Dose-dependent microdystrophin expression enhancement in cardiac muscle by a cardiac specific regulatory element

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease that affects 1:5000 live male births and is characterised by muscle wasting. By the age of 13 years, affected individuals are often wheelchair bound and suffer from respiratory and cardiac failure which results in premature death. Although the administration of corticosteroids and ventilation can relieve the symptoms and extend the patients' lifespan, currently no cure exists for DMD. Among the different approaches under pre-clinical and clinical testing, gene therapy using Adeno-Associated Viral (AAV) vectors is one of the most promising. In this study, we delivered intravenously AAV9 vectors expressing the microdystrophin MD1 (ΔR4-R23/ΔCT) under control of the synthetic muscle specific promoter Spc5-12 and assessed the effect of adding a cardiac-specific cis-regulatory module (designated as CS-CRM4) on its expression profile in skeletal and cardiac muscles. Results show that Spc5-12 promoter, in combination with an AAV serotype that has high tropism for the heart, drives high MD1 expression levels in cardiac muscle in *mdx* mice. The additional regulatory element CS-CRM4 can further improve MD1 expression in cardiac muscles but its effect is dose-dependent and enhancement becomes evident only at lower vector doses.

Introduction

Duchenne muscular dystrophy (DMD), the most common inherited lethal childhood disease, is a severe muscle-wasting disorder caused by gene mutations in the *DMD* gene leading to premature protein truncation and loss of functional dystrophin protein from skeletal and cardiac muscle [1, 2]. The lack of dystrophin results in a progressive muscle wasting, which ultimately leads to loss of independent ambulation and need of a wheelchair often by the age of 13 years[3]. Patients affected by DMD usually die in their mid-thirties and forties due to respiratory failure and cardiomyopathy [4]. The gene addition strategy based on delivering Adeno-Associated Viral (AAV) vectors carrying recombinant cDNA-based micro or mini-dystrophins into diseased tissues is a promising approach for the treatment of DMD. However, AAV vectors have a packaging size limitation, due to the viral capsid, of about 4.7Kb. Therefore, many forms of truncated dystrophins have been produced and tested for their efficacy in various models of dystrophin-deficiency[5]. Over the last few years we have been working to optimize the delivery and expression of a microdystrophin called MD1[6]. This microdystrophin, that is sequence optimized and expressed by a synthetic muscle specific promoter called Spc5-12[7], leads to normalisation of muscle mass, specific force and resistance to exercise induced damage in *mdx* mice[8, 9]. Furthermore, whole body systemic delivery of AAV2/8-Spc5-12-MD1 in canine (GRMD) model of DMD has recently showed to greatly reduce the dystrophic pathology [10] in skeletal muscles.

Obtaining high levels of dystrophin expression is crucial to provide a functional effect to the treated muscles and translate the experimental approach to a clinical benefit [11]. However there is a number of factors that limits the amount of AAV vector injectable in vivo, such as manufacturing constraints and the risk of possible AAV-specific immune responses[12-14]. A promising strategy to increase the transgene expression and therefore reduce the amount of vector to be delivered is optimizing the regulatory elements driving expression of the protein. In the case of DMD, the cardiac muscle is a key target for a gene therapy treatment as cardiac failure is among the main causes of death[15]. Transgene expression in cardiac muscle can be increased by using strong tissue-specific promoters and by including cardiac specific enhancer sequences[16] such as CS-CRM4. Spc5-12, a short (334bp) synthetic

promoter that allows high protein expression in skeletal and cardiac muscles, is made by random assembly of a combination of the myogenic regulatory elements E-box, MEF-2, TEF-1, and SRE[7]. Here we combined the CS-CRM4 enhancer module with the Spc5-12 promoter to drive MD1 expression after systemic delivery in both young and aged *mdx* mice. We show that the effect of the CS-CRM4 element of transgene expression is vector-dose dependent and that an optimal vector dose could be identified (5E+11 vg/mouse) whereby the addition of the CS-CRM4 enhancer significantly increased MD1 expression in cardiac muscles. In contrast, at higher dose of AAV9-Spc5-12-MD1 (1E+12 vg/mouse) Spc5-12 already induces a significant MD1 overexpression in cardiac muscle that cannot be further improved by including the CS-CRM4 enhancer. At very low vector doses (1E+11 vg/mouse), no MD1 is detected in cardiac muscle, regardless of the presence of the CS-CRM4 element. Our data provide novel insights into using cardiac specific regulatory elements for enhanced but controlled dystrophin expression in the heart.

Results

Addition of a CS-CRM4 (CS) enhancer to the muscle-specific promoter Spc5-12 increases microdystrophin expression in both cardiac and tibialis anterior muscles.

To test whether the addition of the cardiac-specific cis-regulatory module 4 (CS-CRM4, named "cs" here) to the promoter increased microdystrophin expression, AAV9-Spc5-12-MD1 and AAV9-csSpc5-12-MD1 constructs where generated and intravenously injected in 8-week old male *mdx* mice at a dose of 5E+11vg/mouse. Heart (H), Diaphragm (DIA) and Tibialis anterior (TA) were collected 12 weeks later. At this dose the cardiac-specific enhancer significantly increased dystrophin expression in the Heart; immunofluorescence for dystrophin and laminin showed homogenous, intense staining after treatment with CS-CRM4 enhanced AAV-csSpc5-12-MD1 while a weaker staining was present in the cardiac muscles of mice treated with AAV-Spc5-12-MD1 (**Figure 1A** and **Supplementary figure 1A)**. Densitometric analysis of immunoblots detecting dystrophin expression confirmed this observation

(1211.0 ± 311.5 and 92.8 ± 72.3 units for cardiac muscles of AAV-csSpc5-12-MD1 and AAV-Spc5-12-MD1 treated mice respectively, unpaired t-test, p=0.006, n=6; **Figure 1B-C**). Immunostaining for dystrophin and laminin in TA muscles showed increased dystrophin expression in mice injected with AAV-csSpc5-12-MD1 compared to AAV-Spc5-12-MD1 injected mice (26.2 ± 2.9% compared to 8.9 ± 2.5% dystrophin positive fibres respectively, Bonferroni test, p<0.01, n=6) (**Figure 1D, E**). Quantification of dystrophin expression by immunoblot showed a non statistically significant trend towards an increase in dystrophin expression after inclusion of the enhancer (232.0 ± 104.1 and 23.4 ± 2.6 units for TA muscles of AAV-csSpc5-12-MD1 and AAV-Spc5-12-MD1 treated mice respectively, unpaired t-test, p=0.07, n=6; **Figure 1F-G**). DIA muscles showed a similar amount of patches of dystrophin positive fibres after injection with either vector. In these patches a similar percentage of dystrophin positive fibres were detected (11.8 ± 1.1% vs 7.6 ± 2.4 with and without the enhancer respectively, Bonferroni test, p>0.05, n=6; **Supplementary figure 1B-C**). *These data show that, in the absence of the CS-CRM4 element, cardiac and TA muscles express a substantial amount of microdystrophin. Furthermore, the data demonstrate that at this dose the inclusion of the CS-CRM4 element in the AAV vector significantly increases MD1 expression in cardiac and TA muscles.*

Addition of a CS-CRM4 (CS) enhancer to the muscle-specific promoter Spc5-12 does not increase microdystrophin expression in muscles when a dose of ~3E+13 vg/Kg is used.

