1-1-2015

# Gamma-Sarcoglycan Mediated Mechanotransduction in Skeletal Muscle

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**Abstract**
Loss of gamma-sarcoglycan (g-SG) is common to both Duchenne and Limb Girdle muscular dystrophies, and is sufficient to induce severe muscle degeneration and signaling defects in response to mechanical load without causing susceptibility to contractile damage. This suggests that disease occurs not by structural deficits, but through aberrant signaling, namely, disruption of normal mechanotransduction signaling through the SG complex. However, the mechanisms of g-SG-mediated mechanical signaling are poorly understood. Here, we identified the muscle-specific protein archvillin as a g-SG and dystrophic interacting protein. Archvillin expression is dependent on g-SG, where levels are significantly unregulated in human LGMD2C patient muscle and at the sarcolemma of murine g-SG-null (gsg-/-) muscle. However, archvillin is delocalized in mdx muscle, indicating dystrophin-dependent sarcolemmal localization. In situ eccentric contraction (ECC) of C57 mouse tibialis anterior (TA) muscles causes ERK1/2 phosphorylation, nuclear activation of P-ERK1/2, and stimulus-dependent archvillin association with P-ERK1/2, whereas TA muscles from gsg-/- and mdx mice exhibit heightened P-ERK1/2 and increased nuclear P-ERK1/2 localization following ECCs, but the archvillin-P-ERK1/2 association is completely ablated. These results position archvillin as a mechanically sensitive component of the dystrophin complex, and demonstrate that signaling defects caused by loss of g-SG occur both at the sarcolemma and in the nucleus. We also extended our previous studies on mechanosensitive, g-SG dependent ERK1/2 phosphorylation to determine whether additional pathways are altered with loss of g-SG. Using a passive stretching protocol to isolate the effects of externally applied tension, we found that in isolated EDL muscles, Akt, S6RP, and p70S6K activation increases with stretch in both C57 and gsg-/- isolated muscles. Treatment of muscles with or without rapamycin during stretch blocks p70S6K activation in stretched C57 muscles, and reduces downstream S6RP phosphorylation, indicating mTOR dependence. However, even though rapamycin treatment decreases p70S6K activation in stretched gsg-/- muscles, S6RP phosphorylation remains elevated. These results suggest that p70S6K is an important component of g-SG-dependent mechanotransduction in skeletal muscle, where loss of g-SG uncouples the response of p70S6K to stretch and implies that g-SG is important for inactivation of this pathway. In sum, this study demonstrates altered load-sensing mechanisms in muscular dystrophies where the sarcoglycans are absent.

**Degree Type**
Dissertation

**Degree Name**
Doctor of Philosophy (PhD)

**Graduate Group**
Cell & Molecular Biology

**First Advisor**
Elisabeth R. Barton

**Second Advisor**
H. Lee Sweeney

**Keywords**
ERK1/2, mechanotransduction, muscular dystrophy, sarcoglycan

**Subject Categories**
Cell Biology | Physiology

**GAMMA-SARCOGLYCAN MEDIATED MECHANOTRANSDUCTION**

**IN SKELETAL MUSCLE**

Janelle Michele Spinazzola

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2015

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GAMMA-SARCOGLYCAN MEDIATED MECHANOTRANSDUCTION IN SKELETAL MUSCLE

## ACKNOWLEDGMENT

First, I would like to thank my thesis advisor Dr. Elisabeth Barton for her incredibly supportive mentorship over the last four years. No matter how many negative results I brought to lab meeting, she would always find something positive and point me in the right direction for success. Her guidance and friendship has been invaluable in my growth as a scientist.

I would also like to thank my thesis committee members: Drs. Michael Ostap, Richard Assoian, Kelly Jordan-Sciutto, and Tejvir Khurana for their mentorship and support. Each member helped foster ideas that are now part of this dissertation, and I am indebted to Mike for giving me much of my fortitude when giving talks.

To past members of the Barton Lab, Becky Brisson and Soo Park, true friends who got me through my first (and last) yeast two-hybrid screen and countless failed western blots, and to Lucas Smith, who has stuck it out with me until the end. You three have been the best labmates anyone could ask for, both scientifically and as lunch buddies. I would also like to thank Hanqin Lei, Dephne Leong, Min Liu, Julia Durzynska, Catherine Moorwood, and the members of the Sweeney Lab, who have generously hosted me the last four months.

I would like to acknowledge the faculty and students of the BGS CBP program, who foster a supportive environment in which to grow as a scientist. Additionally, thank you to the administrative team in the CAMB office without whom the program would surely collapse.

I also want to express my appreciation to my undergraduate research advisor Dr. Herman Vandenburgh and lab manager Janet Shansky at Brown University, who sparked my interest in research and encouraged me to pursue a Ph.D. at UPenn.

To my UPenn friends, with whom I have played endless rounds of board games, watched hours of movies, and eaten countless numbers of Oreos during our weekly gatherings. A special shout out to Joe Zinski, my anime partner in crime, Alex Rohacek, who introduced me to my new love of wood burning, and Jason Diaz, for helping me keep this group together all these years.

My family has been a steadfast source of support and encouragement throughout all my years of education. In particular, my mom Linda has always kept me going in moments of doubt, while also allowing me to find my own path. Lastly, I would like to thank my husband Seth, for deciding to attend UPenn instead of the University of Colorado after we met at interview weekend.

## ABSTRACT

## GAMMA-SARCOGLYCAN MEDIATED MECHANOTRANSDUCTION IN SKELETAL MUSCLE

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Loss of gamma-sarcoglycan (γ-SG) is common to both Duchenne and Limb Girdle muscular dystrophies, and is sufficient to induce severe muscle degeneration and signaling defects in response to mechanical load without causing susceptibility to contractile damage. This suggests that disease occurs not by structural deficits, but through aberrant signaling, namely, disruption of normal mechanotransduction signaling through the SG complex. However, the mechanisms of γ-SG-mediated mechanical signaling are poorly understood. Here, we identified the muscle-specific protein archvillin as a γ-SG and dystrophic interacting protein. Archvillin expression is dependent on γ-SG, where levels are significantly unregulated in human LGMD2C patient muscle and at the sarcolemma of murine γ-SG-null (*gsg*$^{-/-}$) muscle. However, archvillin is delocalized in *mdx* muscle, indicating dystrophin-dependent sarcolemmal localization. *In situ* eccentric contraction (ECC) of C57 mouse tibialis anterior (TA) muscles causes ERK1/2 phosphorylation, nuclear activation of P-ERK1/2, and stimulus-dependent archvillin association with P-ERK1/2, whereas TA muscles from *gsg*$^{-/-}$ and *mdx* mice exhibit heightened P-ERK1/2 and increased nuclear P-ERK1/2 localization following ECCs, but the archvillin-P-ERK1/2 association is completely ablated. These results position archvillin as a mechanically sensitive component of the dystrophin

complex, and demonstrate that signaling defects caused by loss of γ-SG occur both at the sarcolemma and in the nucleus. We also extended our previous studies on mechanosensitive, γ-SG dependent ERK1/2 phosphorylation to determine whether additional pathways are altered with loss of γ-SG. Using a passive stretching protocol to isolate the effects of externally applied tension, we found that in isolated EDL muscles, Akt, S6RP, and p70S6K activation increases with stretch in both C57 and *gsg*$^{-/-}$ isolated muscles. Treatment of muscles with or without rapamycin during stretch blocks p70S6K activation in stretched C57 muscles, and reduces downstream S6RP phosphorylation, indicating mTOR dependence. However, even though rapamycin treatment decreases p70S6K activation in stretched *gsg*$^{-/-}$ muscles, S6RP phosphorylation remains elevated. These results suggest that p70S6K is an important component of γ-SG-dependent mechanotransduction in skeletal muscle, where loss of γ-SG uncouples the response of p70S6K to stretch and implies that γ-SG is important for inactivation of this pathway. In sum, this study demonstrates altered load-sensing mechanisms in muscular dystrophies where the sarcoglycans are absent.

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# CHAPTER 1: Introduction

## 1.1. Mechanotransduction in skeletal muscle

Skeletal muscle accounts for approximately 40% of human body weight and is responsible for force generation, movement, and respiratory function. Not only does skeletal muscle produce force, but it also has the capacity to sense changing workloads and to respond and adapt to physiological demands to meet functional needs. When deformation is imposed on a muscle, mechanical forces initiate changes in cellular and molecular biochemical signals, known as mechanotransduction, altering muscle fiber growth and survival, contractile properties, and metabolism. However, the mechanisms by which mechanical forces acting through skeletal muscle cells generate intracellular signaling are not fully understood.

Research has begun to characterize the sensors involved in relaying the mechanical state of the muscle to the transcriptional and translational machinery during contraction. Both passive stretch and active contraction impose stress on proteins spanning the muscle plasma membrane (sarcolemma) through physical association with the cytoskeleton and the extracellular matrix. In skeletal muscle, three glycoprotein complexes transmit forces across the sarcolemma from the intracellular actin cytoskeleton to the extracellular matrix during contraction: the dystrophin-glycoprotein complex (DGC), the utrophin-glycoprotein complex (UGC), and the $\alpha 7\beta 1$D integrin complex. These complexes are thought to not only have structural function, but also perform mechanical signaling functions critical to the development and maintenance of skeletal muscle.

## 1.2. The Dystrophin Glycoprotein Complex

The dystrophin glycoprotein complex (DGC) is a multimeric assembly of proteins localized at the sarcolemma of skeletal, cardiac, and smooth muscle cells. Its composition of transmembrane, cytoplasmic, and extracellular proteins varies slightly dependent on the type of muscle. Two of the most highly conserved members of the DGC are the dystroglycans (Lapidos et al., 2004). These two proteins are transcribed from a single gene and are proteolytically cleaved to create α- and β-dystroglycan (Deyst et al., 1995). Alpha-dystroglycan (α-DG) is localized at the plasma membrane and undergoes complex glycosylation that enables its function as an extracellular matrix (ECM) receptor for proteins including laminin, agrin, perlecan, and neurexin (Grewal et al., 2001; Michele and Campbell, 2003). At the extracellular surface, the carboxyl-terminal domain of α-DG binds the amino-terminal of β-dystroglycan (β-DG) (Di Stasio et al., 1999), a single pass type-I transmembrane protein that is an integral core component of the DGC. The carboxyl terminal domain of β-DG binds directly to multiple domain sites of dystrophin within the cytosol (Jung et al., 1995; Rentschler et al., 1999; Rosa et al., 1996).

Dystrophin is a 427 kDa cytoskeletal protein organized into four distinct domains: an amino terminal actin-binding domain, a central rod domain with 24 homologous triple helical repeats and four hinge domains, the cysteine-rich domain, and a COOH terminal domain (Koenig et al., 1988). Through its association with actin and the carboxyl terminus of β-DG via its cysteine-rich domain, dystrophin establishes a connection between the cytoskeleton of muscle cells to the membrane bound portion of the DGC, completing a continuous cytoskeletal-ECM linkage via the DGs to laminins (Rybakova

et al., 1996; Sadoulet-Puccio and Kunkel, 1996). The C-terminus of dystrophin associates with α-dystrobrevins and syntrophins, which bind neuronal nitric oxide synthase (nNOS) (Ahn et al, 1996; Brenman et al, 196, Peter et al., 1997).

Another core component of the DGC are the sarocoglycans, which exist within the DGC as a tetrameric subcomplex. Alpha-sarcoglycan (α-SG) (Bonnemann et al., 1995; Roberds et al., 1993), beta-sarcoglycan (β-SG) (Bonnemann et al., 1995; Lim et al., 1995), gamma-sarcoglycan (γ-SG) (Noguchi et al., 1995), and delta-sarcoglycan (δ-SG) (Nigro et al., 1996) compose the SG complex in cardiac and skeletal muscle. In smooth muscle, epsilon-sarcoglycan (ε-SG) (Ettinger et al., 1997) replaces α-SG to form a unique complex (Straub et al., 1999), and zeta-sarcoglycan (ζ-SG), thought to be a functional homologue of γ-SG, is expressed primarily in the brain (Shiga et al., 2006). Associated with the SG complex is sarcospan, a quadruple-pass transmembrane protein of the tetraspanin superfamily that also interacts with β-DG, thereby linking the SG complex to the DGC core. The SG complex also binds to the N-terminus of dystrobrevin (Yoshida et al., 2000). Together, the dystroglycans, sarcospan, dystrobrevin, syntrophins, dystrophin, and the sarcoglycan complex form the DGC, a large complex that serves as an important link between the cytoskeleton and the extracellular matrix surrounding muscle cells. The significance of the DGC is made evident by the fact that mutations in many of its components cause numerous muscle diseases, including several forms of muscular dystrophy.

In the UGC, utrophin, the autosomal homolog of dystophin, replaces dystrophin in the complex, with the other peripheral and integral membrane proteins retained. Utrophin is expressed in several tissues where dystrophin is not such as lung and kidney,

and in normal muscle, the UGC is primarily expressed at the neuromuscular junction (Cartaud et al., 1992; Khurana et al., 1991). In muscles of dystrophin-deficient humans and mice, utrophin expression increases (Khurana et al., 1991; Matsumura et al., 1992), suggesting that the UGC is capable of compensating for the loss of the DGC at non-junctional regions of the sarcolemma, which has been studied extensively as a potential therapeutic strategy (Berthier and Blaineau, 1997; Deconinck et al., 1997b; Tinsley and Davies, 1993).

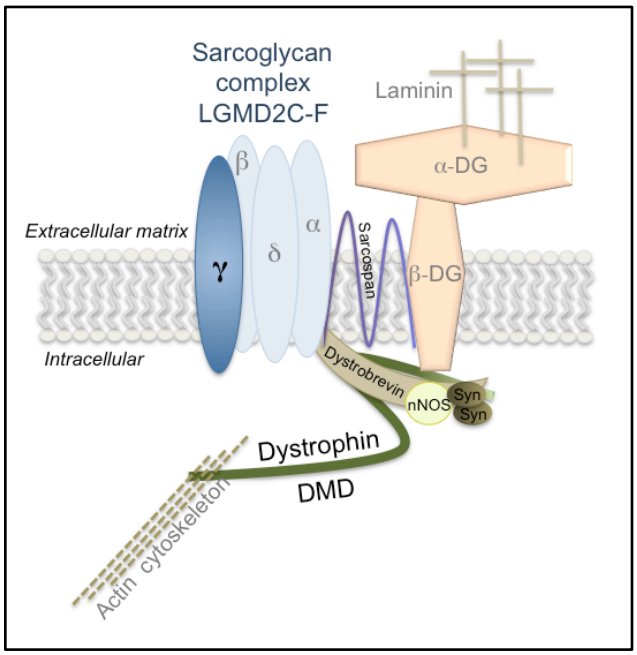


**Figure 1.1. The dystrophin glycoprotein complex**

The dystrophin-glycoprotein complex (DGC) is an assembly of proteins localized at the sarcolemma. Mutation in genes encoding various components of the DGC are associated with several forms of muscular dystrophy.

## 1.3. Muscular dystrophy

The muscular dystrophies are a group of hereditary, progressive muscle disorders characterized by severe muscle weakness and eventual replacement of muscle tissue by adipose and fibrotic connective tissue. There are several classifications of muscular dystrophies that vary in severity, muscles affected, and genetic basis. Some of the most common and severe forms of muscular dystrophy arise from mutation of genes encoding components of the DGC, including Duchenne Muscular Dystrophy (DMD) and Limb Girdle Muscular Dystrophies (LGMD) types 2C-F.

### *Duchenne Muscular Dystrophy*

Duchenne muscular dystrophy is an X-linked recessive disorder caused by out-of-frame deletions of the largest human gene dystrophin. It affects approximately 1 in 3,500 male births (Hoffman et al., 1987), and is one of the most common and severe forms of muscular dystrophy. Initial diagnosis is generally between 2-5 years of age when children begin to demonstrate early hallmarks of the disease including delayed motor milestones and enlarged calf muscles. DMD is characterized by repetitive cycles of muscle degeneration and regeneration that eventually results in replacement of muscle with adipose and fibrotic connective tissue such that the muscle no longer possesses force-generating capacity (Cros et al., 1989; Marshall et al., 1989). Thus, boys generally become wheelchair-dependent between 10-12 years of age, and premature death occurs in the late twenties to early thirties due to respiratory failure and cardiomyopathy (Nigro et al., 1990). Advances in supportive care have improved quality of life and extended lifetime considerably the last decade, but the next step must come from therapeutic

approaches to ameliorate the underlying pathophysiology of DMD. Strategies to address this will require a strong understanding of the genetic, biochemical, and cellular defects that lead to muscle wasting in DMD.

Much of our knowledge about muscular dystrophy has come from studies of animal models. The most frequently used animal model of DMD is the *mdx* mouse, which carries a naturally occurring point mutation in exon 23 of the murine dystrophin gene (Ryder-Cook et al., 1988). This mutation results in a premature stop codon and a complete loss of dystrophin protein expression, and secondary loss of the entire DGC complex from the sarcolemma (Hoffman et al., 1987). *Mdx* mice display severe fiber degeneration, fibrosis and pathological changes in muscle that resemble DMD patients. Despite this, however, compared with human DMD, *mdx* mice display much less severe skeletal muscle and cardiac phenotypes, retain ambulation, and live approximately 80% of normal lifespan (Chamberlain et al., 2007). Although the *mdx* mouse is not a perfect model of the human disease, it has played a vital role in elucidating many of the biochemical and cellular defects that contribute to the disease phenotype.

***Limb Girdle Muscular Dystrophies 2C-F***

Limb-Girdle muscular dystrophies (LGMD) types 2C-F are autosomal recessive disorders caused by mutation of the γ-, α-, β-, δ-SG subunits, respectively, and most severely affect the proximal muscles, including the shoulders, upper arms, pelvic area, and thighs. The general characteristics of LGMD2C-F are similar to DMD, including progressive muscle weakness and replacement of muscle with fibrotic tissue, but can vary greatly in severity and age of onset, with an estimated prevalence of 1 in 10,000 to 1 in

120,000 individuals (Bonnemann et al., 2002). Within LGMD 2C-F, LGMD2C is the most severe and most closely resembles the disease progression and pathology of DMD (Bonnemann et al., 2002; Nowak et al., 2000).

Mouse models of LGMD2C-F include the γ-SG-null mouse (*gsg*$^{-/-}$) (Hack et al., 1998), the α-SG-null mouse (*asg*$^{-/-}$) (Duclos et al., 1998), the β-SG-null mouse (Durbeej et al., 2000), and the δ -SG-null mouse (*dsg*$^{-/-}$) (Hack et al., 2000). These models have been utilized extensively to reveal the pathology associated with primary mutation of each SG subunit. This is of interest given the observation that in LGMD or any of the SG knock-out mice, upon loss of one sarcoglycan subunit, there is secondary loss or reduction of the other sarcoglycans (Bonnemann and Finkel, 2002). Common pathological features include muscle fiber degeneration and fibrosis, though there are differences in the presentation of cardiac involvement (Politano et al., 2001)

## 1.4. Function of the DGC in muscle

The full scope of DGC function and the mechanisms by which mutation of its components causes the pathology of muscle disease is not fully understood. Initially, the DGC was thought to contribute a purely mechanical role in stabilizing the sarcolemma during muscle contraction. However, growing evidence suggests that components of the DGC also play significant signaling roles, that when absent, disrupt signaling pathways involved in cell survival.

### *Mechanical role of the DGC*

During contraction, skeletal muscle undergoes extreme strain and deformation,

making it susceptible to mechanical damage. The DGC is enriched at costameres, transverse rib-like structures that overlie the z-line of the sarcomere, which are thought to function in lateral force transmission from the sarcomere to the sarcolemma, the ECM, and surrounding fibers in both skeletal (Rybakova et al., 2000) and cardiac muscle (Danowski et al., 1992). Given its prime position, the DGC was initially thought to serve a structural role, contributing to this strong mechanical link between the cytoskeleton and the ECM.

Supporting this, early studies demonstrated that muscles of DMD patients (Mokri and Engel, 1998) and *mdx* mouse muscle (Bulfield et al., 1984) have compromised sarcolemma integrity, indicated by highly elevated levels of creatine kinase in DMD patients, and uptake of Procion orange dye by *mdx* muscle, in particular following contraction-induced injury (Moens et al., 1993; Petrof et al., 1993). Additionally, dye was selectively taken up by muscle fibers that appeared necrotic, suggesting that increased membrane permeability eventually resulted in cell death. These observations led to the longstanding "mechanical defect" hypothesis that proposed that mutation in critical structural components of the DGC compromise the mechanical stability of the sarcolemma, exacerbating damage incurred during contraction that leads to death of the myofiber (Cohn and Campbell, 2000; Hack et al., 1998; Petrof et al., 1993).

Indeed, it was later discovered that sarcolemmal tearing allowed leakage of extracellular ions including calcium (MacLennan et al., 1991; Turner et al., 1991) into the myofibers, causing activation of calcium-dependent proteases such as skeletal muscle-specific calpains, resulting in degradation of muscle proteins and fiber degeneration (Alderton and Steinhardt, 2000). This phenotype is further exacerbated with exercise

(Vilquin et al., 1998), but can be improved through immobilization (Mizuno, 1992; Mokhtarian et al., 1999), indicating that the mechanical action of muscle stretch and contraction is at least partially responsible for sarcolemmal damage that occurs in muscle where the DGC is disrupted.

Characterization of the dystrophin homolog, utrophin, has also contributed to revealing the mechanical functions of the DGC. Given utrophin's structural similarity to dystrophin and the discovery that mice lacking both utrophin and dystrophin (*mdx*:utrophin) display a more severe phenotype than *mdx* mice that more closely mimics the progression of DMD (Deconinck et al., 1997a), it was believed that the UGC may perform redundant functions to the DGC. Both DMD patients and *mdx* mice have upregulated utrophin expression that is further increased with exercise, leading to the hypothesis that utrophin may be able to functionally replace dystrophin, acting in a compensatory manner (Gordon et al., 2014; Khurana et al., 1991; Matsumura et al., 1992). Indeed, introduction of full-length or trunctated forms of utrophin has been shown to ameliorate dystrophy in *mdx*:utrophin double knockout mice, eliminating sarcolemma degeneration and ion influx in response to mechanical stimulus, and improving mechanical function (Deconinck et al., 1997b; Tinsley et al., 1998; Tinsley et al., 1996).

These data support a mechanical function for dystrophin and the DGC in protecting the sarcolemma during muscle contraction, where in its absence, the sarcolemma is more susceptible to damage, increasing the permeability of ions and small molecules that ultimately results in cell necrosis and muscle degeneration. Although the importance of the DGC in maintaining sarcolemma integrity is apparent, alternative functions for this complex are becoming recognized, which may reveal mechanisms of

disease where the DGC is compromised.

Although the DGC physically connects the cytoskeleton and the ECM and its structural role in maintaining sarcolemma integrity suggests a primarily mechanical function, these characteristics are also shared by well-known signaling molecules such as cadherins and integrins (Cary et al., 1999; Wheelock and Johnson, 2003). The next section describes the integrins as a basis for addressing the common and distinct features of these complexes in muscle.

## 1.5. Mediators of signal transduction in muscle

### *The alpha7beta1D integrin complex – muscle's classic mechanosensor*

Integrins are glycoprotein heterodimers composed of one α-subunit and one β-subunit, each of which consists of a large extracellular domain, a single α-helical transmembrane region, and a small intracellular C-terminal tail. In skeletal muscle, α7β1D integrin is the major laminin binding integrin and is localized along the sarcolemma, concentrated at costameres and neuromuscular junctions (Berthier and Blaineau, 1997; Burkin and Kaufman, 1999). Similar to the DGC, α7β1D integrin plays a critical role in maintaining stable connections between the actin cytoskeleton and laminin in the ECM, (Boppart et al., 2006; Liu et al., 2012). In addition, α7β1D integrin functions in signal transduction as a mechanoreceptor, serving as bidirectional outside-in and inside-out signaling molecules (Hu and Luo, 2013; Hynes, 2002).

