Antisense pre-treatment increases long-lasting benefit of gene therapy in dystrophic muscles

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Combined treatment for Duchenne muscular dystrophy

AAV vector / Duchenne muscular dystrophy / Exon skipping / Gene therapy

Abstract

In preclinical models for Duchenne muscular dystrophy, dystrophin restoration during AAV-U7-mediated exon-skipping therapy decreases drastically after one year in treated muscles. This decline in benefit is strongly correlated with loss of the therapeutic AAV genomes, probably due to alterations of the dystrophic myofiber membranes. To improve the membrane integrity of the dystrophic myofibers at the time of AAV-U7 injection, *mdx* muscles were pre-treated with a single dose of peptide-phosphorodiamidate morpholino (PPMO) antisense oligonucleotides that induced temporary dystrophin expression at the sarcolemma. The PPMO pre-treatment allowed efficient maintenance of AAV genomes in *mdx* muscles and enhanced the long-term effect of AAV-U7 therapy with a ten-fold increase of the protein level after six months. It is also beneficial to AAV-mediated gene therapy with transfer of micro-dystrophin cDNAs into muscles. This combined approach will allow the use of lower and thus safer vector doses while maximizing long-term therapeutic efficacy for Duchenne patients.

Introduction

The dystrophinopathies are pathologies caused by anomalies in the *DMD* gene that encodes the sub-sarcolemmal protein dystrophin. This protein is absent or drastically diminished in Duchenne muscular dystrophy (DMD) while it is present but qualitatively and/or quantitatively altered in the Becker muscular dystrophy (BMD). The dystrophin structure (central rod-domain made of 24 spectrin-like repeats) tolerates large internal deletions (Harper *et al.*, 2002) which led to the development of two main therapeutic strategies: gene therapy with transfer of micro-dystrophin cDNAs in muscles, and targeted exon skipping. Both approaches have shown encouraging results using adeno-associated viral (AAV) vectors, which allow efficient gene transfer into muscles. AAV-mediated delivery of micro-dystrophins into dystrophin-deficient mice has shown remarkable efficiency (Gregorevic *et al.*, 2006; Koo *et al.*, 2011; Shin *et al.*, 2013) leading to the initiation of an early-phase clinical trial (Mendell *et al.*, 2010).

Exon skipping converts an out-of-frame mutation into an in-frame mutation leading to an internally deleted but partially functional dystrophin. This therapeutic approach has demonstrated some success using antisense oligonucleotides (AONs), but recent studies showed limited clinical benefit (Cirak *et al.*, 2011; Goemans *et al.*, 2011; Mendell *et al.*, 2013; Voit *et al.*, 2014). The novel generation of AON chemistries, in particular tricyclo-DNA (tcDNA) (Goyenvalle *et al.*, 2015) and peptide-phosphorodiamidate morpholino oligonucleotide (PPMO) (Betts *et al.*, 2012; Betts *et al.*, 2015), display unprecedented degrees of dystrophin restoration in skeletal muscles, but also restore dystrophin expression in the heart and, to a lesser extent, in the brain for the tcDNAs. AONs have the enormous advantage of not being immunogenic but require regular administration to maintain therapeutic benefit.

The antisense sequences can be expressed in skeletal or cardiac muscles via a small nuclear RNA such as U7snRNA or U1snRNA (Brun *et al.*, 2003; Denti *et al.*, 2006; Goyenvalle *et al.*, 2004). These therapeutic molecules are vectorised in AAV particles which ensure a permanent production of the antisense in dystrophin-deficient murine models (Denti

et al., 2006;Goyenvalle *et al.*, 2012;Goyenvalle *et al.*, 2004), as well as in the dystrophin-deficient dog GRMD (Bish *et al.*, 2012; Le Guiner *et al.*, 2014; Vulin *et al.*, 2012). In all dystrophic models, a one-shot treatment of AAV-U7snRNA (AAV-U7) was sufficient to attain substantial levels of restored dystrophin, which is associated with a significant improvement of the muscle force (Denti *et al.*, 2006; Goyenvalle *et al.*, 2012; Goyenvalle *et al.*, 2004; Le Guiner *et al.*, 2014; Vulin *et al.*, 2012).

Despite the high efficiency of AAV-U7 strategy, we recently showed that dystrophin levels decreased significantly after one year in various skeletal muscles in the severely dystrophic dystrophin/utrophin knockout (dKO) mouse and in the GRMD dog (Le Hir *et al.*, 2013; Vulin *et al.*, 2012). This decline in dystrophin was strongly correlated with viral genome loss, most likely due to alterations of the dystrophic myofiber membranes. In the context of an AAV-U7 clinical trial for DMD, AAV genome fate in dystrophic muscles is of major importance since the viral capsid immunogenicity currently limits repeated treatment (Lorain *et al.*, 2008). We recently investigated the viral genome fate in muscles of the moderately dystrophic *mdx* mouse and showed that non-therapeutic viral genomes were lost quickly after the injection and that this loss was diminished when high doses of viral genomes restored dystrophin at the sarcolemma (Le Hir *et al.*, 2013).

The goal of the present study was to avoid viral genome loss by pre-conditioning the dystrophic muscles for AAV injections. We showed for the first time that an AAV-U7 threshold dose was required to restore efficiently the dystrophin expression in the *mdx* mouse. We also demonstrated that therapeutic AAV genomes were lost from *mdx* muscle during the time interval between the AAV-U7 injection and the occurrence of dystrophin in sufficient quantity at the sarcolemma. Moreover, induction of transient high dystrophin expression at the sarcolemma of myofibers with peptide-phosphorodiamidate morpholino (PPMO) AONs allowed efficient preservation of AAV genomes in *mdx* muscles. Importantly, the efficacy of AAV-U7-mediated exon skipping as well as AAV-mediated micro-dystrophin gene therapy was markedly improved. Therefore, avoiding viral genome loss after AAV

injection by AON pre-treatment could help achieve a threshold dose of AAV genomes needed for efficient long-term restoration of dystrophin in *mdx* muscles.

