THE UNIVERSITY OF CHICAGO

# RE-ENGINEERING A TRANSMEMBRANE PROTEIN TO TREAT MUSCULAR DYSTROPHY USING EXON SKIPPING

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## ABSTRACT

The dystrophin complex is a multi-protein complex that has both structural and signaling roles in cardiac and skeletal muscle. Loss-of-function mutations in the genes encoding dystrophin, or the associated sarcoglycan proteins, lead to myofiber loss and muscle degeneration. The structure-function correlation between predicted protein structure and clinical outcomes has been extensively cataloged for dystrophin mutations. Studies in animal models also shed light on the dystrophin regions necessary for *in vivo* function. Together, these findings provided the justification for testing exon skipping strategies to restore dystrophin protein in human patients.

Exon skipping uses antisense oligonucleotides as a treatment for genetic diseases. With exon skipping, antisense oligonucleotides target RNA to bypass premature stop codons and restore reading frame disruption. Exon skipping is currently being evaluated in humans with Duchenne Muscular Dystrophy and dystrophin gene mutations. For Duchene Muscular Dystrophy, the rationale for exon skipping derived from observations in patients with naturally occurring dystrophin gene mutations that generated internally deleted but partially functional dystrophin proteins. We now expanded the potential for exon skipping by testing the functionality of an internal, in-frame deletion of a transmembrane protein, γ-sarcoglycan. We generated Mini-gamma by deleting a large portion of the extracellular domain, and showed that Mini-Gamma provided functional and pathological benefit to correct the loss of γ-sarcoglycan in a *Drosophila* model, in heterologous cell expression studies, and in transgenic mice lacking γ-sarcoglycan. Since Mini-Gamma represents removal of four of the seven

coding exons in γ-sarcoglycan, this approach provides a viable strategy to treat the majority of patients with γ-sarcoglycan gene mutations.

**CHAPTER 1**

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

(***Modified from: Gao and McNally, Journal of Comprehensive Physiology, 2015)***

## OVERVIEW

The dystrophin complex stabilizes the plasma membrane of striated muscle cells. Mutations that ablate expression of dystrophin, or the associated sarcoglycan proteins, trigger 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 chapter 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 in order to better define the strategy and assessment of exon skipping for γ-sarcoglycan-mediated muscular dystrophy.

## INTRODUCTION

Muscular dystrophy is a collection of inherited diseases characterized by skeletal muscle weakness and degeneration. Over time, healthy muscle fibers are lost and replaced by fibrosis and fat, making muscle tissues less capable of generating force for everyday activity. As muscle wasting ensues, patients experience muscle weakness, rapid fatigue and pain. Respiratory failure and cardiac complications may limit lifespan in severe forms of muscular dystrophy.

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 (Hoffman et al., 1987). Boys with DMD show signs of muscle weakness early in childhood, typically between 2 and 7 years of age, and often lose ambulation around the time of puberty. Becker muscular dystrophy (BMD) is also caused by mutations in the *DMD* gene. Individuals with BMD share similar signs and symptoms with DMD boys but with later onset and more varied course of disease. The most common type of mutation responsible for DMD and BMD is a deletion spanning one or multiple exons. However, DMD mutations result in frame shifts and leave little or no protein production while BMD mutations produce internally truncated but functional dystrophin proteins.

Current approaches for restoring dystrophin rely on viral-mediated restoration or exon skipping. 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. To circumvent this, a series of mini- and micro-dystrophins has been designed and tested in animal models (Banks et al., 2007; Harper et al., 2002;

Zhang and Duan, 2012) (Figure 1.2). Exon skipping is a distinct therapeutic approach for DMD. Exon skipping uses antisense oligonucleotides to induce alternative splicing that bypasses mutated exons in order to repair protein reading frame, shifting a DMD mutation to a BMD mutation (Figure 1.3). Both approaches require a detailed understanding of structure-function analysis of the DMD gene and dystrophin protein production.

The type2 Limb Girdle muscular dystrophies (LGMD) are caused by autosomal recessive mutations and affect 1 in 15,000 individuals (Nigro and Savarese, 2014). A number of genes that cause LGMD2s encode proteins that are directly associated with dystrophin, forming integral parts of the dystrophin complex. 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 (Danialou et al., 2001; Petrof et al., 1993). In addition to its structural role, dystrophin complex is also thought to mediate cellular signaling such as mechanical force transduction and cell adhesion.

## COMPONENTS OF THE DYSTROPHIN COMPLEX

The dystrophin-associated proteins can be divided into three groups based on their cellular localization: extracellular ($\alpha$-dystroglycan); transmembrane ($\beta$-dystroglycan, sarcoglycans, sarcospan); cytoplasmic (dystrophin, dystrobrevin, syntrophins, neuronal nitric oxide synthase) (Figure 1.1). $\alpha$-Dystroglycan functions as a receptor for the

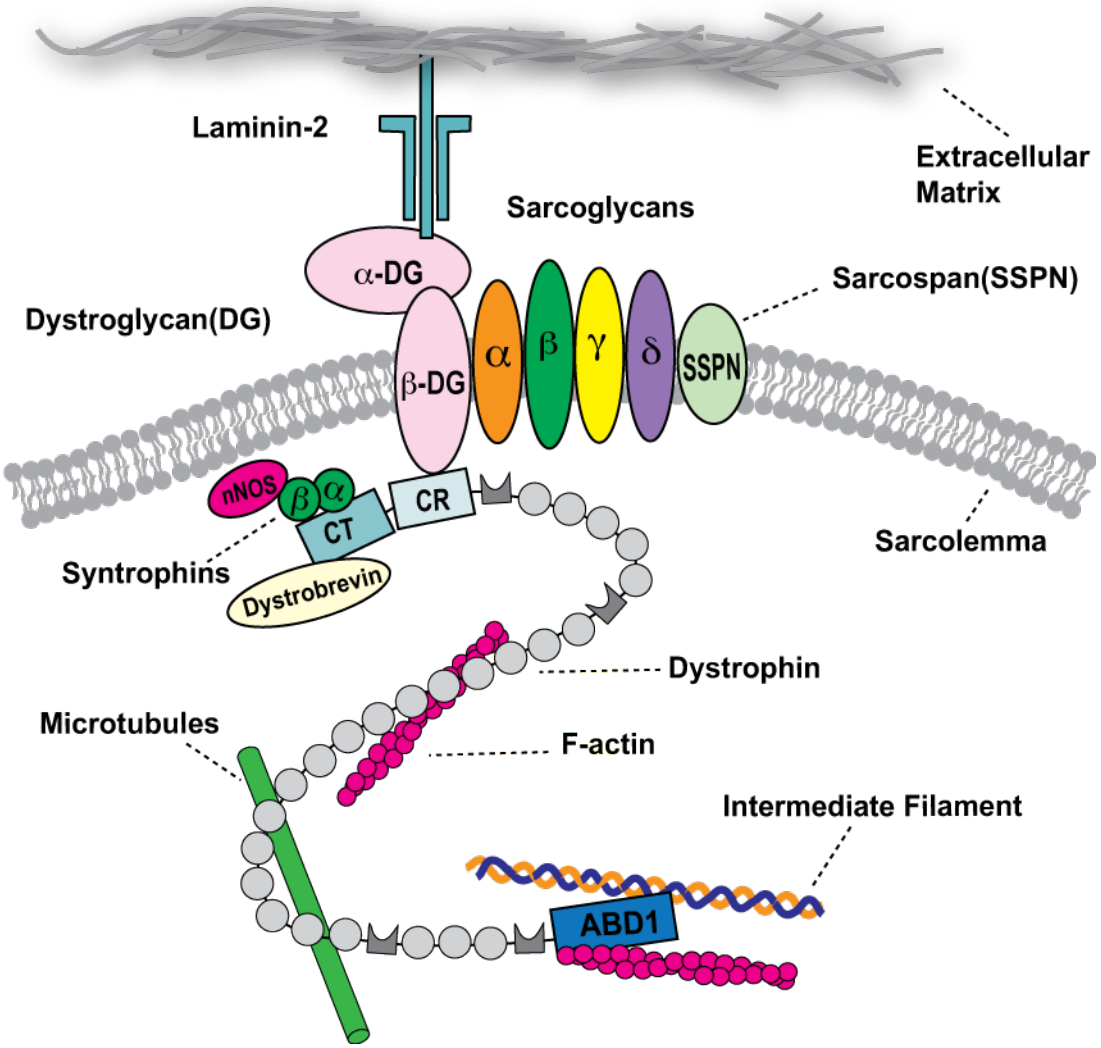


**Figure 1.1. Dystrophin complex. Dystrophin is a large rod-shaped protein with many spectrin repeats (shown in grey).** Dystrophin interacts with intracellular cytoskeleton proteins via its N-terminal actin binding domain (ABD1, in blue) and rod domain. Through its cysteine-rich domain (CR, in cyan) and C-terminus (CT, in light cyan), dystrophin associates with transmembrane components of the DGC, including β-dystroglycan, sarcoglycans and sarcospan. α-Dystroglycan is a peripheral membrane protein and a receptor for extracellular matrix (ECM) proteins such as laminin-2. In the skeletal muscle, the sarcoglycan complex is composed of α-, β-, γ- and δ-sarcoglycans. The sarcoglycan complex is tightly associated with sarcospan, a 25-kDa transmembrane protein that is important for regulating cell adhesion. The syntrophins, dystrobrevin and the neuronal nitric oxide synthase (nNOS) are recruited to the C-terminus of dystrophin and involved in signal transduction.

extracellular ligands such as laminin (Ibraghimov-Beskrovnaya et al., 1992). 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, respectively (Nigro and Savarese, 2014). α-Dystroglycan is tightly associated with β-dystroglycan, a transmembrane protein that also interacts with dystrophin and the sarcoglycans.

The sarcoglycan subcomplex 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. Sarcospan is a small transmembrane protein that is tightly associated with the sarcoglycans. Sarcospan is a member of the tetraspanin family that is known to interact with integrins (Aplin et al., 1998; Crosbie et al., 1997)

At the cytoplasmic face of the sarcolemma, dystrophin binds to β-dystroglycan via its cysteine riche domain (CR). Dystrophin interacts with intracellular cytoskeleton proteins including F-actin, intermediate filaments and microtubules. Other cytoplasmic components associated with dystrophin include α-dystrobrevin, syntrophins and neuronal nitric oxide synthase (nNOS).

## BIOLOGY OF THE DYSTROPHIN COMPLEX IN MUSCLE

Both cardiac and skeletal muscles are striated muscles. Striated muscle cells are marked by transverse dark (Z-line) and light bands (M-line). A sarcomere is defined as the segment between two neighboring Z-lines and serves as the basic structural unit

of striated muscle. The sarcomere is mainly composed of overlapping thin (actin) and thick (myosin) filaments. M-line, the middle area of the sarcomere, is made of myosin filaments that are not superimposed by actin filaments. Striated muscles generate force by shortening individual sarcomeres. The sarcomere contracts when the myosin heads projecting from the myosin filaments bind to and “walk” along the adjacent actin filaments. The Z-line is an important anchor for the actin-myosin filaments from the adjoining sarcomeres. The antiparallel actin filaments from adjacent sarcomeres are cross-linked by layers of $\alpha$-actinin at the Z-line. Titin and nebulin are two giant polymer proteins in muscle that overlap with and form essential parts of the Z-line (Clark et al., 2002). Titin spans half of the sarcomere, connecting the M-line to the Z-line, and consists of many elastic regions. TItin serves as a "molecular spring" and restricts the range of motion of the sarcomere in tension. Similar to the actin filaments, titin polymers are also cross-linked by $\alpha$-actinin at the Z-line (Young et al., 1998). Nebulin protein runs along the thin filaments and has been hypothesized as the template for thin filament assembly (McElhinny et al., 2003).

Force transmission in muscle fibers can occur both longitudinal and lateral to the long axis of the sarcomere. In the longitudinal direction, force is transduced from one sarcomere to the next sarcomere till the end of the fiber is reached. In the lateral direction, force is transduced from intracellular myofibrils to the extracellular matrix. Recent studies have shown that only 20-30% of the total force generated by the sarcomere is transduced longitudinally, suggesting that force transmission occurs mainly in the lateral fashion (Bloch and Gonzalez-Serratos, 2003). Costameres are Z-

line associated membrane-associated structures that are critical for lateral force transmission. Costameres share characteristics of the focal adhesion assemblies in other cell types. Like focal adhesions, costameres are multi-protein containing complexes that serve as linkages from inside of the cell through the membrane to ECM. Focal adhesions are mainly composed of the transmembrane integrins and cytoplasmic adapter proteins. Interestingly, two major large protein complexes are found at the costameres: dystrophin complex and the integrin-vinculin-talin complex (Ervasti, 2003). In dystrophin complex, the dystroglycan serves as the receptor for ECM ligands such as laminin, perlecan, and agrin (Barresi and Campbell, 2006). On the cytoplasmic side, dystrophin links directly to dystroglycan and also connects actin filaments, especially γ-actin. In striated muscle, γ-actin constitutes the subsarcolemmal actin cytoskeleton and has distinct costameric association expression pattern (Rybakova et al., 2000). The enrichment of γ-actin filaments at costamere is disrupted in the absence of dystrophin, suggesting that the dystrophin complex is essential for stabilizing cortical actin network at the Z-line (Rybakova et al., 2000). In addition to the dystrophin complex, the integrin-vinculin-talin complex is also involved in anchoring actin to the sarcolemma at the costameric regions (Peter et al., 2011).

Filamins are a class of actin cross-linkers in cortical cytoplasm and involved in anchoring the actin cytoskeleton to transmembrane proteins such as the integrins (Stossel et al., 2001). Filamin A is the best characterized member of the filamin family. Filamin A is widely expressed and has been shown to protect cells from mechanical stress by increasing the rigidity of the cortical actin network (D'Addario et al., 2003;

D'Addario et al., 2001; Shifrin et al., 2009).

Filamin C is a muscle specific member of the filamins and interacts with both the dystrophin complex and the integrins (Loo DT et al., 1998. PMID: 9722563; Thompson TG et al., 2000. PMID: 10629222). Filamin C interacts with the dystrophin complex through γ- and δ-sarcoglycan and associates with integrins through β1-integrin (Loo et al., 1998; Thompson et al., 2000). Mutations in the human filamin-C gene have been associated with inherited skeletal muscle and cardiac myopathies (Furst et al., 2013). Filamin C deficient mice have defects in myogenesis and live only a short period of time after birth, limiting detailed analysis of filamin C's role in postnatal life (Dalkilic et al., 2006).

Fijita et al. characterized a filamin C null mutant in Medaka fish, referred to as *zac,* that developed dilated heart and progressive skeletal muscle degeneration at later embryonic stages (Fujita et al., 2012). These mutants survive for as long as two weeks after hatching. In *zac* mutant muscle, sarcomere structures become more fragile to mechanical stress caused by muscle contraction. The destabilization of sarcomeres is accompanied by greatly reduced γ-actin filaments in *zac* mutant muscle. Therefore, it is likely that the sarcoglycan-integrin-filamin C network acts to prevent muscle cells from mechanical stress induced damage by regulating the organization of costameric γ-actin filaments.

Both dystroglycan and the integrins serve as receptors for laminins, the major component of the ECM in the muscle tissue, and therefore mediate direct attachment of muscle cells to the ECM (Peter et al., 2011). Mutations in α2 laminin lead to congenital

muscular dystrophy (Wewer and Engvall, 1996). In dystrophin and γ-sarcoglycan deficient mouse models, integrin level increases at the sarcolemma to compensate for the disruption of the dystrophin complex (Allikian et al., 2004; Rooney et al., 2006).

Utrophin is similar to dystrophin in structure and function and acts to partially compensate for dystrophin loss in *mdx* mice (Deconinck et al., 1997). In dystrophin and utrophin double knockout mice, a much more severe muscular dystrophy phenotype similar to that of Duchenne muscular dystrophy patients was observed (Deconinck et al., 1997). The dystrophic phenotype was significantly ameliorated by overexpressing the α7 integrin chain via transgene, suggesting that an increased level of the integrins is sufficient to strengthen the linkage between the cytoskeleton and ECM in the absence of dystrophin/utrophin complex (Burkin et al., 2005).

Sarcospan is a member of the tetraspanin protein family and an integral transmembrane component of the dystrophin complex (Crosbie et al., 1997). Tetraspanins are involved in the clustering of many transmembrane receptors including the integrins (Boucheix and Rubinstein, 2001). Overexpressing sarcospan in *mdx* mice increases the expression level of utrophin and integrins at the sarcolemma in a dosage-dependent manner (transgene expressed at 0.5X, 1.5X, and 3X respectively) (Peter et al., 2008). In sarcospan overexpressing transgenic *mdx* mice, the dystrophic phenotypes are significantly improved. This improvement is dependent on both utrophin and integrins, suggesting that the rescue mechanism of sarcospan is likely to be the upregulation of utrophin and integrins as compensatory adhesion complexes (Marshall et al., 2015). Interestingly, a severe muscular dystrophy phenotype is

observed when sarcospan protein is overexpressed by ten fold in the WT mice. In these transgenic animals, sarcospan protein network clusters the sarcoglycans into insoluble protein aggregates and causes the destabilization of $\alpha$-dystroglycan (Peter et al., 2007).

## DYSTROPHIN

### *Dystrophin functional domain*

Dystrophin is a 427kDa protein that localizes to the cytoplasmic face of the sarcolemma (Porter et al., 1992). Dystrophin protein has four main functional domains; an actin-binding domain (ABD1), a central rod domain, a cysteine-rich domain (CR) and a carboxyl-terminus (CT) (Figure 1.2). ABD1 contains 2 calponin homology domains (CH1 and CH2) (Korenbaum and Rivero, 2002). This conventional CH-actin binding domain binds directly to F-actin (Way et al., 1992). ABD1 also binds to intermediate filament protein cytokeratin 19 (K19), providing additional link to the contractile apparatus in muscle cells (Stone et al., 2005; Stone et al., 2007).

Dystrophin central rod domain contains 24 spectrin repeats and serves as a flexible linker between the N- and C-termini (Broderick and Winder, 2005; Koenig et al., 1988). The rod domain harbors a second actin-binding motif (ABD2) near the middle of the rod (Amann et al., 1998). ABD2 collaborates with ABD1 to form a strong lateral association with actin filaments (Rybakova et al., 1996). The rod domain also interacts with microtubules via spectrin-like repeats 20-23 and is required for the organization of microtubule lattice in skeletal muscle cells (Belanto et al., 2014; Prins et al., 2009).

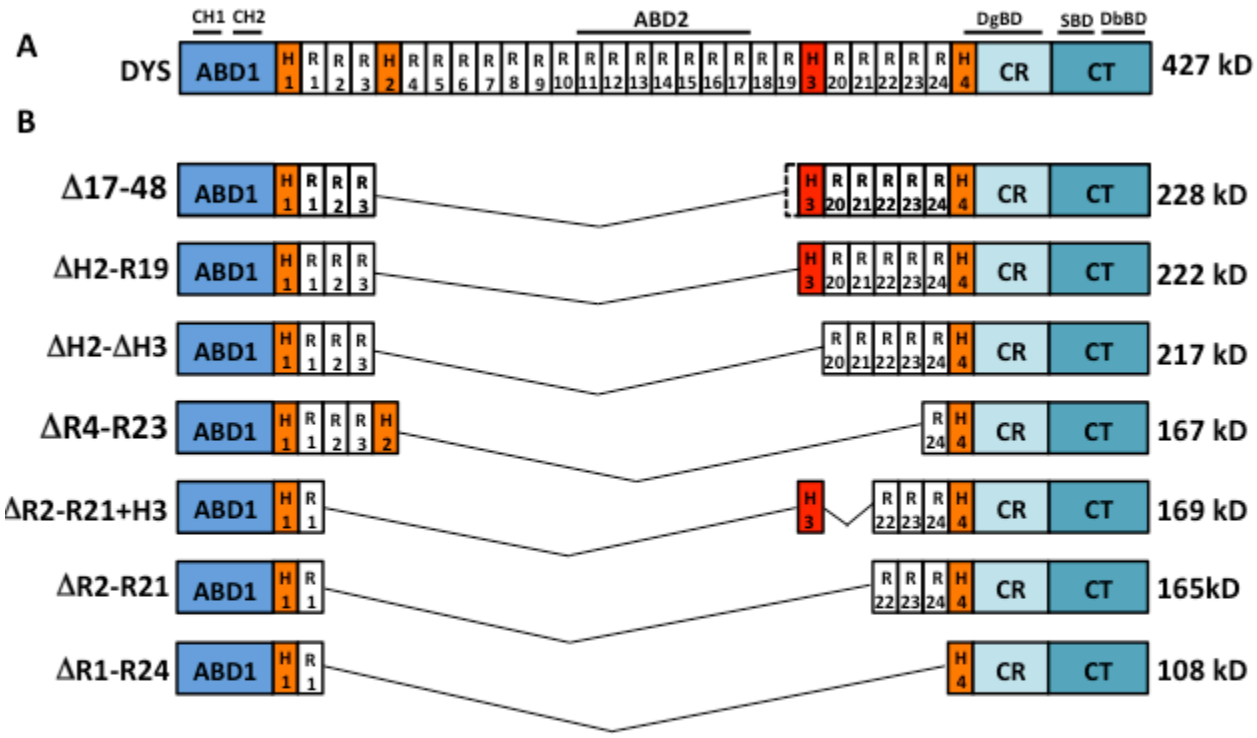


**Figure 1.2. Dystrophin functional domains and mini-/micro-dystrophin constructs. (A) Dystrophin protein has four functional domains.** The N-terminal actin-binding domain (ABD1, shown in blue) contains two calponin-homology (CH) motifs. The central rod domain is composed of 24 spectrin-like repeats (R1-R24, shown in white) interrupted by the proline-rich hinges (H1-H4, shown in orange and red). A second actin-binding domain (ABD2) spans R11-R17. The cysteine-rich domain (CR, shown in light cyan) and part of H4 form the binding site for β-dystroglycan (DgBD). The C-terminus (CT, shown in cyan) 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 deletion (Δ17-48) retains a partial R19. The molecular weights are shown to the right of the construct.

Dystrophin's rod has also been shown to bind membrane phospholipids *in vitro* via the tryptophan residues in the spectrin-like repeats (Le Rumeur et al., 2003). This interaction is thought to strengthen the sarcolemma targeting of dystrophin. The 24 spectrin repeats are interrupted by four short proline-rich spacers, called "hinges" as they provide elasticity to the protein (Koenig and Kunkel, 1990). Hinge 4 is at the end of the rod domain and contains a WW domain, a domain implicated in protein-protein interactions (Ilsley et al., 2002). The WW domain along with two neighboring EF-hands in the cysteine-rich domain bind to β-dystroglycan (Rentschler et al., 1999). The EF-hand motifs have been implicated in calcium binding (Koenig et al., 1988). The cysteine-rich domain also contains a zinc finger (ZZ) that binds to calmodulin in a calcium-dependent manner (Anderson et al., 1996). The cysteine-rich domain in dystrophin has also been shown to bind to ankyrin-B, an adaptor protein that is required for retaining dystrophin at the sarcolemma (Ayalon et al., 2008). 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 (Bhosle et al., 2006). The carboxy-terminal (CT) domain contains two polypeptides that fold into α-helical coiled-coils similar to the spectrin repeats in the rod domain (Blake et al., 1995). The CT domain provides binding sites for dystrobrevin and syntrophins (Sadoulet-Puccio et al., 1997).

*Dystrophin mutations and reading frame rule*

The dystrophin gene is the largest known human gene, containing 79 exons and

spanning > 2,200 Kb, roughly 0.1% of the whole genome (Koenig et al., 1987)(Figure 1.3). 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 (Flanigan et al., 2009; Tuffery-Giraud et al., 2009). DMD is associated with deletions that disrupt the protein's reading frame causing premature stop codons. These mutated transcripts are susceptible to nonsense mediate decay, and the C-terminal truncated protein products are also unstable and subject to degradation, leaving little or no protein production. In contrast, BMD patients usually have in-frame deletions that maintain the correct reading frame. 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. 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. Both the level and content of residual dystrophin protein expression are important for determining phenotype. Patients with less than 10% dystrophin all show severe disease symptoms. When dystrophin level is above 10%, the clinical outcomes are more dependent on the type of the truncated dystrophin (Anthony et al., 2011; van den Bergen et al., 2014).