Unlike classic receptors, integrins do not have inherent kinase or enzymatic activity. Instead, they rely on their association with non-receptor protein tyrosine kinases, such as focal adhesion kinase (FAK), to relay information from the extracellular matrix to

the cell nucleus. The activation of FAK also affects integrin association with several other signaling proteins including Grb2, an adapter protein associated with the Ras-ERK/MAP kinase pathway (Yang et al., 1995), and p85, which leads to activation of the phosphatidylinositol 3-kinase (PI3K) pathway (Rando, 2001). Activation of these pathways via integrins is associated with cell proliferation and survival (Stupack and Cheresh, 2002). α7β1D integrin regulates activation of hypertrophy-associated molecules including AKT, mTOR, and p70S6K (Boppart et al., 2006), and has been found to negatively regulate MAPK activation associated with muscle damage following downhill running. In addition, increasing α7β1 promotes muscle cell proliferation and prevents apoptosis (Boppart et al., 2006; Gurpur et al., 2009; Liu et al., 2008). Thus, integrins not only provide mechanical stability, but also transmit mechanical information into biochemical signals that mediate muscle cell survival.

In skeletal muscle, deficiencies in integrin subunits lead to well-documented signaling disruptions leading to muscle cell death and disease such as congenital myopathy caused by mutation of the gene encoding the α7 subunit (Burkin and Kaufman, 1999; Hayashi et al., 1998; Vachon et al., 1997). Muscles of the *mdx* mouse and DMD patients demonstrate elevated α7β1D integrin at the sarcolemma (Hodges et al., 1997), thought to be a compensatory complex, which has initiated its investigation as a potential therapeutic target. Overexpression of α7 integrin was found to ameliorate dystrophy in *mdx*:utrophin double knockout mice, and expression of β1D integrin rescues the cardiomyopathy of *mdx* mice (Burkin et al., 2005; Burkin et al., 2001; Liu et al., 2012), suggesting that the integrin and dystrophin complexes have overlapping functions. Interestingly, though, enhanced α7β1 integrin expression does not alleviate pathology in

δ-SG-null mice (Milner and Kaufman, 2007). Mice lacking both dystrophin and α7 integrin (*mdx*:α7-null) or γ-SG and α7 integrin develop severe muscular dystrophy and die prematurely before one month of age (Allikian et al., 2004a; Guo et al., 2006; Rooney et al., 2006). Similarly to integrins, dystrophin-glycoprotein complexes do not possess any known inherent enzymatic activity. Thus, like integrins, the DGC could not only serve as a mechanical link between the outside and inside of the cell, but could also serve as an mechanosensor that converts mechanical information into biochemical signals that ultimately lead to regulation of protein synthesis.

***The DGC associates with signaling molecules***

While the integrins may function as laminin-dependent signaling receptors in skeletal muscle (Schwartz, 2010), whether the DGC similarly participates in downstream signal transduction cascades is still under investigation. Growing evidence suggests that the DGC not only has a structural role in muscle, but also functions as a mechanosensor that initiates signaling cascades responsible for muscle maintenance. Studies of dystroglycan function suggest that, similar to the functional binding of integrins to their ECM ligands, binding of the dystroglycan complex to ECM proteins involves both outside-in and inside out signaling (Hynes, 2002). For example, β-DG undergoes adhesion-dependent tyrosine phosphorylation (James et al., 2000), enabling its binding to the SH3 domain of Grb2 (Russo et al., 2000), an adapter protein involved in activation of the MAPK pathway. This site is also where β-DG binds to dystrophin, suggesting that phosphorylation at this site in β-DG may somehow cycle the interaction of β-DG with either Grb2 or dystrophin. Preventing phosphorylation of β-DG improves the dystrophic

phenotype of dystrophin deficient mice (Miller et al., 2012). In addition, inhibition of α-DG expression or its binding to laminin-2 reduces the viability of myotubes in culture (Brown et al., 1999; Montanaro et al., 1999). Similar to integrins, dystroglycans function as scaffolds that mediate ERK signaling by acting as sites for ERK activation, directing ERK signaling to or away from the nucleus, potentially influencing gene expression (Spence et al., 2004). Thus, the association of the dystroglycans with Grb2 suggests that the intracellular signaling pathways activated by integrins may also be activated in a similar fashion by outside-in dystroglycan signaling.

In addition to the β-DG association with Grb2, initial evidence suggesting a signaling role for the DGC is the association of nNOS with several DGC components in skeletal muscle (Brenman et al., 1995). The significance of components of the DGC such as sarcospan, dystrobrevins, and syntrophins that do not appear to have a direct or essential role in the mechanical function of the DGC, but instead appear to be docking sites for other intracellular signaling proteins (Haenggi and Fritschy, 2006), has fueled considerable interest in what other intracellular pathways may be affected in DGC-associated muscular dystrophies. Given the association of dystrobrevins and syntrophins with the carboxyl-terminus of dystrophin in the cytoplasm (Newey et al., 2000; Sadoulet-Puccio et al., 1997), it was hypothesized that they may function as a scaffold for linking nNOS to the DGC. nNOS is a signaling molecule involved in muscle contraction and calcium dynamics, and also influences gene expression. nNOS is associated with the DGC through tight binding with syntrophin (Adams et al., 2001; Miyagoe-Suzuki and Takeda, 2001), which is also dependent on α-dystrobrevin, as demonstrated by the absence of nNOS in α-dystrobrevin-null mice which display dystrophic symptoms

despite an otherwise intact DGC, suggesting that this interaction may be important for nNOS function (Grady et al., 1999).

The significance of nNOS delocalization to dystrophy is a debated topic in the field. Originally, the association of nNOS with the DGC led to the hypothesis that nNOS gene targeting would cause a dystrophic phenotype similar to that observed with other mutations of DGC components. However, knockout of nNOS does not cause overt muscular dystrophy (Huang et al., 1993), nor does knockout of the syntrophins, which also causes loss of nNOS from the sarcolemma (Adams et al., 2004; Kameya et al., 1999). This suggests that the phenotype of the α-dystrobrevin knockout animals is not attributable to the disruption of nNOS alone. Although alteration of the nNOS-syntrophin complex within the DGC does not cause muscular dystrophy, disruption of nNOS does appear to alter other pathways such as muscle growth and regeneration (Anderson, 2000; De Palma et al., 2014), and its overexpression ameliorates inflammation and membrane injury in *mdx* muscle (Wehling et al., 2001). In addition, enhancing nNOS activity through use of phosphodiesterase inhibitors has also been shown to be attenuate functional muscle ischemia, injury, and fatigue following exercise (Kobayashi et al., 2008).

While dystrophin was thought to serve a primarily mechanical role, its association with non-mechanical DGC components elucidated its potential significance in muscle signaling. Similarly, the DGC component sarcospan was initially thought to serve a primarily mechanical role given that its overexpression leads to increased compensatory utrophin and α7β1 integrin expression at the sarcolemma (Hodges et al., 1997) in dystrophin-deficient muscle. Similar to integrins, sarcospan has been found to be a major

regulator of Akt signaling pathways, where sarcospan-deficiency significantly impairs Akt-mediated muscle regeneration via p70S6K following cardiotoxin injury (Marshall et al., 2012). Sarcospan also regulates glycosylation of α-DG, which plays an important role in its association with laminin and its outside-in signaling function (Nguyen et al., 2002).

Despite abundant evidence implicating components of the DGC in mediating muscle signaling pathways, exactly how they regulate action of these signaling molecules, and the relationship between these signaling and mechanical functions and how their misregulation contributes to muscle disease is not fully understood.

### 1.6. Signaling pathways in muscle

Somewhat better understood are the downstream signaling pathways through which mechanical stress affects gene expression in skeletal muscle that activate muscle fiber size, contractile properties, and metabolism. These include the mitogen-activated protein kinase (MAPK) pathway, which encompasses the extracellular regulated kinase (ERK) family (Roux and Blenis, 2004a), the p38 family, and the c-Jun NH2 -terminal kinase (JNK) family, as well as the phosphatidylinositol 3-kinase (PI3K) signaling pathway, all of which are major effectors of mechanotransduction. Investigation of the activation and regulation of these signaling pathways has revealed an emerging role for in the disease progression in muscular dystrophies.

#### *MAPK pathway*

In skeletal muscle, mechanical deformation initiates MAPK activation through tyrosine and threonine phosphorylation events by upstream MAPK kinases. For example,

mechanical events initiate sequential phosphorylation of Raf, MEK, and finally ERK. Critical in the activation of receptor tyrosine kinases and activation of Raf is Grb2, the signal transducer and adaptor protein previously mentioned to strongly associate with the DGC. Ultimately, ERK phosphorylates a number of cytoplasmic targets, including PLA2, p90RSK, and MAP kinase activated protein kinases. Activated ERK also translocates to the nucleus and phosphorylates several transcription factors involved in cell survival and proliferation, including c-Myc, c-Jun, c-Fos, and Elk-1 (Davis, 1995; Garrington and Johnson, 1999), thereby regulating gene expression. ERK phosphorylation has been reported in response to tetanic muscle contraction (Martineau and Gardiner, 2001; Ryder et al., 2000) as well as passive stretch alone (Wretman et al., 2001), and this activation has been shown to be positively correlated with tension, where eccentric contraction elicits the strongest ERK activation, followed by isometric contraction, concentric contraction, and passive stretch (Martineau and Gardiner, 2001). Myotubes from *gsg*$^{-/-}$ mice show increased apoptosis with concomitant activation of ERK1/2 compared to the myotubes prepared from wild-type mice, with activation of ERK1/2 further increased upon application of mechanical stretch (Griffin et al., 2005). In whole EDL muscles from *mdx* and *gsg*$^{-/-}$ mice, ERK1/2 activation is aberrant both at rest and in response to *ex vivo* mechanical stimulation (Barton, 2006), indicating potential misregulation of its target transcription factors. Therefore, the ERK signaling pathway has the potential to be a central mediator of muscle mechanotransduction, integrating mechanical, metabolic, and activity signals.

The JNK pathway is associated with mediating cell differentiation, proliferation, and apoptosis through activation of its downstream targets, including c-Jun, Elk1, and

p53. JNK exists in p46 and p54 isoforms, both of which are mechanosensitive, and its activation is proportional to tension (Martineau and Gardiner, 2001). Grb2 is involved in recruiting the p46 isoform of JNK, which binds syntrophins (Hasegawa et al., 1999) and promotes cell survival (Chamberlain and Rando, 2006; Oak et al., 2001). JNK is highly activated in skeletal and cardiac muscle of *mdx* mice, and expression of the JNK inhibitory protein, JIP1, ameliorates dystrophic pathology in these mice, suggesting that JNK1 contributes to muscle pathogenesis in *mdx* mice (Kolodziejczyk et al., 2001). Of note, ERK1/2 is hyperactivated in younger mice (Kumar et al., 2004), whereas JNK hyperactivation is more pronounced in old mice (Kolodziejczyk et al., 2001) suggesting that variable MAPK are activated at different disease stages and may contribute to disease onset and/or progession differentially. In support of this, expression of constitutively active JNK causes compromised integrity and cell death in cultured myotubes (Kolodziejczyk et al., 2001). However, disruption of the interaction between α-DG and laminin causes decreased JNK p54 activation as well as decreased activation of its downstream target c-Jun and apoptosis (Oak et al., 2003). Overall, these results suggest that there is a narrow window of effective JNK signaling, and that its misregulation may contribute to the pathology of muscular dystrophy.

p38 MAPK signaling mediates skeletal muscle development and maturation, and is associated with the DGC through binding to α-syntrophin (Hasegawa et al., 1999). The most highly expressed isoform in skeletal muscle is p38α, which can affect myoblast fusion to form myotubes (Cuenda and Cohen, 1999; Lechner et al., 1996), and influence activation of transcription factors including MEF2 and MyoD (Keren et al., 2006). The direct role of p38 in the pathogenesis of muscular dystrophy is unclear, although it is

known to be misregulated in *mdx* mice muscle. Interestingly, in *mdx* diaphragm, which exhibits the most severe dystrophic phenotype, p38 activation is significantly reduced, whereas p38 activation is normal in the more mildly affected limb muscles from the same mice (Lang et al., 2004). Given this observation, the effect of upregulating p38 activity has been investigated in several dystrophic mouse models. Activation of p38 signaling has been found to increase utrophin expression in *mdx* diaphragm (Amirouche et al., 2013) as well as in sarcospan-null mice muscle with improved muscle repair following cardiotoxin injury (Marshall et al., 2012). Mice lacking Dusp10, a negative regulator of p38 and JNK, showed attenuated dystrophic pathology in *mdx* muscle suggesting that p38 signaling is protective (Shi et al., 2013). However, there is now contrasting evidence of p38 hyperactivity in dystrophic muscle contributing to pathology. Deletion of *MAPK14* (p38α-encoding gene) in the skeletal muscle of *mdx* or $dsg^{-/-}$ mice was found to reduce pathology, and transgenic mice with heightened muscle-specific p38α signaling presented with severe myofiber necrosis and many hallmarks of muscular dystrophy due to p38 specific phosphorylation of the cell death protein Bax. Further, treatment of $dsg^{-/-}$ mice with a p38 inhibitor has also been found to reduce disease (Wissing et al., 2014). Therefore, similar to JNK, both over-activation and under-activation of p38 may cause dystrophic pathology, suggesting that proper tuning of p38 activity is critical for healthy muscle.

***PI3K/Akt pathway***

The Akt family of serine/threonine-specific protein kinases are activated through several receptor tyrosine kinases associated with the phosphoinositide 3-kinase (PI3K)

pathway. Signaling through the PI3K/Akt cascade leading to downstream activation of p70S6K protein synthesis pathways is important for regulation of muscle strength, hypertrophy, and promotion of cell survival (Otto and Patel, 2010). Akt induces skeletal muscle hypertrophy *in vitro* and *in vivo* through activation of the mammalian target of rapamycin (mTOR) pathway (Bodine et al., 2001; Pallafacchina et al., 2002), which phosphorylates p70S6K (Isotani et al., 1999). Like the MAPK pathways, the PI3K/Akt pathway can interact with the DGC via Grb2 (Leshem et al., 2002).

Disruption of dystroglycan binding to laminin causes PI3K/Akt-mediated apoptotic cell death in muscle cells (Langenbach and Rando, 2002), suggesting a significant role for reduced PI3K/Akt signaling in the dystrophic process. Akt signaling is elevated in DMD patient biopsies as well as *mdx* and *dsg*$^{-/-}$ dystrophic mouse models, and it is proposed that that this elevated Akt activity functions to activate p70S6K in an attempt to increase protein synthesis for myofiber regeneration (Peter and Crosbie, 2006). This hypothesis is supported by evidence showing that over-expression of constitutively active Akt in *mdx* mice results in upregulation of utrophin and a7βl integrin, which leads to improvement in force generation (Kim et al., 2011; Peter et al., 2009). Over-expression of α7 integrin has also been shown to increase Akt signaling and p70S6K protein synthesis (Boppart et al., 2011). Similarly, overexpression of sarcospan has been shown to stabilize the UGC and α7β1 integrin at the sarcolemma in *mdx* mice, activate Akt and downstream p70S6K, and ameliorate dystrophic pathology (Marshall et al., 2012). Taken together, these studies suggest an important role for reduced PI3K/AKkt signaling in the dystrophic process and as a therapeutic target. Differential activation of Akt and p70S6K in C57 and *gsg*$^{-/-}$ muscle in response to mechanical perturbation will be discussed in

Chapter 3.

### 1.7. The sarcoglycans in skeletal muscle

The sarcoglycans (SGs) are a family of homologous transmembrane proteins with single membrane spanning domains (Ozawa et al., 1998). Six sarcoglycan genes have been identified, each of which transcodes a separate sarcoglycan protein: α-, β- δ-, ε-, γ-, and ζ-SG. α-SG and ε-SG share high homology and are type I transmembrane proteins, whereas β-, γ-, δ-, and ζ-SG are type II transmembrane proteins. β-SG shares weak homology with γ- and δ-SG. In cardiac and skeletal muscle, α-, β- δ-, and γ-SG form a tetrameric complex with a 1:1:1:1 stoichiometry (Jung et al., 1995). The complex assembles in the endoplasmic reticulum, where δ-, β-, and γ-SG form a core unit to which α-SG binds (Shi et al., 2004). The complex also co-assembles with β-DG, although this interaction is less well studied (Chan et al., 1998).

As mentioned previously, autosomal recessive mutations in γ-, α-, β-, δ-SG subunits cause LGMD types 2C-F, respectively, and in both humans and mouse models, mutation of a single SG subunit is associated with differential secondary loss or reduction in sarcolemmal expression of the other subunits. For example, in the absence of δ-SG, α-, β-, and γ-SG are quickly degraded before transport from the Golgi and are therefore undetectable at the sarcolemma, whereas upon loss of γ-SG, α-SG is still detectable, albeit at reduced levels (Chen et al., 2006; Hack et al., 2000; Hack et al., 1998; Shi et al., 2004). SG-null mice begin to display dystrophic symptoms at about two weeks of age, including necrosis, immune cell infiltration, fibrosis, and compromised force generation, with variable cardiac involvement (Coral-Vazquez et al., 1999; Durbeej et al., 2000).

### *Function of the sarcoglycan complex*

*Structural/mechanical function*

The role of the sarcoglycan complex alone and as a component of the DGC is not well-defined, but like the DGC itself, the complex appears to have both mechanical and signaling roles. Given its position at the sarcolemma, it was initially thought that the sarcoglycan complex may have a role in stabilizing the DGC. There is a strong association between the sarcoglycan complex and the dystroglycans, though it is unknown whether the sarcoglycan complex binds directly to α-DG, β-DG, both, or possibly through co-interaction with sarcospan (Chan et al., 1998; Marshall and Crosbie-Watson, 2013; Straub et al., 1998). Loss of the sarcoglycan complex results in increased sensitivity of β-DG to cleavage by a matrix metalloproteinase (MMP) disrupting the linkage of the cytoskeleton to the ECM (Yamada et al., 2001). In addition, muscular dystrophies that retain the SG complex do not have cleavage of β-DG, suggesting that physical access to the cleavage site on β-DG by the MMP may be necessary, and the presence of the SG complex inhibits this access. (Matsumura et al., 2005). In the BIO 14.6 Syrian hamster model with primary δ-SG mutation, biochemical analysis reveals that dystroglycan no longer associates with the SG complex (Straub et al., 1998).

In addition to association with β-DG, further evidence that the SGs are involved in DGC stabilization is that the C-terminus of dystrophin appears to directly interact with δ and β-SG in COS- 1 cells, though this interaction appears to be weak (Chen et al., 2006). It is possible that dystrophin binds a fully assembled sarcoglycan complex with greater affinity *in vivo*. However, the association of dystrophin with the DGC does not depend on the SGs, as SG-null mice express normal levels of dystrophin at the

sarcolemma (Hack et al., 2000; Hack et al., 1998). This observation suggests that the SG complex may have an essential function in muscle independent of the primarily mechanical role of dystrophin.

*Mechanosensor/signaling function*

Growing evidence suggests that the SG complex is involved in mechanotransduction. The intracellular regions of α- β- and γ-SG have potential tyrosine phosphorylation sites, and in cell culture studies, adhesion gives rise to phosphorylation of each of these SGs, indicating an attachment-dependent modification, and suggesting that the SG complex may participate in the outside-in signaling of the DGC (Yoshida et al., 1998). The adhesion-dependent tyrosine phosphorylation of α- and γ-SG, possibly by FAK (Yoshida et al., 1998), is analogous to the tyrosine phosphorylation events associated with the outside-in signaling that occurs in response to integrin interactions with extracellular matrix proteins (Schwartz, 2010). The association of the DGC with focal adhesion proteins such as β1 integrin and FAK is modulated by α-SG expression, and the recruitment of these proteins to focal adhesion sites is central to integrin-mediated cell survival signaling (Yoshida et al., 1996). The SG complex may also be involved in the inside-out signaling of the DGC involving α5β1 integrin, which associates with a dystrophin-containing lattice structure during muscle development (Lakonishok et al., 1992). Downregulation of either α-SG or γ-SG reduces α5β1 integrin-mediated muscle cell adhesion and focal adhesion formation (Yoshida et al., 1998). Thus, the affinity of the DGC for its ECM ligand, laminin, may be modulated by other

components of the complex, reminiscent of inside-out signaling of integrins (Disatnik and Rando, 1999).

The SG complex is associated with the N-terminus of dystrobrevin, suggesting that the SG complex is linked to the signaling protein nNOS via α-syntrophin associated with dystrobrevin (Yoshida et al., 2000). Therefore, the signaling defects that have been proposed to underlie muscular dystrophies associated with SG and dystrobrevin mutations may involve these pathways (Grady et al., 1999). Similarly to *mdx* mice, sarcolemmal localization of nNOS is also lost in the mouse models of SG deficiency as well as sarcoglycanopathy patients (Crosbie et al., 2002). However, it has not been shown that the SGs directly alter nNOS-mediated signaling activity.

***Gamma-sarcoglycan and disease***

Gamma-sarcoglycan is a 35 kDa member of the SG complex highly expressed in skeletal, cardiac, and smooth muscle. It is transcoded by the *SGCG* gene located on chromosome 13q12. There have been at least 5 naturally occurring variants and mutations identified in the SGCG gene causing limb-girdle muscular dystrophy type 2C, (Bonnemann and Finkel, 2002; McNally et al., 1996). The most common mutation, Δ521-T, results in a frameshift and premature stop codon, and an unstable truncated protein that causes severe DMD-like muscular dystrophy (Eiris-Punal et al., 2002). Of notable interest is the report of a patient with primary γ-SG mutation resulting in a truncated γ-SG protein absent from the sarcolemma, yet the other three SGs were appropriately expressed and localized to the sarcolemma as was the rest of the DGC. Despite the preservation of nearly the entire DGC, the patient had a severe muscular

dystrophy (Crosbie et al., 2000). This finding suggests that γ-SG is critical for muscle cell survival and possibly a functional interaction with a yet to be identified ligand.

The cytoplasmic domains of γ- as well as δ-SG bind directly to the actin-crosslinking protein filamin C, which is found at both the Z-band and plasma membrane of muscle. Mutation in the Filamin C gene causes human myopathy with cardiac involvement (Furst et al., 2013). Filamin C has been found to be essential in maintaining sarcolemmal integrity during force generation, and is involved in actin reorganization and signal transduction cascades associated with force transduction and cell survival (Dalkilic et al., 2006; Feng and Walsh, 2004; Razinia et al., 2012). In response to the loss of the SG complex, expression of filamin C is significantly upregulated, its distribution concentrated primarily at the plasma membrane (Thompson et al., 2000), though its potential as a compensatory mechanism has not yet been explored.

The LGMD2C γ-SG-null mouse (*gsg*$^{-/-}$) was generated by gene targeting of exon 2 and exhibits an additional loss of β- and δ-SG and decrease of α-SG expression at the sarcolemma. Normal levels of dystrophin remain localized at the plasma membrane, as do the others members of the DGC with the exception of sarcospan (Hack et al., 1998). Mice lacking γ-SG display muscle weakness, fiber degeneration, and fibrosis similar to LGMD2C patients. Early generations of *gsg*$^{-/-}$ mice (C57BL/6 background) displayed severe cardiomyopathy that led to premature mortality at 20 weeks (Hack et al., 1998); however, terminal cardiac defects have since been bred out of this line.

***Emerging role for γ-SG in mechanotransduction***

Initial evidence suggesting γ-SG to be a mechanosensor included studies

demonstrating its stimulus-dependent phosphorylation. In cell culture studies, the intracellular domains of γ-SG as well as α-SG were found to be tyrosine phosphorylated in response to cell adhesion, possibly by FAK (Yoshida et al., 1998). Subsequent studies in mice showed that γ-SG is tyrosine phosphorylated in hindlimb muscle following *ex vivo* eccentric contraction (Barton, 2006). Given this stimulus-dependent modification, the influence of γ-SG in mediated downstream ERK signaling was analyzed. *Gsg*$^{-/-}$ mice display aberrant P-ERK1/2 activation both at rest and with mechanical stimulation (Barton, 2006), indicating that γ-SG plays a role in signal transduction. However, further investigation demonstrated that γ-SG sarcolemmal localization alone is not sufficient to mediate normal signaling, and that phosphorylation of the γ-SG tyrosine 6 residue is required to preserve normal ERK signal transduction patterns (Barton, 2010). The role of γ-SG in mediating mechanosensitive signaling will be discussed in Chapters 2 and 3.

***Does a signaling defect contribute to muscular dystrophy pathology?***

Loss of dystrophin and/or sarcoglycan results in different mechanical consequences, which suggests there are alternative mechanisms leading to muscle degeneration. Dystrophin mutations produce a secondary reduction of the sarcoglycan subunits; therefore, SG deficiency is a feature seen in both DMD and in the *mdx* mouse. In the *mdx* mouse, there is clearly a mechanical defect, where there is a significant loss in force-generating capacity and sarcolemmal degeneration following mechanical stimulation. In contrast, *gsg*$^{-/-}$ mice, which retain dystrophin, do not display these deficits, yet still develop fiber degeneration and fibrosis comparable to *mdx* mice (Hack et al., 1999). Thus, a non-mechanical, perhaps signaling mechanism, may contribute to the

phenotype in muscle with primary SG deficiency as well with primary dystrophin deficiency, highlighting that the SG complex plays more than just a mechanical stabilizing role.