To assess the effect of the CS enhancer on MD1 expression at higher and lower vector doses, vectors were intravenously injected in 8-week old male *mdx* mice at either 1E+12 vg (higher dose, HD, corresponding to ~3E+13 vg/Kg; n=6 mice) or 1E+11 vg (lower dose, LD, corresponding to ~3E+12 vg/Kg; n=6 mice). Age matched *mdx* and C57BL10 mice were injected with saline as controls. Twelve weeks later mouse forelimb strength was assessed using grip strength measurements. Subsequently, TA, DIA, H were harvested and MD1 expression in these muscles was analysed. Quantitative PCR performed on total DNA extracted from cardiac muscles confirmed that the same amount of vectors were initially injected in the HD-treated groups of mice (2577 ± 688.8 vg and 2698 ± 1106 vg

respectively) while a lower amount of vector was detected in LD-injected cohorts (156.6 ± 47.72 vg) (**Figure 2A**). Substantial expression of dystrophin was detected after injection of either vector at HD with most cardiomyocytes expressing dystrophin, although variability was present between representative cardiac sections from different mice (**Figure 2B** and **Supplementary Figure 2**). On the contrary, LD treated mice had almost no dystrophin expression similarly to saline treated *mdx* mice (**Figure 2B**). Dystrophin quantification by immunoblot analysis confirmed this finding and showed that MD1 is well expressed in hearts with (0.169 ± 0.060 intensity unit ratio) or without (0.189 ± 0.060 intensity unit ratio) the enhancer at the higher dose while very low expression level of the transgene was observed in LD (0.002 ± 0.001 intensity unit ratio) treated mice (**Figure 2C**). No statistically significant difference in dystrophin expression levels was observed with or without the enhancer in HD-injected mice and between either HD treated groups and WT mice (0.069 ± 0.003, p=0.672 and p=0.517 respectively for vectors with or without the enhancer, **Figure 2D**). To assess dystrophin expression in skeletal muscles, the percentage of dystrophin positive fibres was calculated (**Figure 3A-B**). No statistically significant difference in dystrophin expression was detected between muscles injected with either AAV-Spc5-12-MD1 (12.1 ± 2.6 % fibres) or AAV-csSpc5-12-MD1 (22.1 ± 7.0 % fibres). Injection of LD of vector showed barely detectable presence of dystrophin positive fibres (1.1 ± 0.3%). (**Figure 3B**). These results correlated with the grip strength analysis that showed similar increase of muscle strength in the groups of mice treated with the HD of vectors, with or without enhancer (**Figure 3C**). By immunoblot MD1 was barely detectable in most muscles suggesting that very little protein was expressed at these doses (**Supplementary Figure 3A**). However a small but significant increase was measured with the enhancer element (**Supplementary Figure 3B**). In DIA only few patches of dystrophin positive fibres were detected per section in these muscles (**Supplementary Figure 4A)**. In those patches 31 ± 12 % of fibres were positive for dystrophin in muscles treated with the AAV-Spc5-12-MD1 while 23.7 ± 5.0 % and 0.7 ± 0.2 % of dystrophin positive fibres were detected in similar patches of fibres of mice injected with AAV-csSpc5-12-MD1 at HD and LD respectively (**Supplementary Figure 4B**). No statistical difference was detected between the two HD treated

groups. *These data show that the injection of the higher dose of AAV-Spc5-12-MD1 (corresponding to 3E+13vg/kg) induces relatively robust MD1 protein expression in mdx mice. In cardiac muscle the amount of MD1 protein expressed with or without CS-CRM4 enhancer is not significantly different compared to the endogenously expressed dystrophin in wild type mice. Hence, at these high vector doses, the addition of the cardiac specific CS-CRM4 element did not further increase MD1 protein expression in either skeletal or cardiac muscles, suggesting a possible saturation effect.*

Discussion

New methodologies have to be developed to reduce the amount of the biological product to be administered in DMD patients to ease the economic and manufacture constraints, and reduce the risk of potential immune responses to the vector and/or transgene. Recently, it was shown that using the chimeric synthetic CS-CRM4/SPc5-12 promoter to deliver luciferase resulted in a nearly three-fold increase in cardiac gene expression compared to SPc5-12 alone while no change in protein expression was shown in skeletal muscle[16]. Increasing dystrophin expression in cardiac muscle is particularly crucial for DMD where cardiac failure is a leading cause of death. Since the aim was seeing an improvement with enhancer inclusion, we tested a range of doses of vector considered suboptimal in dystrophic *mdx* mice [17] [18] and in general lower than the doses used in the current DMD clinical trials (NCT03375164 and NCT03362502). The same dose tested in a canine GRMD model of DMD provided only a small functional effect in skeletal muscle[10] while the effect in the heart was not assessed as this canine model does not develop cardiomyopathy[10]. Systemic delivery of the LD of AAV-csSpc5-12-MD1 produced almost undetectable protein expression in both cardiac and skeletal muscles suggesting that this dose could be below the threshold necessary to achieve a detectable tissue transduction in *mdx* mice. On the other hand, the top dose, irrespective of the inclusion of the cardiac specific element, resulted in a significant amount of dystrophin protein expression in skeletal muscle and a strong expression of dystrophin in the heart at least at the same level of the normal protein expressed by wild type mice. The cardiac-specific effect of the CS-CRM4 regulatory element became evident when

an intermediate vector dose was injected that was sufficient to achieve a substantial protein expression in the heart but not enough to achieve 100% dystrophin restoration. This intermediate dose of the vector including the cardiac specific enhancer was indeed effective in enhancing dystrophin by ~10 times compared to the use of the Spc5-12 promoter without the CS-CRM4 element. The results indicate that the effect of CS-CRM4 on gene expression is dependent on the vector dose and suggest that there is an optimal dose window to enable its enhancing effect. Moreover, the impact of the CS-CRM4 element on gene expression levels may vary depending on the transgene, since gene-dependent post-transcriptional bottlenecks may ultimately determine the net increase in protein levels. The efficiency we observed in the heart is probably due to the combined use of a serotype with high tropism for the heart and the Spc5-12 promoter. The amount of MD1 detected in cardiac muscle using 3E+13 vg/kg of an AAV vector driven by Spc5-12 promoter confirmed previous observations obtained using either AAV8 or AAV9 with either MD1 or the Δ4173-microdystrophin[6, 19]. Here the combination of AAV9 and Spc5-12 promoter partially masked the efficiency of the enhancer in the heart. Indeed, we observed a comparable dystrophin expression in cardiac muscle of HD-treated mice and WT mice suggesting that when a maximal threshold is achieved, the presence of a cardiac specific enhancer may not be able to further enhance expression. In transgenic mice, where minidystrophin was expressed 50 or 100 times higher than the normal dystrophin amount in the heart[20], transgene overexpression resulted in accumulation of minidystrophin vesicles in the sarcoplasm accompanied by worsening of the pathology[20]. However this was likely due to the use of the murine α-myosin heavy chain (αMHC) promoter, that permits supraphysiological transgene expression[20, 21]. *With regard to MD1 expression in skeletal muscles, at the intermediate dose, inclusion of the CS-CRM4 enhancer slightly enhanced dystrophin expression in TA muscles. This increase is in the range previously observed when a reporter gene was driven by the CS-CRM4 enhancer paired with the SPC5-12 promoter*[16]. Notably the observation that the CS-CRM4 enhancer certainly does not decrease and in fact slightly increases expression of dystrophin protein in skeletal muscles is important for the translation of such regulatory element in clinical settings. Other more efficient skeletal muscle cis-regulatory modules

(SK-CRM) have been described that can be used to enhance expression of muscle-specific genes[22]. The inclusion of a 2R5S enhancer sequence, combined with either a highly truncated MCK promoter or with Spc5-12 promoter increased the expression of the minidysΔ3990 gene in skeletal muscles[19]. Our experiments represent a valuable starting point for further studies of MD1 expression in skeletal and cardiac muscles. In the future other enhancers should be tested in conjunction with Spc5-12 promoter: examples include the use of SK-CRMs with Spc5-12 promoter and MD1 transgene. Other options include testing the efficacy of the 2R5S enhancer on expressing micro-dystrophin MD1 in cardiac and skeletal muscles and its comparison with the one offered by CS-CRM4-Spc5-12 after systemic injection in *mdx* mice. Similarly, it would be interesting to assess the efficiency of MD1 expression using CS-CRM4 to control activity of the CK8 promoter, a regulatory sequence currently used in a clinical trial[18, 23].

