Skeletal Muscle-Specific Ablation of γcyto-Actin Does Not Exacerbate the mdx Phenotype

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Abstract

We previously documented a ten-fold increase in γcyto-actin expression in dystrophin-deficient skeletal muscle and hypothesized that increased γcyto-actin expression may participate in an adaptive cytoskeletal remodeling response. To explore whether increased γcyto-actin fortifies the cortical cytoskeleton in dystrophic skeletal muscle, we generated double knockout mice lacking both dystrophin and γcyto-actin specifically in skeletal muscle (ms-DKO). Surprisingly, dystrophin-deficient mdx and ms-DKO mice presented with comparable levels of myofiber necrosis, membrane instability, and deficits in muscle function. The lack of an exacerbated phenotype in ms-DKO mice suggests γcyto-actin and dystrophin function in a common pathway. Finally, because both mdx and ms-DKO skeletal muscle showed similar levels of utrophin expression and presented with identical dystrophies, we conclude utrophin can partially compensate for the loss of dystrophin independent of a γcyto-actin-utrophin interaction.

Introduction

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease affecting approximately 1 in every 3500 males [1]. Afflicted males experience a severe dystrophy marked by wheelchair dependence in the early teens and death due to cardiac and respiratory failure in the mid to late twenties [2]. DMD results from the loss of dystrophin [3], a 427 kDa protein localized to the sub-sarcolemmal space of muscle cells [4]. Dystrophin functions to stabilize muscle cell membranes by binding costameric γcyto-actin [5] and the transmembrane dystroglycan complex [6–8], thereby linking the costameric cytoskeleton to the extracellular matrix (ECM) [9,10]. Dystrophin-deficiency leads to muscle cell necrosis/regeneration and muscle weakness [11] due to a heightened susceptibility to muscle contraction-induced damage [12].

Although the dystrophin-deficient mdx mouse [13] provides a genetic homologue for DMD, the dystrophy of the mdx mouse is less severe than presented by DMD patients. Identification of compensatory proteins responsible for attenuating the phenotype in mdx mice may be useful for developing new therapeutic targets for DMD. For example, utrophin, the autosomal homologue of dystrophin, is upregulated in mdx mice [14–18] and is believed to mitigate the dystrophin-deficient phenotype due to functional overlap between utrophin and dystrophin [7,19]. Accordingly, mice lacking both utrophin and dystrophin (mdx/utrn−/−) exhibit a more severe Duchenne-like dystrophy marked by cardiomyopathy and premature death [20,21]. Moreover, transgenic overexpression of utrophin rescues all known phenotypes of the mdx mouse [22]. Collectively, these results suggest increased utrophin expression can partially compensate for the loss of dystrophin in the mdx mouse.

While utrophin can functionally replace dystrophin, there is evidence suggesting alternative pathways between the ECM and cytoskeleton are fortified in dystrophin-deficient muscle. Levels of α7 integrin, a transmembrane protein that complexes with adapter proteins to link the actin cytoskeleton to the ECM, are increased in both DMD patients and the mdx mouse [23]. The severe phenotype of mice lacking both dystrophin and α7 integrin [24] and the ability of transgenic α7 integrin overexpression to increase lifespan in mdx/utrn−/− mice [25] suggests increased α7 integrin expression fortifies a parallel structural link between the ECM and the cytoskeleton in dystrophin-deficient muscle. In addition, plectin, a large cytoskeletal linker protein, was recently reported to link the dystrophin-glycoprotein complex to the intermediate filament cytoskeleton and plectin levels are also reported to be elevated in dystrophin-deficient muscle [26]. In summary, these data suggest loss of dystrophin results in a cytoskeletal remodeling response to bolster the weakened attachment of the ECM to the cytoskeleton.

