Systemic PPMO-mediated dystrophin expression in the Dup2 mouse model of Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a devastating muscle-wasting disease that arises due to the loss of dystrophin expression, leading to progressive loss of motor and cardiorespiratory function. Four exon-skipping approaches using antisense phosphorodiamidate morpholino oligomers (PMOs) have been approved by the FDA to restore a DMD open reading frame, resulting in expression of a functional but internally deleted dystrophin protein, but in patients with single-exon duplications, exon skipping has the potential to restore full-length dystrophin expression. Cell-penetrating peptide-conjugated PMOs (PPMOs) have demonstrated enhanced cellular uptake and more efficient dystrophin restoration than unconjugated PMOs. In the present study, we demonstrate widespread PPMO-mediated dystrophin restoration in the Dup2 mouse model of exon 2 duplication, representing the most common single-exon duplication among patients with DMD. In this proof-of-concept study, a single intravenous injection of PPMO targeting the exon 2 splice acceptor site induced 45% to 68% exon 2-skipped Dmd transcripts in Dup2 skeletal muscles 15 days post-injection. Muscle dystrophin restoration peaked at 77% to 87% average dystrophin-positive fibers and 41% to 51% of normal signal intensity by immunofluorescence, and 15.7% to 56.8% of normal by western blotting 15 to 30 days after treatment. These findings indicate that PPMO-mediated exon skipping is a promising therapeutic strategy for muscle dystrophin restoration in the context of exon 2 duplications.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe progressive muscle wasting disease caused by the loss of dystrophin expression due to mutations in the X chromosome DMD gene, affecting approximately 1:5,000 live male births worldwide.1 In healthy skeletal and cardiac muscle, dystrophin plays a critical role in sarcolemmal stability during contraction and relaxation. In DMD, the absence of dystrophin leads to muscle degeneration and inflammation that progresses to loss of motor function and eventual cardiorespiratory decline.

Exon skipping is currently the only approved therapeutic strategy for restoring an open reading frame, achieved using antisense oligonucleotides (AOs) that hybridize to pre-mRNA and alter splicing in a manner that produces an in-frame mature DMD transcript.6 Exon skipping may not only allow for expression of an internally deleted but potentially functional dystrophin in patients with deletions, but could also recover full-length dystrophin expression in patients with duplications by skipping a single copy of the duplicated exon.7,8 Currently four AO gene therapies are approved by the Food and Drug Administration for patients with mutations amenable to skipping of exons 45 (casimersen/Amondys 459,10), 51 (eteplirsen/Exondys 5111-14), and 53 (golodirsen/Vyondys 5315 and viltolarsen/Viltepso16,17). These therapies use phosphorodiamidate morpholino oligomers (PMOs) that must be taken up across the sarcolemma to

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reach the cell nucleus and alter splicing. More recently, evidence has pointed toward cell-penetrating peptide-conjugated PMOs (PPMOS) as a potentially more efficient alternative for future therapeutic development due to enhanced cellular uptake.18–24

Along with being the most common single-exon duplication, exon 2 duplication represents an attractive target for exon skipping due to its location immediately upstream of the internal ribosomal entry site (IRES) in exon 5, which fails to be used in the context of exon 2 duplication.25 We have previously described the safety and efficacy of exon skipping in a mouse model of exon 2 duplication (Dup2) using the U7 small nuclear RNA (U7snRNA) delivered via adeno-associated virus (AAV).26 Earlier work has demonstrated that both mRNA transcripts resulting from skipping of either one or both copies of exon 2 are therapeutic, because skipping a single copy results in translation of a full-length dystrophin protein and skipping of both copies (the Del2 transcript) promotes translation initiation from the exon 5 IRES.8,25,26 This IRES-driven mechanism of Del2 transcript expression results in the production of N-terminal-deleted yet highly functional dystrophin,25 allowing exon skipping in the context of exon 2 duplication to have an unusually large therapeutic window.

Here, we evaluate the therapeutic potential of a 31-mer PPMO targeting exon 2 (“PPMO-A”) for rescuing dystrophin expression in the Dup2 mouse model of DMD.27 We demonstrate extensive Dmd exon 2 exclusion and accumulation of the resulting skipped transcripts in a dose-dependent fashion in all skeletal muscles from PPMO-A-treated mice, with peak exon skipping occurring 7 to 15 days after treatment, followed shortly by peak dystrophin protein expression. These results support further investigation into systemic administration of PPMO targeting exon 2 as a strategy for restoring widespread expression of highly functional dystrophin in skeletal muscles of patients with DMD exon 2 duplications.

RESULTS

Single systemic injection of PPMO-A induces dose-dependent exon 2 exclusion

To characterize the dose-response relationship between PPMO-A and exon 2 skipping, we evaluated the efficacy of a single intravenous injection of 20, 40, or 80 mg/kg PPMO-A in 12-week-old Dup2 male mice (Table S1). No clinical signs of toxicity were observed in any dose group, and body weight as a surrogate of health showed no decline (Figure S1, Table S1). Seven days following the injection, RT-PCR demonstrated a dose-dependent increase in skipped transcripts in all tested skeletal muscles (Figures 1, 2, and S2), which include the wild-type (WT) and exon 2-deleted (Del2) transcripts, both of which are at least potentially therapeutic based on their association with correction of pathology and muscle function.25,26 In skeletal muscles, PPMO-A treatment at the low (20 mg/kg) and middle (40 mg/kg) dose levels resulted in an average of 8.8% and 24.8% total skipped transcripts, respectively, whereas the high dose of 80 mg/kg induced an average of 46.7% WT + Del2 transcripts (Figure 2, Table S3). The heart showed lower levels of exon 2 exclusion than skeletal muscles, with 8% average skipped transcripts at the highest PPMO-A dose 7 days after treatment. Animals injected with 80 mg/kg PPMO-Scr, a scrambled control PMMO compound, showed similarly low levels of skipped transcripts as saline-injected Dup2 mice, amounting to approximately 1% to 3% of total transcripts. Such low-level expression has been previously shown in the Dup2 mouse model.26

