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General Article

GENERAL ARTICLE

Loss of sarcospan exacerbates pathology in *mdx* mice, but does not affect utrophin amelioration of disease

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Abstract

The dystrophin–glycoprotein complex (DGC) is a membrane adhesion complex that provides structural stability at the sarcolemma by linking the myocyte's internal cytoskeleton and external extracellular matrix. In Duchenne muscular dystrophy (DMD), the absence of dystrophin leads to the loss of the DGC at the sarcolemma, resulting in sarcolemmal instability and progressive muscle damage. Utrophin (UTRN), an autosomal homolog of dystrophin, is upregulated in dystrophic muscle and partially compensates for the loss of dystrophin in muscle from patients with DMD. Here, we examine the interaction between Utr and sarcospan (SSPN), a small transmembrane protein that is a core component of both UTRN–glycoprotein complex (UGC) and DGC. We show that additional loss of SSPN causes an earlier onset of disease in dystrophin-deficient *mdx* mice by reducing the expression of the UGC at the sarcolemma. In order to further evaluate the role of SSPN in maintaining therapeutic levels of Utr at the sarcolemma, we tested the effect of Utr transgenic overexpression in *mdx* mice lacking SSPN (*mdx*:SSPN $^{-/-}$:Utr-Tg). We found that overexpression of Utr restored SSPN to the sarcolemma in *mdx* muscle but that the ablation of SSPN in *mdx* muscle reduced Utr at the membrane. Nevertheless, Utr overexpression reduced central nucleation and improved grip strength in both lines. These findings demonstrate that high levels of Utr transgenic overexpression ameliorate the *mdx* phenotype independently of SSPN expression but that loss of SSPN may impair Utr-based mechanisms that rely on lower levels of Utr protein.

Introduction

The dystrophin–glycoprotein complex (DGC) is a membrane adhesion complex that provides structural stability at the muscle sarcolemma by linking the myocyte's internal cytoskeleton and external extracellular matrix (1–3). In Duchenne muscular dystrophy (DMD), the absence or reduction of dystrophin leads to the loss of the DGC at the sarcolemma, resulting in sarcolemmal instability and progressive contraction-induced damage (4,5). This progressive loss of membrane integrity causes muscle fiber degeneration and ultimately leads to muscle cell death. Individuals with DMD experience progressive muscle wasting during childhood and typically lose ambulation in early adolescence (6). Although corticosteroid treatment and supportive care can slow disease progression and increase DMD life expectancy, there is currently no curative treatment available and cardiac and respiratory failures are the major causes of morbidity and mortality for individuals with DMD.

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Along with the DGC, two other major adhesion complexes are expressed at the skeletal muscle sarcolemma: the $\alpha 7\beta 1$ integrin complex and the utrophin (UTRN) glycoprotein complex (UGC) (7–9). Both proteins are upregulated in the reduction or absence of dystrophin and can partially protect against damage to the muscle during contraction (9). In healthy muscle, the UGC is primarily localized to the neuromuscular junction, but in DMD, it is upregulated and found throughout the sarcolemma (10). The composition of the DGC and UGC is highly similar and includes the sarcoglycans (α-, β-, γ- and δ-SG), sarcospan (SSPN), dystroglycan (α- and β-DG), the syntrophins and dystrobrevin (11–13). SSPN, a 25-kDa integral membrane protein, forms a tight subcomplex with the SGs that stabilizes α-dystroglycan (α-DG) at the sarcolemma (14–16). Loss of any one of the SGs results in the reduction or loss of the sarcolemmal SG–SSPN subcomplex and causes a spectrum of skeletal and/or cardiac muscle disorders (17). Limb-girdle muscular dystrophy (LGMD) types 2C, 2D, 2E and 2F are caused by recessive mutations in γ-, α-, β- and δ-SG genes, respectively (18–22). LGMD is characterized by progressive proximal skeletal muscle weakness, ranging from mild to severe, and can be accompanied by cardiomyopathy (23,24). Notably, although mutations in SSPN have not been associated with human muscle disease, and SSPN expression is typically reduced or absent in skeletal muscle from LGMD patients (25).

Our group has previously shown that overexpression of SSPN in *mdx* muscle increases the sarcolemmal abundance of all three skeletal muscle adhesion complexes (26–28). Given this key role of SSPN in mediating sarcolemmal integrity in *mdx* pathology, we investigate the loss of SSPN expression in *mdx* skeletal muscle. In order to further evaluate the role of SSPN in maintaining the therapeutic levels of Utr at the sarcolemma, we tested the effect of Utr transgenic overexpression in *mdx* mice lacking SSPN (*mdx*:SN). Transgenic Utr overexpression has been well characterized as a near-complete rescue model in the dystrophin *mdx* mouse, and UTRN-based therapies are currently being tested as a therapeutic approach in DMD. Here, we show that overexpression of Utr restored UGC-associated proteins to the sarcolemma in both *mdx* and *mdx*:SN mice. Furthermore, Utr overexpression reduced central nucleation and improved grip strength in both lines. These findings demonstrate that Utr overexpression can rescue the *mdx* phenotype independently of SSPN expression and suggest that the improvements mediated by SSPN transgenic overexpression may involve pathways in addition to the UGC that are beneficial to muscle.

Results

Loss of SSPN exacerbates dystrophy in *mdx* mice

To determine the effect of SSPN loss on *mdx* dystrophic pathology, we crossed *mdx* mice to *Sspn*$^{-/-}$ mice (29) to generate *mdx*:*Sspn*$^{+/-}$ heterozygous mice. Heterozygous *mdx*:*Sspn*$^{+/-}$ mice were then crossed to produce double knockout *mdx*:*Sspn*$^{-/-}$ mice (abbreviated as *mdx*:SN). Double null mice were born in expected ratios (data not shown), gained weight at approximately the same rate as *mdx* mice (Supplementary Material, Fig. S2) and bred normally. Although survival was not specifically tracked, no excess deaths were noted and some *mdx*:SN animals survived up to at least 18 months.

In *mdx* mice, significant necrosis and inflammation is observed in some limb muscles starting at 3 weeks of age, most prominently in the TA (30–32). This period of damage and myonecrosis is followed by active muscle regeneration, which can be evaluated by the presence of fibers with centralized nuclei. At 3 and 6 weeks of age, hematoxylin and eosin (H&E)-stained transverse cryosections of quadriceps were evaluated for dystrophic pathology (Fig. 1A and B). Few degenerating fibers were seen in 3-week *mdx* quadriceps, and little-to-no inflammation was evident at this timepoint (Fig. 1A). In contrast, at 3 weeks, degenerating fibers were present in the *mdx*:SN quadriceps and regions of inflammatory infiltrates were readily apparent (Fig. 1A). In the quadriceps of *mdx*:SN mice at 3 weeks, there was also a small, but significant increase in central nuclei, indicating an earlier initiation of active regeneration (Fig. 1B). By 6 weeks, both genotypes had a high level of central nucleation in the quadriceps and there was no longer a significant difference in myofiber regeneration (Fig. 1B). However, at 6 weeks, the *mdx*:SN had more severe dystrophic features, including greater fiber size variability, mild endomysial fibrosis and adipose infiltrates (Fig. 1A and C). Staining against collagen I also revealed large patches of fibrotic deposition (Fig. 2A and Supplementary Material, Fig. S1).

We next examined the diaphragm, the most severely affected muscle in the *mdx* mouse. In order to compare diaphragms between animals, sections were taken from the anterolateral right costal muscle in wild-type (WT), *mdx* and *mdx*:SN mice at multiple timepoints (Fig. 1D). Notably, the *mdx*:SN diaphragm exhibited a more severe phenotype at all stages examined. At 4 weeks, there were more cellular infiltrates and fibrosis in *mdx*:SN relative to *mdx*. At 12 weeks, the *mdx*:SN diaphragm was substantially thicker and more fibrotic, with increased regions of necrotic fibers. In aged diaphragms greater than 1 year of age, both *mdx* and *mdx*:SN diaphragms were thinned and displayed substantial fibrosis relative to WT, while the *mdx*:SN diaphragm was thinner and had fewer intact muscle fibers.

In order to further examine the timeline of regeneration in *mdx*:SN, we stained for expression of embryonic myosin heavy chain, which is expressed in newly regenerated fibers (Fig. 2B). As with the regenerative marker of central nucleation, the number of positive fibers was increased in *mdx*:SN relative to *mdx* at 3 weeks, indicating an earlier initiation of active regeneration in the double knockout mice (Fig. 2B–C). There was no longer a significant difference between the groups at 6 weeks (Fig. 2D).

