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DESCRIPTION

FIELD OF THE INVENTION

[0001] The present disclosure relates to an inhibitor of Dynamin 2 or composition comprising the same for use in the treatment of Duchenne's muscular dystrophy.

BACKGROUND OF THE INVENTION

[0002] Duchenne's Muscular Dystrophy (DMD) is the most common myopathy in children. DMD patients develop a dystrophic phenotype in muscle. DMD is a severe X-linked disorder, with progressive muscle wasting and weakness affecting almost all muscles, and normally results in premature death due to cardiorespiratory failure. Despite extensive research into therapies for DMD many of which targeting the expression of dystrophin (reviewed in Al-Zaidy, S., L. Rodino-Klapac and J. R. Mendell (2014). "Gene therapy for muscular dystrophy: moving the field forward." *Pediatr Neurol* 51(5): 607-618), no effective treatment has yet been developed for this devastating disease.

[0003] DMD is due to mutations in dystrophin, resulting in loss of dystrophin protein expression. Dystrophin forms the mechanical link between the contractile apparatus and the plasma membrane of the muscle fiber. In DMD patients the loss of dystrophin expression causes a disruption of the normal force transmission, which places a large amount of stress on the fiber and results in muscle fiber damage. Effective therapies are required for DMD patients which target the physiological muscle force, and therefore improved muscle function. Most therapeutic approaches for DMD have targeted the mutated gene, dystrophin, as to attempt to improve the expression of all/part of dystrophin.

[0004] Recently, it has been disclosed a novel potential therapy for X-linked centronuclear myopathy (XLCNM) patients by reduction of DNM2 expression. This novel approach was based on the findings that reducing dynamin 2 (DNM2) expression can rescue the XLCNM, also called myotubular myopathy, phenotype in *Mtm1^{-y}* mice.

[0005] The inventors have discovered a significant improvement in specific muscle force at several ages by reducing DNM2 in mdx mice, which are the classic mouse model used for preclinical DMD research. Furthermore, an improvement in resistance to contraction-induced muscle injury, confirms the physiological significance of this finding. Therefore, it is herein provided a novel therapeutic approach for the treatment of Duchenne's muscular dystrophy by reducing DNM2 expression.

SUMMARY OF THE INVENTION

[0006] In a first aspect, the present invention concerns an inhibitor of Dynamin 2 for use in the treatment of DMD, as defined by the claims.

[0007] The present invention also concerns a pharmaceutical composition comprising said inhibitor of Dynamin 2 and a pharmaceutically acceptable carrier/excipient for use in the treatment of DMD.

[0008] The Dynamin 2 inhibitor is selected from the group consisting of an antibody directed against Dynamin 2, a nucleic acid molecule interfering specifically with Dynamin 2 expression, a nucleic acid or a nuclease engineered to target the DNM2 gene and to deliver nucleases using genome editing therapy, and a small molecule inhibiting the Dynamin 2 activity, expression or function as defined in claim 1. In a preferred embodiment, the Dynamin 2 inhibitor is selected from the group consisting of a nucleic acid molecule interfering specifically with Dynamin 2 expression. In a particular embodiment, the Dynamin 2 inhibitor is an RNAi, an antisense nucleic acid or a ribozyme interfering specifically with Dynamin 2 expression.

In a more specific embodiment, the Dynamin 2 inhibitor is a siRNA, shRNA or an antisense snRNA. In another particular embodiment, the dynamin 2 inhibitor is a DNA, mRNA or a nuclease engineered to target the DNM2 gene and to deliver nucleases using genome editing therapy

[0009] A further object of the invention relates to a method of screening for identifying compounds useful for the treatment of DMD comprising:

1. a) Providing or obtaining a candidate compound; and
2. b) Determining whether said candidate compound inhibits the activity/expression of Dynamin 2,
3. c) Selecting said candidate compound if it inhibits the activity/expression of Dynamin 2.

[0010] The method for screening or identifying a molecule suitable for the treatment of DMD can optionally further comprise the step of administering *in vivo* or *in vitro* the selected molecule in a DMD non-human animal model or a part thereof (tissue or cells) and analyzing the effect on the myopathy onset or progression.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011]

Figure 1. Specific muscle force is increased in mdx mice with reduced DNM2. Specific muscle force was measured in *mdx^{-ly}* (white) and *mdx^{-ly} Dnm2^{+/-}* (black) mice, at 6 weeks, 3 months, and 1 year of age. Results are displayed as absolute force, relative to muscle mass (sPo/mg).

Statistically significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. Muscle force is improved following contraction-induced muscle injury, in mdx mice with reduced DNM2. Absolute muscle force was measured in *mdx*^{-/*y*} (black, solid line) and *mdx*^{-/*y*} *Dnm2*^{+/-} (black, dashed line) mice at 1 year of age. Results are displayed as a percentage of initial force (%). Statistically significance is indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. (A) Specific muscle force (sPo) was initially measured in the tibialis anterior muscle from *mdx*^{-/*y*} (white) and *mdx*^{-/*y*}*Dnm2*^{+/-} (black) mice. (B) The tibialis anterior muscle was then subjected to 9 lengthening contractions. Results for *mdx*^{-/*y*} (solid black line) and *mdx*^{-/*y*}*Dnm2*^{+/-} (dashed black line) mice are displayed as a percentage of initial force (%), after 0, 3, 6 and 9 lengthening contractions. (C) An intramuscular injection of AAV-shRNA targeting DNM2 (black) or a scrambled control sequence (white) was performed into the tibialis anterior of *mdx*^{-/*y*} mice at 3 weeks of age. Specific muscle force (sPo) was measured in the tibialis anterior muscle at 3 months of age. (D) The tibialis anterior muscle was then subjected to 9 lengthening contractions as in (B), AAV shRNA control (solid black line) and AAV shRNA DNM2 (dashed black line).

DETAILED DESCRIPTION

[0012] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The Dynamin 2 is encoded by the *DNM2* gene (Gene ID 1785). More precisely, the *DNM2* gene is located from base pair 10,919,884 to base pair 10,942,586 on chromosome 19 (GRCh37/hg19 release) or from 10,718,053 to 10,831,910 base pairs on the NC_000019.10 location (GRCh38/hg19). The dynamin 2 gene or gene products are also known by other names, including but not limited to CMTDI1, CMTDIB, DI-CMTB, DYN2, DYN2_HUMAN, dynamin II, DYNII.

Dynamin 2 inhibitors

[0013] As used herein, the term "Dynamin 2 inhibitor" refers to any molecule able to decrease specifically the expression of Dynamin 2 or inhibit the Dynamin 2 activity or function. Preferably, such a Dynamin 2 inhibitor is a direct inhibitor, meaning that it interacts directly with either the Dynamin 2 protein or a nucleic acid encoding said Dynamin 2 or a part thereof. The Dynamin 2 inhibitors according to the invention are capable of inhibiting or decreasing the functional activity of Dynamin 2 *in vivo* and/or *in vitro*. The inhibitor may inhibit the functional activity of Dynamin 2 by at least about 30%, preferably by at least about 50%, preferably by at least about 70, 75 or 80%, still preferably by 85, 90, or 95%. In particular, the inhibitor may inhibit

Dynamin 2 expression by at least about 10%, preferably by at least about 30%, 35%, 40%, 45%, preferably by at least about 50%, preferably by at least about 70, 75 or 80%.

A Dynamin 2 inhibitor of the invention may act by blocking and/or inhibiting the activity or function of Dynamin 2. This may for example be achieved by inhibiting the enzymatic activity of Dynamin 2. Functional or enzymatic activity of Dynamin 2 may be readily assessed by one skilled in the art according to known methods by testing for example the GTPase activity or the function of Dynamin 2 in clathrin-mediated endocytosis (Macia E. et al., Dynasore, a cell-permeable inhibitor of dynamin: Developmental cell 10, 839-850, June 2006). For inhibitors of GTPase activity or lipid binding, subcellular localization, clathrin mediated endocytosis, synaptic vesicle endocytosis, one can use the method described in McCluskey et al, Traffic, 2013 ; McGeachie et al, ACS Chem Biol, 2013. For Dynamin 2 GTPase activity, oligomerisation, lipid binding, one can use the methods described in Wang et al J Biol Chem 2010; or Kenniston and Lemmon, Embo J, 2010.

