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# The Dystrophin Complex: structure, function and implications for therapy

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## Abstract

The dystrophin complex stabilizes the plasma membrane of striated muscle cells. Loss of function mutations in the genes encoding dystrophin, or the associated proteins, triggers instability of the plasma membrane and myofiber loss. Mutations in dystrophin have been extensively cataloged providing remarkable structure-function correlation between predicted protein structure and clinical outcomes. These data have highlighted dystrophin regions necessary for in vivo function and fueled the design of viral vectors and now, exon skipping approaches for use in dystrophin restoration therapies. However, dystrophin restoration is likely more complex, owing to the role of the dystrophin complex as a broad cytoskeletal integrator. This review will focus on dystrophin restoration, with emphasis on the regions of dystrophin essential for interacting with its associated proteins and discuss the structural implications of these approaches.

### Keywords

muscular dystrophy; dystrophin; spectrin repeat; exon skipping; sarcoglycan; sarcolemma

## INTRODUCTION

Muscular dystrophy is a collection of inherited diseases characterized by skeletal muscle weakness and degeneration. Muscular dystrophies are progressive disorders because over time healthy muscle fibers are lost and replaced by fibrosis and fat, making muscle tissues less able to generate force for everyday activity. As muscle wasting ensues, patients experience weakness, although muscle groups may be targeted differently in specific forms of muscular dystrophy. Respiratory failure, resulting from the weakening of breathing muscles, may limit lifespan in muscular dystrophy unless mechanical support is instituted. In some forms of muscular dystrophy, the heart is also affected resulting in cardiac complications including heart failure and irregular heart rhythms.

Duchenne muscular dystrophy (DMD) is one of the most common forms of muscular dystrophy. DMD is caused by recessive mutations in the dystrophin gene on X chromosome, affecting 1 in 3,500 to 5,000 newborn males worldwide (82). Boys with DMD show signs of

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muscle weakness early in childhood, typically between 2 and 7 years of age, and often lose ambulation around the time of puberty. DMD boys may have delayed development of motor skills such as sitting, walking and talking. Becker muscular dystrophy (BMD) is also caused by mutations in the *DMD* gene that encodes dystrophin. Individuals with BMD share similar signs and symptoms with DMD boys but with later onset and more varied time course. Like DMD, the heart can be affected in BMD.

The dystrophin gene is the largest known human gene, containing 79 exons and spanning > 2,200 kb, roughly 0.1% of the whole genome (96). The most common mutation responsible for DMD and BMD is a deletion spanning one or multiple exons. Such deletions account for 60–70% of all DMD cases and 80~85% BMD cases (58, 147). Point mutations are responsible for around 26% of DMD cases and 13% BMD cases. Exonic duplications account for 10 to 15% of all DMD cases and 5% to 10% BMD cases. Subexonic insertions, deletions, splice mutations and missense mutations account for the rest of the cases. DMD is associated with mutations that disrupt the protein's reading frame causing premature stop codons. These mutated transcripts are susceptible to nonsense mediate decay, and the carboxy-terminal truncated protein products are also unstable and subject to degradation, leaving little or no protein produced in cells. In contrast, BMD patients usually have in-frame deletions that maintain the correct reading frame. Furthermore, nonsense mutations have been associated with both BMD and DMD. However, nonsense mutations associated with BMD are more prone to induce exon skipping than those found in DMD (59). The resulting protein products in BMD are internally truncated and expressed at lower levels than normal muscle. However, these internally truncated proteins are expressed at higher levels than in DMD and remain partially functional. Within one BMD affected family, three males carrying the same nonsense mutation in exon 29 displayed phenotypes from severe, mild to asymptomatic. This nonsense mutation is located in an exon recognition sequence in exon 29 and induces partial skipping of exon 29, producing an internally truncated dystrophin. A considerable amount of this alternatively spliced protein product was detected in the muscle tissues from both the mild and the asymptomatic patients (62). Recent studies on BMD patients have showed that while patients with less than 10% dystrophin all show severe disease cause, the correlation between dystrophin level and clinical outcomes is less clear and seems to be more dependent on the type of the truncated dystrophin when dystrophin level is above 10% (10, 149). For example, one study examining genotype-phenotype correlation showed that some in-frame deletions result in earlier onset of cardiomyopathy than others, despite similar expression level of dystrophin protein (91).

Current approaches for restoring dystrophin rely on viral-mediated restoration or exon-skipping, both of which require a detailed understanding of structure-function analysis of the DMD gene and dystrophin protein production. A major barrier for achieving dystrophin restoration using viral gene therapy is the large size of the dystrophin cDNA and the limited capacity of adeno-associated viruses. A series of mini- and micro- dystrophins have been designed, based on clinical observations of internally truncated proteins in BMD patients, and these have been tested in animal models (17, 79, 165). Exon-skipping is a distinct therapeutic approach for DMD also developed based on clinical observations in those predicted to have internally truncated dystrophin. Exon-skipping uses anti-sense

oligonucleotides to induce alternative splicing that bypasses mutated exons in order to repair protein reading frame, shifting a DMD mutation to a BMD mutation. Predicting the function of skipped dystrophin products and designing the optimal skipping plan has relied heavily on observations from patients as well as biochemical characterization dystrophin domains. This will be summarized below.

The Limb-girdle muscular dystrophies (LGMDs) are a collection of over 30 different subtypes of muscular dystrophies. Type1 LGMDs (LGMD1A–H) are dominantly inherited, representing 10% of all LGMD. Affected individuals usually have phenotypic range that overlaps with BMD, although exceptions exist for de novo mutations. Type2 LGMDs are caused by recessive mutations and more common then type1 LGMDs. Taken together, LGMD2A-W affect 1 in 15,000 individuals with some variations among geographic regions, depending on the carrier distribution and the degree of consanguinity within the population (118). A number of genes that cause LGMD2s encode proteins that directly associate with dystrophin, forming integral parts of the dystrophin glycoprotein complex (DGC). In muscle cells, the dystrophin complex localizes at the membrane and connects intercellular cytoskeleton to extracellular matrix. The dystrophin complex has been hypothesized to act as a membrane stabilizer during muscle contraction to prevent contraction-induced damage (48, 124). In addition to its structural role, dystrophin complex is also thought to mediate cellular signaling such as mechanical force transduction and cell adhesion. This review will focus on dystrophin and its associated proteins.

## THE DYSTROPHIN COMPLEX

The dystrophin-associated proteins can be divided into three groups based on their cellular localization: extracellular (α-dystroglycan); transmembrane (β-dystroglycan, sarcoglycans, sarcospan); cytoplasmic (dystrophin, dystrobrevin, syntrophins, neuronal nitric oxide synthase). α-dystroglycan resides on the extracellular surface of sarcolemma due to its heavy glycosylation and peripheral membrane association (Figure 1). α-dystroglycan and β-dystroglycan are translated from a single transcript and the peptide is proteolytically processed into two separate proteins (86). α-dystroglycan functions as a receptor for the extracellular ligands such as laminin, consistent with DGC's role in muscle cell adhesion to the basal lamina (86). α-dystroglycan is tightly associated with β-dystroglycan, a transmembrane protein that also interacts with dystrophin. α-dystroglycan is one of the few proteins that is glycosylated on serine residues, referred to as O-glycosylation, and mutations in genes encoding a series of enzymes involved in α-dystroglycan glycosylation (POMT1, POMT2, POMGnT1, FKTN, FKRP) cause LGMD2I, K, M, N and O (118). Furthermore, mutations in human *LARGE, GTDC2, B3GNT1, POMK, GMPPB, and ISPD* genes have been shown to cause a wide spectrum of congenital muscular dystrophy by disrupting α-dystroglycan glycosylation pathway (34, 35, 50, 104, 121, 157). This subset of muscle diseases are referred to as dystroglycanopathies, featuring reduced dystroglycan glycosylation and a wide spectrum of patient phenotypes ranging from mild congenital muscular dystrophy to dramatic conditions such as brain and eye anomalies, suggesting a role of dystroglycans beyond muscle tissues. Despite its central structural role in mediating muscle stability, mutations in dystroglycan gene itself were not identified in humans until fairly recent when a missense mutation (T192M) was identified that disrupts dystroglycan's

binding to laminin (23, 76). The rare frequency of mutations found in dystroglycan is presumably due to the vital role that dystroglycan plays in early embryonic development, as dystroglycan null mice are embryonic lethal (158).

