Myogenic Akt signaling upregulates the utrophin–glycoprotein complex and promotes sarcolemma stability in muscular dystrophy

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Duchenne muscular dystrophy is caused by dystrophin mutations that lead to structural instability of the sarcolemma membrane, myofiber degeneration/regeneration and progressive muscle wasting. Here we show that myogenic Akt signaling in mouse models of dystrophy promotes increased expression of utrophin, which replaces the function of dystrophin thereby preventing sarcolemma damage and muscle wasting. In contrast to previous suggestions that increased Akt in dystrophy was a secondary consequence of pathology, our findings demonstrate a pivotal role for this signaling pathway such that modulation of Akt can significantly affect disease outcome by amplification of existing, physiological compensatory mechanisms.

INTRODUCTION

Muscular dystrophy describes a group of genetic disorders generally characterized by progressive muscle weakness. The most common form of muscular dystrophy, Duchenne muscular dystrophy (DMD), is caused by primary mutations in the dystrophin gene (1–3). DMD is characterized by progressive muscle weakness eventually leading to cardiac and respiratory failure. Dystrophin deficiency alters the stability of the entire dystrophin-glycoprotein complex (DGC) resulting in reduction of the entire complex at the plasma membrane (for review 4). In skeletal muscle fibers, the core components of the DGC include dystrophin, the dystroglycans (α- and β-DG), the sarcoglycans (α-, β-, γ- and δ-SG), sarcospan (SSPN) and the syntrophins (for review 5). Peripheral and integral membrane components of the DGC provide a physical connection between the extracellular matrix and the intracellular actin cytoskeleton (3,6–10). Loss of dystrophin and the DGC alters the integrity of the sarcolemma, the skeletal muscle plasma membrane, increasing contraction-induced damage. Even though skeletal muscle possesses the innate ability to regenerate, this ability is insufficient to compensate for the structural damage endured during muscle contraction in DMD patients. Thus, the link between the extracellular membrane and the intracellular cytoskeleton provided by the DGC is essential for maintaining sarcolemmal stability.

The regenerative capacity of skeletal muscle is insufficient in combating persistent and widespread degeneration of myofibers with structural defects in the sarcolemma. Akt, a serine/threonine protein kinase, has previously been shown to be altered in both DMD patients and in dystrophin-deficient mdx mice (11,12). Previous reports indicate that activation of the phosphatidylinositol 3-kinase (PI(3)K)/Akt pathway is critical for the activation of muscle hypertrophy (13–17). We and others have established that the same PI(3)K/Akt signaling pathways responsible for inducing skeletal muscle hypertrophy (13) are also activated in many forms of muscular dystrophy (11,12). Furthermore, we have recently demonstrated that direct manipulation of Akt in normal, wild-type (WT) mice induces skeletal muscle hypertrophy that results...
in dramatic improvements in force generation while decreasing adipose mass (18). Taken together, these previous reports indicate activation of Akt in dystrophic muscle may be a key mediator of the hypertrophic response both in the promotion of muscle hypertrophy and in blood vessel recruitment.

The Akt family of protein kinases is activated by numerous extracellular stimuli transduced across the membrane through membrane-bound receptor tyrosine kinases (for review 20). Akt is then recruited to the plasma membrane through activation of PI(3)K. Subsequent phosphorylation releases Akt from the plasma membrane and leads to translocation of phosphorylated Akt (P-Akt) to the cytoplasm and activation of downstream targets. Although recent research has reported Akt translocation to the nucleus following activation (for review 20), Akt remains in the cytosolic fraction following activation in skeletal muscle (11). We have created a skeletal muscle-specific, conditional transgenic mouse expressing a constitutively active form of Akt (18). In this study, an increase in Akt activation in skeletal muscle led to muscle hypertrophy specifically in type IIb fibers. The induction of hypertrophy was also accompanied by increased fiber strength, decreased fat mass and improvement of metabolic parameters (18). These findings indicate that Akt activation alone can increase muscle mass and improve muscle strength.

