Dystrophin and utrophin expression require sarcospan: loss of α7 integrin exacerbates a newly discovered muscle phenotype in sarcospan-null mice

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Sarcospan (SSPN) is a core component of the major adhesion complexes in skeletal muscle, the dystrophin– and utrophin (Utr)–glycoprotein complexes (DGC and UGC). We performed a rigorous analysis of SSPN-null mice and discovered that loss of SSPN decreased DGC and UGC abundance, leading to impaired laminin-binding activity and susceptibility to eccentric contraction-induced injury in skeletal muscle. We show that loss of SSPN increased levels of α7β1 integrin. To genetically test whether integrin compensates for the loss of DGC and UGC function in SSPN-nulls, we generated mice lacking both SSPN and α7 integrin (DKO, double knockout). Muscle regeneration, sarcolemma integrity and fibrosis were exacerbated in DKO mice and were remarkably similar to muscle from Duchenne muscular dystrophy (DMD) patients, suggesting that secondary loss of integrin contributes significantly to pathogenesis. Expression of the DGC and UGC, laminin binding and Akt signaling were negatively impacted in DKO muscle, resulting in severely diminished specific force properties. We demonstrate that SSPN is a necessary component of dystrophin and Utr function and that SSPN modulation of integrin signaling is required for extracellular matrix attachment and muscle force development.

INTRODUCTION

Three adhesion glycoprotein complexes span the sarcolemma and protect muscle from contraction-induced damage by maintaining connections between filamentous actin and the extracellular matrix (ECM): the dystrophin–glycoprotein complex (DGC), the utrophin (Utr)–glycoprotein complex (UGC) and the α7β1 integrin complex (1,2). Dystrophin, located at the subsarcolemma, is a component of the DGC that interacts with filamentous actin and β-dystroglycan (β-DG) (3). α-DG binds to ligands in the ECM through a heavily glycosylated mucin domain and is anchored to the sarcolemma via β-DG (4,5). The sarcoglycan–SSPN (SG-SSPN) subcomplex consists of four transmembrane glycoproteins referred to as the SGs (α-, β-, γ- and δ-SG) and SSPN. The UGC is homologous to the DGC except that Utr replaces dystrophin. The localization of the UGC is restricted to neuromuscular (NMJ) and myotendinous (MTJ) junctions in normal adult muscle (6–8). Similar to the DGC, the α7β1 integrin complex is localized to the extra-synaptic sarcolemma, is enriched at NMJs and MTJs and is also a laminin α2 receptor (9–13). Direct physical interactions between α7β1 integrin and the UGC or DGC have never been demonstrated in adult skeletal muscle. Mutations in most of the components of the three-glycoprotein adhesion complexes result in various forms of muscular dystrophy. The concept of compensatory glycoprotein...
adhesion complexes arose from genetic studies utilizing dystrophin-deficient mdx mice, which serve as a model for DMD (14). Initial observations that mdx mice demonstrate an attenuated form of muscular dystrophy and maintain normal lifespan compared with DMD boys led to the hypothesis that the increased levels of UGC and α7β1 integrin perform redundant functions, thus compensating for the absence of DGC (6–8,15–19). Evidence to support this hypothesis is derived from studies in which additional genetic removal of either Utr or α7 integrin (Itgα7) from mdx mice increased the severity of dystrophic pathology and greatly reduced the lifespan (20–23). Furthermore, over-expression of either Utr or β1 integrin in mdx mice and that of Itgα7 in mdx: Utr-null mice ameliorate dystrophic pathology (24–29), supporting the concept of functional redundancy of the DGC, UGC and α7β1 integrin complexes in murine skeletal muscle.

SSPN is a core component of the DGC and UGC, and its tetraspanin-like properties suggest that it may affect integrin expression (30,31). Thus, SSPN has the potential to regulate cross-talk between all three adhesion glycoprotein complexes. Although young SSPN-deficient mice display a normal muscle phenotype without force deficits (32), we now show that loss of SSPN in aged mice increases α7β1 integrin expression. In the current report, we crossed SSPN-deficient mice with Itgα7-deficient mice to create double-knockout (DKO) mice. Itgα7-deficient mice suffer from a late onset myopathy (33), decrease in force transmission in the diaphragm of 5-month-old mice (34) and altered development of NMJs (35,36) and MTJs (33,37). We test the hypothesis that increased Itgα7 levels in SSPN-null mice compensate for SSPN deficiency.

RESULTS

Levels of α7β1 integrin are increased in SSPN-deficient mice

In DMD patients and mdx mice, increased expression of α7β1 integrin and the UGC at the extra-synaptic sarcolemma compensates for the loss of the DGC (6,7,16,19,38–41). To first investigate whether these proteins functioned in a compensatory fashion in SSPN-deficient mice, we analyzed total skeletal muscle protein lysates from adult 4.5-month-old wild-type and SSPN-null mice. Utr, DGs (α- and β-DGs), SGs (α- and γ-SGs), phosphorylated Akt (Serine 473 and Thr308), phosphorylated p70S6K (Thr386), matrix metalloproteinase 9 (MMP 9), p38 MAPK (p38), phosphorylated p38 MAPK (p-p38), phosphorylated p44 MAPK (p-p44), NF-κB1 p105 (p105), phosphorylated NF-κB1 p105 (p-p105), GAPDH and Coomassie blue (CB) staining serve as loading controls. Quantification of p-Akt/Akt, p-p70S6K and p-IGF-R/IGF-R are provided in Supplementary Material, Figure S1.