Recently, canonical TGFβ signaling was found to be upregulated in *gsg*$^{-/-}$ muscle following *ex vivo* eccentric contraction, with rapid accumulation of nuclear phosphorylated SMAD, which mediates fibrosis in cardiac and skeletal muscle. By introducing a heterozygous mutation of SMAD4 into *gsg*$^{-/-}$ mice to reduce but not ablate SMAD4, Goldstein et al showed that these mice had improved body mass, cardiac function and improved tetanic force (Goldstein et al., 2014). Thus, γ-SG may mediate multiple mechanosensitive signaling pathways that are disrupted in its absence and cause dystrophic pathology.

In summary, the contribution of mechanical transduction pathways to muscle adaptation is particularly pertinent to the manifestation of muscle disease. Current data provide substantial evidence that γ-SG is involved in mediating mechanotransduction in skeletal muscle. However, more work is necessary to identify the mechanisms by which this occurs. To this end, the experiments presented in this dissertation are aimed at testing the hypothesis that γ-SG associates with mechanically sensitive signaling proteins, where understanding of their interaction will help identify the mechanisms underlying mechanical signal transduction disrupted in muscle diseases.

# CHAPTER 2: Gamma-sarcoglycan is Required for the Response of Archvillin to Mechanical Stimulation in Skeletal Muscle

This chapter is adapted from:

Janelle M. Spinazzola, Tara C. Smith, Min Liu, Elizabeth J. Luna, Elisabeth R. Barton. γ-SG is required for the response of archvillin to mechanical stimulation in skeletal muscle. *Human Molecular Genetics* (2015) (Epub ahead of print)

This work was supported by National Institute of Health Grants U54 AR052646 to E.R.B., R01 GM033048-26S1 to E.J.L. and T32 AT-052461 to J.M.S.

## 2.1. Summary

Loss of gamma-sarcoglycan (γ-SG) induces muscle degeneration and signaling defects in response to mechanical load, and its absence is common to both Duchenne and limb girdle muscular dystrophies. Growing evidence suggests that aberrant signaling contributes to the disease pathology; however, the mechanisms of γ-SG-mediated mechanical signaling are poorly understood. To uncover γ-SG signaling pathway components, we performed yeast two-hybrid screens, and identified the muscle-specific protein archvillin as a γ-SG and dystrophin interacting protein. Archvillin protein and message levels were significantly upregulated at the sarcolemma of murine γ-SG-null (*gsg*$^{-/-}$) muscle, but delocalized in dystrophin deficient *mdx* muscle. Similar elevation of archvillin protein was observed in human quadriceps muscle lacking γ-SG. Reintroduction of γ-SG in *gsg*$^{-/-}$ muscle by rAAV injection restored archvillin levels to that of control C57 muscle. *In situ* eccentric contraction of tibialis anterior (TA) muscles from C57 mice caused ERK1/2 phosphorylation, nuclear activation of P-ERK1/2, and stimulus-dependent archvillin association with P-ERK1/2. In contrast, TA muscles from *gsg*$^{-/-}$ and *mdx* mice exhibited heightened P-ERK1/2 and increased nuclear P-ERK1/2 localization following eccentric contractions, but the archvillin-P-ERK1/2 association was completely ablated. These results position archvillin as a mechanically sensitive component of the dystrophin complex, and demonstrate that signaling defects caused by loss of γ-SG occur both at the sarcolemma and in the nucleus.

## 2.2. Introduction

Skeletal muscle mechanotransduction is mediated in part by the dystrophin glycoprotein complex (DGC), an assembly of proteins that also maintains the integrity of the sarcolemma by linking the cytoskeleton and the extracellular matrix (Ervasti and Sonnemann, 2008; Lapidos et al., 2004). Mutations in genes encoding DGC components cause several forms of muscular dystrophy including limb girdle muscular dystrophies (LGMD) types 2C-F from mutation of γ-, α-, β-, and δ-SG subunits of the sarcoglycan (SG) complex, as well as Duchenne muscular dystrophy (DMD) from mutation of dystrophin. Upon loss of dystrophin in DMD patients and in the *mdx* mouse model for the disease, there is a secondary loss of the entire DGC including the sarcoglycans. However, with primary mutations in any of the sarcoglycans, only presence of the SG complex is compromised, with retention of dystrophin and other DGC components (Durbeej and Campbell, 2002; Engel and Franzini-Armstrong, 2004). This holds true for the LGMD2C γ-SG null mouse model (*gsg*$^{-/-}$), where there is secondary reduction or absence of the other three sarcoglycan subunits. Dystrophin loss in skeletal muscle causes compromised force-generating capacity and contraction-induced damage with sarcolemmal tearing and fiber degeneration (Hack et al., 1998; Petrof et al., 1993), which are thought to be primary contributors to the progressive loss of muscle and its replacement with fat and fibrotic tissue (Engel and Franzini-Armstrong, 2004). In contrast, there is a different progression to disease in LGMD2C, where skeletal muscles from *gsg*$^{-/-}$ mice display little contraction-induced damage early in life, yet still develop a similarly severe pathology (Hack et al., 1999; Hack et al., 1998), indicating that γ-SG deficiency alone is sufficient to induce several dystrophic symptoms. Based on this separation between mechanical

fragility and dystrophic pathology, we and others have asserted that loss of the SG complex disrupts proper load sensing in muscle, which then leads to muscle disease.

Previous work in our lab demonstrated that sarcolemmal localization of γ-SG and phosphorylation of its tyrosine 6 residue is essential for normal extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling in muscle subjected to eccentric contraction (ECC) (Barton, 2010), and that loss of γ-SG also uncouples the response of the p70S6K pathway to passive stretch (Moorwood et al., 2014). These results support that γ-SG is an important component of the normal signaling profile in response to mechanical perturbation. However, the mechanisms by which γ-SG mediates mechanical signal transduction are unknown. Because of the importance of γ-SG as a mechanosensor in the absence of any known endogenous enzymatic activity, and the fact that the sequence of its intracellular domain is also critical for normal ERK1/2 signaling, we hypothesized that γ-SG must transmit load-induced signals to downstream pathways via association with binding partners.

Based on this premise, we performed a yeast two-hybrid assay to identify potential binding partners for the intracellular domain of γ-SG, and an intriguing candidate, archvillin, emerged. Archvillin is a 250kDa muscle-specific isoform of supervillin, a known actin- and myosin-II-binding protein (Chen et al., 2003; Oh et al., 2003). Although a series of yeast two-hybrid and proteomics analyses have identified interactors for nonmuscle supervillin isoform 1 (NP_003165.2) (Nebl et al., 2002; Smith et al., 2010; Smith et al., 2013; Takizawa et al., 2007; Takizawa et al., 2006), interaction partners have not been reported for the differentially spliced, muscle-specific insert of amino acids 276-669 in archvillin, which are encoded by coding exons 3, 4, and 5 (Oh et

al., 2003). Thus, even though archvillin is abundantly expressed in cardiac and skeletal muscle (Pope et al., 1998), its role in striated muscle has not been identified. Co-localization at the sarcolemma with dystrophin in hamster skeletal muscle (Oh et al., 2003) suggests that archvillin may serve some function in the DGC, but this has not been confirmed. A related isoform, smooth muscle archvillin (SmAV), has been shown to be an ERK scaffolding protein that associates with ERK in a stimulus-dependent manner in aortic tissue stimulated with the alpha agonist phenylephrine (Gangopadhyay et al., 2009; Gangopadhyay et al., 2004). However, ERK association with the striated muscle archvillin has not been demonstrated to date. Given the known sarcolemmal localization of archvillin, its identification in yeast two-hybrid screens with γ-SG and dystrophin, as well as the ability of the smooth muscle isoform to associate with ERK, the goal of this study was to determine if archvillin is part of the mechanical signaling machinery in skeletal muscle.

## 2.3. Results

### *Archvillin interacts with γ-SG and dystrophin*

To identify binding partners of γ-SG, we performed a yeast-two hybrid assay in which we cloned the intracellular domain of human γ-SG into a pGBKT7 bait plasmid and screened it against a normalized human library. Prey fragments of bait-prey positive interaction colonies were PCR amplified and identified using the BLAST algorithm and included archvillin, the β-sarcoglycan subunit of the sarcoglycan complex, as well as vesicle trafficking proteins (Table 2.1, Supplementary Table 2.1). We identified two identical prey clones encoding the extreme C-terminus of archvillin (aa 2093-2214) (Figure 2.1A), and this fragment was re-transformed into the prey yeast strain for confirmation of the interaction with γ-SG by two methods (Figures 2.1B, 2.1C). First, we performed a binding assay in which yeast containing either the γ-SG/pGBKT7 bait plasmid or empty pGBKT7 plasmid were re-mated with yeast containing the archvillin prey sequence identified in the initial yeast two-hybrid screen, assuring that mated yeast contained both bait and prey plasmids. Mated yeast were plated on selective dropout media plates containing x-α-gal, where positive interaction would activate β-galactosidase expression and yield blue colonies. Yeast mated containing the γ-SG/pGBKT7 plasmid yielded blue colonies, whereas yeast mated containing the negative control empty pGBKT7 plasmid did not grow any colonies on the most stringent quadruple drop-out plates (Figure 2.1B). Second, co-immunoprecipitation (co-IP) with anti-γ-SG was performed on skeletal muscle extracts from wild type (C57) and *gsg*$^{-/-}$ mice, followed by immunoblotting with anti-archvillin or anti-γ-SG. In samples from C57

mice, bands for both γ-SG and archvillin were observed following co-IP (Figure 2.1C, lane 3), whereas co-IP could not pull down archvillin in samples lacking γ-SG even though there was abundant archvillin in the lysate (Figure 2.1C, lane 2, 4). Biochemical confirmation of the archvillin interaction with γ-SG was attempted, but the experiments were unsuccessful due to insolubility of the tagged γ-SG cytoplasmic domain (data not shown). Thus, the interaction between the intracellular portion of γ-SG and the C-terminus of archvillin identified by the yeast two-hybrid screen was sufficient to afford physical association between these proteins in skeletal muscle, but whether this was a direct interaction between the domains of these proteins or an indirect association via intermediate proteins is unknown.

We also used yeast two-hybrid screening and co-sedimentation assays to show a direct interaction between archvillin and dystrophin (Table 2.1, Figure 2.1D). In a separate yeast two-hybrid screen, the bait was the differentially spliced first muscle-specific sequence (aa 277-669) of human archvillin. Thirty unique prey clones from 14 identified interactors were confirmed from this screen (Table 2.1). Five unique dystrophin clones were identified that spanned rod domains 8-13 (aa 1239-1724), as well as two unique clones spanning rod domains 9-13 of the dystrophin ortholog utrophin (aa 1284-1715), with rod domains 11-12 present in all dystrophin and utrophin clones (Figure 2.1A). Other prominent prey fragments from the screens with the archvillin bait were from nexilin and myosin binding protein C, which also play a role in maintaining sarcomeric integrity (Table 2.1). We confirmed the archvillin-dystrophin interaction by co-sedimenting purified recombinant His-tagged dystrophin spectrin repeat domains 10-12 (His-DMD-10-12) with purified GST-tagged archvillin protein fragments. His-DMD-

10-12 was pulled down with the muscle-specific archvillin sequences encoded by exons 3-5 (GST-AV-277-669), but did not co-sediment with either GST-AV-1-171 or GST alone (Figure 2.1D). Thus, based on two independent yeast-two hybrid screens and subsequent co-IP/co-sedimentation experiments, the muscle-specific insert of archvillin binds to dystrophin, and its interaction with γ-SG impacts its association with the DGC. These interactions are schematized in Figure 2.1A.

| Bait | Interactors | Accession Number | | Number of Clones | |
|---|---|---|---|---|---|
| | | Protein | Nucleotide | Total | Individual |
| γ-SG aa 1-35 | Archvillin (SVIL)[a] | NP_003165.2 | NM_003174.3 | 2 | 1 |
| | Coatomer subunit delta isoform 2 (COPD2) | NP_001135753.1 | NM_001142281.1 | 2 | 1 |
| | Beta-sarcoglycan (SGCB) | NP_000223.1 | NM_000232.3 | 1 | 1 |
| | PDZ and LIM domain protein 2 isoform 2 (PDLIM2) | NP_067643.3 | NM_021630.5 | 1 | 1 |
| hAV aa 227-669[c] | Nexilin (NEXN) | NP_001165780 | NM_001172309.1 | 15 | 6 |
| | Myosin binding protein C (MYBPC1) | NP_996555 | NM_206819 | 8 | 6 |
| | Dystrophin (DMD) | NP_004001 | NM_004010 | 8 | 5[b] |
| | Utrophin (UTRN) | NP_009055 | NM_007124 | 2 | 2 |
| | Basic helix-loop-helix family member e40 (BHLHE40) | NP_003661 | NM_003670 | 2 | 1 |
| | Lamin A/C (LMNA) | NP_733822 | NM_170708 | 2 | 1 |
| | A kinase anchor protein 9 (AKAP9) | NP_005742 | NM_005751 | 1 | 1 |
| | Acyl-CoA binding domain containing 3 (ACBD3) | NP_476507 | NM_022735 | 1 | 1 |
| | Collagen, type VI, alpha 3 (COL6A3) | NP_001120959 | NM_057166 | 1 | 1 |
| | Filamin C, gamma (FLNC) | NP_006603 | NM_001127487 | 1 | 1 |
| | Kelch-like family member 7 (KLHL7) | NP_061334 | NM_018846 | 1 | 1 |
| | Kinesin family member 1C (KIF1C) | NP_073572 | NM_006612 | 1 | 1 |
| | Protein inhibitor of activated STAT, 1 (PIAS1) | NP_057250 | NM_016166 | 1 | 1 |
| | Trio Rho guanine nucleotide exchange factor (TRIO) | NP_009049 | NM_007118 | 1 | 1 |

**Table 2.1. Candidate binding partners for human γ-SG intracellular domain aa 1-35 and hAV muscle specific insert 1 aa 277-669.**

Positive interactors were identified in two yeast two-hybrid screens. Names, protein and nucleic acid accession numbers, and the numbers of total and unique clones for each interactor are shown. [a] Archvillin was confirmed as a γ-SG aa 1-35 interactor by secondary assays. [b] Two of the identified dystrophin clones had incomplete sequencing results, and were thus included in the count of total clones, but not in the count of individual, distinct preys. [c] hAV interactors were confirmed by secondary assays. See Table S1 for complete list of candidate interactors from the γ-SG yeast two-hybrid screen.

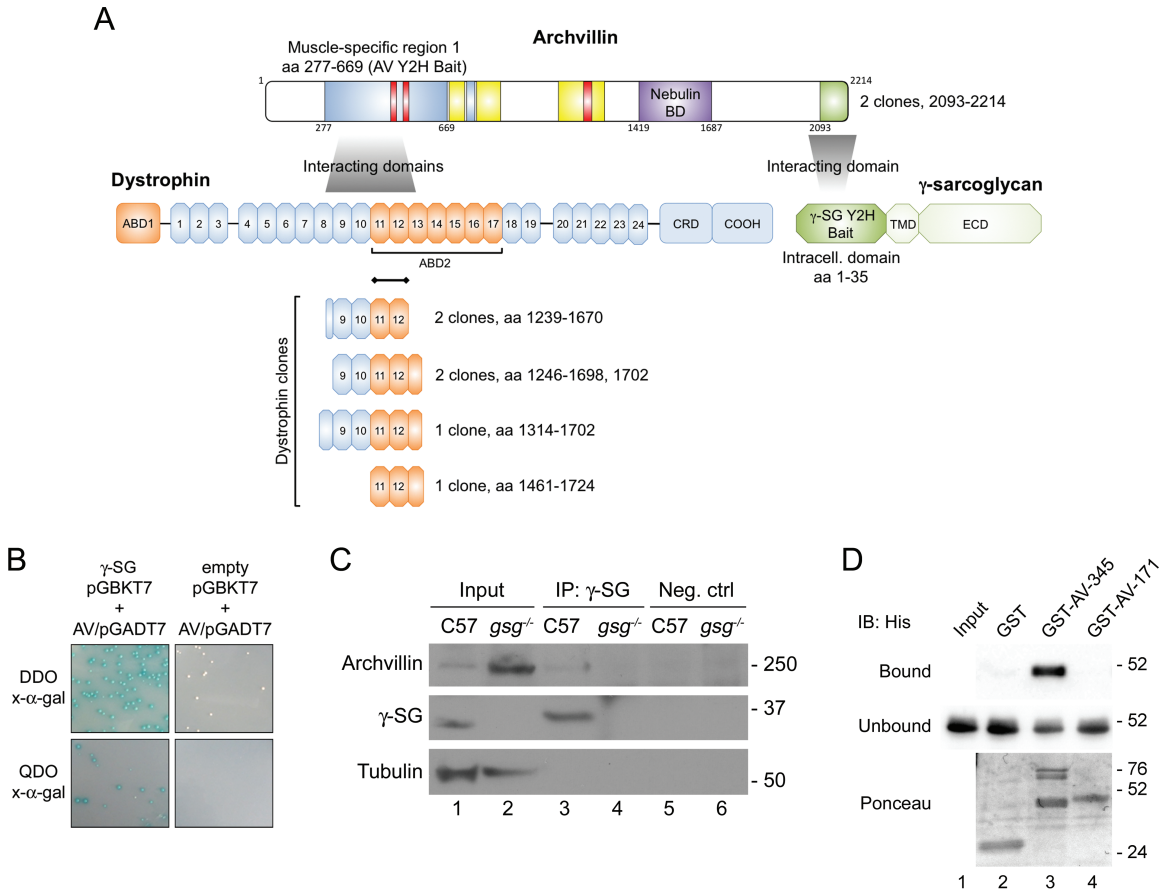


**Figure 2.1. Archvillin binds γ-SG and dystrophin.**

(A) Schematic of interacting domains in human archvillin for dystrophin and γ-SG. The dystrophin preys all contain spectrin-like repeats 11-12; black bar indicates this predicted binding region. The clones of archvillin preys interacting with γ-SG were identical, encoding aa 2093-2214. Sequences of interest within human archvillin are indicated: Blue-shading, muscle-specific differentially spliced sequences encoded by coding exons 3, 4, 5 [aa 277 – 669; yeast two-hybrid (Y2H) bait sequence] or coding exon 9 (aa 750-781); red bars, predicted docking motifs for binding to ERK1/2 or other MAP kinases; yellow bars, F-actin-binding regions; purple shading, nebulin-binding domain; green shading, Y2H prey sequence recovered with the γ-SG bait. Schematics of dystrophin and

γ-SG showing the locations of actin-binding domains 1 and 2 (ABD1, ABD2, orange), numbered spectrin-like repeats 1 – 24, the cysteine-rich domain (CRD), and the carboxy-terminus (COOH) of dystrophin, along with the transmembrane domain (TMD) and extracellular domain (ECD) of γ-SG. The locations of potential ERK-binding sites were predicted by The Eukaryotic Linear Motif (ELM) resource for Functional Sites in Proteins using the default motif probability cutoff of 100 (http://elm.eu.org/), (Dinkel et al., 2014). (B) Confirmation of γ-SG-archvillin interaction. Yeast containing either γ-SG/pGBKT7 or empty pGBKT7 plasmid were mated with yeast containing the archvillin prey sequence (AV/pGADT7) identified in the yeast two-hybrid screen. White colonies indicate the presence of both pGBKT7 and pGADT7 plasmids in diploid yeast. Blue colonies indicate β-galactosidase gene activation from positive bait/prey interaction on double dropout (DDO) and quadruple dropout (QDO) plates. Empty pGBKT7 and AV/pGADT7 matings afforded colony growth on DDO plates, confirming that mated yeast contained both plasmids, but not on QDO plates (negative control). (C) Co-IP of archvillin and γ-SG. C57 and *gsg*$^{-/-}$ tibialis anterior (TA) muscle lysates were immunoprecipitated with anti-γ-SG, and blots were probed with anti-archvillin and anti-γ-SG. Lanes 1 and 2: Archvillin, γ-SG and tubulin in lysates (input). Archvillin co-precipitated with γ-SG in C57 muscle (lane 3), whereas no signal could be seen in *gsg*$^{-/-}$ negative control IP (lane 4) or in negative control IPs without primary antibody (lanes 5 and 6). (D) Archvillin-specific sequence encoded by coding exons 3, 4, and 5 binds to dystrophin spectrin repeats 10-12 *in vitro*. Immunoblots of recombinant His-tagged DMD spectrin repeats (SR) 10-12 co-sedimented with the indicated GST fusion proteins. Blots of bound and unbound fractions were probed with anti-His; Ponceau stain of bound

fractions shows amounts of GST fusion proteins, as well as the bound His-DMD SR 10-12 (aa 1361-1686) in Lane 3. Lane 1: His-DMD-10-12 input; Lane 2: GST (~26 kDa); Lane 3: GST-AV-345 (doublet at 74 - 76 kDa) and His-DMD-10-12 (~45 kDa); and Lane 4: GST-AV-1-171 (~48 kDa).

***Archvillin levels depend on DGC integrity***

Dystrophic muscle presents with an upregulation of membrane-associated proteins involved in maintaining the cytoskeleton/ECM link and signaling; this includes integrins, which are thought to function as a compensatory mechanism (Allikian et al., 2004b; Barton, 2010; Hodges et al., 1997). We examined the expression of archvillin in quadriceps, tibialis anterior (TA), extensor digitorum longus (EDL), soleus, diaphragm, and heart muscles from C57, *gsg*$^{-/-}$, δ-SG-null (*dsg*$^{-/-}$), and *mdx* mice. Archvillin levels were consistently elevated in *gsg*$^{-/-}$ and *dsg*$^{-/-}$ muscles, and were similar to C57 levels in *mdx* muscles (Figure 2.2A). Western blot quantification and quantitative real-time PCR showed that archvillin protein and mRNA were ~2.5-fold higher in *gsg*$^{-/-}$ quadriceps muscle compared to C57 muscle, but were not significantly different in *mdx* muscle (Figures 2.2B, 2.2C). Archvillin localization was first observed in hamster skeletal muscle primarily along the sarcolemma, supporting the interaction with dystrophin (Oh et al., 2003). To determine if archvillin localization was altered in dystrophic muscle, we immunostained for archvillin in EDL muscles from C57, *gsg*$^{-/-}$, and *mdx* mice. Similar to previous observations, archvillin was localized in patches along the sarcolemma in C57 muscle. In *gsg*$^{-/-}$ muscle, archvillin was enriched at the sarcolemma and outlined nearly the entire circumference of the muscle fibers. In *mdx* cross-sections, archvillin was virtually undetectable along the sarcolemma (Figure 2.2D). Thus, localization of archvillin to the sarcolemma depends upon the presence of dystrophin, but does not require γ-SG. However, the compensatory increases of archvillin are apparent only in the absence of the SG complex.

