAUGGGCAG	TTTT	585
Human-Exon 46	32	1	GAACUAUGAAUAACCUAAUGGGCA	TTTC	586
Human-Exon 52	1	-1	UAUUUCCUGUUAAAUUGUUUUCUA	TTTA	587
Human-Exon 52	2	1	GGUUUAUAGAAAACAAUUUAACAG	TTTC	588
Human-Exon 52	3	-1	AUACAGUAACAUCUUUUUUAUUUC	TTTA	589
Human-Exon 52	4	-1	UACAGUAACAUCUUUUUUAUUUCU	TTTT	590
Human-Exon 52	5	1	AUGUUACUGUAUAAGGGUUUAUAG	TTTT	591
Human-Exon 52	6	1	GAUGUUACUGUAUAAGGGUUUAUA	TTTC	592
Human-Exon 52	7	1	CAGCCAAAACACUUUUAGAAAUAA	TTTT	593
Human-Exon 52	8	1	CCAGCCAAAACACUUUUAGAAAUA	TTTT	594
Human-Exon 52	9	1	ACCAGCCAAAACACUUUUAGAAAU	TTTT	595
Human-Exon 52	10	1	GACCAGCCAAAACACUUUUAGAAA	TTTA	596
Human-Exon 52	11	1	GUGAGACCAGCCAAAACACUUUUA	TTTC	597
Human-Exon 52	12	-1	AAUUGUACUUUACUUUGUAUUAUG	TTTA	598
Human-Exon 52	13	-1	AUUGUACUUUACUUUGUAUUAUGU	TTTT	599
Human-Exon 52	14	1	UAAAGUACAAUUGUGAGACCAGCC	TTTT	600
Human-Exon 52	15	1	GUAAAGUACAAUUGUGAGACCAGC	TTTG	601
Human-Exon 52	16	1	GUAUUCCUUUUACAUAAUACAAAG	TTTA	602
Human-Exon 52	17	1	GUUGUGUAUUCCUUUUACAUAAUA	TTTG	603
Human-Exon 52	18	1	AUCCUGCAUUGUUGCCUGUAAGAA	TTTG	604
Human-Exon 52	19	1	UUCCAACUGGGGACGCCUCUGUUC	TTTG	605
Human-Exon 52	20	-1	UUGGAAGAACUCAUUACCGCUGCC	TTTG	606
Human-Exon 52	21	-1	UCAUUACCGCUGCCCAAAAUUUGA	TTTT	607
Human-Exon 52	22	1	CUCUUGAUUGCUGGUCUUGUUUUU	TTTG	608
Human-Exon 52	23	-1	GUUUUUUAACAAGCAUGGGACACA	TTTG	609

Human-Exon 52	24	1	CUUUGUGUGUCCCAUGCUUGUUAA	TTTT	610
Human-Exon 52	25	1	GCUUUGUGUGUCCCAUGCUUGUUA	TTTT	611
Human-Exon 52	26	1	UGCUUUGUGUGUCCCAUGCUUGUU	TTTT	612
Human-Exon 52	27	1	UUGCUUUGUGUGUCCCAUGCUUGU	TTTA	613
Human-Exon 52	28	-1	AGCAAGAUGCAUGACAAGUUUCAA	TTTA	614
Human-Exon 52	29	-1	GCAAGAUGCAUGACAAGUUUCAAU	TTTT	615
Human-Exon 52	30	-1	CAAGAUGCAUGACAAGUUUCAAUA	TTTT	616
Human-Exon 52	31	1	GAUAUAUGAACUUAAGUUUUUAUU	TTTC	617
Human-Exon 50	1	-1	AUAGAAAUCCAAUAAUAUAUUCAC	TTTG	618
Human-Exon 50	2	-1	AUUAAGAUGUUCAUGAAUUAUCUU	TTTG	619
Human-Exon 50	3	-1	UAAGUAAUGUGUAUGCUUUUCUGU	TTTA	620
Human-Exon 50	4	1	AUCUUCUAACUUCCUCUUUAACAG	TTTT	621
Human-Exon 50	5	1	GAUCUUCUAACUUCCUCUUUAACA	TTTC	622
Human-Exon 50	6	-1	AUCUGAGCUCUGAGUGGAAGGCGG	TTTA	623
Human-Exon 50	7	-1	ACCGUUUACUUCAAGAGCUGAGGG	TTTG	624
Human-Exon 50	8	1	CUGCUUUGCCCUCAGCUCUUGAAG	TTTA	625
Human-Exon 50	9	-1	UCUCUUUGGCUCUAGCUAUUUGUU	TTTG	626
Human-Exon 50	10	-1	CUCUUUGGCUCUAGCUAUUUGUUC	TTTT	627
Human-Exon 50	11	1	CACUUUUGAACAAAUAGCUAGAGC	TTTG	628
Human-Exon 50	12	1	UCACUUCAUAGUUGCACUUUUGAA	TTTG	629
Human-Exon 50	13	-1	AUGAAGUGAUGACUGGGUGAGAGA	TTTC	630
Human-Exon 50	14	-1	UGAAGUGAUGACUGGGUGAGAGAG	TTTT	631
Human-Exon 43	1	1	AAGAGAAAAauauauauauauaua	TTTG	632
Human-Exon 43	2	1	GAAUUAGCUGUCUAUAGAAAGAGA	tTTT	633
Human-Exon 43	3	1	UGAAUUAGCUGUCUAUAGAAAGAG	TTTT	634
Human-Exon 43	4	-1	AGCUAAUUCAUUUUUUUACUGUUU	TTTA	635
Human-Exon 43	5	1	AUGAAUUAGCUGUCUAUAGAAAGA	TTTC	636
Human-Exon 43	6	-1	GCUAAUUCAUUUUUUUACUGUUUU	TTTT	637

Human-Exon 43	7	1	AAAAAAAUGAAUUAGCUGUCUAUA	TTTC	638
Human-Exon 43	8	-1	UUAAAAUUUUUAUAUUACAGAAUA	TTTA	639
Human-Exon 43	9	-1	UAAAAUUUUUAUAUUACAGAAUAU	TTTT	640
Human-Exon 43	10	1	AUAUAAAAAUUUUAAAACAGUAAA	TTTT	641
Human-Exon 43	11	1	AAUAUAAAAAUUUUAAAACAGUAA	TTTT	642
Human-Exon 43	12	1	UAAUAUAAAAAUUUUAAAACAGUA	TTTT	643
Human-Exon 43	13	1	GUAAUAUAAAAAUUUUAAAACAGU	TTTT	644
Human-Exon 43	14	1	UGUAAUAUAAAAAUUUUAAAACAG	TTTA	645
Human-Exon 43	15	1	UAUAUUCUGUAAUAUAAAAAUUUU	TTTT	646
Human-Exon 43	16	1	UUAUAUUCUGUAAUAUAAAAAUUU	TTTA	647
Human-Exon 43	17	-1	CAGAAUAUAAAAGAUAGUCUACAA	TTTG	648
Human-Exon 43	18	1	CUAUCUUUUAUAUUCUGUAAUAUA	TTTT	649
Human-Exon 43	19	1	ACUAUCUUUUAUAUUCUGUAAUAU	TTTT	650
Human-Exon 43	20	1	GACUAUCUUUUAUAUUCUGUAAUA	TTTA	651
Human-Exon 43	21	-1	CAUAGCAAGAAGACAGCAGCAUUG	TTTG	652
Human-Exon 43	22	1	CAUUUUGUUAACUUUUUCCCAUUG	TTTC	653
Human-Exon 43	23	-1	CAUAUAUUUUUCUUGAUACUUGCA	TTTC	654
Human-Exon 43	24	1	AAAUCAUUUCUGCAAGUAUCAAGA	TTTT	655
Human-Exon 43	25	1	CAAAUCAUUUCUGCAAGUAUCAAG	TTTT	656
Human-Exon 43	26	1	ACAAAUCAUUUCUGCAAGUAUCAA	TTTC	657
Human-Exon 43	27	1	AUAAAUUCUACAGUUCCCUGAAAA	TTTG	658
Human-Exon 43	28	-1	GAAUUUAUUUCAGUACCCUCCAUG	TTTC	659
Human-Exon 43	29	-1	AAUUUAUUUCAGUACCCUCCAUGG	TTTT	660
Human-Exon 43	30	1	UGAAAUAAAUUCUACAGUUCCCUG	TTTT	661
Human-Exon 43	31	-1	AUUUAUUUCAGUACCCUCCAUGGA	TTTT	662
Human-Exon 43	32	1	CUGAAAUAAAUUCUACAGUUCCCU	TTTC	663
Human-Exon 43	33	-1	UUUAUUUCAGUACCCUCCAUGGAA	TTTT	664
Human-Exon 43	34	-1	UACCCUCCAUGGAAAAAAGACAGG	TTTC	665

