NOVEL MICRO-DYSTROPHINS AND RELATED METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Application No. 62/104,537, filed January 16, 2015, which is hereby incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant No. R01 AG033610, awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

[0003] The present disclosure relates generally to micro-dystrophins. The present disclosure also relates to methods of treating a subject having muscular dystrophy, sarcopenia, heart failure, or cachexia. The present disclosure also relates to methods of prophylactically treating a subject at risk of developing muscular dystrophy, sarcopenia, heart failure, or cachexia. In particular, the methods may include administering a pharmaceutical composition including a micro-dystrophin gene and a delivery vehicle to a subject. More particularly, the methods may include administering the pharmaceutical composition to a subject having Duchenne muscular dystrophy or Becker muscular dystrophy.

BACKGROUND

[0004] Duchenne muscular dystrophy (DMD) is a recessively-inherited muscle wasting disorder that affects approximately 1 in 3500 males. DMD patients carry a mutation in the dystrophin gene that causes aberrant expression or loss of expression of the dystrophin protein. DMD patients experience progressive wasting of skeletal muscles and cardiac dysfunction, which leads to loss of ambulation and premature death, primarily due to cardiac or respiratory failure. Unfortunately,

currently available treatments are generally only able to slow the pathology of DMD. Accordingly, there is an urgent need for compositions and methods for treating DMD.

SUMMARY OF THE INVENTION

[0005] The present disclosure is based, at least in part, on novel micro-dystrophins, compositions thereof, and related methods of use.

[0006] In some embodiments of the present disclosure, the isolated and purified nucleotide sequence, includes: (a) a micro-dystrophin gene encoding a protein including: an amino-terminal actin-binding domain; a β-dystroglycan binding domain; and a spectrin-like repeat domain, including at least four spectrin-like repeats, such that two of the at least four spectrin-like repeats include a neuronal nitric oxide synthase binding domain; and (b) a regulatory cassette.

[0007] In one embodiment, the at least four spectrin-like repeats include spectrin-like repeat 1 (SR1), spectrin-like repeat 16 (SR16), spectrin-like repeat 17 (SR17), and spectrin-like repeat 24 (SR24).

[0008] In another embodiment, the protein encoded by the micro-dystrophin gene further includes at least a portion of a hinge domain.

[0009] In yet another embodiment, the hinge domain is selected from at least one of a Hinge 1 domain, a Hinge 2 domain, a Hinge 3 domain, a Hinge 4 domain, and a hinge-like domain.

[0010] In still another embodiment, the regulatory cassette is selected from the group consisting of a CK8 promoter and a cardiac troponin T (cTnT) promoter.

[0011] In one embodiment, the protein encoded by the micro-dystrophin gene has between five spectrin-like repeats and eight spectrin-like repeats.

[0012] In another embodiment, the protein encoded by the micro-dystrophin gene has at least 80% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0013] In yet another embodiment, the protein encoded by the micro-dystrophin gene has at least 90% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0014] In still another embodiment, the protein encoded by the micro-dystrophin gene has at least 80% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0015] In one embodiment, the protein encoded by the micro-dystrophin gene has at least 90% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0016] In another embodiment, the regulatory cassette is the CK8 promoter, and wherein the CK8 promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0017] In yet another embodiment, the regulatory cassette is the CK8 promoter, and wherein the CK8 promoter has at least 90% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0018] In still another embodiment, the regulatory cassette is the cTnT promoter, and wherein the cTnT promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0019] In one embodiment, the regulatory cassette is the cTnT promoter, and wherein the cTnT promoter has at least 90% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0020] In certain embodiments of the present disclosure, the isolated and purified nucleotide sequence, includes: a micro-dystrophin gene encoding a protein including: an amino-terminal actin-binding domain; and at least two spectrin-like repeats that are directly coupled to each other, wherein the at least two spectrin-like repeats that are directly coupled to each other are selected from at least one of spectrin-like repeat 1 directly coupled to spectrin-like repeat 2, spectrin-like repeat 2 directly coupled to spectrin-like repeat 3, spectrin-like repeat 1 directly coupled to spectrin-like repeat 16, spectrin-like repeat 17 directly coupled to spectrin-like repeat 23, spectrin-like repeat 17 directly coupled to spectrin-like repeat 24, and spectrin-like repeat 23 directly coupled to spectrin-like repeat 24.

[0021] In certain other embodiments of the present disclosure, the isolated and purified nucleotide sequence, includes: a micro-dystrophin gene encoding a protein including, in order: a Hinge 1 domain (H1); a spectrin-like repeat 1 (SR1); a spectrin-

like repeat 16 (SR16); a spectrin-like repeat 17 (SR17); a spectrin-like repeat 24 (SR24); and a Hinge 4 domain (H4).

[0022] In one embodiment, the H1 is directly coupled to the SR1.

[0023] In another embodiment, the SR 1 is directly coupled to the SR16.

[0024] In yet another embodiment, the SR16 is directly coupled to the SR17.

[0025] In still another embodiment, the SR 17 is directly coupled to the SR24.

[0026] In another embodiment, the SR24 is directly coupled to the H4.

[0027] In yet another embodiment, the protein encoded by the micro-dystrophin gene further includes, between the SR1 and the SR16, in order, a spectrin-like repeat 2 (SR2) and a spectrin-like repeat 3 (SR3).

[0028] In still another embodiment, the SR1 is directly coupled to the SR2 and the SR2 is further coupled to the SR3.

[0029] In some embodiments of the present disclosure, the isolated and purified nucleotide sequence, includes: a micro-dystrophin gene encoding a protein including, in order: a Hinge 1 domain (H1); a spectrin-like repeat 1 (SR1); a spectrin-like repeat 16 (SR16); a spectrin-like repeat 17 (SR17); a spectrin-like repeat 23 (SR 23); a spectrin-like repeat 24 (SR24); and a Hinge 4 domain (H4).

[0030] In one embodiment, the H1 is directly coupled to the SR1, the SR1 is directly coupled to the SR16, the SR16 is directly coupled to the SR17, the SR17 is directly coupled to the SR23, the SR23 is directly coupled to the SR24, and the SR24 is directly coupled to the H4.

[0031] In certain embodiments of the present disclosure, the pharmaceutical composition, includes: an isolated and purified nucleotide sequence described herein; and a delivery vehicle.

[0032] In one embodiment, the delivery vehicle includes a recombinant adeno-associated virus vector.

[0033] In another embodiment, the delivery vehicle expresses the micro-dystrophin gene, such that the protein encoded by the micro-dystrophin gene has at least 80% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0034] In yet another embodiment, the delivery vehicle expresses the micro-dystrophin gene, such that the protein encoded by the micro-dystrophin gene has at least 90% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0035] In still another embodiment, the delivery vehicle expresses the micro-dystrophin gene, such that the protein encoded by the micro-dystrophin gene has at least 80% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0036] In another embodiment, the delivery vehicle expresses the micro-dystrophin gene, such that the protein encoded by the micro-dystrophin gene has at least 90% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0037] In some embodiments of the present disclosure, the pharmaceutical compositions described herein include a regulatory cassette, such that the regulatory cassette is the CK8 promoter, and the CK8 promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0038] In certain embodiments of the present disclosure, the pharmaceutical compositions described herein include a regulatory cassette, such that the regulatory cassette is the CK8 promoter, and the CK8 promoter has at least 90% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0039] In some embodiments of the present disclosure, the pharmaceutical compositions described herein include a regulatory cassette, such that the regulatory cassette is the cTnT promoter, and the cTnT promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0040] In certain embodiments of the present disclosure, the pharmaceutical compositions described herein include a regulatory cassette, such that the regulatory cassette is the cTnT promoter, and the cTnT promoter has at least 90% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0041] In some embodiments of the present disclosure, the pharmaceutical composition is configured to reduce a pathological effect or symptom of a muscular dystrophy selected from at least one of myotonic muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy, congenital muscular dystrophy,

oculopharyngeal muscular dystrophy, distal muscular dystrophy, and Emery-Dreifuss muscular dystrophy.

[0042] In certain embodiments of the present disclosure, the pharmaceutical composition is configured to reduce a pathological effect or symptom of a muscular dystrophy selected from at least one of Duchenne muscular dystrophy and Becker muscular dystrophy.

[0043] In some embodiments of the present disclosure, the pharmaceutical composition is configured to reduce a pathological effect or symptom of at least one of sarcopenia, heart disease, and cachexia.

[0044] In particular embodiments of the present disclosure, the pharmaceutical composition, includes: a micro-dystrophin gene including the nucleic acid sequence of SEQ ID NO:16; and an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector. In certain embodiments, the serotype of the AAV vector or the rAAV vector is selected from at least one of serotype 6, serotype 8, and serotype 9.

[0045] In some embodiments of the present disclosure, the pharmaceutical composition, includes: a micro-dystrophin gene encoding a protein, such that the protein includes the amino acid sequence of SEQ ID NO:4; and an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector. In certain embodiments, the serotype of the AAV vector or the rAAV vector is selected from at least one of serotype 6, serotype 8, and serotype 9.

[0046] In certain embodiments of the present disclosure, the pharmaceutical composition, includes: a micro-dystrophin gene including the nucleic acid sequence of SEQ ID NO:18; and an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector. In some embodiments, the serotype of the AAV vector or the rAAV vector is selected from at least one of serotype 6, serotype 8, and serotype 9.

[0047] In particular embodiments of the present disclosure, the pharmaceutical composition, includes: a micro-dystrophin gene encoding a protein, such that the protein includes the amino acid sequence of SEQ ID NO:5; and an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector. In some

embodiments, the serotype of the AAV vector or the rAAV vector is selected from at least one of serotype 6, serotype 8, and serotype 9.

[0048] In some embodiments of the present disclosure, the pharmaceutical compositions suitable for use in the treatment or prophylactic treatment of muscular dystrophy, include: a micro-dystrophin gene including the nucleic acid sequence of SEQ ID NO:16 or SEQ ID NO:18; and an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector, such that the serotype of the AAV vector or the rAAV vector is selected from at least one of serotype 6, serotype 8, and serotype 9.

[0049] In certain embodiments of the present disclosure, the pharmaceutical compositions suitable for the treatment or prophylactic treatment of muscular dystrophy, include: a micro-dystrophin gene including the nucleic acid sequence of SEQ ID NO:16 or SEQ ID NO:18; and an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector, such that the serotype of the AAV vector or the rAAV vector is selected from at least one of serotype 6, serotype 8, and serotype 9.

[0050] In particular embodiments of the present disclosure, the methods for treating a subject having muscular dystrophy, include: administering to the subject a therapeutically effective amount of a pharmaceutical composition including a micro-dystrophin gene operably coupled to a regulatory cassette.

[0051] In one embodiment, the regulatory cassette is selected from the group consisting of a CK8 promoter and a cardiac troponin T (cTnT) promoter.

[0052] In another embodiment, the regulatory cassette is configured to express the micro-dystrophin gene such that a level of expression of the micro-dystrophin gene is at least 100-fold higher in striated muscle cells than the level of expression of the micro-dystrophin gene in non-muscle cells.

[0053] In certain embodiments of the present disclosure, the pharmaceutical compositions described herein further include a recombinant adeno-associated virus vector configured to express the micro-dystrophin gene in the subject.

[0054] In one embodiment, the micro-dystrophin gene encodes a protein having at least 80% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0055] In another embodiment, the micro-dystrophin gene encodes a protein having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0056] In yet another embodiment, the micro-dystrophin gene encodes a protein having at least 80% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0057] In still another embodiment, the micro-dystrophin gene encodes a protein having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0058] In one embodiment, the regulatory cassette is the CK8 promoter, and the CK8 promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0059] In another embodiment, the regulatory cassette is the CK8 promoter, and the CK8 promoter has at least 90% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0060] In yet another embodiment, the regulatory cassette is the cTnT promoter, and the cTnT promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0061] In still another embodiment, the regulatory cassette is the cTnT promoter, and the cTnT promoter has at least 90% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0062] In one embodiment, the micro-dystrophin gene expresses a micro-dystrophin protein in one or more muscles of the subject such that contractility of the one or more muscles is enhanced.

[0063] In another embodiment, the micro-dystrophin gene expresses a micro-dystrophin protein in one or more skeletal muscles of the subject such that a specific-force generating capacity of at least one of the one or more skeletal muscles is increased to within at least 40% of a normal specific-force generating capacity.

[0064] In yet another embodiment, the micro-dystrophin gene expresses a micro-dystrophin protein in one or more cardiac muscles of the subject such that a baseline end-diastolic volume defect is restored to within at least 40% of a normal end-diastolic volume.

[0065] In still another embodiment, the micro-dystrophin gene expresses a micro-dystrophin protein such that localization of the neuronal nitric oxide synthase to the dystrophin-glycoprotein complex is enhanced in the subject.

[0066] In some embodiments, the muscular dystrophy is selected from at least one of myotonic muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy, congenital muscular dystrophy, oculopharyngeal muscular dystrophy, distal muscular dystrophy, and Emery-Dreifuss muscular dystrophy.

[0067] In certain embodiments, the muscular dystrophy is selected from at least one of Duchenne muscular dystrophy and Becker muscular dystrophy.

[0068] In some embodiments of the present disclosure, the pharmaceutical composition reduces a pathological effect or symptom of the muscular dystrophy.

[0069] In particular embodiments, the pathological effect or symptom of the muscular dystrophy is selected from at least one of muscle pain, muscle weakness, muscle fatigue, muscle atrophy, fibrosis, inflammation, increase in average myofiber diameter in skeletal muscle, cardiomyopathy, reduced 6-minute walk test time, loss of ambulation, and cardiac pump failure.

[0070] In some embodiments, the methods described herein include identifying the subject having the muscular dystrophy.

[0071] In certain embodiments, the subject is a mammal.

[0072] In particular embodiments, the subject is a human.

[0073] In some embodiments of the present disclosure, the methods for prophylactically treating a subject at risk of developing muscular dystrophy, include administering to the subject a therapeutically effective amount of a pharmaceutical composition including a micro-dystrophin gene operably coupled to a regulatory cassette.

[0074] In one embodiment, the regulatory cassette is selected from the group consisting of a CK8 promoter and a cardiac troponin T (cTnT) promoter.

[0075] In further embodiments, the regulatory cassette is configured to express the micro-dystrophin gene such that a level of expression of the micro-dystrophin

gene is at least 100-fold higher in striated muscle cells than the level of expression of the micro-dystrophin gene in non-muscle cells.

[0076] In particular embodiments, the pharmaceutical composition further includes a recombinant adeno-associated virus vector configured to express the micro-dystrophin gene in the subject.

[0077] In certain embodiments, the micro-dystrophin gene encodes a protein having at least 80% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0078] In another embodiment, the micro-dystrophin gene encodes a protein having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0079] In some embodiments, the micro-dystrophin gene encodes a protein having at least 80% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0080] In yet another embodiment, the micro-dystrophin gene encodes a protein having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0081] In certain embodiments, the regulatory cassette is the CK8 promoter, and the CK8 promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0082] In another embodiment, the regulatory cassette is the CK8 promoter, and the CK8 promoter has at least 90% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0083] In yet another embodiment, the regulatory cassette is the cTnT promoter, and the cTnT promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0084] In still another embodiment, the regulatory cassette is the cTnT promoter, and the cTnT promoter has at least 90% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0085] In particular embodiments, the micro-dystrophin gene expresses a micro-dystrophin protein in one or more muscles of the subject such that contractility of the one or more muscles is enhanced.

[0086] In another embodiment, the micro-dystrophin gene expresses a micro-dystrophin protein in one or more skeletal muscles of the subject such that a

specific-force generating capacity of at least one of the one or more skeletal muscles is increased to within at least 40% of a normal specific-force generating capacity.