Considering that more than 80% of DMD mutations are eligible for the personalized medicine involving the skipping of a single or of multiple exons (Aartsma-Rus *et al.*, 2009), this combined therapy approach could in theory benefit up to 80% of DMD patients.

Results

Dystrophin rescue kinetics highlight a threshold dose of AAV-U7

We first investigated the kinetics of dystrophin rescue at different doses of therapeutic AAV1 vector in muscles of *mdx* mice that carry a nonsense mutation in exon 23 of the *Dmd* gene (Sicinski *et al.*, 1989). We used an AAV1 vector encoding an U7snRNA (AAV1-U7ex23) allowing efficient exon 23 skipping and therefore dystrophin rescue in *mdx* muscles (Goyenvalle *et al.*, 2004). High (1E+11 viral genome or vg), intermediate (3E+10 vg) and low (1E+10 vg) doses of this vector were injected into *mdx Tibialis anterior* (TA) muscles and dystrophin protein levels were quantified in these muscles by western blotting 3, 6 and 12 weeks post-injection (Fig. 1a&b). The high dose of AAV1-U7ex23 allowed strong dystrophin expression (around 35% of normal dystrophin level) at 3 weeks after the injection, and reached a plateau (50-70%) by six weeks. At the intermediate dose, the kinetics of dystrophin restoration was delayed with only 3% of dystrophin expression at 3 weeks, 40% at 6 weeks and 70% at 12 weeks. At the low dose, dystrophin rescue was around 2% at 3 weeks and remained low even by 12 weeks post-injection. These data showed that the low dose of AAV1-U7ex23 was below a threshold level required for an effective restoration of dystrophin.

The time interval between the AAV injection and the dystrophin rescue is decisive for viral genome maintenance

In the kinetic experiments of dystrophin rescue, the number of viral genomes in TA muscles quantified by quantitative PCR (qPCR) was stable for each AAV1-U7ex23 dose between 3

and 12 weeks post-injection (Fig. 1c). The 3-fold increment applied between the three injected doses correlated to AAV genome levels with 2.5-fold more vg in the intermediate dose injected *mdx* muscles than in the low injected ones. A 5-fold higher factor was quantified between the high and intermediate dose injected muscles indicating that with higher dose more viral particles are retained in the target tissue. These results are consistent with our published observation that AAV1-U7ex23 genomes are maintained at high dose 3 weeks post-injection in *mdx* muscles compared to wild-type (wt) muscles, while they are poorly preserved at low and intermediate doses (Le Hir *et al.*, 2013). Therefore, the viral genome loss observed at low and intermediate doses in the *mdx* muscles occurred principally before three weeks, when dystrophin is not yet present at the sarcolemma, and not during the following weeks. In this sense, at the intermediate dose the delay observed in dystrophin rescue compared to high dose (Fig. 1a&b) might be responsible for the viral genome loss observed at this dose but not at high dose. Hence, the time interval between the AAV injection and the presence of a functional dystrophin in sufficient quantity at the sarcolemma is decisive for viral genome maintenance.

Viral genomes are efficiently maintained in Pip6a-PMO rescued *mdx* muscles

To avoid AAV genome loss following the AAV injection, dystrophin expression was induced temporarily at the sarcolemma of *mdx* TA myofibers by a single injection of Pip6a-PMO AON, a PPMO that is particularly efficient for *mdx* exon skipping (Betts *et al.*, 2012). A non-therapeutic vector carrying non-specific sequence (AAV-U7scr, 1E+11 vg) was injected in the same muscles two weeks after the PPMO injections (Fig. 2a) when dystrophin rescue was already optimal (Supplementary data 1). We previously showed that this high dose of non-therapeutic genomes was drastically lost after three weeks since the sequence is unable to rescue dystrophin expression (Le Hir *et al.*, 2013). Following PPMO pre-treatment and three weeks after AAV1-U7scr injection, immunofluorescence analysis revealed a strong dystrophin restoration with appropriate sarcolemmal location in *mdx* injected muscles (Fig. 2b), between 56 to 98% of normal dystrophin levels when quantified by western blotting

(Fig. 2c), illustrating the high PPMO efficiency for dystrophin restoration. The viral genome content was 6 times lower in non PPMO-treated *mdx* muscles than in wt muscles, as already shown (Le Hir *et al.*, 2013). In contrast, the PPMO-treated *mdx* group had significantly increased numbers of viral genomes, with levels exceeding that of wt muscles, although not to significance (Fig. 2d). Therefore, a significant dystrophin expression induced by PPMO pre-treatment at the time of AAV1-U7scr injection protects against the rapid loss of AAV1-U7scr genomes in *mdx* muscles comparable to what is observed in wt muscles.

Pip6a-PMO pre-treatment allows important dystrophin rescue at low AAV-U7ex23 dose after 6 months

We then evaluated the long-term benefit of an AON pre-treatment on the dystrophin rescue via AAV1-U7ex23. Pip6a-PMO AONs were injected into *mdx* TAs two weeks before injection of low dose of AAV1-U7ex23 vector (1E+10 vg) (Fig. 3a), which without pre-treatment allowed only a weak dystrophin rescue (2%, Fig. 1a&b). Six months later, levels of exon 23 skipping analysed by nested RT-PCR (Fig. 3b) and quantified by qPCR (Fig. 3c) in *mdx* TAs treated with AAV1-U7ex23 or PPMO alone were low as expected, respectively 9 and 6% of skipped transcripts, leading to the synthesis of rescued dystrophin around 2% of the normal level (Fig. 3e). Conversely, TAs treated sequentially with PPMOs then with AAV1-U7ex23 showed 54% of skipped transcripts (Fig. 3c) and a dystrophin at 20% of the normal level of dystrophin (Fig. 3e). Moreover, the viral genome number was 8-fold higher in the combined PPMO/AAV1-U7ex23 treated muscles than in AAV1-U7ex23 only injected muscles (Fig. 3d). These data demonstrate that the PPMO pre-treatment induced maintenance of the therapeutic U7ex23 genomes in *mdx* muscles six months after the AAV-U7 injections and remarkably resulted in a 10-fold increase of the rescued dystrophin amount.