Exon 2 skipping peaks 15 days after a single high-dose PPMO-A injection

To assess the time course of exon 2 skipping following PPMO-A treatment at the highest dose, we measured skipped transcript levels across multiple timepoints ranging from 7 to 60 days post-injection (Tables S2, S3, and S4). No clinical signs of toxicity were observed at any time point, and body weight as a surrogate of health showed no decline (Figure S1, Table S2). The most robust exon 2 skipping was observed 7 to 15 days after a single PPMO-A injection in all tested skeletal muscles, with average skipped transcript levels ranging between 45% and 55% in the gastrocnemius and quadriceps and 62% to 68% in the diaphragm and tibialis anterior (TA) (Figures 3 and S3). PPMO-A-induced exon skipping remained less pronounced in the heart, reaching 14.4% average skipped transcripts 15 days after treatment. The degree of exon skipping declined by 30 days post-injection in all tested tissues except for the gastrocnemius, which showed steady skipped transcript levels until the 60-day time point. By 60 days after PPMO-A injection, skipped transcript levels declined to 4% to 20% in all tested tissues. The RT-PCR time course results in
Figure 2. Transcriptional dose-response for PPMO-A
(A) Quantification analysis of exon 2 skipping in right quadriceps (R. Quad), right gastrocnemius (R. Gas), right tibialis anterior (R. TA), diaphragm (Dia), and heart from Dup2 mice treated with doses of PPMO-A ranging from 20 to 80 mg/kg (20, 40, 80), PPMO-Scr (Scr) at the dose of 80 mg/kg, or saline diluent (Sal) (n = 5–6 per tissue). Bl6 mice injected with saline were used as controls (n = 4–7 per tissue). Dup2 band is shown in white, whereas skipped transcripts, defined as wild-type (WT) and Del2 dystrophin transcripts, are shown in gray and black, respectively. Bl6 mice are represented as 100% WT transcript. Data are presented as mean ± SEM. (B) Linear regression analysis of the percent skipped transcripts (WT + Del2) versus PPMO-A dose for each tissue (n = 4–7 per tissue); **p < 0.01; ***p < 0.001; ****p < 0.0001 for significantly non-zero slopes.

Figure 3 make use of the 7-day time point data from the dose-escalation study that was initially reported in Figure 2.

PPMO-A induces widespread sarcolemmal dystrophin expression in skeletal muscle
Immunofluorescence staining revealed extensive sarcolemmal dystrophin-positive signal throughout muscle sections from PPMO-A-treated Dup2 mice. Dystrophin-positive fibers reached 86% to 87% on average in the TA and 77% to 81% in the diaphragm 15 to 30 days after PPMO-A treatment (Figure 4, Table S6). Dystrophin-positive fibers may begin to decline by 60 days post-treatment, with 65% on average in TA and 63% in the diaphragm, but the small sample size is insufficient to confirm a trend. As expected, muscles from Dup2 mice that received saline and PPMO-Scr injections showed <2.5% average positive fibers, whereas Bl6 muscles had 98% to 99.9% average dystrophin-positive fibers (Table S6).
Dystrophin signal intensity at the sarcolemma of muscle fibers in the TA and diaphragm was also markedly higher in mice that received PPMO-A treatment. In TA sections from Dup2 mice treated with PPMO-A, the mean fiber dystrophin intensity reached 50% to 51% of Bl6 mean dystrophin intensity 15 to 30 days post-treatment, and 38% average dystrophin intensity at 60 days (Figures 5 and S4, Table S7). Diaphragm sections showed a similar trend in fiber dystrophin intensity after PPMO-A treatment, with approximately 41% to 42% average dystrophin intensity 15 to 30 days after PPMO-A and 34% average dystrophin intensity 60 days post-treatment. In contrast to percent dystrophin-positive fibers, the mean dystrophin signal intensity in fibers from saline and PPMO-Scr treatment groups remained ≤19% in TA sections and ≤16% in Dia, reflecting a combination of tissue autofluorescence, very low baseline dystrophin expression in Dup2 mice, and a small number of revertant fibers (Table S7).

PPMO-A efficacy was lower in the hearts, which were quantified using three square regions of interest (ROIs) of cross-sectional myocytes per heart. Aside from a single outlier with 57% dystrophin-positive myocytes, PPMO-A treated hearts showed, on average, 7.1% dystrophin-positive myocytes (15.5% with the outlier included) at 15 days, 4.1% at 30 days, and 1.6% at 60 days after treatment (Figure 6B, Table S6). In contrast, saline and PPMO-Scr treatment groups showed no more than 0.25% average dystrophin-positive myocytes at any time point, although statistical comparisons between groups fell short of the threshold for significance, presumably due to large intra-group variability. Cardiomyocyte dystrophin intensity showed a similarly modest response to PPMO-A treatment, with 16% to 20% dystrophin intensity in treated Dup2 compared with Bl6 hearts at 15 and 30 days, and 13% to 15% in Dup2 that received saline or PPMO-Scr (Figures 6C and S4, Table S7). By 60 days post-treatment, no significant increases in cardiac dystrophin immunofluorescence intensity could be seen in PPMO-A-treated Dup2 mice.

PPMO-A treatment drives increased dystrophin protein expression in muscle

Western blots of TA muscles and hearts corroborated the immunofluorescence (IF) quantification results, showing that a single PPMO-A injection rescued significant dystrophin production in skeletal muscle, but showed reduced efficacy in the heart (Figure 7, Table S5). PPMO-A treatment resulted in 22% to 23% mean dystrophin expression by western blot in the TA 15 to 30 days after treatment, and 18% at 60 days. Interestingly, the diaphragm showed much more robust peak expression of 57% at the 15-day time point, which declined to 16% to 22% at the 30- and 60-day points. The heart displayed a milder treatment effect of 14% mean dystrophin expression at 15 days (6.6% without the highly expressing outlier), 7.6% at 30 days, and 5.8% at 60 days, suggesting relatively stable modestly increased expression over the course of the study. Mean dystrophin expression by western blot in Dup2 control groups treated with PPMO-Scr and saline remained below 7.0% in muscle and 5.2% in the heart at all timepoints.

