Characterization of dystrophin and utrophin diversity in the mouse

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Utrophin is a 400 kDa autosomal homolog of dystrophin and a component of the submembranous cytoskeleton. While multiple dystrophin isoforms have been identified along with alternatively spliced products, to date only two different mRNA species of utrophin have been identified. To determine the degree of evolutionary conservation between dystrophin and utrophin isoforms, we have compared their expression patterns in adult mice. Northern blot analysis of multiple adult tissues confirmed that only two major sizes of transcripts are produced from each gene: 13 and 5.5 kb from utrophin and 14 and 4.8 kb from dystrophin. However, western blot analysis detected several putative short utrophin isoforms that may be homologs of the dystrophin isoforms Dp140, Dp116 and Dp71. We also identified an alternatively spliced utrophin transcript that lacks the equivalent of the alternatively spliced dystrophin exon 71. Finally, we demonstrated that the C-terminal domain of utrophin targeted to neuromuscular junctions in normal mice, but localized to the sarcolemma efficiently only in the absence of dystrophin. Our results provide further evidence for a common evolutionary origin of the utrophin and dystrophin genes.

INTRODUCTION

The submembranous cytoskeleton is composed of a diverse array of molecules that organize cellular structure. One cytoskeletal protein family is comprised of dystrophin and its related proteins [utrophin (1) and DRP2 (2)]. These proteins interact with a large complex of proteins in skeletal muscle known as the dystrophin-associated protein complex (DAPC), which includes the dystroglycans, the sarcoglycans and the syntrophins (for reviews, see refs 3,4).

In muscle, dystrophin, utrophin and the DAPC preserve myofiber integrity by linking actin filaments beneath the sarcolemma to components of the extracellular matrix (ECM).

The importance of dystrophin and utrophin is evident in the phenotypes produced by loss-of-function mutations in these genes. Dystrophin deficiency leads to the muscular dystrophy observed in mdx mice and in patients with Duchenne and Becker muscular dystrophies (DMD/BMD). Without dystrophin, the DAPC is unstable and its levels are severely reduced at the sarcolemma (5). Utrophin-deficient mice have defects in the post-synaptic membrane folds at the neuromuscular junction (NMJ) (6,7). Mice lacking both dystrophin and utrophin display a severe muscular dystrophy with premature death (8,9).

Utrophin and dystrophin are postulated to have arisen from an ancient gene duplication event (10). The dystrophin gene contains seven independent promoters encoding three full-length proteins and four N-terminally truncated proteins (Dp260, Dp140, Dp116 and Dp71) (11–14). There is also extensive alternative splicing of exons 71–74 and 78 that adds to the complexity of dystrophin gene products (15,16). To date, there is little evidence for conserved expression of these alternative transcripts in the utrophin gene. One short utrophin mRNA isoform (G-utrophin) has been cloned and identified as the homolog of Dp116 (17). However, no protein product from this transcript has been identified. Several reports have suggested the presence of smaller utrophin proteins in various tissues and cell lines (18–20).

Like dystrophin (Dp427), utrophin (Up400) contains an N-terminal actin-binding domain, a central spectrin-like repeat region and a C-terminal domain that binds to the DAPC (21). In muscle, utrophin protein is restricted to NMJs and myotendinous junctions, while dystrophin is distributed throughout the sarcolemma (22–24). In other tissues, utrophin is expressed in central nervous system (CNS) blood vessels and membranes (25,26), retinal ganglia and inner nuclear layers (27), small arteries and veins (19), lung (28), platelets (29) and peripheral nerves (20,28).

It is not known if the DAPC is assembled with utrophin at these sites and, in general, little is known about the function of utrophin, dystrophin and the DAPC in non-muscle tissues.

Despite its similarity to dystrophin, utrophin has unique properties that suggest that the utrophin-associated protein complex (UAPC) is distinct from the DAPC. While α1- and β1-syntrophin associate with dystrophin, β1- and β2-syntrophin preferentially associate with utrophin (30). At NMJs, utrophin is

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restricted to the crests of the junctional folds, while dystrophin is found at the troughs (31). The C-terminal region of utrophin is sufficient to localize the protein to junctional patches and to restrict it from extrajunctional sarcolemmal sites (32). Other studies appear to contradict this observation and suggest that utrophin has little intrinsic selectivity toward NMJ binding sites and can co-localize with dystrophin at the sarcolemma (33).