Genetic removal of SSPN from Itgα7-null mice exacerbates muscle atrophy and causes premature lethality

To determine whether increased integrin levels compensate for the loss of SSPN, we crossed Itgα7-null females with SSPN-deficient males to generate mice lacking both Itgα7 and SSPN (DKO). Itgα7-deficient mice exhibit reduced grip strength and a late onset, mild myopathy at ~4 months of age (33,42). As an initial test for the effect of SSPN on integrin function, we investigated whether loss of SSPN affects myopathy in aged Itgα7-deficient mice. We first analyzed body size and skeletal structure by macroscopic evaluation of 4.5- and 6-month-old mice, generated a survival curve and collected data on genotype frequencies. DKO mice demonstrate significant reductions in body weight and wet muscle mass compared with wild-type and SSPN-deficient controls (Supplementary Material, Fig. S2A–F). At 4.5 months,
Itgα7-deficient mice exhibit mild kyphosis relative to SSPN-null and wild-type controls (Fig. 2A). By comparison, DKO mice display severe kyphosis at 4.5 months as evidenced by the steep slope of the spine behind the shoulder blades, which is exacerbated at 6 months of age (Fig. 2A and B). Kyphosis results from weakening of the muscles supporting the back (43), demonstrating that the removal of SSPN from Itgα7-deficient mice increases the severity of skeletal muscle atrophy. As depicted in the Kaplan–Meier survival analysis, 10% of DKO mice do not survive past 4 weeks of age compared with 5% of Itgα7-deficient controls (Fig. 2C). DKO mice failed to thrive throughout the experimental time frame, as evidenced by the significantly reduced viability (50%) of DKO mice at 8 months (Fig. 2C). We observed 3-fold more live DKO births compared with Itgα7-deficient controls (Table 1), suggesting that additional removal of SSPN paradoxically ameliorates embryonic lethality in Itgα7-deficient controls.

DKO mice exhibit muscle pathology

Many forms of muscular dystrophy are characterized by detachment of the sarcolema from either the ECM and/or the intracellular filamentous actin cytoskeleton, leading to cycles of myofiber degeneration and regeneration, satellite cell exhaustion and replacement of muscle with fibrotic tissue and fat (44). To evaluate whether loss of SSPN affects the Itgα7-deficient phenotype, quadriceps and diaphragm muscles were analyzed for muscle regeneration, degeneration, fibrosis deposition and adipose replacement. At 4.5 months of age, Itgα7-deficient mice displayed mild myopathy evidenced by necrosis and centrally placed nuclei in hematoxylin and eosin (H&E)-stained transverse cryosections of quadriceps muscle (Fig. 3A). Pathological symptoms remained absent in SSPN-deficient and Itgα7-deficient muscle at these older ages. Central nucleation, a marker for muscle regeneration, was significantly increased in DKO mice at 4.5 months of age compared with all controls, demonstrating that loss of SSPN and Itgα7 exacerbates myofiber regeneration (Fig. 3A and B).

Sarcolemmal instability is one of the hallmark symptoms of dystrophic pathology. Using an Evan’s blue dye (EBD) tracer assay, we investigated the possibility that the combined loss of both SSPN and Itgα7 caused sarcolemma damage. EBD is a fluorescent dye that is administered via an intraperitoneal route and accumulates in the interior of damaged muscle fibers, where it is detected using fluorescence microscopy (45). Transverse quadriceps muscle sections collected from EBD-treated mice were stained with laminin antibodies (green) as a sarcolemmal marker (Fig. 3A). We demonstrate that EBD accumulation (red) is statistically increased in DKO quadriceps muscle at 4.5 months of age relative to controls (Fig. 3C), whereas the individual loss of either SSPN or Itgα7 was insufficient to cause membrane damage. It is particularly noteworthy that laminin levels are severely diminished in DKO samples (Fig. 3A).

Macroscopic evaluation demonstrated significant differences in myofiber size in DKO mice relative to all controls (Fig. 3A). Quantification of transverse cross-sectional areas (CSAs) of quadriceps muscles revealed that SSPN-deficient and Itgα7-deficient mice exhibit a rightward shift (larger CSAs) in the myofiber distribution profile compared with wild-type controls. These single knockouts exhibit a larger proportion of myofibers ranging between 2500 and 5000 μm² and a corresponding reduction of myofibers with CSAs <2500 μm² (Fig. 3D). Conversely, DKO muscle displays a leftward shift (smaller CSA) in the distribution profile as revealed by the increased percentage of fibers with CSAs between 0 and 2000 μm² and a corresponding decrease in fibers >2000 μm² (Fig. 3D). We provide evidence that the combined loss of Itgα7 and SSPN in quadriceps muscles significantly attenuates the median myofiber CSA, resulting in greater numbers of smaller myofibers (Fig. 3E). We discovered that the trends in myofiber CSA correlate with muscle weight. Quadriceps muscle isolated from SSPN-deficient mice weigh significantly more than wild-type controls,

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Severe kyphosis in SSPN- and α7 integrin-deficient (DKO) mice. (A) Photographs of wild-type (WT), SSPN-null (SSPN−/−), α7 integrin-null (Itgα7−/−) and SSPN-null/α7 integrin-null (DKO) mice at 4.5 months of age (left panels). Macroscopic evaluation of musculoskeletal structure in mice that were injected with EBD (right panels). DKO mice display severe kyphosis compared with control littermates. (B) Photographs of α7 integrin-null (Itgα7−/−) and SSPN-null/α7 integrin-null (DKO) mice at 6 months of age. DKO mice continue to exhibit severe kyphosis compared with control littermates. (C) The viability of DKO (n = 20) mice is severely reduced between 6 and 9 months of age compared with wild-type (n = 20), α7 integrin-null (n = 20) and SSPN-null (n = 20) mice. At 8 months, only 50% of DKO mice remain viable.
whereas DKO quadriceps weigh significantly less than single knockout controls (Supplementary Material, Fig. S2C).

