Mitochondria mediate cell membrane repair and contribute to Duchenne muscular dystrophy

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Dystrophin deficiency is the genetic basis for Duchenne muscular dystrophy (DMD), but the cellular basis of progressive myofiber death in DMD is not fully understood. Using two dystrophin-deficient mdx mouse models, we find that the mitochondrial dysfunction is among the earliest cellular deficits of mdx muscles. Mitochondria in dystrophic myofibers also respond poorly to sarcolemmal injury. These mitochondrial deficits reduce the ability of dystrophic muscle cell membranes to repair and are associated with a compensatory increase in dysferlin-mediated membrane repair proteins. Dysferlin deficit in mdx mice further compromises myofiber cell membrane repair and enhances the muscle pathology at an asymptomatic age for dysferlin-deficient mice. Restoring partial dystrophin expression by exon skipping improves mitochondrial function and offers potential to improve myofiber repair. These findings identify that mitochondrial deficit in muscular dystrophy compromises the repair of injured myofibers and show that this repair mechanism is distinct from and complimentary to the dysferlin-mediated repair of injured myofibers.

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Abbreviations: DMD, Duchenne muscular dystrophy; DAPC, dystrophin-associated protein complex; LC, lengthening contraction; DDKO, Dysferlin-Dystrophin knockout; PO, procion orange; PMO, phosphorodiamidate morpholino oligomer; SILAM, stable isotope labeling in mammal; IM, intramuscular

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increased calcium overload in dystrophic myofibers and mitochondria causes mitochondrial dysfunction, which in turn diminishes the repair ability of the dystrophic myofibers and results in their death.

To investigate the above hypothesis, we have analyzed the involvement of mitochondria in sarcolemmal repair of dystrophin-deficient myofibers. For this, we made use of two mdx mouse models, both lacking dystrophin expression. The first one, mdx-23, has a mutation in the exon 23 of the Dmd gene in the C57BL/10 mouse strain, and the second, mdx-52, lacks exon 52 of the Dmd gene on the C57BL/6 genetic background. We monitored the effect of dystrophin deficit on mitochondrial level, function, and myofiber repair. This showed that both mdx mouse models have reduced mitochondrial activity and myofiber repair ability. These deficits in mdx muscle were accompanied with increased level of dysferlin and associated sarcolemmal repair proteins. Deletion of dysferlin in the mdx muscle showed that dysferlin-mediated sarcolemmal repair complements mitochondria-mediated sarcolemmal repair. By rescuing dystrophin expression using exon skipping and by acutely activating mitochondrial respiration, we evaluate the efficacy of these approaches to improve mdx myofiber repair.

Results

Muscle proteome changes at disease onset in mdx mice. Dystrophin deficiency affects myofiber structure and increases myofiber leakiness by 21 days of age. To monitor the earliest changes in the muscle proteome due to dystrophin deficit, we carried out quantitative proteomic analysis in triplicate of gastrocnemius muscles from mdx mice (C57BL/10ScSn-Dmdmdx/J), henceforth referred to as mdx-23 at postnatal days 21 and 28 and BL/10 wild type (WT) at postnatal day 28 by using the stable isotope labeling approach that we previously described. Across all 9 samples, 1138 proteins were identified and accurately quantified (false detection rate ≥ 0.01, ≥ 2 peptides, protein ratio obtained relative to SILAM spike-in ≥ 50% of the samples – 5 out of 9). By hierarchical cluster analysis of the entire proteome, we described. Across all 9 samples, 1138 proteins were identified and accurately quantified (false detection rate ≥ 0.01, ≥ 2 peptides, protein ratio obtained relative to SILAM spike-in ≥ 50% of the samples – 5 out of 9). By hierarchical cluster analysis of the entire proteome, we found that 21-day-old mdx-23 co-clustered with the BL/10 WT muscle (Supplementary Figure S1a), but 28-day-old mdx-23 clustered independently from the BL/10 WT muscle (Figure 1a). Unlike 21-day-old mdx-23, which did not yield any protein whose level differed significantly (FDR < 0.05) from BL/10 WT, 28-day-old mdx-23 showed 249 proteins with significantly different levels (Supplementary Table S1). Accordingly, comparing average protein expression of 21-day-old mdx-23 and BL/10 WT showed a Pearson correlation coefficient of 0.93 (Supplementary Figure S1b), which was reduced to 0.81 when 28-day-old mdx-23 were compared with BL/10 WT (Figure 1b, proteins with FDR (q) < 0.05 are shown in red). Thus onset of disease pathology at the protein level occurs between 21 and 28 days of age in mdx-23 mouse. To validate this further, we analyzed the mdx-52 mice, which are on C57BL/6 genetic background and have a targeted insertion in exon 52 of the Dmd gene. This insertion results in loss of expression of full-length dystrophin and of the shorter dystrophin isoforms, Dp140 and Dp260. Gastrocnemius muscle of 28-day-old BL/6 WT and mdx-52 were analyzed in triplicate and combined with the analysis of BL/10 and mdx-23 muscle, which resulted in quantitative identification of 1178 proteins using the cutoff criterion listed above and mandatory protein detection in > 50% (7 out of the 12) samples. Hierarchical cluster analysis identified that 28-day-old mdx-52 clustered with age-matched mdx-23 mice but distinct from the age-matched parental WT strain C57BL/6 (Figure 1a). These analyses identify that, independent of genetic background and the mutant allele, the muscle proteome changes due to dystrophin deficiency occurs between postnatal days 21 and 28. These changes corroborate with muscle histological changes, including the presence of small, centrally nucleated, regenerating fibers in both mdx models at 28 days (Figure 1c) but not at 21 days of age (Supplementary Figure S1c). With the similar proteomic changes, histopathology and age of disease onset in mdx-23 and mdx-52 models, we used both models in this study.

