LARGE-MEDIATED GLYCOSYLATION OF DYSTROGLYCAN IN SKELETAL MUSCLE FUNCTION

by

Jessica D. Gumerson

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular and Integrative Physiology)
in the University of Michigan
2012

Doctoral Committee:

Associate Professor Daniel E. Michele, Chair
Professor Gregory D. Cartee
Associate Professor Susan Brooks Herzog
Associate Professor Mark W. Russell

To Jim and Kim

who will always be my inspiration

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

Introduction to Muscular Dystrophies Related to Mutations in the Dystrophin-Glycoprotein Complex

Skeletal muscle is a dynamic tissue that routinely undergoes a significant degree of mechanical strain and cellular deformation with each contraction. In order to preserve normal skeletal muscle function throughout the lifetime of an individual, this complex tissue must be able to routinely undergo cell shortening and generate forces required for movement while at the same time limiting mechanical cellular injury and adapting to changing workloads. In muscular dystrophy, an imbalance between muscle damage or degeneration and muscle repair through stem-cell mediated regeneration is thought to contribute to the disease pathology and consequently results in a progressive decline in muscle function (1). Mutations in a number of distinct genes can cause muscular dystrophy with varying degrees of severity, but precisely how each can negatively affect normal muscle function is unclear (2). Several muscular dystrophies result from mutations that affect the normal assembly of the dystrophin-glycoprotein complex at the sarcolemma in muscle and therefore, highlight the importance of this complex in normal muscle function. Within the DGC, the protein dystroglycan serves as an essential glycosylation-mediated extracellular matrix receptor and this receptor function is impaired in a number of congenital muscular dystrophies caused by mutations in glycosyltransferases. In addition to muscle disease, patients with glycosylation-deficient muscular dystrophy also demonstrate impairments in both the central and peripheral nervous system as a consequence of altered dystroglycan function. While this

suggests that dystroglycan has a critical glycosylation-mediated function in both striated muscle and the nervous system, the molecular mechanism that accounts for the wide clinical spectrum associated with these diseases is poorly understood and the basis for the work described in this dissertation. The goal of this research is to understand how mutations in the putative glycosyltransferase LARGE impair dystroglycan function and cause a multi-system muscle disease. Because the primary function of dystroglycan within the DGC is thought to be predominantly structural in skeletal muscle, initial experiments were focused on understanding how impaired or enhanced LARGE-mediated glycosylation of dystroglycan affected skeletal muscle function. In later experiments, a novel transgenic mouse was used to dissect the functions of dystroglycan in neural tissues that contribute to disease progression in glycosylation-deficient muscular dystrophy. In order to understand how dystroglycan may function in multiple tissues, this introduction will summarize both the literature supporting a mechanical function for the DGC, and the recent evidence highlighting additional functions of the DGC in muscle and non-muscle tissues that when impaired, can result in the spectrum of clinical symptoms associated with glycosylation-deficient muscular dystrophy.

The DGC, Sarcolemma Integrity, and Contraction-Induced Muscle Injury

A longstanding hypothesis to explain the muscle damage and degeneration observed in dystrophic muscle is that mutations affect the function of critical structural proteins in muscle and compromise the mechanical stability of the muscle fiber and/or the integrity of sarcolemma. This exacerbates the damage that occurs during normal muscle contractions and initiates a lethal cascade of events that can cause death of the myofiber (3-5). In support of this, early studies demonstrated an increase in the number of necrotic fibers in muscles from muscular dystrophy patients that likely resulted from irreparable membrane damage as a consequence of normal muscle activity (6). However, rather than dystrophic muscle suffering from an increased susceptibility to external damage, alternative hypotheses exist that could account for increased cell death. For

example, causative mutations may affect either the resting homeostasis of muscle cells or alter the ability of the muscle to adapt and repair following a normal "dose" of muscle injury. Although the importance of the dystrophin-glycoprotein complex in maintaining sarcolemma integrity is well supported, alternative functions for this complex have been proposed through the years and are now gaining significant experimental support (7, 8).

The dystrophin-glycoprotein complex (DGC) is composed of several transmembrane and peripheral components, and is highly expressed in the sarcolemma of skeletal muscle (9-11). Mutations in genes that encode DGC components lead to the loss of either expression and/or function of the DGC in muscle. Dystroglycan is a protein central to this complex that spans the sarcolemma linking the cellular cytoskeleton to the surrounding basal lamina through dystrophin (within the cell) and α-dystroglycan (on the cell surface) (12). Dystrophin in turn binds to the submembrane actin and intermediate filament cytoskeleton within fibers, thereby completing a link between the cytoskeleton and the extracellular matrix (13, 14). Costameres are concentrations of extracellular matrix receptor complexes that reside at the membrane in register with the Z-lines of sarcomeres within muscle fibers (15). The location of the DGC at costameres and the identification of its function as a link between the matrix and the cytoskeleton led to the hypothesis that the DGC might be critical in mechanically stabilizing muscle or the sarcolemma during muscle contraction (12, 16). In addition to dystroglycan and dystrophin, the core of this complex in skeletal muscle includes four sarcoglycans (α, β, γ, and δ) and sarcospan, which are thought to contribute to stabilization of the complex within the sarcolemma. Mutations in either dystrophin or the sarcoglycans are associated with reduced expression or incomplete formation of the DGC (17-19) and are hypothesized to result in muscular dystrophy through a common mechanism which includes a reduction in dystroglycan function. Therefore, reduction in the connections between the cytoskeleton and the extracellular matrix are a critical feature of muscular dystrophies associated with the DGC.

In addition to primary mutations in DGC components, several causative mutations have also been identified in a group of glycosyltransferases, which have been shown to function in a common pathway to glycosylate α-dystroglycan. This *O*-linked glycosylation of α-dystroglycan is essential for enabling α-dystroglycan to function as an extracellular matrix receptor (20). In these glycosylation-deficient muscular dystrophies, dystroglycan and the DGC are expressed and intact at the sarcolemma, but the loss of dystroglycan's ability to bind laminin is sufficient to cause muscular dystrophy (21, 22). These findings highlight specifically the interaction of the DGC with extracellular matrix as a critical function of the DGC in preventing muscular dystrophy.

In dystroglycan glycosylation-deficient mice (LARGE[myd]), electron microscopic analysis showed that the interaction of dystroglycan with laminin in the extracellular matrix appeared to tightly anchor the basal lamina to the sarcolemma (22). This tight and regular interaction of dystroglycan with the basal lamina is proposed to protect the sarcolemma from expansion of small ruptures during mechanical activity. Mutations in dystroglycan itself appear to be quite rare in humans, perhaps related to an essential role of dystroglycan in early development (23). Only recently has a mutation in the dystroglycan gene been identified in muscular dystrophy patients and like previous glycosyltransferase mutations, the mutation appears to impair dystroglycan glycosylation causing loss of function without impacting dystroglycan expression (24).

The heterotrimeric protein laminin-211, which is bound by glycosylated α-dystroglycan, is a major component of the basal lamina surrounding adult muscle fibers. Mutations in the *LAMA2* gene result in loss of laminin α2 expression and the most common form of congenital muscular dystrophy (25-27). The identification of laminin α2 mutations only further reinforces the notion that any disruption of the connection between the muscle fiber cytoskeleton to the extracellular matrix through the DGC, whether it be reduced expression of the DGC, reduced ability of dystroglycan to interact with laminin, or loss of laminin itself from the basal lamina, is sufficient to cause muscular dystrophy.

Despite the first genetic identification of dystrophin as a causal gene in Duchenne muscular dystrophy more than two decades ago (28) and the identification of the DGC in years following (17), whether the DGC contributes purely a mechanical role in stabilizing the sarcolemma during normal contractions or imparts other significant functions in muscle still remains an area of active investigation. The identification of additional 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 (29), has fueled considerable interest in what other intracellular pathways may be affected in DGC-associated muscular dystrophies.

Even normal skeletal muscle is susceptible to mechanical damage, particularly during lengthening contractions, and the resulting defects at the level of the sarcolemma (30), the t-tubules (31), or the contractile machinery (32) contribute to a transient decrease in the isometric force. Following repetitive lengthening contractions, there can also be considerable, prolonged injury and muscle dysfunction that results from muscle degeneration, swelling, and infiltration of inflammatory cells (33). In most cases, muscle dysfunction caused by prolonged injury can be fully repaired over time by active muscle fiber regeneration from resident stem cells, known as satellite cells. This suggests that occasional sarcolemmal injuries, muscle damage and their repair is a critical component of the homeostasis of normal muscle.

Many of the early experiments that sought to identify the mechanism by which mutations affecting the DGC cause muscular dystrophy have utilized the *mdx* mouse model (34). These mice harbor a null mutation in the dystrophin gene and although they do exhibit the clinical features observed in human patients, the severity is milder (35). A role for the function of dystrophin in maintaining the integrity of the sarcolemma during muscle contraction was supported by studies showing that *mdx* muscle was highly susceptible to lengthening contraction-induced injury performed *in vitro,* as compared to healthy

control muscle. Muscle from *mdx* animals demonstrated an increased tendency to take up Procion orange dye from the bathing medium, which suggested an increase in sarcolemma permeability following contraction (4, 36). When injected into *mdx* animals, the membrane-impermeant Evans blue dye was also selectively taken up by muscle fibers that appeared necrotic and hypercontracted, suggesting that increased membrane permeability eventually resulted in cell death (37). This further supported the hypothesis that muscle contractions produce membrane tears, leading to increased permeability of the sarcolemma to calcium and small molecules and resulting in a greater degree of cell death in dystrophic muscle. Muscle from *mdx* mice also demonstrated a measurable deficit in force generation following an *in vivo* lengthening contraction protocol (38, 39). These data support a function for dystrophin and the dystrophin-glycoprotein complex in protecting the sarcolemma during muscle contraction and suggest that, in its absence, the sarcolemma is more susceptible to damage by contractile forces, resulting in increased permeability of ions and small molecules, and eventual cell necrosis and muscle degeneration.

Further support for a critical role of membrane integrity in muscular dystrophies was gained from a different class of muscular dystrophies associated with mutations in the dysferlin gene. Mutations in dysferlin are associated with Miyoshi myopathy and limb-girdle muscular dystrophy 2B in humans (40, 41). Dysferlin is not associated with the DGC but appears to have homology to the vesicle protein, synaptotagmin, and therefore has been predicted to be important for mediating vesicle-mediated membrane repair. While the complete functions of dysferlin are still under investigation, dysferlin has been shown to be required for rapid resealing of the sarcolemma in a calcium-dependent manner following focal membrane damage (42). Although muscle from dysferlin-null animals is not particularly susceptible to contraction-induced damage (43), a recent study demonstrated that recovery of muscle following damage required an immediate and transient membrane resealing event and that dysferlin-deficient muscle consequently took longer to recover (43, 44). The identification of dysferlin as a potential mediator of membrane repair in muscle underlies the importance of

sarcolemmal integrity and its maintenance by repair pathways as important mechanisms in which defects may result in muscle degeneration.

Does contraction-induced injury play a causal role in DGC-associated muscular degeneration and dystrophy?

While the generally accepted dogma is that mutations affecting the DGC render muscle more susceptible to contraction-induced damage during mechanical stress (45), recent evidence from our laboratory suggests that not all muscles of dystrophic animals are equally affected (Chapter 2). We reported that, in the LARGE^{myd} animal model of glycosyltransferase-deficient muscular dystrophy, typical fast-twitch muscles such as the EDL were weaker than in control muscle. Additionally, although LARGE^{myd} EDL demonstrated the increased susceptibility to contraction-induced injury typical of *mdx* mice, a remarkably different phenotype was measured in soleus muscles, which are composed of mixtures of fast and slow fibers. The fact that force deficits measured in wild-type soleus muscle were actually higher than those measured in EDL muscle after two lengthening contractions, suggests that the observed force defect in the EDL muscle of LARGE^{myd} mice was not due to inherent differences in susceptibility to injury between the two muscle groups. However, the soleus of LARGE^{myd} animals was weaker than WT muscle and was dystrophic, as evidenced by an increase in degenerating fibers, accumulation of inflammatory cells, and the presence of centrally nucleated fibers. An explanation that accounts for the dystrophic features and fiber degeneration in the soleus muscle in the absence of any detectable increased susceptibility to contraction-induced injury has not been addressed.

A similar lack of increased susceptibility of muscle to contraction-induced muscle damage was previously demonstrated in the soleus of *mdx* mice, and the authors speculated that dystrophin was not essential for maintaining structural stability in the soleus muscle (38). Utrophin, a homologue of dystrophin, has been shown to be upregulated in the absence of dystrophin (46) and is also more highly expressed in slow-twitch fibers (47) which may therefore confer a degree

7

of stability in the soleus muscle of *mdx* mice that might explain this lack of susceptibility to contraction-induced injury. However, upregulation of utrophin cannot explain the results in LARGE[myd] mice because, other than the loss of dystroglycan glycosylation, the DGC is assembled normally in LARGE[myd] muscle (21, 22). We found that another important laminin receptor in muscle, α7β1 integrin, is expressed at much higher levels in the sarcolemma of soleus muscle as compared to other fast muscles (48). Several reports have demonstrated that when the DGC is impaired, α7 or β1 integrin is upregulated, which suggests that the two receptors may have at least some overlapping functions in muscle (49). Susceptibility to contraction-induced injury was directly compared between α7 integrin-null and LARGE[myd] mice using EDL muscle and only LARGE[myd] muscle was shown to be more susceptible to injury (22). This might suggest that interactions with laminin and dystroglycan are more important for mechanical stability than are the interactions between α7β1 integrin and laminin. However, α7β1 integrin expression in fast muscle is very low, and the comparison was only performed in fast-twitch muscle, and not in the soleus muscle, which remains to be studied. Transgenic overexpression of α7 integrin in dystrophin/utrophin double knock-out mice can significantly improve muscle disease (50), but whether this beneficial effect is due to prevention of mechanical damage or effects on cell signaling has not been fully addressed.

Muscle physiological studies have also been performed in an animal model of congenital muscular dystrophy, the *dy/dy* mouse that harbors a null mutation in the *LAMA2* gene (25). In contrast to the results demonstrated in *mdx* mice, laminin-deficient muscle does not exhibit a defect in sarcolemmal stability (51). Both the EDL and soleus muscles were isolated from laminin-deficient mice and subjected to a moderate lengthening contraction protocol *in vitro* and neither demonstrated an increased susceptibility to injury over that observed in control muscle. As a means to amplify potentially subtle defects in these mice, the anesthetic halothane was used to increase fluidity of the lipid bilayer. Although this caused an increase in force deficit following a lengthening contraction protocol, this deficit was still not any greater in laminin-deficient muscle (51).

8

Because contraction protocols can vary, sarcolemmal permeability was directly compared between three different mouse models of DGC-related muscular dystrophy, *mdx* (34) and two deficient in laminin, *dy/dy* (25) and *dy^{2j}/dy^{2j}* (26). Animals were injected intravenously with Evans blue dye and muscles were analyzed several hours post injection. Muscles from *mdx* demonstrated an increase in dye uptake while dye uptake in laminin-deficient muscle was not different than control animals (52). Additionally, positively stained fibers in *mdx* mice often appeared in groups of neighboring fibers, in contrast to the few individual fibers stained in laminin-deficient muscle, which appeared necrotic. However, laminin-deficient muscle still had dystrophic pathological features, which further supports the notion that, although disruption of the sarcolemma may contribute to the pathology of muscle disease, it is not essential.

Together, these studies demonstrate that membrane damage is not required for muscle degeneration and muscular dystrophy. Although mutations that compromise DGC function can leave muscle vulnerable to membrane damage, this is not true for all muscle groups, since the soleus muscle, despite demonstrating a susceptibility to injury, does not show an increase in susceptibility in the absence of a functional DGC. Additionally, the number of fibers that may be damaged, as evidenced by dye uptake in *mdx* fast muscles, is not sufficient to explain the dramatic loss in force generation following injury (38). Therefore, the DGC likely possesses other cellular functions in skeletal muscle that, when impaired, contribute to muscle degeneration and the dystrophic pathology. Potential alternative pathways and their experimental support are reviewed below.

Role of the DGC in Altered Calcium Homeostasis

Intracellular calcium is a critical mediator of many regulatory processes in skeletal muscle (53). In dystrophic muscle, the concentration of intracellular calcium is elevated, and several potential sources for calcium entry have now been identified (Fig. 1.1). Early studies of *mdx* muscle demonstrated that individual fibers demonstrating an elevation in intracellular calcium were also

9

necrotic, suggesting that calcium entered through membrane tears as a direct result of dystrophin loss (54, 55). Increased intracellular calcium in *mdx* muscle was later explained by an increase in sarcolemmal permeability attributed to calcium leak and stretched-activated channels (56, 57). Stretch-activated channels can be blocked in *mdx* mice via oral delivery of streptomycin resulting in a reduction in intracellular calcium and an improvement in force production (58). In the same study, a decrease in intracellular calcium was not observed in streptomycin-treated control animals, suggesting that the activity of these channels was enhanced in the absence of dystrophin.

Transient receptor potential (TRP) channels are a diverse family of ion channels composed of multiple subunits that have also been identified as potential mediators of altered calcium homeostasis in dystrophic muscle (59). Several TRP channels in the canonical subfamily (TRPC) are expressed in mouse skeletal muscle and TRPC1, TRPC4 and TRPC6 were initially identified as being potentially impaired in *mdx* muscle (60). In a later study, expression of TRPC1 was shown to be increased in *mdx* muscle and the authors speculated that its activity may be increased due to additional upregulation of caveolin-3 and src, which contribute to the translocation of this channel to the sarcolemma (61). Similarly, a stretch-activated TRP channel in the vanilloid receptor subfamily, TRPV2, has also been shown to be more highly expressed at the sarcolemma in *mdx* muscle. Inhibition of TRPV2 using a dominant negative genetic approach resulted in a restoration of normal intracellular calcium levels and an amelioration of dystrophic pathology in *mdx* mice (62).

Although the increased resting calcium concentration observed in the cytoplasm of dystrophic muscle may be the result of increased expression or activity of calcium channels at the sarcolemma, a recent study has demonstrated that an additional source of calcium entry may be due to defects at the level of the sarcoplasmic reticulum (63). RyR1 channels isolated from *mdx* muscle were shown to be hypernitrosylated as a potential consequence of altered nitric oxide signaling downstream of dystrophin loss. This resulted in an increased leak of

calcium ions from the sarcoplasmic reticulum, and pharmacological inhibition of RyR1 was shown to reduce muscle damage in *mdx* muscle.

While several of these studies noted an improvement in muscle health following inhibition of calcium entry in animal models of muscular dystrophy, an important study recently demonstrated that elevated calcium was sufficient to cause muscle damage in the absence of a genetic basis for muscular dystrophy (64). The overexpression of TRPC3 in skeletal muscle resulted in a muscle wasting phenotype with defects similar to those observed in laminin-deficient muscle. Using gene expression profiling, this phenotype was also shown to be associated with altered expression of many genes, in a pattern that was strikingly similar to gene expression changes in δ-sarcoglycan deficient muscle. However, the muscle did not demonstrate any changes in expression of the DGC at the sarcolemma, suggesting that much of the muscle damage observed in DGC-related muscular dystrophies may be attributed to downstream defects in calcium homeostasis.

Elevated intracellular calcium can result in cellular damage in a number of ways that may underlie many of the defects observed in dystrophic muscle. When sustained, abnormal elevations in intracellular calcium can cause mitochondria to undergo permeability transition which can eventually lead to mitochondrially mediated apoptosis, involving swelling of the mitochondria, release of cytochrome c and the activation of caspases (65). Cyclophilin D is a mitochondrial enzyme that is important for regulating the mitochondrial permeability transition and genetic strategies have demonstrated that its absence renders mitochondria insensitive to calcium-induced cell death (66, 67). Both δ-sarcoglycan-null and laminin-null mice have abnormally swollen mitochondria and the muscular dystrophy seen in both models can be partially alleviated by altering the pores mediating the mitochondrial permeability transition (68).

Another important downstream consequence of mitochondrial dysfunction is an increased production of reactive oxygen species (ROS), which can further exacerbate cellular damage (69). Several studies have identified ROS as a

potential mediator of muscle damage in the muscular dystrophies (70-72). Antioxidant treatment of *mdx* mice has demonstrated mixed results but in some cases has lessened the symptoms of muscular dystrophy (72, 73). In a model of muscular dystrophy not associated with DGC defects but instead caused by defective collagen IV, a component of the extracellular matrix, mitochondrial dysfunction was shown to be a major source of ROS. Additionally, when ROS production was suppressed, oxidation of myofibrillar proteins was reduced and improved contractile performance (74). Therefore, cellular mechanisms downstream of mitochondrial defects may be an important step in the process by which DGC mutations eventually result in myofiber damage and cause muscle disease.

Normal excitation-contraction coupling has been suggested to be affected by an increase in cellular calcium concentration and may also be an important contributor to muscle weakness in these diseases (75, 76). Elevated calcium can also directly impair muscle function by increasing the activity of calcium-dependent proteases like calpains which can cleave myofibrillar proteins (77-79). While the genetic and pharmacological inhibition of calpains may alleviate several pathological features in *mdx* muscle (80-82), such experiments have yielded inconsistent results and may be in part due to compensatory increases in calpain activity and/or a lack of efficacy of the proposed inhibitors (83).

Several of these studies have demonstrated that intracellular calcium is elevated in dystrophic muscle and that this can lead to a number of deleterious effects to the cell and contribute to a decline in contractile function. More importantly, these studies have provided several alternatives by which calcium permeability and/or intracellular calcium may be increasing that are independent from direct entry of calcium through sarcolemma tears. However, it is still unclear just how distinct genetic mutations that affect the function of the DGC can alter activity of the various calcium channels that have been proposed. While inhibition of calcium entry has been shown to be beneficial in the animal models addressed, it is important to note that muscle disease was still present in

these animals, albeit it to a lesser degree. This suggests that altered calcium homeostasis, whether it is through membrane tears or from altered activity of calcium channels, may not be the only mechanism downstream of genetic mutations that results in muscle damage.

Laminin-Dependent Intracellular Signaling Through the DGC

Laminins exist as heterotrimeric proteins composed of specific combinations of α, β, and γ subunits and are differentially expressed in multiple cell types. Laminin-211 (α2/β1/γ1) is expressed in the extracellular matrix of adult skeletal muscle and is bound with high affinity by α-DG and α7β1 integrin. Mutations that affect α2 laminin, as is the case in the *dy/dy* mouse and in congenital muscular dystrophy 1A, result in muscular dystrophy as a result of lost interactions with either or both laminin receptors (25-27). Although laminin is the major ligand of dystroglycan by which a connection is forged between the DGC and the extracellular matrix in skeletal muscle, laminin-211 deficient muscle is not susceptible to contraction-induced damage and does not typically show increased uptake of cell impermeant dye (reviewed above). Despite the lack of evidence for increased muscle damage, many fibers of laminin-deficient muscle are apoptotic (84), which is thought to significantly contribute to muscle disease. Both pharmacological and genetic inhibition of apoptosis (e.g. Bcl-2 overexpression, Bax inactivation) has been shown to ameliorate dystrophy in laminin α2 deficient animal models (85-88). Because there appears to be a lack of sarcolemmal damage, the mechanism by which apoptosis occurs in this disease may be independent of elevations in intracellular calcium. Alternatively, increases in apoptosis may be due to disruptions in downstream cell survival signaling as a result of lost interactions between laminin and its two major receptors in skeletal muscle.

Potential increases in survival signaling through laminin receptors in muscle could be the basis for results of a recent study that demonstrated an improvement of dystrophy in *mdx* mice upon injection of soluble laminin-111, a laminin isoform not normally expressed in skeletal muscle (89). While the

mechanism of this effect is unclear, the benefit of laminin-111 injections may be due to either upregulation of α7 integrin mediated signaling or integrin-mediated stabilization of the sarcolemma (89). However, a transgenic approach to deliver laminin-111 to skeletal muscle failed to benefit *mdx* mice (90) despite its rescue of muscular dystrophy in laminin α2 deficient muscle (91, 92). So while laminin α1 and α2 are normally expressed in different tissues, they appear to be functionally similar in promoting muscle cell survival. Whether this important function of laminin in muscle can be targeted therapeutically in all forms of DGC deficient muscular dystrophy is still debatable.

Integrins are formed as heterodimers of α and β subunits, and the predominant alpha isoform expressed in differentiated skeletal muscle, α7 integrin, forms dimers with β1integrin to form a laminin receptor. Mutations in α7 integrin result in muscular dystrophy in patients and in animal models, and a loss of α7 integrin in muscle has been shown to predominantly affect the structure and function of the myotendinous junction, where α7β1 integrin is highly expressed (93-95). α7β1 integrin is also expressed at costameres and, similar to α-dystroglycan, can associate intracellularly with cytoskeletal proteins and may contribute to mechanical stability of the sarcolemma (49). Animals lacking both dystrophin and α7 integrin display a much more severe form of muscular dystrophy than animals lacking either protein alone, which suggests that both laminin receptors may be required at the sarcolemma and can potentially compensate for one another (96). Transgenic overexpression of α7 integrin can partially alleviate muscle disease in dystrophin/utrophin double knock-out mice independently of any change in expression of DGC components (50, 97). However, integrins are associated with a number of signaling pathways and can alter AKT and MAP kinase activity in a contraction-dependent manner (98). Therefore, some of the improvement observed when integrins are overexpressed in dystrophic muscle may be in part due to changes in cell signaling rather than direct prevention of sarcolemma damage.

While the integrins may function as laminin-dependent signaling receptors in skeletal muscle (99), whether dystroglycan and the DGC may similarly participate in downstream signal transduction cascades is less clear. Rather than simply serving as a membrane "stabilizer", dystrophin has also been proposed to serve as a sensor for membrane tension and function early in the development of skeletal muscle (100). The authors of this early study proposed that this function of the DGC may be achieved through either interactions with stretch-activated cation channels or through regulation of a downstream signaling mechanism analogous to integrin signaling. Such signaling would likely be important for either growth of differentiated fibers or for mediating proliferation or fusion of satellite cells with regenerating fibers. Interestingly, when the dystroglycan gene was specifically targeted in differentiated skeletal muscle using cre-loxP mediated recombination, the phenotype was surprisingly mild compared to other models of DGC related muscular dystrophy (101). The residual expression of dystroglycan in satellite cells suggested that dystroglycan might have an unappreciated role in this cell type, which could be important for either cell signaling within satellite cells or for interactions with the basal lamina (101). However, how the DGC may be functioning in muscle regeneration is not known.

Given the critical role of laminin in promoting cell survival signaling in muscle and the existence of two possible receptors that might mediate its effects, dissecting the molecular mechanisms of each would certainly be a key advance towards understanding how disruptions can result in muscle disease. A truncated form of laminin-α1 was recently generated that lacks LG domains 4-5 and can prevent dystroglycan binding while retaining the LG domains necessary for laminin interactions with integrins. In contrast to full length laminin-α1, when this truncated laminin-α1 was transgenically expressed in laminin-α2 deficient muscle, several fibers in select muscle groups were still apoptotic (102). Because transgenic expression of full length laminin-α1 can fully rescue the *dy/dy* phenotype, this suggests that interactions between dystroglycan and laminin are also important for cell survival signaling. This is the first study to our

15

knowledge that directly implicates dystroglycan in laminin-dependent survival signaling in muscle *in vivo* and suggests that disruption of dystroglycan-dependent signaling may also contribute to the pathology of muscular dystrophy.

Several studies using primarily cell culture systems have shown that the DGC may be capable of participating as a scaffold for various signal transduction cascades (Fig. 1.2). β-Dystroglycan is capable of binding multiple signaling and adaptor proteins known to be important for myoblast differentiation and cell survival signaling (103-106). The c-terminus of β-dystroglycan contains a proline rich region that can bind Grb2, a well known adaptor protein (103, 107) and may be important for recruiting additional components of the MAP kinase pathway (105). Dystroglycan can also be phosphorylated at tyrosine892 near the c-terminus of β-DG (104, 108) and may function to regulate interactions between dystrophin and caveolin-3 (108, 109). This phosphorylation has also been shown to be adhesion-dependent and enables dystroglycan to recruit several SH2-domain containing proteins, including c-Src and Fyn, to the sarcolemma (110). In an unrelated study, these two kinases were also shown to be associated with the DGC and functioned to phosphorylate the DGC protein syntrophin (106). In the presence of laminin, syntrophin was shown to associate with the DGC and mediate downstream Rac1 signaling that led to increased activity of c-jun. This result was suggested to explain how increased doses of laminin *in vitro* led to a dose dependent increase in cell proliferation in C2C12 myoblasts (111). An increase in Rac1 signaling was also observed following muscle contraction, which suggested that interactions between laminin and dystroglycan are important for enabling the DGC to function as a laminin-dependent mechanoreceptor (106).

The PI3K/AKT pathway is an important signaling pathway essential for muscle cell survival, growth, and hypertrophy that has been suspected to function downstream of the DGC. Disruption of laminin/dystroglycan binding *in vitro* by antibody blockade results in a decrease in PI3K mediated phosphorylation of AKT and is associated with an increase in apoptosis (112).

16

This result may be mediated in part through interactions of the DGC with heterotrimeric G-proteins, which has also been shown to be laminin-dependent. In the presence of laminin, dystroglycan can be immunopreciptated in a complex with Gβγ and PI3K and leads to an increase in AKT activation (113). Therefore, the authors of this study concluded that, in muscular dystrophies where the DGC is disrupted, loss of an interaction with Gβγ can impair PI3K signaling and may contribute to disease pathology. Several studies have demonstrated perturbations in AKT signaling in *mdx* muscle but generally have demonstrated an increase in AKT activity rather than a decrease that would be predicted by these cell culture studies (113, 114). This potentially could be due to increased AKT signaling downstream of α7β1 integrin, which is upregulated in *mdx* muscle (115) and has been shown to be beneficial when overexpressed in dystrophic muscle either directly (116) or downstream of IGF-1 (117-119).

While the potential loss of PI3K/AKT signaling downstream of laminin binding may impact muscle function through its effect on cell survival or growth, skeletal muscle function may also be compromised due to increases in activity of the ubiquitin-proteasome system (UPS)(120). MuRF1 and MAFbx/atrogin-1 are important mediators of skeletal muscle atrophy that function to ubiquitinate target proteins which subsequently results in their destruction by the proteasome (120). In a recent study, decreased phosphorylation of AKT was demonstrated in laminin-deficient muscle and was associated with an increase in total amount of ubiquitinated protein (121). Additionally, pharmacological inhibition of the ubiquitin-proteasome pathway in laminin-deficient animals resulted in an amelioration of several pathological features of the disease. This led the authors to conclude that impaired laminin-dependent signaling in dystrophic muscle may also be impacting the UPS and contributing to muscle disease. Although a number of signaling pathways are known to be important for skeletal muscle growth, the exact contributions of disrupted dystroglycan-dependent or integrin-dependent signaling in dystrophic muscle still needs to be formally addressed.

Direct Role of the Dystrophin-Glycoprotein Complex in Force Transmission

While muscle damage is hypothesized to be important in the pathogenesis and progression of DGC-associated muscular dystrophy, dystrophic muscle displays considerable muscle weakness even in very early stages of the disease. This weakness, expressed as a reduction in specific force normalized to the cross sectional area of the muscle, occurs prior to muscular atrophy and can even be measured in the presence of pseudohypertrophy in early phases of the disease (122, 123). Our studies and the studies of others, in soleus muscle of DGC-deficient muscular dystrophies, demonstrate that the soleus muscle is also weak and this weakness is completely independent of an increased susceptibility of the muscle to damage (39, 48). Therefore, muscle damage that results as a consequence of contraction-induced injury cannot fully explain the muscle weakness in DGC associated muscular dystrophy.

Given the location of the DGC at costameres in muscle, several investigators have hypothesized that the DGC might contribute to "lateral transmission of force" from the sarcomere to the lateral extracellular matrix (15, 124-126). While the concept of longitudinal force generated in sarcomeres and transmitted down myofibrils in muscle to the tendon is well studied, the concept of lateral force transmission is less well appreciated. This concept of lateral transmission of force in muscle was first described in frog muscle by Street in the early 1980's (127). In these studies, force was shown to be transmitted laterally from a single dissected fiber to the fibers flanking it in a muscle fiber bundle, with little or no decrement. Formal proof that the DGC was important in lateral transmission of force in muscle was lacking. Recently, we developed a novel yoke apparatus to directly measure the transmission of force from the muscle laterally to the extracellular matrix and the epimysium (128). Applying this approach to *mdx* muscle, we showed for the first time that loss of DGC function in tibialis anterior muscles of *mdx* mice was sufficient to cause an approximately 40% loss in lateral force transmission in the muscles. While the precise contribution of each of the other components of the cytoskeleton, the costamere, and the extracellular matrix to the lateral transmission of force in muscle remains

to be elucidated, the loss of lateral transmission of force may help explain how loss of the DGC at the lateral membrane contributes muscle weakness and fragility. Furthermore, the lateral transmission of force might be critical in transmitting force around the sites of focal myofiber injury in whole muscle and may help explain the markedly enhanced force deficits caused by lengthening contractions observed in fast muscles of DGC deficient animals (Fig. 1.3).

DGC Function in Non-Muscle Tissues

Although muscle dysfunction is the primary feature of mutations affecting DGC function, this protein complex is expressed in multiple cell types which highlight the potential for important non-mechanical functions of this complex. While the molecular composition of the DGC demonstrates significant heterogeneity within different cell types and cellular compartments, the presence of dystroglycan within the core of the complex appears critical. Dystroglycan is an important contributor to the formation of basement membranes (23) and in many tissues, this function may be important for the attachment of epithelial cells to form structural barriers (129). In dystroglycan-null brain, discontinuities in the glial limitans basement membrane are observed and this function of dystroglycan does not appear to require the C-terminal domain of β-DG which is responsible for interactions with dystrophin and the actin cytoskeleton (130, 131). Within the brain, dystroglycan is expressed in migrating neurons as well as in radially oriented glia (132), and cell-specific targeted deletions of dystroglycan have delineated many of the distinct functions of dystroglycan within the different cell types. While the absence of neuronal dystroglycan impaired long-term potentiation in the hippocampus, the layering of the cerebral cortex during development was unaffected (131). This was in contrast to dystroglycan deletion in both glial and neuronal cells which resulted in laminar disorganization of the cerebral cortex and extensive neuronal/glial heterotopia. Dystroglycan is also expressed in a specialized DGC in Schwann cells, the myelinating glia of the peripheral nervous system. The gene-targeted deletion of dystroglycan in Schwann cells resulted in impaired nerve conduction, altered structure of the

nodes of Ranvier, and impaired localization of voltage-gated sodium channels (133). Notably, these defects are similar to what has been observed in the dy^{2J}/dy^{2J} model of laminin deficiency (26, 134) which highlights the importance of interactions between dystroglycan and laminin in normal neural development.

The DGC is also uniquely expressed at the neuromuscular junction and the initial identification of dystroglycan as an agrin receptor at the postsynaptic membrane suggested a critical function for the DGC in the assembly of neuromuscular junction (135-137). However, acetylcholine receptor clustering during formation of the synapse can occur in the absence of interactions between dystroglycan and agrin (138, 139), and dystroglycan is suspected to have important functions in the maintenance of this structure in differentiated muscle via its function in basement membrane assembly (140).

Glycosyltransferase-Deficient Muscular Dystrophy

The important functions of dystroglycan are underscored by parallel defects observed in both patients with glycosyltransferase-deficient muscular dystrophy and animal models of these diseases. These diseases share a common biochemical defect in the glycosylation of α-dystroglycan and consequently exhibit muscular dystrophy concomitant with a broad range of clinical symptoms in the CNS and PNS (141). Similar to human patients with either Muscle-eye-brain disease (MEB) (142) or Fukuyama congenital muscle dystrophy (FCMD) (143), LARGE^{myd} animals also display abnormal neuronal migration and disorganized cortical layering in the brain as a consequence of impaired dystroglycan function. Peripherally, LARGE^{myd} mice also demonstrate impaired structure of the NMJ (144), reduced nerve conduction velocity, and abnormal nerve myelination (145), defects shared in fukutin-deficient chimeric mice (146).

Although the identification of mutations in glycosyltransferases has highlighted critical functions of dystroglycan and the DGC in non-muscle tissues, the precise mechanism by which impaired dystroglycan causes disease is

unclear.   Although mutations in these enzymes are rare, the identification of a shared biochemical defect and the increasing frequency of reports describing affected patients have led to an expansion in the spectrum of clinical observations. Perhaps most notable of these diseases that result in a significant range of phenotypic severity are those associated with mutations in fukutin-related protein (FKRP). Mutations in FKRP can cause both congenital muscular dystrophy 1C (CMD1C) and a milder limb-girdle muscular dystrophy 2I (LGMD2I) both initially characterized by elevated serum creatine kinase, muscle weakness, hypotonia, and an absence of any brain involvement (147, 148). More recently, mutations in FKRP have also been identified in patients with severe mental retardation and cerebellar cysts (149). Mutations that cause MEB and Walker-Warburg syndrome (WWS) have been identified in protein-O-mannosyltransferase 1 (POMT1)(150), POMT2 (150, 151), and protein-O-mannose β1,2-N-acetylglucosaminyltranferase (POMGnT) (142) and the broad clinical spectrum for each of these diseases have made it difficult to correlate gene mutations with disease manifestations (152). While diseases like WWS, MEB, and FCMD were once thought to be distinct, the identification of a common genetic basis and the wide range of clinical symptoms associated with each disorder has made it difficult to a determine distinct cellular functions of dystroglycan and thus, ways to prevent or delay the disease progression. Several mutations have now been identified in glycosyltransferases and presently, dystroglycan is the only known shared glycosylation target. Therefore, a potential confounding variable that has made it difficult to attribute the pathologic features of these disorders to the impaired dystroglycan function is the potential existence of additional substrates (153, 154). Moreover, the degree to which dystroglycan glycosylation is impaired in skeletal muscle is not a predictable indicator of disease severity or accompanying clinical symptoms (155).

