Dissecting the signaling and mechanical functions of the dystrophin-glycoprotein complex

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Summary

Duchenne muscular dystrophy is a severe disorder caused by mutations in the dystrophin gene. Dystrophin is required for assembly of the dystrophin-glycoprotein complex and provides a mechanically strong link between the cytoskeleton and the extracellular matrix. Several proteins in the complex also participate in signaling cascades, but the relationship between these signaling and mechanical functions in the development of muscular dystrophy is unclear. To explore the mechanisms of myofiber necrosis in dystrophin-deficient muscle, we tested the hypothesis that restoration of this complex without a link to the cytoskeleton ameliorates dystrophic pathology. Transgenic mice were generated that express Dp116, a non-muscle isoform of dystrophin that assembles the dystrophin-glycoprotein complex, in muscles of dystrophin-deficient *mdx*^{4cv} mice. However, the phenotype of these mice was more severe than in controls. Displacement of utrophin by Dp116 correlated with the severity of dystrophy in different muscle groups. Comparison with other transgenic lines demonstrated that parts of the dystrophin central rod domain were required to localize neuronal nitric oxide synthase to the sarcolemma, but this was not correlated with presence or extent of dystrophy. Our results suggest that mechanical destabilization, rather than signaling dysfunction, is the primary cause of myofiber necrosis in dystrophin-deficient muscle.

Key words: Muscular dystrophy, Dystrophin, Signaling, Neuronal nitric oxide synthase

Introduction

Duchenne muscular dystrophy (DMD) is a lethal X-chromosome-linked genetic disorder that affects approximately 1 in 3500 newborn males (Emery and Muntoni, 2003). The disease course is characterized by progressive wasting of the skeletal musculature with development of profound weakness, joint contractures and kyphoscoliosis (Blake et al., 2002). Most patients die in their late teens or mid-twenties, usually from respiratory or cardiac involvement. The causative gene was identified in 1986 by Kunkel and colleagues, who subsequently named the gene product dystrophin (Monaco et al., 1986; Hoffman et al., 1987; Koenig et al., 1987). Mutations in the murine and canine dystrophin genes have also been identified, leading to the *mdx* and canine X-chromosome-linked muscular dystrophic (*cxmd*) animal models of DMD (Bulfield et al., 1984; Cooper et al., 1988).

The dystrophin isoform in skeletal muscle is 427 kDa and is organized into four general functional domains (reviewed in Abmayr and Chamberlain, 2005). First is the N-terminal actin-binding domain (Koenig et al., 1988; Ervasti and Campbell, 1993). Second is the central rod region that is composed of 24 spectrin-like repeats interrupted by four proline-rich hinges. This region folds into a series of triple-helical coils that create a flexible and elastic structure (Koenig and Kunkel, 1990; Winder et al., 1995). Parts of the rod domain are also able to bind actin through a primarily electrostatic interaction (Amann et al., 1998). The third region has been recently named the dystroglycan-binding domain and is made up of WW, EF hand, and ZZ motifs (Ishikawa-Sakurai et al., 2004). β-dystroglycan spans the sarcolemma and binds to α-dystroglycan, which binds to laminin-α2 in the extracellular matrix (Ibraghimov-Beskrovnaya et al., 1992). Four sarcoglycans and sarcospan also interact with the dystroglycans and may facilitate this link to laminin (Holt and Campbell, 1998; Araishi et al., 1999; Crosbie et al., 1999). Finally, the C-terminal domain mediates interactions with dystrobrevin and syntrophin, the latter of which binds to neuronal nitric oxide synthase (nNOS) (Ahn and Kunkel, 1995; Yang et al., 1995b; Chao et al., 1996; Sadoulet-Puccio et al., 1997). The dystroglycans, sarcoglycans, sarcospan, dystrobrevins, syntrophins and nNOS collectively comprise the dystrophin-glycoprotein complex (DGC). Assembly of the DGC is dependent on specific domains of the dystrophin protein, and accordingly, the DGC is absent from the sarcolemma of dystrophin deficient muscle.

Despite the wealth of knowledge about the dystrophin gene and protein, the precise molecular and cellular events by which dystrophin deficiency leads to muscle fiber degeneration are poorly known (Petrof, 2002). There are two primary hypotheses regarding the essential functions of dystrophin in skeletal muscle. The first is that dystrophin is a structural protein that completes a physical link between the extracellular matrix and the actin cytoskeleton, enabling force transduction from within myofibers to the extracellular matrix (Lynch et al., 2000; Bloch and Gonzalez-Serratos, 2003; Ervasti, 2003). Absence of this mechanical apparatus is thought to destabilize the sarcolemma, making the myofibers more susceptible to

damage during contractions (Sacco et al., 1992; Petrof et al., 1993; Lynch et al., 2000; Dellorusso et al., 2001). It is also clear that dystrophin serves as a scaffold for the assembly of a multi-component signal transduction complex, parts of which are also integral members of the DGC (Madhavan et al., 1992; Yang et al., 1995a; Brenman et al., 1996; Yoshida et al., 2000). A second hypothesis views perturbation of cellular signaling resulting from the absence of dystrophin and many of its associated proteins as a major contributor to myofiber death (Brenman et al., 1995; Grady et al., 1999; Rando, 2001).

Previous studies in patients and in transgenic mice have examined the role of various dystrophin domains and members of the DGC in preventing the development of dystrophy (Abmayr and Chamberlain, 2005). Expression of dystrophins that lack the dystroglycan-binding domain invariably lead to a severe dystrophy, illustrating the importance of the DGC in preventing muscular dystrophy (Hoffman et al., 1991; Bies et al., 1992; Rafael et al., 1996). However, it has been difficult to test the model that restoration of the DGC in the absence of a link to actin might also rescue dystrophic muscle. We previously described lines of transgenic *mdx* mice that expressed the dystrophin isoform Dp260 in muscle. Dp260 contains much of the rod domain and can make a mechanically strong connection to costameric actin (Warner et al., 2002). Whereas those mice expressed the DGC and displayed a mild dystrophy, the finding that Dp260 interacts with the actin cytoskeleton prevented a clear dissection of the mechanical and signaling roles of the dystrophin/DGC network (Warner et al., 2002). By contrast, restoration of the DGC by transgenic expression of Dp71 in skeletal muscles of *mdx* mice or in dystrophin-utrophin double knockout mice failed to improve any aspect of the dystrophic pathology (Cox et al., 1994; Greenberg et al., 1994; Rafael et al., 2000). However, Dp71 lacks the entire rod domain as well as a functional WW domain (Fig. 1), which is an integral component of the dystroglycan-binding domain in dystrophin (Jung et al., 1995; Rentschler et al., 1999; Huang et al., 2000). In fact, Dp71 was not found in the microsomal fraction of skeletal muscle, indicating a weak association with the sarcolemma (Greenberg et al., 1994). β-dystroglycan serves as a ligand for WW domains contained in dystrophin, utrophin and caveolin-3. Furthermore, phosphorylation of β-dystroglycan can modulate its affinity for these various binding partners (Ilsley et al., 2002). It is likely that an intact dystrophin WW domain is required for proper regulation of these interactions, which may underlie important cellular signaling processes.

