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The potential of sarcospan in adhesion complex replacement therapeutics for the treatment of muscular dystrophy

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Abstract

Three adhesion complexes span the sarcolemma and facilitate critical connections between the extracellular matrix and the actin cytoskeleton: the dystrophin- and utrophin-glycoprotein complexes and α7β1 integrin. Loss of individual protein components results in a loss of the entire protein complex and muscular dystrophy. Muscular dystrophy is a progressive, lethal wasting disease characterized by repetitive cycles of myofiber degeneration and regeneration. Protein replacement therapy offers a promising approach for the treatment of muscular dystrophy. Recently, we demonstrated that sarcospan facilitates protein-protein interactions amongst the adhesion complexes and is an important therapeutic target. Here, we review current protein replacement strategies, discuss the potential benefits of sarcospan expression, and identify important experiments that must be addressed for sarcospan to move to the clinic.

Keywords

Duchenne; dystrophin; muscle; integrin; laminin-binding; *mdx*; muscular dystrophy; sarcolemma; sarcospan; utrophin

Introduction

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease caused by mutations in the dystrophin gene leading to a loss of the dystrophin-glycoprotein complex (DGC) at the sarcolemma [1]. DMD occurs in approximately 1 in 3,500 male children, leading to respiratory or cardiac failure in the second decade of life. In 1986, the gene encoding dystrophin was identified, and mutations in the *dystrophin* gene were determined to be responsible for DMD [2, 3]. While dystrophin was identified over 25 years ago, there is still no cure for the disease [2]. The N-terminus of dystrophin binds directly to

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filamentous actin (F-actin) in the cytoplasm of the myofiber and the C-terminus of dystrophin is associated with a group of proteins including neuronal nitric oxide synthase (nNOS), α-syntrophin, α-dystrobrevin, and β-dystroglycan (DG) [4, 5]. Dystrobrevin binds to the intermediate filament protein syncoilin, which provides a connection between desmin and the DGC that is thought to be important for maintaining mechanical strength and structural organization necessary for muscle contractions [6]. nNOS requires binding to both dystrophin and syntrophin for sarcolemmal localization and produces nitric oxide, which stimulates blood flow necessary to meet the metabolic demands of healthy muscle [7-11]. The DGC functions to stabilize the sarcolemma during muscle contractions by providing a critical connection between the extracellular matrix (ECM) and the intracellular actin cytoskeleton [12]. α-DG, a peripheral membrane protein, provides the connection between the transmembrane β-DG and laminin-211 in the ECM [13, 14]. The central mucin domain of α-DG is heavily glycosylated and the extent of glycosylation determines the affinity of DGC attachment to the ECM [5, 13-18]. The sarcoglycan-sarcospan (SG-SSPN) subcomplex stabilizes α-DG's association with β-DG at the cell surface [19-21]. Loss of the DGC in DMD renders the sarcolemma susceptible to membrane ruptures, which initiates the cycles of myofiber degeneration and regeneration characteristic of DMD [1, 12, 15, 22].

Many forms of muscular dystrophy result from a loss of muscle cell attachment to its surrounding ECM. In addition to the DGC, two adhesion complexes span the sarcolemma and facilitate this connection: the utrophin-glycoprotein complex (UGC) and α7β1 integrin. The UGC is homologous to the DGC, where utrophin replaces dystrophin [23, 24]. α7β1 integrin is the main heterodimeric integrin expressed in adult skeletal muscle [25-27]. Mutations in genes encoding the protein components of the DGC/UGC, α7β1 integrin, and ECM cause various forms of muscular dystrophy, which are classified based on the severity of disease and the muscle groups predominantly affected. Autosomal recessive limb-girdle muscular dystrophies (AR-LGMDs) encompass a wide array of genetic disorders and disease onset typically occurs between 10-30 years of age. Mutations in α-, β-, γ-, and δ-SG and mutations in glycosyltransferases resulting in the hypoglycosylation of α-DG cause AR-LGMD subtypes 2C-2F, 2K, and 2N-2O respectively [28-37]. Congenital muscular dystrophies (CMDs) are an autosomal recessive disease characterized by severe muscular dystrophy with onset occurring within the first year of life. Various forms of CMD are also caused by hypoglycosylation of α-DG due to mutations in glycosyltransferases as well as mutations in laminin α2, collagen 6, and α7 integrin [38-42]. Notably, patient mutations in SSPN and β1D integrin have not been identified [43-45].

There are many strategies currently being investigated for the treatment of muscular dystrophy. Several therapeutic approaches focus on adenoviral delivery of gene replacement therapy or exon skipping to produce a shortened, but functional dystrophin protein. This review will focus on therapeutic strategies to replace the DGC with compensatory adhesion complexes, UGC and α7β1 integrin. In particular, this review will discuss the potential of SSPN as a therapeutic target to upregulate both the UGC and α7β1 integrin. SSPN is a tetraspanin-like protein that was discovered as a core-component of the DGC [46]. Although SSPN has been shown to ameliorate the pathology of the *mdx* mouse model of DMD [47, 48], many questions remain to be answered before SSPN-related therapies are suitable for patients.

Restoring Myofiber Function with Compensatory Adhesion Complexes

The adhesion complexes responsible for stabilizing the myofiber membrane have distinct biochemical and functional properties, including sarcolemmal localization and ECM/actin connections in normal muscle. In muscle, the DGC is the most widely expressed adhesion complex, as it is found at all regions of the sarcolemma including the neuromuscular and

myotendinous junctions (NMJs and MTJs) [3, 49-52]. The UGC, a homologous complex to the DGC, is restricted to NMJ and MTJ regions of the sarcolemma, where utrophin replaces dystrophin [53, 54]. Although utrophin and dystrophin are structurally homologous, they bind to F-actin through distinct sites [55, 56] and only dystrophin contains nNOS binding sites [57]. It is thought that muscle ischemia occurs in DMD because nNOS is not anchored to the sarcolemmal membrane [57]. The UGC also differs from the DGC in the glycosylation of α-DG, which is a critical determinant of the binding specificity for ECM ligands [47, 58-60]. α7β1 integrins, which are also highly enriched within NMJ and MTJ structures and expressed at low levels at non-junctional regions of the sarcolemma, are the predominant integrin heterodimers expressed in adult muscle [25-27]. Integrins bind F-actin through a complex of proteins including integrin-linked kinase (ILK), PINCH, and α/β-parvin [61-63]. The ILK, PINCH, and parvin protein complex has been implicated in facilitating cell signaling through Akt/PKB, GSK3β/β-catenin, JNK, and α-Pix/Rac1 pathways [61, 62, 64]. ILK interacts specifically with β1 integrin and deletion of ILK causes muscular dystrophy that resembles the α7 integrin deficient mouse model [65]. Laminin-211 binds α7β1 integrin through laminin-type G domains (LG) 1-3, whereas binding to α-DG is mediated through LG1-3 and 4-5 [66-70]. Given that many differences exist between the three adhesion complexes, the extent to which UGC- and integrin-based therapeutics for DMD fully replace DGC function remains an unanswered question.