In the current report, we examined the effect of constitutive Akt activation on the dystrophic pathology in dystrophin-deficient mdx mice. We demonstrate that activation of the Akt transgene at the prenecrotic (<3.5 weeks of age) stage of disease delays the onset of dystrophic pathology in mdx mice. We exploited the conditional transgene (18) to activate the transgene for a total of 3 weeks before analyzing the tissue for signs of muscular dystrophy. We show an increase in muscle hypertrophy and decreased sarcolemmal fragility in Akt-treated mdx mice compared with the untreated mdx controls. The mechanism of amelioration involves increased expression and wider distribution of adhesion complexes including the utrophin–glycoprotein complex (UGC). Overexpression of the UGC has previously been shown to ameliorate the dystrophic pathology in mdx mice (21,22). These findings suggest that the overexpression and extra-synaptic localization of the UGC and integrin compensate for the loss of the DGC in Akt-transgenic, mdx mice. This current study identifies myogenic Akt signaling as a drug target that could improve the muscle wasting and sarcolemmal fragility associated with muscular dystrophy by increasing expression of the UGC.

RESULTS AND DISCUSSION
Generation of mdx mice with inducible Akt1 expression in skeletal muscle
To investigate whether the beneficial effects of Akt would manifest in dystrophic muscle, we engineered mice with muscular dystrophy to express Akt1 by introducing the TRE-myrAkt1 and MCK-rtTA transgenes (18) into dystrophin-deficient mdx mice (Fig. 1). Offspring that had inherited only one of the TRE-myrAkt1 or MCK-rtTA transgenes are referred to as single-transgenic (STG) mice and lack the means for the conditional activation of Akt1. Double-transgenic (DTG) offspring inherited both of the transgenes and increase expression of Akt1 upon doxycycline (DOX) treatment. All female offspring are heterozygous for dystrophin (X<sup>mdx</sup> Y<sup>Y</sup>) and expressing non-dystrophic phenotypes, are denoted as being either wild-type single-transgenic (WT STG) or wild-type double-transgenic (WT DTG). All male offspring possessed mdx genotypes (X<sup>mdx</sup> Y<sup>Y</sup>) and are denoted as being either mdx STG or mdx DTG. Transgenic offspring were genotyped through two pairs of PCR amplification reactions. In the first pair of reactions, offspring were genotyped for the two transgenes responsible for the activation of constitutively active Akt1. In the first PCR reaction, the transgene for the constitutively active form of Akt under the control of a tetracycline responsive promoter (TRE-myrAkt1, MCK-rtTA) was amplified, yielding a 567 bp product. In the second PCR reaction, the transgene expressing a reverse tetracycline transactivator under the control of a modified muscle creatine kinase promoter (MCK-rtTA) was amplified, yielding a 567 bp product. In the second pair of reactions, offspring were genotyped for the mdx locus. The forward primers used in this pair of reactions are identical. In the first reaction of the pair, the WT allele was amplified using a WT allele-specific reverse primer. In the second reaction, the mdx allele was amplified using an mdx allele-specific reverse primer. Each reaction in this pair yielded a 275 bp product.

DOX-induced Akt overexpression causes muscle hypertrophy in mdx mice
Similar to DMD patients, mdx mice possess a genetic mutation in the dystrophin gene, resulting in loss of dystrophin protein and the entire DGC (1–6). The peak necrotic stage of disease occurs in mdx mice when they are between 3 and 6 weeks of age and when the frequency of degeneration/regeneration is at its greatest (23). In our model system, DOX, required for transgene activation, was administered at the prenecrotic