Materials and methods

AAV preparation

AAV-Spc5-12-MD1 or AAV-csSpc5-12-MD1 were prepared using the standardized double transfection protocol[6, 24]. Briefly, HEK293T cells were seeded in roller bottles and cultured in DMEM at 37°C and 5% CO2. Once 50% confluent, cells were transfected using PEI with the relative pAAV plasmid and pAAV helper cap9 plasmids (pDP9rs). Cells were cultured in roller bottles for 3 more days. Afterwards cells were lysed and recombinant pseudotyped AAV vector particles were purified by iodixanol (Sigma-Aldrich) step-gradient ultracentrifugation. The copy number of vector genomes was quantified by quantitative polymerase chain reaction (qPCR).

In vivo experiments

Groups of 8-week old mdx male mice of similar body weights were randomly allocated to the different groups minimizing as much as possible the number of mice coming from the same litter . Mice were put in a heater and left for 10’ before being restrained and intravenously injected with the vector

diluted in 200 µl sterile saline. Soon after injections mice were put back in the cage. Two experiments were performed; In the first experiment 6, 8-week old mice were injected with5E+11 vg of either AAV-Spc5-12-MD1 or AAV-csSpc5-12-MD1. in the second experiment 6, 8-week old *mdx* male mice per group were injected with the same vectors at either 1E+12 vg (Higher dose, HD) or 1E+11 vg (Lower dose, LD) per mouse. In both experiments, 5-6 age matched *mdx* and C57BL10 male mice were injected with sterile saline as controls. 12 weeks post-injection, mice were sacrificed and Heart (H), Diaphragm (DIA) and Tibialis anterior (TA) muscles were harvested and weighed. All samples were embedded in optimal cutting temperature medium (VWR, Leicestershire, UK) and frozen in liquid nitrogen-cooled isopentane (Sigma, Dorset, UK) for cryosectioning. They were kept at -80°C until use. In vivo experiments were conducted under statutory Home Office recommendation; regulatory, ethics, and licensing procedures and the Animals (Scientific Procedures) Act 1986.

Dystrophin and laminin staining

After cryosectioning on an OTF 5000 cryostat (Bright, Huntingdon, UK), sections (10-µm thickness) were kept at -80°C until use. Frozen sections of TA and DIA were immuno-stained for dystrophin, laminin and 4′,6-diamidino-2-phenylindole (DAPI). Cryosections of H were immunostained for dystrophin and Laminin only. The MOM Immunodetection Kit (VECTOR Laboratories) was used following the standardized protocol. Briefly slides were air dried and fixed with acetone for 5 minutes. Sections were then incubated with MOM blocking solution for 1h at RT. Afterwards, the mouse anti-dystrophin (1:50, manex 1011c DHSB) and rabbit anti-Laminin (1:500, ab11575, Abcam) primary antibodies were incubated for 1 hour at RT diluted in MOM diluent solution. After washings, the secondary antibody for laminin (1:400, goat anti-Rabbit 488 Alexafluor) was added for 1h at RT. After washings the secondary antibody from MOM kit was added for 10 minutes at RT. After further washings slides were incubated for 5 minutes at RT with Streptavidin-568 (1:20, Alexafluor) in PBS (phosphate buffered saline). Sections of TA and DIA were lastly incubated with 1:1000 DAPI in PBS and mounted. All washings were performed with PBST (PBS with 1% Tween 20) three times for five minutes

each. Sections of immunostained tibialis anterior muscles were imaged using a Nikon Ni-E upright microscope. Five, 20X magnification images were randomly taken from a blinded observer and used to calculate the percentage of dystrophin positive fibres.

Western Blot

Proteins were extracted from approximately 75 tissue sections. One 3 mm tungsten carbide bead (QIAGEN) and RIPA (radio immuno precipitation assay) buffer (150 mM sodium chloride, HEPES 5 mM, NP-40 1%, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, EDTA 0,1 Mm, Protease inhibitor tablet (Roche)) was added to each tube (30 µl for TAs and 50 µl for hearts) that was subsequently processed on a TissueLyser II (QIAgen, Manchester, UK) at 25 Hz for 2 x 2 min. Samples were then centrifuged for 10 minutes at 13000g at 4 °C and the supernatant was collected in new pre-chilled tubes and stored at -20 °C. Proteins were quantified using DC Protein Assay (Bio-Rad, Hertfordshire, UK) using the manufacturer's instructions. Fifteen (TAs) or 5 (heart) µg of proteins were diluted in a final volume of 10 µl, denatured for 10 minutes at 100 °C and subsequently loaded in 3-8 % Tris-Acetate Pre-Cast gel (NuPage) alongside with HiMark Pre-Stained Protein Standard (Invitrogen). Proteins were transferred to nitrocellulose blotting membrane (GE Healthcare Life Science) for 2 hours at 30 V by using transfer buffer (NuPage) with 20% methanol and 1 ml antioxidant (NuPage). Blocking was performed with 5% milk in 0.2% PBST for 1 hour at RT. Overnight incubation at 4 °C with the different primary antibodies (1:50 mouse anti-dystrophin manex 1011c (DHSB) and 1:2500 Rabbit α-tubulin (Abcam ab4074)) in 5% milk in 0.2% PBST was performed. Alternatively, the kit “Revert 700 Total Protein Stain for Western Blot Normalization” (Li-cor) was used. Membranes were subsequently incubated with the secondary antibodies (1:5000, Goat anti-mouse 800 or Donkey anti-rabbit 680 (both from Li-cor) in 5% milk in 0.2% PBST for 1 hour at RT. The membranes were scanned using Odyssey CLx Imager (Li-cor) and analysed with Li-cor Image studio Lite.

AAV Genome Copy Number Quantification

The number of AAV viral genome copy number normalized by the total extracted DNA was calculated in cardiac muscles of treated mice. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (QIAgen, UK) and quantified with the ND-1000 NanoDrop spectrophotometer (Thermo Scientific, UK). AAV.Spc512.MD1 plasmid (1000 ng/µl) was diluted in 1:10 serial dilutions and used to generate the qPCR standard curve. Quantitative polymerase chain reaction (qPCR) assay was setup in a mix with water, SYBR Green mastermix (Roche) and the forward (CTACAGAACCGCCATGAAGC) and the reverse (ACGTTCACCAGGTTGTTGTG) primers targeting MD1. qPCR was performed by using the Roche LigthCycler480 II machine with the following setting: 1 cycle of initializing for polymerase activation at 95 °C, 45 cycles of denaturation at 95 °C, 45 cycles of annealing at 60 °C, 45 cycles of extension at 72 °C.

Measurement of forelimb strength

The forelimb strength was measured using a commercial grip strength monitor (Linton Instrumentation, Norfolk, UK). Five measurements per mouse were taken over a 3-day period by the same investigator, who was blind to the treatment of the mice. Mice were allowed to grasp a metal mesh attached to a force transducer using their forelimbs. The force produced during 5 sequential gentle pulls, until the mice release their grip, was recorded. Thirty seconds were waited between consecutive tests per each mouse. Data collected were expressed as gram force normalized by the initial animal body weight.

Statistical Analyses

All results are reported as mean ± SEM. Differences between cohorts were determined using one-way ANOVA with multiple comparison test or unpaired t-test. All data analyses were performed with GraphPad Prism 8 software (San Diego, CA).

Author Disclosure

The authors declare that they have no conflict of interest.