To assess the effect of SSPN loss in the heart, we sectioned hearts from 12-month-old *mdx* and *mdx*:SN mice and stained for HE and with antibody against collagen I (Supplementary Material, Fig. 2G). Consistent with skeletal muscle, we found increased regions of cardiac in the *mdx*:SN heart.

Muscle fiber membrane damage is increased in *mdx*:SN mice

Along with increased regeneration, loss of membrane stability in *mdx* muscle leads to overt membrane damage and infiltration into the muscle fiber (4,5,33). In *mdx*:SN, we measured membrane stability using two different markers of membrane damage, Evans blue dye (EBD) uptake and IgM antibody staining. EBD uptake is an *in vivo* strategy of injecting dye that binds to albumin forming a large complex (34). In healthy muscle, this complex is carried by blood and does not enter the muscle fiber, while fluorescent dye crosses into the sarcoplasm of damaged fibers (35). Similarly, IgM is found in blood in a complex of approximately 800 kDa and does not cross the membrane into healthy muscle fibers (33). Following dissection and sectioning, muscle can be stained with IgM and evaluated for positive fibers. At 3 weeks, quadriceps exhibited a low average percentage (>3%) of EBD positive fibers and no significant difference between groups, although *mdx*:SN had higher values (Fig. 2D). However, at

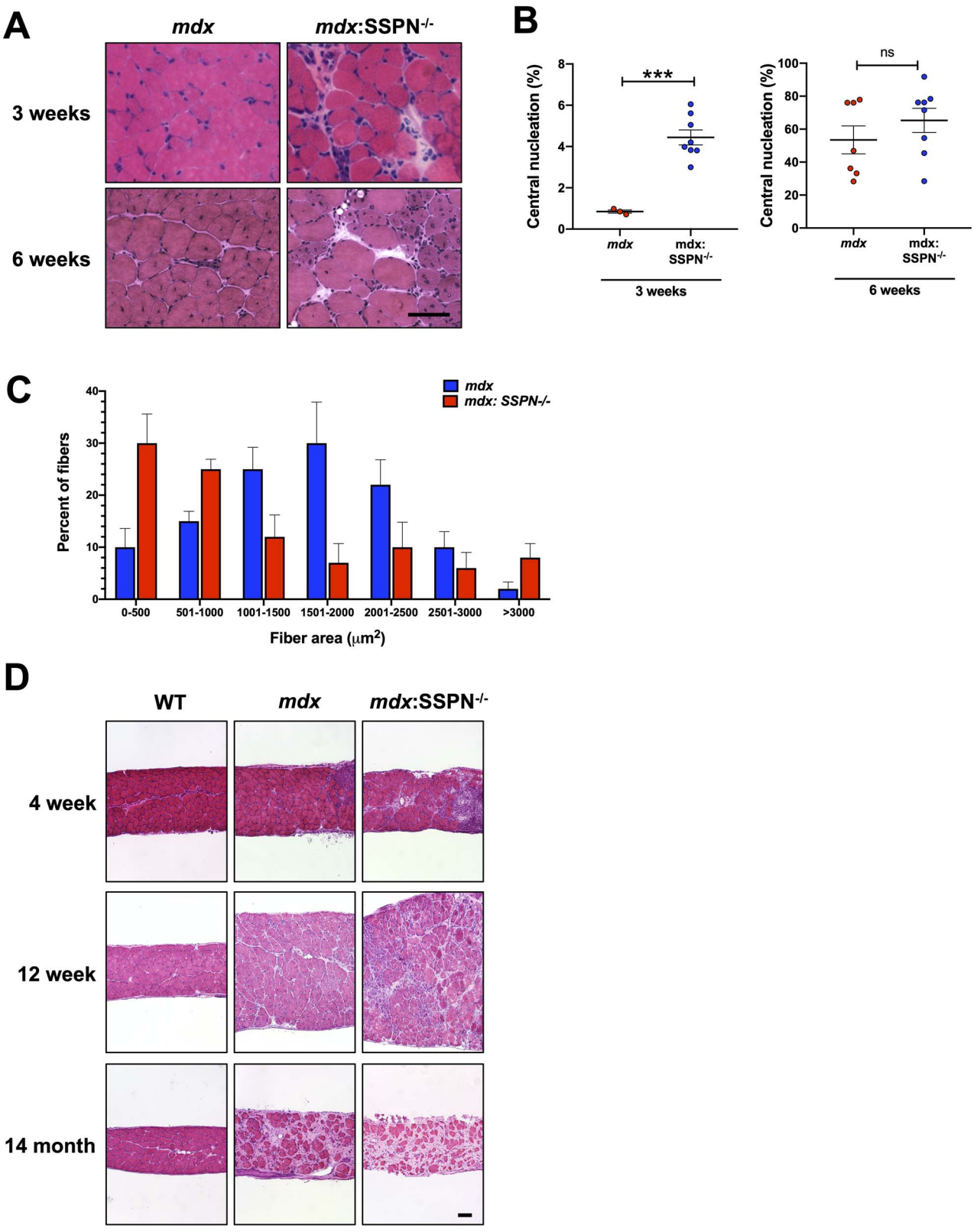

Figure 1. Loss of SSPN results in a more severe dystrophic *mdx* phenotype. (**A**) Transverse sections of quadriceps muscle from 3- and 6-week-old *mdx* and *mdx*:SN mice were stained with H&E to visualize muscle pathology. Bar, 100 μm. (**B**) At 3 weeks of age, sections from *mdx*:SN quadriceps have increased central nucleation relative to *mdx* muscle (*mdx*, $n = 3$; *mdx*:SN, $n = 8$). At 6 weeks, there was no significant difference in central nucleation between *mdx* and *mdx*:SN. Data represent means ± SEM. Statistics were calculated using Student's *t*-test (***$P < 0.001$). (**C**) Fiber size (cross-sectional area) variability in tibialis anterior (TA) at 6–8 weeks of age (*mdx*, $n = 3$; *mdx*:SN, $n = 3$). (**D**) Transverse sections of diaphragm muscle from 4-week-old, 12-week-old and 14-month old WT, *mdx* and *mdx*:SN mice were stained with H&E to visualize muscle pathology. Bar, 100 μm.