Proteins in dysferlin-mediated sarcolemmal repair, including annexins A1 and A2, are altered in mdx muscle. Proteomic analysis confirmed altered annexin expression, but owing to the use of a stringent inclusion cutoff, low abundant proteins such as dysferlin, which we found increased in mdx mice by 20–80%, did not meet criterion for inclusion in the list of altered proteins (Supplementary Table S1). Owing to the known fragility of mdx myofibers and the relevance of dysferlin and associated proteins in sarcolemmal repair, we analyzed the expression of sarcolemmal repair proteins by using western blotting (WB). This showed both mdx-23 and mdx-52, which lack dystrophin expression (Figures 1d and i), have increased abundance of dysferlin and associated membrane repair proteins at 28 days of age. Although dysferlin level in mdx-23 muscle increased by 1.5-fold (Figure 1e), annexin A1 and A2 increased by over 2-fold (Figures 1f and g). A dysferlin-interacting protein, caveolin-3, which did not significantly increase in the 28-day-old mdx-23 muscle proteome, also appeared unchanged even by WB (Figure 1h). The mdx-52 muscle also showed 1.5-fold increase in dysferlin (Figure 1j), a 2-fold increase in annexin A1 and A2 level, and here caveolin-3 level was over 2-fold more (Figures 1k–m) than the matched WT C57BL/6 muscles. The coincident increase in dysferlin and interacting protein levels in mdx models identified that, similar to DMD patients where dysferlin expression is increased, dystrophin deficit increases dysferlin-mediated sarcolemmal repair at the onset of disease symptoms.

Mitochondrial level and response to sarcolemmal injury is reduced in mdx muscle. Mitochondrial respiratory function is known to be reduced in mdx and DMD patient muscles, and this was supported by our previous findings. Of the 79 dysregulated organelle proteins identified, over a third localize to mitochondria (Supplementary Table S2). Proteins from other organelles, including peroxisomes, endosomes, lysosomes, nucleus, and Golgi, accounted for less than a third of the altered organelar proteins. To directly assess whether this reflects altered mitochondrial respiratory activity, we monitored Cytochrome C enzyme activity in mitochondria isolated from 28-day-old
Figure 1  Cell membrane repair proteome changes at disease onset in mdx mice. (a) Hierarchical cluster analysis of the 1178 proteins identified in 28-day-old mdx-23, mdx-52, and their respective age-matched WT strains. (b) Pearson correlation analysis of proteins from 28-day-old mdx-23 and parental WT strain. Significantly altered proteins (FDR q < 0.05) are shown in red. (c) Images showing histological features of gastrocnemius muscle cross-sections from 28-day-old mdx-23, mdx-52, and their respective age-matched WT strains. Note the presence of centrally nucleated, regenerating fibers and inflammatory foci in the muscles on mdx cross-sections. Scale bar = 20 μm. (d) Image showing a WB of proteins from 28-day-old BL/10 WT (N = 3) and mdx-23 mice (N = 4). (e–h) Quantification of the expression level of dysferlin and associated proteins (normalized to the loading control and presented as the fold increase over the normalized average WT level) in the mdx-23 and BL/10 WT muscle. (i) Image showing WB of mdx-52 proteins at 28 days of age from three independent mice. (j–m) Quantification of the expression level of dysferlin and associated proteins (normalized to the loading control and presented as the fold increase over the normalized average WT level) in mdx-52 and BL/6 WT. All graphs are presented as mean ± S.D. *P < 0.05 by t-test.
mdx-52 muscle. Compared with C57BL/6, mitochondria from mdx-52 muscles showed a 2-fold reduction in respiratory activity (Figure 2a). Next we examined whether the mdx muscle show reduced mitochondria level. We quantified two independent mitochondrial structural proteins – voltage-dependent anion channel (VDAC-1) and translocase of outer membrane 20 (TOM20). Compared with the respective parental WT mice, the level of both these proteins was decreased in the 28-day-old mdx-52 (Figures 2b–d) as well as mdx-23 muscle (Figures 2e–g). To monitor the mitochondrial membrane potential in situ in myofibers, we used MitoTracker CM-H2TMRos, which localizes to mitochondria based on mitochondrial membrane potential, and becomes fluorescent upon being oxidized, thus allowing in situ monitoring of mitochondrial membrane potential.37,38 This showed that the 28-day-old mdx-52 myofibers have a significant in situ reduction in mitochondrial membrane potential (Figure 2h).