We recently demonstrated γcyto-actin expression is elevated approximately ten-fold in dystrophin-deficient muscle and hypothesized that the upregulated γcyto-actin functions to reinforce the weakened cytoskeleton by interacting with costameric proteins such as utrophin, the α7 integrin complex, and plectin [27]. Although the function of γcyto-actin in dystrophic muscle has yet to be determined, we showed γcyto-actin expression is required for muscle cell viability as muscle-specific γcyto-actin knockout mice develop a progressive myopathy associated with myofiber necrosis and regeneration along with costamere disorganization and functional deficits [28].
To determine if elevated $\gamma_{cyto}$-actin expression stabilizes parallel linkages between the ECM and cytoskeleton in dystrophin-deficient skeletal muscle, we generated mice lacking both $\gamma_{cyto}$-actin and dystrophin by breeding the conditional Actg1 allele [28] to the mdx background (ms-DKO). No significant differences were measured in dystrophic histological parameters, membrane permeability, and muscle performance when mdx and ms-DKO mice were compared, suggesting $\gamma_{cyto}$-actin and dystrophin function in a common pathway. Increased plectin expression was not found to explain the lack of an exacerbated phenotype in ms-DKO mice. However, utrophin expression was equivalently elevated in mdx and ms-DKO skeletal muscle and co-purified with $\beta$-dystroglycan. These results indicate utrophin can partially abrogate dystrophic phenotypes in mdx skeletal muscle in the absence of a direct link to $\gamma_{cyto}$-actin filaments.

**Results and Discussion**

Expression and localization of cytoplasmic acts in mdx and ms-DKO skeletal muscle

To assess the effects of increased $\gamma_{cyto}$-actin expression in dystrophin-deficient muscle, mice harboring the floxed Actg1 allele [28] and an HSA-Cre transgene [29] were bred to the mdx background to generate mice lacking $\gamma_{cyto}$-actin and dystrophin in skeletal muscle (ms-DKO). Subsequently, expression levels of actin isoforms were determined by western blot analysis of actin extractions from skeletal muscle (Fig. 1A). Consistent with previous findings [27], one-year old mdx mice showed increased $\gamma_{cyto}$-actin expression in skeletal muscle extracts compared to wt (7.1±0.7 fold increase). However, one-year old ms-DKO mice surprisingly showed elevated $\gamma_{cyto}$-actin expression in skeletal muscle extracts compared to wt (3.6±0.8 fold increase, Fig. 1B). Both mdx and ms-DKO mice showed dramatic elevations in $\beta_{cyto}$-actin expression in skeletal muscle extracts when compared to wt (13.8±1.4 and 8.1±0.9 fold respectively) (Fig. 1 B). However, no changes in expression levels of either $\alpha_{cyto}$- or $\gamma_{cyto}$-actin were measured (Fig. 1 A and B).

To determine what cell type was responsible for increased $\gamma_{cyto}$-actin and $\beta_{cyto}$-actin expression in mdx and ms-DKO skeletal muscle extracts, immunofluorescence analysis of quadriceps cross sections was conducted. Small pockets of strong immunoreactivity were observed for both cytoplasmic acts in mdx and ms-DKO skeletal muscle, which appeared to be macrophages invading necrotic fibers (Fig. S1 and Fig. 1 C). Colocalization between a macrophage marker (Mac-1) and both cytoplasmic acts was observed (Fig. S1 and Fig. 1 D), suggesting the elevated cytoplasmic actin expression detected in mdx and ms-DKO skeletal muscle extracts was in part due to macrophage infiltration.

We next quantified the number of macrophages present in wild type, $\gamma_{cyto}$-actin muscle-specific knockout [28] (Actg1 ms-KO), mdx, and ms-DKO skeletal muscle to determine if $\gamma_{cyto}$-actin expression correlates with inflammatory cell infiltration. Because wt skeletal muscle shows low levels of $\gamma_{cyto}$-actin expression and Actg1 ms-KO skeletal muscle shows no $\gamma_{cyto}$-actin expression, we expected to observe low levels of acid-phosphatase positive macrophages in wt and Actg1 ms-KO skeletal muscle. As expected, the number of macrophages present in wt and Actg1 ms-KO skeletal muscle was significantly less than the number of macrophages present in mdx and ms-DKO skeletal muscle (Fig. 2 A and B). Because mdx and ms-DKO skeletal muscle showed identical levels of macrophage infiltration, we conclude that approximately half of the $\gamma_{cyto}$-actin expressed in mdx skeletal muscle extracts (mdx 7.1 fold increase vs. ms-DKO 3.6 fold increase over wt) can be attributed to macrophage infiltration.