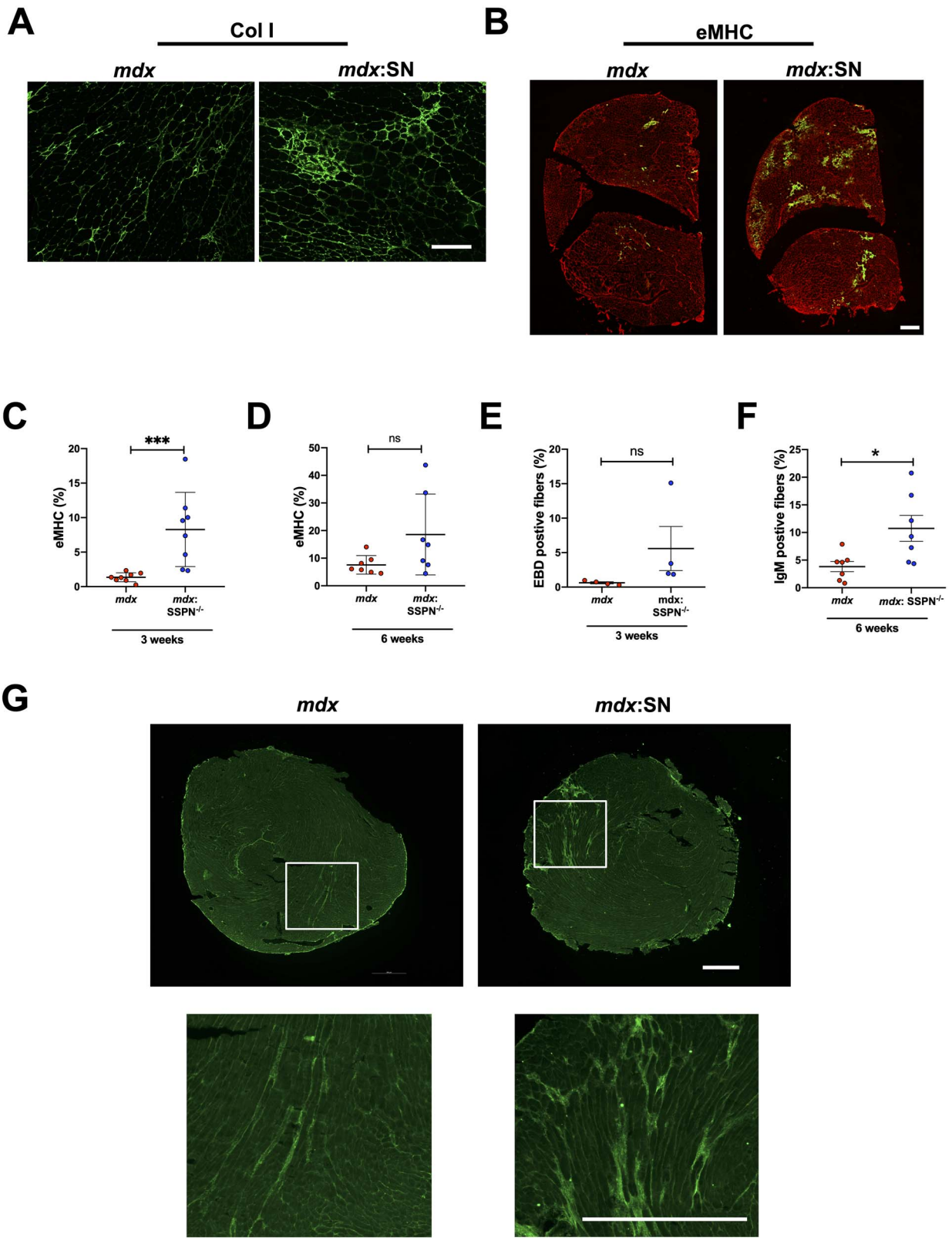

Figure 2. Increased fibrosis and loss of membrane stability in *mdx*:SN muscle. (**A**) Transverse sections of quadriceps muscle from 6-week-old *mdx* and *mdx*:SN mice were stained with antibody against collagen I to assess fibrosis. Bar, 250 µm. (**B**) Transverse sections of quadriceps muscle from 3- and 6-week-old *mdx* and *mdx*:SN mice were stained with eMHC. Representative images from 3-week-old mice are shown here. Bar, 400 µm. (**C**, **D**) Fibers positive for eMHC were quantified relative to total number of fibers. (**E**) In 3-week-old mice, the number of positive EBD fibers was counted in order to quantify sarcolemmal damage. (**F**) In 6-week-old mice, sections were stained for IgM and positive fibers were counted. (**G**) Hearts from 12-month-old mice were sectioned and stained with antibody against collagen I. Magnified regions of fibrosis are shown in the figure. Bar, 500 µm.

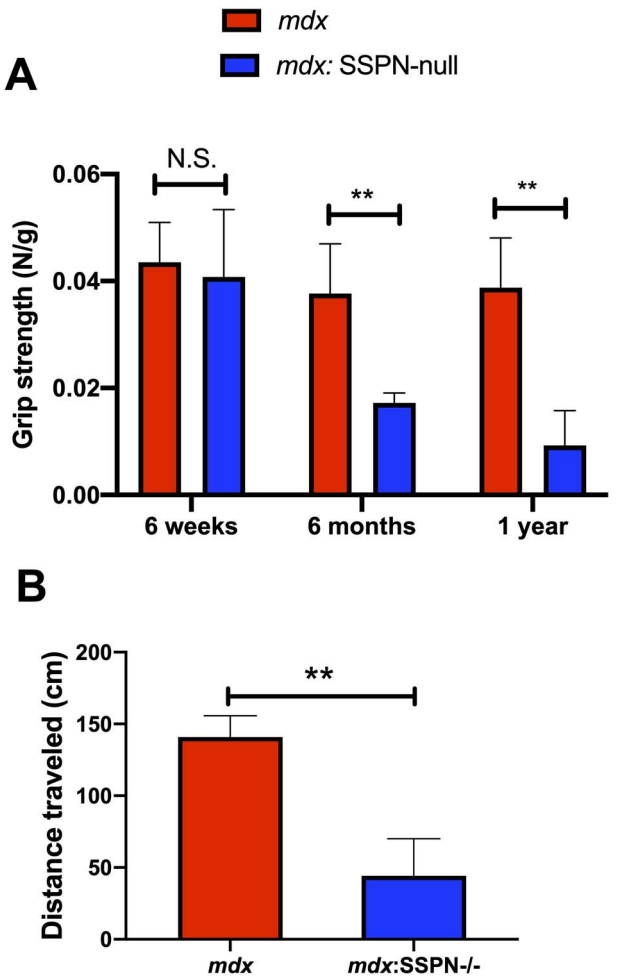

Figure 3. Loss of SSPN exacerbates weakness in aged *mdx*:SN mice. (**A**) Forelimb grip strength normalized to body weight at 6 weeks of age, 6 months and 1 year (fifth consecutive trial reported). (**B**) Post-exercise 6 min walking distances at 6 months of age were recorded using an open field activity chamber ($n = 3$ for both conditions). Data represent means ± *SEM*. Statistics calculated using one-way ANOVA followed by Tukey's multiple comparison's test ($^{*}P < 0.05$ and $^{**}P < 0.01$).

6 weeks, there was a significant increase in IgM-positive fibers, suggesting that loss of SSPN expression leads to more fiber damage at this timepoint (Fig. 2F).

Grip strength and voluntary ambulation are decreased in *mdx*:SN mice

To determine the effect of SSPN loss on *mdx* muscle strength, forelimb grip strength was tested at 6 weeks for *mdx* and *mdx*:SN mice. Each mouse was tested in five consecutive trials, and all animals performed progressively worse over the five trials. At 6 weeks, there was no significant difference between *mdx* and *mdx*:SN, and both genotypes were weaker at all timepoints compared with WT (Fig. 3A). We further tested the mice at a more advanced timepoints and found that there was a significant decrease in *mdx*:SN grip strength relative to age-matched *mdx* at both 6 months and 1 year of age (Fig. 3A).

In addition to grip strength, voluntary ambulation was used as a further metric of muscle function. Following mild exercise, *mdx* mice show reduced activity (36). Our laboratory has developed an ambulation assay that can be readily coupled with the mild exertion of grip strength testing (28,37). Immediately following grip strength testing, mice are placed in an open field chamber and are allowed to freely explore for 6 min. The movement of the mouse is recorded, and the total distance traveled by each mouse is calculated. We have previously shown that after grip strength testing trials, *mdx* mice typically remain motionless for a few minutes and then begin to slowly explore the field chamber at a much slower rate than WT mice (28,37). Here, we found that at the 6-month timepoint, *mdx*:SN mice moved far less than age-matched *mdx* mice, and the total distance traveled was reduced by 69% relative to *mdx* controls (Fig. 3B), revealing that recovery from a grip strength exercise protocol is much slower in *mdx* mice lacking SSPN. Taken together, these data reveal that SSPN contributes to the compensatory response in *mdx* muscle (Table 1).

Consistent with the *mdx*:SN genotype, SSPN and dystrophin (Fig. 4) were not detected in skeletal muscle by immunofluorescent staining. Since UTRN and integrin are upregulated at the sarcolemma in *mdx* muscle overexpressing SSPN (26,28), we examined the expression of UGC components and β1D integrin to determine if additional ablation of SSPN in the *mdx* context would affect the expression of the compensatory adhesion complexes that are upregulated in dystrophin deficiency. SSPN forms a tight subcomplex with the SGs and loss of any one of the SGs at the sarcolemma results in the absence of the entire SG–SSPN subcomplex within the DGC, although cases of partial loss of the subcomplex in human LGMD cases have been reported. Ablation of SSPN in *mdx* muscle caused reduction of α-sarcoglycan (α-SG) in *mdx*:SN samples, although no major change in β-SG was noted (Fig. 4). Furthermore, we found that β-dystroglycan (β-DG) and integrin β1 levels were decreased in in *mdx*:SN muscles relative to controls (Fig. 4). Taken together, these results support that SSPN contributes to the compensatory mechanisms in *mdx* muscle (Table 1).

Rescue of *mdx* pathology by UTRN overexpression is not dependent on SSPN

Given the increased severity of pathology in *mdx* mice lacking SSPN, we were interested in whether overexpression of Utr could rescue the *mdx*:SN phenotype. The *mdx*:utr-Tg rescue model ('Fiona' line) is a well-established transgenic model expressing high levels of Utr (38). These mice exhibit near-normal muscle histology and improved physiological measures (38–40). No differences in body weight were observed at 6 weeks between *mdx*:utr-Tg and *mdx*:SN:utr-Tg mice, and mice from both lines appeared similar to WT control mice (Supplementary Material, Fig. S3).