Human-Exon 43	35	-1	ACCCUCCAUGGAAAAAAGACAGGG	TTTT	666
Human-Exon 43	36	-1	CCCUCCAUGGAAAAAAGACAGGGA	TTTT	667
Human-Exon 43	37	1	UUUUUUCCAUGGAGGGUACUGAAA	TTTA	668
Human-Exon 43	38	1	UGUCUUUUUUCCAUGGAGGGUACU	TTTC	669
Human-Exon 6	1	1	CCUUGAGCAAGAACCAUGCAAACU	TTTA	670
Human-Exon 6	2	-1	UGCUCAAGGAAUGCAUUUUCUUAU	TTTC	671
Human-Exon 6	3	-1	GCUCAAGGAAUGCAUUUUCUUAUG	TTTT	672
Human-Exon 6	4	1	UGCAUUCCUUGAGCAAGAACCAUG	TTTG	673
Human-Exon 6	5	-1	GAAAAUUUAUUUCCACAUGUAGGU	TTTG	674
Human-Exon 6	6	-1	AAAAUUUAUUUCCACAUGUAGGUC	TTTT	675
Human-Exon 6	7	-1	AAAUUUAUUUCCACAUGUAGGUCA	TTTT	676
Human-Exon 6	8	1	CAUGUGGAAAUAAAUUUUCAUAAG	TTTT	677
Human-Exon 6	9	1	ACAUGUGGAAAUAAAUUUUCAUAA	TTTC	678
Human-Exon 6	10	-1	CCACAUGUAGGUCAAAAAUGUAAU	TTTC	679
Human-Exon 6	11	-1	CACAUGUAGGUCAAAAAUGUAAUG	TTTT	680
Human-Exon 6	12	-1	ACAUGUAGGUCAAAAAUGUAAUGA	TTTT	681
Human-Exon 6	13	1	ACAUUUUUGACCUACAUGUGGAAA	TTTA	682
Human-Exon 6	14	1	CAUUACAUUUUUGACCUACAUGUG	TTTC	683
Human-Exon 6	15	-1	AAAAAUAUCAUGGCUGGAUUGCAA	TTTG	684
Human-Exon 6	16	-1	GCUGGAUUGCAACAAACCAACAGU	TTTC	685
Human-Exon 6	17	-1	CUGGAUUGCAACAAACCAACAGUG	TTTT	686
Human-Exon 6	18	1	CCUAUGACUAUGGAUGAGAGCAUU	TTTG	687
Human-Exon 6	19	-1	UAGGUAAGAAGAUUACUGAGACAU	TTTA	688
Human-Exon 6	20	-1	AUUACUGAGACAUUAAAUAACUUG	TTTA	689
Human-Exon 6	21	-1	UUACUGAGACAUUAAAUAACUUGU	TTTT	690
Human-Exon 6	22	1	GGGGAAAAAUAUGUCAUCAGAGUC	TTTA	691
Human-Exon 6	23	1	CAUGAUCUGGAACCAUACUGGGGA	TTTT	692
Human-Exon 6	24	1	ACAUGAUCUGGAACCAUACUGGGG	TTTT	693

Human-Exon 6	25	1	GACAUGAUCUGGAACCAUACUGGG	TTTC	694
Human-Exon 7	1	1	uacacacauacacaAAGACAAAUA	TTTA	695
Human-Exon 7	2	1	uacacauacacacauacacaAAGA	TTTG	696
Human-Exon 7	3	1	aacacauacacauacacacauaca	TTtg	697
Human-Exon 7	4	1	AUUCCAGUCAAAUAGGUCUGGCCU	ttTT	698
Human-Exon 7	5	1	UAUUCCAGUCAAAUAGGUCUGGCC	tTTA	699
Human-Exon 7	6	1	GCUGGCAAACCACACUAUUCCAGU	TTTG	700
Human-Exon 7	7	1	AGUCGUUGUGUGGCUGACUGCUGG	TTTG	701
Human-Exon 7	8	-1	CGCCAGAUAUCAAUUAGGCAUAGA	TTTC	702
Human-Exon 7	9	-1	AAACUACUCGAUCCUGAAGGUUGG	TTTA	703
Human-Exon 7	10	1	CAUACUAAAAGCAGUGGUAGUCCA	TTTC	704
Human-Exon 7	11	1	GAAAACAUUAAACUCUACCAUACU	TTTT	705
Human-Exon 7	12	1	UGAAAACAUUAAACUCUACCAUAC	TTTA	706
Human-Exon 8	1	-1	UUGUUCAUUAUCCUUUUAGAGUCU	TTTG	707
Human-Exon 8	2	1	AAAGGAUAAUGAACAAAUCAAAGU	TTTA	708
Human-Exon 8	3	-1	UAUCCUUUUAGAGUCUCAAAUAUA	TTTC	709
Human-Exon 8	4	1	ACUCUAAAAGGAUAAUGAACAAAU	TTTG	710
Human-Exon 8	5	-1	UUUUAGAGUCUCAAAUAUAGAAAC	TTTG	711
Human-Exon 8	6	-1	UUUAGAGUCUCAAAUAUAGAAACC	TTTT	712
Human-Exon 8	7	-1	UUAGAGUCUCAAAUAUAGAAACCA	TTTT	713
Human-Exon 8	8	1	UUGAGACUCUAAAAGGAUAAUGAA	TTTG	714
Human-Exon 8	9	1	UUUGGUUUCUAUAUUUGAGACUCU	TTTT	715
Human-Exon 8	10	1	UUUUGGUUUCUAUAUUUGAGACUC	TTTA	716
Human-Exon 8	11	-1	AGCAUUGAAGCCAUCCAGGAAGUG	TTTC	717
Human-Exon 8	12	1	GCUUCAAUGCUCACUUGUUGAGGC	TTTT	718
Human-Exon 8	13	1	GGCUUCAAUGCUCACUUGUUGAGG	TTTG	719
Human-Exon 8	14	-1	AGUGGAAAUGUUGCCAAGGCCACC	TTTA	720
Human-Exon 8	15	-1	GUUGCCAAGGCCACCUAAAGUGAC	TTTA	721

Human-Exon 8	16	-1	GAAGAACAUUUUCAGUUACAUCAU	TTTG	722
Human-Exon 8	17	-1	AUCAAAUGCACUAUUCUCAACAGG	TTTA	723
Human-Exon 8	18	1	AUAGUGCAUUUGAUGAUGUAACUG	TTTT	724
Human-Exon 8	19	1	AAUAGUGCAUUUGAUGAUGUAACU	TTTC	725
Human-Exon 8	20	-1	ACUAUUCUCAACAGGUAAAGUGUG	TTTA	726
Human-Exon 8	21	1	UACCUAAAAAUGCAUAUAAAACAG	TTTT	727
Human-Exon 8	22	1	AUACCUAAAAAUGCAUAUAAAACA	TTTC	728
Human-Exon 8	23	1	CACGUAAUACCUAAAAAUGCAUAU	TTTT	729
Human-Exon 8	24	1	GCACGUAAUACCUAAAAAUGCAUA	TTTA	730
Human-Exon 8	25	1	auauauauGUGCACGUAAUACCUA	TTTT	731
Human-Exon 8	26	1	uauauauauGUGCACGUAAUACCU	TTTT	732
Human-Exon 8	27	1	auauauauauGUGCACGUAAUACC	TTTA	733
Human-Exon 55	1	-1	CUGCACAAUAUUAUAGUUGUUGCU	TTTA	734
Human-Exon 55	2	1	AUAAAAAGAGAAAGAUGGAGGAAC	TTTA	735
Human-Exon 55	3	1	CACCUAGUGAACUCCAUAAAAAGA	TTTC	736
Human-Exon 55	4	1	AUGGUGCACCUAGUGAACUCCAUA	TTTT	737
Human-Exon 55	5	1	AAUGGUGCACCUAGUGAACUCCAU	TTTT	738
Human-Exon 55	6	1	GAAUGGUGCACCUAGUGAACUCCA	TTTA	739
Human-Exon 55	7	1	GACCAAAUGUUCAGAUGCAAUUAU	TTTA	740
Human-Exon 55	8	1	UCGCUCACUCACCCUGCAAAGGAC	TTTG	741
Human-Exon 55	9	-1	AGUGAGCGAGAGGCUGCUUUGGAA	TTTC	742
Human-Exon 55	10	1	GCAGCCUCUCGCUCACUCACCCUG	TTTG	743
Human-Exon 55	11	1	UUGCAGUAAUCUAUGAGUUUCUUC	TTTG	744
Human-Exon 55	12	-1	CUGCAACAGUUCCCCCUGGACCUG	TTTC	745
Human-Exon 55	13	-1	UGCAACAGUUCCCCCUGGACCUGG	TTTT	746
Human-Exon 55	14	-1	UUUCUUGCCUGGCUUACAGAAGCU	TTTC	747
Human-Exon 55	15	1	UUUCAGCUUCUGUAAGCCAGGCAA	TTTC	748
Human-Exon 55	16	-1	GUCCUACAGGAUGCUACCCGUAAG	TTTC	749