At the sarcolemma, the sarcoglycan subcomplex is tightly associated with β-dystroglycan. The most prevalent form of sarcoglycan complex in skeletal muscle is composed of four single-pass transmembrane proteins: α-sarcoglycan β-sarcoglycan γ-sarcoglycan and δ-sarcoglycan. Mutations in the genes encoding α-, β-, γ- and δ-sarcoglycan cause LGMD2C-2F, respectively. The sarcoglycan subunits assemble and translocate within the myofiber as a complex, and loss of any individual subunit from loss of function mutation adversely affects the stability and trafficking of the unmutated sarcoglycan proteins. Sarcospan is a small transmembrane protein that is tightly associated with the sarcoglycans. The role of sarcospan has been more clearly delineated using mice engineered to lack sarcospan (102). The myopathy present in sarcospan null mice is mild but is exacerbated by loss of α7-integrin (107). Sarcospan is a member of the tetraspanin family that is known to interact with integrins (12, 44)

At the cytoplasmic face of the sarcolemma, dystrophin maintains its membrane localization by interacting with β-dystroglycan. Dystrophin binds to the intracellular actin network to link the cytoskeleton to DGC, which in turns connects to basal lamina by interacting with ECM ligands. Other cytoplasmic components of DGC include α-dystrobrevin, syntrophins and neuronal nitric oxide synthase (nNOS). The α-dystrobrevin/syntrophin triplet associates with dystrophin and anchors nNOS to the sarcolemma. In response to exercise, nNOS produces NO which signals blood vessels to dilate to increase blood flow to provide sufficient oxygen and nutrition to muscle cells.

## DYSTROPHIN

### Dystrophin structure overview

Dystrophin is a 427kDa cytoskeletal protein that localizes to the cytoplasmic face of the sarcolemma and is enriched at costameres in muscle fibers (127). Dystrophin protein has four main functional domains; an actin-binding amino-terminal domain (ABD1), a central rod domain, a cysteine-rich domain and a carboxyl-terminus (Figure 2A). ABD1 contains 2 calponin homology domains (CH1 and CH2) (99). This conventional CH-actin binding domain binds directly to F actin, linking dystrophin to the subsarcolemmal actin network (153). Dystrophin, and the dystrophin complex, also serve as broader cytoskeletal integrator, critical for muscle membrane stability. For example, ABD1 also binds to costamere-enriched intermediate filament protein cytokeratin 19 (K19), connecting dystrophin to the contractile apparatus in skeletal muscle cells (139, 140). Dystrophin's central rod domain contains 24 spectrin repeats, which are ~110 aa motifs consisting of triple α-helices folded into small ~5 nm rods (32, 98). The rod domain also harbors a second actin-binding motif (ABD2) that spans a unique collection of spectrin repeats enriched in basic amino acids, suggesting that an electrostatic interaction underlies the interaction with acidic actin filaments (6). ABD2 falls near the middle of the rod and collaborates with ABD1 to form a strong lateral association with actin filaments (132). The rod domain also mediates dystrophin interaction with microtubules via spectrin-like repeats 20–23 and is required for

the organization of microtubule lattice in skeletal muscle cells (21, 128). This disordered microtubule network in *mdx* mouse skeletal muscle cells has been linked to excess ROS signaling and increased intracellular calcium, contributing to the pathophysiological phenotype in *mdx* mice (92).

Dystrophin's rod has also been shown to bind membrane phospholipids in vitro via the tryptophan residues in the spectrin-like repeats (101). This interaction is thought to further facilitate the sarcolemma targeting of dystrophin, in addition to dystrophin's association with F-actin. In addition, the rod forms a flexible linker between the amino- and carboxy-termini. The 24 spectrin repeats are interrupted by four short proline-rich spacers, called "hinges" as they provide elasticity to the protein (97). Hinge 4 is at the end of the rod domain and contains a WW domain, a domain implicated in protein-protein interactions (88). The WW domain along with two neighboring EF-hands binds the carboxy-terminus of β-dystroglycan, anchoring the dystrophin at sarcolemma (130). The EF-hand motifs consist of two α-helices, linked by a short loop region that has been implicated in calcium binding (98). In dystrophin, the two EF-hands are located in the cysteine-rich domain, which resides between the central rod and C-terminus. The cysteine rich domain also contains a zinc finger (ZZ) domain that contains conserved cysteine residues and folds to form domain structure in the presence of divalent metal cations such as $Zn^{2+}$ (126). The ZZ domain of dystrophin binds to calmodulin in a calcium-dependent manner (8). The cysteine rich domain in dystrophin has also been shown to bind to ankyrin-B, an adaptor proteins that is required for retaining dystrophin at the sarcolemma (15). In addition, the cysteine rich domain and certain repeats in the rod have been shown to bind to intermediate filament protein synemin, further strengthening the link between costameric regions and myofibrils (24). The carboxy-terminal (CT) domain contains two polypeptides that fold into α-helical coiled coils similar to the spectrin repeats in the rod domain (28). Coiled coils are common protein motifs that are involved in protein-protein interaction. The CT domain provides binding sites for dystrobrevin and syntrophins, mediating their sarcolemma localization (133)

## Mutations and insights into gene therapies

Two thirds of *DMD* mutations are deletions that span one or multiple exons. These large deletions tend to cluster around two mutational hotspots (103). The most common hotspot spans from exon 45 to exon 55 and removing a central portion of the rod domain. The second most common deletional hotspot spans from exon 3 to exon 19, removing some or all of ABD1 along with a portion of the rod. When a deletion disrupts the reading frame, this truncated protein usually expresses at extremely low levels and associates with DMD phenotype. Molecularly, DMD is defined as producing no detectable dystrophin and clinically DMD is defined by loss of ambulation at or before age 12. If a *DMD* gene deletion maintains the correct reading frame, a smaller but functional internally truncated protein can be produced, although may be at lower than normal levels. Both the level and content of residual dystrophin protein expression are important for determining phenotype. Given the range of both type and amount of residual dystrophin, it is not surprising that the clinical phenotype associated with these mutations is similarly broad. BMD spans a clinical phenotype ranging from loss of ambulation in the mid second decade to those that remain ambulatory into the $5^{th}/6^{th}$ decade. This "reading frame" rule was consistent with 92% of the

cases in an early study of 258 independent deletions in the dystrophin locus (95). In a more recent study of 354 deletions, the prediction value of the reading frame rule was 82.5%. 254/286 out-of-frame deletions were associated with DMD while 38/68 in-frame deletions are associated with BMD or intermediate phenotype (58). The relative low prediction value for in-frame deletions may result from sample bias since mild/asymptomatic individuals with in-frame mutations are less like to participate in the study than the more severe patients. It may also suggest that different regions in dystrophin have variable significance for its function (58). The rod domain is particularly tolerant with large in-frame deletions. One dramatic example of an in-frame *DMD* gene mutation was described that removed 46% of the coding sequence (exon 17 to exon 48) (53). This deletion encompassed hinge 2, spectrin repeats 4 to 18, and a portion of spectrin repeat 19. The disease phenotype associated with this mutation was strikingly mild with one family member remaining ambulant at age 61. A major barrier for viral gene replacement therapy for dystrophin is the large size of the coding sequence. The most commonly used adeno-associated viruses for human gene therapy are restricted to less than 4–5 Kb (14). The observation of a severely truncated but highly functional dystrophin prompted the development of mini-dystrophins not only for viral gene delivery but also supported the concepts behind antisense mediated exon skipping as a “gene correction” strategy.