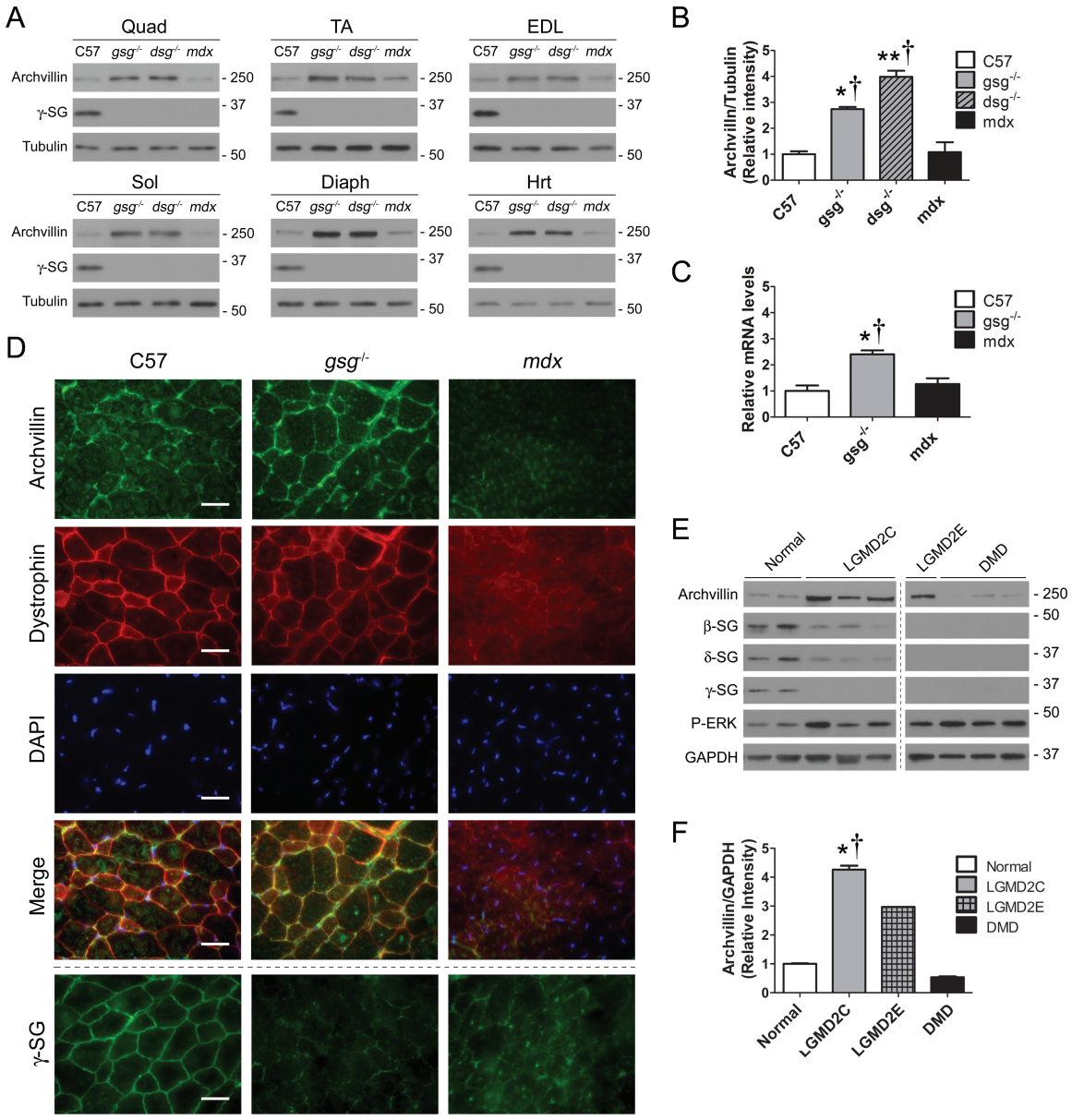


**Figure 2.2. Archvillin expression is altered in dystrophic muscle.**

(A) Archvillin protein levels in quadriceps (Quad), tibialis anterior (TA), extensor digitorum longus (EDL), soleus (Sol), diaphragm (Dia) and heart (Hrt) muscle of 12-14 week old C57/Bl6, *gsg*$^{-/-}$, *dsg*$^{-/-}$, and *mdx* mice. Increased archvillin is apparent in all striated muscles from *gsg*$^{-/-}$ and *dsg*$^{-/-}$ mice. (B) Quantification of archvillin protein in quadriceps confirms 2.7-4 fold increase of archvillin in *gsg*$^{-/-}$ and *dsg*$^{-/-}$ mouse muscles. $^{*}$,

$P < 0.05$; **, $P < 0.01$ vs. C57; †, $P < 0.05$ vs. *mdx* by one-way ANOVA with Bonferroni post-hoc test, n = 3. (C) Quantitative RT-PCR for archvillin expression shows significant upregulation in *gsg*$^{-/-}$ quadriceps muscles, as compared with C57 and *mdx* muscles. Bars represent fold change; means ± SEM relative to C57. *, $P < 0.05$; **, $P < 0.01$ vs. C57; †, $P < 0.05$ vs. *mdx* by one-way ANOVA with Bonferroni post-hoc test; n = 3-5. (D) Immunostaining of EDL muscles shows that archvillin is localized primarily along the sarcolemma in control C57 muscle, where it is present with increased intensity when γ-SG is absent in *gsg*$^{-/-}$ muscle. Archvillin localization is disrupted by the absence of dystrophin in *mdx* muscle. Dotted line indicates γ-SG staining on an adjacent section. Bars: 25μm. (E) Archvillin protein levels in quadriceps muscles from normal healthy control, LGMD2C, LGMD2E, and DMD patient biopsies. Increased archvillin is apparent in biopsies with primary sarcoglycan deficiency, whereas DMD patients with primary dystrophin deficiency have reduced archvillin expression. All dystrophic biopsies demonstrate elevated P-ERK activation vs. normal. Relative archvillin (AV) values represent archvillin/tubulin relative to normal patients. Panels are from one membrane with one lane removed for clarity. (F) Quantification of archvillin protein in human quadriceps confirms ~4-fold increase of archvillin in LGMD2C patients. *, $P < 0.05$ vs. Normal; †, $P < 0.05$ vs. DMD by one-way ANOVA with Bonferroni post-hoc test, n = 2-3; LGMD2E, n = 1 (not included in one-way ANOVA). See Table S2 for gene mutations and age at time of biopsy of human patient biopsies.

To determine if archvillin levels were also altered in diseased human muscle, we examined quadriceps muscle biopsies from LGMD2C, LGMD2E, and DMD patients with primary deficiency in γ-SG, β-SG, and dystrophin, respectively. Immunoblotting showed increased archvillin in LGMD2C biopsies, similar to observations in *gsg*$^{-/-}$ muscle, whereas DMD muscle expressed slightly lower archvillin relative to normal biopsies (Figures 2.2E, 2.2F). Therefore, in both mouse and human muscle samples, archvillin levels increased in the absence of γ-SG, but were normal or reduced when dystrophin was missing. We have previously shown that reintroduction of γ-SG in *gsg*$^{-/-}$ muscle by recombinant adeno-associated virus (AAV) injection restores normal ERK1/2 signaling (Barton, 2010). To assess the dependence of archvillin on the presence of γ-SG, we injected 3-week old *gsg*$^{-/-}$ mice with AAV expressing γ-SG and harvested tissue for analysis 1-month post-injection. AAV injection restored γ-SG expression to C57 levels in the injected limb and normalized archvillin expression levels (Figures 2.3A, 2.3B). Thus, the level of archvillin in muscle appears inversely proportional to the presence of γ-SG.

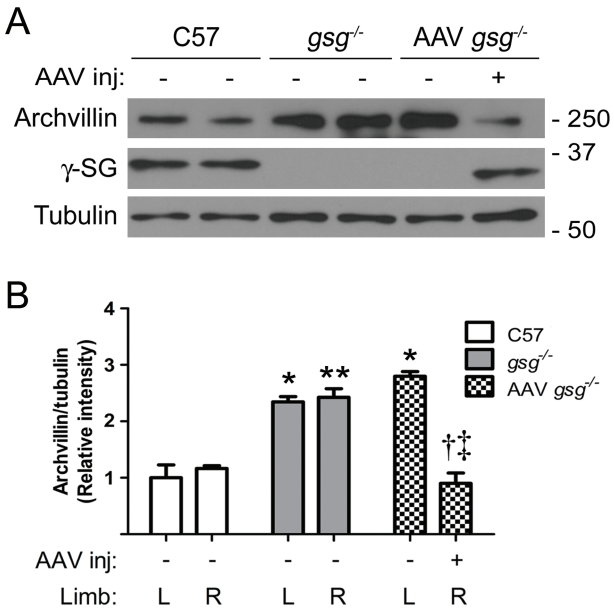


**Figure 2.3. γ-SG restoration normalizes archvillin expression.**

(A) Immunoblots of tibialis anterior (TA) muscles with (+) and without (-) injection of adeno-associated virus (AAV) expressing human γ-SG show that archvillin expression decreases with restoration of γ-SG expression (lane 6). (B) Western blot quantification shows archvillin levels significantly decrease in *gsg*$^{-/-}$ TA muscles with γ-SG AAV injection. Bars represent means ± SEM relative to C57 L. $^{*}$, $P < 0.05$; $^{**}$, $P < 0.01$ vs. C57 corresponding limb; $^{\dagger}$, $P < 0.05$ vs. *gsg*$^{-/-}$ R-; $^{\ddagger}$, $P < 0.01$ vs. AAV *gsg*$^{-/-}$ L- by two-way ANOVA and Bonferroni post-hoc test, n = 4-5.

***Archvillin association with P-ERK is stimulus-dependent***

Mechanical perturbation evokes transient increases in P-ERK1/2 in skeletal muscle (Martineau and Gardiner, 2001), and aberrant ERK signaling is common to many mouse models of muscular dystrophies (Barton, 2006; Muchir et al., 2013; Wu et al., 2014). Increased basal P-ERK is not only a phenotype of diseased murine muscle, but in probing our biopsy samples from human patients, we found that P-ERK1/2 was also elevated in all of the LGMD and DMD samples compared to biopsies from healthy subjects (Figure 2.2E). Thus, identification of potential mediators of ERK signaling may provide insight into disease mechanisms. Interestingly, the smooth muscle archvillin isoform, which lacks the differentially spliced sequences by *SVIL* exons 3, 5, and 9, has been shown to bind ERK in aortic tissue in a stimulus-dependent manner (Gangopadhyay et al., 2009; Gangopadhyay et al., 2004). Candidate ERK docking sites exist in archvillin (Figure 2.1A), raising the potential for a similar response to occur for this protein.

To determine if archvillin also associates with ERK, we subjected TA muscles to a series of eccentric contractions (ECC) *in situ* during which the muscle was simultaneously stimulated to contract and lengthened to induce maximal strain on the membrane. Measurements of maximal isometric tetanic force showed that *gsg*$^{-/-}$ and *mdx* muscle generated less force compared to C57 muscle (Figure 2.4A). However, following ECC stimulation, the loss of force production in *gsg*$^{-/-}$ muscles was comparable to that in C57 muscles, whereas *mdx* muscles displayed a significant drop in force generation following ECCs (Figure 2.4B). This is consistent with studies performed on isolated muscles from the *gsg*$^{-/-}$ mouse (Hack et al., 1999), and distinguishes the functional phenotypes of *gsg*$^{-/-}$ and *mdx* muscles.

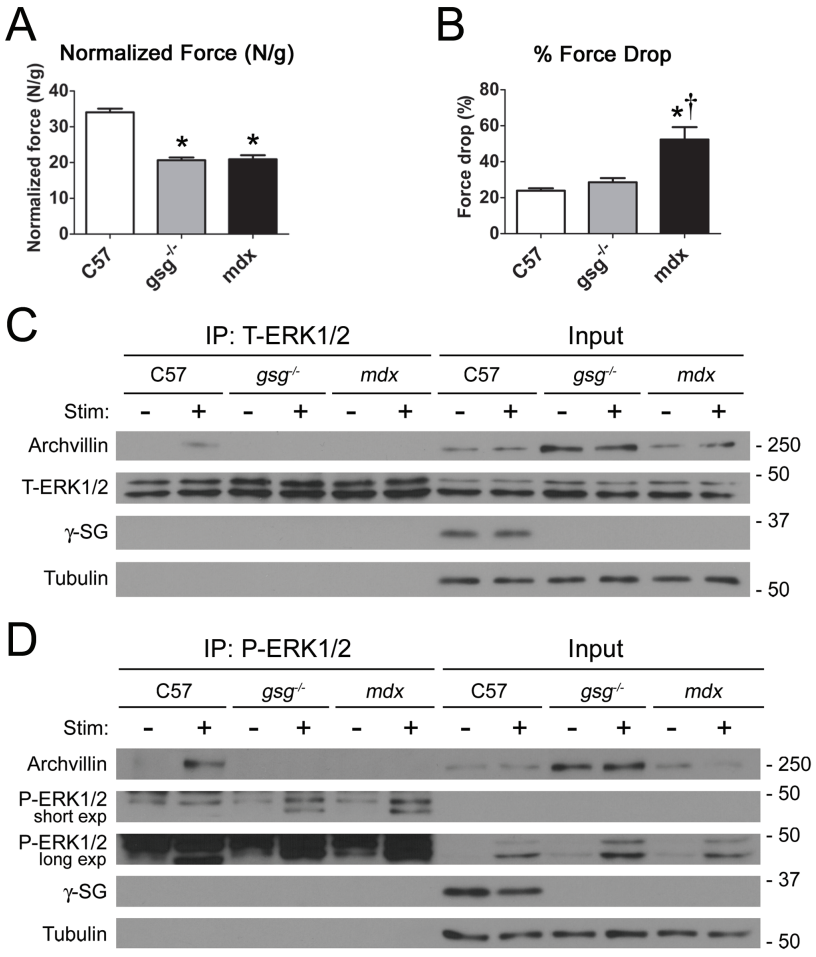


**Figure 2.4. Archvillin associates with ERK1/2 in a stimulation- and sarcoglycan-dependent manner.**

**Figure 2.4. Archvillin associates with ERK1/2 in a stimulation- and sarcoglycan-dependent manner.**

(A) Dystrophic *gsg*$^{-/-}$ and *mdx* tibialis anterior (TA) muscles generate less force than C57 muscles when subjected to *in situ* isometric mechanical stimulation. $^{*}$, $P < 0.001$ vs. C57 by one-way ANOVA with Bonferroni post-hoc test, n = 5-7. (B) Mice with primary γ-SG deficiency do not show compromised force generating capacity following *in situ* eccentric contraction mechanical injury, whereas *mdx* mice show a significant increase in force drop. $^{*}$, $P < 0.001$ vs. C57; $^{\dagger}$, $P < 0.01$ vs. *gsg*$^{-/-}$ by one-way ANOVA with Bonferroni post-hoc test, n = 5-7. (C) Immunoprecipitation assay of TA muscles with (+) and without (-) *in situ* eccentric contraction stimulation (Stim). Immunoprecipitation with total ERK1/2 (T-ERK1/2) antibody shows that archvillin associates with ERK1/2 in stimulated C57 muscle, but this association is lost in *gsg*$^{-/-}$ and *mdx* muscle. Without stimulation, archvillin does not associate with ERK1/2 in any strain, indicating a stimulation-dependent, sarcoglycan-dependent interaction. Immunoblotting for γ-SG demonstrates that it does not associate tightly with ERK1/2. Tubulin serves as a loading control for the inputs. (D) Immunoprecipitation with phospho-specific anti-P-ERK1/2 shows that archvillin associates with P-ERK1/2 only in stimulated C57 muscle. Results were consistent from three independent experiments.

Muscle lysates were immunoprecipitated with anti-ERK1/2 and probed for archvillin, ERK1/2, and γ-SG. Archvillin associated with ERK1/2 in stimulated TA muscles from C57 mice, but not in the non-stimulated contralateral control limb. However, this association was eliminated in stimulated TA muscles from *gsg*$^{-/-}$ or *mdx* mice, even though there was archvillin present in the lysates of all muscle samples (Figure 2.4C). Interestingly, there was no direct interaction between ERK1/2 and γ-SG, supporting that ERK1/2 associated with archvillin, but only when γ-SG was present. We extended this evaluation to distinguish between archvillin association with phosphorylated or non-phosphorylated ERK1/2, employing the same procedures, but with an antibody specific to P-ERK1/2. The eccentric contractions produced a robust increase in P-ERK1/2 in TAs from all three mouse lines. However, only stimulated C57 muscles displayed an association between P-ERK1/2 and archvillin, whereas stimulation of dystrophic muscles failed to cause this interaction (Figure 2.4D). Taken together, P-ERK1/2 associates with archvillin in a stimulation- and γ-SG-dependent manner.

***Nuclear P-ERK1/2 is elevated in dystrophic muscle***

A central component of P-ERK1/2 activity is to phosphorylate transcription factors in the nucleus, which then positively regulates target genes (Roux and Blenis, 2004b). Because we have previously established aberrant ERK1/2 signaling in dystrophic muscles, we determined if this also altered the distribution of P-ERK1/2 between cytosolic and nuclear fractions from C57, *gsg*$^{-/-}$ and *mdx* muscles. Fractioned lysates from muscles subjected to eccentric contraction and from non-stimulated control muscles were probed for P-ERK1/2. Eccentric contractions of TA muscles caused a dramatic increase

in P-ERK1/2 that was most pronounced in the dystrophic muscles (Figure 2.5). Not only was P-ERK1/2 elevated in the cytosolic fraction of muscles subjected to eccentric contractions, there was enhanced localization of P-ERK1/2 in the nuclear fraction in both *gsg*$^{-/-}$ and *mdx* muscles, suggesting that the aberrant signaling observed in whole muscle lysates led to increased nuclear accumulation. Of note was the increase of lamin A/C in the nuclear extracts of the dystrophic muscles. We performed additional probes with a second nuclear marker (histone 3), which revealed that lamin A/C was elevated, as opposed to an increase in nuclear content in these tissues, suggesting some compensatory expression of lamin A/C associated with these dystrophies. In sum, the aberrant P-ERK1/2 signaling in *gsg*$^{-/-}$ and *mdx* muscle was associated with enhanced localization of this signaling molecule in nuclear fractions.

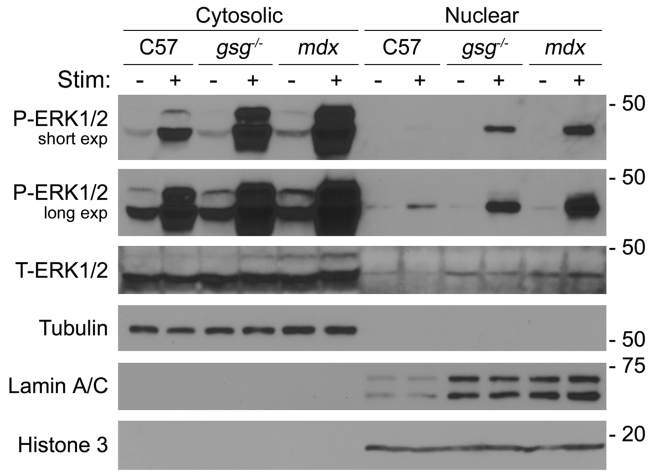


**Figure 2.5. Nuclear P-ERK1/2 is elevated in dystrophic muscle following stimulation.**

Immunoblotting of cytosolic and nuclear fractions of tibialis anterior (TA) muscle with (+) and without (-) *in situ* eccentric contraction stimulation (Stim). Stimulation led to elevated P-ERK1/2 in both fractions, which was augmented in dystrophic muscles. Nuclear accumulation of P-ERK1/2 was most pronounced in *gsg*$^{-/-}$ and *mdx* muscles with stimulation. Lamin A/C was also elevated in nuclear fractions from dystrophic muscles. Tubulin and histone 3 were used as loading controls and as cytosolic and nuclear markers, respectively.

***ERK1/2 was not detectable in muscle cross-sections by immunofluorescence***

Immunofluorescent staining of P-ERK1/2 and T-ERK1/2 was performed in 10um TA muscle cross-sections from C57, *gsg*$^{-/-}$ and *mdx* mice with and without ECC stimulation. Several conditions were tested in 4% formaldehyde and methanol/acetone fixation, horse serum/BSA and 0.2% triton x-100 blocking solutions, and primary antibody diltutions ranging from 1:5 to 1:500 applied either for 1 hour or overnight, expanding upon the conditions recommended in the commercial specifications, as well as those from papers detecting ERK1/2 in cell culture. However, none of these were successful in detecting ERK1/2 in the sarcoplasm or the nucleus. Images from C57 muscle samples (Figure 2.6) are representative of all genotypes for ERK1/2.

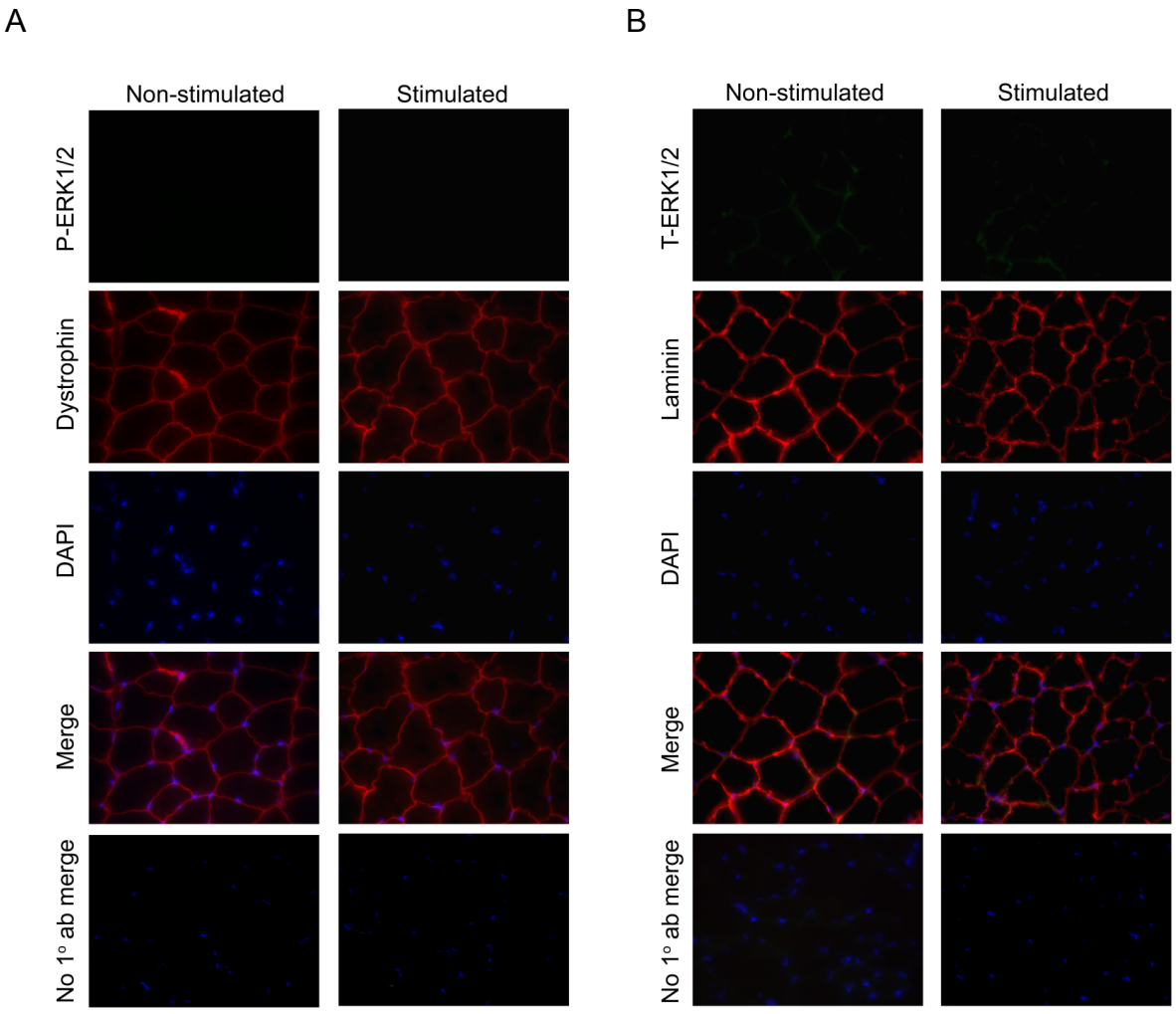


**Figure 2.6. ERK1/2 is not detectable in cross-sections by immunofluorescence.** Immunostaining of 10μm cross-sections of C57 TA muscles with and without ECC stimulation. Neither P-ERK1/2 (A) nor T-ERK1/2 (B) was detectable in the sarcoplasm or the nucleus despite testing several staining conditions.

## 2.3. Discussion

Growing evidence suggests that signaling defects associated with the loss of DGC components contribute to the pathology of muscular dystrophy. In our previous work, γ-SG was shown to be a mediator of ERK1/2 signaling in response to mechanical perturbation in skeletal muscle (Barton, 2010). However, the proteins involved in γ-SG-mediated signaling are unknown, as the SG complex possesses no known enzymatic activity. In the present study, we have identified the muscle-specific protein archvillin as a γ-SG and dystrophin interacting protein through two independent screens and biochemical assays. These results position archvillin as an intriguing component of the mechanical signal transduction pathway associated with the DGC.

