Title
Isolation of Primary Myoblasts and Characterization of the Endoplasmic Reticulum Stress Response in Wild-type and mdx Mice

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Publication Date
2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

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A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Physiological Sciences

by

Yuk Wah Kwok

2014

ABSTRACT OF THE THESIS

Isolation of Primary Myoblasts and Characterization of the Endoplasmic Reticulum Stress Response in Wild-type and *mdx* Mice

by

Yuk Wah Kwok

Master of Science in Physiological Sciences

University of California, Los Angeles, 2014

Professor Rachelle H. Crosbie-Watson, Chair

Duchenne Muscular Dystrophy (DMD) is a devastating muscle disease caused by a recessive X-linked mutation in the dystrophin gene. This mutation leads to a loss of the dystrophin-glycoprotein complex (DGC) and destabilization of the sarcolemmal membrane. In the past, it has been assumed that mutant dystrophin protein is rapidly degraded in DMD muscle due to the lack of dystrophin detection in DMD muscle lysates. A recent study in the Crosbie-Watson group showed the accumulation of truncated dystrophin in the ER/Golgi of the *mdx* mice, leading our lab to examine the unfolded protein response (UPR) in these mice. The UPR is a cascade of molecular events that function to stabilize the cell as either mis-folded or unfolded proteins overload the ER by attenuating transcription and up-regulating chaperones to assist with the accumulation of proteins. We hypothesize that loss of dystrophin and accumulation of compensatory proteins in the ER lead to the UPR in DMD muscle. We performed a cell viability

assay to examine isolated myoblasts' ability to tolerate stress when treated with tunicamycin, a known ER stressor, and found *mdx* myoblasts displayed a lower percentage of viable cells when compared to wild-type. Many UPR proteins, such as BiP, PERK, and cleaved ATF6 also display an increased abundance in immunoblots from *mdx* myotubes as well as total skeletal muscle when compared to wild-type myotubes. Curcumin treatment decreased protein expression of BiP and ATF6, but up-regulated the PERK pathway. When analyzing the UPR before and after the onset of pathology at 3 and 10 weeks of age in *mdx* mice, we found 3-week old *mdx* mice had increased abundance of UPR proteins compared to wild-type, but this difference dissipated in 10-week old mice. ATF6 also increased in abundance in 10-week old mice. Interestingly, we also found an increase in the accumulation of α-dystroglycan (DG), a dystrophin binding protein, in the ER after the onset of pathology in *mdx* mice compared to the wild-type controls. The effects of the accumulation of α-DG and the physiological effects of UPR activation in dystrophin-deficient muscle remains to be understood and ER function needs to be further analyzed in future studies.

The thesis of Yuk Wah Kwok is approved.

Jim Tidball

Mark Frye

Carmen Bertoni

Rachelle H. Crosbie-Watson, Committee Chair

University of California, Los Angeles

2014

DEDICATION

I would like to dedicate this thesis to my parents, whose hard work and personal sacrifices have allowed me to pursue my dreams. I would also like to dedicate my work to my best friend and boyfriend, Dylan Hand, and my fellow Quaker sidekick, Dr. Debbie Hand. Their unwavering support during my times of stress has helped me excel and be the best that I can be.

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ACKNOWLEDGEMENTS

I would like to thank my mentor and committee chair, Dr. Rachelle Crosbie-Watson, for her guidance throughout my graduate career at UCLA. Her mentorship has helped me advance as a scientist and educator, as well as grow my passion for research and undergraduate education, which I hope to carry on as a future veterinarian. I would also like to thank the other members of my committee, Dr. Jim Tidball, Dr. Mark Frye, and Dr. Carmen Bertoni. Thank you Jim for sharing your expertise in DMD to help me further my project. Thank you Carmen for providing your help and expertise on the isolation of primary myoblasts; this project would not have been possible without all of your advice and countless hours teaching me how to isolate and purify primary myoblasts. Thank you Mark for your support, as well as your fresh insight and perspective into my work.