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References

1. Hoffman EP, Brown RH, Jr., Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919-928.
2. Monaco AP, Bertelson CJ, Liechti-Gallati S et al. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics 1988;2:90-95.
3. Bushby K, Finkel R, Birnkrant DJ et al. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. Lancet Neurol 2010;9:77-93.
4. Landfeldt E, Thompson R, Sejersen T et al. Life expectancy at birth in Duchenne muscular dystrophy: a systematic review and meta-analysis. Eur J Epidemiol 2020.
5. Goyenvalle A, Seto JT, Davies KE et al. Therapeutic approaches to muscular dystrophy. Hum Mol Genet 2011;20:R69-78.
6. Foster H, Sharp PS, Athanasopoulos T et al. Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer. Mol Ther 2008;16:1825-1832.
7. Li X, Eastman EM, Schwartz RJ et al. Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. Nat Biotechnol 1999;17:241-245.
8. Foster H, Sharp PS, Athanasopoulos T et al. Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer. Mol Ther 2008;16:1825-1832.
9. Koo T, Malerba A, Athanasopoulos T et al. Delivery of AAV2/9-microdystrophin genes incorporating helix 1 of the coiled-coil motif in the C-terminal domain of dystrophin improves muscle pathology and restores the level of alpha1-syntrophin and alpha-dystrobrevin in skeletal muscles of mdx mice. Hum Gene Ther 2011;22:1379-1388.
10. Le Guiner C, Servais L, Montus M et al. Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. Nat Commun 2017;8:16105.
11. Wells DJ. What is the level of dystrophin expression required for effective therapy of Duchenne muscular dystrophy? J Muscle Res Cell Motil 2019;40:141-150.
12. Mingozzi F, Maus MV, Hui DJ et al. CD8(+) T-cell responses to adeno-associated virus capsid in humans. Nat Med 2007;13:419-422.
13. Mingozzi F, Meulenberg JJ, Hui DJ et al. AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells. Blood 2009;114:2077-2086.
14. VandenDriessche T, Chuah MK. Vector decoys trick the immune response. Sci Transl Med 2013;5:194fs128.
15. Trucco F, Domingos JP, Tay CG et al. Cardiorespiratory Progression Over 5 Years and Role of Corticosteroids in Duchenne Muscular Dystrophy: A Single-Site Retrospective Longitudinal Study. Chest 2020.

16. Rincon MY, Sarcar S, Danso-Abeam D et al. Genome-wide computational analysis reveals cardiomyocyte-specific transcriptional Cis-regulatory motifs that enable efficient cardiac gene therapy. Mol Ther 2015;23:43-52.
17. Bostick B, Shin JH, Yue Y et al. AAV-microdystrophin therapy improves cardiac performance in aged female mdx mice. Mol Ther 2011;19:1826-1832.
18. Ramos JN, Hollinger K, Bengtsson NE et al. Development of Novel Micro-dystrophins with Enhanced Functionality. Mol Ther 2019;27:623-635.
19. Wang B, Li J, Fu FH et al. Construction and analysis of compact muscle-specific promoters for AAV vectors. Gene Ther 2008;15:1489-1499.
20. Yue Y, Wasala NB, Bostick B et al. 100-fold but not 50-fold dystrophin overexpression aggravates electrocardiographic defects in the mdx model of Duchenne muscular dystrophy. Molecular therapy Methods & clinical development 2016;3:16045.
21. Robbins J, Palermo J, Rindt H. In vivo definition of a cardiac specific promoter and its potential utility in remodeling the heart. Ann N Y Acad Sci 1995;752:492-505.
22. Sarcar S, Tulalamba W, Rincon MY et al. Next-generation muscle-directed gene therapy by in silico vector design. Nat Commun 2019;10:492.
23. Duan D. Systemic AAV Micro-dystrophin Gene Therapy for Duchenne Muscular Dystrophy. Mol Ther 2018;26:2337-2356.
24. Koo T, Malerba A, Athanasopoulos T et al. Delivery of AAV2/9-microdystrophin genes incorporating helix 1 of the coiled-coil motif in the C-terminal domain of dystrophin improves muscle pathology and restores the level of alpha1-syntrophin and alpha-dystrobrevin in skeletal muscles of mdx mice. Hum Gene Ther 2011;22:1379-1388.

Figure legends

Figure 1: Systemic delivery of 5E+11vg/mouse AAV9-csSpc5-12-MD1 in young mdx mice increase dystrophin expression in both skeletal and cardiac muscles compared to the delivery of AAV9-Spc5-12-MD1. Eight-week old *mdx* or C57BL10 mice were intravenously injected with a mid-dose (5E+11 vg/mouse) of AAV vectors carrying the transgene MD1 under control of Spc5-12 or csSpc5-12 promoters or with saline solution. All samples were harvested 16 weeks later. (A) Representative images of immuno-stained cardiac muscle cryosections for dystrophin (red) and laminin (green) show higher expression of MD1 protein in muscles treated with AAV-csSpc5-12-MD1. (B) Representative immunoblot for dystrophin (green) and corresponding REVERT total protein stain bands (red) suggests that in muscles treated with AAV-csSpc5-12-MD1 higher amount of MD1 protein is expressed. (C) Quantification of dystrophin expression by densitometric analysis of immunoblot. The intensity of dystrophin bands (green), quantified and normalized to the intensity of corresponding REVERT total protein stain bands (red), show increased MD1 expression after administration of AAV-csSpc5-12-MD1 compared to AAV-Spc5-12-MD1 in cardiac muscles. (D) Representative images of immuno-stained TA

muscle cryosections for dystrophin (red), laminin (green) and DAPI (blue) show higher expression of MD1 protein in muscles treated with AAV9-csSpc5-12-MD1 compared to the one in muscles treated with AAV9-Spc5-12-MD1. (E) Quantification of dystrophin positive fibres in TAs sections. The graphs show the percentages of dystrophin positive fibres for each treated group of mice (*mdx* injected with saline, AAV-Spc5-12-MD1 or AAV-csSpc5-12-MD1). AAV-csSpc5-12-MD1 administration increases MD1 expression compared with AAV-Spc5-12-MD1 administration. (F) Representative immunoblots for dystrophin (green) and the REVERT staining (red) suggest that in both HD treated groups a significant amount of MD1 protein is expressed. (G) Quantification of dystrophin expression by densitometric analysis of immunoblot. The intensity of dystrophin bands, quantified and normalized to the intensity of corresponding REVERT bands, show that AAV-csSpc5-12-MD1 administration induce higher expression of MD1 compared with AAV-Spc5-12-MD1. Scale bar = 200 µm. Statistical analysis was performed by One-way ANOVA test with Bonferroni analysis in b and student t-test in g (ns: not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 2: Systemic delivery of AAV9-Spc5-12-MD1 in young mdx mice restores high levels of dystrophin expression in cardiac muscles irrespective of inclusion of a CS-CRM4 enhancer. Eight-week old *mdx* or C57BL10 mice were intravenously injected with high or low doses (HD and LD respectively) of AAV vectors carrying the transgene MD1 under control of Spc5-12 or csSpc5-12 or with saline solution. Cardiac muscles were harvested 16 weeks later. (A) The quantification of vector genomes by qPCR in cardiac muscles of mice treated with AAVs or saline indicates that a similar amount of AAV were used for the 2 top doses with/without enhancer. (B) Representative images of immuno-stained cardiac muscle cryosections for dystrophin and laminin show significant expression of MD1 protein in muscles treated with either AAV vector at the high dose. (C) Representative immunoblot for dystrophin (green) and α-tubulin (red) suggests that in both HD treated groups a significant amount of MD1 protein is expressed. (D) Quantification of dystrophin expression by densitometric analysis of immunoblot. The intensity of dystrophin bands was quantified and normalized to the intensity of

corresponding α-tubulin bands (n=4-6 per group). Scale bar = 200 µm. Statistical analysis was performed by One-way ANOVA test with Bonferroni analysis, (ns: not significant).