The proteins encoded by the *Svil* gene (supervillin, archvillin, smooth muscle archvillin) are involved in many cellular processes, predominantly through their association with the actin cytoskeleton. Archvillin has several associations with the sarcolemma and cytoskeleton including binding to F-actin and non-muscle myosin (Chen et al., 2003), localization with dystrophin at costameres (Oh et al., 2003), as well as association with the C-terminus of nebulin (Lee et al., 2008). We show here that the N-terminal muscle-specific insert of archvillin binds to dystrophin, and also may interact with the same domains of its ortholog utrophin. The dual interactions of archvillin with both dystrophin and γ-SG suggest a strong association with the DGC that appears to be independent of nebulin binding.

Given this association, we did not anticipate the alterations in archvillin levels and localization observed in the absence of γ-SG and/or dystrophin. Because archvillin interacts with nebulin and has multiple F-actin binding domains, one would predict that

its localization near the membrane would be preserved in the absence of members of the DGC. However, in the absence of dystrophin in *mdx* tissue, there was diminished concentration of archvillin at the sarcolemma, suggesting that dystrophin is required for archvillin to remain at the membrane. The fact that archvillin is de-localized, but not de-stabilized or degraded in *mdx* muscle, may be due to the many additional binding partners for archvillin in the cell.

More surprising to us was the increased expression and accumulation of archvillin at the sarcolemma in the absence of γ-SG. Given the upregulated γ-SG expression in *gsg*$^{-/-}$ muscle, it is possible that γ-SG positively regulates the degradation of archvillin. However, archvillin is not upregulated in *mdx* muscle, suggesting that archvillin expression may be mediated by another mechanism in the absence of dystrophin. Loss of the SG complex also results in a compensatory increase in the integrin complex (Burkin et al., 2001; Hodges et al., 1997), and so one possibility is that increased archvillin levels are associated with integrin-associated proteins. While the related protein, supervillin, regulates integrin function, there is no known direct binding of either protein to integrin, though supervillin links to focal adhesions through binding to LIM domain proteins TRIP6 and LPP (Takizawa et al., 2006). Ultimately, we cannot discount that the increased archvillin in *gsg*$^{-/-}$ or in LGMD2C patients is correlated with integrin levels, but even so, the stimulation and mechanically dependent ERK binding to archvillin is completely eliminated in these muscles regardless of the 3-fold increase in the protein levels. This implicates the SG complex in mediating archvillin-ERK association in response to mechanical perturbation.

Smooth muscle archvillin, a highly homologous isoform of archvillin, has been implicated as an ERK scaffolding protein that binds ERK in a stimulus-dependent manner (Gangopadhyay et al., 2009; Gangopadhyay et al., 2004; Li et al., 2009). Archvillin also contains predicted ERK docking motifs, two of which are located within the first muscle-specific insert (Dinkel et al., 2014), suggesting that it also has the potential to bind ERK and mediate its activation in skeletal muscle, where there is significant strain imposed on the sarcolemma. To investigate a potential archvillin-ERK1/2 interaction, we subjected muscles to an *in situ* eccentric contraction protocol, which elicits dramatic P-ERK1/2 activation. Our results suggest that archvillin also associates with ERK1/2, specifically with P-ERK1/2, in C57 muscle upon eccentric contraction, through we cannot discern whether this interaction is direct or indirect involving a yet to be identified intermediate. In either scenario, however, this association is lost in *gsg*$^{-/-}$ and *mdx* muscle, indicating a γ-SG and dystrophin-dependent archvillin-P-ERK1/2 association. Complementary to this, we also observed increased nuclear P-ERK1/2 in stimulated *gsg*$^{-/-}$ and *mdx* muscles, presenting the possibility that loss of the archvillin-ERK1/2 association in dystrophic muscle may contribute to the elevated nuclear P-ERK1/2, and consequently, activation of downstream nuclear targets. From this result, it is possible that in wild type muscle, archvillin may be acting as a P-ERK sponge, and absence of its binding partners in dystrophic muscle disrupts their interaction. Because there was also an increase in cytosolic P-ERK1/2 in stimulated dystrophic muscle, we cannot distinguish between a model where there is a proportional increase in nuclear translocation of P-ERK1/2, and the loss of the P-ERK1/2 interaction is simply a parallel occurrence, or if indeed the loss of the archvillin-ERK1/2 association

is contributing to aberrant P-ERK1/2 signaling. Ablation of archvillin would help to evaluate the contribution of archvillin to P-ERK1/2 signaling and to the development of muscle disease, but attempts at deleting the *Svil* gene have been unsuccessful. Targeting the muscle specific insert may preserve the vital functions of supervillin in the rest of the body, and afford examination of archvillin specific properties.

The potential for archvillin to play a role in mechanical signal transduction is high given the known functions of this protein family. In addition to the ERK docking properties of smooth muscle archvillin, nonmuscle supervillin controls stress-dependent thrombus formation of platelets (Edelstein et al., 2012; Senetar et al., 2007), promotes cell survival by suppressing p53 protein expression (Fang and Luna, 2013), and increases the rapid recycling of integrins and other motile processes (Bhuwania et al., 2012; Crowley et al., 2009; Fang et al., 2010; Smith et al., 2013; Takizawa et al., 2007). The isoform switch from nonmuscle supervillin to archvillin occurs early in differentiation of C2C12 murine skeletal myoblasts (Senetar et al., 2007), and suggests a muscle-specific role for the protein, which is supported by its necessity to afford myotube formation of these cells (Lee et al., 2008; Lim et al., 2007; Oh et al., 2003). While these properties could be associated solely with actin cytoskeleton binding, the additional mechanically sensitive ERK1/2 interaction with archvillin may contribute to its actions in muscle.

We were intrigued to find that the nuclear fractions from both dystrophic muscle samples displayed increased lamin A/C. Because dystrophies exhibit heightened degeneration and regeneration, as well as increased immune infiltration, the nuclear content is elevated compared to healthy tissue (Evans et al., 2009). To control for this, we loaded equal protein from each fraction and used histone 3 as a nuclear marker pointed to

the specific elevation of lamin A/C. Lamin A/C gene (*LMNA*) mutations are one cause of Emery-Dreifuss muscular dystrophy, and a mouse model for the *LMNA* H222P mutation exhibits progressive dystrophic pathology and heightened ERK1/2 phosphorylation in skeletal and cardiac muscle (Arimura et al., 2005; Muchir et al., 2013). Whether or not the elevated lamin A/C in *mdx* and *gsg*$^{-/-}$ muscles is a compensatory measure to counter nuclear accumulation of P-ERK1/2 is not known. Further, the potential interaction between lamin A/C and archvillin identified in our yeast two-hybrid screen could implicate archvillin-ERK1/2 binding at both the sarcolemma and nucleus. Ultimately, these independent observations raise the potential for a common pathway – aberrant ERK signaling – that contributes to a range of dystrophies. This is substantiated by the upregulated P-ERK1/2 we observed in the LGMD and DMD human samples. Because there is now evidence that pharmacologic ERK inhibition improves muscle function in *LMNA* H222P EDMD mice (Muchir et al., 2013; Wu et al., 2014), application of this strategy to other dystrophies is warranted.

A yeast two-hybrid screen using the intracellular domain of γ-SG as bait has been performed previously (Blandin et al., 2013). Surprisingly, no preys emerged in common between our two screens, suggesting that there may be many γ-SG interactors. Because, to our knowledge, none of the prey hits from the previous screen have been pursued, the interactions with archvillin characterized here may represent the first of many new insights into processes involved in the dystrophic phenotype.

Based on our results, we provide a model in which archvillin sarcolemmal expression and localization are dependent on both the SG complex and dystrophin, where *gsg*$^{-/-}$ muscle has strongly upregulated archvillin levels concentrated at the sarcolemma

(Figure 2.7). In the absence of both sarcoglycan and dystrophin binding partners in *mdx* muscle, archvillin is no longer detected at the sarcolemma. The archvillin-P-ERK1/2 interaction is stimulus- and γ-SG-dependent, and we posit the possibility that the absence of the SG complex or the entire DGC may contribute to the aberrant ERK signaling observed in dystrophic muscle by an archvillin-mediated mechanism.

To our knowledge, this work in the first application of an *in situ* eccentric contraction protocol on tibialis anterior muscles of γ-SG-null mice. Consistent with previous *ex vivo* reports in EDL muscles, γ-SG deficiency does not cause contraction-induced injury, and is sufficient to compromise contractile force and cause muscle degeneration. Further experiments will be required to determine the contribution of archvillin to ERK signaling, and manipulation of ERK activity will provide insight into its impact on pathology. If archvillin proves to have a substantial impact on ERK signaling in muscle, it may be a viable target for muscle-specific manipulation of ERK activation. Given the role of ERK signaling on cell survival and proliferation, targeting archvillin may normalize the aberrant ERK activation and potential misregulation of its targets in dystrophic muscle. In conclusion, we have implicated archvillin as a new player in mechanical signal transduction in muscle, and that its localization and actions require the presence of dystrophin and the sarcoglycans, respectively.

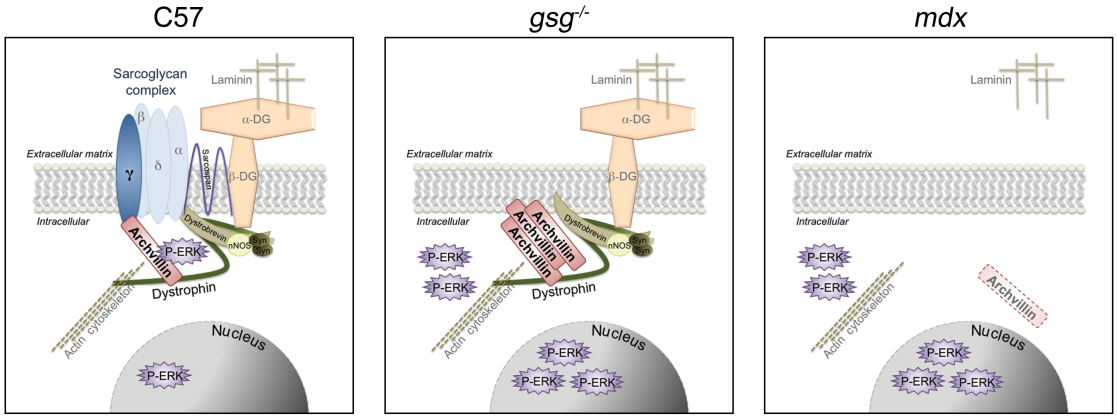


**Figure 2.7. Model of archvillin-γ-SG signaling.** Archvillin sarcolemmal localization is dependent on both the sarcoglycan complex and dystrophin. Muscle deficient in the sarcoglycan complex alone in *gsg*$^{-/-}$ mice has strongly upregulated archvillin levels concentrated at the sarcolemma. However, in the absence of both sarcoglycan and dystrophin binding partners in *mdx* muscle, archvillin is no longer detected at the sarcolemma. Archvillin exhibits stimulus- and γ-SG-dependent association with P-ERK1/2, where archvillin only associates with P-ERK in C57 muscle subjected to *in situ* eccentric contraction mechanical stimulation, but this association is absent in *gsg*$^{-/-}$ and *mdx* muscle with or without stimulation.

| Bait | Interactors | Accession Number | | Number of Clones | |
|---|---|---|---|---|---|
| | | Protein | Nucleotide | Total | Individual |
| γ-SG aa 1-35 | 78 kDa glucose-regulated protein precursor (GRP78) | NP_005338.1 | NM_005347.4 | 4 | 1 |
| | Coatomer subunit delta isoform 1 (COPD1) | NP_001646.2 | NM_001655.4 | 4 | 1 |
| | Archvillin (SVIL)† | NP_003165.2 | NM_003174.3 | 2 | 1 |
| | Coatomer subunit delta isoform 2 (COPD2) | NP_001135753.1 | NM_001142281.1 | 2 | 1 |
| | DNA damage-regulated autophagy modulator protein 1 (DRAM1) | NP_060840.2 | NM_018370.2 | 2 | 1 |
| | Na/K-transporting ATPase subunit beta-3 (ATP1B3) | NP_001670.1 | NM_001679.2 | 2 | 1 |
| | 60 kDa heat shock protein, mitochondrial (HSPD1) | NP_002147.2 | NM_002156.4 | 1 | 1 |
| | Adenosylhomocysteinase isoform 1 (AHCY) | NP_000678.1 | NM_000687.2 | 1 | 1 |
| | AP-2 complex subunit beta isoform b (AP2B1) | NP_001273.1 | NM_001282.2 | 1 | 1 |
| | Beta-sarcoglycan (SGCB) | NP_000223.1 | NM_000232.3 | 1 | 1 |
| | COP9 signalosome complex subunit 5 (COPS5) | NP_006828.2 | NM_006837.2 | 1 | 1 |
| | Cubilin precursor (CUBN) | NP_001072.2 | NM_001081.3 | 1 | 1 |
| | DnaJ homolog subfamily B member 4 (DNAJB4) | NP_008965.2 | NM_007034.3 | 1 | 1 |
| | Dynein heavy chain 14, axonemal isoform 1 (DNAH14) | NP_001364.1 | NM_001373.1 | 1 | 1 |
| | Glutamate--cysteine ligase regulatory subunit (GCLM) | NP_002052.1 | NM_002061.2 | 1 | 1 |
| | Sodium/potassium-transporting ATPase subunit beta-1 (ATP1B1) | NP_001668.1 | NM_001677.3 | 1 | 1 |
| | Neurexin-1-beta isoform alpha1 precursor (NRXN1) | NP_004792.1 | NM_004801.4 | 1 | 1 |
| | PDZ and LIM domain protein 2 isoform 2 (PDLIM2) | NP_067643.3 | NM_021630.5 | 1 | 1 |
| | Phospholipid scramblase 1 (PLSCR1) | NP_066928.1 | NM_021105.2 | 1 | 1 |
| | Probable G-protein coupled receptor 115 precursor (GPR115) | NP_722580.3 | NM_153838.3 | 1 | 1 |
| | Propionyl-CoA carboxylase alpha chain, mitochondrial isoform a precursor (PCCA) | NP_000273.2 | NM_000282.3 | 1 | 1 |
| | Propionyl-CoA carboxylase alpha chain, mitochondrial isoform b (PCCA) | NP_001121164.1 | NM_001127692.2 | 1 | 1 |
| | Protein transport protein Sec31A isoform 1 (SEC31A) | NP_055748.2 | NM_014933.3 | 1 | 1 |
| | Protein transport protein Sec31A isoform 2 (SEC31A) | NP_057295.2 | NM_016211.3 | 1 | 1 |
| | Protein transport protein Sec31A isoform 3 (SEC31A) | NP_001070676.1 | NM_001077208.2 | 1 | 1 |
| | Protein transport protein Sec31A isoform 5 (SEC31A) | NP_001177978.1 | NM_001191049.1 | 1 | 1 |
| | Sperm-associated antigen 17 (SPAG17) | NP_996879.1 | NM_206996.2 | 1 | 1 |
| | Splicing factor 3B subunit 1 isoform 1 (SF3B1) | NP_036565.2 | NM_012433.2 | 1 | 1 |
| | Splicing factor 3B subunit 3 (SF3B3) | NP_036558.3 | NM_012426.4 | 1 | 1 |
| | Thioredoxin-like protein 1 (TXNL1) | NP_004777.1 | NM_004786.2 | 1 | 1 |
| | Translation initiation factor IF-2, mitochondrial precursor (MTIF2) | NP_002444.2 | NM_002453.2 | 1 | 1 |
| | Uncharacterized protein C17orf46 (SPATA32) | NP_689556.2 | NM_152343.2 | 1 | 1 |
| | WD repeat-containing protein 5B (WDR5B) | NP_061942.2 | NM_019069.3 | 1 | 1 |
| | WD repeat-containing protein 61 (WDR61) | NP_079510.1 | NM_025234.1 | 1 | 1 |

**Supplemental Table 2.1. Complete list of candidate interactors from γ-SG yeast two-hybrid assay**

# CHAPTER 3: Absence of γ-sarcoglycan Alters the Response of p70S6 Kinase to Passive Stretch in Murine Skeletal Muscle

This chapter is adapted from and excerpt of:

Catherine Moorwood, Anastassios Philippou, Janelle M. Spinazzola, Benjamin Keyser, Edward J. Macarak, Elisabeth R. Barton. 2014. Absence of γ-sarcoglycan alters the response of p70S6 kinase to mechanical perturbation in murine skeletal muscle. *Skeletal Muscle* 4:13.

## 3.1 Summary

Deficiency in the sarcoglycan (SG) complex alone is sufficient to cause muscular dystrophy including severe myofiber degeneration, yet without causing susceptibility to contractile damage. This suggests that disease occurs not by structural deficits, but through aberrant signaling, namely, disruption of normal mechanotransduction signaling through the SG complex. In this chapter, we extended our previous studies on mechanosensitive, γ-sarcoglycan (γ-SG) dependent ERK1/2 phosphorylation to determine whether additional pathways are altered with the loss of γ-SG. We examined mechanotransduction in the presence and absence of γ-SG, where signaling protein phosphorylation was determined by immunoblotting of lysates from C2C12 myotubes and isolated muscles from C57Bl/6 (C57) and γ-SG-null (*gsg*$^{-/-}$) mice with and without cyclic passive stretch. C2C12 myotube stretch caused a robust increase in P-p70SK, but decreased P-FAK and P-ERK2. Neither Akt nor ERK1 were responsive to passive stretch. In isolated muscles, Akt, S6RP, and p70S6K activation increased with stretch in both C57 and *gsg*$^{-/-}$ isolated muscles. Dependence on mTOR was determined by stretching isolated muscles in the presence or absence of rapamycin, which blocked all of p70S6K activation in stretched C57 muscles, and reduced downstream S6RP phosphorylation. However, even though rapamycin treatment decreased p70S6K activation in stretched *gsg*$^{-/-}$ muscles, S6RP phosphorylation remained elevated. Our results suggest that p70S6K is an important component of γ-SG-dependent mechanotransduction in skeletal muscle, where loss of γ-SG uncouples the response of p70S6K to stretch and implies that γ-SG is important for inactivation of this pathway.

Overall, we assert that altered load-sensing mechanisms exist in muscular dystrophies where the sarcoglycans are absent.

## 3.2 Introduction

The dystrophin glycoprotein complex (DGC) is found at the sarcolemma of skeletal, cardiac, and smooth muscle cells, where it physically links the extracellular matrix (ECM) with the intracellular cytoskeleton, providing structural support (Lapidos et al., 2004). Mutations in DGC components cause different types of muscular dystrophy; for example, mutations in dystrophin cause Duchenne muscular dystrophy (DMD), while mutations in α-, β-, γ-, or δ-sarcoglycan (SG) cause limb girdle muscular dystrophy (LGMD) (Durbeej and Campbell, 2002; Engel and Franzini-Armstrong, 2004). When dystrophin is mutated in DMD or in the *mdx* mouse model of DMD, the entire DGC is substantially reduced at the sarcolemma. In contrast, when any one of the SGs is mutated, in LGMD or any of the SG knock-out mice, the other three SGs are either absent or reduced at the sarcolemma, but the rest of the DGC remains, including the link formed by dystrophin and dystroglycan between the cytoskeleton and ECM and (Blake et al., 2002; Durbeej and Campbell, 2002). Unlike the skeletal muscles of the *mdx* mouse, muscles of the γ-SG-null ($gsg^{-/-}$) mouse display no mechanical fragility, at least until 4 months of age, as shown by a minimal loss of force-generating capacity following a series of eccentric contractions (ECCs) (Hack et al., 1999; Petrof et al., 1993). In spite of this, the $gsg^{-/-}$ mouse exhibits a severe dystrophic phenotype on histological examination, with extensive myofiber degeneration and regeneration, fibrosis, and disruption of sarcolemmal integrity, similar to the *mdx* mouse (Hack et al., 1998). The lack of mechanical fragility suggests that aberrant signaling may contribute to the muscle degeneration seen in the $gsg^{-/-}$ mouse. Indeed, our previous studies demonstrated that localization of the SG complex to the sarcolemma and phosphorylation of the tyrosine 6

residue of γ-SG are essential for normal signaling by extracellular signal-regulated kinases 1 and 2 (ERK1/2), in response to ECCs (Barton, 2006; Barton, 2010). Based upon these data, we have asserted that the SG complex acts as a mechanosensor in skeletal muscle because of its position within the DGC, the modifications that occur to γ-SG with mechanical perturbation, and the necessity of the complex for normal signaling.

One pathway of interest involves p70S6K, which is canonically activated in response to mitogens via the phosphoinositide 3-kinase (PI3K) pathway (Fenton and Gout, 2011) and is known to respond to mechanical load (Hornberger et al., 2004). Activation of p70S6K involves a hierarchical series of phosphorylation events, beginning with phosphorylation of multiple sites in the C-terminal autoinhibitory domain, followed by mammalian target of rapamycin (mTOR)-dependent phosphorylation of sites in the linker region, which allows for full activation of the kinase via phosphorylation of threonine 229 (T229) in the catalytic domain by phosphoinositide-dependent kinase 1 (PDK1) (Dufner and Thomas, 1999). Although phosphorylation of T229 is required for p70S6K activation, phosphorylation of T389 in the linker region has been found to correlate most closely with *in vivo* activity (Pearson et al., 1995), and can be used as a measure of kinase activation. p70S6K has a multitude of downstream targets, with roles in protein synthesis, growth, proliferation, survival, and more (Fenton and Gout, 2011), including S6 ribosomal protein (S6RP), which closely correlates with protein translation rates (Nielsen et al., 1982).

In this chapter, we examined ERK1/2, Akt, focal adhesion kinase (FAK), and p70S6K responses to passive stretch in C57 and *gsg*$^{-/-}$ skeletal muscle to further elucidate the importance of the SG complex for mechanotransduction. We used a passive

stretching protocol to isolate the effects of externally applied tension in the absence of active contraction, in order to examine the downstream signaling in more detail. The p70S6K response to stretch in isolated extensor digitorum longus (EDL) muscles was differentially regulated in the absence of γ-SG. Specifically, experiments in isolated muscles suggest that γ-SG is required for inactivation of p70S6K. The findings increase our understanding of the contribution of aberrant load-sensing to the pathology of muscular dystrophies where the SG complex is deficient.

## 3.3 Results

### *p70S6K, but not ERK1/2 or Akt, responds to passive stretch in vitro*

Studies using whole muscles from animal models of the dystrophies are made more complex by the presence of multiple cell types, as well as pathological processes such as fibrosis. Therefore, we initially investigated mechanotransduction signaling in passively stretched C2C12 myotubes. We found that passive stretching *in vitro* did not alter phosphorylation of ERK1 or Akt, and that ERK2 and FAK phosphorylation decreased following stretch (Figure 3.1A,C-F, H-I). However, we found an increase in p70S6K phosphorylation at T389, which reflects activity, in response to passive stretching of C2C12 myotubes (Figure 3.1B,G). Therefore, this *in vitro* model reflected some, but not all, stretch responses observed in muscle *in vivo* (Hornberger et al., 2005; Martineau and Gardiner, 2001), and highlighted p70S6K as a pathway of interest. The lack of Akt phosphorylation suggests that p70S6K phosphorylation occurred through an Akt-independent pathway, while the lack of FAK phosphorylation supports an integrin-independent mechanism.

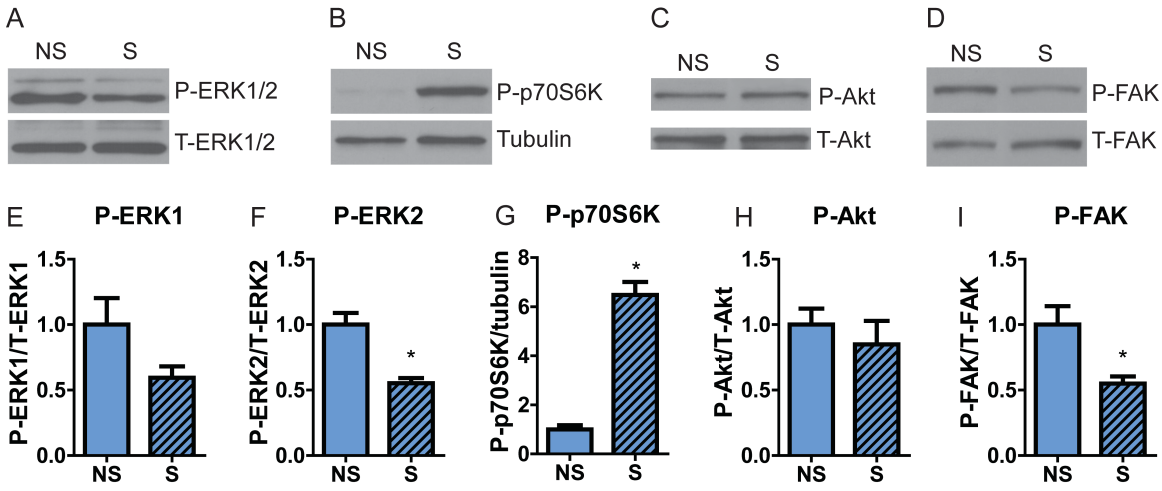


**Figure 3.1. p70S6K responds to stretch in C2C12 cells.**

C2C12 myotubes were cultured on silicone membranes and subjected to passive stretching for 30 min. (A-D) Representative immunoblots for P-ERK1/2, T-ERK1/2, P-p70S6K (T389 site), tubulin, P-Akt, T-Akt, P-FAK, and T-FAK in non-stretched (NS) and stretched (S) C2C12 cells. (E-I) Quantification of phosphorylation levels. P-ERK1 and 2 were normalized to T-ERK1 and 2, respectively, P-p70S6K was normalized to tubulin, P-Akt was normalized to T-Akt, and P-FAK was normalized to T-FAK. n = 5-6 wells of C2C12 cells per group. Bars represent mean ± standard error. * Significantly different from non-stretched myotubes by unpaired Student's t-test. NS, non-stretched; S, stretched.