In the mdx mouse model of DMD, the diaphragm is the most severely affected muscle, as evidenced by robust fibrosis and fat deposition (46). To further investigate the relationship between SSPN and Itg7, diaphragm muscles were analyzed for markers of dystrophic pathology at 4.5, 6 and 9 months of age. H&E histological analysis of transverse diaphragm cryosections revealed increased interstitial connective tissue between myofibers in Itg7-deficient mice, which was more evident in DKO muscle (Fig. 4A). At 4.5 months of age, SSPN-deficient and Itg7-deficient mice do not display signs of fibrosis; however, DKO mice exhibit a dramatic increase in collagen deposition as revealed by Van Geison staining that was remarkably analogous to DMD muscle. Representative images of H&E-stained whole diaphragms from 4.5-month-old Itg7-deficient and DKO mice are provided to illustrate widespread fibrosis and adiposity in DKO samples (Supplementary Material, Fig. S3). The Itg7-deficient mice exhibit detectable collagen deposition at 6 months; however, the myopathy in the DKO mice is more severe than that in the Itg7 single knockout. Collagen deposition was undetectable in SSPN-deficient mice at all ages examined. At 9 months, the DKO diaphragms display significant fibrotic collagen deposition and fat replacement in the diaphragm (Fig. 4A). Oil Red staining, a marker for adipose cells and lipid deposits, is not detectable in mice at 4.5 months of age. However, Itg7 single null and DKO mice display Oil Red staining at 6 months, which is exacerbated in the DKO at 9 months of age (Fig. 4A).

Quantification of central nucleation in the diaphragm (4.5 months) revealed a 16-fold increase in regeneration in DKO mice relative to all controls (Fig. 4B). Embryonic myosin heavy chain (eMHC) staining, which identifies newly regenerated fibers, was detected in DKO diaphragms, suggesting that markers for early stages of regeneration are normally expressed (Supplementary Material, Fig. S4A). SSPN-null and Itg7-null mice display wild-type levels of regeneration in the diaphragm at this age (Fig. 4B). CSA distribution profiles for single-mutant muscle were similar to wild-type (Fig. 4C and D). DKO diaphragms exhibit greater numbers of small fibers (0–500 μm²) and fewer large fibers with CSAs greater than 500 μm² compared with all controls (Fig. 4C). Similar to the quadriceps muscle, DKO diaphragms display more than a 2-fold increase in the percentage of very small (0–500 μm²) myofibers (Fig. 4D), consistent with the observed increases in collagen deposition and regeneration.

### Akt signaling and levels of DGC/UGC are diminished in DKO muscle

Several recent studies have uncovered the important role of intracellular signaling in regulating muscle strength, hypertrophy and pathophysiology. One of the main pathways to govern these physiological events is the stimulation of the phosphatidylinositol-3 kinase/Akt signaling pathway leading to downstream activation of p70S6K protein synthesis pathways (47,48). Activation of the Akt signaling pathway occurs through many different membrane receptors, including insulin-like growth factor 1 (IGF-1), growth and differentiation factor 8 or myostatin and β1 integrin-associated integrin-linked kinase (ILK) (47–49). Loss of Akt signaling in mice results in severe muscle atrophy, fetal growth retardation and neonatal lethality (50). Over-expression of constitutively active Akt in dystrophin-deficient mdx mice results in a functional amelioration of the sarcolemma through up-regulation of Utr and α7β1 integrin, which leads to an improvement in force generation (51,52). To determine whether defects observed in the DKO mice involve deficiencies in the Akt pathway, we performed immunoblotting on total skeletal muscle lysates to determine relative levels of phosphorylated proteins within the signaling axis. Phosphorylation of Akt, p70S6K and IGF-1 receptor was decreased in DKO muscle, accompanied by an increase in myostatin levels (Fig. 5A; Supplementary Material, Fig. S1A–D). Myostatin has been shown to negatively attenuate the activated Akt/p70S6K pathway, which is associated with skeletal muscle atrophy (47,53). Furthermore, IGF-1 levels decrease with age and are correlated with muscle cachexia. When over-expressed, IGF-1 augments force generation capacity, reduces fibrosis in the diaphragms of aged mdx mice and prevents normal loss of muscle mass associated with senescence in 2-year-old wild-type mice (54–56).

In addition to the Akt pathway, MMPs, MAPK signaling and NF-κB1 signaling are important in tissue remodeling and disease progression in several pathophysiological conditions in skeletal muscle. MMP 9 has been shown to be deleteriously increased in the mdx mouse model of DMD because inhibition of MMP 9 alleviates dystrophic pathology in young mdx mice and reduces myofiber injury in the diaphragm of old mdx mice (57–59). In muscle, over-expression of MMP 9 increased the levels of contractile proteins and force production in isometric contractions while reducing the level of collagen deposition in the ECM (57), demonstrating the importance of MMP 9 in remodeling the ECM. Although the role of p38 MAPK in skeletal muscle disease remains unclear, it has been hypothesized that activated p38 MAPK stimulates the transcriptional activity of NF-κB1 which promotes an increase in an increase in the transcription of MMP 9 (60). NF-κB signaling has been shown to be persistently elevated

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**Table 1. Improved embryonic survival in DKO mice compared with α7 integrin-null mice**