Central nucleation shows a significant improvement 60 days after PPMO-A treatment

Central nucleation in both TA and diaphragm sections showed a modest but statistically significant response to PPMO-A treatment in Dup2 mice by 60 days post-injection (Figure S5). Mean central nucleation in Dup2 TA muscles 60 days after receiving saline or PPMO-Scr injections was 65.9% and 62.6%, respectively, while the PPMO-A treatment group showed 56.2% centrally nucleated fibers. Similarly, diaphragms from Dup2 mice that received saline or PPMO-Scr showed 44.8% and 47.8% centrally nucleated fibers at 60 days, while PPMO-A-treated diaphragms showed 36.9% central nucleation. Interestingly, central nucleation in the diaphragm showed an earlier and more pronounced response to PPMO-A treatment than in TA sections. Fiber size was also assessed in TA and diaphragm sections, measured as the minimum Feret’s diameter, but no significant treatment effect on fiber size could be detected at the tested timepoints (Figure S6).

DISCUSSION

In the present study, we provide encouraging evidence that PPMO-A, a cell-penetrating peptide-conjugated PMO targeting the splice acceptor site of DMD exon 2, is a promising therapeutic strategy for patients with exon 2 duplication. Our group has previously shown robust PMO-mediated exon skipping in patient-derived DMD fibroblast cell lines, including cells with the exon 2 duplication mutation. With recent progress in PMO chemistry enabling the development of this new generation of AOs with improved uptake in skeletal muscle, we confirm potent skipping of Dmd exon 2 and restoration of widespread muscle dystrophin expression after a single systemic injection in Dup2 mice, supporting continued preclinical and clinical development.

Our previous in vivo work has confirmed that exon 2 skipping using a U7snRNA delivered via rAAV9.U7.ACCA is safe and efficient, avoiding toxicity in non-human primates and inducing extensive dystrophin expression in Dup2 mice. These studies paved the way for an ongoing clinical trial (ClinicalTrials.gov NCT04240314) in patients with exon 2 duplication, but the current limitation of large-scale AAV production and pre-existing AAV immunity in some patients.

Figure 3. Transcriptional analysis confirms Dmd exon 2 exclusion in PPMO-A time course study

Right quadriceps (R. Quad), right gastrocnemius (R. Gas), right tibialis anterior (R. TA), diaphragm (Dia), and hearts from Dup2 mice systemically injected with PPMO-A (80) or PPMO-Scr (Scr) at the dose of 80 mg/kg, or saline (Sal) showing skipping of Dmd exon 2 at 7, 15, and 30 days after treatment. C57Bl/6 (Bl6) mice are displayed as 100% WT transcript. Data are represented as mean ± SEM from each group in all tissues (n = 4–7 per group). Where appropriate, the total skipped transcripts (WT + Del2) were compared for 80 mg/kg PPMO-A (80) and control groups with a Welch’s ANOVA and Dunnnett’s T3 multiple comparison test. Groups without variation—all Bl6 and some Sal and Scr—were excluded from statistical analysis. Statistical comparison markers indicate which groups were analyzed. ns, not significant; *p < 0.05; **p < 0.01.
Figure 4. Dystrophin expression in muscles 15, 30, and 60 days following PPMO-A injection

Immunofluorescence (IF) quantification showing the percentage of dystrophin-positive fibers in (A) left tibialis anterior (L. TA) and (B) diaphragm from Dup2 mice injected with either PPMO-A or scrambled PPMO (PPMO-Scr) at a dose of 80 mg/kg, or saline (Sal) at all tested timepoints. Wild-type (Bl6) mice injected with saline were used as controls. The dystrophin-positive fiber content is plotted for each treatment group and time point, with individual data points showing the percent dystrophin-positive fibers for each mouse. The data are presented as mean ± SEM, n = 3–6 per tissue. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Statistically significant differences identified by Welch’s
continue to present challenges. The results presented here offer support for PPMO-A as an alternative agent for accomplishing the goal of skipping one or both copies of the duplicated exon 2, effectively inducing expression of full-length and highly functional Del2 dystrophin isoforms in skeletal muscles. This approach is especially attractive for patients with existing AAV immunity who are currently unable to receive AAV-based gene therapies or patients who have been previously treated with AAV-based therapies with a limited improvement in dystrophin expression. A previous study reported that combination therapy with AAV and PPMO showed some additive benefits in mdx mice, confirming the feasibility of this combined treatment approach. We note that this approach has theoretical advantages over delivery of engineered microdystrophin transgenes, as there are no patients who express the microdystrophin proteins from whom one can infer likely maximal benefit; this is in contrast to either full-length dystrophin or the IRES-driven dystrophin isoform, which confers the ability to walk into the seventh or eighth decade. We also note that PPMO approaches are likely to be safer than somatic gene editing approaches, which carry risk of significant off-target effects.

Here we report the dose- and time-dependent effects of a single systemic injection of PPMO-A on expression of skipped, presumptively therapeutic dystrophin transcripts and associated protein isoforms in Dup2 mice. These results demonstrate that skipped transcripts and dystrophin production in muscle reached their maximal levels 15 to 30 days after intravenous PPMO-A administration, and that the high 80 mg/kg dose is associated with 45% to 68% average skipped Dmd transcripts. This degree of exon skipping resulted in 68% to 76% dystrophin-positive fibers by immunofluorescence and 23% to 57% peak average dystrophin levels by western blot in the TA and diaphragm. Most of the assayed muscles showed a decline in skipped transcripts at the 30-day time point, with the exception of the gastrocnemius. Such minor differences in exon skipping between individual muscle groups may be due a variety of potential factors, including differential uptake in different muscles or inherent variability in the RT-PCR based assay. We find this observation to be of limited significance in this short-term, single-injection study. The immunofluorescence and western blot results do not clearly define the time point at which dystrophin protein expression meaningfully declines after a single PPMO-A injection, but previous work has suggested that increased dystrophin expression induced by AOs may last well beyond 60 days. Future studies should aim to investigate the kinetics of dystrophin expression, muscle function improvement, and any potential toxicity using repeated high-dose administrations of PPMO-A. Further work also will be necessary to determine whether efficiency continues to improve with even higher doses, along with the appropriate dosing regimen for maintaining therapeutic dystrophin levels long-term.