We have previously shown that sarcolemmal injury causes mitochondrial accumulation at the site of repair, which is compromised by mitochondrial depolarization.28 Owing to the reduced mitochondrial potential in mdx muscle, we monitored...
mitochondrial response to sarcolemmal injury in mdx myofibers. Within 20 s of sarcolemmal injury, mitochondria accumulated at the repair site (Figure 2i), but the extent of accumulation was significantly reduced in mdx myofibers (Figure 2j). Recently, increase in autophagy has been linked to early compensatory stage of DMD progression.39 Thus we examined whether reduced mdx mitochondrial potential and response to sarcolemmal injury is associated with increased mitophagic response. The level of the lysosomal membrane protein LAMP-1 is increased in mdx muscle (Supplementary Figures S2a and b) and an increasing trend was observed for the autophagic marker — lipidation of LC3 (Supplementary Figures S2a and c). Thus disease onset in mdx muscle involves mitochondrial dysfunction and enhanced autophagy may aid to clear these mitochondria.

Dystrophin-deficient mdx myofibers repair poorly in response to sarcolemmal injury. With the increased fragility of mdx myofibers and compromised mitochondrial response to sarcolemmal injury, we hypothesized that increased dysferlin-dependent membrane repair proteins aid with the repair of mdx myofibers. To assess sarcolemmal repair, following laser injury, we imaged entry of a membrane impermeable lipid-binding dye FM1-43 into the myofiber of 28-day-old mdx-52, as before.33,41 Unlike the myofibers from the parental BL/6 WT, dye entry into mdx myofibers continued steadily (green staining) eventually causing myofiber hypercontraction (Figures 3a and b). Most of the mdx-52 myofibers hypercontracted, preventing quantification of the FM-dye entry kinetics as we have carried out in other studies.30,41 Only 25±9% of mdx-52 myofibers (N=34) showed the same low FM dye entry that was seen in the WT myofibers (Figures 3b and c). This is in contrast to BL/6 WT myofibers where 66±8% (N=22) of myofibers repaired (Figures 3b and c).

Muscle contraction induces greater sarcolemmal damage to mdx-23 myofibers,4 but this has not been tested for the mdx-52 muscles. We thus evaluated the ability of mdx-52 mice myofibers to repair from ex vivo eccentric injury caused by 10% lengthening contraction (LC). After the very first eccentric injury, the extensor digitorum longus (EDL) muscles from 28-day-old mdx-52 mice exhibited a greater loss in the contractile force as compared with the parental BL/6 WT muscle (Figure 3d). This loss was even greater following subsequent round of LC-induced injuries and by the ninth LC injury, the loss in contractile force was 35% greater in mdx-52 muscle as compared with the BL/6 WT muscle (Figure 3d). To assess whether greater force loss owing to LC injury correlated with similar increase in sarcolemmal damage, the muscles were incubated in cell-impermeable dye procion orange (PO). A single LC injury to EDL muscles caused PO labeling of 8.8±8.6 mdx myofibers/muscle section, which increased with additional LC injuries (Figures 3e and f). In contrast, even after 9 LC injuries only 4.3±2.6 BL/6 WT myofibers were PO labeled in each EDL muscle section (Figure 3g). Thus two independent methods of myofiber injury show greater loss in sarcolemmal integrity in the dystrophin-deficient mdx myofibers. This suggests that absence of dysferlin and subsequent mitochondrial pathology impairs repair of mdx myofibers despite the upregulation of dysferlin and associated membrane repair proteins.