***Passive stretch is sufficient to induce tyrosine phosphorylation of γ-SG***

Previous studies have shown that the SG complex is sensitive to mechanical stimulation. Each of the sarcoglycans undergoes adhesion-dependent phosphorylation in cell culture studies (Yoshida et al., 1998). In addition, whole muscles also exhibit post-translational modification of intracellular tyrosine(s) following eccentric contractions (Barton, 2006). However, it was not clear if the strain imposed by passive stretch alone could generate the same response in muscles. A passive stretching protocol comprised of a 15% strain, 20 times per min, for 30 min in high-glucose DMEM was sufficient to cause increased γ-SG phosphorylation in the EDL, as is the case for eccentric contraction of the EDL (Figure 3.2A (Barton, 2006)).

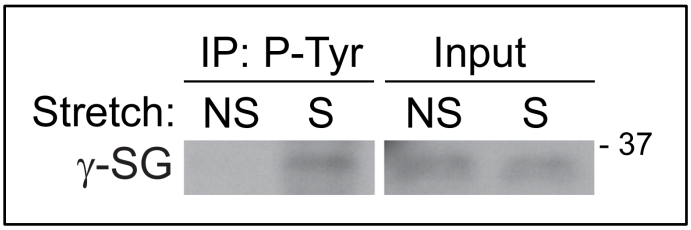


**Figure 3.2. γ-SG is tyrosine phosphorylated in response to passive stretch in isolated C57 EDL muscle.**

EDL muscles from C57 mice were maintained in oxygenated high glucose DMEM and subjected to passive stretching. (A) Immunoblot of γ-SG following immunoprecipitation with anti-P-Tyr or lysate only, showing γ-SG phosphorylation in response to 30 min of stretch. Protein loading: 200μg IP lanes, 50μg Input lanes. NS, non-stretched; S, stretched.

***Stretch response of p70S6K T389, but not S6RP, is rapamycin-sensitive in gsg$^{-/-}$ muscles***

Because mTOR is a key mediator of p70S6K activation, we examined the effect of the mTOR inhibitor rapamycin on stretch responses in isolated C57 and *gsg*$^{-/-}$ muscles. EDL muscles were subjected to cyclic stretch as described above. Unlike in C2C12 cells, P-Akt showed a trend to increase on stretching, which was statistically significant in *gsg*$^{-/-}$ muscles. As anticipated, P-Akt was unaffected by rapamycin (Figure 3.3A,B). Rapamycin treatment completely blocked the increase in p70S6K T389 phosphorylation after passive stretch of C57 muscles, consistent with previous studies (Dufner and Thomas, 1999; Hornberger et al., 2004). In *gsg*$^{-/-}$ muscles, rapamycin abrogated most p70S6K T389 phosphorylation, but residual phosphorylation remained in stretched muscles (Figure 3.3A,C). T421/S424 showed a trend to increase in response to stretch in both C57 and *gsg*$^{-/-}$ muscles. Surprisingly, rapamycin blunted the p70S6K T421/S424 stretch response in C57 muscles, which is inconsistent with previous studies (Hornberger et al., 2004). However, the T421/S424 response to stretch persisted in *gsg*$^{-/-}$ muscles in the presence of rapamycin (Figure 3.3A,D). S6RP phosphorylation increased in response to stretch in both C57 and *gsg*$^{-/-}$ muscles. Interestingly, while rapamycin blocked stretch-induced phosphorylation of S6RP in C57 muscles, phosphorylation in response to stretch was preserved in *gsg*$^{-/-}$ muscles (Figure 3.3A,E). Taken together, these results suggest either that the level of active p70S6K remaining in *gsg*$^{-/-}$ muscles is sufficient to phosphorylate S6RP regardless of rapamycin or that an alternate pathway bypasses p70S6K to phosphorylate S6RP in muscles lacking γ-SG.

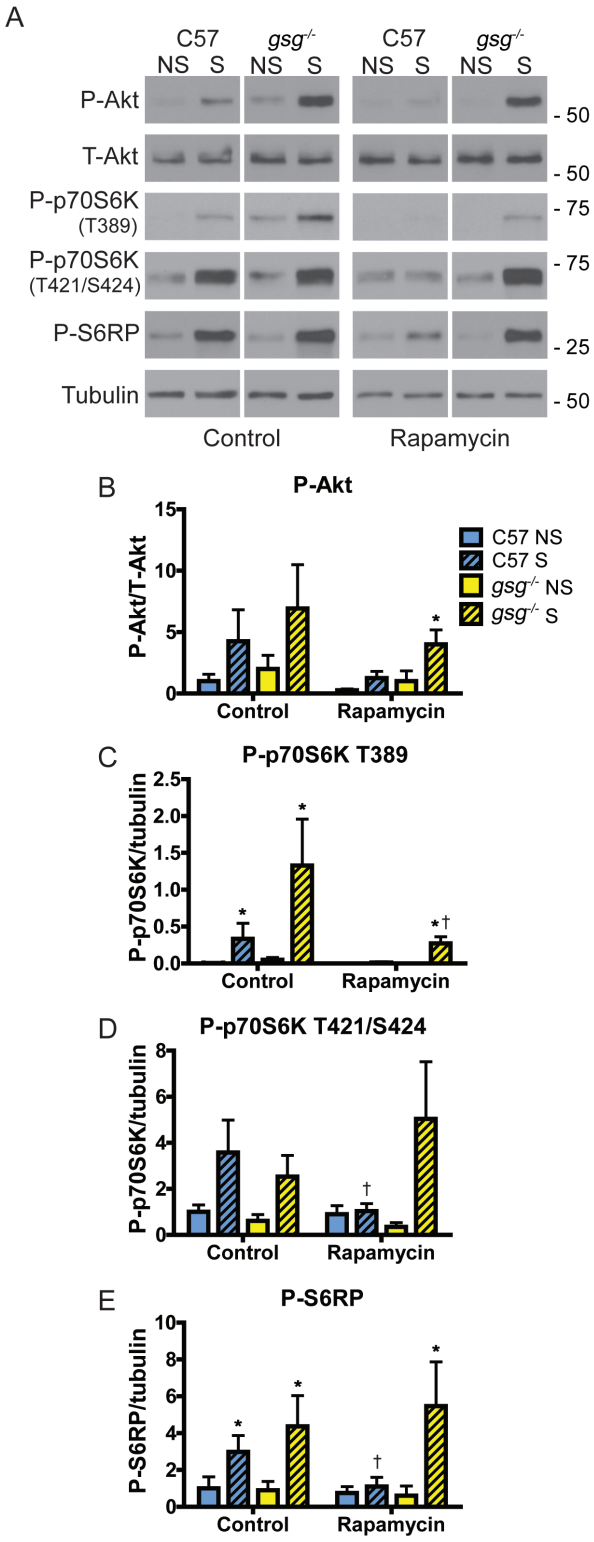


**Figure 3.3 Stretch response of p70S6K T389, but not S6RP, is rapamycin-sensitive in *gsg*$^{-/-}$ muscles**.

**Figure 3.3. Stretch response of p70S6K T389, but not S6RP, is rapamycin-sensitive in *gsg*$^{-/-}$ muscles.**

EDL muscles from C57 and *gsg*$^{-/-}$ mice were maintained in oxygenated high glucose DMEM supplemented with or without rapamycin and subjected to passive stretching. (A) Immunoblots of P-Akt, T-Akt, P-p70S6K (T389 and T421/S424 sites), P-S6RP, and tubulin. Left panels DMEM alone; right panels DMEM + rapamycin. (B-E) Quantification of P-Akt (B), P-p70S6K T389 (C), P-p70S6K T421/S424 (D), and P-S6RP (E). Legend in B applies to all graphs. P-Akt was normalized to T-Akt; all other proteins were normalized to tubulin. Bars represent mean ± standard error. $^{*}P < 0.05$ vs. respective NS; $^{\dagger}P < 0.05$ vs. respective control without rapamycin by two-way ANOVA with Tukey's multiple comparisons test; n = 2-3 muscles per genotype and condition. NS, non-stretched; S, stretched.

### 3.4. Discussion

Skeletal muscle has the ability to adapt to changes in workload. Almost all muscle properties can be modulated, such as muscle fiber size, contractile properties and metabolism. Changes in patterns of gene expression as well as shifts in the balance between protein synthesis and degradation are required to mediate the response. Identification of major pathways that directly regulate gene expression and protein synthesis/degradation demonstrate that multiple inputs (mechanical, chemical, and metabolic) can converge on final common pathways for muscle growth and adaptation (Glass, 2003). However, sorting out the contribution of the wide variety of inputs on muscle adaptation has been more difficult. In our previous work, we used an eccentric contraction protocol to investigate the dependence of ERK1/2 mechano-sensing on phosphorylation of γ-SG. However, this protocol alters multiple factors, including externally applied tension and internally generated tension, which potentially have effects on mechanosensitive signaling pathways. In this study, we used a passive stretching protocol to isolate the effects of externally applied tension in the absence of active contraction, in order to examine the downstream signaling in more detail.

Passive stretching protocols can be performed in both cell cultures and whole muscle preparations, and the reductionist approach of utilizing cultures can eliminate some of the physiological complexities associated with intact or isolated muscles. As such, our initial experiments using C2C12 cells served in identifying p70S6K as being activated in response to stretch, in contrast to the lack of response by ERK1/2, Akt, or FAK. Using isolated muscles from C57 and *gsg*$^{-/-}$ mice to investigate γ-SG-dependent mechanotransduction pathways, we observed a modest increase of P-p70S6K in *gsg*$^{-/-}$

muscles at rest, and a prolonged activation of p70S6K following stretch. These results support a role for γ-SG in mechanical signal transduction, where the loss of this protein leads to inappropriate activation and/or deactivation. Given the dependence of our findings on the experimental platform utilized, verification of the results in an *in situ* muscle model should be included in future directions.

A potential explanation for our *in vivo* results is that γ-SG is required for dephosphorylation and deactivation of p70S6K. There is considerable evidence that p70S6K is directly dephosphorylated by protein phosphatase 2A (PP2A), independently of mTOR (Cho et al., 2006; Hahn et al., 2010; Peterson et al., 1999; Petritsch et al., 2000). The phosphatase PHLPP has also been shown to target p70S6K (Liu et al., 2011), and γ-SG may mediate the activation of these phosphatases in response to sustained mechanical stimulation. Alternatively, γ-SG may regulate pathways that deactivate p70S6K indirectly. For example, the phosphatase SHP-2 can cause mTOR-dependent dephosphorylation of p70S6K (Ketroussi et al., 2011; Zito et al., 2007). Further studies will be required to define the inactivation pathway disrupted by γ-SG loss.

In this study, we sought to identify mechanosensitive signaling that is attributable to the SG complex rather than other processes occurring during mechanical perturbation. By using passive stretch models, we eliminated the contribution of active contraction or SR calcium fluxes. However, this does not eliminate the effects of extracellular $Ca^{2+}$ fluxes through mechanically sensitive channels, and it has been shown previously that passive stretch causes greater $Ca^{2+}$ influx into myotubes lacking members of the SG complex (Sampaolesi et al., 2001). This raises the possibility that the mechanical signal transduction pathways we evaluated previously may be modulated not only by the SG

complex, but also by additional channels in the sarcolemma. To address this, additional work not presented in this dissertation was performed in this study examining the contribution of calcium to the elevated p70S6K and ERK1/2 activity found in muscles. By maintaining isolated muscles in calcium-free of tetracaine-supplemented Ringer's solution, it was found that the elevation of P-ERK1/2 in the absence of γ-SG was dependent on both internal and external sources of calcium. In contrast, the difference in basal P-p70S6K between C57 and *gsg*$^{-/-}$ muscles was not calcium dependent (data not shown). This suggests that while ERK1/2 activation may lie downstream of the calcium misregulation that occurs in SG-deficient muscle, changes in p70S6K activation may be a more direct consequence of the absence of the SG complex. This is of considerable interest given that γ-SG has been shown to be important for mechanotransduction, but the downstream signaling pathways are uncharacterized (Barton, 2006; Barton, 2010).

The pattern of differential p70S6K phosphorylation in response to stretch in *gsg*$^{-/-}$ muscles was similar both for phosphorylation of T389, which correlates with kinase activity, and for T421/S424, two of the four phosphorylation sites in the autoinhibitory domain. Phosphorylation of T389 is mTOR-dependent, while phosphorylation of the autoinhibitory domain is carried out by proline-directed kinases. Furthermore, it is thought that phosphorylation of the autoinhibitory domain is necessary for phosphorylation of T389 (Dufner and Thomas, 1999). Therefore, the correlation between phosphorylation of T421/S424 and T389 in our isolated muscle model suggests that phosphorylation of the autoinhibitory domain was the rate-limiting step for p70S6K activation, which is of interest given that the autoinhibitory domain may be targeted by ERK1/ 2 (Mukhopadhyay et al., 1992). Therefore, a future hypothesis to test is that

differential p70S6K activation is a downstream consequence of differential ERK1/2 activation in *gsg*$^{-/-}$ muscle.

Our experiments with rapamycin showed that, for both C57 and *gsg*$^{-/-}$ muscles, phosphorylation of p70S6K at T389 is mTOR-dependent, consistent with previous studies (Dufner and Thomas, 1999; Hornberger et al., 2004). The T421/S424 autoinhibitory domain sites were phosphorylated in response to stretch in both C57 and *gsg*$^{-/-}$ muscles. However, in the presence of rapamycin, this response was not present. This is surprising given that the T421/S424 sites are not thought to be targeted by mTOR. Previous studies have shown these sites to be rapamycin-insensitive (Hornberger et al., 2004), but recent evidence suggests a modest mTOR dependence (Axelrod et al., 2014). In *gsg*$^{-/-}$ muscles, rapamycin had no effect on T421/S424 phosphorylation. Further experiments are needed to fully understand the role of mTOR on phosphorylation of the p70S6K autoinhibitory domain in C57 and *gsg*$^{-/-}$ skeletal muscle. Interestingly, the stretch induced phosphorylation of S6RP was rapamycin sensitive in C57 muscles, but not in *gsg*$^{-/-}$ muscles. This suggests that an alternative pathway can bring about S6RP phosphorylation in *gsg*$^{-/-}$ muscles when p70S6K is not activated. One possibility is that S6RP is phosphorylated by p90 ribosomal S6 kinase, which is activated by ERK. This is consistent with the increase in basal ERK1/2 in *gsg*$^{-/-}$ muscle, and the over-response of ERK2 with mechanical stimulation by eccentric contraction (Barton, 2006).

We did not observe a strong Akt response to passive stretch or any difference between C57 and *gsg*$^{-/-}$, implying that mTOR and/or p70S6K were being activated through Akt-independent pathways. This is consistent with previous studies showing that Akt does not respond to mechanical stimulation in skeletal muscle, and that p70S6K

phosphorylation in response to stretch is independent of PI3K (Hornberger and Chien, 2006). We also did not see increased phosphorylation of FAK in response to passive stretch in C2C12 cells. Although integrins can participate in mechanotransduction, it appears that our cyclic passive stretch protocols did not cause integrin activation (data not shown). Further studies will be needed to elucidate the details of crosstalk between SG-dependent and integrin-dependent signaling pathways.

Based on our findings, we position γ-SG as a mechanosensor important for transient ERK1/2 activation during active contractions, as well as for modulation of p70S6K activation during passive stretch. Because passive stretch does not appear to increase P-FAK or P-Akt, γ-SG is likely to regulate p70S6K through other pathways. These may include regulation of ERK1/2, which can promote p70S6K activation indirectly via mTOR or directly by phosphorylation of the autoinhibitory domain, and/or phosphatases such as PP2A that dephosphorylate p70S6K. Loss of γSG uncouples the response to stretch, which may contribute to muscle pathology.

The stability of the SG complex is directly affected in several LGMDs and in DMD, and growing evidence suggests that inappropriate load sensing contributes to the pathology of these diseases. The first step in therapeutic development is identifying and understanding the target, but little is currently known about the role of the SG complex in load sensing. Therefore, understanding the functions that are disrupted and the pathways that are involved in mechanotransduction involving the SG complex will help in the design of therapies for LGMDs and DMD. While restoration of a completely normal SG complex either through gene correction or protein replacement would also normalize mechanical signal transduction, this may not be possible for all mutations responsible for

DMD and LGMD. It is known that localization of the SG complex is not the sole criterion for appropriate signaling (Barton, 2010), and downstream pathways may emerge as more feasible therapeutic targets.

We do not know whether the enhanced basal and stretch-responsive activation of p70S6K in *gsg*$^{-/-}$ muscle contributes to pathology or compensates for it. Likewise, it is not clear whether inhibition of p70S6K would have a beneficial or a detrimental effect in dystrophic muscle. Muscle-specific gene targeting of mTORC1 components induces myopathy (Bentzinger et al., 2008; Risson et al., 2009), and overexpression of integrin α7 can improve the dystrophic phenotype through increased survival signaling via p70S6K (Boppart et al., 2011), suggesting that p70S6K inhibition would not be advantageous. However, treatment of *mdx* mice with the mTOR inhibitor rapamycin improves the dystrophic phenotype (Eghtesad et al., 2012). It is also interesting to note that p70S6K is inhibited by glucocorticoids, which are used in the treatment of DMD and LGMD (Shah et al., 2000). Based on these observations, it is clear that there is a narrow window of effective p70S6K activation and that any intervention to alter its activity would require considerable fine-tuning.

We have identified p70S6K as part of a novel SG-dependent mechanosensitive signaling pathway in skeletal muscle. Our results suggest that γ-SG is required for the inactivation of p70S6K following its activation in response to mechanical stimulation. In addition to increasing our understanding of the normal function of the SG complex, these results begin to provide mechanistic insight into how mechanical signaling is disrupted and altered in the absence of γ-SG. As we learn more about the mechanisms by which signaling defects may contribute to dystrophic pathology, there is potential to identify

more refined targets that could be beneficial to patients either in isolation or in combination with other therapeutic approaches.

# CHAPTER 4: PILOT STUDY

## EFFECTS OF SHORT-TERM TREATMENT WITH ERK1/2 INHIBITOR SCH772984 IN MOUSE MUSCLE

This chapter describes pilot experiments to determine the utility of the ERK1/2 inhibitor SCH772984 in mouse muscle subjected to mechanical stimulation

## 4.1 Summary

Growing evidence suggests that disrupted signaling pathways associated with the loss of the dystrophin-glycoprotein complex contribute to muscle disease. Heightened P-ERK1/2 activity in skeletal muscle is a feature of several muscular dystrophies. In this chapter, we assess the utility of a newly developed ERK1/2 specific inhibitor SCH772984 in C57 and *gsg*$^{-/-}$ mice for future use in determining the effect of reducing ERK1/2 activity to prevent or ameliorate dystrophic pathology. Five days of treatment with 12.5mg/kg SCH772984 in 6-7 week old mice decreased P-ERK1/2 in mouse heart, diaphragm, and liver, but not in tibialis anterior muscle with or without *in situ* eccentric contraction. Treatment with SCH772984 did not affect force generation or contraction-induced injury in either strain. C57 and *gsg*$^{-/-}$ mice treated with SCH772984 lost significant of body weight compared to their respective untreated controls, which gained weight. These results position SCH772984 as a potentially effective ERK1/2 inhibitor in select tissues, and address potential pitfalls for future investigation.

## 4.2 Introduction

Mitogen-activated protein kinase (MAPK) cascades are key signaling pathways involved in the regulation of normal cell proliferation, survival and differentiation (Roux and Blenis, 2004a). The extracellular signal-regulated kinase (ERK) MAPK pathway is of particular interest given that hyperactivation of this pathway is a feature of muscle diseases such as Duchenne Muscular Dystrophy (DMD) and Limb-Girdle Muscular Dystrophies (LGMD) (Barton, 2006). These diseases are caused by mutation and loss of members of the dystrophin glycoprotein complex (DGC) such as γ-sarcoglycan (γ-SG), which has been shown to be a mediator of ERK1/2 signaling (Barton, 2006; Barton, 2010). Growing evidence suggests that in addition to mechanical perturbations, disrupted signaling pathways associated with the loss of DGC components contribute to disease pathology (Barton, 2010; Hack et al., 1999). However, ERK1/2 misregulation has not yet been directly linked to manifestation of dystrophic pathology in DMD and LGMDs.

ERK1/2 are downstream components of an evolutionarily conserved signaling module that is activated by the Raf serine/threonine kinases. Raf activates the MAPK/ERK kinase (MEK)1/2 dual-specificity protein kinases, which then activate ERK1/2 (McCubrey et al., 2007). In skeletal muscle, ERK1/2 activation is positively correlated to tension (Martineau and Gardiner, 2001). Chronic activation of this signaling pathway has been implicated in the development and perpetuation of a number of different pathologies, such as diabetes, cachexia, and cancer (McCubrey et al., 2012). However, ERK1/2 is also stimulated by exercise, promoting improvements in fuel homeostasis, and can prevent skeletal muscle atrophy (Salto et al., 2014; Shi et al., 2009).

Thus, appropriate tuning of this signaling pathway seems to be critical for maintaining healthy muscle.

Most research targeting ERK1/2 inhibition as a therapeutic agent has been in cancer (Figure 4.1). Several Ras inhibitors have reached clinical testing, but have been unsuccessful due to off-target effects, reducing interest in Ras as a therapeutic target (Rusconi et al., 2012). However, MEK is considered an attractive drug target since ERK1/2 are the only known targets (Kolch, 2000; Wei et al., 2011). Although the first two MEK inhibitors, PD98059 and U0126, were highly specific (Davies et al., 2000), their pharmacological characteristics rendered them unsuitable for clinical development (Dudley et al., 1995). Use of the first clinically tested MEK inhibitor, Cl-1040, was discontinued due to lack of reduced ERK phophorylation in a phase II study (Rinehart et al., 2004), though there have been promising phase I clinical trial results with a second generation MEK inhibitor PD-0325901 (Haura et al., 2010).

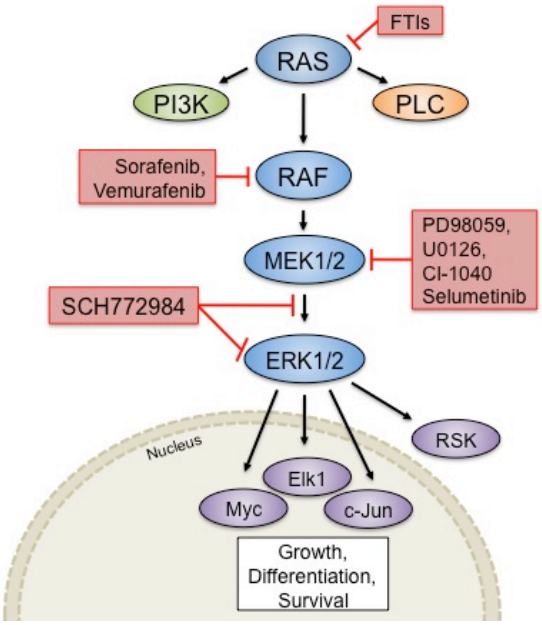


**Figure 4.1. Inhibitors of the MAPK/ERK signaling pathway**

Recently, there have been promising results in the use of MEK inhibitors to treat muscular dystrophy. Autosomal Emery-Dreifuss muscular dystrophy is caused by mutations in the lamin A/C gene (*LMNA*) encoding A-type nuclear lamins, resulting in muscle weakness, cardiomyopathy and heightened ERK1/2 activity. Treatment of mice with the MEK inhibitor selumetinib ameliorated skeletal muscle histopathology, reduced centrally nucleated fibers, and improved muscle function as assessed by *in vivo* grip strength testing (Muchir et al., 2013). Given these results, the potential use of an ERK1/2 specific inhibitor to treat SG-deficient muscular dystrophies, which also present with heightened ERK1/2 activity, is an attractive prospect.