[0087] In some embodiments, the micro-dystrophin gene expresses a micro-dystrophin protein in one or more cardiac muscles of the subject such that a baseline end-diastolic volume defect is restored to within at least 40% of a normal end-diastolic volume.

[0088] In certain embodiments, the micro-dystrophin gene expresses a micro-dystrophin protein such that localization of the neuronal nitric oxide synthase to the dystrophin-glycoprotein complex is enhanced in the subject.

[0089] In particular embodiments, the muscular dystrophy is selected from at least one of myotonic muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy, congenital muscular dystrophy, oculopharyngeal muscular dystrophy, distal muscular dystrophy, and Emery-Dreifuss muscular dystrophy.

[0090] In some embodiments, the muscular dystrophy is selected from at least one of Duchenne muscular dystrophy and Becker muscular dystrophy.

[0091] In certain embodiments, the pharmaceutical compositions described herein reduce a risk of developing a pathological effect or symptom of the muscular dystrophy.

[0092] In one embodiment, the pathological effect or symptom of the muscular dystrophy is selected from at least one of muscle pain, muscle weakness, muscle fatigue, muscle atrophy, fibrosis, inflammation, increase in average myofiber diameter in skeletal muscle, cardiomyopathy, reduced 6-minute walk test time, loss of ambulation, and cardiac pump failure.

[0093] In some embodiments of the present disclosure, the methods described herein further include identifying the subject at risk of developing the muscular dystrophy.

[0094] In one embodiment, the subject is a mammal.

[0095] In another embodiment, the subject is a human.

[0096] Other features and advantages of the disclosure will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0100] The embodiments disclosed herein will become more fully apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

[0101] FIG. 1A depicts protein structure diagrams of embodiments of truncated dystrophin constructs as disclosed herein. NT, amino terminal domain; H, hinge; R, spectrin-like repeat; nNOS BD, neuronal nitric oxide synthase binding domain; CR, cysteine-rich domain; CT, carboxyl terminal domain; Syn, syntrophin binding domain; Db BD, dystrobrevin binding domain; the unlabeled region marks 20-amino acids between R15 and R16; aa, amino acid; and kDa, kilodalton.

[0102] FIG. 1B is a Western blot illustrating the results of injecting dystrophic *mdx*^{4cv} mice with 5x10^{10} vector genomes (vg) of rAAV/CMV-μDys into one tibialis anterior (TA) muscle while, the contralateral muscle served as an internal, untreated control. Expression of all tested constructs was verified at 4 weeks after treatment by Western blot analysis of TA muscle lysates, along with wild type and untreated *mdx*^{4cv} controls. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal loading control.

[0103] FIGS. 1C and 1D are graphs depicting quantification of myofibers from TA cross sections for dystrophin expression and central nucleation at 4 or 12 weeks post-treatment, respectively (N=3-5 per cohort for each time point, mean ± S.E.M.). μDysH3 served as a comparative gauge of performance. μDys6 and μDys7 were too large to be cloned into AAV-expression vectors using the ubiquitous cytomegalovirus (CMV) promoter, consequently, the CMV promoter was replaced with the myogenic-specific CK8 promoter to allow efficient packaging and *in vivo* evaluation. Accordingly, μDysH3 was re-evaluated with the CK8 regulatory expression cassette. Characters denote significance from wild type mice. *P<0.05, **P<0.01, ***P<0.001, #P<0.0001.

[0104] FIG. 2 is a series of micrographs depicting representative gastrocnemius cross sections at six months post-treatment. Dystrophin and DAPI-stained nuclei are shown in the left column, β-dystroglycan and DAPI are shown in the middle column,

and neuronal nitric oxide synthase (nNOS) is shown in the right column, as indicated. Each row depicts representative results from cohorts of wild type, treated *mdx*^{4cv}, and untreated *mdx*^{4cv} mice. Scale bar, 200 μm. Recruitment of dystrophin glycoprotein complex (DGC) members is generally dependent on binding domains within μDys constructs. Dystrophic *mdx*^{4cv} mice were injected retro-orbitally with 1x10^{13} vg of rAAV6/CK8-μDys at 14 days of age. Three and six months post-treatment, skeletal muscles were immunostained for DGC members.

[0105] FIGS. 3A-3D are graphs depicting evaluation of systemic treatment at 3 months post-treatment. Gastrocnemius muscles (FIGS. 3A and 3B) and diaphragm muscles (FIGS. 3C and 3D) were evaluated to determine the performance of novel μDys constructs. Muscle cross sections were quantified for dystrophin expression and centrally nucleated myofibers. Levels of myofibers exhibiting dystrophin expression and/or exhibiting central nucleation are represented as percentages (FIGS. 3A and 3C). Specific force generation was measured *in situ* for gastrocnemius (FIG. 3B) and *in vitro* for diaphragm strips (FIG. 3D). The n value for each cohort is listed in columns of FIG. 3D. For non-bracketed characters, *P<0.05, **P<0.01, ***P<0.001 from wild type. ^P<0.05, ^^P<0.01, ^^^P<0.001 from μDys2-treated mice. #P<0.05, ##P<0.01, ###P<0.001 from μDys5-treated mice.

[0106] FIGS. 4A-4D are graphs depicting evaluation of systemic treatment at 6 months post-treatment. Gastrocnemius muscles (FIGS. 4A and 4B) and diaphragm muscles (FIGS. 4C and 4D) were evaluated as described in FIGS. 3A-3D. The n value for each cohort is listed in columns of FIG. 4D. *P<0.05, **P<0.01, ***P<0.001 from wild type. ^P<0.05, ^^P<0.01, ^^^P<0.001 from μDys2-treated mice. #P<0.05, ##P<0.01, ###P<0.001 from μDys7-treated mice.

[0107] FIGS. 5A and 5B are graphs depicting the extent of sarcolemmal protection from eccentric contraction in skeletal muscles. Systemically treated mice, as described in FIGS. 2 and 4A-4D, were subjected to eccentric contractions of increasing length. Gastrocnemius (FIG. 5A) and diaphragm strips (FIG. 5B) were measured for the maximum isometric force generated prior to an eccentric contraction. During stimulating contractions, muscles were lengthened at a defined distance beyond their optimum fiber lengths. Distances are reported as percentage

beyond optimal fiber length (L_O). *P<0.05, ***P<0.001, ****P<0.0001 from wild type at 45% beyond L_O. ^^^P<0.001, ^^^^P<0.0001 from μDys2-treated mice at 45% beyond L_O. ∇∇∇∇P<0.0001 from μDys7-treated mice at 45% beyond L_O.

[0108] FIG. 6 is a series of micrographs illustrating that systemically tested novel μDys constructs do not induce ringbinden phenotype in skeletal muscle. Dystrophic *mdx*^{4cv} mice were injected retro-orbitally with 1x10^{13} vg at 14 days of age. Six months post-treatment, cross sections of gastrocnemius muscles were immunostained for dystrophin, DAPI, and α-sarcomeric actin. One representative section is shown from cohorts of wild type (panel "a") and *mdx*^{4cv} treated with μDysH3 (panel "b"), μDys1 (panel "c"), μDys2 (panel "d"), μDys5 (panel "e"), μDys6 (panel "f"), μDys7 (panel "g"), or untreated *mdx*^{4cv} mice (panel "h"). Gastrocnemius from transgenic mice expressing ΔR4-R23/ΔCT (*see* Harper, S. Q., *et al.*, Nature Medicine 8, 253-261, (2002)) on *mdx*^{4cv} background (panel "i") was also immunostained as a positive control. Arrowheads mark examples of ringbinden formation around myofibers. Scale bar, 50 μm.

[0109] FIG. 7 depicts protein structure diagrams of embodiments of novel micro-dystrophin constructs as disclosed herein. The top protein structure diagram is of full-length dystrophin showing many of the known functional domains: NT, amino terminal actin-binding domain; H, hinge; R, spectrin-like repeat; nNOS BD, neuronal nitric oxide synthase binding domain; CR, cysteine-rich domain; CT, carboxyl terminal domain; Dg BD, dystroglycan binding domain; Syn, syntrophin binding domain; Db BD, dystrobrevin binding domain; and the unlabeled region marks 20-amino acids between R15 and R16. The WW domain is within Hinge 4. On the left are shown the micro-dystrophin protein structures, with the designated name to the left of the protein structure diagram, and the domain structure listed to the right of the schematic diagram.

[0110] FIG. 8 is two graphs depicting left ventricle (LV) ejection fraction at 2 weeks (left) and 3 weeks (right) for untreated (UN; n=S) vs. low (L; n=3) or high (H; n=3) dose of AAV6-L48Q.

[0111] FIG. 9 is an anti-cTnC Western blot for AAV6-L48Q cTnC injected mouse cardiac tissue (left) and uninjected control (right), as indicated

[0112] FIG. 10A is a Western blot for RI, with GAPDH as a loading control.

[0113] FIG. 10B is a Western blot for R2, with GAPDH as a loading control.

[0114] FIG. 10C is a graph depicting HPLC of transfected cardiomyocytes [dATP].

[0115] FIG. 11 is two graphs. The graph at the left depicts the percentage fractional shortening (FS) increase in R1R2 over-expressing mice vs. control littermates. The graph at the right depicts the change in left ventricular inner diameter (LVID) in R1R2 over-expressing mice vs. control littermates. d-diastole, s-systole.

[0116] FIG. 12A depicts mouse aortic smooth muscle contraction traces with ATP and dATP.

[0117] FIG. 12B is a graph depicting a summary of the data in FIG. 12A.

[0118] FIG. 13A depicts Western blots for R1 and R2.

[0119] FIG. 13B depicts α-tubulin as a loading control for the Western blots of FIG. 13A.

[0120] FIG. 14 shows preliminary Western blot evidence for the expression levels of RI and R2 subunits in the skeletal muscle, lung, and heart of rAAV6-R1R2^{cTnT455} injected ($4.5x10^{13}$) mice and control mice (panel "A"). FIG. 14 also provides data for heart tissue from non-injected (panel "B") vs. AAV6-alkaline phosphatase (panel "C") injected mice (*see* Rafael, J. A., *et al.*, The Journal of Cell Biology 134, 93-102 (1996)) after 20 months, suggesting AAV6-R1R2^{cTnT455} may provide stable, long-term R1R2 over-expression.

[0121] FIG. 15 is a graph showing the effect of $1.5x10^{13}$, $4.5x10^{13}$, and $1.35x10^{14}$ rAAV6-RIR2^{cTnT455} vector genomes or saline (control) injected systemically over an approximate 10-fold range into 3 month old mice (n=6 per group) on LV function.

[0122] FIG. 16 is two graphs showing the change in fractional shortening in rats given direct cardiac injections of rAAV6-R1R2 on the fifth day post-infarct as measured by echocardiography in comparison with untreated infarct rats and untreated sham-operated rats.

[0123] FIG. 17 is a graph showing the *in vitro* Neely working heart measurements of the rat hearts assessed in FIG. 16. Power on the y-axis is given in units of

g•cm/min. A loss of pre-load responsiveness of hearts (heart failure) that have been infarcted (no treatment) and a recovery of pre-load responsiveness of the infarcted hearts receiving the vectors to the level of control, uninfarcted hearts were observed, thereby demonstrating a restoration of cardiac function.

[0124] FIG. 18 illustrates miniaturization of human-cTnT (Enh + Promoter) regulatory cassettes based on deleting sequences hypothesized to have relatively low activities.

[0125] FIG. 19 illustrates transcription tests of the FIG. 18 deletions relative to a native human-cTnT enhancer/promoter; the 320 bp version retains ~95% of the activity.

[0126] FIG. 20 illustrates increased activity of human-cTnT455 via adding a second miniaturized enhancer compared to the native enhancer/promoter, the 320 bp version, and to a Chicken cTnT promoter/enhancer (*see* American Journal of Physiology - Cell Physiology 280, C556-C564 (2004)).

[0127] FIG. 21 is a series of schematic illustrations of the structure of dystrophin spectrin-like repeats and the juxtaposition with hinge domains. Top left, interdigitated folding of individual spectrin-like repeats is illustrated to show how 3 adjacent repeats can fold together, with the different alpha-helical segments highlighted (a, b, c; a', b', c'; and a'', b'', c'' representing the helical domains of the three different spectrin-like repeats). Top right, in native dystrophin and utrophin, some spectrin-like repeats are separated by hinge domains that disrupt the normal interdigitated folding of adjacent spectrin-like repeats. Shown at the top right is the folding pattern of Spectrin-like repeats 18, 19, and 20 and their separation by Hinge 3. Middle left, Optimized mini- and micro-dystrophins typically display maximal functional activity when the spectrin-like repeats domains are arranged in such a way as to preserve normal folding patterns; this normal folding is disrupted when non-integral units of spectrin-like repeats are present in a mini- or micro-dystrophin proteins, such as when a natural occurring deletion that removes whole exons occurs in a Becker muscular dystrophy patient. This latter situation is illustrated in the schematic illustration at the middle right, which represents the predicted structure of the junctional domain of dystrophin from a patient with a genomic deletion

removing exons 17-48. The bottom schematic illustrations show the folding pattern predicted in the μDysH2 (left) and μDysH3 (right) proteins, and also illustrate the unpredictable nature of the functional activity of miniaturized dystrophin proteins. While μDysH2 and μDysH3 have similar folding patterns, μDysH2 leads to ringbinden when expressed in *mdx* mouse skeletal muscles, whereas μDysH3 does not lead to ringbinden (*see* Banks, G. B., *et al.*, PLoS Genetics 6, e1000958, (2010)).

[0128] FIG. 22 is an image of an mdx^{4cv} mouse muscle cryosection that was stained for dystrophin expression using an anti-dystrophin antibody.

DETAILED DESCRIPTION

[0129] The present disclosure features compositions and methods for treating Duchenne muscular dystrophy (DMD). More particularly, the present disclosure relates to methods for producing mini-dystrophin proteins for treating a subject having muscular dystrophy, DMD, sarcopenia, heart failure, and/or cachexia. As described in detail below, the present disclosure is based, at least in part, on the unexpected discovery that mini-dystrophin proteins comprising specific combinations of protein domains (*e.g.*, a mini-dystrophin protein including an N-terminal domain, H1 domain, SR1 domain, SR16 domain, SR17 domain, SR 23 domain, SR24 domain, H4 domain, and CR domain) from the dystrophin protein are able to restore dystrophin function to levels sufficient to treat muscular dystrophy, DMD, sarcopenia, heart failure, and/or cachexia.

[0130] Duchenne muscular dystrophy (DMD) is a recessively-inherited muscle wasting disorder afflicting approximately 1 in 3500 males. DMD patients carry a mutation in the *dystrophin* gene, resulting in aberrant or absent expression of the dystrophin protein. DMD patients experience progressive wasting of skeletal muscles and cardiac dysfunction, leading to loss of ambulation and premature death, primarily due to cardiac or respiratory failure. Current available treatments are generally only able to slow the pathology of DMD (*see* Emery, A. E. H. and Muntoni, F., Duchenne Muscular Dystrophy, Third Edition (Oxford University Press, 2003)). Gene therapy approaches for DMD have been demonstrated in dystrophic animal models by either directly targeting a class of mutations, as with exon skipping, or

replacing the mutated gene with viral-vector mediated delivery (*see* Koo, T. and Wood, M. J. Human Gene Therapy 24, (2013); Benedetti, S., *et al.*, The FEBS Journal 280, 4263-4280, (2013); and Seto, J. T., *et al.*, Current Gene Therapy 12, 139-151 (2012)). Recombinant adeno-associated virus (rAAV) vectors are a potential vehicle for gene therapy, being already tested in clinical trials for both DMD and limb-girdle muscular dystrophies (*see* Mendell, J. R., *et al.*, The New England Journal of Medicine 363, 1429-1437, (2010); Mendell, J. R., *et al.*, Annals of Neurology 68, 629-638 (2010); and Herson, S., *et al.*, Brain: A Journal of Neurology 135, 483-492, (2012)). Several serotypes of adeno-associated virus (AAV) demonstrate a high degree of tropism for striated muscles (*see* Seto, J. T., *et al.*, Current Gene Therapy 12, 139-151 (2012)).