Dystrophin-deficient myofibers rely on dysferlin for sarcolemmal repair. In view of the reduced mitochondrial function and poor repair of mdx myofiber sarcolemmal injury, we hypothesized that the increased expression of the dysferlin-mediated sarcolemmal repair proteins offsets some of repair deficit of dystrophin-deficient myofibers. To test this, we crossed mdx-52 and B6A/J mice to generate congenic C57BL/6 mice lacking both dysferlin and dystrophin protein expression (Dysferlin-Dystrophin Knockout (DDKO)). PCR genotyping and WB analysis confirmed that dystrophin and dysferlin genes are mutated in these mice and neither of these proteins are expressed in the skeletal muscle of the DDKO mice (Figures 4a and b). These mice grow to adulthood and breed to produce healthy litters. Loss of dysferlin in B6A/J mice does not cause histological and functional deficit for the first 2 months of life.42 However, histological analysis showed that DDKO muscle exhibit greater pathology than the dystrophin-and-dystrophin-deficient BL/6 muscle (Figure 4c) and have high level of pro-inflammatory marker interleukin-1β (Figure 4d).

To investigate the relevance of dysferlin expression for the repair of injured mdx myofibers, we monitored sarcolemmal repair kinetics of DDKO mice at 28 days of age, when lack of dysferlin alone does not result in muscle pathology.43 In contrast to the mdx myofibers where 21±8% of myofibers repaired (replicating the findings in Figure 3), only 8±4% of the DDKO myofibers repaired (N=29 myofibers) (Figure 4e). This –3-fold decrease in myofiber repair ability suggests that increased dysferlin expression in mdx muscle is crucial for the limited ability of mdx myofiber repair. To further assess the role of dysferlin in mdx myofiber repair, we examined the contractile force and the recovery of EDL muscle from LC injury. DDKO muscles have lower contractile force than C57BL/6 and mdx-52 mice (Figure 4f) and showed increased susceptibility to LC-induced damage. Following 9 successive LC injuries, DDKO muscles lost >80% of original contractile force as compared with the loss of 60% in mdx-52 and of <30% in the WT EDL muscle (Figure 4g). By PO labeling following LC injury, we found that greater loss of contractile force in DDKO muscle matches with nearly 2-fold and 10-fold more PO-labeled DDKO fibers as compared with mdx and WT EDL muscles, respectively (Figure 4h). To assess whether the poor repair of DDKO myofibers involves further reduction in the ability of mitochondria to accumulate at the site of injury, we monitored mitochondrial response to sarcolemmal injury in DDKO myofibers and found it was similar to what we observed in mdx myofibers (Figures 4i and j versus Figures 2i and j). The above results demonstrate that presence of dysferlin in the young mdx mice is critical for myofiber repair and thus dysferlin-mediates sarcolemmal repair independently of mitochondria. Poor sarcolemmal repair of the dystrophin-deficient skeletal muscle due to the mitochondrial deficit is offset in part by the increase in dysferlin-mediated repair machinery.
Effect of exon skipping and increased mitochondrial respiration on mdx sarcolemmal repair. As lack of dystrophin is the primary defect responsible for the mitochondrial dysfunction and poor repair of mdx myofibers, we tested whether acute expression of a dystrophin protein by exon skipping therapy would improve the ability of mdx myofibers...
to repair. We have established that a single high bolus (800 mg/kg) systemic delivery of antisense oligonucleotide (phosphorodiamidate morpholino oligomer (PMO)) re-expresses dystrophin in mdx-23 mice. One month after administering PMO to 28-day-old mdx-23 mice (N=6), the highest dystrophin expression in the EDL muscle was 2% of the WT (C57BL/10) (Supplementary Figures S3a and b). This increase in protein expression only marginally (5.9%)
increased EDL specific force but not its recovery from LC injury (Supplementary Figure S3c).

To further enhance exon skipping efficacy, flexor digitorum brevis (FDB) muscles from mdx-23 mice were treated with three, weekly intramuscular (IM) PMO injections at 20 mg/muscle. Two weeks after the last injection, dystrophin level was measured and was 8 ± 2.12% of the BL/10 WT level (Figures 5a and b). Using laser injury, we assessed the effect of this increased dystrophin expression on sarcolemmal repair. As with the mdx-52 myofibers (Figure 3), 21 ± 2% of mdx-23 fibers (N = 30 myofibers) repaired following focal sarcolemmal injury (Figure 5c), which doubled to 42 ± 12% (N = 26 myofibers) upon dystrophin re-expression (Figure 5c). The repair response between the muscles from different
PMO-treated mice was significantly heterogeneous owing to which, despite the 2-fold improvement in myofiber repair, the increase was not statistically significant.