Pip6a-PMO pre-treatment significantly increases the efficacy of AAV1 mediated micro-dystrophin gene therapy

To evaluate the efficacy of an AON pre-treatment on AAV-micro-dystrophin gene therapy, we injected Pip6a-PMO AONs into *mdx* TAs two weeks before injection of AAV1-MD1 vector (1E+10 vg) expressing a murine micro-dystrophin under the control of the muscle-restrictive Spc5-12 promoter (Foster *et al.*, 2008) (Fig.4a). Four weeks later, a strong dystrophin restoration was observed in PPMO-treated *mdx* TAs induced by the PPMO pre-treatment (Fig. 4c). AAV genome copy number and micro-dystrophin expression were 3-fold higher in the PPMO/AAV1-MD1 treated muscles than in AAV1-MD1 only treated muscles (Fig. 4b&c), illustrating the PPMO pre-treatment benefit on AAV-micro-dystrophin gene therapy. This experiment demonstrates that the AON pre-treatment is capable of enhancing all AAV-based gene therapies for DMD.

Discussion

We previously showed that therapeutic AAV genomes are rapidly lost from dystrophic muscles during AAV-U7-mediated exon-skipping therapy (Le Hir *et al.*, 2013). However, a strong dystrophin rescue induced by high dose (1E+11 vg) of AAV-U7 in *mdx* muscles prevents this viral genome loss. We show here that, at this high dose, the dystrophin restoration was not only maximal but also rapid after the therapeutic injection. With a 3-fold lower viral genome dose (intermediate dose) injected in the dystrophic muscles, the dystrophin synthesis was delayed but became maximal with albeit a massive genome loss occuring during the first weeks post-injection. These data demonstrated that a delay in dystrophin synthesis is sufficient to cause a substantial viral genome loss that could significantly limit the long-term therapeutic benefit of AAV therapies for DMD. Therefore, the initial period between the therapeutic injection and the presence of a functional dystrophin at the myofiber sarcolemma is decisive for viral genome maintenance in dystrophic muscles; the sooner dystrophin is present at the sarcolemna, the more AAV genomes are maintained in *mdx* muscles.

We demonstrate also that a low dose of AAV-U7ex23 was not sufficient to induce significant dystrophin restoration while the intermediate dose that contained only 3-fold more viral

genomes induced a high level of dystrophin restoration as just described. This observation suggests that the low dose of therapeutic vector is below a threshold necessary to restore significant dystrophin expression and thus to allow long-term maintenance of viral genomes. The existence of this threshold level is crucial for AAV-U7 therapy meaning that our goal is to reach and maintain this threshold dose of therapeutic viral genomes inside the dystrophic myofibers in order to obtain a sustainable therapeutic benefit.

To slow down the loss of therapeutic viral genomes from dystrophic muscles and achieve long-term restoration of dystrophin expression, we previously proposed recurrent systemic injections of AONs to prevent the progressive reappearance of a dystrophic phenotype caused by the partial loss of AAV genomes over time (Le Hir *et al.*, 2013). Instead of these successive therapies, we demonstrate here the synergistic benefit of the reciprocal treatment combination with first a single AON injection to restore dystrophin at the myofiber sarcolemma and secure membrane integrity followed by a single systemic injection of AAV-U7 vector to induce a strong and long-lasting expression of dystrophin in muscles. A significant dystrophin rescue by PPMO pre-treatment at the time of AAV-U7 injections allows an efficient maintenance of the viral genomes in *mdx* muscles three weeks later. Additionally, this initial maintenance of viral genomes increases dystrophin restoration by AAV-U7, around 8-fold at RNA level and 10-fold at protein level up to six months later. Of course, this does not rule out our previous hypothesis that AON administration subsequent to AAV treatment could supplement AAV-mediated dystrophin restoration and act so as to maintain or even increase dystrophin levels, especially as life-long treatment will be required in DMD patients.
We showed also that an AON pre-treatment confers an increased benefit to AAV-mediated micro-dystrophin cDNA transfer into *mdx* muscles. Indeed, four weeks later, micro-dystrophin expression was already 3-fold higher in PPMO-treated *mdx* mouse compared to untreated one.
The PPMO pre-treatment results in substantial dystrophin expression at the time of AAV-U7 injection that likely reduces the membrane abnormalities leading to AAV genome loss before

AAV-U7 induced dystrophin expression occurs. Once established, an AAV-U7 mediated high dystrophin expression will be maintained because it will by itself prevent transgene loss (Le Hir *et al.*, 2013). The same scenario is conceivable for micro-dystrophin gene therapy. By allowing the maintenance of high viral genome content in the decisive period between AAV injection and AAV-mediated transgene expression in the treated dystrophic muscles, PPMO-mediated dystrophin restoration guarantees a higher therapeutic benefit of the AAV based therapy compared to direct AAV injection. Additionally, in condition of poor AAV transduction, especially with systemic delivery some muscles are less transduced than others (Louboutin *et al.*, 2005), PPMO pre-treatment could help reaching in these muscles the threshold dose of AAV genomes needed to restore efficiently dystrophin in *mdx* muscles.