Resolving these differences and understanding the isoform diversity of utrophin will be important if utrophin is to be utilized in gene therapy for dystrophin deficiency in DMD patients as proposed (33). To compare and contrast the diversity of dystrophin and utrophin gene products, we have performed a comprehensive study of their expression in the adult mouse.

RESULTS

Utrophin expression in adult mouse tissues

While examining the expression of the 3′ end of the murine dystrophin gene (15), we also isolated 2.1 kb of the 3′ end of the mouse utrophin cDNA using RT–PCR with degenerate primers based on the human utrophin sequence. Our sequences closely matched the reported full-length mouse utrophin sequence (the base pair numbers given are based on this latter sequence) (32). To compare the diversity of utrophin expression in the mouse with that of dystrophin, we probed a northern blot of RNA samples from adult C57BL/10 tissues with a fragment from the 3′ end of the mouse utrophin cDNA (bp 9382–9943) (Fig. 1). We detected a 13 kb transcript in adult brain, heart, liver, testis and kidney which corresponded to the full-length utrophin mRNA. An additional 5.5 kb message was detected primarily in brain, with lower levels in testis and kidney. This latter band corresponded to the G-utrophin transcript previously described (17). The identical blot was stripped and reprobed with the corresponding 3′ fragment of the mouse dystrophin cDNA (bp 10 280–10 960) (data not shown). In contrast to utrophin, the 14 kb full-length dystrophin message was only detected in brain and heart. A smaller 4.8 kb mRNA, corresponding to Dp71 transcripts, was observed in all non-muscle tissues sampled (liver, testes and kidney).

To analyze utrophin proteins, we generated polyclonal antibodies against a C-terminal region of utrophin (amino acids 3123–3429). The specificity of these antibodies for utrophin was determined by negative selection of the antisera against a bacterial fusion protein containing the equivalent region of dystrophin, followed by positive selection on a utrophin fusion protein column. These affinity-purified antibodies detected the full-length 400 kDa utrophin protein (Up400) in all tissues sampled (testes, spleen, kidney, liver, brain and muscle), in agreement with the northern blots (Fig. 2). In addition, our antibodies detected multiple smaller bands, suggesting a diversity of utrophin isoforms. Proteins of 78 and 82 kDa were detected in brain extracts of both wild-type C57 and mdx<sup>cv</sup> mice. This result agrees with other reports that detected an 80 kDa utrophin protein (Up80) in neuronal tissues (19,20). Up80 is the putative utrophin homolog of Dp71. mdx<sup>cv</sup> mice express extremely low levels of all dystrophin isoforms due to a mutation in intron 65 (34); therefore, the 78–82 kDa bands are not due to cross-reactivity with Dp71. Our antibodies also detected proteins of 90 kDa in testis, spleen and liver (Up90) and of ≈97 kDa in testes. Our antibodies did not detect a protein corresponding to the full-length G-utrophin (predicted mol. wt of 110 kDa).

![Figure 1.](image1) Expression of utrophin mRNA in adult mouse tissues. Northern analysis of utrophin expression. RNA (20 µg) from adult C57BL/10 tissues was separated on an agarose–formaldehyde gel and transferred to nylon membranes. The blot was first probed with a 3′ fragment of utrophin (bp 9382–9943) (Fig. 1). We detected a 13 kb transcript in adult brain, heart, liver, testis and kidney which corresponded to the full-length utrophin mRNA. An additional 5.5 kb message was detected primarily in brain, with lower levels in testis and kidney. This latter band corresponded to the G-utrophin transcript previously described (17). The identical blot was stripped and reprobed with the corresponding 3′ fragment of the mouse dystrophin cDNA (bp 10 280–10 960) (data not shown).

![Figure 2.](image2) Multiple utrophin isoforms detected by immunoblot. A 100 µg aliquot of total protein extracts from adult C57BL/10 tissues and from mdx<sup>cv</sup> brain was electrophoresed by SDS–PAGE and transferred to nitrocellulose. Blots were probed with affinity-purified C-terminal utrophin antibodies. Major bands are identified by arrows.

For comparison, the same tissue panel was probed with antibodies against the C-terminus of dystrophin (NCL-DYS2) (data not shown). Full-length Dp427 was detected only in brain and muscle extracts, while Dp71 was expressed in all non-muscle tissues analyzed, with the highest levels in testes and brain. As
expected, brain extracts from mdx<sup>2<sup>C</sup></sup> mice did not have detectable dystrophin. A 140 kDa protein was observed in both the utrophin and dystrophin immunoblots, which may be a non-specific product or could correspond to either Dp140 or a novel 140 kDa utrophin isoform.