[0014] The Dynamin 2 inhibitor of the invention may also act by blocking and/or inhibiting the Dynamin 2 expression (including transcription, splicing, transcript maturation, or translation). The decrease or inhibition of Dynamin 2 expression can be evaluated by any means known to those skilled in the art including but not limited to assessing the level of Dynamin 2 protein using for instance Western Blot analysis (such as shown by figure 1) or ELISA, for example using an Anti-Dynamin 2 antibody, and/or assessing the level of mRNA for Dynamin 2 (such as shown by figure 2) using any available technique such as quantitative PCR for example.

[0015] The Dynamin 2 inhibitor is selected from the group consisting of an antibody directed against Dynamin 2, a nucleic acid molecule interfering specifically with Dynamin 2 expression, and a small molecule inhibiting the Dynamin 2 enzymatic activity (i.e., inhibition of the GTPase activity), expression (such as by inhibiting promoter, splicing or translation), or function (such as inhibition of oligomerisation, activation, lipid binding, or partner binding).

[0016] According to a particular embodiment, the Dynamin 2 inhibitor is selected from the group consisting of an antibody directed against Dynamin 2 or a nucleic acid molecule (or nucleotide) interfering specifically with Dynamin 2 expression. In a preferred embodiment, the Dynamin 2 inhibitor is selected from the group consisting of a nucleic acid molecule interfering specifically with Dynamin 2 expression. According to the invention, the nucleic acid molecule interfering specifically with Dynamin 2 expression is usually a non-naturally occurring nucleic acid. In a particular embodiment, the Dynamin 2 inhibitor is a RNAi, an antisense nucleic acid or a ribozyme interfering specifically with Dynamin 2 expression.

In a particular embodiment, the Dynamin 2 inhibitor is a siRNA or shRNA.

[0017] In the present invention, the nucleic acid is capable of hybridizing specifically to a gene or transcripts coding for Dynamin 2. By "hybridizing specifically", is intended hybridized in stringent conditions. In particular, stringent conditions can be defined by salt concentration, the concentration of organic solvent, for example, formamide, temperature, and other conditions well known in the art. Typical stringent hybridisation conditions include temperatures above 30° C, preferably above 35°C, more preferably in excess of 42°C, and/or salinity of less than about

500 mM, preferably less than 200 mM. Nevertheless, it is understood that the nucleic acid according to the invention does not need to have 100% complementarity with the target sequence to hybridize specifically. In particular, a nucleic acid with a degree of complementarity at least equal to approximately 90% is capable of hybridizing specifically. Preferably, the degree of complementarity between the nucleic acid according to the invention and the target sequence is equal to at least 95%, 96%, 97%, 98%, 99% or 100%.

[0018] The term "complementary" or "complementarity" refers to the ability of polynucleotides to form base pairs with another polynucleotide molecule. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands. Complementary polynucleotide strands can base pair in the Watson-Crick manner (e.g., A to T, A to U, C to G), or in any other manner that allows for the formation of duplexes. As persons skilled in the art are aware, when using RNA as opposed to DNA, uracil rather than thymine is the base that is considered to be complementary to adenosine. However, when a U is denoted in the context of the present invention, the ability to substitute a T is implied, unless otherwise stated. Perfect complementarity or 100 percent complementarity refers to the situation in which each nucleotide unit of one polynucleotide strand can bind to a nucleotide unit of a second polynucleotide strand. Less than perfect complementarity refers to the situation in which some, but not all, nucleotide units of two strands can bind with each other. For example, for two 20-mers, if only two base pairs on each strand can bind with each other, the polynucleotide strands exhibit 10 percent complementarity. In the same way, if 18 base pairs on each strand can be bond with each other, the polynucleotide strands exhibit 90 percent complementarity.

[0019] As used herein, the term "iRNA", "RNAi" or "interfering RNA" means any RNA which is capable of down-regulating the expression of the targeted protein. It encompasses small interfering RNA (siRNA), double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and short hairpin RNA (shRNA) molecules. RNA interference designates a phenomenon by which dsRNA specifically suppresses expression of a target gene at post-transcriptional level. In normal conditions, RNA interference is initiated by double-stranded RNA molecules (dsRNA) of several thousands of base pair length. *In vivo*, dsRNA introduced into a cell is cleaved into a mixture of short dsRNA molecules called siRNA. The enzyme that catalyzes the cleavage, Dicer, is an endo-RNase that contains RNase III domains (Bernstein, Caudy et al. 2001 Nature. 2001 Jan 18;409(6818):363-6). In mammalian cells, the siRNAs produced by Dicer are 21-23 bp in length, with a 19 or 20 nucleotides duplex sequence, two-nucleotide 3' overhangs and 5'-triphosphate extremities (Zamore, Tuschl et al. Cell. 2000 Mar 31;101(1):25-33; Elbashir, Lendeckel et al. Genes Dev. 2001 Jan 15;15(2):188-200; Elbashir, Martinez et al. EMBO J. 2001 Dec 3;20(23):6877-88). According to a particular embodiment, iRNAs do not encompass microRNAs.

[0020] A number of patents and patent applications have described, in general terms, the use of siRNA molecules to inhibit gene expression, for example, WO 99/32619. RNA interference therapy by siRNA and shRNA is also detailed in the review by Z. Wang et al., Pharm Res (2011) 28:2983-2995.

[0021] siRNA or shRNA are usually designed against a region 19-50 nucleotides downstream the translation initiator codon, whereas 5'UTR (untranslated region) and 3'UTR are usually avoided. The chosen siRNA or shRNA target sequence should be subjected to a BLAST search against EST database to ensure that the only desired gene is targeted. Various products are commercially available to aid in the preparation and use of siRNA or shRNA.

In a preferred embodiment, the RNAi molecule is a siRNA of at least about 10-40 nucleotides in length, preferably about 15-30 base nucleotides.

siRNA or shRNA can comprise naturally occurring RNA, synthetic RNA, or recombinantly produced RNA, as well as altered RNA that differs from naturally-occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end of the molecule or to one or more internal nucleotides of the siRNA, including modifications that make the siRNA resistant to nuclease digestion.

Some Dynamin 2 inhibitory nucleic acids are commercially available. One can cite for example, but not limited to: Abnova-Novus Biologicals, Dynamin 2 RNAi with references: H00001785-R05-H00001785-R08; Santa Cruz Biotechnology, Dynamin II siRNA (h) with reference: sc-35236, Dynamin II (h)-PR with reference: sc-35236-PR, Dynamin II shRNA Plasmid (h) with reference: sc-35236-SH, Dynamin II shRNA (h) Lentiviral Particles with reference: sc-35236-V). In a particular embodiment, the nucleic acid molecule interfering specifically with Dynamin 2 is a nucleic acid interfering specifically with at least one part of the full length muscle human cDNA sequence of dynamin 2 (as shown in SEQ ID No 1, transcript variant 1 (NM_001005360.2)(exon 10a, 13ter) with 12b added). According to this embodiment, and more specifically, the RNAi molecule is a siRNA or shRNA of at least about 10-40 nucleotides in length, preferably about 15-30 base nucleotides iRNA. In a particular embodiment, siRNA or shRNA targets at least one exon of Dynamin2 mRNA, and more specifically at least one of exon 1, 4, 5, 12b, 13, 15, 17 and 21 of Dynamin2 mRNA.

[0022] In a particular embodiment, the nucleic acid molecule specifically interfering with Dynamin 2 comprises or consists of a sequence selected from the group consisting of

- iRNA sequence of SEQ ID No 2 : 5'- AAGGACATGATCCTGCAGTTCAT - 3'(or shRNA seq N°C, below),
- iRNA sequence of SEQ ID No 3: 5'- AAGAGGCTACATTGGCGTGGTGA- 3'
- iRNA sequence of SEQ ID No 4: 5'- AGGTGGACACTCTGGAGCTCTCC - 3',
- iRNA sequence of SEQ ID No 5: 5'- AAGAAGTACATGCTGCCTCTGGA - 3',
- iRNA sequence of SEQ ID No 6: 5'- AACGTCTACAAGGACCTGCGGCA - 3',
- iRNA sequence of SEQ ID No 7: 5'- AGGAGAACACCTTCTCCATGGAC - 3',
- iRNA sequence of SEQ ID No 8: 5'- AACTGTTACTATACTGAGCAG - 3',
- iRNA sequence of SEQ ID No 9: 5'- TGCCAACTGTTACTATACT - 3',
- iRNA sequence of SEQ ID No 10: 5' - GAAGAGCTGATCCCGCTGG -3'
- iRNA sequence of SEQ ID No 11: 5' - GCACGCAGCTGAACAAGAA -3'
- iRNA sequence of SEQ ID No 12: 5'-GGACTTACGACGGGAGATC-3'
- iRNA sequence of SEQ ID No 13: 5' -GGATATTGAGGGCAAGAAG-3'
- iRNA sequence of SEQ ID No 14: 5'-GGACCAGGCAGAAAACGAG-3'