The fact that the rescue effect on AAV genomes was relatively lower for AAV-MD1 as compared to AAV-U7 mediated treatment can likely be explained by the direct transcription and overexpression of the micro-dystrophin cDNA resulting in more rapid dystrophin-mediated membrane sealing. In contrast, the U7-mediated exon skipping is dependent on the U7-antisense transcription and its action on intrinsically produced dystrophin mRNA which delays membrane sealing and thereby favours increased AAV genome loss.

The viral genome loss is certainly due to the fragility of the dystrophic muscle fibers that undergo cycles of necrosis/generation as we observed a similar loss of vector genomes in cardiotoxin-treated normal muscles (Le Hir *et al.*, 2013). However, other causes could participate to the process of viral genome loss from the dystrophic muscles. Dystrophic myofibers present abnormally leaky membranes that could passively loose the viral genomes, as exemplified by the creatine kinase (CK) activity greatly elevated in sera of Duchenne patients and preclinical animal models or conversely by Evans blue uptake into dystrophic muscle (Straub *et al.*, 1997). In addition, AAV vectors were also found associated with exosomes termed vexosomes in culture media of AAV producer cells (Maguire *et al.*, 2012). Interestingly, lack of dystrophin at the sarcolemma of *mdx* myofibers was shown to lead to an increased excretion of exosomes that was partially restored by dystrophin rescue

(Duguez *et al.*, 2013), suggesting that therapeutic viral vectors might also be lost through excretion via exosomes from the dystrophic myofibers. However, such secretion mechanisms of AAV vectors remain to be further investigated *in vivo.* Therefore, AON-mediated dystrophin restoration could reduce these membrane abnormalities and thus further preserve the therapeutic viral genomes in the dystrophic myofibers.

Recently, transcripts derived from AAV vectors have been shown to be destabilized by oxidative damage caused by free radicals, reducing the transgene expression in dystrophic muscles (Dupont *et al.*, 2015). Interestingly, high expression of micro-dystrophin was able to significantly reduce the proportion of oxidized transcripts in GRMD dog muscles showing a direct correlation between the oxidative stress of the myofibers and the dystrophic phenotype. Hence, lowering the oxidative status by AON pre-treatment might also facilitate a high AAV transgene expression and help reaching the threshold of therapeutic viral genome number necessary to allow a long-lasting benefit of AAV therapies.

On the eve of clinical trials using AAV-based therapies for DMD patients, this study underscores the strong impact of combined approaches to improve the benefit of AAV-based therapies allowing the use of lower and thus safer vector doses for a larger level of dystrophin expression in the long term.

Materials and Methods

Viral vector production and animal experiments

A three-plasmid transfection protocol was used with pAAV(U7smOPT-SD23/BP22) (Goyenvalle *et al.*, 2004), pAAV(U7smOPT-scr) (Le Hir *et al.*, 2013) and codon optimized pΔR4-R23/ΔCT (MD1) (Foster *et al.*, 2008) plasmids for generation of single-strand AAV1-U7ex23, AAV1-U7scr and AAV1-MD1 vectors. Three-month-old *mdx* mice were injected into the *Tibialis anterior* (TA) muscles with 1 nmole of Pip6a-PMO oligonucleotides (GGCCAAACCTCGGCTTACCTGAAAT) (Betts *et al.*, 2012). Additionally, 50µl of AAV1-U7scr, AAV1-U7ex23 or AAV1-MD1 containing 1E+10, 3E+10 or 1E+11 viral genomes (vg) were injected into C57BL/6 (wt) or *mdx* TAs. These animal experiments were performed at

the Myology Research Center, Paris, France, according to the guidelines and protocols approved by the Institutional Review Board. A minimum of four mice were injected per group for each experiment. At sacrifice, muscles were collected, snap-frozen in liquid nitrogen-cooled isopentane and stored at -80°C.

Viral genome quantification

Genomic DNA was extracted from mouse muscles using Puregene Blood kit (Qiagen). Copy number of AAV genomes and genomic DNA were measured on 100ng of genomic DNA by absolute quantitative real-time PCR on a StepOnePlus™ (Applied Biosystems) using the Taqman® Universal Master Mix (Applied Biosystems). Primers (forward: CTCCATCACTAGGGGTTCCTTG and reverse: GTAGATAAGTAGCATGGC) and probe (TAGTTAATGATTAACCC) were used to specifically amplify the viral genome sequence. As a reference sample, a pAAV plasmid was 10-fold serially diluted (from 10^7 to 10^1 copies). All genomic DNA samples were analysed in duplicates.

RT-PCR analysis

Total RNA was isolated from mouse muscle with NucleoSpin® RNA II (Macherey-Nagel), and reverse transcription (RT) performed on 200ng of RNA by using the Superscript™ II and random primers (Life technologies). Non-skipped and skipped dystrophin transcripts were detected by nested PCR and quantified as described (Goyenvalle *et al.*, 2012).

Western blot analysis

Protein extracts were obtained from pooled muscle sections treated with 125 mM sucrose, 5 mM Tris-HCl pH 6.4, 6% of XT Tricine Running Buffer (Bio-Rad), 10% SDS, 10% Glycerol, 5% β-mercaptoethanol. The samples were purified with the Pierce Compat-Able™ Protein Assay Preparation Reagent Set (Thermo Scientific) and the total protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific). Samples were denatured at 95°C for 5 minutes and 100 µg of protein were loaded onto Criterion XT Tris-

acetate precast gel 3-8% (Bio-Rad). Membrane was probed with primary monoclonal antibodies directed against dystrophin (NCL-DYS1, 1:50, Leica Biosystems; or MANEX1011B, 1:50, kindly gifted by The Muscular Dystrophy Association Monoclonal Antibody Resource (Bartlett *et al.*, 2000)) and α-actinin (1:1000, Sigma-Aldrich), followed by incubation with a sheep anti-mouse secondary antibody (horseradish peroxidase conjugated; 1:15000) and Pierce ECL Western Blotting Substrate (Thermo Scientific).