![Figure 1. Expression and localization of actin isoforms in skeletal muscle.](image)

To more definitely test the hypothesis that residual $\gamma_{cyto}$-actin expression in ms-DKO skeletal muscle was solely due to macrophage infiltration, we measured $\gamma_{cyto}$-actin expression in muscle extracts from 2.5 week old mice, which is prior to the onset of dystrophy (Fig. 2C). Western blot analysis of actin extractions of skeletal muscle from 2.5 week mice showed detectable $\gamma_{cyto}$-actin.
expression in mdx skeletal muscle and not ms-DKO skeletal muscle (Fig. 2D). These results demonstrate that γcyt-actin expression detected in ms-DKO skeletal muscle extracts was due to macrophage infiltration.

The mdx phenotype is not exacerbated by muscle-specific ablation of γcyt-actin

To determine if ms-DKO mice were more dystrophic than mdx mice, muscle cell death and regeneration was quantified by determining the proportion of centrally nucleated fibers in quadriceps muscles at 1, 3, and 12 months of age. At all timepoints examined, mdx and ms-DKO mice showed comparable levels of histopathology (Fig. 3A) and equivalent levels of muscle cell death and regeneration (Fig. 3B). Accordingly, the mean muscle-fiber diameter of mdx (31.13±0.499 µm) and ms-DKO (29.99±0.479 µm) triceps muscles was significantly smaller than the mean muscle-fiber diameter in wt triceps muscle (38.37±0.464 µm) (Fig. 3C). Muscle cell membrane fragility of mdx and ms-DKO did not differ as demonstrated by comparable levels of serum phosphocreatine kinase (Fig. 3D).

To determine how loss of both γcyt-actin and dystrophin affected muscle performance in vivo, whole body tension analysis was conducted on mice at three months and twelve months of age. At three months of age, wt mice (WBT1 122.2±7.7 mN/g and WBT1–5 111.8±6.9 mN/g) produced significantly more pulling force than both mdx (WBT1 67.2±2.7 mN/g and WBT1–5 61.8±2.7 mN/g) and ms-DKO mice (WBT1 76.8±11.1 mN/g and WBT1–5 69.6±10.8 mN/g). At twelve months of age, wt mice (WBT1 105.5±7.2 mN/g and WBT1–5 94.7±5.3 mN/g) generated significantly more pulling force than both mdx (WBT1 67.6±5.4 mN/g and WBT1–5 54.3±6.1 mN/g) and ms-DKO mice (WBT1 79.5±7.3 mN/g and WBT1–5 67.7±6.4 mN/g). The force generated by mdx mice was not significantly different from the force generated by ms-DKO mice at either age examined (Fig. 4).

To more precisely probe muscle function, contractile properties of isolated extensor digitorum longus muscles were determined. Consistent with the results from in vivo force analysis, mdx and ms-DKO muscle showed similar deficiencies in twitch force (Fig. 5A), maximal force production (Fig. 5B), and normalized maximal force production (Fig. 5C) when compared to wt. In addition, susceptibility to damage caused by eccentric contractions (Fig. 5D) was comparable in mdx and ms-DKO muscle. Taken together, the finding that mdx and ms-DKO muscle function is indistinguishable both in vivo and ex vivo indicates increased γcyt-actin expression does not improve mdx muscle function. Collectively, the results of Figs. 3–5 demonstrate that the established parameters of dystrophin-deficiency in mdx mice were not significantly worsened by skeletal muscle-specific ablation of γcyt-actin.