Rationale and Experimental Approach

Several studies have suggested that mutations affecting the DGC result in muscular dystrophy due to the importance of this complex in preserving sarcolemmal integrity. While several reports have demonstrated deficits in force production in dystrophic muscle in response to contraction-induced damage, it is not clear whether these defects are directly due to changes in sarcolemma integrity. Additionally, while it is clear that dystroglycan has important functions in development and function of striated muscle and the nervous system, how impaired function of dystroglycan as an extracellular matrix receptor contributes to glycosylation-deficient muscular dystrophy is poorly understood.

The overall working hypothesis of this thesis is the following: **LARGE-mediated glycosylation is essential for normal skeletal muscle function, the complete loss of which results in muscular dystrophy due to the combined and specific effects on the function of dystroglycan in differentiated skeletal muscle, at the neuromuscular junction, and in motor neurons.** This hypothesis was tested with the following specific aims:

Specific Aim 1). Determine whether the primary defect in LARGE-deficient muscular dystrophy is due to impaired interactions between the sarcolemma and the extracellular matrix which predisposes skeletal muscle to injury during muscle contraction.

The purpose of this set of experiments, described in Chapter 2, was to investigate the functional defects in slow-twitch and fast-twitch muscles of glycosylation-deficient LARGE^{myd} mice. While a partial alteration in glycosylation of dystroglycan in heterozygous Large$^{myd/-}$ mice was not sufficient to alter muscle function, homozygous LARGE$^{myd/myd}$ mice demonstrated a marked reduction in specific force in both soleus and extensor digitorum longus (EDL) muscles. Although EDL muscles from LARGE$^{myd/myd}$ mice were highly susceptible to lengthening contraction-induced injury, LARGE$^{myd/myd}$ soleus muscles surprisingly showed no greater force deficit compared with wild-type soleus muscles even after five lengthening contractions. Despite the lack of increased susceptibility to

injury, LARGE^myd/myd soleus muscles showed loss of dystroglycan glycosylation and laminin binding activity and dystrophic pathology. Interestingly, soleus muscles have a markedly higher sarcolemma expression of β1-containing integrins compared with EDL and gastrocnemius muscles. Therefore, β1-containing integrins may play an important role as alternative matrix receptors in protecting muscles containing slow-twitch fibers from contraction-induced injury in the absence of dystroglycan function. Additionally, this study demonstrates that contraction-induced injury appears to be a separable phenotype from the dystrophic pathology of muscular dystrophy.

Specific Aim 2). Determine whether overexpression of LARGE in skeletal muscle is sufficient to enhance dystroglycan function and protect skeletal muscle from contraction-induced injury.

To further determine the importance of dystroglycan glycosylation in skeletal muscle function, transgenic mice were generated that direct overexpression of LARGE specifically in differentiated muscle fibers. I tested the hypothesis that overexpression of LARGE would result in enhanced dystroglycan glycosylation and function above that observed even in normal muscle. These results, described in Chapter 3, demonstrate that hyperglycosylation of dystroglycan in skeletal muscle of MCK-LARGE transgenic mice coincided with a significantly elevated laminin binding affinity compared to that of wild-type muscles. Additionally, fast-twitch skeletal muscle from transgenic mice demonstrated enhanced protection from contraction-induced injury such that force deficits following injury were significantly less when compared to muscle from wild-type mice. This suggests that hyperglycosylation and enhanced function of dystroglycan via increased activity of the enzyme LARGE may be therapeutic not only in inherited glycosylation-deficient muscular dystrophy but also in acquired diseases or disability resulting from muscle injury.

Specific Aim 3). Determine how loss of LARGE-mediated glycosylation of dystroglycan in neural tissues contributes to muscle dysfunction and disease progression in LARGE^myd mice.

Although dystroglycan glycosylation is essential for normal skeletal muscle function, the nature of LARGE-mediated glycosylation of dystroglycan in non-muscle tissues is unclear. Dystroglycan is expressed ubiquitously in the brain and in the peripheral nervous system, but the specific mechanistic role of dystroglycan glycosylation in these tissues and how it affects muscle function and motor coordination has not been determined. Chapter 4 describes experiments where MCK-LARGE transgenic animals were crossed to LARGE$^{myd/myd}$ mice to produce animals that demonstrate impaired dystroglycan receptor function in all tissues except differentiated muscle fibers. This resulted in the successful rescue of skeletal muscle function as evidenced by a reduction in serum creatine kinase, restoration of normal muscle structure, suppression of muscle degeneration, and an increase in both specific force and protection from contraction-induced injury. Additionally, structural defects observed at the neuromuscular junction in LARGE$^{myd/myd}$ mice were corrected in transgenic LARGE$^{myd/myd}$ animals and correspondingly were associated with the rescue of the neurotransmission and nerve conduction deficits observed in LARGE$^{myd/myd}$ animals. These data suggest that skeletal muscle weakness observed in LARGE$^{myd/myd}$ mice is the result of combined defects in both muscle and nerve function and that neuronal defects associated with impaired dystroglycan function may be compounded by primary deficits in muscle function that affect communication at the neuromuscular synapse.

The final chapter will summarize the main findings of this thesis and describe how they have expanded upon what we previously knew about dystroglycan function. Finally, this thesis will conclude with some of the remaining questions regarding dystroglycan function and how a complete understanding of both glycosylation dependent and independent functions of dystroglycan in various tissues where the DGC is expressed may be important for generating better therapeutics and potential cures for muscular dystrophy.

Acknowledgements
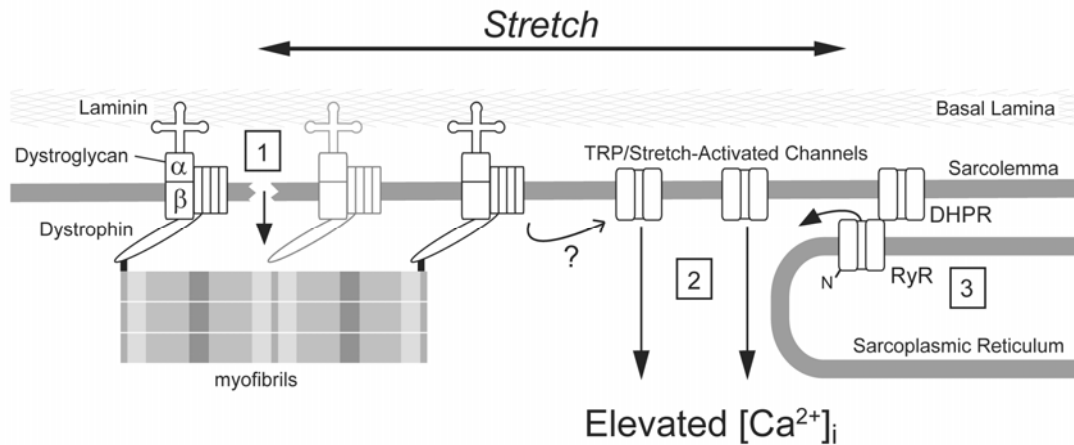
Figure 1-1) Sources of calcium entry in dystrophic muscle.
(1) Disruptions to the DGC can result in instability of the sarcolemma that permits calcium entry through membrane tears when the sarcolemma is stretched during lengthening muscle contractions. (2) The activity of TRP and other stretch-activated channels have been shown to be increased in dystrophin-deficient muscle and their inhibition *in vivo* can improve dystrophic pathology. (3) The ryanodine receptor has recently been shown to be hypernitrosylated in *mdx* muscle which may result in an increased leak of calcium from the sarcoplasmic reticulum.
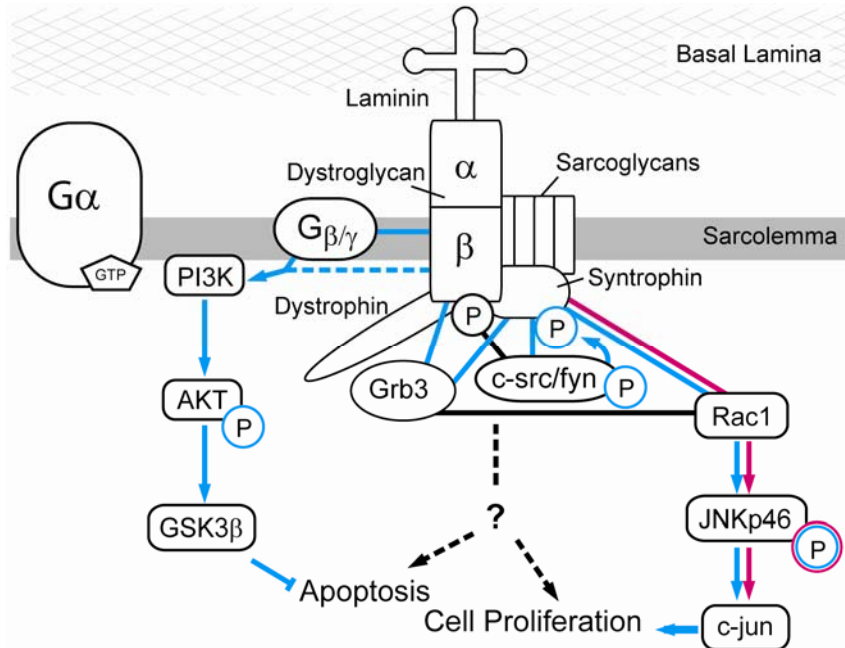
Figure 1-2) Potential signaling pathways disrupted in muscular dystrophy.
The dystrophin-glycoprotein complex may participate in laminin-dependent
signaling in skeletal muscle. Interactions shown in blue indicate interactions that
have been shown to be increased when laminin is bound to dystroglycan.
Interactions in pink indicate those that have been shown to be increased
following a muscle contraction protocol. Since the phosphorylation of β-
dystroglycan can bind a number of other SH2-domain containing proteins and
can also interact with Grb2, it may participate in additional signal transduction
cascades that have not yet been identified. It is important to remember that in
many cases, these interactions have been studied in cell culture systems and the
relevance of these interactions in muscle *in vivo* has not been extensively
characterized.
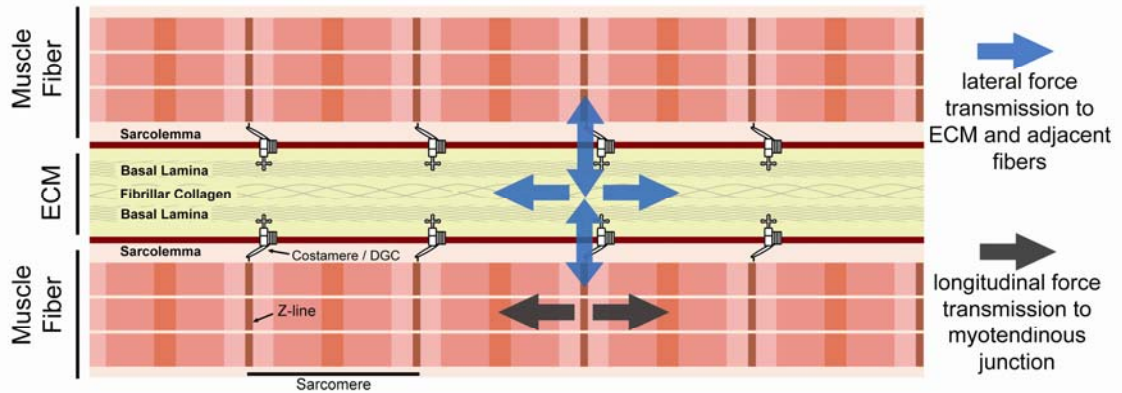
Figure 1-3) Lateral force transmission in skeletal muscle.
The dystrophin-glycoprotein complex transmits forces laterally at costameres in muscle. Longitudinal forces generated in sarcomeres are transmitted down myofibrils in muscle, and forces are also transmitted laterally to the ECM and neighboring muscle fibers at costameres, at least in part through the dystrophin-glycoprotein complex (DGC). Recent data showing a loss of lateral force transmission in dystrophin-deficient muscle explains how the reduction or improper assembly of the DGC at the lateral membrane may contribute to overall muscle weakness and fragility.

CHAPTER 2

Soleus muscle in glycosylation-deficient muscular dystrophy is protected from contraction-induced injury

ABSTRACT

The glycosylation of dystroglycan is required for its function as a high affinity laminin receptor, and loss of dystroglycan glycosylation results in congenital muscular dystrophy. The purpose of this study was to investigate the functional defects in slow and fast muscles of glycosylation-deficient LARGE^{myd} mice. While a partial alteration in glycosylation of dystroglycan in heterozygous LARGE$^{myd/+}$ mice was not sufficient to alter muscle function, homozygous LARGE$^{myd/myd}$ mice demonstrated a marked reduction in specific force in both soleus and EDL muscles. Although EDL muscles from LARGE$^{myd/myd}$ mice were highly susceptible to lengthening contraction-induced injury, LARGE$^{myd/myd}$ soleus muscles surprisingly showed no greater force deficit than wild-type soleus muscles even after 5 lengthening contractions. Despite no increased susceptibility to injury, LARGE$^{myd/myd}$ soleus muscles showed loss of dystroglycan glycosylation and laminin binding activity, and dystrophic pathology. Interestingly, we show that soleus muscles have a markedly higher sarcolemma expression of β1 containing integrins compared with EDL and gastrocnemius muscles. Therefore, we conclude that β1 containing integrins play an important role as matrix receptors in protecting muscles containing slow-twitch fibers from contraction-induced injury in the absence of dystroglycan function, and that

contraction-induced injury appears to be a separable phenotype from the dystrophic pathology of muscular dystrophy.

INTRODUCTION

The muscular dystrophies comprise a heterogeneous group of genetic diseases characterized by progressive degeneration of myofibers, severe weakness, and impaired skeletal muscle function (2). Although these diseases can arise from a single mutation in any of several known causative genes, a subset of congenital muscular dystrophies known as the "dystroglycanopathies" are caused by distinct mutations that result in loss of function of the membrane protein, dystroglycan, as an extracellular matrix receptor (156). Mutations have been identified in several genes that appear to encode glycosyltransferases (157). While specific glycan structures have been proposed to be important for the function of dystroglycan as an extracellular matrix receptor (158), the precise pathway by which each of these glycosyltransferases functions is unclear. In each of the dystroglycanopathies, the glycosylation of dystroglycan is reduced or lost completely (21). The reduction or loss of the glycosylation of dystroglycan impairs the function dystroglycan, which is thought to lead to the observed phenotypes in the many tissues where dystroglycan is expressed.

Dystroglycan is transcribed from the *DAG1* gene and the protein is cleaved post-translationally into α and β subunits (159) which then remain associated with one another at the cell membrane (160). The β-dystroglycan (β-DG) subunit contains a single transmembrane domain and associates with dystrophin at its intracellular C-terminus. In contrast, the extracellular α-DG subunit localizes to the exterior of the sarcolemma due to its non-covalent association with β-DG. The extensive glycosylation enables α-DG to function as a receptor for several ligands in the extracellular matrix (ECM) that include laminin, agrin, & perlecan (12, 137, 161). The interaction of α-DG with laminin appears to be critical for the normal functioning of skeletal muscles in that both reduction of α-DG glycosylation leading to loss of laminin binding affinity, and null mutations in laminin α2, leading to loss of the predominant laminin expressed in

30

skeletal muscle, result in severe congenital muscular dystrophy (21, 27). Dystroglycan is an essential component of the dystrophin-glycoprotein complex (DGC) in muscle, a multi- subunit complex that links the intracellular actin cytoskeleton to the extracellular matrix (ECM) through the interactions of β-DG with dystrophin and α-DG with the ECM (162, 163). Null mutations in several components of the DGC, including dystrophin, that disrupt DGC expression also cause several forms of muscular dystrophy in humans.

While the complete function of the DGC in muscle is unknown, dystroglycan is hypothesized to provide a mechanical link that helps maintain integrity of the sarcolemma during cycles of contraction and relaxation (12). Evidence that the DGC plays a role in stabilizing the sarcolemma during contraction comes from observations that dystrophic muscle, with genetic disruption of the DGC, is highly susceptible to contraction-induced injury (164). The *mdx* mouse contains a null mutation in dystrophin, and *mdx* muscle demonstrates an increased propensity for muscle injury as measured by a significantly elevated force deficit when maximal force production is measured before and after a series of lengthening contractions (39). Additionally, increased force deficit in *mdx* muscle following injury is associated with an increase in membrane permeability when lengthening contractions are performed in the presence of a membrane impermeant dye (165). Such defects at the membrane are thought to either directly or indirectly contribute to the observed degeneration of myofibers that ultimately results in impaired muscle function and weakness. The increased fragility of the sarcolemma in *mdx* mice is also supported by the observation that treatment with membrane sealants *in vitro* leads to a decrease in the magnitude of injury produced by repetitive isometric contractions (166). Therefore, susceptibility to contraction-induced injury appears to be a hallmark feature in many forms of DGC-related muscular dystrophy. Although the presence of dystroglycan is hypothesized to be essential for normal function of the DGC, the role of dystroglycan glycosylation which specifically regulates its function as a matrix receptor in the preservation of sarcolemma integrity during lengthening contractions is not as well defined. Therefore, our goal was to study

the functional deficits in a mouse model of glycosylation-deficient muscular dystrophy to provide further insights into the role of the interaction of dystroglycan with the ECM in skeletal muscle function.

The LARGE^{myd} mouse is a spontaneously arising mouse model of muscular dystrophy that contains an autosomal recessive mutation in the gene that encodes the glycosyltransferase LARGE and consequently demonstrates reduced glycosylation of α-dystroglycan (167, 168). Although enzymatic activity for LARGE has not been confirmed, it contains two predicted catalytic domains (167) and has been shown to directly bind to the N-terminus of dystroglycan (169). Additionally, overexpression of LARGE in myoblasts from patients with defects in other glycosyltransferases restores dystroglycan function (170). Although several studies have identified specific glycan structures that may be necessary for dystroglycan function (171-174), the precise glycan on dystroglycan that is transferred by LARGE and its role in normal skeletal muscle function is presently unknown.

While mice provide important genetic models of human disease, the composition of fiber types in muscles of humans and mice differ significantly. The limb muscles most commonly studied in dystrophic mice, the extensor digitorum longus and tibialis antierior, are composed almost exclusively of fast-twitch fibers with an approximately equal proportion of glycolytic and oxidative fibers. However, the soleus muscle is one of the few skeletal muscles in the mouse that contains a large percentage of slow fibers (>50% of the total) (175). In that regard it is interesting to note that many human skeletal muscles involved in locomotion are similarly composed of a mixture of both fast-twitch and slow-twitch fiber types. Mouse soleus muscle was also shown to bear a greater molecular resemblance to human muscle and was therefore more representative of human muscle than other commonly studied mouse muscles such as the EDL and gastrocnemius muscle which are composed of predominantly fast-twitch fibers (176). Muscle fiber types are determined by activation of distinct genetic programs that regulate both myosin isoform composition and oxidative capacity.

Whether the complex glycosylation pathway necessary for dystroglycan glycosylation and function, or the impact of the loss of its function is similar in both fast and slow muscle fiber types is not known. Therefore, we compared the contractile defects associated with loss of LARGE-dependent glycosylation of dystroglycan in fast and slow muscles. In doing so, we identified differences in expression of key matrix receptors between fast-twitch and slow-twitch fibers that might underlie fiber type specific differences in the susceptibility to contraction-induced injury of the skeletal muscles of the dystrophic LARGE[myd] mouse. Furthermore, while susceptibility to contraction-induced injury is often considered a hallmark of DGC related muscular dystrophies, our data shows that dystrophic pathology and muscle weakness is a clearly separable phenotype from contraction-induced injury in muscles containing a mixture of fast and slow muscle fibers.

METHODS

Animals. All animals were housed in a specific pathogen free (SPF) barrier facility in the Unit for Laboratory Animal Medicine at the University of Michigan, and all procedures were approved by the University of Michigan Committee for the Use and Care of Animals. LARGE[myd] and WT littermates used for all experiments were aged 12-36 weeks and taken from a maintained colony. Sprague-Dawley rats were received from Charles River Laboratory.

Measurement of Contractile Properties. Contractile measurements were performed as previously described (177). For *in vitro* measurements, the EDL or soleus muscle was isolated from anesthetized mice. A 5-0 silk suture was tied to the proximal and distal tendons. The muscle was placed in Krebs mammalian Ringer solution maintained at 25°C and bubbled continuously with 95% O_2 and 5% CO_2 to stabilize pH at 7.4. One tendon was tied to a servo motor (Aurora Scientific, model 300) and the other to a force transducer (Kulite Semiconductor, model BG-50). The muscle was stimulated by square wave pulses delivered between two platinum electrodes connected to a high-power biphasic current stimulator (Aurora Scientific, model 701B). An IBM–compatible personal

33

computer and custom-designed software (LabVIEW 7.1, National Instruments, Austin, TX) controlled electrical pulse properties and servomotor activity and recorded data from the force transducer. The voltage of pulses was increased, and optimal muscle length (L_o) was subsequently adjusted to the length that resulted in maximum twitch force (Brooks and Faulkner, 1988). The L_o was measured with digital calipers. Muscles were held at L_o and subjected to trains of pulses to generate an isometric contraction. Pulse trains were 300 ms for EDL muscles and 900 ms for soleus muscles. Stimulus frequency was increased until maximum isometric force (P_o) was achieved (Brooks and Faulkner, 1988). The muscle was weighed and the mean cross sectional area was estimated by dividing the muscle wet mass by the product of fiber length and the density of mammalian muscle $1.06g/cm^3$. Specific force (sP_o) was determined by dividing P_o by the cross-sectional area (CSA).

Muscle Injury Protocol. Following measurement of maximum twitch force and P_o, muscles were stimulated and held at L_o for 100 ms for EDL muscles and 300ms for soleus muscles to allow muscles to develop P_o. Following the isometric contraction, muscles were stretched through a 30% strain relative to L_f. The velocity of the stretch was 1 L_f/s. The total time of stimulation was 400 ms for EDL muscles and 600 ms for soleus muscles. Following stimulation, muscles were returned to L_o, then were subjected to four additional 30% stretches, each with 12 seconds in between, for a total of 5 stretches per muscle. The muscle was allowed to rest one minute and P_o was measured. The force deficit was calculated as the decrease in P_o observed after the stretch protocol as a percentage of P_o prior to the protocol.

Membrane Preparation, SDS-PAGE, and Western Blotting. Mouse and rat EDL and soleus muscles were dissected from anesthetized animals, the myotendinous regions were removed, and samples were immediately frozen on dry ice until further processing. Individual (rat) or pooled (mouse) samples were homogenized in ice-cold homogenization buffer containing 20 mM sodium pyrophosphate, 20 mM sodium phosphate (monobasic), 1 mM magnesium

chloride, 0.303 M sucrose, 5 mM EDTA, pH 7.1. Samples were subjected to a low speed (10,000 g) and high speed (45,000 g) spin in order to isolate the membrane fraction and final pellets were resuspended in Buffer I (0.303 M sucrose, 20 mM Tris-Maleate, pH 7.0) and quantified using the Biorad Bradford assay. All buffers contained protease inhibitors (0.5 µg/ml Pepstatin A, 2 kallikrein inhibitor units/ml Aprotinin, 1 ug/ml Leupeptin, 0.4 mM PMSF, 0.6 mM Benzamidine). Samples were separated using 3-15% gradient polyacrylamide gels and were transferred via Western blot to polyvinylidene fluoride membrane (Millipore). After blocking membranes in TBS + 0.05% Tween 20 (TBS-T) + 5% non-fat dry milk for 1 hour, membranes were incubated with primary antibody for at least 2 hours up to overnight. Primary antibodies included rabbit polyclonal antibodies to β-dystroglycan and α7 integrin (H40) (Santa Cruz), α5 integrin and β1 integrin (Chemicon/Millipore), laminin (L-9393, Sigma), mouse monoclonal antibody to dysferlin (Hamlet, Novacastra), slow myosin (A4.840, DSHB, Iowa City, IA), and glycosylated dystroglycan (IIH6, gift from Dr. Kevin Campbell), rat monoclonal antibodies to α6 integrin (eBiosciences) and β1 integrin (BD Pharmingen). Following 3 x 10 minute washes in TBS-T, membranes were incubated with secondary antibody conjugated to HRP for 1.5 hours. Membranes were washed 3 x 10 minutes with TBS-T and incubated in chemiluminescent substrate (Thermo Scientific) 1-2 minutes prior to exposure. Membranes used for re-probing were washed in TBS and incubated in a stripping buffer (TBS + 2% SDS + 7ul/ml β-mercaptoethanol) for 30 minutes. Membranes were rinsed several times in TBS and were re-blocked for 1-2 hours in TBS-T + 5% NFDM prior to a second round of antibody staining.

Solid-Phase Laminin Binding Assays. WGA-purified skeletal muscle samples were diluted in TBS and coated onto 96-well microplates at a final concentration of 0.1ug/well overnight. After washing in binding buffer (TBS + Ca^{2+}), plates were coated with a blocking buffer of 3% bovine serum albumin (BSA) in binding buffer for 1 hour. Wells were aspirated, rinsed in binding buffer, and replaced with dilutions of 0.02 - 20 nM laminin (Invitrogen) and diluted in blocking buffer with and without the presence of 20 mM EDTA for 2 hours. After 4 washes in

binding buffer, wells were replaced with anti-laminin antibody (L-9393) diluted 1:5000 in blocking buffer and incubated at room temperature for 1.5 hours. Wells were aspirated, washed 4 times in binding buffer, and replaced with goat anti-rabbit IgG HRP diluted 1:5000 in blocking buffer for 1 hour at room temperature. Again, plates were washed 4 times in binding buffer. For developing, 100ul of OPD/CPB was added to each well and incubated 5-25 minutes. The reaction was stopped with 50ul 2M H_2SO_4 and plates were read at 495nm.

Immunofluorescence & Histology. Soleus, EDL, and gastrocnemius muscles were dissected together from anesthetized mice, mounted in OCT and immediately frozen in liquid nitrogen cooled isopentane. Frozen samples were cut into 8 µm cross sections using a cryostat and stored at -80° until the slides were later used for immunofluorescent microscopy or stained with either hematoxylin and eosin or 0.1% Sirius Red/0.1% Fast Green in picric acid. For immunohistochemical experiments, slides were rehydrated with PBS and blocked 1-2 hours in 5% BSA in PBS. Slides were then incubated with primary and secondary antibody for 1-2 hours each with 4 x 5 minutes washes of PBS in between incubations. Final slides were mounted in Permafluor (Invitrogen) and imaged with an Olympus BX-51 fluorescence microscope.

RESULTS

Partial loss of dystroglycan glycosylation in heterozygous LARGE[myd/+] mice. Homozygous LARGE[myd/myd] mice have previously been described as having a complete loss of alpha dystroglycan glycosylation as demonstrated by the loss of reactivity with the glycosylation specific antibody IIH6 (21). Western blot analysis from skeletal muscle whole lysates (WT, LARGE[myd/+], and LARGE[myd/myd]) revealed altered glycosylation in mice heterozygous for the LARGE mutation as indicated by a slight reduction in the molecular weight of α-dystroglycan (Fig. 2.1A). Partial loss of α-dystroglycan glycosylation has also been detected in human patients with mild limb-girdle muscular dystrophy (156). Although heterozygous LARGE[myd/+] mice did not show pathology consistent with muscular dystrophy (Fig. 2.1B, Fig. 2.6), the potential functional effects of the

36

partial glycosylation of dystroglycan remain unknown. Therefore, the contractile function of heterozygous mice was studied to determine whether the altered glycosylation due to the presence of a single functional LARGE gene was sufficient to cause changes in skeletal muscle function. Although absolute and specific forces were both reduced in the soleus and EDL muscles of homozygous LARGE$^{myd/myd}$ mice, heterozygous mice exhibited no significant differences when compared to wild-type littermates (Fig. 2.2A, B). In response to muscle injury induced by two lengthening contractions of 30% strain, force deficits measured in heterozygous mice displayed no differences from WT mice for either the EDL muscle (17%, WT; 18%, heterozygous) or the soleus muscle (22%, WT; 21% heterozygous) (Fig. 2.2C). Therefore, while the loss of a single functional LARGE gene was sufficient to reduce the glycosylation of dystroglycan in skeletal muscle slightly, this alteration was not sufficient to cause a change in muscle function or susceptibility to contraction-induced injury following lengthening contractions.

Effects of complete loss of functional dystroglycan glycosylation on muscle function are fiber-type specific. The complete loss of dystroglycan glycosylation in LARGE$^{myd/myd}$ mice leads to dystrophic pathology (Fig. 2.1B, Fig. 2.6), and results in a profound reduction in both absolute and specific force production (Fig. 2.2A, B). The maximum specific force measured for muscles from homozygous LARGE$^{myd/myd}$ mice was only 67% of that measured in EDL muscle and 66% of that measured in soleus muscle of WT mice indicating that maximal force production in skeletal muscle requires the presence of LARGE-mediated glycosylation of dystroglycan. Additionally, a significantly elevated force deficit of 36% was measured in the EDL muscle of homozygous LARGE$^{myd/myd}$ mice after two lengthening contractions which was nearly 2-fold greater than the force deficit observed in EDL muscles from WT and heterozygous mice (Fig. 2.2C). Interestingly, the degree of injury due to lengthening contractions in muscles containing functionally impaired dystroglycan seemed to be fiber type specific. A force deficit of 22% measured in the soleus muscle of homozygous LARGE$^{myd/myd}$ mice following two lengthening

37

contractions was not significantly different from that of WT mice. To further stress the muscle we performed three additional lengthening contractions and after a total of five lengthening contractions, force deficits measured in EDL muscles from WT and heterozygous mice were not significantly different (35%, WT; 36%, heterozygous) nor were those in soleus muscles (26%, WT; 30%, heterozygous) (Fig. 2.2D). While EDL muscles from homozygous LARGE$^{myd/myd}$ mice showed a further increase in force deficit to 88% (compared to 32% in WT), the 30% force deficit measured in the soleus of LARGE$^{myd/myd}$ mice after five lengthening contractions was still not significantly different from WT measurements. These results suggest that while the complete loss of functional glycosylation of dystroglycan in LARGE$^{myd/myd}$ skeletal muscle results in reduced maximal force production in both soleus and EDL muscle, the soleus muscle of LARGE$^{myd/myd}$ mice is protected completely from the high degree of susceptibility to lengthening contraction-induced injury observed in the EDL muscle.

LARGE$^{myd/myd}$ soleus is dystrophic and demonstrates reduced glycosylation of dystroglycan concomitant with a reduction in laminin binding activity. Because susceptibility to contraction-induced injury in skeletal muscle is considered to be a hallmark of DGC-related muscular dystrophies, we sought to determine whether the soleus of LARGE$^{myd/myd}$ mice was also protected from dystrophy. Hematoxylin and eosin staining of gastrocnemius, EDL, and soleus sections of LARGE$^{myd/myd}$ mice revealed that similar to gastrocnemius and EDL muscles, the soleus contained several features of muscular dystrophy including a high percentage of fibers with internalized nuclei, heterogeneity in fiber size, and infiltration of mononuclear cells (Fig. 2.1B).   Additionally, all three muscles isolated from LARGE$^{myd/myd}$ animals contained increased Sirus Red staining indicative of interstitial fibrosis (Fig. 2.6).

An alternative hypothesis for the lack of susceptibility to contraction-induced injury in the soleus muscle of LARGE$^{myd/myd}$ animals was that perhaps a partial glycosylation of dystroglycan is preserved in soleus muscle through the actions of either the LARGE homologue, LARGE2, or alternative

glycosyltransferases (178). In support of this hypothesis, we observed that although α-dystroglycan isolated from membrane preparations of rat EDL and soleus muscles ran as a broad smear, the range in the molecular weight of α-dystroglycan was different between the two muscles (Fig. 2.4A). Since the soleus muscle in the rat is composed almost exclusively of slow muscle fibers, this suggests potential fiber-type specific differences in glycosylation of dystroglycan. However, immunofluorescent staining of LARGE$^{myd/myd}$ soleus and gastrocnemius muscles confirmed that although dystroglycan is retained at the sarcolemma of fibers in the soleus, the functional glycosylation of α-dystroglycan, as indicated by reactivity with IIH6, was lost completely (Fig. 2.3A). Despite the loss of glycosylated dystroglycan in the soleus, laminin was still detected at comparable levels in the ECM. Using WGA-purified glycoproteins isolated from dissected muscles, we also show that soleus muscles from LARGE$^{myd/myd}$ homozygous mice have markedly reduced high-affinity laminin binding activity similar to glycoprotein preparations isolated from EDL muscles (Fig. 2.3B) and gastrocnemius (not shown), and similar to what has been previously reported in pooled hindlimb muscles from LARGE$^{myd/myd}$ mice (21). Similar reductions in high affinity laminin binding activity are also observed in dystroglycan gene-targeted mice demonstrating that this high affinity activity reflects dystroglycan-dependent activity (101). Together, these results demonstrate that the protection of the soleus muscle from contraction-induced injury in LARGE$^{myd/myd}$ mice is not due to residual dystroglycan glycosylation and function. Therefore, additional experiments were aimed at determining whether there were differences in expression of other laminin receptors between the two muscle types that might contribute to the protection from contraction-induced injury in soleus muscles of LARGE$^{myd/myd}$ mice.

β1 containing integrins are highly expressed in the sarcolemma of soleus muscles. Integrins, which exist as dimers of alpha and beta subunits, are ECM receptors that are expressed in multiple tissues (179). The α7β1 integrin is a laminin receptor expressed in skeletal muscle and loss of α7 integrin expression has been associated with impaired muscle function and myopathic diseases in

mouse models and in human patients (49). To overcome the limitation in size of mouse EDL and soleus muscles and the need for high amounts of tissue in order to isolate sufficient quantities of membrane preparations, we first utilized muscle from the rat. Because the soleus muscle of the rat is almost exclusively composed of slow muscle fibers compared to the mouse which is approximately 50-60% slow fibers, this offered a higher contrast in fiber type distribution when comparing the soleus muscles to the EDL muscles and more easily allowed us to determine fiber type specific expression of several proteins involved in sarcolemma integrity. In order to identify the alternative extracellular receptors that are expressed primarily at the lateral membrane, the tendons and myotendinous regions of the muscle were trimmed away. In rat, membrane associated $\beta1$ integrin expression was markedly higher in soleus than EDL muscle (Fig. 2.4A). We also examined the expression of dysferlin, a protein involved in membrane repair and associated with limb-girdle muscular dystrophy but did not see a difference in its expression between EDL and soleus muscle in either the rat (Fig. 2.4A) or the mouse (not shown). Consistent with these results in rat muscle, separately pooled soleus and EDL muscle from mice also demonstrated a much higher expression of $\beta1$ integrin in the soleus than in the EDL muscle (Fig. 2.4B). Additionally, immunofluorescence microscopy performed on gastrocnemius, soleus, and EDL muscle sections from mice demonstrated a higher degree of staining for $\beta1$ integrin at the sarcolemma in soleus muscle fibers compared to fibers in the gastrocnemius and EDL muscle (Fig. 2.4C, D).

In skeletal muscle, the function of $\beta1$ integrin changes during development due to differential pairing with distinct alpha integrin isoforms. During development, $\beta1$ integrin dimerizes with $\alpha5$ integrin to form a receptor for fibronectin. However, in adult muscle, $\alpha5$ integrin expression is down-regulated and replaced with $\alpha7$ integrin which, when dimerized with $\beta1$ integrin, forms a laminin receptor that is predominantly localized to the myotendinous junction (180). Using both a WGA-purified fraction (Fig. 2.5A) and a microsome fraction (Fig. 2.5B) isolated from EDL and soleus muscles of rats, a heavy chain specific

α7 integrin antibody detected bands at both 100 kDa and 120 kDa. The 120 kDa isoform is reported to be a glycosylated form of α7 integrin (181) and in support of this conclusion, only this isoform was detected in the WGA-purified fraction. While no differences were observed in expression levels of the 100 kDa α7 integrin isoform in microsome fractions from either EDL or soleus, α7 integrin detected in the WGA-purified fraction appeared to be more highly expressed in soleus muscle. Additionally, immunofluorescent staining detected α7 integrin expression predominantly at the sarcolemma in soleus in a pattern consistent with previously shown β1 expression (Fig. 2.4C, 2.5C). To determine whether it may be possible that β1 integrin combines with other alpha integrins in the soleus to form receptors for additional extracellular ligands, Western blots and/or soleus/gastrocnemius muscle cross sections were also stained for α5 and α6 integrin. The α6 integrin was not expressed at detectable levels in either fibers of the soleus or gastrocnemius of WT muscle and instead appeared to be concentrated only in blood vessels (not shown). The α5 integrin appeared to be expressed in both the EDL and soleus muscles but levels were lower than those in neonatal muscles and did not differ between the two muscle types (Fig. 2.5A).