At 6 weeks, H&E staining of quadriceps in both *mdx*:utr-Tg and *mdx*:SN:utr-Tg histologically resembled WT muscle, with no obvious dystrophic features such as fibrosis, regenerating fibers or fiber size variation (Fig. 5A). Notably, the more severe pathology seen in *mdx*:SN muscle at 6 weeks, including fat deposition and inflammatory infiltrates, was not observed with expression of the Utr transgene. Central nucleation was counted across whole quadriceps in order to evaluate degeneration/regeneration. All conditions had little-to-no central nucleation, demonstrating that at 6 weeks of age, the Utr transgene was able to fully rescue this feature of *mdx* pathology with or without SSPN expression (Fig. 5B).

When forelimb grip strength was tested, both *mdx*:utr-Tg and *mdx*:SN:utr-Tg mice performed significantly better that *mdx* alone (Fig. 5C). There was no significant difference between *mdx*:utr-Tg and *mdx*:SN:utr-Tg, suggesting that the functional

Table 1. SSPN is necessary for compensatory responses in muscular dystrophy

	SSPN-null mice crossed with:			
Genotypes:	Itga mice (α7 integrin null mice)	*mdx* mice (*Dmd*-deficient mice)	*mdx*:Itga mice (α7 integrin and dystrophin double null mice)	myd mice (LARGE-deficient mice)
Question:	Does elevation of α7 integrin compensate for reduction of other known laminin-binding complexes (UGC and DGC) in SSPN-nulls? SSPN-null and Itga-null mice exhibit very mild muscle phenotypes at baseline with normal lifespans. α7 integrin expression is increased in SSPN-null muscle relative to WT controls, while the UGC and DGC expression levels are decreased in the SSPN-nulls	Is the increased UGC and $\alpha7\beta1$ expression at the *mdx* sarcolemma affected by loss of SSPN? The UGC and $\alpha7\beta1$ integrin are increased at the sarcolemma in *mdx* muscle as part of a compensatory mechanism in response to the loss of dystrophin in *mdx* muscle	Does additional loss of SSPN in DKO muscle affect DGC expression? $\alpha7\beta1$ integrin is increased at the sarcolemma in *mdx* muscle as part of a compensatory mechanism in response to the loss of dystrophin in *mdx* muscle. Loss of α7 integrin in *mdx* muscle exacerbates disease pathology, even though levels of UGC are increased in the DKO muscle	Does loss of SSPN affect UGC and DGC expression in myd mice? Spontaneous mutation in the LARGE gene causes muscular dystrophy in mice and humans, resulting in hypoglycosylation of α-DG that diminishes laminin-binding (47). LARGE-deficient (myd) mice exhibit increased UGC expression (27)
Outcome:	Yes. DKO mice die prematurely and DKO muscle exhibits histopathology. Loss of SSPN in α7 integrin nulls further decreases DGC and UGC expression in addition to abolishing Akt signaling (45)	Yes. Loss of SSPN affects expression of the UGC and $\alpha7\beta1$ integrin at the *mdx* sarcolemma (27; this study). DKO mice exhibit earlier onset of pathology and increased membrane fragility relative to *mdx* mice (this study)	Yes. Loss of SSPN diminishes Utr expression and abolishes association of SGs and Utr with DG. SSPN is necessary for protein–protein interactions within the UGC (45)	Yes. Loss of SSPN from myd muscle decreases expression of all adhesion complexes (27)

improvements resulting from the Utr transgene are independent of SSPN expression. It is worth noting that, in our assay, the performance of *mdx*:utr-Tg was significantly decreased relative to WT mice. While some studies have reported that utr-Tg improves *mdx* physiological performance to WT levels in whole body tension escape assays (38,41), Duan and colleagues (42) demonstrated that *mdx*:utr-Tg are susceptible to injury following treadmill exercise. Ervasti and colleagues (40) later showed that *mdx*:utr-Tg mice have reduced activity after mild exercise and that *mdx*:utr-Tg muscle is susceptible to eccentric contraction-induced force loss *in vivo*. Of note, there was no difference between *mdx*:utr-Tg and *mdx*:SN:utr-Tg performance in any of the five trial repetitions.

Indirect immunofluorescence analysis of transverse quadriceps cryosections revealed that dystrophin and SSPN were absent from the sarcolemma *mdx*:SN:utr-Tg muscle, as expected (Fig. 6). The Utr transgenic expression was robustly evident at the *mdx* sarcolemma, as expected, and also increased SSPN at the sarcolemma, but not completely back to WT levels (Fig. 6). Interestingly, ablation of SSPN in *mdx*:utr-Tg muscle decreased Utr localization to the sarcolemma relative to *mdx*:utr-Tg controls, but we conclude that the levels of Utr remaining at the sarcolemma are sufficient to ameliorate disease pathology. The observed reduction in Utr expression in cases of SSPN deficiency is consistent with other double knockout studies with SSPN (Table 1). Both full length and truncated Utr transgenes are able to ameliorate disease in *mdx* and in *mdx*:utr-null mice, and loss of SSPN does not hamper Utr function in preventing muscle degeneration or improving grip strength (Table 2). Thus, we conclude that SSPN is not required for Utr transgenic rescue of dystrophin deficiency at these high levels of Utr overexpression. It is feasible that loss of SSPN may impair Utr-based mechanisms that rely on lower levels of Utr protein.

Discussion

In this study, we examined the effect of SSPN loss on muscle pathology and function in *mdx* mice (Table 1). Our analysis revealed that while *mdx*:SN mice are viable and fertile, they have a more severe dystrophic phenotype than *mdx* mice, with an earlier onset of both histological and functional changes in skeletal muscle. H&E histology revealed an increase in several hallmarks of dystrophic pathology including inflammation, muscle fiber degeneration, lipid deposits and fibrosis. By 6 weeks, *mdx*:SN mice show a decrease in membrane stability, while regeneration (measured both by percent of fibers with central nuclei and eMHC) is no longer significantly increased relative to *mdx*. This may indicate that the acute phase of damage has stabilized by the 6 week timepoint in *mdx*:SN muscle; alternatively, this could be due to a reduced regenerative capacity as seen in more severe dystrophic models such as D2-*mdx* mouse (43,44). Interestingly, a previous study from our laboratory demonstrated that loss of SSPN in WT muscle impairs regeneration following acute muscle injury (27). This suggests that, in addition to the increased membrane fragility observed in *mdx*:SN fibers, reduced regeneration may play a role in the progression of *mdx*:SN dystrophy.

The initial characterization of SSPN knockout mice reported no effect on muscle physiology in mice up to 4 weeks of age (29).

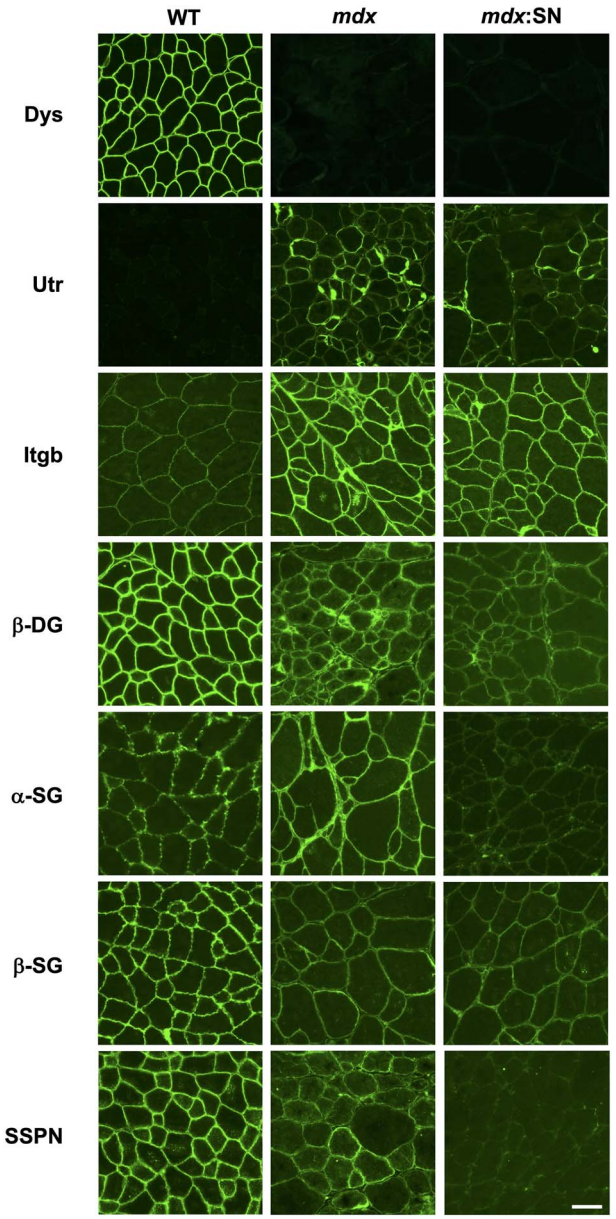

Figure 4. Localization of major muscle adhesion complexes in *mdx*:SN muscle. Transverse sections of whole quadriceps muscle from 6-week-old WT, *mdx* and *mdx*:SN were probed with antibodies against DMD, UTRN, β1D integrin (Itgb), β-DG, sarcoglycans (α- and β-SG) and SSPN and were visualized using indirect fluorescence microscopy. The fluorescence signal was adjusted to visualize the reduced levels of sarcolemmal proteins in *mdx* muscle for comparison to *mdx*:SN samples. Bar, 100 µm.