![Figure 1. Generation of Akt-transgenic mdx mice.](image-url)
stage of disease and mice were analyzed during peak necrosis (Fig. 2A). Increased Akt1 signaling induced obvious hind limb hypertrophy and hypervascularization in mdx mice (Fig. 2B) and increased quadriceps mass by 60% in WT (Fisher’s *P*, 0.00001) and by 35% in mdx DTG mice (Fisher’s *P*, 0.005) relative to their respective STG controls (Fig. 2C). Muscles from both male and female mice displayed similar levels of increased muscle mass upon transgene activation (Supplementary Material, Fig. S1). Immunoblotting skeletal muscle protein lysates revealed comparable expression and functionality of the Akt transgene in treated WT and mdx DTG mice. Induction of transgene in DTG muscle significantly increases total quadriceps weight when compared to their WT (Fisher’s, *P < 0.00001) and mdx STG (Fisher’s, **P < 0.005) counterparts. Quadriceps weights are represented as an average of the left and right quadriceps of each animal. Bars represent mean quadriceps weights (± SEM; *n* = 21 mdx STG, *n* = 4 mdx DTG. *n* = 18 WT STG, *n* = 11 WT DTG). (D) Immunoblotting for Akt pathway proteins. Akt pathway proteins were detected from total skeletal muscle lysates of six-week-old WT STG, WT DTG, mdx STG, and mdx DTG mice. Identical membranes were probed with antibodies against Akt, P-Akt, P-70S6K, P-GSK3β, P-MDM2, and the HA-tag engineered onto the Akt1 transgene, as indicated. Coomassie blue staining of total protein is shown on the bottom panel (CB Stain) as a loading control. Activation of Akt for three weeks is sufficient for activation of P-70S6K in the Akt signaling axis. Furthermore, P-MDM2 levels increase upon Akt activation whereas P-GSK3β levels remain constant.

We found that Akt activation increased cross-sectional myofiber area in quadriceps muscle from both WT and mdx DTG mice (Fig. 3A and B). The distribution of fiber cross-sectional areas (CSAs) reveals increases in larger fibers (4500–7500 μm²) for DTG mice (Fig. 3B). Central nucleation, a marker for fiber regeneration, was elevated in mdx muscle (ANOVA, *P < 0.005) and Akt activation in mdx mice did not significantly alter the frequency of central nucleation in mdx mice (Fig. 3C).

**Improved sarcolemmal integrity upon Akt1 expression in mdx DTG mice**

Loss of dystrophin and the DGC in mdx mice and in DMD patients results in contraction-induced sarcolemmal disruption and instability. Sarcolemmal integrity in mdx DTG mice was
examined by the Evan’s Blue Dye (EBD) tracer assay, which allows assessment of blood serum albumin infiltration into damaged muscle fibers. The quadriceps muscles of \textit{mdx} STG mice displayed elevated levels of EBD infiltration compared with WT STG and DTG controls (Fig. 4A) due to the sarcolemmal fragility in \textit{mdx} muscle. In contrast, Akt induction ameliorated sarcolemmal damage in \textit{mdx} DTG mice to nearly WT DTG control levels (Fig. 4B; Student’s t-test with Bonferroni adjustment, \(P < 0.03\)).

Akt activates muscle regeneration

The observation that central nucleation was unaffected in \textit{mdx} DTG mice suggested that myofibers were undergoing regeneration despite restoration of membrane stability. Based on previous observations that Akt is involved in proliferation of satellite cells (25), we postulated that regeneration in \textit{mdx} DTG muscle resulted from activation of satellite cells in Akt over-expressing muscle. In order to test this observation, we determined the myofiber CSA for WT DTG muscle after 2, 4 and 6 weeks of Akt1 transgene activation. For these studies, we chose to induce Akt activation in adult WT mice to avoid any complications of disease pathology that occur in \textit{mdx} muscles (i.e. diaphragm, soleus, cardiac and EDL muscles do not express the Akt1 transgene) in this model (18). Myofiber CSA doubled after 2 weeks of Akt1 activation (Fig. 5A and B). An additional 2 weeks of Akt1 transgene activation increased the CSA by an additional 0.5-fold and no further changes in myofiber size were detected after 6 weeks of transgene overexpression (Fig. 5B). We found that the levels of central nucleation were not affected by 2 weeks of transgene activation nor were the number of nuclei per fiber changed at this time point. Interestingly, central nucleation in WT DTG muscle increased to over 20% after 4 and 6 weeks of DOX treatment (Fig. 5C), which correlated with an increase in the number of nuclei per CSA (Fig. 5D). Taken together, this data suggests that Akt1 expression in muscle activates normally quiescent satellite cells, which is further supported by the observation of elevated levels of mRNA transcripts for many satellite cell markers (myoD, pax7, PCNA, myf6 and myogenin) in WT DTG muscle (Table 1; data not shown).