- iRNA sequence of shRNA 15: 5'- GCGAATCGTCACCACTTAC-3'

| shRNA against DNM2 | Target sequence | Dnm2 Exon target | SEQ ID No : |
|--------------------|-----------------------------|------------------|-------------|
| A | AACCGCGGGATGGAAG AGCT | 1 | 16 |
| B | AACTTGACCCATCATCG ACCTC | 4 | 17 |
| C | AAGGACATGATCCTGC AGTTCAT | 4 | 2 |
| D | TCGGTGTCATCACCAA GCT | 5 | 18 |
| E | TGCCAACTGTTTCTATA CT | 12b | 19 |
| F | AACTGTTTCTATACTGA GGAG | 12b | 20 |
| G | TTTCTATACTGAGGAG CTGGT | 12b | 21 |
| H | GCACGCAGCTGAACAA GAA | 13 | 22 |
| I | AAGAAGTACATGCTGC CTCTGGA | 15 | 23 |
| J | AACACCTTCTCCATGG ACCC | 17 | 24 |
| K | CCATTATCCGCCCAGC CGAGC | 21 | 25 |

[0023] Antisense nucleic acid can also be used to down-regulate the expression of Dynamin 2. The antisense nucleic acid can be complementary to all or part of a sense nucleic acid

encoding Dynamin 2, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence, and it is thought to interfere with the translation of the target mRNA. The antisense nucleic acids used in the invention interfere specifically with Dynamin 2 expression.

[0024] According to an embodiment, the antisense nucleic acid is a RNA molecule complementary to a target mRNA encoding Dynamin 2.

[0025] According to another embodiment, the antisense nucleotide denotes a single stranded nucleic acid sequence, either DNA or RNA, which is complementary to a part of a pre-mRNA encoding Dynamin 2. In particular, the antisense nucleotide of the present invention is designed to block a splice acceptor (SA) site and/or an exon splicing enhancer (ESE) and/or a branch point in the Dynamin2 pre-mRNA and/or any sequence which could modulate pre-mRNA splicing, i.e. it is designed to be complementary to a part of the Dynamin 2 pre-mRNA comprising an SA, an ESE, a branch point sequence or any sequence which could modulate pre-mRNA splicing. More specifically, the antisense nucleotide is used for inducing exon-skipping within a Dynamin 2 pre-mRNA, thereby leading to a frameshift which produces a truncated cDNA containing a premature stop codon in the resulting mRNA. This strategy thus allows the reduction of the level of DNM2 protein. In a particular embodiment, the antisense nucleotide is used for inducing exon-skipping within a Dynamin 2 pre-mRNA. For example, the implemented antisense nucleotide is designed to specifically induce exon 2 or exon 8 skipping. In a particular embodiment, the antisense nucleotide of the present invention is able to induce the inclusion of a premature stop codon in the human DNM2 mRNA. Skipping of exon 2 or exon 8 was shown to lead to an absence of the Dynamin 2 protein (as mentioned in "Reducing dynamin 2 expression rescues X-linked centronuclear myopathy". Cowling BS, Chevremont T, Prokic I, Kretz C, Ferry A, Coirault C, Koutsopoulos O, Laugel V, Romero NB, Laporte J., J Clin Invest. 2014 Mar 3;124(3):1350-63. doi: 10.1172/JCI71206. Epub 2014 Feb 24; and Tinelli E, Pereira JA, Suter U. Hum Mol Genet. 2013 Nov 1;22(21):4417-29. doi: 10.1093/hmg/ddt292. Epub 2013 Jun 27).

[0026] In a particular embodiment, the antisense nucleotide is designed to specifically induce DNM2 exon 2 or exon 8 skipping, and comprises or consists of one of the following sequences: U7-Ex2 (target skipping of DNM2 exon 2 with an antisense U7 snRNA), comprising the following sequence:

SEQ ID No 26: GTCACCCGGAGGCCTCTCATTCTGCAGCTC

U7-Ex8 (target skipping of DNM2 exon 8 with an antisense U7 snRNA), comprising the following sequence:

SEQ ID No 27: ACACACTAGAGTTGTCTGGTGGAGCCCGCATCA

[0027] An antisense nucleic acid can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. Particularly, antisense RNA molecules are usually 15-50 nucleotides in length. An antisense nucleic acid for use in the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. Particularly, antisense RNA can be chemically synthesized, produced by *in vitro* transcription from linear

(e.g. PCR products) or circular templates (e.g., viral or non-viral vectors), or produced by *in vivo* transcription from viral or non-viral vectors. Antisense nucleic acid may be modified to have enhanced stability, nuclease resistance, target specificity and improved pharmacological properties. For example, antisense nucleic acid may include modified nucleotides or/and backbone designed to increase the physical stability of the duplex formed between the antisense and sense nucleic acids.

[0028] In the context of the invention "Ribozymes" are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes can be used to catalytically cleave mRNA transcripts to thereby inhibit translation of the protein encoded by the mRNA. Ribozyme molecules specific for functional Dynamin 2 can be designed, produced, and administered by methods commonly known to the art (see e.g., Fanning and Symonds (2006) RNA Towards Medicine (Handbook of Experimental Pharmacology), ed. Springer p. 289-303).

[0029] Genome editing can also be used as a tool according to the invention. Genome editing is a type of genetic engineering in which DNA is inserted, replaced, or removed from a genome using artificially engineered nucleases, or "molecular scissors". The nucleases create specific double-stranded break (DSBs) at desired locations in the genome, and harness the cell's endogenous mechanisms to repair the induced break by natural processes of homologous recombination (HR) and non-homologous end-joining (NHEJ). There are currently four families of engineered nucleases being used: Zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), the CRISPR/Cas system (more specifically Cas9 system, as described by P. Mali et al., in Nature Methods, vol. 10 No. 10, October 2013), or engineered meganuclease re-engineered homing endonucleases. Said nucleases can be delivered to the cells either as DNAs or mRNAs, such DNAs or mRNAs are engineered to target the DNM2 gene, according to the invention. According to an embodiment, Dynamin 2 inhibitor is a DNA or mRNA engineered to target the DNM2 gene and to deliver nucleases using genome editing therapy or is a nuclease engineered to target the DNM2 using genome editing therapy.

[0030] The nucleotides as defined above used according to the invention can be administered in the form of DNA precursors or molecules coding for them.

For use *in vivo*, the nucleotides of the invention may be stabilized, via chemical modifications, such as phosphate backbone modifications (e.g., phosphorothioate bonds). The nucleotides of the invention may be administered in free (naked) form or by the use of delivery systems that enhance stability and/or targeting, e.g., liposomes, or incorporated into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, bioadhesive microspheres, or proteinaceous vectors, or in combination with a cationic peptide. They can also be coupled to a biomimetic cell penetrating peptide. They may also be administered in the form of their precursors or encoding DNAs. Chemically stabilized versions of the nucleotides also include "Morpholinos" (phosphorodiamidate morpholino oligomers - PMO), 2'-O-Methyl oligomers, AcHN-(RXRRBR)2XB peptide-tagged PMO (R, arginine, X, 6-aminohexanoic acid and B, ®-alanine) (PPMO), tricyclo-DNAs, or small nuclear (sn) RNAs. The latter forms of nucleotides that may be used to this effect are small nuclear RNA molecules including U1, U2, U4, U4atac,

U5, U7, U11, and U12 (or other UsnRNPs), preferably U7snRNA (as identified above for SEQ ID No 26 and 27, in particular in combination with a viral transfer method based on, but not limited to, lentivirus, retrovirus, adenovirus or adeno-associated virus. All these techniques are well known in the art.

[0031] The nucleic acid molecule interfering specifically with Dynamin 2 expression of the invention may be delivered *in vivo* alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the nucleotide to the cells and preferably cells expressing DN2. Preferably, the vector transports the nucleotide to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, and other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleotides of the invention. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: lentivirus such as HIV-1, retrovirus, such as moloney murine leukemia virus, adenovirus, adeno-associated virus; SV40-type viruses; Herpes viruses such as HSV-1 and vaccinia virus. One can readily use other vectors not named herein but known in the art. Among the vectors that have been validated for clinical applications and that can be used to deliver the nucleotides, lentivirus, retrovirus and Adeno-Associated Virus (AAV) show a greater potential for exon skipping strategy.