[0131] Pre-clinical studies designing and testing newer generations of therapeutic constructs for DMD can be confined by the approximately 4.9 kb size of a single-stranded rAAV vector genome (*see* Dong, B., *et al.*, Molecular Therapy: The Journal of the American Society of Gene Therapy 18, 87-92, (2010) and Wu, Z., *et al.*, Molecular Therapy: The Journal of the American Society of Gene Therapy 18, 80-86, (2010)). Packaging the entire approximately 13.9 kb cDNA of the muscle-specific isoform of dystrophin into a single rAAV capsid cannot be achieved, accordingly, miniaturized, synthetic versions of the muscle-specific isoform of dystrophin cDNA may be used. Although *in vivo* recombination of two and three rAAV vector genomes has been demonstrated to deliver a mini- or full-length dystrophin coding sequence (*see*, Odom, G. L., *et al.*, Molecular Therapy: The Journal of the American Society of Gene Therapy 19, 36-45, (2011); Lostal, W., *et al.*, Human Gene Therapy, (2014); and Koo, T., *et al.*, Human Gene Therapy 25, 98-108, (2014)), the efficiency of delivering multiple vectors for reconstituting full-length dystrophin may be suboptimal and can increase the overall dose of viral capsid proteins needed for delivering vectors. However, beneficial rAAV-mediated gene therapy has been achieved using rationally-designed miniature versions of the dystrophin cDNA based in part on mRNA expressed in mild Becker muscular dystrophy patients carrying in-frame deletions within the gene (*see* Beggs, A. H., *et al.,* American Journal of Human Genetics 49, 54-67 (1991); Koenig, M., *et al.*, American Journal of Human

Genetics 45, 498-506 (1989); Goldberg, L. R., *et al.*, Annals of Neurology 44, 971-976, (1998); and England, S. B., *et al.*, Nature 343, 180-182 (1990)). Studies in transgenic and vector treated dystrophic mice expressing various dystrophin truncations have identified several elements of the *dystrophin* gene that may be present in a functional micro-dystrophin (μDys) (*see* Harper, S. Q., *et al.*, Nature Medicine 8, 253-261, (2002)).

[0132] The full-length striated muscle isoform of dystrophin can play a role in transmitting contractile force through the sarcolemma and out to the extracellular matrix. In addition to maintaining the mechanical link between the intracellular cytoskeleton and the membrane bound dystrophin glycoprotein complex (DGC), dystrophin can also be a scaffold for signaling proteins (*see* Ozawa, E. in Myology (ed. Franzini-Armstrong C Engel A) 455-470 (McGraw-Hill, 2004); Winder, S. J. Journal of Muscle Research and Cell Motility 18, 617-629 (1997); and Campbell, K. P. and Kahl, S. D. Nature 338, 259-262, (1989)). The amino-terminal domain of dystrophin can bind to F-actin filaments of the intracellular cytoskeleton (*see* Way, M., *et al.*, FEBS Letters 301, 243-245 (1992); Hemmings, L., *et al.*, The Journal of Cell Biology 116, 1369-1380 (1992); Fabbrizio, E., *et al.*, Biochemistry 32, 10457-10463 (1993); and Pavalko, F. M. and Otey, C. A. Proceedings of the Society for Experimental Biology and Medicine 205, 282-293 (1994)). The middle, rod domain is the largest and is composed of 24 spectrin-like repeats (SRs) that are flanked and interspersed with at least four hinge sub-domains. The rod domain can give dystrophin elasticity and flexibility for maintaining the integrity of the sarcolemma during muscle contractility (*see* Winder, S. J. Journal of Muscle Research and Cell Motility 18, 617-629 (1997)). Various SRs provide unique regions that can serve as additional binding sites for the intracellular cytoskeleton, the sarcolemma, as well as members of the DGC (*see* Rybakova, I. N., *et al.*, The Journal of Cell Biology 135, 661-672 (1996); Warner, L. E., *et al.*, Human Molecular Genetics 11, 1095-1105 (2002); Metzinger, L., *et al.*, Human Molecular Genetics 6, 1185-1191 (1997); Lai, Y., *et al.*, The Journal of Clinical Investigation 119, 624-635, (2009)). In particular, the cysteine-rich domain and the adjacent Hinge 4 region form the β-dystroglycan binding domain (Dg BD) (*see* Blake, D. J., *et al.*, Physiological Reviews 82, 291-329,

(2002); Ishikawa-Sakurai, M., *et al.*, Human Molecular Genetics 13, 693-702, (2004)), while the carboxy-terminal domain is a scaffold for additional DGC components (*see* Abmayr S, in Molecular Mechanisms of Muscular Dystrophies (ed. Winder, S. J.) 14-34 (Landes Biosciences, 2006)).

[0133] Partially functional micro-dystrophins can improve the dystrophic pathology in striated muscle by protecting the sarcolemma from contraction-induced injury and increasing the capacity to generate force. These parameters can be achieved by binding to F-actin filaments and β-dystroglycan through the amino-terminal domain and the Dg BD (*see* Harper, S. Q., *et al.,* Nature Medicine 8, 253-261, (2002); Warner, L. E., *et al.*, Human Molecular Genetics 11, 1095-1105 (2002); Cox, G. A., *et al.*, Nature Genetics 8, 333-339, (1994); Greenberg, D. S., *et al.*, Nature Genetics 8, 340-344, (1994); Gardner, K. L., *et al.*, Gene Therapy 13, 744-751, (2006); Corrado, K., *et al.*, The Journal of Cell Biology 134, 873-884 (1996); and Rafael, J. A., *et al.*, The Journal of Cell Biology 134, 93-102 (1996)). Without being bound by any one particular theory, prior studies indicate these two domains must be connected by at least four SRs from the central rod domain, but there are numerous ways in which miniaturized dystrophins containing at least four SRs can be constructed. While some combinations of SRs have been shown to improve the dystrophic pathophysiology, other combinations have not yielded proteins with significant functional capacity (*see* Harper, S. Q., *et al.*, Nature Medicine 8, 253-261, (2002) and Abmayr S, in Molecular Mechanisms of Muscular Dystrophies (ed. Winder, S. J.) 14-34 (Landes Biosciences, 2006)). Selection of specific SRs in μDys design can restore additional DGC components to the sarcolemma. Neuronal nitric oxide synthase (nNOS) is a signaling protein that can be involved in vasodilation in response to muscle contractile activity (*see* Stamler, J. S. and Meissner, G. Physiological Reviews 81, 209-237 (2001); Brenman, J. E., *et al.*, Cell 82, 743-752 (1995); Kobayashi, Y. M., *et al.*, Nature 456, 511-515, (2008); and Torelli, S., *et al.*, Neuropathology and Applied Neurobiology 30, 540-545, (2004)), and the presence of SRs 16 and 17 can be involved in proper association of nNOS with the DGC (*see* 28 Lai, Y. *et al.*, The Journal of Clinical Investigation 119, 624-635, (2009) and Lai, Y.,

et al., Proceedings of the National Academy of Sciences of the United States of America 110, 525-530, (2013)).

[0134] Sequences within spectrin-like repeats 20-24 as well as Hinge 4 can play a role in proper association of dystrophin with microtubules, which can be important for maintaining the intracellular architecture and torque production in skeletal muscle (*see* Prins, K. W. *et al.*, The Journal of Cell Biology 186, 363-369, (2009) and Belanto, J. J., *et al.*, Proceedings of the National Academy of Sciences of the United States of America 111, 5723-5728, (2014)). Nonetheless, the carboxy-terminal domain and most of the SR domains have been found dispensable without severely compromising the health of striated muscles (*see* McCabe, E. R., *et al.*, The Journal of Clinical Investigation 83, 95-99, (1989); Crawford, G. E., *et al.*, The Journal of Cell Biology 150, 1399-1410 (2000); and Dunckley, M. G., *et al.*, FEBS Letters 296, 128-134 (1992)).

[0135] Several of the best micro-dystrophins tested to date can protect muscles from contraction-induced injury and restore some, but generally not all, of the specific force generating capacity to dystrophic mouse and canine models for DMD (*see* Seto, J. T., *et al.*, Current Gene Therapy 12, 139-151 (2012) and Wang, Z., *et al.*, Frontiers in Microbiology 2, 201, (2011)). Other micro-dystrophins carrying different combinations of SRs and hinges may function less well in dystrophic muscles, and the reasons for differences in functionality are not clear. However, without being bound by any one particular theory, they may relate to effects on micro-dystrophin elasticity, folding, stability, and the ability to assemble sub-portions of the DGC without steric hindrance.

[0136] The present disclosure relates generally to micro-dystrophins. The micro-dystrophins may be operatively linked to a regulatory cassette. The present disclosure also relates to methods of treating a subject having muscular dystrophy, sarcopenia, heart failure, or cachexia. Further, the present disclosure relates to methods of prophylactically treating a subject at risk of developing muscular dystrophy, sarcopenia, heart failure, or cachexia. The methods for treating a subject having, or at risk of developing, muscular dystrophy, sarcopenia, heart failure, or

cachexia may comprise administering a pharmaceutical composition including a micro-dystrophin gene and a delivery vehicle to the subject.

[0137] It will be readily understood that the embodiments, as generally described herein, are exemplary. The following more detailed description of various embodiments is not intended to limit the scope of the present disclosure, but is merely representative of various embodiments. Moreover, the order of the steps or actions of the methods disclosed herein may be changed by those skilled in the art without departing from the scope of the present disclosure. In other words, unless a specific order of steps or actions is required for proper operation of the embodiment, the order or use of specific steps or actions may be modified.

[0138] Unless specifically defined otherwise, the technical terms, as used herein, have their normal meaning as understood in the art. The following terms are specifically defined with examples for the sake of clarity.

[0139] As used herein, "peptide" and "polypeptide" may be used in their broadest senses to refer to a sequence of subunit amino acids. The peptides or polypeptides of the disclosure may comprise L-amino acids, D-amino acids (which can be resistant to L-amino acid-specific proteases *in vivo*), or a combination of D- and L-amino acids. The terms peptide and polypeptide can be used interchangeably. The peptides and polypeptides described herein may be chemically synthesized or recombinantly expressed. The peptides and polypeptides may be linked to any other moiety as deemed useful for a given purpose. Such linkage can comprise covalent linkages or non-covalent linkages as is understood by those of skill in the art.

[0140] Amino acid residues as disclosed herein can be modified by conservative substitutions to maintain, or substantially maintain, overall polypeptide structure and/or function. As used herein, "conservative amino acid substitution" indicates that: hydrophobic amino acids (*i.e.*, Ala, Cys, Gly, Pro, Met, Val, Ile, and Leu) can be substituted with other hydrophobic amino acids; hydrophobic amino acids with bulky side chains (*i.e.*, Phe, Tyr, and Trp) can be substituted with other hydrophobic amino acids with bulky side chains; amino acids with positively charged side chains (*i.e.*, Arg, His, and Lys) can be substituted with other amino acids with positively charged side chains; amino acids with negatively charged side chains (*i.e.*, Asp and

Glu) can be substituted with other amino acids with negatively charged side chains; and amino acids with polar uncharged side chains (*i.e.*, Ser, Thr, Asn, and Gln) can be substituted with other amino acids with polar uncharged side chains.

[0141] Treating a subject can comprise delivering an effective amount or delivering a prophylactic treatment and/or a therapeutic treatment to a subject (*e.g.*, a patient). An "effective amount" is an amount of a compound that can result in a desired physiological change in a subject. Effective amounts may also be administered for research purposes.

[0142] A "prophylactic treatment" comprises a treatment administered to a subject who does not display signs or symptoms of a disease or condition, or a subject who displays only early signs or symptoms of a disease or condition, such that treatment is administered for the purpose of diminishing, preventing, and/or decreasing the risk of further developing the disease or condition or of diminishing, preventing, and/or decreasing the risk of developing the disease or condition. Thus, a prophylactic treatment may function as a preventive treatment against a disease or condition.

[0143] A "therapeutic treatment" comprises a treatment administered to a subject who displays symptoms or signs of a disease or a condition and the therapeutic treatment is administered to the subject for the purpose of diminishing or eliminating the symptoms or the signs of the disease or the condition.

[0144] "Therapeutically effective amounts" comprise amounts that provide prophylactic treatment and/or therapeutic treatment. Therapeutically effective amounts need not fully prevent or cure the disease or the condition but can also provide a partial benefit, such as a delay of onset or an alleviation or an improvement of at least one symptom of the disease or the condition.

[0145] For administration, effective amounts and therapeutically effective amounts (also referred to herein as doses) can be initially estimated based on results from *in vitro* assays and/or animal model studies. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture. Such information can be used to more accurately determine useful doses in subjects of interest.

[0146] The actual dose amount administered to a particular subject can be determined by a physician, a veterinarian, or a researcher, taking into account parameters such as, but not limited to, physical and physiological factors including body weight, severity of condition, type of disease, previous or concurrent therapeutic interventions, idiopathy of the subject, and/or route of administration.

[0147] Doses can range from $1x10^{8}$ vector genomes per kg (vg/kg) to $1x10^{15}$ vg/kg, from $1x10^{9}$ vg/kg to $1x10^{14}$ vg/kg, from $1x10^{10}$ vg/kg to $1x10^{13}$ vg/kg, or from $1x10^{11}$ vg/kg to $1x10^{12}$ vg/kg. In other non-limiting examples, a dose can comprise about $1x10^{8}$ vg/kg, about $1x10^{9}$ vg/kg, about $1x10^{10}$ vg/kg, about $1x10^{11}$ vg/kg, about $1x10^{12}$ vg/kg, about $1x10^{13}$ vg/kg, about $1x10^{14}$ vg/kg, or about $1x10^{15}$ vg/kg. Therapeutically effective amounts can be achieved by administering single or multiple doses during the course of a treatment regimen (*i.e.*, days, weeks, months, etc.).

[0148] Pharmaceutically acceptable salts, tautomers, and isomers of the compounds disclosed herein can also be used. Exemplary salts can include, but are not limited to, sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, besylate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, and pamoate (*i.e.*, 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts.

[0149] The formulations described herein can be administered by, without limitation, injection, infusion, perfusion, inhalation, lavage, and/or ingestion. Routes of administration can include, but are not limited to, intravenous, intradermal, intraarterial, intraperitoneal, intralesional, intracranial, intraarticular, intraprostatic, intrapleural, intratracheal, intranasal, intravitreal, intravaginal, intrarectal, topically, intratumoral, intramuscular, intravesicular, intrapericardial, intraumbilical, intraocularal, mucosal, oral, subcutaneous, and/or subconjunctival. In other non-limiting examples, administration can be performed by intramuscular injection,

intravascular injection, intraperitoneal injection, or any other method suitable for delivery of vector to musculature.

[0150] In some embodiments, for injection, formulations can be made as aqueous solutions, such as in buffers including, but not limited to, Hanks' solution, Ringer's solution, and/or physiological saline. The solutions can contain formulatory agents such as suspending, stabilizing, and/or dispersing agents. Alternatively, the formulation can be in lyophilized and/or powder form for constitution with a suitable vehicle control (*e.g.*, sterile pyrogen-free water) before use.