The concept of the UGC and integrins acting as compensatory adhesion complexes and their potential as therapeutic targets to replace the DGC arose from studies in the *mdx* mouse model of DMD. Muscle pathology in *mdx* muscle is less severe than that observed in boys with DMD and the *mdx* mouse maintains a fairly normal lifespan [71]. It was hypothesized that increased abundance of the UGC and α7β1 integrin at extra-junctional regions of the sarcolemma in *mdx* muscle may partially compensate for the loss of the DGC, resulting in moderate dystrophic pathology without affecting lifespan, which is in stark contrast with the severe dystrophic pathology and premature lethality observed in the DMD population. To experimentally address this hypothesis, *mdx*:utrophin-null and *mdx*:α7 integrin-null double knockout mice were created. The additional loss of utrophin or α7 integrin in *mdx* mice exacerbated dystrophic symptoms to more closely resemble DMD. Furthermore, lifespan was shortened to 20- and 4-weeks, respectively for utrophin-deficient and α7 integrin-deficient *mdx* mice [72-75]. Significantly, over-expression of either utrophin or β1D integrin in *mdx* muscle prevented muscular dystrophy, demonstrating that the UGC and integrins are capable of replacing the DGC in the mouse model of DMD [76, 77]. Mental retardation and brain abnormalities have been detected in about 30% of patients with DMD [78]. Utrophin and its smaller isoforms are expressed in the blood vessels in all brain regions, walls of the lateral ventricle, cortex, subiculum, thalamus, brain stem nuclei, superior colliculus, and deep cerebellar nuclei [79]. Although utrophin stabilizes the sarcolemma in *mdx* mice, utrophin was not upregulated in a compensatory fashion in the brain of *mdx* mice [79]. Arginine butyrate alleviated *mdx* muscle disease through utrophin upregulation and increased all utrophin isoforms in the brain of *mdx* mice, but did not rescue cognitive deficits observed in behavioral assays including exploration, emotional reactivity, and spatial and fear memories [79]. These results suggest that utrophin cannot fully compensate for the loss of dystrophin in all tissues. The ability of α7β1 integrin to ameliorate cognitive deficits in *mdx* mice has not been tested.

Although the UGC and α7β1 integrin do not fully replace the DGC, they remain excellent candidates for protein replacement therapies. The packaging limit of adeno-associated virus (AAV) capsids prevents the delivery of the most ideal protein for the treatment of DMD, the entire *dystrophin* gene [80]. Additionally, recent evidence that the expression of AAV delivered mini-dystrophin is circumvented by an immune response to dystrophin in patient clinical trials [81], strengthens the importance of UGC and α7β1 integrin protein

replacement therapies. A thorough investigation of the biochemical, structural, and functional differences between the UGC, α7β1 integrin, and the DGC may lead to the future development of combinatorial therapies designed to replace the DGC. The first goal is to determine if UGC- and α7β1 integrin-based therapies are safe and beneficial to patients with DMD. To date, only three therapies designed to replace the DGC with the UGC or integrins are near clinical trials. Two therapies designed to upregulate utrophin are currently in or near Phase 1 clinical trials: BMN195 (Summit plc) and biglycan (Trivorsan Pharmaceuticals). BMN195, a 5-(ethylsulfonyl)-2-(naphthalene-2-yl) benzodoxazole, was identified in a screen for small molecules, which upregulated utrophin mRNA 25% in human myoblasts, increased utrophin protein levels two-fold in DMD patient cells, and demonstrated efficacy in treatment of *mdx* mice [82]. Specifically, BMN195 reduced regeneration, inflammation, serum CK levels, and fibrosis, and prevented membrane damage due to eccentric contractions in treated *mdx* mice [82]. Although BMN195 was shown to be safe at all doses in a Phase 1 clinical trial by BioMarin Pharmaceuticals, it did not achieve plasma concentrations, even at the highest doses, required to increase utrophin expression[1]. Summit plc has since reformulated BMN195 to allow for better absorption and initiated Phase 1 clinical trials. The second utrophin upregulation therapy, biglycan, is an extracellular protein that is highly expressed, similar to utrophin, in regenerating and developing muscle [83, 84]. Injection of recombinant human biglycan protein improves muscle pathology in the *mdx* mouse by increasing cell surface expression of utrophin and γ-SG [85]. Furthermore, the inability of biglycan to ameliorate dystrophic pathology of the utrophin-deficient *mdx* mouse demonstrates the requirement of utrophin for biglycan therapies [85]. Biglycan is currently being developed for Phase 1 clinical trials by Trivorsan Pharmaceuticals. The only therapeutic designed to upregulate α7 integrin near Phase 1 clinical trials is laminin-111 protein. Laminin-111 is an ECM protein present in cardiac and skeletal muscles during embryonic development that is replaced by laminin-211 in adult muscle [69, 70]. Injection of Engelbreth-Holt-Swarm-derived purified natural mouse laminin-111 protein improves dystrophic pathology in the *mdx* and laminin α2-deficient dy^W mouse models [86-89] through the upregulation of both utrophin and integrins at the sarcolemma. Prothelia is currently developing laminin-111 for clinical trials and a Phase 1 trial will occur upon the completion of preclinical trials. Laminin-111 has the potential for therapeutic use in merosin-deficient congenital muscular dystrophy (MDC1A), DMD, and LGMD2I (mutations in fukutin-related protein (FKRP)). The outcome of these clinical trials may illuminate whether utrophin and integrin therapeutics will alleviate the symptoms of DMD (Figure 1).

Although introduction of recombinant laminin-111 protein was initially discovered to increase α7β1 integrin protein levels, it has also been shown to increase the levels of utrophin in *mdx* muscle [89]. Given that neither the over-expression of utrophin or α7β1 integrin fully rescue dystrophic symptoms, it would be interesting to determine whether the upregulation of both adhesion complexes would be more beneficial than targeting a single complex alone. Experiments to directly test UGC and integrin association have not been performed and numerous murine genetic experiments are often complicated with compensatory actions of other proteins. Migration of the three adhesion complexes in the same sucrose gradient fractions after biochemical isolation of DG bound proteins using succinylated wheat germ agglutinin (sWGA) lectin raises the interesting question of whether integrins and the UGC/DGC physically interact at the sarcolemma [90, 91]. If laminin-111 is successful in Phase 1 trials, the potential benefit of targeting both the UGC and α7β1 integrin may be addressed in DMD patients. Similar to laminin-111, Adam12 and SSPN also secondarily upregulate both the UGC and integrins at extra-junctional regions of the

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sarcolemma when over-expressed in *mdx* mice [47, 48, 92] (Figure 1). Adam12 is an active metalloproteinase that is expressed during muscle development and regeneration. The mechanism by which it promotes cell adhesion through the stabilization of the UGC and α7β1 integrin is currently unknown [93-95].