[0032] As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE, and humanized or chimeric antibody. In certain embodiments, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and they are most easily manufactured. The term "antibody" is used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, F(ab')₂, single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, e.g., Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, ed., Cold Spring Harbor Laboratory).

[0033] A "humanized" antibody is an antibody in which the constant and variable framework region of one or more human immunoglobulins is fused with the binding region, e.g. the CDR, of an animal immunoglobulin. "Humanized" antibodies contemplated in the present invention are chimeric antibodies from mouse, rat, or other species, bearing human constant and/or variable region domains, bispecific antibodies, recombinant and engineered antibodies and fragments thereof. Such humanized antibodies are designed to maintain the binding specificity of the non-human antibody from which the binding regions are derived, but to avoid an immune reaction against the non-human antibody.

[0034] A "chimeric" antibody is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or

species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

Antibodies directed against Dynamin 2 are commercially available, such as antibodies sold or made by Novus Biologicals: catalogue numbers: Dynamin 2 Antibody NB300-617, Dynamin 2 Antibody NBP2-16244, Dynamin 2 Antibody (6C9) H00001785-M01, by Santa Cruz Biotechnology: catalogue number: sc-81150, sc-6400, sc-166525, sc-166669, sc-166526, by BD-Biosciences: anti-DNM2 (mouse ab, 610264), or by IGBMC-IlkKirch: anti-DNM2 : R2679, R2680, R2865, R2866, R2640, or R2641.

[0035] In another particular embodiment, the Dynamin 2 inhibitor is a small molecule inhibiting the Dynamin 2 enzymatic activity or function.

[0036] As used herein, the term "small molecule inhibiting Dynamin 2 activity, expression or function" refers to small molecule that can be an organic or inorganic compound, usually less than 1000 daltons, with the ability to inhibit or reduce the activity, expression or function of Dynamin 2. This small molecule can be derived from any known organism (including, but not limited to, animals, plants, bacteria, fungi and viruses) or from a library of synthetic molecules. Small molecules inhibiting Dynamin 2 activity, expression or function can be identified with the method described in this document.

[0037] Dynamin inhibitors are described in Harper CB et al., Trends Cell Biol. 2013 Feb;23(2):90-101. Review. The small molecule according to the invention is selected from the group consisting of:

- Dynasore (a non-competitive, cell-permeable semicarbazone compound inhibitor of Dynamin 1 and Dynamin 2. - N° CAS 304448-55-3), its chemical name is 3-Hydroxynaphthalene-2-carboxylic acid (3,4-dihydroxybenzylidene)hydrazide,
- Hydroxy-Dynasore (a highly potent inhibitor of dynamin 2 (IC₅₀ = 2.6 μM)) (Hydroxy-Dynasore is a cell-permeable hydroxylated analog of Dynamin Inhibitor, Dynasore - N° CAS 1256493-34-1), its chemical name is 3-Hydroxy-N'-[(2,4,5-trihydroxyphenyl)methylidene]naphthalene-2-carbohydrazide,
- Tetradecyltrimethylammonium bromide (N° CAS 1119-97-7), sold under the name MiTMAB™ (ab120466) by Abcam (a Cell permeable dynamin 1 and dynamin 2 inhibitor (IC₅₀ = 8.4 μM for inhibition of dynamin II). It targets the pleckstrin homology (PH) (lipid binding) domain. It inhibits receptor-mediated and synaptic vesicle endocytosis (IC₅₀ values 2.2 μM),
- Phthalodyn-23 (a cell-permeable phthalimide compound that is reported to inhibit Dynamin 2 GTPase activity (IC₅₀ = 63 μM)), the chemical name of Phthalodyn-23 is 4-Chloro-2-((2-(3-nitrophenyl)-1,3-dioxo-2,3-dihydro-1H-isoindole-5-carbonyl)-amino)-benzoic acid,
- Dynole 34-2, it is a Dynamin inhibitor V (scbt.com) and acts on GTPase activity, non-competitive for GTP, chemical name of Dynole 34-2 is 2-Cyano-N-octyl-3-[1-(3-

- dimethylaminopropyl)-1*H*-indol-3-yl]acrylamide,
- M-divi 1 (mitochondrial division inhibitor, IC₅₀ = 10μM) (scbt.com), the chemical name of M-divi-1 is 3-(2,4-Dichloro-5-methoxyphenyl)-2-sulfanylquinazolin-4(3H)-one,
 - Iminodyn-22/17 (scbt.com) (Iminodyn 22 : IC₅₀ = 390nM acting on a GTPase allosteric site and displays uncompetitive antagonism with respect to GTP), the chemical name of Iminodyn 22 is *N,N'*-(Propane-1,3-diyl)bis(7,8-dihydroxy-2-imino-2*H*-chromene-3-carboxamide), the chemical name of Iminodyn 17 is *N,N'*-(Ethane-1,2-diyl)bis(7,8-dihydroxy-2-imino-2*H*-chromene-3-carboxamide).
 - OcTMAB, i.e., OctadecylTriMethylAmmonium Bromide, (abcam.com), it targets the PH domain,
 - Dynamin inhibitory peptide (Tocris Biosciences 1774): with aminoacid sequence: SEQ ID No 28: QVPSRPNRAP,
 - Dyngo-4a (IC₅₀ -2.5μM), it acts on a GTPase allosteric site, chemical name of Dyngo-4a is 3-Hydroxy-*N'*-[(2,4,5-trihydroxyphenyl)methylidene]naphthalene-2-carbohydrazide,
 - RTIL-13 (IC₅₀ -2.3μM), it is a norcantharidin scaffold targeting the PH domain, chemical name of RTIL-13 is 4-(*N,N*-Dimethyl-*N*-octadecyl-*N*-ethyl)-4-aza-10-oxatricyclo-[5.2.1]decane-3,5-dione bromide.

Uses of Dynamamin 2 inhibitors

[0038] The invention relates a Dynamamin 2 inhibitor as described herein for use in the treatment of DMD.

[0039] Moreover, the present invention relates to a pharmaceutical composition comprising said Dynamamin 2 inhibitor, and a pharmaceutically acceptable carrier, for use in the treatment of DMD.

[0040] In a particular embodiment of the invention, Duchenne's Muscular Dystrophy (DMD) can be treated by increasing muscle force of DMD patients and/or by improving the resistance of muscles to contraction induced injury.

[0041] As used herein, the term "therapeutically effective amount" is intended an amount of therapeutic agent, administered to a patient that is sufficient to constitute a treatment of a DMD. In a particular embodiment, the therapeutically effective amount to be administered is an amount sufficient to reduce the Dynamamin 2 expression, activity or function in a level equal or less than the normal level. The normal level is the Dynamamin 2 expression, activity or function of subjects that do not present DMD. The amount of Dynamamin 2 inhibitor to be administered can be determined by standard procedure well known by those of ordinary skill in the art. Physiological data of the patient (e.g. age, size, and weight), the routes of administration and the disease to be treated have to be taken into account to determine the appropriate dosage, optionally compared with subjects that do not present DMD. One skilled in the art will recognize