[0151] Any formulation disclosed herein can advantageously comprise any other pharmaceutically acceptable carrier or carriers which comprise those that do not produce significantly adverse, allergic, or other untoward reactions that may outweigh the benefit of administration, whether for research, prophylactic, and/or therapeutic treatments. Exemplary pharmaceutically acceptable carriers and formulations are disclosed in Remington's Pharmaceutical Sciences, 18th Ed., Mack Printing Company, 1990, which is incorporated by reference herein for its teachings regarding the same. Moreover, formulations can be prepared to meet sterility, pyrogenicity, general safety, and purity standards as required by the United States FDA's Division of Biological Standards and Quality Control and/or other relevant U.S. and foreign regulatory agencies.

[0152] Exemplary, generally used pharmaceutically acceptable carriers may comprise, but are not limited to, bulking agents or fillers, solvents or co-solvents, dispersion media, coatings, surfactants, antioxidants (*e.g.*, ascorbic acid, methionine, and vitamin E), preservatives, isotonic agents, absorption delaying agents, salts, stabilizers, buffering agents, chelating agents (*e.g.*, EDTA), gels, binders, disintegration agents, and/or lubricants.

[0153] Exemplary buffering agents may comprise, but are not limited to, citrate buffers, succinate buffers, tartrate buffers, fumarate buffers, gluconate buffers, oxalate buffers, lactate buffers, acetate buffers, phosphate buffers, histidine buffers, and/or trimethylamine salts.

[0154] Exemplary preservatives may comprise, but are not limited to, phenol, benzyl alcohol, meta-cresol, methylparaben, propyl paraben,

octadecyldimethylbenzyl ammonium chloride, benzalkonium halides, hexamethonium chloride, alkyl parabens (such as methyl or propyl paraben), catechol, resorcinol, cyclohexanol, and/or 3-pentanol.

[0155] Exemplary isotonic agents may comprise polyhydric sugar alcohols comprising, but not limited to, trihydric or higher sugar alcohols, (*e.g.*, glycerin, erythritol, arabitol, xylitol, sorbitol, and/or mannitol).

[0156] Exemplary stabilizers may comprise, but are not limited to, organic sugars, polyhydric sugar alcohols, polyethylene glycol, sulfur-containing reducing agents, amino acids, low molecular weight polypeptides, proteins, immunoglobulins, hydrophilic polymers, and/or polysaccharides.

[0157] Formulations can also be depot preparations. In some embodiments, such long-acting formulations may be administered by, without limitation, implantation (*e.g.*, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, compounds can be formulated with suitable polymeric and/or hydrophobic materials (*e.g.*, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives (*e.g.*, as a sparingly soluble salt).

[0158] Additionally, in various embodiments, compounds can be delivered using sustained-release systems, such as semipermeable matrices of solid polymers comprising at least one compound. Various sustained-release materials have been established and are well known by those of ordinary skill in the art. Sustained-release capsules may, depending on their chemical nature, release the compound following administration for a few weeks up to over 100 days.

[0159] Gene therapy methods can be used for delivering (*e.g.*, at sustained levels) specific proteins into patients or subjects. These methods allow practitioners to introduce DNA coding for a gene of interest directly into a patient or subject (*in vivo* gene therapy) or into cells isolated from a patient, a subject, or a donor (*ex vivo* gene therapy). The introduced DNA then directs the patient's or subject's own cells or grafted cells to produce the desired protein product. Gene delivery, therefore, can obviate the need for daily injections. Gene therapy may also allow practitioners to select specific organs or cellular targets (*e.g.*, muscle, liver, blood cells, brain cells, etc.) for therapy.

[0160] DNA may be introduced into a subject's cells in several ways. There are transfection methods, including chemical methods such as calcium phosphate precipitation and liposome-mediated transfection, and physical methods such as electroporation. In general, transfection methods are not suitable for *in vivo* gene delivery. There are also methods that use recombinant viruses. Current viral-mediated gene delivery methods include, but are not limited to, retrovirus, adenovirus, herpes virus, pox virus, and adeno-associated virus (AAV) vectors.

[0161] One viral system that has been used for gene delivery is adeno-associated virus (AAV). AAV is a parvovirus which belongs to the genus *Dependoparvovirus*. AAV has several attractive features not found in other viruses. First, AAV can infect a wide range of host cells, including non-dividing cells. Second, AAV can infect cells from different species. Third, AAV has not been associated with any human or animal disease and does not appear to alter the biological properties of the host cell upon integration. Indeed, it is estimated that 80-85% of the human population has been exposed to the virus. Finally, AAV is stable at a wide range of physical and chemical conditions which lends itself to production, storage, and transportation requirements.

[0162] The AAV genome is a linear, single-stranded DNA molecule containing 4681 nucleotides. The AAV genome generally comprises an internal non-repeating genome flanked on each end by inverted terminal repeats (ITRs). The ITRs are approximately 145 base pairs (bp) in length. The ITRs have multiple functions, including as origins of DNA replication and as packaging signals for the viral genome.

[0163] The internal non-repeated portion of the genome includes two large open reading frames, known as the AAV replication (*rep*) and capsid (*cap*) genes. The *rep* and *cap* genes code for viral proteins that allow the virus to replicate and package the viral genome into a virion. In particular, a family of at least four viral proteins are expressed from the AAV *rep* region, Rep78, Rep68, Rep52, and Rep40, named according to their apparent molecular weight. The AAV cap region encodes at least three proteins, VP1, VP2, and VP3.

[0164] AAV is a helper-dependent virus; that is, it requires co-infection with a helper virus (*e.g.*, adenovirus, herpesvirus, or vaccinia) in order to form AAV virions. In the absence of co-infection with a helper virus, AAV establishes a latent state in which the viral genome inserts into a host cell chromosome, but infectious virions are not produced. Subsequent infection by a helper virus "rescues" the integrated genome, allowing it to replicate and package its genome into infectious AAV virions. While AAV can infect cells from different species, the helper virus must be of the same species as the host cell. Thus, for example, human AAV will replicate in canine cells co-infected with a canine adenovirus.

[0165] "Gene transfer" or "gene delivery" comprises methods or systems for inserting foreign DNA into host cells. Gene transfer can result in transient expression of non-integrated transferred DNA, extrachromosomal replication, and expression of transferred replicons (*e.g.*, episomes), or integration of transferred genetic material into the genomic DNA of host cells.

[0166] A "vector" comprises any genetic element, such as, but not limited to, a plasmid, phage, transposon, cosmid, chromosome, artificial chromosome, virus, virion, etc., which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

[0167] An "AAV vector" comprises a vector derived from an adeno-associated virus serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, and AAV9. AAV vectors can have one or more of the AAV wild-type genes deleted in whole or part, *e.g.*, the *rep* and/or *cap* genes, but retain functional flanking ITR sequences. Functional ITR sequences are necessary for the rescue, replication, and packaging of the AAV virion. Thus, an AAV vector is defined herein to include at least those sequences required in *cis* for replication and packaging (*e.g.*, functional ITRs) of the virus. The ITRs need not be the wild-type nucleotide sequences, and may be altered, *e.g.*, by the insertion, deletion or substitution of nucleotides, so long as the sequences provide for functional rescue, replication and packaging.

[0168] A "recombinant AAV vector" or "rAAV vector" comprises an infectious, replication-defective virus composed of an AAV protein shell encapsulating a heterologous nucleotide sequence of interest that is flanked on both sides by AAV ITRs. An rAAV vector is produced in a suitable host cell comprising an AAV vector, AAV helper functions, and accessory functions. In this manner, the host cell is rendered capable of encoding AAV polypeptides that are required for packaging the AAV vector (containing a recombinant nucleotide sequence of interest) into infectious recombinant virion particles for subsequent gene delivery.

[0169] A first aspect of the disclosure relates to nucleotide sequences including a micro-dystrophin gene encoding a protein. The nucleotide sequences may also include a regulatory cassette. Additionally, the nucleotide sequences may be isolated and/or purified.

[0170] In some embodiments, the protein encoded by the micro-dystrophin gene may include an amino-terminal actin-binding domain, a dystroglycan-binding domain, and/or a spectrin-like repeat domain. The spectrin-like repeat domain may include at least four spectrin-like repeats or portions of at least four spectrin-like repeats. Two of the at least four spectrin-like repeats may comprise a neuronal nitric oxide synthase binding domain. Stated another way, the at least four spectrin-like repeats may include spectrin-like repeats 16 and 17 or portions thereof. In some embodiments, the at least four spectrin-like repeats may include spectrin-like repeats 1 and 24 or portions thereof. In alternative embodiments, the at least four spectrin-like repeats may include other suitable spectrin-like repeats or portions thereof (*e.g.*, spectrin-like repeats 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22, and/or 23).

[0171] In certain embodiments, the spectrin-like repeat domain may include four, five, six, seven, eight, or more spectrin-like repeats or portions thereof. In certain other embodiments, the protein encoded by the micro-dystrophin gene may include between five spectrin-like repeats and eight spectrin-like repeats (*e.g.*, five, six, seven, or eight spectrin-like repeats). In yet certain other embodiments, the spectrin-like repeat domain may include another suitable number of spectrin-like repeats or portions thereof.

[0172] In some embodiments, the protein encoded by the micro-dystrophin gene may further comprise a hinge domain or a portion thereof. For example, the protein encoded by the micro-dystrophin gene may include at least a portion of a hinge domain selected from at least one of a Hinge 1 domain, a Hinge 2 domain, a Hinge 3 domain, a Hinge 4 domain, and/or a hinge-like domain (such as the hinge-like domains encoded by the sequences downstream from spectrin-like repeat 15 (SEQ ID NO:20) and within spectrin-like repeat 23 (SEQ ID NO:21)).

[0173] In various embodiments, the micro-dystrophin gene may include a portion of the nucleic acid sequence of SEQ ID NO:16. In various other embodiments, the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:16. In yet various other embodiments, the micro-dystrophin gene may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:16.

[0174] In some embodiments, the protein encoded by the micro-dystrophin gene may include a portion of the amino acid sequence of SEQ ID NO:4. In some other embodiments, the protein encoded by the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:4. In yet some other embodiments, the protein encoded by the micro-dystrophin gene may have 100% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0175] In certain embodiments, the micro-dystrophin gene may include a portion of the nucleic acid sequence of SEQ ID NO:18. In certain other embodiments, the

micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:18. In yet various other embodiments, the micro-dystrophin gene may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:18.

[0176] In various embodiments, the protein encoded by the micro-dystrophin gene may include a portion of the amino acid sequence of SEQ ID NO:5. In various other embodiments, the protein encoded by the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:5. In yet various other embodiments, the protein encoded by the micro-dystrophin gene may have 100% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0177] Further, the micro-dystrophin gene may include a portion of one or more of the nucleic acid sequences of SEQ ID NOs:11-18. In certain embodiments, the protein encoded by the micro-dystrophin gene may include a portion of one or more of the amino acid sequences of SEQ ID NO:3-10. In certain other embodiments, the protein encoded by the micro-dystrophin gene may include a portion of one or more of the proteins depicted in the protein structure diagrams of FIG. 7 (*e.g.*, μDysH3 and μDys1-μDys16).

[0178] In some embodiments, the regulatory cassette may be selected from at least one of a CK8 promoter, a cardiac troponin T (cTnT) promoter, and/or another suitable regulatory cassette. In certain embodiments, the regulatory cassette may be the CK8 promoter and the CK8 promoter may comprise a portion of the nucleic

acid sequence of SEQ ID NO:19. In certain other embodiments, the regulatory cassette may be the CK8 promoter and the CK8 promoter may have at least at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:19. In yet certain other embodiments, the regulatory cassette may be the CK8 promoter and the CK8 promoter may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0179] In various embodiments, the regulatory cassette may be the cTnT promoter and the cTnT promoter may comprise a portion of the nucleic acid sequence of SEQ ID NO:1. In various other embodiments, the regulatory cassette may be the cTnT promoter and the cTnT promoter may have at least at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:1. In yet various other embodiments, the regulatory cassette may be the cTnT promoter and the cTnT promoter may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0180] Another aspect of the disclosure relates to pharmaceutical compositions comprising nucleotide sequences as discussed above. In some embodiments, the pharmaceutical compositions may further include a delivery vehicle. For example, the pharmaceutical compositions may comprise a nucleotide sequence including a regulatory cassette and a micro-dystrophin gene encoding a protein and the pharmaceutical compositions may further comprise a delivery vehicle. The

nucleotide sequences of the pharmaceutical compositions may be isolated and purified nucleotide sequences.

[0181] In various embodiments, the delivery vehicle may comprise an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector. The AAV vector may be a serotype 6 AAV (AAV6). Likewise, the rAAV vector may be a serotype 6 rAAV (rAAV6). The AAV vector may be a serotype 8 AAV (AAV8). Likewise, the rAAV vector may be a serotype 8 rAAV (rAAV8). The AAV vector may be a serotype 9 AAV (AAV9). Likewise, the rAAV vector may be a serotype 9 rAAV (rAAV9). The rAAV vector may be comprised of AAV2 genomic inverted terminal repeat (ITR) sequences pseudotyped with capsid proteins derived from AAV serotype 6 (rAAV2/6). Other suitable serotypes of the AAV or rAAV are also within the scope of this disclosure.

[0182] In some embodiments, as discussed above, the delivery vehicle may express, or be configured to express, the micro-dystrophin gene. In various embodiments, the micro-dystrophin gene may include a portion of the nucleic acid sequence of SEQ ID NO:16. In various other embodiments, the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:16. In yet various other embodiments, the micro-dystrophin gene may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:16.

[0183] In some embodiments, the protein encoded by the micro-dystrophin gene may include a portion of the amino acid sequence of SEQ ID NO:4. In some other embodiments, the protein encoded by the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least

93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:4. In yet some other embodiments, the protein encoded by the micro-dystrophin gene may have 100% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0184] In certain embodiments, the micro-dystrophin gene may include a portion of the nucleic acid sequence of SEQ ID NO:18. In certain other embodiments, the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:18. In yet certain other embodiments, the micro-dystrophin gene may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:18.

[0185] In various embodiments, the protein encoded by the micro-dystrophin gene may include a portion of the amino acid sequence of SEQ ID NO:5. In various other embodiments, the protein encoded by the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:5. In yet various other embodiments, the protein encoded by the micro-dystrophin gene may have 100% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0186] Also, as discussed above, the regulatory cassette may be selected from at least one of a CK8 promoter, a cardiac troponin T (cTnT) promoter, and/or another suitable regulatory cassette. In certain embodiments, the regulatory cassette may be the CK8 promoter and the CK8 promoter may comprise a portion of the nucleic acid sequence of SEQ ID NO:19. In certain other embodiments, the regulatory

cassette may be the CK8 promoter and the CK8 promoter may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:19. In yet certain other embodiments, the regulatory cassette may be the CK8 promoter and the CK8 promoter may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0187] In various embodiments, the regulatory cassette may be the cTnT promoter and the cTnT promoter may comprise a portion of the nucleic acid sequence of SEQ ID NO:1. In various other embodiments, the regulatory cassette may be the cTnT promoter and the cTnT promoter may have at least at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:1. In yet various other embodiments, the regulatory cassette may be the cTnT promoter and the cTnT promoter may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0188] In some embodiments, the pharmaceutical composition may be configured to reduce a pathological effect or symptom of a muscular dystrophy. The muscular dystrophy may be selected from at least one of myotonic muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy, congenital muscular dystrophy, oculopharyngeal muscular dystrophy, distal muscular dystrophy, Emery-Dreifuss muscular dystrophy, and/or another suitable muscular dystrophy. In some other embodiments, the pharmaceutical composition may be configured to reduce a

pathological effect or symptom of a muscular dystrophy selected from at least one of Duchenne muscular dystrophy and/or Becker muscular dystrophy. In certain embodiments, the pharmaceutical composition may be configured to reduce a pathological effect or symptom of at least one of sarcopenia, heart disease, and/or cachexia.