An important limitation revealed by the present study is inefficient cardiac dystrophin induction, which has been documented in previous in vivo studies using PMOs for dystrophin restoration and hypothesized to be related to differences in endocytosis in cardiomyocytes. This study revealed that exon 2 skipping at the highest PPMO-A dose reached a peak of 14% average skipped transcripts, 14% average dystrophin protein by western blot, and 16% average dystrophin-positive myocytes by IF in the heart. It is worth noting that one mouse in particular showed a markedly higher degree of exon 2 skipping in the heart (41.6% total skipped transcripts) by RT-PCR and displayed high cardiac dystrophin expression by both IF (57% positive myocytes) and western blot (42% of normal) 15 days after treatment. This mouse also showed the highest exon skipping in all skeletal muscles and the highest diaphragm dystrophin expression by western blot, but displayed otherwise similar muscle dystrophin levels compared with the rest of its treatment cohort. This interesting biological outlier was not omitted from the study in order to accurately represent the range of outcomes observed in individual animals treated with PPMO-A. Evidence from other studies using higher doses and biochemical modifications to augment PMO uptake has shown potential for higher expression in the heart, demonstrating the existence of multiple avenues for addressing limited cardiac efficacy in future studies. Such improvements would be vital for ensuring that increased cardiac workload due to prolonged ambulation and greater motor activity does not accelerate cardiac decline in patients receiving PPMO therapies for DMD.

Taken together, our results suggest that systemic delivery of PPMO-A shows significant promise as a therapeutic agent in the context of exon 2 duplications, resulting in a significant rescue of dystrophin in skeletal muscles. Animal health monitoring did not reveal any clinical signs of toxicity following PPMO treatment, and no mice were lost in the course of the study except for exclusion due to a spontaneous second Dmd mutation. Although this study was not designed to assess safety, the absence of any indications of toxicity suggests that the described PPMO-A likely has an improved safety profile compared with earlier PPMOs used in previously published mdx mouse studies for skipping exon 23,34,44. More recent work has also demonstrated well-tolerated PPMO treatment up to 80 mg/kg in mdx mice, confirming relative safety of similar PPMOs. For exon 2 skipping, further efforts in confirming safety and identifying the optimal dose and administration approaches for improving muscle function and cardiac efficacy will provide important groundwork for future clinical development.

MATERIALS AND METHODS

Animal studies and injections

Dup2 mice generated in the laboratory and age-matched WT control C57Bl/6 (Bl6) male mice were used in all experiments starting at...
Figure 5. Dystrophin immunofluorescence intensity in muscle after PPMO-A treatment
Cumulative histograms of muscle fiber dystrophin intensities in (A) left TA (L. TA) muscles and (B) diaphragm (Dia) from Dup2 mice treated with either PPMO-A or PPMO-Scr at the dose of 80 mg/kg, or saline (Sal) showing a dystrophin intensity across time points of 15, 30, and 60 days post-treatment in comparison with wild-type (Bl6) mice treated with saline. All intensity measurements are normalized to the Bl6 cohort at each time point, and the mean (line) ± range (shaded) is plotted for each group. Indicated significant differences, identified by Welch’s ANOVA with Dunnett’s T3 multiple comparison test, reflect comparisons based on the mean fiber intensity per tissue for all groups (n = 3–6 per group), shown in Figure S4. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Representative 1 mm² regions of (C) left TA and (D) diaphragm sections with laminin in green and dystrophin in red showing localization and intensity of dystrophin expression. Color-coded heatmaps of each region display the normalized dystrophin intensity at the perimeter of each muscle fiber. Color key in the bottom corner shows color to percent intensity conversion for dystrophin intensity heatmaps, where 100% corresponds to the median fiber intensity in Bl6 muscle.
12 weeks of age. C57Bl/6 mice were purchased from The Jackson Laboratory (Stock # 000664). All animal studies were performed according to the guidelines and approval of the Institutional Animal Care and Use Committee of the Abigail Wexner Research Institute at Nationwide Children’s Hospital. All animal studies were performed in a blinded fashion.

For the dose-escalation study, PPMO-A or PPMO-Scr were reconstituted in saline and injected into the tail vein of 12-week-old Dup2 mice at final doses of 20, 40, and 80 mg/kg, followed by euthanasia 7 days later. For the time course study, 12-week-old Dup2 mice were injected at a dose of 80 mg/kg and killed 15, 30, and 60 days post-injection. Age-matched Bl6 and Dup2 mice were injected with saline and killed at the same timepoints to be used as controls. Mice were killed using a lethal dose of ketamine/xylazine cocktail (Nationwide Children’s Hospital [NCH] pharmacy) for tissue collection. TA, gastrocnemius (Gast), quadriceps (Quad), diaphragm (Dia), and heart were snap-frozen in liquid nitrogen-cooled isopentane using standard methods and stored at −80°C until use. Tissue sections for RT-PCR, IF, and western blot analysis were cut using Cryostat CM1950 (Leica).

After completion of the study, the three animals in the 60-day PPMO-A treatment group were found to have a second, spontaneous Dmd mutation resulting in the deletion of exons 18 to 41 (del18-41). These Dup2Del18-41 mice displayed a complete absence of dystrophin protein expression and were omitted from the results, leaving only three mice in the PPMO-A 60-day treatment group for all assays. All remaining mice in the study were confirmed negative for this additional unintended mutation, including those in the negative control groups.

PPMO compounds production

The PPMO-A and PPMO-Scr peptide conjugates were synthesized and purified to >90% purity at Sarepta Therapeutics, Inc. These consist of a proprietary peptide conjugated to a 31-nt PMO sequence (GTATCTATTATTCGTCTATTCTTTCT) targeting the exon 2 splice acceptor site of the DMD gene. The development of this exon 2-targeting sequence has been described in our previous work. Due to 100% homology of the target region, the PPMO-A sequence is applicable to be used in both mouse and human DMD gene correction studies. PPMO-Scr sequence (GTATCTATTATTCGTCTATTCTTTCT) was designed as an antisense 30-nt long control for PPMO-A using online GenScript Bioinformatics software (https://www.genscript.com/tools/create-scrambled-sequence).