DISCUSSION

Our results demonstrate for the first time that, although the skeletal muscles of LARGE[myd/myd] mice are weaker than WT controls, the soleus is protected from contraction-induced injury despite the loss of the glycosylation dependent function of dystroglycan as an extracellular matrix receptor. We also show that soleus muscle has markedly higher expression of β1 integrin compared to other limb muscles composed of fast muscle fibers. This observation suggests that α7β1 integrin may be playing an important role as an alternative matrix receptor capable of protecting the sarcolemma from contraction-induced injury in slow muscle. Finally, although susceptibility to contraction-induced injury is often considered a hallmark of DGC-related muscular dystrophies, our results indicate that a susceptibility to contraction-

induced injury is not required, and that muscle weakness and dystrophy can occur in the soleus of LARGE$^{myd/myd}$ homozygous mice in its absence.

The exact mechanism by which the DGC protects muscle from injury following lengthening contractions and the relationship of muscle injury to the development of dystrophy is still unclear. In whole muscle, lengthening contractions may produce injury by straining sarcomeres with pre-existing heterogeneity in length within muscle fibers, or strain lateral connections from fiber to fiber resulting in fiber damage and damage to sarcomeres (182). Alternatively, lengthening contractions may impart large mechanical strain on connections of muscle fibers to the matrix, disrupting those connections that stabilize the sarcolemma. Recently, Han et al. demonstrated that dystroglycan is important for enabling skeletal muscle fibers to bind the sarcolemma tightly to the basal lamina, the loss of which results in muscle that is prone to contraction-induced sarcolemma injury (22). However, our results show that this is not universally true for all muscles. The loss of glycosylation and function of dystroglycan is not sufficient to result in increased susceptibility to contraction-induced injury in LARGE$^{myd/myd}$ soleus muscle. Interestingly, the soleus muscles of LARGE$^{myd/myd}$ mice still have pathological features of muscular dystrophy including increased variability of fiber size and centrally nucleated fibers consistent with ongoing muscle degeneration and regeneration. The soleus showed marked deficits in specific force and contained dystroglycan that lacked functional glycosylation and was unable to bind extracellular laminin. This implies that contraction-induced injury is not a primary cause for dystrophy and weakness observed in this muscle. While dystroglycan may have an important role in mechanically stabilizing fast-twitch muscle against contraction-induced injury, our results suggest that dystroglycan may also have additional essential functions in muscle that when impaired significantly contribute to the dystrophic phenotype and weakness in muscles containing slow-twitch fibers.

Several groups have hypothesized that defects in cell signaling might contribute to the dystrophic pathology but the physiological significance has not

42

been demonstrated. Muscles isolated from laminin-211 deficient *dy/dy* mice are dystrophic and weak but have been shown to be no more susceptible to contraction-induced injury when compared to wild-type animals (51). *Dy/dy* animals have increased activation of apoptotic death pathways and it was suggested that altered signaling may contribute to the dystrophic pathology in laminin-211 deficiency (84, 87). Consistent with this hypothesis, several studies have demonstrated that defects in the specific interaction of laminin with dystroglycan results in altered cellular signaling important for normal cell growth. The disruption of the laminin/dystroglycan interaction *in vitro* using blocking antibodies against α-dystroglycan resulted in altered AKT and GSK-3β activation and an increase in apoptotic cell death (112). More recently, the DGC has also been shown to interact with subunits of heterotrimeric G proteins in a laminin dependent manner (113) which has been suggested to underlie the altered Ca^{2+} homeostasis observed in several forms of muscular dystrophy. While the precise mechanism by which defects in dystroglycan result in impaired signaling is unclear, our results suggest that such signaling may be physiologically relevant and may have significant implications for other dystrophies associated with genetic disruption of the DGC that lead to a concomitant reduction in dystroglycan expression at the sarcolemma.

Previous studies have shown that lengthening contractions of soleus muscles of *mdx* mice fail to cause higher force deficits or sarcolemma damage when compared to WT mice (36). While this has led to the predominant use of muscles composed primarily of fast fibers such as EDL in studying contraction induced injury in the *mdx* mouse, an underlying molecular mechanism that accounts for the lack of increased contraction-induced injury in soleus muscle has not yet been elucidated. Muscle impairment due to the loss of dystrophin expression in *mdx* mice can be compensated by the upregulation of utrophin and differences in utrophin expression might be a reasonable hypothesis to explain the lack of susceptibility of the *mdx* soleus muscles to contraction-induced injury. However, in the present study, the LARGE[myd/myd] sarcolemma contains normal expression of all DGC components including dystrophin and only the post-

43

translational processing of α-dystroglycan and its laminin binding activity is compromised (22). Therefore, we hypothesized that differences in expression of other extracellular matrix receptors between the EDL and soleus muscles may be responsible for the protection against contraction-induced injury. In addition to dystroglycan, α7β1 integrin has also been shown to be an important laminin receptor essential for normal skeletal muscle function and mutations have been associated with muscular dystrophy in patients (93) and in animal models. Although the β1 integrin knockout mouse is embryonically lethal due to its essential role in embryogenesis (183, 184), the tissue specific loss of α7 integrin in skeletal muscle results in myopathy and initial experiments demonstrated that α7 integrin-deficient animals exhibited a myopathy predominantly affecting the myotendinous junction (94). While most of the α7 integrin null skeletal muscle evaluated exhibited very few histological characteristics of muscular dystrophy, the authors noted that only the soleus showed histological signs of myopathy. These results support our observation that the α7β1 receptor, being more highly expressed in soleus, functions as an important laminin receptor along the lateral membrane of skeletal muscle fibers that can function in the prevention of contraction-induced injury specifically in slow-twitch fibers.

Han et al. recently showed that loss of either the DGC or α7β1 integrin can result in deficits in force production but that only the loss of the DGC resulted in susceptibility to contraction-induced injury (22). This was based on the observation that although a greater force deficit was observed in EDL muscles taken from LARGE$^{myd/myd}$ animals when compared to WT controls, no such difference existed in EDL muscles from α7 integrin-null animals. Although the soleus was not studied in these experiments, based on our results we would hypothesize that α7 integrin-null mice would indeed have a measured force deficit following contraction-induced injury due to the increased expression and requirement of α7β1 integrin in this muscle.

Since our data indicates that soleus muscles of LARGE$^{myd/myd}$ mice are protected from contraction-induced injury and that this is correlated with

increased α7β1 integrin expression in soleus muscle, we would predict that increased expression of α7β1 integrin in fast muscles might also provide protection against contraction-induced injury. While this hypothesis remains to be formally tested in LARGE[myd/myd] mice, previous studies have shown that transgenic overexpression of integrins in dystrophin-deficient mouse models can partially alleviate the dystrophic pathology. Expression of rat α7 integrin in *mdx:utr* [-/-] animals resulted in an improvement in muscle integrity, a decrease in mononuclear cell infiltrate, and correlated with an increase in lifespan (50). Additionally, when α7 integrin was overexpressed in muscles of healthy animals, exercised-induced muscle injury was minimized as demonstrated by a reduction in Evans Blue Dye uptake post-exercise (98). This suggests that an increase in α7 integrin expression can provide protection against sarcolemma damage in fast muscles. Finally, the combined loss of dystrophin and α7 integrin causes an even more severe muscular dystrophy than the loss of either alone (96) which suggests that the DGC and α7β1 integrin have compensatory functions in skeletal muscle.

The striking differences in susceptibility to contraction-induced damage in LARGE[myd/myd] soleus and EDL muscles, and the markedly higher expression of α7β1 integrin in soleus muscle compared to EDL and gastrocnemius muscles indicates that there are important fiber type specific differences in the interactions between the basal lamina and the cytoskeleton in muscle fibers. Specifically, the functions of the DGC and α7β1 integrin complexes may be fiber type specific. PGC-1α is a transcriptional activator that has been shown to play a critical role in driving the slow muscle fiber gene program. This protein is highly expressed in slow fibers and when transgenically overexpressed throughout skeletal muscle, results in a conversion to a slow-twitch fiber phenotype (185). Interestingly, overexpression of PGC-1α in *mdx* mice partially improved the muscular dystrophy phenotype although the mechanism of action was not clearly identified (186). However, *mdx*/PGC-1α mice were shown to have decreased levels of serum creatine kinase and decreased levels of Evans Blue Dye uptake following downhill running which supports the hypothesis that part of the effect may be

mediated by an increase in protection of the sarcolemma. This suggests that although most of the downstream transcriptional events related to PGC-1α expression has focused on metabolic genes, the induction of a slow muscle gene program may also produce downstream changes that affect expression of ECM receptors, particularly that of α7β1 integrin resulting in its increased sarcolemmal expression and leading to protection from contraction-induced injury. While the overexpression of PGC-1α likely results in a numbers of changes in gene transcription, the specific overexpression of α7β1integrin in skeletal muscle can increase muscle fiber viability without significantly affecting the transcription of other proteins and may be beneficial as a potential therapeutic for muscular dystrophy patients (187). This also supports the conclusions that the alleviation of muscle damage and dystrophic phenotype observed when α7 integrin is transgenically expressed in animal models is due to the specific function of α7β1integrin at the sarcolemma and not due to altered expression of other proteins that may also contribute to enhanced viability of muscle fibers.

While the soleus muscle of LARGE$^{myd/myd}$ mice contains a substantial number of fast-twitch muscle fibers, we did not observe a partial deficit in contraction-induced injury. This suggests that interactions of slow muscle fibers with the extracellular matrix through β1containing integrins may also help stabilize neighboring fast muscle fibers from contraction-induced injury. Previous studies have shown that striated muscle from animals with an impaired DGC typically have a higher degree of uptake of Evans Blue Dye in large patches of neighboring fibers rather than randomly distributed in single fibers throughout the muscle (52). This supports the hypothesis that strong lateral interactions of muscle fibers with the extracellular matrix between neighboring fibers, through either dystroglycan or integrin, are critical for the protection of both individual fibers and the integrity of the entire muscle during normal muscle contractile activity. Finally, it is important to remember that human skeletal muscle is composed of a mixture of fiber types and in many respects, the mouse soleus better models this typical composition of human skeletal muscle (188). Therefore, understanding the complete functions of dystroglycan in both fast-

twitch and slow-twitch skeletal muscle and the relative functional contributions of dystroglycan and integrins in the different fiber types will have significant implications for future therapeutics aimed at treating muscular dystrophy.

ACKNOWLEDGEMENTS
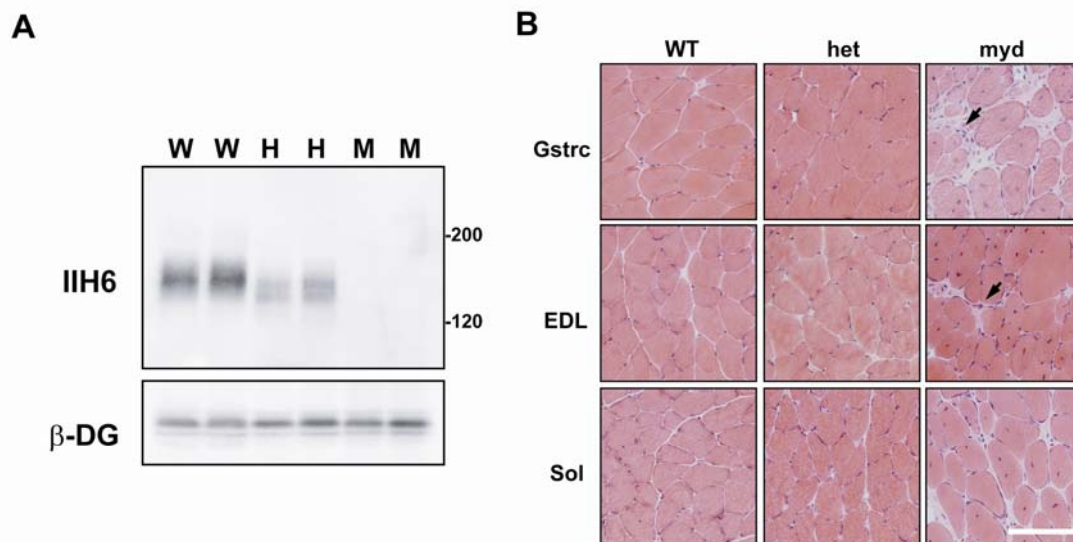
Figure 2-1) Impaired dystroglycan glycosylation and muscle histology in LARGE^myd mice. Altered dystroglycan glycosylation is not sufficient to cause muscular dystrophy in heterozygous LARGE^myd mice. Immunoblots of whole muscle lysates (A) from quadriceps muscle of wild-type (W), heterozygous (H), or LARGE^myd/myd (M) mice were stained with antibodies against glycosylated α-dystroglycan (IIH6) or β-dystroglycan (β -DG). Altered glycosylation of mice heterozygous for the LARGE^myd mutation is demonstrated by a downward shift in the molecular weight of α-dystroglycan. Hematoxylin and eosin staining (B) of mouse gastrocnemius, EDL and soleus muscles shows that while all three muscles of LARGE^myd/myd (myd) mice are dystrophic, heterozygous (het) muscles are indistinguishable from healthy wild-type littermates (WT). LARGE^myd/myd soleus, EDL and gastrocnemius muscles contain fibers of heterogeneous size, several regenerating fibers indicated by internalized nuclei, and mononuclear cell infiltrate (indicated by black arrows). All muscles were dissected from wild-type, heterozygous, and LARGE^myd/myd mice aged 30 weeks and frozen in liquid nitrogen cooled isopentane. Scale bar represents 100 μm.
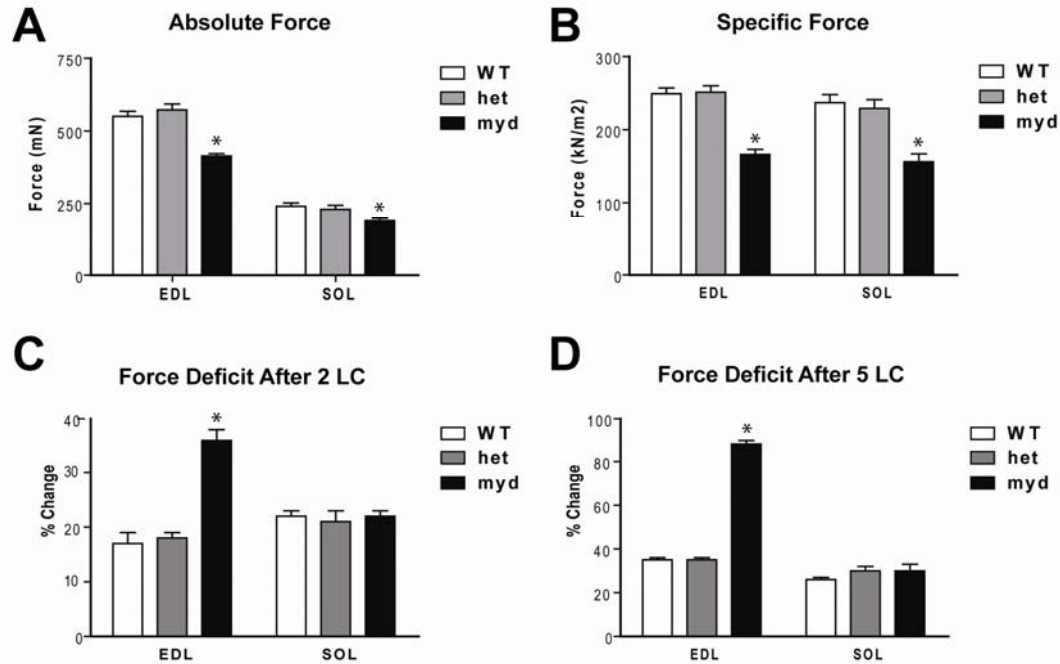
Figure 2-2) *In vitro* **contractile function of LARGE^{myd} muscle EDL and soleus muscle.** LARGE$^{myd/myd}$ soleus muscle is weak but not sensitive to contraction-induced injury. Absolute (A) and specific (B) force was measured *in vitro* using soleus and EDL muscles isolated from 4-5 month-old wild-type (WT), heterozygous (het), or LARGE$^{myd/myd}$ (myd) mice. While absolute and specific force was significantly reduced in both LARGE$^{myd/myd}$ soleus and EDL muscles, heterozygous mice showed no difference in either muscle when compared to wild-type animals. Contraction-induced injury was performed by subjecting each muscle to two (C) or five (D) lengthening contractions of 30% strain. In both soleus and EDL muscles from heterozygous mice, measured force deficits were not different from wild-type muscle. Although force deficits measured in EDL muscles of LARGE$^{myd/myd}$ mice were significantly higher than wild-type, no such difference was observed in the soleus muscle.
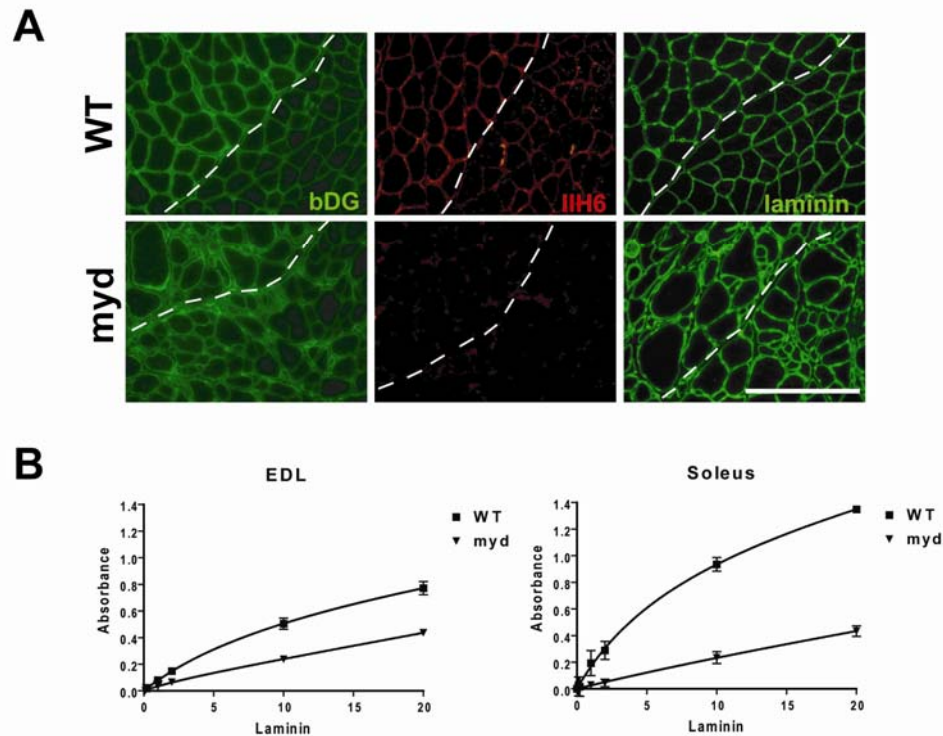
Figure 2-3) Laminin binding activity and immunofluorescent staining of LARGE^{myd} gastrocnemius and soleus muscle. Loss of glycosylated dystroglycan occurs in both soleus and gastrocnemius muscle of LARGE$^{myd/myd}$ mice and results in decreased laminin binding activity. Gastrocnemius and soleus muscles were dissected from 36- week old wild-type and LARGE$^{myd/myd}$ mice. Frozen sections were stained separately for β-dystroglycan (bDG), glycosylated α-dystroglycan (IIH6), or laminin. Sections were imaged at regions where the gastrocnemius (bottom right) and soleus (top left) muscles lie adjacent to one another, and the boundary of each is indicated by the dashed line (A). Loss of dystroglycan glycosylation in both muscles is indicated by a loss of reactivity with the IIH6 antibody. Despite loss of dystroglycan glycosylation, the presence of laminin, an important dystroglycan ligand in skeletal muscle, is retained. Scale bar represents 200 μm. Loss of dystroglycan glycosylation results in loss of laminin binding activity in both soleus and EDL muscles (B). WGA-purified fractions of pooled muscle tissue from wild-type (WT) and LARGE$^{myd/myd}$ (myd) mice were isolated, quantified, and coated onto 96-well plates. Laminin binding activity was determined by overlaying wells with a range of concentrations of laminin between 0.2 - 20 nM.
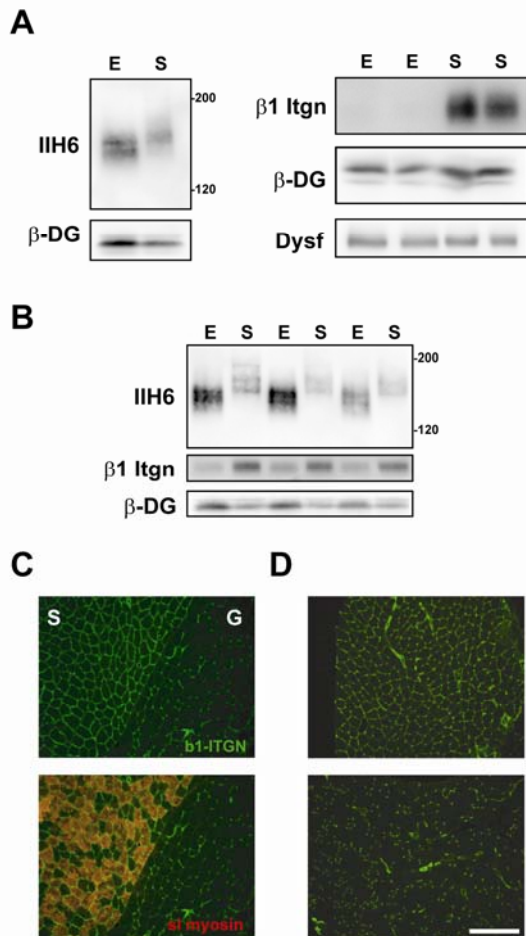
Figure 2-4) β1 integrin expression in fast-twitch versus slow-twitch muscle. β1 integrin is highly expressed at the sarcolemma of muscle containing a large percentage of slow fibers. Immunoblots were stained with IIH6 and anti-β-dystroglycan. In both rat (A) and mouse (B), α-dystroglycan from soleus muscle appears to run at a higher molecular weight than α-dystroglycan from EDL muscle. Immunoblots were also stained for β1 integrin (β1 Itgn) and dysferlin (Dysf). In both rat and mouse, β1 integrin appears to be more highly expressed in soleus muscle. No differences were observed in the expression of dysferlin, a protein thought to be involved in membrane repair. Immunofluorescent staining of soleus (S)/ gastrocnemius (G) muscle sections shows that the increased expression of β1 integrin observed in the soleus muscle is due to increased expression at the sarcolemma (C). Co-staining with an antibody specific to slow myosin demonstrates that increased expression of β1 integrin in the soleus muscle corresponds to muscle that contains a high proportion of slow fibers. The decreased expression of β1 integrin in predominantly fast fibers is also demonstrated by reduced staining in the EDL muscle (D, lower panel) when compared to the soleus (D, upper panel) when both sections were imaged at the same exposure. Scale bar represents 200 μm.

51
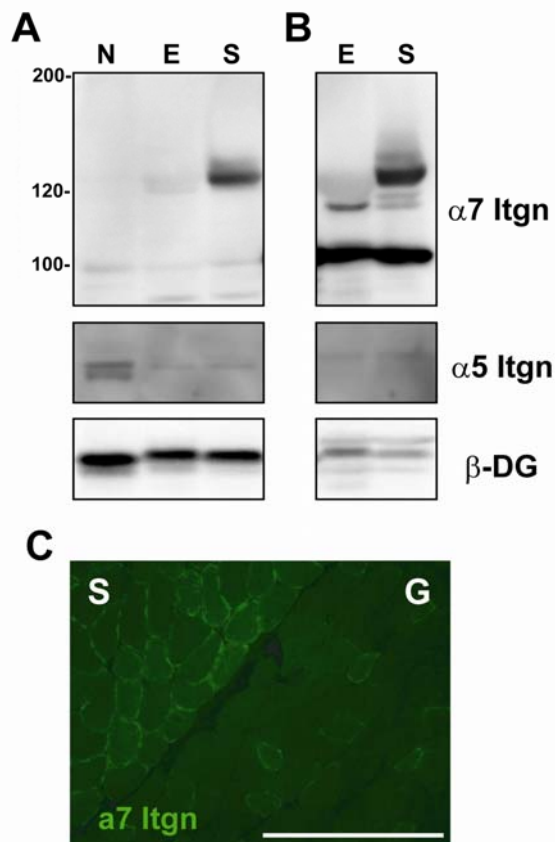
Figure 2-5) α7 integrin expression in fast-twitch versus slow-twitch muscle. Skeletal muscle contains multiple α integrin isoforms capable of forming dimers with β1 integrin. Microsome or WGA-purified fractions were isolated from either soleus and EDL muscle from Sprague-Dawley rats or whole muscle from neonatal mice. Staining with an antibody to α7 integrin resulted in the detection of multiple bands, two of which have molecular weights corresponding to a non-glycosylated (100 kDa) and glycosylated (120 kDa) form of α7 integrin (A, B). While no difference in expression of α7 integrin was detected in the 100 kDa band, the 120 kDa band appeared to be more highly expressed in soleus (S) muscle than in EDL (E) muscle. Additionally, α5 integrin was expressed at equally low levels in both muscles when compared to neonatal muscle (A, B). In a pattern similar to β1 integrin, immunofluorescent staining of mouse soleus (S)/ gastrocnemius (G) muscle sections showed that α7 integrin is more highly expressed at the sarcolemma of soleus muscle (C). Scale bar represents 200 μm.
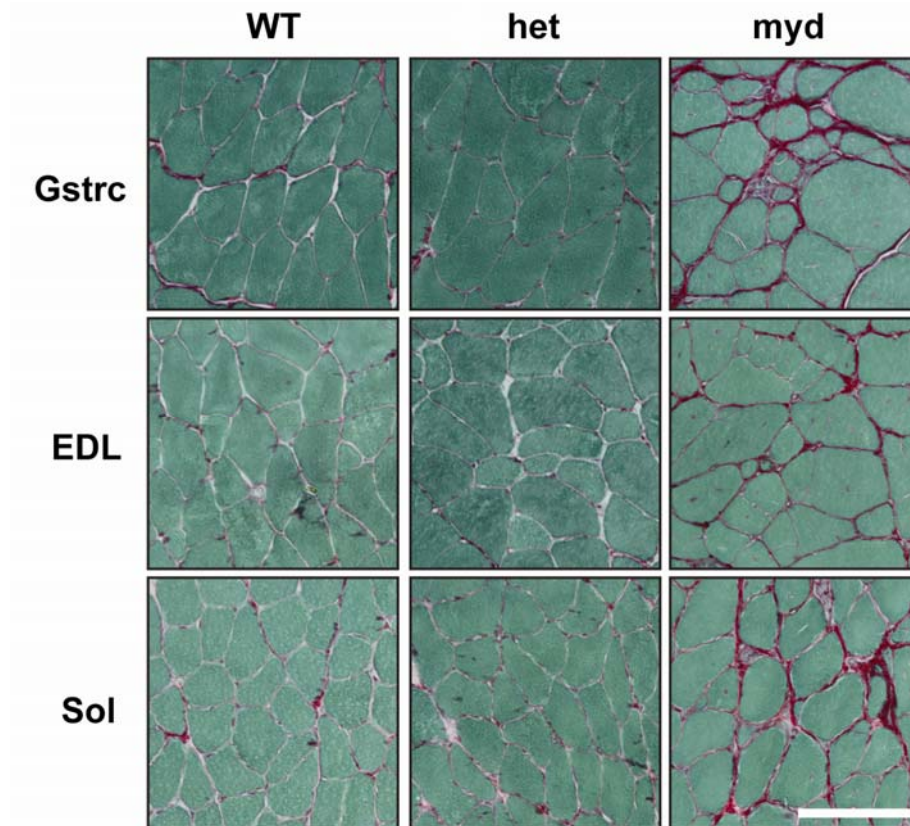
Figure 2-6) Interstitial fibrosis in LARGE^{myd} skeletal muscles.
Sirius Red staining of mouse gastrocnemius (Gstrc), EDL, and soleus (Sol)
muscles shows that all three muscles of LARGE$^{myd/myd}$ (myd) mice demonstrate
an increase in interstitial fibrillar collagen deposition (red staining) while
heterozygous (het) muscles are indistinguishable from that of healthy wild-type
littermates (WT). Additionally, fibers of heterogenous size are apparent in
LARGE$^{myd/myd}$ muscle. All muscles were dissected from 30-week old wild-type,
heterozygous and LARGE$^{myd/myd}$ mice and frozen in liquid nitrogen cooled
isopentane. Scale bar represents 100 µm.

CHAPTER 3

Hyperglycosylation by LARGE protects mouse skeletal muscle from contraction induced-injury

ABSTRACT

Dystroglycan is a glycosylation-dependent extracellular matrix receptor essential for maintaining lateral connections between the sarcolemma and basal lamina in skeletal muscle. Several glycosyltransferases have been identified in which mutations result in muscle disease and are associated with a reduction in dystroglycan glycosylation in skeletal muscle. We have previously demonstrated that in an animal model of glycosylation-deficient muscle dystrophy, loss of glycosylation and ligand binding activity of dystroglycan renders muscle susceptible to contraction-induced damage. To further determine the importance of dystroglycan glycosylation in skeletal muscle function, transgenic mice were generated that direct overexpression of Like-acetylglucosaminyltransferase (LARGE) in differentiated muscle fibers. We tested the hypothesis that overexpression of LARGE would result in enhanced dystroglycan glycosylation and function above that observed in normal muscle. Skeletal muscle of MCK-LARGE transgenic mice demonstrated hyperglycosylation of dystroglycan that coincided with a significantly elevated laminin binding affinity compared to that of wild-type muscles. Additionally, fast-twitch skeletal muscle from transgenic mice demonstrated enhanced protection from contraction-induced injury such that force deficits following injury were significantly reduced as compared to muscle of wild-type littermates. This suggests that hyperglycosylation and enhanced function of dystroglycan via increased activity of the enzyme LARGE may be

therapeutic not only in inherited glycosylation-deficient muscular dystrophy but also in acquired diseases or disability resulting from muscle injury.

INTRODUCTION

Dystroglycan is an extracellular matrix receptor and central component of the dystrophin-glycoprotein complex (DGC), a protein complex essential for skeletal muscle function, yet one that is disrupted in several forms of muscular dystrophy. Dystroglycan is transcribed from the *DAG1* gene and post-translational cleavage produces two subunits that remain tightly associated at the plasma membrane (159). β-dystroglycan contains a single transmembrane domain and interacts intracellularly with dystrophin and extracellularly with α-dystroglycan. Anchored to the extracellular face of the sarcolemma by β-dystroglycan, α-dystroglycan can interact with a number of extracellular ligands, including agrin (137), neurexin (189), perlecan (190), and laminin (12, 191). Through high affinity binding of α-dystroglycan to laminin and β-dystroglycan to dystrophin, dystroglycan acts as a bridge between the basal lamina and the cellular cytoskeleton and mutations that disrupt this link can result in muscular dystrophy (12). Dystrophin mutations account for one of the most common forms of muscular dystrophy, Duchenne muscular dystrophy (DMD) with the loss of dystrophin function in a mouse model of DMD rendering muscle highly susceptible to contraction-induced muscle damage (38, 39). This has led to the hypothesis that one of the primary functions of dystroglycan and the DGC is to stabilize the sarcolemma during successive muscle contractions (12).

In order to bind laminin and other extracellular ligands, α-dystroglycan requires extensive *O*-linked glycosylation (160), the reduction or complete absence of which is the basis for a group of diseases referred to as the "dystroglycanopathies". These diseases are characterized by progressive muscle dysfunction and often include additional clinical symptoms such as developmental delay, cobblestone lissencephaly and occular abnormalities. In many of these diseases, the mutation responsible has been identified in one of several glycosyltransferase genes which are required for the extensive *O*-linked

glycosylation of dystroglycan within its central mucin domain. Mutations resulting in the development of Walker-Warburg syndrome, one of the most severe of the dystroglycanopathies, have been identified in the enzymes protein-*O*-mannosyltransferase 1 (POMT1) and POMT2 (150, 151). These enzymes have been shown to catalyze the first step, an addition of mannose, in the synthesis of a tetrasaccharide, NeuAcα2-3Galβ1-4GlcNAcβ1-2Man-S/T, identified on α-dystroglycan (171). The second step, an addition of a *N*-acetylglucosamine (GlcNAc) residue to mannose is catalyzed by protein-*O*-mannose β1,2-*N*-acetylglucosaminyltranferase (POMGnT), the enzyme mutated in muscle-eye brain-disease (142). In addition to the tetrasaccharide that is synthesized in part by the enzymes POMT1, POMT2, and POMGnT, the mucin domain of α-dystroglycan has been shown to contain several additional glycans (171-173, 192, 193); however, the enzymes required for their synthesis are still unknown. Additional mutations have been identified in fukutin that cause Fukuyama congenital muscular dystrophy, fukutin-related peptide (FKRP) that can result in either limb-girdle muscular dystrophy 2I (147) or congenital muscular dystrophy 1C (148) and like-acetylglucosaminyltransferse (LARGE) that can cause both congenital muscular dystrophy 1D (194) and Walker-Warburg syndrome (195). However, the catalytic activity and the precise nature of the reaction catalyzed by fukutin, FKRP, and LARGE is unclear. Additionally, in many forms of dystroglycanopathy, the causative gene remains unidentified (196).

LARGE is a putative glycosyltransferase that contains two potential catalytic DXD motifs and has homology to both 1,3-*N*-acetylglucosaminyltransferase and bacterial glycosyltransferase (167). In order for LARGE-mediated glycosylation of dystroglycan to occur, LARGE must be able to directly bind to the N-terminus of α-dystroglycan (169), and it appears that this is essential for modifying laminin-binding glycans on specific threonines within the mucin domain nearest the N-terminus (197). Additionally, α-dystroglycan was recently shown to contain a transient phosphorylated mannose residue that was present in muscle containing a mutation in LARGE, suggesting that LARGE may modify phosphorylated mannose structures in order to form

laminin binding glycans (174). Viral-mediated overexpression of LARGE in myoblasts cultured from dystroglycanopathy patients was capable of restoring dystroglycan glycosylation and laminin binding, despite that the cells harbored mutations in enzymes other than LARGE (170). This suggests that LARGE may be able to circumvent defects in the stalled synthesis of glycans due to mutations in either POMT1 or POMGnT and moreover, may be able to add novel laminin-binding glycans to dystroglycan.

The LARGE^{myd} mouse model of glycosylation-deficient muscle dystrophy demonstrates hypoglycosylation of α-dystroglycan (167), and we and others have shown that loss of LARGE-mediated glycosylation of α-dystroglycan renders muscle susceptible to contraction-induced injury (22, 48). Specific interactions between dystroglycan and laminin within the extracellular matrix are critical for tightly anchoring the basal lamina to the sarcolemma (22) and therefore, LARGE-mediated glycosylation of dystroglycan appears to be essential for conferring the laminin binding activity of dystroglycan necessary for maintaining tight association with the basal lamina. Consequently, the reduction in these interactions due to mutations in glycosyltransferases known to modify dystroglycan leaves the sarcolemma vulnerable to mechanical injury. Therefore, we hypothesized that enhanced interactions between dystroglycan and laminin would further protect the sarcolemma from contraction-induced injury. Here, we demonstrate that overexpression of a muscle-specific LARGE transgene causes dramatic hyperglycosylation of dystroglycan, significantly increases laminin binding activity, and protects muscle from lengthening-contraction induced mechanical injury .

METHODS

Generation of Transgenic Mice. A construct containing the human LARGE cDNA, a C-terminal myc sequence and a BGH polyadenylation signal was amplified via PCR from LARGEmyc-pCDNA3 and cloned into the pCR 2.1 TOPO vector. An MCK promoter/enhancer sequence (received from Jeffrey Chamberlain, Department of Neurology, University of Washington School of

Medicine, Seattle, WA) was amplified and digested with *NdeI* and *XhoI* to generate two pieces that were ligated in a two step ligation protocol 5' to the LARGEmyc coding sequence. The resulting construct containing the transgene was linearized via *NotI* digestion and the University of Michigan Transgenic Animal Model Core was utilized to perform injections into C57Bl/6 mouse eggs. The generated pups were screened and two founders were identified by PCR of genomic DNA from tail biopsies using two sets of transgene specific primers. All animals were housed in a specific pathogen free (SPF) barrier facility in the Unit for Animal Medicine at the University of Michigan and all experiments were approved by the University of Michigan Committee for the Use and Care of Animals (UCUCA). Transgenic and wild-type littermates used in all experiments described were 31-32 weeks of age.