Akt1 increases levels of several compensatory adhesion complexes

To determine the mechanism of rescue for sarclemma fragility in DTG \textit{mdx} mice, we investigated expression of several adhesion complexes that are known to rescue dystrophin deficiency and restore membrane stability (1–6,16). We found that induction of Akt signaling in \textit{mdx} muscle increased protein expression of the sarcoglycan and dystroglycan subunits to WT levels (Fig. 6). Akt activation increased utrophin protein levels by a minimum of 5- and 10-fold, respectively, in WT and \textit{mdx} DTG muscle, relative to their STG controls.
Figure 4. Akt1 activation ameliorates sarcolemmal stability in mdx DTG mice. (A) Detection of blood serum protein infiltration with Evans Blue dye (EBD) tracer assay. Transverse sections of quadriceps muscle fibers with damaged sarcolemma are shown with red fluorescence. Laminin in the basement membrane is shown with green fluorescence to delineate each myofiber. EBD infiltration was absent in WT STG, detected in low levels in WT DTG and mdx DTG mice, and elevated in mdx STG mice. Bar, 50 μm. (B) Quantification of EBD-positive fibers in whole transverse quadriceps sections. Bars represent percentage of EBD infiltrated fibers in a whole quadriceps section (±SEM; Student’s t-test with Bonferroni adjustment (α = 0.025), N.S. WT STG versus WT DTG, *P < 0.02, mdx DTG versus mdx STG, n = 3 mice).

(FIG. 6). Utophin, an autosomal homologue of dystrophin, replaces dystrophin at the post-synaptic face of the neuromuscular junction to form the UGC (21). In addition to utrophin, integrin α7β1D, which are normally restricted to the neuromuscular junction, are broadly localized throughout the extrasynaptic sarcolemma upon Akt expression (Fig. 7). Like utrophin, the skeletal muscle-specific β1D isoform of integrin is enriched at the neuromuscular and myotendinous junctions in WT mice, but has been found to be up-regulated in mdx mice (26). Overexpression of α7β1 integrin promotes muscle hypertrophy and regeneration in mdx/utr−/− mice (28). We found integrin β1 expression homogenously at the sarcolemma in DTG skeletal muscle (Fig. 7).

To further assess the regulation of compensatory complexes, quantitative real-time PCR was performed from skeletal muscle of STG and DTG mice. mRNA levels of several genes including β-DG, α7 integrin, dysferlin and MyoD were significantly elevated in DTG muscle relative to STG controls (Table 1). The observation that mRNA levels of the muscle-specific transcription factor MyoD were tripped in mdx DTG muscle supports a role for transcriptional regulation of gene expression upon Akt1 activation. Akt1 regulates skeletal muscle differentiation, at least in part, by enhancing transcriptional activity of MyoD via co-activators p300 and P/CAF (29). In turn, MyoD controls transcription of genes expressed during differentiation including α7 integrin, and several components of the DGC and the UGC (30).

Here we demonstrate that myogenic expression of Akt1 in dystrophin-deficient muscle ameliorates crucial pathologies of muscular dystrophy. Our studies suggest that Akt up-regulates utrophin expression to levels that are sufficient to stabilize the sarcolemma in the absence of dystrophin and thus ameliorate muscular dystrophy. These findings provide a rationale for identification of mechanisms to increase utrophin protein stability or translation as a means to increase utrophin protein expression, in addition to current strategies to identify methods of augmenting utrophin transcription. We demonstrate for the first time that overexpression of a signaling molecule that has been suggested to control hypertrophy (13–17) can diminish the progressive muscle wasting associated with dystrophin deficiency. Akt may represent a critical ‘master switch’ for myofiber regeneration and modulation of these pathways may have therapeutic value for a broad-range of inherited and acquired muscle wasting disorders.