However, further studies have revealed skeletal muscle and cardiac phenotypes that are exacerbated by age or injury (27,45,46). While there are no pathological changes, or loss of force production seen in limb muscles tested at 4 weeks or 4.5 months, diaphragm muscle had a decrease in specific force and was more susceptible to eccentric contraction damage relative to WT at 4.5 months (29,45). In the present study, we found that although grip strength was not affected at the 6 weeks timepoint, *mdx*:SN mice are significantly weaker than *mdx* mice at both 6 months and 1 year of age, and they have reduced open field activity following mild exercise. Previously, our laboratory showed that UGC complexes from SSPN-null muscle are more readily dissociated by fractionation than UGC complexes from WT muscle (27), and some components of the DGC/UGC complexes are less abundant at the sarcolemma in skeletal and cardiac muscle (45,46). Given that SSPN is a core component of the UGC as well as the DGC (16,26), a likely explanation for this finding is that the loss of SSPN reduces the stability of compensatory UGC complexes at the sarcolemma. The reduced expression of the UGC is a common theme that is observed upon SSPN deficiency in many double knockout mouse studies (Table 1). Examples include reduced sarcolemma expression of the UGC in muscle from mice lacking both integrin α7 and SSPN (45) as well as double knockout mice lacking both SSPN and LARGE (27), the putative GlcNAC glycosyltransferase that causes muscular dystrophy in the myd mouse (47). Interestingly, loss of SSPN does not appear have as much of an effect on the localization of β-SG to the sarcolemma in *mdx* muscle. However, the loss of α-SG and reduction of β-DG are expected to completely disrupt protein–protein interactions within the UGC as we have demonstrated in both skeletal and cardiac muscles (26,27,45,46).

In order to further examine the association of SSPN and UTRN in protecting the *mdx* sarcolemma, we tested whether Utr overexpression could rescue *mdx* pathology in the absence of SSPN. Interestingly, we detected no difference in dystrophic pathology between *mdx*:utr-Tg muscle with or without SSPN expression (Table 2). This suggests that Utr-mediated rescue is not dependent on SSPN expression. This finding is of particular interest, given that UTRN overexpression and SSPN overexpression have both been shown to ameliorate *mdx* pathology and are both ongoing targets for therapeutic development in DMD (48,49). Our findings raise the possibility that Utr and SSPN may alleviate muscle damage through different mechanisms and that simultaneously targeting Utr and SSPN could have additive therapeutic benefits for patients with DMD. For example, while Utr overexpression ameliorates both *mdx* and utr$^{-/-}$:*mdx* mice (Table 2), SSPN overexpression is only able to rescue *mdx* pathology. One major benefit of both SSPN and UTRN as single or combined therapeutic targets in DMD is their potential to treat patients regardless of specific dystrophin mutation.

Drug-mediated upregulation of UTRN expression has been extensively investigated in pre-clinical studies and represents an appealing therapeutic strategy that could be utilized regardless of DMD mutation. Although the small molecule Utr modulator ezutromid failed to meet endpoints in a recent Phase 2 trial (50,51), there is new focus on developing second-generation Utr modulators with more favorable pharmacokinetic profiles (52,53) and repurposing existing drugs for Utr upregulation (49,54). Our laboratory has similarly developed a high-through screening platform for identifying novel enhancers of SSPN expression (48). Given our finding that SSPN expression is not required for UTRN rescue in *mdx*, it is possible that a combinatorial therapy approach for these small molecule modulators may hold promise for the long-term protection of muscle in DMD. It is currently unknown whether Utr upregulation would benefit other muscular dystrophies, including the sarcoglycanopathies or the dystroglycanopathies (Table 2).

An important caveat to this part of our study is that the *mdx*:utr-Tg mouse expresses transgenic Utr at a high level, reported to be between 3- and 10-fold over WT levels of Utr (40). It may be that, at more modest levels of Utr overexpression, loss of SSPN would reveal deficits that are not seen at this high, non-physiologic level of Utr transgene. Our findings demonstrate that Utr transgenic overexpression can rescue the *mdx* phenotype independently of SSPN expression, providing further insight

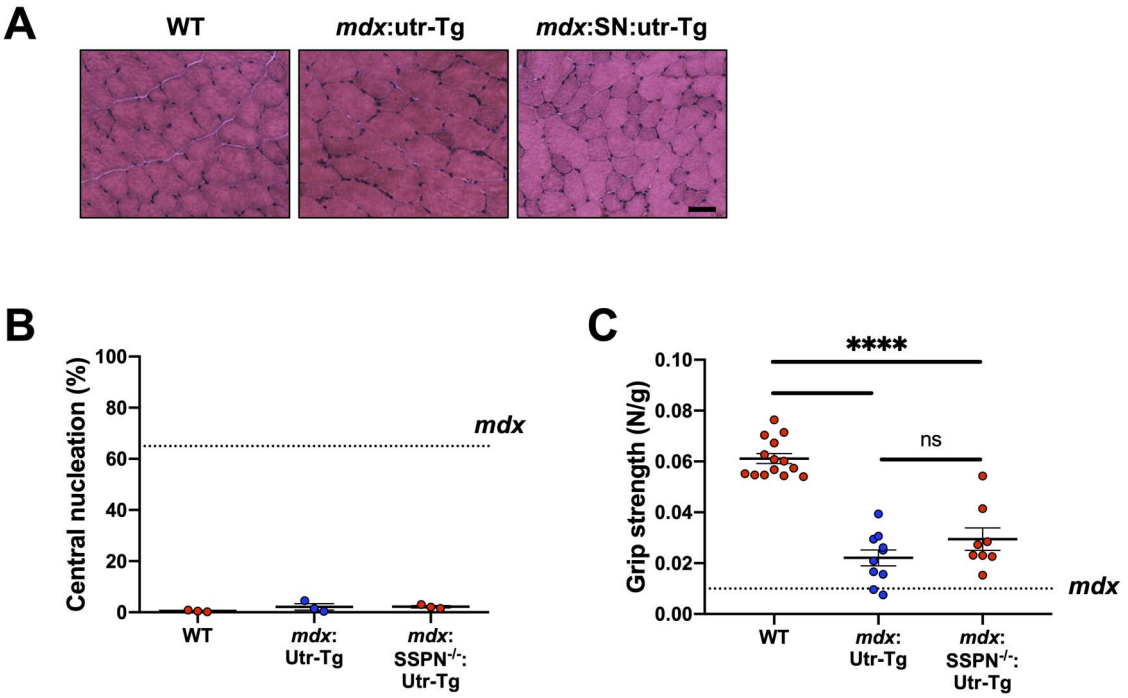

Figure 5. Transgenic expression of Utr improves dystrophic phenotype in *mdx* and *mdx*:SN mice. (**A**) Transverse sections of whole quadriceps muscle from 6-week-old WT, *mdx*:utr-Tg and *mdx*:SN:Utr-Tg mice were stained with H&E to visualize muscle pathology. Bar, 100 µm. (**B**) Fibers with central nucleation were quantitated in quadriceps muscle and expressed as relative to total fibers for each genotype. (**C**) Forelimb grip strength was measured across five trials and normalized to body weight at 6 weeks of age (fifth consecutive trial reported). Statistics calculated using one-way ANOVA followed by Tukey's multiple comparison's test.

into the mechanism of Utr-mediated rescue of dystrophic pathology.