Human-Exon 55	17	-1	GGCUCCUAGAAGACUCCAAGGGAG	TTTA	750
Human-Exon 55	18	-1	GCUCCUAGAAGACUCCAAGGGAGU	TTTT	751
Human-Exon 55	19	-1	CUCCAAGGGAGUAAAAGAGCUGAU	TTTC	752
Human-Exon 55	20	1	UGGAUCCACAAGAGUGCUAAAGCG	TTTC	753
Human-Exon 55	21	1	GUUCAAUUGGAUCCACAAGAGUGC	TTTA	754
Human-Exon 55	22	-1	UACUUGUAACUGACAAGCCAGGGA	TTTG	755
Human-Exon 55	23	-1	ACUUGUAACUGACAAGCCAGGGAC	TTTT	756
Human-Exon 55	24	-1	GUAACUGACAAGCCAGGGACAAAA	TTTG	757
Human-Exon 55	25	-1	UAACUGACAAGCCAGGGACAAAAC	TTTT	758
Human-Exon 55	26	1	UCCCUGGCUUGUCAGUUACAAGUA	TTTG	759
Human-G1-exon51		1	CAGAGUAACAGUCUGAGUAGGAGc	TTTA	760
Human-G2-exon51		1	uacuuuguuuagcaauacauggua	TTTC	761
Human-G3-exon51		-1	uggcucaaauuguuacucuucaau	TTTA	762
mouse-Exon23-G1		1	CUUUCAAagaacuuugcagagccu	TTTG	763
mouse-Exon23-G2		1	guugaaGCCAUUUUGUUGCUCUUU	TTTG	764
mouse-Exon23-G3		1	guugaaGCCAUUUUAUUGCUCUUU	TTTG	765
mouse-Exon23-G4		-1	uuuugagGCUCUGCAAAGUUCUUU	TTTC	766
mouse-Exon23-G5		-1	aguuauuaaugcauagauauucag	TTTA	767
mouse-Exon23-G6		-1	uauaauaugcccuguaauauaaua	TTTC	768
mouse-Exon23-G7		1	uaaaggccaaaccucggcuuaccU	TTTC	769
mouse-Exon23-G8		1	ucaauaucuuugaaggacucuggg	TTTA	770

* In this table, upper case letters represent sgRNA nucleotides that align to the exon sequence of the gene. Lower case letters represent sgRNA nucleotides that align to the intron sequence of the gene.

VI. Sequence Tables

Table 3 - Sequence of primers for sgRNA targeting *Dmd* Exon 50 and Exon 79 to generate the mice models

ID	Mouse Model	Sequence (5'-3')	SEQ ID NO.
exon 50_F1	Δex50	CACCGAAATGATGAGTGAAGTTATAT	1
exon 50_R1	Δex50	AAACATATAACTTCACTCATCATTTC	2
exon 50_F2	Δex50	CACCGGTTTGTTCAAAAGCGTGGCT	3
exon 50_R2	Δex50	AAACAGCCACGCTTTTGAACAAAC	4
exon79_F1	*Dmd*-KI-Luciferase	CACCGGACACAATGTAGGAAGCCT	5
exon79_R1	*Dmd*-KI-Luciferase	AAACAGGCTTCCTACATTGTGTCC	6

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Table 4 - Sequence of primers for *in vitro* transcription of sgRNA

ID	Mouse Model	Sequence (5'-3')	SEQ ID NO.
exon 50_T7-F1	Δex50	GAATTGTAATACGACTCACTATAGGAATGATGAGTGAAGTTATAT	7
exon 50_T7-F2	Δex50	GAATTGTAATACGACTCACTATAGGGTTTGTTCAAAAGCGTGGCT	8
exon 50_T7-Rv	Δex50	AAAAGCACCGACTCGGTGCCAC	9
exon 50_R2	Δex50	AAACAGCCACGCTTTTGAACAAAC	10
exon 79_T7-F1	*Dmd*-KI-Luciferase	GAATTGTAATACGACTCACTGGACACAATGTAGGAAGCCT	11

exon 79_T7-Rv	*Dmd*-KI-Luciferase	AAAAGCACCGACTCGGTGCCAC	12

Table 5 - Sequence of primers for genotyping

ID	Mouse Model	Sequence (5'-3')	SEQ ID NO.
Geno50-F	Δex50	GGATTGACTGAAATGATGGCCAAGG	13
Geno50-R	Δex50	CTGCCACGATTACTCTGCTTCCAG	14
GenoKI/WT-F	*Dmd*-KI-Luciferase	AGCAGGCAGAGAAGGTGGTA	15
GenoKI-R	*Dmd*-KI-Luciferase	GGGCGTATCTCTTCATAGCCTT	16
GenoWT-R	*Dmd*-KI-Luciferase	GCGTGTGTGTTTGTTTAGG	17

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Table 6 – Sequence of primers for sgRNA targeting *Dmd* Exon 51 for correction of reading frame

ID	Mouse Model	Sequence (5'-3')	SEQ ID NO.
exon 51_F1	ex51-SA-Top	CACCGCACTAGAGTAACAGTCTGAC	771
exon 51_F1	ex51-SA-Bottom	AAACCCAGTCAGACTGTTACTCTC	772

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Table 7 – Sequence of primers for Amplicon Deep Sequencing Analysis

ID	Mouse Model	Sequence (5'-3')	SEQ ID NO.
Amplicon Deep Sequencing	M-ex51-Mi-seq-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAAATTTTACCTCAAACTGTTGCTTC	773
Amplicon Deep Sequencing	M-ex51-Mi-seq-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAGGGAAATGGAAAGTGACAATATAC	774
Amplicon Deep Sequencing	Univ-Miseq-BC-Fw-LA	AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTC	775
Amplicon Deep Sequencing	BC1-LA	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTCTCGTGGGCTCGG	776
Amplicon Deep Sequencing	BC2-LA	CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTCTCGTGGGCTCGG	777
Amplicon Deep Sequencing	BC3-LA	CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTCTCGTGGGCTCGG	778
Amplicon Deep Sequencing	BC4-LA	CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GTCTCGTGGGCTCGG	779
Amplicon Deep Sequencing	BC5-LA	CAAGCAGAAGACGGCATACGAGAT**GATCTG**GTCTCGTGGGCTCGG	780
Amplicon Deep Sequencing	BC6-LA	CAAGCAGAAGACGGCATACGAGAT**TACAAG**GTCTCGTGGGCTCGG	781
Amplicon Deep Sequencing	BC7-LA	CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTCTCGTGGGCTCGG	782
Amplicon Deep Sequencing	BC8-LA	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTCTCGTGGGCTCGG	783
Amplicon Deep Sequencing	BC9-LA	CAAGCAGAAGACGGCATACGAGAT**TCAAGT**GTCTCGTGGGCTCGG	784
Amplicon Deep Sequencing	BC10-LA	CAAGCAGAAGACGGCATACGAGAT**AGCTAG**GTCTCGTGGGCTCGG	785

VII. Examples

The following examples are included to demonstrate preferred embodiments of the disclosure. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the disclosure, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the disclosure.

EXAMPLE 1 - Materials and Methods

Study Approval. All experimental procedures involving animals in this study were reviewed and approved by the University of Texas Southwestern Medical Center's Institutional Animal Care and Use Committee.

CRISPR/Cas9-mediated exon 50 deletion in mice. Two single-guide RNA (sgRNA) specific intronic regions surrounding exon 50 sequence of the mouse *Dmd* locus were cloned into vector px330 using the primers from Table 3. For the *in vitro* transcription of sgRNA, T7 promoter sequence was added to the sgRNA template by PCR using the primers from Table 4. The gel purified PCR products were used as template for *in vitro* transcription using the MEGAshortscript T7 Kit (Life Technologies). sgRNA were purified by MEGAclear kit (Life Technologies) and eluted with nuclease-free water (Ambion). The concentration of guide RNA was measured by a NanoDrop instrument (Thermo Scientific).

CRISPR/Cas9-mediated Homologous Recombination in Mice. A single-guide RNA (sgRNA) specific to the exon 79 sequence of the mouse *Dmd* locus was cloned into vector px330 using the primers from Table 3. For the *in vitro* transcription of sgRNA, T7 promoter sequence was added to the sgRNA template by PCR using the primers from Table 4. A donor vector containing the protease 2A and luciferase reporter sequence was constructed by incorporating short 5' and 3' homology arms specific to the *Dmd* gene locus.

Genotyping of ΔEx50 Mice and *Dmd*-Luciferase Mice. ΔEx50, *Dmd*-Luciferase and ΔEx50-*Dmd*-Luciferase mice were genotyped using primers encompassing the targeted region from Table 5. Tail biopsies were digested in 100 μL of 25-mM NaOH, 0.2-mM EDTA (pH 12)

for 20 min at 95 °C. Tails were briefly centrifuged followed by addition of 100 µL of 40-mM Tris·HCl (pH 5) and mixed to homogenize. Two microliters of this reaction was used for subsequent PCR reactions with the primers below, followed by gel electrophoresis.