Figure 3: Systemic delivery of AAV9-Spc5-12-MD1 in young mdx mice restores significant percentage of dystrophin positive fibres in tibialis anterior muscles and improves muscle strength irrespective of inclusion of a CS-CRM4 enhancer. Eight-week old *mdx* or C57BL10 mice were intravenously injected with high or low doses (HD and LD respectively) of AAV vectors carrying the transgene MD1 under control of Spc5-12 or csSpc5-12 or with saline solution. Tibialis anterior (TA) muscles were harvested 16 weeks later. (A) Representative images of immune-stained TAs cryosections of injected *mdx* for dystrophin (red), laminin (green) and DAPI (blue) show significant percentage of dystrophin positive fibres in muscles treated with the high doses. (B) Percentage of dystrophin positive fibres in TAs sections of young mice. The graphs show the percentages of dystrophin positive fibres for each treated group of mice. No differences in the number of dystrophin positive fibres was observed between the groups MD1 HD and csMD1 HD. (C) Evaluation of forelimb strength by grip strength test. Assessment was performed one week before the harvest of samples. Forelimb strength, normalized to the initial body weight and expressed as gram force per gram of body weight, shows an improvement in muscle force for the mice treated with the HD. Scale bar = 200 µm. In B, C statistical analysis is performed by One-way ANOVA test with Bonferroni analysis, (ns: not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Supplementary figures

Supplementary figure 1: Systemic delivery of 5E+11 vg/mouse of AAV9-Spc5-12-MD1 in young mdx mice restores dystrophin expression in diaphragm muscles irrespective of inclusion of a CS-CRM4 enhancer. Eight-week old *mdx* or C57BL10 mice were intravenously injected with a mid-dose (5E+11 vg/mouse) of AAV vectors carrying the transgene MD1 under control of Spc5-12 or csSpc5-12 or with saline solution. (A) Representative low magnification images of immune-staining for dystrophin of

cardiac muscles treated with either AAV-Spc5-12-MD1 or AAV-csSpc5-12-MD1 show higher expression of MD1 protein in cardiac muscles of AAV-csSpc5-12-MD1 treated mice. (B) Diaphragm muscles were harvested 16 weeks later. (A) Representative immunostaining images of a patch of dystrophin positive fibres in Diaphragm cryosections of injected *mdx*. Dystrophin, laminin and DAPI show expression of MD1 protein in muscles treated with the mid dose. (C) Percentage of dystrophin positive fibres in the regions positive for dystrophin expression in Diaphragm sections of young mice. No difference in the number of dystrophin positive fibres was observed between the groups treated with AAV-Spc5-12-MD1 and AAV-csSpc5-12-MD1. Scale bar = 200 µm. Statistical analysis was performed by One-way ANOVA test with Bonferroni analysis (ns: not significant, * $p<0.05$, *** $p<0.001$).

Supplementary figure 2: Systemic delivery of either AAV9-Spc5-12-MD1 or AAV9-csSpc5-12-MD1 in young mdx mice restores similar (though variable among samples) dystrophin expression in cardiac muscle. Eight-week old *mdx* or C57BL10 mice were intravenously injected with higher or lower doses (HD and LD respectively) of AAV vectors carrying the transgene MD1 under control of Spc5-12 or csSpc5-12 or with saline solution. Cardiac muscles were harvested 16 weeks later. Representative low magnification images of immune-staining for dystrophin of all 6 cardiac muscles per group treated with HD of either AAV-Spc5-12-MD1 or AAV-csSpc5-12-MD1 show variable expression of MD1 protein in cardiac muscles. Scale bar = 200 µm.

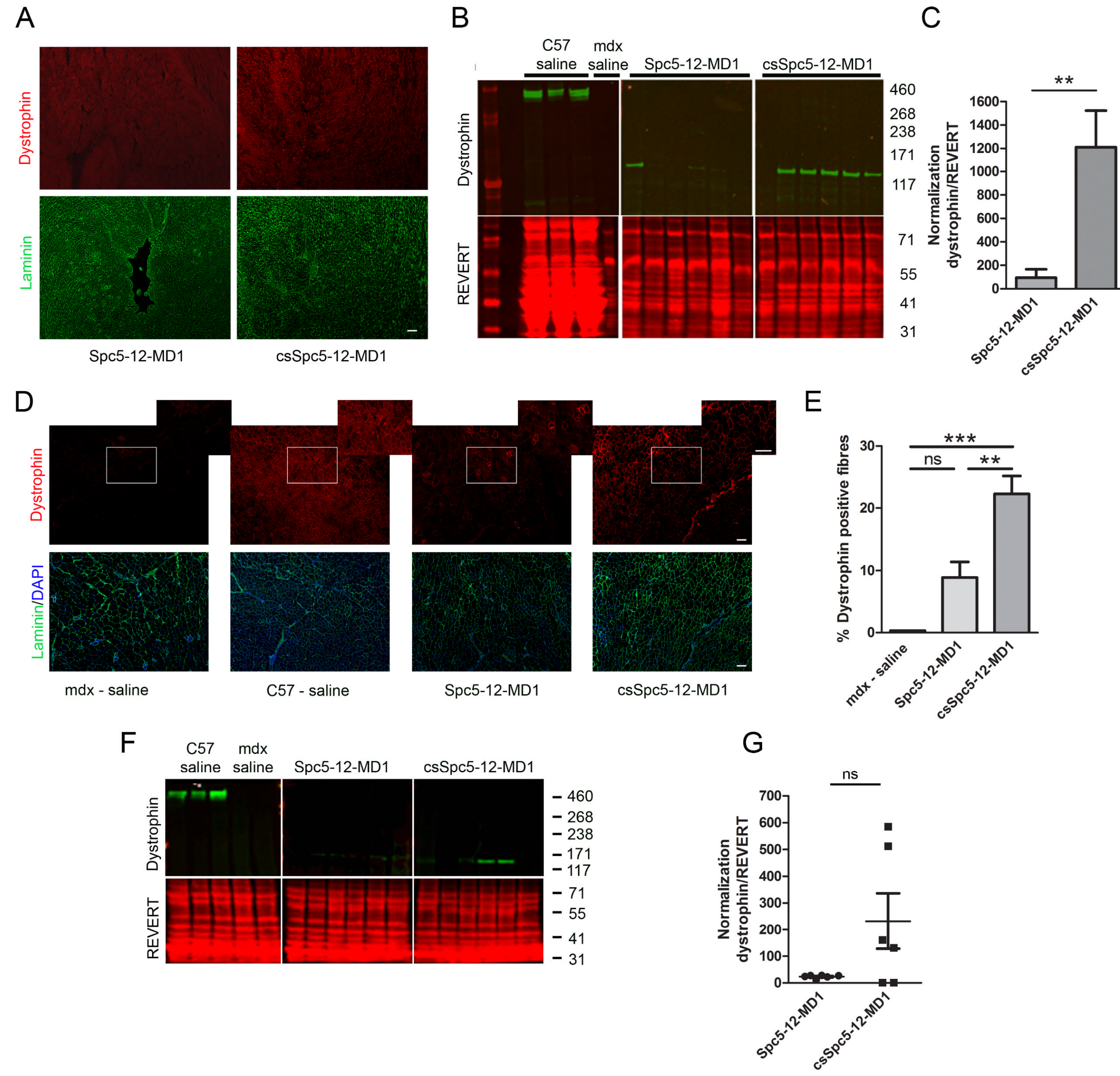
Supplementary figure 3: Systemic delivery of AAV9-Spc5-12-MD1 either with or without a CS-CRM4 enhancer restores dystrophin expression in tibialis anterior muscles of young mdx mice. Eight-week old *mdx* or C57BL10 mice were intravenously injected with high or low doses (HD and LD respectively) of AAV vectors carrying the transgene MD1 under control of Spc5-12 or csSpc5-12 or with saline solution. TAs were harvested 16 weeks later. (A) Representative immunoblot for dystrophin (green) and α-tubulin (red) shows that in TA muscles treated with AAVs there is a small amount of MD1 expression. (B) Quantification of dystrophin expression by densitometric analysis of immunoblot.