The "reading frame" rule for BMD and DMD is consistent with 80~90% of the cases (Flanigan et al., 2009; Koenig et al., 1989). One dramatic example of an in-frame *DMD* gene mutation was described that removed 46% of the coding sequence (exon 17 to exon 48) (England et al., 1990). This deletion encompassed hinge 2, spectrin

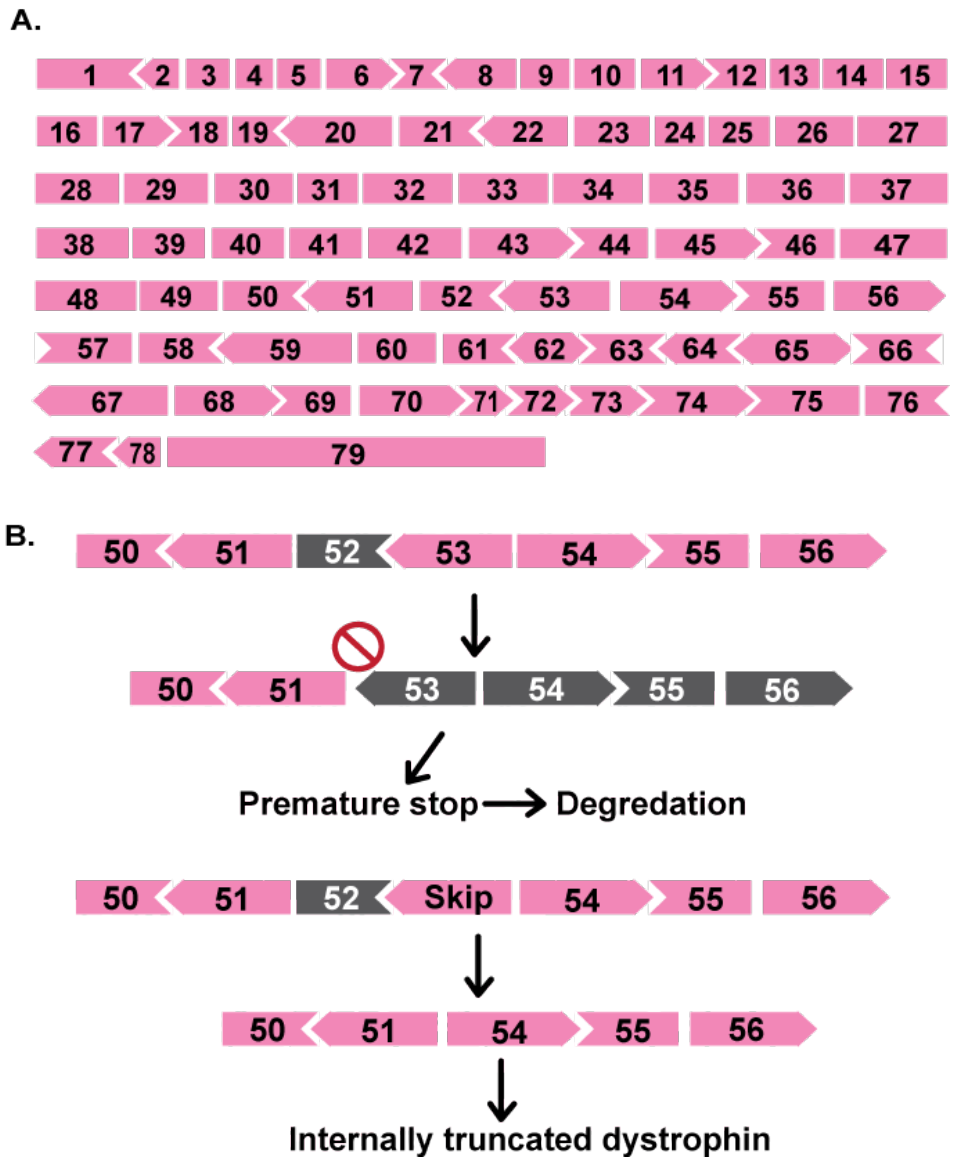


**Figure 1.3. Mechanism of applying exon skipping to restore dystrophin expression in DMD.** (A) The dystrophin gene is the largest known human gene, containing 79 exons and spanning > 2,200 Kb, roughly 0.1% of the whole genome (Koenig et al., 1987). Two thirds of *DMD* mutations are deletions that span one or multiple exons. These large deletions tend to cluster around two mutational hotspots (Liechti-Gallati et al., 1989). The most common hotspot spans from exon 45 to exon 55. The second hotspot spans from exon 3 to exon 19. (B) In patients carrying exon 52 deletion, exon 51 and exon 53 are joined up together, resulting in reading frame disruption and no protein production. When exon 53 is excluded via exon skipping inducing antisense oligos, exon 51 and exon 54 are joined up together and the reading frame is maintained, producing a truncated but functional dystrophin protein.

repeats 4 to 18, and a portion of spectrin repeat 19 (Figure 1.2). 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 (Athanasopoulos et al., 2004). 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. Exon skipping uses antisense oligos to induce the bypass of target exons and restores the reading frame and dystrophin production (Figure 1.3).

*Functionality of mini- and micro-dystrophins*

Mini-dystrophins, defined as containing more than four spectrin repeats, and micro-dystrophins, defined as those with four or fewer than four spectrin repeats, are designed and tested in mouse models (Figure 1.2). *Mdx* mice carry a nonsense mutation in exon 23 in the dystrophin gene and do not express dystrophin protein. Expressing a mini-dystrophin transgene, which encoded a protein missing regions encoded by exons 17-48 ($\Delta$17-48), in *mdx* mice reversed many of the dystrophic changes (Phelps et al., 1995). 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, central nucleation of the quadriceps muscles were only slightly improved. 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 folds of control levels (Phelps et al., 1995). Consistent with the clinical data, 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.

The phasing of the spectrin repeats within the rod region affects the functionality of truncated dystrophins. Notably, the Δ17-48 construct expressed a dystrophin that contain 8.5 spectrin repeats (Figure 1.2). A second construct that was tested was referred to as ΔH2-R19, containing an even eight perfectly phased spectrin repeats (Figure 1.2). The ΔH2-R19 transgenic *mdx* mice were not different from wild type mice by specific force measurement in both EDL and diaphragm muscles (Harper et al., 2002). 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. The important of correct phasing of the spectrin repeats is also supported by an *in vivo* approach 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 (Kaspar et al., 2009). 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 (Ruszczak et al., 2009). 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.

Dystrophin central hinge also appears to be important for protein function. ΔH2-H3 construct was generated and differed from ΔH2-R19 by the absence or presence of hinge 3 (Figure 1.2) (Harper et al., 2002). 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 1.2) (Harper et al., 2002). Among the three different micro-dystrophins, ΔR4-R23 had four repeats plus hinge2 and was the most effective since ΔR4-ΔR23 transgenic *mdx* had almost normal histology. ΔR2-R21+H3 contains four repeats but a different central hinge (hinge 3). ΔR2-R21+H3 restored a normal histology in diaphragm muscle, yet the limb muscles retained dystrophic pathology, suggesting less rescue efficiency. ΔR2-R21 micro-dystrophin 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.

Importantly, the variable expression level of the constructs must be considered when interpreting the rescue data. For example, ΔH2-R19 construct was expressed at a higher level than that of ΔH2-ΔH3 and this higher expression level 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 (Harper et al., 2002).

*Insights into exon skipping design*

Deletions that span exon 45 to exon 55 (del45-55) are associated with very mild or even asymptomatic cases (Ferreiro et al., 2009; Nakamura et al., 2008). 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 (Flanigan et al., 2009). Although generally mild, variations of disease severity exist between patients carrying del45-55, and this variable phenotype correlates with nNOS sarcolemma localization (Flanigan et al., 2009). 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 patients with a deletion (Beroud et al., 2007). 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 (Aoki et al., 2012). 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 previously discussed studies showing a threshold effect of dystrophin. 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 (Goemans et al., 2011).

The N-terminal actin-binding domain (ABD1) appears to be less dispensable than the rod domain when considering exon skipping strategy. Deletions within the ABD1 usually results in reduction of dystrophin level and is associated with more severe BMD (Vainzof et al., 1993). Furthermore, more than 50% of missense mutations that cause disease occur in the ABD1 (Singh et al., 2010). The effects of these missense mutations were tested *in vitro* and suggest that ABD1 is not only essential for actin

binding, but also for proper protein folding and protein stability throughout the dystrophin protein (Banks et al., 2010; Singh et al., 2010).

Curiously C-terminal truncated dystrophins have been described and produced where they have been observed at the sarcolemma (Bies et al., 1992; Recan et al., 1992). The deletions in the above cases encompass both cysteine-rich domain and C-terminus, suggesting that neither is required for plasma membrane localization. However, these patients developed DMD, indicating additional function can be ascribed to this region. Interestingly, the micro-dystrophin lacking a C-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 (Yue et al., 2006). 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 dystrophin complex (Yoshida et al., 1994). Six mammalian sarcoglycans have been identified so far: α (50kDa), β (43kDa), γ (35kDa), δ (35kDa), ε (50kDa), and ζ (40kDa), (Figure 1.4). All sarcoglycans are single pass transmembrane proteins with at least one asparagine-linked glycosylated residue (Figure 1.4, branches). Both α/ε-sarcoglycan are closely related proteins with similar gene and protein structure, and these are both type I transmembrane proteins with a cleaved amino-terminal signal

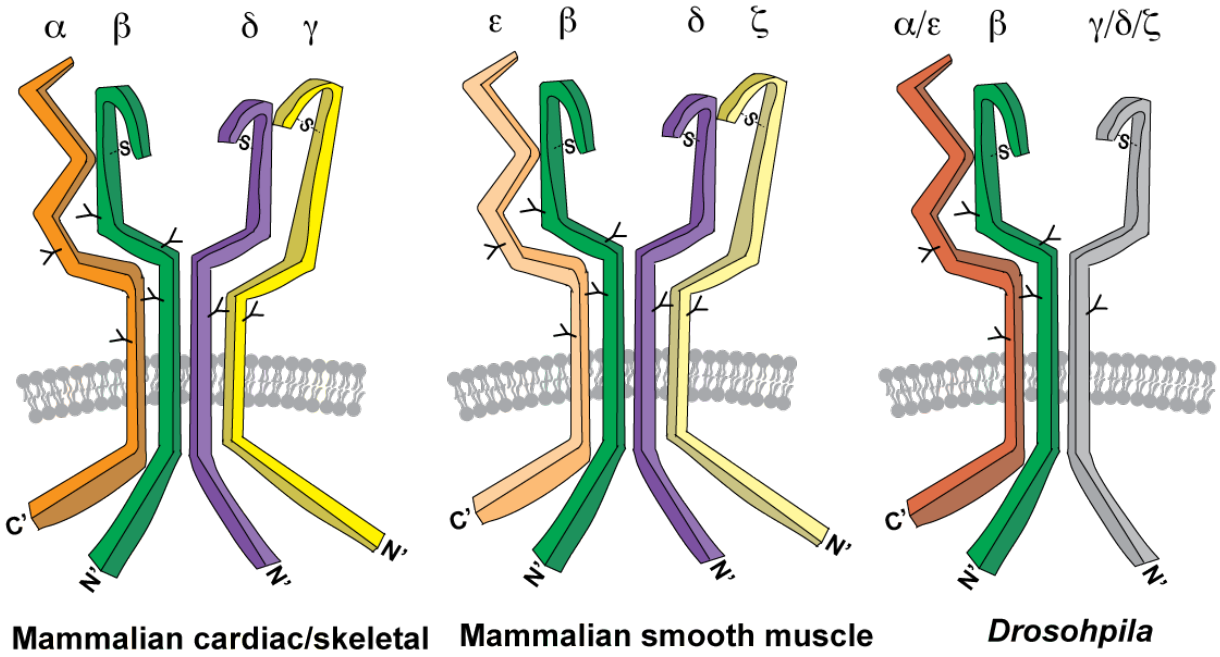


**Figure 1.4. The sarcoglycan complex.** Six sarcoglycans have been identified in mammals. α-Sarcoglycan and ε-sarcoglycans are type I transmembrane proteins and are ~60% similar. α-Sarcoglycan and ε-sarcoglycan genes likely arose from a single gene duplication event since they also have an identical intron-exon structure. There is a single gene related to both α- and ε-sarcoglycan in invertebrates. γ-Sarcoglycan, δ-sarcoglycan, and ζ-sarcoglycan are type II transmembrane proteins. These three sarcoglycans have identical gene structure and are ~70% similar. There is a single gene related to γ-, δ- and ζ-sarcoglycan genes 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 intra-molecular disulfide bond formation (Chan et al., 1998). 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/ε-, γ/δ/ζ- and β-sarcoglycan (right).

sequence. γ/δ/ζ-Sarcoglycans are also highly related to each other with similar gene and protein structure, but these sarcoglycans, like β-sarcoglycan, are type II transmembrane sequences with an intracellular amino-terminal domain and extracellular C-terminal domain. There is weak similarity between β-sarcoglycan and γ/δ/ζ- sarcoglycans, especially in the distal portion of the C-terminus with conserved cysteines that form disulfide bonds. The sarcoglycan complex is evolutionarily conserved. In zebrafish, five out of the six sarcoglycans have ortholog (Chambers et al., 2003). 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 (Allikian et al., 2007; Grisoni et al., 2002).

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-sarcoglycans are maintained (Imamura et al., 2005; Wheeler et al., 2002). 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 (Imamura et al., 2005). Non-muscle forms of the sarcoglycan complex are present with varying composition of sarcoglycan subunits (Anastasi, 2010; Waite et

al., 2012). 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 (Zimprich et al., 2001).

*Sarcoglycan complex assembly in muscle*

Correct assembly of the sarcoglycan complex is required for its stability and sarcolemma targeting. 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 (Chan et al., 1998). A complex containing α-, β, γ-, and δ-sarcoglycan was co-immunoprecipitated from myotubes. With increasing stringency of the immunoprecipitation, α-SG was completely dissociated from the complex at 0.3% SDS. 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 (Shi et al., 2004). 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 1.5). Supporting this model, during myoblast differentiation β-sarcoglycan and δ-sarcoglycan protein expression is detected prior to α- and γ-sarcoglycan protein (Noguchi et al., 2000). 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 (Noguchi et al., 2000) (Figure 1.5).

This assembly model is also supported by observations in muscle from patients with sarcoglycan gene mutations and sarcoglycan mutant animals. 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 (Hack et al., 2000). In muscles from *SGCB* mutated patients and primary culture derived from the biopsy, complete loss of the other three sarcoglycans was observed (Fanin and Angelini, 2002). In patients with *SGCA* mutations, the other three sarcoglycans are often still present at the sarcolemma though at a reduced level, suggesting α-sarcoglycan association may not be required for the membrane trafficking

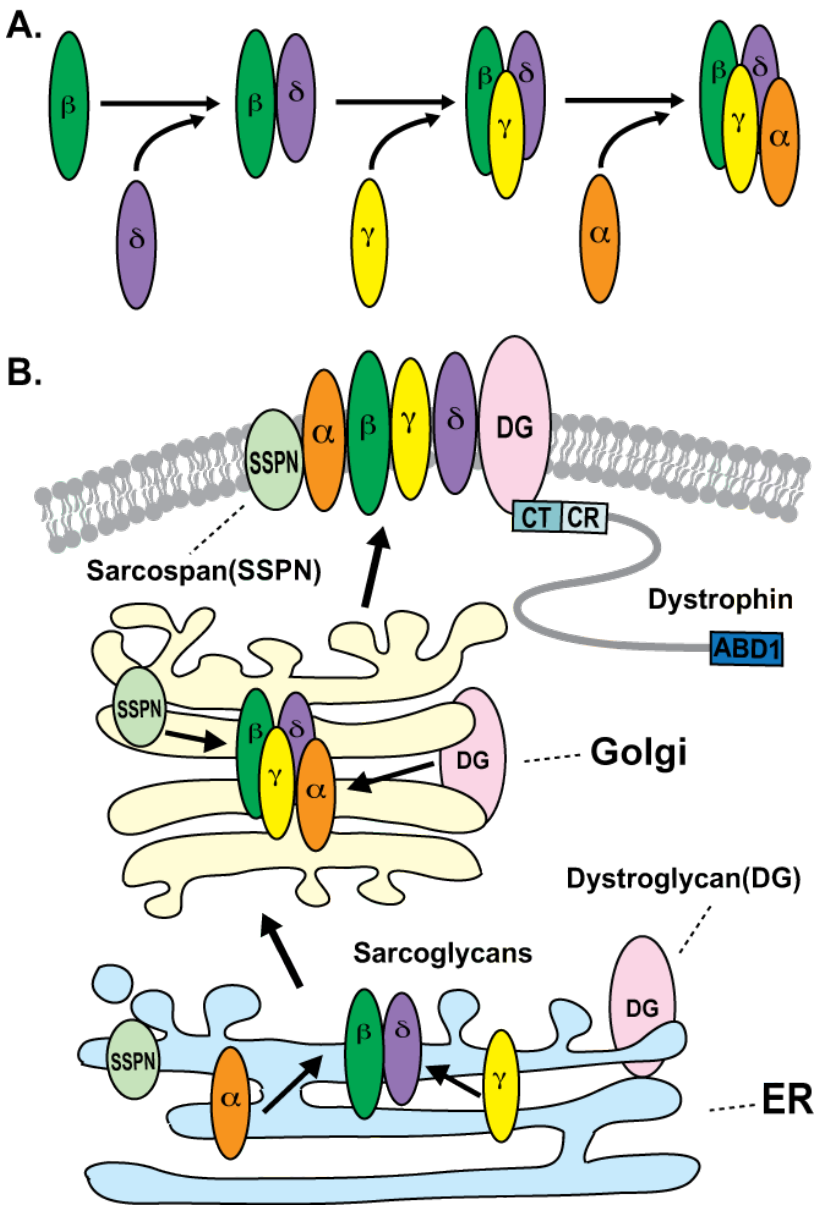


**Figure 1.5. Sarcoglycan complex assembly and sarcolemmal targeting.** (A) The assembly of the sarcoglycan complex follows a specific order. First, β-sarcoglycan associates with δ-sarcoglycan to form the core of the complex. Next, γ-sarcoglycan is added to the β-δ core. Finally, α-sarcoglycan is attached, completing complex assembly. Deficiency in any sarcoglycan gene impairs the complex formation and plasma membrane translocation. (B) The sarcoglycan complx assembly occurs from the ER en route to the Golgi complex. From the Golgi to the sarcolemma, the sarcoglycans become associated with dystrophin, dystroglycan and sarcospan. At the sarcolemma, dystrophin and dystroglycan strengthens the membrane localization of the sarcoglycans. In the absence of dystrophin, the sarcoglycan complex is also lost from the sarcolemma.

of the SG complex (Hackman et al., 2005). 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 and ER stress (Dressman et al., 2002; Zhu et al., 2001).

Functional domains have been identified in sarcoglycans that are required for their interaction and membrane targeting (Chen et al., 2006). 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 (Chen et al., 2006).

*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. Sarcoglycan deficient mice develop membrane disruptions and sarcolemmal leak in muscle. This is demonstrated by both the abnormal uptake of Evans blue dye and the elevated serum creatine kinase levels, similar to muscles lacking dystrophin (Araishi et al., 1999; Hack et al., 2000; Hack et al., 1998). 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 (Hack et al., 2000). The exact role of the sarcoglycan complex is not fully understood, yet both structural and signaling roles are proposed.

The sarcoglycan complex stabilizes the dystrophin complex through at least three mechanisms. Firstly, the sarcoglycan complex is tightly linked to dystroglycan. The sarcoglycan complex and dystroglycan can be dissociated together as a unit from purified dystrophin complex (Yoshida et al., 2000). Furthermore, δ-sarcoglycan was shown to interact directly with β-dystroglycan in mouse myotubes (Chan et al., 1998). The BIO14.6 hamster is a muscular dystrophy model that is deficient in δ-sarcoglycan. In BIO14.6 hamster, dystroglycan was shown to be dissociated from the sarcoglycan complex and the cell surface anchorage of α-dystroglycan was also shown to be disrupted, while dystrophin expression was similar to WT (Straub et al., 1998). Secondly, the sarcoglycans have been shown to interact directly with dystrobrevin (Yoshida et al., 2000). Dystrobrevin is a dystrophin-related protein that heterodimerizes with dystrophin through coiled-coil motifs (Sadoulet-Puccio et al., 1997). The sarcoglycan-dystrobrevin-dystrophin interaction further holds the complex together. Thirdly, the sarcoglycans are very tightly associated with sarcospan, a 25kDa transmembrane protein that belongs to the tetraspan family (Crosbie et al., 1999). 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 (Miller et al., 2007). In all, interactions between the sarcoglycans and other complex members contribute to the integrity and membrane

anchorage/organization of the larger complex.

Eccentric contraction (ECC) is an *ex vivo* muscle mechanic protocol where muscles are stimulated in a stretched condition (110% of optimal muscle length). Multiple contractions are induced with resting periods in between and force measurements are taken for each contraction. Muscles lacking dystrophin or δ-sarcoglycan showed a substantial force drop after several rounds of eccentric contractions that was coupled to increased dye uptake, supporting the previous notion that these membranes became less stable when the dystrophin complex is disrupted (Hack et al., 2000). In γ-sarcoglycan null muscle however, the force drop and percentage of dye positive fibers after eccentric contraction was similar to that of WT (Hack et al., 1999). However, LGMD 2C patients and γ-sarcoglycan mutant animals still suffer from substantial muscle cell damage/death and severe muscle degeneration, indicating that γ-sarcoglycan has other important roles in addition to stabilizing the dystrophin complex.

γ- and δ-sarcoglycans are involved in the mechanoprotection process through their interaction with filamin C. Filamin C (FLNC) protein binds to the cytoplasmic tails of γ- and δ-sarcoglycan (Thompson et al., 2000). FLNC is the only muscle specific member of the filamin protein family. The filamins are known for their roles in actin organization and mechanoprotection, a process protecting cells from external stress (Stossel et al., 2001). 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 may contribute to a disrupted actin network and impairs resistance to strain at sarcolemma. Follow up studies showed that Calpain-3 regulates the interaction between the sarcoglycan and FLNC by cleaving the C-terminus of FLNC, which contains binding site for the sarcoglycans (Guyon et al., 2003). Calpain-3 is the muscle-specific member of the calcium-dependent protease family. Mutations in the calpain-3 gene cause LGMD 2A. The sarcoglycan-FLNC-calpain-3 interaction provides additional insight in disease mechanism underlying muscular dystrophy. It would be interesting to further examine the significance of the cleavage events of filamin-C by calpain-3 in muscle cells.

γ-Sarcoglycan mediates mechanical signal transduction via phosphorylation of the tyrosine residue at the 6$^{th}$ amino acid in γ-sarcoglycan. Isolated muscle fibers from *Sgcg* null mice showed higher phosphorylation levels of both ERK-1 and ERK2 at resting state than WT (Barton, 2006). When subject to eccentric contractions, 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 phosphorylation level was seen in *Sgcg* null muscle compared to WT, suggesting a compensatory response. Failure of the ERK-1 phosphorylation response is independent of contractile damage since the force-drop after eccentric contractions 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 (Barton, 2010). More recently, γ-

sarcoglycan was shown to regulate another mechanical-responsive kinase, p70S6K (Hornberger et al., 2004; Moorwood et al., 2014). Upon stretch, p70S6K was activated in both *Sgcg* null and WT muscles but failed to return to normal level in mutant muscle. Both ERK-1 and p70S6 kinases are implicated in cell survival and growth (Cheung and Slack, 2004; Harada et al., 2001). Aberrant regulation of the kinases can contribute to muscle cell death in γ-sarcoglycan mutants (Griffin et al., 2005). 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 regulates cell-cell adhesion via interacting with the integrin complex. In cultured myocytes, the sarcoglycans co-precipitated with integrin α5β1 and other focal adhesion proteins (Yoshida et al., 1998). Tyrosine phosphorylation of α- and γ-sarcoglycan occurred when the cells were exposed to known integrin ligands. 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 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 (Groh et al., 2009). Loss of β- or δ-sarcoglycan resulted in the loss of the sarcoglycan complex and great reduction of dystroglycan and sarcospan in the adipocytes. β-sarcoglycan null mice exhibited glucose-intolerant and whole body insulin resistance (Groh et al., 2009). 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 (Allikian et al., 2007). Studies have shown that both adipose-derived and muscle-derived stem cells tend to differentiate into adipocytes when exposed to high glucose level (Aguiari et al., 2008). 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 (Yamanouchi et al., 2006).

$\alpha$-Sarcoglycan has its unique properties and functions. $\alpha$-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 (Sandona et al., 2004). High levels of extracellular ATP can occur when muscle cells are damaged and act as pro-apoptotic stimuli (Allen, 2001). Hence $\alpha$-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, $\alpha$-sarcoglycan plays a role in regulating proliferation ability of the myogenic progenitor cells by binding to and stabilizing fibroblast growth factor receptor 1 (FGFR1) (Cassano et al., 2011). $\alpha$-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.

## OUTLINE OF DISSERTATION

The chapters of this thesis focus on the functionality of an internally truncated γ-sarcoglycan protein (Mini-Gamma) and potential application of exon skipping strategy to Limb Girdle Muscular Dystrophy type 2C. We generated Mini-Gamma by deleting a large portion of the extracellular domain of γ-sarcoglycan. Despite the large truncation, Mini-Gamma may still be functional because the cytoplasmic tail, the transmembrane domain and the conserved cysteines at the extreme C-terminus are kept intact. The sarcoglycan complex is conserved in *Drosophila*. There is only one fly homology *Sgcd* that is equally related to mammalian γ- and δ-sarcoglycan. In Chapter 2, I will describe work in a *Drosophila* model of muscular dystrophy, focusing on the functional rescue by Mini-Gamma. *Sgcd*$^{840}$ flies lack endogenous γ/δ-sarcoglycan (*Sgcd*) and develop defects in both cardiac and skeletal muscle (Allikian et al., 2007). Expressing Mini-Gamma in muscle restores heart contractility and ameliorates the skeletal muscle weakness of *Sgcd*$^{840}$ flies, indicating that Mini-Gamma is functional in this *Drosophila* model of muscular dystrophy.

In Chapter 3, I will present experiments examining the function of Mini-Gamma in a heterologous cell expression system and a mouse model of muscular dystrophy. The sarcoglycan complex is composed of α-, β-, γ-, and δ-sarcoglycans. Interaction among these sarcoglycan subunits is important for complex assembly and membrane trafficking. Mini-Gamma is able to incorporate into the sarcoglycan complex and translocate to the plasma membrane in the cell expression system and in mice. Furthermore, expressing Mini-Gamma in mice deleted for endogenous γ-sarcoglycan

demonstrates pathological benefits, reduced sarcolemmal leak, and functional rescue *in vivo*.

In Chapter 4, I will cover the following three topics. First, I will propose future directions and describe preliminary data showing that multiple exon skipping could be induced in RNA encoding mutant human γ-sarcoglycan (work from Eugene Wyatt, Ph.D., a postdoctoral fellow with whom I have worked closely). Secondly, I will summarize structural and functional roles of γ-sarcoglycan protein and explain the mechanisms of rescue by Mini-Gamma. Lastly, I will discuss the efforts and challenges to apply antisense oligonucleotides as drugs to human patients.