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<thead>
<tr>
<th>Genotype</th>
<th>Predicted</th>
<th>Observed</th>
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<tr>
<td>WT</td>
<td>6.3%</td>
<td>21.0% (11/52)</td>
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<tr>
<td>SSPN−/−</td>
<td>6.3%</td>
<td>17.0% (9/52)</td>
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<tr>
<td>Itg7−/−</td>
<td>6.3%</td>
<td>4.0% (2/52)</td>
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<tr>
<td>DKO</td>
<td>6.3%</td>
<td>12.0% (6/52)</td>
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Predicted and observed genotype probabilities for wild-type (WT), SSPN-null (SSPN−/−), α7 integrin-null (Itg7−/−) and SSPN-null:α7 integrin-null (DKO) mice are represented. α7 integrin and SSPN heterozygous mice (Itg7+/−:SSPN−/−) were crossed, yielding a 6.25% probability for each genotype in the resultant litters. Genotypes and their relative representation (Itg7+/−:SSPN−/−) were determined when mice were 1.5 weeks of age. α7 integrin-null mice display more than 50 progeny were determined when mice were 1.5 weeks of age. α7 integrin-null mice display partial embryonic lethality, which is consistent with previous observations (75). The additional loss of SSPN doubled the expected survival rate of α7 integrin-null mice (DKO), although the survival rate remains less than the numbers observed for wild-type and SSPN-null controls.
in the immune infiltrate and regenerating myofibers of DMD patients and mdx mice. Specifically, NF-κB signaling functions to inhibit muscle progenitor cells and to promote inflammation and myofiber necrosis in activated macrophages (61). Preventing the activity of NF-κB ameliorates dystrophic pathology in mdx mice, and constitutive activation of NF-κB
results in muscle wasting, highlighting the importance of NF-κB signaling in muscle disease (60–63). To determine whether this pathway is partly responsible for the alterations in myopathy observed in DKO muscle, we immunoblotted for the MAPK, NF-κB and MMP signaling proteins. We discovered that the additional loss of SSPN in Itgα7-deficient muscle results in greater activation of p38 MAPK, p44 MAPK and p105 NF-κB1 (Fig. 5A). Consistently, the protein levels of downstream MMP 9 were elevated (Fig.5A). These results support a role for the MAPK–NF-κB1–MMP pathway in contributing to the exacerbated pathology observed in DKO muscle.

To determine whether the combined loss of Itgα7 and SSPN affects the localization of adhesion complexes at the sarcolemma and thus contributes to muscle pathology, indirect immunofluorescence assays were performed on transverse cryosections of quadriceps muscle from 4.5-month-old mice. Although young SSPN-deficient mice express normal levels of the DGC (32), we found a notable decrease in dystrophin, β-DG, α-SG and γ-SG and an increase in Itgα7 in aged SSPN-deficient mice (Fig. 5B). Similarly, we discovered that aged DKO muscles exhibit defects in dystrophin, DGs (α- and β-) and the SGs (α-, β- and γ-) at the sarcolemma compared with controls (Fig. 5B). Aged Itgα7-deficient mice were similar to wild-type, except that levels of α-SG were reduced at the sarcolemma (Fig. 5B). Ut was normally enriched at NMJs, although we noted a decrease in Ut in DKO muscle (Fig. 5B and C). Specificity of NMJ staining was confirmed by co-staining muscle sections with Ut antibodies and α-bungarotoxin (α-BTX) (Fig. 5C).

Enhanced Akt signaling has been shown to improve expression of the DGC, UGC and α7β1 integrin adhesion complexes, restore defective regeneration by increasing UGC expression in SSPN-deficient mice and ameliorate mdx pathology by improving UGC and α7β1 integrin density at the sarcolemma (51,52,64). To determine whether the combined loss of SSPN and Itgα7, in addition to suppressed Akt signaling, affected relative levels of the major adhesion complexes,
protein lysates were prepared from skeletal muscle and enriched for adhesion complexes, using succinylated wheat germ agglutinin (sWGA) agarose (30). The integrity of protein interactions within macromolecular complexes was preserved using a mild digitonin buffer followed by lectin affinity chromatography, which enables a rigorous analysis of intact adhesion complexes. By immunoblotting equivalent concentrations of sWGA-enriched samples, we demonstrate that loss of SSPN decreased expression of DGC and UGC components (Fig. 6A). Furthermore, laminin binding to α-DG in SSPN-null muscle is diminished relative to wild-type as revealed by laminin overlay assays (Fig. 6A). We now provide evidence that DGC levels are reduced in aged SSPN-deficient muscle, causing reduced laminin binding. The DGC represents the major structural component of the sarcolemma and our finding that SSPN-deficiency reduces levels of dystrophin (Fig. 6A) as well as the entire DGC complex at the sarcolemma (Fig. 5B), in addition to decreasing laminin binding (Fig. 6A), is likely to have significant impact on muscle function and membrane stability.

Consistent with total skeletal protein immunoblots, β1D integrin is significantly increased in SSPN-deficient muscle compared with wild-type controls (Fig. 6A; Supplementary Material, Fig. S5C), suggesting that integrins may compensate not only for the loss of SSPN, but additionally for secondary reductions in DGC and UGC. In comparison with wild-type mice, Itgα7-deficient mice display mildly decreased levels of laminin, dystrophin, the DGs (α- and β-DG), SGs (α-, β- and γ-SG) and SSPN. Arrows denote NMJ structures. Bar, 50 μm. (C) Transverse cryosections of quadriceps muscle from 4.5-month-old mice were co-stained with Utr antibody and α-BTX, which serve as a marker for NMJs. Merged images (right panels) reveal that Utr protein is localized to NMJ structures in all samples. However, NMJs in DKO muscle appear to be smaller in size with faint Utr staining. Bar, 50 μm.
Figure 6. SSPN and integrin stabilize protein interactions within the DGC and UGC. (A) Skeletal muscle lysates from 4.5-month-old wild-type (WT), SSPN-null (SSPN2/2), α7 integrin-null (Itgα72/2) and SSPN-null:α7 integrin-null (DKO) mice were prepared in digitonin buffer and enriched by sWGA lectin chromatography. Equal protein samples (10 μg) were resolved by SDS–PAGE. Immunoblotting was performed with antibodies against laminin (Lam), dystrophin (Dys), Utr, α7 integrin (α7 Intg), β1D integrin (β1D Intg), DGs (α- and β-DG), SGs (α-, β-, γ-SG) and SSPN. Laminin protein was overlaid on α-DG and visualized by immunoblotting with antibody against laminin (Lam O/L). Quantification of lectin-purified adhesion complexes is provided in Supplementary Material, Figure S5. (B) Utr mRNA was measured from the quadriceps muscle of 4.5-month-old wild-type (WT), SSPN-null (SSPN2/2), α7 integrin-null (Itgα72/2) and SSPN-null:α7 integrin-null (DKO) mice and normalized to the GAPDH internal control. Data are presented as an average normalized to wild-type, and error bars represent standard deviation of the mean (n = 3 mice per genotype). Levels of Utr mRNA were not statistically altered in any genotype.
wild-type and Itgα7-null controls. Quantitative real-time PCR (qRT-PCR) analysis revealed equal quantities of Utr mRNA in all genotypes, suggesting that regulation of Utr protein occurs at the post-transcriptional level (Fig. 6B). Utr is unlikely to be a major contributor to sarcolemmal force production and stabilization since its expression is restricted to the NMJs in DKO mice (Fig. 5B and C). Therefore, exacerbated muscle pathology in DKO mice likely results from the dramatic decrease in the entire DGC and associated laminin binding capacity, in addition to reduced Akt signaling that renders the sarcolemma vulnerable to myofiber damage. Similar to SSPN-deficient mice, further reduction in the DGC, along with reduced laminin binding and loss of α7β1 integrins, is likely to have significant impact on muscle pathophysiology and strength.