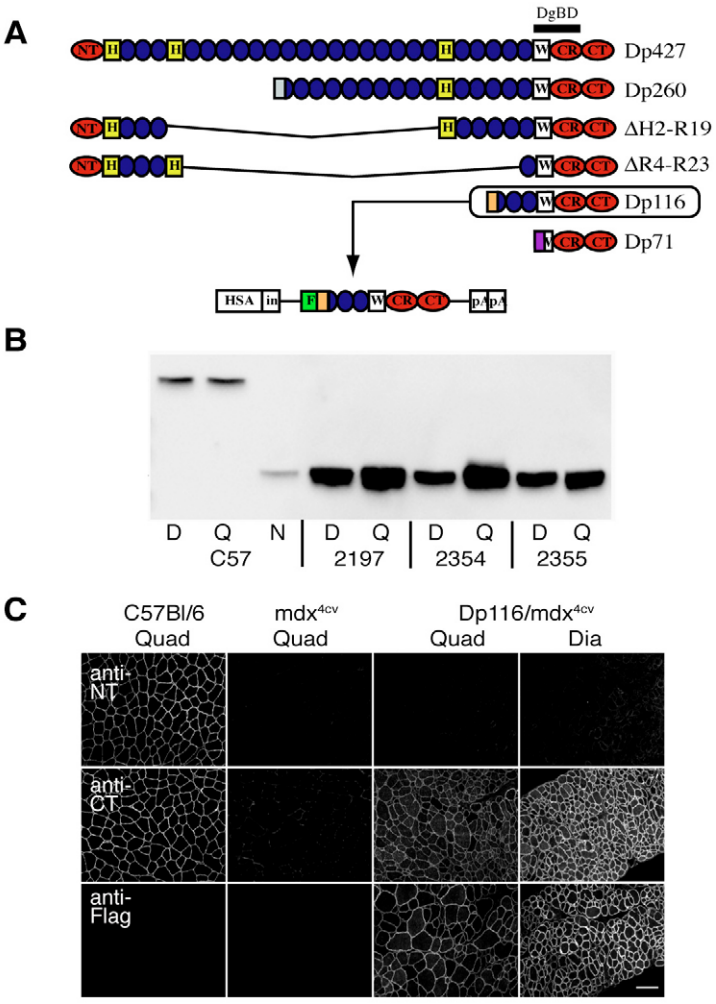

Fig. 1. Generation of Dp116/*mdx*^{4cv} transgenic mice. (A) Design of the Dp116 expression construct with the domain structure of Dp116 compared with other dystrophin isoforms and mini-genes. NT, N-terminal domain; H, hinge; W, WW domain; CR, cysteine rich domain; CT, C-terminal domain; DgBD, dystroglycan-binding domain; HSA, human skeletal α-actin promoter; in, hybrid HSA/SV40 vp1 intron; F, Flag epitope tag; pA, SV40 polyadenylation sequence. (B) Western analysis using a monoclonal antibody specific for the dystrophin C-terminal domain (Dys-2) shows expression of Dp116 in diaphragm (D) and quadriceps (Q) muscles from three independent lines of Dp116/*mdx*^{4cv} transgenic mice. Samples from control C57Bl/6 mice show full-length dystrophin in diaphragm and quadriceps, and Dp116 in peripheral nerve (N). (C) Immunofluorescence staining with antisera to the dystrophin N-terminal domain detects full-length dystrophin only in control C57Bl/6 muscles. Polyclonal antibodies to the dystrophin C-terminal domain or the Flag epitope demonstrate uniform expression of the Dp116 transgene in quadriceps and diaphragm muscles (line 2197).

To differentiate more carefully between the mechanical and signaling roles of dystrophin and the DGC, we generated transgenic mice that express Dp116 in skeletal muscles of *mdx*^{4cv} mice. Dp116 contains the entire WW domain but only two complete repeats from the rod domain (Fig. 1). We reasoned that this protein could reconstitute the signaling functions of dystrophin and the DGC without contributing to mechanical reinforcement of the sarcolemma. Despite expression of Dp116 and an intact DGC, transgenic *mdx*^{4cv} mice were more severely dystrophic than their *mdx*^{4cv} littermates. The worsened phenotype was likely caused by displacement of utrophin from the sarcolemma. Analysis of nNOS expression in several transgenic lines expressing different truncated dystrophin isoforms demonstrated that specific parts of the dystrophin rod domain are necessary for localization of nNOS at the sarcolemma. However, there was no correlation between nNOS expression and phenotype in these various lines. These data clearly demonstrate that loss of

mechanical function is the primary contributor to the dystrophic phenotype.

Results

Generation of transgenic mice

A full-length Dp116 cDNA carrying an N-terminal Flag tag was generated by PCR and cloned downstream of the human skeletal α-actin promoter prior to injecting into C57Bl/6 mouse embryos. Fig. 1 shows the domain structure of Dp116 compared with full-length dystrophin, Dp260, Dp71, and mini- and micro-dystrophins with internal deletions of the rod domain. All of these latter constructs have been previously tested in transgenic mice with a variety of phenotypic consequences (Cox et al., 1993; Cox et al., 1994; Greenberg et al., 1994; Harper et al., 2002; Warner et al., 2002). Dp116 is unique among these various dystrophin proteins because it contains the complete dystroglycan-binding domain but lacks the actin-binding domain of both the N-terminal and rod domains.

Transgene-positive F_0 mice were identified by a PCR screen and backcrossed onto the *mdx*^{4cv} background, which has a nonsense mutation in exon 53 of the murine dystrophin gene (Im et al., 1996). The *mdx*^{4cv} mutation does not affect normal expression of Dp116 or Dp71, but it does eliminate expression of all of the larger dystrophin isoforms (Im et al., 1996). Three independent lines of Dp116 transgenic mice were backcrossed to *mdx*^{4cv} for multiple generations. Skeletal muscles from all three of these transgenic lines expressed Dp116 protein at high levels as visualized by western blot (Fig. 1B). Immunofluorescence labeling using antibodies to the Flag epitope and the dystrophin C-terminal domain demonstrated uniform staining of the muscle fiber sarcolemma throughout the quadriceps and diaphragm (Fig. 1C) as well as the tibialis anterior and soleus muscles (data not shown).