Immunohistochemistry

TA sections of 12 µm were cut and examined for dystrophin expression using the NCL-DYS2 monoclonal antibody (1:50; Leica Biosystems) and a goat anti-mouse secondary antibody Alexa 488 (1:1000; Life technologies).

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Author Contributions

CP, AM and MLH performed the experiments; LJ prepared AAV productions; GD, SJ and ALH provided the MD1 construct; GM and MJW provided the PPMO oligonucleotides; SL and TV conceived the project; CP, GM, MJW, SBZ, FPR, TV helped design and supervise the study; SL designed the experiments, supervised the entire study and wrote the manuscript with input from the other authors.

Conflict of Interest

The authors declare no competing financial interests.

The Paper Explained

PROBLEM:

AAV-U7-mediated exon-skipping strategy is a very promising therapy for Duchenne muscular dystrophy. However, in preclinical models dystrophin restoration decreases drastically after one year in treated muscles. This decline in benefit is strongly correlated with loss of the therapeutic AAV genomes, probably due to alterations of the dystrophic myofiber membranes. In the context of an AAV-U7 clinical trial for DMD, AAV genome fate in dystrophic muscles is of major importance since the viral capsid immunogenicity currently limits repeated treatment.

RESULTS:

To improve the membrane integrity of the dystrophic myofibers at the time of AAV-U7 injection, *mdx* muscles were pre-treated with a single dose of peptide-phosphorodiamidate morpholino (PPMO) antisense oligonucleotides that induced temporary dystrophin expression at the sarcolemma. The PPMO pre-treatment allowed efficient maintenance of AAV genomes in *mdx* muscles and enhanced the long-term effect of AAV-U7 therapy with a ten-fold increase of the protein level after six months. It is also beneficial to AAV-mediated gene therapy with transfer of micro-dystrophin cDNAs into muscles.

IMPACT:

This combined approach will allow the use of lower and thus safer vector doses while maximizing long-term therapeutic efficacy for Duchenne patients. Considering that more than 80% of DMD mutations are eligible for the personalized medicine involving the skipping of a

single or of multiple exons, this combined therapy approach could in theory benefit up to 80% of DMD patients.

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Figure legends

Fig. 1. Kinetics of dystrophin restoration at different AAV1-U7ex23 doses. *Tibialis anterior* (TA) muscles of *mdx* mice were injected with 1E+11 viral genomes (vg) (High dose), 3E+10 vg (Intermediate dose) or 1E+10 vg (Low dose) of AAV1-U7ex23. Three *mdx* TAs were injected per group. The mice were sacrificed 3, 6 and 12 weeks later (3, 6 or 12w). (a) Dystrophin restoration was evaluated by western blotting with NCL-DYS1 monoclonal antibodies (upper panel) on whole protein extracts from the treated muscles (lower panel: α-actinin). The result of one representative TA is shown per condition. (b) Dystrophin restoration was quantified by ImageJ software and expressed as the percentage of dystrophin expression in wild-type (wt) muscle. (c) Quantification of AAV vg by absolute Taqman qPCR in injected *mdx* TAs. AAV genome content is expressed as the AAV genome

number relative to the value obtained for the *mdx* muscles injected with low dose of AAV1-U7ex23 at three weeks post-injection. The data presented in (b) and (c) represent the mean values of the three TAs per group ± SEM. One of two representative experiments is shown. n.s., non-significant, ***P≤0.0001, Student's t-test.

Fig. 2. Effect of dystrophin restoration by Pip6a-PMO pre-treatment on viral genome maintenance. (a) TAs from *mdx* and wt mice were injected with 1 nmole of Pip6a-PMO two weeks (-2w) before the injection of 1E+11 vg of the non-therapeutic AAV1-U7scr vector (day 0, d0). Control *mdx* and wt TAs were injected with AAV1-U7scr vector alone. Four TAs were injected per group. The mice were sacrificed 3 weeks later (3w). (b) Dystrophin rescue monitored by immunostaining with the NCL-DYS2 monoclonal antibody on transverse sections of TA muscles. One representative immunostained section is shown per condition. (c) Dystrophin restoration evaluated by western blotting with NCL-DYS1 monoclonal antibodies (upper panel) on whole protein extracts from the PPMO-treated muscles (lower panel: α-actinin). Dystrophin restoration was quantified by ImageJ software and expressed as the percentage of dystrophin expression in wt muscle. (d) Quantification of AAV viral genomes by absolute Taqman qPCR. AAV genome content is expressed as the AAV genome number relative to the value obtained for the non PPMO-treated *mdx* muscles. The data represent the mean values of 4 muscles per group ± SEM. n. s.: non-significant, ***p < 0.001, Student's t-test. One of two representative experiments is shown.

Fig. 3. Effect of Pip6a-PMO pre-treatment on long-term dystrophin rescue by low dose of AAV-U7ex23. (a) *Mdx* TAs were injected with 1 nmole of Pip6a-PMO two weeks (-2w) before the injection of 1E+10 vg of therapeutic AAV1-U7ex23 vector (day 0, d0). Control *mdx* TAs were injected with PPMO or AAV1-U7ex23 vector alone. Four TAs were injected per group. The mice were sacrificed 6 months later (6m). (b) Level of exon 23 skipping estimated by nested RT-PCR. The 901 bp PCR product corresponds to full-length dystrophin transcripts whereas the 688 bp product corresponds to transcripts lacking exon 23. (c)

Quantification of exon 23 skipping performed by relative TaqMan qPCR and expressed as a percentage of total dystrophin transcripts. (d) Quantification of AAV viral genomes by absolute Taqman qPCR. AAV genome content is expressed as the AAV genome number relative to the value obtained for the non PPMO-treated *mdx* muscles. The data presented in (c) and (d) represent the mean values of the four TAs per group ± SEM. *p < 0.05, ***p < 0.001, Student's t-test. (e) Dystrophin restoration evaluated by western blotting with NCL-DYS1 monoclonal antibodies (upper panel) on whole protein extracts from the treated muscles (lower panel: α-actinin). Dystrophin restoration was quantified by ImageJ software and expressed as the percentage of dystrophin expression in wt muscle.