## CHAPTER 2

# Mini-Gamma rescues a *Drosophila* model of muscular dystrophy

***(Figure 2.1 to Figure 2.4 are modified from Gao, QQ et al., Journal of Clinical Investigation, 2015)***

## OVERVIEW

Limb Girdle muscular dystrophy type 2C is caused by mutations in the γ-sarcoglycan gene. Human γ-sarcoglycan gene (*SGCG*) is composed of 8 exons. The most common mutation in *SGCG* is a deletion of one thymine in exon 6, referred to as 521ΔT (Leiden muscular dystrophy pages Variation Database, http://www.dmd.nl/nmdb/home.php). To bypass exon 6 and restore the protein's reading frame, exons 4, 5, 6 and 7 need to be skipped, creating an internally truncated protein encoded by exon 2, 3 and 8, which we have termed Mini-Gamma. Mini-Gamma retains the cytoplasmic tail, the transmembrane domain and the extracellular C-terminus of the full-length γ-sarcoglycan protein. To test the functionality of Mini-Gamma, a *Drosophila* LGMD2C model was used. *Drosophila* has one ortholog, *Sgcd*, that is equally related to mammalian γ- and δ-sarcoglycan. $Sgcd^{840}$ flies lack endogenous sarcoglycan and develop defects in both heart and skeletal muscle (Allikian et al., 2007). The UAS/Gal4 system was used to express Mini-Gamma in $Sgcd^{840}$ flies. Mini-Gamma was able to translocate to the plasma membrane in both heart and skeletal muscle cells in $Sgcd^{840}$ flies. Mini-Gamma fully restored the cardiac function and significantly

improved the spontaneous activity level of *Sgcd*$^{840}$ flies. These results indicate that Mini-Gamma protein is able to traffic with other fly sarcoglycans and functionally replace fly *Sgcd* in both heart and skeletal muscles.

## CONTRIBUTIONS

The work in this chapter was done in collaboration with along another graduate student Dr. Jeffrey Goldstein, along with Dr. Matthew Wolf at Duke University. Dr. Goldstein created the construct to express Mini-Gamma and worked with Rainbow Transgenic Flies (San Diego, CA) to create *UAS-Min-Gamma* and *UAS-mGSG* transgenic flies. Dr. Matt Wolf performed the OCT studies in flies blinded to genotype (Figure 2.4C). I characterized the expression of Mini-Gamma protein in the cardiac and skeletal muscle in flies (Figure 2.2 and Figure 2.4B). I also performed the fly breeding and spontaneous activity studies (Figure 2.3 and Figure 2.5). Alec Gazda and Natalie Petrossian, University of Chicago undergraduate students, assisted in fly husbandry. I designed the experiments with Elizabeth McNally, and wrote the manuscript, which she edited.

## INTRODUCTION

Muscular dystrophy is a collection of inherited diseases characterized by skeletal muscle weakness and degeneration. The 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. In some

forms of muscular dystrophy, the heart is also affected. The dystrophin complex localizes to the muscle plasma membrane, acting as a linker between the intracellular cytoskeleton and the extracellular matrix (Cohn and Campbell, 2000; Ervasti and Campbell, 1993). Mutations in the *DMD* gene, which encodes dystrophin, result in Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). The sarcoglycan subcomplex within the dystrophin complex is composed of four single pass transmembrane subunits: α, β, γ, and δ-sarcoglycan (Ervasti and Campbell, 1991; Ozawa et al., 2005). Loss-of-function mutations in genes encoding α, β, γ, and δ-sarcoglycan cause the Limb Girdle Muscular Dystrophies type 2D, 2E, 2C, 2F, respectively (Bonnemann et al., 1995; Nigro et al., 1996; Noguchi et al., 1995; Roberds et al., 1994).

*Drosophila melanogaster* has been used as a genetic model organism for over 100 years, with the whole genome fully sequenced in 2000 (Adams et al., 2000). The sarcoglycan complex is well conserved between flies and humans. There is a single common ortholog of vertebrate α- and ε-sarcoglycan (fly α/ε-sarcoglycan) and a single orthologue of vertebrate γ- and δ-sarcoglycan (fly γ/δ-sarcoglycan) in *Drosophila*. The availability of gene manipulation tools and transgenic/mutant libraries provides rich resources for gene functional studies. In addition, *Drosophila* and human muscle share many structural and functional features, which makes flies a great model for genetic muscle diseases such as muscular dystrophies.

Previously work in our lab generated a muscular dystrophy model in *Drosophila* by deleting the gene encoding γ/δ-sarcoglycan, referred to as *Sgcd*, using P element

excision (Allikian et al., 2007). *Sgcd* null flies develop normally but suffer from mobility defects and heart tube dilation with age. This reflects what is seen in human muscular dystrophy patients and mouse disease models. Unlike the muscular dystrophy phenotype in mammals, *Sgcd* flies do not develop fibrosis and fat deposition in muscle. Other *Drosophila* models of muscular dystrophy such as dystrophin (*Dys*) and dystroglycan (*Dg*) mutants have also been characterized. These mutants are modified by deletions, hypomorphic alleles, RNAi, or mutations in enzymes that are involved in dystroglycan glycosylation (Christoforou et al., 2008; Haines et al., 2007; Mosqueira et al., 2010; Shcherbata et al., 2007; Taghli-Lamallem et al., 2008). These mutants also display age-dependent heart and skeletal muscle defects at adult stage, supporting the functional conservation of the dystrophin complex in muscle.

We now used the *Sgcd* null flies to examine the functionality of murine Mini-Gamma protein in heart and skeletal muscle. We found that Mini-Gamma protein was able to traffic correctly to the plasma membrane in both heart and skeletal muscle. More importantly, Mini-Gamma expression prevented the dilation of heart tube and ameliorated the activity deficits of *Sgcd* null flies.

## MATERIALS AND METHODS

### *Drosophila breeding and husbandry*

Flies were raised on standard medium at 25°C with 12-hour light and 12-hour dark cycling. To express murine γ-sarcoglycan in $Sgcd^{840}$ mutants, $Sgcd^{840}$ allele (on X chromosome) and *Mef2-Gal4* transgene (on 3rd chromosome) were first combined into

one fly strain *Sgcd*$^{840}$*; Mef2-Gal4*. *Sgcd*$^{840}$*; Mef2-Gal4* virgin females were collected and mated with either *UAS-Sgcg* or *UAS-Mini-Gamma* males. Since the *Sgcd*$^{840}$ allele is on X chromosome, all male progeny from this cross were null for fly γ/δ-sarcoglycan and expressed either murine full-length γ-sarcoglycan or Mini-Gamma in muscle. Five to fifteen newly eclosed males were collected everyday over the course of 3 to 7 days. Flies were flipped into fresh vials every three days during the aging process. yw and *Sgcd*$^{840}$ males were collected at the same time and aged in the same manner.

*Drosophila strains*

To generate *UAS-Sgcg* and *UAS-Mini-Gamma* transgenic flies, murine *Sgcg* and *Mini-Gamma* coding sequences were ligated into pUAST vector (Brand and Perrimon, 1993). An Xpress epitope tag was added to the N-terminus of Mini-Gamma. *pUAST-Sgcg* and *pUAST-Mini-Gamma* plasmids were integrated using P-element insertion (Rainbow Transgenics, Camarillo, CA). Founder males were mated to *y[1]w[1118]* (yw) females, and their progeny were screened for the presence of w[+mC]. The *TinCΔ4-Gal4* strain was a gift from Manfred Frasch (Lo and Frasch, 2001). *Mef2-Gal4* and *MHC-Gal4* were gifts from Ron Dubreuil (Ranganayakulu et al., 1996; Schuster et al., 1996). The *Sgcd*$^{840}$ strain was previously described (Allikian et al., 2007). The *Drosophila* strain *y[1]w[1118]* (yw) was used as the wild type control in all studies (Bloomington Stock Center, Bloomington, IN). *Sgcd*$^{840}$ and all transgenic fly strains were backcrossed with the yw strain for at least six generations to allow homogenization across the whole genome.

*Drosophila immunofluorescence microscopy*

One drop of Tissue Freezing Medium (TFM, Triangle Bioscience, Durham NC) was placed on the surface of a piece of wooden block. Fifteen to twenty-five flies were anesthetized and embedded in the medium in the same orientation. The block was then immersed in liquid nitrogen-chilled isopentane for 5 minutes, followed by liquid nitrogen for another 5 minutes. Ten-μm sections were cut from frozen flies, fixed in ice-cold methanol for 2 minutes and briefly rinsed in cold phosphate buffered saline (PBS) immediately afterwards. The sections were blocked in PBS containing 5% fetal bovine serum and 0.1% Triton-X for 2 hours at 4°C. The sections were then incubated with primary antibodies diluted in blocking solution at 4°C overnight, followed by three 15-minute washes with PBS containing 0.1% Triton-X at 4°C on a shaker. The sections were incubated with secondary antibody for 2 hours at 4°C in dark. Samples were washed again before mounted with VECTASHIELD Mounting Medium with DAPI H-1200 (Vector Labs, Youngstown, OH). Images were collected using an Axiophot microscope with iVision software and edited using Adobe Photoshop CS4 and Image J in concert with NIH policy on appropriate image manipulation.

*Drosophila activity assay*

The MB5 MultiBeam Activity Monitor (TriKinetics, Waltham, MA) was used to quantify fly basal activity. All activity assays were performed on flies that were aged to 20 days after eclosion. After anesthetization by CO2, individual flies were loaded into

glass detection tubes. One end of the glass tube was dipped in standard fly food and sealed with a rubber cap. The other end of the glass tube was loosely sealed with KIMTECH's Kimwipes to allow airflow (Irving, Texas). Up to sixteen flies can be evaluated for activity in independent tubes simultaneously by monitoring infrared beam breaks. The DAMSystemMB 106X software was used to record activity at 1-minute intervals over 24-48 hours, and the DAMFileScan 108X was used to process the raw data (Trikinetics). Prism (Graphpad, San Diego, CA) was used for data analysis. 30~50 files were recorded for each genotype. Student's t-test was used to compare results between two groups.

*Optical coherence tomography (OCT)*

OCT was performed as previously described (Wolf et al., 2006; Wolf and Rockman, 2008). Ten to twelve male flies from each group were assessed at 7 days after eclosion. The end systolic and end diastolic diameters for individual flies were entered into Prism (Graphpad, San Diego, CA). One-way ANOVA analysis was done to compare different groups.

*Antibodies*

A rabbit polyclonal antibody was raised to the Xpress epitope and affinity-purified (Pocono Rabbit Farms, Canadensis, PA). Mouse full-length γ-sarcoglycan was detected using a mouse monoclonal antibody NCL-g-SARC (CAT#G-SARC-CE, Leica Biosystems, Nussloch, Germany). Secondary antibodies were Alexa Fluor® 488 Goat

Anti-Rabbit and AlexaFluor®488 Goat Anti-mouse (Invitrogen, Carlsbad, CA). Primary antibodies were typically used at 1:1K dilution and the secondary antibodies were used at 1:5K dilution.

## RESULTS

*Mini-Gamma reflects the deletion of exons 4, 5, 6 and 7 of γ-sarcoglycan*

Human γ-sarcoglycan gene (*SGCG*) is composed of 8 exons. To target the 521ΔT mutation in exon 6 and restore the correct reading frame, skipping exon 4, 5, 6 and 7 is required (Fig 2.1 A). The resulting product is composed of exon 2, 3 and 8, referred to as *Mini-Gamma.* γ-Sarcoglycan is a type II transmembrane protein with a short intracellular domain, a single transmembrane pass and a large carboxyl-terminal extracellular domain. Mini-Gamma protein carries an internally truncation in the extracellular domain while keeping other function domains intact (Fig 2.1 A). Shared amino acids between fly γ/δ-sarcoglycan and mouse γ-sarcoglycan are marked in colors (Fig 2.1 B, left panel). Note that the conserved cysteine residues and tyrosine phosphorylation sites remain in the Mini-Gamma protein while the N-linked glycosylation site is lost (Fig 2.1 B, right panel).

*Mini-Gamma localizes at the plasma membrane in a fly model of LGMD2C*

To examine the function of Mini-Gamma *in vivo*, the GAL4/UAS system was used to express Mini-Gamma in a previously established *Drosophila* model for LGMD2C (Allikian et al., 2007; Brand and Perrimon, 1993). *Sgcd*$^{840}$ flies carry a large deletion in

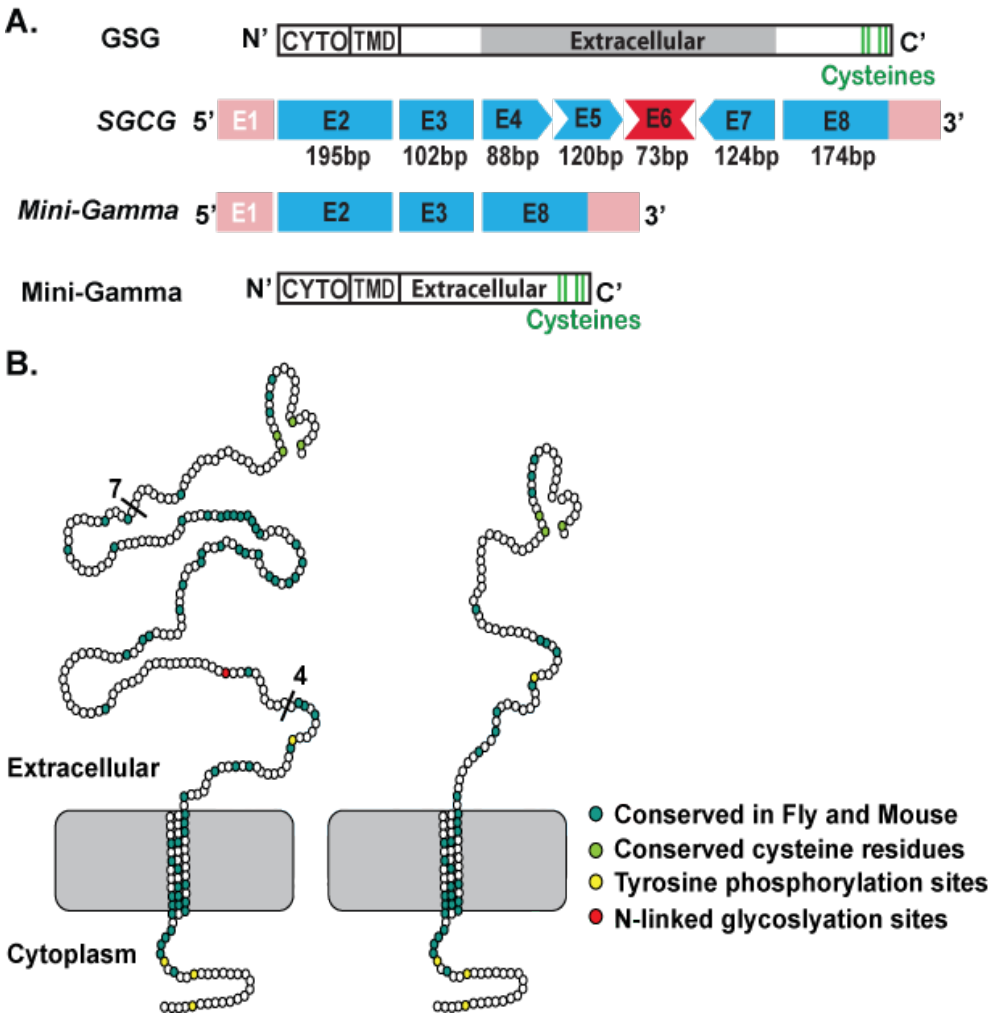


**Figure 2.1. Reading frame and topology of γ-sarcoglycan and Mini-Gamma**. (A) γ-Sarcoglycan (GSG) is a type II single-pass transmembrane protein. The *SGCG* gene encoding γ-sarcoglycan is composed of 8 exons, and the most common mutation falls in exon 6. This mutation disrupts the reading frame and is considered a null mutation. To restore the reading frame, skipping exon 4, 5, 6 and 7 is required. This removes a portion of the extracellular domain, producing an internally truncated protein, referred to as Mini-Gamma. (B) Protein topology of full-length γ-sarcoglycan and Mini-Gamma are shown. The intracellular N-terminus interacts with Filamin-C and archvillin (Spinazzola et al., 2015; Thompson et al., 2000). The cytoplasmic tail also contains tyrosine phosphorylation sites (in yellow) that are implicated in signaling transduction (Barton, 2006; Barton, 2010; Moorwood et al., 2014; Spinazzola et al., 2015; Yoshida et al., 1998). The extracellular membrane-proximal region interacts with other sarcoglycans (Chen et al., 2006). The extreme C-terminus contains conserved cysteines (in bright green) that are essential for protein stability and membrane localization (Chan et al., 1998; Chen et al., 2006; Shi et al., 2004). These essential functional sites, together with the transmembrane domain, are kept intact in the Mini-Gamma protein. γ-Sarcoglycan is conserved between mouse and *Drosophila* (35% identical, 56% similar).

the fly γ/δ-sarcoglycan gene (*Sgcd*). The sarcoglycan complex is normally found at the muscle plasma membrane (sarcolemma) in WT animals. In humans or mouse models, loss of function mutations in any single sarcoglycan result in absence of sarcolemma staining of other sarcoglycans at the plasma membrane (Duclos et al., 1998; Durbeej et al., 2000; Hack et al., 2000; Mizuno et al., 1994; Vainzof et al., 1996). Full-length murine γ-sarcoglycan (mGSG) localized to the sarcolemma when expressed in *Sgcd*$^{840}$ muscle (Figure 2.2, bottom left, arrow heads), indicating that the murine γ-sarcoglycan normally translocates in *Drosophila* muscle. Expression of murine Mini-Gamma showed the same distinct plasma membrane localization as full-length sarcoglycan, consistent with normal plasma membrane trafficking (Fig 2.2, bottom right, arrow heads). Interestingly, when Mini-Gamma was expressed in WT fly muscle, both sarcolemma staining (arrow head) and peri-nuclear staining (arrow) were observed (Fig 2.2, upper right). The peri-nuclear staining indicates that a subfraction of Mini-Gamma protein accumulates in the endoplasmic reticulum (ER) and likely reflects competition from the endogenous Sgcd protein. Non-specific staining in fly muscle without the transgenes is shown (Fig 2.2, upper left).

*Mini-Gamma improves skeletal muscle function of Sgcd*$^{840}$ *flies*

Adult *Sgcd*$^{840}$ flies display locomotive defects as a result of skeletal muscle weakness and degeneration (Allikian et al., 2007). The MB5 MultiBeam *Drosophila* activity monitor (TriKinetics) was used to measure fly spontaneous activity over 24~48 hours at 20 days after eclosion (Fig 2.3 A). Nocturnal activity (from midnight to 8 am)

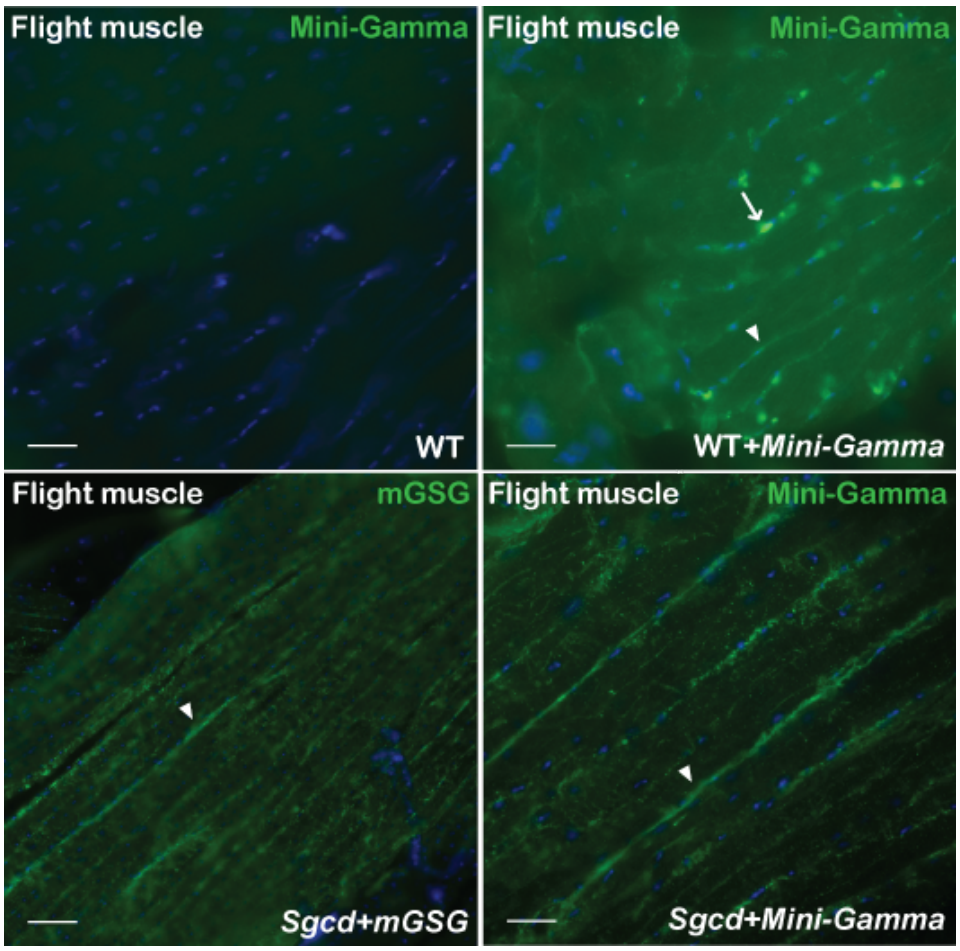


**Figure 2.2. Mini-Gamma localizes to the plasma membrane of fly skeletal muscle cells.** The UAS-Gal4 system was used to express murine sarcoglycans as transgenes in flies. When Mini-Gamma was expressed in WT fly muscle, both plasma membrane staining (arrow head) and peri-nuclear staining (arrow) were observed. *Sgcd* flies lack endogenous sarcoglycan and serve as a model of muscular dystrophy (Allikian et al., 2007). When expressed in the *Sgcd* flies, Mini-Gamma protein localized mainly to the plasma membrane of muscle cells (*Mef-Gal4*, *UAS-Mini-Gamma*) (arrowhead), same as the full-length murine γ-sarcoglycan (*Mhc-Gal4*, *UAS-mGSG*). The peri-nuclear staining of Mini-Gamma in WT flies indicates that a fraction of Mini-Gamma protein accumulates in the endoplasmic reticulum and likely reflects from the endogenous Sgcd protein. Scale bar = 20μm.

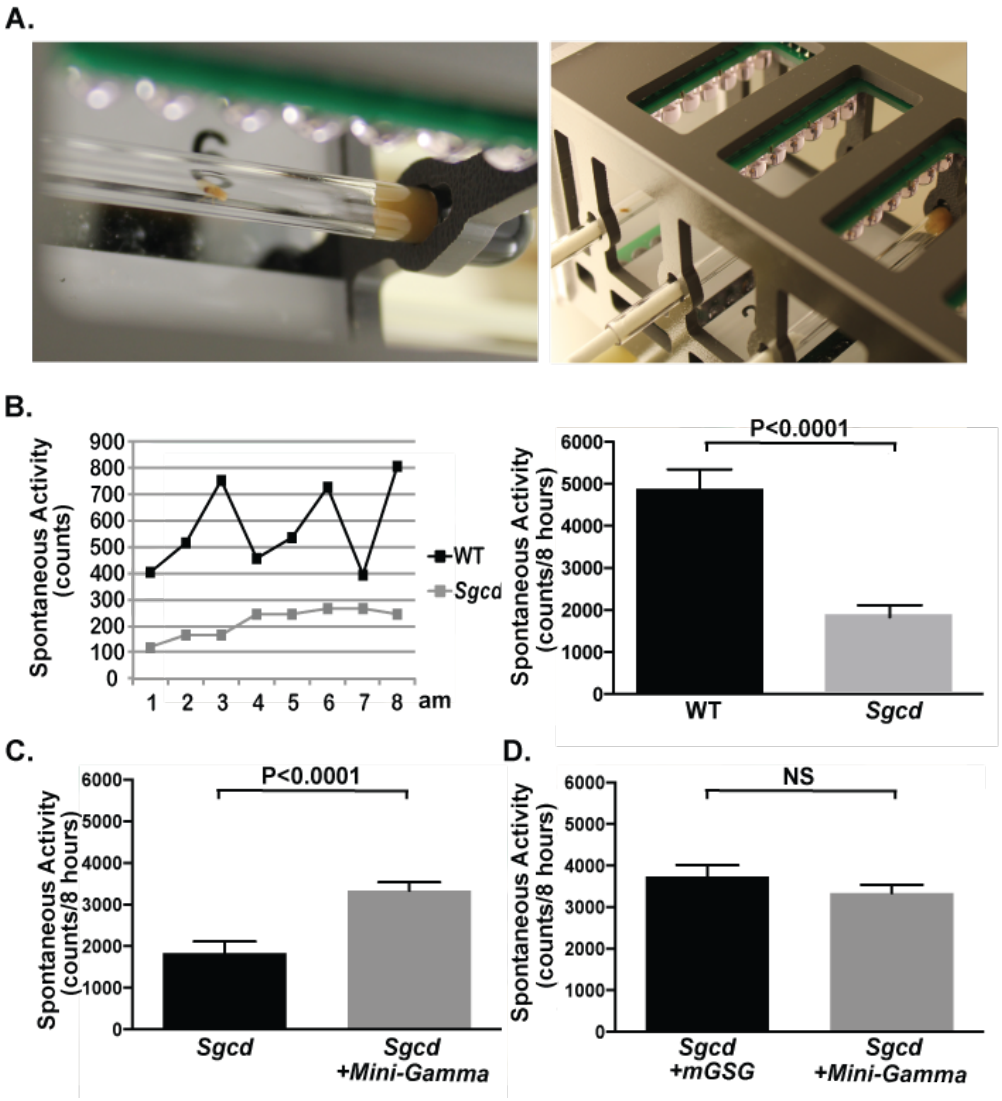


**Figure 2.3. Mini-Gamma improves spontaneous activity level of *Sgcd* flies.** (A) *Drosophila* MB5 MultiBeam activity monitor (MB5) was used to record fly spontaneous activity. Each fly was placed into a glass tube supplied with food at one end. 17 Infrared beams from the purple bulbs per tube detected fly movements. 16 tubes were loaded into one apparatus and recorded simultaneously. (B) *Sgcd* flies had reduced nocturnal activity compared to WT. The nocturnal activity levels of WT and *Sgcd* from midnight to 8 AM are shown on the left (each dot represents the average value of 30~40 flies at the given time point). The sum of activity counts from midnight to 8AM of WT and *Sgcd* are compared on the right. (C) Expression of Mini-Gamma improved nocturnal activity of *Sgcd* flies. (D) The degree of rescue was similar between mGSG and Mini-Gamma. Standard error of the mean (SEM) is shown for comparisons in B, C, and D. N = 20~35 flies per genotype.

was used to compare between groups. *Sgcd*$^{840}$ flies showed greatly reduced activity compared WT flies, consistent with other mobility assays (Fig 2.3 B) (Allikian et al., 2007). Expression of Mini-Gamma in *Sgcd*$^{840}$ flies significantly improved the activity of *Sgcd*$^{840}$ flies (Fig 2.3 C) but did not fully restore *Sgcd840* fly mobility to wild type activity level. However, we noted that expression of mGSG (murine full-length γ-sarcoglycan) resulted in similar level of rescue of activity as Mini-Gamma (Fig 2.3 D), suggesting that at least part of the failure to fully restore activity derives from the differences between *Drosophila* and mammalian sarcoglycans. All flies compared here were age, sex and background matched.