Clinical observations from patients with dystrophin gene mutations stimulated the development of mini-dystrophins, defined as containing more than four spectrin repeats, and micro-dystrophins, defined as those with four or fewer than four spectrin repeats. Both transgenic and viral delivery approaches were used to rescue the phenotype of the *mdx* mouse model, and provided consistent results and have provided additional support for exon-skipping approaches. Expressing a mini-dystrophin transgene, which encoded a protein missing regions encoded by exons 17–48 (Δ17–48), in *mdx* mice reversed many of the dystrophic changes (125). These $Tg^{+}$ *mdx* mice had reduced central nucleation, improved specific force generation and decreased creatine kinase level. However, the truncated protein did not function as well as the full-length protein and required higher protein content to reach similar rescue effects. When expressed at 20% of control level, full-length dystrophin was able to fully rescue the central nucleation and specific force in diaphragm muscle while the truncated form provided only partial rescue. Another interesting observation is that limb muscle appears to require a higher level of dystrophin than the diaphragm. When full-length protein was expressed at 15% of control levels, the quadriceps muscles were only slightly improved by measuring the percentage of fibers with central nucleation. Only when 70% of control level was achieved, did the dystrophic histology of quadriceps match that of normal muscle. To achieve a similar rescue effect in quadriceps, the truncated dystrophin needed to be expressed at several fold of control levels (125). This pioneering study highlighted that the functionality of internally truncated dystrophin molecules is not only dependent on the protein structure, but also affected by the expression level.

Notably, this Δ17–48 construct expressed a dystrophin that contain 8.5 spectrin repeats (Figure 3C). The number and phasing of the spectrin repeats within the rod region is likely critical as a second construct that was tested was referred to as ΔH2-R19, containing an even eight perfectly phased spectrin repeats (Figure 3D). The ΔH2-R19 transgenic *mdx* mice were

not different from wild type mice by specific force measurement in both EDL and diaphragm muscles (79). ΔH2-R19-rescued muscles had fewer centrally nucleated fibers (<1%) than those from Δ17–48 transgenic *mdx*, suggesting the significance of correct phasing of spectrin repeats. However, as central nucleation is only one measure with unclear meaning for muscle function, these comparative findings should be interpreted with caution.

An in vivo approach to examine the effect of in frame *DMD* gene mutations queried mutations in patients with cardiomyopathy and BMD. This approach was designed to evaluate the effect of internal deletions on dystrophin protein function. Notably, BMD subjects with out-of-phase deletions in the rod developed dilated cardiomyopathy about a decade earlier than patients with in-phase deletions (91). These data are further complemented by In vitro studies showing that incorrect phasing of spectrin repeats may result in increased misfolding and instability of the protein (131). Together, these findings have significant implications for exon skipping, because it may be preferable to skip more exons to create an in-phase and in-frame protein, referring to spectrin repeat phasing and open reading frame. These data do not take into account the level of protein expression, which can vary significantly in BMD and between heart and muscle. However, a more stable protein, generated from maintaining spectrin repeat phase, may also yield improved protein stability and therefore higher levels of protein production. Quantitative measures of dystrophin production in biopsies, both before and after exon skipping or viral correction, are needed. These quantitative measures are challenging since current methods rely on muscle biopsies and for these biopsies there may considerably variability across the muscle, which is not adequately assessed by a single biopsy. The inability to accurately quantify dystrophin protein production remains problematic for the gene editing/correction/rescue field since genetic evidence indicates that levels of expression may be equally important and interrelated to spectrin repeat phasing.

The importance of dystrophin's central hinge was also shown by generating ΔH2–H3 construct (79). This construct differed from ΔH2–R19 by the absence or presence of hinge 3 (Figure 2B). ΔH2–ΔH3 transgenic mdx demonstrate nearly normal histology but slightly elevated central nucleated fibers. Importantly, force generation was less with ΔH2–ΔH3 mice compared to WT or ΔH2–R19 transgenic *mdx*. Since the eight-repeat ΔH2–R19 was highly functional in rescuing the dystrophic phenotype in *mdx*, four-repeat micro-dystrophins were generated to test the function of even smaller proteins (Figure 2B). Among the three different micro-dystrophins, ΔR4–R23 had four repeats plus one central hinge (hinge2) and was the most effective since ΔR4–ΔR23 transgenic *mdx* had almost normal histology. ΔR2–R21+H3 is another construct that also contains four repeats but a different central hinge (hinge 3). This construct, ΔR2–R21+H3 restored a normal histology in diaphragm muscle, yet the limb muscles retained dystrophic pathology, suggesting less rescue efficiency. ΔR2–R21 microdystrophin is a construct that lacked both hinge 2 and hinge 3. This construct displayed a higher capacity in rescuing pathology and force generation than ΔR2–R21+H3. Micro-dystrophin construct with no repeats (ΔR1–R24) was unable to rescue by histology, indicating absolute requirement to maintain some rod domain. Interestingly, the protective role of hinge 3 in mini-dystrophins is reversed in micro-dystrophins, suggesting the effects of internal hinges may be context dependent. In two

follow-up studies, the authors found that expression of the ΔR4–R23 micro-dystrophin in *mdx* mice led to chronic myotendinous injury and this defect was rescued by replacing the hinge 2 in ΔR4–R23 with hinge 3 (16, 18). Hinge 2 and hinge 3 are similar in size and contain six proline residues while the distribution of the proline residues is different (98). In contrast to the evenly distributed proline residues in hinge 3, five consecutive proline residues are present in hinge 2. Deletion of this polyproline site in hinge 2 also prevented the development of the chronic myotendinous injury in ΔpolyP/ΔR4–R23 expressing *mdx* mice (18). Importantly, one needs to take the variable expression level of the constructs into consideration as well when interpreting the rescue data. For example, ΔH2–R19 construct was expressed at a higher level than that of ΔH2–ΔH3 and may also contribute to its superior rescue ability in addition to the presence of central hinge. Conversely, ΔR2–R21+H3 was overexpressed in the quadriceps and formed large protein inclusions that may result in cytotoxicity (79).

Deletion spanning exon 45 to exon 55 (del45–55) is associated with very mild or even asymptomatic cases (57, 116). This deletion removes spectrin repeats 17 to 22, and this truncated dystrophin lacks part of the binding sites for neuronal nitric oxide synthase (nNOS), mediated by spectrin repeats 16 and 17 (100). Although generally mild, variations of disease severity exist between patients carrying del45–55, and this variable phenotype correlates with nNOS sarcolemma localization (100). In a study of 12 patients with del45–55, the mild/asymptomatic group had mainly sarcolemma nNOS, while the more severe group exhibited an exclusive cytosolic nNOS. Nevertheless, the generally benign clinic feature of del45–55 provides an optimal goal for exon skipping strategies. Since exon 45 to exon 55 is a mutational hotspot, skipping exon 45–55 can theoretically apply to 63% of all DMD cases or 75% of all deletion mutations (22). Current clinic trials for exon skipping have focused on the skipping of single exons, due to the technical difficulty of skipping multiple exons. Recently, body wide restoration of dystrophin in mice engineered with exon 52 deletion (*mdx52*) was achieved after five biweekly injections of anti-sense oligonucleotides targeting exons 45–55 (11). After antisense induced multi-exon skipping, the level of dystrophin protein expression in multiple skeletal muscle groups was approximately 8~15% of normal level by immunoblotting. Antisense treated mice had significantly ameliorated muscle histopathology with fewer centrally nucleated fibers compared to untreated mice. The antisense treated mice also showed reduced serum CK levels, improved maximum forelimb grip force. The benefit of internally truncated yet relatively low level of dystrophin protein production in the treated mice is consistent with previous studies showing a threshold effect of dystrophin. In dystrophin/utrophin double null mice, very low levels (up to 5%) were found to improve survival, and levels between 5%~15% further improved survival as well as histology. At dystrophin levels above 15%, survival was normal and histology was further corrected towards normal (150). A similar threshold phenomenon can also be inferred from human patient data. DMD patients and very severe BMD patients typically have dystrophin levels below 10%, while mild Becker patients have a wide range of dystrophin level with the lowest above 10% (83). In a group of 13 patients with exon 45–47 deletion, dystrophin levels varied from 13% to 76%, however no correlation between dystrophin protein level and disease severity was found (149). Similar threshold effects were observed in another study comparing dystrophin levels in out-

of-frame to those in in-frame deletions within the exon 42–49 region (9). The fact that even low level of truncated dystrophin can be beneficial is encouraging, as up to 15.6% dystrophin restoration has been achieved in patients in exon skipping clinical trials (63).