Both PPMO-A and PPMO-Scr compounds were dissolved in sterile saline and their concentrations were measured in the Sarepta Therapeutics facility before overnight shipping to a laboratory at NCH for experiments. Compounds were stored at 4°C and used 30 to 45 days after resuspension.

RNA extraction and exon 2 skipping analysis

Total RNA was extracted from frozen skeletal muscles and hearts through standard TRIzol/chloroform extraction (Life Technologies, cat # 15596018, Carlsbad, CA; Fisher Bioreagents, cat # C297-4, Hampton, NH) and then purified using RNA Clean & Concentrator-25 according to the manufacturer’s instruction (Zymo Research, cat # R1018, Tustin, CA). Reverse transcription (RT) was performed on 1,000 ng of total RNA with RevertAid RT Kit and random hexamer primers according to the manufacturer’s protocol (Thermo Scientific, cat #K1691, Waltham, MA). cDNA was amplified via PCR (Thermo Fisher, cat #K0171, Waltham, MA) using primers specific to the DMD 5’UTR and the exon 3–4 junction. Digital images of DNA bands on gels stained with ethidium bromide as an intercalating agent were quantified using ImageJ software (Version 1.46r; NIH, Bethesda, MD) to determine relative amounts of different amplicons. Primer sequences (IDT, Coralville, IA) and PCR program are available upon request.

Protein extraction and western blotting

Mouse tissue lysates were prepared by mechanically disrupting tissue in a TissueLyser II (Qiagen, USA) in lysis buffer containing 4M urea lysis buffer, pH 6.8; 150 μL of lysis buffer was added to 10 sections of 20-μm-thick tissue. Tissue was lysed using a metal bead (2 min at 30 Hz, TissueLyser II, Qiagen) with following samples incubation at room temperature for 30 min prior to a second lysis step (1 min at 30 Hz, TissueLyser II, Qiagen). The lysate was centrifuged at 14,000g for 20 min at room temperature and supernatants were collected for analysis. Total protein was quantified by using a Bio-Rad DC assay kit (Bio-Rad, cat # 5000112) following the manufacturer’s protocol. Calibration curve was made by diluting normal...
(WT) dystrophin from pooled C57Bl/6 mice (n = 6) into dystrophin-null lysate from age-matched Dup2Del18-41 mice (n = 3), due to a complete absence of dystrophin protein in Dup2Del18-41 tissue. The supernatant was mixed with a 4x Laemmli sample buffer and then boiled for 5 min at 90–100°C, and 30 μg of total protein was run on a precast 3% to 8% Tris-Acetate NuPage gel (Invitrogen, cat # EA0378BOX, USA) for 1 h at 80 V followed by 2 h at 120 V. Protein was transferred from gels to a 0.45-μm PVDF membrane (Cytiva Life Sciences Amersham Hybond, cat # 10600023) at a constant 55 mA current overnight with slow stirring at 4°C. HiMark pre-stained protein standard (Invitrogen, cat # LC5699) and/or precision plus protein dual color standards (Bio-Rad, cat # 1610394) were used to

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**Figure 7. Restoration of dystrophin detected by western blot following PPMO-A injection**

(A) Western blot images of left tibialis anterior (L. TA) muscles, diaphragm, and hearts showing dystrophin expression in Dup2 PPMO-A-treated mice at 15, 30, and 60 days after treatment. Individual treated samples are numbered 1 to 6, and the right six lanes of every blot contain a 6-point standard curve of pooled Bl6 (n = 6) lysate diluted in dystrophin-null muscle lysate, ranging from 0% to 100%. Sample numbers 1, 5, and 6 from 60-day cohort were found to have a spontaneous deletion of exons 18 to 41 in the Dmd gene, and were excluded from quantification. (B) Normalized dystrophin levels in Dup2 mice treated with PPMO-A, scrambled PPMO (PPMO-Scr), or saline (Sal). Data reported as mean ± SEM for each group and time point, with individual points reflecting individual samples. The dashed line indicates 100% based on the standard curve. Statistical analysis was performed using Welch’s ANOVA with Dunnett’s T3 multiple comparison test; *p < 0.05; **p < 0.01; ***p < 0.001.
determine the size of proteins of interest during gel running and protein transferring. Membranes were probed with dystrophin anti-rabbit polyclonal antibody (Abcam, cat # ab154168) at 1:1000 dilution in 5% non-fat dry milk in PBS buffer with 0.1% Tween 20 (PBST) for 2 h. Blots were then washed 5 × 5 min with 0.1% Tween in PBS, followed by 1 × 5 min wash in PBS. Membranes then were exposed to the secondary antibody, goat anti-rabbit HRP (1:5000) for 1 h at room temperature followed by 5 × 5 min washes with PBST and 1 × 5 min wash with PBS. Membranes were incubated with 2 mL of ECL reagent (Thermo Scientific, cat # 34580) prior to visualization on Chemidoc MP Imaging System (Bio-Rad).

Dystrophin signal was quantified as the area under the curve of the peak in the lane intensity profile line at the expected molecular weight using Image Lab software with rolling ball background subtraction (Bio-Rad, Version 6.0.0 build 25). The lanes with the six-point (0%-10%-20%-40%-80%-100%) standard curve of pooled WT and dystrophin-null Dup2Del18-41 lysates were quantified first, and the best-fit line of the standard curve was used to complete quantification of individual samples. The standard curve acceptance criteria required that R² ≥ 0.90, and the final standard curves had R² ≥ 0.927.

**Immunohistochemistry and microscopy**

Frozen muscles were cut at 10 μm, air-dried, permeabilized in 0.1% Triton X-100, and blocked in 1X PBS with 10% normal goat serum and 0.1% Tween 20. Following blocking, sections were co-stained in 1:400 rabbit monoclonal anti-dystrophin antibody (Abcam, cat # ab218198) and 1:400 rat anti-laminin (R&D Systems, cat # MAB4656) for 2 h at room temperature. Slides were washed 4 × 5 min, incubated in the appropriate Alexa Fluor 488 (Jackson Labs, cat # 712-546-153) or 568 (Invitrogen, cat # A-21069) conjugated secondary antibodies (1:500) for 1 h, and washed again 3 × 5 min. Cover slips were affixed with Vectashield Hard Set anti-fade mounting medium with DAPI (Vector Laboratories, cat # H-1500).