[0189] Another aspect of the disclosure relates to methods for treating a subject having muscular dystrophy, sarcopenia, heart disease, and/or cachexia. The methods may comprise administering to the subject a pharmaceutical composition comprising a micro-dystrophin gene coupled to a regulatory cassette. The methods may comprise administering to the subject a therapeutically effective amount of the pharmaceutical composition. Furthermore, the micro-dystrophin gene may be operably coupled to the regulatory cassette.

[0190] In some embodiments, the method may comprise administering to the subject a pharmaceutical composition wherein the pharmaceutical composition further comprises an AAV vector, an rAAV vector, and/or another suitable delivery vehicle. The delivery vehicle may express, or be configured to express, the micro-dystrophin gene in the subject.

[0191] As discussed above, in various embodiments, the micro-dystrophin gene may include a portion of the nucleic acid sequence of SEQ ID NO:16. In various other embodiments, the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:16. In yet various other embodiments, the micro-dystrophin gene may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:16.

[0192] In some embodiments, the protein encoded by the micro-dystrophin gene may include a portion of the amino acid sequence of SEQ ID NO:4. In some other embodiments, the protein encoded by the micro-dystrophin gene may have at least

20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:4. In yet some other embodiments, the protein encoded by the micro-dystrophin gene may have 100% sequence identity to the amino acid sequence of SEQ ID NO:4.

[0193] In certain embodiments, the micro-dystrophin gene may include a portion of the nucleic acid sequence of SEQ ID NO:18. In certain other embodiments, the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:18. In yet certain other embodiments, the micro-dystrophin gene may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:18.

[0194] In various embodiments, the protein encoded by the micro-dystrophin gene may include a portion of the amino acid sequence of SEQ ID NO:5. In various other embodiments, the protein encoded by the micro-dystrophin gene may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence of SEQ ID NO:5. In yet various other embodiments, the protein encoded by the micro-dystrophin gene may have 100% sequence identity to the amino acid sequence of SEQ ID NO:5.

[0195] In some embodiments, the regulatory cassette may express, or be configured to express, the micro-dystrophin gene such that a level of expression of the micro-dystrophin gene is at least 100-fold higher in striated muscle cells than the level of expression of the micro-dystrophin gene in non-muscle cells. For example, the level of expression of the micro-dystrophin gene may be at least 100-fold higher in the striated muscle cells of the subject than in lung cells of the subject. In some other embodiments, the regulatory cassette may express, or be configured to express, the micro-dystrophin gene such that a level of expression of the micro-dystrophin gene is between at least 50-fold higher and 150-fold higher, between at least 75-fold higher and 125-fold higher, or between at least 90-fold higher and 110-fold higher in striated muscle cells than the level of expression of the micro-dystrophin gene in non-muscle cells.

[0196] As discussed above, the regulatory cassette may be selected from at least one of a CK8 promoter, a cardiac troponin T (cTnT) promoter, and/or another suitable regulatory cassette. In certain embodiments, the regulatory cassette may be the CK8 promoter and the CK8 promoter may comprise a portion of the nucleic acid sequence of SEQ ID NO:19. In certain other embodiments, the regulatory cassette may be the CK8 promoter and the CK8 promoter may have at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:19. In yet certain other embodiments, the regulatory cassette may be the CK8 promoter and the CK8 promoter may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:19.

[0197] In various embodiments, the regulatory cassette may be the cTnT promoter and the cTnT promoter may comprise a portion of the nucleic acid sequence of SEQ ID NO:1. In various other embodiments, the regulatory cassette may be the cTnT promoter and the cTnT promoter may have at least at least 20%, at

least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence of SEQ ID NO:1. In yet various other embodiments, the regulatory cassette may be the cTnT promoter and the cTnT promoter may have 100% sequence identity to the nucleic acid sequence of SEQ ID NO:1.

[0198] In some embodiments, the micro-dystrophin gene may express, or be configured to express, a micro-dystrophin protein in one or more muscles of the subject such that contractility of the one or more muscles is enhanced or increased. In certain embodiments, the micro-dystrophin gene may express, or be configured to express, a micro-dystrophin protein in one or more skeletal muscles of the subject such that a specific-force generating capacity of at least one of the one or more skeletal muscles is enhanced or increased to within at least 10%, at least 20%, at least 30%, or at least 40% of a normal specific-force generating capacity. In certain other embodiments, the micro-dystrophin gene may express, or be configured to express, a micro-dystrophin protein in one or more cardiac muscles of the subject such that a baseline end-diastolic volume defect is restored to within at least 10%, at least 20%, at least 30%, or at least 40% of a normal end-diastolic volume. In various embodiments, the micro-dystrophin gene may express, or be configured to express, a micro-dystrophin protein such that localization of the neuronal nitric oxide synthase to the dystrophin-glycoprotein complex is enhanced or increased in the subject.

[0199] In some embodiments, as discussed above, the methods may comprise treating a subject having at least one of myotonic muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy, congenital muscular dystrophy, oculopharyngeal muscular dystrophy, distal muscular dystrophy, Emery-Dreifuss muscular dystrophy, and/or another suitable muscular dystrophy. In some other

embodiments, the methods may comprise treating a subject having at least one of Duchenne muscular dystrophy and/or Becker muscular dystrophy.

[0200] In certain embodiments, the pharmaceutical composition may reduce, or be configured to reduce, a pathological effect or symptom of the muscular dystrophy, sarcopenia, heart disease, and/or cachexia. The pathological effect or symptom of the muscular dystrophy may be selected from at least one of muscle pain, muscle weakness, muscle fatigue, muscle atrophy, fibrosis, inflammation, increase in average myofiber diameter in skeletal muscle, cardiomyopathy, reduced 6-minute walk test time, loss of ambulation, cardiac pump failure, and/or one or more other suitable pathological effects or symptoms. The pathological effect or symptom of sarcopenia may be selected from at least one of muscle wasting and/or muscle weakness. The pathological effect or symptom of heart disease may be selected from at least one of cardiomyopathy, reduced hemodynamics, and/or arrhythmia. The pathological effect or symptom of cachexia may be selected from at least one of muscle wasting and/or muscle weakness.

[0201] The methods of treating a subject having muscular dystrophy may further comprise identifying a subject having muscular dystrophy. Similarly, the methods of treating a subject having sarcopenia, heart disease, and/or cachexia may further comprise identifying a subject having sarcopenia, heart disease, and/or cachexia, respectively. In some embodiments, the subject may be a mammal. In certain embodiments, the subject may be a human.

[0202] Another aspect of the disclosure relates to methods for prophylactically treating a subject at risk of developing muscular dystrophy, sarcopenia, heart disease, and/or cachexia. The methods may comprise administering to the subject a pharmaceutical composition as described above in reference to the methods of treating a subject having a muscular dystrophy, sarcopenia, heart disease, and/or cachexia.

[0203] In some embodiments, the methods may comprise treating a subject at risk of developing at least one of myotonic muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy, congenital muscular dystrophy,

oculopharyngeal muscular dystrophy, distal muscular dystrophy, Emery-Dreifuss muscular dystrophy, and/or another suitable muscular dystrophy. In some other embodiments, the methods may comprise treating a subject at risk of developing at least one of Duchenne muscular dystrophy and/or Becker muscular dystrophy.

[0204] In certain embodiments, the pharmaceutical composition may reduce, or be configured to reduce, a risk of developing a pathological effect or symptom of a muscular dystrophy, sarcopenia, heart disease, and/or cachexia. The methods of treating a subject at risk of developing muscular dystrophy, sarcopenia, heart disease, and/or cachexia may further comprise identifying a subject at risk of developing muscular dystrophy, sarcopenia, heart disease, and/or cachexia, respectively. In some embodiments, the subject may be a mammal. In certain embodiments, the subject may be a human.

[0205] Another aspect of the disclosure relates to regulatory cassettes including enhancers and/or promoters that enhance and/or target expression of a pharmaceutical composition (*e.g.*, a micro-dystrophin gene). In some embodiments, the enhancers or promoters for enhancing and/or targeting expression of a pharmaceutical composition may include at least a portion of a gene, a peptide, a polypeptide, and/or a regulatory RNA. Targeting expression of the pharmaceutical composition may include expression the pharmaceutical composition in a specific cell type, tissue, and/or organ of a subject. For example, cTnT455 (SEQ ID NO:1) may be used for cardiac-specific expression.

[0206] In certain embodiments, the enhancers or promoters may express, or be configured to express, a pharmaceutical composition comprising a peptide. In various embodiments, the enhancers or promoters may express, or be configured to express, the peptide in developing, injured, and/or diseased muscle (*i.e.*, muscle that may be undergoing regeneration). The hum-cTnT455 RC (SEQ ID NO:1) may not be transcriptionally active in steady state mature skeletal muscle.

[0207] As discussed above, the enhancers and/or promoters may be operatively linked to a pharmaceutical composition, *i.e.*, for enhancing expression and/or targeting of the pharmaceutical composition. Additionally, the pharmaceutical composition may be operatively linked to one or enhancers and/or promoters. In

some embodiments, expression of the pharmaceutical compositions disclosed herein may assist in regenerating cardiac muscle. For example, the hum-cTnT455 RC (SEQ ID NO:1) may enhance or target the transient expression of the pharmaceutical composition in wounded and/or regenerating cardiac muscle. In some embodiments, expression of the pharmaceutical compositions disclosed herein may assist in preventing loss of cardiac muscle and/or of cardiomyocytes. In certain embodiments, expression of the pharmaceutical compositions disclosed herein may assist in regenerating skeletal muscle. In various embodiments, expression of the pharmaceutical compositions disclosed herein may assist in preventing necrosis and/or wasting of skeletal muscle.

[0208] Another aspect of the disclosure relates to nucleotide sequences comprising a micro-dystrophin gene, wherein the micro-dystrophin gene may encode a protein comprising at least two spectrin-like repeats that are directly coupled to each other. In some embodiments, the at least two spectrin-like repeats that are directly coupled to each other may be selected from at least one of a spectrin-like repeat 1 (SR1) directly coupled to a spectrin-like repeat 2 (SR2), an SR2 directly coupled to a spectrin-like repeat 3 (SR3), an SR1 directly coupled to a spectrin-like repeat 16 (SR16), a spectrin-like repeat 17 (SR17) directly coupled to a spectrin-like repeat 23 (SR23), an SR17 directly coupled to a spectrin-like repeat 24 (SR24), and/or an SR23 directly coupled to an SR24. The micro-dystrophin gene may also encode a protein comprising an amino-terminal actin-binding domain and/or a β-dystroglycan binding domain.

[0209] Another aspect of the disclosure relates to nucleotide sequences comprising a micro-dystrophin gene, wherein the micro-dystrophin gene may encode a protein comprising, in order, a Hinge 1 domain (H1), an SR1, an SR16, an SR17, an SR24, and/or a Hinge 4 domain (H4). In some embodiments, the H1 may be directly coupled to the SR1. In various embodiments, the SR 1 may be directly coupled to the SR16. In certain embodiments, the SR16 may be directly coupled to the SR17. In some embodiments, the SR17 may be directly coupled to the SR24. In various embodiments, the SR24 may be directly coupled to the H4.

[0210] In some embodiments, the protein encoded by the micro-dystrophin gene may further comprise, in order, an SR2 and an SR3, wherein the SR2 and the SR3 may be disposed between the SR1 and the SR16. Furthermore, the SR1 may be directly coupled to the SR2 and the SR2 may be further coupled to the SR3.

[0211] Another aspect of the disclosure relates to nucleotide sequences comprising a micro-dystrophin gene encoding a protein, wherein the micro-dystrophin gene may encode a protein comprising, in order, a H1, an SR1, an SR16, an SR17, an SR 23, an SR24, and/or a H4. In some embodiments, the H1 may be directly coupled to the SR1, the SR1 may be directly coupled to the SR 16, the SR 16 may be directly coupled to the SR 17, the SR 17 may be directly coupled to the SR 23, the SR 23 may be directly coupled to the SR 24, and/or the SR 24 may be directly coupled to the H4.

[0212] Another aspect of the disclosure relates to pharmaceutical compositions that may comprise a micro-dystrophin gene comprising the nucleic acid sequence of SEQ ID NO:16 and an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector. In some embodiments, a serotype of the AAV vector or the rAAV vector may selected from at least one of serotype 6, serotype 8, serotype 9, or another suitable serotype.

[0213] Another aspect of the disclosure relates to pharmaceutical composition that may comprise a micro-dystrophin gene encoding a protein, wherein the protein may comprise the amino acid sequence of SEQ ID NO:4 and an AAV vector or an rAAV vector. In certain embodiments, a serotype of the AAV vector or the rAAV vector may be selected from at least one of serotype 6, serotype 8, serotype 9, or another suitable serotype.

[0214] Another aspect of the disclosure relates to pharmaceutical compositions that may comprise a micro-dystrophin gene comprising the nucleic acid sequence of SEQ ID NO:18 and an AAV vector or an rAAV vector. In various embodiments, a serotype of the AAV vector or the rAAV vector is selected from at least one of serotype 6, serotype 8, serotype 9, or another suitable serotype.

[0215] Another aspect of the disclosure relates to pharmaceutical compositions that may comprise a micro-dystrophin gene encoding a protein, wherein the protein

may comprise the amino acid sequence of SEQ ID NO:5 and an AAV vector or an rAAV vector. In some embodiments, a serotype of the AAV vector or the rAAV vector is selected from at least one of serotype 6, serotype 8, serotype 9, or another suitable serotype.

[0216] Another aspect of the disclosure relates to pharmaceutical compositions for use in the treatment or prophylactic treatment of muscular dystrophy, sarcopenia, heart failure, and/or cachexia. In some embodiments, the pharmaceutical compositions may comprise a micro-dystrophin gene. In certain embodiments, the micro-dystrophin gene may comprise the nucleic acid sequence of SEQ ID NO:16 or SEQ ID NO:18 and an AAV vector or an rAAV vector. In various embodiments, a serotype of the AAV vector or the rAAV vector may be selected from at least one of serotype 6, serotype 8, serotype 9, or another suitable serotype.

[0217] Another aspect of the disclosure relates to pharmaceutical compositions for the treatment or prophylactic treatment of muscular dystrophy sarcopenia, heart failure, and/or cachexia. In some embodiments, the pharmaceutical compositions may comprise a micro-dystrophin gene. In certain embodiments, the micro-dystrophin gene may comprise the nucleic acid sequence of SEQ ID NO:16 or SEQ ID NO:18 and an AAV vector or an rAAV vector. In various embodiments, a serotype of the AAV vector or the rAAV vector may be selected from at least one of serotype 6, serotype 8, serotype 9, or another suitable serotype.

EXAMPLES

[0218] The following examples are illustrative of disclosed methods and compositions. In light of this disclosure, those of skill in the art will recognize that variations of these examples and other examples of the disclosed methods and compositions would be possible without undue experimentation.

Example 1 - Development of Micro-dystrophins

[0219] To develop micro-dystrophins with improved performance a variety of structural modifications of the dystrophin central rod domain, which accounts for approximately 80% of the coding region, were assessed. Novel constructs were generated that comprise unique combinations of between four and six of the 24

spectrin-like repeats (SRs) present in the full-length protein as well as the presence or absence of internal hinge domains. These novel micro-dystrophins were evaluated by rAAV-mediated delivery to dystrophic mdx mice followed by pathophysiologic analysis of skeletal muscles after three and six months.