Fig. 4. Effect of Pip6a-PMO pre-treatment on AAV1 mediated micro-dystrophin gene therapy. (a) *Mdx* TAs were injected with 1 nmole of Pip6a-PMO two weeks (-2w) before injection of 1E+10 vg of AAV1-MD1 micro-dystrophin expressing vector (day 0, d0). Control *mdx* TAs were injected with PPMO or AAV1-MD1 vector alone. Five TAs were injected per group. The mice were sacrificed 4 weeks later (4w). (b) Quantification of AAV viral genomes by absolute Taqman qPCR. AAV genome content is expressed as the AAV genome number relative to the value obtained for the non PPMO-treated *mdx* muscles. The data represent the mean values of the 5 muscles per group ± SEM. *p < 0.05, Student's t-test. (c) Expression of PPMO-induced dystrophin (DYS, 427kDa) and micro-dystrophin (μDYS, 132kDa) evaluated by western blotting with MANEX1011B monoclonal antibodies (upper panel) on whole protein extracts from the treated muscles (lower panel: α-actinin).

Figure 1

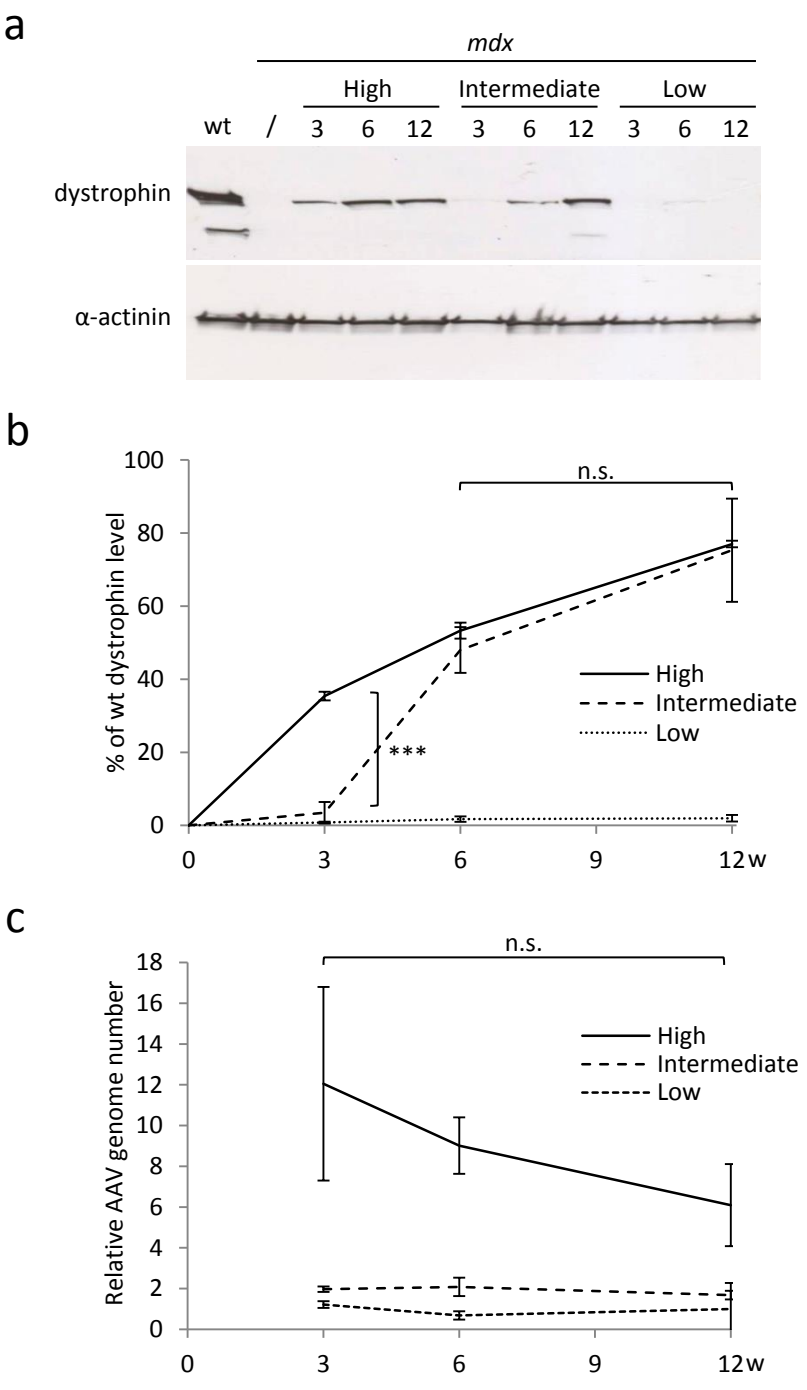
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Figure 2