that the amount of Dynamin 2 inhibitor or of a vector containing or expressing the nucleic acid interfering specifically with Dynamin 2 expression to be administered will be an amount that is sufficient to induce amelioration of unwanted DMD symptoms or to induce alleviation of one or more symptoms or characteristics of DMD. An alleviation of one or more symptoms or characteristics may be assessed by any of the following assays on a myogenic cell or muscle cell from a patient: reduced calcium uptake by muscle cells, decreased collagen synthesis, altered morphology, altered lipid biosynthesis, decreased oxidative stress, and/or improved muscle fiber function, integrity, and/or survival. These parameters are usually assessed using immunofluorescence and/or histochemical analyses of cross sections of muscle biopsies. An alleviation of one or more symptoms or characteristics may also be assessed by any of the following assays on the patient self: prolongation of time to loss of walking, improvement of muscle strength, improvement of the ability to lift weight, improvement of the time taken to rise from the floor, improvement in the nine-meter walking time or 6 minute walk, improvement in the time taken for four-stairs climbing, improvement of the leg function grade, improvement of the pulmonary function, improvement of cardiac function, improvement of the quality of life. Each of these assays is known to the skilled person. For each of these assays, as soon as a detectable improvement or prolongation of a parameter measured in an assay has been found, it will preferably mean that one or more symptoms of Duchenne Muscular Dystrophy has been alleviated in an individual using the method of the invention. Detectable improvement or prolongation is preferably a statistically significant improvement or prolongation as described in Hodgetts et al (Hodgetts S., et al, (2006), *Neuromuscular Disorders*, 16: 591-602.2006). Alternatively, the alleviation of one or more symptom(s) of Duchenne Muscular Dystrophy may be assessed by measuring an improvement of a muscle fiber function, integrity and/or survival as later defined herein. The improvement of muscle fiber function, integrity and/or survival may be assessed using at least one of the following assays: a detectable decrease of creatine kinase in blood, a detectable decrease of necrosis of muscle fibers in a biopsy cross-section of a muscle suspected to be dystrophic, and/or a detectable increase of the homogeneity of the diameter of muscle fibers in a biopsy cross-section of a muscle suspected to be dystrophic. Each of these assays is known to the skilled person. The amount of Dynamin 2 inhibitor or of a vector containing or expressing the nucleic acid interfering specifically with Dynamin 2 expression may vary inter alia depending on such factors as the type of selected dynamin 2 inhibitors, the gender, age, weight, overall physical condition of the patient, etc. and may be determined on a case by case basis. The amount may also vary according to other components of a treatment protocol (e.g. administration of other medicaments, etc.). Generally, when the Dynamin 2 inhibitor is a nucleic acid, a suitable dose is in the range of from about 1 mg/kg to about 100 mg/kg, and more usually from about 2 mg/kg/day to about 10 mg/kg. If a viral-based delivery of the nucleic acid is chosen, suitable doses will depend on different factors such as the virus that is employed, the route of delivery (intramuscular, intravenous, intra-arterial or other), but may typically range from 10^{-9} to 10^{-15} viral particles/kg. If the inhibitor is a small molecule inhibiting the Dynamin 2 activity, expression or function, each unit dosage may contain, for example, from 2 to 300 mg/kg of body weight, particularly from 5 to 100 mg/kg of body weight. If the inhibitor is an antibody, each unit dosage may contain, for example, from 0.1 to 20 mg/kg of body weight, particularly from 4 to 10 mg/kg of body weight. Those of skill in the art will recognize that such parameters are normally worked out during

clinical trials. Further, those of skill in the art will recognize that, while disease symptoms may be completely alleviated by the treatments described herein, this need not be the case. Even a partial or intermittent relief of symptoms may be of great benefit to the recipient. In addition, treatment of the patient may be a single event, or the patient is administered with the Dynamin 2 inhibitor on multiple occasions, that may be, depending on the results obtained, several days apart, several weeks apart, or several months apart, or even several years apart. A treatment in a method according to the invention may have a duration of at least one week, at least one month, at least several months, at least one year, at least 2, 3, 4, 5, 6 years or more. The frequency of administration may be ranged between at least once in a two weeks, or three weeks or four weeks or five weeks or a longer time period.

[0042] Each Dynamin 2 inhibitor as defined herein for use according to the invention may be suitable for direct administration to a cell, tissue and/or an organ *in vivo* of individuals affected by or at risk of developing DMD and may be administered directly *in vivo*, *ex vivo* or *in vitro*. An oligonucleotide as used herein may be directly or indirectly administered to a cell, tissue and/or an organ *in vivo* of an individual affected by or at risk of developing DMD, and may be administered directly or indirectly *in vivo*, *ex vivo* or *in vitro*. As Duchenne muscular dystrophy has a pronounced phenotype in muscle cells, it is preferred that said cells are muscle cells, it is further preferred that said tissue is a muscular tissue and/or it is further preferred that said organ comprises or consists of a muscular tissue. A preferred organ is the heart. Preferably said cells are cells of an individual suffering from DMD.

[0043] A Dynamin 2 inhibitor as defined herein can be delivered as is to a cell. When administering said inhibitor to an individual, it is preferred that it is dissolved in a solution that is compatible with the delivery method. For intravenous, subcutaneous, intramuscular, intrathecal and/or intraventricular administration, it is preferred that the solution is a physiological salt solution. Particularly preferred for a method of the invention is the use of an excipient that will further enhance delivery of said inhibitor as defined herein, to a cell and into a cell, preferably a muscle cell.

[0044] The pharmaceutical composition of the invention is formulated in accordance with standard pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, Lippincott Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York) known by a person skilled in the art. Preferably, Dynamin 2 inhibitor as defined herein is dissolved in a solution that is compatible with the delivery method. For intravenous, subcutaneous, intramuscular, intrathecal and/or intraventricular administration, it is preferred that the solution is a physiological salt solution.

More generally, possible pharmaceutical compositions include those suitable for oral, rectal, mucosal, topical (including transdermal, buccal and sublingual), or parenteral (including subcutaneous, intramuscular, intravenous, intra-arterial and intradermal) administration. For these formulations, conventional excipient can be used according to techniques well known by those skilled in the art.

More particularly, in order to provide a localized therapeutic effect, specific muscular

administration routes are preferred. In particular, intramuscular administration is preferred.

[0045] Pharmaceutical compositions according to the invention may be formulated to release the active drug substantially immediately upon administration or at any predetermined time or time period after administration.

[0046] Within the context of the invention, the term treatment denotes curative, symptomatic, and preventive treatment. As used herein, the term "treatment" of a disease refers to any act intended to extend life span of subjects (or patients) such as therapy and retardation of the disease progression. The treatment can be designed to eradicate the disease, to stop the progression of the disease, and/or to promote the regression of the disease. The term "treatment" of a disease also refers to any act intended to decrease the symptoms associated with the disease, such as hypotonia and muscle weakness. Prolongation of time to loss of walking, improvement of muscle strength, improvement of the ability to lift weight, improvement of the time taken to rise from the floor, improvement in the 6 minute walk or nine-meter walking time, improvement in the time taken for four-stairs climbing, improvement of the leg function grade, improvement of the pulmonary function, improvement of cardiac function, or improvement of the quality of life of subjects (or patients) are also within the definition of the term "treatment". More specifically, the treatment according to the invention is intended to delay the appearance of the DMD phenotypes or symptoms, ameliorate the motor and/or muscular behavior and/or lifespan, in particular by improving muscle force and/or resistance to contraction-induced muscle injury.

[0047] The subject (or patient) to treat is any mammal, preferably a human being. Preferably the subject is a human patient, whatever its age or sex. New-borns, infants, children are included as well. More preferably, the patient or subject according to the invention is a Duchenne patient or is suspected to be a Duchenne patient (a patient susceptible to develop DMD because of his or her genetic background).

Screening of Dynamin 2 inhibitors

[0048] The present invention also concerns a method for identifying or screening molecules useful in the treatment of DMD, based on the ability of such molecules to inhibit the expression, activity and/or function of Dynamin 2.

[0049] Said method for screening comprises the steps of:

1. a) providing or obtaining a candidate compound; and
2. b) determining whether said candidate compound inhibits the activity, function and/or expression of Dynamin 2,
3. c) wherein the ability of said candidate compound to inhibit the expression, function or activity of said Dynamin 2 candidate compound is indicative of the usefulness of said candidate compound for the treatment of DMD.

[0050] The candidate compound to be tested in the frame of this method may be of any molecular nature, for example it may correspond to a chemical molecule (preferably a small molecule), an antibody, a peptide, a polypeptide, an aptamer, a siRNA, a shRNA, a snRNA, a sense or antisense oligonucleotide, a ribozyme, or a targeted endonuclease.

The ability of said candidate compound to inhibit the expression, activity or function of Dynamin 2 may be tested using any of the methods known to those skilled in the art, such as those identified above or described in the examples.

The method for screening or identifying a molecule suitable for the treatment of DMD can optionally further comprise the step of administering *in vivo* or *in vitro* selected molecule in DMD non-human animal model or a part thereof (tissue or cells, such as muscle tissue or cells) and analyzing the effect on the myopathy onset or progression.

As DMD non-human animal models, one can cite the dystrophin-deficient mdx mice. The dystrophin-deficient mdx mice is a classic model used for medical research into Duchenne's muscular dystrophy, which was first discovered 30 years ago (Bulfield, G., W. G. Siller, P. A. Wight and K. J. Moore (1984). "X chromosome-linked muscular dystrophy (mdx) in the mouse." Proc Natl Acad Sci U S A 81(4): 1189-1192.). Mdx mice have a spontaneous mutation resulting in a premature stop codon in exon 23 of the dystrophin gene, and total loss of protein expression. The mdx mouse model has been extensively used for research into the pathophysiology of DMD, and in testing potential therapies in preclinical approaches (Manning, J. and D. O'Malley (2015). "What has the mdx mouse model of duchenne muscular dystrophy contributed to our understanding of this disease?" J Muscle Res Cell Motil.). This model mice was used in the following examples.