Recent studies have implicated a role for SSPN in facilitating interactions between the UGC and α7β1 integrin. SSPN, a tetraspanin-like protein, functions with the SGs to stabilize the association of α-DG with β-DG in the DGC [19, 20, 46]. Over-expression of threefold levels of SSPN ameliorates *mdx* muscle by increasing the levels of the UGC, α7β1 integrin, and laminin-binding to α-DG [47, 48]. Interestingly, the increase in laminin-binding to α-DG was not observed with 1.5-fold levels of SSPN over-expression in *mdx* mice, demonstrating that there is a minimum threshold of SSPN expression required for restoring normal ECM binding to DG [47]. To determine if laminin-binding to α-DG is required for SSPN-mediated amelioration of dystrophic symptoms, threefold SSPN transgenic mice were crossed with LARGE^{myd} mice. LARGE, like-acetylglucosaminyltransferase, is a glycosyltransferase responsible for elongating O-mannose glycans in the mucin domain of α-DG and mutations in LARGE abolish laminin-binding to α-DG [96]. Over-expression of SSPN does not ameliorate muscular dystrophy in the LARGE^{myd} mouse model of hypoglycosylated α-DG (MDC1D) despite increasing levels of the UGC at the sarcolemma, demonstrating that functionally glycosylated α-DG capable of binding to laminin is a requisite for SSPN-mediated amelioration of dystrophic pathology [47]. Recent genetic studies demonstrated that the combined loss of SSPN and α7 integrin results in extensive muscle pathology and decreased specific force production possibly due to decreased protein levels of the DGC and UGC in 4.5 month old diaphragm muscle [90]. These studies suggest that SSPN and integrins genetically interact and affect protein abundance of the DGC/UGC, but do not establish a direct interaction between these adhesion complexes. Investigation of the dependence of SSPN-mediated amelioration of *mdx* mice on α7 integrin and utrophin would clarify whether both adhesion complexes are essential for SSPN-based therapeutics. Interestingly, α7 integrin over-expression in *mdx*:utrophin-null mice causes a 10% reduction in regeneration, a 3-fold increase in mean survival age, and reduced severity of joint contractures [97]. These results suggest that α7 integrin reduces the severity of muscular dystrophy in *mdx* mice partially independent of utrophin, although the muscle pathology was not significantly reduced. It is possible that utrophin is required for the full rescue effect of α7 integrin in *mdx* muscle or that higher than 2-fold levels of α7 integrin are needed to rescue *mdx* muscle independently of utrophin. The ability of utrophin alone to rescue *mdx*:α7 integrin-null muscle has not been tested. Thus, additional evidence is needed to establish a direct SSPN-mediated interaction between the UGC and α7β1 integrin at the sarcolemma while the benefit of upregulating both adhesion complexes for the treatment of DMD remains undetermined.

Glycosylation Of α-DG and Amelioration of Muscular Dystrophy

Appropriate glycosylation of sarcolemmal proteins is integral for proper muscle function. Over fifty percent of all known CMDs result from hypoglycosylation of α-DG and therefore are known as dystroglycanopathies [98]. Mutations in thirteen genes are known to cause aberrant glycosylation of α-DG. These gene products include: protein O-mannosyl transferase-1 and -2 (POMT1 and POMT2), protein O-linked mannose β-1,2-N-acetylglucosaminyltransferase (POMGnT1), LARGE, fukutin, FKRP, β-1,3-*N*-acetylgalactosaminyltransferase 2 (B3GALNT2), Isoprenoid Synthase Domain Containing (ISPD), Dolichyl-phosphate-mannosyltransferase subunits -2 and -3 (DPM2 and 3), Dolichol Kinase (DOLK), Glycosyltransferase-like domain containing (GTDC2), and Transmembrane protein 5 (TMEM5) [36, 37, 39, 99-113]. POMT1/2 act collaboratively to add the initiating mannose to serine or threonine residues of the mucin domain of α-DG

[114, 115]. Classical mannose glycans are then elongated by the addition of a β-1,2-*N*-acetylglucosamine (β1,2GlcNAc) residue, while laminin-binding mannose glycans are elongated by the addition of a β-1,4-*N*-acetylglucosamine (β1,4GlcNAC) residue (Figure 2) [37, 99, 116]. It was recently proposed that B3GALNT2 may be the glycosyltransferase responsible for the addition of the GalNAc residue following β1,4GlcNAc addition to laminin-binding mannose glycans, however, this precise activity remains to be proven [108]. On laminin-binding glycan structures, LARGE creates the unique moiety required for binding of α-DG to laminin as well as other ECM components (Figure 2) [39, 117, 118]. Binding of α-DG to laminin has been shown to be dependent upon the xylosyl and glucuronyl transferase activities of LARGE [119]. Furthermore, knockout of LARGE activity causes a decrease in the molecular weight of α-DG [116]. Precise enzymatic functions of fukutin, FKRP, ISPD, GTDC2, and TMEM5 remain elusive; however, mutations in these genes lead to hypoglycosylation of α-DG and subsequent CMD phenotypes [100, 102-104, 107, 120]. DOLK and DPM-2/-3 are required for synthesis of the glycosylation precursors dolichol-phosphate and dolichol-phosphate-mannose respectively [121-123]; mutations in all three give rise to dystroglycanopathies as both glycosylation precursors are required for synthesis of N-glycans and O-mannosyl glycans [124, 125].

The dystroglycanopathies represent a diverse group of muscular dystrophies with broad phenotypic severities and wide ranging causative gene mutations. Walker-Warburg syndrome (WWS) cases result from the largest group of genetic mutations and can be attributed to mutations in POMT1, POMT2, LARGE, fukutin, FKRP, ISPD, β-1,3-*N*-acetylglucosaminyltransferase (B3GNT1), B3GALNT2, GTDC2, and TMEM5 [103, 107, 126, 127]. Patients presenting with muscle-eye-brain disease (MEB) represent a similarly heterogeneous population of phenotypic severities and genetic mutations. MEB has been reported to result from mutations in POMGnT1, fukutin and FKRP [37, 127, 128]. LGMD types 2K, N, M, I, and O result from mutations in POMT1, POMT2, fukutin, FKRP, and POMGnT1, respectively [28, 127-129]. CMD types 1C and 1D result from mutations in FKRP and LARGE, respectively [39, 130]. Furthermore, an insertion of a retrotransposon in the *fukutin* gene is known to cause Fukuyama congenital muscular dystrophy (FCMD) [102]. The genetic basis for the various dystroglycanopathies is varied and the resulting phenotypes are overlapping, which reinforces the need for a thorough understanding of the connection between specific genetic mutations and resulting biochemical dysfunctions that underlie or cause muscular dystrophy.