The integrity of glycoprotein adhesion complexes was determined by subjecting equal concentrations of sWGA enrichments to high-speed linear sucrose centrifugation, which separates proteins by molecular mass and frictional coefficient. In wild-type muscle, all three-adhesion glycoprotein complexes migrate to fractions 9–11, which correspond to the heaviest fractions in the sucrose gradient (Fig. 6C). In agreement with previously reported data, Utr was not detectable in sucrose gradient fractions, and the DGC remains intact despite loss of SSPN (32,64). Removal of SSPN from wild-type muscle also does not alter the migration profile of α7β1 integrin (Fig. 6D). Migration of the UGC and DGC remains unchanged in comparison with wild-type profiles in Itgα7-deficient muscle (Fig. 6E). Similar to SSPN-deficient muscle, Utr was not detectable, and the DGC components remained intact in DKO fractions (Fig. 6F). To discern differences between the DKO and SSPN-deficient muscles, immunoblots of sucrose gradient fractions of SSPN-deficient and DKO mice were subjected to identical blotting conditions and exposures. Dystrophin migrates to fractions 9–11, which also contain DGs (α- and β-DG) as well as laminin-binding activity in SSPN-deficient muscle. In DKO muscle, dystrophin levels were drastically reduced and restricted to fraction 11. Additional reductions were also observed in the DGs (α- and β-DG) and laminin binding to α-DG in the dystrophin-containing fraction 11 of DKO muscle (Fig. 6G). To determine relative levels of intact DGC and laminin binding to α-DG, densitometry was performed on immunoblots shown for SSPN-deficient and DKO mice. Analysis was restricted to the dystrophin-containing fractions, and the percentage of total signal intensity of intact fractions was plotted relative to the SSPN-null controls. DKO mice exhibit an 87% reduction in dystrophin and an 83% reduction in laminin binding to α-DG compared with SSPN-deficient mice (Fig. 6G). In addition to the reductions in functional DGC, DKO mice also lack α7β1 integrin complexes, which further impacts the stability of the sarcolemma.

Loss of SSPN diminishes specific force in single-knockout and DKO mice

In order to determine whether the observed morphological and biochemical changes affected muscle function, we investigated force production in diaphragm muscle isolated from mice at 4.5 months of age. The diaphragm muscles were subjected to maximum isometric tetanus following eccentric contractions, and the average specific force and percent force drop are presented for each genotype. Diaphragms from SSPN- and Itgα7-single nulls, which possess similar reductions in laminin binding to α-DG, exhibited a 30% reduction in specific force values compared with wild-type (Fig. 7A), confirming results observed in a previous study on Itgα7-null diaphragm muscles (34). Combined loss of both SSPN and Itgα7 further reduced specific force. The effect was additive as DKO diaphragms exhibited 51% reduction in force relative to wild-type (Fig. 7A). A previous study demonstrated that SSPN-deficient extensor digitorum longus (EDL) and soleus muscles isolated from young (1 month of age) mice maintained normal force and power generation capabilities (32) and we now demonstrate that these parameters are also normal in EDL and soleus muscles from 4.5-month SSPN-nulls (Supplementary Material, Fig. S4B–E). Evaluation of muscle fragility in the diaphragm was conducted by measuring the percent drop in force between the first and fifth eccentric contraction (66). Both SSPN-deficient and DKO diaphragms exhibit increased susceptibility to eccentric contraction-induced damage as measured by the percent drop in force (Fig. 7B). Loss of Itgα7 alone did not alter the percent drop in force after eccentric contractions compared with wild-type controls, suggesting that although integrins contribute to force production, they do not contribute to membrane fragility, which is consistent with other studies (67). Our report provides evidence that aged SSPN-deficient mice exhibit impaired force production and increased susceptibility to eccentric contraction-induced injury in diaphragm muscles, demonstrating an important role for SSPN in the maintenance of muscle strength and membrane stability. Furthermore, we demonstrate that expression of both SSPN and Itgα7 is critical for normal muscle function as loss of both proteins weakens muscle, suggesting that they function in a cooperative manner.