Histopathology of Dp116 transgenic muscle

Dp116/*mdx*^{4cv} transgenic mice and *mdx*^{4cv} littermates from line 2197 were sacrificed at 3, 4, 12 and 28 weeks of age along with age-matched C57Bl/6 controls. Mice from lines 2354 and 2355 were also sacrificed at 12 weeks to verify a consistent phenotype. Hematoxylin and eosin stained cross sections from quadriceps, tibialis anterior, extensor digitorum longus, soleus and diaphragm muscles were examined at each age to evaluate the extent of dystrophic pathology. In muscles from *mdx*^{4cv} mice the dystrophic pathology developed significantly between 3 and 4 weeks of age (Fig. 2 and data not shown). At 3 weeks, relatively small areas of focal necrosis and infiltration of mononuclear cells were apparent (Fig. 2). Regenerating fibers, marked by the presence of centrally located nuclei, were rare or absent. At 4 weeks, there was continued evidence of degeneration and inflammation, but large numbers of regenerating fibers with central nuclei were now present (Figs 2, 3). Surprisingly, Dp116/*mdx*^{4cv} muscle displayed an earlier onset of a more severe dystrophy. Degeneration and inflammation were much more extensive in transgenic muscle of mice aged 3 and 4 weeks, particularly in the diaphragm and soleus (Figs 2, 3). Unlike in *mdx*^{4cv} muscles, clusters of small regenerating fibers were already visible at 3 weeks in Dp116 transgenic muscles.

At age 12 weeks, the quadriceps, tibialis anterior and extensor digitorum longus muscles appeared qualitatively similar between transgenic/*mdx*^{4cv} and *mdx*^{4cv} mice. However, the diaphragm and soleus muscles continued to be more severely affected in the transgenic/*mdx*^{4cv} mice (Fig. 3). The *mdx*^{4cv} soleus showed a majority of relatively normal, successfully regenerated muscle fibers at this age, with focal areas of continued necrosis and inflammation. By contrast, Dp116/*mdx*^{4cv} soleus muscles showed widespread inflammation and loss of normal muscle architecture. Immunostaining against CD11b, a surface marker for the monocyte/macrophage lineage, demonstrated focal immunoreactivity clustered around necrotic fibers in *mdx*^{4cv} muscles, but diffuse staining surrounding even apparently normal fibers in muscles of transgenic *mdx*^{4cv} mice (Fig. 3). Histopathology was very similar between all three transgenic lines at 12 weeks. At 28 weeks, both Dp116/*mdx*^{4cv} and *mdx*^{4cv} muscles appeared very similar to those at 12 weeks, but with less inflammatory infiltrate and more development of fibrotic tissue (data not shown).

Our breeding scheme also produced Dp116/wild-type transgenic mice, some of which were sacrificed at age 12 weeks. Overexpression of Dp116 on the wild-type background

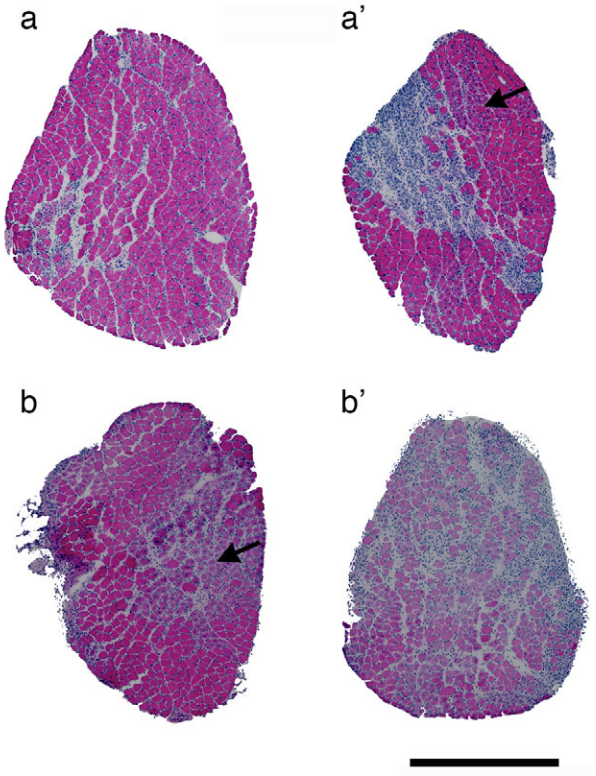

Fig. 2. Soleus muscle is dramatically affected at an early age in Dp116/*mdx*^{4cv} transgenic mice. Complete cross sections of hematoxylin- and eosin-stained soleus muscles from Dp116/*mdx*^{4cv} (a',b') and *mdx*^{4cv} (a,b) mice at both 3 weeks (a,a') and 4 weeks (b,b') of age are shown. At 3 weeks the *mdx*^{4cv} muscle has small areas of degeneration but minimal numbers of fibers with central nuclei. At this age, Dp116 transgenic muscle shows extensive degeneration and inflammation with clusters of regenerated fibers containing central nuclei (arrow). At 4 weeks the *mdx*^{4cv} muscle has many successfully regenerated fibers (arrow) and large areas of normal tissue. By contrast, the transgenic muscle at 4 weeks has little normal muscle tissue remaining. Bar, 500 μm.

had dominant-negative effects in the quadriceps, tibialis anterior and soleus muscles. Dp116/wild-type muscles displayed increased numbers of fibers with central nuclei without overt evidence of necrosis or inflammation (Fig. 4). Immunostaining with the anti-Flag antibody demonstrated strong expression of Dp116 at the sarcolemma, wheras immunostaining with an antibody to the dystrophin N-terminal domain revealed decreased expression of the full-length dystrophin isoform compared with controls (Fig. 4A). Western analysis confirmed a decrease in the total amount of full-length dystrophin protein in Dp116/wild-type skeletal muscles (Fig. 4B).

Two standard histological parameters used to quantitatively asses the extent of dystrophic pathology in mouse skeletal muscle are the percentage of fibers with central nuclei and the variance in fiber cross-sectional diameter (Harper et al., 2002; Briguet et al., 2004). Dystrophic muscle is characterized by a high percentage of centrally nucleated fibers and a large variance in fiber cross sectional size. Transgenic expression of Dp116 on the wild-type background caused an increase in the percentage of myofibers with central nuclei to over 20% compared with <1% in C57Bl/6 (Fig. 5). Transgenic expression of Dp116 on the *mdx*^{4cv} background resulted in a slight, but statistically significant, decrease in the percentage