Providing pyruvate as the substrate for mitochondrial respiration improves force production by dystrophin-and-utrophin-deficient muscle. To test whether pyruvate treatment increases mitochondrial respiration in the mdx myofibers, we again made use of the CM-H2TMRos dye. Although depolarizing mitochondria with CCCP decreased, 100 mM pyruvate increased CM-H2TMRos fluorescence (Figure 5d).

Independently, using a genetically encoded ATP sensor ATeam, we monitored change in ATP production at single cell level. Pyruvate increased, whereas the respiratory chain poison Azide decreased ATP production as indicated by the ATeam FRET signal (Figures 5e and f). Pyruvate-mediated increase in mitochondrial potential and ATP production established that 100 mM pyruvate treatment is sufficient to boost energy production by mdx mitochondria (Figure 5d). We next tested whether increasing mitochondrial bioenergetics can improve mdx sarcolemmal repair. Treating muscles with 100 mM pyruvate increased CM-H2TMRos intensity by 2-fold (Figures 5g and h) but did not significantly increase the EDL-specific force: 126.7 ± 25.94 in untreated versus 141.33 ± 14.83 KN/m² in pyruvate-treated (N=6 muscles each), nor improved mitochondrial accumulation at the site of injury. We next assessed the effect of pyruvate treatment on myofiber repair following laser injury. A total of 22.2 ± 7.7% of untreated mdx-23 myofibers (N=36) repaired and pyruvate treatment did not significantly improve myofiber repair (29.2 ± 7.65% myofibers; N=49) (Figure 5i). Thus, while dystrophin re-expression holds the potential for improving mdx myofiber repair ability, once mitochondrial dysfunction sets in, an acute increase in respiratory activity of these dysfunctional mitochondria is insufficient to improve dystrophic myofiber repair.

**Discussion**

Our investigation of early events during disease initiation due to dystrophin-deficit has identified poor sarcolemmal repair as an early consequence of dystrophin deficit. We propose a model to explain the underlying mechanism for the poor repair (Figure 6). Calcium entry owing to sarcolemmal tears caused by the dystrophin-deficient sarcolemma fragility and by the calcium leak channels results in calcium overload in the dystrophic muscle. Mitochondria buffers part of this calcium overload. This causes decline in mitochondrial function and mitochondrial loss by autophagy. Dysfunction and loss of mitochondria causes poor myofiber repair, which further increases calcium overload setting up a positive feedback loop for dystrophic myofiber necrosis (Figure 6). A compensatory increase in dysferlin-mediated sarcolemmal repair offers relief but fails to rescue the poor repair of dystrophic sarcolemmal caused by the decline in mitochondrial function and their ability to accumulate at the site of sarcolemmal damage.

In agreement with early changes in mdx muscle, we find that mdx muscle proteome is altered by 3–4 week of age and includes reduced level and function of mitochondria. In addition to energetic deficit and oxidative damage that has

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**Figure 6** A model for the contribution of mitochondria in the damage of dystrophin-deficient myofiber. Absence of dystrophin increases susceptibility of myofiber sarcolemma to contraction-induced damage. Frequent sarcolemmal damage repeatedly increases cellular calcium load, which is taken up in part by the mitochondria. This calcium overload contributes to the decrease in mitochondrial activity, in accumulation at the site of injury and in mitochondrial level (potentially by mitophagy). These mitochondrial pathologies compromise the repair ability of the dystrophin-deficient myofibers, creating a positive feedback loop that results in greater necrosis of the dystrophin-deficient myofibers and increased muscle degeneration. Uregulation of dysferlin and dysferlin-related proteins is a compensatory response to this poor sarcolemmal repair, which activates the vesicular machinery for the repair of injured dystrophic sarcolemma. This increase in dysferlin-mediated repair machinery slows but fails to rescue the poor repair of dystrophic sarcolemma caused by the decline in mitochondrial function and their ability to accumulate at the site of sarcolemmal damage.