Western Blots. Muscle tissue used for western blot analysis was collected from deeply anesthetized animals and immediately frozen in dry ice prior to processing. Microsome fractions were created by homogenizing samples in ice-cold homogenization buffer (20 mM sodium pyrophosphate, 20 mM sodium phosphate (monobasic), 1 mM magnesium chloride, 5 mM EDTA, and 0.303 M sucrose, pH 7.1). Homogenate was subjected to a low-speed (10,000 g) centrifugation to remove cellular debris and a high-speed (45,000 g) spin in order to isolate the membrane fraction. Resulting pellets were resuspended in Buffer I (0.303 mM sucrose, 20 mM Tris-maleate, pH 7.0) and quantified using the Bio-Rad Bradford assay. Additional tissues were homogenized in a Triton-X 100 lysate buffer and subjected to a low-speed spin (10,000 g) to clear cellular debris. Lysates were quantified using the Bio-Rad DC assay. All buffers contained protease inhibitors (0.0 µg/ml pepstatin A, 2 kallikrein inhibitor units/ ml aprotinin, 1 µg/ml leupeptin, 0.4 mM PMSF, 0.6 mN benzamidine). Samples were separated on a 3-15% polyacrylamide gel and transferred to polyvinylidene fluoride membrane (Millipore). Membranes were initially reversibly stained with PonceauS in order to verify equal loading and incubated in a blocking solution containing in 5% nonfat dry milk in TBS-T (120-150 mM sodium chloride, 50 mM Tris, 0.05% Tween-20). All blots were blocked for 1-2 hours and incubated in

58

primary antibody for 2 hours to overnight. Primary antibodies included IIH6 (gift from Dr. Kevin Campbell, University of Iowa, Iowa City, IA) and the anti-myc 9E10 antibody (Sigma). After three 5-10 minute washes, membrane were incubated in secondary antibodies conjugated to horseradish peroxidase (HRP) for 1-2 hours. Following three 5-10 minutes washes, membrane were incubated in chemiluminescent substrate (Thermo scientific) and digitally developed using an Alpha Innotech Fluorchem.

Immunofluorescence and Histology. Muscles were carefully dissected from deeply anesthetized animals and immediately frozen in liquid nitrogen-cooled isopentate. Frozen samples were cut into 8-10 uM cross or longitudinal sections with a cryostat and stored at -80° until further processed for hematoxylin and eosin or immunofluorescent staining. For immunofluorescent staining, slides were rehydrated in PBS and incubated in a blocking buffer containing 5% bovine serum albumin and 0.05% Triton-X 100 in PBS. Slides were incubated in primary antibody for 2-3 hours and secondary antibody for 1-1.5 hours with three 5 min washes in between incubations. Primary antibodies included an anti-myc A-14 polyclonal (Santa Cruz), IIH6 (gift from Dr. Kevin Campbell, University of Iowa, Iowa City, IA), and anti-laminin L-9393 (Sigma). Slides were mounted in Permafluor (Thermo Scientific) and imaged on an Olympus BX-51fluorescence microscope.

Solid-phase Laminin Binding Assay. Laminin binding assays were performed as described previously (my paper). Wheat germ agglutinin (WGA)-purified muscle fractions were coated onto 96-well microplate and incubated with increasing concentrations of laminin (0.02 – 2nM) from Invitrogen. Laminin binding was detected using the primary L-9393 (Sigma) antibody and a secondary antibody conjugated to horseradish peroxidase (HRP). Antibody binding was detected using o-phenylenediamine (OPD)-citrate phosphate buffer (CPB) and stopped with 2M H_2SO_4.

In Vitro **Muscle Function and Injury Protocol.** Contractile measurements were performed as previously described (177). EDL and soleus muscle were carefully

dissected from anesthetized mice and tied to a servomotor (Aurora Scientific, model 300) and a force transducer (Kulite Semiconductor) using 5.0 silk suture. Muscle were maintained in 25°C bath containing Krebs mammalian Ringer solution bubbled continuously with 95% O_2 and 5% CO_2 to stabilize pH at 7.4. Each muscle was stimulated by square wave pulses delivered between two platinum electrodes connected to a high-power biphasic current stimulator (Aurora Scientific, model 701B). Electrical pulse properties and servomotor activity was controlled and force transducer recorded from an IBM-compatible personal computer with custom-designed software (LabVIEW 7.1, National Instruments, Austin, TX). Upon determination of L_o (optimal muscle length) and optimal voltage and frequency, muscle were held at L_o and subjected to trains of pulses (300 ms for EDL, 900 ms for soleus) to generate maximum isometric contraction (P_o). Mean cross-sectional area (CSA) was calculated by dividing the muscle wet mass by the product of fiber length (L_f) and the density of mammalian muscle (1.06 g/cm^3) and specific force (sP_o) was determined by dividing P_o by CSA. The muscle injury protocol was performed as described previously (48). Following contractile measurements, muscle were stimulated to achieve maximum twitch force (P_o) and immediately stretched through a strain of 30% relative to L_f at a velocity of 1 L_f/s. Total stimulation time was 400ms for EDL muscle, 600 ms for soleus muscle. After stimulation, muscles were returned to L_o and subjected to 4 additional 30% stretches, each with 12 seconds in between for a total of five stretches per muscles. Force deficit was calculated as the decrease in P_o observed after each stretch as a percentage of the P_o immediately prior to the lengthening contraction.

Statistics. For contractile function experiments, unpaired t-tests were used to compare differences in results obtained between wild-type and MCK-LARGE littermates. Statistical significance was established for P-value less than 0.05. Error bars represent SEM.

RESULTS

MCK-LARGE transgenic animals demonstrate hyperglycosylation of α-dystroglycan and increased laminin binding activity. Transgenic mice that express full length LARGE exclusively in striated muscle were generated using a muscle creatine kinase (MCK) promoter/enhancer sequence to drive expression of the human *LARGE* cDNA sequence (Fig. 3.1A). The transgene contained a c-terminal myc epitope used to detect LARGE protein expression. Two founders were generated that demonstrated expression of the LARGE-myc in cardiac and skeletal muscle. Western blot analysis was performed on Triton-X lysates from heart and quadriceps muscle of MCK-LARGE and wild-type littermates to determine whether human LARGE was capable of glycosylating α-dystroglycan as determined by increased reactivity with the glycosylation specific IIH6 antibody. Although α-dystroglycan has a predicted molecular weight of ~74 kDa based on the amino acid sequence, it has been observed to run as a broad smear on SDS-PAGE with a molecular weight of 120-156 kDa as a result of extensive glycosylation. The molecular weight of α-dystroglycan from both lines of transgenic animals was dramatically increased, suggesting that LARGE is capable of either extending or adding additional glycans to α-dystroglycan (Fig. 3.1B). No difference in expression of β-dystroglycan between wild-type and transgenic muscle was observed (not shown). Western blots of muscle microsome preparations from wild-type and MCK-LARGE skeletal muscle confirmed expression of LARGE-myc as detected by the anti-myc 9E10 antibody (Fig. 3.1C). Since glycosylation of α-dystroglycan is essential for enabling dystroglycan to function as an extracellular matrix receptor, we sought to determine how hyperglycosylation of α-dystroglycan affected laminin binding affinity. Laminin binding assays performed on wheat germ agglutinin (WGA)-purified fractions of gastrocnemius muscle isolated from two transgenic and two wild-type animals demonstrated that transgene expression resulted in a nearly 5-fold increase in laminin binding activity of the muscle (Fig. 3.1D). These data demonstrate that LARGE overexpression in skeletal muscle results in the

addition of laminin binding glycans on α-dystroglycan and significantly enhances the overall laminin binding activity of the skeletal muscle.

Representative muscle sections from wild-type and MCK-LARGE mice also demonstrate the expression and localization of transgenic LARGE-myc. Native LARGE is localized to the golgi apparatus (198) and detection of myc in muscle using the polyclonal A-14 antibody identified transgenic LARGE in all fibers in a pattern consistent with golgi localization (Fig. 3.2B). The creatine kinase gene has been shown to be differentially expressed in fast-twitch and slow-twitch fibers (199). Therefore, to verify that all MCK-LARGE muscles expressed the transgene and demonstrated hyperglycosylation of dystroglycan, several muscle groups were analyzed. IIH6 stained cross sections of extensor digitorum longus (EDL) (Fig. 3.2D) and soleus muscle (Fig. 3.2F) of MCK-LARGE mice showed increased reactivity beyond that observed in wild-type muscle (Fig. 3.2C, EDL; Fig. 3.2E, soleus) indicating that hyperglycosylation of α-dystroglycan in transgenic animals was not restricted to either fast-twitch or slow-twitch muscle. Additionally, hematoxylin and eosin stained muscle sections from wild-type (Fig. 3.2G) and transgenic (Fig. 3.2H) animals revealed that increased LARGE expression and consequent hyperglycosylation of α-dystroglycan does not affect normal muscle morphology since sections from MCK-LARGE mice were indistinguishable from wild-type sections. Laminin staining in skeletal muscle sections from both wild-type and transgenic animals did not appear to be altered between the two tissues (Fig. 3.2I, wild-type: 3.2J, MCK-LARGE), suggesting that the enhanced laminin binding activity of transgenic muscle does not result in changes in laminin retention in the extracellular matrix.

Contractile function in MCK-LARGE muscle is comparable to wild-type muscle. In order to demonstrate that LARGE overexpression in skeletal muscle is not deleterious to skeletal muscle function, *in vitro* contractile measurements were performed on EDL and soleus muscles from wild-type and MCK-LARGE animals. Neither the EDL nor the soleus muscle from MCK-LARGE mice was different in mass (EDL: 10.72 mg ± 0.58, soleus: 9.98 mg ± 0.47) from the

corresponding muscle in wild-type animals (EDL: 10.33 ± 0.54, soleus: 8.95 mg ± 0.34) indicating that LARGE overexpression did not result in significant changes to muscle mass (Fig. 3.3A). Additionally, absolute forces measured in EDL (WT: 439 mN +/- 19, TG: 405 mN +/- 13) and soleus muscle (WT: 231 mN ± 6, TG: 229 mN ± 7.4) did not reveal any significant differences between transgenic and wild-type muscle (Fig. 3.3B). However, specific force calculations resulted in significant differences between transgenic and wild-type mice in both EDL and soleus muscle (Fig. 3.3C). No differences in body weight were observed between the different genotypes which suggests that muscle from MCK-LARGE does not undergo significant hypertrophy (Fig. 3.3D).

Muscles of MCK-LARGE mice display increased protection from contraction-induced injury. Previously we reported that mice containing a mutation in the *LARGE* gene demonstrate reduced glycosylation of α-dystroglycan concomitant with a susceptibility to lengthening contraction-induced damage in muscles composed of predominantly fast-twitch fibers (48). Susceptibility to contraction-induced injury is a common feature of muscular dystrophies associated with mutations in the dystrophin-glycoprotein complex and interactions between laminin and α-dystroglycan are particularly critical for providing stability during mechanical injury by anchoring the basal lamina to the sarcolemma (22). Therefore, we hypothesized that enhanced α-dystroglycan glycosylation and increased laminin binding affinity would provide additional stability to the sarcolemma and as a result, MCK-LARGE muscle would be more resistant to lengthening contraction-induced injury than wild-type muscle. Lengthening contractions of 30% strain were performed *in vitro* on both EDL and soleus muscles from MCK-LARGE and wild-type littermates. Injury was determined by calculating the difference in maximal force production prior to and after each contraction and the force deficit was expressed as a percentage of the maximal force production. With each successive contraction, an increase in the force deficit was observed in both wild-type and transgenic muscle. The mean force deficits measured in the MCK-LARGE EDL muscle after the first and second lengthening contraction (LC1: 7.08% +/- 0.42, LC2: 14.29% +/- 0.39)

63

were respectively 22.5% and 17.3% less than the mean force deficits measured in wild-type EDL muscle (LC1: 9.14% +/- 0.45, LC2:17.27% +/- 0.94) which indicated significant differences in susceptibility to contraction-induced injury between the two muscles (Fig. 3.4). Furthermore, although the data was not statistically significant, force deficits trended lower in MCK-LARGE EDL after the additional 3rd, 4th and 5th lengthening contraction (contraction 3-5 not shown). In contrast to the results obtained in the EDL muscle, significant differences were not observed between force deficits measured in wild-type and transgenic soleus muscle (Fig. 3.4).

DISCUSSION

Lengthening contractions are particularly injurious to skeletal muscle and can disrupt force generating/ transmitting structures and damage the sarcolemma. The dystrophin-glycoprotein complex (DGC) is essential for binding proteins within the basal lamina that surrounds each muscle fiber and is thought to provide structural support to the sarcolemma during cycles of contraction and relaxation. Consequently, several forms of muscular dystrophy are thought to result in part from compromised sarcolemma integrity due to impaired function or loss of components in the DGC. Recently, it has also been shown that the DGC can function to transmit forces laterally through costameres to the epimysium and by doing so, may also serve to protect muscle fibers from contraction-induced injury (128). Here, we demonstrate that enhancing connections with the basal lamina via increased affinity for extracellular laminin can result in significant protection of the muscle from lengthening-contraction induced injury.

In the present study, we observed a protection from mechanical injury only in muscle of predominantly fast-twitch fibers despite having found that both fast-twitch and slow-twitch muscle were hyperglycosylated as a result of transgene expression. This was particularly apparent after the first and second lengthening contraction and force deficits measured in transgenic muscle trended lower than those in wild-type muscle after three additional contractions. We also demonstrated that α-dystroglycan, as detected by the glycosylation specific IIH6

64

antibody, ran at a higher molecular weight than α-dystroglycan from wild-type muscle. This suggests that human LARGE is capable of either extending glycans already present or adding additional novel glycans on the ~50 serine and threonine residues located within the mucin domain of α-dystroglycan. In either case, this results in a nearly 5-fold increase in laminin binding affinity in transgenic muscle. Since high-affinity laminin binding activity is reduced in dystroglycan gene-targeted mice (101), it is likely that this increase in laminin binding activity can be attributed to enhanced dystroglycan glycosylation in MCK-LARGE mice. This results in a strengthening of the connections between the basal lamina and the sarcolemma and underlies the effects we observe after subjecting the muscle to a series of lengthening contractions – namely, that enhanced laminin binding coincides with a greater degree of protection from contraction-induced injury. In support of this, muscle lacking functional glycans on α-dystroglycan, as a result of LARGE mutation, are highly susceptible to contraction-induced injury (48) and exhibit defects in the basal lamina (22).

A recent similar study postulated that hyperglycosylation of dystroglycan via a LARGE transgene does not provide protection from contraction-induced injury and demonstrated that muscle containing hyperglycosylated dystroglycan was more susceptible to mechanical injury (200). This study utilized the tibialis anterior muscle, also a predominantly fast-twitch muscle, and performed lengthening-contractions *in situ.* Although the specific muscle utilized and the protocol was different from our study, the animals were 8 months of age, approximately the same age and genetic background as those used in our study. An explanation that accounts for the increased susceptibility of LARGE transgenic muscle to mechanical injury by the authors of this study was the possibility that the hyperglycosylation of α-dystroglycan may negatively affect turnover of the basal lamina. Since we observed an opposite result despite hyperglycosylation of α-dystroglycan, the most probable explanation that accounts for the differences we observed in our study is related to the method of transgene expression. While the Brockington study employed a ubiquitous promoter to drive expression of LARGE in all tissues, we used the muscle

creatine kinase (MCK) promoter to direct LARGE expression exclusively in differentiated muscle fibers. This promoter/enhancer construct has also been used to drive muscle-specific expression of dystrophin in *mdx* muscle, and the dystrophin transgene was not detected in uterine smooth muscle, liver, or brain (201). Additionally, the muscle creatine kinase gene is not expressed until later in development once proliferating myoblasts exit the cell cycle and commit to a muscle cell lineage (202). Therefore, it is possible that dystroglycan has additional functions during muscle development, and that early hyperglycosylation of dystroglycan by ubiquitous overexpression of LARGE may negatively impact skeletal muscle function.

While the EDL muscle from transgenic animals demonstrated a protection from contraction-induced injury greater than that observed in wild-type muscle, we did not observe any difference between the two genotypes in soleus muscle. In some respects, this was expected since we previously demonstrated that in LARGE-deficient animals, only the EDL muscle was highly susceptible to lengthening contraction-induced injury (48). Force deficits measured in LARGE^{myd} soleus were not different from those measured in wild-type animals despite a lack of dystroglycan glycosylation in this muscle. As a partial explanation for this, we showed that soleus muscle contained a much higher expression of an alternative laminin receptor, α7β1 integrin, as compared to muscle composed primarily of fast-twitch fibers. Therefore, it appears that slow-twitch muscle may utilize different or additional laminin receptors as a means to forge connections with the sarcolemma. This would imply that in our transgenic mice, hyperglycosylated dystroglycan accounts for a greater percentage of laminin bound receptors in the EDL than it does in the soleus and could explain the observed diminished effect in the soleus. While we did not see a protection in the soleus muscle, we cannot rule out the possibility that small increases were too low to be detected in our study.

Although symptoms observed in glycosyltransferase-deficient muscular dystrophy are thought to result from reduced dystroglycan glycosylation and

consequent impaired function, recent data suggests that the enzymes identified in these diseases may have multiple substrates in addition to dystroglycan (153, 154, 203). Zhang et al. showed that overexpression of LARGE in a clonal population of neural stem cells lacking the dystroglycan gene, still demonstrated an increase in IIH6 reactivity (153). Additionally, in cells that were null for both dystroglycan and POMT2, LARGE overexpression resulted in the formation of a IIH6 binding epitope that could be removed when incubated with PNGase F which removes *N*-linked glycans. This suggests that LARGE is capable of modifying both *O*-linked and *N*-linked glycans on additional substrates (154). In light of these studies, we cannot definitively confirm that the effects we observed are specific to the enhanced glycosylation of α-dystroglycan. However, as these studies were performed using neuronal cell populations, it is still unclear whether or not additional substrates for LARGE exist in skeletal muscle. When LARGE was overexpressed using the ubiquitous CMV promoter (200), no additional IIH6 reacting bands were present in transgenic brain, intestine, liver or kidney despite the detection of transgenic LARGE protein in these tissues. This suggests that if there are additional LARGE substrates in these tissues, either a significant elevation in LARGE expression, beyond that expressed by the transgene, is required to generate IIH6 reactive glycans or that IIH6 reactive glycans are present in amounts too low to be detected using immunoblot methods.

We have demonstrated that hyperglycosylation of LARGE in otherwise normal muscle does not result in deleterious effects to skeletal muscle function or overall animal health. Although we do not observe any differences in maximal force production or in muscle mass, we see a slight decrease in calculated specific force of muscle from transgenic mice. This appears to result from slightly elevated muscle mass in transgenic animals that did not result in a greater increase in total force production. While the predominant function of dystroglycan is hypothesized to be related to its important function in basal lamina formation and stabilization of the sarcolemma during muscle contractions, several reports have highlighted a potential role in survival signaling downstream of laminin binding. Inhibition of the binding between laminin and dystroglycan

results in a decrease in PI3K/AKT signaling, an important pathway mediating cell growth (112). Since we observe a significant increase in laminin binding affinity which is most likely due to enhanced glycosylation of dystroglycan, this has the potential for enhancing PI3K/AKT signaling or additional pathways that may be downstream of dystroglycan and could result in muscle hypertrophy. However, we do not observe any differences in body weight nor difference in ratio of wet mass to dry mass (data not shown), which suggests that muscle from MCK-LARGE muscle is not undergoing significant hypertrophy or edema as a result of dystroglycan hyperglycosylation. This suggests that hyperglycosylation of dystroglycan might have a slight negative effect on force production that is not accounted for by any structural changes within the muscles examined.

Our results demonstrate that overexpression of human *LARGE* in both fast-twitch and slow-twitch mouse muscle results in hyperglycosylation of dystroglycan and significantly enhances the laminin binding activity of the muscle. Additionally, this increase in laminin binding affinity of the muscle can significantly protect muscle from injury caused by lengthening contractions. Previously, it was demonstrated that in cell lines derived from patients with mutations in distinct glycosyltransferases involved in dystroglycan glycosylation, overexpression of LARGE was sufficient to bypass the genetic defect and restore functional glycosylation and laminin binding affinity of α-dystroglycan (170). Here, we demonstrate that this hyperglycosylation of dystroglycan by LARGE may also be beneficial in muscle diseases unrelated to the dystroglycanopathies by enhancing lateral connections in the muscle and providing protection from contraction-induced injury.

ACKNOWLEDGEMENTS
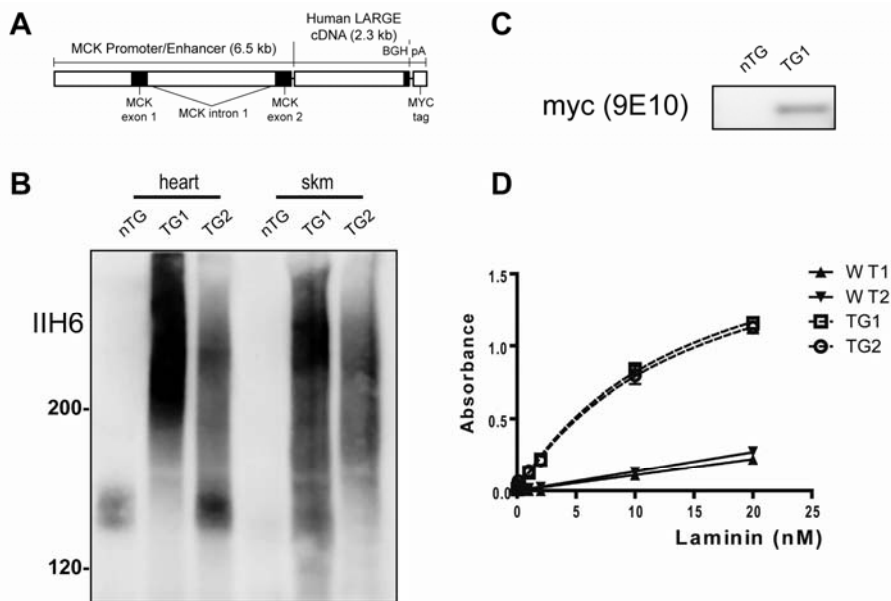
Figure 3-1) Muscle-specific expression of LARGE results in hyperglycosylation of α-dystroglycan and enhances laminin binding affinity. The transgene was generated by fusing a myc-tagged human LARGE cDNA sequence upstream of a bovine growth hormone (BGH) polyadenylation signal and downstream of a muscle creatine kinase (MCK) promoter/enhancer sequence (A). An immunoblot of Triton-X lysates (200 µg per lane) from heart and quadriceps muscle (skm) of wild-type (nTG) and two different lines of transgenic animals (TG1, TG2) was stained with the glycosylation-specific IIH6 antibody to α-dystroglycan. In both heart and skeletal muscle, expression of the transgene results in a dramatic increase in the molecular weight of α-dystroglycan as evidenced by an upward shift of glycosylated dystroglycan in both transgenic lines (B). Because of the high reactivity of the IIH6 antibody with transgenic dystroglycan, the blot was imaged at a low exposure and consequently, α-dystroglycan in wild-type skeletal muscle is barely visible. Microsomes from wild-type and transgenic (line 1) skeletal muscle (50 µg per lane) were stained with the 9E10 antibody that recognizes transgenic myc-tagged LARGE (C). Wheat germ agglutinin (WGA)-purified fractions of gastrocnemius muscle taken from two transgenic and two wild-type animals were coated onto 96-well plates, overlaid with increasing concentrations of laminin, and detected using the L-9393 anti-laminin antibody. Compared to wild-type muscle, hyperglycosylated transgenic muscle demonstrated a nearly 5-fold increase in laminin binding activity (D).
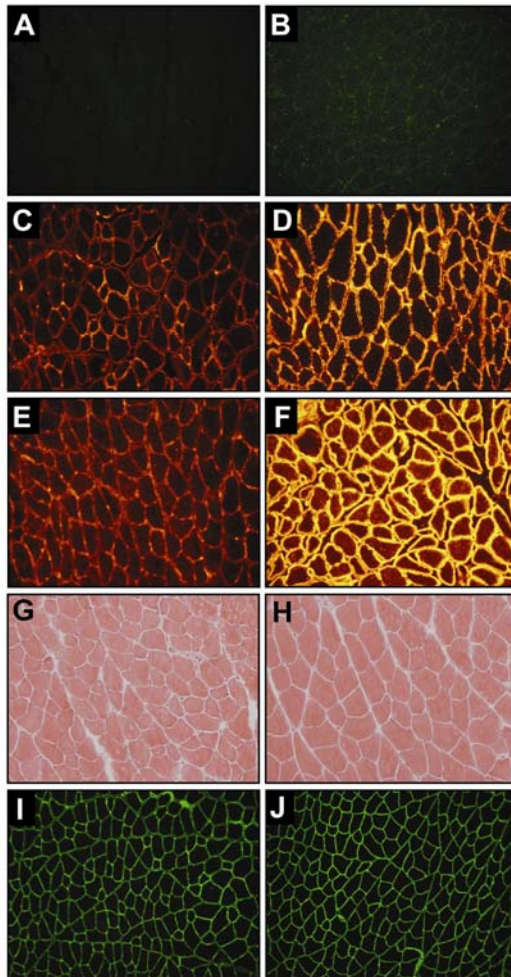
Figure 3-2) Hyperglycosylation of α-dystroglycan is not restricted by fiber type and does not cause muscle pathology. Frozen sections of wild-type and MCK-LARGE quadriceps muscle stained with the anti-myc A-14 antibody demonstrate ubiquitous expression of LARGE only in transgenic fibers (B) in a pattern consistent with localization in the golgi apparatus. Reactivity is not detected in wild-type sections (A). Cross sections of either EDL (C,D) or soleus muscle (E, F) from wild-type (C,E) and transgenic (D,F) animals stained with IIH6 demonstrate that expression of the transgene results in a ubiquitous hyperglycosylation of α-dystroglycan in both fast-twitch and slow-twitch muscle. Hematoxylin and eosin stained sections of EDL muscle from wild-type (G) and transgenic (H) animals also show that muscle of transgenic animals is indistinguishable from wild-type muscle and does not display pathology as a result of transgene expression. Additionally, transgenic (J) and wild-type (I) EDL muscle stained for laminin shows that despite enhanced laminin binding activity of transgenic muscle, laminin concentration in the extracellular matrix is not altered.

70
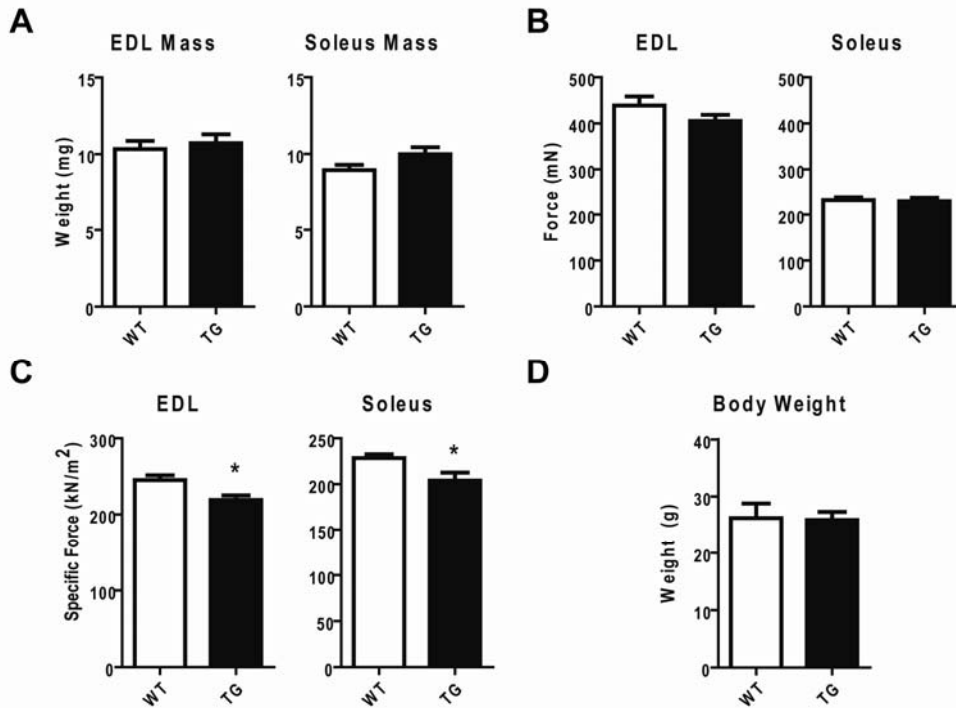
Figure 3-3) Muscle function is similar in both wild-type and MCK-LARGE transgenic mice. EDL and soleus muscles (n=6) were dissected from wild-type and transgenic animals of 31-32 weeks of age. Neither the mass of the EDL nor the soleus muscle of transgenic animals differed significantly from that of wild-type littermates (A). Similarly, contractile measurements performed *in vitro* demonstrated that transgenic EDL and soleus muscles generated maximal forces similar that observed in wild-type muscle (B). However, specific forces calculated for each muscle revealed that for both the EDL and soleus muscle, specific forces were lower in transgenic animals than those measured in wild-type muscle (C). Body weight was not significantly different between wild-type and transgenic animals (D). Data are presented as means ± SEM.
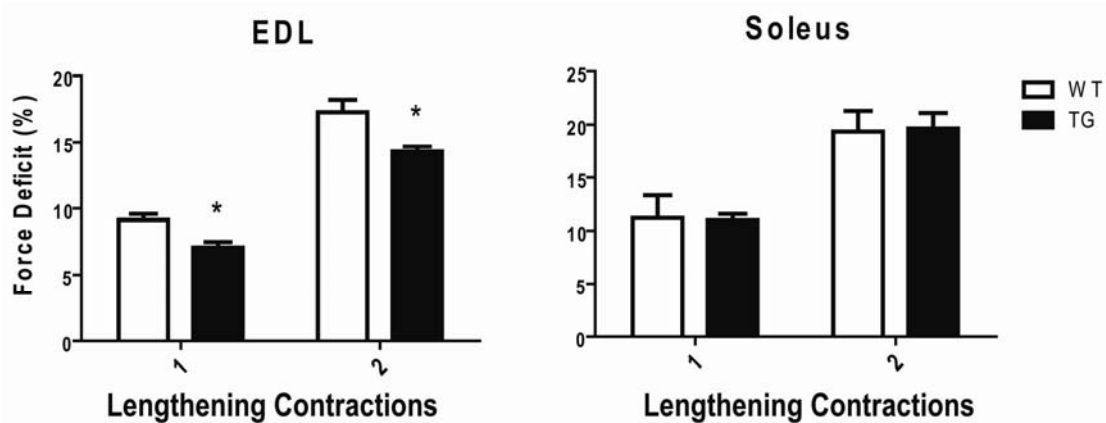
Figure 3-4) Fast-twitch muscle in MCK-LARGE animals is protected from contraction-induced injury. Contraction-induced injury was performed *in vitro* by subjecting EDL and soleus muscle from either wild-type or transgenic littermates (line 1) of 31-32 weeks of age to a series of 5 lengthening contractions of 30% strain. Force deficits measured in the EDL were consistently lower in MCK-LARGE muscle as compared to wild-type and were significantly different after the first and second contractions. Force deficits measured in the soleus muscle were not different between the two genotypes. Force deficit was calculated as the difference in maximal force production immediately prior to and following each contraction and expressed as a percentage of maximal force production.

CHAPTER 4

Muscle-specific expression of LARGE restores neurotransmission

deficits in LARGE^{myd} mice

ABSTRACT

Mutations in several glycosyltransferases underlie a group of muscular dystrophies known as glycosylation-deficient muscular dystrophy. A common feature of these diseases is loss of glycosylation and consequent dystroglycan function that is correlated with severe pathology in muscle, brain, and additional tissues. While glycosylation of dystroglycan is essential for function in skeletal muscle, whether glycosylation-dependent function of dystroglycan is sufficient to explain all complex pathological features associated with these diseases is less clear. Dystroglycan glycosylation is impaired in LARGE^{myd} mice as a result of a mutation in like-acetylglucosaminyltransferase (LARGE), a putative glycosyltransferase known to cause muscle disease in humans. We generated animals with restored dystroglycan function exclusively in skeletal muscle by crossing LARGE^{myd} animals to a recently created transgenic line that expresses LARGE selectively in differentiated muscle. Transgenic LARGE^{myd} mice were indistinguishable from wild-type littermates and demonstrated a complete amelioration of muscle disease as evidenced by an absence of muscle pathology, restored contractile function, and a reduction in serum creatine kinase activity. Moreover, while deficits in nerve conduction and neuromuscular transmission were observed in LARGE^{myd} animals, these deficits were fully rescued by muscle-specific expression of LARGE and resulted in restored

73

structure of the neuromuscular junction. These data demonstrate that impaired neurotransmission contributes to muscle weakness in LARGE[myd] mice and that the noted defects are primarily due to the effects of LARGE in stabilizing the endplate of the neuromuscular junction.

INTRODUCTION

The muscular dystrophies are a heterogeneous group of genetic diseases characterized by muscle degeneration, progressive weakness, and often a reduced lifespan. Several severe forms of muscular dystrophy, such as Walker-Warburg Syndrome (WWS) or Muscle-eye-brain disease (MEB) can also include hypotonia, mental retardation and eye malformations (204). WWS, MEB, Fukuyama CMD, MDC1C and several forms of milder limb-girdle muscular dystrophy (LGMD 2I, 2K, 2M, 2N) share a defect in the post-translational processing of the cell surface protein dystroglycan, and are sometimes termed "dystroglycanopathies" (141). Dystroglycan is encoded by the *DAG1* gene, producing a single polypeptide sequence that is cleaved to form two functional subunits (α and β) which remain associated at the plasma membrane (159). α-dystroglycan is heavily glycosylated and functions as a receptor for several components in the extracellular matrix including laminin (12, 191), agrin (137), and neurexin (189). α-dystroglycan is anchored to the extracellular face of the plasma membrane through its non-covalent association with β-dystroglycan, a type I membrane protein (160). β-dystroglycan, in turn, binds to dystrophin and the rest of the dystrophin glycoprotein complex, thereby creating a transmembrane link that is critical for dystroglycan function. In order to function as an extracellular matrix receptor, glycosylation of α-dystroglycan is essential and has been shown to be reduced or completely absent in tissues of dystroglycanopathy patients (21). Mutations in the *DAG1* gene are rare and nearly all causative mutations that result in disease have been identified in genes thought to encode glycosyltransferases (156). Consequently, these mutations abolish the function of dystroglycan as a receptor for extracellular ligands in the

various tissues where dystroglycan is expressed which is thought to underlie the broad clinical spectrum observed in patients.

In addition to dystrophin, dystroglycan associates with other proteins within the dystrophin-glycoprotein complex (DGC), and mutations in a number of DGC components have been shown to disrupt the normal assembly or function of the entire complex, resulting in multiple forms of muscular dystrophy. Muscle fibers undergo a significant degree of mechanical stress and the DGC is hypothesized to function, at least in part, in the stabilization the sarcolemma during cycles of contraction and relaxation (12). Within the DGC, dystroglycan functions as a transmembrane bridge between the basal lamina surrounding each muscle fiber (22), via binding laminin, and the intracellular cytoskeleton, through associations with dystrophin, thereby providing structural support to the sarcolemma. While the importance of this complex in skeletal muscle is unequivocal, dystroglycan is ubiquitously expressed and its functions in non-muscle tissues are not as well understood.

The targeted gene deletion of dystroglycan is embryonic lethal (23) and several studies have utilized tissue-specific deletions to dissect discrete functions of dystroglycan in neural cell types. Dystroglycan is expressed in a specialized DGC in Schwann cells of peripheral nerves (205, 206) where it can serve as a receptor for both laminin (207) and agrin (208). Schwann cell specific deletion of dystroglycan leads to the development of a progressive neuropathy as evidenced by reduced nerve conduction velocity, altered structure and reduced staining of voltage-gated sodium channels at nodes of Ranvier, and dysmyelination defects (133). Dystroglycan is also expressed at the neuromuscular junction (NMJ), and mice chimeric for deletion of dystroglycan demonstrate impaired organization/structure of NMJs (209). Agrin is an essential organizer of the NMJ during muscle development and although dystroglycan can serve as an agrin receptor in muscle, interactions between agrin and dystroglycan are dispensable for acetylcholine receptor aggregation at the postsynaptic membrane during formation of this synapse (139, 210). Rather, dystroglycan appears to function

both in the assembly of the synaptic basement membrane (140) and the localization of additional DGC components to the synapse (211) which contribute to maintenance of the synapse in adult muscle.

While dystroglycan appears to have important functions in both peripheral nerve and the NMJ, whether these functions are dependent upon its ability to bind extracellular ligands is not well understood. The LARGE^{myd} mouse model contains a mutation in the like-acetylglucosaminyltransferase (LARGE) gene (167, 168), a putative glycosyltransferase, and display a muscular dystrophy similar to that observed in patients with LARGE mutations (194, 195). The structure of the neuromuscular junction in LARGE^{myd} muscle is also impaired (144, 145), resembling defects observed in dystroglycan-null NMJs (209), and appears to be result from impaired NMJ maintenance rather than initial assembly (140). Additionally, peripheral nerve conduction velocity is reduced in both LARGE^{myd} and the allelic variant LARGE^{enr} animal model and coincides with defects in distal nerve myelination (145). These data suggest that dystroglycan glycosylation is essential not only in skeletal muscle but also for peripheral nerve function and NMJ stabilization.