Although many in-frame deletions in the central rod domain are associated with mild phenotypes, deletions within the ABD1 usually results in reduction of dystrophin level and is associated with more severe BMD (148). The importance of ABD1 is further demonstrated by missense mutations in ABD1 that associated with severe phenotypes. In fact, it has been suggested that more than 50% of missense mutations that cause disease occur in the ABD1 (136). The effects of four missense mutations, L54R, A168D, A171P, and Y231N, were examined (136). All four mutants were found to have decreased thermodynamic stability and increased misfolding. In another study by DM Henderson et al, the K18N, L54R, D165V, A168D, L172H, and Y231N mutations were characterized (18). Similar to the aforementioned study, all six mutants were less stable and were more prone to aggregation. Interestingly, only K18N and L54R also displayed decreased actin-binding affinity and are associated with the more severe disease progressions. The results suggest that ABD1 is not only essential for actin binding, but also for proper protein folding and protein stability throughout the dystrophin protein. To dissect the effect of low-level protein expression from the functionality of truncated dystrophin, micro-dystrophin with deletions in ABD1 were overexpressed in *mdx* mice by viral delivery (17). Deletion of actin binding sequence in ABD1 impaired the function of the micro-dystrophin to prevent muscle degeneration and was maintained less well in vivo over time. Surprisingly, an N-truncated isoform of dystrophin missing the first half of the ABD1 appeared to be highly functional in both humans and a mouse model of DMD (154). The high functionality of this N-truncated dystrophin might be explained by the presence of second half of ABD1 and of ABD2 in the rod domain while ABD2 is absent in the micro-dystrophin. In BMD, deletions involving amino-terminal domain predict an earlier onset dilated cardiomyopathy (DCM) in mid-20s, while deletions within the rod domain are associated with a later onset DCM in mid-40s (90).

Curiously carboxy-terminal truncated dystrophins have been described and produced where they have been observed at the sarcolemma (25, 129). The deletions in the above cases encompass both cysteine-rich domain and carboxy terminus, suggesting that neither is required for plasma membrane localization. However, these patients developed DMD, indicating additional function can be ascribed to this region, such as interacting with β-dystroglycan and dystrobrevin. Interestingly, the micro-dystrophin lacking a carboxy-terminus, which retains the cysteine-rich region (ΔR4–R23/ΔCT), appeared highly functional in rescuing the dystrophin/utrophin double null mice after AAV mediated gene transfer (164). This result highlights the crucial role of the cysteine-rich region while also suggesting that the very distal end of dystrophin may be dispensable.

## Sarcoglycans

**Overview of the sarcoglycan complex—**The sarcoglycan complex was first characterized as a transmembrane subunit of the DGC (162). Six mammalian sarcoglycans have been identified so far: α (50kDa), β(43kDa), γ (35kDa), δ (35kDa), ε (50kDa), and ζ

(40kDa), (Figure 4). All sarcoglycans are single pass transmembrane proteins with at least one asparagine-linked glycosylated residue. Both α/ε-sarcoglycan are closely related proteins with similar gene and protein structure, and these are both type 1 transmembrane proteins with a cleaved amino-terminal signal sequence. γ/δ/ζ-sarcoglycan are also highly related to each other with similar gene and protein structure, but these sarcoglycans like β-sarcoglycan are type 2 transmembrane sequences with an intracellular amino-terminal domain and extracellular carboxy-terminal domain. There is weak similarity between β-sarcoglycan and γ/δ/ζ-sarcoglycans, especially in the distal portion of the carboxy-terminus with conserved cysteines that form disulfide bones. The sarcoglycan complex is evolutionarily conserved. In zebrafish, five out of the six sarcoglycans have orthologs (38). Three sarcoglycan orthologs have been identified in both *Drosophila* and *C. elegans*: one gene similar to mammalian β-sarcoglycan, one gene equally related to mammalian α/ε-sarcoglycan and one to mammalian γ/δ/ζ-sarcoglycan (4, 68).

In mammals, four types of sarcoglycan are present in the dystrophin complex with a 1:1:1:1 ratio. In striated muscle, the major sarcoglycan complex is composed of α, β, γ and δ sarcoglycan. In vascular smooth muscle, ε and ζ sarcoglycan replace α - and γ-sarcoglycan, while β- and δ-sarcoglycan are present in multiple cell and tissue types (89, 155). ε-sarcoglycan is also present in striated muscle but at a much lower level than that of α-sarcoglycan. Interestingly, overexpression of ε sarcoglycan in striated muscle was sufficient to rescue the dystrophic phenotype of α sarcoglycan null mice, suggesting that ε sarcoglycan can functionally replace α-sarcoglycan (89). Nonmuscle forms of the sarcoglycan complex are present with varying composition of sarcoglycan subunits (7, 152). Mutations in the gene encoding ε-sarcoglycan cause myoclonus-dystonia syndrome, and this syndrome is thought to reflect its expression in the central nervous systems rather than in the muscle itself (167).

**Sarcoglycan complex assembly in muscle—**Correct assembly of the sarcoglycan complex is required for its stability and sarcolemma targeting. In human patients with sarcoglycan gene mutations and mice engineered with null alleles of sarcoglycan genes, mutations in any one of the sarcoglycan genes result in the deficiency of the entire SG complex at the sarcolemma. How the sarcoglycan complex is assembled and trafficked to the plasma has been examined in multiple cell culture systems, animal models and human patients. Early observations in cultures mouse myotubes suggested a preferential association between β-sarcoglycan and δ-sarcoglycan as a central step in the normal assembly of the sarcoglycan complex (39). A complex containing α-, β-γ-, and δ-sarcoglycan was co-immunoprecipitated from myotubes. With increasing stringency of the immunoprecipitated, 0.3% SDS, α-SG was completely dissociated from the complex. At 0.4% SDS, γ-SG was greatly reduced from the complex while β-SG and δ-SG remained tightly associated. Cross-linking experiments showed that β-SG and δ-SG were in close physical proximity at the plasma membrane. Cross-linking products containing β-, δ-, and γ-sarcoglycan were also observed while α-sarcoglycan was not cross-linked to other sarcoglycans, suggesting a partial separation of α-sarcoglycan from the remainder of the sarcoglycan complex. The especially tight link between β- and δ-sarcoglycan is consistent with the β/δ acting as the structural core of the sarcoglycan complex. To determine the assembly order of the sarcoglycan subunits, a heterologous expression system was established (135). COS-1 is a

fibroblast-like cell that lacks endogenous sarcoglycans. When expressed individually, none of the four sarcoglycans translocates with significant efficiency to the plasma membrane. Co-expression of β- and δ-sarcoglycan is the minimal requirement for plasma membrane localization. These data suggest a model in which β- and δ-sarcoglycan associate to form the complex core while γ- and then α-sarcoglycan become associated in subsequent steps (Figure 5A). Supporting this model, during myoblast differentiation β-sarcoglycan and δ-sarcoglycan protein expression is detected prior to α and γ sarcoglycan protein (119). The sarcoglycan complex is assembled in the endoplasmic reticulum (ER) but it does not associate with β-dystroglycan or sarcospan in the ER. Instead, β-dystroglycan and sarcospan become associated *en route* from the Golgi apparatus to the plasma membrane (119) (Figure 5B).

This assembly model is also supported by observations in muscle from patients with sarcoglycan gene mutations and sarcoglycan mutant animals. Primary mutations in *SGCB* and *SGCD* usually result in greater reduction of all sarcoglycan subunits at the sarcolemma compared to muscle with primary mutations in *SGCA* and *SGCG*. While deletion of *Sgcd* eliminates the sarcolemmal sarcoglycan complex, *Sgcg* null mice have only partially reduced sarcoglycan expression level and the residual sarcoglycan protein expression is associated with normal glycosylation consistent with normal trafficking (73). In muscles from *SGCB* mutated patients and primary culture derived from the biopsy, complete loss of the other three sarcoglycans was observed (56). The R77C *SGCA* mutation is a common mutation associated with LGMD 2D (75). R77C α-sarcoglycan protein has been shown to have membrane trafficking defects (75). In these patients, the other three sarcoglycans are still present at the sarcolemma though at a reduced level, suggesting α-sarcoglycan association may not be required for the membrane trafficking of the SG complex. Overexpression of α-sarcoglycan or γ-sarcoglycan have been associated with cytotoxicity and pathology in mice muscle, presumably due to the disruption of the SG assembly process in the ER presumably leading to ER stress (51, 166).