*Mini-Gamma restores cardiac function of Sgcd*$^{840}$ *flies*

*Drosophila* has an open circulation system and a thin-walled heart tube structure localizes at the dorsal side (Fig 2.4 A). Expression of Mini-Gamma in *Sgcd*$^{840}$ hearts showed distinct plasma membrane-associated staining (Fig 2.4 B). To monitor *Drosophila* heart function, optical coherence tomography (OCT) was used to measure heart tube dimension during both contraction and relaxation (Wolf et al., 2006). *Sgcd*$^{840}$ flies had dilated heart tubes with significantly increased end systolic dimension (ESD) compared to wild type (Fig 2.4 C, left panel). Expression of Mini-Gamma in the heart tube by Mef2-Gal4 driver was sufficient to restore ESD to wild type dimensions (Fig 2.4 C, left panel). A representative OCT tracing demonstrates the dilated nature in *Sgcd*$^{840}$ heart tubes and rescue of this phenotype by transgenic expression of Mini-Gamma (Fig 2.4 C, right panel)

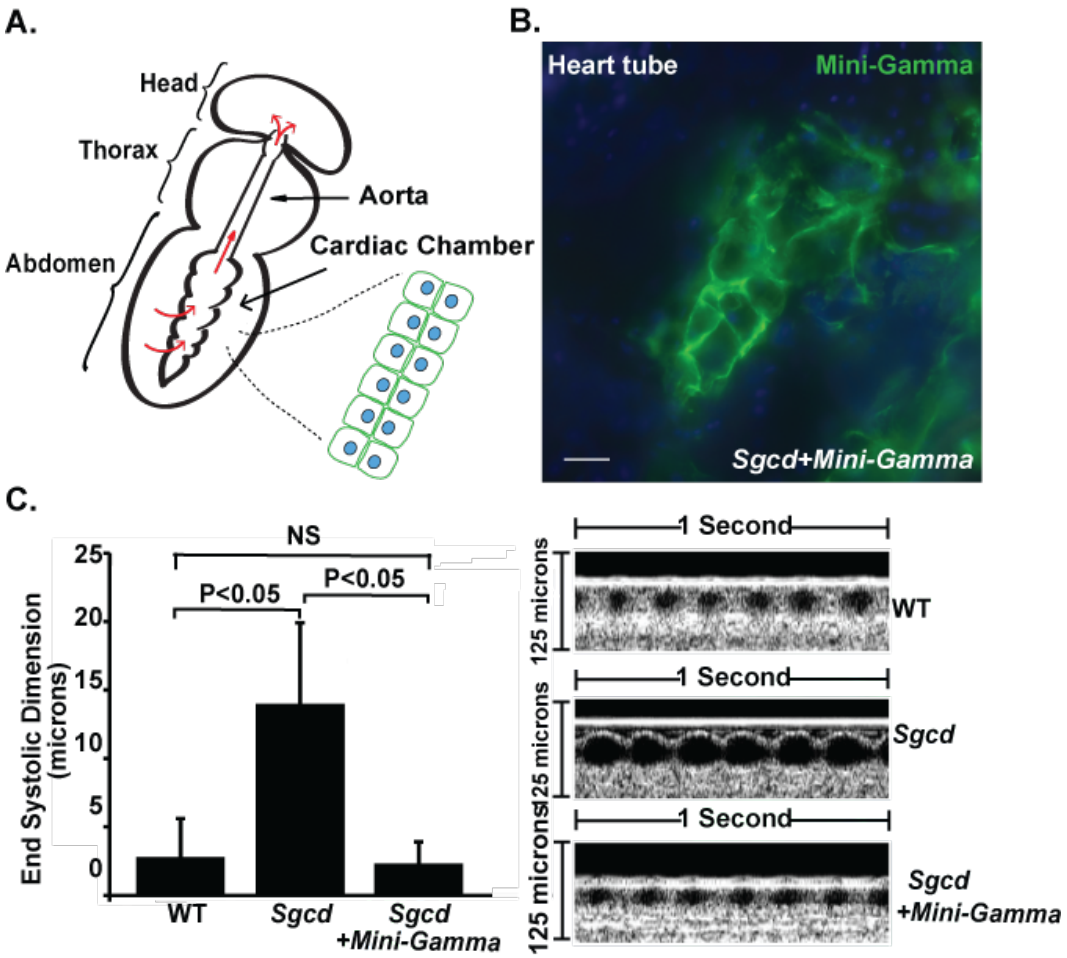


**Figure 2.4. Mini-Gamma improves heart function of *Sgcd* flies.** (A) Diagram of *Drosophila* heart and circulation system. The adult fly has an open circulatory system. The long tubular cardiac chamber is localized on the dorsal side of the abdomen, directly beneath the cuticle. The cardiac chamber is composed of a single layer of cardiomyocytes, shown in green. Circulation of hemolymph (*Drosophila* blood) is shown in red arrows. (B) Mini-Gamma localized at the membrane of the cardiomyocytes in *Sgcd* flies (*ΔTin-Gal4, UAS-Mini-Gamma*). Scale bar = 20μm. (C) Optical coherence tomography (OCT) was used to measure fly heart function (Wolf et al., 2006; Wolf and Rockman, 2008). *Sgcd* flies had dilated heart tubes with increased end systolic dimension (ESD) compared to wild type flies. Expression of Mini-Gamma in the *Sgcd* heart tube reduced ESD to wild type level (*Mef-Gal4*, *UAS-Mini-Gamma*). Representative M-mode images are shown on the right. N = 10~12 flies per genotype

*Mouse δ-sarcoglycan fully rescues the activity defects of Sgcd$^{840}$ flies*

Murine δ-sarcoglycan (mDSG) and γ-sarcoglycan are equally related to the fly *Sgcd.* When expressed in *Sgcd*$^{840}$ flies by transgenes, both mDSG and fly *Sgcd* were able to fully restore the nocturnal spontaneous activity level similar to that of wild type (Fig 2.5 and data not show). The rescue efficiency of mDSG was significantly higher than that of either mGSG or Mini-Gamma, suggesting that mDSG might be more similar to fly *Sgcd* in skeletal muscle than mGSG (Fig 2.5). This result is consistent with the assembly model where murine δ-sarcoglycan has a pivotal role in interacting with β-sarcoglycan to initiate sarcoglycan complex assembly.

**DISCUSSION**

*Drosophila is a useful model for genetic muscle diseases and therapeutics*

The comparatively more rapid ability to genetic manipulate flies combined with available muscle/heart functional assays and well-conserved muscle genes make *Drosophila* a highly useful model for dissecting functional pathways and testing potential therapeutics for muscle diseases. Here we show that the exon skipping product, Mini-Gamma, is functional in both heart and skeletal muscle using a fly muscular dystrophy model lacking endogenous sarcoglycan (*Sgcd*). The encouraging data in flies prompted the further testing of Mini-Gamma function in mouse and human cell culture model (see next chapters) but also emphasizes the utility of this model system.

Using the same *Sgcd* fly model, Goldstein et al. demonstrated that SMAD signaling is pathogenic in muscular dystrophy and reduction of this pathway is sufficient

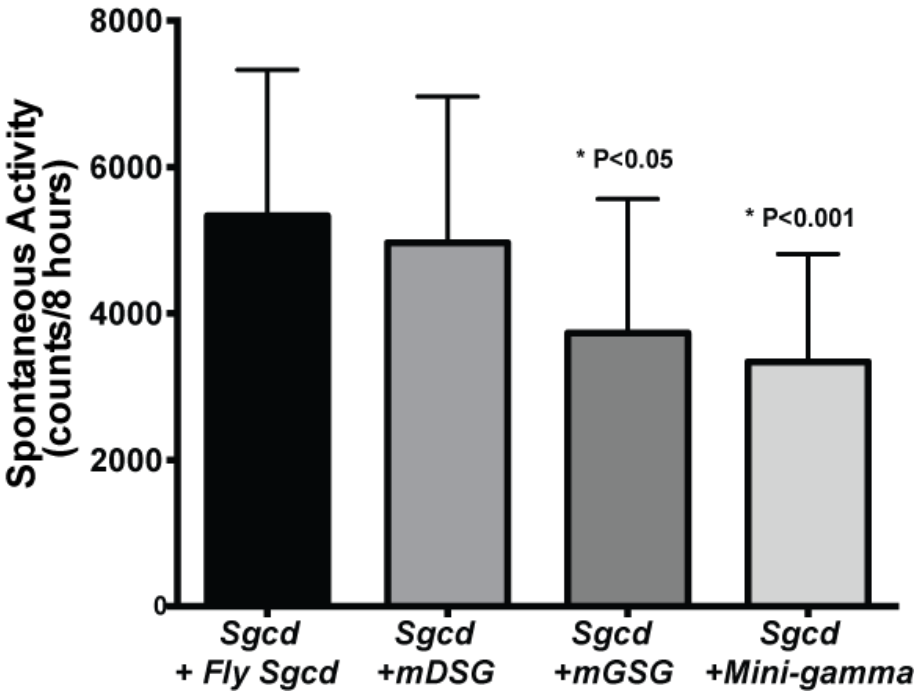


**Figure 2.5. Murine δ-sarcoglycan is more efficient in rescuing *Sgcd* activity deficiency than murine γ-sarcoglycan and Mini-Gamma.** Expressing either the fly endogenous γ/δ-sarcoglycan (Fly Sgcd) or murine δ-sarcoglycan (mDSG) fully rescued the activity deficits of *Sgcd* flies to WT level (WT = 4878±463.1, n=37). Both Mini-Gamma and murine γ-sarcoglycan (mGSG) were less efficient in restoring the activity of *Sgcd* flies when compared to murine δ-sarcoglycan ($p<0.001$, $p<0.05$ respectively). These results suggest that the function of fly γ/δ-sarcoglycan in skeletal muscle is more similar to murine δ-sarcoglycan than γ-sarcoglycan. One-way ANOVA was used.

to correct both heart and muscle dysfunction in *Sgcd* flies (Goldstein et al., 2011). This notion is further supported by a later study in a mouse model of muscular dystrophy (*Sgcg*) showing similar functional rescue effects as a result of reduction of the SMAD signaling pathway, indicating that SMAD signaling is a potential target for therapeutic intervention (Goldstein et al., 2014) and further re-emphasizing the conserved nature of muscle and muscle degeneration between mammals and *Drosophila*.

Similar to *Sgcd* flies, dystrophin (Dys) and dystroglycan (Dg) mutant flies also display muscle wasting and dysfunction (Shcherbata et al., 2007). Using a genetic modifier screen, Kurcherenko et al. found that Dg-Dys complex interacted with members of Notch, TGFβ and EGFR signaling pathways and components of pathways involved in cytoskeletal regulation (Kucherenko et al., 2008). Pantoja et al further characterized the suppression mechanism of one of the modifiers, *wunen* (Pantoja et al., 2013). *Wunen* is a homolog of lipid phosphate phosphatase 3. The authors discovered that loss of *wunen* suppressed the muscle degeneration phenotype in *Dys* mutants by up regulating the bioactive lipid Sphingosine 1-phosphate (SIP), which activates cell proliferation and differentiation in various tissues including muscle. Treating flies with drugs reported to increase SIP signaling suppressed muscle wasting phenotype in *Dys* mutants. These and other studies have demonstrated that *Drosophila* is a powerful system to study genetic diseases and potential therapeutics for human patients.

*Sgcd muscular dystrophy phenotype is affected by genetic background*

*Sgcd* flies were created by inducing a large deletion at the *Sgcd* locus using P

element imprecise excision (Allikian et al., 2007). The genetic background of *Sgcd* flies is a mix of the P element insertion line (KG5430), transposase transgenic line and balancer lines, referred to as the "original" background. A negative geotaxis assay was used to characterize the motility defects and showed a significant reduction of climbing ability of *Sgcd* flies (originally background) at 20 days after eclosion (Allikian et al., 2007). Since genetic background is known to affect fly behavior assays, I backcrossed all the transgenic lines and *Sgcd* null flies into the yellow white (yw) background when measuring the rescue effects of Mini-Gamma. Curiously, *Sgcd* null flies in yw background displayed significantly higher climbing ability than *Sgcd* flies in the original background and were not different from WT flies (data not shown). Heart tube dilation phenotype measured by OCT is preserved in *Sgcd* null flies in yw background. However, the degree of dilation is also reduced; the end systolic dimension (ESD) is around 40μm in *Sgcd* in the original background while ESD is around 15μm in *Sgcd* in the yw background.

The phenotypic differences observed in the two different genetic backgrounds are consistent with findings from human patients and mouse models of muscular dystrophy. Patients carrying the same γ-sarcoglycan mutation can display either mild or severe forms of muscular dystrophy (McNally et al., 1996b). When a null allele of γ-sarcoglycan was introduced into four different inbred strains in mice, a spectrum of phenotypes from mild to severe was observed (Heydemann et al., 2005). Using QTL mapping, latent TGFβ-binding protein 4 (LTBP4) and annexin A6 were found to be modifiers of muscular dystrophy in mice (Heydemann et al., 2009; Swaggart et al.,

2014). In a later study, the *LTBP4* genotype was also found to correlate with age of ambulatory loss in Duchenne muscular dystrophy patients (Flanigan et al., 2013). It would be interesting to perform a similar QTL mapping study in flies to look for modifiers of the dystrophic phenotype. QTL mapping is cheaper and faster in flies and is likely to shed light on muscle disease mechanisms and provide therapeutic insight.

# CHAPTER 3

## Mini-Gamma is incorporated into the sarcoglycan complex and rescues a mouse model of muscular dystrophy

(***modified from Gao QQ et al., Journal of Clinical Investigation, 2015)***

## OVERVIEW

Exon skipping uses antisense oligonucleotides as a treatment for genetic diseases. With exon skipping, antisense oligonucleotides target RNA to bypass premature stop codons and restore reading frame disruption. Exon skipping is currently being tested in humans with Duchenne Muscular Dystrophy and dystrophin gene mutations. For Duchene Muscular Dystrophy, the rationale for exon skipping derived from observations in patients with naturally occurring dystrophin gene mutations that generated internally deleted but partially functional dystrophin proteins. We now expanded the potential for exon skipping by testing whether an internal, in-frame truncation of a transmembrane protein γ-sarcoglycan was functional. We generated Mini-gamma by deleting a large portion of the extracellular domain. In the previous chapter, we showed that Mini-Gamma provided functional benefit to correct the loss of γ-sarcoglycan in a *Drosophila* model. In Chapter 3, we demonstrated that Mini-Gamma was incorporated into the sarcoglycan complex both in a heterologous cell expression system and in transgenic mice. More importantly, expression of Mini-Gamma in mice lacking γ-sarcoglycan (*Sgcg*) stabilized the sarcoglycan complex at the membrane,

improved muscle pathology, reduced muscle membrane leak, and ameliorated heart function deficit of *Sgcg* mice. Since Mini-Gamma represents removal of four of the seven coding exons in γ-sarcoglycan, this approach provides a viable strategy to treat the majority of patients with γ-sarcoglycan gene mutations.

## CONTRIBUTIONS

I performed all the experiments in HEK293 cells. Judy Earley performed the H&E and Masson's Trichrome staining (Figure 3.10A, Figure 3.11). Dr. Alexis Demonbreun assisted with serum CK measurements and acquiring images of EBD injected diaphragm and abdominal muscles (Figure 3.9A, Figure 3.9B, and Figure 3.9E). Dr. David Barefield performed mouse echocardiography (Figure 3.12C). Michele Hadhazy assisted in mouse breeding and husbandry. Alec Gazda and Natalie Petrossian were undergraduate research assistants under my mentorship who helped with mouse genotyping. I analyzed the histology data and quantified EBD data. I performed cell and tissue fractionations, immunoprecipitation, immunofluorescence microscopy, and immunoblotting. I performed data analysis. I designed the experiments with Elizabeth McNally, and wrote this manuscript, which she edited.

## INTRODUCTION

Mutations that disrupt the dystrophin glycoprotein complex (DGC) cause muscular dystrophy (Durbeej and Campbell, 2002; Ervasti, 2007; Rahimov and Kunkel, 2013). Dystrophin and its associated proteins localize to the muscle plasma membrane,

acting as a linker between the intracellular cytoskeleton and the extracellular matrix (Cohn and Campbell, 2000; Ervasti and Campbell, 1993). Large deletions in the dystrophin gene account for Duchenne muscular dystrophy (DMD). Mutations that result in internal deletions and maintain the reading frame of dystrophin cause the milder Becker muscular dystrophy (BMD). These observations in BMD provided the basis for developing antisense oligonucleotide (AON) therapies for the treatment of DMD with the goal of inducing exon skipping events to restore reading frame. DMD exon skipping, by design, generates an internally truncated and partially functional protein. Clinically, exon skipping is expected to convert severe DMD patients into milder BMD patients resulting prolonged ambulation and better maintenance of muscle strength. Clinical trials that test exon skipping in DMD are advancing (Cirak et al., 2011; Goemans et al., 2011; Kinali et al., 2009; Lu et al., 2014; van Deutekom et al., 2007). Dystrophin, with its highly repetitive internal structure composed of 24 spectrin repeats, is ideal for exon skipping.

The sarcoglycan subcomplex within the DGC is composed of four single pass transmembrane subunits: α, β, γ, and δ-sarcoglycan (Ervasti and Campbell, 1991; Ozawa et al., 2005). Recessive loss-of-function mutations in genes encoding α, β, γ, and δ-sarcoglycan cause the Limb Girdle Muscular Dystrophies type 2D, 2E, 2C, 2F, respectively (Bonnemann et al., 1995; Nigro et al., 1996; Noguchi et al., 1995; Roberds et al., 1994). Here, we examined the plausibility of applying an exon skipping strategy to treat LGMD 2C patients with mutations in *SGCG*, the gene encoding γ-sarcoglycan. The most common mutation in LGMD2C patients is a deletion of a thymine from a string

of 5 thymines at nucleotide bases 521-525 in exon 6 of the γ-sarcoglycan gene, referred to as 521ΔT (Noguchi et al., 1995). This mutation shifts the reading frame and results in the absence of γ-sarcoglycan protein and secondary reduction of β- and δ-sarcoglycans (Noguchi et al., 1995). To skip this mutation and restore reading frame requires skipping of exons 4, 5, 6 and 7 together. This internally truncated protein, which we refer to as "Mini-Gamma", retains the intracellular, transmembrane and extreme carboxyl-terminus.

Previous studies have established heterologous cell expression systems for studying sarcoglycan trafficking and impacts of sarcoglycan mutations on protein stability and function (Chen et al., 2006; Shi et al., 2004). COS-1 is a fibroblast-like cell that lacks endogenous sarcoglycans, and when expressed individually in COS-1 cells, all four sarcoglycans remain in the cytoplasm without significant plasma membrane translocation (Shi et al., 2004). The minimal requirement for membrane targeting in COS-1 cells is the co-expression of β- and δ-sarcoglycan (Shi et al., 2004). Mutations affecting interactions between sarcoglycans disrupt the membrane trafficking of the sarcoglycan complex (Chen et al., 2006). Human embryonic kidney cells (HEK293T) are similar to COS-1 cells and do not express sarcoglycan proteins. Murine sarcoglycan constructs were expressed in HEK293 cells to compare the function of Mini-Gamma and full-length γ-sarcoglycan. I found that Mini-Gamma interacted directly with β- and δ-sarcoglycan and was able to translocate to the membrane when co-expressed with β- and δ-sarcoglycans, similar to when full-length γ-sarcoglycan was expressed. These data suggest that Mini-Gamma can functionally replace γ-

sarcoglycan in this heterologous cell expression system.

Previous work in the lab has established a γ-sarcoglycan null mouse model (*Sgcg*) (Hack et al., 1998). The loss of γ-sarcoglycan resulted in secondary reduction of β- and δ-sarcoglycan at the plasma membrane and muscle heavy microsomal fraction (Hack et al., 2000; Hack et al., 1998). *Sgcg* mice display severe skeletal muscle degeneration with pseudohypertrophy, increased membrane leak, elevated central nucleation and fibrosis (Hack et al., 1998). *Sgcg* mice also develop cardiomyopathy as they age (Hack et al., 1998). Mini-Gamma expression in *Sgcg* mice increased the expression of β- and δ-sarcoglycan at the plasma membrane, ameliorated skeletal muscle pathology, reduced sarcolemmal leak and improved heart function. These data indicate that Mini-Gamma is a functional replacement of the full-length protein γ-sarcoglycan *in vivo*.

## MATERIALS AND METHODS

*Plasmids for in vitro transfection*

To generate the *CMV-Mini-Gamma* construct for expression in HEK293T cells, *pUAST-Mini-Gamma* plasmid was digested and inserted into pcDNA3.0 vector at EcoR1 and Xho1 sites. *CMV-Sgcg* construct was generated from the *pUAS-Sgcg* plasmid in similar fashion. Mouse *Sgcb* (MR204617) and mouse *Sgcd* (MR221060) cDNA ORF clones were purchased from OriGene (Rockville, MD). Both *Sgcb* and *Sgcd* vectors contain a CMV promoter and Myc-DDK tags at the C-terminus.

*Transfection of HEK293T cells*

Human Embryonic Kidney (HEK 293T) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), to which 10% fetal bovine serum and 1% penicillin-streptomycin was added. FuGene® HD transfection reagent (Promega, Fitchburg, WI) was used to transfect HEK cells with sarcoglycan plasmids using the manufacturer's protocol. Cells were harvest 40 hours post transfection, briefly washed with ice-cold PBS once before 300μL pre-chilled co-IP buffer per 10cm plate was applied. The co-IP buffer contains 50mM Tris pH7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM PMSF, 0.1% Triton, 0.01% SDS and cOmplete EDTA-free tablet from Roche. Cell were collected and transferred to 1.5mL centrifuge tubes on ice, titurated three times with insulin syringes. The lysates were then centrifuged at 14K rpm for 10 minutes at 4°C and the supernatant was used for co-IP experiments.

*Co-immunoprecipitation from HEK293T cells*

Co-immunoprecipitation (co-IP) was performed according to published protocols (Hack et al., 2000) with modifications. Bio-Rad Bradford assay was performed to determine the protein concentration and cell lysates containing 600~1000μg protein was used as co-IP input. The lysates were first pre-cleared with 45 μL Protein G Plus/Protein A Agarose Suspension (EMD Millipore Chemicals, Billerica, MA) for 1h at 4°C. The protein G/A beads were washed 3 times with co-IP buffer before adding to the lysates. Pre-cleared samples were then incubated with antibodies (15 μL NCL-b-SARC or 5 μL Xpress antibody) or no antibodies (as negative control) at 4°C for 3 hours with

light agitation. After antibody incubation, the samples were incubated with 60μL protein G/A beads for 2 hours at 4°C. The samples were then centrifuged at 4000rpm for 10 minutes at 4°C, and the supernatant was discarded. The beads were then washed for 7 times using ice-cold wash buffer (co-IP buffer minus SDS). 30 μL 2XLaemmli SDS buffer (supplemented with 5% BME) was mixed with the beads and boiled at 95°C for 5 minutes. The beads were then centrifuged at 14000rpm for 2 minutes at room temperature and the supernatant was saved as the co-IP fraction.

*Immunofluorescence microscopy for HEK293T cells*

A sterilized cover slip was place in each well of 6-well cell culture plates before cells were plated. Transfection was performed on the next day and cells were harvested 40 hours after transfection. Cell culture media was aspirated and cells were rinsed once with cold PBS. The cells were then immediately fixed in ice-cold methanol for 5 minutes and briefly rinsed in cold phosphate buffered saline (PBS) for 3 times. The cells were blocked in PBS containing 5% fetal bovine serum and 0.1% Triton-X for 2 hours at 4°C. The cells were then incubated with primary antibodies diluted in blocking solution overnight at 4°C, followed by three 10-minute washes with PBS containing 0.1% Triton at 4°C. The cells were incubated with secondary antibody for 2 hours at 4°C. Samples were washed again and then mounted with VECTASHIELD Mounting Medium with DAPI H-1200 (Vector Labs, Youngstown, OH). Images were collected using an Axiophot microscope with iVision software and edited using Adobe Photoshop CS4 and Image J in concert with NIH policy on appropriate image

manipulation.