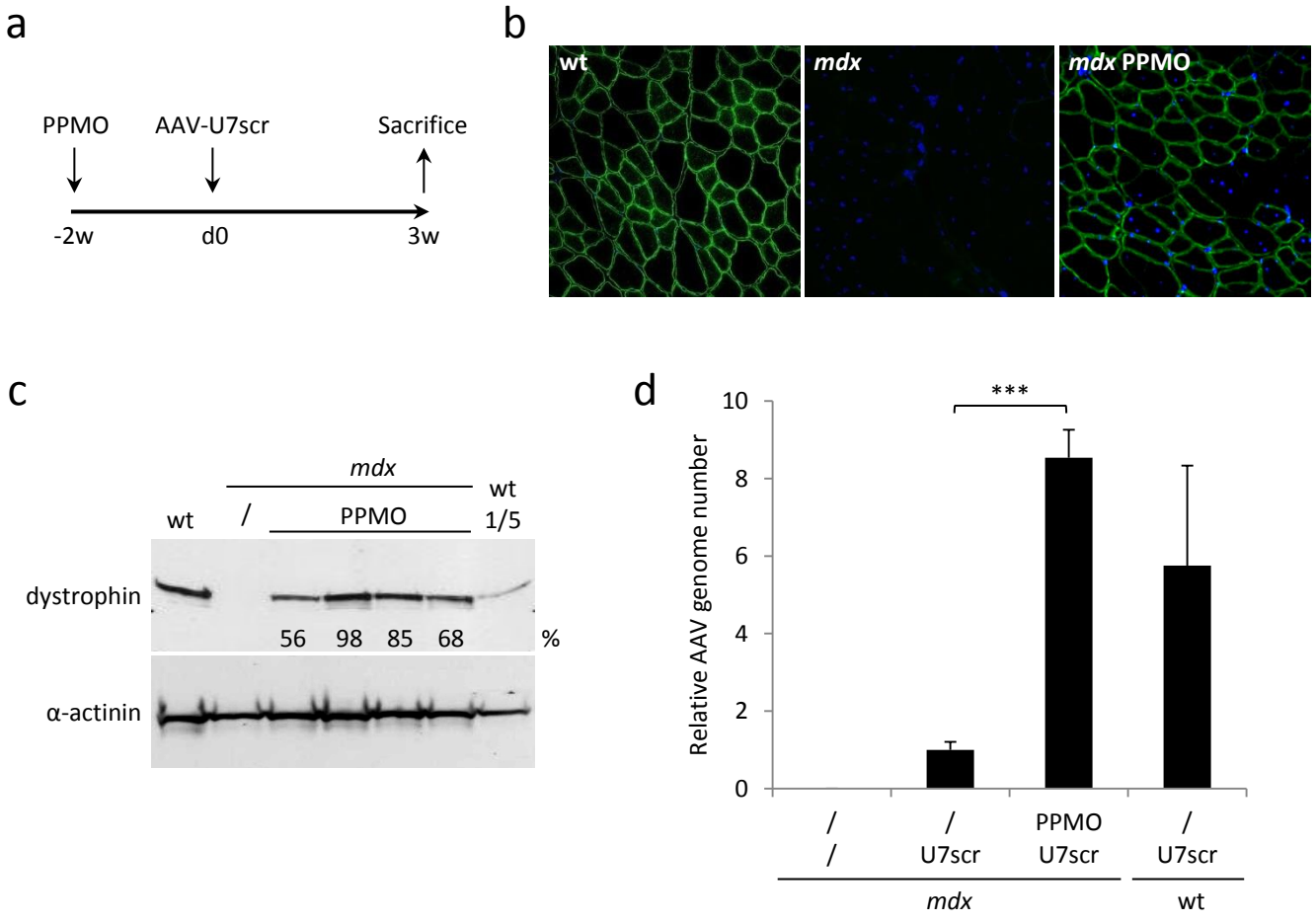
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Figure 3