While DMD results from mutations in dystrophin, it is noteworthy that significant changes in glycosylation have been reported in the mouse model for DMD. The lectin *Wisteria floribunda* agglutinin (WFA) recognizes terminal β1,4 GalNAc residues of α-DG in skeletal muscle [60, 131] and binding is normally restricted to the NMJ and MTJ in wild-type myofibers [59]. However, WFA binds to the extrasynaptic sarcolemma in addition to the NMJ on *mdx* muscle cryosections [47, 58]. This redistribution of WFA staining to the extrasynaptic sarcolemma is concomitant with the redistribution of utrophin and the associated UGC in *mdx* mouse muscle.

Over-expression of both full length and truncated utrophin rescues the dystrophic phenotype in *mdx* mice [77, 132-135]. As WFA preferentially binds to α-DG in the UGC, increasing GalNAc modification of α-DG has been one approach to increase extrasynaptic utilization of utrophin as a potential therapy for DMD. The over-expression of terminal β-GalNAc glycosyltransferase *galgt2* in wild-type mice has been shown to increase levels of UGC proteins, laminin-binding and reactivity of α-DG to WFA [59, 60]. Similar results were obtained from *galgt2* over-expression in *mdx* mice, where rescue of laminin-binding and the dystrophic phenotype were additionally noted [59, 60]. Interestingly, transgenic over-expression of SSPN also increases the WFA reactivity of α-DG in *mdx* and LARGE^{myd}

muscle while loss of SSPN reduces UGC levels and the reactivity of α-DG with WFA [47]. Importantly, no mitigation of dystrophic pathology or rescue of laminin-binding is observed in LARGE^{myd} mice over-expressing SSPN [47]. These studies are significant as they demonstrate that laminin-binding is required for SSPN-mediated amelioration of dystrophic pathology and that the glycans detected by increasing WFA reactivity are distinct from the structure created by LARGE activity. It has been proposed that SSPN over-expression mediates changes in glycosylation via increased galgt2 activity [47]; however, this mechanism requires further validation through the creation of galgt2-deficient *mdx* SSPN transgenic mice. Interestingly, over-expression of *galgt2* has also been demonstrated to be effective in ameliorating the dystrophic phenotype in mouse models of CMD 1A (dy^{W}) and α-SG-deficient LGMD 2D [136, 137]. It is noteworthy, however, that the over-expression of a neuronal homolog *galgt1*, which adds terminal β-GalNAc residues distinctly to ganglioside glycolipids, caused muscle pathology including decreased myofiber diameter and increased central nucleation in wild-type mice [138]. These stark differences resulting from the over-expression of either *galgt2* or *galgt1* demonstrate the tissue and acceptor substrate specificity of glycosylation and reinforce the importance of thoroughly evaluating the effects of manipulating glycosylation.

While genetic over-expression of a glycosyltransferase provides one potential therapeutic approach for manipulating glycosylation of sarcolemmal glycoproteins, pharmacologic approaches also provide potential therapeutics. Using high throughput screening, the small molecule lobeline was identified as a pharmacological treatment that altered C2C12 glycosylation *in vitro* [139]. Compounds from the Prestwick library of about 1200 FDA approved small molecules were added to myoblasts in differentiation media and changes in glycosylation were measured after two days of treatment [139]. Specifically, binding of terminal β-GalNAc modifications as detected by WFA binding increased in C2C12 cells as well as isolated wild-type and *mdx* myoblasts following treatment with lobeline during differentiation [139]. Lobeline treatment increased abundance of UGC proteins and laminin-binding in wild-type and *mdx* primary cell cultures [139]; these results are similar to changes resulting from *galgt2* over-expression. It is noteworthy that the increase in WFA binding following lobeline treatment was dependent upon complex N-glycans and not O-mannose glycans such as those required for laminin-binding [139]. Furthermore, deoxymannojirimycin (DMNJ) inhibition of complex N-glycans necessary for WFA binding caused a decrease in laminin-binding as detected by laminin overlay [139]. While lobeline was first FDA approved for smoking cessation, *in vitro* studies have shown that lobeline may potentially act as a protein folding chaperone or nicotinic antagonist through the dopamine or vesicular monoamine transporter [140-143]. In the C2C12 studies, the molecular mechanism by which lobeline increased WFA binding *in vitro* was not determined. While the ability of lobeline to alter muscle glycosylation *in vivo* remains to be demonstrated, these studies represent strong proof of principle for the pharmacologic manipulation of sarcolemmal protein glycosylation as a potential therapeutic.

Protein Aggregation and Amelioration in Muscular Dystrophies

The *mdx* mouse contains a point mutation in exon 23 [144], resulting in a premature termination codon that is predicted to result in a 115 kDa protein [145]. It has previously been shown that dystrophin mRNA levels are decreased in skeletal muscle, cardiac muscle, and brains of three *mdx* mouse strains [146]. However, dystrophin mRNA levels vary widely, depending on the causative mutation in human DMD and Becker muscular dystrophy (BMD) patients [147]. It was previously thought that the 115 kDa dystrophin protein is rapidly degraded after synthesis in *mdx* muscle, but studies suggest that truncated dystrophin may be expressed at the sarcolemma [148-150] or retained in the ER/Golgi [47].

A 70-80 kDa dystrophin reactive to a C-terminal antibody was found in a group of Japanese Spitz dogs displaying progressive Duchenne-like muscular dystrophy [149]. These dogs displayed exercise intolerance, an abnormal gait, and pain upon handling of muscles beginning at 10 to 12 weeks of age. The symptoms worsened over time, suggesting that the 70-80 kDa dystrophin was unable to restore sarcolemmal stability. In a separate study, immunohistochemical assays revealed expression of truncated dystrophin in myotubes isolated from three 12-week old aborted fetuses at risk for DMD, suggesting that mutant dystrophin is synthesized in human DMD patients [148]. Recently, the truncated form of dystrophin was detected within the ER/Golgi compartments of *mdx* mice [47], suggesting that the 115 kDa dystrophin may be retained in the ER/Golgi compartments.

The mutation in *mdx* mice leads to the loss of the β-DG binding domain, leaving only the actin binding domains in the dystrophin fragment [145]. The N-terminus of dystrophin binds directly to F-actin in the cytoplasm of the myofiber, while the C-terminus of dystrophin contains cysteine-rich domains that bind the C-terminus of β-DG [4, 151]. Recent studies utilizing truncated dystrophin have provided more insight into which segments of the protein are required for sarcolemmal stability. One study utilized rAAV6-microdystrophin to evaluate the role of the actin-binding domains in sarcolemmal stabilization [152]. Four months after injection into the tibialis anterior, muscles treated with micro-dystrophins lacking actin-binding domains displayed decreased specific force and a decreased ability to protect against contraction-induced injury [152], indicating the importance of an intact actin-binding domain in maintaining sarcolemmal stability.