*Generation of Mini-Gamma transgenic mice and mouse breeding*

The desmin (Des) promoter was amplified from human genomic DNA to obtain the short promoter previously characterized by (Pacak et al., 2008). The Des promoter sequence was then inserted into the CMV-Mini-Gamma vector at SpeI and EcoR1 sites, replacing the CMV promoter while keeping the start codon, the Xpress tag and Mini-Gamma coding sequence intact. To construct the Kozak consensus sequence for optimal transcription, site directed mutagenesis was used to add "ACC" before the start codon. The primers used for site mutagenesis were Forward: 5'-CGCGCCGTCGAA TTCACCATGGATCT-3' and KK Reverse: 5'-GCGCGGCAGCTTAAGTGGTACCTAG-3'. The Des-Mini-Gamma sequence was amplified and introduced into pCR2.1-TOPO via TA cloning, then digested at the BamH1 and Not1 sites. The sequence was verified by Sanger sequencing. The digestion product was purified and injected into C57BL/6J embryos at the University of Chicago Transgenic Core. Founders were screened by PCR on genomic DNA isolated from tail clippings. Two transgenic lines were generated and bred as heterozygotes. The primers used for genotyping were mini-Forward: 5'-CGAATTCACCATGGATCTGTACGACGA-3' and mini-Reverse: 5'-CTAGATGCATGCT CGAGTCAAAGACAG-3'. Transgenic positive animals show a single band at 530bp. The targeted deletion to generate a null mutation of *Sgcg* was previously described (Hack et al., 1998), and this allele was previously bred into C57BL/6J for more than ten generations (Heydemann et al., 2005). The Des-Mini-Gamma transgenic mice were

bred to *Sgcg* null mice in the C57BL/6J background. Transgenic positive *Sgcg* null mice and transgenic negative *Sgcg* null littermates were compared. Animal work was conducted under the approval of the University of Chicago and Northwestern IACUCs.

*Microsomal preparation*

Membrane-bound proteins were isolated following the protocol of (Ohlendieck and Campbell, 1991) with modifications. Seven distinct muscle groups were dissected and combined, including triceps, diaphragm, abdominal muscle, quadriceps, gluteus, hamstring and gastrocnemius muscles. Muscles from one animal were homogenized in 12mL pre-chilled Buffer A on ice (20mM sodium pyrophosphate, 20mM sodium phosphate monohydrate, 1mM MgCl2, 0.303M sucrose, 0.5mM EDTA, 1mM PMSF, Roche cOmplete protease inhibitor tablet) using a Tissue Tearor Homogenizer (Model 985-370 Type II, with 7mm probe, Biospec products, Bartlesville, OK). Homogenized muscles were then transferred to a 15mL Dounce tissue grinder (Sigma-Aldrich) and grinded 40 times using a tightness “B” pestle on ice. One hundred µL lysate was removed as “total protein” (T). Lysates were centrifuged at 9000 rpm for 18 minutes at 4°C using Beckman SW41T1 rotor (13,900g). The pellet was discarded and 100µL supernatant was removed as “cytoplasmic protein” (C). The remainder of the supernatant was transferred to a new tube and centrifuged again at 13.200rpm for 30 minutes at 4°C (30,000g). One hundred µL supernatant was removed as “light microsomes” (L). The pellet was re-suspended in 12mL prechilled KCL wash buffer (0.6M KCl, 0.303M sucrose, 50mM Tris-HCl pH 7.4, 1mM PMSF, Roche cOmplete

protease inhibitor tablet), incubated for 30 minutes on ice to remove actomyosin contamination. The suspension was then centrifuged again at 28,800rpm for 30 minutes at 4°C (142,000g). The pellet was re-suspended in 300 to 500µL co-IP buffer and saved as "heavy microsomes" (H). All fractions were stored in -80°C freezer. For co-IP experiments, fresh heavy microsomes were used without freeze-thaw cycles.

*Co-immunoprecipitation from mouse muscles*

Co-immunoprecipitation from muscle followed protocol similar to the one used for co-IP from HEK293T cells with following modifications. Five hundred µg of skeletal muscle heavy microsomes were used as input. 45µL protein G/A beads were used for pre-clear and 100µL beads were used for pull down. Pre-cleared samples were incubated 25µL NCL-b-SARC or 10µL polyclonal Xpress antibodies overnight at 4°C.

*Immunoblotting*

Protein samples were denatured, resolved on pre-cast 14% Tris-glycine protein gels (Novex, San Diego, CA) and transferred to Immobilon-P membranes (Millipore, Bedford, CA). Reversible protein stain (Thermo Scientific, Waltham, MA) was performed on the membranes to evaluate transfer efficiency and equal protein loading. Membranes were blocked for 1h at room temperature with Starting Block T20 blocking buffer (Thermo Scientific, Waltham, MA), followed by incubation with primary antibody diluted in T20 blocking buffer for either 1h at room temperature or overnight at 4°C. After primary incubation, the membranes were washed times, 10 minutes each at room

temperature with TBS containing 0.1% Tween-20. The membranes were then incubated with secondary antibodies conjugated with Horseradish Peroxidase (Jackson ImmunoResearch, West Grove, PA) diluted in T20 blocking buffer for 1h at room temperature, followed by 3 washes. The blots were developed using Clarity™ Western ECL Blotting Substrate (BioRad, Hercules, CA) and imaged with UVP-GelDoc-IT® Imageer (UVP, upland, CA)

*Immunofluorescence microscopy for mice*

Mouse muscles were harvested and snap-frozen in liquid nitrogen. The samples were kept frozen in -80°C freezer until sectioning. Ten µm sections were cut from frozen tissues and immediately fixed in ice-cold methanol for 2 minutes and briefly rinsed in cold phosphate buffered saline (PBS) immediately afterwards. The rest of the staining and imaging methods were the same as previously described for HEK293T cells.

*Antibodies for HEK cells and mice*

To detect full-length murine γ-sarcoglycan (SGCG) protein, rabbit polyclonal anti-SGCG antibody was used (McNally et al., 1996b). To detect Mini-Gamma protein, a rabbit polyclonal antibody was raised to the Xpress epitope (Pocono Rabbit Farms, Canadensis, PA) and affinity-purified. The rabbit polyclonal anti-SGCG antibody NBP1-90298 recognized both SGCG and Mini-Gamma (Novus Biologicals, Littleton, CO). β-Sarcoglycan was detected with NCL-b-SARC (CAT#B-SARC-CE, Leica Biosystems, Nussloch, Germany) and δ-sarcoglycan was detected with a polyclonal antibody (Hack

et al., 2000). Secondary antibodies were Alexa Fluor® 488 Goat Anti-Rabbit and Alexa Fluor®594 Goat Anti-Rabbit (Invitrogen, Carlsbad, CA). For microscopy, primary antibodies were used at 1:500 dilutions, and the secondary antibodies were used at 1:8000 dilutions. For immunoblotting, primary antibodies were used at 1:1000 dilutions. Secondary antibodies were Goat anti-mouse or Goat anti-rabbit Horseradish Peroxidase conjugated antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and used at 1:8000 dilution.

*Additional antibodies for immunofluorescence microscopy*

The anti-dystrophin antibody was Cat# RB-9024-P (Thermo, Waltham, MA). The anti-β-sarcoglycan antibody was Cat# NBP1-19782 (Novus Biologicals, Littleton, CO). The anti-γ- sarcoglycan antibody used to detect Mini-Gamma was Cat # NBP1-90298 Novus Biologicals.

*Evans Blue Dye Imaging and Quantification*

Evans Blue Dye (EBD) (Sigma, E-2129) was dissolved in phosphate buffered saline (PBS) at 10 mg/ml. Each animal received an intraperitoneal injection of EBD at 5µl/g body weight. Approximately 24 hours after injection, tissues were harvested and imaged. Gross images were acquired with a Canon Digital Rebel T5i. For quantification, quadriceps, abdominal and diaphragm muscles were harvested from 4~5 month-old age- and sex-matched mice. The excised muscle was immediately frozen in liquid nitrogen and stored at -80°C. Seven µm sections from the center of the muscle

were fixed in 4% paraformaldehyde for 5 minutes, blocked in 10% fetal bovine serum in PBS with 0.1% Triton X-100, and stained with anti-dystrophin antibody (Cat#RB-9024-P, Thermo, Waltham, MA) for 1 hour at room temperature at a dilution of 1:100. A secondary antibody, donkey anti-rabbit conjugated to Alexa-488 (Life Technologies, Calsbad, CA) was used at 1:5000. Sections were mounted in Vectashield with DAPI. Images were acquired on a Zeiss Axio Imager M2. Three to five images per muscle section were taken at 10x from 3 animals per genotype and analyzed. Statistics were performed with Prism (Graphpad, La Jolla, CA) using student t-test.

*Creatine Kinase Assay*

Serum was collected mid-morning on the same day from age- and sex- matched (2-5m) *Sgcg* and *Sgcg, Tg+* animals from retro-orbital bleeds using heparinized capillary tubes (Cat#22-362-566, Fisher, Pittsburgh, PA) into serum separator tubes (Cat#02-675-185, Becton Dickinson, Franklin Lakes, NJ) and centrifuged for 10 minutes at 8000 x *g*. The plasma fractions were frozen and stored at -80°C and then assayed later using the Enzy-Chrom CK Assay kit (ECPK-100; BioAssay Systems, Hayward, CA). CK activity was measured at 37°C in the Synergy|HTX Multi-mode plate reader (BioTek, Winooski, VT) in Costar 96-well plates (Corning Inc, Corning, NY). Statistical analysis was performed with a one-way ANOVA (Prism, Graphpad, La Jolla, CA).

*Histology*

A cross-sectional strip was obtained from midline of the diaphragm muscle. The

strip was then fixed in formalin, dehydrated and embedded in paraffin. Seven μm sections were obtained and stained with hematoxylin and eosin (H&E). For central nucleated fiber analysis, three random fields each were obtained at 20x magnification from six animals per genotype. For diaphragm thickness calculations, three evenly spaced fields along the length of the strip each were taken at 10x magnification from six animals per genotype. The ruler tool in Photoshop (Adobe, San Jose, CA) was used to calculate the thickness of each field. Diaphragm thickness of each animal was the average of the three different fields. For quadriceps muscle histology, a cross-sectional middle piece (quad discs) was cut out using razor blades. Five random fields were obtained for central nucleation analysis. For fibrosis analysis, Masson's Trichrome staining was performed and percent of fibrotic area (Blue) was calculated using ImageJ.

*Hydroxyproline (HOP) assay*

Hydroxyproline content was determined as previously described (Heydemann et al., 2005).

*Echocardiography*

Echocardiography was performed as previously described (Goldstein JA et al., 2014). All measurements were done by the same operator, who was blinded to the genotype at the time of measurements.

**RESULTS**

*Mini-Gamma translocates to membrane with β- and δ-sarcoglycan*

Murine sarcoglycan proteins were transiently expressed in the human embryonic kidney 293T (HEK293T) cells to examine their intracellular localization. A diagram of the sarcoglycan plasmids used for *in vitro* transfection assays is shown in Fig 3.1 A. It was previously shown that β- and δ-sarcoglycan form a core subunit, followed by the addition of γ-sarcoglycan to the complex (Hack et al., 2000; Noguchi et al., 2000; Shi et al., 2004). Expression of the individual sarcoglycan subunits, β-, γ-, or δ-sarcoglycan or Mini-Gamma, produced little accumulation of immunoreactivity at the plasma membrane (Fig 3.1.B, upper panels), consistent with prior reports of interdependency for normal intracellular trafficking (Shi et al., 2004). Co-expression of β- and δ-sarcoglycan along with γ-sarcoglycan resulted in plasma membrane enrichment of γ-sarcoglycan (Fig 3.1.B, lower panel left). Similarly, expression of β- and δ-sarcoglycan and Mini-Gamma also resulted in plasma membrane associated Mini-Gamma staining (Fig 3.1.B, lower panel left).

*Mini-Gamma interacts with β- and δ-sarcoglycan*

Immunoprecipitation of expressed sarcoglycan subunits using an anti-β-sarcoglycan antibody confirmed the presence of complexes containing β-sarcoglycan, γ-sarcoglycan and δ-sarcoglycan (Fig 3.2, upper panel). Likewise, immunoprecipitation with an anti-β-sarcoglycan antibody demonstrated an interaction among β-sarcoglycan, δ-sarcoglycan and Mini-Gamma (Fig 3.2, bottom panels). Immunoprecipitation for Mini-

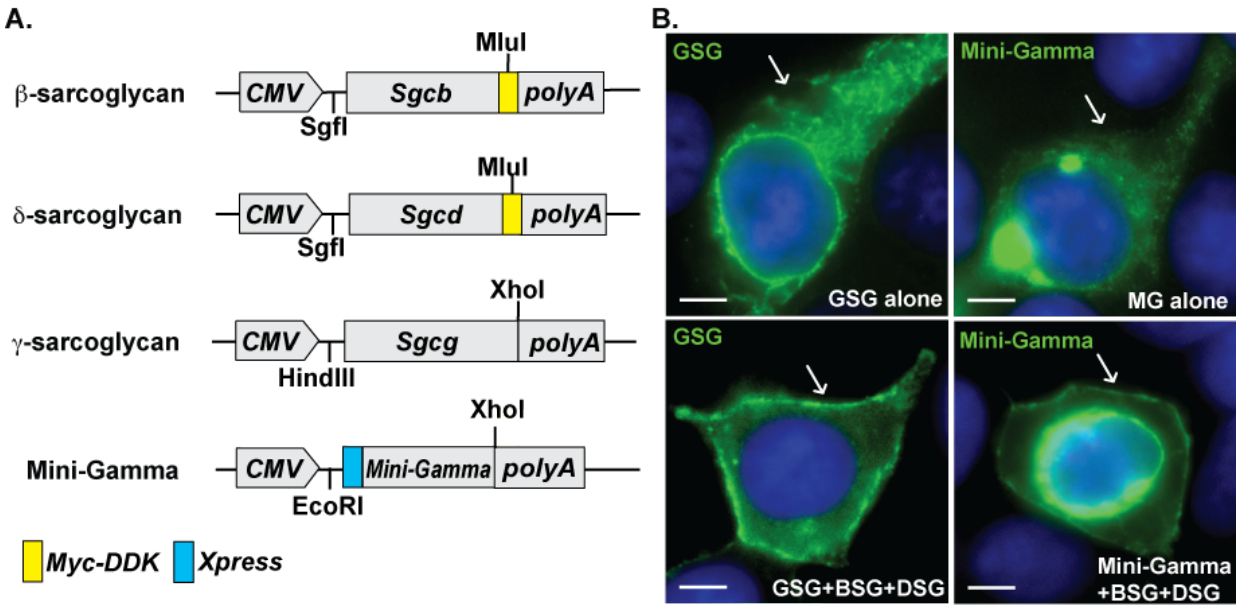


**Figure 3.1. Mini-Gamma translocates to the membrane in the presence of β- and δ-sarcoglycan *in vitro*.** Plasmids encoding mammalian sarcoglycans were expressed in HEK293T cells. (A) Diagram of sarcoglycan constructs for transfecting human embryonic kidney cells (HEK293T). All four constructs shared a human cytomegalovirus (CMV) promoter. Note that Mini-Gamma construct was tagged with an Xpress epitope tag at the N-terminus. (B) Expression of γ-sarcoglycan (GSG) or Mini-Gamma alone resulted in cytoplasmic and peri-nuclear accumulation (arrows show little to no plasma membrane trafficking). This observation is consistent with previous reports that association with the β/δ-sarcoglycan core is required for membrane targeting (Shi et al., 2004). Co-expression of γ-sarcoglycan with β- and δ-sarcoglycan resulted in plasma membrane translocation of γ-sarcoglycan (arrow in bottom left panel). Similarly, expression of Mini-Gamma with β- and δ-sarcoglycan resulted in plasma membrane translocation of Mini-Gamma (arrow in bottom right panel). Scale bar = 5μm.

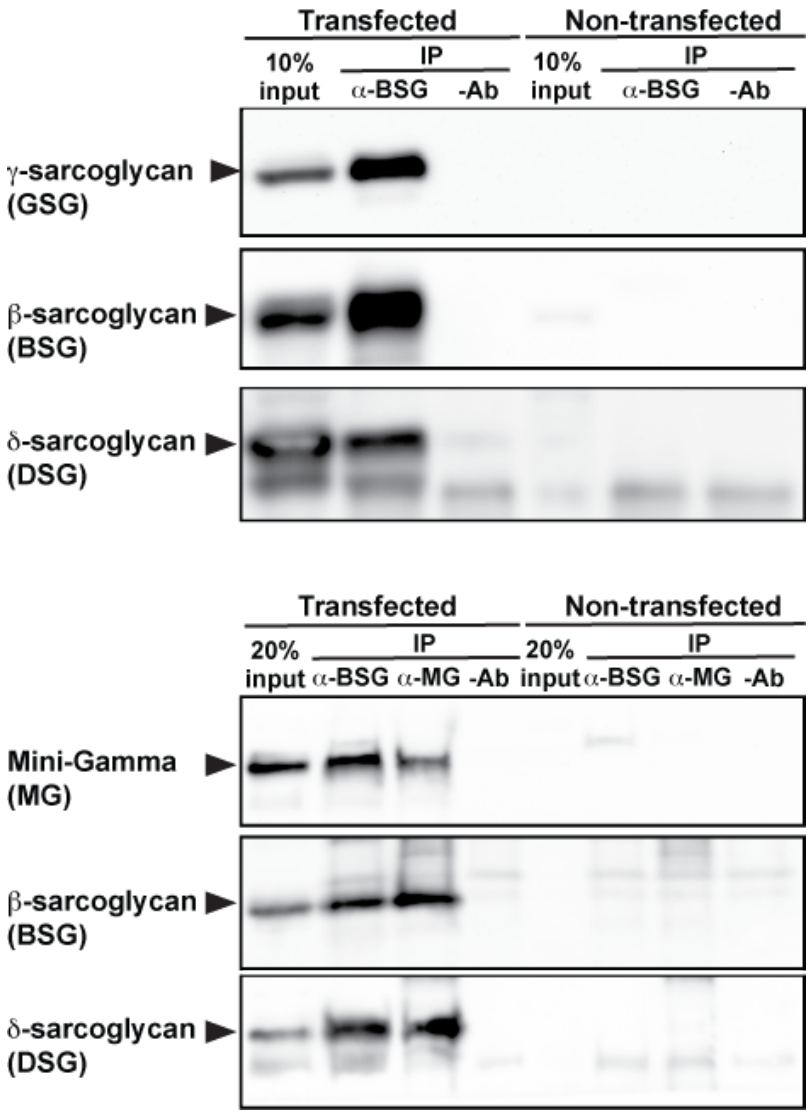


**Figure 3.2. Mini-Gamma interacts with β-sarcoglycan and δ-sarcoglycan in a heterologous cell expression system.** Co-immunoprecipitation was performed to examine sarcoglycan complex formation from HEK293T cells. After immunoprecipitation with an anti-β-sarcoglycan antibody, a complex containing β-, δ- and γ-sarcoglycan was detected in β /δ/ γ co-expressing cells (upper panels). Likewise, immunoprecipitation with the same anti-β-sarcoglycan antibody demonstrated an interaction among β-, δ- and Mini-Gamma (lower panels). Immunoprecipitation for Mini-Gamma using an antibody against the Xpress tag also detected β- and δ-sarcoglycan.

Gamma also detected β- and δ-sarcoglycan (Fig 3.2, bottom panels). These data demonstrate that Mini-Gamma formed a complex with β- and δ-sarcoglycan like full-length γ-sarcoglycan.

*Generation of Mini-Gamma transgenic lines*

To test the function of Mini-Gamma *in vivo*, transgenic mice expressing Mini-Gamma under the control of the desmin promoter were generated. The desmin promoter is active in both heart and skeletal muscle (Pacak et al., 2008). The transgene construct is shown (Fig 3.3 A). Two lines were characterized. Line Tg50 demonstrated high expression and Line Tg84 had lower expression. A rabbit polyclonal antibody (NBP1-90298) was raised by immunizing the animal with a peptide containing the 100 amino acids at the carboxyl-terminal of γ-sarcoglycan (Novus Biologicals); about half of them remained in Mini-Gamma. NBP1-90298 is able to recognize both γ-sarcoglycan and Mini-Gamma. By western blot using this antibody, Mini-Gamma protein band is two times brighter than the endogenous γ-sarcoglycan protein band in Tg50 while it is 6-9% as bright as γ-sarcoglycan protein band in Tg84 (Fig 3.3 B and data not shown). However, the affinity of this antibody to the full-length protein and Mini-Gamma is likely to be different since half of the immunogen peptide is deleted in Mini-Gamma. Therefore, a precise quantification of the protein expression level of the transgene relative to the endogenous γ-sarcoglycan can be challenging.

*Mini-Gamma is enriched in the membrane-bound fraction in muscle*

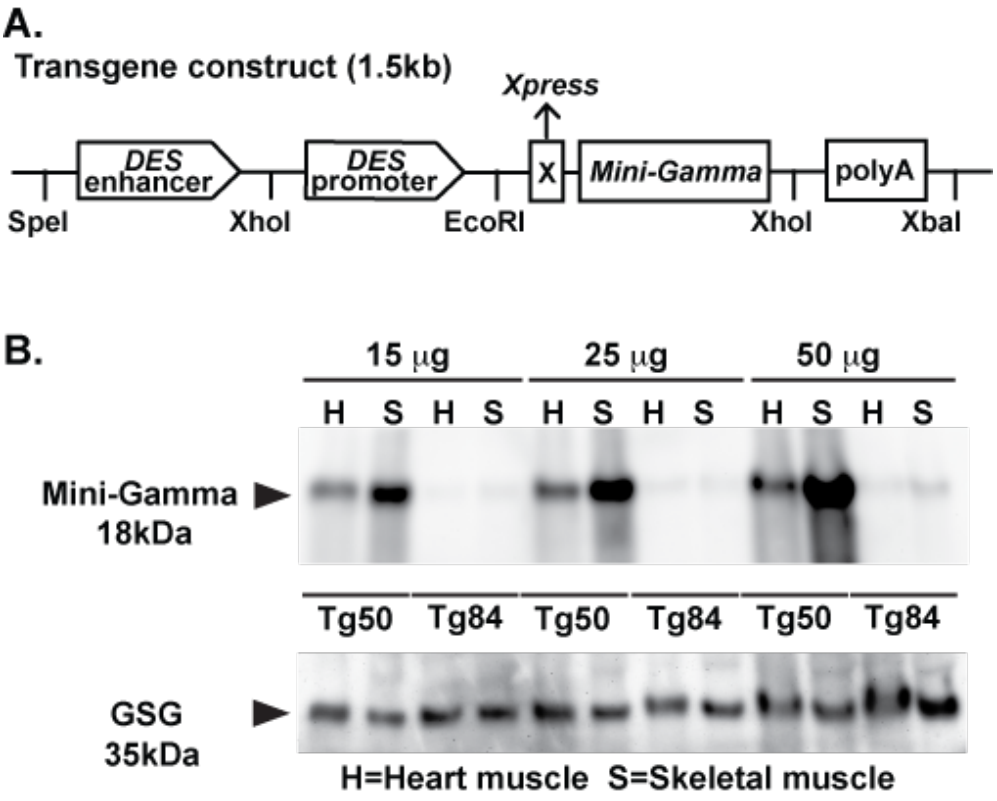


**Figure 3.3. Transgenic mice expressing Mini-Gamma protein were generated** (A) Human desmin (DES) regulatory sequences were used to direct expression of Mini-Gamma in a muscle-specific manner. Note that an Xpress-tag was placed at the N-terminus of Mini-Gamma. (B) Two independent lines of Mini-Gamma were characterized; Tg50 had high expression, while Tg84 had lower expression. Both cardiac and skeletal muscle expression were achieved for the transgene, shown by western blotting. 15 μg, 25 μg, and 50 μg represent how much protein was loaded onto the gel. A rabbit polyclonal antibody against Xpress (Pocono Farms) was used to recognize Mini-Gamma and a mouse monoclonal antibody (NCL-gSARC) was used to detect γ-sarcoglycan (GSG).

Muscle microsomal fractionation was used to monitor the expression of Mini-Gamma in muscle by separating fractions of crude total muscle lysates (T) into cytoplasmic fraction (C), light microsomal (L) and heavy microsomal (H) fractions (Fig 3.4 A). Sarcolemma, endoplasmic reticulum and Golgi-associated membrane proteins are enriched in the heavy microsomal fraction. In wild type animals, sarcoglycan proteins and other membrane-bound DGC components are mainly found in the muscle heavy microsomal fraction (Ohlendieck and Campbell, 1991). Similar to the endogenous γ-sarcoglycan, Mini-Gamma protein was highly enriched in heavy microsomes from both transgenic lines indicating its normal trafficking in muscle (Fig 3.4 B, H lanes). Low amounts of Mini-Gamma were detected in the other membrane fractions, possibly consistent with competition between Mini-Gamma and full-length γ-sarcoglycan protein.

*Mini-Gamma is localized at the sarcolemma in mouse skeletal muscle*

To document intracellular localization, immunofluorescence microscopy was used to demonstrate the plasma membrane localization of Mini-Gamma protein in skeletal muscle (Fig 3.5, top panels). Interestingly, endogenous γ-sarcoglycan was slightly diminished in Mini-Gamma transgenic animals compared to identically and simultaneously processed muscle sections from wild type animals, suggesting competition for plasma membrane localization between Mini-Gamma and the endogenous γ-sarcoglycan protein (Fig 3.5, compare bottom two panels).