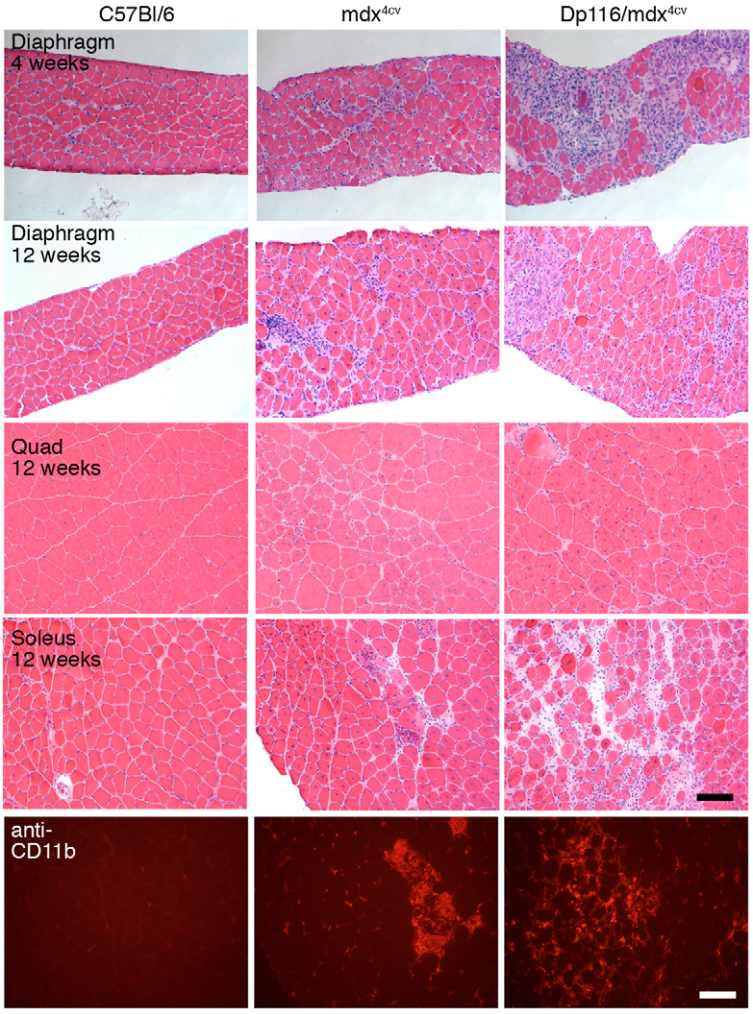

Fig. 3. Histopathology of Dp116/*mdx*^{4cv} transgenic muscle is more severe than that of *mdx*^{4cv}. Hematoxylin and eosin stained sections from the diaphragm muscle at 4 weeks of age and diaphragm, quadriceps, and soleus muscles at 12 weeks age are shown for C57Bl/6, *mdx*^{4cv}, and Dp116/*mdx*^{4cv} mice. Widespread necrosis of muscle fibers and mononuclear cell infiltrates are more prominent in the diaphragm and soleus muscles of Dp116/*mdx*^{4cv} transgenic mice at all ages compared with *mdx*^{4cv}. The quadriceps muscles from Dp116/*mdx*^{cv} and *mdx*^{4cv} mice were not noticeably different at 12 weeks. Immunofluorescence staining of serial sections of 12 week soleus muscles with anti-CD11b antibody demonstrates infiltration of inflammatory cells in *mdx*^{4cv} and transgenic/*mdx*^{4cv} muscles. Inflammation in the transgenic/*mdx*^{4cv} soleus muscle was widespread and diffuse, in contrast to the focal pattern in *mdx*^{4cv} soleus muscle. Bars, 100 μm.

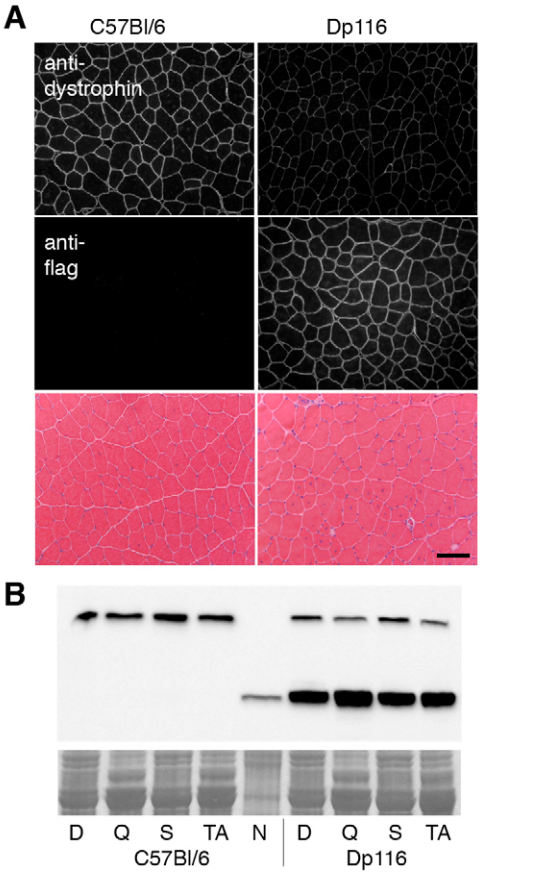

Fig. 4. Overexpression of Dp116 on a wild-type background has dominant-negative effects. (A) Cross sections of quadriceps muscle from Dp116 transgenic mice and C57Bl/6 controls. Immunofluorescence staining with antibodies specific to the dystrophin N-terminal domain and the Flag epitope demonstrate decreased expression of full-length dystrophin but high levels of Dp116 on the sarcolemma of transgenic muscles. Hematoxylin and eosin staining shows increased numbers of muscle fibers with central nuclei in the transgenic quadriceps. Bar, 100 μm. (B) Western analysis using a monoclonal antibody specific for the dystrophin C-terminus confirms reduced amounts of full-length dystrophin protein in various skeletal muscles of transgenic mice compared with controls. An identical gel stained in parallel with Coomassie Blue is shown as a loading control. D, diaphragm; Q, quadriceps; S, soleus; TA, tibialis anterior; N, peripheral nerve.

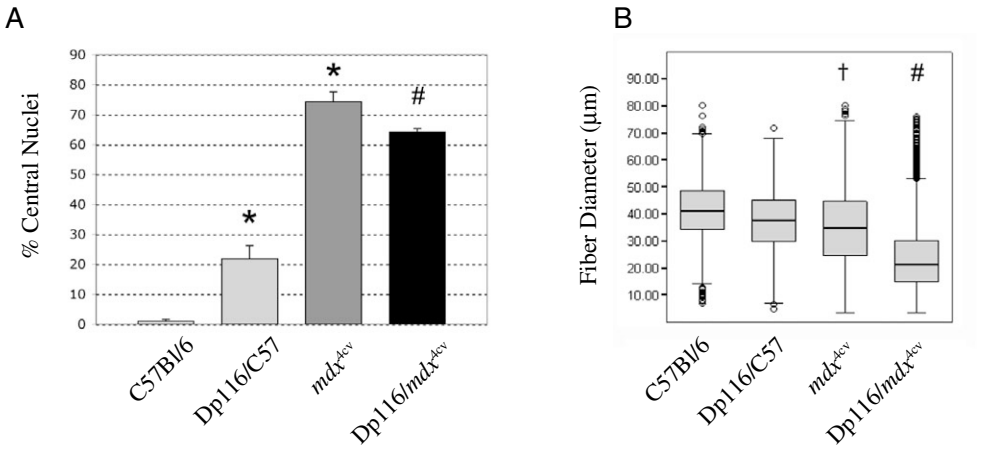

Fig. 5. Quantitative assessment of histopathology in tibialis anterior muscles of 12-week-old mice. (A) Percentage of fibers with central nuclei. (B) Box plots show variance in the muscle fiber diameter. Boxes represent the middle quartiles from the 25th to 75th percentiles, bars represent high and low values (statistical outliers are shown as circles). Each data set is composed of >1000 fibers of n=3-6 mice each. ANOVA statistical tests were performed on measurements of % central nuclei and the median fiber diameters. *P<0.01, statistical difference compared with C57Bl/6; $^{\#}P$<0.01, statistical difference compared with *mdx*^{4cv}; $^{\dagger}P$<0.05, statistical difference compared with C57Bl/6.