We used muscle-specific overexpression of LARGE to rescue extracellular matrix receptor function of dystroglycan exclusively in LARGE^{myd} muscle to determine the degree to which dystroglycan glycosylation in non-muscle tissues contributes to the neuromuscular and muscle function in muscular dystrophy. Here we demonstrate that rescue of dystroglycan function exclusively in striated muscle is sufficient to rescue several symptoms of muscular dystrophy in LARGE^{myd} animals including amelioration of muscle pathology, restoration of force production, and extension of lifespan. Furthermore, we demonstrate that structural defects at the neuromuscular junction and functional deficits in neuromuscular transmission observed in LARGE^{myd} mice can be completely restored via rescued glycosylation of dystroglycan in muscle fibers.

METHODS

Animals. MCK-LARGE transgenic animals were generated previously on a C57BL/5 background (Chapter 3) and bred onto the LARGE[myd] strain from our maintained colony. Wild-type, TG-LARGE[myd], and LARGE[myd] mice used for all experiments were age/sex-matched littermates aged 20-40 weeks unless otherwise noted. Animals were housed in a specific pathogen free (SPF) barrier facility in the Unit for Laboratory Animal Medicine at the University of Michigan and all procedures were approved by the University of Michigan Committee for the Use and Care of Animals.

Western Blotting. Tissues were removed from deeply anesthetized animals and immediately frozen on dry ice until further processing. Samples of sciatic nerve were pooled from 5 mice per genotype. All samples were minced and homogenized in a buffer containing TBS (120-150 mM sodium chloride, 50 mM Tris pH 7.5) and 1% Triton-X 100. Samples were cleared via centrifugation and quantified using the DC Assay (Bio-Rad). Wheat germ agglutin (WGA) enrichment was performed by incubating Triton-X lysates overnight with WGA-conjugated sepharose (Vector Biolabs) at 4 degrees while rotating. Beads were washed and protein was eluted in batch by incubating beads with a buffer containing 500 mM *N*-acetylglucosamine and 0.1% Triton-X 100 in TBS. All buffers contained protease inhibitors (0.5 μg/ml Pepstatin A, 2 kallikrein inhibitor units/ml Aprotinin, 1 ug/ml Leupeptin, 0.4 mM PMSF, 0.6 mM Benzamidine). Samples were separated on 3-15% gradient polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). Immunoblotting was performed using a blocking/incubation buffer that contained 5% nonfat dry milk dissolved in TBS-T (TBS, 0.05% Tween-20). Primary antibodies included a rabbit polyclonal antibodies to β-dystroglycan (Santa Cruz), and mouse monoclonal antibodies to the myc epitope (9E10, Sigma) and glycosylated α-dystroglycan (IIH6, gift from Dr. Kevin Campbell).

Immunofluorescent Microscopy and Histology. Brain samples were carefully removed, cut in half along the sagittal axis, and immediately frozen on a plastic

77

cover slip placed on dry ice. Sciatic nerves were removed from deeply anesthetized animals and fixed overnight in 2.5% glutaraldehyde / 0.1M Cacodylate buffer, pH 7.4. Samples were further processed by the Microscopy and Image Analysis Core (MIL) at the University of Michigan. Muscles were dissected and mounted in OCT and immediately frozen in liquid nitrogen cooled isopentane. Frozen samples were cut into 8 µm cross sections using a cryostat and stored at -80° until later being used for immunofluorescent microscopy or staining with hematoxylin and eosin. For immunfluorescent staining, slides were rehydrated with PBS and blocked 1-2 hours in an incubation buffer containing 5% BSA in PBS. Slides were incubated at room temperature with primary and secondary antibody for 1-2 hours each with 4 x 5 minutes washes of PBS in between incubations. Final slides were mounted in Permafluor (Thermo Scientific) and imaged with an Olympus BX-51 fluorescence microscope.

Creatine Kinase Activity. Serum was collected from the saphenous vein of restrained animals and stored at -80 degrees. Creatine kinase activity was measured in duplicate from 13-16 animals per genotype using CK NADP Reagent (RAICHEM).

In Situ **Contractile Function Measurements.** For *in vitro* measurements, the EDL and soleus muscles were carefully dissected from deeply anesthetized mice. A 5-0 silk suture was tied to the proximal and distal tendons. One tendon was tied to a servo motor (Aurora Scientific, model 300), the other to a force transducer (Kulite Semiconductor, model BG-50). The muscle was bathed in Krebs mammalian Ringer solution maintained at 25°C and bubbled continuously with 95% O_2 and 5% CO_2 to stabilize pH at 7.4. The muscle was stimulated by square wave pulses delivered between two platinum electrodes connected to a high-power biphasic current stimulator (Aurora Scientific, model 701B) and controlled via an IBM–compatible personal computer and custom-designed software (LabVIEW 7.1, National Instruments, Austin, TX). Pulses were delivered with increasing voltage until maximum isometric twitch was determined, at which muscle length was adjusted and optimal muscle length (L_o) was

determined. The L_o was measured with digital calipers and recorded. Stimulus frequency was then increased until maximum isometric force (P_o) was achieved. Muscles were held at L_o and subjected to trains of pulses to generate an isometric contraction (300 ms for EDL, 900 ms for soleus). Cross sectional area was estimated by dividing the muscle wet mass (mg) by the product of fiber length (L_f, mm) and the density of mammalian skeletal muscle ($1.06 g/cm^3$). Specific force (sP_o) was calculated by dividing P_o by the total fiber cross-sectional area (CSA) for each muscle. A total of 3 mice and 6 muscles per genotype were tested between the ages of 27 and 31 weeks.

Muscle Injury Protocol. Following measurement of maximum twitch force and P_o, muscles were stimulated (100 ms for EDL, 300 ms for soleus) and held at L_o to allow muscles to develop P_o. Immediately following the isometric contraction, muscles were stretched through a 30% strain relative to L_f and a velocity of 1 L_f/s. Total stimulation time was 400 ms for EDL muscles and 600 ms for soleus muscles. Muscles were then returned to L_o, and subjected to four additional 30% lengthening contractions, each with 12 seconds in between, for a total of 5 stretches per muscle. The muscle was allowed to rest one minute and a final P_o was measured. The force generated after each stretch was recorded during the isometric contraction that immediately preceded the subsequent stretch. Force deficit was calculated as the decrease in P_o observed after each stretch as a percentage of P_o measured prior to the first lengthening contraction. A total of 3 mice and 6 muscles per genotype were tested between the ages of 27 and 31 weeks.

In Situ **Contractile Function Measurements.** Contractile function in gastrocnemius muscle was measured *in situ* in anesthetized mice that were placed on a temperature-controlled platform warmed to 37 degrees. The muscle was carefully dissected from the surrounding environment and a 4-0 silk suture was tied around the distal tendon that was subsequently severed and tied to the lever arm of a servo motor (model 305B, Aurora Scientific). The hindlimb was tied securely to a fixed post at the knee. A continuous drip of warmed saline was

administered to the muscle to maintain a temperature of 37 degrees for the entirety of the procedure. A bipolar platinum wire electrode was used to directly stimulate the tibial nerve and optimal voltage, frequency and muscle length (L_o) were determined. Muscle was then held at L_o and 300 ms trains of pulses were applied to determine maximum isometric tetanic contraction (P_o). This process was repeated with the exception that a cuff electrode was placed around the proximal and distal ends of the muscle in order to stimulate the muscle. When force measurements were completed, the muscle was removed and cross sectional area and specific force was calculated as described above. A total of 5 mice per genotype were tested between the ages of 20 and 40 weeks.

Neuromuscular Junction Staining. Sternocleidomastoid muscles were dissected from anesthetized animals and incubated in 1% paraformaledhyde for 20 minutes at room temperature. Muscles were then rinsed in PBS and incubated in 30% sucrose overnight at 4 degrees. Muscle were mounted and a cryostat was used to generated 30 μm longitudinal sections. Immunofluorescent staining was performed similar to that described above using α-bungarotoxin conjugated to AlexaFluor 488 (Invitrogen) and a rabbit polyclonal anti-neurofilament antibody (Millipore).

Nerve Conduction Velocity Measurements. Mice were anesthetized with isofluorane (5% induction, 1-2% maintenance) and temperature was maintained at 34 degrees using a heat lamp. Sterile electrodes were placed in the ankle and the dorsum of the foot. Sural nerve conduction velocity (SNCV) was determined by antidromically stimulating at the ankle and recording at the foot. SNCV was calculated by dividing the distance by the onset latency. Sciatic-tibial motor nerve conduction velocity (SMNCV) was determined by placing recording electrodes at the dorsum of the foot and orthodromically stimulating at the ankle and sciatic notch. SMNCV was calculated by dividing the distances between the onset latencies. A total of 10 mice were tested per genotype between the ages of 20 and 40 weeks.

Tail Flick Assay. Tail flick responses were measured using an adjustable red light emitter. The time it took for the mouse to move their tail after the beam was activated was recorded electronically. The light source was set at 25 °C and the temperature increased to 70 °C over the course of 10 seconds. A threshold of 10 seconds was applied to prevent injury to the mice. A total of 6 mice were tested per genotype between the ages of 20 and 40 weeks.

Rotarod Test. Animals were tested using an Accelerating Rotarod (Economex, Columbus Instruments). Mice were place on the rod and allowed to remain stationary for 10 seconds, after which the speed was set at 5 rpm. After rotating at a constant speed for 60 seconds, the rod began accelerating at a rate of 0.1 rpm/second and continued until the animal was unable to remain on the rod. Total time spent on the rod was recorded from the time the rod began rotating. Mice were given 3 trials daily for 5 consecutive days. In between daily trials, animals were returned to their cage for a minimum of 15 minutes. A total of 6 animals were tested for each genotype between the ages of 22 and 30 weeks.

Clasping Behavior. Clasping behavior was scored blindly according to Guyenet et. al (212). Briefly, animals of unknown genotype were suspended by the base of the tail for 10 seconds and the time each animal spent with one or both legs partially or completely retracted was used as the basis for a score between 0 (unaffected) and 3 (severely affected). A total of 10-16 mice per genotype were tested for each genotype. LARGE^{myd} animals were 12-30 weeks whereas wild-type and TG-LARGE^{myd} were as old as 62 weeks.

RESULTS

Muscle specific expression of LARGE-myc results in hyperglycosylation of dystroglycan in LARGE^{myd} skeletal muscle. We generated a line of transgenic mice in that express human LARGE exclusively in striated muscle tissues through the use of a muscle creatine kinase (MCK) promoter/enhancer sequence (Chapter 3). These mice demonstrate hyperglycosylation of dystroglycan in skeletal muscle concomitant with significantly enhanced laminin binding activity.

Transgenic MCK-LARGE animals demonstrated muscle-specific expression of the myc-tagged LARGE protein as evidenced by reactivity with the anti-myc 9E10 antibody in whole lysates from both skeletal and cardiac muscle (Fig. 4.1A). LARGE-myc was not detected in non-muscle tissues including brain, spinal cord, and lung. In order to reveal aspects of the disease in LARGE^{myd} mice that result from impaired dystroglycan function in non-muscle tissues, MCK-LARGE transgenic mice were crossed onto the LARGE^{myd} strain to generate transgenic animals homozygous for the LARGE mutation. Western blot analysis of WGA-purified lysates isolated from wild-type (WT), LARGE^{myd} (myd) and transgenic LARGE^{myd} (mydTG) animals demonstrated that the transgene was capable of glycosylating α-dystroglycan in LARGE^{myd} cardiac and skeletal muscle (Fig. 4.1B). Staining with the glycosylation specific IIH6 antibody demonstrated that while glycosylation is absent in LARGE^{myd} tissues, tissues from TG-LARGE^{myd} animals exhibited significant levels of glycosylation, above that observed in wild-type muscle. Native dystroglycan runs as a broad band between 120 and 156 kDa and similar to what is observed in MCK-LARGE mice (Chapter 3), α-dystroglycan from TG-LARGE^{myd} muscle ran at a much higher molecular weight demonstrating that LARGE overexpression was capable of adding or extending additional glycans on α-dystroglycan. Dystroglycan glycosylation was not detected in non-muscle tissues such as brain (Fig. 4.1C) or sciatic nerve (Fig. 4.1D) from LARGE^{myd} and TG-LARGE^{myd} mice confirming that the transgene was not active in these tissues. Because LARGE^{myd} mice exhibit neuronal migration defects in the cerebellum (21), brain sections were stained to confirm that this defect was still present in transgenic LARGE^{myd} animals. Sagittal sections of cerebellum stained with IIH6 and DAPI demonstrated a lack of dystroglycan glycosylation in both LARGE^{myd} and TG-LARGE^{myd} brain (Fig. 4.2A). Additionally, groups of densely stained granule cells were detected in the molecular layer, indicating a neuronal migration failure during cerebellar development (Fig. 4.2B). IIH6 stained gastrocnemius muscle sections from the same animals demonstrated that dystroglycan glycosylation was absent in LARGE^{myd} animals and restored at the sarcolemma in transgenic LARGE^{myd}

mice (Fig. 4.2C). These data demonstrate that the LARGE transgene is able to selectively glycosylate and restore function to dystroglycan in skeletal muscle while non-muscle tissues remain impaired.

Hyperglycosylation of skeletal muscle dystroglycan in LARGE^{myd} mice ameliorates muscle disease. LARGE^{myd} mice demonstrate a progressive muscle disease characterized by ongoing cycles of muscle degeneration and regeneration and an increase in fibrosis as a result of impaired interactions with the extracellular matrix that predispose the muscle to contraction-induced injury (22, 168). Therefore, we hypothesized that the selective rescue of dystroglycan function in differentiated muscle would restore interactions with the extracellular matrix that serve to protect skeletal muscle from mechanical injury. Hematoxylin and eosin stained gastrocnemius sections showed that while LARGE^{myd} muscle exhibited several parameters of muscle pathology including fibers of heterogeneous size, infiltration of inflammatory cells, and multiple regenerating fibers, muscle from TG-LARGE^{myd} animals was indistinguishable from wild-type muscle (Fig. 4.3A). In addition to decreased histological signs of dystrophy, TG-LARGE^{myd} mice also displayed a marked reduction in fibers containing internalized nuclei, which indicated a reduction in fibers undergoing regeneration (Fig. 4.3B). Because muscle pathology was not evident, we hypothesized that TG-LARGE^{myd} animals would also have lower levels of serum creatine kinase activity. While creatine kinase activity was nearly 3-fold higher in LARGE^{myd} animals compared to wild-type levels, expression of LARGE in skeletal muscle was sufficient to reduce creatine kinase activity to levels of wild-type littermates (Fig. 4.3C). Additionally, although LARGE^{myd} mice begin to lose significant muscle mass in the later stages of life as a consequence of muscle wasting (Fig. 4.7B), TG-LARGE^{myd} animals continued to gain weight similar to wild-type littermates (Fig. 4.3D).

To confirm that muscle-specific overexpression of LARGE in LARGE^{myd} mice rescued muscle function, soleus and extensor digitorum longus (EDL) muscles were used to assess muscle contractile function *in vitro.* Consistent with

our previous report (Chapter 2), soleus and EDL muscles from LARGE^{myd} animals demonstrated a reduction in both absolute and specific force production (Fig. 4.4A). Additionally, total force production in both TG-LARGE^{myd} EDL and soleus muscle was fully restored to values similar to those measured in wild-type littermates. Specific force values calculated for EDL muscle of LARGE^{myd} mice was approximately 63% of that measured in wild-type animals.   While specific force measured in TG-LARGE^{myd} EDL muscle was significantly higher than that measured in LARGE^{myd} animals, this value was approximately 85% of that measured in wild-type animals. This appeared to be due to an elevation in mass of TG-LARGE^{myd} EDL muscle that did not result in an equivalent increase in total force production (data not shown). Although specific forces measured in the soleus muscle were significantly reduced in LARGE^{myd} animals, TG-LARGE^{myd} values were fully rescued and not different from values measured in wild-type muscle.

We previously demonstrated that LARGE^{myd} muscles composed of predominantly fast-twitch fibers are highly susceptible to contraction-induced injury as a result of impaired interactions between dystroglycan and laminin (48). We hypothesized that restoration of dystroglycan glycosylation specifically in skeletal muscle would be sufficient to restore extracellular matrix receptor function and reduce the susceptibility of LARGE^{myd} muscle to contraction-induced injury. Muscle injury was performed by subjecting EDL and soleus muscles to a series of 5 lengthening contractions of 30% strain *in vitro*. In agreement with our previous study, force deficits were significantly greater in LARGE^{myd} EDL muscle than in wild-type muscle after each successive lengthening contraction. After 5 lengthening contractions, force production in LARGE^{myd} EDL muscle was roughly 20% of the value measured prior to injury (Fig. 4.4C). In contrast, force deficits measured in TG-LARGE^{myd} EDL muscle were no different than values measured in wild-type muscle after each lengthening contraction.

In addition to significant improvements in function, expression of the LARGE transgene in LARGE^myd mice also dramatically improved the health of LARGE^myd animals and restored breeding capacity. While LARGE^myd animals in our colony rarely survive past 40 weeks of age, transgenic LARGE^myd are presently as old as 70 weeks (Fig. 4.7A). Additionally, TG-LARGE^myd animals do not demonstrate the hindlimb clasping behavior exhibited by LARGE^myd littermates (Fig. 4.7C). Because glycosylation of dystroglycan remains impaired in the central and peripheral nervous system of transgenic LARGE^myd animals, we next wanted to assess motor performance in the absence of muscle disease.

Transgenic LARGE^myd mice do not demonstrate deficits in neuronal function. Deficits in motor coordination as a result of either impaired cerebellar architecture or deficits in peripheral nerve function were first tested using an accelerating rotarod protocol. Animals were placed on a stationary rod and the time each animal was able to remain on the rod once it began rotating was recorded daily for 5 consecutive days. On each day, fall latencies were significantly reduced in LARGE^myd animals but were not different between TG-LARGE^myd and wild-type animals (Fig. 4.5A). Additionally, all three genotypes were able to significantly increase the amount of time they spent on the rod by day 5. Although TG-LARGE^myd demonstrated impaired cerebellar development (Fig. 4.2B), these results suggest that the defect does not significantly affect motor function or task learning since the performance of TG-LARGE^myd animals was not difference from wild-type mice.

Deficits in nerve structure and function have been reported in both LARGE^myd mice and an additional strain containing a mutation in LARGE, which suggests that dystroglycan glycosylation is required for normal peripheral nerve function (145, 213). Therefore, we wanted to determine whether TG-LARGE^myd mice similarly demonstrated impaired nerve function as a consequence of disrupted dystroglycan glycosylation in neuronal tissues. A tail flick assay was performed which measures the ability of an animal to respond to a heated light beam stimulus focused on the tail. As expected, time latencies measured in

LARGE^{myd} animals were significantly longer than those measured in wild-type animals. Values obtained for TG-LARGE^{myd} animals however, were similar to wild-type (Fig. 4.5B). Because performance during a tail flick assay can be dependent upon both nerve and muscle function, we wanted to assess nerve function independently. Although conduction velocity measured in the sural nerve was not different between the three different genotypes (not shown), sciatic motor nerve conduction velocity was significantly reduced only in LARGE^{myd} animals (Fig. 4.5C). However, semi-thin stained sections of sciatic nerve did not identify any pathological defects in myelination in either 30-week old LARGE^{myd} or TG-LARGE^{myd} animals (Fig. 4.8).

Neurotransmission deficits present in LARGE^{myd} animals are restored in TG-LARGE^{myd} mice. Neuromuscular junction structure is disrupted in LARGE^{myd} and DG-deficient muscle (144, 209). Although dystroglycan is an agrin receptor, interactions with agrin are dispensable during formation of the neuromuscular junction and instead, dystroglycan is hypothesized to function in the maintenance and stabilization of the synapse (140, 214). Because the neuromuscular junction is formed from both presynaptic and postsynaptic components, we investigated whether rescue of sarcolemmal dystroglycan function was sufficient to restore NMJ architecture in transgenic LARGE^{myd} mice. Alexa-488 conjugated α-bungarotoxin and an antibody to neurofilament were used to label presynaptic and postsynaptic regions of NMJs in whole-fixed sternocleidomastoid muscles. NMJs in wild-type muscle exhibited the characteristic pretzel shape while this structure in LARGE^{myd} muscle was fragmented in appearance, consistent with previous studies (144) (Fig. 4.6A). However, expression of full length LARGE was sufficient to restore NMJs in TG-LARGE^{myd} muscle so that they were indistinguishable from those in wild-type muscle. This suggests a critical importance of sarcolemmal dystroglycan, as opposed to dystroglycan expressed in either presynaptic neurons or perisynaptic Schwann cells, for normal maintenance of NMJ structure. In order to address the functional consequence of impaired NMJ structure in LARGE^{myd} mice, a contractile protocol was performed that utilized paired measurements of force

production in the gastrocnemius muscle. This allowed for comparisons to be made in each muscle between maximum forces produced following stimulation to either the sciatic nerve or to the muscle directly. If neurotransmission was impaired, direct muscle stimulation would produce higher maximal force values than when the muscle was stimulated via the nerve. Consistent with observed measurements in the EDL and soleus muscle, specific force values measured in LARGE[myd] gastrocnemius muscle were significantly lower than wild-type and transgenic LARGE[myd] values (Fig. 4.6B). Additionally, while forces measured during direct muscle stimulation were slightly lower than values obtained following nerve stimulation in wild-type animals, the opposite was observed in all LARGE[myd] animals. Direct stimulation of LARGE[myd] muscle resulted in a mean 16% increase in maximal force production compared to values measured during nerve stimulation (Fig. 4.6C). However, no such increase was measured in transgenic LARGE[myd] muscle, likely due to improved NMJ structure. These results suggest that aberrant structure of the neuromuscular junction as a consequence of impaired dystroglycan function causes a functional denervation in muscle fibers, and that restoration of dystroglycan function at the postsynaptic membrane of skeletal muscle alone is sufficient to restore these functional defects in neurotransmission.

DISCUSSION

Although muscle disease is the prominent and shared feature of all muscular dystrophies, patients with mutations in glycosyltransferases also suffer from severe central and peripheral nervous system impairments as a result of disrupted dystroglycan function (150, 151, 194, 215). Several studies have highlighted the importance of glycosylation-dependent interactions between dystroglycan and laminin at the sarcolemma that are important for providing critical structural support during muscle contractions (22, 216). Disruption of this mechanical link can result in a high susceptibility to contraction-induced damage and is hypothesized to underlie the eventual decline in muscle function observed in DGC-related muscular dystrophies. However, dystroglycan is ubiquitously

expressed and a mechanical role for the DGC in non-muscle tissue is less apparent.

Here we demonstrate that muscle-specific restoration of the ligand binding activity of dystroglycan rescues several features of muscular dystrophy in LARGE^{myd} animals. Overexpression of MCK-LARGE in LARGE^{myd} muscle resulted in significant hyperglycosylation of sarcolemmal α-dystroglycan beyond that observed in wild-type animals, reestablishing dystroglycan as an extracellular matrix receptor in skeletal muscle. This resulted in a complete attenuation of muscle pathology in LARGE^{myd} animals and coincided with a recovery of muscle contractile performance. Additionally, we observed that restored function of dystroglycan at the motor endplate was sufficient to restore normal NMJ architecture and this corresponded to a functional rescue of neurotransmission deficits observed in LARGE^{myd} animals. These results suggest that muscle weakness observed in LARGE^{myd} mice as a consequence of primary muscle dysfunction is compounded by a failure in neurotransmission.

LARGE^{myd} muscle demonstrates altered structure of the neuromuscular junction and our results demonstrate that this defect can also be reversed via selective restoration of dystroglycan function at the sarcolemma. Although dystroglycan is a glycosylation-dependent agrin receptor (137) and can bind rapsyn (217), interactions with these critical NMJ proteins are not thought to be essential for initial formation of the neuromuscular junction. Instead, dystroglycan and the DGC that is present at the NMJ appear to function in NMJ maintenance in adult muscle by contributing to the formation of the surrounding basal lamina and serving as a scaffold for additional proteins (211). Because the structure of the NMJ is more disrupted in LARGE^{myd} animals than in other mouse models of muscular dystrophy (144) this suggests that the altered structure is not simply a product of degenerating muscle but rather due to the distinct requirement of glycosylated dystroglycan in this structure.

In addition to an amelioration of muscle dysfunction, an overall increase in health was observed that included improvements in longevity. While the cause

of death is unknown in LARGE^{myd} mice, severe muscle weakness/paralysis leading to failure of food intake and cardiomyopathy (216) may contribute. Unexpectedly, a complete recovery of motor performance was also observed in TG-LARGE^{myd} animals despite evidence of impaired cerebellar and brain development that was not rescued by the transgene. Because the cerebellum and motor cortex participate in the coordination of balance and movement, the functional consequence of defective neuronal migration in TG-LARGE^{myd} animals was tested using an accelerating rotarod. Ordinarily, comparisons between wild-type and LARGE^{myd} animals using this assay would be confounded by the profound muscle weakness present in LARGE^{myd} animals. However, because muscle function was restored in TG-LARGE^{myd} animals and was comparable to wild-type mice, comparisons were able to be made with wild-type mice. Surprisingly, no differences in performance were detected and both groups of animals were able to improve significantly over the five day period. Although LARGE^{myd} mice performed poorly, they were also able to improve their performance by the fifth day. These results suggest that despite defects in cerebellar architecture, motor learning and coordination is not severely impaired in LARGE^{myd} animals.

Deficits in rotarod performance have been documented in a Schwann-cell specific deletion of dystroglycan, which also demonstrates defects in myelination and conduction velocity (133). In a related model of LARGE deficiency, conduction velocity in the sciatic nerve was reduced and coincided with the presence of large clusters of unmyelinated axons in the nerve (145). In order to determine whether these same defects existed in LARGE^{myd} animals, sciatic nerves were collected from 40-week old animals. Because electron microscopy did not reveal any differences between the three genotypes (not shown), this suggests that the nerve defect is either not 100% penetrant in all animals or is variable along the length of the nerve and missed in our analysis. Nerve conduction velocity was measured in both sciatic and sural nerves, and deficits were only observed in sciatic nerves of LARGE^{myd} animals while sural conduction velocity was not different between the three genotypes. Because peripheral

nerve function appeared normal in TG-LARGE^{myd} animals, despite evidence demonstrating that dystroglycan was hypoglycosylated, these data suggest that LARGE-mediated glycosylation of dystroglycan is not essential for normal peripheral nerve function. While this conflicts with several studies demonstrating the importance of interactions between dystroglycan and laminin in myelination of peripheral nerves (218-220), not all nerve defects reported in DG-null animals are consistent with the exclusive glycosylation-dependent function of dystroglycan in the PNS. LARGE^{enr} animals do not demonstrate the defects in node elongation and sodium channel clustering that have been reported for Schwann cell specific dystroglycan-null animals (133, 145) which suggests that the formation of axonal nodal domains does not require glycosylation of dystroglycan by LARGE.

In order to verify the tissue specific nature of the promoter, multiple tissues were stained for LARGE-myc protein expression and the transgene was not detected in either wild-type or transgenic non-muscle tissues. Because α-dystroglycan is capable of being cleaved and can be detected in serum (221), we also confirmed that hyperglycosylated dystroglycan was restricted to striated muscle tissues of TG-LARGE^{myd} animals, and reactivity with the glycosylation specific IIH6 antibody was not detected in neuronal tissues. Additionally, neuronal migration defects were observed in the cerebellum of transgenic LARGE^{myd} animals which confirmed that functional deficits caused by impaired dystroglycan glycosylation in non-muscle tissues (222) were still present in transgenic LARGE^{myd} animals. Therefore, absence of neuronal defects in transgenic LARGE^{myd} mice is not explained by expression of the transgene resulting in residual glycosylation of dystroglycan in neuronal tissues.

An interesting explanation that might account for the observed rescue of nerve function may be related to the improvement in either muscle function or restored structure of the neuromuscular synapse. While we observed a deficit in nerve conduction velocity of LARGE^{myd} sciatic nerve, sural nerve function was not different from wild-type animals. If the impaired muscle function of LARGE^{myd}

animals negatively influenced motor neuron function, this might explain impaired conduction velocity in the motor sciatic nerve but not in the sensory sural nerve. Maintenance of neuronal connections can be dependent upon target-derived retrograde signals such as neurotrophins (223). Skeletal muscle is a source of several neurotrophins including brain-derived neurotrophic factor (BDNF), NT-3, NT-4 and glia-derived neurotrophic factor (GDNF) (224-228) which may contribute to motor neuron survival and/or differentiation (229). There is also evidence to suggest that expression of neurotrophins and their receptors might be altered in muscular dystrophy (230, 231). While it is intriguing to speculate that disruptions in neurotrophin related signaling may contribute to muscle weakness in muscular dystrophy, this hypothesis remains to be addressed.

In this study, we demonstrate via selective restoration of dystroglycan function in skeletal muscle that extracellular matrix function of dystroglycan in cardiac and skeletal muscle is sufficient to ameliorate several characteristics of muscular dystrophy. Additionally, we show that structural defects reported at the LARGE^{myd} neuromuscular junction result in a functional deficit in neurotransmission as a result of impaired sarcolemmal dystroglycan function. Because we no longer observe deficits in peripheral nerve function, we hypothesize that impaired function of dystroglycan in skeletal muscle may cause reciprocal deficits in nerve function as a consequence of either impaired communication at the neuromuscular junction or through retrograde signaling from diseased myofibers.

ACKNOWLEDGEMENTS

91
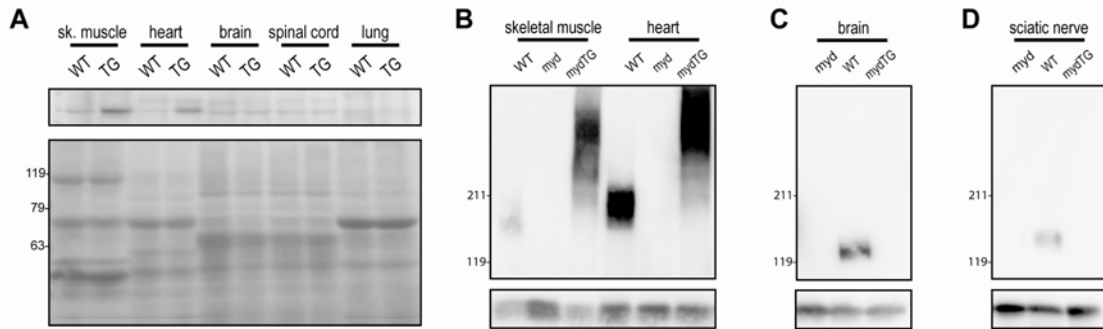
Figure 4-1) Selective expression of LARGE-myc in striated muscle results in hyperglycosylation of α-dystroglycan in LARGE^{myd} mice. Triton-X whole lysates from wild-type (WT) and transgenic MCK-LARGE (TG) mice stained with the 9E10 anti-myc antibody demonstrate LARGE-myc expression exclusively in skeletal and cardiac muscle (A, upper panel). Equal loading of the gel is indicated by Ponceau S staining of the blot prior to immunostaining (A, lower panel). WGA-enriched lysates demonstrate a loss of α-dystroglycan glycosylation in LARGE^{myd} (myd) animals as evidenced by a lack of reactivity with the glycosylation specific IIH6 antibody (B,C,D, upper panels). Expression of the LARGE-myc transgene in LARGE^{myd} mice (mydTG) results in hyperglycosylation of α-dystroglycan selectively in cardiac and skeletal muscle (25 µg per lane) as demonstrated by a dramatic increase in molecular weight of the α-dystroglycan. Glycosylation of α-dystroglycan was absent in brain (75 µg per lane) and sciatic nerve (60 µg) of LARGE^{myd} transgenic animals owing to the lack of transgene expression in these tissues. In contrast to altered glycosylation of α-dystroglycan between the three different genotypes, expression levels of dystroglycan was unaltered as evidenced by similar reactivity with an anti-β-dystroglycan antibody using stripped blots (B,C,D, lower panels).
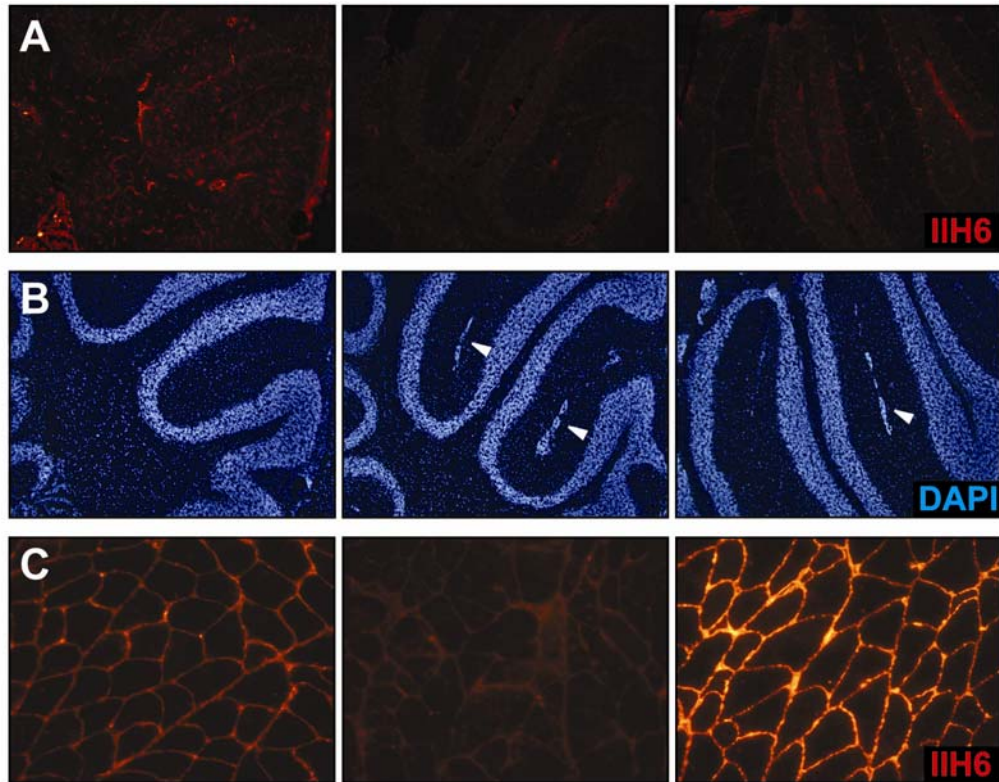
Figure 4-2) Transgenic LARGE^{myd} mice demonstrate absence of functional dystroglycan in non-muscle tissue. Parasagittal sections of cerebellum from wild-type (left), LARGE^{myd} (middle) and transgenic LARGE^{myd} (right) stained with IIH6 demonstrate that only wild-type brain expresses glycosylated dystroglycan (A). Absence of glycosylation in LARGE^{myd} and transgenic LARGE^{myd} cerebellum resulted in neuronal migration failure during development as indicated by densely stained DAPI stained cells (white arrows) within the molecular layer (B). Although abnormal dystroglycan glycosylation and function in the cerebellum was evident in both LARGE^{myd} and transgenic LARGE^{myd} brains, skeletal muscle from transgenic LARGE^{myd} animals contained glycosylated dystroglycan at the sarcolemma (C).
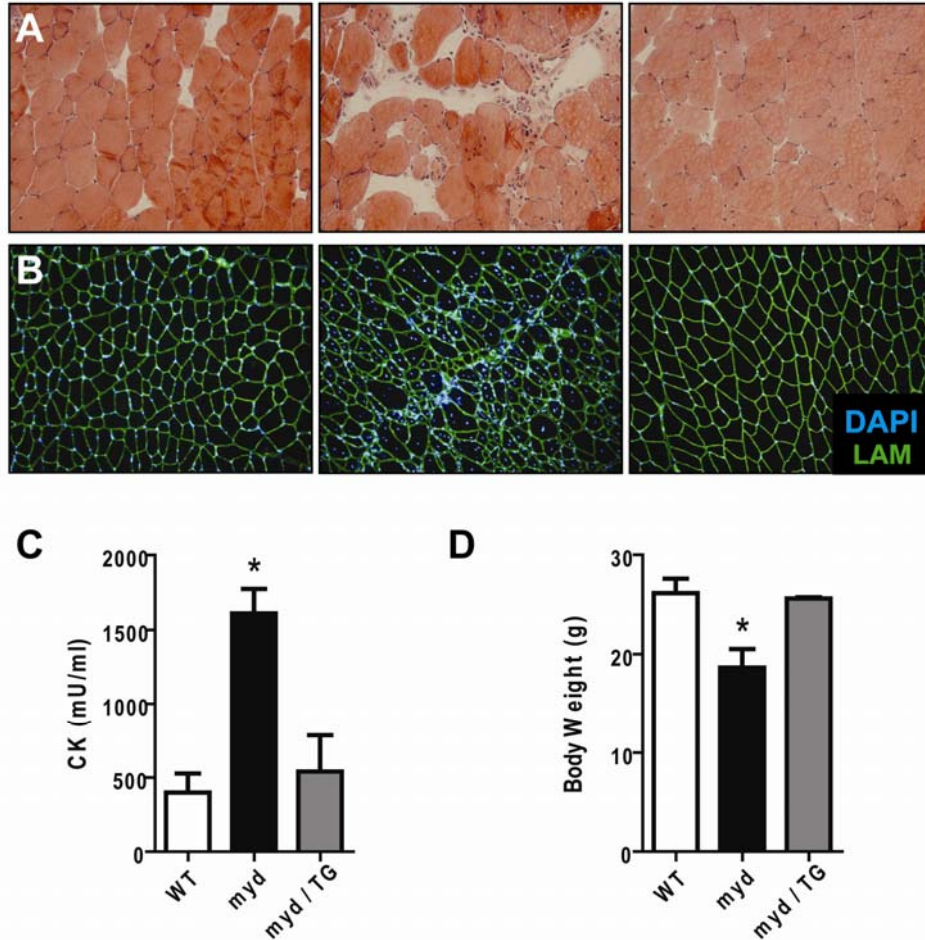
Figure 4-3) Muscle disease is ameliorated in transgenic LARGE^{myd} animals.
Hematoxylin and eosin stained skeletal muscle sections demonstrate amelioration of dystrophy when the MCK-LARGE transgene is expressed in LARGE^{myd} muscle as indicated by an absence of inflammatory cells and a normalization of fiber size (A). Muscle sections co-stained with the anti-laminin L-9393 antibody and DAPI demonstrate restoration of normal muscle architecture and a dramatic reduction in fibers undergoing degeneration or regeneration as indicated by a reduction in fibers with internalized nuclei (B). Plasma creatine kinase levels were significantly reduced in transgenic LARGE^{myd} mice compared to LARGE^{myd} animals (n = 13-16 mice) (C). Although body weights of LARGE^{myd} animals were significantly reduced due to the progressive loss of muscle mass, weights of transgenic LARGE^{myd} were not different from wild-type animals (D).
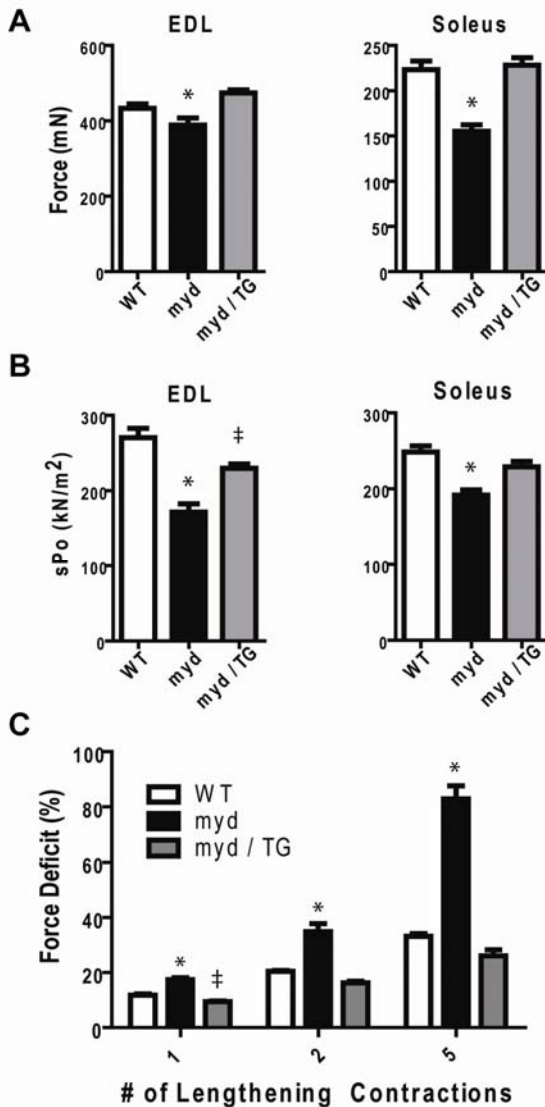
Figure 4-4) Muscle function is improved in transgenic LARGE^{myd} mice. Contractile function of EDL and soleus muscle was measured *in vitro* (n=6 muscles). While force production in both LARGE^{myd} EDL and soleus muscle was significantly reduced compared to wild-type, values for transgenic LARGE^{myd} muscles were not different from wild-type (A). Although expression of the transgene in LARGE^{myd} muscle resulted in a significant improvement in specific force, values for transgenic LARGE^{myd} EDL were below that of wild-type muscle (B). Contraction-induced injury was performed by subjecting EDL muscles to a series of lengthening contractions of 30% strain. LARGE^{myd} muscle demonstrated an elevated susceptibility to contraction-induced damage as indicated by an increase in force deficit following 1, 2, and 5 lengthening contractions compared to wild-type muscle (C). Muscles from transgenic LARGE^{myd} animals did not display a susceptibility to injury any more so that wild-type animals following 2 and 5 lengthening contraction.