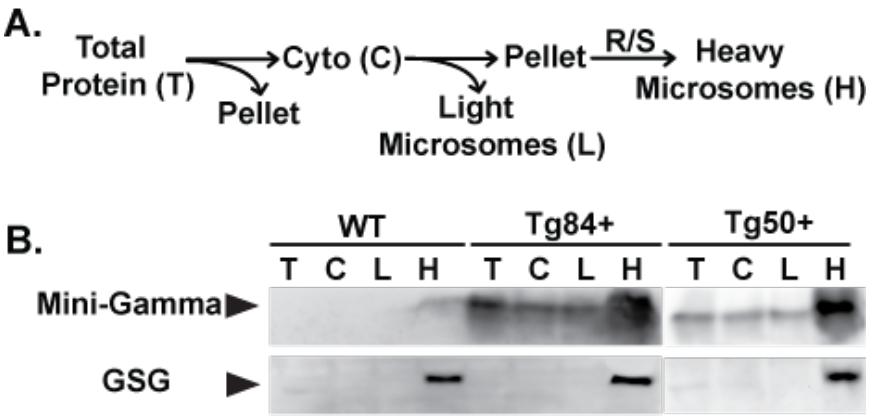


**Figure 3.4. Mini-Gamma is highly enriched in the muscle membrane fraction.** (A) Microsomal preparation was performed to isolate membrane-associated proteins. Briefly, muscles were dissected and homogenized to produce total lysates (T). After a low speed spin (9000RPM), the pellet (containing nuclei, mitochondria, lysosomes and et cetera) was discarded and the supernatant was saved as cytoplasmic fraction (C). After a high speed spin (13500RPM), the supernatant containing the soluble fraction (Light microsomes, L) was discarded. The pellet was washed and spun down again at 28800RPM. Finally, the pellet was re-suspended and saved as heavy microsomes (H). The sarcoglycan complex is known to enrich in the heavy microsomal fraction, which contains the secretory system and plasma membrane. (B) Mini-Gamma protein was found mainly in the muscle heavy microsomes purified from both transgenic mouse lines, similar to the endogenous γ-sarcoglycan. This result suggested that Mini-Gamma shared cellular localization and secretion pathways with other endogenous sarcoglycans. 25μg protein was loaded for all Tg50+ lanes while 100μg protein was loaded for Tg84+ lanes and WT lanes

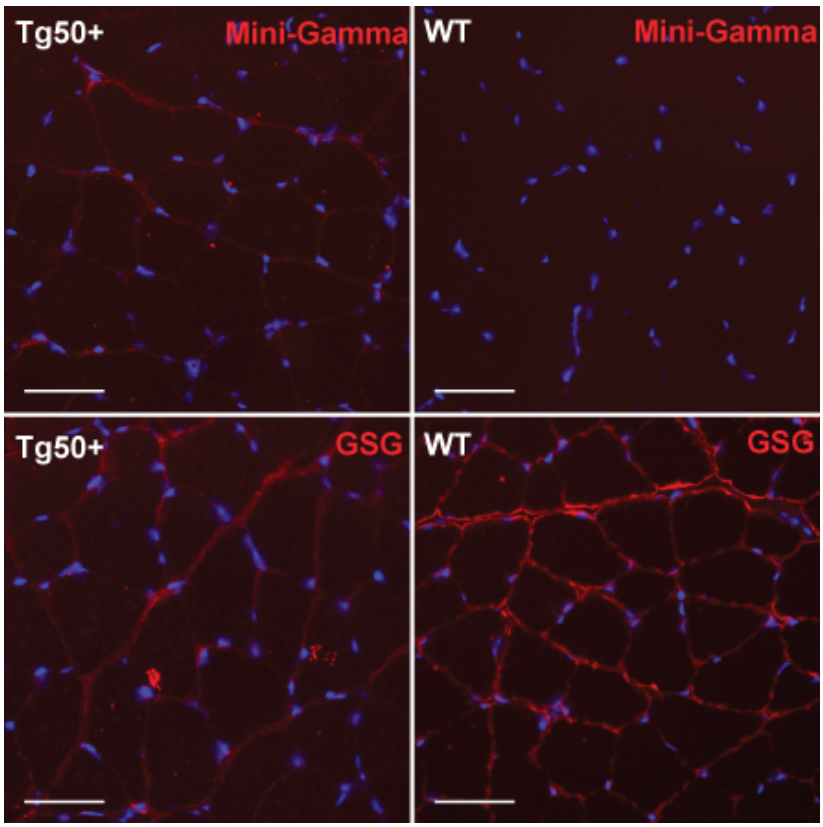


**Figure 3.5. Mini-Gamma localizes at the plasma membrane of skeletal muscle in transgenic mice.** Mini-Gamma showed distinct membrane immunostaining in cross sections from Tg50+ mouse muscle, suggesting that Mini-Gamma was able to incorporate into the sarcoglycan complex and translocates to the membrane *in vivo*. Interestingly, endogenous γ-sarcoglycan was slightly diminished in Tg50+ animals compared to identically and simultaneously processed muscle sections from WT, suggesting competition for plasma membrane localization between Mini-Gamma and endogenous γ-sarcoglycan. Scale bar = 50 μm

*Mini-Gamma is incorporated into the sarcoglycan complex in vivo*

To test the interaction between Mini-Gamma and the other sarcoglycans *in vivo*, co-immunoprecipitation was performed from the heavy microsomal fraction. Mini-Gamma was co-immunoprecipitated along with β-sarcoglycan (Fig 3.6 A). The epitope tag antibody to Mini-Gamma also resulted in co-immunoprecipitation of β-sarcoglycan (Fig 3.6 A). This result indicates that complexes containing α-sarcoglycan, β-sarcoglycan, δ-sarcoglycan and Mini-Gamma can be formed *in vivo* (Fig. 3.6 B).

*Mini-Gamma improves membrane association of β- and δ-sarcoglycan in Sgcg mice*

Proper assembly of sarcoglycan complex is essential for its translocation to the plasma membrane in the muscle cells. In the absence of γ-sarcoglycan, sarcolemma targeting of β- and δ-sarcoglycan is impaired, reducing β- and δ-sarcoglycan content in the heavy microsomal fraction (Fig 3.7 B, left drawing) (Hack et al., 2000). Tg50+ mice were crossed with *Sgcg* null animals to assess the capacity of Mini- Gamma to rescue the absence of *Sgcg*. In *Sgcg, Tg50+* animals, β- and δ-sarcoglycan protein levels were increased in the heavy microsomal fraction compared to those from *Sgcg* null animals (Fig 3.7 A**,** H lanes). Consistent with this observation, immunostaining of β- and δ-sarcoglycan at the muscle membrane was also improved in *Sgcg, Tg50+* animals compared to that of *Sgcg* (Fig 3.8). Taken together, these data implicate that Mini-Gamma forms a complex with other sarcoglycan and facilitate the membrane translocation of the sarcoglycan complex in the absence of γ-sarcoglycan (Fig 3.7 B).

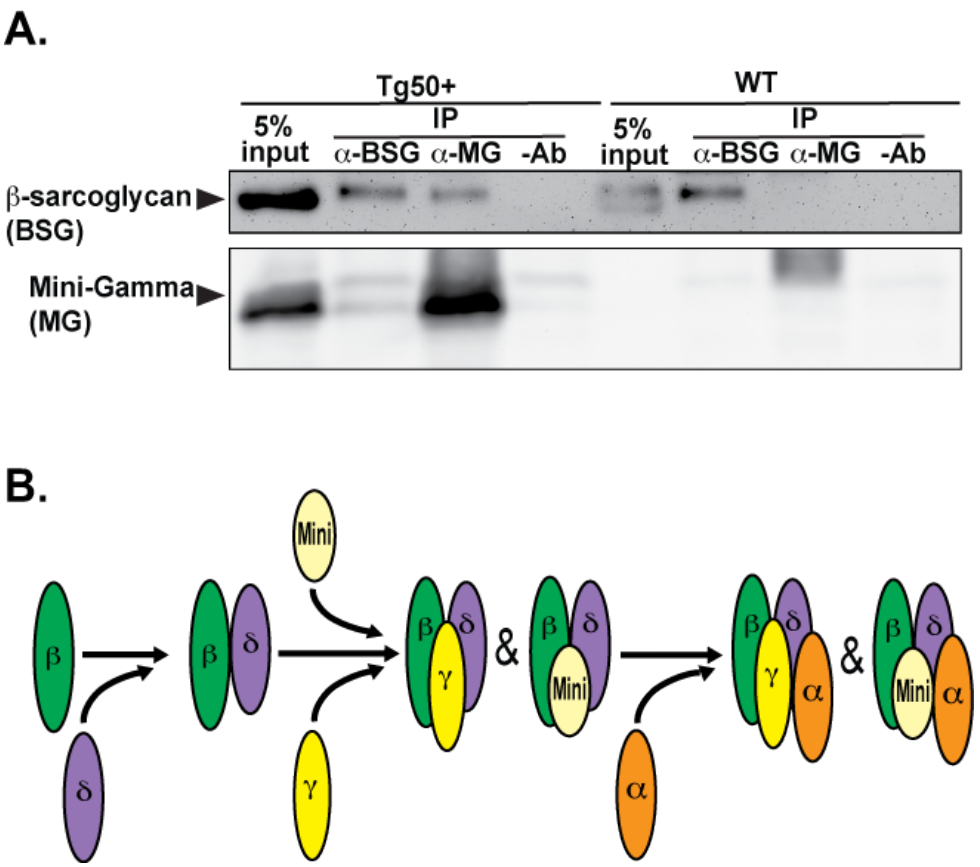


**Figure 3.6. Mini-Gamma is incorporated into the sarcoglycan complex *in vivo***. (A) Co-immunoprecipitation from the heavy microsomal fraction was performed to test the interaction between Mini-Gamma and other sarcoglycans. Mini-Gamma was precipitated using an antibody against β-sarcoglycan (α-BSG). The Xpress tag antibody to Mini-Gamma (α-MG) also resulted in precipitation of β-sarcoglycan. (B) Sarcoglycan assembly model in Tg+ mouse muscle. Sarcoglycan complexes composed of either β-, δ-,α- and γ- sarcoglycan or β-, δ-,α- and Mini-Gamma were formed and translocated to the sarcolemma.

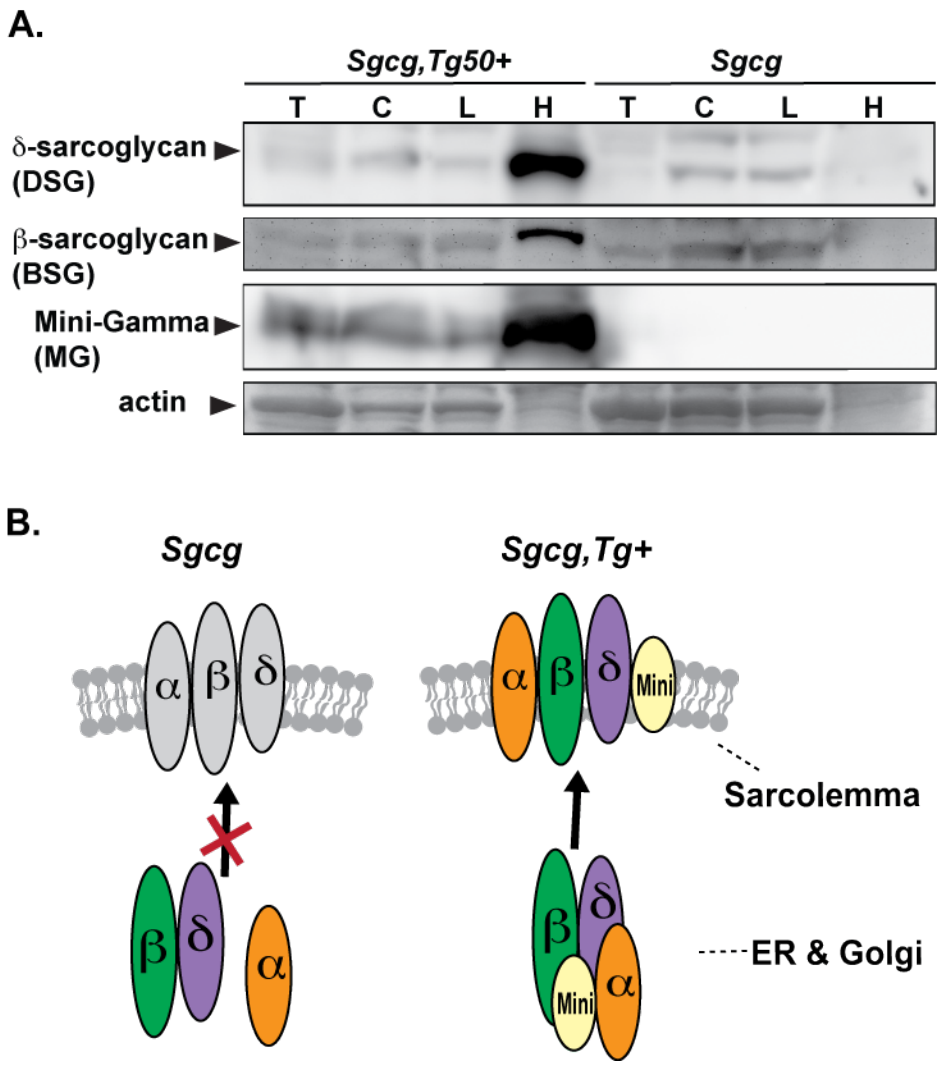


**Figure 3.7. Mini-Gamma rescues the expression of β- and δ-sarcoglycans in *Sgcg* skeletal muscle.** (A) In the absence of γ-sarcoglycan, β- and δ-sarcoglycan content in the heavy microsomal fraction is reduced due to impaired sarcolemma targeting, consistent with previous reports (Hack et al., 2000). In *Sgcg, Tg50+* mice, β- and δ-sarcoglycan protein levels were increased in the heavy microsomal fraction compared to those from *Sgcg* muscle without the Mini-Gamma transgene. (B) Diagram of Mini-Gamma improving the trafficking of the sarcoglycan complex in the *Sgcg* mice is shown.

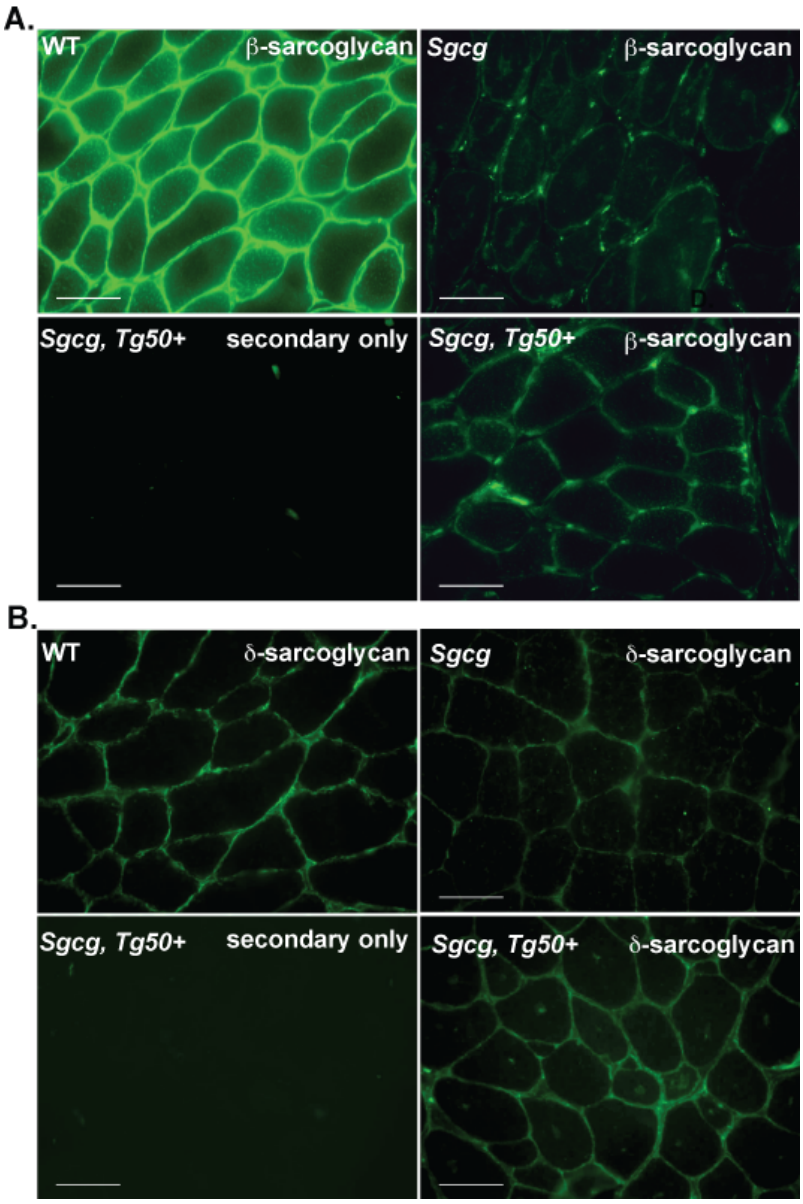


**Figure 3.8. Mini-Gamma improves the impression of β- and δ-sarcoglycan at the sarcolemma.** (A) Compared to WT, *Sgcg* mouse muscle showed greatly reduced β-sarcoglycan staining at the muscle membrane. In the presence of Mini-Gamma, β-sarcoglycan membrane localization was increased compared to identically and simultaneously processed muscle sections from *Sgcg*. (B) δ-Sarcoglycan membrane localization was improved in *Sgcg, Tg50+* compared to identically and simultaneously processed muscle sections from *Sgcg*. Scale bar = 50μm.

*Mini-Gamma reduce muscle membrane leak in Sgcg mice*

Evans blue (EBD) is a dye that binds albumin, the most common protein found in blood. Healthy muscle fibers are impermeable to albumin-bound dye. Muscle fibers in *Sgcg* mice have impaired membrane stability and increased dye permeability (Hack et al., 1998). To measure sarcolemmal fragility and leak, EBD was injected into *Sgcg, Tg50+* and *Sgcg* null mice. By gross inspection, reduced uptake of dye was seen in *Sgcg, Tg50+* compared to *Sgcg* null muscle, and this was seen in diaphragm and abdominal muscles (Fig 3.9 A and B). Microscopically, dye uptake (red fluorescence) was reduced in quadriceps muscle (Fig 3.9 C). The percent of the diaphragm and abdominal muscles opacified by dye uptake was reduced in *Sgcg, Tg50+* compared to *Sgcg* null muscle (Fig 3.9 D). Creatine Kinase (CK) is an enzyme that is normally found in high concentrations in the muscle cells. Membrane fragility of *Sgcg* null muscle allows the enzyme to leak into the bloodstream. Elevated serum CK level has been widely as indicator for muscle membrane damage in both animal studies and clinical diagnosis. Consistent with reduced EBD uptake, serum CK was reduced in *Sgcg, Tg50+* mice compared to *Sgcg* null mice (Fig 3.9 E).

*Mini-Gamma improve skeletal muscle pathology in Sgcg mice*

In many models of muscular dystrophy, the diaphragm muscle is one of the most severely diseased muscles, and the diaphragm muscle in *Sgcg* null mice is markedly thickened with an increase in centrally nucleated myofibers (Hack et al., 1998). In *Sgcg, Tg50+* mice, the thickness of the diaphragm muscle was reduced to normal size (Fig

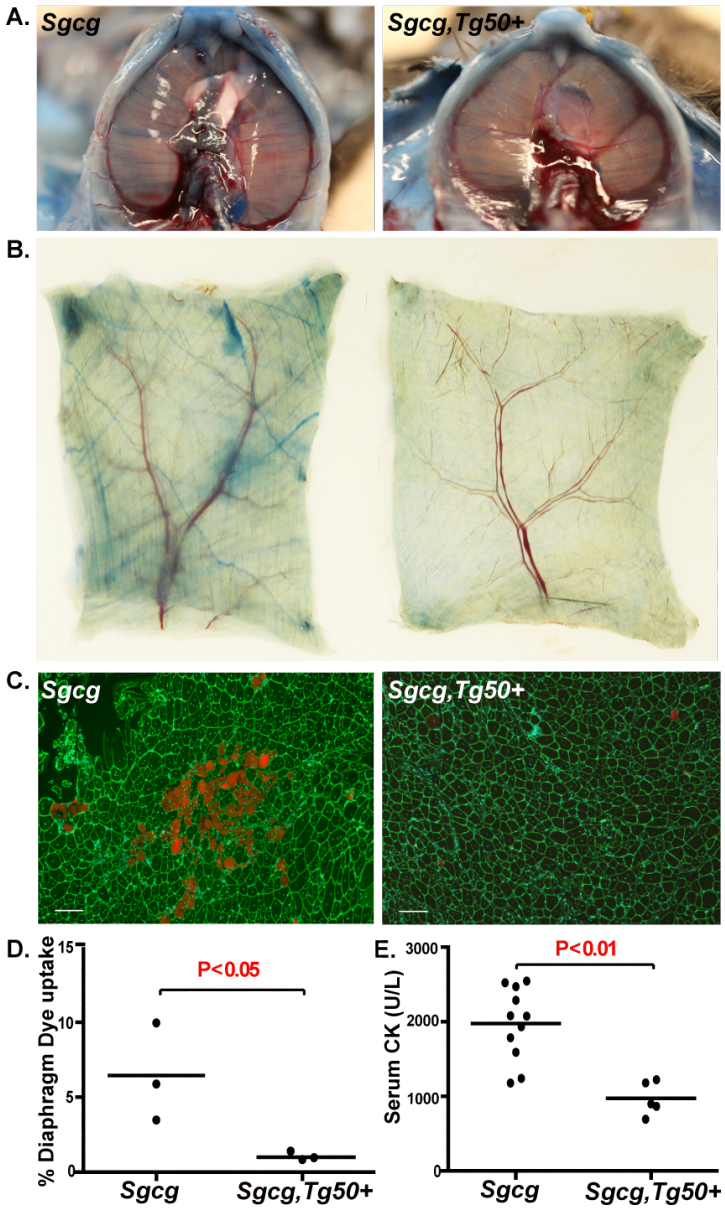


**Figure 3.9. Reduction of sarcolemmal leak with Mini-Gamma expression in *Sgcg* null mice.** Evans blue dye was injected into *Sgcg* null mice to monitor sarcolemmal fragility and subsequently leak. (A) & (B) Gross images are shown from the diaphragm muscle (top row), abdominal muscle (middle row) demonstrating dye uptake (blue). (C) Immunofluorescence images of quadriceps muscle sections with dystrophin staining (green) and dye uptake marked as red. Scale bar = 200 μm. The reduction of dye uptake was observed in *Sgcg, Tg50* mice across multiple muscle tissues. WT mice have very little or no EBD uptake. (D) The percent of the diaphragm muscle that was opacified by dye was measured and was reduced in *Sgcg, Tg50* muscle compared to *Sgcg* null muscle. (E) Serum CK was reduced in *Sgcg, Tg50* compared to *Sgcg* null mice. Reduction of CK is another reflection of improved muscle membrane stability. CK in WT mice is about 300 U/L ((Hack et al., 1998).

3.10 A and B). Centrally nucleation is a sign of muscle regeneration in response to repeated injury and degeneration. The percentage of centrally nucleated fibers was reduced in diaphragm muscle from *Sgcg, Tg50+* mice compared to *Sgcg* null mice (Fig 3.10 C), consistent with less injury and therefore reduced need for regeneration from the presence of Mini-Gamma.

Quadriceps is another muscle group that is severely affected in *Sgcg* null mice. Fibrosis is the abnormal deposition of collagen in the muscle tissue and another hallmark for muscle degeneration. Collagen deposition is represented by blue staining in the Masson's trichrome staining. Compare to *Sgcg* null, *Sgcg, Tg50+* mice have reduced fibrosis in the quadriceps (Fig 3.11). Central nucleated fibers are also reduced in quadriceps muscle from *Sgcg, Tg50+* mice compared to *Sgcg* null mice (Fig 3.11).