of myofibers with central nuclei (64%) compared with *mdx*^{4cv} (74%). This difference stems probably from the large increase in small caliber, regenerating myotubes and myofibers in the Dp116/*mdx*^{4cv} muscles, in which it is very difficult to determine whether the cell has central or peripherally located myonuclei. The analysis of muscle-fiber diameter demonstrated a striking difference between Dp116/*mdx*^{4cv} transgenic and *mdx*^{4cv} tibialis anterior muscles. The *mdx*^{4cv} muscles showed greater variation in muscle-fiber diameter and a smaller median-fiber diameter (34.1 μm) compared with wild type (40.9 μm). The Dp116/*mdx*^{4cv} transgenic muscles showed less variation in fiber diameter than *mdx*^{4cv} muscles but both the median (21.3 μm) and overall distribution were markedly skewed in favor of fibers with small diameters.

Dp116 assembles the dystrophin-glycoprotein complex and displaces utrophin

To explore the mechanism by which overexpression of Dp116 led to a severe phenotype on the *mdx*^{4cv} background, we examined the dystrophin-glycoprotein complex in Dp116/*mdx*^{4cv} skeletal muscle. Endogenous Dp116 expressed in Schwann cells is associated with a glycoprotein complex that differs from that found in skeletal muscle. In Schwann cells, ϵ-sarcoglycan is highly expressed, wheras α-sarcoglycan and γ-sarcoglycan are not (Imamura et al., 2000). Immunofluorescence analysis showed that Dp116 transgenic mice express the normal skeletal-muscle complex on the sarcolemma, including β-dystroglycan as well as α-, β- and γ-sarcoglycans (Fig. 6A). Overexpression of Dp116 resulted in upregulation of members of the glycoprotein complex on the sarcolemma, as seen by western blots of KCl-washed skeletal-muscle microsomes (Fig. 6B). The sub-sarcolemmal proteins α_1-syntrophin and α-dystrobrevin 2 were restored to levels similar to wild type (Fig. 6A,B).

In normal muscle, the PDZ domain of α_1-syntrophin binds to nNOS, localizing it to the inner face of the

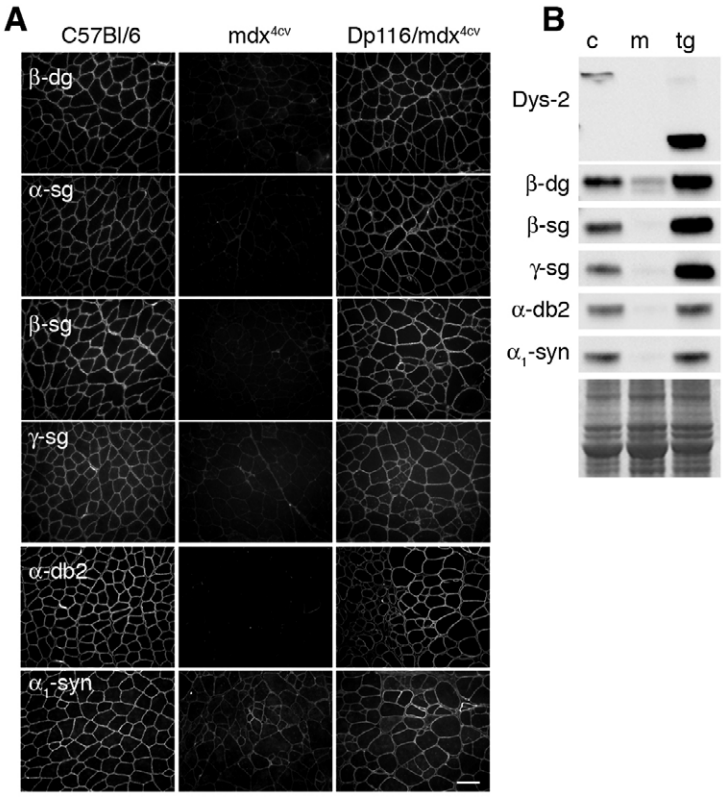

Fig. 6. Expression of dystrophin-associated proteins is restored in Dp116/*mdx*^{4cv} transgenic muscle. (A) Immunofluorescence staining of quadriceps muscles with antibodies specific for β-dystroglycan, α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, α-dystrobrevin 2, and α_1-syntrophin. Bar, 100 μm. (B) Western blots from KCl-washed microsomes using antibodies against the dystrophin C-terminus (Dys-2), β-dystroglycan, β-sarcoglycan, γ-sarcoglycan, α-dystrobrevin 2, and α_1-syntrophin. An identical gel stained in parallel with Coomassie Blue is shown as a loading control. c, C57Bl/6; m, *mdx*^{4cv}; tg, Dp116/*mdx*^{4cv}.

sarcolemma (Brenman et al., 1996; Adams et al., 2001). In dystrophin-deficient muscle nNOS expression is lost from the membrane along with other members of the DGC (Brenman et al., 1995). However, no nNOS immunoreactivity was found on the sarcolemma of Dp116/*mdx*^{4cv} muscle despite the presence of α_1-syntrophin (Fig. 7). This observation is consistent with previous data indicating that various dystrophin mutations are associated with a loss of nNOS from the sarcolemma despite persistence of α_1-syntrophin expression (Chao et al., 1996). We also examined nNOS expression in several transgenic mouse lines expressing various truncated dystrophin proteins in an attempt to identify the part of dystrophin that is required for nNOS expression and localization (Fig. 7). Immunofluorescence staining showed that transgenic *mdx* mice expressing mini-dystrophin (ΔH2-R19) or micro-dystrophin (ΔR4-R23) had minimal amounts of membrane-associated nNOS, even though these mice do not show signs of dystrophic pathology (Harper et al., 2002). Transgenic *mdx* mice expressing Dp260, which results in a phenotype intermediate between wild type and *mdx* (Warner et al., 2002), showed greater nNOS immunoreactivity at the sarcolemma, although still less than wild type.