Functional domains have been identified in sarcoglycans that are required for their interaction and membrane targeting (40). The membrane proximal portion of the extracellular domains of δ-sarcoglycan (aa57-92) and γ-sarcoglycan (aa94-157) are required for their interaction with β-sarcoglycan. The cysteine-rich motif and asparagine-linked glycosylation in δ-sarcoglycan are essential for its membrane trafficking but do not appear to have effect on its binding to β-sarcoglycan. Consistent with the importance of sarcoglycan assembly and trafficking, these functional domains are also hot spots for disease-causing mutations (40).

**Function of the sarcoglycans—**Recessive mutations in the sarcoglycan genes result in a wide range of muscular dystrophy phenotypes in patients, with a range of severity similar to DMD and BMD. In sarcoglycan deficient mice, like in the absence of dystrophin, the plasma membrane of muscle cells becomes fragile and susceptible to contraction-induced damage. This is demonstrated by both the abnormal uptake of Evans blue dye and the increased force drop after *ex vivo* eccentric contractions (ECC) in sarcoglycan deficient muscles, similar to muscles lacking dystrophin (13, 73, 74). In DMD patients or *mdx* mice, the sarcoglycans are undetectable at the sarcolemma. In the absence of sarcoglycans

however, dystrophin is still intact or only slightly reduced at the sarcolemma, suggesting that the sarcoglycans may act as a mediator for dystrophin function in muscle (73). The exact role of the SG complex is not fully understood, yet clearly both mechanical and non-mechanical roles are at play.

The sarcoglycan complex has important structural role in stabilizing the DGC through at least two mechanisms. First, the sarcoglycans enforce the link between α- and β-dystroglycan. The sarcoglycan complex is tightly linked to dystroglycan in that they can be dissociated together as a unit from purified dystrophin complex (161). Furthermore, δ-sarcoglycan was shown to interact directly with β-dystroglycan in mouse myotubes (39). The BIO14.6 hamster is a muscular dystrophy model that is deficient in δ-sarcoglycan. In BIO14.6 hamster, dystroglycan was dissociated from the sarcoglycan complex and the cell surface anchorage of α-dystroglycan was also disrupted, while dystrophin expression was similar to WT (142). Second, the sarcoglycans have been shown to interact directly with dystrobrevin (161).

Dystrobrevin is a dystrophin-related protein that heterodimerizes with dystrophin through coiled-coil motifs (133). The sarcoglycan-dystrobrevin-dystrophin interaction further holds the complex together. The sarcoglycans are very tightly associated with sarcospan, a 25kDa transmembrane protein that belongs to the tetraspan family (45). Sarcospans are hypothesized to form homo-oligomers that act to cluster the components of the dystrophin complex within the sarcolemma and facilitate its sarcolemma anchorage (113). In all, interactions between the sarcoglycans and other complex members contribute to the integrity and membrane anchorage/organization of the larger complex. Muscles lacking dystrophin or δ-sarcoglycan showed a substantial force drop after the eccentric contraction with increased dye uptake, supporting the previous notion that these membranes became susceptible to contraction-induced damage when the dystrophin complex is disrupted (73). In γ-sarcoglycan null muscle however, the force drop and percentage of dye positive fibers after eccentric contraction was similar to that of WT (72). However, LGMD 2C patients and γ-sarcoglycan mutant animals still suffer from substantial muscle cell damage/death and severe muscle degeneration, indicating that sarcoglycans may involve in functions other than stabilizing the DGC.

The sarcoglycans may participate in the mechanoprotection process through their interaction with filamin C. Filamin C (FLNC) protein binds to the cytoplasmic tails of γ- and δ-sarcoglycan (145). The filamin protein family is composed of three different types (FLN1, b-FLN and FLNC) and FLNC is the only muscle specific member of the family. The filamins are known for their roles in actin polymerization, membrane receptor organization and mechanoprotection, a process protecting cells from external stress (141). Two populations of FLNC exist in muscle cells, cytoplasmic and plasma membrane-associated. In the absence of γ- or δ-sarcoglycan, the membrane-bound pool of FLNC increased by almost ten fold (3% to >20%). This abnormal localization of FLNC is likely to contribute to a disrupted actin cytoskeleton network and impairs resistance to strain at sarcolemma. Furthermore, the interaction between the sarcoglycans and FLNC is regulated by calpain-3, the muscle-specific member of the calcium-dependent protease family (71). Calpain 3 regulates the interaction between the sarcoglycan and FLNC by cleaving the carboxy-

terminus of FLNC, which contains binding site for the sarcoglycans. Calpain 3 is an important regulator for muscle function as mutations in the calpain 3 gene cause LGMD 2A. The sarcoglycan-FLNC-calpain 3 interaction may provide additional insight in disease mechanism underlying muscular dystrophy.

γ-sarcoglycan was also found to mediate mechanical signal transduction. When plated on collagen-coated surface, *Sgcg* null muscle cells displayed significantly increased apoptosis and hypercontractility despite normal adhesion (67). In the resting *Sgcg* null cells, activity level of ERK-1 and several other phosphoproteins were greatly increased compared to WT. Hyperactive ERK-1 was associated with cell death in other terminally differentiated cells (42). Consistent with this finding, isolated muscle from *Sgcg* null mice showed a higher phosphorylation levels of both ERK-1 and ERK2 at resting state than WT (19). When subject to the eccentric contraction, WT muscle responded with increased phosphorylation of ERK-1 and ERK-2 while *Sgcg* null muscle failed to increase ERK-1 phosphorylation. Interestingly, a bigger increase of ERK-2 level was seen in *Sgcg* null muscle compared to WT, suggesting a compensatory response. Failure of the ERK-1 response is independent of contractile damage since the force-drop after eccentric contraction in *Sgcg* null muscles was normal. Expression of WT γ-sarcoglycan was able to restore the ERK-1 response while expression of γ-sarcoglycan engineered with Y6A mutation failed to do so despite its normal sarcolemma localization, supporting that tyrosine 6 phosphorylation mediates ERK-1 response (20). Recently, γ-sarcoglycan was shown to regulate another mechanical-responsive kinase, p70S6 (84, 114). Upon stretch, p70S6K was activated in both *Sgcg* null and WT muscles but failed to return to normal level in mutant muscle. p70S6 kinase is implicated in cell survival and growth and its aberrant regulation may contribute to muscle pathogenesis in γ-SG mutants (78). In all, γ-sarcoglycan is tyrosine phosphorylated in response to external mechanical perturbations and mediates the intracellular signaling events that are important for cell survival.

The sarcoglycan complex may also regulate cell-cell adhesion via interacting with the integrin complex. In cultured myocytes, the sarcoglycans co-precipitated with integrin α5β1 and other focal adhesion proteins (163). Tyrosine phosphorylation of α- and γ-sarcoglycan occurred when the cells were exposed to known integrin ligands, similar to their phosphorylation induced by cell adhesion to fibronectin matrix. When α- and γ-sarcoglycan were removed by antisense treatment, the associated adhesion proteins were also greatly reduced. These data suggest a bidirectional signaling between the integrins and sarcoglycans. The 16 kDa subunit c (16K) of vacuolar H(+)-ATPase may mediate this communication between integrins and sarcoglycans (41). The 16K subunit directly binds to γ- and δ-sarcoglycan, forming a complex with the sarcoglycans and integrins at the plasma membrane. The 16K subunit was also shown to regulate the expression level of β-integrin at sarcolemma by binding to β-integrin directly in ER to inhibit its maturation. In δ-sarcoglycan deficient muscle, the16K subunit was mislocalized at the sarcolemma and might explain the increased level of integrin since less of the 16K subunit was available at ER to inhibit β-integrin. This may explain the upregulation of integrins observed in sarcoglycan deficient animal models and human patients (5, 70, 81).

The sarcoglycan complex is also implicated in the metabolic defects in muscular dystrophies. In white adipocytes, a cell specific sarcoglycan complex composed of β-, δ- and ε-sarcoglycan is expressed at the cell surface, together with dystroglycan and sarcospan (69). Loss of β- or γ-sarcoglycan resulted in the loss of the sarcoglycan complex and great reduction of dystroglycan and sarcospan in the adipocytes. Furthermore, β-sarcoglycan null mice exhibited glucose-intolerant and whole body insulin resistance due to compromised glucose uptake in response to insulin. The metabolic defects in sarcoglycan deficient mice may contribute to the abnormal fat deposition in the skeletal muscle, a common feature shared among many muscular dystrophies arising from a disrupted sarcoglycan complex (110). Studies have shown that both adipose-derived and muscle-derived stem cells tend to differentiate into adipocytes when exposed to high glucose level (2). Supporting this, the adipogenesis-competent cells within the skeletal muscle are activated during the degeneration/regeneration cycles in the dystrophic muscle tissue, linking muscle degeneration to fat infiltration (159).