The small molecule SCH772984 is a selective inhibitor of ERK1/2 that has been shown to effectively inhibit MAPK signaling and cell proliferation in MEK-inhibitor resistant tumor cells. *In vivo*, tumors in nude mice showed diminished ERK1/2 activity and suspended growth following 14 days of twice-daily i.p. injection with 12.5-50mg/kg SCH772984 (Morris et al., 2013). Therefore, a similar strategy may reduce the ERK1/2 activity in γ-SG-deficient dystrophic muscle. In this chapter, we assess the utility of SCH772984 in γ-SG-null ($gsg^{-/-}$) mice for future investigation as a potential treatment to prevent or ameliorate dystrophic pathology associated with sarcoglycan deficiency. The goal was to determine if SCH772984 treatment was effective in lowering P-ERK1/2 without harmful side effects.

## 4.3 Results

### *SCH772984 inhibits ERK1/2 in C57 and gsg$^{-/-}$ heart and liver*

Mechanical stimulation evokes ERK1/2 activation in skeletal muscle (Martineau and Gardiner, 2001), and dystrophic muscle displays aberrant ERK1/2 signaling both at rest and with stimulation (Barton, 2006; Barton, 2010). We examined the phosphorylation of ERK1/2 in tibialis anterior (TA), heart, and liver lysates from C57 and γ-SG-null (*gsg*$^{-/-}$) mice with and without SCH772984 ERK1/2 inhibitor treatment. P-ERK1/2 was elevated in *gsg*$^{-/-}$ TA muscle at rest compared to muscles from C57 mice, but SCH772984 did not reduce ERK1/2 at rest or following eccentric contraction (ECC) stimulation (Figure 4.1A,B,F). Even though TA muscles did not appear to respond to SCH772984, the treatment effectively inhibited ERK1/2 phosphorylation in heart, diaphragm, and liver lysates from both C57 and *gsg*$^{-/-}$ mice (Figure 4.2A,C-E,G-I). These results support that the 12.5mg/kg dose of SCH772984 is sufficient to modulate P-ERK1/2 in multiple tissues, but suggest that skeletal muscles may be unresponsive to the actions of the inhibitor, or require a different dosing regimen.

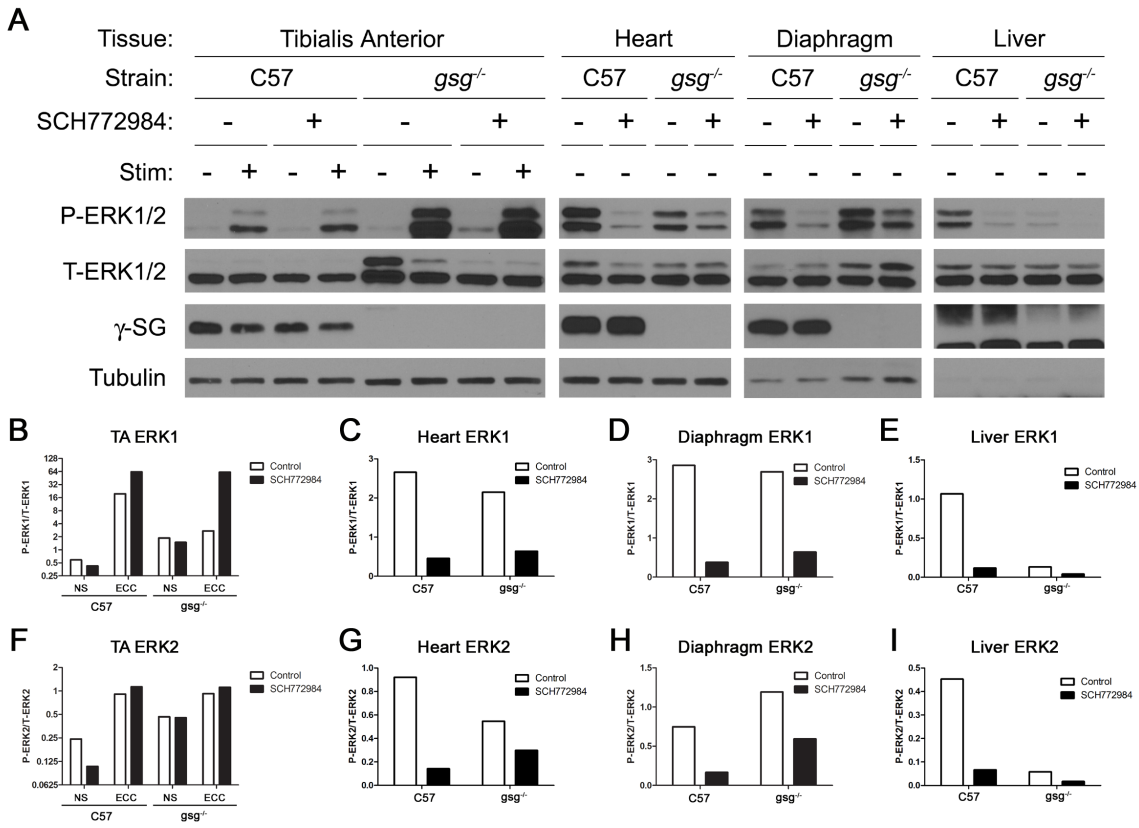


**Figure 4.2. SCH772984 inhibits ERK1/2 in murine heart, diaphragm, and liver, but not in TA muscle with or without ECC.**

6-7 week old C57 and *gsg*$^{-/-}$ mice were treated for 5 consecutive days with 12.5mg/kg SCH772984 delivered by i.p. injection. (A) Immunoblotting of tissue lysates from mice with (+) and without (-) SCH772984 treatment, and with (+) and without (-) *in situ* eccentric contraction stimulation (Stim). (B,F) SCH772984 treatment did not inhibit ERK1/2 activation in tibialis anterior (TA) muscle +/- stimulation. (C-E,G-I) SCH772984 treatment decreased ERK1/2 activation in heart, diaphragm, and liver lysates. TA bar graphs (B,F) are represented on a $\log_2$ scale. n = 1-2.

***SCH772984 does not compromise TA force-generating capacity***

To determine the effect of SCH772984 on muscle function, we subjected TA muscles of mice with and without treatment to a series of eccentric contractions *in situ*. Measurements of maximal isometric tetanic force showed that *gsg*$^{-/-}$ muscles generated less force compared to C57 muscle as observed previously (Spinazzola et al., 2015), and that SCH772984 did not affect force in either strain. Following ECC stimulation, the loss of force production in control *gsg*$^{-/-}$ muscles was comparable to that of control and treated C57 muscle (Figure 4.3). Interestingly, SCH772984 treatment seemed to increase percent force drop in *gsg*$^{-/-}$ mice, though this was not statistically significant. Thus, the inhibitor does not appear to negatively affect muscle force production.

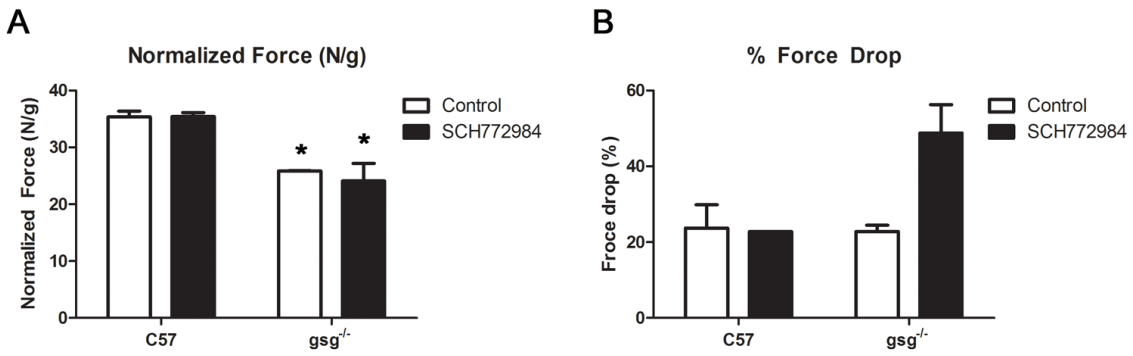


**Figure 4.3. Treatment with SCH772984 does not affect TA response to mechanical stimulation.** (A) Dystrophic *gsg*$^{-/-}$ TA muscles generate less force than C57 muscles when subjected to *in situ* isometric mechanical stimulation. SCH772984 treatment does not affect force generation in either strain. (B) *gsg*$^{-/-}$ mice with and without SCH772984 treatment do not show compromised force generating capacity following *in situ* eccentric contraction mechanical injury. $^{*}$, $P < 0.05$ vs. C57 by two-way ANOVA with Bonferroni post-hoc test, n = 3. Bars represent means ± SEM.

***Mice treated with SCH772984 lose body weight***

To determine the effect of SCH772984 treatment on overall wellness, we tracked body weight before and throughout treatment. Both C57 and *gsg*$^{-/-}$ mice treated with SCH772984 showed significant difference in weight compared to their respective untreated controls (Figure 4.4). Treated mice lost an average of 5.5% of their initial body weight compared to untreated controls, which gained an average of 2.6% of initial body weight. This suggests that this drug may compromise animal health and not be usable for long-term treatment.

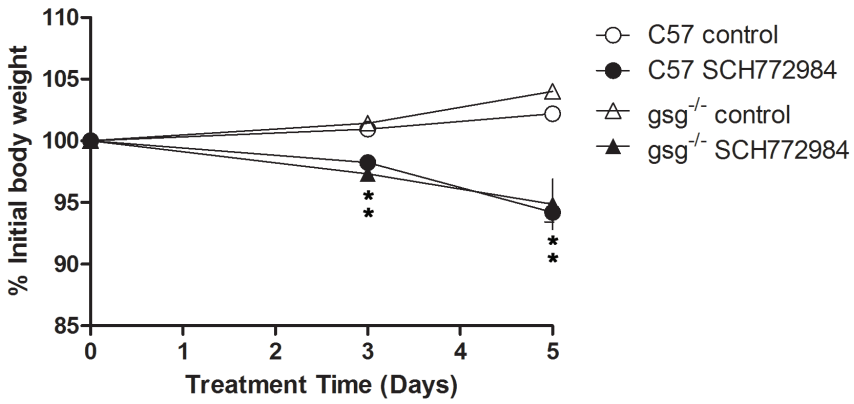


**Figure 4.4. Short-term treatment with SCH772984 causes mice to lose body weight.** Both C57 and *gsg*$^{-/-}$ mice treated with 5 days of 12.5mg/kg SCH772984 (black) lost weight compared to untreated controls (white), which gained weight. $^{*}$, $P < 0.05$ vs. strain-matched control by two-way ANOVA with Bonferroni post-hoc test, $n = 3$. Symbols represent means ± SEM.

## 4.4 Discussion

Growing evidence suggests that disrupted signaling pathways associated with the loss of DGC components contribute to muscle disease. In our previous work, γ-SG was shown to be a mediator of ERK1/2 signaling, which is heightened in several forms of muscular dystrophy (Barton, 2006; Barton, 2010). In this chapter, we assessed the utility of a novel ERK1/2 specific inhibitor SCH772984 in C57 and *gsg*$^{-/-}$ mice for future investigation as a strategy to prevent or ameliorate dystrophic pathology. Our results showed that SCH772984 can inhibit ERK1/2 in a subset of tissues at the dose tested, but also revealed potential pitfalls to consider with extended treatment.

The reason for lack of effect of SCH772984 in inhibiting ERK1/2 in TA muscle as opposed to in the heart, diaphragm, and liver is unclear. In accord with this, it was not unexpected that SCH772984 did not alter maximal tetanic force generated in *gsg*$^{-/-}$ following stimulation. Given that the circulation to limb muscles is at a minimum in resting mice, it is possible that the biodistribution of the inhibitor was low to these muscle groups. Altering the treatment regimen may help to improve the exposure to inhibitor, or incorporation of increased cage activity to the study would increase circulation to the limbs and provide better biodistribution. However, given its effect in the heart, it would be of interest to see if SCH772984 treatment yielded any cardiac improvements given that *gsg*$^{-/-}$, *dsg*$^{-/-}$ and *mdx* mice develop cardiomyopathy (Coral-Vazquez et al., 1999; Hack et al., 2000; Quinlan et al., 2004). Long-term treatment with the MEK1/2 inhibitor PD98059 has been found to prevent cardiomyopathy, including left ventricular end-systolic dilation, increase ejection fraction, and decrease myocardial fibrosis in the H222P mouse model of autosomal Emery-Dreifuss muscular dystrophy (Wu et al., 2014).

In addition, long-term SCH772984 treatment on the diaphragm would also be of interest, as it is the most affected muscle in *mdx* mice and its failure is often the cause of death in DMD patients.

The significant loss of body weight in treated animals with just five days of once-daily 12.5mg/kg SCH772984 raises concern about potential long-term and high-dose testing. Treated mice lost an average of 5.5% of their initial body weight compared to untreated controls, which gained an average of 2.6% of initial body weight (Figure 5.3). This was unexpected given that no weight loss or lethality was reported in nude mice given twice-daily doses up to 50mg/kg for two weeks (Morris et al., 2013). Future investigation with SCH772984 should include body weight monitoring to assess secondary effects exasperated with long-term or high-dose treatment.

The ERK MAPK pathway is critical in mediating cell proliferation, differentiation, and survival. ERK1/2 has several cytoplasmic and nuclear targets including P90RSK and transcription factors Elk-1 and myc that are phosphorylated in response to mechanical perturbation (Yu et al., 2001). If SCH772984 did prove to be a beneficial in ameliorating or preventing pathology, the next steps would include investigation of these downstream ERK1/2 targets to determine if they are differentially regulated in normal versus dystrophic muscle. Given findings of aberrant p70S6K activation in γ-SG-deficient muscle subjected to passive stretch (Moorwood et al., 2014), it is likely that multiple pathways, in addition to ERK1/2, are disregulated.

With growing evidence of the importance of signaling cascades mediated by the DGC in muscle, and aberrations in the ERK cascade identified in several forms of muscular dystrophy, targeting this pathway may be a viable component of a multifaceted

therapeutic strategy. Despite variable success with small molecules inhibitors such as SCH772984 in the clinical setting, they have been invaluable academic research tools for dissecting the ERK pathway. Even if SCH772984 itself cannot be utilized as a clinical treatment, it may reveal insight into the contribution of disrupted ERK signaling in the development of dystrophic pathology.

# CHAPTER 5: Conclusions and Future Directions

## Conclusions

The DGC is a key player in muscle adaptation to load. Mutations in genes encoding the DGC cause several forms of muscular dystrophy, emphasizing its importance in maintaining healthy, functional muscle. However, the mechanisms by which each of these components contributes to the development of disease pathology is unclear. With the discovery of dystrophin as the protein associated with Duchenne Muscular Dystrophy nearly thirty years ago, early observations suggested that dystrophy was a consequence of a mechanical deficit. However, increasing evidence has revealed that signaling pathways disrupted with mutation of components of the DGC contribute to the development of dystrophic pathology. In the present work, we investigated the contribution of γ-SG to signaling in normal and γ-SG-deficient muscle. We reaffirm the position of γ-SG as a mechanosensor and expand on its role in regulating signaling in skeletal muscle. Further, we introduce the protein archvillin as a mechano-sensitive protein associated with the DGC.

Yeast two-hybrid screens identified archvillin as a γ-SG- and dystrophin-interacting protein. We verified that archvillin associates with γ-SG in murine skeletal muscle, and that muscle-specific region of archvillin binds to the rod domain of dystrophin. We confirmed with immunostaining that archvillin is localized at the sarcolemma, and that it is delocalized from the sarcolemma in the absence of dystrophin. Moreover, we show that archvillin expression is dependent on γ-SG expression. Muscle lacking γ-SG displayed increased archvillin expression concentrated at the sarcolemma,

and reintroduction of γ-SG in *gsg*$^{-/-}$ muscle by AAV injection reduced archvillin expression to wild type levels. To test our hypothesis that archvillin, like its closely related isoform smooth muscle archvillin, associates with ERK1/2, we immunoprecipitated muscle lysates with and without *in situ* ECC mechanical stimulation with anti-ERK1/2 and probed for archvillin. Archvillin associated with P-ERK1/2 in C57 stimulated muscle, but not on *gsg*$^{-/-}$ or *mdx* muscle. These findings led us to propose a model in which γ-SG and dystrophin mediate archvillin expression, localization, and association with P-ERK1/2 in skeletal muscle.

In addition, we extended our previous studies on mechanosensitive, γ-SG dependent ERK1/2 phosphorylation to determine whether additional pathways are altered with the loss of γ-SG. We examined mechanotransduction in lysates from C2C12 myotubes and isolated muscles from C57 *gsg*$^{-/-}$ mice with and without cyclic passive stretch. C2C12 myotube stretch caused a robust increase in P-p70SK, but decreased P-FAK and P-ERK2, and neither Akt nor ERK1 were stretch responsive. In isolated muscles, Akt, S6RP, and p70S6K activation increased with stretch in both C57 and *gsg*$^{-/-}$ isolated muscles. Treatment with the mTOR inhibitor, rapamycin, blocked all of p70S6K activation in stretched C57 muscles, and reduced downstream S6RP phosphorylation. However, even though rapamycin treatment decreased p70S6K activation in stretched *gsg*$^{-/-}$ muscles, S6RP phosphorylation remained elevated, suggesting that γ-SG may be important for inactivation of this pathway and that loss of γ-SG uncouples the response of p70S6K to stretch. These data suggest that there are differentially altered load-sensing mechanisms in muscular dystrophies where the sarcoglycans are absent. Overall, this work provides further evidence that γ-SG impacts mechanical signal transduction in

skeletal muscle, and present archvillin as a new DGC-interacting protein and further evidence for a role of γ-SG as a mechanosensor in skeletal muscle. In this chapter, I discuss the significance of these findings and provide insight into directions for future research.

## Remaining Questions and Future Directions

### *The role of archvillin in signaling*

We have shown that archvillin associates with P-ERK1/2 in a γ-SG- and stimulus-dependent manner. Previous studies on the isoform smooth muscle archvillin showed that it serves as a scaffold and impacts ERK1/2 phosphorylation in aortic tissue (Gangopadhyay et al., 2009; Gangopadhyay et al., 2004), posing the question if archvillin plays a similar role in skeletal muscle. Knockdown of smooth muscle archvillin was achieved by delivery of antisense oligonucleotides by a four-day organ culture "chemical loading procedure" (Gangopadhyay et al., 2009). While this long-term technique is not a feasible strategy in skeletal muscle, knockdown of archvillin in skeletal muscle would be of considerable interest. Attempts to generate a knockout mouse by our collaborators were unsuccessful, but other methods to knock down archvillin in muscle could include delivery of siRNA or antisense oligos by alternative methods. For example, gene silencing in limb muscles of old and young mice has been reported in a developing technique involving siRNA delivery via intravascular injection with and without electropulsation (Golzio et al., 2005; Hagstrom et al., 2004).

Using this or alternative methods, knockdown of archvillin in wild type mice would allow us to determine the contribution of archvillin alone to skeletal muscle

signaling defects and/or pathology. Knockdown of archvillin in *gsg*$^{-/-}$ muscle would also determine whether γ-SG mediates ERK1/2 signaling through its association with archvillin, providing evidence of this potential mechanism of regulation posed in Chapter 2. If archvillin does prove to be a primary mediator of ERK1/2 signaling, its manipulation could be a strategy by which to influence ERK1/2 signaling in a skeletal muscle-specific manner. Smooth muscle archvillin lacks multiple muscle insert sequences encoded by exons 3 and 5, as well as the second muscle-specific archvillin insert encoded by exon 9 (Gangopadhyay et al., 2004; Oh et al., 2003). By targeting these archvillin exclusive sequences, one could direct deletion strategies to archvillin specifically, and avoid interference of smooth muscle archvillin or supervillin. In addition to differences in muscle insert sequences, archvillin and smooth muscle archvillin also have different predicted ERK1/2 binding sites. Two of the three predicted ERK1/2 binding sites of archvillin are within the first muscle-specific region (aa 277 – 669) (Dinkel et al., 2014), whereas both smooth muscle archvillin sites, P219 and P774, are outside of the muscle-specific region. This may influence how ERK1/2 associates with each isoform and alter the downstream effects.

***Association of archvillin with the other sarcoglycans***

The work in Chapter 2 used the intracellular domain of γ-SG as bait in a yeast two-hybrid screen to identify archvillin as a binding partner. Given the homology of β-SG, and especially δ-SG, to γ-SG, this poses the question of whether archvillin might also associate with them. Similar immunoprecipitation experiments to those described in Chapter 2 could be performed using antibodies against the other SGs and immunoblotting

with anti-archvillin. These could be followed up with yeast two-hybrid experiments to reaffirm archvillin and also identify other potential binding partners. The secondary loss or reduction of the other complex members from loss of one subunit suggests that the SGs may work cooperatively, but each individual subunit's function versus as a complex is not known. Given the importance of each SG in the formation of the complex, it is possible that they work in a coordinated manner to mediate interactions with intracellular binding partners and associated signaling pathways. Evidence of either shared or individual binding partners or functions would be of interest, and perhaps shed light onto the mechanisms underlying the variability in disease progression and pathology amongst LGMD types 2C-F.

***Impact of γ-SG phosphorylation***

Phosphorylation is a versatile post-translational modification, as it is readily added and removed from substrates by kinases and phosphatases, respectively, allowing for a dynamic regulation of protein function in response to an ever changing cellular environment. In addition to acting as a molecular mark whose presence or absence can be recognized by other proteins, the negative charge inherent to the phosphate moiety can be utilized to alter protein structure or interaction/affinity with other proteins.

*Does γ-SG tyrosine 6 mediate its association with archvillin?*

Previous work in our lab demonstrated that γ-SG is tyrosine phosphorylated in response to mechanical stimulation and that the γ-SG tyrosine 6 affects ERK1/2 signaling, where expression of a WTgsg construct, but not a mutant Y6A construct in

*gsg*$^{-/-}$ muscle was able to restore normal ERK1/2 signaling (Barton, 2010). Having identified archvillin as γ-SG-associated protein that also binds P-ERK1/2 in a stimulus-dependent manner in Chapter 2, whether γ-SG Y6 phosphorylation impacts binding dynamics is of interest. By similarly reintroducing either normal γ-SG or mutant Y6A γ-SG by AAV expression, we could determine whether this residue is important for γ-SG-archvillin association. Further, we could determine if γ-SG Y6 phosphorylation impacts archvillin association with P-ERK1/2 in muscle subjected to *in situ* ECC. This would demonstrate that Y6 phosphorylation not only affects P-ERK1/2 signaling, but also provide evidence of a γ-SG-mediated ERK1/2 signaling mechanism through archvillin. If γ-SG Y6 does impact the γ-SG-archvillin and archvillin-P-ERK1/2 associations, it would suggest that binding γ-SG may impart some conformational change that affects its ability to bind P-ERK1/2.

*Does phosphorylation of the other Y residues of g-SG affect (ERK1/2) signaling?*

Since the initial experiments investiating the significance of γ-SG Y6 phosphorylation on ERK1/2 signaling, updated algorithms have also identified other tyrosine residues of the γ-SG intracellular domain as predicted phosphorylation sites. Therefore, it would be of interest to assess the significance of each of these residues by expressing γ-SG constructs with individual and combinatorial mutations in *gsg*$^{-/-}$ muscle, and assessing their influence on ERK1/2 activation with and without mechanical stimulation. This would tell us which specific tyrosine residues are actually phosphorylated in response to active contraction, and which are important for mediating

ERK1/2 signaling and for γ-SG interaction with archvillin. In addition to γ-SG, the intracellular regions of the α- and β-SG subunits of the SG complex also have poential phosphorylated tyrosine residues (Yoshida et al., 1998). Therefore, it would be interesting to see if tyrosine phosphorylation of the other SGs imparts effects on ERK1/2 signaling.