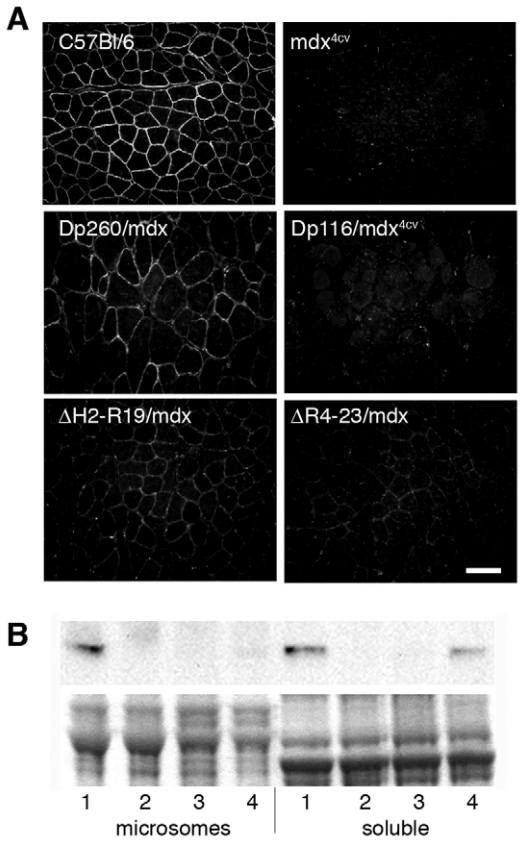

Fig. 7. Expression of nNOS is not restored by expression of Dp116. (A) Immunofluorescence staining of quadriceps muscles with a polyclonal antibody against nNOS. Dp116/*mdx*^{4cv} and transgenic/*mdx* muscles expressing Dp260, ΔH2-R19 (mini-dystrophin), and ΔR4-23 (micro-dystrophin) are shown. Bar, 100 μm. (B) Western analysis of nNOS from pellet (microsomal) and supernatant (soluble) fractions of KCl-washed microsome preparations from C57Bl/6 (1), *mdx*^{4cv} (2), Dp116/*mdx*^{4cv} (3), and ΔR4-23/*mdx* (4) skeletal muscles. An identical gel stained in parallel with Coomassie Blue is shown as a loading control.

Western blots of both the pellet (membrane fraction) and supernatant (cytosolic fraction) from muscle microsome preparations showed nNOS protein in both cellular compartments in C57Bl/6, but nNOS was nearly undetectable in both fractions from *mdx*^{4cv} and Dp116/*mdx*^{4cv} (Fig. 7B). Microsome pellets from ΔR4-23 muscles had barely detectable nNOS levels, confirming the immunofluorescence data. Interestingly, the soluble fraction from the ΔR4-23 preparation did contain significant levels of nNOS, although less than wild type. It was previously shown that nNOS was present in Dp260/*mdx* muscle microsomes (Warner et al., 2002). This analysis indicates that expression of α_1-syntrophin and the complete DGC are not sufficient for localization of nNOS to the sarcolemma, but that parts of the central rod domain that are present in Dp260 but lacking in the micro- and mini-dystrophins are also important. However, nNOS expression was not correlated with the extent of muscle pathophysiology in these various transgenic lines.

Utrophin-A, normally restricted to the neuromuscular junction in mouse skeletal muscle, is broadly distributed on the sarcolemma in *mdx* muscle (Weir et al., 2002). Upregulation of utrophin may partially compensate for dystrophin deficiency, so we investigated whether overexpression of Dp116 could displace utrophin from the sarcolemma of *mdx*^{4cv} muscle in a manner similar to its competition with dystrophin in wild-type muscle. Immunostaining with an antibody specific to the Utrophin-A isoform demonstrated increased immunoreactivity at the sarcolemma in *mdx*^{4cv} quadriceps muscle compared with wild-type controls. This upregulation of utrophin was mosaic in nature, with some fibers showing strong staining of the sarcolemma and others showing little (Fig. 8). Dp116/*mdx*^{4cv} quadriceps showed similar mosaic staining for utrophin, making comparison difficult. By contrast, the *mdx*^{4cv} soleus muscle showed a dramatic and uniform increase in utrophin immunoreactivity at the sarcolemma that was almost completely absent in Dp116/*mdx*^{4cv} soleus muscle. This is consistent with previous data demonstrating greater upregulation of utrophin in slow soleus compared to fast extensor digitorum longus muscles (Gramolini et al., 2001). Transgenic *mdx* mice expressing the highly functional ΔR4-R23 micro-dystrophin protein displayed utrophin immunostaining that was very similar to that of wild-type mice in both quadriceps and soleus (Fig. 8). It is notable that when comparing *mdx*^{4cv} and Dp116/*mdx*^{4cv} skeletal muscles, the soleus showed the greatest difference in both histopathology and utrophin expression.

Discussion

Increasing evidence suggests that dystrophin and the DGC act as a scaffold that anchors signaling proteins to the sarcolemma (Rando, 2001). Dystroglycan, sarcoglycans and short isoforms of dystrophin are expressed in a wide variety of tissues that do not have the unique contractile properties of muscle. Thus, non-structural functions of the DGC in other cell types are probably also active in muscle cells. In cultured myoblasts, dystroglycan binding to laminin in the extracellular matrix activates the phosphoinositide 3-kinase (PI 3-kinase)-Akt pathway, which regulates cell growth and survival (Langenbach and Rando, 2002). Abnormal activity of various

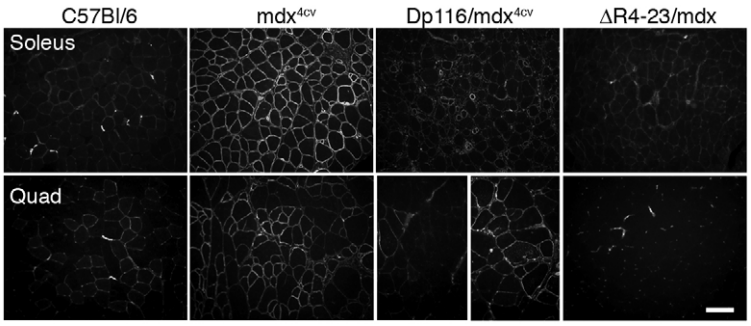

Fig. 8. Dp116 expression displaces utrophin from the sarcolemma in soleus muscle. Immunofluorescence staining of quadriceps and soleus muscles using an antibody specific for the utrophin A isoform. The split panels shown for Dp116/*mdx*^{4cv} quadriceps muscle represent extremes in utrophin staining. Bar, 100 μm.

kinases in this and other signaling pathways have also been reported in dystrophic muscle in vivo (Kolodziejczyk et al., 2001; Kumar and Boriek, 2003; Kumar et al., 2004; Lang et al., 2004). However, it is not clear whether these abnormalities are primary contributors to the dystrophic phenotype or whether they are secondary consequences. In some cases they may be adaptive responses that reflect increased regeneration and hypertrophy of muscle fibers.