[0051] The following examples are given for purposes of illustration.

EXAMPLES

Example 1

Materials:

[0052] Animal experiments. Animals were housed in a temperature-controlled room (19-22°C) with a 12:12-hour light / dark cycle. When required mice were humanely sacrificed by CO₂ inhalation followed by cervical dislocation, according to national and European legislations on animal experimentation, and the tibialis anterior muscles were dissected and frozen in nitrogen-cooled isopentane and liquid nitrogen. Animal experimentation was approved by the institutional ethical committee Com'Eth IGBMC-ICS, and the French Ministry (approval number N°01594.01).

[0053] Mice breedings: Mdx mice, a spontaneously occurring mouse model for DMD which does not express dystrophin and present a mild dystrophic phenotype, were obtained by breeding males Homozygous for *Dmd^{mdx}* and females Homozygous for *Dmd^{mdx}* from Charles River Laboratories. Mdx mice were bred with *Dnm2^{+/-}* mice to genetically downregulate DNM2. *Dnm2^{+/-}* mice were generated and characterized by the host laboratory, as described previously (Cowling, B. S., T. Chevremont, I. Prokic, C. Kretz, A. Ferry, C. Coirault, O. Koutsopoulos, V. Laugel, N. B. Romero and J. Laporte (2014). "Reducing dynamin 2 expression rescues X-linked centronuclear myopathy." J Clin Invest 124(3): 1350-1363).

[0054] In situ muscle force: Tibialis anterior maximal muscle force was measured in situ in *mdx^{-ly}* and *mdx^{-ly} Dnm2^{+/-}* mice at 6 weeks, 3 months, and at one year of age, using the Whole Mouse Test System (Aurora 1300A). The sciatic nerve was stimulated, and the total force produced by contraction of the tibialis anterior muscle was measured, as described previously (Cowling et al. 2014). Specific muscle force was calculated as a ratio of maximal muscle force relative to muscle mass.

[0055] Contraction-induced muscle injury: Susceptibility to contraction-induced muscle injury was measured using the Whole Mouse Test System (Aurora 1300A) at one year of age, as described previously (Hourde et al. 2013). The decrease in absolute muscle force resulting from several damage-inducing lengthening contractions was measured. Initially the sciatic nerve was stimulated (700 milliseconds, frequency 150 Hz), and the isometric muscle force produced was measured, followed by 8 lengthening (+10% length) contractions, in which isometric muscle force was measured (at the initial length) after each contraction. Force is represented at a percentage of initial force produced. Data is represented as the percentage of initial force, after 2, 5, and 8 lengthening contractions. After contractile measurements, the animals were euthanized with an overdose of pentobarbital, and tibialis anterior muscles were weighed.

Results:

[0056] Physiological improvement in muscle function was tested by measurement of specific muscle force of the tibialis anterior muscle, in situ. When the sciatic nerve was stimulated, absolute muscle force was measured. Specific muscle force was then calculated as a ratio of total muscle force compared to muscle fiber mass. In *mdx^{-ly}* mice with reduced DNM2 expression (*mdx^{-ly}Dnm2^{+/-}*), specific muscle force was increased at 6 weeks and 3 months of age, compared to control *mdx^{-ly}* mice (Figure 1).

[0057] In order to confirm the physiological significance of this improvement in muscle force. Similar to patients with Duchenne muscular dystrophy (DMD), skeletal muscles from *mdx^{-ly}* mice lack dystrophin, and are known to be more susceptible to contraction-induced injury than

healthy controls. Mice were therefore submitted to lengthening muscle contractions which induces damage to muscle fibers, to analyze the susceptibility of mdx dystrophic muscles to damage, when DNM2 expression is reduced.

[0058] 8 lengthening contractions, inducing muscle fiber damage, were performed, and maximum muscle force was measured after each contraction. Importantly *mdx^{-ly} Dnm2^{+/-}* mice exhibited a 2-fold increase in resistance to contraction-induced injury, compared to *mdx^{-ly}* mice (figure 2). Therefore reducing DNM2 improves the resistance of mdx muscles to contraction induced injury.

Conclusions:

[0059] Reducing DNM2 expression has thus therapeutic potential in Duchenne's Muscular Dystrophy. It was indeed shown a significant improvement in specific muscle force at several ages by reducing DNM2 in mdx mice, which are the classic mouse model used for preclinical DMD research. Furthermore, a two-fold improvement is seen in resistance to contraction-induced muscle injury, confirming the physiological significance of this finding. Therefore, reducing DNM2 is a novel therapeutic target for Duchenne's muscular dystrophy.

Example 2

Materials and Methods:

Production and Purification of AAV:

[0060] AAV2/9 vectors were generated by a triple transfection of AAV-293 cell line with pAAV2 insert containing the insert under the control of the CMV promoter and flanked by serotype 2 inverted terminal repeats, pXR1 containing rep and cap genes of AAV serotype 9, and pHelper encoding the adenovirus helper functions. Cell lysates were subjected to 3 freeze/thaw cycles, then treated with 50 U/mL of Benzonase (Sigma) for 30 minutes at 37°C, and clarified by centrifugation. Viral vectors were purified by iodixanol gradient ultracentrifugation followed by dialysis and concentration against Dulbecco's Phosphate Buffered Saline using centrifugal filters (Amicon Ultra-15 Centrifugal Filter Devices 30K, Millipore, Bedford). Physical particles were quantified by real-time PCR using a plasmid standard pAAV-eGFP, and titers are expressed as viral genomes per milliliter (vg/mL). rAAV titers used in these experiments were 5 to 7 10¹¹ vg/mL.

AAV Transduction of mdx-ly Tibialis Anterior (T.A) Muscles of Mice:

[0061] 3-week-old, male, mdx-/y mice were anesthetized by i.p. injection of 5µl/g of ketamine (20 mg/mL; Virbac, Carros, France) and xylazine (0.4%, Rompun; Bayer, Wuppertal, Germany). An intramuscular injection of AAV2/9-shRNA targeting DNM2 (black) or a AAV2/9 scrambled control sequence (white) was performed into the tibialis anterior of mdx-/y mice at 3 weeks of age. Animals were housed in a temperature-controlled room (19°C to 22°C) with a 12:12-hour light/dark cycle.

[0062] Mice breedings were as described in example 1.

Results:

[0063] Muscle force following contraction-induced muscle injury, in mdx mice with reduced DNM2.

[0064] Mdx-/y and mdx-/yDnm2+/- mice were analyzed for muscle force. Specific muscle force (sPo) was initially measured in the tibialis anterior muscle from mdx-/y (white) and mdx-/yDnm2+/- (black) mice (see figure 3A). Following this the tibialis anterior muscle was subjected to 9 lengthening contractions. Results for mdx-/y (solid black line) and mdx-/yDnm2+/- (dashed black line) mice are displayed as a percentage of initial force (%), after 0, 3, 6 and 9 lengthening contractions (see figure 3B).

Mdx-/y mice at 3 weeks of age were injected with AAV2/9-shRNA targeting DNM2 (black) or an AAV2/9 scrambled control sequence (white) into the tibialis anterior muscles. Specific muscle force (sPo) was measured in the tibialis anterior muscle at 3 months of age (see figure 3C). The tibialis anterior muscle was then subjected to 9 lengthening contractions as in figure 3B (see figure 3D), AAV shRNA control (solid black line) and AAV shRNA DNM2 (dashed black line).

All mice were 3 months old at age of analysis. n=7-10 mice/group. All graphs represent mean +s.e.m.

Both figures 3B and 3D mdx-/y mice with reduced DNM2 (dashed black line) display a trend towards resistance to contraction-induced injury.

Conclusions:

[0065] Mdx mice were crossed with *Dnm2*+/- mice or subjected to intramuscular injection of AAV expressing shRNA targeting *Dnm2* to reduce the level of DNM2. At 3 months of age, while there was no significant difference in specific muscle force between the groups, a trend for increased resistance to contraction-induced injury was observed for both techniques.