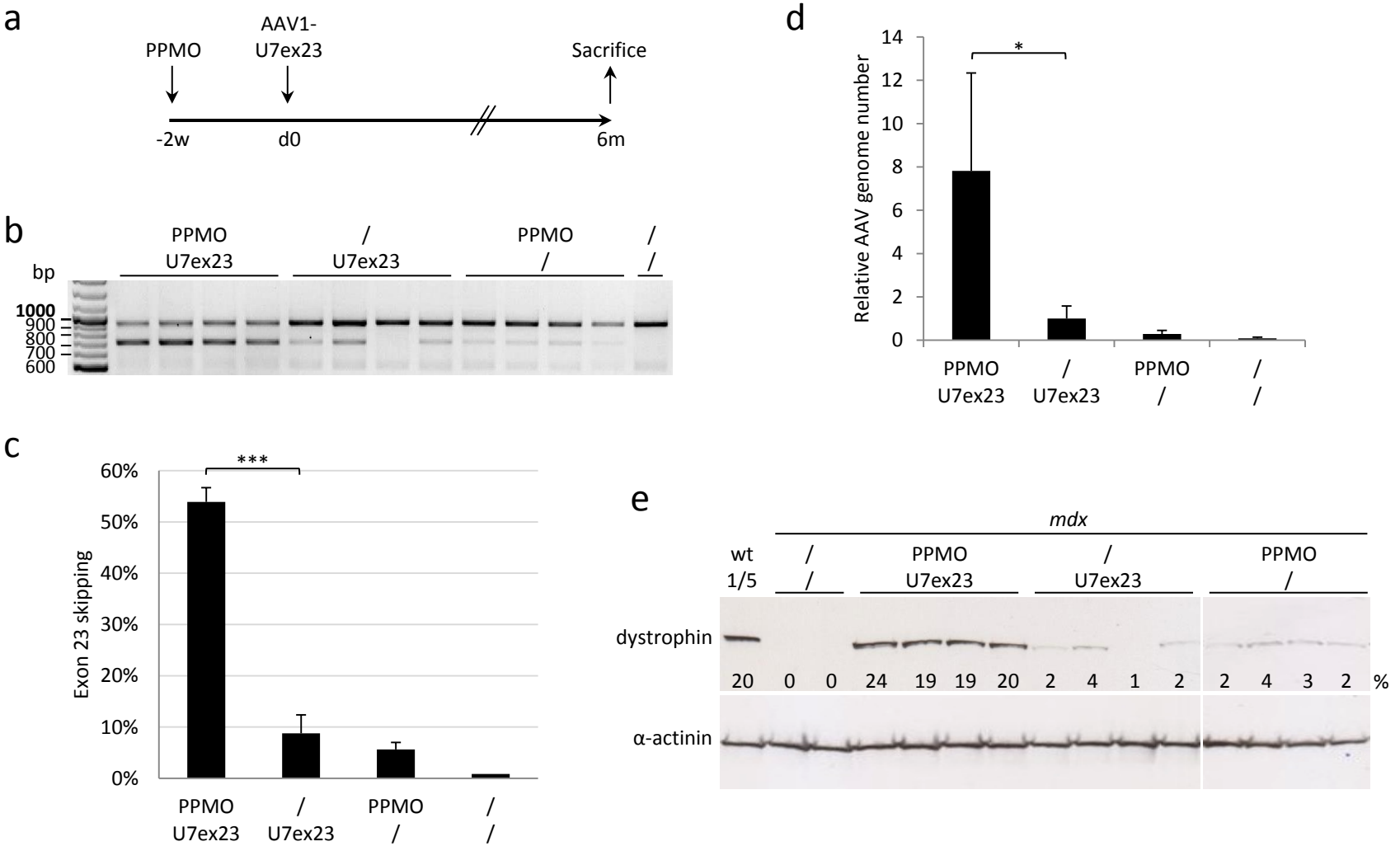
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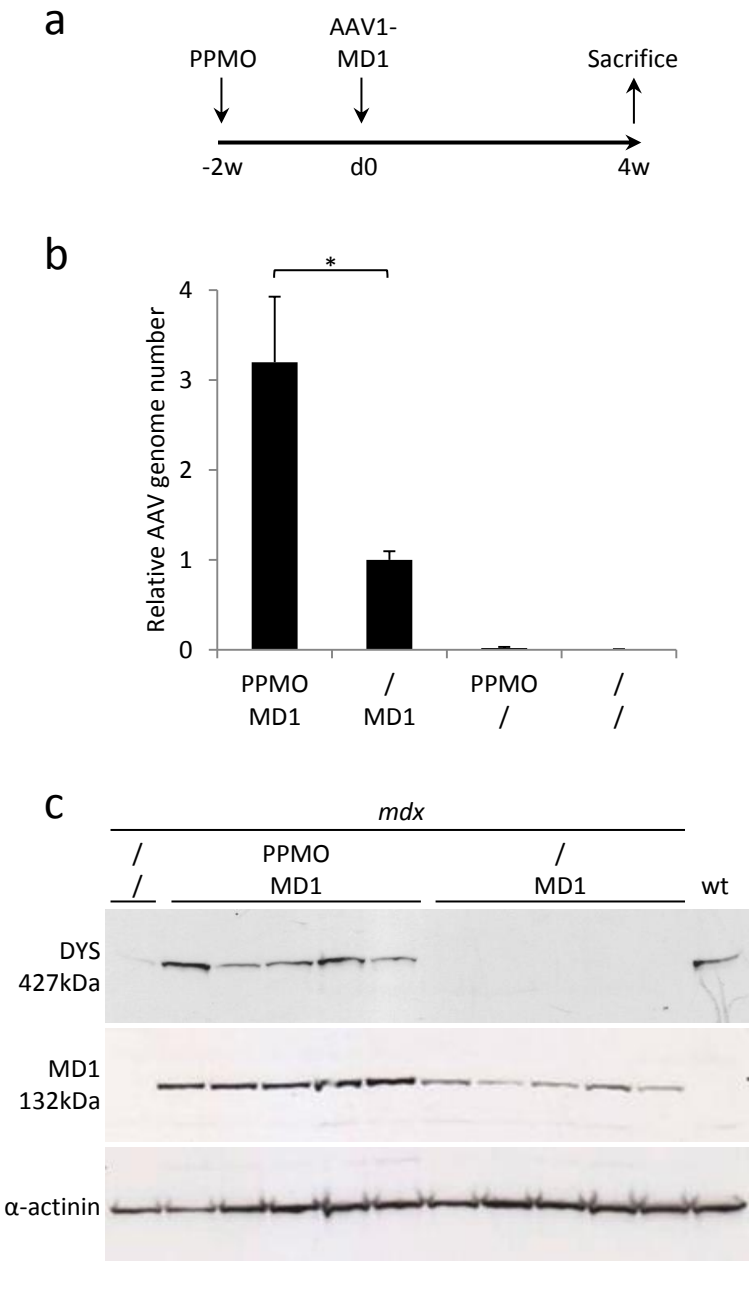
Figure 4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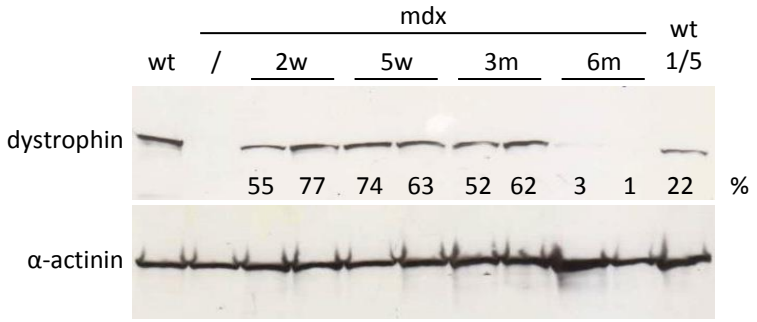

Supplementary data 1. Kinetics of dystrophin restoration by a single Pip6a-PMO intramuscular injection

Two *Tibialis anterior* (TA) muscles of *mdx* mice were injected per group with 1nmole of Pip6a-PMO. Dystrophin expression was evaluated after 2 weeks (2w), 5 weeks (5w), 3 months (3m) and 6 months (6m), by western blotting with NCL-DYS1 monoclonal antibodies (upper panel) on whole protein extracts from the treated muscles (lower panel: α-actinin). Dystrophin restoration was quantified by ImageJ software and expressed as the percentage of wild-type (wt) dystrophin expression.