**Alternative splicing in utrophin and dystrophin C-terminal domains**

To expand on previous observations of alternative splicing in dystrophin, we examined splicing across exons 71–74 in a panel of adult mouse RNAs using RT–PCR (Fig. 3A). Alternatively spliced transcripts were detected in embryonic muscle and brain, and adult muscle, brain and heart. The major splice variants were Δ39 bp (removal of exon 71), Δ159 bp (removal of exon 74) and Δ330 bp (removal of exons 71–74) (Fig. 3E). Analysis of additional tissues indicated that there is little or no alternative splicing in adult liver, kidney and spleen. However, in adult testes, we detected both the Δ39 and Δ330 bp splice variants. Based on the previous northern and western blots, the splice variants in non-muscle tissues are most likely derived from Dp71 transcripts.

We hypothesized that alternative splicing would occur in the 3′ end of the utrophin gene, given its homology with dystrophin. Previous studies did not detect alternative splicing in the C-terminal domain of utrophin (35). We examined the utrophin region equivalent to dystrophin exons 71–74 for alternative splicing by RT–PCR using primers designed to amplify the utrophin cDNA between bp 9382 and 9943. A 561 bp band corresponding to full-length utrophin was observed in all tissues examined. However, an alternative transcript was observed in the spleen sample as a smaller PCR product (Fig. 3B). Subcloning and sequencing of the smaller product identified a 39 bp deletion between bp 9486 and 9524. By sequence alignments, the deleted segment corresponded precisely to the 39 bp dystrophin exon 71 (bp 10 423–10 461), indicating that alternative splicing of this exon has been conserved between the genes.

Alternative use of dystrophin exon 78 results in different dystrophin C-termini (16). The inclusion of exon 78 generates a hydrophilic tail to the protein, while exclusion of exon 78 generates an alternative hydrophobic C-terminus frequently detected in fetal tissues. To examine exon 78 splicing in adult mouse tissues, we performed RT–PCR using primers flanking dystrophin exon 78 (bp 10 936–11 336) (Fig. 3C). Full-length transcripts produce a 400 bp band, while a 368 bp product (Δ32 bp) results from products that have spliced around exon 78. Doublets were clearly observed in samples from adult liver, kidney and spleen, and faint Δ32 bp products were present in the brain and heart lanes. A Δ32 bp product was not detected in skeletal muscle. This result indicates that alternative usage of exon 78 is widespread in many adult tissues except skeletal muscle.

To examine the extreme C-terminus of utrophin for alternative splicing, primers were made against sequences in the coding region and 3′ untranslated region (3′-UTR; bp 9996–10 363) (Fig. 3D). To insure high sensitivity, the primers were radiolabeled before PCR. In all samples examined, only the full-length 367 bp product was detected. As a control, dystrophin primers flanking exon 78 were used for RT–PCR on brain RNA samples and correctly detected the full-length and Δ32 bp alternatively spliced products. Therefore, on the basis of size, the final coding exons of utrophin were not alternatively spliced in any tissues examined.

**Expression of the utrophin C-terminal domain in skeletal muscle**

Since the C-terminal domain of dystrophin alone (Dp71) can localize to the sarcolemma (36), we investigated the ability of the
Figure 4. Expression of the utrophin C-terminus in muscle. (A) Schematic illustration of pCMV-FcDRP. The promoter is a 524 bp fragment of the CMV enhancer/promoter linked to the minx intron (47). Dp71 sequences included are the 5′-UTR and the first 10 amino acids (shaded region). A FLAG epitope (F) is incorporated at the N-terminus of the utrophin sequence. (B) Immunoblot of transfected 293 cells extracts with anti-FLAG antibodies detects a 75 kDa protein. Cells were transfected with pCMV-FcDRP or no DNA and harvested in TBS + 1% Triton. (C and D) FcDRP localizes to NMJs in wild-type mice. pCMV-FcDRP + pcDNA-lacZ were injected into BALB/c TA muscles. Sections were double-labeled with biotinylated anti-FLAG antibodies followed by streptavidin-Alexa488 (C) and TRITC-conjugated α-bungarotoxin (D) and visualized by epifluorescence. (E and F) FcDRP localizes to the sarcolemma in mdx mice. pCMV-FcDRP + pcDNA-lacZ were injected into mdx TA muscles. Adjacent sections were stained with biotinylated anti-FLAG antibodies and visualized by epifluorescence (E) or with X-gal (F). Bar, 50 µm.