*Mini-Gamma reduces fibrosis and improves function of Sgcg hearts*

Because Mini-Gamma transgenic mice also expressed protein in cardiac muscle, improvement of heart function was also examined. Protein expressed from the Mini-Gamma transgene was detected at the sarcolemma of cardiomyocytes from *Sgcg, Tg50+* mice, similar to the γ-sarcoglycan protein (GSG) in WT hearts (Fig 3.12 A). *Sgcg* mice develop cardiac dysfunction and fibrosis as they age (Hack et al., 1998). Fibrosis, as monitored by hydroxyproline content, was reduced in the hearts of *Sgcg, Tg+* mice compared to *Sgcg* null mice ($p<0.05$) (Fig 3.12 B). In addition, *Sgcg, Tg84+* animals had improved fractional shortening compared to *Sgcg* null animals ($p<0.05$) (Fig 3.12 C). *Sgcg, Tg50+* animals only showed trends of improvements in fractional shortening. This might be explained by previous studies showing that overexpression of γ-

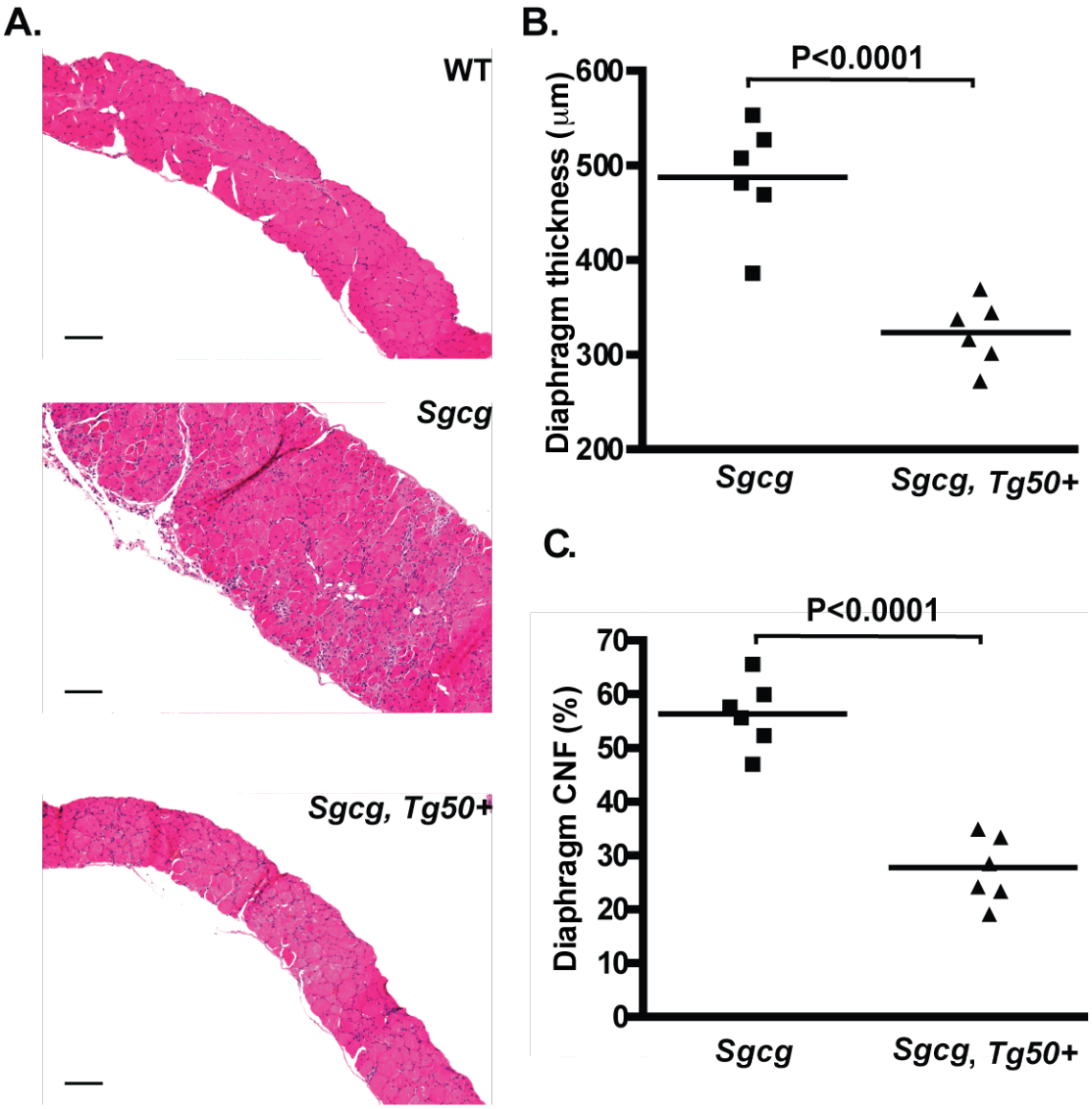


**Figure 3.10. Mini-Gamma expression improves diaphragm muscle pathology in *Sgcg* mice.** (A) The diaphragm muscle is severely affected by the dystrophic process in *Sgcg* mice, as it is in other mouse models of muscular dystrophy, and this is seen as marked thickening, referred to as pseudohypertrophy (Hack et al., 1998). This pseudohypertrophy process was reversed in *Sgcg, Tg50+* mice. (B) Quantification of diaphragm thickness is shown. In *Sgcg, Tg50+* mice, the thickness of the diaphragm muscle was reduced to WT level. (C) Central nucleation, another feature of dystrophic muscle, is increased in *Sgcg* mice, reflecting accelerated pathological regeneration. The percentage of centrally nucleated fibers was reduced in diaphragm muscle from *Sgcg, Tg50* mice compared to *Sgcg* mice, consistent with reduced degeneration and therefore a decreased need for regeneration. Percentage of central nucleated fibers in WT diaphragm is 5%~10%. Scale bar = 100μm.

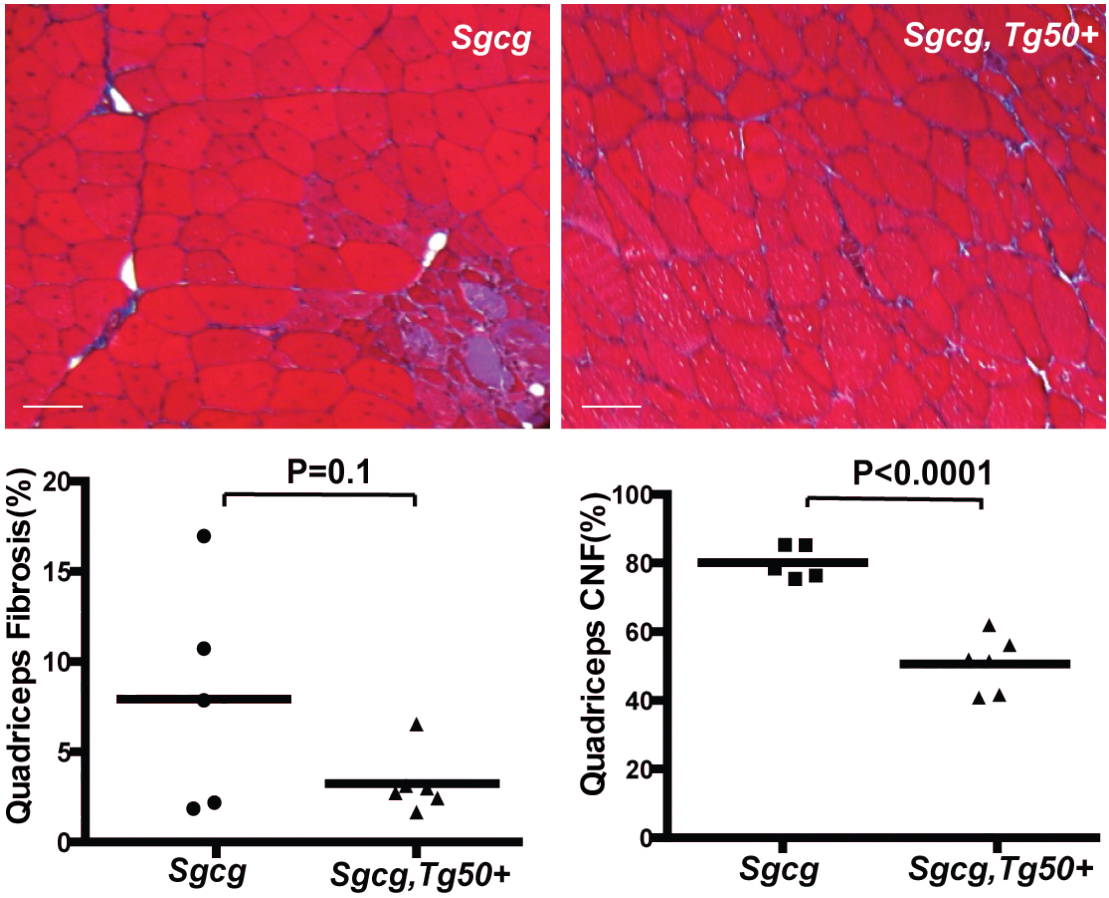


**Figure 3.11. Mini-Gamma expression improves quadriceps muscle pathology in *Sgcg* mice.** Quadriceps pathology is also improved in *Sgcg, Tg50* mice compared to *Sgcg* mice. Masson's Trichrome staining was performed on quadriceps muscle sections and fibrotic areas were marked by blue staining. Muscle fibrosis is the replacement of healthy muscle tissue by scar tissue and is another hallmark of muscular dystrophy. *Sgcg, Tg50+* mice trend towards having reduced fibrotic area in quadriceps compared to *Sgcg* mice (upper panels and lower panel left). WT mice at this age do not develop fibrosis. Central nucleation in quadriceps is significantly reduced in *Sgcg, Tg50+* mice (lower panel right). The percentage of central nucleated fibers in WT quadriceps is 2%~3%. Scale bar = 50μm. Student's t-test was used to compare results between two groups. Age- and sex matched littermates were compared.

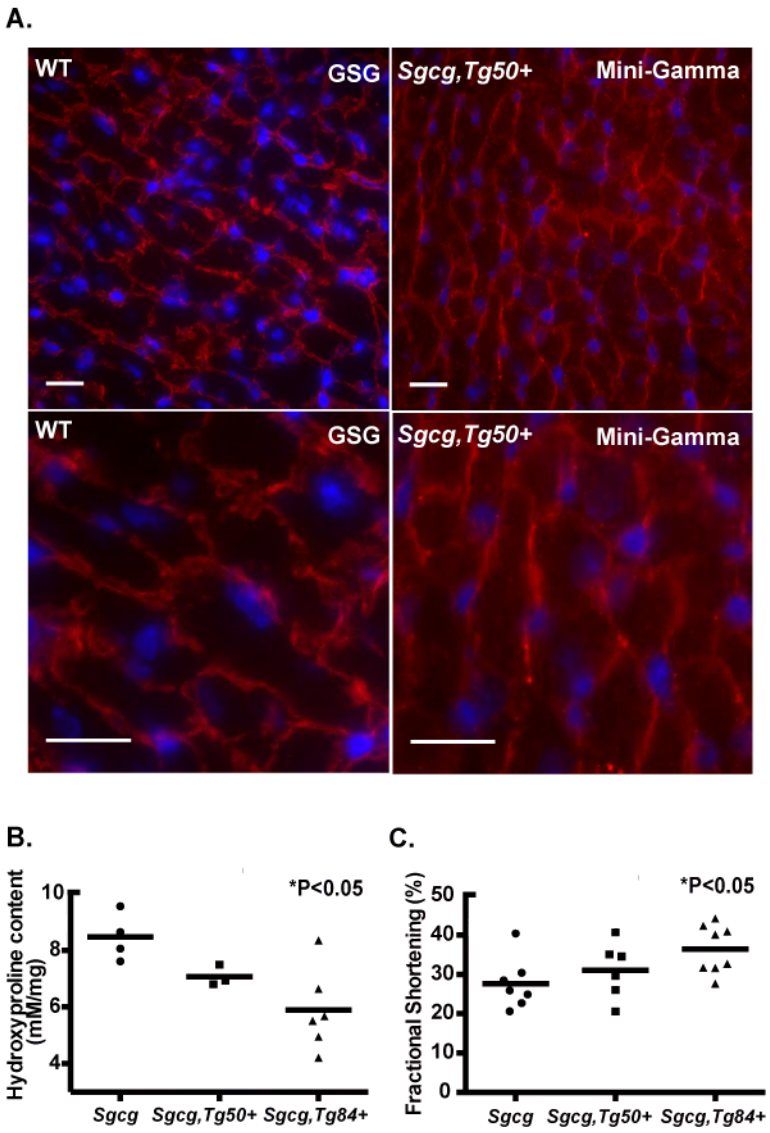


**Figure 3.12. Mini-Gamma reduces fibrosis and improves function of *Sgcg* hearts.** (A) Mini-Gamma protein was detected at the sarcolemma of cardiomyocytes from *Sgcg, Tg50+* mice, similar to that of the endogenous γ-sarcoglycan in wild type animals. Scale bar = 20μm. (B) *Sgcg* mice develop fibrosis and impaired cardiac function (Hack et al., 1998). Cardiac fibrosis was monitored by hydroxyproline content. In *Sgcg, Tg84+* mice, heart fibrosis was reduced compared to *Sgcg* mice ($p<0.05$). Fibrosis in WT heart is around 6mM/mg. (C) Cardiac function was evaluated by echocardiography. Compared to *Sgcg* mice, *Sgcg, Tg84+* mice had improved fractional shortening ($p<0.05$). *Sgcg, Tg50+* trended towards improvement in fibrosis and fractional shortening, and this may be due to toxic overexpression of Mini-Gamma as overexpression of γ-sarcoglycan in skeletal muscle can induce muscular dystrophy (Zhu et al., 2001). Fractional shortening in WT mice averages around 35% to 40%. Fibrosis and fractional shortening measurements shown here are of males only. Females showed the same trends but not significant (data not shown). Comparisons were made using a one-way ANOVA.

sarcoglycan is associated with cellular toxicity in muscle (Zhu et al., 2001). These observations were made in males since female *Sgcg* mice have greater variability in physiological measurements. These data demonstrate that Mini-Gamma improved cardiac dysfunction caused by loss of full-length γ-sarcoglycan.

## DISCUSSION

Gene therapy for muscular dystrophy has often relied on viral strategies for gene replacement. For DMD, this has been especially problematic due to the large size of the dystrophin gene and protein and the limited capacity of viral vectors. For Limb Girdle Muscular Dystrophy 2C, which is caused by *SGCG* mutations and results in the loss of much smaller γ-sarcoglycan protein, viral gene therapy has been initiated (Herson et al., 2012). However, viral gene therapy for any muscle disease is challenged by the need to deliver the product to all muscle groups. Because of these hurdles, exon skipping is emerging as a genetic correction strategy of clinical utility. The progress in clinical trials for DMD suggests that exon skipping could be applied to other disorders, including neuromuscular disorders. The premise for exon skipping for DMD derives from observations in BMD patients with in-frame deletions that preserve the functionally important amino- and carboxyl-termini of dystrophin. The X-linked recessive nature of DMD, combined with the repetitive structure of the dystrophin rod domain, made DMD an ideal target for exon skipping. Although an attractive method for genetic correction, exon skipping in DMD typically yields a much lower level of dystrophin protein compared to endogenous dystrophin levels. This lower level of

expression of what is essentially an abnormal protein likely explains the partial correction observed in clinical trials (Voit et al., 2014). For DMD, detection of the protein product produced from exon skipping has been challenging raising questions whether dystrophin protein production is a suitable biomarker (Wilton et al., 2014).

Whether exon skipping is useful for other disorders requires documentation that internally truncated proteins are functional. Here we show that Mini-Gamma protein is able to biochemically replace full-length γ-sarcoglycan in flies, mouse heart and muscle, and in a heterologous cell expression system by forming a complex with the β- and δ-sarcoglycan and promoting translocation to the membrane. Furthermore, we showed evidence for functional improvement by Mini-Gamma in a *Drosophila* model of sarcoglycan deficiency where mobility and heart function were each improved. In the *Sgcg* null mouse, we found that Mini-Gamma improved cardiac dysfunction. Of note, this model does not allow us to detect functional improvement using *ex vivo* muscle mechanics. *Sgcg* null skeletal muscle differs from dystrophin-deficient muscle in that dystrophin-deficient muscle displays contraction-induced damage while *Sgcg* null muscle does not have increased force deficit from eccentric contraction (Hack et al., 1999; Petrof et al., 1993). Dystrophin, with its multi-spectrin repeat nature, creates a mechanically strong link for the sarcolemma (Rybakova et al., 2000), and this is in contradistinction to *Sgcg* null muscle in which dystrophin is normally localized (Hack et al., 1999).

Although the strategy to generate Mini-Gamma removes half of γ-sarcoglycan, it retains the components integral to γ-sarcoglycan function. γ-Sarcoglycan is a type II

transmembrane protein with a 37 aa intracellular amino-terminus, a 21 aa transmembrane domain and a 233 aa extracellular domain. Exon 2 encodes the initiator methionine and the entire intracellular and transmembrane domains, and these domains remained intact in Mini-Gamma. Notably, the existing mouse model for LGMD 2C was deleted for exon 2 and is therefore not a suitable model for testing exon skipping (Hack et al., 1998). The intracellular amino-terminus of γ-sarcoglycan contains tyrosine phosphorylation consensus sequences, and tyrosine phosphorylation is seen with cell attachment and contraction and is required for proper mechanosignaling (Barton, 2006; Barton, 2010; Moorwood et al., 2014; Spinazzola et al., 2015; Yoshida et al., 1998). The intracellular domain has also been shown to interact directly with intermediate filament protein filamin-C and actin-associated protein archvillin (Spinazzola et al., 2015; Thompson et al., 2000). The amino-terminal half of the extracellular domain is important for interacting with other sarcoglycans during complex assembly (Chen et al., 2006). The interaction of Mini-Gamma with the other sarcoglycans and its translocation to the plasma membrane indicate that the residual extracellular portion of γ-sarcoglycan was sufficient for membrane targeting. The carboxyl-terminal extracellular region of γ-sarcoglycan contains an EGF-like cysteine-rich domain that is conserved among β-, δ- and γ-sarcoglycan, and notably this region remained intact in Mini-Gamma (Bonnemann et al., 1995; McNally et al., 1996b; Nigro et al., 1996). This cysteine-rich motif found at the distal carboxyl-terminus of γ-, β- and δ-sarcoglycans has been shown to form intra-molecular disulfide bridges and is required for plasma membrane targeting (Chan et al., 1998; Chen et al., 2006; Shi et al., 2004).

Missense mutations of these cysteines and small deletions in this region cause severe forms of human muscular dystrophy (McNally et al., 1996a; Piccolo et al., 1996).

The most common mutation in the *SGCG* gene is a frameshift in exon 6, Δ521-T (McNally et al., 1996b; Noguchi et al., 1995) (Leiden muscular dystrophy pages variation database, http://www.dmd.nl/nmdb/home.php). An exon skipping strategy that includes exon 6 is expected to benefit not only patients carrying the Δ521-T mutation, which accounts for about half of all LGMD2C patients, but also patients carrying missense, nonsense or frame shifting mutations spanning from exon 4 to exon 7.

Like exon skipping for DMD, the presence of an internally truncated protein, likely at much lower than normal protein levels as exon skipping remain an inefficient process, is anticipated to produce only partial rescue of phenotype. This partial rescue is consistent with what is seen in the milder Becker Muscular Dystrophy patients, and still represents a significant improvement in phenotype. Improved chemistries for antisense approaches should lead to more efficient skipping and higher levels of appropriate RNA templates. Permanent genetic therapy may one day be achievable through gene editing.

# CHAPTER 4

# Discussion

## OVERVIEW

Exon skipping has emerged as a promising approach for treatment of Duchenne muscular dystrophy and a number of other genetic diseases. Now we have expanded the potential of exon skipping to treat LGMD2C patients with γ-sarcoglycan mutations. I have shown that the exon skipping product of γ-sarcoglycan, Mini-Gamma, is functional in both fly and mouse models of muscular dystrophy. These data provide evidence for applying exon skipping strategy to LGMD2C patients. In this chapter, I will discuss a proof-of-principle study showing that multi-exon skipping can be induced in RNA encoding mutant human γ-sarcoglycan. This work was done by and presented here with permission from Dr. Eugene Wyatt, a postdoctoral fellow with whom I worked closely. I will also compare the structure and function of γ-sarcoglycan and Mini-Gamma. Afterwards, I will summarize the potential applications of exon skipping strategy to other genetic mutations. Finally, I will propose future experiments and draw general conclusions.

## EXON SKIPPING IN LGMD2C MYOGENIC CELLS

To produce Mini-Gamma protein, four out of the seven coding exons in the γ-sarcoglycan gene have to be skipped. Multi-exon skipping on mutant γ-sarcoglycan transcripts can be technically challenging. Simultaneous skipping four exons requires significant work to screen for optimal combinations of AONs with high skipping

efficiency and minimal production of intermediate skipped products. Moreover, these mutant transcripts are likely subject to nonsense-mediated decay, leaving a small number of RNA transcripts for AON to act on. To prove that multi-exon skipping can be achieved for mutant γ-sarcoglycan transcripts, Dr. Wyatt, established a muscle culture system derived and transformed from patient fibroblasts and demonstrated that multi-exon skipping can be accomplished for the mutant γ-sarcoglycan transcripts deleted for exon 6.

*Establish patient muscle cell culture*

Fibroblasts were obtained from an individual with LGMD2C. This individual carried a large deletion of 1.4 MB spanning 7 genes, including *SGCG* encoding γ-sarcoglycan on one allele. The other allele was deleted for 14,000 bp that encompassed only exon 6 of *SGCG* (Figure 4.1A), leading to a premature stop codon and disrupting the reading frame (red triangle). The individual has clinically diagnosed LGMD2C with progressive muscle weakness and elevated serum CK, which began in early childhood. A muscle biopsy confirmed absence of γ-sarcoglycan protein and reduction of the other sarcoglycan proteins (data not shown). Fibroblasts were obtained and induced into a myogenic lineage using a tamoxifen (Tam) inducible MyoD, following similar methods used to examine DMD cells (Kendall et al., 2012; Kimura et al., 2008). After induction, MyoD-reprogrammed fibroblasts entered into the myogenic lineage seen as desmin expression along with the appearance of elongated myotube-like structures (Figure 4.1B). LGMD 2C fibroblasts entered into the myogenic lineage similar to wild-type

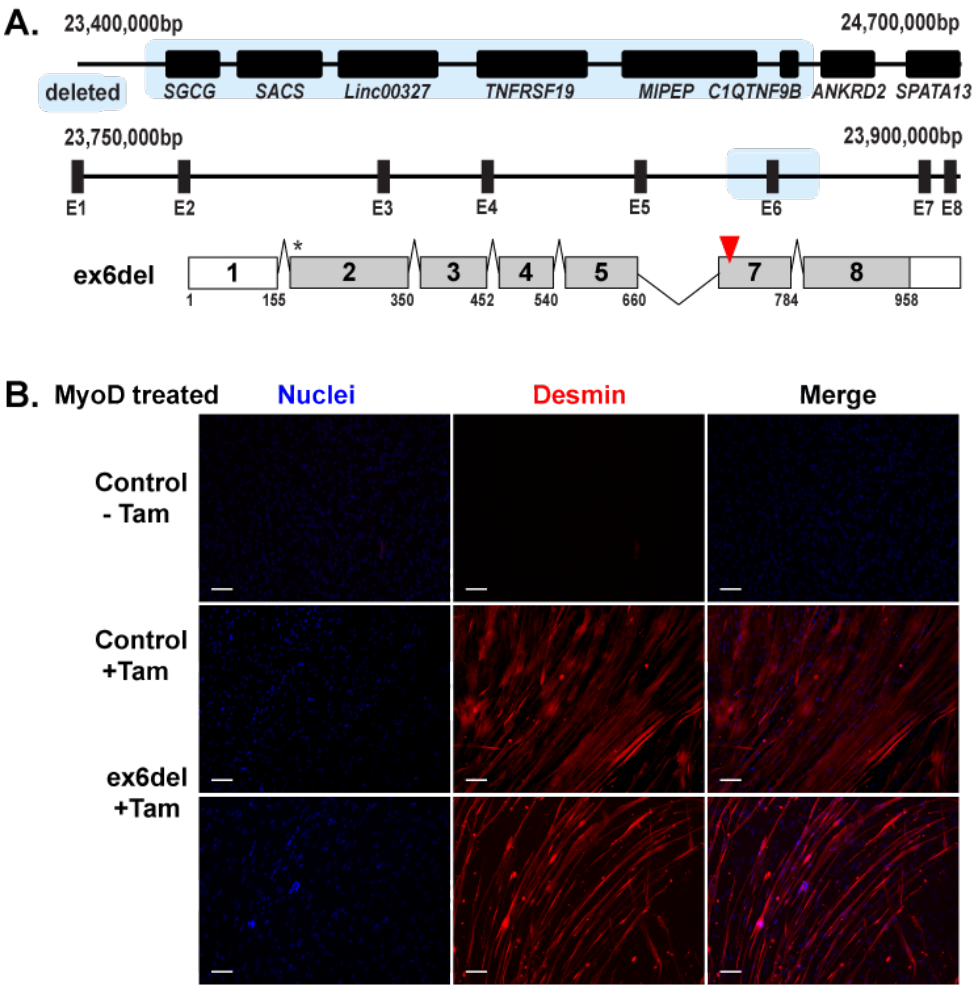


**Figure 4.1. Establish human *SGCG* mutant muscle cell system for AON testing**
(A) Shown are the two different alleles from an individual with Limb Girdle Muscular Dystrophy 2C affecting the *SGCG* locus, a 1.4 MB deletion encompassing multiple genes (blue shaded area top) and a smaller deletion encompassing SGCG exon 6 in its entirety (blue shaded area, middle schematic). Numbers refer to genome position in hg19. The exon organization for exon 6 deletion (ex6del) mutant *SGCG* transcripts is shown in the lower schematic. * Translation start site. Red triangle:premature stop codon. (B) Skin fibroblasts from control and ex6del were obtained and reprogrammed to the myogenic lineage using a tamoxifen-inducible MyoD (Kendall et al., 2012; Kimura et al., 2008). Desmin expression (red) and multinucleated myotube formation indicated myogenic reprogramming after 4OH-tamoxifen exposure (5μM, 48h) and culture in differentiation media. Nuclei were marked with Hoechst 3342 (blue). Scale bar = 10μM.

control cells. *SGCG* RNA expression was detected in MyoD reprogrammed fibroblasts (Figure 4.2A) from both control and the LGMD 2C patient (ex6del). The degree of *SGCG* RNA expression was qualitatively less in ex6del, consistent with the presence of a single functional *SGCG* allele and/or nonsense mediated decay.

The ability to take patient fibroblasts and transform them into muscle cells provides a powerful platform to screen for optimal AONs for individual or specific groups of patients without painful muscle biopsies. More recently, it was shown that induced pluripotent stem cells (iPSCs) can be generated from human urine and differentiated into beating cardiomyocytes (Guan et al., 2014). Preliminary work in our lab has shown that urine cells can be transformed into skeletal muscle-like cells with the overexpression of MyoD gene (E. Kim and E. Wyatt, unpublished results). The accessibility of urine samples opens up a new venue for testing AONs and other drugs in a non-invasive and painless manner.