***Consequences of aberrant ERK1/2 signaling in skeletal muscle***

We have demonstrated here and previously the aberrant ERK1/2 signaling in murine skeletal muscle lacking γ-SG in response to both passive and active stretch in *ex vivo* and *in vitro* models. Given the significance of ERK1/2 signaling in mediating cell proliferation and survival pathways, we pose the question of whether its misregulation is sufficient to cause dystrophic pathology, and if inhibition may prevent or ameliorate pathology. Inhibition of ERK1/2 signaling with the small molecule MEK1/2 inhibitor, selumetinib, has been found to prevent pathology in a mouse model of Emery-Dreifuss muscular dystrophy with primary lamin A/C gene mutation, which also exhibits heightened ERK1/2 activity (Muchir 2013, Wu 2014). Using a similar approach, we performed pilot experiments utililizing a new ERK1/2-specific inhibitor, SCH772984 (see Chapter 5). Short-term intra-periotoneal injection of the drug at 12.5 mg/kg was effective in decreasing basal ERK1/2 activation in both the heart and liver. This is of interest because cardiomyopathy is a feature of LGMD in both humans and animal models lacking the SGs. Going forward, it will be important to address the long-term delivery potential of this drug and perhaps other ERK1/2 inhibitors.

Amelioration of pathology by inhibition of another aberrant signaling pathoway has recently been reported in γ-SG-deficient mice. *Ex vivo* ECC stimulation elicits increased accumulation of nuclear phosphorylated SMAD2/3 in $gsg^{-/-}$ mice EDL muscles. By introducing a heterozygous mutation of SMAD4 into $gsg^{-/-}$ mice to reduce but not ablate SMAD4, Goldstein et al. showed that these mice had improved body mass, cardiac function and improved tetanic force (Goldstein et al., 2014). Given these positive effects by normalizing an aberrant signaling pathway in the $gsg^{-/-}$ mouse, we believe the preventative and ameliorative capacity of SCH772984 or another MEK or ERK inhibitor is worthy of further investigation.

In addition to histopathological and functional consequences of aberrant ERK1/2 signaling, it would also be of interest to look at expression differences of ERK1/2 targets in dystrophic muscles. In chapter 2, we reported an increase in nuclear P-ERK1/2 in $gsg^{-/-}$ and *mdx* muscle, indicating the potential for differentially regulated transcription factors. Therefore, an assessment of genes differentially expressed in $gsg^{-/-}$ and *mdx* muscle, with and without mechanical stimulation, focused on ERK1/2 mediated targets would be of interest. Studies of differentially expressed genes using microarray data has been performed comparing biopsies from unaffected control, DMD, and LGMD2A/B patients (Eisenberg et al., 2007; Haslett et al., 2002), but not in muscle with primary SG mutation.

***p70S6K activation with active stretch***

In Chapters 2 and 3, we demonstrated differential activation of signaling pathways including ERK1/2 in response to passive and active stretch models. Therefore, the analysis of signaling pathways induced by different mechanical stimuli is important to

fully understand their function. Expanding our investigation of mTOR effector p70S6K activation in dystrophic muscle to include its response to our *in situ* ECC protocol is of interest given its role in skeletal muscle in mediating muscle protein synthesis. Parallel or synergic pathways can be activated through different stimuli; therefore, like ERK1/2, p70S6K may also be differentially activated with active stretch in dystrophic muscle.

Similarly, we also posit whether γ-SG Y6 tyrosine phosphorylation impacts p70S6K activation as it does ERK1/2. This could be analyzed by AAV-mediated reintroduction of either WTgsg or Y6Agsg in $gsg^{-/-}$ mice muscle with and without ECC. If p70S6K activation does depend on γ-SG phosphorylation, this raises the possibility that differential p70S6K activation is a downstream consequence of differential ERK1/2 activation in $gsg^{-/-}$ muscle. Contrary to this, recent work by others has shown that stretch-induced activation of mTOR and p70S6K at T389 is independent of ERK1/2 (Carriere et al., 2011; You et al., 2014). Whether γ-SG is modulating ERK1/2 and p70S6K in parallel or sequentially is important for understanding if one or both pathways leads to pathology, and ultimately can aid in designing therapeutic strategies that target signaling.

**Concluding remarks**

Under healthy conditions, skeletal muscle is a highly plastic tissue, capable of adapting to countless adverse conditions and impositions. However, in several muscular dystrophies in which the stability of the SG complex compromised, inappropriate load sensing appears to contribute significantly to disease pathology. The first step in therapeutic development is identifying and understanding the target, but little is currently known about the role of the SG complex in load sensing. Therefore, understanding the

functions that are disrupted and the pathways that are involved in mechanotransduction involving the SG complex will help in the design of therapies for LGMDs and DMD, perhaps by revealing more feasible therapeutic targets. To this end, the findings described in this dissertation begin to provide mechanistic insight into how mechanical signaling is disrupted and altered in the absence of γ-SG. In addition to increasing our understanding of the normal function of the SG complex, there is potential to provide more refined targets that could be beneficial to patients either in isolation or in combination with other therapeutic approaches.

# CHAPTER 6: Materials and Methods

## *Yeast two-hybrid assays*

Two independent yeast two-hybrid assays were performed to identify binding partners for the intracellular domain of γ-SG, and binding partners for the muscle-specific insert of archvillin. The Matchmaker 3 system (Clontech, Mountain View, CA) was used to perform the γ-SG yeast two-hybrid assay. The bait plasmid was generated by cloning the cDNA sequence corresponding to the intracellular domain of human γ-SG (NG_008759) (aa 1-35) into the pGBKT7 bait vector containing the DNA binding domain (BD) of Gal4. The bait plasmid was electroporated into AH109 yeast. The normalized human cDNA library prey, fused to the activation domain (AD) of Gal4, was pre-transformed in AH109 yeast. The mated yeast were screened on trp-, leu-, his-, ade-, x-alpha-$Gal^+$ media plates (dropout media for positive selection of bait and prey plasmids and their interaction), and colonies that grew and were blue were considered hits. All appropriate control tests were performed as described in the assay kit. Prey fragments from all positive hits were amplified by PCR, sequenced, and identified using the GenBank database (NCBI). A confirmation of binding assay for the archvillin prey was performed as described in the Matchmaker 3 system. In short, AH109 yeast containing either empty pGBKT7 plasmid or the γ-SG/pGBKT7 bait constructs were mated with Y187 yeast containing the archvillin/pGADT7 plasmid prey identified from the original yeast two-hybrid screen. The mated yeast were screened on either double drop out (DDO) trp-, leu-, x-alpha-$Gal^+$ plates or quadruple dropout (QDO) trp-, leu-, his-, ade-, x-alpha-$Gal^+$ media plates.

Archvillin yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S., Paris, France (http://www.hybrigenics-services.com). The sequence from the differentially spliced coding exons 3, 4, and 5, which encodes a large N-terminal insert (amino acids 276 – 669) in human supervillin isoform 2 (archvillin, NP_068506) and in supervillin isoform 4 (AGE81989.1) was generated by PCR (Fang and Luna, 2013; Oh et al., 2003). This PCR-amplified sequence was cloned into pB27, a derivative of pBTM116 (Vojtek and Hollenberg, 1995), as a C-terminal fusion to LexA (N-LexA-SVIL-C) and into pB66, a derivative of pAS2ΔΔ (Fromont-Racine et al., 1997), as a C-terminal fusion to the Gal4 DNA-binding domain (N-Gal4-SVIL-C). The constructs were verified by sequencing and used as baits for screening a random-primed Human Adult and Fetal Skeletal Muscle cDNA library in pP6, a derivative of pGADGH (Bartel et al., 1993). The LexA bait construct was used to screen 61 million clones (6-fold coverage of the library), using a mating approach with YHGX13 (matα) and L40ΔGal4 (mata) yeast strains, as previously described (Fromont-Racine et al., 1997) yielding a total of 17 positive colonies. The Gal4 construct and the same mating approach were used to screen 50 million clones (5-fold library coverage) with HGX13 (matα) and CG1945 (mata) yeast strains, yielding a total of 42 positive colonies. The prey fragments of the positive clones were confirmed for interaction, amplified by PCR, sequenced, and identified using the GenBank database (NCBI).

***Generation of His-DMD-10-12***

An intein-tagged plasmid encoding dystrophin spectrin repeats (aa 1361-1686; pTYB1-DMD-10-12), a kind gift from Dr. Elisabeth Le Rumeur (Université de Rennes),

was used as template for PCR using the following primers: sense: 5'-GAATTCATGAGGCAAAAGTTGCTTGAACAGAGCATC; antisense: 5'-CTCGAGACTGGTCAAAAGTTTCCATGTGTTTCTGGTA. The resulting fragment was cloned into the pET30a+ vector using the EcoRI and XhoI restriction sites as described previously (Takizawa et al., 2006). The vector was verified by sequencing.

***Recombinant protein purification***

GST fusion proteins and His-DMD-10-12 were purified from Rosetta 2 pLysS (DE3) cells (EMD-Millipore Biosciences, Billerica, MA), grown and extracted as described previously (Smith et al., 2010). Briefly, cells were induced with 0.2 mM IPTG at 30$^{o}$C for 18 h and collected by centrifugation before resuspension and extraction. The GST proteins (Smith et al., 2010) and His-tagged DMD 10-12 (Takizawa et al., 2003) was retrieved, as described. His-tagged DMD 10-12 beads were washed five times with 10 ml of wash buffer B (10 mM phosphate buffer, pH 7.5, 60 mM NaCl, 2% glycerol, 0.01% DTT, and 20 mM imidazole) (Takizawa et al., 2003). Three 1-ml fractions were eluted using wash buffer B containing 250 mM imidazole. All purified proteins were analyzed by SDS-PAGE.

***GST pulldown experiments and His immunoblot analysis***

Immediately prior to use, the purified His-DMD-10-12 solution in wash buffer B was modified to contain 90 mM NaCl, 2 mM DTT, 10 mg/ml BSA, and 1% Tween-20 and clarified by centrifugation at 15,000 x *g* for 15 minutes. After the His-DMD-10-12 supernatant was transferred to a fresh tube, 100 μl aliquots were mixed with 200 μl of

GST or GST fusion proteins, and incubated for 2 h at 4°C with rotation. Aliquots of glutathione-Sepharose 4B (50 μl slurry volume; GE Healthcare Bio-Sciences, Piscataway, NJ) were washed three times with wash buffer before addition of the GST/His protein mixtures, then incubated at 4°C with agitation for 1.5 hours. The Sepharose beads were collected by centrifugation, and the supernatants were saved as unbound fractions. Beads were then washed five times with 500 μl of 0.5x TBST (83.5 mM NaCl, 5 mM Tris, 0.025% Tween-20, pH 7.5); at the second wash, the bead slurry was moved to a fresh tube. Bound fractions were eluted with 100 μl of 10 mM glutathione in wash buffer B. All samples were solubilized in Laemmli Sample Buffer (Laemmli, 1970), resolved on 12% SDS-PAGE gels, and transferred to 0.45 μm nitrocellulose (Whatman GmBH, Dassel, Germany). Immunoblots were probed using rabbit polyclonal anti-His (Cell Signaling, Beverly, MA) and HRP conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA), developed using SuperSignal WestFemto ECL (Thermo Scientific; Rockford, IL) and imaged on a BioRad Gel Doc with Image Lab 4.1 software (BioRad, Hercules, CA). The experiment was repeated twice with comparable results.

***Immunohistochemistry***

10 μm frozen cross-sections from the midbelly of EDL muscles were fixed for 10 minutes in 4% formaldehyde and washed three times with PBS, followed by staining using the mouse-on-mouse (M.O.M) staining kit (Vector Laboratories, Burlingame, CA). Sections were blocked for 1 hour at room temperature in blocking buffer plus 2% horse serum and 2% BSA in phosphate buffered saline (PBS), then incubated overnight at 4°C

in M.O.M. diluent with rabbit polyclonal anti-archvillin (Sigma Life Sciences, St. Louis, MO, A1355) and anti-dystrophin (Vector Laboratories; VP-D505) or anti-γ-SG (Vector Laboratories VP-G803), all at 1:10 dilution or anti-laminin (Thermo Scientific RB-082-A) at 1:500. Secondary antibodies were applied at 1:500 (Life Technologies, Grand Island, NY; A11034, S32354, S32356), and slides were mounted using Vectashield with DAPI (Vector). Images were acquired at 400x on a Leica DMR microscope and Leica DFC300 CCD camera, using Improvision OpenLab software (Perkin Elmer, Waltham, MA).

***Immunoprecipitation***

Immunoprecipitation experiments were carried out using the Pierce Classic IP kit (Thermo Scientific). Muscle lysates containing 100 μg or 200 μg total protein were immunoprecipitated with anti-P-ERK1/2 (Thr202/Tyr204) (Cell Signaling; #9101 1:100), anti-ERK1/2 (Cell Signaling; #9107 1:100), anti-γ-SG (Vector Laboratories; VP-G803 1:20), or anti-P-Tyr (Cell Signaling; #9411 1:50) overnight at 4$^{o}$C with end-over-end mixing. The immune complex was eluted with non-reducing sample buffer and boiled at 100$^{o}$C for 5 minutes before being applied to a SDS-PAGE gel and immunoblotted as described below.

***Immunoblotting***

Snap frozen tissues were ground using a mortar and pestle over dry ice. Both snap frozen tissues and C2C12 myotubes were lysed in RIPA buffer RIPA buffer (50 mM HEPES pH7.5, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 15 mM p-nitrophenyl

phosphate disodium hexahydral, 1% NP-40, 0.1% SDS, 1% deoxycholate, 0.025% sodium azide) with protease and phosphatase inhibitor cocktails added (Sigma). Lysates were incubated on ice for 1 hour, vortexing half way through, centrifuged at 14K rpm for 30 minutes at 4$^{o}$C and the supernatants retained. Protein concentration was measured using a Bradford method protein assay kit (Bio-Rad). Lysates were separated by SDS-PAGE on Tris-HCl polyacrylamide gels (Bio-Rad) and transferred to PVDF membranes. Membranes were blocked in 5% milk and 2% BSA in TTBS, then probed with antibodies to the following: anti-archvillin (Oh et al., 2003) 1:500, anti-P-ERK1/2 (Thr202/Tyr204) (Cell Signaling; #9101 1:1,000), anti-total-ERK1/2 (Cell Signaling; #9107 1:1,000), anti-γ-SG (Vector Laboratories; VP-G803, 1:300), anti-β-SG (Vector Laboratories; VP-B206 1:500), anti-δ-SG (Vector Laboratories; VP-D501 1:50), anti-histone 3 (Cell Signaling; #9715 1:2,000), anti-tubulin (Sigma; T5168 1:20,000), anti-lamin A/C (Cell Signaling; #2032 1:500), anti-GAPDH (Santa Cruz; sc-32233 1:5,000), phospho (P)-p70S6K (T389) (Cell Signaling #9234 1:250), P-p70S6K (T421/S424) (Cell Signaling; #9204 1:1,000), P-S6RP (Ser235/236) (Cell Signaling; #2211 1:2,000), P-Akt (Ser473) (Cell Signaling; #9271 1:300), T-Akt (Cell Signaling; #2920 1:2,000), P-FAK (Tyr397) (Millipore; 05-1140, 1:500), T-FAK (Millipore; 06-543, 1:1,000). Membranes were then incubated with HRP-conjugated secondary antibodies (Cell Signaling; #7074, #7076 1:2,000), and visualized using ECL Plus reagent (Thermo Scientific) and autoradiography film (BioExpress, Kaysville, UT).

For human samples, 25 pooled, 10 μm frozen sections of quadriceps biopsies from LGMD2 patients, DMD patients and healthy male controls were lysed in RIPA buffer, and 5% of the lysate was used for immunoblotting, as described above. Samples

were obtained from the Wellstone Muscular Dystrophy Tissue and Cell Repository at the University of Iowa Carver College of Medicine (Iowa City, IA). LGMD patients were between 9 and 17 years, DMD patients between 5 and 8 years, and controls between 8 and 13 years at the time of biopsy (Supplemental Table II).

***Quantitative RT-PCR***

Total RNA was isolated from frozen quadriceps muscles using TRIzol (Life Technologies). Equal amounts of total RNA from each sample were subjected to single-strand reverse transcription (Applied Biosystems, Foster City, CA-now part of Life Technologies). The resultant cDNA was utilized for quantitative real-time PCR with primers specific for archvillin exon 4: sense - 5' AAAGAGGAGAGTGCTCGCAG 3'; antisense: - 5' GCTGGTGACCCTATCAAAGGT 3', using the Applied Biosystems 7300 Real-Time PCR System and reagents (Power SYBR Green PCR Master Mix, Life Technologies). All samples were loaded in duplicate in 96-well plates. Expression of 18S was used to control for cDNA content.

***Animals***

All experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. C57Bl/6 (C57), γ-SG-null (*gsg*$^{-/-}$), δ-SG-null (*dsg*$^{-/-}$), and *mdx* mice were used. Three-week old mice were used for viral injections; all other experiments were done with 12-14 week old mice. The *gsg*$^{-/-}$ mouse lacks γ-SG due to gene targeting, resulting in an additional loss of β- and δ-SG and a decrease of α-SG (Hack et al., 1998), and were backcrossed for more than 10 generations onto the C57Bl/6

strain. The *dsg*$^{-/-}$ mouse lacks δ-SG also due to gene targeting, resulting in an additional loss of α-, β-, and γ-SG on a 129SvJ/129SvEms-+Ter/J background (Hack et al., 2000). *Mdx* mice lacking dystrophin were obtained from The Jackson Laboratory (Bar Harbor, ME), and were maintained as a colony of homozygous mutant animals (C57Bl/10 background strain).

***In situ TA muscle mechanics***

*In situ* muscle function was examined on the tibialis anterior (TA) using a protocol developed from methods described previously (Dellorusso et al., 2001). Briefly, mice were deeply anesthetized by i.p. injection of ketamine-xylazine (80 and 10 mg/kg), with body temperature maintained at 37$^{o}$C, and monitored throughout the experiment. For each preparation, the distal tendon of the TA was carefully dissected and tied with 4.0 braided surgical silk. The sciatic nerve was exposed and all of its branches were cut except for the common peroneal nerve. The foot was secured to a platform and the knee immobilized using a stainless steel pin, with care taken not to interfere with the blood supply to the muscles. The suture from the TA tendon was attached to the lever arm of a 305B dual-mode servomotortransducer (Aurora Scientific, Ontario, Canada). Isometric muscle contractions were then elicited by stimulating the distal part of the sciatic nerve via bipolar electrodes, using supramaximal square-wave pulses of 0.02 msec (701A stimulator; Aurora Scientific). Data acquisition and control of the servomotor were conducted using a Lab-View-based DMC program (version 5.202; Aurora Scientific). Optimal muscle length ($L_o$) was determined by incrementally stretching the muscle until the maximum isometric twitch force was achieved ($P_o$). Muscle length was measured

using digital calipers based on well-defined anatomical landmarks. Three maximum isometric tetanic forces were acquired using a train of 150Hz, 500msec supramaximal electrical pulses at the optimal length in the muscles and highest force was recorded. A 2-minute resting period was allowed between each tetanic contraction. Following isometric contractions, muscles were subjected to two eccentric contractions (ECC) with the muscle stimulated at 150 Hz for a total of 300 ms. TA muscles were stretched 24% $L_o$ in the final 200 ms stimulation. The second ECC was administrated after a 10 s rest period under the same parameters. Post-ECC muscle isometric forces were measured after 1min, 5min and 15 min. The drop in force between the isometric force during eccentric contraction and recovery time served as an index of susceptibility to eccentric contraction-induced injury.

***Viral constructs and injections***

Human γ-SG cDNA was utilized to generate recombinant adeno-associated virus serotype 2/8 (rAAV) as described previously (Barton, 2010), and expression was regulated by a truncated desmin promoter (Li et al., 1993).Vector production was performed at the University of Pennsylvania Vector Core. Solutions containing 1 x $10^{11}$ viral particles diluted in 50µl of phosphate-buffered saline (PBS) were injected into the anterior compartment of the lower hindlimbs of 3-week old *gsg*$^{-/-}$ mice, targeting the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles as described previously. After injection, mice were housed in the animal facility until time of analysis, 1-month post-injection. The TA muscles were dissected and rapidly frozen in liquid

nitrogen for biochemical analysis, while the EDL muscles were frozen in OCT (Sakura Finetek, Torrance, CA) for immunohistochemical analysis.

***Cytosolic/Nuclear Fractionation***

Snap frozen TA muscles were ground using a mortar and pestle and processed as described previously (Dimauro et al., 2012). In short, nuclear fractions were isolated by lysates undergoing a series of centrifugations and resuspensions in STM buffer (250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, protease and phosphatase inhibitors) followed by resuspension in NET buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl2, 0.5M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton-x-100, protease and phosphatase inhibitors) and lysing of nuclei by passage though an 18-gauge needle and sonication. Cytosolic fractions were isolated by centrifugation, precipitation in 100% acetone at -20$^{o}$C for 1 hour, and resuspension in STM buffer. Processed lysates were then analyzed by immunoblotting as described above.

***C2C12 myotube culture***

Flexible silicone membranes (Specialty Manufacturing, Inc.) were stretched across the bottom of custom cylinders which acted as a culture chamber. The membranes were held in place using an O-ring as described previously (He et al., 2004). Membranes were then coated with a thin layer of 2 mg/mL GFR Matrigel (BD #354230). C2C12 myoblasts ($3.5 \times 10^5$ / cylinder) were seeded onto the membranes and maintained at 5% $CO_2$ at 37°C in growth media (10% FBS, 100 U penicillin, 100 µg streptomycin, 100 µg/mL gentamycin in DMEM) for approximately 24 h until 70% to 80% confluent, then switched to differentiation media (2% HS, 100 U penicillin, 100 µg streptomycin, 100

μg/mL Gentamycin in DMEM). Myoblasts were allowed to differentiate into multinucleated myotubes for 5 days, during which media was changed every other day before stretching as described below.

***Myotube stretching protocol***

C2C12 myotubes were stretched using an apparatus that produces isotropic two-dimensional strain of cells *in vitro*, as described previously (He et al., 2004). Briefly, myotubes were subjected to 10% strain, 40 times per minute, for 30 min, at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Control myotubes were treated identically but were not stretched. Lysates were harvested immediately as described below. Primary myotubes were stretched using the protocol described for C2C12 myotubes. Stretched myotubes were then incubated without stretch for a further 1, 2, or 4 h at 37°C before harvesting lysates for immunoblotting as described below. Control (non-stretched) myotubes were harvested immediately after the end of the stretching protocol used for the stretched myotubes.

***In vitro EDL muscle mechanics***

Mice were anaesthetized using ketamine and xylazine. EDL muscles were dissected and placed in an organ bath containing oxygenated high-glucose (25 mM) DMEM with HEPES (25 mM) (Life Technologies), at room temperature. For rapamycin sensitivity experiments, high-glucose DMEM was supplemented with 150 nM rapamycin (Sigma) or vehicle only (0.1% DMSO). Muscles were adjusted to 9.3 mN of resting tension, approximately equivalent to optimal length, based on our previous experiments

(Moorwood et al., 2013). After a 10-min equilibration period, the length of the muscle was measured using calipers, and the muscle was subjected to a stretching protocol of 15% strain (held for 100 ms with ramp times of 50 ms), 20 times per minute, for either 30 or 90 min, using an in vitro muscle test system (Aurora Scientific). Muscles were snap-frozen immediately following the end of the stretch protocol. Contralateral muscles were used as controls and were adjusted to the same length as the stretched muscles, then incubated in oxygenated high-glucose DMEM with HEPES at room temperature for the equivalent length of time, before snap-freezing.

***SCH772984 Treatment***

SCH772984 (Selleck Chemicals, S7101) was prepared in 20% HPβCD to pH 4.5, then diluted in 20% HPβCD for dosing at 12.5mg/kg at 5mL/kg. 6-7 week old C57 and *gsg*$^{-/-}$ mice were injected daily by i.p. injection for 5 consecutive days. Mice were weighed prior to treatment, on day 3, and on day 5 before *in situ* TA mechanical stimulation described above.

***Statistics***

Chapters 2+4: All results are shown as the means ± S.E.M. unless stated otherwise. Statistical analyses of the data were performed using one-way or two-way ANOVA followed by Bonferroni post hoc test. P-values of less than 0.05 were considered statistically significant.

Chapter 3: Comparisons between non-stretched and stretched C2C12 cells performed using unpaired Student's t-test. Comparisons between C57 and *gsg*$^{-/-}$ muscles

with and without rapamycin were performed by two-way ANOVA with Tukey's multiple comparisons test.

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