equivalent utrophin C-terminal domain to localize to the membrane in vivo. For these studies, we constructed a vector that would express the utrophin C-terminal domain (bp 8533–10,610) and evaluated its expression pattern by direct DNA injection into muscle. The vector (pCMV-FcDRP) contained the utrophin C-terminal domain fused to the unique first exon of Dp71 driven by the cytomegalovirus (CMV) promoter (Fig. 4A). For detection, a FLAG epitope tag was incorporated at the 5′ end of the cDNA. pCMV-FcDRP was tested in cell culture by transient transfection into 293 cells (Fig. 4B). On immunoblots of transfected cell extracts, we observed a single band of 75 kDa, indicating that the recombinant protein (FcDRP) was properly expressed.

pCMV-FcDRP (50 µg) was injected into the tibialis anterior (TA) of 2-week-old mdx and BALB/c mice along with a β-galactosidase expression vector (5 µg of pcDNA-lacZ) as a marker for transduced areas. After 1 week, animals were sacrificed and their muscles analyzed for FcDRP expression by immunofluorescence with anti-FLAG monoclonal antibodies. Injections into dystrophin-positive BALB/c mice showed little sarcolemmal staining in Xgal-positive regions. FcDRP was restricted to NMJs as detected by co-localization with α-bungarotoxin (Fig. 4C and D). In contrast, anti-FLAG antibodies detected expression of FcDRP throughout the sarcolemma in lacZ-positive regions of injected mdx mice (Fig. 4E and F). These experiments showed that, like Dp71, the C-terminal domain of utrophin is sufficient for associations with the sarcolemma and NMJs. These data also suggested that FcDRP preferentially localized to junctional regions and did not compete efficiently with dystrophin for sarcolemma sites of attachment.

DISCUSSION

This study characterized C-terminal utrophin expression in adult mouse tissues and suggests a more complex array of utrophin gene products than has been described previously. Analysis of utrophin mRNA and protein in a panel of tissues highlighted several significant differences and similarities between utrophin and dystrophin. The full-length utrophin mRNA and protein (Up400) were expressed in every tissue sampled and were the predominant utrophin species in each tissue. Our observations of Up400 distribution agree with previous reports of utrophin expression in mdx mouse tissues (35). In contrast, Dp427 was observed only in brain and muscle. The shorter Dp71 protein was the major dystrophin protein outside of muscle. These data suggest that, in non-muscle tissues, utrophin functions as the major link between actin filaments and the ECM.
Our utrophin antibodies also detected a variety of smaller putative utrophin gene products analogous to the known short dystrophin isoforms. Several other groups have observed small gene products using antibodies raised against the utrophin C-terminus (19, 20, 28). Up80 has been detected by us and others in the CNS, small peripheral arteries and sciatic nerve. Such an isoform may be a relative of the Dp71 non-muscle dystrophin product. In our immunoblots, Up80 appeared as a doublet that resembled the broad molecular weight range for Dp71 caused by differential phosphorylation (34). We predict that, like Dp71, Up80 is transcribed from an internal promoter and a unique first exon. However, our attempts to use PCR-based methods (5′ RACE and library screens) to identify and clone a Up80 cDNA have not been successful (data not shown).

G-utrophin, with a predicted mol. wt of 110 kDa, is the only short utrophin gene product that has been identified by cDNA cloning (17). While our northern blots clearly demonstrate the presence of a G-utrophin-sized transcript in brain, testes and kidney, our antibodies do not detect a 110 kDa protein. Blake et al. (17) reported that the G-utrophin open reading frame contains multiple in-frame AUG codons, but that none displayed a close match with the Kozak consensus sequence. The inability to detect a G-utrophin protein may be because of poor translation or instability of the protein product. Since the translation initiation site of the G-utrophin mRNA is unclear, it is also possible that some, or all, of the smaller protein isoforms we detected arose from the 5.5 kb utrophin mRNA.

Our data have revealed further the pattern of alternative splicing in the 3′ end of the dystrophin gene. As reported previously, dystrophin exons 71–74 are spliced in a complex pattern in embryonic and adult brain and muscle, with the major products being Δ39, Δ159 and Δ330 bp isoforms (15). We now show that similar products are produced in adult testes, but no alternative splicing was observed in adult liver, kidney or spleen. The splicing pattern we observed in adult mouse brain and kidney agreed with the pattern we observed in adult mouse brain and kidney (15, 16, 37). We have now also detected alternative splicing of exon 78 in adult liver, kidney and spleen, suggesting that the phenomenon is widespread. Notably, in skeletal muscle, exclusion of exon 78 does not occur in adult animals. Our detection of exon 78 splicing in mouse kidneys agreed with the observations of similar splicing in human kidney (37).