α-sarcoglycan has its unique properties and functions. α-sarcoglycan has been characterized as a $Ca^{2+}$, $Mg^{2+}$-dependent ecto-ATPase, contributing to the increased extracellular ATP-hydrolyzing activity of differentiated C2C12 myotubes (134). High levels of extracellular ATP can occur when muscle cells are damaged and act as pro-apoptotic stimuli (3). Hence α-sarcoglycan may act to protect muscle fibers from tissue injury caused by exercise or muscle degeneration by reducing extracellular ATP levels. Before muscle cell differentiation, α-sarcoglycan plays a role in regulating proliferation ability of the myogenic progenitor cells by binding to and stabilizing fibroblast growth factor receptor 1 (FGFR1) (37). α-sarcoglycan deficient myogenic progenitor cells lost FGFR1 at the plasma membrane and had impaired proliferation in response to basic fibroblast growth factor. Impaired proliferative ability of muscle stem cells is likely to worsen the muscle degeneration in dystrophic muscle.

## Sarcospan

**SSPN overview—**Sarcospan (SSPN) is an integral transmembrane component of the DGC (44). Sarcospan is also part of the utrophin-glycoprotein complex (UGC) that is enriched at the neuromuscular junction (45). Sarcospan is preferentially associated with the sarcoglycans, and this association is required for the stable expression SSPN at the sarcolemma. SSPN is completely devoid or greatly reduced from the sarcolemma in sarcoglycan deficient human patients and animal models (46). Unlike mutations in the SG genes, to date mutations in the *SSPN* gene have not been associated with human diseases (108). *SSPN* knockout mice do not display any apparent dystrophic phenotype until aged and after exposure to cellular stress (102, 109).

**SSPN structure and function—**SSPN is a member of the tetraspan family and is 25-kDa in size. SSPN is a highly hydrophobic protein that contains four transmembrane domains (TMs) with both N-terminal (NT) and C-terminal (CT) regions located inside of the cell. A large extracellular loop (LEL) resides between TM3 and TM4 (44). SSPN plays an important mechanical role in stabilizing the DGC. Within the sarcolemma, SSPN forms homo-oligomers to establish SSPN enriched microdomains to enhance protein-protein

interactions. Oligomerization of SSPN is mediated by several points of contact, including NT-NT, NT-CT and LEL-LEL. The cysteine residues within the LEL are important for SSPN-SG interactions and DGC stability (113). An appropriate level of SSPN in the cell is also essential for the integrity of DGC. When overexpressed in mice by 10 fold, SSPN clusters the sarcoglycans into insoluble protein aggregates and causes the destabilization of α-dystroglycan (123). Microspan is an alternatively spliced product of the *SSPN* gene. Microspan is half the size of the SSPN, containing TM1, TM2 but lacks TM3, TM4 as well as LEL. Microspan is not part of the DGC and is enriched at the sarcoplasmic reticulum (SR), consistent with the notion that LEL is required for SSPN-SG interactions and sarcolemma localization (112).

An emerging role for SSPN is to regulate muscle cell survival and regeneration by modulating the abundance of laminin-binding complexes, including DGC, the utrophin glycoprotein complex (UGC), and the integrin complex. In aged sarcospan null mice, DGC and UGC levels are reduced, accompanied by decreased laminin-binding affinity (109). SSPN null mice also show delayed regeneration after cardiotoxin injury due to the loss of utrophin and Akt signaling in the regenerating fibers (109). Activation of Akt signaling increases protein synthesis and improves survival of damaged muscle cells (94). Viral delivery of Akt was able to restore the UGC and rescue the muscle repair in SSPN null cardiotoxin-injured muscle, suggesting SSPN-Akt-UGC signaling contributes to muscle regeneration. In transgenic animals overexpressing SSPN at different levels, utrophin, dystrophin and α7β1 integrin were increased at the sarcolemma in a dose-dependent manner. In *mdx* mice, upregulation of utrophin is the main compensation mechanism for maintaining their relatively mild phenotype (49, 146). When SSPN is overexpressed in *mdx* mice by three fold, levels of utrophin and integrins are both increased, accompanied by the restoration of sarcoglycans and dystroglycans. In the SSPN $Tg^+$ *mdx*, mRNA level of utrophin is unchanged, while utrophin protein is decreased in the ER/Golgi fraction of the cell, suggesting that SSPN may facilitate the transportation of utrophin from ER/Golgi to the sarcolemma (109). Dystrophic pathology of these SSPN $Tg^+$ *mdx* mice is significantly improved compared to $Tg^-$ *mdx*, suggesting SSPN overexpression might be a potential therapeutic strategy (122).

**Dystroglycans—**Dystroglycan was first identified as an integral transmembrane component of the DGC purified from skeletal muscle membranes (55). Later, dystroglycan was detected in a broad range of tissues during both developmental and adult stage, suggesting roles for dystroglycan beyond muscle (16). In the DGC, dystroglycan connects the cytoskeletal proteins to the extracellular matrix (ECM) (86). Proper glycosylation is essential for the function of dystroglycan. Mutations in the genes that participate in the dystroglycan glycosylation pathway cause a subgroup of muscular dystrophy referred to as dystroglycanopathies. The disease severity ranges from mild ones with little or no central nervous system involvement, to severe forms with structural brain defects (115). So far, up to sixteen glycosyltransferase genes have been associated with the dystroglycanopathies, including LARGE, fukutin, FKPP, POMT1, POMT2 and POMGnT1 (26).

In humans, dystroglycan is encoded by one gene *Dag1* and post-translationally cleaved into two subunits, α and β (86). α-dystroglycan is an extracellular peripheral membrane protein

that contains three potential N-glycosylation sites in the N- and C-terminus. The globular N- and C-terminal domains are connected by a highly *O*-glycosylated central mucin domain (29, 30). Although the predicted molecular weight of α-dystroglycan is ~40 kDa, α-dystroglycan in skeletal muscle migrates at 156 kDa on electrophoresis, due to the heavy glycosylation (86). α-dystroglycan serves as a receptor for a variety of ECM ligands such as laminins, perlecan and agrin (61, 86, 143). Mutations in the genes encoding LARGE, fukutin, FKPP, POMT1, POMT2 and POMGnT1 result in the production of a species of α-dystroglycans that has impaired glycosylation and altered ligand-binding ability (93, 111, 156). Furthermore, full chemical deglycosylation of α-dystroglycan results in the complete loss of ligand-binding activity. In contrast, elimination of only *N*-linked sugars in α-dystroglycan by *N*-glycanases does not affect its activity as an ECM receptor, suggesting that the ligand-binding ability is dependent on the *O*-linked sugars in the mucin domain (54). Specifically, a recent study has shown that *O*-glycosylation and subsequent modification by LARGE at Thr-317 and -319 within the first 18 amino acids of the central mucin domain is both sufficient and necessary for laminin-binding (77).

The 43 kDa β-dystroglycan is a single-pass transmembrane protein with potential *N*-glycosylation sites. The extracellular N-terminal domain of β-dystroglycan associates tightly with α dystroglycan, anchoring α dystroglycan at the sarcolemma. The cytoplasmic C-terminus of β-dystroglycan binds to the WW and EF hands domain in dystrophin (43). Caveolin-3 is a scaffolding protein and the main component of caveolae in muscle cells (60). Caveolin-3 also directly binds to the C-terminus of β-dystroglycan and may compete with dystrophin for this interaction and sarcolemma localization (137). Other binding partners for β-dystroglycan include growth factor receptor-bound protein 2 (Grb2) and rapsyn (36, 160). Grb2 is an adaptor protein and dystroglycan-Grb2 interaction may participate in the ERK-MAP kinase pathway involving MEK and ERK (138). Rapsyn is an Src-like kinase that is involved in the clustering of the acetylcholine receptor at the synapse in response to agrin binding to β-dystroglycan (36).