95
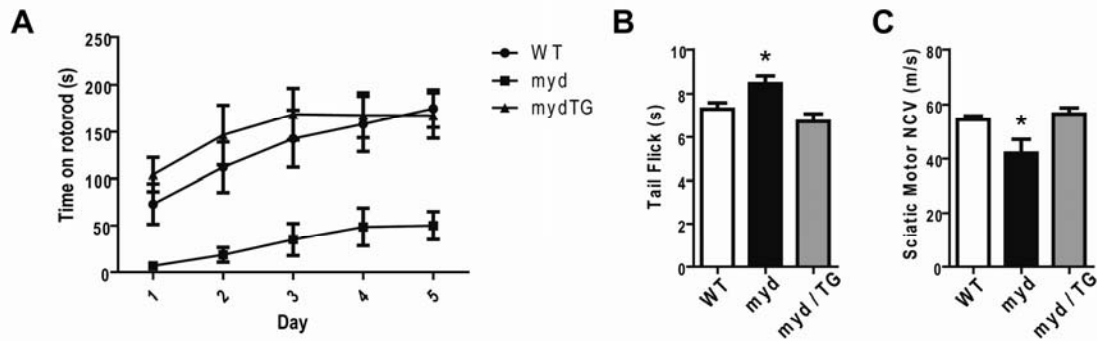
Figure 4-5) Neuronal function is improved in transgenic LARGE^myd animals.
Motor coordination was tested using an accelerating rotorod. Animals (n= 6 per
genotype) were placed on a stationary rod that rotated at a constant speed of 5
rpm for 60 seconds and began accelerating at a rate of 0.1 rpm/second. The
time each animal was able to stay on the rod beginning at rotation onset was
recorded for 3 daily trials over 5 days. Wild-type and transgenic LARGE^myd
managed to stay on significantly longer than LARGE^myd mice during each trial
and values measured for each trial were not statistically different from one
another (A). Each data point is representative of the mean latency for all mice of
each genotype. A tail flick assay was used to assess whether neurological
dysfunction was evident in LARGE^myd and transgenic LARGE^myd animals. Tail
flick responses (n= 6 animals per genotype) were measured as the time it took
for each mouse to remove their tail from a heated beam of light. Only LARGE^myd
animals demonstrated a significant delay compared to control animals (B).
Nerve conduction velocity was measure in sciatic nerve (n = 10 animals per
genotype). Values measured in LARGE^myd animals were significantly slower
while values in transgenic LARGE^myd were not different from values recorded in
wild-type littermates (C). Error bars for all graphs indicate SEM.
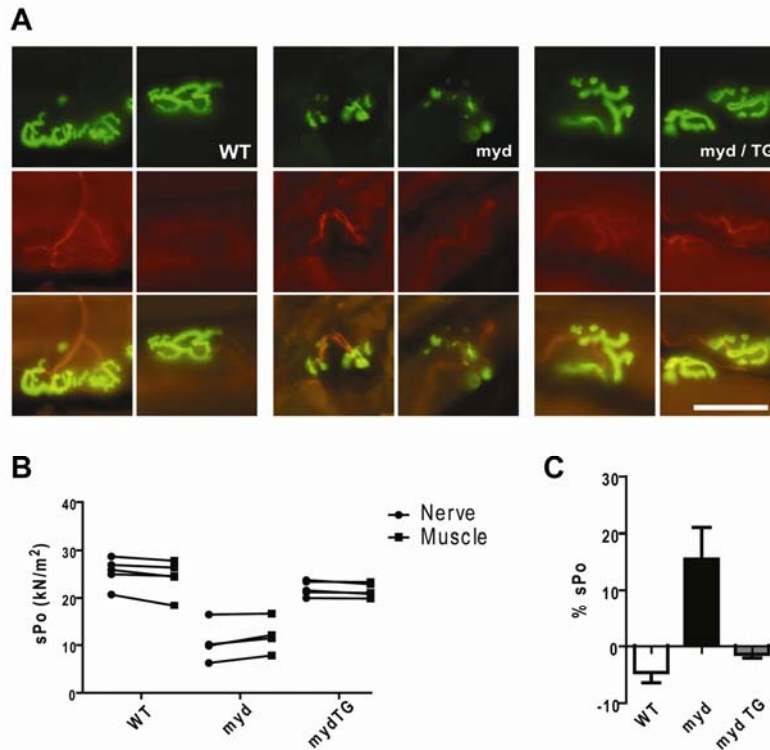
Figure 4-6) Neuromuscular junction structure and neurotransmission defects are restored in transgenic LARGE^{myd} mice. Sternocleidomastoid muscles were fixed whole to stain the presynaptic and postsynaptic regions of the neuromuscular junction. Acetylcholine receptors were labeled with Alexa488 conjugated α-bungarotoxin (green) and an antibody to neurofilament (red) was used to label the motor neuron. Representatives images of NMJs in wild-type and transgenic LARGE^{myd} muscle demonstrate the characteristic pretzel shape whereas NMJs in LARGE^{myd} muscle appear abnormal and fragmented (A). To determine the functional consequence of disrupted NMJ structure, force production of gastrocnemius muscle was measured *in situ* and stimulated by either the tibial nerve or directly using a cuff electrode surrounding the muscle. Specific force values measured following muscle stimulation were always slightly lower than those measured after nerve stimulation for each wild-type and transgenic LARGE^{myd} animal (n= 4-5 animals per genotype) (B). In contrast, specific force values were always higher for LARGE^{myd} animals following direct muscle stimulation indicating a partial functional denervation of fibers. The difference in specific force measured for either nerve or direct muscle stimulation is shown as a percentage of total specific force produced from nerve (C).
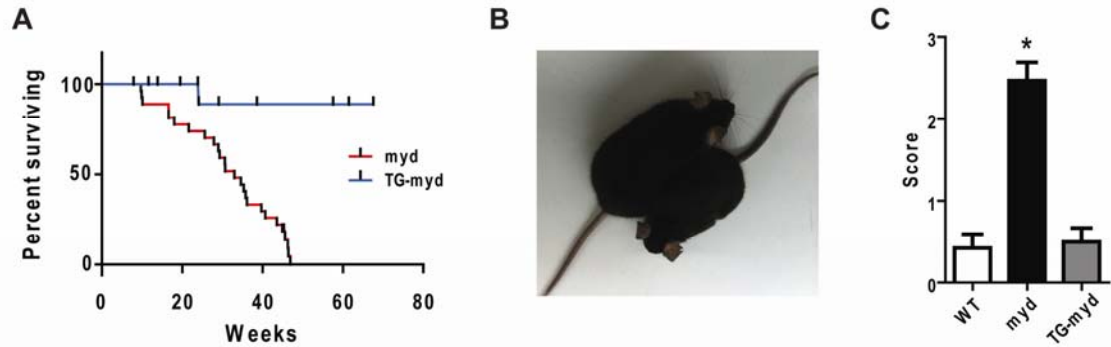
Figure 4-7) Restoration of dystroglycan glycosylation in skeletal muscle significantly improves overall health. A survival curve depicts survival data from a LARGE^{myd} colony maintained by our lab with transposed survival data from the recently generated TG-LARGE^{myd} strain that are presently 70 weeks of age (A). While LARGE^{myd} animals lose significant muscle mass in the later stages of life, TG-LARGE^{myd} mice gain weight comparable to wild-type littermate. Shown is a photograph of a 68 week-old TG-LARGE^{myd} mouse (top left), the oldest currently surviving in the colony, alongside a 33-week old LARGE^{myd} mouse (bottom right) (B). LARGE^{myd} mice demonstrate an abnormal hindlimb clasping behavior that is also rescued in TG-LARGE^{myd}(C). Mice were briefly suspended by the tail and a score from 0 to 3 was assigned according to severity (n= 10-16 mice).
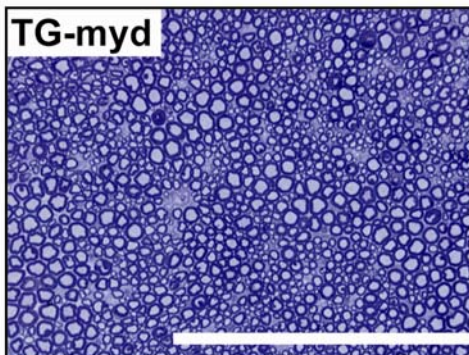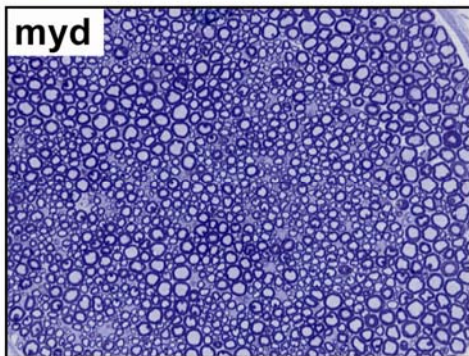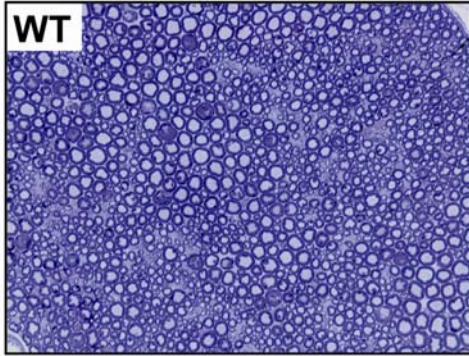
Figure 4-8) Lack of pathology in LARGE^{myd} and TG-LARGE^{myd} sciatic nerve. Although impaired conduction velocity was detected in the sciatic nerve of LARGE^{myd} animals, pathology was not evident. Toluidine blue stained semi-thin sections of sciatic nerve from wild-type (WT), LARGE^{myd}(myd), and TG-LARGE^{myd} (TG-myd) animals demonstrate the absence of myelination defects in LARGE^{myd} and TG-LARGE^{myd} mice. Scale bar represents 200 µm.

CHAPTER 5

Conclusions and Future Directions

Summary of Thesis Work

The occurrence of muscle degeneration in what we now refer to as muscular dystrophy was first described nearly 150 years ago (232, 233). Despite the identification of a genetic basis nearly 120 years later (28), the precise mechanism by which muscle progressively degenerates remains a fervent topic of scientific research. Although muscular dystrophy can arise from mutations in any of several distinct genes, a subset of these diseases are defined by a common defect in the post-translational modification of dystroglycan, and the causative gene mutations have been identified in a group of glycosyltransferases. This hypoglycosylation impairs the function of dystroglycan as an extracellular matrix receptor and consequently disrupts interactions with its multiple extracellular ligands. The critical interaction between laminin and dystroglycan in skeletal muscle is thought to function in the preservation of sarcolemmal integrity. Therefore, when the interaction of dystroglycan with the extracellular matrix is abnormally disrupted, micro-tears in the sarcolemma that occur as a consequence of normal muscle contraction are exacerbated and allow for an increased flux of ions and small molecules across the membrane. The membrane tears and flux of ions are thought to disrupt normal homeostasis and eventually result in death of the myofiber. The importance of the interaction of dystroglycan and matrix to normal muscle function was the basis for chapters 2 and 3 which explored the physiological consequence of either reduced or

enhanced laminin binding activity of dystroglycan in skeletal muscle. Dystroglycan is ubiquitously expressed and can also serve as a receptor for the neural-derived ligands, agrin and neurexin. Because patients with mutations in glycosyltransferases exhibit both progressive muscle disease in addition to severe neurological deficits, this suggests that dystroglycan has additional essential functions in non-muscle tissues. Potential deficits in peripheral nerve function that result from dystroglycan hypoglycosylation and the effects of loss of dystroglycan function on motor performance were investigated in chapter 4. Taken together, the experiments described in this thesis have attempted to dissect the distinct functions of dystroglycan in different tissues as a means to understand how mutations affecting dystroglycan function ultimately result in a multi-system muscle disease. The work described in the preceding chapters and summarized below support the overall hypothesis that LARGE-mediated glycosylation is essential for normal skeletal muscle function with distinct functions at the lateral membrane and at the neuromuscular junction.

Chapter 2 described the functional and molecular consequences resulting from impaired dystroglycan glycosylation in both slow-twitch and fast-twitch muscles of LARGE$^{myd/myd}$ mice. While the partial reduction of dystroglycan glycosylation observed in heterozygous LARGE$^{myd/-}$ mice was not sufficient to alter muscle function, homozygous LARGE$^{myd/myd}$ mice demonstrated a marked reduction in specific force in both soleus and EDL muscles. Additionally, although EDL muscles from LARGE$^{myd/myd}$ mice were highly susceptible to lengthening contraction-induced injury, LARGE$^{myd/myd}$ soleus muscle surprisingly showed no greater force deficit compared to wild-type soleus muscle even though the muscle demonstrated a significant reduction in laminin binding activity and dystrophic pathology. Interestingly, soleus muscles were shown to display a markedly higher expression of β1-containing integrins compared with EDL and gastrocnemius muscles. This suggests that β1-containing integrins play an important role as alternative matrix receptors that can protect muscles containing slow-twitch fibers from contraction-induced injury in the absence of dystroglycan

101

function. More importantly, these results reveal that contraction-induced injury is a separable phenotype from the dystrophic pathology of muscular dystrophy.

To further determine the importance of dystroglycan glycosylation in skeletal muscle function, a novel transgenic mouse was created that directs the overexpression of LARGE exclusively in striated muscle through the use of a MCK promoter/enhancer sequence. Chapter 3 outlined the experiments designed to test the hypothesis that overexpression of LARGE in skeletal muscle could enhance lateral connections between the sarcolemma and surrounding basal lamina to provide additional protection from lengthening contraction-induced injury in otherwise normal muscle. Skeletal muscle of MCK-LARGE transgenic mice demonstrated hyperglycosylation of dystroglycan that coincided with a significantly elevated laminin binding affinity compared to that of wild-type muscles. More importantly, fast-twitch skeletal muscle from transgenic mice demonstrated enhanced protection from mechanical injury such that force deficits following injury were significantly lower than those measured in wild-type littermates. These results indicate that hyperglycosylation and enhanced function of dystroglycan via increased activity of the enzyme LARGE may be therapeutic not only in inherited glycosylation-deficient muscular dystrophy but also in acquired diseases or disability resulting from muscle injury.

Chapter 4 discussed the experiments used to dissect the glycosylation-specific functions of dystroglycan at the neuromuscular junction and in peripheral nerve function. MCK-LARGE animals were crossed onto the LARGE^{myd} strain to generate mice homozygous for the *myd* mutation with restored LARGE function exclusively in cardiac and skeletal muscle.   These TG-LARGE^{myd} animals displayed a successful rescue of skeletal muscle function as evidenced by a reduction in serum creatine kinase activity, restoration of normal muscle structure, suppression of muscle degeneration, and an increase in protection from contraction-induced injury. Notably, structural defects observed at the neuromuscular junction in LARGE$^{myd/myd}$ mice were corrected in transgenic LARGE$^{myd/myd}$ animals and were correspondingly associated with the rescue of

neurotransmission and nerve conduction deficits. These results demonstrate that skeletal muscle weakness in LARGE$^{myd/myd}$ mice results from combined defects in both skeletal muscle and neuromuscular function. Importantly, these data suggest that neuronal deficits associated with impaired dystroglycan function may be exacerbated by impaired muscle function and/or communication at the neuromuscular junction.

The results outlined in this thesis expand the current body of knowledge in regard to the importance of dystroglycan glycosylation in normal muscle function and motor performance. Because dystroglycan is central to the DGC, these results have important implications for the mechanisms by which dystrophy results in additional forms of DGC-related muscular dystrophies and are therefore critical for the eventual design of therapeutics aimed at treating the disease. The implications of these results will be discussed in greater detail in the remainder of this chapter and future experiments that can clarify the discrete functions of dystroglycan in both muscle and neuronal tissues will be highlighted.

Implications and Future Directions

Mechanical Functions of the Dystrophin-Glycoprotein Complex

Within the dystrophin-glycoprotein complex, dystroglycan serves as an essential transmembrane link between dystrophin and laminin in an interaction that has largely been hypothesized to be mechanical in nature. Consequently, mutations that affect the assembly or function of the complex are thought to cause a destabilization of the sarcolemma which can disrupt myofiber homeostasis and initiate a destructive signaling cascade leading to cell death. Support for this hypothesis comes from studies that have shown increased membrane permeability of dystrophic muscle concomitant with increased concentrations of intracellular calcium (6, 54). Additionally, studies have cited the susceptibility of dystrophic muscle to injury induced by lengthening contractions as evidence for impaired sarcolemmal integrity in dystrophic muscle resulting from mutations in DGC components (4, 30, 36). Although the results

obtained in EDL muscle and described in chapters 2 and 3 support this claim for a mechanical function of dystroglycan, the LARGE[myd] soleus muscle was resistant to mechanical injury but still exhibited pathological features of muscular dystrophy. These results demonstrate that susceptibility to damage is not an initiating step that is sufficient to cause the dystrophy and weakness observed in this muscle.

While a lack of susceptibility of the soleus to mechanical injury has also been reported in other models of muscular dystrophy (39, 51, 234), an explanation that accounts for this result is lacking. In contrast to mutations in either dystrophin or sarcoglycan, mutations affecting the glycosylation of dystroglycan do not alter the composition or expression level of the DGC and therefore, the defects observed in muscle are a direct consequence of impaired dystroglycan function. Consequently, the observed lack of impaired sarcolemma integrity in soleus muscle of LARGE[myd] mice suggests that the interaction between dystroglycan and laminin is dispensable as a means of providing membrane stability to myofibers within this muscle. In support of this conclusion, an alternative laminin receptor, α7β integrin, was shown to be highly enriched in the soleus muscle which highlights the possibility that these two primary laminin receptors in skeletal muscle function in a fiber-type specific manner (Fig. 5.1). Surprisingly, the targeted deletion of α7 integrin was shown to be only mildly dystrophic and largely affected the myotendinous junction, though evidence of dystrophy was observed almost exclusively in the soleus muscle (94). In a more recent study, integrin-deficient and dystroglycan-deficient EDL muscles were directly compared and though both muscles demonstrated deficits in force production, only the loss of dystroglycan function resulted in susceptibility to mechanical injury.   This led the authors to conclude that only the DGC was required for anchoring the basal lamina to the sarcolemma in order to provide protection from mechanical injury (22). An important caveat of the study was that all experiments were performed in fast-twitch muscle. Based on the observation that α7β1integrin is highly enriched in slow-twitch muscle, directly testing the susceptibility of α7 integrin-null soleus muscle to contraction-induced injury would

be a critical experiment to test the hypothesis that dystroglycan and α7β1integrin have fiber-type specific functions. Integrin-deficient soleus muscle would be predicted to be more susceptible to contraction-induced injury than a comparable fast-twitch muscle such as the EDL. Additionally, the contribution of integrins to the prevention of muscle injury in fast-twitch muscle could also be directly tested in LARGE[myd] animals by overexpression of α7 integrin using either an adenovirus vector or by crossing LARGE[myd] animals to the reported α7 integrin transgenic strain (97). Increased expression of α7β1 integrin at the sarcolemma of fast-twitch fibers concomitant with a protection from contraction-induced injury would support a fiber-type specific laminin receptor hypothesis.

As a means to further understand the importance of dystroglycan glycosylation in skeletal muscle function, a novel transgenic was created in which dystroglycan was hyperglycosylated exclusively in skeletal and cardiac muscle. This resulted in a dramatic elevation in laminin binding activity and enhanced protection from contraction-induced injury as compared to normal muscle. Similar to the results obtained in LARGE[myd] animals, this effect was only observed in the EDL muscle and force deficits measured in wild-type and MCK-LARGE soleus muscle were not different. Because neither the absence nor the enhanced affinity of dystroglycan for laminin in soleus muscle altered the susceptibility of the soleus muscle to mechanical damage, this suggests that interactions between laminin and dystroglycan do not play a significant role in preventing mechanical injury in this muscle type. This is especially noteworthy in the context of human disease since the mouse soleus is more representative of human muscle (188). Although LARGE[myd] soleus muscle is resistant to mechanical injury, it is important to note that the muscle still demonstrates pathological features of muscular dystrophy, and this implies that sarcolemmal disruptions are not an initiating event leading to dystrophy in this muscle. This result supports the hypothesis that the DGC has important non-mechanical functions in skeletal muscle that when impaired, contribute to muscle disease.

Non-Mechanical Functions of the Dystrophin-Glycoprotein Complex

Several reports have identified interactions between dystroglycan and known signaling and adaptor proteins but how impaired dystroglycan function affects cell signaling and whether or not altered intracellular signaling contributes to the dystrophic pathology is not well understood. The c-terminal domain of β-dystroglycan can bind the well known adapter protein Grb2 (103, 107) and also contains a tyrosine residue that when phosphorylated (108), can recruit several additional SH2-domain containing adaptor proteins (110). Disruptions in cell signaling have also been hypothesized to be dependent upon the binding of dystroglycan to laminin. In one example, the DGC has been shown to interact with subunits of heterotrimeric G proteins in a laminin-dependent manner (113) which might underlie the altered Ca^{2+} homeostasis observed in several forms of muscular dystrophy and originally attributed to membrane tears. Additionally, the disruption of the laminin/dystroglycan interaction *in vitro* using antibodies against α-dystroglycan resulted in decreased AKT and GSK-3β activation and an increase in apoptotic cell death (112). Notably, laminin-211 deficient *dy/dy* mice also had increased activation of apoptotic death pathways and it has been hypothesized that altered signaling may contribute to the dystrophic pathology in laminin-211 associated muscle disease (84, 87). While the precise mechanism by which defects in dystroglycan result in impaired signaling is unclear, our results suggest that such signaling may be physiologically relevant and have significant implications for other DGC-associated dystrophies that lead to a concomitant reduction in dystroglycan expression at the sarcolemma.

In light of these observations, it is important to note that while the hyperglycosylation of dystroglycan in fast-twitch muscles resulted in an enhanced protection from contraction-induced injury, a significant elevation in laminin binding activity may also affect potential laminin-mediated signaling pathways. Though deleterious effects as a result of dystroglycan hyperglycosylation in MCK-LARGE mice were generally not observed, muscles examined from these animals were slightly larger than wild-type littermates which resulted in a slight reduction in specific force as compared to wild-type animals. However, since

body weights were not significantly different between the two genotypes, this suggests that the quality of force that is produced in muscles containing dystroglycan hyperglycosylation is somehow reduced. This does not appear to result from any structural abnormalities in the muscle since histological examination did not detect any differences from wild-type muscle. Hyperglycosylation of dystroglycan and potential unidentified substrates of LARGE could cause an increase in fluid retention in the extracellular space contributing to an increase in mass without affecting force production. However, ratios of wet mass to dry mass did not reveal differences between transgenic and wild-type muscle (not shown). Because the DGC functions in the transmission of forces at the lateral membrane (128), it is possible that enhanced glycosylation of dystroglycan by LARGE can affect mechanisms of force production which might explain the observed reduction in specific force. However, the lateral membrane is often the site of muscle injury in dystrophic muscle and because the transgene was capable of completely restoring susceptibility to muscle injury, this suggests that any defect at the lateral membrane has been corrected. Further detailed examination of the muscle basal lamina and myotendinous junction structure by electron microscopy may lend some insight into the mechanisms underlying the slight loss of specific force production in MCK-LARGE mice.

Since laminin-mediated increases in AKT signaling have been reported, this may warrant a deeper investigation into the effect of dystroglycan hyperglycosylation on muscle growth. One possibility that remains to be tested is the importance of dystroglycan glycosylation during muscle regeneration. The muscle-specific deletion of dystroglycan in skeletal muscle yielded an unexpected result in that while animals were dystrophic, they were also much larger that littermate controls, demonstrating significant muscle hypertrophy as a consequence of retained dystroglycan function in satellite cells (101). Because TG-LARGE^{myd} mice express functional LARGE only in differentiated muscle and not in muscle precursors, the importance of dystroglycan glycosylation in satellite cells could be tested using this animal model.

107

Although wild-type and TG-LARGE^myd animals did not demonstrate differences in response to contraction-induced injury when measured in isolated muscles *in vitro*, an *in situ* injury protocol could be used in order to determine whether the recovery from muscle injury, which requires the proliferation and differentiation of satellite cells, is abnormal. As a means to test whether TG-LARGE^myd animals demonstrate deficits in muscle regeneration as a consequence of impaired dystroglycan glycosylation in satellite cells, lengthening contractions could be performed in anesthetized mice using EDL muscle in a protocol similar to that used by Lockhart et al. (235). This would allow for an immediate measurement of force production and force deficit following a lengthening contraction-induced injury. Force production could also be measured at later time points (5-7 days post-injury) to determine whether the timeline for recovery of muscle function was prolonged in TG-LARGE^myd animals due to deficits in satellite cell function resulting from impaired dystroglycan glycosylation. In parallel with these studies, a more severe muscle injury could be achieved using injection of either cardiotoxin or barium chloride. Because cardiotoxin causes severe muscle injury and destroys local satellite cells, muscle regeneration in this model would require satellite cell proliferation and substantial cell migration into the injury site. Alternatively, barium chloride destroys muscle fibers without harming the satellite cell pool and provides a milder model of muscle injury (236). In order to quantify regeneration, injured muscle could be collected at multiple time points and the persistence of internalized nuclei and expression of muscle differentiation markers (myogenin, MyoD, Pax7) could be analyzed. Reduced staining of proteins required for differentiation might suggest a critical function for LARGE-mediated glycosylation in satellite cell populations. If differences are observed, satellite cells could be isolated from LARGE^myd mice and the potential for proliferation, migration, and differentiation could be studied *in vitro* before and after LARGE overexpression. An inability to recover from muscle injury similar to wild-type controls would highlight essential functions of dystroglycan glycosylation in the process of muscle regeneration that may contribute to muscle disease in cases of glycosyltransferase deficiency.

Dystroglycan Function in Neural Tissues

In addition to being required for normal skeletal muscle function, tissue-specific dystroglycan deletions have also demonstrated the importance of dystroglycan in neurological function (131, 133). In order to understand the physiological consequences of impaired interactions between dystroglycan and its various ligands in neuronal tissues, TG-LARGE[myd] mice were generated that have impaired dystroglycan function in all tissues except skeletal muscle. Animals exhibited an apparent amelioration of muscle pathology, allowing for more subtle physiological defects in other tissues to be assessed, the results of which were described in chapter 4 and outlined in figure 5.2.

Dystroglycan was initially identified as a receptor for agrin in postsynaptic membranes isolated from the Torpedo electric organ (135). Although dystroglycan can bind both neural and muscle-derived agrin, agrin-dependent clustering of acetylcholine receptors in the postsynaptic membrane during the formation of the neuromuscular junction (NMJ) does not require interactions with dystroglycan (138, 139). Instead, dystroglycan is suspected to function in the maintenance of the NMJ which may be mediated by interactions with laminin in the synaptic basement membrane (140, 237). Impaired structure of the NMJ has been reported in both dystroglycan-deficient (209) and LARGE-deficient muscle (144) which suggests that glycosylation of dystroglycan is critical for normal maintenance of this structure in muscle. In order to test whether restoration of dystroglycan ligand binding activity in the postsynaptic membrane was sufficient for the maintenance of neuromuscular junction architecture, these structures were examined in TG-LARGE[myd] mice and compared to those in wild-type and LARGE[myd] muscle. While all NMJs observed in LARGE[myd] muscle were fragmented, NMJs in both wild-type and LARGE[myd] muscle demonstrated the characteristic pretzel-like appearance which indicated that only postsynaptic dystroglycan glycosylation was essential for maintenance of this synapse.

In order to determine the physiological consequence of impaired NMJ structure in LARGE[myd] muscle, a paired-analysis approach was utilized in order

to assay for deficits in neurotransmission.   Maximal force production was measured twice in each gastrocnemius muscle, once during stimulation of the sciatic nerve and again following direct stimulation of the muscle. In wild-type animals, maximum force production was achieved during nerve stimulation, and direct muscle stimulation yielded only a slight decrement in force production. However, nerve stimulation in LARGE^{myd} muscle resulted in less force production than was capable of being generated during direct muscle stimulation. In direct contrast, maximum forces generated in TG-LARGE^{myd} animals occurred during nerve stimulation which indicated a complete structural and functional rescue of the NMJ in these animals. These results indicate that the impaired structure of the NMJ observed in LARGE^{myd} mice yields a functional deficit that can be rescued via specific glycosylation of dystroglycan at the postsynaptic membrane.

In addition to a complete rescue of the structure and function of the NMJ in TG-LARGE^{myd} animals, the peripheral neuropathy that has also been reported in LARGE-deficient animals was also rescued. Using both assays of nerve function and motor performance, in addition to direct conduction velocity measurements in the sciatic and sural nerves, no deficits were observed in TG-LARGE^{myd} animals. This was an unexpected finding considering several studies that have demonstrated a critical function of dystroglycan as a laminin receptor in normal Schwann cell function. During peripheral nerve development and following nerve crush, expression of both laminin and dystroglycan is increased in Schwann cells which suggests the importance of their interaction during myelinogenesis (238). Interactions between dystroglycan and laminin are also hypothesized to be important for radial sorting of axons which would explain the presence of clusters of unmyelinated axons in LARGE^{enr} nerves (145). Mutations in either laminin-211 or laminin-411 impair radial sorting of axons (218, 219) and radial sorting defects are more severe in the absence of both laminin-211 and laminin-411 (220), which suggests that different aspects of the radial sorting process may be mediated by multiple laminin isoforms and their different receptors which include both dystroglycan and integrins.   Additionally, loss of function of laminin receptors may perturb laminin-mediated signaling required for

Schwann cell proliferation (220). The dy^{2J}/dy^{2J} model of laminin deficiency demonstrates both radial sorting and myelination defects in conjunction with impaired nerve conduction velocity, clustering of sodium channels, and nodal structure (134) which is quite similar to defects observed in the Schwann cell specific deletion of dystroglycan (133). While these studies highlight an importance of dystroglycan glycosylation for normal radial sorting and myelination of neurons during development, it is important to note that not all nerve defects reported in DG-null animals are consistent with the exclusive glycosylation-dependent function of dystroglycan in the PNS. Although Schwann cell-specific dystroglycan-null animals demonstrate defects in node elongation and sodium channel clustering (133, 145), this has not been reported in either LARGE^{myd} or LARGE^{enr} animals which suggests that glycosylation of dystroglycan is not essential for the formation of nodal domains.

Although impaired peripheral nerve function was not detected in transgenic LARGE^{myd} animals, this does not rule out the importance of glycosylation-dependent functions of dystroglycan since defects in myelination have been observed in LARGE^{enr} mice (145) and in other models of glycosyltransferase deficiency (146). Rather, defects in myelination are likely variable along the length of the axon and would be expected to be progressive in nature. This could be further tested in LARGE^{myd} animals by analyzing additional or more proximal regions of peripheral nerve. Because LARGE^{myd} animals do not often survive past 40 weeks of age, this time point was used to test for both functional and histological evidence of neuropathy in all animals analyzed. However, because TG-LARGE^{myd} animals are presently surviving much longer than LARGE^{myd} animals, these mice can be analyzed at later time points and compared to wild-type littermates. Anecdotally, the hindlimb paralysis that is commonly observed in older LARGE^{myd} animals has not yet been observed in TG-LARGE^{myd}, despite their having survived nearly twice as long as LARGE^{myd} animals, which suggests that the onset of neuropathy is either delayed or completely absent.

111

A possible explanation that might account for the observed rescue of nerve function may be related to the improvement in either muscle function or restored structure of the neuromuscular synapse. While a deficit in nerve conduction velocity was observed in LARGE^{myd} sciatic nerve, sural nerve function was not different from wild-type animals. Therefore, if the impaired muscle function of LARGE^{myd} animals negatively influenced motor neuron function, this might explain impaired conduction velocity specific to the motor sciatic nerve. Maintenance of neuronal connections can be dependent upon target-derived retrograde signals such as neurotrophins (223) that may act on peripheral nerves to maintain normal structure and function. Skeletal muscle is a source of several neurotrophins including brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and neurotrophin-3/4 (224-228) which may contribute to motor neuron survival and/or differentiation (229). There is also evidence to support the hypothesis that impaired neurotrophin signaling may be relevant in the muscular dystrophies. Brains from *mdx* mice demonstrate increased staining of both nerve growth factor (NGF) and its receptor (230). Additionally, NGF is more highly expressed in regenerating fibers of patients with Duchenne/Becker muscular dystrophy as compared to healthy controls (231). More recently, the NGF precursor, pro-NGF, was also shown to promote death of motor neurons, though the source was thought to be from astrocytes (239). In addition to promoting neuron survival, neurotrophins can also regulate myelination of the peripheral nervous system (240-242). Neuregulin-1 is a growth factor produced in both the central and peripheral nervous system that binds receptors on myelinating Schwann cells to promote growth and differentiation during myelination (243).