MATERIALS AND METHODS

Animal models

Mdx female retired breeders were purchased from Jackson Laboratories (Bar Harbor, Maine). DTG mice which were produced by crossing 1256 [3Emut] MCK-rtTA transgenic mice expressing the tetracycline transactivator controlled by a mutated skeletal muscle creatine kinase promoter with TRE-myrAkt1 transgenic mice harboring the constitutively active form of the mouse Akt1 transgene controlled by a tetracycline-responsive promoter. Male DTG mice were bred with mdx females to produce four groups of mice used for comparison: (i) Akt STG (either MCK-rtTA or TRE-myrAkt1) females on an mdx heterozygous background, (ii) Akt DTG females on an mdx heterozygous background, (iii) Akt STG (either MCK-rtTA or TRE-myrAkt1) mdx males and (iv) Akt...
To activate Akt transgene expression in DTG males, to activate Akt transgene expression in DTG mice, three-week-old mice were treated with DOX administered in drinking water (0.5 mg/ml) for a three week period. STG mice (TRE-myrAkt1 or MCK-rtTA) treated with DOX were utilized as controls to eliminate any effect directly related to DOX treatment. After DOX treatment, quadriceps muscles and total skeletal muscles were harvested, weighed and snap-frozen in liquid nitrogen or mounted in 10.2% polyvinyl alcohol/4.3% polyethylene glycol and rapidly frozen in liquid nitrogen-cooled isopentane. All tissues were stored at −80°C until analyzed. Mice were housed in the Life Sciences Vivarium. All procedures were carried in accordance with guidelines set by the UCLA Institutional Animal Care and Use Committee.

Genotyping of Akt-transgenic mdx mice

Akt genotyping. PCR amplifications for transgenes were carried out as previously described (18). Briefly, the TRE-myrAkt1 transgene was amplified using the following primers: Tet-Akt #1 (5'-CTGGACTACTTGCACTCCGAGAAG-3') and Tet-Akt #2 (5'-CTGTGTAGGGTCCTTCTTCGCAG-3'). The cycling conditions were 95°C, 5 min; then 95°C, 30 s and 62°C, 30 s for 30 cycles, followed by 72°C, 10 min. The reaction yielded a 380 bp product. PCR amplifications of the MCK-rtTA transgene was amplified using the following primers: MCK-rtTA #1 (5'-CATCTGCGGACTGGA AAAACAAC-3') and MCK-rtTA #2 (5'-GACATCGGTAAAC ATCTGCTCAAAC-3'). The cycling conditions were 95°C, 5 min; then 94°C, 30 s; 62°C, 30 s and 72°C, 1 min. for 30

Table 1. Akt1-transgene activation mediates changes in mRNA levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative mRNA levels</th>
<th>Wild-type</th>
<th>STG</th>
<th>mdx</th>
<th>STG</th>
<th>DTG</th>
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<tr>
<td>Utrophin</td>
<td>1.00 ± 0.10</td>
<td>1.09 ± 0.18</td>
<td>0.57 ± 0.22</td>
<td>0.72 ± 0.15</td>
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<tr>
<td>β-DG</td>
<td>1.00 ± 0.16</td>
<td>1.41 ± 0.17</td>
<td>0.61 ± 0.10</td>
<td>0.91 ± 0.09</td>
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<tr>
<td>γ-SG</td>
<td>1.00 ± 0.11</td>
<td>1.57 ± 0.30</td>
<td>0.83 ± 0.26</td>
<td>1.01 ± 0.15</td>
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<tr>
<td>Itga-7</td>
<td>1.00 ± 0.14</td>
<td>1.93 ± 0.20</td>
<td>0.78 ± 0.07</td>
<td>1.81 ± 0.22</td>
<td></td>
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<tr>
<td>Dysf</td>
<td>1.00 ± 0.14</td>
<td>2.17 ± 0.25</td>
<td>0.96 ± 0.11</td>
<td>1.86 ± 0.52</td>
<td></td>
<td></td>
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<tr>
<td>MyoD</td>
<td>1.00 ± 0.10</td>
<td>2.08 ± 0.33</td>
<td>0.63 ± 0.11</td>
<td>1.87 ± 0.25</td>
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Relative mRNA expression levels of utrophin, β-DG, γ-SG, integrin α7, dysferlin (dysf), MyoD as measured by qRT–PCR. mRNA levels were normalized to internal control gene 36B4 and values are represented relative to WT STG controls. Results are presented as mean ± SEM (n = 4). Values in italics are statistically significant in comparisons for STG and DTG control sets (Fisher’s, P < 0.05).
cycles, followed by 72°C, 10 min. The reaction yielded a 567 bp product.