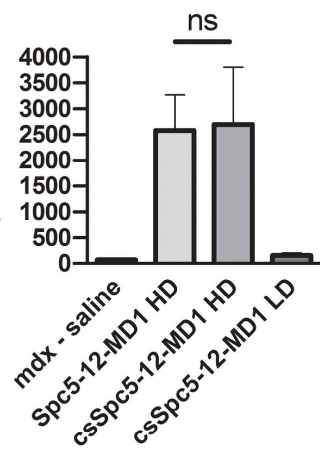
Treatment with AAV-csSpc5-12-MD1 HD, provides significantly higher MD1 expression than the treatment with AAV-Spc5-12-MD1 HD. The intensity of dystrophin bands was quantified and normalized to the intensity of corresponding α-tubulin bands. Statistical analysis was performed by One-way ANOVA test with Bonferroni analysis, (*$p<0.05$).

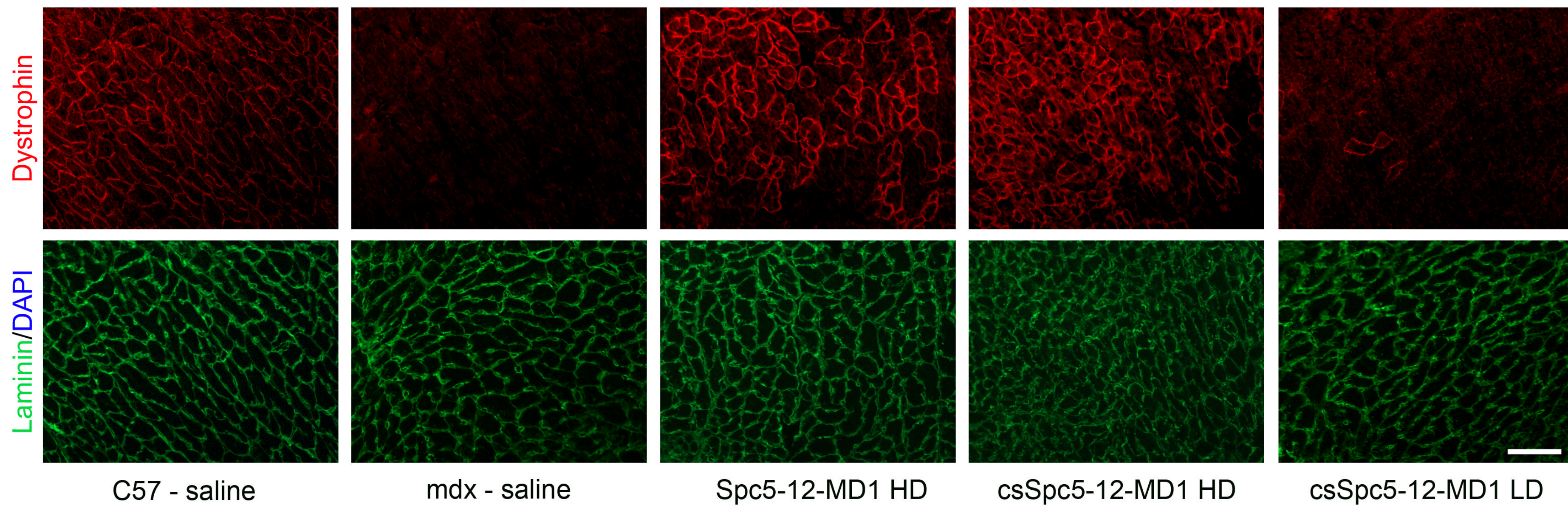
Supplementary figure 4: Systemic delivery of AAV9-Spc5-12-MD1 in young mdx mice restores dystrophin expression in diaphragm muscles irrespective of inclusion of a CS-CRM4 enhancer. Eight-week old *mdx* or C57BL10 mice were intravenously injected with high or low doses (HD and LD respectively) of AAV vectors carrying the transgene MD1 under control of Spc5-12 or csSpc5-12 or with saline solution. Diaphragm muscles were harvested 16 weeks later. (A) Representative immunostaining images of dystrophin positive fibres in DIA cryosections from injected *mdx* mice. Dystrophin (red), laminin (green) and DAPI (blue) show expression of MD1 protein in muscles treated with the high doses. (B) Percentage of dystrophin positive fibres measured in the regions positive for dystrophin expression in Diaphragm sections of young mice. No differences in the number of dystrophin positive fibres was observed between the groups treated with AAV-Spc5-12-MD1 and AAV-csSpc5-12-MD1. Scale bar = 200 µm.

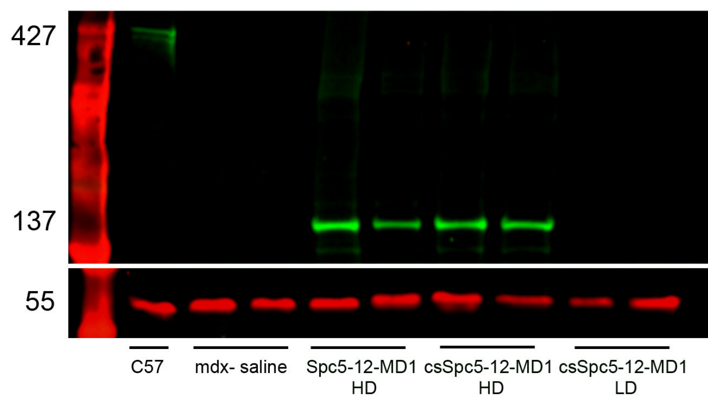
Figures:

Figure 1




A
ns
AAV vg/µg extracted DNA
4000
3500
3000
2500
2000
1500
1000
500
0
mdx - saline
Spc5-12-MD1 HD
csSpc5-12-MD1 HD
csSpc5-12-MD1 LD


B
Dystrophin
Laminin/DAPI
C57 - saline
mdx - saline
Spc5-12-MD1 HD
csSpc5-12-MD1 HD
csSpc5-12-MD1 LD