Assessment of compensatory proteins in mdx and ms-DKO skeletal muscle

To assess expression levels of dystrophin-associated glycoproteins (DAG), KCl-washed microsomes were prepared from wt, mdx, and ms-DKO mice for western blot analysis. α-Sarcoglycan, γ-sarcoglycan, and β-dystroglycan expression levels were similarly reduced approximately 75–80% in mdx and ms-DKO microsomes when compared to wt microsomes (Fig. 6). The finding that utrophin and not plectin expression levels were increased in mdx and ms-DKO microsomes when compared to wt microsomes (3.7±1.0 and 4.5±1.5 fold increases respectively), suggested that increased utrophin expression stabilizes low levels of DAG at mdx.
and ms-DKO membranes (Fig. 6). In agreement with a previous report [27], we observed elevated \( \gamma_{cyto} \)-actin levels in \( mdx \) microsomes when compared to wt microsomes (9.8 \( \pm \) 3.0 fold increase). However, \( \gamma_{cyto} \)-actin was also elevated in ms-DKO microsomes when compared to wt microsomes (5.1 \( \pm \) 2.6 fold increase), which suggested nonmuscle cell types contributed to approximately half of the observed increase in \( \gamma_{cyto} \)-actin expression in \( mdx \) microsomes. Contaminants by nonmuscle cell types also likely explains the elevated levels of \( \beta_{cyto} \)-actin in \( mdx \) and ms-DKO microsomes when compared to wt microsomes (18.3 \( \pm \) 2.2 and 18.1 \( \pm \) 2.0 fold increases respectively) (Fig. 6).

To further investigate whether plectin or utrophin may compensate for the loss of dystrophin, relative expression levels in wt, \( mdx \), and ms-DKO skeletal muscle was examined by western blot analysis of SDS-extracted skeletal muscle. At all ages examined, only utrophin expression was elevated in \( mdx \) and ms-DKO skeletal muscle when compared to wt skeletal muscle (Fig. 7 A and B). Next, we subjected solubilized \( mdx \) and ms-DKO skeletal muscle extracts to wheat-germ agglutinin chromatography to determine if plectin or utrophin co-purified with \( \beta \)-dystroglycan (Fig. 7C). Only utrophin co-purified with \( \beta \)-dystroglycan in \( mdx \) skeletal muscle and ms-DKO skeletal muscle (Fig. 7D). In summary, these data suggest utrophin compensates for the loss of dystrophin through a \( \gamma_{cyto} \)-actin independent mechanisms.

**Concluding remarks**

Identifying proteins that can functionally compensate for the loss of dystrophin is important because strategies to upregulate such proteins may have therapeutic potential for the treatment of DMD. Recently, we identified a ten-fold increase in \( \gamma_{cyto} \)-actin expression in dystrophin-deficient muscle and hypothesized that elevated \( \gamma_{cyto} \)-actin expression may partially strengthen dystro-
Materials and Methods

Generation of mice

Mice harboring the conditional Actg1 allele were described previously [28] and subsequently backcrossed a minimum of five generations to the C57BL/6 background. These mice were crossed to mice expressing cre recombinase under control of the human α-actin promoter (HSA-Cre mice were provided by Dr. Judith Melki, INSERM, France) [29] to generate mice that were homozygous for the floxed Actg1 allele and hemizygous for HSA-Cre. These mice were backcrossed to the mdx background two generations to isolate mice with the genotype Actg1flox/flox HSA-Cre mdx or Actg1flox/lox HSA-Cre mdx. Both mice showed similar phenotypes so their results were pooled. Genotypes of the Actg1 locus and presence of the cre transgene were determined using PCR as described [28]. All animals were housed and treated in accordance with the standards set forth by the University of Minnesota Institutional Animal Care and Use Committee.

Antibodies

Antibodies to γc-syn-actin were described earlier (pAb 7577 and mAb 2-4I) [27]. A polyclonal antibody (pAb) to βc-syn-actin was generated by injecting rabbits with a peptide mimicking the amino terminus of βc-syn-actin (2963). pAb 2963 was then affinity purified using platelet actin (Cytoskeleton Inc catalogue number A4PH199) as described previously [30]. Monoclonal antibodies to βc-syn-actin (AC-15 catalogue number A1978), α-sm-actin (1A4 catalogue number A5228), α-sm-actin (5C5 catalogue number A2172), α-tubulin (B512 catalogue number T6074), and laminin (4H8-2 catalogue number L0663) were purchased from Sigma. The monoclonal γc-sm-actin antibody (B4) was provided as a kind gift from Dr. James Lessard. The monoclonal Mac-1 antibody (CD11b) was provided as a kind gift from Dr. Melissa Spencer. The polyclonal utrophin antibody (103) was provided from Dr. Stanley Froehner. The polyclonal plectin antibody (46) was a kind gift from Dr. Gerhard Wiche. The dystrophin (catalogue number NCL-DYS2), α-sarcoglycan (catalogue number NCL-a-SARC), and the γ-sarcoglycan (catalogue number NCL-g-SARC) antibodies were purchased from Novacasta. The β-dystroglycan antibody (mAb 43DAG-1, catalogue number VP-B205) was purchased from Vector Laboratories. The monoclonal Na+/K+ ATPase antibody (McB2) was described previously [31]. Infrared dye-conjugated anti-mouse and anti-rabbit antibodies were purchased from LI-COR Biosciences (catalogue numbers 926-32223 and 926-32210). Alexa-488- or 568-conjugated anti-rabbit and anti-rat 2º antibodies were purchased from Molecular Probes (catalogue numbers A11034 and A11077).