Materials and Methods

Animal models

WT and *mdx* mice were purchased from Jackson Laboratories (Bar Harbor, ME), and SSPN-null mice (Sspntm1Kcam, MGI:2387936) were a generous gift from Kevin P. Campbell, PhD (University of Iowa Carver College of Medicine, Howard Hughes Medical Institute, Iowa City, IA) (29). *mdx*:Utr-Tg (*mdx*:utr-Tg) mice were a gift from James M. Ervasti, PhD (University of Minnesota, Minneapolis, MN), with permission from Kay E. Davies, PhD (University of Oxford, Oxford, UK), and were maintained on the *mdx* background. To generate *mdx*:SN and *mdx*:SN:utr-Tg mice, *mdx*:utr-Tg females (C57Bl/10ScSn background) were crossed with SSPN-null males (C57BL/6 J background), generating heterozygous *mdx*:SSPN-het or *mdx*:SSPN-het:utr-Tg males and females. Littermates were crossed to generate homozygous *mdx*:SN or *mdx*:SN:utr-Tg and were maintained by sibling crosses (B6/B10ScSn background). Double knockouts (DKOs) were viable and fertile, and the line was maintained with homozygous crosses (*mdx*:SN to *mdx*:SN:utr-Tg). Mice were maintained in the Terasaki Life Sciences Vivarium, and all procedures were carried out in accordance with guidelines set by the University of California, Los Angeles Institutional Animal Care and Use Committee.

Grip strength test

Forelimb grip strength was measured using a digital force gage (Columbus Instruments, Columbus, OH) using methods described previously (28,37). Five trials were performed with 1 min of rest between each trial. For each trial, the mouse grasped the pull bar connected to the grip strength meter and was gently pulled back until the pull bar was released. This procedure was repeated five times per trial and the peak tension (*N*) was recorded for each trial.

Open field activity

Ambulation in 6-month-old mice was recorded immediately following grip strength testing (mild exercise protocol, described in (28,37)). Mice were placed in individual recording chambers and were allowed to freely move for 6 min. Distance traveled by each animal was determined using frame-by-frame object tracking in Kinovea open source video analysis software (Version 0.8.15, www.kinovea.org).

Histology and immunofluorescence assays

Muscles were mounted in OCT (Tissue-Tek) and were flash frozen in liquid nitrogen-cooled isopentane and were stored at −80°C until further processing. Transverse 10 µm transverse cryosections were stained with H&E as described previously (28). In H&E-stained sections, centrally nucleated and total fibers were counted in order to determine the level of regeneration (number of fibers with central nuclei divided by total number of fibers). For antibody staining, sections were blocked with 3% BSA in PBS for 30 min at room temperature. Avidin/biotin blocking kit (Vector Laboratories) was used according to manufacturer's instructions. For antibodies raised in mouse, Mouse on Mouse blocking reagent (Vector Laboratories) was used according to manufacturer's instructions. Sections were incubated in primary antibody in PBS at 4°C overnight with the following antibodies:

Table 2. UTRN ameliorates dystrophin-deficient muscular dystrophy in an SSPN-independent manner

	Utr-Tg mice crossed with:				
Genotypes:	*mdx* mice (dystrophin-deficient mice)	utr$^{-/-}$:*mdx* mice (Utr and dystrophin double null mice)	*mdx*:SSPN-null mice (SSPN and dystrophin double null mice)	Sarcoglycan-null mice (α-, β-, γ- or δ-SG null mice are models for LGMD)	myd mice (LARGE-deficient mice represent one of many models for dystroglycanopathy)
Question:	Does Utr rescue skeletal and cardiac muscle disease in the DMD murine model?	Can Utr replace both for Utr and Dys to rescue disease?	Can Utr 'rescue' *mdx* disease in the absence of SSPN?	Can Utr rescue muscular dystrophy caused by absence of the SG–SSPN subcomplex?	Can Utr address hypoglycosylation deficiency of α-DG?
Outcome:	Yes. Expression of full length and mini-Utr reduces membrane instability, prevents degeneration/regeneration cycles and reduces fibrosis (38,55,56)	Yes. Overexpression of a truncated Utr transgene in utr$^{-/-}$:*mdx* skeletal muscle prevented premature death, decreased central nucleation and supported expression of the UGC at the sarcolemma (58)	Yes. *mdx*:SSPN-null:utr-Tg mice were identical to Utr-Tg:*mdx* mice in amelioration of pathology. Ablation of SSPN reduced localization of Utr at the sarcolemma (this study)	Unknown	Unknown

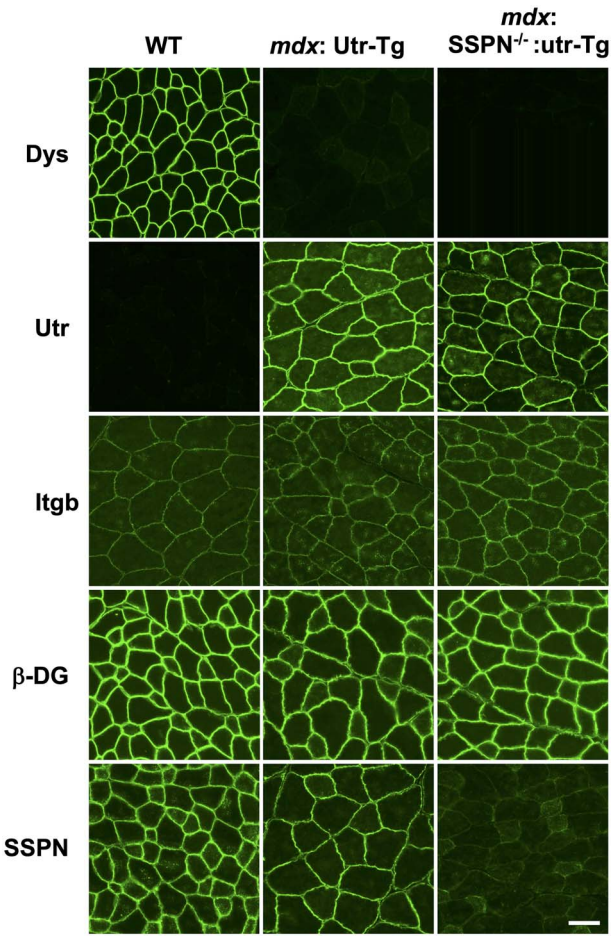

Figure 6. Utr localization to the sarcolemma is diminished in the absence of SSPN. Transverse sections of whole quadriceps muscle from 6-week-old WT, *mdx*:utr-Tg and *mdx*:SSPN$^{-/-}$:utr-Tg were stained with antibodies against Dys, Utr, integrin β1D (Itgb), β-DG and SSPN. Loss of dystrophin and SSPN in the *mdx*:SSPN$^{-/-}$:utr-Tg confirms expected expression across genotypes. Bar, 50 µm.

dystrophin (MANDYS1; 1:5; Development Studies Hybridoma Bank), Utr (NCL-DRP2, 1:100; Leica), β-DG (MANDAG2; 1:50; Development Studies Hybridoma Bank), α-SG (VP-A105; 1:30; Vector Laboratories), β-SG (VP-B206; 1:30; Vector Laboratories), α7 integrin (C-20; 1:100; Santa Cruz), laminin (L9393; 1:500; Sigma-Aldrich), β1D integrin (MAB1900; 1:20; Millipore) and SSPN (E2; 1:100). Primary antibodies were detected by biotinylated anti-rabbit (BA-1000; 1:500; Vector Laboratories) and biotinylated anti-mouse (BA-9200; 1:500; Vector Laboratories). Fluorescein-conjugated avidin D (A-2001; 1:500; Vector Laboratories) was used to detect biotinylated secondary antibodies. All sections were mounted in Vectashield (Vector Laboratories) and were visualized using an Axioplan 2 fluorescence microscope with Axiovision 3.0 software (Zeiss Inc.). In quadriceps sections from mice treated with EBD, both EBD-positive fibers and total and total fibers outlined with anti-laminin were counted and percent of positive fibers was calculated (positive fibers divided by the total number of fibers in the section).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., Version 8.3.0). All data are presented as mean ± SEM. Significance was determined by two-tailed Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison's test. $P < 0.05$ was considered statistically significant.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