Thanks to all members past and present of the Crosbie-Watson lab, especially Dr. Jamie Marshall, whose insightful advice and technical assistance has helped me progress as a graduate student and move my project forward. This project would not have been possible without her help and expertise, and I am glad we became good friends and colleagues.

I also extend my thanks to Michael Carr, who made the program easier to navigate with reminders of deadlines and tasks to do that would have completely slipped my mind as a graduate student! I also thank the wonderful faculty and staff in the Life Science Core for allowing me to teach and inspire students in LS2.

Chapter 3 is a version of the following article: Marshall JL, Kwok Y, McMorran BJ, Baum LG, Crosbie-Watson RH. "The potential of sarcospan in adhesion complex replacement therapeutics for the treatment of muscular dystrophy." I would like to thank the co-authors for their contributions. The authors thank A.W. Kwok, J. Lee and J. Oh for critically reading this

manuscript. This work was supported by grants from the Genetic Mechanisms Pre-doctoral Training Fellowship USPHS National Research Service Award GM07104, the Edith Hyde Fellowship, the Eureka Pre-doctoral Training Fellowship, and the Ruth L. Kirschstein National Service Award T32AR059033 from the National Institute of Arthritis and Musculoskeletal and Skin Diseases to JLM; Muscular Dystrophy Association RG 135449 and NIH P30 AR057230 to LGB and NIH/NIAMS (R01 AR048179) to RCW.

Lastly, thanks to all of my awesome classmates in the Integrative Biology and Physiology Department. This experience would not have been the same without them. I am truly grateful for everything UCLA has done for me.

CHAPTER 1: The isolation of primary myoblasts from the whole muscle of wild-type and *mdx* mice

INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is a devastating muscle disease caused by a recessive X-linked mutation leading to a change in the DMD gene [1]. Dystrophin is a 427 kDa protein that is undetectable or completely absent in patients with DMD [2]. DMD occurs in approximately 1 in 3,500 male children, leading to respiratory or cardiac failure in the second decade of life. In 1986, the gene encoding dystrophin was identified, and mutations in the *dystrophin* gene were determined to be responsible for DMD [3,4]. While dystrophin was identified over 25 years ago, there is still no cure for the disease [3]. The N-terminus of dystrophin binds directly to filamentous actin (F-actin) in the cytoplasm of the myofiber and the C-terminus of dystrophin is associated with a group of proteins including neuronal nitric oxide synthase (nNOS), α-syntrophin, α-dystrobrevin, and β-dystroglycan (DG) [5,6]. Dystrobrevin binds to the intermediate filament protein syncoilin, which provides a connection between desmin and the DGC that is thought to be important for maintaining mechanical strength and structural organization necessary for muscle contractions [7]. nNOS requires binding to both dystrophin and syntrophin for sarcolemmal localization and produces nitric oxide, which stimulates blood flow necessary to meet the metabolic demands of healthy muscle [8-12]. The DGC functions to stabilize the sarcolemma during muscle contractions by providing a critical connection between the extracellular matrix (ECM) and the intracellular actin cytoskeleton [13]. α-Dystroglycan (α-DG), a peripheral membrane protein, provides the connection between the transmembrane β-DG and laminin-211 in the ECM [14,15]. The central mucin domain of α-DG is heavily glycosylated and the extent and type of glycosylation determines the affinity of DGC

attachment to the ECM [6,14-19]. The sarcoglycan-sarcospan (SG-SSPN) subcomplex stabilizes α-DG's association with β-DG at the cell surface [20-22]. Loss of the DGC in DMD renders the sarcolemma susceptible to membrane ruptures, which initiates the cycles of myofiber degeneration and regeneration characteristic of DMD [13,16,23,24].