SEQUENCE LISTING

[0066]

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<120> Dynamin 2 inhibitor for the treatment of Duchenne\222s muscular dystrophy

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<170> PatentIn version 3.5

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Patentkrav

1. Inhibitor af Dynamin 2 til anvendelse i behandlingen af Duchennes muskeldystrofi, valgt fra gruppen bestående af:

- et antistof rettet mod Dynamin 2,
- 5 - et nukleinsyremolekyle der interfererer specifikt med Dynamin 2-ekspression,
- et nukleinsyre eller en nuklease manipuleret til at ramme DNM2-genet og til at levere nukleaser under anvendelse af genom-redigerende terapi, og
- et lille molekyle der hæmmer Dynamin 2-aktiviteten, -ekspressionen eller
- 10 -funktionen, hvor nævnte lille molekyle er valgt fra gruppen bestående af 3-Hydroxynaphthalen-2-carboxylsyre (3,4-dihydroxybenzyliden)hydrazid, 3-Hydroxy-N'-[(2,4,5-trihydroxyphenyl)methyliden]naphthalen-2-carbohydrazid, Tetradecyltrimethylammoniumbromid, 4-Chlor-2-((2-(3-nitrophenyl)-1,3-dioxo-2,3-dihydro-1H-isoindol-5-carbonyl)-amino)-
- 15 benzoinsyre, 2-Cyano-N-octyl-3-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]acrylamid, 3-(2,4-Dichlor-5-methoxyphenyl)-2-sulfanylquinazolin-4(3H)-on, N,N'-(Propan-1,3-diyl)bis(7,8-dihydroxy-2-imino-2H-chromen-3-carboxamid), N,N'-(Ethan-1,2-diyl)bis(7,8-dihydroxy-2-imino-2H-chromen-3-carboxamid), OctadecylTriMethylAmmoniumBromid, det Dynamin-
- 20 hæmmende peptid af aminosyresekvens SEQ ID NO: 28, 3-Hydroxy-N'-[(2,4,5-trihydroxyphenyl)methyliden]naphthalen-2-carbohydrazid, og 4-(N,N-Dimethyl-N-octadecyl-N-ethyl)-4-aza-10-oxatricyclo-[5.2.1]decan-3,5-dionbromid.

25 **2.** Inhibitoren af Dynamin 2 til anvendelse ifølge krav 1, hvor Dynamin 2-inhibitoren er valgt fra gruppen bestående af et antistof rettet mod Dynamin 2, eller et nukleinsyremolekyle der interfererer specifikt med Dynamin 2-ekspression.

30 **3.** Inhibitoren af Dynamin 2 til anvendelse ifølge krav 1 eller 2, hvor Dynamin 2-inhibitoren er et RNAi, en antisense-nukleinsyre eller et ribozym der interfererer specifikt med Dynamin 2-ekspression, fortrinsvis Dynamin 2-inhibitoren er et siRNA eller shRNA.

- 4.** Inhibitoren af Dynamin 2 til anvendelse ifølge et hvilket som helst af kravene 1-3, hvor Dynamin 2-inhibitoren er et antisense-nukleotid, som inducerer exon-skipning i et Dynamin 2 præ-mRNA.
- 5 **5.** Inhibitoren af Dynamin 2 til anvendelse ifølge krav 4, hvor antisense-nukleotidet er designet til specifikt at inducere DNM2-exon 2- eller exon 8-skipning, og fortrinsvis omfatter eller består af en af de følgende sekvenser:
- U7-Ex2 (målskipning af DNM2 exon 2), omfattende den følgende sekvens:
SEQ ID No 26: GTCACCCGGAGGCCTCTCATTCTGCAGCTC
- 10 U7-Ex8 (målskipning af DNM2 exon 8), omfattende den følgende sekvens:
SEQ ID No 27: ACACACTAGAGTTGTCTGGTGGAGCCCGCATCA.
- 6.** Inhibitoren af Dynamin 2 til anvendelse ifølge et hvilket som helst af kravene 1-5, hvor Dynamin 2-inhibitoren er et nukleinsyremolekyle der specifikt
- 15 interfererer med Dynamin 2 omfatter eller består af en sekvens valgt fra gruppen bestående af SEQ ID 2-25.
- 7.** Inhibitoren af Dynamin 2 til anvendelse ifølge et hvilket som helst af kravene 1-6, hvor Dynamin 2-inhibitoren er et DNA, mRNA eller en nuklease manipuleret
- 20 til at ramme DNM2-genet og til at levere nukleaser under anvendelse af genom-redigerende terapi.
- 8.** Inhibitoren af Dynamin 2 til anvendelse ifølge et hvilket som helst af kravene 1-7, hvor inhibitoren er administreret i en mængde tilstrækkelig til at reducere
- 25 Dynamin 2-ekspressionen eller Dynamin 2-aktiviteten, -ekspressionen eller -funktionen i et niveau lig med eller fortrinsvis mindre end det normale niveau.
- 9.** Fremgangsmåde til at identificere eller screene molekyler der hæmmer
- 30 Dynamin 2 anvendelig i behandlingen af Duchennes muskeldystrofi, omfattende trinnene:
- a. at tilvejebringe eller opnå en kandidatforbindelse; og

b. at bestemme hvorvidt nævnte kandidatforbindelse hæmmer aktiviteten, funktionen og/eller ekspressionen af Dynamamin 2,

c. hvor evnen af nævnte kandidatforbindelse til at hæmme ekspressionen, funktionen eller aktiviteten af nævnte Dynamamin 2 indikerer

5 anvendeligheden af nævnte kandidatforbindelse i behandlingen af Duchennes muskeldystrofi.

10. Farmaceutisk sammensætning omfattende en inhibitor af Dynamamin 2 som defineret i et hvilket som helst af kravene 1 til 8 og en farmaceutisk acceptabel
10 bærer/excipients til anvendelse i behandlingen af Duchennes muskeldystrofi.