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been associated with mitochondrial deficit in dystrophin-deficient muscle, we show that this also results in poor sarcolemmal repair. Poor repair is evident not only in isolated myofibers but also in intact muscles, such that 28-day-old mdx mouse muscle suffers greater sarcolemmal damage by eccentric contraction (Figure 3). Enhanced dye uptake by dystrophin-deficient myofibers upon a single eccentric contraction identifies that poor sarcolemmal repair contributes to muscle force loss by eccentric contractions (Figure 3). Mitochondrial role in sarcolemmal repair is in agreement with reports that upregulating PGC1α in mdx mice improves myofiber recovery from LC injury and reduces muscle degeneration.47–50 The underlying mechanism for the role of mitochondria has not been elucidated but may involve regulation of myofiber bioenergetics and calcium handling. This is supported by studies showing that increased PGC1α expression in mdx mice improves metabolism and mitochondrial capacity to buffer Ca2+ and reduces Ca2+-dependent protease activity.47,50,51

Our results show that mitochondria-mediated sarcolemmal repair is distinct from dysferlin-mediated repair. Although mitochondria-mediated repair declines within the first 4 week of age in mdx mice, dysferlin-mediated repair is increased by this age. Previous studies with 6-month-old DDKO mice have found more severe muscle pathology of these mice than either of the parental knockouts.12,52 But, as dysferlin deficit causes muscle pathology at both ages, mitochondrial deficit increases muscle pathology of the mdx mice at 28 days of age, when lack of dysferlin alone does not cause detectable muscle pathology.53 Thus, owing to poor mitochondria-mediated repair, dysferlin-mediated repair is increased to facilitate repair of dystrophin-deficient myofibers. Simultaneous upregulation of dysferlin-related membrane repair proteins, including annexin A1 and annexin A2 in the mouse, and of dysferlin, annexin A1 and mitsugumin 53 in DMD patients independently supports this mechanism and explains how genetic ablation of dysferlin can significantly worsen the phenotype of dystrophin-deficient muscle.12,13,52,53

To investigate whether dystrophin re-expression could reverse the sarcolemmal repair deficit, we used exon skipping therapy to restore dystrophin, which is known to improve dystrophic muscle strength in a dystrophin expression level-dependent manner.54,55 Antisense oligonucleotides conjugated to a cell-penetrating peptide restores dystrophin expression up to 15% of the healthy level and protects dystrophic mdx muscle against eccentric contraction-induced injury.56 However, with reduced toxicity, FDA approved use of PMOs as an exon skipping therapy for DMD. We observed that PMO caused an 8% increase in dystrophin level, which increased sarcolemmal repair by 2-fold. However, large heterogeneity in AO-induced dystrophin re-expression between myofibers causes significant variability in myofiber repair. These results provide a proof-of-principle demonstration that a large and/or homogenous increase in dystrophin expression could improve the repair ability of dystrophic myofibers. Further, we identified that merely increasing the mitochondrial respiratory activity (by the use of pyruvate, the limiting substrate for the TCA cycle) does not improve mdx myofiber repair. Our findings that exon skipping-mediated restoration of dystrophin expression shows its potential to improve membrane repair, but this requires reduced dysfunction and increased biogenesis of mitochondria, which is achieved using exercise mimetics.47,50 Thus combined use of exercise mimetics and exon skipping with PMOs may be a potent therapy for improving sarcolemmal repair. In summary, our findings demonstrate a nexus between dystrophin deficit, mitochondrial dysfunction, and myofiber necrosis and identify this nexus as a novel therapeutic target to improve myofiber survival in DMD.

Materials and Methods
Animal husbandry and live imaging of myofiber repair. All animal procedures were conducted in accordance with guidelines for the care and use of laboratory animals as approved by the Institutional Animal Care and Use Committee. C57BL/6 and C57BL/10 control mice and mdx-52 and mdx-23 mice were bred in-house and used for all experiments. All animal experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Institutional Animal Care and Use Committee. Dysferlin-deficient-B6A/J and dystrophin-deficient-mdx-52 mice were each created on the BL/6 genetic background and were used here to generate DDKO mice. The F1 offspring were intercrossed, and progeny that were null at both alleles and those animals were used to generate the DDKO colony. To identify mutant dysferlin and dystrophin genes, PCR genotyping was performed as previously described.39,43 Immunoblotting was also performed to confirm the absence of proteins in the skeletal muscle. To assess histological features, gastrocnemius muscle tissues were obtained from C57BL/10, mdx-23, C57BL/6, mdx-52, and DDKO mice (N = 3–5/group) and flash frozen in isopentane chilled in liquid nitrogen. These tissue samples were sectioned and stained with hematoxylin and eosin as previously described.39 Images were obtained using an Olympus BX61 microscope with DP71 camera (Center Valley, PA, USA).