**Mdx genotyping.** The *mdx* point mutation was screened using a modified version of an amplification-resistant mutation system (ARMS) assay (31). Two separate PCR reactions were performed. The WT PCR amplifies the WT dystrophin allele using the following oligonucleotide primers: p260E (5'-GTCACTCAGATAGTGGAAGCCATTAG-3') and p306F (5'-CATAGTTAATGCAATAGATTCAG-3'). The *mdx* PCR amplifies the *mdx* allele using the following oligonucleotide primers: p259E (5'-GTCACTCAGATAGTGGAAGCCATTAA-3') and p306F (5'-CATAGTTAATGCAATAGATTCAG-3'). PCR cycling conditions for both reactions were 95°C, 4 min; then 95°C, 1 min; 55°C, 1 min and 72°C, 1 min for 34 cycles, followed by 72°C, 10 min. Each reaction yielded a 275 bp product. All PCR reactions utilized the GoTaq DNA Polymerase kit (Promega, Madison, WI, USA).

**Protein preparation**
Snap-frozen skeletal muscles were crushed with liquid nitrogen and a mortar and pestle. Ice-cold RIPA lysis buffer [1%

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**Figure 6.** Akt increases expression of compensatory proteins in *mdx* mice. Immunoblotting for several glycoprotein complexes (DGC, UGC, integrin) on skeletal muscle lysates from six-week-old WT STG, WT DTG, *mdx* STG, and *mdx* DTG mice. Identical membranes were probed with antibodies against dystrophin (Dys), utrophin (Utrn), alpha- and beta-dystroglycan (α-DG, β-DG), alpha-, beta- and gamma-sarcoglycan (α-SG, β-SG, γ-SG), β1D integrin and dysferlin (Dysf). GAPDH immunoblotting and Coomassie blue (CB) staining of total protein are shown on the bottom panels as a loading controls. Constitutive Akt activation increased the expression of the DGC and UGC in WT mice. Urophin levels increased in *mdx* mice compared to those of WT mice. Increased expression of β1D integrin and dysferlin was observed upon Akt activation in both WT mice and in *mdx* mice.

**Figure 7.** Broad sarcolemmal distribution of compensatory proteins upon Akt activation. Immunohistochemical analyses on transverse quadriceps sections in WT STG, WT DTG, *mdx* STG and *mdx* DTG mice. Sections were stained with antibodies to dystrophin (Dys), utrophin (Utrn), β1D integrin, alpha- and beta-dystroglycan (α-DG, β-DG), alpha-, beta- and gamma-sarcoglycan (α-SG, β-SG, γ-SG) and sarcospan (SSPN), and visualized using indirect immunofluorescence. Increased expression of the DGC and UGC in WT mice was observed upon constitutive activation of Akt. Akt activation increased expression of only the UGC in *mdx* mice. An increase in utrophin levels was observed in *mdx* mice relative to levels in WT mice. In both WT mice and in *mdx* mice, Akt activation increased the expression of β1D integrin. Bar, 50 μm.

Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid, 5 mM N-ethylmaleimide, 50 mM sodium fluoride, 2 mM β-glycerophosphate, 1 mM sodium orthovanadate, 100 mM okadaic acid, 5 mM microcystin LR and 20 mM Tris–HCl, pH 7.6) was utilized for homogenization. Immediately before addition of crushed tissue, protease inhibitors (0.6 μg/ml pepstatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.75 mM benzamidine and 0.1 mM phenylmethylsulfonyl fluoride) were added to the lysis buffer. Homogenates were rocked at 4°C for 1 h. Clarified lysates were obtained following centrifugation at 15 000g for 15 min. Clarified tissue lysates were stored at −80°C until analyzed by immunoblot analysis.
Immunoblot analysis