Other studies have analyzed different isoforms of dystrophin. The dystrophin isoform Dp116 is expressed in Schwann cells within the peripheral nervous system [153]. This isoform lacks the actin-binding domains while retaining the complete dystroglycan-binding domain [153]. Although expression of Dp116 in *mdx*:utrophin-null mice increased muscle mass, life span, and maximal force, the specific force and histopathology did not improve [153]. It is hypothesized that Dp116, or the dystroglycan binding domain, attempts to stabilize the DGC at the sarcolemma through other interactions with the cytoskeleton, although specifics have yet to be elucidated [153]. The expression of a non-muscle isoform of dystrophin, Dp71, which lacks N-terminal actin-binding domains and spectrin-like repeats, restores the DGC in muscle, suggesting that the β-DG binding domain is required for assembly of the DGC [154]. Thus, it would be interesting to examine other mice with C-terminal mutations in dystrophin. Such experiments would assess the ability of truncated dystrophin to be incorporated and expressed with the DGC at the sarcolemma and restore functionality of dystrophic muscle. This provides an exciting opportunity to examine ways to express fragmented dystrophin at the sarcolemma to improve dystrophic pathology. Other studies involving protein retention within cellular compartments in different muscular dystrophies may provide insights into the therapeutic translation of these findings.

Studies in FCMD, which is caused by mutations in *fukutin* [102], have provided insight into the importance of protein processing within the ER/Golgi. The exact role of fukutin remains unclear, but mutations in *fukutin* lead to abnormal glycosylation of α-DG [155], a reduction in laminin-binding activity [155], and the mislocalization and retention of fukutin in the ER [156]. A recent study analyzed thirteen missense *fukutin* constructs in C2C12 cells and discovered that four were mislocalized to the ER. To understand whether mutant fukutin still leads to the production of functionally glycosylated α-DG, site-directed mutagenesis was used to generate mutants for transfection into fukutin-null mouse embryonic stem cells [156]. The expression of the four missense fukutin mutants restored α-DG reactivity against the IIH6C4 antibody and laminin-binding to α-DG, suggesting α-DG is functionally glycosylated with the expression of mutant fukutin [156]. Using treatments to improve protein folding, including curcumin, a molecule isolated from the spice turmeric, and low

temperature culturing conditions, ER retention of fukutin was corrected in the four mutants shown to be mislocalized in the ER. In order to elucidate the reasons for aberrant fukutin trafficking, brefeldin A was used to examine anterograde transport and nocodazole was used to examine retrograde transport [156]. After incubation with brefeldin A, the fukutin mutants remain accumulated in the ER [156]. The results suggest that while mutant fukutin proteins are processed in the ER, they are unable to be transported to the Golgi through the anterograde pathway, providing valuable insight into how the protein trafficking process is affected upon the mislocalization of a mutant protein. Thus, it is possible that truncated dystrophin may be retained within the ER/Golgi compartments and is not transported to the cell surface in *mdx* muscle. It will be important for future studies to utilize a similar strategy to elucidate the effects of 115 kDa dystrophin on retrograde and anterograde transport in *mdx* cells. Additionally, treatment with pharmacological agents, such as curcumin or exposure to low temperature, may potentially alleviate accumulation of truncated dystrophin in the ER/Golgi compartments and improve the transport of DGC and UGC components to the sarcolemmal membrane.

Curcumin was found to target the NF-κB pathway [157] and has been used to treat dystrophic muscle. The activation of NF-κB, which is involved in the modulation of immune responses and regulation of myogenesis, is increased in DMD patient muscle [158]. Intraperitoneal administration of curcumin inhibits NF-κB activation and reduces dystrophic pathology in *mdx* mice [159]. However, a different study reports that curcumin treatment is unable to inhibit the NF-κB pathway and does not improve specific force in the diaphragm muscle of *mdx* mice [160]. Future studies examining the effect of curcumin on the accumulation of truncated dystrophin in the ER/Golgi will be important in determining what role, if any, it plays in pathogenesis.

Protein mislocalization has also been implicated in LGMDs 2C-F pathologies caused by mutations in the SG genes [161]. The severity of pathology in LGMDs 2C-F and cellular fate of the affected SG varies according to the mutation [161]. According to data compiled from the Leiden University mutation database, the most common mutations are α-p.R77C, β-p.S114F, and γ-p.C283Y, which cause a mild to severe phenotype [161]. These data led to an examination of the noted point mutations and their effect on intracellular fate and expression of the SGs at the cell surface. Following treatment with kifunensine, an α-mannosidase I inhibitor that prevents ER-associated degradation [162], the expression of sarcoglycans with mild mutations thought to cause the least amount of structural modification was restored at the cell surface. Future studies manipulating ER quality control in wild-type and *mdx* cells might provide more information on how changes in the ER can affect DGC and UGC expression at the cell surface.

Heat Shock Proteins and Proteasome Inhibition in the Treatment of Muscular Dystrophy

Heat shock proteins (Hsp) are induced to combat cellular stress when organisms are under environmental strains such as heat, or during disease and infection [163]. Analysis of skeletal muscle from young DMD patients revealed an induction of Hsp72 and Hsp65 in hypercontracted fibers and Hsp90 in regenerating muscle [164]. Hsp72 is also increased at the mRNA level following electrical stimulation for tetanic contractions in isolated single skeletal muscle fibers from *Xenopus laevis* [165] and after high-intensity exercise in normal Wistar rats [166], suggesting a role for Hsp72 in protection against muscle stress. Treatment of *mdx* and utrophin-deficient *mdx* mice with BGP-15, a pharmacologic inducer of Hsp72, showed that an increase in Hsp72 expression improved SERCA function and dystrophic pathology, decreased kyphosis, and ultimately extended lifespan [167]. Targeting Hsp72 to improve protein folding and quality control during cellular stress and disease progression

provides a possible avenue to improve muscle function while prolonging the lifespan of patients with muscular dystrophy.

Ubiquitin is an important component in proteasome regulation and can direct proteins towards degradation [168]. Ubiquitin was also found to be elevated in hypercontracted, regenerating, and necrotic myofibers in the skeletal muscle of young DMD patients [164]. The increase in ubiquitin protein may suggest misregulation of the ubiquitin-proteasome pathway as well as an increase in protein degradation in DMD patients, which may contribute to the pathogenesis of DMD. Recent studies attempting to ameliorate dystrophic pathology using proteasome inhibitors have produced mixed results. Velcade and MLN273, two FDA-approved proteasome inhibitors, were injected into the gastrocnemius muscle of *mdx* mice [169]. A truncated 97 kDa dystrophin product, as well as an increase in α-DG, β-DG, and α-SG, were detected using immunoblotting and immunohistochemistry 24 hours post treatment [169]. Similarly, another proteasome inhibitor, MG-132, rescued the membrane localization of dystrophin, α-DG, β-DG, and α-SG [170]. However, physiological functionality was not examined and experiments such as grip strength and force production were not performed. This information is critical for the pursuit of such pharmacological agents as treatments for DMD (Fig. 3A).