DISCUSSION

Emerging evidence supports a model whereby SSPN interacts with each of the major adhesion complexes in skeletal muscle as a common component of laminin-binding receptors. SSPN is a core-component of the DGC and UGC (30,31). It is well established that tetraspanins interact with many transmembrane receptors, most notably the α-subunit of integrins, to regulate signaling functions. Furthermore, disruption of the
tetranspin–integrin association reduced the laminin-binding activity of integrins (68,69). In the current report, we investigated whether SSPN, like tetranspins, influences the activity of α7β1 integrin, the predominantly expressed integrin in adult skeletal muscle. We have demonstrated novel interactions between SSPN and Itgα7 that are important for maintaining expression of the DGC at the sarcolemma. Initial observations revealed that the additional loss of SSPN from Itgα7-null mice improved the embryonic lethality observed in Itgα7-null mice. We did not pursue this paradoxical discovery in the current manuscript, but it is interesting to speculate whether reduction in DKO embryonic lethality may be due to the expression of SSPN in non-muscle tissue such as the placenta or vasculature. Schematic diagrams are provided to illustrate the localization of glycoprotein adhesion complexes at the sarcolemma and their effects on laminin binding based on our analysis of lectin-enriched lysates and sucrose gradient profiles of intact complexes isolated from single- and double-null mice (Fig. 8). We show that SSPN-null mice are deficient in DGC expression and that α7β1 integrin levels are elevated to compensate for the reduction of the DGC and laminin binding. The combined removal of SSPN and Itgα7 results in undetectable levels of the UGC, similar to SSPN-deficient mice, and a reduction in the abundance of DGC complexes at the sarcolemma. The >85% reduction in the DGC results in a >80% loss of laminin-binding activity. The diminution of connections between the intracellular actin cytoskeleton and ECM renders the sarcolemma more susceptible to contraction-induced damage and results in muscle with reduced force-generating capacity, as evidenced by experiments on the diaphragm muscle. We provide evidence that SSPN interacts with α7β1 integrin at the muscle membrane, an interaction characteristic of all tetranspans.

Our data are consistent with co-immunoprecipitation experiments from cultured myotubes, suggesting that the α5β1 integrin, the embryonic form of the integrin complex, associates with the DGC (70). It is also reasonable to speculate that the pathophysiology of DKO diaphragm muscles is affected by reductions in IGF-R signaling as well as increased myostatin. Together, these molecular events contribute to decreased activation of Akt and p70S6K, resulting in reduced protein synthesis, muscle wasting and collagen accumulation giving rise to fibrosis. Similarly, the elevated levels of active MAPK and NF-κB signaling as well as MMP 9 likely contribute to the extensive necrosis and alterations in ECM deposits observed in DKO diaphragms.

The SG-SSPN subcomplex has been well described in relation to the DGC (71). Redundant functions between γ-SG and Itgα7 have been described in DKO mice displaying exacerbated myopathy, severe kyphosis and drastically shortened lifespan (72). Although the proteins levels of SSPN were not reported in γ-SG and Itgα7 DKO mice, SSPN- and Itgα7-deficient mice have reduced protein levels of the DGC, including γ-SG. γ-SG-deficient mice display muscle pathology that is more severe than SSPN-deficient mice with reduced protein levels of α- and β-SG and SSPN (73,74). Similar to SSPN-deficient mice, γ-SG-deficient mice do not exhibit deficits in the percent drop in force following eccentric contractions in the EDL muscle. It would be interesting to examine the diaphragm muscle of SG-deficient mice to determine whether reductions in force production and susceptibility to muscle damage following eccentric contractions are observed.

In addition to affecting the stability of the DGC with integrin, SSPN also determines the stability of the UGC. Over-expression of SSPN in dystrophin-deficient mdx mice ameliorates dystrophic pathology by stabilizing the UGC at the extra-synaptic sarcolemma (31). We have recently shown
that the UGC is severely reduced in SSPN-deficient mice, rendering the muscle more susceptible to cardiotoxin injury (64). Although SSPN-deficient mice were reported to display a normal muscle phenotype without force deficits (32), we recently discovered that SSPN-deficient mice exhibit reductions in the UGC and activated Akt signaling, a failure to repair efficiently after cardiotoxin injury in an Akt-dependent manner and an earlier onset of dystrophic pathology when crossed with \(mdx\) mice (64). These findings mimic those observed in knockouts of other tetraspanins, where the phenotype is revealed under pathological conditions such as cardiotoxin injury and dystrophin deficiency.

The reduced and restricted localization of the UGC at the NMJ in SSPN-deficient mice makes it difficult to determine whether the loss of SSPN disrupts the integrity of the UGC. To overcome this obstacle, future studies will focus on generation and analysis of SSPN-deficient \(mdx\) mice. Furthermore, to directly test the ability of Utr alone to alleviate symptoms of dystrophy in the \(mdx\) mice, we plan to generate triple-knockout mice lacking SSPN, dystrophin and \(\alpha\)7\(\beta\)1 integrin. Our studies represent the first demonstration of molecular and physiological defects resulting from the absence of SSPN and highlight the importance of interactions between SSPN and the adhesion complexes for muscle function. The use of genetic models to understand compensatory overlap between adhesion glycoprotein complexes reveals important molecular mechanisms related to the treatment of muscular dystrophy.

**MATERIALS AND METHODS**

**Animal models**

SSPN-deficient mice (32) were a generous gift from Kevin P. Campbell (University of Iowa Medical School, Iowa City, IA), and \(\text{Itg}{\alpha}7\)-deficient mice (75) were transferred from UNR to UCLA. SSPN-null males were crossed to \(\text{Itg}{\alpha}7\)-null homozygous females. The resulting \(\text{Itg}{\alpha}7\) heterozygous, SSPN heterozygous sibling males and females were mated to generate wild-type, SSPN-null, \(\text{Itg}{\alpha}7\)-null and \(\text{Itg}{\alpha}7\)-null:SSPN-null (DKO) males, which were analyzed at 18, 24 and 36 weeks of age. Genotyping protocols have been previously reported (32,75). Mice were maintained in the Life Sciences Vivarium, and all procedures were carried out in accordance with guidelines set by the Institutional.
Animal Care and Use Committee at the University of California, Los Angeles.