[0220] Several versions of μDys clones were designed with a focus on increasing functional activity while allowing more complete restoration of the dystrophin glycoprotein complex (DGC). The designed μDys clones were compared with a previously characterized ΔH2-R23+H3/ΔCT clone, μDysH3, which can be highly functional in striated muscles of *mdx* mice (*see* Banks, G. B., *et al.*, PLoS Genetics 6, e1000958, (2010)). The design of these constructs focused, at least in part, on the central rod domain in efforts to improve the contractility of muscles expressing the constructs and to restore neuronal nitric oxide synthase (nNOS) localization to the DGC (*see* Lai, Y., *et al.*, The Journal of Clinical Investigation 119, 624-635, (2009) and Lai, Y., *et al.*, Proceedings of the National Academy of Sciences of the United States of America 110, 525-530, (2013)). Also tested, were the functional capacity and the ability to deliver larger constructs carrying 4, 5, or 6 SRs. To allow stable packaging of these larger μDys clones, a small gene regulatory cassette (RC) modified from the muscle creatine kinase gene was incorporated. This CK8 RC can display strong, muscle-restricted expression, yet this CK8 RC is less than 500 bps in size (*see* Goncalves, M. A., *et al.*, Molecular Therapy: The Journal of the American Society of Gene Therapy 19, 1331-1341, (2011) and Martari, M., *et al.*, Human Gene Therapy 20, 759-766, (2009)).

Example 2 - Design of Micro-dystrophin Clones

[0221] Seven novel micro-dystrophin (μDys) clones were designed to test variations of the rod domain structure. Each of the seven micro-dystrophin clones retained coding sequences for the N-terminal actin-binding domain (N-ABD) and the dystroglycan-binding domain (Dg BD), however, each μDys clone incorporated novel combinations of SR and hinge domains, with a goal of generating μDys clones with improved functional properties that may be delivered and expressed from an rAAV vector. Each of the μDys clones were also tested in a mouse model for Duchenne muscular dystrophy (DMD), as described below. The SEQ ID NOs of the amino acid

sequences and nucleic acid sequences of μDysH3 and these seven novel μDys constructs are listed in Table 1.

Table 1: Micro-dystrophin Construct Sequences

Micro-dystrophin Construct	Amino Acid Sequence	Nucleic Acid Sequence
μDysH3	SEQ ID NO:3	SEQ ID NO:11
μDys1	SEQ ID NO:6	SEQ ID NO:12
μDys2	SEQ ID NO:7	SEQ ID NO:13
μDys3	SEQ ID NO:8	SEQ ID NO:14
μDys4	SEQ ID NO:9	SEQ ID NO:15
μDys5	SEQ ID NO:4	SEQ ID NO:16
μDys6	SEQ ID NO:10	SEQ ID NO:17
μDys7	SEQ ID NO:5	SEQ ID NO:18

[0222] Previous studies suggest that the choice of hinge domains within a μDys clone can impact the function of the protein (*see* Banks, G. B., *et al.*, PLoS Genetics 6, e1000958, (2010)). It was assessed whether alternative and/or shorter hinge domains could be substituted for the Hinge 3 domain, which was used in the μDys clone, μDysH3 (*see id.*). It has been indicated that inclusion of SRs 16 and 17 can improve the function of some μDys clones (*e.g.*, by recruiting nNOS to the DGC). Accordingly, SRs 16 and 17 were also tested in the context of various hinge domains and other SRs. Creation of novel junctions was also minimized (*i.e.*, junctions wherein domains not normally adjacent to one another in the full-length protein are brought together). Additionally, the effect of the inclusion of combinations of either 5 or 6 SRs on μDys-clone function was also assessed. The structure of the seven novel μDys clones, in comparison to the μDysH3 clone and the full-length protein, are illustrated in FIG. 1A.

[0223] Two regions in the dystrophin were tested for their ability to substitute for Hinge 3. The hinge regions of the rod domain are proline rich and lack alpha-helical signature motifs that compose the triple-helical coiled-coil of a spectrin-like repeat (*see* Winder, S. J., *et al.*, FEBS Letters 369, 27-33 (1995)). SR23 contains a proline-rich linker between alpha-helices b and c (*see*, *e.g.*, FIG. 21). It was assessed if this sequence (with alpha-helix c of SR23) could be used as a hinge domain either by itself (μDys1), adjoining SR16-17 (μDys2), or together with H3 (μDys4). One additional construct replaced Hinge 3 with the entire SR23 (μDys5; *see* FIG. 1A). A

second hinge-like region (SEQ ID NO:20) composed of a 20 amino acid insertion previously noted to be located between SR15 and SR16 was also tested (μDys6) (*see* Winder, S. J., *et al.*, FEBS Letters 369, 27-33 (1995)). Additional constructs were designed to test various combinations of the SR domains in the context of these hinges. It has been suggested that the context of SR domains can be important for their function, as such, it was tested whether a hybrid SR, composed of the first half of SR20 and the final half of SR24, would improve μDys function (μDys3). This hybrid SR merges the portion of SR20 normally adjacent to Hinge 3 with the portion of SR24 that merges into Hinge 4 (*see* FIG. 1A). Similar considerations influenced the design of the μDys6 construct noted above, where the novel hinge located between SR15 and SR16 was used in its normal context adjacent to the nNOS location region in SR16-17. This latter construct was also compared directly with a similar construct but which used Hinge 3 instead of the short hinge-like region from between SR15 and SR16 (*see* FIG. 1B). It was also noted that μDys clones 5-7, which incorporate either 5 or 6 SR domains, potentially increase the overall function of the protein (*see* Harper, S. Q. *et al.*, Nature Medicine 8, 253-261, (2002)).

Example 3 - Functionality of Partial Spectrin-like Repeats Can be Dependent on the Rod Domain Composition

[0224] An initial functional screen of μDys clones 1-7 was made in comparison to the μDysH3 clone by generating rAAV6 vectors regulated by the CMV promoter. A dose of $5x10^{10}$ vector genomes (vg) was intramuscularly injected into one tibialis anterior (TA) muscle of 5-6 week old dystrophic *mdx*^{4cv} male mice (*see* Chapman, V. M., *et al.*, Proceedings of the National Academy of Sciences of the United States of America 86, 1292-1296 (1989)), with the contralateral muscle serving as an internal negative control (N=4-5 mice per construct).

[0225] Dystrophin expression and central nucleation, a hallmark of degeneration/regeneration, was measured at 4 weeks and 12 weeks post-injection (*see* FIGS. 1C and 1D) to determine how well each construct was expressed, whether expression persisted, and whether the constructs were able to prevent or reduce ongoing myofiber necrosis. All constructs generated μDys proteins of the

predicted sizes, as shown by Western blot analysis (*see* FIG. 1B). At this age and vector dose per injected TA muscle, all treated *mdx*^{4cv} cohorts had significantly fewer dystrophin-positive (Dys+) myofibers compared to wild type C57BL/6 mice ($P<0.001$), yet differences of functionality were observed among the micro-dystrophins. Constructs μDys3 and μDys4 performed less well than μDysH3, as evidenced by a reduction in dystrophin-positive myofibers between 4 and 12 weeks post-injection. Constructs μDys-1, 2, and 5 exhibited more dystrophin-positive myofibers than μDysH3 by 12 weeks post-injection (*see* FIG. 1D). An initial screen of μDys6 and μDys7 was made against μDysH3 driven by the CK8 promoter. Both the new constructs generated comparable levels of transduced (Dys+) and centrally nucleated (CNF+) myofibers by 12 weeks post-injection relative to μDysH3 (*see* FIG. 1D). Myofibers exhibiting both dystrophin expression and central nucleation were quantified at both time points (*see* FIGS. 1C and 1D). Levels of Dys+ and CNF+ myofibers decreased from 4 to 12 weeks post-injection in the treated cohorts, yet remained higher than in wild type muscles. Whether this was the result of poor functionality or sub-optimal dose of a micro-dystrophin construct remained uncertain with the initial screen alone, which prompted a systemic administration for further evaluation.

Example 4 - Novel μDys Constructs Attenuate Pathology in Respiratory and Hind Limb Skeletal Muscles

[0226] The μDys-1, 2, 3, 4, 5, and μDysH3 vectors were re-cloned to replace the CMV with the smaller and muscle-specific CK8 promoter, enabling a direct comparison with the larger six SR-containing constructs (μDys6 and 7). For systemic treatment, a bolus of 10^{13} vg was delivered to 14-day old *mdx*^{4cv} male mice via retro-orbital injection. Treated mice were assessed at either 3 or 6 months post-injection, along with age matched untreated and wild type controls. This experiment was designed to monitor expression of the μDys constructs and assess the relative extent to which they may halt dystrophic pathophysiology. Persistence of μDys expression was measured by immunofluorescence staining of gastrocnemius muscle and diaphragm muscle cryosections. The recruitment of DGC members, β-

dystroglycan and nNOS (for applicable constructs), to the sarcolemma was also verified (*see* FIG. 2).

[0227] At three months post-injection, all treated groups had greater than 60% expression of dystrophin at the sarcolemma in both the gastrocnemius and diaphragm myofibers. The percentage of dystrophin-positive myofibers that were centrally nucleated was not significantly different from wild type controls (*see* FIGS. 3A and 3C). At this time point, μDys2 was observed to be expressed at significantly lower levels compared with μDys5 in the gastrocnemius and the diaphragm (*see* FIGS. 3A and 3C). The μDys2 treated mice also had significantly fewer transduced myofibers in the diaphragm compared to μDys1, μDys5, and μDysH3 injected animals (*see* FIG. 3C). Conversely, μDys5 injected mice displayed significantly higher numbers of transduced myofibers in the gastrocnemius compared with all other treated groups (*see* FIG. 3A). Morphological analysis of the same muscles demonstrated that all treated groups had significantly reduced percentages of centrally nucleated myofibers. In the diaphragm, there were no significant differences in the percentages of centrally nucleated myofibers between the wild type and treated groups. However, μDys2 and μDysH3 injected mice displayed significantly higher levels of central nucleation (19% and 20%, respectively; $P<0.001$) than wild type (0%) in the gastrocnemius.

[0228] The absence of a functional dystrophin can impair assembly of the DGC. This can result in a loss of mechanical force transmission as well as increased susceptibility to contraction-induced injury (*see* Emery, A. E. H. and Muntoni, F., Duchenne Muscular Dystrophy, Third Edition (Oxford University Press, 2003) and Ozawa, E. in Myology (ed. Franzini-Armstrong C Engel A) 455-470 (McGraw-Hill, 2004)). Expression of some rAAV-μDys vectors has demonstrated an ability to increase specific force generation and resistance to contraction-induced injury in dystrophic animal models (*see* Seto, J. T., *et al.*, Current Gene Therapy 12, 139-151 (2012)). It was assessed which novel μDys constructs could improve these metrics at three months post-injection (*see* FIGS. 3C and 3D).

[0229] Gastrocnemius muscles and diaphragm muscle strips were prepared for *in situ* and *in vitro* measurement of mechanical properties, respectively. The specific

force generation in the gastrocnemius muscle increased in all treated groups compared to untreated dystrophic controls (*see* FIG. 3B). Only μDys1 and μDys2 injected mice displayed increased specific force in diaphragm muscle strips (156 kN/m^2 and 110 kN/m^2, respectively, compared to 98 kN/m^2 in untreated mice) (*see* FIG. 3D). Additionally, expression of all the novel μDys constructs increased resistance to contraction-induced injury, yet there were no significant differences in comparison to each other. The dystrophic pathology appeared halted by three months post-injection, yet the physiological performance was not significantly improved. Longer time points may be used to further assess the functionality of the μDys constructs.

Example 5 - Long-term Expression Exposes Functional Discrepancies of μDys Constructs

[0230] By six months post-treatment, most treated groups exhibited reduced expression of dystrophin and a concomitant increase in the percentage of myofibers displaying central nucleation, albeit to varying degrees, compared to analysis at three months post-treatment. However, the percentage of myofibers exhibiting both dystrophin expression and central nucleation was not significantly different from wild type controls (*see* FIGS. 4A and 4C). The μDys1, -5, -6, -7 and -H3 injected mice displayed ≥60% dystrophin positive myofibers in the gastrocnemius and ≥74% in the diaphragm at 6 months. Transduction levels of μDys2 decreased approximately 2-fold in the gastrocnemius over the course of three months (from 63% to 31% positive myofibers), and decreased 20% in the diaphragm, making its performance the worst of the constructs tested ($P<0.001$; *see* FIGS. 4A and 4C). The degree of degeneration/regeneration had increased in both muscles for all treated cohorts, with the exception of two tested constructs. Central nucleation for μDys1 remained at 3% in the diaphragm, and μDysH3 decreased from 20% to 8% in the gastrocnemius (*see* FIGS. 4A and 4C).

[0231] Despite the morphological trend observed with the six month post-treatment data, the specific force generation was still higher than in muscles from untreated controls. Injection of one construct, μDys5, led to force generation levels close to those in wild type mice in both the gastrocnemius (225 versus 226 kN/m^2;

see FIG. 4B) and the diaphragm (148 versus 160 kN/m^2; *see* FIG. 4D). Based on previous studies with mini-dystrophins containing six to eight SRs, it was predicted that the μDys constructs containing six SRs would generate the most specific force as well as provide the greatest protection from contraction-induced injury (*see* Harper, S. Q., *et al.*, Nature Medicine 8, 253-261, (2002)). However, specific force generation in the gastrocnemius muscles of μDys6 and μDys7 treated mice was significantly higher than untreated controls ($P<0.01$ and $P<0.0001$, respectively), but were not the highest (*see* FIGS. 4B and 4D). Instead, μDys5 injected mice displayed the highest levels of specific force generation. The larger constructs were also not necessarily the best at protecting from contraction-induced injury. For example, μDys6 injected mice had the largest force deficit while μDys7 provided the highest protection from contraction-induced injury in the gastrocnemius (*see* FIG. 5A). However, resistance to contraction-induced injury in diaphragm muscle strips was the highest in mice expressing μDys6 and μDys7 as well as μDys5 and μDysH3 (*see* FIG. 5B). The contrasting results among μDys6 and μDys7 between muscle groups and the significant difference in force deficits within the gastrocnemius ($P<0.0001$) suggest that the performance of a particular μDys construct may be influenced by the regulatory expression cassette and the muscle assessed (*see* Harper, S. Q., *et al.*, Nature medicine 8, 253-261, (2002) and Salva, M. Z., *et al.*, Molecular Therapy: The Journal of the American Society of Gene Therapy 15, 320-329, (2007)). This point was also exemplified with μDys2 treatment, where the susceptibility to contraction-induced injury was reduced in the gastrocnemius but unexpectedly exacerbated in the diaphragm, relative to untreated controls (*see* FIGS. 5A and 5B).

Example 6 - Gene Delivery Via rAAV6 + Cardiac-Specific Promoter

[0232] Vectors for WT and L48Q cTnC (rAAV6-WT cTnC and rAAV6-L48Q cTnC, respectively) were produced. Plasmids can be produced with rAAV genomes containing a cardiac specific promoter (cTnT455) and a C-terminal c-Myc tag. cTnC variant transgene expression cassettes (with an mCherry fluorescent reporter) can be co-transfected into HEK293 cells with a packaging/helper plasmid pDGM6 by $CaPO_4$ precipitation methodology. Vectors can be collected from culture, freeze-thawed, and the supernatant can be collected. Affinity purification can use a

HITRAP™ heparin column (GE HEALTHCARE LIFE SCIENCES™, Piscataway, NJ). The virus can be concentrated on a sucrose gradient (40%), spun at 27,000 rpm (18 hours, 4 °C), and resolubilized in Hanks' balanced solution. Vector genomes can be determined relative to plasmid standards using a SV40 polyadenylation region oligonucleotide ^{32}P end-labeled probe with Southern blot hybridization and confirmed by qPCR.