Plasmids. The pSpCas9(BB)-2A-GFP (PX458) plasmid containing the human codon optimized SpCas9 gene with 2A-EGFP and the backbone of sgRNA was purchased from Addgene (Plasmid #48138). Cloning of sgRNA was done using Bbs I site.

AAV9 strategy and delivery to ΔEx50-KI-Luciferase mice. *Dmd* exon 51 sgRNAs were selected using crispr.mit.edu. sgRNA sequences were cloned into px330 using primers in Table 4. sgRNAs were tested in tissue culture using 10T1/2 cells as previously described (Long *et al.*, 2016) before cloning into the rAAV9 backbone.

Prior to AAV9 injections, ΔEx50-KI-Luciferase mice were anesthetized by intraperitoneal (IP) injection of ketamine and xylazine anesthetic cocktail. For intramuscular (IM) injection, tibialis anterior (TA) muscle of P12 male ΔEx50 mice was injected with 50 µl of AAV9 (1E12 vg/ml) preparations, or saline solution.

Targeted deep DNA sequencing. PCR of genomic DNA from 10T1/2 mouse fibroblast was performed using primers designed against the respective target region and off-target sites (Table 5). A second round of PCR was used to add Illumina flowcell binding sequences and experiment-specific barcodes on the 5' end of the primer sequence (Table 2). Before sequencing, DNA libraries were analyzed using a Bioanalyzer High Sensitivity DNA Analysis Kit (Agilent). Library concentration was then determined by qPCR using a KAPA Library Quantification Kit for Illumina platforms. The resulting PCR products were pooled and sequenced with 300 bp paired-end reads on an Illumina MiSeq instrument. Samples were demultiplexed according to assigned barcode sequences. FASTQ format data was analyzed using the CRISPResso software package version 1.0.8 (Pinello *et al.*, 2016).

Western blot analysis. Western blot was performed as described previously (Long *et al.*, 2016). Antibodies to dystrophin (1:1000, D8168, Sigma-Aldrich), luciferin (1:1000, Abcam ab21176), vinculin (1:1000, V9131, Sigma-Aldrich), goat anti-mouse and goat-anti rabbit HRP-conjugated secondary antibodies (1:3000, Bio-Rad) were used for the described experiments.

EXAMPLE 2 - Results

New Humanized model recapitulates muscle dystrophy phenotype. The first hot spot mutation region in DMD patients is the region between exon 45 to 51 where skipping of exon 51 would apply to the largest group (i.e., 13-14% of DMD patients). To investigate CRISPR/Cas9-mediated exon 51 skipping *in vivo*, a mimic of the human "hot spot" region was generated in a mouse model by deleting the exon 50 using CRISPR/Cas9 system directed by 2 single guide RNA (sgRNA) (FIG. 1A). The deletion of exon 50 was confirmed by DNA sequencing (FIG. 1B). The deletion of exon 50 placed the dystrophin gene out of frame leading to the absence of dystrophin protein in skeletal muscle and heart (FIG. 1C). Mice lacking exon 50 showed pronounced dystrophic muscle changes in 2 months-old mice. Serum analysis of delta-exon 50 mice shows a significant increase of creatine kinase (CK) level, which is a sign of muscle damage. Taken together, dystrophin protein expression, muscle histology and serum validated dystrophic phenotype of ΔEx50 mouse model.

Humanized DMD reporter line. In an effort to facilitate the analysis of exon skipping strategies *in vivo* in a non-invasive way, reporter mice were generated by insertion of a Luciferase expression cassette into the 3' end of the *Dmd* gene so that Luciferase would be translated in-frame with exon 79 of dystrophin, referred as *Dmd*-KI-Luciferase as shown in FIGS. 2A-B. To avoid the possibility that Luciferase might destabilize the dystrophin protein, a protease 2A was engineered at cleavage site between the proteins, which is auto-catalytically cleaved (FIG. 2A). Thus, the reporter protein will be released from dystrophin after translation. The reporter *Dmd*-luciferase reporter line were successfully generated and validated by DNA sequencing. The bioluminescence imaging of mice shows a high-expression level and muscle-specificity of Luciferase expression in the *Dmd*-Luciferase mice (FIG. 2B). To generate a ΔEx50-*Dmd*-luciferase reporter line mouse, 2 sgRNA were used to delete exon 50 in *Dmd*-luciferase reporter line (FIG. 3A). The deletion of exon 50 was confirmed by DNA sequencing. The deletion of exon 50 placed the dystrophin gene out of frame leading to the absence of dystrophin protein and decreased bioluminescence signal (FIG. 3C). Deletion of exon 50 placed the *Dmd* gene out of frame, preventing production of dystrophin protein in skeletal muscle and heart (FIG. 3D). Thus, since the Luciferase reporter protein expression is linked to the dystrophin translation the deletion of exon 50 leads to the absence of luciferin protein expression in ΔEx50-KI-Luciferase mice (FIG. 3D).

***In vivo* monitoring of correction of the dystrophin reading frame in ΔEx50-KI-Luciferase mice by a single DNA cut.** To correct the dystrophin reading frame in ΔEx50-KI-Luciferase mice (FIG. 4A), sgRNA were designed to target a region adjacent to the exon 51 splice acceptor site (referred to as sgRNA-SA) (FIG. 4B). *S. pyogenes* Cas9 that requires NAG/NGG as a proto-spacer adjacent motif (PAM) sequence to generate a double-strand DNA break was used for the *in vivo* correction.

First, the DNA cutting activity of Cas9 coupled with sgRNA-SA was evaluated in 10T1/2 mouse fibroblasts. To investigate the type of mutations generated by Cas9 coupled with sgRNA-SA, genomic deep-sequencing analysis was performed. The sequencing analysis revealed that 9.3% of mutations contained a single adenosine (A) insertion 4 nucleotides 3' of the PAM sequence and 7.3% contained deletions covering the splice acceptor site and a highly-predicted ESE site for exon 51 (FIG. 4C).

For the *in vivo* delivery of Cas9 and sgRNA-SA to skeletal muscle and heart tissue, adeno-associated virus 9 (AAV9) was used, which displays preferential tropism for these tissues. To further enhance muscle-specific expression, an AAV9-Cas9 vector (CK8e-Cas9-shortPolyA), which contains a muscle-specific creatine kinase (CK) regulatory cassette was used, referred to as the CK8e promoter, which is highly specific for expression in muscle and heart (FIG. 4D). This 436 bp muscle-specific cassette and the 4101 bp Cas9 cDNA, together, are within the packaging limit of AAV9. Expression of each sgRNA was driven by three RNA polymerase III promoters (U6, H1 and 7SK) (FIG. 4D).

Following intra-muscular (IM) injection of mice at postnatal day (P) 12 with 5E10 AAV9 viral genomes (vg) in left tibialis anterior (TA) muscles were analyzed and monitored by bioluminescence for 4 weeks (FIG. 5A). The *in vivo* bioluminescence analysis showed appearance of signal in the injected leg 1 week after injection. The signal progressively increased over the following weeks expanding to the entire hindlimb muscles (FIG. 5B).

Histological analysis of AAV9-injected TA muscle was performed to evaluate the number of fibers that expressed dystrophin and the correlation with the bioluminescence signal. Dystrophin immunohistochemistry of muscle from ΔEx50-KI-Luciferase mice injected with AAV9-SA revealed restoration of dystrophin (FIGS. 5C-D). Taken together, these results demonstrate an *in vivo* assessment of dystrophin reading frame correction in ΔEx50-KI-

Luciferase mice. ΔEx50-KI-Luciferase mice will be useful as a platform for testing many different strategies for amelioration of DMD pathogenesis.

* * * * * * * * * * * * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this disclosure have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the disclosure. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the disclosure as defined by the appended claims.

VII. References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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WHAT IS CLAIMED:

1. A composition comprising a sequence encoding a Cas9 polypeptide, a sequence encoding a first guide RNA (gRNA) targeting a first genomic target sequence, and a sequence encoding a second gRNA targeting a second genomic target sequence, wherein the first and second genomic target sequences each comprise an intronic sequence surrounding an exon of the murine dystrophin gene.

2. The composition of claim 1, wherein the exon comprises exon 50 of the murine dystrophin gene.

3. The composition of claim 1 or 2, wherein the sequence encoding a Cas9 polypeptide is isolated or derived from a sequence encoding a *S. aureus* Cas9 polypeptide.

4. The composition of any one of claims 1-3, wherein at least one of the sequence encoding the Cas9 polypeptide, the sequence encoding the first gRNA, or the sequence encoding the second gRNA comprises an RNA sequence.