Whole-section images of skeletal muscles and hearts were collected on a fully motorized Nikon Ti2-E inverted microscope with Plan Apochromat Lambda objectives and a Hamamatsu ORCA Fusion camera within 6 h of completion of staining. Skeletal muscles were imaged using ×10 magnification at a resolution of 0.64 μm/pixel and hearts were imaged using ×20 magnification at a resolution of 0.32 μm/pixel.

**Microscope image analysis**

Analysis was carried out in Nikon NIS-Elements AR software using the General Analysis 3 software module and a custom analysis workflow developed for mouse tissue. Skeletal muscles were analyzed as whole tissue sections, using thresholds for dystrophin-positive and laminin-positive pixels that were empirically derived from the intensity profiles of both signals in untreated Dup2 tissue sections. Dystrophin-positive fibers were quantified by identifying all individual muscle fibers using laminin-positive boundaries, measuring the total length of dystrophin-positive segments around each muscle fiber, and normalizing it to the total length of the laminin-positive segment around the muscle fiber perimeter. The criterion for identifying a muscle fiber as overall positive for dystrophin was set at 50% or more of the perimeter. Fiber dystrophin intensity was analyzed by measuring the mean intensity of all pixels in the dystrophin channel within a 5-μm-thick boundary region around each muscle fiber.

Muscle fiber size was measured in terms of area and minimum Feret’s diameter automatically as a part of the dystrophin analysis. In a separate analysis, centrally nucleated fibers were identified as those having at least one DAPI nucleus overlapping with the interior region of the fiber that was eroded in from the laminin-positive sarcolemma by 5 μm, and the % centrally nucleated fibers was calculated for each tissue.

Hearts were analyzed as three-square ROIs sampled from the transverse myocyte regions of each heart section, with each ROI measuring 0.5 × 0.5 mm. The ROI sampling was performed using the laminin channel by an operator blinded to the dystrophin channel. The analysis workflow used to quantify dystrophin-positive myocytes and myocyte dystrophin intensity in heart ROIs followed the same approach as the skeletal muscle analysis described above. Cardiac myocytes touching the edges of the ROI field were excluded from analysis. Quantification results from the three ROIs for each heart were pooled to produce a single result for dystrophin-positive myocytes and myocyte dystrophin intensities for each heart.

All figure representative images were selected to appropriately reflect the center of the group’s distribution rather than any extreme values or outliers.

**Statistical analysis**

All results are presented as mean value ±SEM. Differences between groups within a given time point were assessed by one-way ANOVA with Sidak’s multiple comparison test for groups with comparable standard deviations, or Welch’s ANOVA with Dunnett’s T3 multiple comparison test for groups with significantly different standard deviations. Individual comparisons were performed for PPMO-A versus each control group (C57Bl/6, PPMO-Scr, and saline). Dose-escalation results were analyzed by linear regression. Significance was determined based on α = 0.05.

**DATA AVAILABILITY STATEMENT**

Qualified researchers may request access to the data that support the findings of this study from the corresponding author by contacting kevin.lanigan@nationwidechildrens.org.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.10.025.

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AUTHOR CONTRIBUTIONS
L.V.G. designed the study, acquired data, interpreted data, created figures, and drafted the manuscript. T.A.V. developed the quantitative image analysis, acquired data, interpreted data, created figures, and drafted the manuscript. E.C.F., A.J.B., K.M.G., and J.L. acquired data, interpreted data, and created figures. N.H. performed the mammalian experiments and prepared tissues. A.S. prepared tissues and acquired data. F.J.S. and G.H. designed and produced reagents and designed experiments. T.R.S. designed experiments and reviewed the manuscript. N.W. and K.M.F. designed the study, reviewed and interpreted the data, and reviewed the manuscript.

DECLARATION OF INTERESTS
K.M.F. has received research support for a clinical trial from Sarepta Therapeutics. K.M.F. and N.W. hold a patent related to therapeutic exon 2 skipping, and along with Nationwide Children’s Hospital receive royalties from Astellas Gene Therapy for that patent, which is not directly related to the data presented here.

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Supplemental information

Systemic PPMO-mediated dystrophin expression in the Dup2 mouse model of Duchenne muscular dystrophy

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Supplemental Figures

Figure S1. Animals body weights. (A) Dup2 mice in dose escalation and (B) time-point studies before injections and prior to necropsies. (A) Dup2 mice treated with saline (Sal), PPMO-A at the dose of 20 mg/kg (A-20), 40 mg/kg (A-40) and 80 mg/kg (A-80), PPMO-Scr (Scr-80) and saline treated C57Bl/6 (Bl6) at 7 days treatment. (B) Dup2 mice treated with saline (Sal), PPMO-A (PPMO-A) and PPMO-Scramble (PPMO-Scr) at the dose of 80 mg/kg and saline treated Bl6 at 15, 30, and 60 days after. Data reported as absolute value for each group and timepoint, with individual points representing individual mice.
Figure S2. Gel images of dose-dependent PPMO-induced exon 2 skipping. Exon 2 exclusion in Dup2 mice (n=5-6) treated for 7 days with a dose escalation of PPMO-A (20, 40 and 80 mg/kg), PPMO-Scr (80 mg/kg) or saline (Dup2-Sal), and C57Bl/6 (Bl6-Sal) (n=5) injected with saline as a control. Right side of gastrocnemius (R. Gas), tibialis anterior (R. TA), diaphragm (Dia) and heart in all groups was used in RT-PCR analysis. Dup2, wild type (WT), and Del2 mRNA transcripts are represented as 340 bp, 278 bp and 216 bp bands, respectively. The stars indicate the lanes with drop-outs due to technical error. † is RT(–) and ‡ is PCR(–) negative controls, in which water was used as a template for RT and PCR steps to ensure that the tested samples were free of DNA contamination and non-specific amplification. Ladder, 1kb Plus DNA ladder.
PPMO-mediated therapy in Dup2 (Gushchina)
Figure S3. Gel images of the time course of PPMO-induced exon 2 skipping. Exon 2 exclusion in Dup2 mice at 15, 30 and 60 days post injection with (n=5-6) treated with PPMO-A (80mg/kg), PPMO-Ser (80 mg/kg) or saline (Dup2-Sal), and C57Bl/6 (Bl6-Sal) (n=5) injected with saline as a control. Right side of gastrocnemius (R. Gas), tibialis anterior (R. TA), diaphragm (Dia) and heart in all groups was used in RT-PCR analysis. Dup2, wild type (WT) and Del2 mRNA transcripts are represented as 340 bp, 278 bp and 216 bp bands, respectively. The stars indicate the lanes with drop-outs due to technical error. † is RT(–) and ‡ is PCR(–) negative controls, in which water was used as a template for RT and PCR steps to ensure that the tested samples were free of DNA contamination and non-specific amplification. Ladder, 1kb Plus DNA ladder.
Figure S4. Mean sarcolemmal dystrophin intensities after PPMO-A treatment.