While it is intriguing to speculate that disruptions in neurotrophin related signaling may contribute to muscle weakness in muscular dystrophy (244), this hypothesis has yet to be formally addressed. One possibility to explain the rescue of nerve function in transgenic LARGE^{myd} muscle might be that poor muscle function in LARGE^{myd} muscle results in impaired secretion of neurotrophins important for maintenance of the neuromuscular synapse. BDNF

is secreted from skeletal muscle in an activity dependent manner (245) and contributes to synaptic potentiation at the neuromuscular junction (246). Additionally, muscle-specific expression or systemic injections of GDNF can cause multiple innervation of muscle fibers or slow the process of NMJ elimination (247). To determine whether BDNF or GDNF expression is altered in LARGE[myd] muscle and contributes to impaired neurotransmission deficits, whole muscle lysates or muscle sections from LARGE[myd], TG-LARGE[myd], and wild-type littermates could be used to screen for changes in BDNF of GDNF expression using RT-PCR or western blotting similar to what has been shown in *dy/dy* and *mdx* mice (230, 248). In addition to potential changes in BDNF expression in the skeletal muscle of LARGE[myd] mice, it would also be important to determine whether the expression of its known receptors, both TrkB and p75[NTR], or activation of known downstream signaling molecules was altered in LARGE[myd] neurons. A reduction in BDNF or a downregulation of its receptors only in LARGE[myd] muscle would suggest that an absence of BDNF-mediated signaling at the neuromuscular junction may contribute to impaired neurotransmission defects in LARGE[myd] mice. To more directly determine whether retrograde signaling mediated by neurotrophins is defective in LARGE[myd] mice contributing to impaired function of the neuromuscular junction, specific neurotrophins could be directly applied *in vivo* and the consequences on gene transcription could be measured. For example, Pazyra-Murphy et al. showed that in sensory neurons, the binding of neurotrophins to receptors on distal axons activated a large set of cell survival genes termed "retrograde response genes" including MEF2D and *bcl-w* in the dorsal root ganglia (249). Similarly, the transcription of these or other retrograde response genes could also be analyzed following muscle injection of specific neurotrophins in LARGE[myd] mice by using either RT-PCR or *in situ* hybridization in the dorsal horn of the spinal cord where motor neuron cell bodies are located.

To more generally confirm that impaired neurotrophin signaling is a consequence of muscle degeneration and not a direct result of impaired glycosylation, it would be important to show that any neurotrophins identified as

being altered in LARGE^{myd} mice were similarly altered in other models of acute or chronic muscle damage. Despite the known roles of NGF, BDNF and GDNF as neurotrophic factors, the mechanisms underlying retrograde signaling from the muscle to motor neurons have not been fully elucidated. Therefore, gene expression profiling using microarrays from muscles of LARGE^{myd} and transgenic LARGE^{myd} mice or additional mouse models of muscle disease may be a useful screen to identify secreted factors with known neurotrophic activity in other systems that may be important candidates for future study.

Conclusions

The results described in this thesis demonstrate that dystroglycan glycosylation is required for normal muscle function not only at the lateral membrane of muscle fibers but also at the postsynaptic membrane of the neuromuscular junction. While the important mechanical function of DGC has been demonstrated in numerous studies, these results indicate that susceptibility to contraction-induced damage is not a characteristic of all DGC-related muscle dystrophies. Additionally, this work is the first to demonstrate that dystroglycan has an essential glycosylation-mediated function at the neuromuscular junction that, when impaired, contributes to skeletal muscle weakness.

Although mutations that affect dystroglycan function are rare, these results have significant implications regarding the molecular mechanisms underlying disease progression in other forms of muscular dystrophy. A primary defect in sarcolemmal integrity is an attractive hypothesis to account for the observed dystrophy in DGC-related muscular dystrophy. Mutations that affect the DGC render myofibers permeable to calcium, creatine kinase and impermeant dyes and this is correlated with increased susceptibility to mechanical injury. However, the resistance of LARGE^{myd} soleus muscle to mechanical injury, despite the observation of several pathological features of muscular dystrophy, indicates that mechanical injury is not the initiating event in this muscle that eventually causes death of myofibers. This observation supports several alternative hypotheses that purport a direct function of the DGC in either calcium

114

being altered in LARGE[myd] mice were similarly altered in other models of acute or chronic muscle damage. Despite the known roles of NGF, BDNF and GDNF as neurotrophic factors, the mechanisms underlying retrograde signaling from the muscle to motor neurons have not been fully elucidated. Therefore, gene expression profiling using microarrays from muscles of LARGE[myd] and transgenic LARGE[myd] mice or additional mouse models of muscle disease may be a useful screen to identify secreted factors with known neurotrophic activity in other systems that may be important candidates for future study.

Conclusions

The results described in this thesis demonstrate that dystroglycan glycosylation is required for normal muscle function not only at the lateral membrane of muscle fibers but also at the postsynaptic membrane of the neuromuscular junction. While the important mechanical function of DGC has been demonstrated in numerous studies, these results indicate that susceptibility to contraction-induced damage is not a characteristic of all DGC-related muscle dystrophies. Additionally, this work is the first to demonstrate that dystroglycan has an essential glycosylation-mediated function at the neuromuscular junction that, when impaired, contributes to skeletal muscle weakness.

Although mutations that affect dystroglycan function are rare, these results have significant implications regarding the molecular mechanisms underlying disease progression in other forms of muscular dystrophy. A primary defect in sarcolemmal integrity is an attractive hypothesis to account for the observed dystrophy in DGC-related muscular dystrophy. Mutations that affect the DGC render myofibers permeable to calcium, creatine kinase and impermeant dyes and this is correlated with increased susceptibility to mechanical injury. However, the resistance of LARGE[myd] soleus muscle to mechanical injury, despite the observation of several pathological features of muscular dystrophy, indicates that mechanical injury is not the initiating event in this muscle that eventually causes death of myofibers. This observation supports several alternative hypotheses that purport a direct function of the DGC in either calcium

114

homeostasis independent of membrane tears, or in disrupted survival and growth signaling pathways. Many strategies aimed at treating these disorders have been hindered by the mechanical nature of the mutated gene and the obstacle of safely restoring expression of a corrected version of the diseased gene throughout skeletal muscle. Therefore, the identification of critical non-mechanical functions of the DGC may highlight additional targets aimed at treating and eventually curing disease. In addition to the direct prevention of muscular dystrophy, targeting muscle fiber rescue may also have a significant indirect impact on the function of nerves, and therefore may help prevent the overall progressive decrease in neuromuscular function with age.
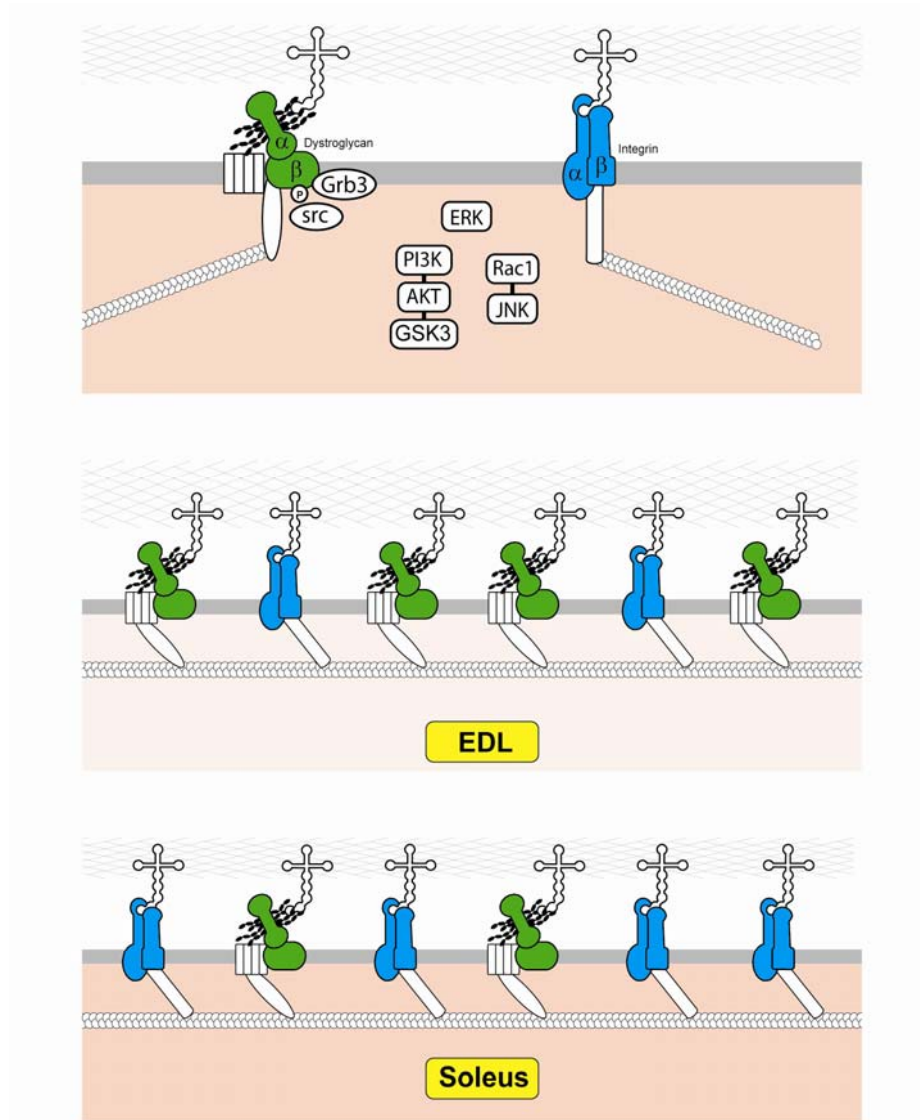
Figure 5-1) Fiber-type specific functions of laminin receptors in skeletal muscle. Dystroglycan and α7β1 integrin are differentially expressed in extensor digitorum longus (EDL) and soleus muscle and have been implicated in several shared signaling pathways related to cell survival and growth.

	LARGE^{myd}	WT	TG-LARGE^{myd}
Dystroglycan Function			
Skeletal Muscle	Absent	Normal	Enhanced
Brain	Absent	Normal	Absent
Nerve	Absent	Normal	Absent
Phenotypic Outcome			
Skeletal Muscle	Dystrophic	Normal	Normal
Brain Development	Abnormal	Normal	Abnormal
Nerve Conduction	Slowed	Normal	Normal
Neuromuscular Junction	Abnormal	Normal	Normal
Neurotransmission	Reduced	Normal	Normal
Motor Performance	Poor	Normal	Normal

Figure 5-2) Phenotypic outcome as a result of impaired dystroglycan function in neuronal tissues. The structure of the dystrophin-glycoprotein complex (DGC) in LARGE^{myd}, wild-type, and TG-LARGE^{myd} in skeletal muscle is depicted. The mechanical link formed by the DGC between the basal lamina and the cytoskeleton is disrupted in LARGE^{myd} skeletal muscle and restored in TG-LARGE^{myd} mice. Despite the loss of function of dystroglycan as a laminin receptor in brain and peripheral nerve of TG-LARGE^{myd} animals, several parameters of neurological function are restored.

BIBLIOGRAPHY

1. Wallace, G. Q., and McNally, E. M. (2009) Mechanisms of muscle degeneration, regeneration, and repair in the muscular dystrophies. *Annu Rev Physiol* **71**, 37-57

2. McNally, E. M., and Pytel, P. (2007) Muscle diseases: the muscular dystrophies. *Annu Rev Pathol* **2**, 87-109

3. Cohn, R. D., and Campbell, K. P. (2000) Molecular basis of muscular dystrophies. *Muscle Nerve* **23**, 1456-1471

4. Petrof, B. J., Shrager, J. B., Stedman, H. H., Kelly, A. M., and Sweeney, H. L. (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc Natl Acad Sci U S A* **90**, 3710-3714

5. Petrof, B. J. (1998) The molecular basis of activity-induced muscle injury in Duchenne muscular dystrophy. *Mol Cell Biochem* **179**, 111-123

6. Moser, H. (1984) Duchenne muscular dystrophy: pathogenetic aspects and genetic prevention. *Hum Genet* **66**, 17-40

7. Allen, D. G., and Whitehead, N. P. (2011) Duchenne muscular dystrophy--what causes the increased membrane permeability in skeletal muscle? *Int J Biochem Cell Biol* **43**, 290-294

8. Batchelor, C. L., and Winder, S. J. (2006) Sparks, signals and shock absorbers: how dystrophin loss causes muscular dystrophy. *Trends Cell Biol* **16**, 198-205

9. Yoshida, M., and Ozawa, E. (1990) Glycoprotein complex anchoring dystrophin to sarcolemma. *J Biochem* **108**, 748-752

10. Campbell, K. P., and Kahl, S. D. (1989) Association of dystrophin and an integral membrane glycoprotein. *Nature* **338**, 259-262

11. Minetti, C., Beltrame, F., Marcenaro, G., and Bonilla, E. (1992) Dystrophin at the plasma membrane of human muscle fibers shows a costameric localization. *Neuromuscul Disord* **2**, 99-109

12. Ervasti, J. M., and Campbell, K. P. (1993) A role for the dystrophin-glycoprotein complex as a transmembrane linker between laminin and actin. *J Cell Biol* **122**, 809-823

13. Campbell, K. P. (1995) Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. *Cell* **80**, 675-679

14. Stone, M. R., O'Neill, A., Catino, D., and Bloch, R. J. (2005) Specific interaction of the actin-binding domain of dystrophin with intermediate filaments containing keratin 19. *Mol Biol Cell* **16**, 4280-4293

15. Bloch, R. J., Reed, P., O'Neill, A., Strong, J., Williams, M., Porter, N., and Gonzalez-Serratos, H. (2004) Costameres mediate force transduction in healthy skeletal muscle and are altered in muscular dystrophies. *J Muscle Res Cell Motil* **25**, 590-592

16. Rybakova, I. N., Patel, J. R., and Ervasti, J. M. (2000) The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin. *J Cell Biol* **150**, 1209-1214

17. Ervasti, J. M., and Campbell, K. P. (1991) Membrane organization of the dystrophin-glycoprotein complex. *Cell* **66**, 1121-1131

18. Ozawa, E., Mizuno, Y., Hagiwara, Y., Sasaoka, T., and Yoshida, M. (2005) Molecular and cell biology of the sarcoglycan complex. *Muscle Nerve* **32**, 563-576

19. Ohlendieck, K., and Campbell, K. P. (1991) Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. *J Cell Biol* **115**, 1685-1694

20. Muntoni, F., Torelli, S., and Brockington, M. (2008) Muscular dystrophies due to glycosylation defects. *Neurotherapeutics* **5**, 627-632

21. Michele, D. E., Barresi, R., Kanagawa, M., Saito, F., Cohn, R. D., Satz, J. S., Dollar, J., Nishino, I., Kelley, R. I., Somer, H., Straub, V., Mathews, K. D., Moore, S. A., and Campbell, K. P. (2002) Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* **418**, 417-422

22. Han, R., Kanagawa, M., Yoshida-Moriguchi, T., Rader, E. P., Ng, R. A., Michele, D. E., Muirhead, D. E., Kunz, S., Moore, S. A., Iannaccone, S. T., Miyake, K., McNeil, P. L., Mayer, U., Oldstone, M. B., Faulkner, J. A., and Campbell, K. P. (2009) Basal lamina strengthens cell membrane integrity via the laminin G domain-binding motif of alpha-dystroglycan. *Proc Natl Acad Sci U S A* **106**, 12573-12579

23. Williamson, R. A., Henry, M. D., Daniels, K. J., Hrstka, R. F., Lee, J. C., Sunada, Y., Ibraghimov-Beskrovnaya, O., and Campbell, K. P. (1997) Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice. *Hum Mol Genet* **6**, 831-841

24. Hara, Y., Balci-Hayta, B., Yoshida-Moriguchi, T., Kanagawa, M., Beltran-Valero de Bernabe, D., Gundesli, H., Willer, T., Satz, J. S., Crawford, R. W., Burden, S. J., Kunz, S., Oldstone, M. B., Accardi, A., Talim, B., Muntoni, F., Topaloglu, H., Dincer, P., and Campbell, K. P. (2011) A dystroglycan mutation associated with limb-girdle muscular dystrophy. *N Engl J Med* **364**, 939-946

25. Xu, H., Wu, X. R., Wewer, U. M., and Engvall, E. (1994) Murine muscular dystrophy caused by a mutation in the laminin alpha 2 (Lama2) gene. *Nat Genet* **8**, 297-302

26. Sunada, Y., Bernier, S. M., Utani, A., Yamada, Y., and Campbell, K. P. (1995) Identification of a novel mutant transcript of laminin alpha 2 chain gene responsible for muscular dystrophy and dysmyelination in dy2J mice. *Hum Mol Genet* **4**, 1055-1061

27. Helbling-Leclerc, A., Zhang, X., Topaloglu, H., Cruaud, C., Tesson, F., Weissenbach, J., Tome, F. M., Schwartz, K., Fardeau, M., Tryggvason, K., and et al. (1995) Mutations in the laminin alpha 2-chain gene (LAMA2) cause merosin-deficient congenital muscular dystrophy. *Nat Genet* **11**, 216-218

28. Hoffman, E. P., Brown, R. H., Jr., and Kunkel, L. M. (1987) Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* **51**, 919-928

29. Haenggi, T., and Fritschy, J. M. (2006) Role of dystrophin and utrophin for assembly and function of the dystrophin glycoprotein complex in non-muscle tissue. *Cell Mol Life Sci* **63**, 1614-1631

30. McNeil, P. L., and Khakee, R. (1992) Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage. *Am J Pathol* **140**, 1097-1109

31. Ingalls, C. P., Warren, G. L., Williams, J. H., Ward, C. W., and Armstrong, R. B. (1998) E-C coupling failure in mouse EDL muscle after in vivo eccentric contractions. *J Appl Physiol* **85**, 58-67

32. Newham, D. J., McPhail, G., Mills, K. R., and Edwards, R. H. (1983) Ultrastructural changes after concentric and eccentric contractions of human muscle. *J Neurol Sci* **61**, 109-122

33. Clarkson, P. M., and Hubal, M. J. (2002) Exercise-induced muscle damage in humans. *Am J Phys Med Rehabil* **81**, S52-69

34. Bulfield, G., Siller, W. G., Wight, P. A., and Moore, K. J. (1984) X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc Natl Acad Sci U S A* **81**, 1189-1192

35. Pastoret, C., and Sebille, A. (1995) mdx mice show progressive weakness and muscle deterioration with age. *J Neurol Sci* **129**, 97-105

36. Moens, P., Baatsen, P. H., and Marechal, G. (1993) Increased susceptibility of EDL muscles from mdx mice to damage induced by contractions with stretch. *J Muscle Res Cell Motil* **14**, 446-451

37. Matsuda, R., Nishikawa, A., and Tanaka, H. (1995) Visualization of dystrophic muscle fibers in mdx mouse by vital staining with Evans blue: evidence of apoptosis in dystrophin-deficient muscle. *J Biochem* **118**, 959-964

38. Consolino, C. M., and Brooks, S. V. (2004) Susceptibility to sarcomere injury induced by single stretches of maximally activated muscles of mdx mice. *J Appl Physiol* **96**, 633-638

39. Dellorusso, C., Crawford, R. W., Chamberlain, J. S., and Brooks, S. V. (2001) Tibialis anterior muscles in mdx mice are highly susceptible to contraction-induced injury. *J Muscle Res Cell Motil* **22**, 467-475

40. Bashir, R., Britton, S., Strachan, T., Keers, S., Vafiadaki, E., Lako, M., Richard, I., Marchand, S., Bourg, N., Argov, Z., Sadeh, M., Mahjneh, I., Marconi, G., Passos-Bueno, M. R., Moreira Ede, S., Zatz, M., Beckmann, J. S., and Bushby, K. (1998) A gene related to Caenorhabditis elegans spermatogenesis factor fer-1 is mutated in limb-girdle muscular dystrophy type 2B. *Nat Genet* **20**, 37-42

41. Liu, J., Aoki, M., Illa, I., Wu, C., Fardeau, M., Angelini, C., Serrano, C., Urtizberea, J. A., Hentati, F., Hamida, M. B., Bohlega, S., Culper, E. J., Amato, A. A., Bossie, K., Oeltjen, J., Bejaoui, K., McKenna-Yasek, D., Hosler, B. A., Schurr, E., Arahata, K., de Jong, P. J., and Brown, R. H., Jr. (1998) Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy. *Nat Genet* **20**, 31-36

42. Bansal, D., Miyake, K., Vogel, S. S., Groh, S., Chen, C. C., Williamson, R., McNeil, P. L., and Campbell, K. P. (2003) Defective membrane repair in dysferlin-deficient muscular dystrophy. *Nature* **423**, 168-172

43. Roche, J. A., Lovering, R. M., and Bloch, R. J. (2008) Impaired recovery of dysferlin-null skeletal muscle after contraction-induced injury in vivo. *Neuroreport* **19**, 1579-1584

44. Roche, J. A., Lovering, R. M., Roche, R., Ru, L. W., Reed, P. W., and Bloch, R. J. (2010) Extensive mononuclear infiltration and myogenesis characterize recovery of dysferlin-null skeletal muscle from contraction-induced injuries. *Am J Physiol Cell Physiol* **298**, C298-312

45. Davies, K. E., and Nowak, K. J. (2006) Molecular mechanisms of muscular dystrophies: old and new players. *Nat Rev Mol Cell Biol* **7**, 762-773

46. Tanaka, H., Ishiguro, T., Eguchi, C., Saito, K., and Ozawa, E. (1991) Expression of a dystrophin-related protein associated with the skeletal muscle cell membrane. *Histochemistry* **96**, 1-5

47. Gramolini, A. O., Belanger, G., Thompson, J. M., Chakkalakal, J. V., and Jasmin, B. J. (2001) Increased expression of utrophin in a slow vs. a fast muscle involves posttranscriptional events. *Am J Physiol Cell Physiol* **281**, C1300-1309

48. Gumerson, J. D., Kabaeva, Z. T., Davis, C. S., Faulkner, J. A., and Michele, D. E. (2010) Soleus muscle in glycosylation-deficient muscular dystrophy is protected from contraction-induced injury. *Am J Physiol Cell Physiol* **299**, C1430-1440

49. Hodges, B. L., Hayashi, Y. K., Nonaka, I., Wang, W., Arahata, K., and Kaufman, S. J. (1997) Altered expression of the alpha7beta1 integrin in human and murine muscular dystrophies. *J Cell Sci* **110 (Pt 22)**, 2873-2881

50. Burkin, D. J., Wallace, G. Q., Nicol, K. J., Kaufman, D. J., and Kaufman, S. J. (2001) Enhanced expression of the alpha 7 beta 1 integrin reduces muscular dystrophy and restores viability in dystrophic mice. *J Cell Biol* **152**, 1207-1218

51. Head, S. I., Bakker, A. J., and Liangas, G. (2004) EDL and soleus muscles of the C57BL6J/dy2j laminin-alpha 2-deficient dystrophic mouse are not vulnerable to eccentric contractions. *Exp Physiol* **89**, 531-539

52. Straub, V., Rafael, J. A., Chamberlain, J. S., and Campbell, K. P. (1997) Animal models for muscular dystrophy show different patterns of sarcolemmal disruption. *J Cell Biol* **139**, 375-385

53. Chin, E. R. (2010) Intracellular Ca2+ signaling in skeletal muscle: decoding a complex message. *Exerc Sport Sci Rev* **38**, 76-85

54. Turner, P. R., Westwood, T., Regen, C. M., and Steinhardt, R. A. (1988) Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. *Nature* **335**, 735-738

55. Valentine, B. A., Cooper, B. J., and Gallagher, E. A. (1989) Intracellular calcium in canine muscle biopsies. *J Comp Pathol* **100**, 223-230

56. Franco, A., Jr., and Lansman, J. B. (1990) Calcium entry through stretch-inactivated ion channels in mdx myotubes. *Nature* **344**, 670-673

57. Fong, P. Y., Turner, P. R., Denetclaw, W. F., and Steinhardt, R. A. (1990) Increased activity of calcium leak channels in myotubes of Duchenne human and mdx mouse origin. *Science* **250**, 673-676

58. Yeung, E. W., Whitehead, N. P., Suchyna, T. M., Gottlieb, P. A., Sachs, F., and Allen, D. G. (2005) Effects of stretch-activated channel blockers on [Ca2+]i and muscle damage in the mdx mouse. *J Physiol* **562**, 367-380

59. Clapham, D. E. (2003) TRP channels as cellular sensors. *Nature* **426**, 517-524

60. Vandebrouck, C., Martin, D., Colson-Van Schoor, M., Debaix, H., and Gailly, P. (2002) Involvement of TRPC in the abnormal calcium influx observed in dystrophic (mdx) mouse skeletal muscle fibers. *J Cell Biol* **158**, 1089-1096

61. Gervasio, O. L., Whitehead, N. P., Yeung, E. W., Phillips, W. D., and Allen, D. G. (2008) TRPC1 binds to caveolin-3 and is regulated by Src kinase - role in Duchenne muscular dystrophy. *J Cell Sci* **121**, 2246-2255

62. Iwata, Y., Katanosaka, Y., Arai, Y., Shigekawa, M., and Wakabayashi, S. (2009) Dominant-negative inhibition of Ca2+ influx via TRPV2 ameliorates muscular dystrophy in animal models. *Hum Mol Genet* **18**, 824-834

63. Bellinger, A. M., Reiken, S., Carlson, C., Mongillo, M., Liu, X., Rothman, L., Matecki, S., Lacampagne, A., and Marks, A. R. (2009) Hypernitrosylated ryanodine receptor calcium release channels are leaky in dystrophic muscle. *Nat Med* **15**, 325-330

64. Millay, D. P., Goonasekera, S. A., Sargent, M. A., Maillet, M., Aronow, B. J., and Molkentin, J. D. (2009) Calcium influx is sufficient to induce muscular dystrophy through a TRPC-dependent mechanism. *Proc Natl Acad Sci U S A* **106**, 19023-19028

65. Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003) Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* **4**, 552-565

66. Baines, C. P., Kaiser, R. A., Purcell, N. H., Blair, N. S., Osinska, H., Hambleton, M. A., Brunskill, E. W., Sayen, M. R., Gottlieb, R. A., Dorn, G. W., Robbins, J., and Molkentin, J. D. (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* **434**, 658-662

67. Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H., Inohara, H., Kubo, T., and Tsujimoto, Y. (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* **434**, 652-658

68. Millay, D. P., Sargent, M. A., Osinska, H., Baines, C. P., Barton, E. R., Vuagniaux, G., Sweeney, H. L., Robbins, J., and Molkentin, J. D. (2008) Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy. *Nat Med* **14**, 442-447

69. Shkryl, V. M., Martins, A. S., Ullrich, N. D., Nowycky, M. C., Niggli, E., and Shirokova, N. (2009) Reciprocal amplification of ROS and Ca(2+) signals in stressed mdx dystrophic skeletal muscle fibers. *Pflugers Arch* **458**, 915-928

70. Rando, T. A. (2002) Oxidative stress and the pathogenesis of muscular dystrophies. *Am J Phys Med Rehabil* **81**, S175-186

71. Whitehead, N. P., Yeung, E. W., and Allen, D. G. (2006) Muscle damage in mdx (dystrophic) mice: role of calcium and reactive oxygen species. *Clin Exp Pharmacol Physiol* **33**, 657-662

72. Tidball, J. G., and Wehling-Henricks, M. (2007) The role of free radicals in the pathophysiology of muscular dystrophy. *J Appl Physiol* **102**, 1677-1686

73. Buetler, T. M., Renard, M., Offord, E. A., Schneider, H., and Ruegg, U. T. (2002) Green tea extract decreases muscle necrosis in mdx mice and protects against reactive oxygen species. *Am J Clin Nutr* **75**, 749-753

74. Menazza, S., Blaauw, B., Tiepolo, T., Toniolo, L., Braghetta, P., Spolaore, B., Reggiani, C., Di Lisa, F., Bonaldo, P., and Canton, M. (2010) Oxidative stress by monoamine oxidases is causally involved in myofiber damage in muscular dystrophy. *Hum Mol Genet* **19**, 4207-4215

75. Lovering, R. M., Michaelson, L., and Ward, C. W. (2009) Malformed mdx myofibers have normal cytoskeletal architecture yet altered EC coupling and stress-induced Ca2+ signaling. *Am J Physiol Cell Physiol* **297**, C571-580

76. Woods, C. E., Novo, D., DiFranco, M., and Vergara, J. L. (2004) The action potential-evoked sarcoplasmic reticulum calcium release is impaired in mdx mouse muscle fibres. *J Physiol* **557**, 59-75

77. Zhang, B. T., Yeung, S. S., Allen, D. G., Qin, L., and Yeung, E. W. (2008) Role of the calcium-calpain pathway in cytoskeletal damage after eccentric contractions. *J Appl Physiol* **105**, 352-357

78. Spencer, M. J., Croall, D. E., and Tidball, J. G. (1995) Calpains are activated in necrotic fibers from mdx dystrophic mice. *J Biol Chem* **270**, 10909-10914

79. Alderton, J. M., and Steinhardt, R. A. (2000) Calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. *J Biol Chem* **275**, 9452-9460

80. Spencer, M. J., and Mellgren, R. L. (2002) Overexpression of a calpastatin transgene in mdx muscle reduces dystrophic pathology. *Hum Mol Genet* **11**, 2645-2655

81. Briguet, A., Erb, M., Courdier-Fruh, I., Barzaghi, P., Santos, G., Herzner, H., Lescop, C., Siendt, H., Henneboehle, M., Weyermann, P., Magyar, J. P., Dubach-Powell, J., Metz, G., and Meier, T. (2008) Effect of calpain and proteasome inhibition on Ca2+-dependent proteolysis and muscle histopathology in the mdx mouse. *FASEB J* **22**, 4190-4200

82. Badalamente, M. A., and Stracher, A. (2000) Delay of muscle degeneration and necrosis in mdx mice by calpain inhibition. *Muscle Nerve* **23**, 106-111

83. Selsby, J., Pendrak, K., Zadel, M., Tian, Z., Pham, J., Carver, T., Acosta, P., Barton, E., and Sweeney, H. L. (2010) Leupeptin-based inhibitors do not improve the mdx phenotype. *Am J Physiol Regul Integr Comp Physiol* **299**, R1192-1201

84. Mukasa, T., Momoi, T., and Momoi, M. Y. (1999) Activation of caspase-3 apoptotic pathways in skeletal muscle fibers in laminin alpha2-deficient mice. *Biochem Biophys Res Commun* **260**, 139-142

85. Erb, M., Meinen, S., Barzaghi, P., Sumanovski, L. T., Courdier-Fruh, I., Ruegg, M. A., and Meier, T. (2009) Omigapil ameliorates the pathology of muscle dystrophy caused by laminin-alpha2 deficiency. *J Pharmacol Exp Ther* **331**, 787-795

86. Girgenrath, M., Beermann, M. L., Vishnudas, V. K., Homma, S., and Miller, J. B. (2009) Pathology is alleviated by doxycycline in a laminin-alpha2-null model of congenital muscular dystrophy. *Ann Neurol* **65**, 47-56

87. Girgenrath, M., Dominov, J. A., Kostek, C. A., and Miller, J. B. (2004) Inhibition of apoptosis improves outcome in a model of congenital muscular dystrophy. *J Clin Invest* **114**, 1635-1639

88. Vachon, P. H., Xu, H., Liu, L., Loechel, F., Hayashi, Y., Arahata, K., Reed, J. C., Wewer, U. M., and Engvall, E. (1997) Integrins (alpha7beta1) in muscle function and survival. Disrupted expression in merosin-deficient congenital muscular dystrophy. *J Clin Invest* **100**, 1870-1881

89. Rooney, J. E., Gurpur, P. B., and Burkin, D. J. (2009) Laminin-111 protein therapy prevents muscle disease in the mdx mouse model for Duchenne muscular dystrophy. *Proc Natl Acad Sci U S A* **106**, 7991-7996

90. Gawlik, K. I., Oliveira, B. M., and Durbeej, M. (2011) Transgenic Expression of Laminin alpha1 Chain Does Not Prevent Muscle Disease in the mdx Mouse Model for Duchenne Muscular Dystrophy. *Am J Pathol* **178**, 1728-1737

91. Gawlik, K., Miyagoe-Suzuki, Y., Ekblom, P., Takeda, S., and Durbeej, M. (2004) Laminin alpha1 chain reduces muscular dystrophy in laminin alpha2 chain deficient mice. *Hum Mol Genet* **13**, 1775-1784

92. Gawlik, K. I., and Durbeej, M. (2010) Transgenic overexpression of laminin alpha1 chain in laminin alpha2 chain-deficient mice rescues the disease throughout the lifespan. *Muscle Nerve* **42**, 30-37

93. Hayashi, Y. K., Chou, F. L., Engvall, E., Ogawa, M., Matsuda, C., Hirabayashi, S., Yokochi, K., Ziober, B. L., Kramer, R. H., Kaufman, S. J., Ozawa, E., Goto, Y., Nonaka, I., Tsukahara, T., Wang, J. Z., Hoffman, E. P., and Arahata, K. (1998) Mutations in the integrin alpha7 gene cause congenital myopathy. *Nat Genet* **19**, 94-97

94. Mayer, U., Saher, G., Fassler, R., Bornemann, A., Echtermeyer, F., von der Mark, H., Miosge, N., Poschl, E., and von der Mark, K. (1997) Absence of integrin alpha 7 causes a novel form of muscular dystrophy. *Nat Genet* **17**, 318-323

95. Nakashima, H., Kibe, T., and Yokochi, K. (2009) 'Congenital muscular dystrophy caused by integrin alpha7 deficiency'. *Dev Med Child Neurol* **51**, 245

96. Rooney, J. E., Welser, J. V., Dechert, M. A., Flintoff-Dye, N. L., Kaufman, S. J., and Burkin, D. J. (2006) Severe muscular dystrophy in mice that lack dystrophin and alpha7 integrin. *J Cell Sci* **119**, 2185-2195

97. Burkin, D. J., Wallace, G. Q., Milner, D. J., Chaney, E. J., Mulligan, J. A., and Kaufman, S. J. (2005) Transgenic expression of {alpha}7{beta}1 integrin maintains muscle integrity, increases regenerative capacity, promotes

hypertrophy, and reduces cardiomyopathy in dystrophic mice. *Am J Pathol* **166**, 253-263

98. Boppart, M. D., Burkin, D. J., and Kaufman, S. J. (2006) Alpha7beta1-integrin regulates mechanotransduction and prevents skeletal muscle injury. *Am J Physiol Cell Physiol* **290**, C1660-1665

99. Schwartz, M. A. (2010) Integrins and extracellular matrix in mechanotransduction. *Cold Spring Harb Perspect Biol* **2**, a005066

100. Brown, S. C., and Lucy, J. A. (1993) Dystrophin as a mechanochemical transducer in skeletal muscle. *Bioessays* **15**, 413-419

101. Cohn, R. D., Henry, M. D., Michele, D. E., Barresi, R., Saito, F., Moore, S. A., Flanagan, J. D., Skwarchuk, M. W., Robbins, M. E., Mendell, J. R., Williamson, R. A., and Campbell, K. P. (2002) Disruption of DAG1 in differentiated skeletal muscle reveals a role for dystroglycan in muscle regeneration. *Cell* **110**, 639-648

102. Gawlik, K. I., Akerlund, M., Carmignac, V., Elamaa, H., and Durbeej, M. (2010) Distinct roles for laminin globular domains in laminin alpha1 chain mediated rescue of murine laminin alpha2 chain deficiency. *PLoS One* **5**, e11549

103. Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G., and Campbell, K. P. (1995) SH3 domain-mediated interaction of dystroglycan and Grb2. *J Biol Chem* **270**, 11711-11714

104. James, M., Nuttall, A., Ilsley, J. L., Ottersbach, K., Tinsley, J. M., Sudol, M., and Winder, S. J. (2000) Adhesion-dependent tyrosine phosphorylation of (beta)-dystroglycan regulates its interaction with utrophin. *J Cell Sci* **113 (Pt 10)**, 1717-1726

105. Spence, H. J., Dhillon, A. S., James, M., and Winder, S. J. (2004) Dystroglycan, a scaffold for the ERK-MAP kinase cascade. *EMBO Rep* **5**, 484-489

106. Zhou, Y., Jiang, D., Thomason, D. B., and Jarrett, H. W. (2007) Laminin-induced activation of Rac1 and JNKp46 is initiated by Src family kinases and mimics the effects of skeletal muscle contraction. *Biochemistry* **46**, 14907-14916