C
427
137
55
C57
mdx- saline
Spc5-12-MD1 HD
csSpc5-12-MD1 HD
csSpc5-12-MD1 LD

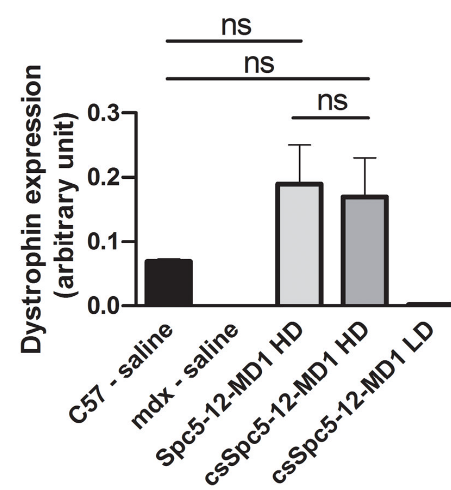
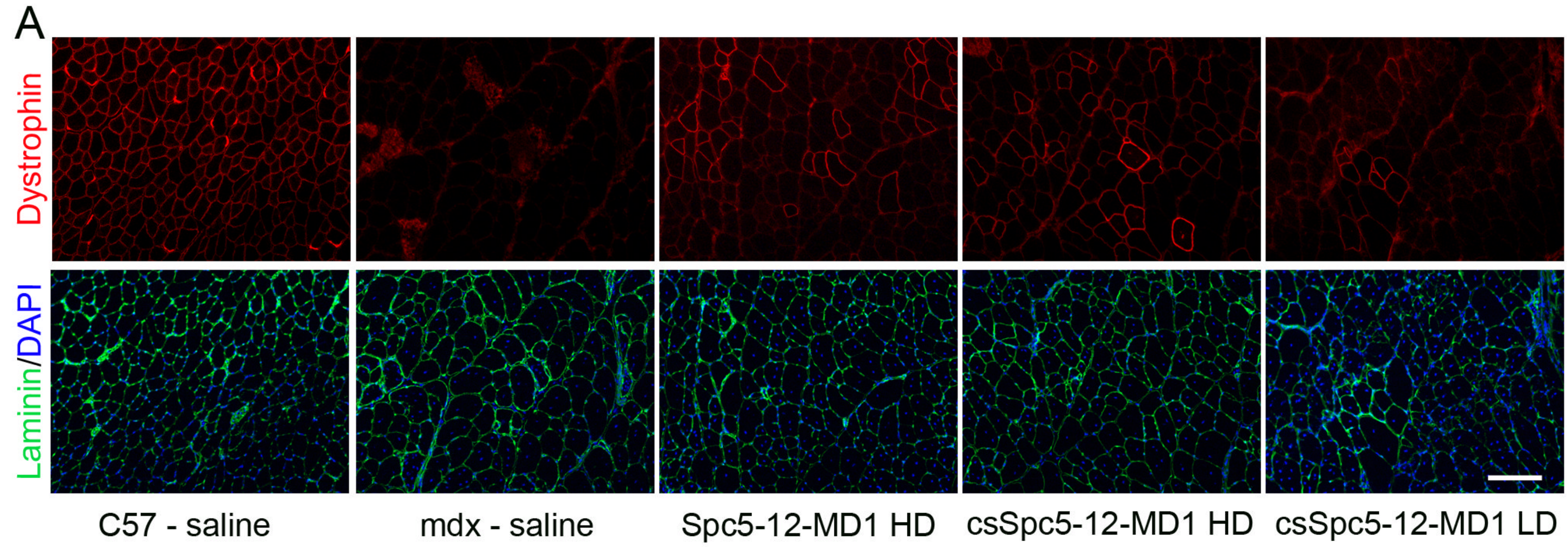
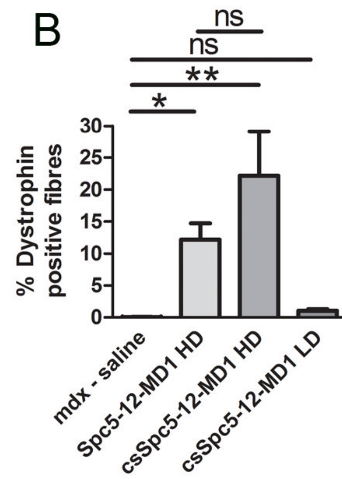
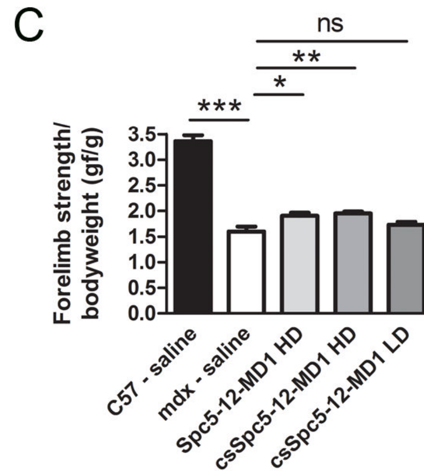

D
ns
ns
ns
Dystrophin expression (arbitrary unit)
0.3
0.2
0.1
0.0
C57 - saline
mdx - saline
Spc5-12-MD1 HD
csSpc5-12-MD1 HD
csSpc5-12-MD1 LD