*Achieve multi-exon skipping using AON mixture*

AONs using 2'-*O*-methyl phosphorothioate (2OMePS) chemistry were targeted to intra-exonic regions in *SGCG* exons 4, 5, and 7, in accordance with previously described principles for antisense design (Aartsma-Rus, 2012). To generate the multi-exon skipping reading frame corrected ex6del transcript, reprogrammed cells were treated with a cocktail of AONs targeting exons 4, 5, and 7 (100nM/AON, 300nM total). Analysis of PCR amplified transcripts 3 days after treatment demonstrated the

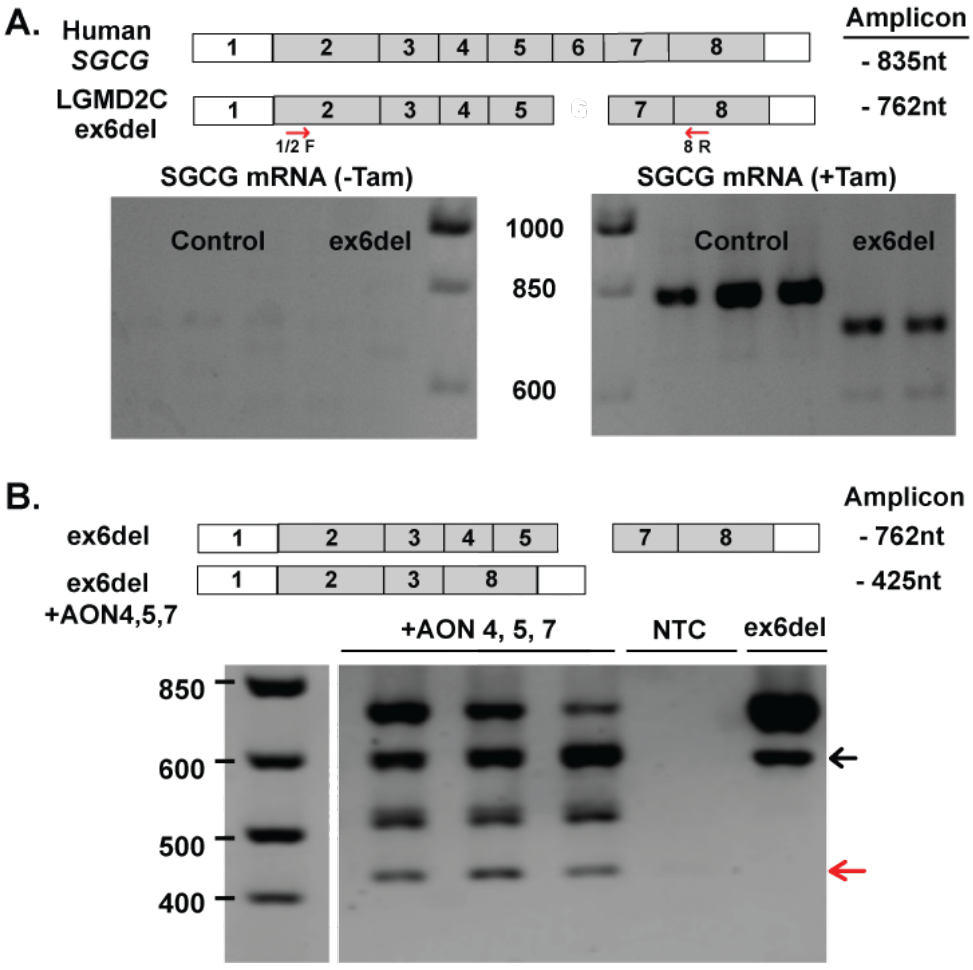


**Figure 4.2. Antisense oligonucleotide (AON)-mediated reading frame correction in human *SGCG* mutant cells** (A) RT-PCR demonstrated SGCG transcripts from control and ex6del reprogrammed (right) fibroblasts after differentiation (5μM 4OH-tamoxifen, 48h; 12d differentiation). (B) MyoD-reprogrammed fibroblasts were treated with AONs targeting exons 4, 5, and 7 (100nM/AON, 300nM total). RT-PCR demonstrated the expected skipped products, including the smallest product representing exons 2, 3 and 8 and deleted for exons 4, 5, and 7 (red arrow). Results from 3 independent replicates are shown for AON treatment. NTC = no-template control. Black arrow indicates the single exon skipping of exon 7 in the ex6del *SGCG* transcript, which occurs in the absence of AON treatment.

generation of an internally truncated transcript with the desired reading frame correction of ex6del *SGCG* (Figure 4.2B), in addition to the intermediate skipped products. The three-exon skipped product was confirmed by sequencing (data not shown). Collectively, these data demonstrate the potential of correcting *SGCG* frameshift mutations with a multi-exon skipping strategy.

AONs vary in length between 16 and 22 nucleotides and are chemically modified to be resistant to intracellular nucleases. Different AON chemistries have been developed to enhance tissue uptake, increase stability and reduce toxicity. Current exon skipping clinical trails by Sarepta Therapeutics and Prosensa/GSK are conducted with two different AON chemistries, phosphorodiamidate morpholino oligomers (PMO) and 2'O-methylated phosphorothioates (2'OMePs), respectively. One major difference between the two chemistries is that the PMO drug (eteplirsen) has been administered systemically up to 50mg/kg. This is eight times higher than the 2'OMePs drug (drisapersen). The limited dose of drisapersen is attributed to kidney toxicity whereas no clear toxicity has been reported for eteplirsen. PMO chemistry also seems to possess a higher efficiency of exon skipping than 2'OMePS in most preclinical studies with equivalent level of dosing (Lu et al., 2005; Tanganyika-de Winter et al., 2012; Wu et al., 2010). Tolerance for higher dosing and superior efficiency can at least partially explain why outcomes from current clinical trials seem to favor eteplirsen verses drisapersen.

## MOLECULAR ROLES OF γ-SARCOGLYCAN & FUNCTIONALITY OF MINI-GAMMA

*Stabilization of sarcoglycan complex at the sarcolemma*

Primary mutations in β-sarcoglycan (*SGCB*) and δ-sarcoglycan (*SGCD*) consistently result in the complete loss of all sarcoglycan subunits at the sarcolemma in patient muscle samples (Draviam et al., 2001; Fanin and Angelini, 2002; Nigro et al., 1996). In contrast, variable levels of β- and δ-sarcoglycan are retained at the sarcolemma in muscle sections from patients with γ-sarcoglycan mutations (Crosbie et al., 2000; Draviam et al., 2006; McNally et al., 1996b; Vainzof et al., 1999; Vorgerd et al., 2001). These data are consistent with the observations in mouse models. While mice lacking δ- or β-sarcoglycan exhibit complete loss of other sarcoglycans, γ-sarcoglycan null mice (*Sgcg*) only have partially reduced δ-and β-sarcoglycan at the sarcolemma (Durbeej et al., 2000; Hack et al., 2000). Heterologous expression studies have shown that the minimal requirement for sarcoglycan complex trafficking is the formation of the β-δ-sarcoglycan core (Draviam et al., 2006; Shi et al., 2004). In these studies, γ-sarcoglycan is restricted to the intracellular compartments until the co-expression with β- and δ-sarcoglycan. Taken together, these results argue for an absolute necessity for δ-and β-sarcoglycan for sarcoglycan complex assembly and trafficking, while γ-sarcoglycan assumes a secondary yet still important role to stabilize the complex at the sarcolemma.

The sarcoglycans share potential N-glycosylation sites. When expressed individually in cells, the sarcoglycans are not correctly glycosylated (Shi et al., 2004). Improper glycosylation affects protein folding in the endoplasmic reticulum (ER) and

subsequent export out of ER (Ermonval et al., 2000). The residual β- and δ-sarcoglycans in *Sgcg* muscle are properly glycosylated in the absence of γ-sarcoglycan (Hack et al., 2000). These data suggest that the formation of β-δ-sarcoglycan core may induce conformational changes that are important for their proper glycosylation, folding and exiting ER. The N-glycosylation site in γ-sarcoglycan is deleted in Mini-Gamma. Interestingly, my data have shown that Mini-Gamma is able to interact and traffic with β-δ-sarcoglycan core *in vivo* and *in vitro*. These observations suggest that the N-glycosylation site is not required for γ-sarcoglycan protein folding and subsequent trafficking in the presence of β-δ-sarcoglycan. Moreover, expressing Mini-Gamma increases the membrane expression of β-δ-sarcoglycan at the sarcolemma in *Sgcg* mice, indicating that the addition of Mini-Gamma stabilizes the sarcoglycan complex and facilitates its trafficking to the membrane.

*Additional protein-protein interactions for γ-sarcoglycan*

γ-Sarcoglycan interacts with filamin-C, the muscle specific form of filamin protein (Thompson et al., 2000). This interaction is mediated through the cytoplasmic amino-terminal of γ-sarcoglycan, which is intact in Mini-Gamma (Thompson et al., 2000). Absence of γ-sarcoglycan results in the mislocalization of filamin-C in muscle cells, which may contribute to cytoskeletal instability. It would be interesting to examine whether expression of Mini-Gamma can restore the normal cellular localization of filamin-C.

γ-Sarcoglycan and α-sarcoglycan bind to different motifs on biglycan, the small leucine-rich repeat proteoglycan localizes at the muscle cell surface (Rafii et al., 2006). This interaction is rather specific since biglycan does not interact with β- or δ-sarcoglycan. Biglycan is highly expressed during muscle development. The authors showed that biglycan regulates the expression of γ-sarcoglycan in immature mice but not adult mice (Rafii et al., 2006). In adult mice, biglycan is transiently activated during skeletal muscle regeneration (Casar et al., 2004). It is not too surprising for the adult regeneration process to recapitulate some of the developmental properties. It is possible that biglycan activation also promotes the expression of γ-sarcoglycan during muscle regeneration. Many forms of muscular dystrophies are characterized by repeated cycles of degeneration and regeneration. A more detailed understanding of how biglycan regulates γ-sarcoglycan expression in muscle development and regeneration may shed light on novel avenues to treat the muscular dystrophies.

*Mechanical signaling transduction*

γ-Sarcoglycan is required for mechanical signaling transduction in muscle cells (Figure 4.3). Isolated muscle fibers from *Sgcg* null mice show higher phosphorylation levels of ERK-1 and ERK-2 at resting state than WT fibers (Barton, 2006). When subject to the eccentric contraction, WT muscle responds with increased phosphorylation of ERK-1/2 while *Sgcg* null muscle fails to respond. The ERK-1 response is mediated by the tyrosine 6 phosphorylation site in the cytoplasmic domain of γ-sarcoglycan (Barton, 2010). More recently, Spinezzola et al. identified a novel

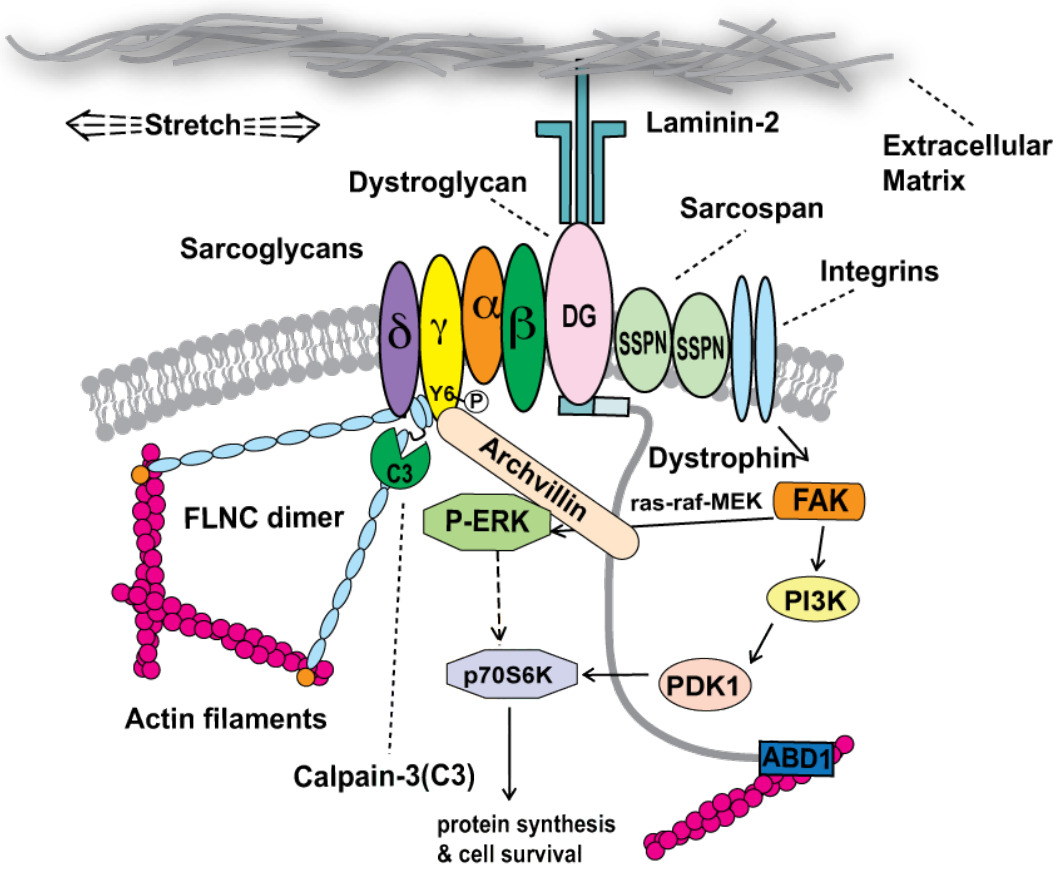


**Figure 4.3. γ-Sarcoglycan mediates signaling transduction**. Tyrosine-6 (Y6) phosphorylation in γ-sarcoglycans is important for mechanical signal transduction. Upon stretch, WT muscle responds with increased ERK phosphorylation while γ-sarcoglycan deficient muscle fails to mount a response (Barton, 2010). P70S6 kinase is another mechanical-responsive kinase that is regulated by γ-sarcoglycan (Hornberger et al., 2004; Moorwood et al., 2014). Both ERK-1 and p70S6 kinases are implicated in cell survival and growth (Cheung and Slack, 2004; Harada et al., 2001). Filamin-C (FLNC) proteins dimerize and plays an important role in crosslinking actin filaments. Filamin-C interacts with the cytoplasmic domain of γ- and δ-sarcoglycans through its carboxyl-terminal dimerization domain and hinge region (Thompson et al., 2000). 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 may contribute to a disrupted actin network and impairs resistance to strain at sarcolemma. Calpain-3 (C3) is muscle-specific member of the calcium-dependent protease. C3 cleaves filamin-C near the carboxyl-terminal hinge region and regulates its interaction with the sarcoglycans (Guyon et al., 2003).

interaction between γ-sarcoglycan and archvillin using a yeast two-hybrid screen (Spinazzola et al., 2015). Archivillin becomes associated with phosphorylated ERK-1/2 upon muscle stimulus. The archivillin-P-ERK1/2 association fails to occur in γ-sarcoglycan null muscle. In addition, γ-sarcoglycan has been shown to regulate another mechanical-responsive kinase, p70S6 (Hornberger et al., 2004; Moorwood et al., 2014). Since Mini-Gamma contains an intact cytoplasmic domain, I expect this signaling transduction role is maintained in the Mini-Gamma.

## EXON SKIPPING STRATEGIES FOR OTHER MUTATIONS

*Knock down Tau protein in neurodegenerative diseases*

One common feature shared between Alzheimer's and a number of other neurodegenerative diseases is the accumulation of neurofibrillary tangles in brain cells (Lee et al., 2001). Tau protein is a microtubule stabilizing protein highly expressed in neurons and the chief component of these tangles (Grundke-Iqbal et al., 1986; Weingarten et al., 1975). Missense mutations in the gene encoding Tau protein, *MAPT*, cause familiar dementia FTDP-17, suggesting aggregations of tau protein is pathogenic (Hutton et al., 1998). Therefore, targeting *MAPT* gene provides an avenue to prevent abnormal Tau aggregation and reverse the courses of these neurodegenerative diseases. To reduce the expression *MAPT,* Sud et al. designed and tested oligonucleotides to induce exon skipping in the *MATP* gene in human neuroblastoma cell lines and human *MAPT* gene transgenic mice (Sud et al., 2014). One of the most potent morpholinos induces the skipping of exon 5 and significantly decreases tau

protein level up to 70~80% *in vitro* and *in vivo* (Sud et al., 2014). However, the *in vivo* experiments were done in mice leg muscles instead of the brain. It remains a significant hurdle for the oligonucleotides to cross the blood brain barrier and achieve adequate levels in the brain.

*Modify huntingtin protein in the Huntington Disease*

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a CAG repeat expansion in the *HTT* gene (Andrew et al., 1993; Duyao et al., 1993; Snell et al., 1993). The CAG expansion translates into a polyglutamine stretch at the N-terminal end of huntingtin protein. Inhibition of mutant huntingtin protein using RNAi technologies has been proposed as a therapeutic strategy (Sah and Aronin, 2011). However, RNAi knockdown strategy may not be ideal since it affects both wild type and mutant *HTT* alleles. Huntingtin is an essential protein in neurons with many functions. *Htt* gene knockout in mice leads to embryonic lethality (Zeitlin et al., 1995). Inactivation of *Htt* gene after developmental stage leads to neurodegenerative disease in adult mice (Dragatsis et al., 2000).

Studies have shown that the proteolytic cleavage of huntingtin protein by caspase-6 contributes to HD pathogenesis (Ehrnhoefer et al., 2011; Graham et al., 2006). The brain aggregates observed in HD patients contain N-terminal fragments but not full-length protein, suggesting that these N-terminal fragments are more toxic than the full-length protein or C-terminal fragments ((Lunkes et al., 2002). Consistent with these findings, inhibition of caspase-6 cleavage by administering caspase inhibitor

reduces toxicity and aggregate formation in cultured neuronal cells (Wellington et al., 2000). Furthermore, mutation in caspase-6 cleavage site in huntingtin protein leads to the reversal of HD phenotype a mouse model of HD (Pouladi et al., 2009). This critical cleavage site in huntingtin protein is encoded by exon 12 of the *HTT* gene. To prevent the production of toxic N-terminal fragments from mutant alleles, Evers et al. designed AONs to induce skipping of exon 12 in *HTT* pre-mRNA and showed the production of a modified huntingtin protein with higher resistance to caspase-6 *in vitro* (Evers et al., 2014). This exon skipping strategy successfully transforms a pathogenic huntingtin protein into a less toxic one without altering protein expression level, offering an alternative method with fewer side effects than RNAi strategy and caspase inhibitors. However, the *in vivo* rescue effects of this strategy still need to be characterized before developing therapeutic strategies. Also it remains to be addressed whether this truncation of huntingtin (45 amino acids removed as a result of skipping exon 12) affects its normal function in the brain cells.

*Induce exon inclusion in SMN2 gene in spinal muscular dystrophy*

Spinal muscular dystrophy (SMA) is an autosomal recessive neuromuscular disease characterized by progressive loss of motor neurons. Loss of function mutations in the survival of motor neuron 1 (*SMN1*) gene cause SMA (Lefebvre et al., 1995). Survival of motor neuron 2 (*SMN2*) is a homolog of the *SMN1* gene. *SMN2* and *SMN1* are almost identical except for 11 nucleotide substitutions. One of the substitutions, a silent C6T transition in *SMN2* exon 7, results in the disruption of the SF2/ASF-binding

site and a significant skipping of this exon during pre-mRNA splicing (Lim and Hertel, 2001). The limited amount of full-length protein produced from *SMN2* is not sufficient to fully rescue the loss of *SMN1,* but is essential for survival in the absence of *SMN1* (Monani et al., 2000). Furthermore, *SMN2* gene copy number was found to inversely correlate with disease severity in both SMA patients and mouse models (Mailman et al., 2002; Monani et al., 2000). These attributes make *SMN2* a significant disease modifier and a great target for therapeutics. Several strategies have been developed to enhance the inclusion of exon 7 in the *SMN2* mRNA transcripts (Cartegni and Krainer, 2003; Hua et al., 2007; Skordis et al., 2003). Skordis et al. used AONs that were complementary to exon 7 and containing exonic splicing enhancer motifs to restore the disrupted binding site (Skordis et al., 2003). Cartegni et al. designed serine-arginine peptide conjugated AONs to recruit serine/arginine-rich splicing factor (SF2/ASF) to the exonic splicing enhancer site (Cartegni and Krainer, 2003). Both of these strategies achieved increased level of full-length *SMN2* transcripts. Similar results were obtained by targeted inhibition of exonic splicing silencer sites using complementary AONs (Hua et al., 2007). These studies not only offer putative therapeutic strategies for SMA patients but also provide opportunities to identify cis-regulatory elements that can be further studied to shed light on splicing mechanisms.

*Correct splicing in CEP290 gene in retinal dystrophy*

Mutations in the *CEP290* gene cause Leber congenital amaurosis (LCA) (den Hollander et al., 2006). LCA is the most severe form of retinal dystrophies and causes

childhood blindness. The most prevalent disease-causing mutation in *CEP290* localizes in intron 26 (c.2991+1655A>G) (den Hollander et al., 2006). This mutation creates a strong splice donor site and inserts a cryptic exon encoding a premature stop codon (den Hollander et al., 2006). CEP290 protein is important for cilia assembly and maintenance (Craige et al., 2010). Using an exon skipping strategy, Gerard et al. successfully induced efficient skipping of this cryptic exon and restored cilia expression in patient fibroblasts (Gerard et al., 2012). Since the eye is a small, confined, and easily accessed organ, it is an ideal target for AONs treatment. Currently, the only FDA approved 2'-OMePS AON drug is Vitravene, a treatment for acute cytomegalovirus retinitis. The efficiency and lack of major side effects of Vitravene further support that exon skipping is a promising therapy for *CEP290* splicing mutations.

**FUTURE DIRECTIONS**

The experiments presented here have proven that Mini-Gamma protein is highly functional in γ-sarcoglycan deficient animal models and in a heterologous cell expression system. Preliminary studies in the lab by Eugene Wyatt and Ellis Kim have established cellular models of LGMD2C either from patient fibroblasts or urine samples. Multi-exon skipping is technically challenging and the cellular models provide great tools to test different combinations and chemistries of AONs before applying them to animal models and human patients. The current available γ-sarcoglycan null mouse model carries a deletion of exon 2 that contains the transcription start site (Hack et al., 1998). Therefore this mouse model is not suitable for testing AONs *in vivo*. To create a

preclinical model for exon skipping testing, I propose to create a 521ΔT or similar frame shifting mutations in exon 6 in mice using the CRISPR/Cas9 technology. The severity of muscular dystrophy phenotype caused by the same γ-sarcoglycan gene mutation varies greatly across different genetic background in mice (Heydemann et al., 2005). Ideally, the 521ΔT mutation will be created in a severe genetic background of muscular dystrophy such as the DBA/2J strain since the phenotype matches better to human patients and allows a more comprehensive characterization of rescue effects.

Another interesting future area to explore is the homology between mammalian and fly sarcoglycans. In the *Drosophila* model of LGMD2C, we found that Mini-Gamma and γ-sarcoglycan only partially rescued the activity defects of *Sgcd* flies while δ-sarcoglycan and fly Sgcd restored the activity level back to normal. Mammalian γ-sarcoglycan and δ-sarcoglycan have identical intron exon structure and are thought to result from a gene duplication event. At protein sequence level, murine γ-sarcoglycan and δ-sarcoglycan are 56% identical and 74% similar. Fly Sgcd is equally related to γ-sarcoglycan and δ-sarcoglycan (35% identical and 56% similar). In the mammalian sarcoglycan trafficking model, δ-sarcoglycan and β-sarcoglycan has a more pivotal role in initiating complex assembly while γ-sarcoglycan is added to the β-δ core at later stages. Since Sgcd protein is the sole counterpart of δ-sarcoglycan and γ-sarcoglycan in flies, I hypothesized that higher ability of δ-sarcoglycan to interact with fly β-sarcoglycan (Sgcb) contributes to its more complete rescue effects than γ-sarcoglycan. To test this idea, I would first co-express either δ-sarcoglycan & fly Sgcb, or γ-

sarcoglycan & fly Sgcb in fly S2 cells. I would perform co-immunoprecipitation assays at different stringency to test which murine sarcoglycan forms a tighter bond with the fly Sgcb. I would also examine the cellular localization and look for membrane translocation. Co-expression of fly Sgcd and fly Sgcb will serve as a positive control for these experiments. I predict that δ-sarcoglycan exhibits a higher affinity to fly Sgcb than γ-sarcoglycan by co-IP assays. Also co-expression of δ-sarcoglycan and fly Sgcb results in a higher membrane translocation than co-expression of γ-sarcoglycan and fly Sgcb. Next, I would clone hybrid versions of γ-sarcoglycan and δ-sarcoglycan with swapped domains responsible for each to interact with β-sarcoglycan. Deletion experiments have shown that the amino-terminal half of the extracellular domain (regions proximal to the plasma membrane) is important for sarcoglycan interaction (Chen et al., 2006). Specifically, residues #57-92 in γ-sarcoglycan and residues #94-157 in δ-sarcoglycan are responsible for interacting with murine β-sarcoglycan. I would switch residues #57-157 between γ- and δ-sarcoglycan and examine whether the properties of the hybrids change in cell culture studies. I would also express them in *Sgcd* flies and test if their abilities to rescue activity reversed. I expect to see γ-sarcoglycan with δ-β interacting domain behaves more like δ-sarcoglycan in these assays and vice versa.

**CONCLUDING REMARKS**

In conclusion, we demonstrated the high functionality of Mini-Gamma protein in γ-sarcoglycan deficiency animal models and a heterologous cell expression system. The

ability of a severely truncated transmembrane protein to interact with its normal cellular partners and translocate to the plasma membrane provides important insights on future studies on protein functional domain, protein-protein interaction, and trafficking of transmembrane proteins. Furthermore, this study encourages the further development of exon skipping as a therapeutic strategy to treat half of the LGMD2C patients. This work falls within the context of a larger effort to expand the potential of exon skipping strategy to treat a variety of genetic diseases. Pioneer studies and clinical trials of exon skipping therapy in Duchenne muscular dystrophy have paved way for the future development of more AON drugs and eventually provide cures for life-threatening diseases.

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