5. The composition of claim 4, wherein the RNA sequence comprises an mRNA sequence.

6. The composition of claim 4 or 5, wherein the RNA sequence comprises at least one chemically-modified nucleotide.

7. The composition of any one of claims 1-3, wherein at least one of the sequence encoding the Cas9 polypeptide, the sequence encoding the first gRNA, or the sequence encoding the second gRNA comprises a DNA sequence.

8. The composition of any one of claims 1-7, wherein a first vector comprises the sequence encoding the Cas9 polypeptide and a second vector comprises at least one of the sequence encoding the first gRNA or the sequence encoding the second gRNA.

9. The composition of claim 8, wherein the first vector or the sequence encoding the Cas9 polypeptide further comprises a first polyA sequence.

10. The composition of claim 8, wherein the second vector or the sequence encoding the first gRNA or the sequence encoding the second gRNA encodes a second polyA sequence.

11. The composition of claim 8, wherein the first vector or the sequence encoding the Cas9 polypeptide further comprises a first promoter sequence.

12. The composition of claim 8, wherein the second vector or the sequence encoding the first gRNA or the sequence encoding the second gRNA comprises a second promoter sequence.

13. The composition of claim 11 or 12, wherein the first promoter sequence and the second promoter sequence are identical.

14. The composition of claim 11 or 12, wherein the first promoter sequence and the second promoter sequence are not identical.

15. The composition of any of claims 11-14, wherein the first promoter sequence or the second promoter sequence comprises a CK8 promoter sequence.

16. The composition of any of claims 11-14, wherein the first promoter sequence or the second promoter sequence comprises a CK8e promoter sequence.

17. The composition of any of claims 11-14, wherein the first promoter sequence or the second promoter sequence comprises a constitutive promoter.

18. The composition of any of claims 11-14, wherein the first promoter sequence or the second promoter sequences comprises an inducible promoter.

19. The composition of any of claims 1-7, wherein one vector comprises the sequence encoding the Cas9 polypeptide, the sequence encoding the first gRNA and the sequence encoding the second gRNA.

20. The composition of claim 19, wherein the vector further comprises a polyA sequence.

21. The composition of claim 20 or 21, wherein the vector further comprises a promoter sequence.

22. The composition of claim 21, wherein the promoter sequence comprises a constitutive promoter.

23. The composition of claim 21, wherein the promoter sequence comprises an inducible promoter.

24. The composition of claim 21, wherein the promoter sequence comprises a CK8 promoter sequence.

25. The composition of claim 21, wherein the promoter sequence comprises a CK8e promoter sequence.

26. The composition of any one of claims 1-25, wherein the composition comprises a sequence codon optimized for expression in a mammalian cell.

27. The composition of any one of claims 1-16, wherein the composition comprises a sequence codon optimized for expression in a human cell or a mouse cell.

28. The composition of claim 27, wherein the sequence encoding the Cas9 polypeptide is codon optimized for expression in human cells or mouse cells.

29. The composition of any one of claims 8-18, wherein at least one of the first vector and the second vector is a non-viral vector.

30. The composition of claim 29, wherein the non-viral vector is a plasmid.

31. The composition of claim 29 or 30, wherein a liposome or nanoparticle comprises the non-viral vector.

32. The composition of any one of claims 8-18 wherein at least one of the first vector and the second vector is a viral vector.

33. The composition of any of claims 19-28, wherein the vector is a viral vector.

34. The composition of claim 32 or 33, wherein the viral vector is an adeno-associated viral (AAV) vector.

35. The composition of claim 34, wherein the AAV vector is replication-defective or conditionally replication defective.

36. The composition of claim 34 or 35, wherein the AAV vector is a recombinant AAV vector.

37. The composition of any of claims 34-36, wherein the AAV vector comprises a sequence isolated or derived from an AAV vector of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or any combination thereof.

38. The composition of any one of claims 1-37, further comprising a pharmaceutically carrier.

39. A cell comprising the composition of any one of claims 1 to 38.

40. The cell of claim 39, wherein the cell is a murine cell.

41. The cell of claim 39 or 40, wherein the cell is an oocyte.

42. A composition comprising the cell of any one of claims 39-41.

43. A genetically engineered mouse comprising the cell of any of claims 39-41.

44. A method of creating a genetically engineered mouse comprising contacting the cell of any of claims 39-41 with a mouse.

45. A method of creating a genetically engineered mouse comprising contacting a cell of the mouse with a composition of any one of claims 1-38.

46. A genetically engineered mouse generated by the method of claim 44 or 45.

47. A genetically engineered mouse, wherein the genome of the mouse comprises a deletion of exon 50 of the dystrophin gene resulting in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene.

48. The genetically engineered mouse of claim 47, further comprising a reporter gene located downstream of and in frame with exon 79 of the dystrophin gene, and upstream of a dystrophin 3'-UTR, wherein the reporter gene is expressed when exon 79 is translated in frame with exon 49.

49. The genetically engineered mouse of claim 48, wherein the reporter gene is luciferase.

50. The genetically engineered mouse of any of claims 47-49, further comprising a protease coding sequence upstream of and in frame with the reporter gene, and downstream of and in frame with exon 79.

51. The genetically engineered mouse of claim 50, wherein the protease is autocatalytic.

52. The genetically engineered mouse of claim 50 or 51, wherein the protease is 2A protease.

53. The genetically engineered mouse of any of claims 47-52, wherein the mouse is heterozygous for the deletion.

54. The genetically engineered mouse of any of claims 47-52, wherein the mouse is homozygous for the deletion.

55. The genetically engineered mouse of any of claims 47-54, wherein the mouse exhibits increased creatine kinase levels compared to a wildtype mouse.

56. The genetically engineered mouse of any of claims 47-55, wherein the mouse does not exhibit detectable dystrophin protein in heart or skeletal muscle.

57. A method of producing the genetically engineered mouse of any of claims 47-56 comprising:

(a) contacting a fertilized oocyte with CRISPR/Cas9 elements and two single guide RNA (sgRNA) targeting sequences flanking exon 50 of the dystrophin gene, thereby creating a modified oocyte, wherein deletion of exon 50 by CRISPR/Cas9 results in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene;

(b) transferring the modified oocyte into a recipient female.

58. The method of claim 57, wherein the oocyte comprises a dystrophin gene having a reporter gene located downstream of and in frame with exon 79 of the dystrophin gene, and upstream of a dystrophin 3'-UTR, wherein the reporter gene is expressed when exon 79 is translated in frame with exon 49.

59. The method of claim 58, wherein the reporter gene is luciferase.

60. The method of any of claims 57-59, further comprising a protease coding sequence upstream of and in frame with the reporter gene, and downstream of and in frame with exon 79.

61. The method of claim 60, wherein the protease is autocatalytic.

62. The method of claim 60 or 61, wherein the protease is 2A protease.

63. The method of any of claims 57-62, wherein the mouse is heterozygous for the deletion.

64. The method of any of claims 57-62, wherein the mouse is homozygous for the deletion.

65. The method of any of claims 57-64, wherein the mouse exhibits increased creatine kinase levels compared to a wildtype mouse.

66. The method of any of claims 57-65, wherein the mouse does not exhibit detectable dystrophin protein in heart or skeletal muscle.

67. An isolated cell obtained from the genetically engineered mouse of any of claims 46-56.

68. The cell of claim 67, further comprising a reporter gene located downstream of and in frame with exon 79 of the dystrophin gene, and upstream of a dystrophin 3'-UTR, wherein the reporter gene is expressed when exon 79 is translated in frame with exon 49, in particular wherein the reporter is luciferase.

69. The cell of any of claims 66-68, further comprising a protease coding sequence upstream of and in frame with the reporter gene, and downstream of and in frame with exon 79.

70. The cell of claim 69, wherein the protease is autocatalytic.

71. The cell of claims 69 or 70, cell of claim 25, wherein the protease is 2A protease.

72. The cell of any of any of claims 69-71, wherein the cell is heterozygous for the deletion.

73. The cell of any of any of claims 67-71, wherein the cell is homozygous for the deletion.

74. A genetically engineered mouse produced by a method comprising the steps of:

(a) contacting a fertilized oocyte with CRISPR/Cas9 elements and two single guide RNA (sgRNA) targeting sequences flanking exon 50 of the dystrophin gene, thereby creating a modified oocyte, wherein deletion of exon 50 by CRISPR/Cas9 results in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene;

(b) transferring the modified oocyte into a recipient female.

75. A method of screening a candidate substance for DMD exon-skipping activity comprising:

(a) contacting a mouse according to any of claims 43, 46, 47, or 74 with the candidate substance; and

(b) assessing in frame transcription and/or translation of exon 79 of the dystrophin gene,

wherein the presence of in frame transcription and/or translation of exon 79 indicates the candidate substance exhibits exon-skipping activity.