DRAWINGS

Specific muscle force

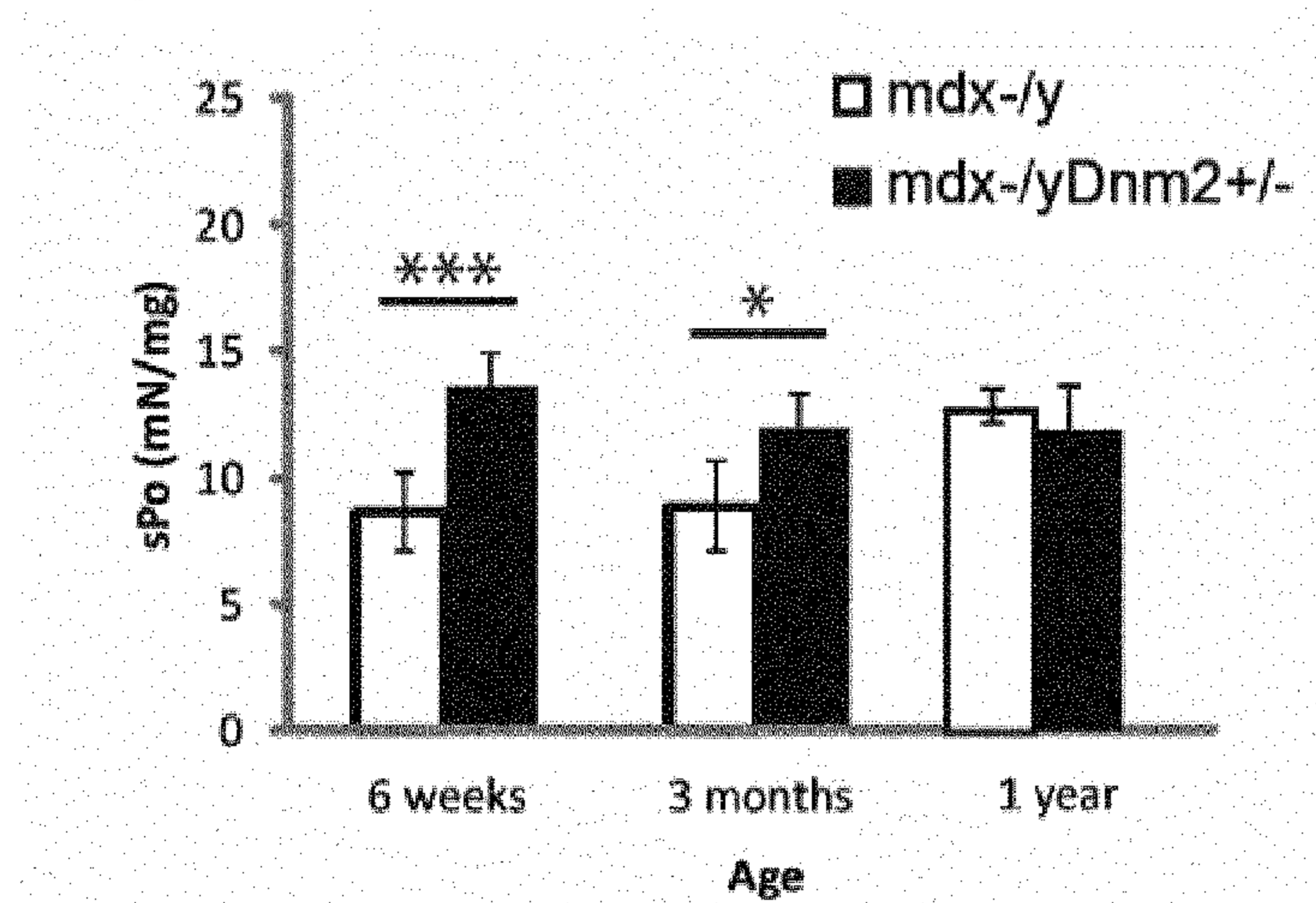


Figure 1

Contraction induced muscle injury

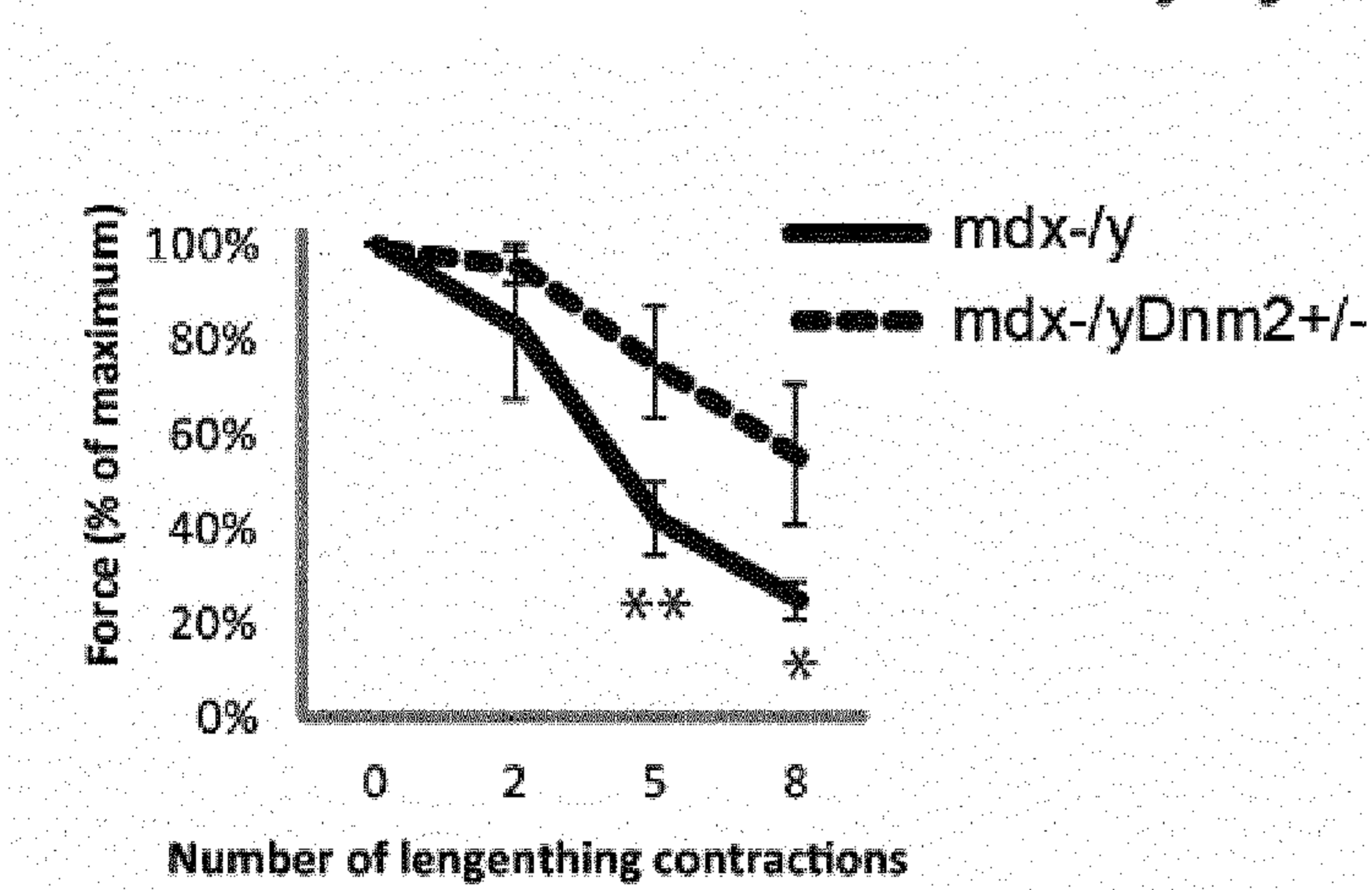


Figure 2

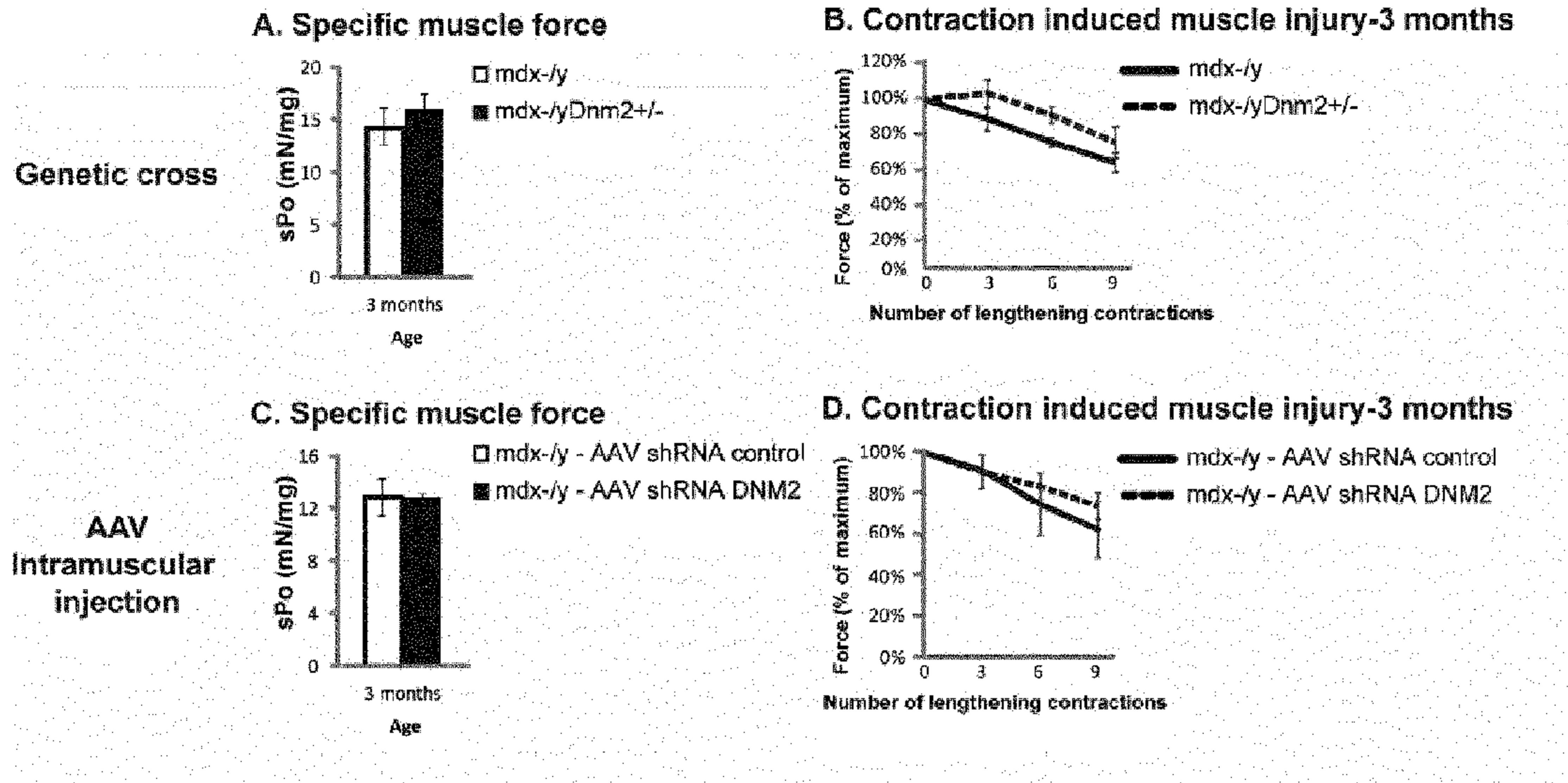


Figure 3