References

1. Yoshida, M. and Ozawa, E. (1990) Glycoprotein complex anchoring dystrophin to sarcolemma. *J. Biochem.*, **108**, 748–752.
2. Ibraghimov-Beskrovnaya, O., Ervasti, J.M., Leveille, C.J., Slaughter, C.A., Sernett, S.W. and Campbell, K.P. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature*, **355**, 696–702.
3. Ervasti, J.M. and Campbell, K.P. (1993) A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.*, **122**, 809–823.
4. Petrof, B.J., Shrager, J.B., Stedman, H.H., Kelly, A.M. and Sweeney, H.L. (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. U. S. A.*, **90**, 3710–3714.
5. Weller, B., Karpati, G. and Carpenter, S. (1990) Dystrophin-deficient *mdx* muscle fibers are preferentially vulnerable to necrosis induced by experimental lengthening contractions. *J. Neurol. Sci.*, **100**, 9–13.
6. Verhaart, I.E.C. and Aartsma-Rus, A. (2019) Therapeutic developments for Duchenne muscular dystrophy. *Nat. Rev. Neurol.*, **15**, 373–386.
7. von der Mark, H., Durr, J., Sonnenberg, A., von der Mark, K., Deutzmann, R. and Goodman, S.L. (1991) Skeletal myoblasts utilize a novel beta 1-series integrin and not alpha 6 beta 1 for binding to the E8 and T8 fragments of laminin. *J. Biol. Chem.*, **266**, 23593–23601.
8. Song, W.K., Wang, W., Foster, R.F., Bielser, D.A. and Kaufman, S.J. (1992) H36-alpha 7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis. *J. Cell Biol.*, **117**, 643–657.
9. Boppart, M.D., Burkin, D.J. and Kaufman, S.J. (2006) Alpha7beta1-integrin regulates mechanotransduction and prevents skeletal muscle injury. *Am J Physiol Cell Physiol*, **290**, C1660–C1665.
10. Love, D.R., Hill, D.F., Dickson, G., Spurr, N.K., Byth, B.C., Marsden, R.F., Walsh, F.S., Edwards, Y.H. and Davies, K.E. (1989) An autosomal transcript in skeletal muscle with homology to dystrophin. *Nature*, **339**, 55–58.
11. Campbell, K.P. and Kahl, S.D. (1989) Association of dystrophin and an integral membrane glycoprotein. *Nature*, **338**, 259–262.
12. Ervasti, J.M., Ohlendieck, K., Kahl, S.D., Gaver, M.G. and Campbell, K.P. (1990) Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature*, **345**, 315–319.
13. Pearce, M., Blake, D.J., Tinsley, J.M., Byth, B.C., Campbell, L., Monaco, A.P. and Davies, K.E. (1993) The utrophin and dystrophin genes share similarities in genomic structure. *Hum. Mol. Genet.*, **2**, 1765–1772.
14. Yoshida, M., Hama, H., Ishikawa-Sakurai, M., Imamura, M., Mizuno, Y., Araishi, K., Wakabayashi-Takai, E., Noguchi, S., Sasaoka, T. and Ozawa, E. (2000) Biochemical evidence for association of dystrobrevin with the sarcoglycan-sarcospan complex as a basis for understanding sarcoglycanopathy. *Hum. Mol. Genet.*, **9**, 1033–1040.
15. Crosbie, R.H., Lebakken, C.S., Holt, K.H., Venzke, D.P., Straub, V., Lee, J.C., Grady, R.M., Chamberlain, J.S., Sanes, J.R. and Campbell, K.P. (1999) Membrane targeting and stabilization of sarcospan is mediated by the sarcoglycan subcomplex. *J. Cell Biol.*, **145**, 153–165.
16. Crosbie, R.H., Heighway, J., Venzke, D.P., Lee, J.C. and Campbell, K.P. (1997) Sarcospan: the 25kDa transmembrane component of the dystrophin-glycoprotein complex. *J. Biol. Chem.*, **272**, 31221–31224.
17. Nalini, A., Gayathri, N., Thaha, F., Das, S. and Shylashree, S. (2010) Sarcoglycanopathy: clinical and histochemical characteristics in 66 patients. *Neurol. India*, **58**, 691–696.
18. Nigro, V., de Sa Moreira, E., Piluso, G., Vainzof, M., Belsito, A., Politano, L., Puca, A.A., Passos-Bueno, M.R. and Zatz, M. (1996) Autosomal recessive limb-girdle muscular dystrophy, LGMD2F, is caused by a mutation in the delta-sarcoglycan gene. *Nat. Genet.*, **14**, 195–198.
19. Lim, L.E., Duclos, F., Broux, O., Bourg, N., Sunada, Y., Allamand, V., Meyer, J., Richard, I., Moomaw, C., Slaughter, C. *et al.* (1995) Beta-sarcoglycan: characterization and role in limb-girdle muscular dystrophy linked to 4q12. *Nat. Genet.*, **11**, 257–265.
20. Bonnemann, C.G., Modi, R., Noguchi, S., Mizuno, Y., Yoshida, M., Gussoni, E., McNally, E.M., Duggan, D.J., Angelini, C. and Hoffman, E.P. (1995) Beta-sarcoglycan (A3b) mutations cause autosomal recessive muscular dystrophy with loss of the sarcoglycan complex. *Nat. Genet.*, **11**, 266–273.
21. Noguchi, S., McNally, E.M., Othmane, K.B., Hagiwara, Y., Mizuno, Y., Yoshida, M., Yamamoto, H., Bonnemann, C.G., Gussoni, E., Denton, P.H. *et al.* (1995) Mutations in the dystrophin-associated protein gamma-sarcoglycan in chromosome 13 muscular dystrophy. *Science*, **270**, 819–822.
22. Bueno, M.R., Moreira, E.S., Vainzof, M., Chamberlain, J., Marie, S.K., Pereira, L., Akiyama, J., Roberds, S.L., Campbell, K.P. and Zatz, M. (1995) A common missense mutation in the adhalin gene in three unrelated Brazilian families with a relatively mild form of autosomal recessive limb-girdle muscular dystrophy. *Hum. Mol. Genet.*, **4**, 1163–1167.
23. Trabelsi, M., Kavian, N., Daoud, F., Commere, V., Deburgrave, N., Beugnet, C., Llense, S., Barbot, J.C., Vasson, A., Kaplan, J.C. *et al.* (2008) Revised spectrum of mutations in sarcoglycanopathies. *Eur. J. Hum. Genet.*, **16**, 793–803.
24. Nigro, V. and Savarese, M. (2014) Genetic basis of limb-girdle muscular dystrophies: the 2014 update. *Acta Myol.*, **33**, 1–12.
25. Crosbie, R.H., Lim, L.E., Moore, S.A., Hirano, M., Hays, A.P., Maybaum, S.W., Collin, H., Dovico, S.A., Stolle, C.A., Fardeau, M. *et al.* (2000) Molecular and genetic characterization of sarcospan: insights into sarcoglycan-sarcospan interactions. *Hum. Mol. Genet.*, **9**, 2019–2027.
26. Peter, A.K., Marshall, J.L. and Crosbie, R.H. (2008) Sarcospan reduces dystrophic pathology: stabilization of the utrophin-glycoprotein complex. *J. Cell Biol.*, **183**, 419–427.
27. Marshall, J.L., Holmberg, J., Chou, E., Ocampo, A.C., Oh, J., Lee, J., Peter, A.K., Martin, P.T. and Crosbie-Watson, R.H. (2012) Sarcospan-dependent Akt activation is required for utrophin expression and muscle regeneration. *J. Cell Biol.*, **197**, 1009–1027.
28. Gibbs, E.M., Marshall, J.L., Ma, E., Nguyen, T.M., Hong, G., Lam, J.S., Spencer, M.J. and Crosbie-Watson, R.H. (2016) High levels of sarcospan are well tolerated and act as a sarcolemmal stabilizer to address skeletal muscle and pulmonary dysfunction in DMD. *Hum. Mol. Genet.*, **25**, 5395–5406.
29. Lebakken, C.S., Venzke, D.P., Hrstka, R.F., Consolino, C.M., Faulkner, J.A., Williamson, R.A. and Campbell, K.P. (2000) Sarcospan-deficient mice maintain normal muscle function. *Mol. Cell. Biol.*, **20**, 1669–1677.
30. Grounds, M.D., Radley, H.G., Lynch, G.S., Nagaraju, K. and De Luca, A. (2008) Towards developing standard operating procedures for pre-clinical testing in the *mdx* mouse model of Duchenne muscular dystrophy. *Neurobiol. Dis.*, **31**, 1–19.