Protein concentrations of clarified tissue lysates were determined using the DC Protein Assay (Bio-Rad). Equal concentrations of protein samples (60 μg) were resolved by 4–20% gradient SDS–PAGE (Pierce, Rockford, IL, USA) and transferred to nitrocellulose membranes (Millipore Corp., Billerica, MA, USA) for subsequent immunoblot experiments. Primary antibodies against Akt, phosphorylated Akt (Ser 473) and phosphorylated GSK3β (Cell Signaling Technologies, Beverly, MA, #9272, #9271 and #9336, respectively) were diluted 1:750. Phosphorylated p70S6K (Cell Signaling Technologies, #9205) was used at a 1:250 dilution. β1D Integrin (Temecula California; International, MAB1900) was diluted 1:100. Primary antibodies against proteins in the DGC and UGC and their respective concentrations include dystrophin (Vector Laboratories, Burlingame, CA, USA; VP-D507, 1:2), utrophin (University of Iowa, Hybridoma Facility; MANCHO3, 1:200), α-DG (Upstate Cell Signaling Solutions, Lake Placid, NY, USA; IIH6, 1:700), β-DG (University of Iowa, Hybridoma Facility; MANDAG2, 1:10), α-SG (Vector Laboratories; VP-A105, 1:20), β-SG (Vector Laboratories; VP-B206, 1:100), γ-SG (Vector Laboratories; VP-G803, 1:200) and SSPN (Rabbit 3 (described previously in 32), 1:50). α7 Integrin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; L-17) was diluted 1:100 and α5 integrin (Abcam, Cambridge, UK; ab55988) was diluted 1:50. Anti-dysferlin antibody (Abcam; ab15108) was diluted 1:600. Goat polyclonal antibodies were detected using horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology, Inc.; SC-2033). Rabbit polyclonal antibodies were detected using horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ, USA, #NA934). Mouse monoclonal primary antibodies were detected by incubating with horseradish peroxidase-conjugated anti-mouse IgG or IgM (Amersham Pharmacia Biotech, 1:3000; Roche Applied Science, Indianapolis, IN, USA; 1:1000, respectively) for 1 h. Immunoblots were developed using enhanced chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce, # 34080).

Histology

Hematoxylin and eosin (H&E) staining was used for visualization of fibrosis, central nucleation and cross-sectional fiber area as previously described (11). Transverse quadriceps sections were left at RT for 15 min prior to staining. Briefly, muscle sections were incubated with hematoxylin for 3 min, washed with water for 1 min, incubated with eosin for 3 min, dehydrated in solutions of 70, 80, 90 and 100% ethanol and then incubated in xylene for a total of 10 min. Stained sections were mounted with permount. All supplies for the H&E staining were purchased from Fisher Scientific (Fairlawn, NJ, USA). Centrally nucleated fibers and cross-sectional fiber area were measured from digitized images captured under identical conditions with an Axiosplan 2 fluorescent microscope (Carl Zeiss Inc., Thornwood, NY, USA) and Axiovision 4.5 software (Carl Zeiss Inc.). Central nucleation was quantified as a percentage of centrally nucleated fibers over the total number of fibers in an entire transverse quadriceps section. CSA of fiber diameters was calculated using the outline spline function on the Axiovision 4.5 software. For adult studies, DOX treatment was started at the age of 10 weeks.

Evan’s blue tracer assay

To assess sarcolemmal permeability, 6-week-old mice were intraperitoneally injected (50 μl per 10 g of body weight) with sterilized EBD (10 mg/ml in sterile 10 mM phosphate buffer, 150 mM NaCl, pH 7.4). Twenty-four hours post-injection, muscles were excised and frozen in liquid nitrogen-cooled isopentane. Transverse quadriceps cryosections (8 μm) were prepared using a CM 3050S cryostat (Leica Microsystems, Bannockburn, IL, USA). Sections were incubated with ice-cold acetone, washed with PBS and blocked for 1 h at RT with 3% BSA diluted in PBS. For sarcolemmal visualization of EBD infiltrated fibers, sections were incubated at 4°C for 18 h with an antibody to laminin (Sigma, St Louis, MO, USA; L 9393) diluted at 1:25 in 1% BSA in PBS. Sections were incubated at RT for 1 h with biotinylated anti-rabbit antibody (Vector Laboratories; BA-1000, 1:250) and then with fluorescein avidin D (Vector Laboratories; A-2001, 1:250). Sections were mounted in Vectashield (Vector Laboratories; H-1000) and imaged using the Axioplan 2 fluorescent microscope with green and blue excitation filters and the Axiovision 4.5 software (Carl Zeiss Inc.). Images were merged using ImageJ software (available on http://rsbweb.nih.gov/ij/). Quantification of sarcolemmal integrity was quantified as a percentage of EBD-positive fibers over the total number of fibers counted in an entire transverse quadriceps section. Data represented is the average percentage of EBD-positive fibers in both quadriceps of each animal.