**Dystrobrevin, syntrophin and NO synthase—**α-Dystrobrevin was originally isolated as an 87 kDa phosphorylation substrate enriched in the postsynaptic membranes in the Torpedo electric organ (151). α-Dystrobrevin shares significant homology with the C-terminal domains of dystrophin (27). α-Dystrobrevin interacts with dystrophin through the C-terminal coiled-coil regions in each protein (28). Association with dystrophin anchors α-dystrobrevin to the sarcolemma, and α-dystrobrevin is greatly reduced from the sarcolemma in dystrophin-deficient mice (27). Other binding-partners for α-dystrobrevin in the DGC include the sarcoglycans and the syntrophins (117, 161). α-Dystrobrevin-deficient mice develop mild dystrophic phenotype in both cardiac and skeletal muscle (65). The DGC is assembled correctly at the sarcolemma and the muscle cells remain resistant to damage in α-dystrobrevin-deficient mice. However, α-dystrobrevin-deficient mice display defects of neuromuscular junction (NMJ) maturation and abnormal myotendinous junctions (64, 66). Despite an intact DGC at the sarcolemma, nNOS is greatly reduced at the muscle membrane in α-dystrobrevin-deficient mice. It is not clear whether the mislocalization of nNOS results in the pathology in α-dystrobrevin-deficient mice since nNOS knockout mice remain asymptomatic (85). So far, mutations in the dystrobrevin gene have not been described to

associate with skeletal muscle disease. However, a missense mutation in the dystrobrevin protein (P121L) was reported in a family with left ventricular non-compaction and congenital heart disease (87).

Syntrophin was also isolated as a cytoplasmic component of the acetylcholine receptor-riche domains in the Torpedo electric tissues (33). Syntrophin associates with both dystrophin and α-dystrobrevin in the DGC (52). Syntrophin is involved in the coordination of a number of important signaling molecules including nNOS, Grb2, stress-activated protein kinase-3 and calmodulin (31, 80, 105, 106, 120). Syntrophin-deficient mice show disrupted NMJ with reduced acetylcholine receptor and absence of utrophin. nNOS is also depleted from the sarcolemma in the mutant mice. Despite these molecular defects, syntrophin deficient mice do not develop dystrophic phenotype (1).

Neuronal nitric oxide synthase (nNOS) produces nitric oxide (NO) from L-arginine in many different cell types throughout the body. nNOS is anchored at the sarcolemma by the DGC and controls local blood flow by antagonizing sympathetic vasoconstriction (144). Mislocalization of nNOS has been observed in many types of muscular dystrophies, resulting in blood vessel over constriction and focal lesions (47).

## CONCLUSIONS

The dystrophin complex has both mechanical and nonmechanical roles in stabilizing the sarcolemma and protecting the muscle cells from contraction-induced damage. Mutations in the genes encoding DGC components destabilize the dystrophin complex producing muscle weakness and muscular dystrophy. The information derived from dystrophin's primary sequence, combined with extensive knowledge of the effects of a wide variety of dystrophin mutations, have led to remarkable understanding of the precise components required to re-stabilize the sarcolemma through partial restoration of dystrophin function. The original "reading frame hypothesis" put forward more than 20 years ago provided initial clinical utility in predicting long term phenotypic outcome. Observations from BMD patients provided substantial hope for viral gene replacement studies, limited by the capacity of adeno-associated viruses and challenged by the large size the dystrophin open reading frame. In more recent years, this structure function analysis, combined with clinical phenotype data, provides the impetus for exon skipping strategy. Now in clinical trials, this approach is designed to restore partial expression of an internally truncated protein, with the goal of converting a severe phenotype to a more mild phenotype. Although this approach, by design, will yield only partial correction, this is still perceived to be clinically extremely meaningful. The functionality of a variety of internally-truncated dystrophin constructs has been tested in the *mdx* mouse model, including the highly functional mini- and micro-dystrophins. At the sarcolemma, dystrophin binds to dystroglycan, which in turn act as receptor for multiple ECM ligands. The sarcoglycans are also transmembrane components of the DGC. Assembly of the sarcoglycan complex follows specific steps and requires the presence of all sarcoglycans. The intracellular components of complex include dystrobrevin, syntrophins and nNOS, and these proteins play important roles in protein-protein interaction and signal transduction. As more is learned from exon skipping, the full functionality of the dystrophin complex will be learned in humans.

## Abbreviations

| | |
|---|---|
| **DGC** | dystrophin-glycoprotein complex |
| **DMD** | Duchenne muscular dystrophy |
| **BMD** | Becker muscular dystrophy |
| **ECM** | extracellular matrix |
| **LGMD** | Limb-girdle muscular dystrophy |
| **UGC** | utrophin glycoprotein complex |

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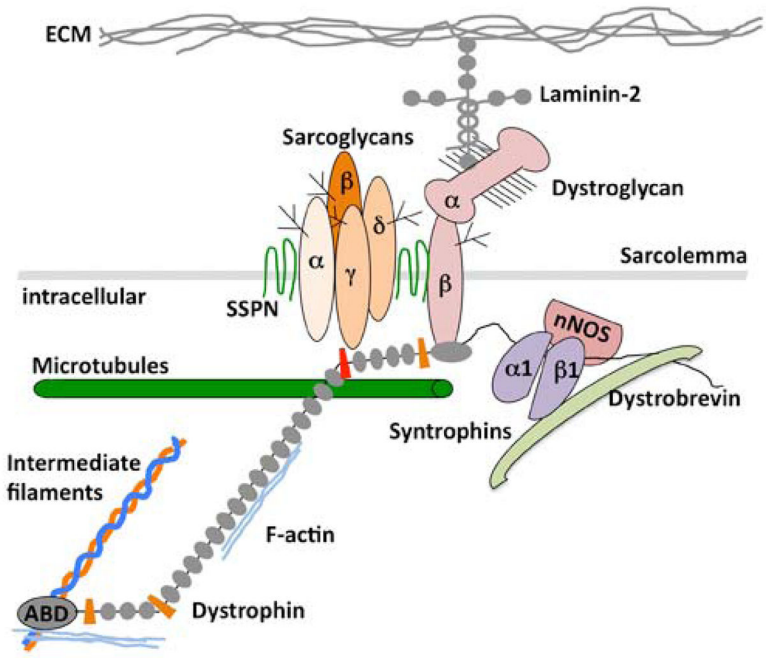


**Figure 1.**

Dystrophin-glycoprotein complex (DGC). Dystrophin is a rod shape protein that links intracellular cytoskeleton network to transmembrane components of the DGC, including dystroglycan, sarcoglycans and sarcospan. Dystroglycan is composed of two subunits, α and β. α-Dystroglycan is an extracellular peripheral membrane protein and a receptor for laminin-2, linking the DGC to the ECM. The sarcoglycans form a tight complex with sarcospan, strengthening the connection between α and β-dystroglycans. Besides a structural role, the sarcoglycan-sarcospan subcomplex is also involved in signal transduction and mechanoprotection. α-Dystroglycan is heavily O-glycosylated (straight lines) in the central mucin domain. β-Dystroglycan and the sarcoglycans contain potential N-glycosylation sites (branch). The syntrophins, dystrobrevins, and nNOS are recruited to the C-terminus of dystrophin and participate in signal transduction pathways.

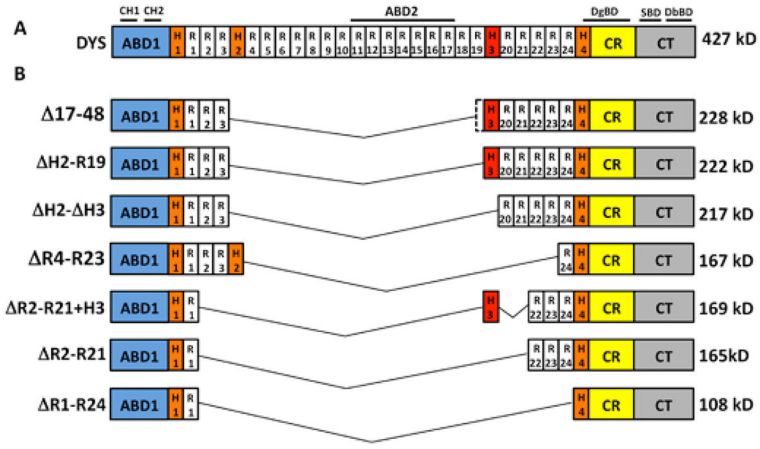


**Figure 2.**

Dystrophin functional domains and mini-/micro-dystrophin constructs. (A) Dystrophin protein has four major functional domains. The N-terminal actin-binding domain (ABD1, shown in blue) contains two calponin-homology (CH) motifs. The central rod domain is composed of 24 spectrin-like repeats (R1-R24, shown in white) interrupted by the proline-rich hinges (H1–H4, shown in yellow). A second actin-binding domain (ABD2) spans R11–R17. The cysteine-rich domain (CR, shown in yellow) and part of H4 form the binding site for β-dystroglycan (DgBD). The C-terminus (CT, shown in grey) contains binding sites for syntrophins (SBD) and dystrobrevin (DbBD). (B) Domain structure of the internally-truncated dystrophin constructs discussed in the text. Note that exon 17–48 deletions (Δ17–48) retains a partial R19. The molecular weights are shown to the right of the constructs.