For live myofiber imaging, mice were killed with CO2 asphyxiation and then muscles were dissected and intact FDB, biceps or EDL muscles were imaged directly as intact muscles or individual myofibers were isolated. For the latter, EDL muscle was placed in a sterile solution of collagenase type 1 (2 mg/ml in Dulbecco’s modified Eagle medium (DMEM)) and incubated at 35 °C shaking water bath for 1–2 h. Individual muscle fibers were separated by gentle trituration with a fire-polished wide-mouth Pasteur pipette. The individual fibers were washed three times with DMEM and placed in 35 mm glass bottom petri dishes that were precoated with matrigel and supplied with growth medium (DMEM; 20% PBS, 2% chicken embryo extract, 1:100 dilution of Penicillin/streptomycin stock solution). The isolated fibers were maintained for up to 2 days at 37 °C and 5% CO2 for further live imaging.

Prior to imaging, growth medium was removed and the myofibers were washed once with prewarmed cell imaging media (CM; HBSS with 10 mM HEPES pH 7.4 and 2 mM Ca2+) at 37 °C and then transferred to CIM containing 1.66 μg/μl of FM-43 dye. Sarcolemmal was injured by irradiating a 2–4 μm² area for 10 ms with the pulsed laser (Ablate, Intelligent Imaging Innovations, Inc., Denver, CO, USA). Repair kinetics was monitored by time-lapse imaging at 10 s intervals for 5 min using an inverted Olympus IX81 microscope (Olympus America, Center Valley, PA, USA) custom equipped with a CSU1X spinning dichroic confocal unit (Yokogawa Electric Corp., Tokyo, Japan), pulsed laser Ablate, and diode laser of 561 nm (Coherent, Stockholm, Sweden). Images were acquired using Evolve S12 EMCCD (Photometrics, Tucson, AZ, USA) at 1 Hz. Image acquisition and laser injury was controlled using Slidebook 5.0 (Intelligent Imaging Innovations). The fibers that were repaired were counted and presented as percentage. To monitor repair kinetics, the FM-43 dye fluorescence in the myofiber was measured and plotted as change in intensity (FFC; average ± S.D.) for each group.

Proteomic profiling and immunoblotting. Gastrocnemius mouse muscles were homogenized in radioimmunoprecipitation assay buffer (RIPA) buffer (50 mm Tris-HCl, pH 8.0, with 150 mm sodium chloride, 1.0% Igepal CA-630 (Nonidet P-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (Teknova, Hollister, CA, USA) containing protease inhibitors (Halt protease inhibitor mixture 10X; Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were measured using the Thermo BCA Microplate Protein Assay (Thermo Fisher Scientific) according to the manufacturer’s protocol. Approximately 50 μg of each sample RIPA extract was mixed 1:1 with RIPA quadruplex protein extract from a...
After the protocol of LCs, muscles were pinned on cork at optimal length and placed in 0.2% PO dye Ringer-solution for 1 h at RT. After removing the excess dye by washing with Ringer’s solution, the muscle was frozen using isopentane prechilled in liquid nitrogen. Frozen tissues were sectioned (10 μm) and imaged under red channel using a Nikon Eclipse E800 (Nikon, Sendai, Japan) microscope that was fitted with a SPOT digital color camera with SPOT advanced software (Diagnostic Instruments, Sterling Heights, MI, USA). Representative pictures (≥20) were taken and PO-positive fibers/image were quantified for each section. Data are represented as the number of PO-positive fibers per image (mean ± S.D.).

Measurement of inflammation marker. IL-1β inflammatory marker was monitored using real-time quantitative PCR assay as previously described. 29 In brief, quadriceps muscle tissue was obtained from 28-day-old C57BL/6, mdx-52, and DDKO mice. RNA was isolated using TRIzol according to the protocol recommended by the manufacturer (Life Technologies, Carlsbad, CA, USA). Complementary DNA was synthesized using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies). Quantitative PCR was performed using an Applied Biosystems 7900HT Real-Time PCR machine. TaqMan probe sets IL-1β (Mm00434228_m1) and hypoxantine guanine phosphoribosyl transferase (HPRT; Mm01543939_m1) were obtained from Applied Biosystems (Foster City, CA, USA). The relative expression of the transcript was calculated according to the ΔΔCt method with HPRT as the internal reference, and fold change is expressed as the mean ± S.D.

Monitoring the mitochondrial dynamics and activity. For monitoring in situ mitochondrial dynamics, isolated or intact myofibers and cultured C2C12 myoblasts were stained for 15 min at 37°C with 100 nM MitoTracker Orange CM-H2TMRos (Thermo Fisher Scientific). The excess dye was washed off, and the fibers were transferred to CIM. Myofibers were injured using a pulsed laser and imaged as described above. In cell culture experiments, C2C12 myoblasts were incubated in 100 mM pyruvate for 10 min or 5 μM CCCP for 45 min at 37°C prior to labeling, and following washes, the cells were transferred to CIM containing the indicated drug for imaging. The fluorescence intensity of MitoTracker dye at the site of injury or in the whole cell (as indicated) was measured and the data are presented as mean ± S.D.