Although some drugs such as Velcade and MLN273 have shown promise for the attenuation of dystrophic pathology in *mdx* mice, other studies have described conflicting results. In a separate study, long-term administration of a MG-132 failed to restore dystrophin expression in *mdx* muscle and ultimately increased susceptibility to contraction-induced damage [171]. The effects of proteasome inhibitors on grip strength, force production, and DGC protein expression need to be analyzed more rigorously to better determine their efficacy in improving muscle function and dystrophic pathology.

A Possible Function for Sarcospan as a Chaperone Protein

New data suggests a role for SSPN in protein trafficking to the sarcolemma. A recent study isolated ER/Golgi membranes from *mdx* muscle and immunoblots revealed an increase in α-DG and utrophin compared to wild-type muscle [47]. This suggests a possible compensatory mechanism is at play, whereby the system attempts to utilize utrophin upon the loss of dystrophin (Fig. 3B). Interestingly, SSPN transgenic *mdx* mice revealed decreased levels of WFA reactive α-DG and utrophin in ER/Golgi preparations, while immunofluorescence assays showed an increased abundance of these proteins at the sarcolemma [47]. SSPN over-expression increased WFA binding 1.8-fold per α-DG molecule as detected by WFA overlay [47]. Increased extrasynaptic binding of WFA may be the result of general chaperone trafficking of glycoprotein complexes by SSPN. However, the increase in WFA-reactive glycosylation per α-DG protein demonstrates a role for SSPN in specific changes in glycosylation of α-DG. This modification in α-DG glycosylation may result from downstream transcriptional effects of increased Akt signaling [47] or could additionally be a more direct result of chaperone activities of SSPN in trafficking. SSPN might act directly as a chaperone to stabilize α-DG during ER/Golgi trafficking and addition of glycans resulting in increased glycan modification per α-DG molecule. Importantly, over-expression of SSPN drives changes in glycosylation of α-DG, which aid in the amelioration of *mdx* pathology [47]. These studies highlight a promising role for SSPN as a therapeutic and it would be interesting to examine SSPN over-expression in dystroglycanopathy mouse models where direct manipulation of glycosylation ameliorates dystrophic pathology [136, 137]. SSPN may possess chaperone-like functions, and the over-expression of SSPN may improve overall protein folding and quality control as well as transport to the cell surface.

How to Achieve Forced over-Expression of SSPN

Membrane proteins are synthesized by ribosomes on the ER and disulfide bonds are synthesized and rearranged in the ER lumen [172]. Since SSPN is an integral membrane protein with disulfide bonds requiring processing through the ER, it most likely cannot be administered systemically. The small size of SSPN makes it an excellent gene to be delivered through AAV. AAV delivery of α-SG in α-SG-deficient patients and γ-SG in γ-SG-deficient patients resulted in no adverse events, demonstrating that intramuscular AAV delivery is likely to be safe in adult patients [173, 174]. The only patients that did not express SG following AAV delivery had pre-existing immunity to the AAV serotype used, demonstrating the need for pre-screening of AAV serotypes [173, 174]. However, there are many challenges to be overcome before systemic delivery of AAV is feasible. Systemic delivery of therapeutics will be required for amelioration of fatal dystrophic symptoms in the diaphragm and heart muscles. Surprisingly, a T-cell mediated immune reaction against dystrophin prevented the expression of mini-dystrophin following AAV delivery in DMD patients [81]. The precise reason for the varied T-cell immune responses (against mini-dystrophin and against self revertant dystrophin) in the 6 trial patients is unknown. These results warrant caution in future AAV mini/micro-dystrophin and exon skipping trials. Importantly, immune responses should not be a problem in secondary approaches to upregulate utrophin or α7β1 integrin for protein replacement therapeutics. A drug screen for compounds that upregulate SSPN offers an additional approach to AAV-mediated SSPN therapy. A similar approach led to the discovery of BMN195 for utrophin upregulation and is currently in Phase 1 clinical trials.

Unanswered Questions for SSPN-Based Therapeutics

Over-expression of SSPN under the human skeletal actin promoter results in a 60% reduction in regeneration in the *mdx* mouse model of DMD by replacing the DGC with the UGC and α7β1 integrin [47, 48]. As with most therapeutic targets, there are many questions that remain to be answered for SSPN-based therapeutics. For SSPN to become a viable therapeutic for the treatment of DMD, the following questions remain to be addressed:

1. *Does SSPN delivery with AAV prevent dystrophic pathology in the mdx mouse model?* This is an important question that needs to be answered, as it will determine the delivery system used for SSPN-based therapeutics. It would also be useful to further determine the feasibility of AAV delivery of SSPN in the golden retriever model of DMD (GRMD), as the dog model provides a more realistic clinical model for systemic delivery.
2. *Does SSPN eliminate/reduce dystrophic pathology in the heart and diaphragm?* The human skeletal actin promoter is not highly expressed in the diaphragm or heart muscles. Since death occurs from respiratory and/or cardiac failure in DMD patients, an ideal therapeutic target should prevent dystrophic pathology in the heart and diaphragm. This question can be addressed with systemic delivery of AAV6-SSPN in the *mdx* mouse model. AAV6 has been shown to infect the heart and the diaphragm [175, 176].
3. *Can SSPN reverse or prevent dystrophic pathology after the onset of dystrophy?* The human skeletal actin promoter is turned on early in muscle development. SSPN is likely preventing the onset of dystrophic pathology rather than reversing pathology. Boys with DMD are often diagnosed with the disease well after the onset of dystrophic pathology, so effective treatments should reverse or halt pathology already in progress. To determine if SSPN is effective after the onset of disease, a murine inducible transgenic system could be engineered to turn SSPN

expression on later in life. Intramuscular or systemic injection of AAV-SSPN after the onset of pathology in *mdx* mice will also address this question.

4. *Is widespread expression of SSPN safe?* The promoter used in murine studies of SSPN over-expression in the *mdx* mouse model restricted SSPN expression to striated skeletal muscle [177]. An effective treatment in patients with DMD would require systemic delivery of SSPN in order to target the heart and diaphragm. It is important to determine whether long-term systemic delivery of SSPN is safe. This question can also be addressed with systemic delivery of AAV-SSPN in *mdx* mice. Since AAV infection of particular tissues depends on the AAV serotype, it would be important to test SSPN delivery with the serotypes approved for use in patients.