Evans blue dye assay
Sarcolemmal membrane damage was assessed using an EBD tracer analysis that was performed by an intraperitoneal injection of mice with 50 μl of EBD (10 mg/ml in 10 mM of sterile phosphate buffer and 150 mM NaCl, pH 7.4) per 10 g of body weight at least 8 h prior to dissection as described previously (45). Quadriceps muscles were processed as described below.

Immunofluorescence assays
Muscles were mounted in 10.2% polyvinyl alcohol/4.3% polyethylene glycol and flash-frozen in liquid nitrogen-cooled isopentane. Muscles were stored at −80°C until further processing. Transverse sections (8 μm) were placed onto positively charged glass slides (Thermo Fisher Scientific) and stored at −80°C. Sections were acclimated to room temperature for 15 min and blocked with 3% BSA diluted in PBS for 30 min at room temperature. The avidin/biotin blocking kit (Vector Labs) was used according to manufacturer’s instructions. Mouse primary antibodies were prepared with the M.O.M. blocking reagent (Vector Labs) as described by the manufacturer’s protocol. Sections were incubated in primary antibody in PBS at 4°C overnight with the following antibodies: dystrophin (MANDYS1, 1:5; Development Studies Hybridoma Bank), Utr (MANCHO3, 1:5; Development Studies Hybridoma Bank), α-DG IIH6 (sc-53987, 1:500; Santa Cruz Biotechnology, Inc.), β-DG (MANDAG2, 1:50; Development Studies Hybridoma Bank), α-SG (VP-A105, 1:30; Vector Labs), β-SG (VP-B206, 1:30; Vector Labs), γ-SG (VP-G803, 1:30; Vector Labs), laminin (L9393, 1:25; Sigma), β1D integrin (MAB1900, 1:20; Chemicon), α7A integrin (affinity purified, rabbit A2 345, 1:500) (23), eMH C (F1.652, 1:25; Developmental Studies Hybridoma Bank), α-BTX conjugated to AlexaFluor® 555 (B35451, 1:200; Molecular ProbesTM) and SSPN (affinity purified rabbit 18, 1:200). Polyclonal antibodies to endogenous mouse SSPN have been described previously (76). Primary antibodies were detected by biotinylated anti-rabbit (BA-1000, 1:500; Vector Labs), fluorescein anti-IGM (AP128F, 1:500, Chemicon) and biotinylated anti-mouse (BA-9200, 1:500; Vector Labs). Fluorescein (A-2001, 1:500; Vector Labs)- conjugated avidin D was used to detect biotinylated secondary antibodies. Both secondary and tertiary probes were diluted in PBS and incubated with sections for 1 h at room temperature. Sections were mounted in VECTASHIELD (Vector Labs) to prevent photobleaching. Sections were incubated with secondary and tertiary antibodies alone as a control for specificity. Antibody-stained sections were visualized using a fluorescent microscope (Axioplan 2; Carl Zeiss, Inc.) equipped with a Plan Neofluor 40× NA 1.3 oil differential interference contrast objective, and images were captured under identical conditions using the Axiovision Rel 4.5 software (Carl Zeiss, Inc.).

Histology
H&E staining was used for the visualization of centrally placed nuclei as described previously (77). Transverse quadriceps and diaphragm sections (8 μm) were acclimated to room temperature for 15 min before beginning the staining procedure. Slides were incubated in hematoxin for 5 min, washed in water for 2 min, incubated in eosin for 5 min and dehydrated in 70, 80, 90 and 100% ethanol. Sections were then incubated in xylene for 10 min and mounted in Permount. All supplies for this procedure were purchased from thermo Fisher Scientific. Van Geison staining (adapted from 78) was used to compare collagen infiltration around transverse muscle fibers. Slides were incubated for 5 min in Celestion Blue (206342, Sigma-Aldrich) diluted in 5% ammonium ferric sulfate (221260, Sigma-Aldrich), rinsed with distilled water, incubated for 5 min in hematoxylin, rinsed in distilled water, incubated for 5 min in Curtis stain and dehydrated in 70, 80, 90 and 100% ethanol. Sections were then incubated in xylene for 10 min and mounted in Permount. Oil Red staining was performed according to instructions in the NovaUltra Oil Red O Stain Kit (IW-3008, IHCWORLD). Images of quadriceps/diaphragms were captured using a Plan Neofluor 40× fluorescent microscope equipped with an Plan Neofluor 40× NA 1.3 oil differential interference contrast objective and the Axiovision Rel 4.5 software (Carl Zeiss, Inc.). The percentage of centrally nucleated fibers was assessed from three to four quadriceps/diaphragms of each genotype. The data are represented as an average percentage of the total number of fibers in each whole-muscle section.

Protein preparation from skeletal muscle
Total skeletal muscle was snap-frozen in liquid nitrogen and stored at −80°C. Tissues were ground to a fine powder using a mortar and pestle and then added to ice-cold radioimmunoprecipitation assay buffer (RIPA) (89901; Thermo Scientific) with phosphatase inhibitors (78420; Thermo Scientific) or DGC buffer (50 mM Tris–HCl, pH 7.8, 500 mM NaCl and 0.1% digitonin) containing fresh protease inhibitors (0.6 μg/ml peptatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.75 mM benzamidine, 0.2 mM PMSF, 5 μM calpain I and 5 μM calpeptin). Homogenates were rotated at 4°C for 1 h. Lysates were clarified by centrifugation at 15 000g for 20 min at 4°C, protein concentration was determined using the DC Protein Assay (Bio-Rad) and lysates were stored at −80°C.