While dystrophin has multiple alternatively spliced exons, we observed only one case of alternative exon usage in utrophin. A 39 bp segment that corresponds precisely to dystrophin exon 71 (in-frame deletion of 13 amino acids) was alternatively spliced in the spleen. These data show that the mechanisms that regulate splicing around dystrophin exon 71 have been conserved in the utrophin gene. The conservation of splicing regulation between dystrophin and utrophin suggests the close evolutionary relationship between the two genes. The splicing of dystrophin exons 72–74 may have developed after its genetic separation from the utrophin gene.

We also explored the localization of the C-terminal domain of utrophin when expressed in muscle. Previous reports presented seemingly conflicting data on the ability of utrophin to localize at NMJs when constitutively expressed. In cultured muscle cells, Guo et al. (32) showed that the C-terminal region of utrophin, from bp 7735 to the end, can localize utrophin to junctional patches and does not recognize dystrophin-binding sites at extrajunctional regions. However, in utrophin transgenic mdx mice, utrophin co-localized with dystrophin throughout the sarcolemma, suggesting that utrophin has little inherent specificity toward NMJ binding sites (33).

We observed that the utrophin C-terminal domain from the cysteine-rich domain (bp 8532) to the end localized only at NMJs when expressed in wild-type mice, but localized to the sarcolemma in mdx mice. These data support the hypothesis that the C-terminal domain of utrophin has a specific affinity for NMJ binding sites. They also suggest that dystrophin has a higher affinity for extrajunctional binding sites (DAPC binding) than does utrophin. Such qualitative differences in affinity may explain why utrophin protein is not detectable at extrajunctional sites despite the production of utrophin transcripts from extrajunctional nuclei (41), and may account for some of the differential distributions of dystrophin and utrophin at the NMJ (31). In the absence of dystrophin, variable amounts of utrophin are detected at non-junctional sites. This phenomenon may result from the release of dystrophin’s competition with utrophin for DAPC sites.

The presence of multiple utrophin isoforms adds to the complexity of dystrophin-related protein complexes outside of muscle. Functional correction of the mdx phenotype by ectopic expression of dystrophin clearly demonstrates a functional overlap between these related proteins (33, 42). However, their distinct localization patterns and similar, but not identical, isoform expression pattern indicate that these proteins have unique functions in both muscle and non-muscle tissues. Investigation of utrophin complex composition in non-muscle cells will aid in further understanding its function.

**MATERIALS AND METHODS**

**Cloning and RT–PCR**

All cloning, PCR and sequencing were performed as described (43). Reverse transcriptase reactions was performed using 1 μg of total RNA as previously described (15). Degenerate primers were used to PCR amplify the 3′ end of murine utrophin from C57Bl/10 adult skeletal muscle cDNA. The nucleotide sequences obtained were homologous to bp 8496–10 610 (amino acids 3071–3429) of the mouse utrophin cDNA previously reported (32) (GenBank accession no. Y12229). To facilitate cloning, an NruI site was engineered at bp 9375 and a NotI site was added at bp 10 290 by PCR. RT–PCR of dystrophin was performed as previously described (15).

**Utophin and dystrophin bacterial fusion proteins**

A bacterial expression vector was constructed to express a fusion of *Escherichia coli* maltose-binding protein (MBP) and the C-terminal 305 amino acids of mouse utrophin (amino acids 3123–3428). An NruI–NotI fragment (bp 9374–10 290) was blunt-end ligated into pMAL-c (New England Biolabs, Beverly, MA) at the EcoRI site (pMAL-DRP-EF). Another expression vector was constructed in pMAL-c in a similar manner expressing the equivalent region (bp 10 311–11 320, amino acids 3362–3678) of the mouse dystrophin cDNA (pMAL-DYS-EF) (36). Both fusion proteins were expressed and purified according to the manufacturer’s specifications. Fusion protein affinity columns were made by coupling purified MBP-DRP-EF and MBP-DYS-EF fusion proteins (2–4 mg) to Affigel-10 (Bio-Rad, Hercules, CA) according to the manufacturer’s specifications.
Generation and affinity purification of utrophin-specific antisera