Muscle Extracts

Muscle was harvested from anesthetized mice immediately following cervical dislocation and snap frozen in liquid N2. SDS-extracts of muscle were performed as described [27]. Protein concentration was determined using the BioRad DC Protein Assay (catalogue numbers 500-0111, 500-0112, and 500-0116). KCl-washed microsomal preparations were generated as described [32] and protein concentration was quantified using a Lowry Assay (Pierce catalogue number 25240). Actin preparations from skeletal muscle were collected by subjecting muscle to a low-salt extraction and DNeasy-1 amplification protocol [27].

Western blot analysis and quantification

To determine changes in protein expression in total muscle and microsomal fractions, 25 μg of protein was subjected to SDS-PAGE and transferred to nitrocellulose. Nitrocellulose membranes

Figure 4. Analysis of force production in vivo. Whole body tension analysis on 3 month old (A) and 11–12 month old mice (B). Averages of the maximal pulling force (WBT1) and the top five pulling forces (WBT1–5) are represented graphically. Error bar represents the SEM. Asterisk indicates a statistically significant different outcome from wt (p<0.05).

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phosphodeficient membranes by fortifying parallel links between the ECM and cytoskeleton [27]. To determine if increased γc-syn-actin expression stabilizes the cortical actin cytoskeleton in dystrophin-deficient skeletal muscle, we generated mice lacking dystrophin and conditionally lacking γc-syn-actin in skeletal muscle (ms-DKO). Surprisingly, the phenotype of msdx mice was identical as demonstrated by comparable levels of muscle cell death and regeneration, muscle membrane fragility, and muscle weakness. These results suggest increased γc-syn-actin expression does not fortify dystrophin-deficient muscle cell membranes, but γc-syn-actin and dystrophin lie in the same functional pathway. Consistent with previous results, utrophin co-purified with β-dystroglycan [15] and showed increased expression in msdx skeletal muscle [14–18]. We also showed utrophin co-purified with β-dystroglycan and showed expression levels comparable to mdsx in ms-DKO skeletal muscle. Collectively, these results imply utrophin does not need to interact with γc-syn-actin to attenuate dystrophic phenotypes in msdx skeletal muscle.
were washed/block in a 5% milk solution in phosphate-buffered saline (PBS) for one hour at room temperature. Membranes were then incubated with primary antibodies overnight at room temperature (primary antibody dilutions: mAb 2-4 (1:1000), mAb AC-15 (1:500), mAb 1A4 (1:250), mAb B4 (1:250), mAb 5C5 (1:2000), mAb DYS2 (1:50), pAb 103 (1:250), pAb 46 (1:3000), mAb 43DAG-1 (1:50), mAb NCL-a-SARC (1:50), mAb NCL-g-SARC (1:50), mAb McB2 (1:100), and mAb B512 (1:1000)). Membranes were washed in 5% milk solution in PBS ten minutes two times at room temperature and then incubated with IR-dye conjugated secondary antibodies (1:10,000 dilution) for 30 minutes at room temperature. Then, membranes were washed/block with 0.1% triton solution in PBS for ten minutes two times to remove excess secondary antibody. Western blots were imaged and quantified with an Odyssey Infrared Imaging System (LI-COR Biosciences catalogue number 9201-01). The normalizing signal in SDS-extracts was α-tubulin while Na+/K+ ATPase was used in microsomal fractions.