Immunoblot analysis
Equal quantities (10 μg for sWGA and 60 μg for total protein) of protein samples were resolved on 4–20% Pierce precise protein gels (Thermo Scientific) by SDS–PAGE and transferred to nitrocellulose membranes (Millipore). An identical protein gel was stained with Coomassie blue stain to visualize levels of total protein. 5% BLOTTO (non-fat dry milk in TBS with 0.2% Tween-20) was used to block membranes for 30 min at room temperature and incubate in primary antibodies overnight at 4°C. Incubations were performed with the following primary antibodies: dystrophin (MANDYS1,
sWGA enrichment and sucrose gradient ultracentrifugation

Protein samples (7 mg) were incubated with 1.2 ml of succinylated WGA-conjugated agarose slurry (AL-1023S, Vector labs) and gently rotated overnight at 4°C. sWGA agarose was washed four times in 10 ml of cold DGC solubilization buffer (50 mM Tris–HCl, pH 7.8, 500 mM NaCl and 0.1% digitonin) containing fresh protease inhibitors (0.6 µg/ml pepstatin A, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.75 mM benzamidine, 0.2 mM PMSF, 5 µM calpain I and 5 µM calpeptin) to remove unbound proteins. Bound proteins were eluted with 0.3 M N-acetylglucosamine (sWGA) (Sigma-Aldrich) and concentrated using Centricron Ultrafiltration columns (Millipore) by centrifugation at 4000g for 75 min. Protein concentration was determined with the DC Protein Assay (Bio-Rad). Equal concentrations of eluates (10 µg) were resolved by SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted as described earlier. Samples of sWGA enrichments (200 µg) were loaded on a 5–20% sucrose gradient column. The column was prepared by adding 6 ml of 5% sucrose solution into an open-top polycarbonate centrifuge tube (7030, Seton), using a 14-gauge Hamilton syringe and underlaying with an equal volume of 20% sucrose solution. Gradients were mixed using the Gradient IP station (BioComp). Sucrose gradients were centrifuged overnight at 4°C at 35 000g in an ultra-centrifuge (Optima L-90K; Beckman Coulter), using an SW41 rotor (Beckman Coulter). Twelve 1 ml fractions were collected and concentrated at 14 000g at 4°C for 2.5 h, using Amicon Ultra-0.5 filters (UFC500324, Millipore). Each fraction (18 µl) was resolved by SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted as described in a previous section.

Membranes were prepared as described in sWGA enrichment of protein lysates and blocked with 5% BSA in laminin-binding buffer (LBB; 10 mM triethanolamine, 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, pH 7.6) followed by incubation of mouse ultrapure Engelbreth–Holm–Swarm laminin (354239, BD Biosciences) in LBB for 6 h at 4°C. Membranes were washed and incubated with rabbit anti-laminin (L9393, 1:5000; Sigma) overnight at 4°C followed by horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (GE Healthcare) at room temperature for 3 h. Blots were developed using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific).

Quantitative RT-PCR

The RNeasy Fibrous Tissue Kit (Qiagen, Valencia, CA, USA) was used according to the manufacturer’s instructions to isolate total RNA from quadriceps of 3–6-week-old mice. Dissected muscles were stored in RNA later (Ambion, Austin, TX, USA). RNA integrity was verified by visualization of RNA after electrophoresis through agarose. RNA concentrations were determined using a NanoDrop 1000 instrument (Thermo Scientific, Wilmington, DE, USA). Reverse transcription was performed using the Maxima™ First Strand cDNA Synthesis Kit (Fermentas, Inc.) with 3 µg of total RNA following the recommendations of the manufacturer. qRT-PCR was performed in triplicates on a TaqMan ABI PRISM 7900 (Applied Biosystems) according to the instructions in the Maxima™ SYBR Green Kit (Fermentas, Inc.). GAPDH mRNA was used as an internal control. PCR conditions and primers for Utr have been described previously (31, 79). Relative gene expression was quantified by the 2^(-ΔΔCT) method.

Specific force measurements

The contractile properties of the diaphragm, EDL and soleus muscles were measured as described previously (66). After mice were anesthetized with ketamine/xylazine (80 and 10 mg/kg body weight, respectively), muscles were dissected, removed and placed in a bath of Ringer’s solution gas-equilibrated with 95% O2/5% CO2. Sutures were attached to the central tendon and the rib in the diaphragm preparations.
Muscles were subjected to isolated mechanical measurements, using a previously described apparatus (Aurora Scientific, Ontario, Canada) (80). After determining optimum length (Lo) by supramaximal twitch stimulation, maximum isometric tetanus was measured. Upon completion of these measurements, muscles were then subjected to a series of five eccentric contractions with a 5 min rest between contractions. Average specific force of maximal isometric tetanus was displayed per genotype. Susceptibility to eccentric contraction-induced injury was measured by calculating the percent drop in force between the first and the last contraction. Other EDL muscles were stimulated once per second for 10 min (200 μs pulse, 100 Hz, 330 ms duration) in order to determine resistance to fatigue. Plots contain standard deviation, n-values and P-values calculated by performing an analysis of variance (ANOVA) with Bonferroni’s correction or Tukey’s multiple comparison tests to determine significance. Upon completion of analysis, muscles were rapidly frozen in OCT Quadriceps, EDL, soleus and tibialis anterior, and gastrocnemius muscles were dissected from the same mice and muscle weights were recorded. Additionally, body weight of each mouse was recorded prior to beginning the dissections. Data were analyzed by determining average muscle weight per genotype, calculating standard deviation and performing an ANOVA with Bonferroni’s correction or Tukey’s multiple comparison tests to determine significance.

Statistics
All statistics were analyzed using the one-way ANOVA with a Bonferroni correction or Tukey’s multiple comparison tests to determine differences between groups. n- and P-values are indicated on the plots. Statistical significance was accepted for P < 0.05.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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