76. A method of producing the genetically engineered mouse of any of claims 47-56 comprising:

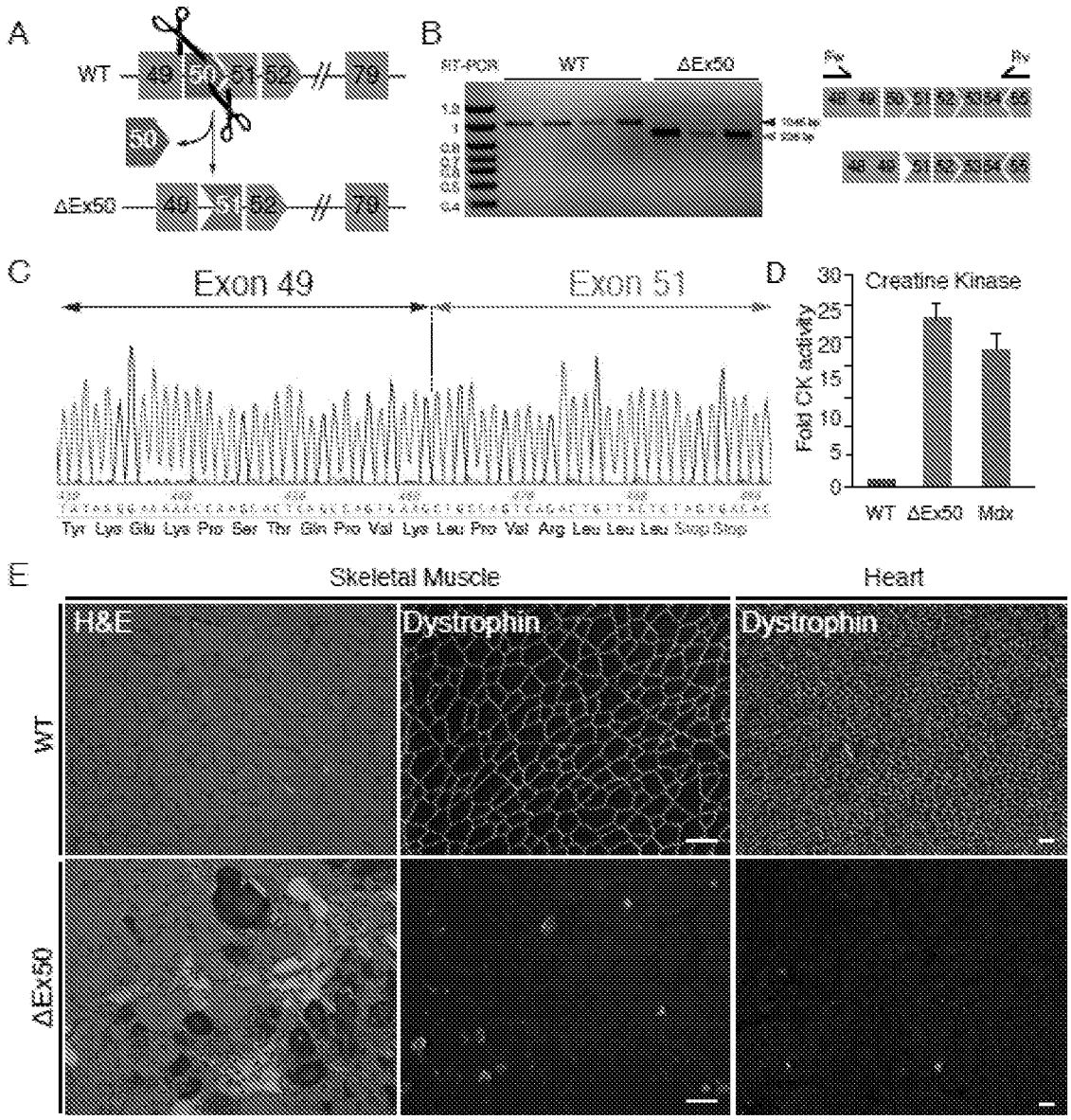
(a) contacting a fertilized oocyte with CRISPR/Cpf1 elements and two single guide RNA (sgRNA) targeting sequences flanking exon 50 of the dystrophin gene, thereby creating a modified oocyte, wherein deletion of exon 50 by CRISPR/Cpf1 results in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene;

(b) transferring the modified oocyte into a recipient female.

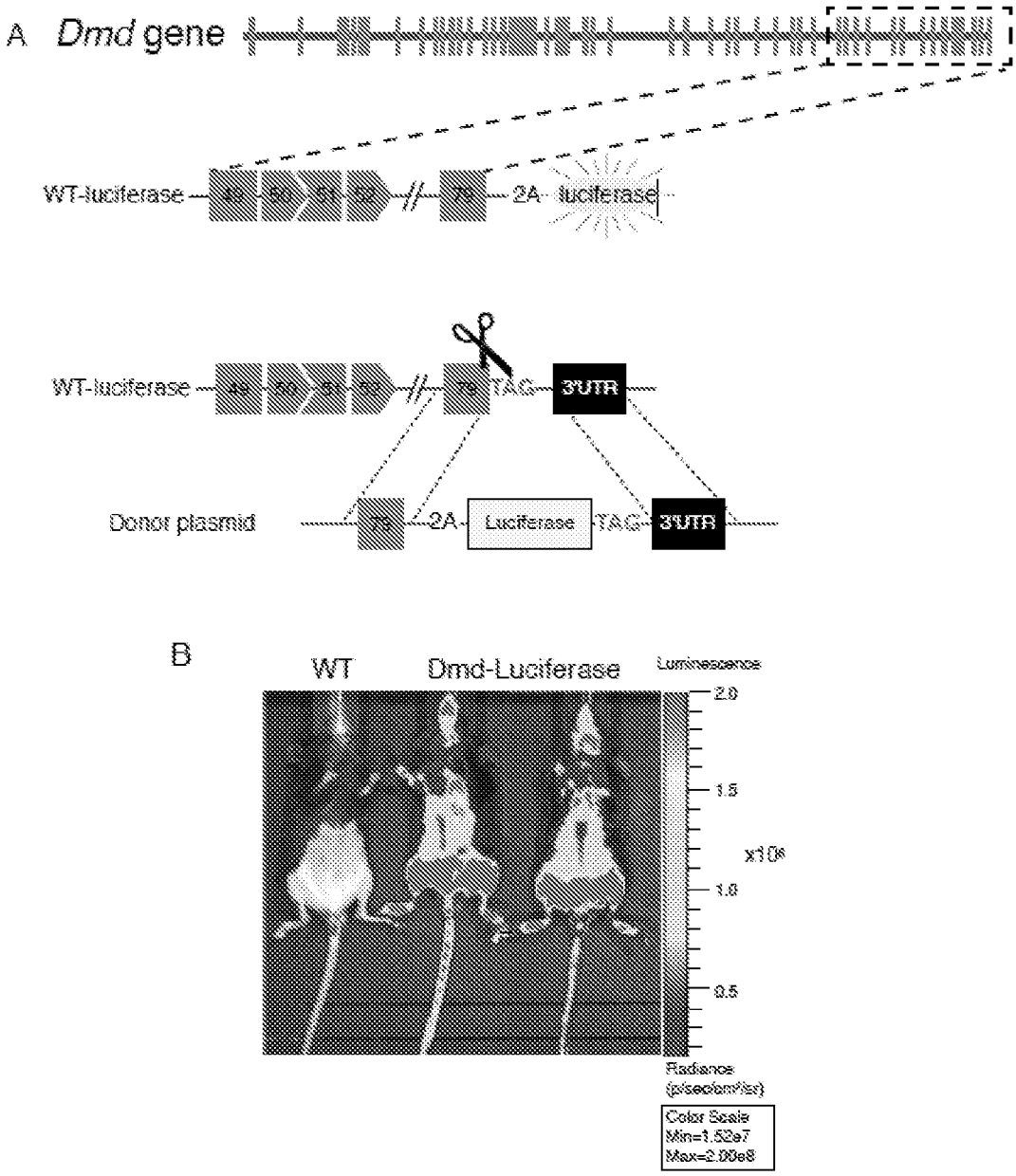
77. A genetically engineered mouse produced by a method comprising the steps of:

(a) contacting a fertilized oocyte with CRISPR/Cpf1 elements and two single guide RNA (sgRNA) targeting sequences flanking exon 50 of the dystrophin gene, thereby creating a modified oocyte, wherein deletion of exon 50 by CRISPR/Cpf1 results in an out of frame shift and a premature stop codon in exon 51 of the dystrophin gene;