**Immunofluorescence microscopy**

Individual muscles were dissected, coated in OCT (TissueTek catalogue number 4583), and then frozen in melting isopentane. Ten micron transverse sections were cut on a Leica CM3050 cryostat, air dried, then fixed in 4% paraformaldehyde for 10 minutes. Sections were washed with PBS, blocked in 5% goat serum for 30 min, and incubated with 1° antibodies overnight at 4°C (primary antibody dilutions: pAb 7577 (1:50), pAb 2963 (1:1) mAb 4H8-2 (1:250), and mAb Mac-1 (1:100)). Sections were then washed/blocking with 5% goat serum for 10 min 3 times and then incubated with Alexa-488- or 568-conjugated 2° antibodies (1:1000 dilution) for 30 min at 37°C. Then, sections were washed with PBS and coverslips were applied with a drop of Anti-Fade Reagent (Molecular Probes catalogue number P36930). Confocal images were collected on a Bio-Rad MRC 1000 scan head mounted on an upright Nikon Optiphot microscope at the University of Minnesota Biomedical Image Processing Lab. Images were equivalently processed using Adobe Photoshop.

**Assessment of dystrophic parameters**

Ten micron thick cryosections of quadriceps, tibialis anterior, and triceps were stained with hematoxylin and eosin-phloxine as described [33]. Four images from different areas of the muscle section were collected on a Zeiss Axiovert 25 microscope using ImagePro Software. These images were imported into Scion Image to determine the proportion of centrally nucleated fibers (800–1000 fibers counted/muscle) at 1, 3, and 12 month old mice (n = 4 for each genotype at each timepoint). Fiber diameter distribution was determined in 12 month old triceps sections as described [33] from at least 700 fibers/genotype. Membrane permeability was determined by quantifying serum creatine kinase levels on Vitros CK DT slides (Ortho-Clinical Diagnostics catalogue number DT1975580) using a Kodak Ektachem DT60 analyzer.

**Figure 5. Contractile properties of isolated extensor digitorum longus muscles.** Examination of normalized twitch force (A), maximal tetanic force (B), normalized maximal tetanic force (C), and susceptibility to damage caused by eccentric contractions (D). Both mdx and ms-DKO extensor digitorum longus muscles showed significant decrements when compared to wt extensor digitorum longus muscles. (*) indicates p<0.05 when compared to wt. doi:10.1371/journal.pone.0002419.g005
Quantification of inflammation

Ten micron thick cryosections from either quadriceps or gastrocnemius (4 sections per genotype) were subjected to an Acid Phosphatase stain. Briefly, sections were incubated in Acid Phosphatase buffer (98 mM Napthol AS-BI Phosphate, 0.046 M Sodium Acetate, 0.15% Pararosaniline, and 0.58 M Sodium Nitrite pH 5.0) for two hours at 37°C. Sections were washed extensively and then counterstained with Gills Hematoxylin and mounted in Permount (Fisher Scientific catalogue number SP15-100). Montages of muscle sections were collected on a Zeiss Microscope mounted with a Leica DFC300 FX camera. The entire muscle section was examined and the number of Acid Phosphatase-positive macrophage were counted and normalized to section area (mm²) using Image Pro Plus Software.

Whole body tension

Mice were subjected to whole body tension as described [28]. At 3 months of age 5 wild type, 5 mdx, and 4 ms-DKO mice were subjected to analysis. At 11 to 12 months of age 5 wild type mice, 7 mdx, and 7 ms-DKO mice were analyzed.