[0233] FIG. 8 depicts the first use of the rAAV6-L48Q cTnC systemically injected (intraocular) into 3 mice each at low (L; $0.6x10^{12}$) and high (H; $1.2x10^{12}$) viral particle dose. Echocardiography indicated about a 20% increase in left ventricular (LV) ejection fraction compared with uninjected (UN) controls two weeks after injection, and a 30-40% increase at 3 weeks. Systemic injections with control adenoviral vectors have not altered LV function. Myofibrils from one rAAV6-L48Q cTnC-myc transfected mouse (and uninjected control) were separated by SDS-PAGE and Western blots were probed with anti-cTnC. The presence of myc-tag caused slower migration of cTnC (*see* FIG. 9), and the ratio of cTnC-myc to native cTnC was densitometrically determined to be about 40% (similar to that seen with adenovirus and transgenic animals, *see* below).

Example 7 - Acute and Chronic Effects of cTnC Variants on Cardiac Function

[0234] Acute and chronic effects of cTnC variants on cardiac function can be assessed using rAAV6-cTnC vectors and transgenic mice. To determine the response to acute changes in myofilament function, normal adult mice can be transfected via tail vein or intraocular orbit injection of rAAV6-cTnC variants (*e.g.*, WT, L48Q, L57Q, or 161Q) with a cardiac specific promoter (cTnT455). Parallel experiments can be performed with the L48Q cTnC and 161Q cTnC transgenic mice by repressing the MHC promoter until adulthood. Additional studies can be conducted with mice without repression of the promoter to determine the effects of these cTnC variants on normal cardiac development and function. Echocardiographic assessments at 1, 2, 3, and 6 months of age can be conducted to determine onset and progression of any changes in function. Some animals may be stressed via β-adrenergic stimulation with isoproterenol. Following

echocardiography, some animals may undergo hemodynamic measurements using MILLAR™ catheter protocols, others may be euthanized and hearts dissected for working heart protocols or for intact or skinned trabeculae preparations, cultured cardiomyocytes, or myofibril preparations.

Example 8 - Tissue-Specific Targeting with rAAV6 Constructs

[0235] Tissue specificity was assessed using alkaline phosphatase driven by various gene promoters in rAAV6 constructs. Table 2 (*see* below) compares two cardiac specific promoters (creatine kinase 7 (CK7) and cardiac troponin T (cTnT455)) to the non-specific cytomegalovirus (CMV) promoter), with values normalized to CK7 in the TA. cTnT455 can lead to high expression in the heart but little to no expression in other tissue. This specificity may reduce potential for effects of R1R2 over-expression in non-cardiac tissues.

[0236] dATP has no significant effect on mouse aortic smooth muscle force development. To study potential systemic effects of elevated dATP it was determined if dATP affects mouse aortic smooth muscle contraction. FIG. 12A shows that back to back contractions in skinned muscle strips did not differ for dATP vs. ATP as the contractile substrate, and the data for multiple experiments is summarized in FIG. 12B. Additionally, control measurements demonstrated that dATP did not change the level of myosin light chain phosphorylation, which controls smooth muscle myosin binding to actin.

Example 9 - Recombinant AAV6-R1R2 for Cardiac-Specific Targeting

[0237] rAAV6 vectors were used to acutely increase cardiac levels of R1R2 (and [dATP]). Plasmids can be produced with recombinant rAAV genomes containing a cardiac specific promoter (cTnT455). R1R2 transgene expression cassettes can be co-transfected into HEK293 cells with a packaging/helper plasmid pDGM6 by $CaPO_4$ precipitation methodology. Vectors can be collected from culture, freeze-thawed, and the supernatant can be collected. Affinity purification can use a HITRAP™ heparin column (GE HEALTHCARE LIFE SCIENCES™, Piscataway, NJ). The vector can be concentrated on a sucrose gradient (40%), spun at 27,000 rpm (18 hours, 4 °C), and resolubilized in Hanks' balanced solution. Vector genomes can be determined relative to plasmid standards using a SV40 polyadenylation region

oligonucleotide ^{32}P end-labeled probe with Southern blot hybridization and confirmed by qPCR.

Table 2: Comparison of CK7, CMV, and cTnT455 promoters

	CK7	CMV	cTnT455
Tibialis Anterior	1	3.1	0
Heart	1.9	5.1	1.6
Lung	0.02	0.09	0.01
Liver	0.02	0.09	0.004
Aorta	0.01	0.13	0.005

[0238] Selection of the cardiac targeting construct was assessed using alkaline phosphatase driven by various gene promoters in rAAV6 constructs. Table 2 compares two striated muscle specific promoters (creatine kinase 7 (CK7) and cardiac troponin T (cTnT455)) to the nonspecific cytomegalovirus (CMV) promoter, with values normalized to CK7 in the TA. cTnT455 can lead to high expression in the heart, but little to no expression in other tissue, thus reducing the potential for effects of R1R2 over-expression in non-cardiac tissues. FIG. 14A shows Western blot evidence for this, where heart tissue from a rAAV6-RIR2cTnT455 injected (4.5 e^{13}) mouse expressed high RI & R2 subunits compared to control mouse heart. Note that upper bands are nonspecific staining, with arrows pointing to RI and R2 protein (identified by molecular weight markers). R1 & R2 expression in lung was extremely low in comparison with heart and was not changed in skeletal muscle. This is demonstrated in FIG. 14 for heart tissue from non-injected (panel "B") vs. rAAV6-alkaline phosphatase (panel "C") injected mice, suggesting rAAV6-RIR2cTnT455 may provide stable, long-term R1R2 over-expression. Stable rAAV6 transgene expression has also been shown to persist for 12 or more weeks in rat and at least 6 or more months in dogs.

[0239] Studies may determine the relationship between rAAV6-R1R2cTnT455 injection dose, time course, and stability of increased LV pump function, cardiac tissue R1R2 levels, and [dATP]. FIG. 15 shows the effect of 3 vector doses, *i.e.*, $1.5x10^{13}$, $4.5x10^{13}$, and $1.35x10^{14}$ rAAV6-RIR2cTnT455 vector genomes or saline (control) injected into 3 month old mice (n=6 per group) on LV function. LV fractional

shortening (FS) was significantly increased at the high dose after one week and at all doses after two weeks, with equivalent effects by 6 weeks. The magnitude increase in FS is 25%-50%, indicating the effect that may be achievable with a relatively low vector dose.

Example 10 - Transgenic R1R2 Over-Expression Mice (TG-R1R2)

[0240] Bi-transgenic mice that over-express both subunits (Rrm1 & Rrm2) of RR can be utilized. FIG. 13 depicts over-expression of both subunits in cardiac muscle, with densitometric calculation values for these TG-R1R2 mice that are 33.7 ± 7.6 (Rrm1) and 23.7 ± 3.4 (Rrm2) fold greater than corresponding values for wild type (WT) mice. Note that for Rrm2 the upper band (*) is non-specific. The endogenous Rrm2 protein is not detectable in WT tissue, but in TG-R1R2 mice it appears as the band below the background band. While dATP levels for cardiac tissue have not yet been assessed, [dATP] is increased 10-fold in skeletal muscle, which had corresponding 3.3 ± 2.1 (Rrm1) and 35.7 ± 11.1 (Rrm2) fold increases in the enzyme subunits. This magnitude of increase in dATP is similar to what has been determined for cardiomyocytes transfected with adenovirus-R1R2 in culture (*see* FIG. 10). Preliminary echocardiography of these TG-R1R2 mice at 6-8 months of age (measured on 3 successive weeks) revealed an average >50% increase in fractional shortening (FS) and a 15% reduction in diastolic LV inner diameter (LVIDd). As shown in FIG. 11, these differences (from WT controls) are similar in magnitude to values for the preliminary adenovirus-R1R2 injection experiments.

Example 11 - Acute Effects of Elevated Cellular R1R2 and [dATP] on Cardiac Function

[0241] Acute R1R2 over-expression (via rAAV6-R1R2 vectors) may increase [dATP] in mouse hearts, resulting in increased systolic and diastolic function. This may be reflected in: 1) increased cardiomyocyte and myofibril contraction with faster relaxation (due in part to increased crossbridge cycling kinetics); 2) an increase in basal cardiac metabolism without compromising energetic reserves; and 3) no change or a decrease in action potential duration (due to enhanced Ca^{2+} sequestration).

[0242] Normal adult FVB/N mice can be transfected via tail vein or intraocular orbit injection with rAAV6-R1R2 vectors with the cardiac specific promoter cTnT455 (as described above), with sham injections and with rAAV6 containing only cTnT455 as controls. Following injection, echocardiography can be performed weekly (out to 6 weeks) to determine the optimal (maximal effect) time point for further assessments. Initial studies may characterize cardiac function *in vivo* with echocardiography, followed by *in situ* hemodynamic measures, or *ex vivo* using Langendorff perfused hearts for energetic studies and a working heart apparatus to assess pump performance. At selected time-points, other mice can be euthanized and hearts dissected for intact or skinned trabeculae preparations, isolated cardiomyocytes, myofibril preparations, protein analysis, and (immuno)histology. These measurements may provide molecular mechanisms for alterations in cardiac function with acute R1R2 over-expression.

Example 12 - Myofilament and SR Protein Profiling

[0243] Changes in contractile function, Ca^{2+} transients, SR spark activity, and/or Ca^{2+} load under all conditions may be correlated with isoform, abundance, and phosphorylation of myofilament proteins (cTn1, cTnT, MLC-2, cMyBP-C, and Tm), SR proteins (PLB, RyR), and sarcolemmal proteins (NCX, PMCA, and L-type Ca^{2+} channel). Changes in mRNA and protein expression may be determined using RT-PCR and Western blot analysis. SR protein fractions can be prepared. If electrophysiological measurements indicate changes, ion channels can be assessed with specific antibodies. Analysis of R1R2 expression can be made via Western blots (*see* FIG. 10) or immunohistochemistry, and correlated with experimental endpoints. Specificity of the cTnT455 promoter can be assessed by determining R1R2 expression in non-cardiac tissues such as skeletal muscle and lung. Phosphorylation can be profiled using PRO-Q® Diamond phosphoprotein gel stain (with SYPRO® Ruby Protein Gel Stain) and Western blot analysis. For site specific serine and threonine residue phosphorylation, mass spectrometry can be performed.

[0244] R1R2 over-expression and increased [dATP] may improve cardiac performance of infarcted hearts at the selected time point for analysis. Response to high Ca^{2+} challenge, β-adrenergic stimulation, and increasing pre-loads may be

improved. *In vitro* Neely working heart measurements of the hearts assessed in FIG. 16 showed a loss of pre-load responsiveness of hearts (heart failure) that have been infarcted (no treatment), but showed a recovery of pre-load responsiveness of the infarcted hearts receiving the vectors to the level of control, uninfarcted hearts, thereby demonstrating a restoration of cardiac function. With reference to FIG. 17, where power is given in units of g•cm/min, the effect may have occurred by lessening chronic β-adrenergic stimulation (which can be assessed by monitoring plasma hormones). This may be reflected in the multi-scale analysis as improved: 1) Ca^{2+} transients; 2) myofilament contraction and relaxation magnitude and kinetics; and 3) energetic profile. A difference between treated and untreated hearts in α- and β-adrenergic mediated cardiomyocyte protein phosphorylation may also be seen.

Example 13 - AAV9 Vector Carrying CK8-μDys5

[0245] An 8 week old *mdx*^{4cv} mouse was injected (intramuscularly) into the TA muscle with $2.5x10^{11}$ vector genomes of an AAV9 vector carrying a CK8-μDys5 expression cassette. Two weeks later, the mouse was sacrificed and muscle cryosections were stained for dystrophin expression using an anti-dystrophin antibody. As shown in FIG. 22, widespread and robust expression of the μDys5 protein was observed in the injected muscle.

[0246] An AAV9 vector can comprise an expression cassette (*e.g.*, a promoter, a cDNA, and a Poly(A) site) linked to an AAV inverted terminal repeat (ITR). The ITRs may be from AAV2. The AAV9 vector can include the genomic DNA comprising the expression cassette and the ITR packaged into a vector using the capsid proteins from AAV9. SEQ ID NO:22 is an exemplary nucleic acid sequence of a CK8-μDys5 cassette with an inverted terminal repeat (ITR) attached. Such a sequence may be used to generate AAV6, AAV9, etc. Different introns, poly(A) sites, spacers, etc. may also be added to the sequence.

Example 14 - Animal Experiments

[0247] Male wild type and dystrophic *mdx*^{4cv} mice bred on a C57BL/6 inbred strain were used in this study. Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of the University of Washington. For initial screening, 5-6 week-old dystrophic *mdx*^{4cv} mice were

administered $5x10^{10}$ vg of rAAV6 vector into the TA muscle. Control mice were injected with Hanks' balanced saline solution as a sham manipulation. In systemic analysis, 14-day old *mdx*^{4cv} males were administered 10^{13} vg of rAAV6 vector intravenously via retro-orbital injection. Mice were sacrificed at either three or six months post-treatment for further evaluation.

Example 15 - Vector Cloning and Virus Production

[0248] All micro-dystrophin transgenes were engineered using standard cloning techniques (*see* Chamberlain, J., PCR-mediated Mutagenesis, doi:10.1038/npg.els.0003766 (2004)). Modified regions were subcloned into μDysHinge3 (ΔH2-R23/ΔCT, +H3) within the AAV vector genome backbone plasmid, pARAP4, using MfeI/XhoI or NheI/XhoI restriction sites flanking the majority of the central rod domain (*see* Banks, G. B., *et al.*, PLoS Genetics 6, e1000958, (2010)). The polyadenylation signal from the rabbit beta-globin gene was subcloned immediately after the μDys cDNA carboxy terminus. The CMV promoter composed of the cytomegalovirus immediate early promoter and enhancer drove expression of micro-dystrophin cDNA. The CK8 regulatory cassette (*see* Goncalves, M. A., *et al.*, Molecular Therapy: The Journal of the American Society of Gene Therapy 19, 1331-1341, (2011)) was subcloned in SphI/SacII sites to replace the CMV promoter and drive expression of micro-dystrophin cDNA in myogenic cells. Recombinant AAV6 vectors were made as previously described (*see* Gregorevic, P., *et al.*, Nature Medicine 12, 787-789, (2006)). Briefly, expression constructs were co-transfected into HEK293 cells with pDGM6 packaging plasmid and later harvested and purified by a combination of filtration, heparin affinity chromatography, and ultracentrifugation. Viral preparations were quantified by Southern blot and quantitative PCR analysis and always in comparison to other preparations used in this study to ensure equal dosing in treating dystrophic mice.

Example 16 - Histological Analysis

[0249] After physiological analysis, mice were sacrificed for necropsy. Muscles were embedded in TISSUE-TEK® O.C.T. Compound, an optimum cutting temperature formulation of water-soluble glycols and resins (SAKURA FINETEK USA™, Torrance, CA) and frozen in liquid nitrogen-cooled isopentane. Transverse

sections approximately 10 μm thick were used for immunofluorescence studies. Sections were blocked in 2% gelatin and 1% Tween-20 in potassium phosphate buffered saline (KPBS). Sections were washed with 0.2% gelatin in potassium phosphate buffered saline (KPBS-G) and followed an incubation of primary antibodies diluted in 2% normal goat serum in KPBS-G. Sections were then rinsed in KPBS-G three times before incubation with secondary antibodies and DAPI, 4′,6-Diamidine-2′-phenylindole dihydrochlorid (SIGMA-ALDRICH®, St. Louis, MO). After washing three more times in KPBS-G, slides were mounted in PROLONG® GOLD ANTIFADE MOUNTANT, a liquid mountant (LIFE TECHNOLOGIES™, Grand Island, NY). Primary antibodies included rabbit polyclonal N-terminal anti-dystrophin antibody (*see* Harper, S. Q., *et al.*, Nature Medicine 8, 253-261, (2002)), mouse monoclonal anti-dystrophin (MANEX1011B clone 1C7, Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa, Iowa City, IA) conjugated to ALEXA FLUOR® 488 DYE, a green-fluorescent dye (LIFE TECHNOLOGIES™), mouse anti-β-dystroglycan (MANDAG2 clone 7D11, DSHB) conjugated to DYLIGHT™ 594, an amine-reactive dye (THERMO FISHER SCIENTIFIC™, Rockford, IL), rat anti-α2-laminin (clone 4H8-2, SIGMA-ALDRICH®, St. Louis, MO), and rabbit anti-nNOS (Z-RNN3, LIFE TECHNOLOGIES™). Secondary antibodies were goat anti-rabbit or anti-rat conjugated to ALEXA FLUOR® 660 far-red dye or ALEXA FLUOR® 594 red-fluorescent dye, respectively (LIFE TECHNOLOGIES™). Images were captured on an OLYMPUS™ SZX16™ dissection fluorescent microscope with DP™ software (OLYMPUS™, Center Valley, PA).