Immunofluorescence (IF) quantification showing the mean sarcolemmal dystrophin intensities in left tibialis anterior (L. TA), diaphragm (Dia), and hearts from Dup2 mice injected with either PPMO-A, PPMO-Scr, or saline (Sal). All values are normalized to wild type (Bl6) mean intensity at the respective timepoint. The bars represent the mean ± SEM, with individual data points showing the mean fiber dystrophin intensity for each sample. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Statistically significant differences identified by Welch’s ANOVA with Dunnett’s T3 multiple comparison test.
Figure S5. Central nucleation in muscle after treatment with PPMO-A. The percent centrally nucleated (CN) fibers in Dup2 (A) left TA muscles and (B) diaphragms is shown as mean ± SEM for each treatment group and timepoint, with individual data points showing the percent CN fibers for each mouse. Statistically significant differences identified by ordinary one-
way ANOVA with Sidak’s multiple comparison test. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$. Representative 0.5 × 0.5 mm regions of (C) left TA and (D) diaphragm sections with laminin in green and DAPI in blue show peripheral and internalized nuclei. Color-coded fiber maps of each region display the automated categorization of each fiber as centrally nucleated (CN) or not (Non-CN) based on detected DAPI signal location.
Figure S6. Effects of PPMO-A treatment on muscle fiber size. Histograms of fiber minimum Feret’s diameter in (A) left TA muscles and (B) diaphragms show fiber size in Dup2 mice treated with PPMO-A, scrambled PPMO (PPMO-Scr), or saline (Sal) in comparison to wild type (Bl6) muscle. (C) Summary quantification of the fiber size as minimum Feret’s diameter mean ± SEM for each treatment group and timepoint, with individual data points showing the mean for each mouse. Statistically significant differences identified by ordinary one-way ANOVA with Sidak’s multiple comparison test; *p<0.05; **p<0.01.
Supplemental Tables

**Table S1.** Dose Escalation study design.

| Strain   | Sex | Treatment | n  | Doses (mg/kg) | In-Life Duration (days) | Body Weight Before Injections (g) (mean ± SD) | Body Weight Prior to Necropsy (g) (mean ± SD) |
|----------|-----|-----------|----|---------------|-------------------------|-----------------------------------------------|-----------------------------------------------|
| Dup2     | Male| Saline    | 7  | N/A           | 7                       | 28.91 ± 2.57                                  | 27.30 ± 2.1                                   |
| Dup2     | Male| PPMO-A    | 6  | 20            | 7                       | 27.35 ± 2.34                                  | 27.80 ± 2.29                                  |
| Dup2     | Male| PPMO-A    | 5  | 40            | 7                       | 29.40 ± 3.22                                  | 30.18 ± 3.44                                  |
| Dup2     | Male| PPMO-A    | 5  | 80            | 7                       | 25.28 ± 0.58                                  | 25.58 ± 0.69                                  |
| Dup2     | Male| PPMO-Scr  | 6  | 80            | 7                       | 27.02 ± 1.05                                  | 27.15 ± 0.82                                  |
| C57Bl/6  | Male| Saline    | 6  | N/A           | 7                       | 27.87 ± 0.89                                  | 28.37 ± 0.82                                  |
**Table S2.** Time-Point study design.

| Strain    | Sex | Treatment | n  | Doses (mg/kg) | In-Life Duration (days) | Body Weight Before Injections (g) (mean ± SD) | Body Weight Prior to Necropsy (g) (mean ± SD) |
|-----------|-----|-----------|----|---------------|------------------------|-----------------------------------------------|-----------------------------------------------|
| Dup2      | Male| Saline    | 6  | N/A           | 15                     | 27.90 ± 0.69                                  | 30.25 ± 0.85                                  |
| Dup2      | Male| PPMO-A    | 6  | 80            | 15                     | 27.60 ± 3.09                                  | 27.88 ± 2.69                                  |
| Dup2      | Male| PPMO-Scr  | 5  | 80            | 15                     | 27.90 ± 2.44                                  | 29.94 ± 3.27                                  |
| C57Bl/6   | Male| Saline    | 7  | N/A           | 15                     | 26.50 ± 2.13                                  | 26.56 ± 1.88                                  |


Table S3. Summary of transcript quantification in Dose Escalation study. Table provided as separate Excel file.

Table S4. Summary of transcript quantification in Time-Point study. Table provided as separate Excel file.
### Table S5. Summary of western blot results in Time-Point study.