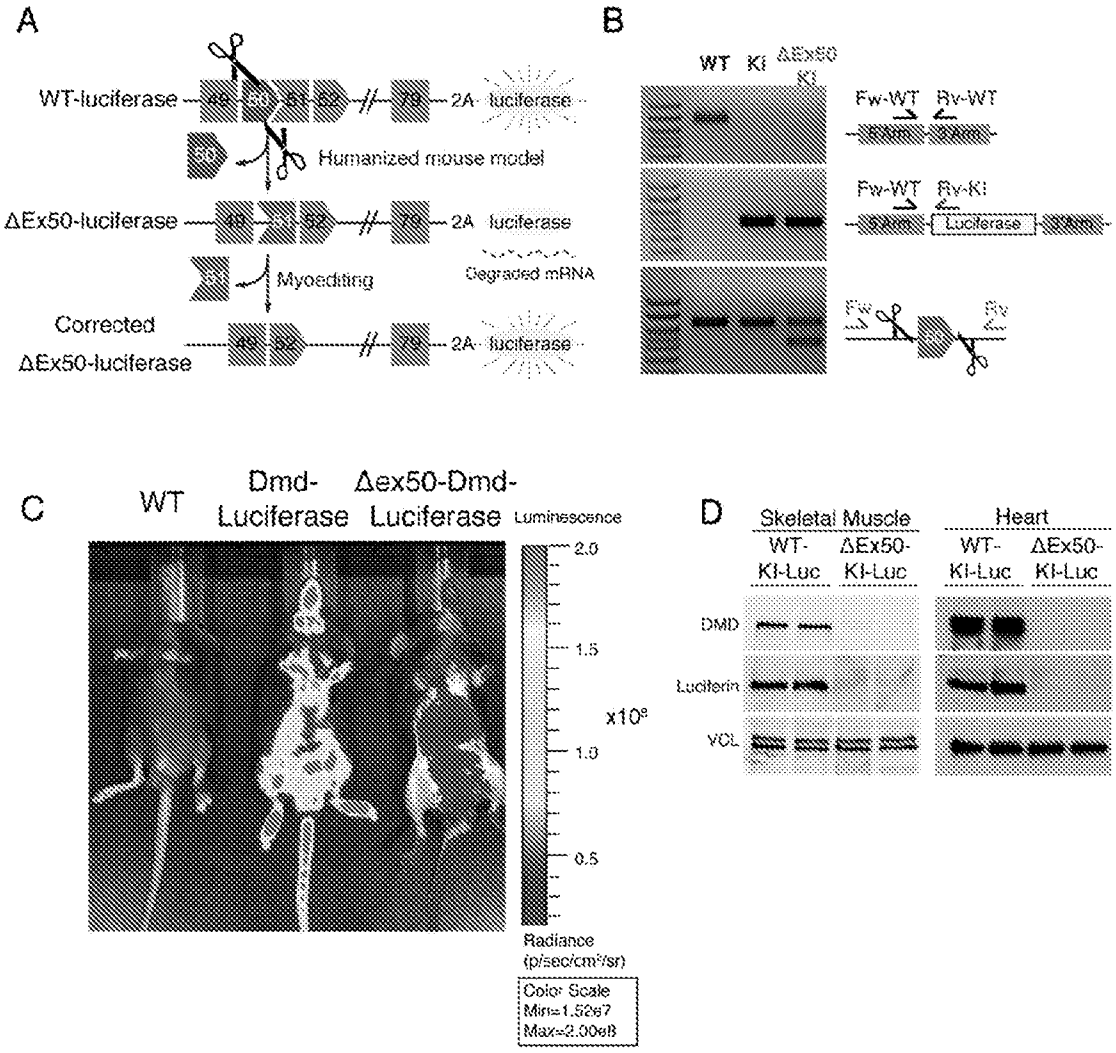
(b) transferring the modified oocyte into a recipient female.


A
WT
49
50
51
52
79
ΔEx50
B
RT-PCR
WT
ΔEx50
C
Exon 49
Exon 51
Tyr Lys Glu Lys Pro Ser Thr Gln Pro Val Lys Leu Pro Val Arg Leu Leu Leu Stop Stop
D
Creatine Kinase
Fold CK activity
WT
ΔEx50
Mdx
E
Skeletal Muscle
Heart
H&E
Dystrophin
Dystrophin
WT
ΔEx50

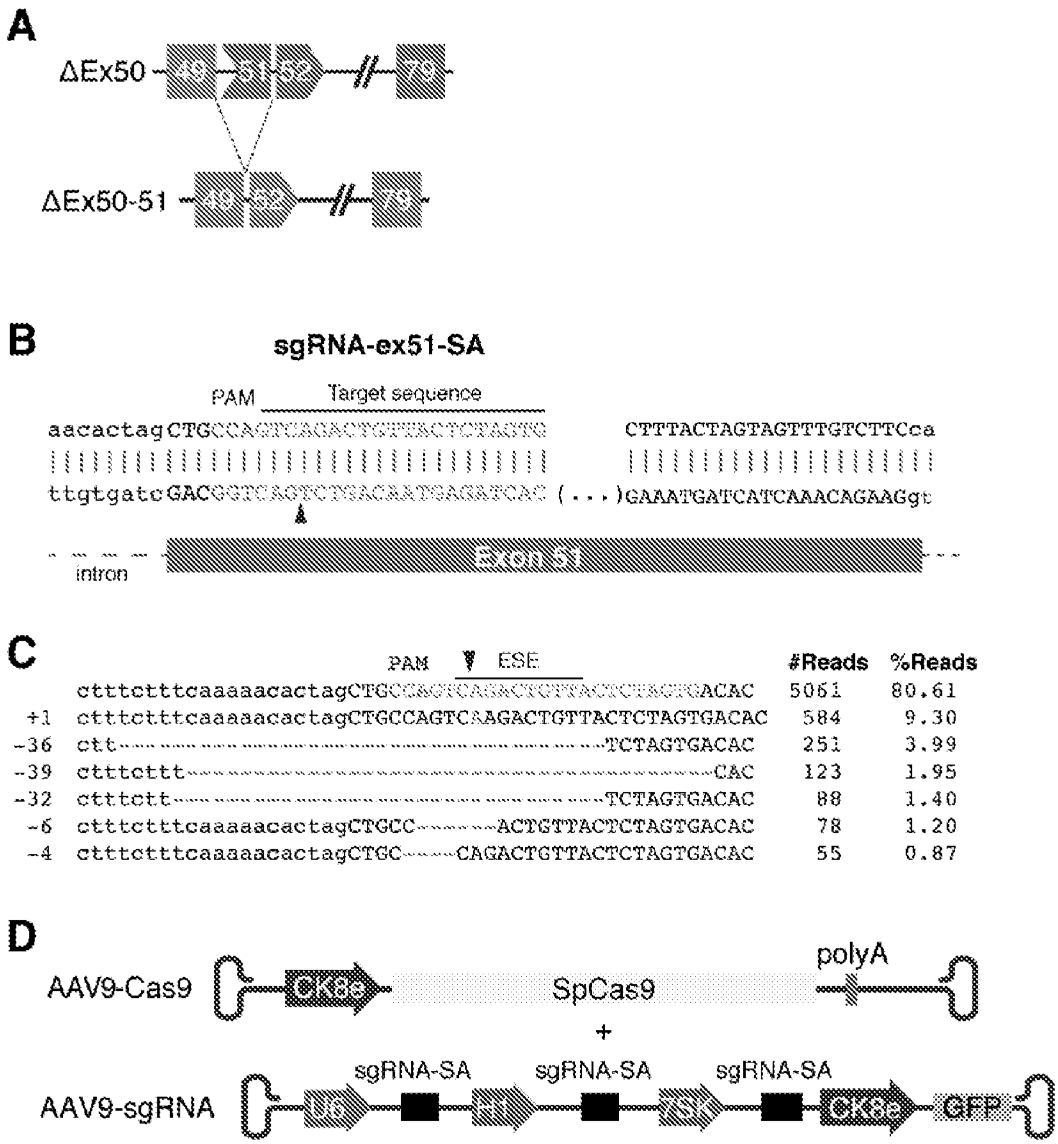
FIGS. 1A-E


A
Dmd gene
WT-luciferase
2A
luciferase
WT-luciferase
TAG
3'UTR
Donor plasmid
2A
Luciferase
TAG
3'UTR
B
WT
Dmd-Luciferase
Luminescence
2.0
1.5
x10⁶
1.0
0.5
Radiance
(p/sec/cm²/sr)
Color Scale
Min=1.52e7
Max=2.00e8