107. Russo, K., Di Stasio, E., Macchia, G., Rosa, G., Brancaccio, A., and Petrucci, T. C. (2000) Characterization of the beta-dystroglycan-growth factor receptor 2 (Grb2) interaction. *Biochem Biophys Res Commun* **274**, 93-98

108. Ilsley, J. L., Sudol, M., and Winder, S. J. (2001) The interaction of dystrophin with beta-dystroglycan is regulated by tyrosine phosphorylation. *Cell Signal* **13**, 625-632

109. Ilsley, J. L., Sudol, M., and Winder, S. J. (2002) The WW domain: linking cell signalling to the membrane cytoskeleton. *Cell Signal* **14**, 183-189

110. Sotgia, F., Lee, H., Bedford, M. T., Petrucci, T., Sudol, M., and Lisanti, M. P. (2001) Tyrosine phosphorylation of beta-dystroglycan at its WW domain binding motif, PPxY, recruits SH2 domain containing proteins. *Biochemistry* **40**, 14585-14592

111. Oak, S. A., Zhou, Y. W., and Jarrett, H. W. (2003) Skeletal muscle signaling pathway through the dystrophin glycoprotein complex and Rac1. *J Biol Chem* **278**, 39287-39295

112. Langenbach, K. J., and Rando, T. A. (2002) Inhibition of dystroglycan binding to laminin disrupts the PI3K/AKT pathway and survival signaling in muscle cells. *Muscle Nerve* **26**, 644-653

113. Xiong, Y., Zhou, Y., and Jarrett, H. W. (2009) Dystrophin glycoprotein complex-associated Gbetagamma subunits activate phosphatidylinositol-3-kinase/Akt signaling in skeletal muscle in a laminin-dependent manner. *J Cell Physiol* **219**, 402-414

114. Dogra, C., Changotra, H., Wergedal, J. E., and Kumar, A. (2006) Regulation of phosphatidylinositol 3-kinase (PI3K)/Akt and nuclear factor-kappa B signaling pathways in dystrophin-deficient skeletal muscle in response to mechanical stretch. *J Cell Physiol* **208**, 575-585

115. Boppart, M. D., Burkin, D. J., and Kaufman, S. J. (2011) Activation of AKT signaling promotes cell growth and survival in alpha7beta1 integrin-mediated alleviation of muscular dystrophy. *Biochim Biophys Acta* **1812**, 439-446

116. Blaauw, B., Mammucari, C., Toniolo, L., Agatea, L., Abraham, R., Sandri, M., Reggiani, C., and Schiaffino, S. (2008) Akt activation prevents the force drop induced by eccentric contractions in dystrophin-deficient skeletal muscle. *Hum Mol Genet* **17**, 3686-3696

117. Kumar, A., Yamauchi, J., Girgenrath, T., and Girgenrath, M. (2011) Muscle-specific expression of insulin-like growth factor 1 improves outcome in Lama2Dy-w mice, a model for congenital muscular dystrophy type 1A. *Hum Mol Genet* **20**, 2333-2343

118. Barton, E. R., Morris, L., Musaro, A., Rosenthal, N., and Sweeney, H. L. (2002) Muscle-specific expression of insulin-like growth factor I counters muscle decline in mdx mice. *J Cell Biol* **157**, 137-148

119. Shavlakadze, T., White, J., Hoh, J. F., Rosenthal, N., and Grounds, M. D. (2004) Targeted expression of insulin-like growth factor-I reduces early myofiber necrosis in dystrophic mdx mice. *Mol Ther* **10**, 829-843

120. Glass, D. J. (2003) Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. *Nat Cell Biol* **5**, 87-90

121. Carmignac, V., Quere, R., and Durbeej, M. (2011) Proteasome inhibition improves the muscle of laminin alpha2 chain-deficient mice. *Hum Mol Genet* **20**, 541-552

122. Grange, R. W., Gainer, T. G., Marschner, K. M., Talmadge, R. J., and Stull, J. T. (2002) Fast-twitch skeletal muscles of dystrophic mouse pups are resistant to injury from acute mechanical stress. *Am J Physiol Cell Physiol* **283**, C1090-1101

123. Lowe, D. A., Williams, B. O., Thomas, D. D., and Grange, R. W. (2006) Molecular and cellular contractile dysfunction of dystrophic muscle from young mice. *Muscle Nerve* **34**, 92-100

124. Ervasti, J. M. (2003) Costameres: the Achilles' heel of Herculean muscle. *J Biol Chem* **278**, 13591-13594

125. Bloch, R. J., and Gonzalez-Serratos, H. (2003) Lateral force transmission across costameres in skeletal muscle. *Exerc Sport Sci Rev* **31**, 73-78

126. Paul, A. C., Sheard, P. W., Kaufman, S. J., and Duxson, M. J. (2002) Localization of alpha 7 integrins and dystrophin suggests potential for both lateral and longitudinal transmission of tension in large mammalian muscles. *Cell Tissue Res* **308**, 255-265

127. Street, S. F. (1983) Lateral transmission of tension in frog myofibers: a myofibrillar network and transverse cytoskeletal connections are possible transmitters. *J Cell Physiol* **114**, 346-364

128. Ramaswamy, K. S., Palmer, M. L., van der Meulen, J. H., Renoux, A., Kostrominova, T. Y., Michele, D. E., and Faulkner, J. A. (2011) Lateral transmission of force is impaired in skeletal muscles of dystrophic mice and very old rats. *J Physiol* **589**, 1195-1208

129. Durbeej, M., Henry, M. D., Ferletta, M., Campbell, K. P., and Ekblom, P. (1998) Distribution of dystroglycan in normal adult mouse tissues. *J Histochem Cytochem* **46**, 449-457

130. Moore, S. A., Saito, F., Chen, J., Michele, D. E., Henry, M. D., Messing, A., Cohn, R. D., Ross-Barta, S. E., Westra, S., Williamson, R. A., Hoshi, T., and Campbell, K. P. (2002) Deletion of brain dystroglycan recapitulates aspects of congenital muscular dystrophy. *Nature* **418**, 422-425

131. Satz, J. S., Ostendorf, A. P., Hou, S., Turner, A., Kusano, H., Lee, J. C., Turk, R., Nguyen, H., Ross-Barta, S. E., Westra, S., Hoshi, T., Moore, S. A., and Campbell, K. P. (2010) Distinct functions of glial and neuronal dystroglycan in the developing and adult mouse brain. *J Neurosci* **30**, 14560-14572

132. Henion, T. R., Qu, Q., and Smith, F. I. (2003) Expression of dystroglycan, fukutin and POMGnT1 during mouse cerebellar development. *Brain Res Mol Brain Res* **112**, 177-181

133. Saito, F., Moore, S. A., Barresi, R., Henry, M. D., Messing, A., Ross-Barta, S. E., Cohn, R. D., Williamson, R. A., Sluka, K. A., Sherman, D. L., Brophy, P. J., Schmelzer, J. D., Low, P. A., Wrabetz, L., Feltri, M. L., and Campbell, K. P. (2003) Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. *Neuron* **38**, 747-758

134. Occhi, S., Zambroni, D., Del Carro, U., Amadio, S., Sirkowski, E. E., Scherer, S. S., Campbell, K. P., Moore, S. A., Chen, Z. L., Strickland, S., Di Muzio, A., Uncini, A., Wrabetz, L., and Feltri, M. L. (2005) Both laminin and Schwann cell dystroglycan are necessary for proper clustering of sodium channels at nodes of Ranvier. *J Neurosci* **25**, 9418-9427

135. Bowe, M. A., Deyst, K. A., Leszyk, J. D., and Fallon, J. R. (1994) Identification and purification of an agrin receptor from Torpedo postsynaptic membranes: a heteromeric complex related to the dystroglycans. *Neuron* **12**, 1173-1180

136. Campanelli, J. T., Roberds, S. L., Campbell, K. P., and Scheller, R. H. (1994) A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. *Cell* **77**, 663-674

137. Gee, S. H., Montanaro, F., Lindenbaum, M. H., and Carbonetto, S. (1994) Dystroglycan-alpha, a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell* **77**, 675-686

138. Sugiyama, J., Bowen, D. C., and Hall, Z. W. (1994) Dystroglycan binds nerve and muscle agrin. *Neuron* **13**, 103-115

139. Gesemann, M., Cavalli, V., Denzer, A. J., Brancaccio, A., Schumacher, B., and Ruegg, M. A. (1996) Alternative splicing of agrin alters its binding to heparin, dystroglycan, and the putative agrin receptor. *Neuron* **16**, 755-767

140. Jacobson, C., Cote, P. D., Rossi, S. G., Rotundo, R. L., and Carbonetto, S. (2001) The dystroglycan complex is necessary for stabilization of acetylcholine receptor clusters at neuromuscular junctions and formation of the synaptic basement membrane. *J Cell Biol* **152**, 435-450

141. Martin, P. T. (2005) The dystroglycanopathies: the new disorders of O-linked glycosylation. *Semin Pediatr Neurol* **12**, 152-158

142. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T., and Endo, T. (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev Cell* **1**, 717-724

143. Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., Hamano, K., Sakakihara, Y., Nonaka, I., Nakagome, Y., Kanazawa, I., Nakamura, Y., Tokunaga, K., and Toda, T. (1998) An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature* **394**, 388-392

126

144. Herbst, R., Iskratsch, T., Unger, E., and Bittner, R. E. (2009) Aberrant development of neuromuscular junctions in glycosylation-defective Large(myd) mice. *Neuromuscul Disord* **19**, 366-378

145. Levedakou, E. N., Chen, X. J., Soliven, B., and Popko, B. (2005) Disruption of the mouse Large gene in the enr and myd mutants results in nerve, muscle, and neuromuscular junction defects. *Mol Cell Neurosci* **28**, 757-769

146. Saito, F., Masaki, T., Saito, Y., Nakamura, A., Takeda, S., Shimizu, T., Toda, T., and Matsumura, K. (2007) Defective peripheral nerve myelination and neuromuscular junction formation in fukutin-deficient chimeric mice. *J Neurochem* **101**, 1712-1722

147. Brockington, M., Yuva, Y., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Herrmann, R., Anderson, L. V., Bashir, R., Burgunder, J. M., Fallet, S., Romero, N., Fardeau, M., Straub, V., Storey, G., Pollitt, C., Richard, I., Sewry, C. A., Bushby, K., Voit, T., Blake, D. J., and Muntoni, F. (2001) Mutations in the fukutin-related protein gene (FKRP) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. *Hum Mol Genet* **10**, 2851-2859

148. Brockington, M., Blake, D. J., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Ponting, C. P., Estournet, B., Romero, N. B., Mercuri, E., Voit, T., Sewry, C. A., Guicheney, P., and Muntoni, F. (2001) Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin alpha2 deficiency and abnormal glycosylation of alpha-dystroglycan. *Am J Hum Genet* **69**, 1198-1209

149. Topaloglu, H., Brockington, M., Yuva, Y., Talim, B., Haliloglu, G., Blake, D., Torelli, S., Brown, S. C., and Muntoni, F. (2003) FKRP gene mutations cause congenital muscular dystrophy, mental retardation, and cerebellar cysts. *Neurology* **60**, 988-992

150. Beltran-Valero de Bernabe, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W. B., Cormand, B., Lehesjoki, A. E., Cruces, J., Voit, T., Walsh, C. A., van Bokhoven, H., and Brunner, H. G. (2002) Mutations in the O-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. *Am J Hum Genet* **71**, 1033-1043

151. van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabe, D., Sabatelli, P., Merlini, L., Boon, M., Scheffer, H., Brockington, M., Muntoni, F., Huynen, M. A., Verrips, A., Walsh, C. A., Barth, P. G., Brunner, H. G., and van Bokhoven, H. (2005) POMT2 mutations cause alpha-dystroglycan hypoglycosylation and Walker-Warburg syndrome. *J Med Genet* **42**, 907-912

152. Godfrey, C., Clement, E., Mein, R., Brockington, M., Smith, J., Talim, B., Straub, V., Robb, S., Quinlivan, R., Feng, L., Jimenez-Mallebrera, C., Mercuri, E., Manzur, A. Y., Kinali, M., Torelli, S., Brown, S. C., Sewry, C. A., Bushby, K., Topaloglu, H., North, K., Abbs, S., and Muntoni, F. (2007) Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. *Brain* **130**, 2725-2735

153. Zhang, Z., Zhang, P., and Hu, H. (2011) LARGE expression augments the glycosylation of glycoproteins in addition to alpha-dystroglycan conferring laminin binding. *PLoS One* **6**, e19080

154. Zhang, P., and Hu, H. (2011) Differential glycosylation of {alpha}-dystroglycan and proteins other than {alpha}-dystroglycan by LARGE. *Glycobiology*

155. Jimenez-Mallebrera, C., Torelli, S., Feng, L., Kim, J., Godfrey, C., Clement, E., Mein, R., Abbs, S., Brown, S. C., Campbell, K. P., Kroger, S., Talim, B.,

Topaloglu, H., Quinlivan, R., Roper, H., Childs, A. M., Kinali, M., Sewry, C. A., and Muntoni, F. (2009) A comparative study of alpha-dystroglycan glycosylation in dystroglycanopathies suggests that the hypoglycosylation of alpha-dystroglycan does not consistently correlate with clinical severity. *Brain Pathol* **19**, 596-611

156. Muntoni, F., Brockington, M., Torelli, S., and Brown, S. C. (2004) Defective glycosylation in congenital muscular dystrophies. *Curr Opin Neurol* **17**, 205-209

157. Moore, C. J., and Hewitt, J. E. (2009) Dystroglycan glycosylation and muscular dystrophy. *Glycoconj J* **26**, 349-357

158. Martin, P. T. (2003) Dystroglycan glycosylation and its role in matrix binding in skeletal muscle. *Glycobiology* **13**, 55R-66R

159. Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W., and Campbell, K. P. (1992) Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* **355**, 696-702

160. Michele, D. E., and Campbell, K. P. (2003) Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. *J Biol Chem* **278**, 15457-15460

161. Peng, H. B., Xie, H., Rossi, S. G., and Rotundo, R. L. (1999) Acetylcholinesterase clustering at the neuromuscular junction involves perlecan and dystroglycan. *J Cell Biol* **145**, 911-921

162. Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G., and Campbell, K. P. (1990) Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* **345**, 315-319

163. Ervasti, J. M., and Sonnemann, K. J. (2008) Biology of the striated muscle dystrophin-glycoprotein complex. *Int Rev Cytol* **265**, 191-225

164. Lynch, G. S. (2004) Role of contraction-induced injury in the mechanisms of muscle damage in muscular dystrophy. *Clin Exp Pharmacol Physiol* **31**, 557-561

165. Barton-Davis, E. R., Cordier, L., Shoturma, D. I., Leland, S. E., and Sweeney, H. L. (1999) Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice. *J Clin Invest* **104**, 375-381

166. Ng, R., Metzger, J. M., Claflin, D. R., and Faulkner, J. A. (2008) Poloxamer 188 reduces the contraction-induced force decline in lumbrical muscles from mdx mice. *Am J Physiol Cell Physiol* **295**, C146-150

167. Grewal, P. K., Holzfeind, P. J., Bittner, R. E., and Hewitt, J. E. (2001) Mutant glycosyltransferase and altered glycosylation of alpha-dystroglycan in the myodystrophy mouse. *Nat Genet* **28**, 151-154

168. Holzfeind, P. J., Grewal, P. K., Reitsamer, H. A., Kechvar, J., Lassmann, H., Hoeger, H., Hewitt, J. E., and Bittner, R. E. (2002) Skeletal, cardiac and tongue muscle pathology, defective retinal transmission, and neuronal migration defects in the Large(myd) mouse defines a natural model for glycosylation-deficient muscle - eye - brain disorders. *Hum Mol Genet* **11**, 2673-2687

169. Kanagawa, M., Saito, F., Kunz, S., Yoshida-Moriguchi, T., Barresi, R., Kobayashi, Y. M., Muschler, J., Dumanski, J. P., Michele, D. E., Oldstone, M. B., and Campbell, K. P. (2004) Molecular recognition by LARGE is essential for expression of functional dystroglycan. *Cell* **117**, 953-964

170. Barresi, R., Michele, D. E., Kanagawa, M., Harper, H. A., Dovico, S. A., Satz, J. S., Moore, S. A., Zhang, W., Schachter, H., Dumanski, J. P., Cohn, R. D., Nishino, I., and Campbell, K. P. (2004) LARGE can functionally bypass alpha-dystroglycan glycosylation defects in distinct congenital muscular dystrophies. *Nat Med* **10**, 696-703

171. Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997) Structures of sialylated O-linked oligosaccharides of bovine peripheral nerve alpha-dystroglycan. The role of a novel O-mannosyl-type oligosaccharide in the binding of alpha-dystroglycan with laminin. *J Biol Chem* **272**, 2156-2162

172. Sasaki, T., Yamada, H., Matsumura, K., Shimizu, T., Kobata, A., and Endo, T. (1998) Detection of O-mannosyl glycans in rabbit skeletal muscle alpha-dystroglycan. *Biochim Biophys Acta* **1425**, 599-606

173. Smalheiser, N. R., Haslam, S. M., Sutton-Smith, M., Morris, H. R., and Dell, A. (1998) Structural analysis of sequences O-linked to mannose reveals a novel Lewis X structure in cranin (dystroglycan) purified from sheep brain. *J Biol Chem* **273**, 23698-23703

174. Yoshida-Moriguchi, T., Yu, L., Stalnaker, S. H., Davis, S., Kunz, S., Madson, M., Oldstone, M. B., Schachter, H., Wells, L., and Campbell, K. P. (2010) O-mannosyl phosphorylation of alpha-dystroglycan is required for laminin binding. *Science* **327**, 88-92

175. Warren, G. L., Hayes, D. A., Lowe, D. A., Williams, J. H., and Armstrong, R. B. (1994) Eccentric contraction-induced injury in normal and hindlimb-suspended mouse soleus and EDL muscles. *J Appl Physiol* **77**, 1421-1430

176. Saltin, B., Henriksson, J., Nygaard, E., Andersen, P., and Jansson, E. (1977) Fiber types and metabolic potentials of skeletal muscles in sedentary man and endurance runners. *Ann N Y Acad Sci* **301**, 3-29

177. Brooks, S. V., and Faulkner, J. A. (1988) Contractile properties of skeletal muscles from young, adult and aged mice. *J Physiol* **404**, 71-82

178. Fujimura, K., Sawaki, H., Sakai, T., Hiruma, T., Nakanishi, N., Sato, T., Ohkura, T., and Narimatsu, H. (2005) LARGE2 facilitates the maturation of alpha-dystroglycan more effectively than LARGE. *Biochem Biophys Res Commun* **329**, 1162-1171

179. Hynes, R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25

180. Nawrotzki, R., Willem, M., Miosge, N., Brinkmeier, H., and Mayer, U. (2003) Defective integrin switch and matrix composition at alpha 7-deficient myotendinous junctions precede the onset of muscular dystrophy in mice. *Hum Mol Genet* **12**, 483-495

181. Schober, S., Mielenz, D., Echtermeyer, F., Hapke, S., Poschl, E., von der Mark, H., Moch, H., and von der Mark, K. (2000) The role of extracellular and cytoplasmic splice domains of alpha7-integrin in cell adhesion and migration on laminins. *Exp Cell Res* **255**, 303-313

182. Gao, Y., Wineman, A. S., and Waas, A. M. (2008) Mechanics of muscle injury induced by lengthening contraction. *Ann Biomed Eng* **36**, 1615-1623

183. Fassler, R., and Meyer, M. (1995) Consequences of lack of beta 1 integrin gene expression in mice. *Genes Dev* **9**, 1896-1908

184. Stephens, L. E., Sutherland, A. E., Klimanskaya, I. V., Andrieux, A., Meneses, J., Pedersen, R. A., and Damsky, C. H. (1995) Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev* **9**, 1883-1895

185. Lin, J., Wu, H., Tarr, P. T., Zhang, C. Y., Wu, Z., Boss, O., Michael, L. F., Puigserver, P., Isotani, E., Olson, E. N., Lowell, B. B., Bassel-Duby, R., and Spiegelman, B. M. (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* **418**, 797-801

186. Handschin, C., Kobayashi, Y. M., Chin, S., Seale, P., Campbell, K. P., and Spiegelman, B. M. (2007) PGC-1alpha regulates the neuromuscular junction program and ameliorates Duchenne muscular dystrophy. *Genes Dev* **21**, 770-783

187. Liu, J., Burkin, D. J., and Kaufman, S. J. (2008) Increasing alpha 7 beta 1-integrin promotes muscle cell proliferation, adhesion, and resistance to apoptosis without changing gene expression. *Am J Physiol Cell Physiol* **294**, C627-640

188. Kho, A. T., Kang, P. B., Kohane, I. S., and Kunkel, L. M. (2006) Transcriptome-scale similarities between mouse and human skeletal muscles with normal and myopathic phenotypes. *BMC Musculoskelet Disord* **7**, 23

189. Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K., and Sudhof, T. C. (2001) A stoichiometric complex of neurexins and dystroglycan in brain. *J Cell Biol* **154**, 435-445

190. Peng, H. B., Ali, A. A., Daggett, D. F., Rauvala, H., Hassell, J. R., and Smalheiser, N. R. (1998) The relationship between perlecan and dystroglycan and its implication in the formation of the neuromuscular junction. *Cell Adhes Commun* **5**, 475-489

191. Smalheiser, N. R., and Schwartz, N. B. (1987) Cranin: a laminin-binding protein of cell membranes. *Proc Natl Acad Sci U S A* **84**, 6457-6461

192. Stalnaker, S. H., Hashmi, S., Lim, J. M., Aoki, K., Porterfield, M., Gutierrez-Sanchez, G., Wheeler, J., Ervasti, J. M., Bergmann, C., Tiemeyer, M., and Wells, L. (2010) Site mapping and characterization of O-glycan structures on alpha-dystroglycan isolated from rabbit skeletal muscle. *J Biol Chem* **285**, 24882-24891

193. Nilsson, J., Larson, G., and Grahn, A. (2010) Characterization of site-specific O-glycan structures within the mucin-like domain of alpha-dystroglycan from human skeletal muscle. *Glycobiology* **20**, 1160-1169

194. Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R. K., Voit, T., Merlini, L., Sewry, C. A., Brown, S. C., and Muntoni, F. (2003) Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of alpha-dystroglycan. *Hum Mol Genet* **12**, 2853-2861

195. van Reeuwijk, J., Grewal, P. K., Salih, M. A., Beltran-Valero de Bernabe, D., McLaughlan, J. M., Michielse, C. B., Herrmann, R., Hewitt, J. E., Steinbrecher, A., Seidahmed, M. Z., Shaheed, M. M., Abomelha, A., Brunner, H. G., van Bokhoven, H., and Voit, T. (2007) Intragenic deletion in the LARGE gene causes Walker-Warburg syndrome. *Hum Genet* **121**, 685-690

196. Manzini, M. C., Gleason, D., Chang, B. S., Hill, R. S., Barry, B. J., Partlow, J. N., Poduri, A., Currier, S., Galvin-Parton, P., Shapiro, L. R., Schmidt, K., Davis, J. G., Basel-Vanagaite, L., Seidahmed, M. Z., Salih, M. A., Dobyns, W. B., and Walsh, C. A. (2008) Ethnically diverse causes of Walker-Warburg syndrome (WWS): FCMD mutations are a more common cause of WWS outside of the Middle East. *Hum Mutat* **29**, E231-241

197. Hara, Y., Kanagawa, M., Kunz, S., Yoshida-Moriguchi, T., Satz, J. S., Kobayashi, Y. M., Zhu, Z., Burden, S. J., Oldstone, M. B., and Campbell, K. P. (2011) Like-acetylglucosaminyltransferase (LARGE)-dependent modification of dystroglycan at Thr-317/319 is required for laminin binding and arenavirus infection. *Proc Natl Acad Sci U S A*

198. Brockington, M., Torelli, S., Prandini, P., Boito, C., Dolatshad, N. F., Longman, C., Brown, S. C., and Muntoni, F. (2005) Localization and functional analysis of the LARGE family of glycosyltransferases: significance for muscular dystrophy. *Hum Mol Genet* **14**, 657-665

199. Yamashita, K., and Yoshioka, T. (1991) Profiles of creatine kinase isoenzyme compositions in single muscle fibres of different types. *J Muscle Res Cell Motil* **12**, 37-44

200. Brockington, M., Torelli, S., Sharp, P. S., Liu, K., Cirak, S., Brown, S. C., Wells, D. J., and Muntoni, F. (2010) Transgenic overexpression of LARGE induces alpha-dystroglycan hyperglycosylation in skeletal and cardiac muscle. *PLoS One* **5**, e14434

201. Cox, G. A., Cole, N. M., Matsumura, K., Phelps, S. F., Hauschka, S. D., Campbell, K. P., Faulkner, J. A., and Chamberlain, J. S. (1993) Overexpression of dystrophin in transgenic mdx mice eliminates dystrophic symptoms without toxicity. *Nature* **364**, 725-729

202. Jaynes, J. B., Chamberlain, J. S., Buskin, J. N., Johnson, J. E., and Hauschka, S. D. (1986) Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts. *Mol Cell Biol* **6**, 2855-2864

203. Stalnaker, S. H., Aoki, K., Lim, J. M., Porterfield, M., Liu, M., Satz, J. S., Buskirk, S., Xiong, Y., Zhang, P., Campbell, K. P., Hu, H., Live, D., Tiemeyer, M., and Wells, L. (2011) Glycomic analyses of mouse models of congenital muscular dystrophy. *J Biol Chem* **286**, 21180-21190

204. Godfrey, C., Foley, A. R., Clement, E., and Muntoni, F. (2011) Dystroglycanopathies: coming into focus. *Curr Opin Genet Dev* **21**, 278-285

205. Matsumura, K., Yamada, H., Shimizu, T., and Campbell, K. P. (1993) Differential expression of dystrophin, utrophin and dystrophin-associated proteins in peripheral nerve. *FEBS Lett* **334**, 281-285

206. Saito, F., Masaki, T., Kamakura, K., Anderson, L. V., Fujita, S., Fukuta-Ohi, H., Sunada, Y., Shimizu, T., and Matsumura, K. (1999) Characterization of the transmembrane molecular architecture of the dystroglycan complex in schwann cells. *J Biol Chem* **274**, 8240-8246

207. Yamada, H., Shimizu, T., Tanaka, T., Campbell, K. P., and Matsumura, K. (1994) Dystroglycan is a binding protein of laminin and merosin in peripheral nerve. *FEBS Lett* **352**, 49-53

208. Yamada, H., Denzer, A. J., Hori, H., Tanaka, T., Anderson, L. V., Fujita, S., Fukuta-Ohi, H., Shimizu, T., Ruegg, M. A., and Matsumura, K. (1996) Dystroglycan is a dual receptor for agrin and laminin-2 in Schwann cell membrane. *J Biol Chem* **271**, 23418-23423

209. Cote, P. D., Moukhles, H., Lindenbaum, M., and Carbonetto, S. (1999) Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. *Nat Genet* **23**, 338-342

210. Hopf, C., and Hoch, W. (1996) Agrin binding to alpha-dystroglycan. Domains of agrin necessary to induce acetylcholine receptor clustering are overlapping but not identical to the alpha-dystroglycan-binding region. *J Biol Chem* **271**, 5231-5236

211. Grady, R. M., Zhou, H., Cunningham, J. M., Henry, M. D., Campbell, K. P., and Sanes, J. R. (2000) Maturation and maintenance of the neuromuscular synapse: genetic evidence for roles of the dystrophin--glycoprotein complex. *Neuron* **25**, 279-293

212. Guyenet, S. J., Furrer, S. A., Damian, V. M., Baughan, T. D., La Spada, A. R., and Garden, G. A. (2010) A simple composite phenotype scoring system for evaluating mouse models of cerebellar ataxia. *J Vis Exp*

213. Kelly, D., Chancellor, K., Milatovich, A., Francke, U., Suzuki, K., and Popko, B. (1994) Autosomal recessive neuromuscular disorder in a transgenic line of mice. *J Neurosci* **14**, 198-207

214. Jacobson, C., Montanaro, F., Lindenbaum, M., Carbonetto, S., and Ferns, M. (1998) alpha-Dystroglycan functions in acetylcholine receptor aggregation but is not a coreceptor for agrin-MuSK signaling. *J Neurosci* **18**, 6340-6348

215. Clement, E., Mercuri, E., Godfrey, C., Smith, J., Robb, S., Kinali, M., Straub, V., Bushby, K., Manzur, A., Talim, B., Cowan, F., Quinlivan, R., Klein, A., Longman, C., McWilliam, R., Topaloglu, H., Mein, R., Abbs, S., North, K., Barkovich, A. J., Rutherford, M., and Muntoni, F. (2008) Brain involvement in muscular dystrophies with defective dystroglycan glycosylation. *Ann Neurol* **64**, 573-582

216. Kabaeva, Z., Meekhof, K. E., and Michele, D. E. (2011) Sarcolemma instability during mechanical activity in Largemyd cardiac myocytes with loss of dystroglycan extracellular matrix receptor function. *Hum Mol Genet* **20**, 3346-3355

217. Cartaud, A., Coutant, S., Petrucci, T. C., and Cartaud, J. (1998) Evidence for in situ and in vitro association between beta-dystroglycan and the subsynaptic 43K rapsyn protein. Consequence for acetylcholine receptor clustering at the synapse. *J Biol Chem* **273**, 11321-11326

218. Bradley, W. G., and Jenkison, M. (1975) Neural abnormalities in the dystrophic mouse. *J Neurol Sci* **25**, 249-255

219. Wallquist, W., Plantman, S., Thams, S., Thyboll, J., Kortesmaa, J., Lannergren, J., Domogatskaya, A., Ogren, S. O., Risling, M., Hammarberg, H., Tryggvason, K., and Cullheim, S. (2005) Impeded interaction between Schwann cells and axons in the absence of laminin alpha4. *J Neurosci* **25**, 3692-3700

220. Yang, D., Bierman, J., Tarumi, Y. S., Zhong, Y. P., Rangwala, R., Proctor, T. M., Miyagoe-Suzuki, Y., Takeda, S., Miner, J. H., Sherman, L. S., Gold, B. G., and Patton, B. L. (2005) Coordinate control of axon defasciculation and myelination by laminin-2 and -8. *J Cell Biol* **168**, 655-666

221. Saito, F., Saito-Arai, Y., Nakamura, A., Shimizu, T., and Matsumura, K. (2008) Processing and secretion of the N-terminal domain of alpha-dystroglycan in cell culture media. *FEBS Lett* **582**, 439-444

222. Qu, Q., and Smith, F. I. (2005) Neuronal migration defects in cerebellum of the Largemyd mouse are associated with disruptions in Bergmann glia organization and delayed migration of granule neurons. *Cerebellum* **4**, 261-270

223. Zweifel, L. S., Kuruvilla, R., and Ginty, D. D. (2005) Functions and mechanisms of retrograde neurotrophin signalling. *Nat Rev Neurosci* **6**, 615-625

224. Maisonpierre, P. C., Belluscio, L., Friedman, B., Alderson, R. F., Wiegand, S. J., Furth, M. E., Lindsay, R. M., and Yancopoulos, G. D. (1990) NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron* **5**, 501-509

225. Koliatsos, V. E., Clatterbuck, R. E., Winslow, J. W., Cayouette, M. H., and Price, D. L. (1993) Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. *Neuron* **10**, 359-367

226. Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., Simmons, L., Moffet, B., Vandlen, R. A., Simpson, L. C., and et al. (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* **266**, 1062-1064

227. Funakoshi, H., Belluardo, N., Arenas, E., Yamamoto, Y., Casabona, A., Persson, H., and Ibanez, C. F. (1995) Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. *Science* **268**, 1495-1499

228. Nagano, M., and Suzuki, H. (2003) Quantitative analyses of expression of GDNF and neurotrophins during postnatal development in rat skeletal muscles. *Neurosci Res* **45**, 391-399

229. Henderson, C. E., Camu, W., Mettling, C., Gouin, A., Poulsen, K., Karihaloo, M., Rullamas, J., Evans, T., McMahon, S. B., Armanini, M. P., and et al. (1993) Neurotrophins promote motor neuron survival and are present in embryonic limb bud. *Nature* **363**, 266-270

230. Nico, B., Mangieri, D., De Luca, A., Corsi, P., Benagiano, V., Tamma, R., Annese, T., Longo, V., Crivellato, E., and Ribatti, D. (2009) Nerve growth factor and its receptors TrkA and p75 are upregulated in the brain of mdx dystrophic mouse. *Neuroscience* **161**, 1057-1066

231. Toti, P., Villanova, M., Vatti, R., Schuerfeld, K., Stumpo, M., Barbagli, L., Malandrini, A., and Costantini, M. (2003) Nerve growth factor expression in human dystrophic muscles. *Muscle Nerve* **27**, 370-373

232. Duchenne (1867) The Pathology of Paralysis with Muscular Degeneration (Paralysie Myosclerotique), or Paralysis with Apparent Hypertrophy. *Br Med J* **2**, 541-542

233. Meryon, E. (1866) On Granular Degeneration of the Voluntary Muscles. *Med Chir Trans* **49**, 45-50 41

234. Hack, A. A., Cordier, L., Shoturma, D. I., Lam, M. Y., Sweeney, H. L., and McNally, E. M. (1999) Muscle degeneration without mechanical injury in sarcoglycan deficiency. *Proc Natl Acad Sci U S A* **96**, 10723-10728

235. Lockhart, N. C., and Brooks, S. V. (2008) Neutrophil accumulation following passive stretches contributes to adaptations that reduce contraction-induced skeletal muscle injury in mice. *J Appl Physiol* **104**, 1109-1115

236. Hall, J. K., Banks, G. B., Chamberlain, J. S., and Olwin, B. B. (2010) Prevention of muscle aging by myofiber-associated satellite cell transplantation. *Sci Transl Med* **2**, 57ra83

237. Montanaro, F., Gee, S. H., Jacobson, C., Lindenbaum, M. H., Froehner, S. C., and Carbonetto, S. (1998) Laminin and alpha-dystroglycan mediate acetylcholine receptor aggregation via a MuSK-independent pathway. *J Neurosci* **18**, 1250-1260

238. Masaki, T., Matsumura, K., Saito, F., Yamada, H., Higuchi, S., Kamakura, K., Yorifuji, H., and Shimizu, T. (2003) Association of dystroglycan and laminin-2 coexpression with myelinogenesis in peripheral nerves. *Med Electron Microsc* **36**, 221-239

239. Domeniconi, M., Hempstead, B. L., and Chao, M. V. (2007) Pro-NGF secreted by astrocytes promotes motor neuron cell death. *Mol Cell Neurosci* **34**, 271-279

240. Cosgaya, J. M., Chan, J. R., and Shooter, E. M. (2002) The neurotrophin receptor p75NTR as a positive modulator of myelination. *Science* **298**, 1245-1248

241. Chan, J. R., Cosgaya, J. M., Wu, Y. J., and Shooter, E. M. (2001) Neurotrophins are key mediators of the myelination program in the peripheral nervous system. *Proc Natl Acad Sci U S A* **98**, 14661-14668

242. Nave, K. A., and Salzer, J. L. (2006) Axonal regulation of myelination by neuregulin 1. *Curr Opin Neurobiol* **16**, 492-500

243. Birchmeier, C., and Nave, K. A. (2008) Neuregulin-1, a key axonal signal that drives Schwann cell growth and differentiation. *Glia* **56**, 1491-1497

244. Angelucci, F., and Colantoni, L. (2010) Facioscapulohumeral muscular dystrophy: do neurotrophins play a role? *Muscle Nerve* **41**, 120-127

245. Tao, X., West, A. E., Chen, W. G., Corfas, G., and Greenberg, M. E. (2002) A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. *Neuron* **33**, 383-395

246.   Yang, F., Je, H. S., Ji, Y., Nagappan, G., Hempstead, B., and Lu, B. (2009) Pro-BDNF-induced synaptic depression and retraction at developing neuromuscular synapses. *J Cell Biol* **185**, 727-741

247.   Keller-Peck, C. R., Feng, G., Sanes, J. R., Yan, Q., Lichtman, J. W., and Snider, W. D. (2001) Glial cell line-derived neurotrophic factor administration in postnatal life results in motor unit enlargement and continuous synaptic remodeling at the neuromuscular junction. *J Neurosci* **21**, 6136-6146

248.   Sakuma, K., Watanabe, K., Totsuka, T., Sano, M., Nakano, H., Nakao, R., Nishikawa, J. J., Sorimachi, Y., Yoshimoto, K., and Yasuhara, M. (2002) The reciprocal change of neurotrophin-4 and glial cell line-derived neurotrophic factor protein in the muscles, spinal cord and cerebellum of the dy mouse. *Acta Neuropathol* **104**, 482-492

249.   Pazyra-Murphy, M. F., Hans, A., Courchesne, S. L., Karch, C., Cosker, K. E., Heerssen, H. M., Watson, F. L., Kim, T., Greenberg, M. E., and Segal, R. A. (2009) A retrograde neuronal survival response: target-derived neurotrophins regulate MEF2D and bcl-w. *J Neurosci* **29**, 6700-6709