31. Shavlakadze, T., White, J., Hoh, J.F., Rosenthal, N. and Grounds, M.D. (2004) Targeted expression of insulin-like growth factor-I reduces early myofiber necrosis in dystrophic *mdx* mice. *Mol. Ther.*, **10**, 829–843.
32. Whitehead, N.P., Yeung, E.W. and Allen, D.G. (2006) Muscle damage in *mdx* (dystrophic) mice: role of calcium and reactive oxygen species. *Clin. Exp. Pharmacol. Physiol.*, **33**, 657–662.
33. Straub, V., Rafael, J.A., Chamberlain, J.S. and Campbell, K.P. (1997) Animal models for muscular dystrophy show different patterns of sarcolemmal disruption. *J. Cell Biol.*, **139**, 375–385.
34. Reeve, E.B. (1957) The contribution of I 131-labeled proteins to measurements of blood volume. *Ann. N. Y. Acad. Sci.*, **70**, 137–147.
35. Matsuda, R., Nishikawa, A. and Tanaka, H. (1995) Visualization of dystrophic muscle fibers in *mdx* mouse by vital staining with Evans blue: evidence of apoptosis in dystrophin-deficient muscle. *J. Biochem.*, **118**, 959–964.
36. Kobayashi, Y.M., Rader, E.P., Crawford, R.W. and Campbell, K.P. (2012) Endpoint measures in the *mdx* mouse relevant for muscular dystrophy pre-clinical studies. *Neuromuscul. Disord.*, **22**, 34–42.
37. Gibbs, E.M. and Crosbie-Watson, R.H. (2017) A simple and low-cost assay for measuring ambulation in mouse models of muscular dystrophy. *J. Vis. Exp.* **(130)**: 56772.
38. Tinsley, J., Deconinck, N., Fisher, R., Kahn, D., Phelps, S., Gillis, J.M. and Davies, K. (1998) Expression of full-length utrophin prevents muscular dystrophy in *mdx* mice. *Nat. Med.*, **4**, 1441–1444.
39. Guiraud, S., Edwards, B., Squire, S.E., Moir, L., Berg, A., Babbs, A., Ramadan, N., Wood, M.J. and Davies, K.E. (2019) Embryonic myosin is a regeneration marker to monitor utrophin-based therapies for DMD. *Hum. Mol. Genet.*, **28**, 307–319.
40. Belanto, J.J., Mader, T.L., Eckhoff, M.D., Strandjord, D.M., Banks, G.B., Gardner, M.K., Lowe, D.A. and Ervasti, J.M. (2014) Microtubule binding distinguishes dystrophin from utrophin. *Proc. Natl. Acad. Sci. U. S. A.*, **111**, 5723–5728.
41. Gillis, J.M. (2002) Multivariate evaluation of the functional recovery obtained by the overexpression of utrophin in skeletal muscles of the *mdx* mouse. *Neuromuscul. Disord.*, **12**, S90–S94.
42. Li, D., Bareja, A., Judge, L., Yue, Y., Lai, Y., Fairclough, R., Davies, K.E., Chamberlain, J.S. and Duan, D. (2010) Sarcolemmal nNOS anchoring reveals a qualitative difference between dystrophin and utrophin. *J. Cell Sci.*, **123**, 2008–2013.
43. Rodrigues, M., Echigoya, Y., Maruyama, R., Lim, K.R., Fukada, S.I. and Yokota, T. (2016) Impaired regenerative capacity and lower revertant fibre expansion in dystrophin-deficient *mdx* muscles on DBA/2 background. *Sci. Rep.*, **6**, 38371.
44. van Putten, M., Putker, K., Overzier, M., Adamzek, W.A., Pasteuning-Vuhman, S., Plomp, J.J. and Aartsma-Rus, A. (2019) Natural disease history of the D2-*mdx* mouse model for Duchenne muscular dystrophy. *FASEB J.*, **33**, 8110–8124.
45. Marshall, J.L., Chou, E., Oh, J., Kwok, A., Burkin, D.J. and Crosbie-Watson, R.H. (2012) Dystrophin and utrophin expression require sarcospan: loss of alpha7 integrin exacerbates a newly discovered muscle phenotype in sarcospan-null mice. *Hum. Mol. Genet.*, **21**, 4378–4393.
46. Parvatiyar, M.S., Marshall, J.L., Nguyen, R.T., Jordan, M.C., Richardson, V.A., Roos, K.P. and Crosbie-Watson, R.H. (2015) Sarcospan regulates cardiac isoproterenol response and prevents Duchenne muscular dystrophy-associated cardiomyopathy. *J. Am. Heart*, **4**, e002481.
47. Grewal, P.K., Holzfeind, P.J., Bittner, R.E. and Hewitt, J.E. (2001) Mutant glycosyltransferase and altered glycosylation of alpha-dystroglycan in the myodystrophy mouse. *Nat. Genet.*, **28**, 151–154.
48. Shu, C., Kaxon-Rupp, A.N., Collado, J.R., Damoiseaux, R. and Crosbie, R.H. (2019) Development of a high-throughput screen to identify small molecule enhancers of sarcospan for the treatment of Duchenne muscular dystrophy. *Skelet. Muscle*, **9**, 32.
49. Loro, E., Sengupta, K., Bogdanovich, S., Whig, K., Schultz, D.C., Huryn, D.M. and Khurana, T.S. (2020) High-throughput identification of post-transcriptional utrophin up-regulators for Duchenne muscle dystrophy (DMD) therapy. *Sci. Rep.*, **10**, 2132.
50. Proof of Concept Study to Assess Activity and Safety of SMT C1100 (Ezutromid) in Boys With Duchenne Muscular Dystrophy (DMD). *Retrieved from http://clinicalTrials.gov (Identifier: NCT02858362; date last accessed December 17, 2020)*.
51. Muntoni, F., Tejura, B., Spinty, S., Roper, H., Hughes, I., Layton, G., Davies, K.E., Harriman, S. and Tinsley, J. (2019) A phase 1b trial to assess the pharmacokinetics of ezutromid in pediatric Duchenne muscular dystrophy patients on a balanced diet. *Clin. Pharmacol. Drug Dev.*, **8**, 922–933.
52. Babbs, A., Berg, A., Chatzopoulou, M., Davies, K.E., Davies, S.G., Edwards, B., Elsey, D., Emer, E., Guiraud, S., Harriman, S. *et al.* (2020) 2-Arylbenzo[d]oxazole phosphinate esters as second-generation modulators of utrophin for the treatment of Duchenne muscular dystrophy. *J. Med. Chem.*, **2020 Jul 23;63(14)**, 7880–7891.
53. Guiraud, S., Squire, S.E., Edwards, B., Chen, H., Burns, D.T., Shah, N., Babbs, A., Davies, S.G., Wynne, G.M., Russell, A.J. *et al.* (2015) Second-generation compound for the modulation of utrophin in the therapy of DMD. *Hum. Mol. Genet.*, **24**, 4212–4224.
54. Peladeau, C., Adam, N., Bronicki, L.M., Coriati, A., Thabet, M., Al-Rewashdy, H., Vanstone, J., Mears, A., Renaud, J.M., Holcik, M. *et al.* (2020) Identification of therapeutics that target eEF1A2 and upregulate utrophin A translation in dystrophic muscles. *Nat. Commun.*, **11**, 1990.
55. Tinsley, J.M., Potter, A.C., Phelps, S.R., Fisher, R., Trickett, J.I. and Davies, K.E. (1996) Amelioration of the dystrophic phenotype of *mdx* mice using a truncated utrophin transgene. *Nature*, **384**, 349–353.
56. Deconinck, N., Tinsley, J., De Backer, F., Fisher, R., Kahn, D., Phelps, S., Davies, K. and Gillis, J.M. (1997) Expression of truncated utrophin leads to major functional improvements in dystrophin-deficient muscles of mice. *Nat. Med.*, **3**, 1216–1221.
57. Marshall, J.L., Oh, J., Chou, E., Lee, J.A., Holmberg, J., Burkin, D.J. and Crosbie-Watson, R.H. (2015) Sarcospan integration into laminin-binding adhesion complexes that ameliorate muscular dystrophy requires utrophin and alpha7 integrin. *Hum. Mol. Genet.*, **24**, 2011–2022.
58. Rafael, J.A., Tinsley, J.M., Potter, A.C., Deconinck, A.E. and Davies, K.E. (1998) Skeletal muscle-specific expression of a utrophin transgene rescues utrophin-dystrophin deficient mice. *Nat. Genet.*, **19**, 79–82.