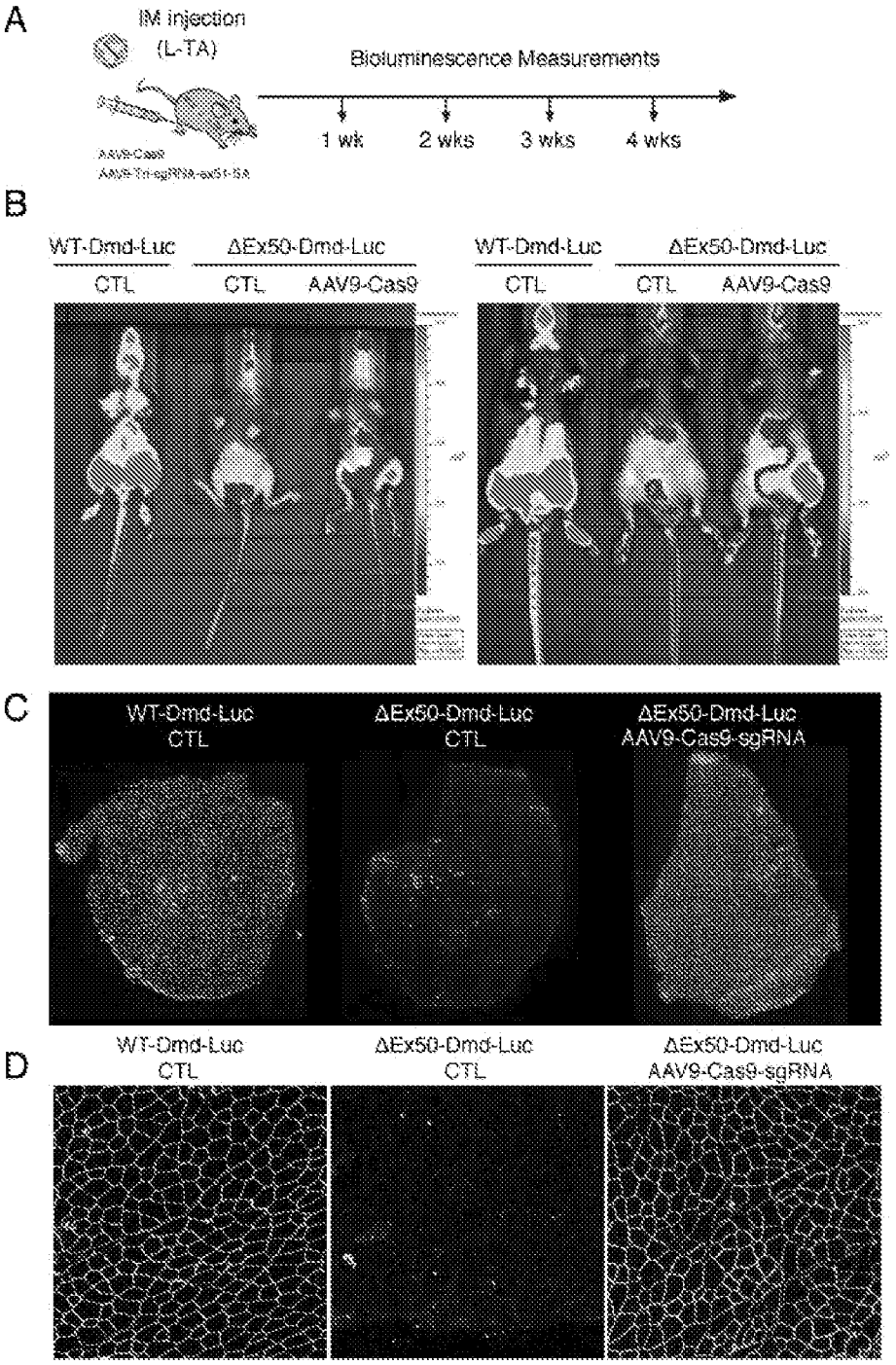
FIGS. 2A-B


A
WT-luciferase
2A luciferase
Humanized mouse model
ΔEx50-luciferase
2A luciferase
Myoediting
Degraded mRNA
Corrected
ΔEx50-luciferase
2A luciferase
B
WT
KI
ΔEx50 KI
Fw-WT
Rv-WT
Fw-WT
Rv-KI
Luciferase
Fw
Rv
C
WT
Dmd-Luciferase
Δex50-Dmd-Luciferase
Luminescence
2.0
1.5
x10⁸
1.0
0.5
Radiance (p/sec/cm²/sr)
Color Scale
Min=1.52e7
Max=2.00e8
D
Skeletal Muscle
Heart
WT-KI-Luc
ΔEx50-KI-Luc
WT-KI-Luc
ΔEx50-KI-Luc
DMD
Luciferin
VCL

FIGS. 3A-D


A
ΔEx50
49
51
52
79
ΔEx50-51
49
52
79
B
sgRNA-ex51-SA
PAM
Target sequence
aacactagCTGCCAGTCAGACTGTTACTCTAGTG CTTTACTAGTAGTTTGTCTTCca
ttgtgatcGACGGTCAGTCTGACAATGAGATCAC (...)GAAATGATCATCAAACAGAAGgt
intron
Exon 51
C
PAM
ESE
#Reads
%Reads
ctttctttcaaaaacactagCTGCCAGTCAGACTGTTACTCTAGTGACAC 5061 80.61
+1 ctttctttcaaaaacactagCTGCCAGTCAAGACTGTTACTCTAGTGACAC 584 9.30
-36 ctt TCTAGTGACAC 251 3.99
-39 ctttcttt CAC 123 1.95
-32 ctttctt TCTAGTGACAC 88 1.40
-6 ctttctttcaaaaacactagCTGCC ACTGTTACTCTAGTGACAC 78 1.20
-4 ctttctttcaaaaacactagCTGC CAGACTGTTACTCTAGTGACAC 55 0.87
D
AAV9-Cas9
CK8e
SpCas9
polyA
+
AAV9-sgRNA
sgRNA-SA
sgRNA-SA
sgRNA-SA
U6
H1
7SK
CK8e
GFP

FIGS. 4A-D


A
IM injection
(L-TA)
Bioluminescence Measurements
1 wk
2 wks
3 wks
4 wks
B
WT-Dmd-Luc
ΔEx50-Dmd-Luc
CTL
CTL
AAV9-Cas9
WT-Dmd-Luc
ΔEx50-Dmd-Luc
CTL
CTL
AAV9-Cas9
C
WT-Dmd-Luc
CTL
ΔEx50-Dmd-Luc
CTL
ΔEx50-Dmd-Luc
AAV9-Cas9-sgRNA
D
WT-Dmd-Luc
CTL
ΔEx50-Dmd-Luc
CTL
ΔEx50-Dmd-Luc
AAV9-Cas9-sgRNA

FIGS. 5A-D

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/065268

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/22 C12N15/10 C12N15/11 C12N15/90
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. W. MCGREEVY ET AL: "Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy", DISEASE MODELS & MECHANISMS, vol. 8, no. 3, 1 March 2015 (2015-03-01), pages 195-213, XP055235296, GB ISSN: 1754-8403, DOI: 10.1242/dmm.018424 abstract pages 195-196 Supplementary Table 1 ----- -/--	1-77

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2018	09/03/2018

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EIICHI ARAKI ET AL: "Targeted Disruption of Exon 52 in the Mouse Dystrophin Gene Induced Muscle Degeneration Similar to That Observed in Duchenne Muscular Dystrophy", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 238, no. 2, 1 September 1997 (1997-09-01), pages 492-497, XP055452618, AMSTERDAM, NL ISSN: 0006-291X, DOI: 10.1006/bbrc.1997.7328 abstract figure 1 -----	1-77
A	KATSUYUKI NAKAMURA ET AL: "Generation of muscular dystrophy model rats with a CRISPR/Cas system", SCIENTIFIC REPORTS, vol. 4, no. 1, 9 July 2014 (2014-07-09), XP055452622, DOI: 10.1038/srep05635 the whole document -----	1-77
A	ECHIGOYA YUSUKE ET AL: "Dystrophin Exon 52-Deleted Pigs as a New Animal Model of Duchenne Muscular Dystrophy: Its Characterization and Potential as a Tool for Developing Exon Skipping Therapy", MOLECULAR THERAPY, vol. 24, no. Suppl. 1, May 2016 (2016-05), page S247, XP002778344, & 19TH ANNUAL MEETING OF THE AMERICAN-SOCIETY-OF-GENE-AND-CELL-THERAPY (ASGCT); WASHINGTON, DC, USA; MAY 04 -07, 2016 abstract -----	1-77
A	Xinran Yu ET AL: "Dystrophin-deficient large animal models: translational research and exon skipping", Am J Transl Res Accepted July, 26 March 2015 (2015-03-26), pages 1314-1331, XP055452626, Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4568789/pdf/ajtr0007-1314.pdf [retrieved on 2018-02-20] the whole document ----- -/--	1-77

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GEMMA L. WALMSLEY ET AL: "A Duchenne Muscular Dystrophy Gene Hot Spot Mutation in Dystrophin-Deficient Cavalier King Charles Spaniels Is Amenable to Exon 51 Skipping", PLOS ONE, vol. 5, no. 1, 13 January 2010 (2010-01-13), page e8647, XP055452627, DOI: 10.1371/journal.pone.0008647 the whole document	1-77
X,P	LEONELA AMOASII ET AL: "Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy", SCIENCE TRANSLATIONAL MEDICINE, vol. 9, no. 418, 29 November 2017 (2017-11-29), page eaan8081, XP055452628, US ISSN: 1946-6234, DOI: 10.1126/scitranslmed.aan8081 the whole document	1-77