To assess mitochondrial respiratory activity, muscle tissues (gastrocnemius) were harvested and homogenized in ice-cold H medium (70 mM sucrose, 220 mM mannitol, 2.5 mM ATP, 2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and 2 mM EDTA). The homogenate was centrifuged at 1500 × g for 10 min at 4°C. Supernatants were removed and centrifuged at 10 000 × g for 10 min at 4°C. The mitochondria pellets were resuspended in H medium and centrifuged at 10 000 × g for 10 min at 4°C. Pellets were again resuspended in H medium, and mitochondrial protein concentration was determined by the Protein Assay Kit (Bio-Rad).

Cytochrome c oxidase kinetics was determined spectrophotometrically, using the method of Smith, in which the rate of oxidation of ferrocytochrome c was measured by following the decrease in absorbance at 550 nm. An average of 1.5 μg of mitochondria was resuspended in reaction buffer (1 M phosphate buffer, 2% dodecyl maltoside) and the reaction was started by addition of reduced ferrocytochrome c at 2–80 μM. Specific activity was calculated as the average of four measurements using 21.1 nM/cm as the extinction coefficient of ferrocytochrome c at 550 nm. 36

Live cell ATP Imaging. To measure mitochondrial ATP, C2C12 myoblasts were transfected with the FRET-based ATP biosensor Ateam.36 Cells were first imaged in CM (untreated) and then allowed to incubate in 100 mM pyruvate for 10 min at 37°C. After incubation, same cells were imaged to determine the change in Ateam FRET emission for each cell. Three-channel FRET imaging was performed by monitoring the donor (445 laser excitation), acceptor (515 laser excitation), and transfer (FRET) channels. Emission from these three channels was corrected for bleed through and used to measure corrected FRET. Fc = Transfer − Acceptor − Donor, which generated a corrected FRET image and the FRET quantification.

Antisense oligonucleotide and pyruvate treatment. As previously described, mice were anesthetized using 4% isoflurane and 0.5% l/min 100% oxygen and maintained using 2% isoflurane and 0.5 l/min oxygen delivered via a nose cone with a passive exhaust system on a warming device.36 The AO of choice was the morpholinophospholipid chemistry, PMO mExon 23-07 (5′-GGCATAACCTCCGCTT CCTGAAAT-3′) against the boundary sequences of exon 23 and intron 23 of the mouse dystrophin gene (Gene Tools, Philomath, OR, USA). PMO was either

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administered via a single 800 mg/kg dose through an IV injection via the retro-orbital sinus as previously described\(^1\) or through weekly IM injections to the FDB muscle for 3 weeks (20 mg PMO/dose). After each injection, the mouse was monitored for pain or distress. Age-matched control mdx mice were left untreated. Uninjected C57BL/10 mice were used as dystrophic-positive controls. For pyruvate treatment, biceps muscle from mdx-23 mice were dissected and incubated with pyruvate (100 mM salt, Sigma, St. Louis, MO, USA) for 25 min at 37 °C before laser injury in the presence of FM1-43 dye (as described earlier). The same procedure was carried out for FDB muscles and mitochondria potential measured using MitoTracker Red (CM-H2TMRos) as above. Contralateral muscles were used as untreated controls.

**Statistical analyses.** Statistical analyses were performed using either Student’s \(t\)-test or one-way analysis of variance (with Bonferroni correction posttest) where appropriate. When data are not normally distributed, non-parametric tests were used. Significant differences for each condition were determined with Tukey’s \(t\)-test, where appropriate. Where indicated, specific statistics are noted in the figure legends. When data are not normally distributed, non-parametric tests were used. Significant differences for each condition were determined with a paired \(t\)-test. Data are expressed as means ± standard error of the mean (SEM). 0.05 was considered significant.

**Conflict of Interest**

The authors declare no conflict of interest.

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**Author contributions**

MCV and SR conducted the work described here with help from MH in the therapeutic and histological analysis, from JVM in muscle physiology studies, from AD in live myofiber imaging, from AH in live myofiber and live cell studies and from KJB and YH in proteomic studies. ST contributed new reagents; KN and JKJ conceived this study and provided oversight. JKJ designed the experimental approaches with help from all authors and participated in data collection, analysis and interpretation. MCV and JKJ wrote the manuscript with help from all the authors.

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