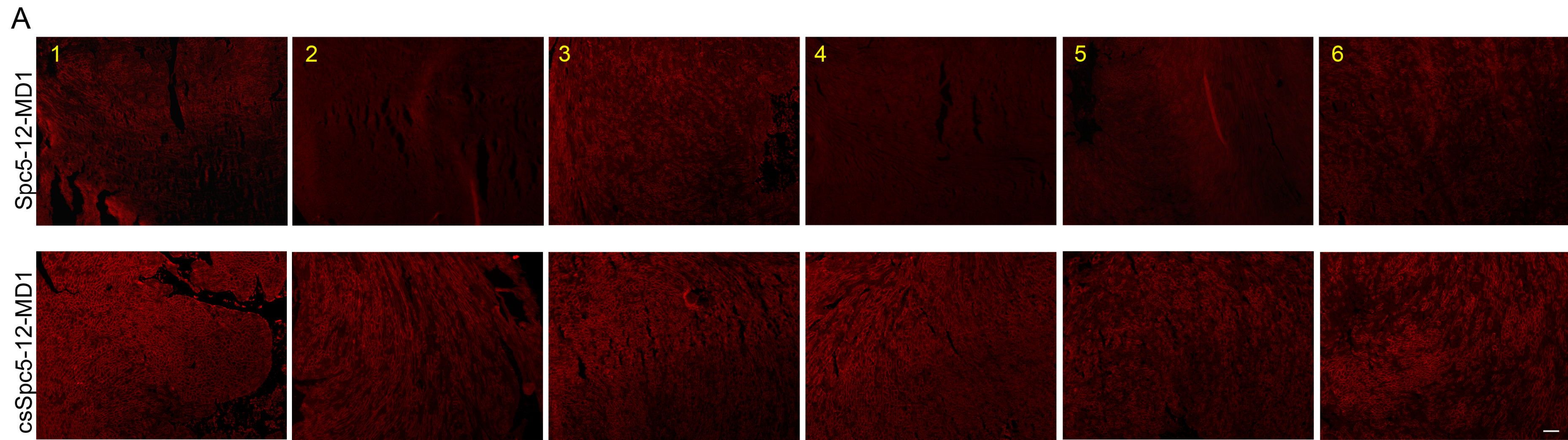
Figure 3

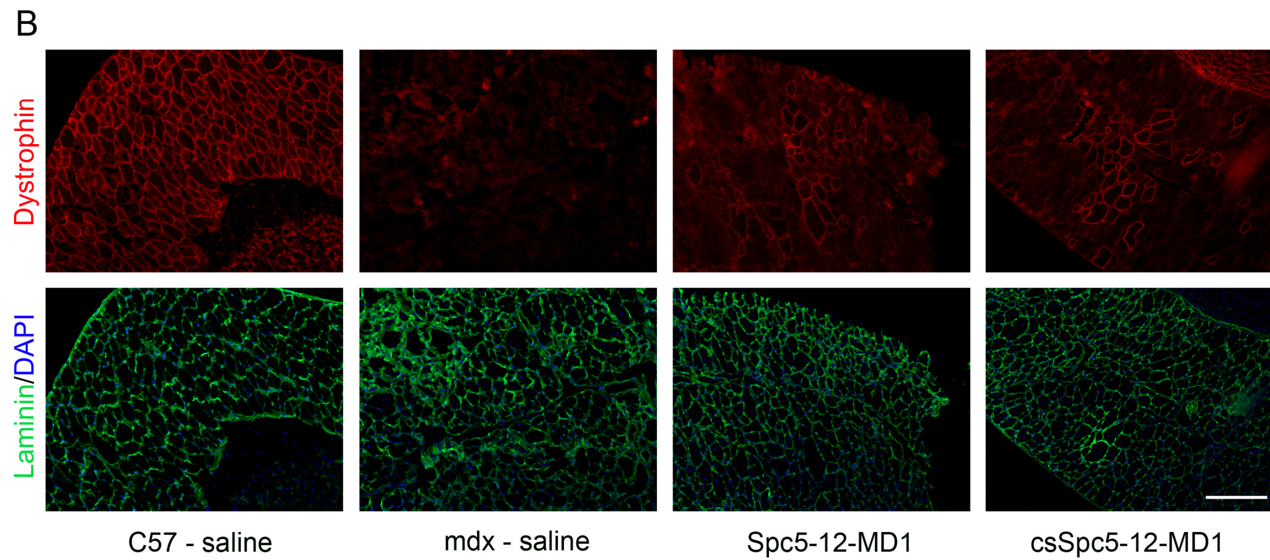


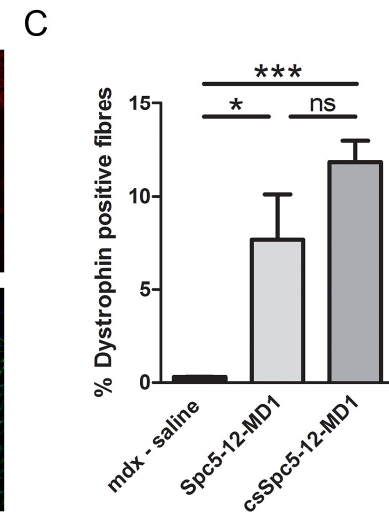




Supplementary figure 1







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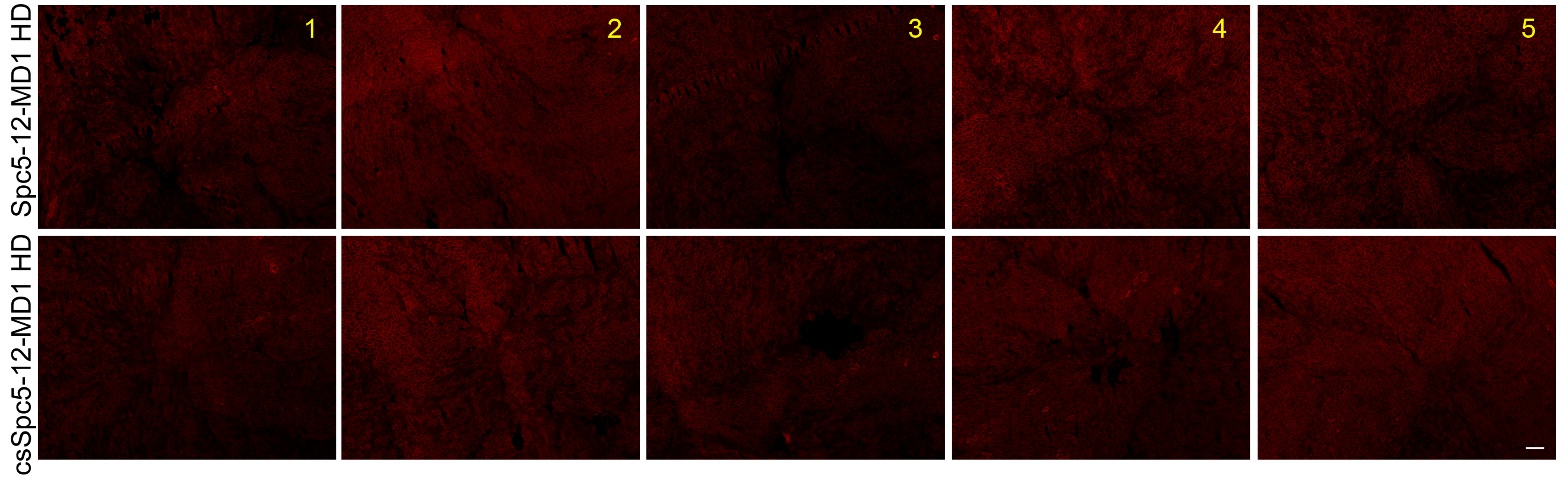

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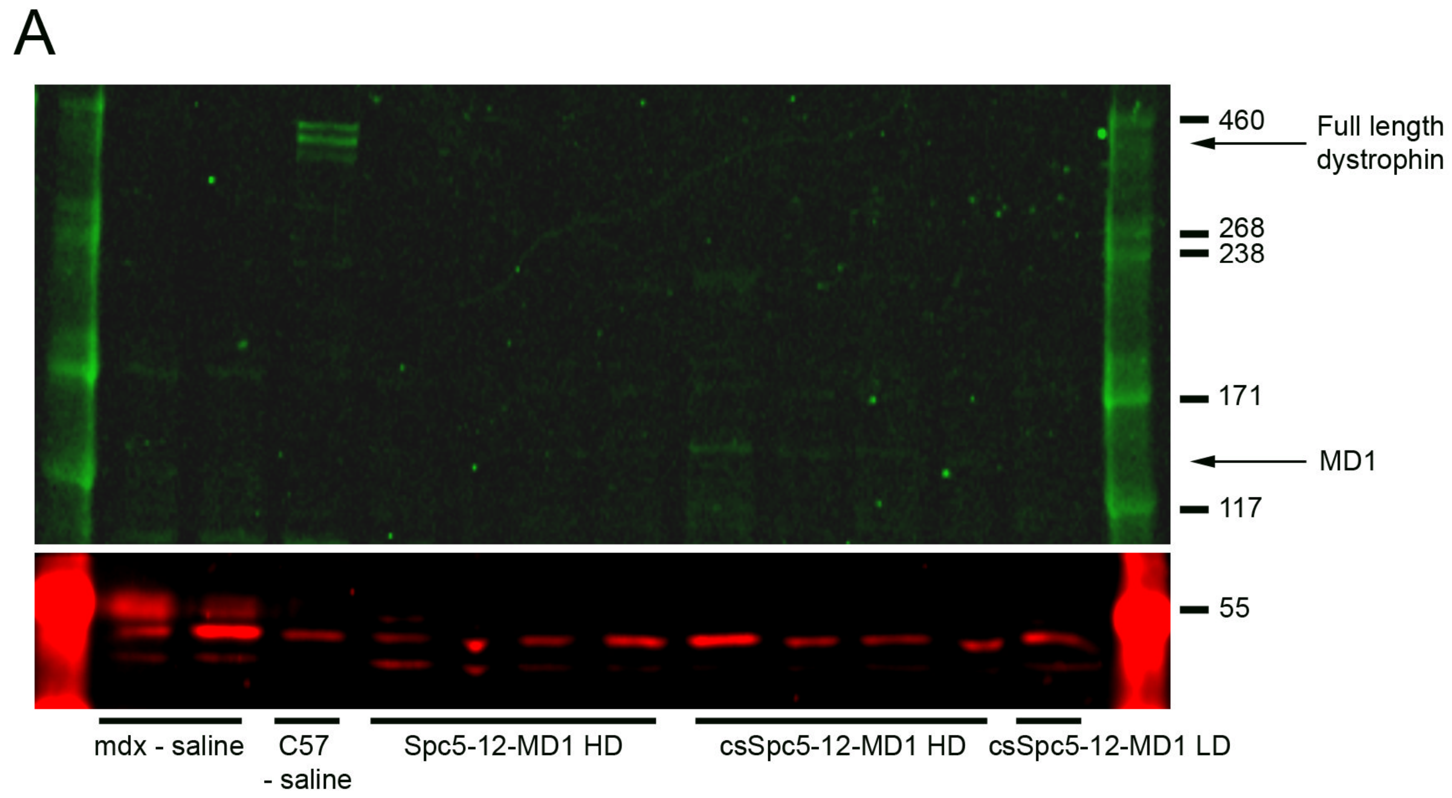

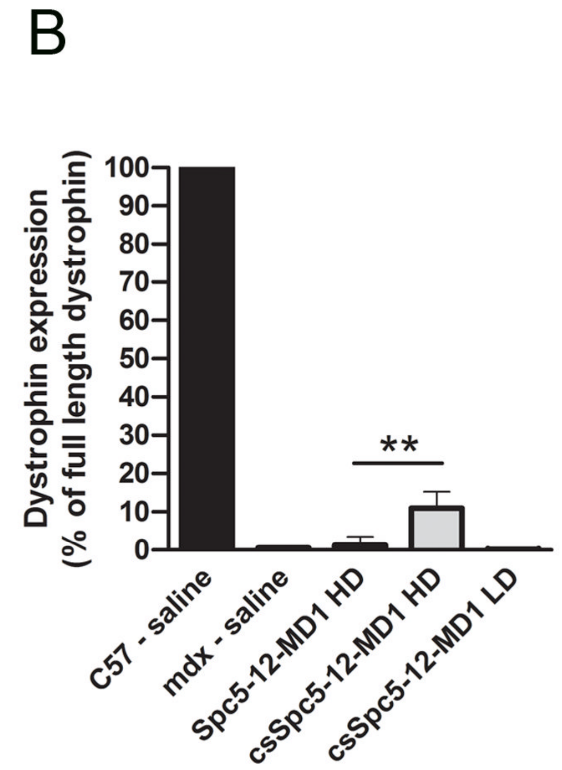

Supplementary figure 4