5. *Does SSPN upregulate the UGC and integrins in human myoblasts?* Many recent treatment strategies have been tested in DMD myoblasts, including BMN195 and dantrolene [82, 178]. The ability to demonstrate that a therapy acts through similar targets in human cells offers a nice proof-of-principle for the approach being tested.

6. *Which patient populations will benefit from SSPN-based therapeutics?* It is important when developing therapies for muscular dystrophy to determine how many different muscle diseases will benefit from the treatment. This will allow for the design and recruitment of appropriate patient populations for clinical trials. SSPN is effective in ameliorating pathology in the *mdx* model of DMD [47, 48]. SSPN did not reduce dystrophic pathology in the LARGE^{myd} model of hypoglycosylation of α-DG (MDC1D) [47]. It would be interesting to determine if SSPN can ameliorate various limb-girdle and congenital muscular dystrophies.

Conclusions

In the *mdx* model of DMD, the SSPN transgene ameliorates dystrophic pathology by increasing the UGC and α7β1 integrin extra-synaptic sarcolemma, the synapse specific GalNAc glycosylation of α-DG, and the transport of utrophin and α-DG from the ER/Golgi to the cell surface [47, 48, 179]. These studies raise important questions about the mechanisms by which SSPN over-expression accomplishes these observed effects. Biochemical evidence that SSPN is a component of both the DGC and UGC complexes and genetic analysis of mice lacking both SSPN and α7 integrin have led to the proposal that SSPN stabilizes the UGC and integrins through direct interactions at the sarcolemma [46, 47, 90, 179]. More experiments are needed to address the requirement of utrophin and α7 integrin for SSPN-mediated amelioration of *mdx* muscle, as well as the possibility that the UGC and α7β1 integrin function synergistically at the sarcolemma. Additionally, SSPN may act either directly or indirectly as a chaperone protein to facilitate the efficient assembly and export of the UGC to the cell surface [47]; however, more studies are needed to elucidate the role of SSPN in the ER/Golgi. Over-expression of constitutively active Akt signaling has been shown to be beneficial in *mdx* muscle by increasing the UGC and integrins at the sarcolemma, reducing membrane damage, and improving the force generating capacity of muscle [180, 181]. SSPN-mediated amelioration of *mdx* muscle results in a similar increase in active Akt signaling and downstream muscle growth pathways [47]. Many therapeutic approaches for DMD concentrate on a single target. Although experimental strategies for the treatment of DMD have been developed for over 25 years, steroids remain the only approved drugs to slow the progression of the disease. Thus, the best treatment strategy remains an open question. SSPN is unique because it incorporates several beneficial therapeutic targets into a single protein that is small and easily packaged in AAV delivery systems. SSPN is also ubiquitously expressed in other tissues throughout the body so systemic delivery and immune response should not be an issue [46]. We are currently experimentally addressing the unanswered questions for SSPN therapeutics.

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Abbreviations

AAV	adeno-associated virus
AR-LGMD	autosomal recessive limb-girdle muscular dystrophy
B3GALNT2	β-1,3-*N*-acetylgalactosaminyltransferase 2
B3GNT1	β-1,3-*N*-acetylglucosaminyltransferase
BMD	Becker muscular dystrophy
CMD	congenital muscular dystrophy
DMNJ	deoxymannojirimycin
DG	dystroglycan
DGC	dystrophin-glycoprotein complex
DMD	Duchenne muscular dystrophy
DOLK	Dolichol Kinase
DPM2/3	Dolichyl-phosphate-mannosyltransferase subunits -2 and -3
ECM	extracellular matrix
ER	endoplasmic reticulum
F-actin	filamentous actin
FCMD	Fukuyama congenital muscular dystrophy
FKRP	fukutin-related protein
GRMD	golden retriever muscular dystrophy
GTDC2	Glycosyltransferase-like domain containing
Hsp	heat shock protein
ILK	integrin-linked kinase
ISPD	isoprenoid synthase domain containing
LARGE	like-acetylglucosaminyltransferase
MDC1A	merosin-deficient congenital muscular dystrophy 1A
MEB	muscle-eye-brain disease
MTJ	myotendinous junction
NMJ	neuromuscular junction
nNOS	neuronal nitric oxide synthase
POMT	protein O-mannosyl transferase
POMGnT1	protein O-linked mannose β-1,2-acetylglucosaminyltransferase

SG	sarcoglycan
SSPN	sarcospan
sWGA	succinylated wheat germ agglutinin
TMEM5	Transmembrane protein 5
UGC	utrophin-glycoprotein complex
Utr	utrophin
WFA	*Wisteria floribunda* agglutinin

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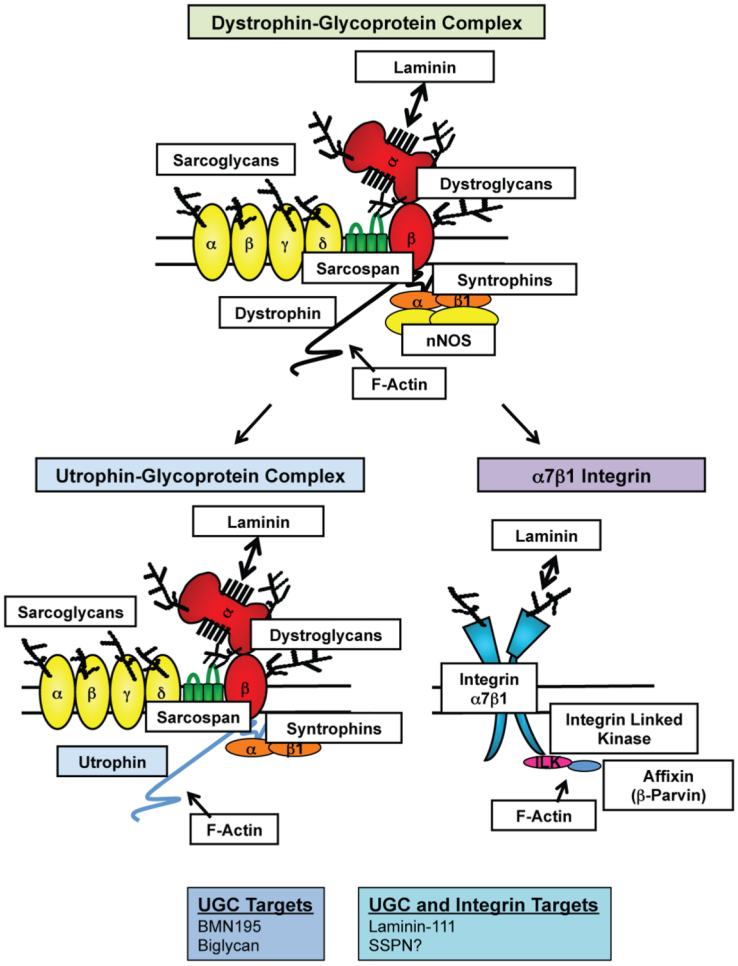