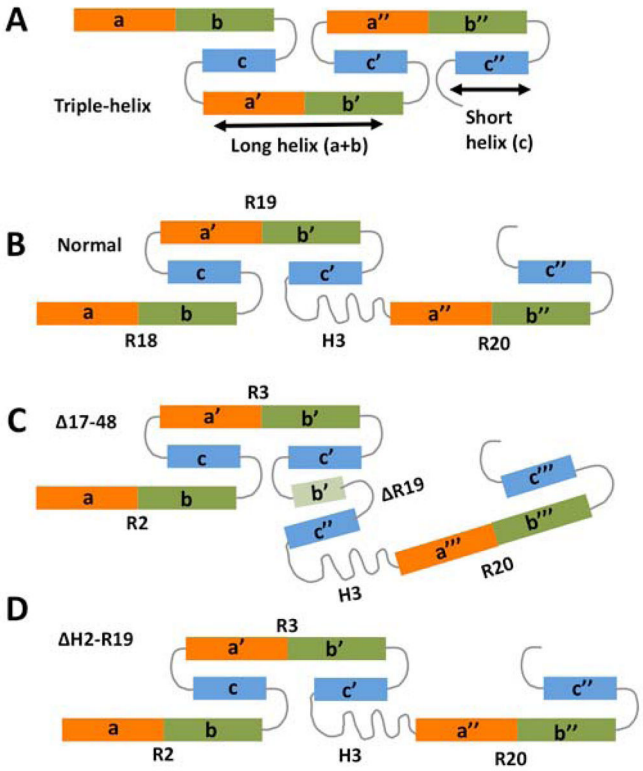


**Figure 3.**

Phasing of spectrin repeats with dystrophin has functional consequences. (A) The central rod of dystrophin is composed of 24 spectrin-like repeat. Each repeat unit is ~110 aa in size and forms a triple α-helical bundles; a and b form the long helix while c forms the short helix. (B) In the normal dystrophin protein, repeat 19 and repeat 20 is separated by hinge 3. (C) The exon Δ17–48 deletion retains the last half of b helix and full c helix from R19 in the protein, producing an extra helical region that may disrupt the folding pattern of the protein. (D) The ΔH2–R19 constructs removes the partial R19 that is retained in the Δ17–48 deletions, resulting in an overall structure identical to that of the normal protein. Maintaining the triple helical structure of each repeat is important for its molecular function. When expressed in the *mdx* mice, ΔH2–R19 construct has a better rescue effects than the Δ17–48 construct (79). Furthermore, clinical observations show that BMD patients carrying deletions that disrupt repeat phasing develop cardiomyopathy ten years earlier than those carrying deletions that retain the correct phasing of the repeat (91).

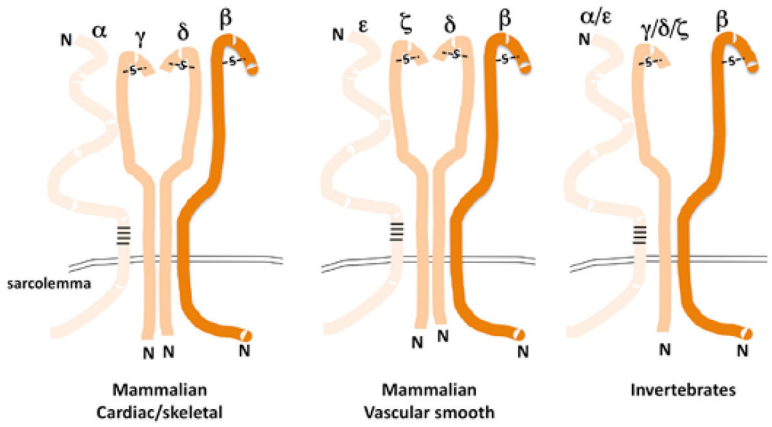


**Figure 4.**

The sarcoglycan complex. Six sarcoglycans have been identified in mammals. α-and ε-sarcoglycans are type I transmembrane proteins and are ~60% related. α- and ε-sarcoglycan genes likely arose from a single gene duplication event since they also have an identical intron-exon structure. There is a single gene related to both α- and ε-sarcoglycan in invertebrates. γ-, δ- and ζ- are type II transmembrane proteins. These three sarcoglycans have identical gene structure and are ~70% similar in protein sequence. There is a single gene related to γ-, δ- and ζ-sarcoglycan in invertebrates, suggesting that they arose from multiple gene duplication events. β-sarcoglycan is also a type II transmembrane protein but is only weakly related to these sarcoglycans. Conserved cysteine residues at the C-terminus of β-, δ-, γ- and ζ- are necessary for intramolecular disulfide bond formation (39). In striated muscle, the major sarcoglycan complex is composed of α-, β-, γ- and δ-sarcoglycan (left). In vascular smooth muscle, the major sarcoglycan complex contains ε-, β-, ζ- and δ-sarcoglycan (middle). In invertebrates (*Drosophila* and *C elegans*), there are only three sarcoglycans, α/ζ -, γ/δ /ζ- and β-sarcoglycan (right).

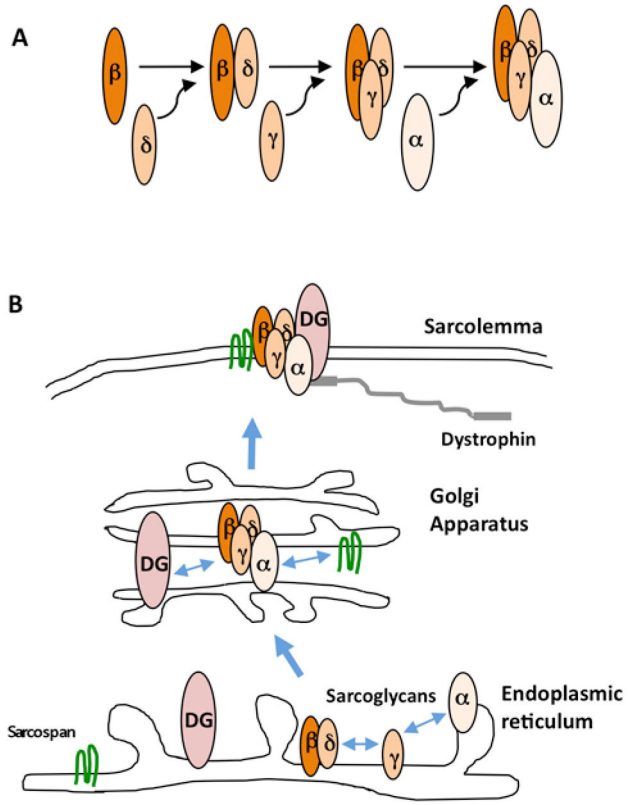


**Figure 5.**

Sarcoglycan complex assembly and sarcolemmal targeting. (A) The assembly of the sarcoglycan complex follows a specific path after protein translation. First, β-sarcoglycan interacts with δ-sarcoglycan to form the complex core. γ-sarcoglycan then associates with the β-δ core. Finally, α-sarcoglycan completes the formation of the complex. Deficiency in any sarcoglycan gene impairs the complex formation and plasma membrane translocation. (B) The sarcoglycan complex formation occurs in the ER. From Golgi to the sarcolemma, the sarcoglycans become associates with dystroglycan and sarcospan. At the sarcolemma, dystrophin reinforces the membrane localization of the sarcoglycans. In the absence of dystrophin, the sarcoglycan complex is also lost from the sarcolemma.