Contractile properties of isolated extensor digitorum longus

11–12 month old mice were anesthetized and the extensor digitorum longus muscle was removed (n = 4 mice per genotype). The proximal tendon was attached to 4-0 suture silk to a dual-mode muscle lever system (model 300B-LR Aurora Scientific, Aurora, ON, Canada). Muscles were equilibrated for 10 minutes in a bath assembly containing Krebs-Ringer-bicarbonate buffer (119 mM NaCl, 5.0 KCl mM, 1.0 MgSO₄ mM, 12.25 NaHCO₃ mM, 1.0 CaCl₂ mM, 1.0 mM KH₂PO₄, 10.0 mM glucose, 0.17 mM leucine, 0.10 mM isoleucine, 0.20 mM valine plus 10 μg/ml gentamicin sulfate and 0.10 U/ml insulin) at 25°C while being constantly oxygenated with 95% O₂/5% CO₂ gas. Then, the resting tension was set to 0.4 g and twitch force was determined by stimulating the muscle with a 0.5 ms pulse at 150 V (Grass stimulator, Grass Telefactor, Warwick, RI). Thirty seconds later the muscle was stimulated to twitch again and the greater of two contractions was recorded. Tetanic contractions were elicited by stimulating the muscle with 150 V for 200 ms at 180 Hz. The greater of two contractions separated by two minutes of recovery time was recorded. To determine damage caused by eccentric contractions, muscles were subjected to five lengthening contractions at three minute intervals. Each ECC consisted of a maximal tetanic stimulation for 200 ms at 180 Hz. The greater of two contractions separated by two minutes of recovery time was recorded. To determine damage caused by eccentric contractions, muscles were subjected to five lengthening contractions at three minute intervals. Each ECC consisted of a maximal tetanic stimulation for 200 ms at 180 Hz. The greater of two contractions separated by two minutes of recovery time was recorded. To determine damage caused by eccentric contractions, muscles were subjected to five lengthening contractions at three minute intervals. Each ECC consisted of a maximal tetanic stimulation for 200 ms at 180 Hz. 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Author Contributions
Conceived and designed the experiments: JE KP. Performed the experiments: KP DL. Analyzed the data: JE KP. Contributed reagents/materials/analysis tools: DL. Wrote the paper: JE KP.

References
1. Emery AE (1993) Duchenne muscular dystrophy–meryon's disease. Neuromuscul Disord 3(4): 263–266.
2. Moser H (1984) Duchenne muscular dystrophy: Pathogenetic aspects and genetic prevention. Hum Genet 66(1): 17–40.
3. Hoffman EP, Brown RH Jr, Kunkel LM (1987) Dystrophin: The protein product of the duchenne muscular dystrophy locus. Cell 51(6): 919–928.
4. Matsumura K, Campbell KP (1994) Dystrophin-glycoprotein complex: Its role in the molecular pathogenesis of muscular dystrophies. Muscle Nerve 17(1): 2–15.
5. Rybakova IN, Patel JR, Ervasti JM (2000) The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin. J Cell Biol 150(5): 1209–1214.
6. Chung W, Campanelli JT (1999) WW and EF hand domains of dystrophin-family proteins mediate dystroglycan binding. Mol Cell Biol Res Commun 2(3): 162–171.
7. Ishikawa-Sakurai M, Yoshida M, Isamura M, Davies KE, Ozawa E (2004) ZZ domain is essentially required for the physiological binding of dystrophin and utrophin to beta-dystroglycan. Hum Mol Genet 13(7): 693–702.
8. Suzuki A, Yoshida M, Yamamoto H, Ozawa E (1992) Glycoprotein-binding site of dystrophin is confined to the cysteine-rich domain and the first half of the carboxy-terminal domain. FEBS Lett 308(2): 154–160.
9. Ervasti JM, Campbell KP (1993) A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. J Cell Biol 122(4): 889–893.
10. Ervasti JM (2005) Costameres: The achilles’ heel of herculean muscle. J Biol Chem 27(16): 13591–13594.
11. Blake DJ, Weir A, Newey SE, Davies KE (2002) Function and genetics of dystrophin and dystrophin-related proteins in muscle. Physiol Rev 82(2): 291–329.
12. Petrof BJ, Shragger JB, Stedman HH, Kelly AM, Sweeney HL (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. Proc Natl Acad Sci U S A 90(8): 3710–3714.
13. Bullfield G, Siller WG, Wight PA, Moore KJ (1984) X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc Natl Acad Sci U S A 81(4): 1189–1192.
14. Khurana TS, Watkins SC, Chafey P, Chelly J, Tome FM, et al. (1991) Immunolocalization and developmental expression of dystrophin related protein in skeletal muscle. Neuromuscul Disord 1(3): 185–194.
15. Matsumura K, Ervasti JM, Ohlendieck K, Kuhl S, Campbell KP (1992) Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. Nature 360(6404): 588–591.
16. Porter JD, Rafael JA, Godwin AJ, Bruckner JK, Trickett JI et al. (1998) The sparing of extraocular muscle in dystrophinopathy is lost in mice lacking utrophin and dystrophin. J Cell Sci 111 (Pt 13): 1081–1081.
17. Rybakova IN, Patel JR, Davies KE, Yurchenco PD, Ervasti JM (2002) Utrophin binds laterally along actin filaments and can couple costameric actin with sarcolemma when overexpressed in dystrophin-deficient muscle. Mol Biol Cell 13(1): 1512–1521.
18. Weir AP, Morgan JE, Davies KE (2004) A-utrophin up-regulation in mdx skeletal muscle is independent of regeneration. Neuromuscul Disord 14(1): 19–23.