Example 17 - Immunoblotting

[0250] TA muscles of mice from an initial screen were snap frozen in liquid nitrogen and then ground by dry ice-chilled mortar and pestle. Muscles were homogenized in kinase assay lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA supplemented with COMPLETE™ MINI protease inhibitor cocktail tablet (ROCHE™, Indianapolis, IN). Protein concentration of lysate was determined using the PIERCE™ Coomassie Plus (Bradford) Assay (PIERCE™, Rockford, IL). 40 μg of protein was suspended in NUPAGE® LDS sample buffer (LIFE TECHNOLOGIES™) supplemented with 100 mM dithiothreitol and loaded

onto a NuPAGE® 4-12% Bis-Tris polyacrylamide gel (LIFE TECHNOLOGIES™). After running the gels and transferring samples onto AMERSHAM™ HYBOND™ P polyvinylidene fluoride membrane (GE HEALTHCARE LIFE SCIENCES™, Piscataway, NJ), blots were blocked with 10% nonfat dry milk in PBS. Blots were then incubated with primary antibodies in 5% nonfat dry milk, 0.1% Tween-20 in PBS (PBST). After washing three times in PBST, secondary antibodies were incubated in 5% nonfat milk in PBST and followed by four washes in PBST. Primary antibodies included mouse anti-dystrophin (MANEX1011B clone 1C7, DSHB) and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (G9545, SIGMA-ALDRICH®) as a loading control. Secondary antibodies included donkey anti-rabbit or mouse (JACKSON IMMUNORESEARCH LABORATORIES™, West Grove, PA). Blots were developed with PIERCE™ ECL Plus Western blotting substrate (THERMO FISHER SCIENTIFIC™) and scanned using a STORM™ 860 imaging system (GE HEALTHCARE LIFE SCIENCES™).

Example 18 - Functional Analyses of Skeletal Muscles

[0251] Muscles were assayed *in situ* (gastrocnemius) and *in vitro* (diaphragm) for force generation and susceptibility to contraction-induced injury as previously described with the noted modifications (*see* Banks, G. B., *et al.*, Human Molecular Genetics 17, 3975-3986, (2008) and Gregorevic, P., *et al.*, The American Journal of Pathology 161, 2263-2272, (2002)). The maximum isometric force was determined at optimal muscle fiber length and then the muscle was subjected to a series of progressively increasing length changes under stimulation (model 701C™, high-power, bi-phase stimulator, AURORA SCIENTIFIC™). Maximum isometric tetanic force was measured by stimulating at 150 Hz and 180 Hz for the gastrocnemius and diaphragm, respectively. Eccentric contractions were performed at thirty-second intervals, each comprising stimulation at a fixed length to allow peak isometric force of either 150 ms (gastrocnemius) or 100 ms (diaphragm), followed by a continued 200 ms (gastrocnemius) or 300 ms (diaphragm) of stimulation during physical lengthening of the muscle. A series of length changes, or strains, of 0-45% of the optimum length was applied to potentiate overloading of the contractile properties and damage to the muscle architecture. The result from an eccentric contraction

was measured in the peak isometric force generated just prior to the subsequent eccentric contraction.

[0252] Mice were anesthetized with 2,2,2-tribromethanol (SIGMA-ALDRICH®) to be unresponsive to tactile stimuli and then prepped for *in situ* analysis of the gastrocnemius. The Achilles' tendon was exposed by incision at the ankle, sutured with 3-0 braided silk (ETHICON™, Cincinnati, OH), severed, and secured to the lever arm of a dual-mode force transducer-servomotor (model 305B-LR™, AURORA SCIENTIFIC™, Ontario, CA). Mice were immobilized and secured to the apparatus by a stainless steel pin inserted through the knee, and by taping the hind paw to a customized PLEXIGLAS®, poly(methyl methacrylate), platform. Gastrocnemius muscle was stimulated via two needle electrodes that were inserted through the skin on either side of the peroneal nerve in the region between the knee and hip. The servomotor's position was manipulated on three axes to help determine the optimal muscle fiber length. The servomotor was controlled by LABVIEW™ software that also allowed data acquisition (NATIONAL INSTRUMENTS®, Austin, TX).

[0253] For *in vitro* preparation of diaphragm, the anesthetized mouse was sacrificed after gastrocnemius analysis and the entire diaphragm muscle and surrounding ribcage was quickly excised to a dish containing oxygenated Tyrode's solution (*see* Lannergren, J., Bruton, *et al.*, The Journal of Physiology 526 Pt 3, 597-611 (2000)) containing (mM): NaCl 121, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.4, $NaHCO_3$ 24, glucose 5.5 solution as bubbled by 5% CO_2-95% O_2 mixture (pH 7.3). A diaphragm strip composed of longitudinally arranged full-length muscle fibers, a portion of the central tendon, and a portion of rib bones and intercostal muscle on the distal end of the strip was isolated under a microscope. The muscle strip was tied with needle-lead braided surgical silk (6-0, P1; ETHICON™) at the central tendon, sutured through the rib bone portion (5-0; ETHICON™) and then secured to an *in situ* mouse apparatus with a temperature controlled, horizontal bath (model 809A™, AURORA SCIENTIFIC™). Apparatus bath was filled with the bubbled Tryode's solution described above and maintained at 25 °C. Optimal fiber length was determined and isometric and eccentric contractile properties were assessed in a manner similar to gastrocnemius muscle analysis, with the conditions specified

above for the diaphragm muscle. Specific force of both muscle groups was determined by normalizing maximum isometric force to the mass of the gastrocnemius muscle or diaphragm strip, respectively. The following equation was used: specific force = maximum force × pennation × muscle length × 1.04 density/muscle weight (*see* Burkholder, T. J., *et al.*, Journal of Morphology 221, 177-190, (1994)). Pennation is the angle at which bundles of skeletal muscle fibers orient themselves between the tendons of the muscle. For the gastrocnemius muscle, this angle was determined by a previous study (*see* Banks, G. B., *et al.*, PLoS Genetics 6, e1000958, (2010)). Diaphragm muscle strips were isolated in such a way that the myofibers would contract in a direct line between the semitendinosus junction to the myotendinous junction at the rib (*see* Gregorevic, P., *et al.*, The American Journal of Pathology 161, 2263-2272, (2002)). Pennation for the gastrocnemius and diaphragm equals 0.45 and 1, respectively.

Example 19 - Construction of the cTnT455 Regulatory Cassette

[0254] The cTnT455 regulatory cassette (SEQ ID NO:1; 455 indicates the number of base pairs in the RC) was constructed as described herein. DNA was prepared from human cells. PCR primers were used to amplify the cTnT enhancer/promoter region based on sequence similarity to rat and chicken cTnT sequences. The wildtype cTnT enhancer/promoter was ligated to a human placental alkaline phosphatase (AP) cDNA, and plasmid DNA was produced. cTnT-AP plasmids were transfected into newborn rat cardiomyocytes and into differentiating mouse skeletal muscle cells.

[0255] The wild type human cTnT RC (SEQ ID NO:2) had high activity in both cardiac and skeletal muscle cell cultures. cTnT's expression in skeletal muscle was initially unanticipated. Without being bound by any one particular theory, however, cTnT expression in skeletal muscle may be due to the normal activation of cardiac gene expression during early skeletal muscle development and during muscle regeneration. This property may be potentially beneficial for some gene therapy applications, for example, such as in the transient expression of a therapeutic protein only during muscle regeneration.

[0256] The wild type cTnT enhancer was then miniaturized by removing non-conserved base sequences (based on comparisons between human, rat, dog, and chicken) as well as some conserved sequence motifs, followed by transfection tests, as discussed above, to verify that the deletions did not decrease transcriptional activity (*see* FIGS. 18 and 19)

[0257] To obtain higher activity, it was tested whether the addition of multiple miniaturized cTnT enhancers to the cTnT promoter would increase activity. These tests were carried out in cardiac and skeletal muscle cultures and cTnT455 (containing one extra enhancer) was found to be the most active (*see* FIG. 20).

[0258] To determine whether cTnT455 was active *in vivo*, the cTnT455-AP construct was packaged in rAAV6, and the vectors were administered via retro-orbital systemic delivery to mice. Four weeks later, the mice were euthanized and assays were carried out for RC expression levels in cardiac as well as skeletal muscles and non-muscle tissues. The data showed that cTnT455 had high transcriptional activity in cardiac muscle and was transcriptionally silent in both skeletal muscles and all non-muscle tissues (*see* Table 2; *see also,* PCT Application No. PCT/US2012/039897 entitled "Cell and Gene Based Methods to Improve Cardiac Function", the entirety of which is incorporated by reference herein).

Example 20 - Statistical Analysis

[0259] All results are reported as mean ± standard error mean. Differences between cohorts were determined using one-way and two-way ANOVA with Tukey's *post hoc* multiple comparison test. All data analyses were performed with GRAPHPAD™ PRISM™ 6 software (San Diego, CA).

[0260] As will be understood by one of ordinary skill in the art, each embodiment disclosed herein can comprise, consist essentially of, or consist of its particular stated element, step, ingredient, or component. As used herein, the transition term "comprise" or "comprises" means includes, but is not limited to, and allows for the inclusion of unspecified elements, steps, ingredients, or components, even in major amounts. The transitional phrase "consisting of" excludes any element, step, ingredient or component not specified. The transition phrase "consisting essentially

of" limits the scope of the embodiment to the specified elements, steps, ingredients or components, and to those that do not materially affect the embodiment.

[0261] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. When further clarity is required, the term "about" has the meaning reasonably ascribed to it by a person skilled in the art when used in conjunction with a stated numerical value or range, *i.e.*, denoting somewhat more or somewhat less than the stated value or range, to within a range of ±20% of the stated value; ±19% of the stated value; ±18% of the stated value; ±17% of the stated value; ±16% of the stated value; ±15% of the stated value; ±14% of the stated value; ±13% of the stated value; ±12% of the stated value; ±11% of the stated value; ±10% of the stated value; ±9% of the stated value; ±8% of the stated value; ±7% of the stated value; ±6% of the stated value; ±5% of the stated value; ±4% of the stated value; ±3% of the stated value; ±2% of the stated value; or ±1% of the stated value.

[0262] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0263] The terms "a," "an," "the" and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely

intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0264] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0265] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The applicants expect skilled artisans to employ such variations as appropriate, and the applicants intend for the various embodiments of the disclosure to be practiced otherwise than specifically described herein. Accordingly, this disclosure includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the disclosure unless otherwise indicated herein or otherwise clearly contradicted by context.

[0266] Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above-cited

references and printed publications are individually incorporated herein by reference in their entirety.

[0267] It is to be understood that the embodiments of the present disclosure are illustrative of the principles of the present disclosure. Other modifications that may be employed are within the scope of the disclosure. Thus, by way of example, but not of limitation, alternative configurations of the present disclosure may be utilized in accordance with the teachings herein. Accordingly, the present disclosure is not limited to that precisely as shown and described.

[0268] The particulars shown herein are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present disclosure only and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of various embodiments of the disclosure.

[0269] Definitions and explanations used in the present disclosure are meant and intended to be controlling in any future construction unless clearly and unambiguously modified in the examples or when application of the meaning renders any construction meaningless or essentially meaningless in cases where the construction of the term would render it meaningless or essentially meaningless, the definition should be taken from Webster's Dictionary, 3rd Edition or a dictionary known to those of ordinary skill in the art, such as the Oxford Dictionary of Biochemistry and Molecular Biology (Ed. Anthony Smith, Oxford University Press, Oxford, 2004).

[0270] It will be apparent to those having skill in the art that many changes may be made to the details of the above-described embodiments without departing from the underlying principles of the invention. The scope of the present invention should, therefore, be determined only by the following claims.

CLAIMS

1. An isolated and purified nucleotide sequence, comprising:

a micro-dystrophin gene encoding a protein comprising:

an amino-terminal actin-binding domain;

a β-dystroglycan binding domain; and

a spectrin-like repeat domain, consisting of five spectrin-like repeats, including spectrin-like repeat 1 (SR1), spectrin-like repeat 16 (SR16), spectrin-like repeat 17 (SR17), spectrin-like repeat 23 (SR23), and spectrin-like repeat 24 (SR24);

in which the micro-dystrophin gene is operatively linked to a regulatory cassette.

2. An isolated and purified nucleotide sequence according to Claim 1, in which the protein encoded by the micro-dystrophin gene further comprises at least a portion of a hinge domain.

3. An isolated and purified nucleotide sequence according to Claim 2, in which the hinge domain is selected from at least one of a Hinge 1 domain, a Hinge 2 domain, a Hinge 3 domain, a Hinge 4 domain, and a hinge-like domain.

4. An isolated and purified nucleotide sequence according to Claim 1, in which the regulatory cassette is selected from the group consisting of a CK8 promoter and a cardiac troponin T (cTnT) promoter.

5. An isolated and purified nucleotide sequence according to Claim 1, in which the regulatory cassette is a CK8 promoter, and in which the CK8 promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO: 19.

6. An isolated and purified nucleotide sequence according to Claim 1, in which the regulatory cassette is a cTnT promoter, and in which the cTnT promoter has at least 80% sequence identity to the nucleic acid sequence of SEQ ID NO: 1.

7. An isolated and purified nucleotide sequence according to Claim 1, in which the micro-dystrophin gene encodes a protein comprising:

at least one of spectrin-like repeat 1 directly coupled to spectrin-like repeat 16, spectrin-like repeat 17 directly coupled to spectrin-like repeat 23, and spectrin-like repeat 23 directly coupled to spectrin-like repeat 24.

8. An isolated and purified nucleotide sequence according to Claim 3, which comprises a Hinge 1 domain (H1) and a Hinge 4 domain (H4).

9. A pharmaceutical composition, comprising:

an isolated and purified nucleotide sequence according to Claim 1; and

a delivery vehicle.

10. A pharmaceutical composition according to Claim 9, in which the delivery vehicle comprises an adeno-associated virus (AAV) vector or a recombinant adeno-associated virus (rAAV) vector.

11. A pharmaceutical composition according to Claim 10, in which delivery vehicle comprises an adeno-associated virus (AAV) vector which consists of AAV6, AAV8, or AAV9.

12. A pharmaceutical composition according to Claim 10, in which the delivery vehicle comprises a recombinant adeno-associated virus (rAAV) vector which consists of rAAV6, rAAV8, rAAV9 or rAAV2/6.

13. A pharmaceutical composition according to Claim 10, in which the delivery vehicle comprises an adeno-associated virus (AAV) vector which is serotype AAV9.

14. A pharmaceutical composition according to Claim 10, in which the delivery vehicle comprises a recombinant adeno-associated virus (rAAV) vector which is rAAV9.