We hypothesized that transgenic expression of a dystrophin isoform containing the entire dystroglycan-binding domain, including the complete WW domain, can restore not only expression of the DGC but also normal regulation of the interactions within the complex. Our results clearly show that expression of Dp116 in skeletal muscle actually exacerbated the dystrophic phenotype, particularly in muscles such as the diaphragm and soleus. Two explanations for this result are possible. First, instead of restoring normal signaling through the DGC we may have merely pushed the balance too far in the opposite direction. We did observe significant overexpression not only of Dp116, but also of β-dystroglycan and the sarcoglycan complex (which might contain a clue to the regulation and assembly of this complex). However, this explanation is incompatible with previous studies showing that marked overexpression of full-length or mechanically functional mini-dystrophins fully corrected the dystrophic phenotype (Cox et al., 1993; Crawford et al., 2000; Harper et al., 2002). These latter studies showed no negative consequences resulting from increased levels of β-dystroglycan and the sarcoglycan complex. Furthermore, overexpression of Dp116 in the wild-type background only caused a very mild phenotype. Such dominant-negative phenotypes have previously been observed only in mice expressing functionally impaired dystrophins (Crawford et al., 2001; Leibovitz et al., 2002; Warner et al., 2002). Our data support the second explanation, that overexpression of Dp116 competes with utrophin and displaces it from the sarcolemma, further destabilizing the mechanical connection between the DGC and the cytoskeleton.

Utrophin is normally restricted to the neuromuscular junction, but is expressed throughout the sarcolemma in dystrophin-deficient muscle (Weir et al., 2002). Mice that are deficient for both utrophin and dystrophin (double knockout or *dko* mice) have a much more severe dystrophy than *mdx* mice (Deconinck et al., 1997; Grady et al., 1997). Further, overexpression of utrophin in either *mdx* or *dko* mice can effectively compensate for the lack of dystrophin (Tinsley et al., 1998; Wakefield et al., 2000). In sum, these data highlight the fact that utrophin and dystrophin are functionally redundant to some degree and that upregulation of utrophin partially compensates for the lack of dystrophin in *mdx* mice. These observations further underscore the importance of the mechanical functions of these proteins in that, low level expression of full-length utrophin is more effective at maintaining myofiber integrity than high level expression of Dp116 (Figs 3, 8). Differences in utrophin expression might also explain the fact that the soleus and diaphragm muscles were most notably affected by expression of Dp116. These two muscles have a high proportion of slow fibers, which contain the highest levels of utrophin in *mdx* mice (Gramolini et al., 2001). Interestingly, this significant upregulation of utrophin in the *mdx*^{4cv} soleus was almost completely reversed by Dp116 expression. Conversely, larger muscles, such as the quadriceps, showed greater variability in both utrophin expression and dystrophic pathology. It might also be that the mechanical properties of the soleus and diaphragm predispose them to dystrophic injury, because they are two of the most severely affected muscles in *mdx* mice.

The best studied signaling protein linked to the DGC is nNOS. This enzyme produces the second messenger nitric oxide, which can have diverse effects including the activation of guanylate cyclase and inhibition of cytochrome-*c* oxidase (Stamler and Meissner, 2001). Transgenic overexpression of nNOS in *mdx* skeletal muscle ameliorated the dystrophic phenotype without restoring expression of other DGC members, apparently by attenuating the inflammatory response in the dystrophic muscles (Wehling et al., 2001; Tidball and Wehling-Henricks, 2004). Another function of nNOS in skeletal muscle is to regulate blood flow during exercise. DMD patients and mice with primary deficiencies of either dystrophin or nNOS are unable to attenuate the vasoconstriction produced by adrenergic stimulation during muscle contraction (Thomas et al., 1998; Sander et al., 2000). Recent data also showed the same defect in regulation of blood flow in mice lacking α_1-syntrophin or expressing a transgenic syntrophin with the PDZ domain deleted (Thomas et al., 2003). These results indicate that nNOS must be localized to the sarcolemma to serve this particular function, because association with the PDZ domain of α_1-syntrophin is necessary to target nNOS to the inner face of the sarcolemma (Adams et al., 2001).

It is also clear that expression of α_1-syntrophin alone is not sufficient to localize nNOS to the sarcolemma. Transgenic *mdx* mice and Becker muscular dystrophy (BMD) patients, which express dystrophin with deletions of the rod domain, display loss of sarcolemma-associated nNOS despite normal expression of other DGC components, including α_1-syntrophin

(Chao et al., 1996; Crosbie et al., 1998; Wells et al., 2003; Torelli et al., 2004). Our data support these results, demonstrating that deletion of the central-rod-domain region in the ΔH2-R19 or ΔR4-23 transgenic mice results in the loss of nNOS from the sarcolemma. Dp116 also failed to restore nNOS expression in either the membrane-associated or cytoplasmic fractions. The Dp260 transgene was the most successful at restoring nNOS to the sarcolemma in our studies, although nNOS levels were still reduced compared with wild type. These observations confirm that the central rod domain has some role in localizing nNOS to the sarcolemma. Direct binding of nNOS to dystrophin has not been detected, so the mechanism of this effect remains uncertain.

The majority of data suggest that the loss of nNOS from the sarcolemma is not by itself a primary contributor to the dystrophic phenotype. Disruption of either the nNOS or α_1-syntrophin genes in mice did not produce myopathy (Chao et al., 1998; Adams et al., 2000). Analysis of BMD patients that express various internally truncated dystrophin proteins did not show a correlation between sarcolemmal nNOS expression and the severity of phenotype (Chao et al., 1996). Similarly, our data show that nNOS expression at the sarcolemma is not correlated with the presence or the degree of the dystrophic phenotype in mice expressing various types of dystrophin. For example, *mdx* mice expressing ΔH2-R19 display no phenotypic differences from wild-type mice, despite a dramatic reduction of nNOS at the sarcolemma (Fig. 7) (Harper et al., 2002). However, examination of all of the previous data reveals one potential pattern. Mutations in dystrophin that result in a severely dystrophic phenotype are invariably associated with the loss of nNOS from the sarcolemma, independent of the extent of mutant dystrophin protein or DGC expression, e.g. transgenic mice expressing either Dp116 (Fig. 7) or dystrophins lacking various parts of the dystroglycan-binding domain (Rafael et al., 1996). Conversely, mutations that result in a very mild or not detectable phenotype can be associated with either loss or persistence of nNOS, depending on the exact nature of the mutant protein. It is possible that two phenomena are being observed. First, there might be abnormalities of dystrophic muscle that promote the degradation of nNOS or inhibit its production. Second, regions of the rod domain appear to be necessary, in addition to α_1-syntrophin, to localize nNOS to the sarcolemma. The combination of these hypotheses could explain our observation that the ΔR4-23 transgenic mice do express some nNOS in the cytoplasm of their skeletal muscles. Prevention of the dystrophic phenotype by expression of micro-dystrophin apparently partially corrects the defect in overall nNOS expression. However, these mice maintain a defect in nNOS localization to the sarcolemma, presumably because of a requirement for parts of the rod domain.