Figure 7. Comparison of utrophin and plectin in dystrophic muscle. (A) Representative western blots from SDS-extracted skeletal muscle from wt, mdx, and ms-DKO mice. (B) Quantification of utrophin and plectin expression in skeletal muscle of mdx and ms-DKO mice. Fold difference is determined by comparing mdx and ms-DKO to wt. Error bars represent standard error of the mean. (C) Schematic representation of method used to determine what proteins co-purify with β-dystroglycan. (D) Western blot analysis of the input fraction and wheat germ agglutinin elution fraction of digitonin solubilized mdx and ms-DKO skeletal muscle. Utrophin, and not plectin, co-purified with β-dystroglycan.
doi:10.1371/journal.pone.0002419.g007
19. Rybakova IN, Humston JL, Sonnemann KJ, Ervasti JM (2006) Dystrophin and utrophin bind actin through distinct modes of contact. J Biol Chem 281(15): 9996–10001.

20. Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, et al. (1997) Utrophin-dystrophin-deficient mice as a model for duchenne muscular dystrophy. Cell 90(4): 717–727.

21. Grady RM, Teng H, Nichol MC, Cunningham JC, Wilkinson RS, et al. (1997) Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: A model for duchenne muscular dystrophy. Cell 90(4): 729–738.

22. Tinsley J, Deconinck N, Fisher R, Kahn D, Phelps S, et al. (1998) Expression of full-length utrophin prevents muscular dystrophy in mdx mice. Nat Med 4(12): 1441–1444.

23. Hodges BL, Hayashi YK, Nonaka I, Wang W, Arahata K, et al. (1997) Altered expression of the alpha7beta1 integrin in human and murine muscular dystrophies. J Cell Sci 110 (Pt 22): 2873–2881.

24. Rooney JE, Welser JV, Dechert MA, Flintoff-Dye NL, Kaufman SJ, et al. (2006) Severe muscular dystrophy in mice that lack dystrophin and alpha7 integrin. J Cell Sci 119(Pt 11): 2185–2195.

25. Hanft LM, Rybakova IN, Patel JR, Rafael-Fortney JA, Ervasti JM (2006) Cytoplasmic gamma-actin contributes to a compensatory remodeling response in dystrophin-deficient muscle. Proc Natl Acad Sci U S A 103(14): 5383–5388.

26. Sonnemann KJ, Fitzsimons DP, Patel JR, Liu Y, Schneider MF, et al. (2006) Cytoplasmic gamma-actin is not required for skeletal muscle development but its absence leads to a progressive myopathy. Dev Cell 11(3): 387–397.

27. Minou P, Tizzano D, Friapter T, Roblot N, Le Meur M, et al. (1999) Gene targeting restricted to mouse striated muscle lineage. Nucleic Acids Res 27(19): e27.

28. Sharp AH, Campbell KP (1989) Characterization of the 1,4-dihydropyridine receptor using subunit-specific polyclonal antibodies. evidence for a 32,000-da subunit. J Biol Chem 264(5): 2016–2025.

29. Ohlendieck K, Ervasti JM, Snook JB, Campbell KP (1991) Dystrophin-glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. J Cell Biol 112(1): 135–148.

30. Harper SQ, Hauser MA, DelloRusso C, Duan D, Crawford RW, et al. (2002) Modular flexibility of dystrophin: Implications for gene therapy of duchenne muscular dystrophy. Nat Med 8(3): 253–261.