| Strain  | Treatment | Tissue                | % Dystrophin expression by WB | 15 days # of mice | Mean ± SEM | 30 days # of mice | Mean ± SEM | 60 days # of mice | Mean ± SEM |
|---------|-----------|-----------------------|------------------------------|-------------------|------------|-------------------|------------|-------------------|------------|
| Dup2    | Saline    | Tibialis Anterior (TA)|                              | 5                 | 2.4 ± 0.3  | 3                 | 2.1 ± 0.6  | 6                 | 3.9 ± 0.5  |
| Dup2    | PPMO-A    | Diaphragm (Dia)       |                              | 6                 | 22.1 ± 2.4 | 6                 | 22.8 ± 4.0 | 3                 | 17.8 ± 5.4 |
| Dup2    | PPMO-Scr  | Heart                 |                              | 5                 | 1.9 ± 0.2  | 6                 | 4.3 ± 0.4  | 6                 | 3.4 ± 0.3  |
|         | Saline    | Tibialis Anterior (TA)|                              | 6                 | 5.7 ± 0.8  | 6                 | 2.7 ± 0.3  | 6                 | 3.9 ± 0.5  |
| Dup2    | PPMO-A    | Diaphragm (Dia)       |                              | 6                 | 56.8 ± 4.6 | 6                 | 15.7 ± 1.8 | 3                 | 22.3 ± 8.1 |
| Dup2    | PPMO-Scr  | Heart                 |                              | 5                 | 5.7 ± 0.3  | 6                 | 4.7 ± 0.6  | 5                 | 7.0 ± 0.8  |
|         | Saline    | Heart                 |                              | 5                 | 2.6 ± 0.6  | 4                 | 4.8 ± 0.5  | 6                 | 3.8 ± 1.0  |
| Dup2    | PPMO-A    | Heart                 |                              | 6                 | 13.8 ± 5.7 | 6                 | 7.6 ± 1.2  | 3                 | 5.8 ± 0.3  |
| Dup2    | PPMO-Scr  | Heart                 |                              | 5                 | 5.2 ± 1.0  | 6                 | 4.9 ± 0.3  | 6                 | 4 ± 0.4    |
Table S6. Summary of Dystrophin Positive Fibers in Time-Point Study.

| Strain  | Treatment | Tissue                  | 15 days | 30 days | 60 days |
|---------|-----------|-------------------------|---------|---------|---------|
|         |           |                         | # of mice | Mean ± SEM | # of mice | Mean ± SEM | # of mice | Mean ± SEM |
| Dup2    | Saline    | Tibialis Anterior (TA)  | 6       | 0.4 ± 0.1 | 6       | 0.8 ± 0.4 | 6       | 2.4 ± 0.9 |
| Dup2    | PPMO-A    | Diaphragm (Dia)         | 5       | 0.6 ± 0.3 | 6       | 2.1 ± 1.1 | 6       | 0.7 ± 0.3 |
| Dup2    | PPMO-Scr  | Heart                   | 5       | 0.8 ± 0.3 | 6       | 0.3 ± 0.2 | 6       | 1.7 ± 0.5 |
| C57Bl/6 | Saline    |                         | 6       | 99.8 ± 0.0 | 6       | 99.8 ± 0.0 | 6       | 99.5 ± 0.2 |
| Dup2    | Saline    |                         | 6       | 87.2 ± 2.8 | 5       | 85.6 ± 6.6 | 3       | 64.5 ± 23.7 |
| Dup2    | PPMO-A    |                         | 6       | 81.2 ± 2.8 | 6       | 76.9 ± 6.2 | 3       | 62.5 ± 16.0 |
| Dup2    | PPMO-Scr  |                         | 5       | 97.9 ± 0.7 | 5       | 97.5 ± 0.5 | 5       | 98.7 ± 0.3 |
| C57Bl/6 | Saline    |                         | 5       | 0.2 ± 0.1 | 6       | 0.2 ± 0.1 | 6       | 0.1 ± 0.1 |
| Dup2    | PPMO-A    |                         | 6       | 15.5 ± 8.5 | 6       | 4.1 ± 1.2 | 3       | 1.6 ± 0.3 |
| Dup2    | PPMO-Scr  |                         | 5       | 0.0 ± 0.0 | 6       | 0.1 ± 0.1 | 6       | 0.1 ± 0.0 |
| C57Bl/6 | Saline    |                         | 6       | 99.9 ± 0.0 | 6       | 99.9 ± 0.1 | 6       | 99.9 ± 0.0 |
**Table S7. Summary of Dystrophin Intensities in Time-Point Study.**

| Strain   | Treatment | Tissue          | Dystrophin Intensities by IF |
|----------|-----------|----------------|-----------------------------|
|           |           |                | 15 days | 30 days | 60 days |
|           |           |                | # of mice | Mean ± SEM | # of mice | Mean ± SEM | # of mice | Mean ± SEM |
| Dup2     | Saline    | Tibialis Anterior (TA) | 6 | 18.5 ± 0.5 | 6 | 18.5 ± 0.9 | 6 | 18.5 ± 0.2 |
| Dup2     | PPMO-A    |                | 6 | 51.3 ± 2.8 | 5 | 50.3 ± 4.4 | 3 | 38.2 ± 11.7 |
| Dup2     | PPMO-Scr  |                | 5 | 17.3 ± 0.6 | 6 | 19.1 ± 0.5 | 6 | 18.0 ± 0.7 |
| C57Bl/6  | Saline    |                | 6 | 100.0 ± 2.3 | 6 | 100.0 ± 4.6 | 6 | 100.0 ± 2.0 |
| Dup2     | Saline    | Diaphragm (Dia) | 6 | 14.1 ± 0.6 | 5 | 13.1 ± 1.2 | 6 | 12.9 ± 1.3 |
| Dup2     | PPMO-A    |                | 5 | 40.9 ± 2.1 | 6 | 41.9 ± 4.4 | 3 | 34.2 ± 3.7 |
| Dup2     | PPMO-Scr  |                | 5 | 13.1 ± 1.0 | 6 | 11.8 ± 0.9 | 6 | 16.0 ± 1.0 |
| C57Bl/6  | Saline    |                | 5 | 100.0 ± 8.8 | 5 | 100.0 ± 6.2 | 5 | 100.0 ± 4.8 |
| Dup2     | Saline    | Heart          | 5 | 13.6 ± 0.7 | 6 | 14.3 ± 0.5 | 6 | 15.3 ± 0.4 |
| Dup2     | PPMO-A    |                | 6 | 20.5 ± 3.3 | 6 | 15.8 ± 0.5 | 3 | 15.2 ± 0.6 |
| Dup2     | PPMO-Scr  |                | 5 | 12.7 ± 0.3 | 6 | 13.1 ± 0.2 | 6 | 15.2 ± 0.4 |
| C57Bl/6  | Saline    |                | 6 | 100.0 ± 7.1 | 6 | 100.0 ± 2.1 | 6 | 100.0 ± 2.2 |