In summary, we found that overexpression of Dp116 in skeletal muscle of *mdx*^{4cv} mice did not ameliorate the dystrophic phenotype. Our results support previous data suggesting that a mechanically functional dystrophin is absolutely required to effect a significant improvement in the *mdx* muscle phenotype. Our results do not rule out the possibility that subtle signaling defects contribute to the phenotype to a lesser degree. For example, a transgenic dystrophin construct containing a deletion in the cysteine-rich domain partially corrected abnormalities in fiber-type composition and neuromuscular junction topology when tested in *dko* mice (Rafael et al., 2000). The effect in these studies was presumably due to restored signaling, because the deletion included crucial parts of the dystroglycan-binding domain, rendering the protein mechanically non-functional. Nonetheless, the myofibers in these transgenic *dko* mice remained dystrophic.

Finally, it is still not clear whether nNOS plays a role in the pathophysiology of muscular dystrophy. An ideal strategy for gene replacement of dystrophin in DMD patients would result in restoration of nNOS, given its importance in maintaining perfusion of the exercising muscle. However, current micro-dystrophins that were tested for delivery to skeletal muscle by adeno-associated viral vectors do not restore nNOS. Further understanding of the factors that regulate the expression, localization and activity of this enzyme may help us to design more effective strategies for gene replacement.

Materials and Methods

Generation of transgenic mice

The Dp116 transgene was prepared by PCR amplification from a full-length mouse dystrophin cDNA clone using the following oligonucleotides, the first of which encodes the Flag-epitope tag in-frame with the unique first exon from human Dp116: 5′-cgccggccgATGGACTACAAGGACGACGACGACAAGTTACACAGG-AAGACATACCATGTAAAGGATCTCCAAGGAGAAATTGAAAC-3′ and 5′-gc-gcggccgCCAAATCATCTGCCATGTGG-3′ (uppercase letters indicate sequence of Dp116 cDNA, lowercase letters indicate restriction sites added for sub-cloning). The PCR product was cloned into the pGEM-T Easy Vector (Promega) and sequenced. The Dp116 cDNA was subcloned into the previously described pBSX-HSAvpA expression vector (Crawford et al., 2000). The linearized expression cassette was injected into C57Bl/6 embryos and the progeny screened by PCR. Five transgene-positive founders were identified and crossed with *mdx*^{4cv} mice as independent lines. Three of these lines (2197, 2354, 2355) expressed Dp116 at high levels in skeletal muscle and were crossed onto the *mdx*^{4cv} background for a minimum of two generations. Dp116/wild-type transgenic mice were obtained from the male offspring of Dp116/heterozygous *mdx*^{4cv} transgenic females. The *mdx*^{4cv} mutation was genotyped by an ARMS assay (Pearson-White, 2002). All experimental protocols involving live mice were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Western analysis

Whole tissue lysates were prepared by homogenization in extraction buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0) with protease inhibitor cocktail (Roche) and cleared by centrifugation. KCl-washed skeletal muscle microsomes were prepared as previously described (Ohlendieck et al., 1991). Protein concentrations were determined with the Coomassie Plus Protein Assay Reagent (Pierce). Equivalent amounts of total protein were separated on 4-12% NuPAGE gradient gels (Invitrogen Life Technologies) and electrophoretically transferred to PVDF membranes or stained with Coomassie Blue. Membranes were blocked with 5% nonfat dry milk in TBS and probed with antibodies to dystrophin C-terminus, β-dystroglycan, β-sarcoglycan, γ-sarcoglycan (Novocastra Laboratories Ltd.), α-dystrobrevin 2 (Peters et al., 1998), α_1-syntrophin (Peters et al., 1998), and nNOS (Zymed Laboratories). The membranes were washed with TBS containing 0.1% Tween 20 and probed with horseradish peroxidase-conjugated donkey anti-mouse or donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch). After further washing, detection of immunoblotted proteins was performed with the Enhanced Chemiluminescent Plus reagent (Amersham Biosciences) and visualization on a GeneGnome chemiluminescent imaging system (SynGene Bioimaging).

Immunofluorescence

Skeletal muscles were dissected, embedded in OCT (Sakura Finetek, USA) on cork support and frozen in liquid-nitrogen-cooled isopentane. Cryosections of 7-10 μm thickness were blocked in KPBS (20 mM potassium phosphate pH 7.4, 150 mM sodium chloride) containing 0.3 mg/ml BSA, 2% gelatin, and 1% Tween 20. The blocked sections were incubated with primary antibodies diluted in KPBS containing 0.2% gelatin (KPBS-G) and 2% normal goat serum. Rabbit polyclonal antibodies were used to label Flag (Sigma), dystrophin N-terminus (Rafael et al., 1996), dystrophin C-terminus (Cox et al., 1994), nNOS (Zymed Laboratories), α_1-syntrophin (Peters et al., 1997), α-dystrobrevin 2 (Peters et al., 1998), and utrophin A (S. Froehner). After several washes with KBPS-G the sections were incubated with goat anti-rabbit Alexa Fluor 488 (Molecular Probes). Monoclonal rat anti-CD11b (BD Pharmingen) was used in combination with goat anti-rat Alexa Fluor 594 (Molecular Probes). Monoclonal antibodies to β-dystroglycan and α, β, γ-

sarcoglycans (Novocastra Laboratories Ltd) were used with the M.O.M. Immunodetection kit (Vector Laboratories). Immunostained slides were washed repeatedly with KBPS-G and mounted with Vectashield (Vector Laboratories). All photomicrographs were obtained with a Spot II CCD camera (Diagnostic Instruments, Inc.) and Spot Advanced software connected to a Nikon Eclipse E1000 using a 20× Plan-Apochromat objective (numerical aperture, 0.75). Images for all the sections probed with a given antibody were acquired under identical exposure conditions.

Histological analysis

Quadriceps, tibialis anterior, extensor digitorum longus, soleus, and diaphragm muscles were dissected, embedded in OCT (Sakura Finetek USA) on cork support and frozen in liquid nitrogen-cooled isopentane. Cryosections of 10 μm thickness were stained with Gill's hematoxylin and eosin-phyloxine. Photomicrographs were obtained with a Spot II CCD camera (Diagnostic Instruments, Inc.) and Spot Advanced software connected to a Nikon Eclipse E1000 microscope using a 20× Plan-Apochromat objective (numerical aperture, 0.75). Images of entire muscle cross sections were obtained using the Montage Explorer software (Syncroscopy) driving a JVC 3-CCD color video camera. ImagePro software (Media Cybernetics) was used to measure muscle fiber diameters and count fibers with central nuclei. The percentage of central nuclei was determined by dividing the number of fibers with central nuclei by the total number of fibers. Muscle fiber diameter was determined by measuring the minimal diameter through the center of each fiber. Fibers from randomly located fields in the tibialis anterior muscle were scored until the total number of fibers exceeded 1000.

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