Immunofluorescence

Transverse sections were prepared from quadriceps muscles as described earlier. All sections, except for those used for detecting α-DG and SSPN, were acclimated to RT for 15 min and then blocked with 3% BSA diluted in PBS for 30 min. The Vector® M.O.M.™ Immunodetection Kit (Vector Laboratories) was then used on these sections following manufacturer’s instructions. Primary antibodies diluted in M.O.M. diluent were incubated at 4°C for 18 h against their respective proteins as follows: dystrophin (University of Iowa, Hybridoma Facility; MANDYS1, 1:30), utrophin (Vector Laboratories; VP-U579, 1:5), β1D integrin (Chemicon International; MAB1900, 1:25), β-DG (Vector; VP-B205 1:15), α-SG (Vector Laboratories; VP-A105, 1:30), β-SG (Vector Laboratories; VP-B206, 1:30), γ-SG (Vector Laboratories; VP-G803, 1:15). Afterwards, the sections were incubated with biotinylated anti-mouse antibody (1:250) provided in the M.O.M. kit and then with fluorescein avidin D (Vector Laboratories; A-2001, 1:250). Sections used for detecting α-DG and SSPN were blocked in 3% BSA diluted in PBS followed by primary antibody incubations at 4°C for 18 h as follows: α-DG (Upstate Cell Signaling Solutions; VIA4-1, 1:20) and SSPN (Rabbit 3, 1:5). These primary antibodies were detected by incubating for 1 h with FITC-conjugated anti-mouse secondary antibody or with FITC-conjugated anti-
rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA; each, 1:500) for α-DG and SSPN, respectively. Sections were mounted in VectaShield (Vector Laboratories). Images were obtained using the Axioplan 2 fluorescent microscope and Axiovision 4.5 software (Carl Zeiss Inc.).

Statistical analyses

Central nucleation, EBD and quadriceps mass data were represented as means ± SEM. The mean for each variable is calculated as means of the average between the values from the left and right quadriceps of each animal. A two-way ANOVA followed by Fisher’s protected least significant difference (Fisher’s PLSD) method and the Kruskal–Wallis nonparametric test was used for statistical analysis (Origin 7.0 Software; OriginLab Corporation; Northampton, MA). Values were considered significant when \( P < 0.05 \). For EBD analysis, the Student’s t-test with Bonferroni adjustment to significance at \( P < 0.025 \) was used. Decreases in EBD fibers in WT DTG relative to WT STG mice could not be observed because levels of EBD-positive fibers were already near zero. Because of this, the two-way ANOVA was not sufficient in assessing the decrease in EBD-positive fibers seen in the mdx mice.

Quantitative real-time PCR

Total RNA from whole skeletal muscle was prepared by QIAGEN RNeasy kit using manufacturer protocols. cDNA was produced using the ThermoScript RT–PCR System (Invi- 

trogen). Real-time PCR was performed as described previ- 

ously (18). Transcript levels were determined relative to 36B4 and normalized to the mean value of each gene are as follows: utrophin (forward, 5'-GTGGCTTCCTCAGC-3'; reverse, 5'-CTGTCC-3'); dysferlin (forward, 5'-CCGGATGTGAGGCAGCAG-3'; reverse, 5'-GGCTCTTCAGTGCT-3'); MyoD (forward, 5'-TGTTGAGTGGCTTCCCTCAGC-3'; reverse, 5'-GGGCTAGGCTCCA CCTTCT-3'); TCCATC-3'; reverse, 5'-TGTGCTAGGCTCCA CCTTCT-3').

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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