Figure 1. UGC- and α7β1 integrin-mediated replacement therapy for the DGC in DMD

The DGC, UGC, and α7β1 integrins function to prevent contraction-induced damage of the sarcolemma by maintaining connections between the actin cytoskeleton and ECM. The DGC is composed of dystrophin, the dystroglycans (α- and β-DG), the sarcoglycans (α-, β-, γ-, and δ-SG), sarcospan (SSPN), and the syntrophins (α- and β-subunits). Neuronal nitric oxide synthase (nNOS) requires dystrophin and syntrophin to be anchored to the sarcolemmal membrane, where it is thought to function in preventing functional muscle ischemia. The UGC is homologous to the DGC, where utrophin replaces dystrophin. However, many differences exist between the UGC and DGC, including the glycosylation of α-DG, the domains in which actin binding occurs, and the lack of nNOS binding sites on utrophin. α7β1 integrin differs from the UGC/DGC in the globular domains by which laminin binds and the presence of adaptor proteins that facilitate actin binding, including ILK and β-parvin. BMN195 and biglycan are two utrophin upregulation therapeutics that are near/in clinical trials and laminin 111 is the only dual (utrophin and integrin) target therapy that is near clinical trials. This review proposes that AAV delivery of SSPN should be considered as an additional dual target therapy.

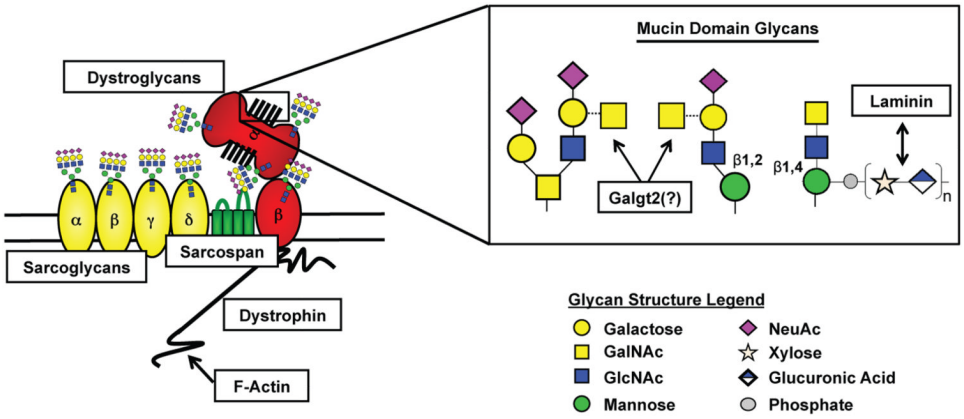

Figure 2. Glycosylation of the DGC

Known and putative sites of glycosylation of DGC component proteins are depicted. Inset of laminin-binding glycan is provided along with potential site of Galgt2 modification. Colored symbols used to represent glycan structures are in accordance with the guidelines outlined by the Consortium of Functional Glycomics.

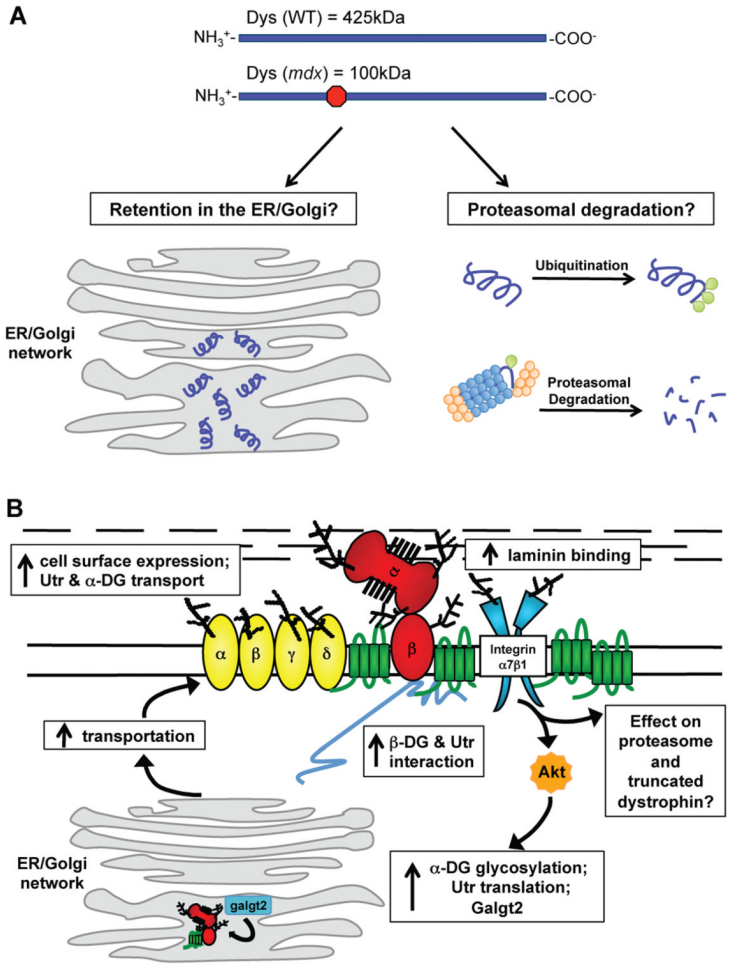

Figure 3. Effects of SSPN over-expression in *mdx* mice on the cell surface protein expression and protein processing and possible outcomes of truncated dystrophin within the cell

(A) Wild-type mice express full-length dystrophin, which is 425 kDa. The *mdx* mutation leads to a premature stop codon that results in a truncated dystrophin of 100 kDa. Dystrophin is depicted in purple, the proteasome is blue and orange, and ubiquitin is green. Truncated dystrophin can be retained in the ER/Golgi. Alternatively, truncated dystrophin is ubiquitinated and sent to the proteasomal degradation pathway. (B) The over-expression of SSPN in *mdx* muscle leads to molecular events resulting in the restoration of laminin-binding and rescue of *mdx* pathology. SSPN activates Akt, which leads to an increase in utrophin and integrins. Galgt2, one enzyme responsible for GalNAc modification of α-DG, is also increased in isolated ER/Golgi membranes. SSPN also improves utrophin-DG transportation to the sarcolemma while simultaneously restoring laminin-binding and membrane stability. SSPN's effect on the trafficking of truncated dystrophin and proteasomal degradation is still unknown. DGs (red), SGs (yellow), SSPN (green), integrins (blue), and Akt (orange) are shown. Utrophin (Utr) is depicted in gray.