Purified MBP-DRP-EF was injected into female New Zealand white rabbits as previously described (36). Antisera from bleeds 4–6 were diluted 1:5–10 and passed at least four times over the MBP-DYS-EF column to remove any cross-reacting activity. For further purification, the flow-through from the MBP-DYS-EF column was passed over an MBP-DRP-EF column to select for utrophin-specific antibodies. The negatively and positively selected affinity-purified antibodies were eluted from the second column in 20 mM glycine pH 2.5 and neutralized with 1 M Tris base. Fractions were concentrated using Centricron-30 microconcentrators (Millipore, Bedford, MA). The specificity of the affinity-purified utrophin antisera was demonstrated by an inability to detect dystrophin fusion proteins on immunoblots and an inability to detect sarcolemmal staining in wild-type muscle frozen sections (data not shown).

Northern analysis and western blotting

Northern analysis was performed by electrophoresis of 20 µg of total RNA (44) on 1.2% agarose–formaldehyde gels. The RNA was transferred to Nytran membranes (Schleicher and Schuell, Keene, NH) and pre-hybridized at 42°C overnight [50% formamide, 5x SSC, 5x Denhardt’s, 50 mM sodium phosphate pH 7.4, 1% glycine, 5 mM EDTA, 0.2% SDS, 0.1 mg/ml poly(A)]. Hybridization was performed overnight at 42°C using 3 x 106 c.p.m. of probe per ml of hybridization solution [50% formamide, 5x SSC, 1x Denhardt’s, 20 mM sodium phosphate pH 7.4, 5 mM EDTA, 0.2% SDS, 0.1 mg/ml poly(A)]. The utrophin probe was generated by PCR and spanned bp 9382–9943. The dystrophin probe was also generated by PCR and spanned bp 10 280–10 960. Membranes were washed at 9382–9943. The dystrophin probe was also generated by PCR and spanned bp

Urophin expression cassette

pCMV-FcDRP was constructed using a modification of the Dp71 cDNA and replaces dystrophin from a SacI site to the end (bp 9470–11 320) with utrophin sequences (bp 8533–10 610). The expressed protein (FcDRP) contains the first 10 amino acids of Dp71 at its N-terminus. An NcoI site was incorporated at the Dp71 initiator methionine by PCR. A primer (5’-CTATGCACTAGTACTACAAGGA CGACCATGAC AAGAGGGAAAC ACCTCTAAAGG CC) was designed to incorporate a FLAG epitope tag at the N-terminus of Dp71. The PCR product generated from this primer and a reverse C-terminal dystrophin primer was digested with NcoI and SacI to release the 5'-coding region of Dp71 up to the SacI site (bp 8530) and subcloned into pBluescript-II (pFtLAgDys). The 5’-UTR of Dp71 (HincII–NcoI fragment) was inserted upstream of the FLAG sequences (p5’FLAGtGd). After insertion of a NotI linker (NEB) upstream of the 5’-UTR, the utrophin C-terminal region was added sequentially as SacI–NruI (bp 8533–9372) and NruI–NotI (bp 9373–10 610) fragments. The entire cDNA was then cloned into the NotI site of pCMVαM (46) to generate pCMV-FcDRP.

Plasmid DNA injections into muscle

Mice were anaesthetised using 1% Avertin in phosphate-buffered saline (PBS) at 15 µl/g body wt (100% Avertin stock = 10 g of 2,2,2-tribromoethanol in 10 ml of tert-isooamyl alcohol). A 50 µl aliquot of pCMV-FcDRP was mixed with 5 µg of pCDNA-lacZ (Invitrogen, Carlsbad, CA) and injected into the right tibialis anterior (TA) muscles of 2-week-old mdx and BALB/c mice in a volume of 50 µl. Four mice of each strain were injected. After 1 week, animals were killed and TAs were dissected, embedded in OCT and cut into 7 µm sections using a cryotome. Contralateral TAs were used as negative controls. Sections were fixed in 0.5% glutaraldehyde in PBS for 5 min at room temperature and incubated with X-gal staining solution [5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6-3H2O, 1 mg/ml X-gal, 1 mM MgCl2 in PBS] overnight at 37°C. Adjacent sections were incubated with BioM2 anti-FLAG antibodies (Kodak, Rochester, NY) at 10 µg/ml for 2 h with subsequent incubation with streptavidin–fluorescein isothiocyanate (Molecular Probes, Eugene, OR). Tetramethylrhodamine isothiocyanate (TRITC)-conjugated α-bungarotoxin (Molecular Probes) was used at 1:200 dilution of a 1 mg/ml stock to label NMJs.

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