The most commonly used mouse model for DMD is the *mdx* mouse, which lacks full-length dystrophin expression at the sarcolemma [25]. Muscle pathology in *mdx* muscle is less severe than that observed in boys with DMD and the *mdx* mouse maintains a fairly normal lifespan [26]. However, this mouse model has led to the realization of many other physiological dysfunctions caused by this disease, including deficits in mitochondrial localization and ATP synthesis [27], cardiac dysfunction [28,29], and increased respiratory dysfunction with age [30]. Interestingly, data from our lab has also suggested that there is an accumulation of truncated dystrophin in the ER/Golgi of *mdx* mice [31]. Prolonged accumulation of proteins within the ER has been shown to be detrimental in many other diseases, but the mechanisms of dystrophin accumulation and its cellular consequences are unclear. Protein transportation is challenging to investigate in whole skeletal muscle due to the complex intracellular membrane systems, including T-tubules and sarcoplasmic reticulum coupled with the less characterized ER/Golgi in muscle. Thus, cell culture models offer advantages to analyze dystrophin aggregation and intracellular protein transport, since conditions can be more easily manipulated when compared to whole skeletal muscle.

In addition to stronger control of experimental parameters, the use of a cell culture model offers the ability to reduce the numbers of mice utilized, which leads to significant financial advantages and savings. In addition, with further development, a cell culture model could lead to replacement of mouse models. A cell culture model also facilitates more collaboration with

other labs in the muscle field, as cells are much easier to ship than mice. The isolation of cells from different lines of mice affords us to experiment *in vitro* prior to utilizing our resources on *in vivo* experiments. This first chapter details the techniques utilized to isolate primary myoblasts from whole skeletal muscle.

MATERIALS AND METHODS

Animal Use

Experiments were performed in accordance with rules and regulations of the NIH guidelines for research animals, as approved by the Institutional Animal Care and Use Committee of the University of California.

Preparation of Extracellular Matrix Coated Plates

Extracellular matrix (ECM) gel-coated plates were made using a 1:500 dilution of ECM gel from Engelbreth-Holm-Swarm murine sarcoma (Sigma Aldrich) in Dulbecco's phosphate-buffered saline (DPBS) with calcium and magnesium (GIBCO). 6 ml of the dilution is used for a 10 cm plate (Corning), while 5 ml of the dilution is used for a 60 mm plate (Corning). Plates were wrapped in plastic and rocked overnight at room temperature. DPBS was changed the following day and plates were stored for up to 2 weeks at 4°C.

Isolation of Primary Myoblasts

Primary myoblasts were isolated from two to six-day old neonatal mice from: $WT^{SSPN3.0}$ and $WT^{SSPN1.5}$ (wild-type mice over-expressing human sarcospan [SSPN] 3-fold and 1.5-fold, respectively), $WT^{SSPN-/-}$, *mdx*, $mdx^{SSPN3.0}$ (*mdx* mice overexpressing human sarcospan 3-fold), and wild-type colonies. Neonatal mice were anesthetized by inducing hypothermia over ice for 15 minutes. After anesthesia, mice were cervically dislocated and sterilized in a 70% ethanol solution for 10 minutes in order to prevent fungal contamination of cultures. The total skeletal muscle from the hind limbs and forelimbs were carefully dissected and placed in a petri dish with

several drops of DPBS with calcium and magnesium to keep the tissues moist over ice as other mice were dissected.

Muscles were minced using razor blades until the consistency of dissected muscle was even. Collagenase D (Roche) was dissolved in DPBS with calcium and magnesium for a final working concentration of 20 mg/ml, and dispase (Stem Cell Technologies) was added to create a 1:1 collagenase D to a dispase enzyme mixture. This mixture was added to the muscle with mincing to further dissociate the samples into a slurry. The slurry was transferred into a sterile 15 ml falcon tube (Genemate) for incubation at 37°C for 45 minutes, with gentle pipetting every 15 minutes to break up large clumps of tissue.

After incubation, the mixture was strained through a 60 μm mesh (BD Biosciences) to separate large clumps. The filtrate was centrifuged at 3000 rpm for 5 minutes at room temperature. The cell pellet was resuspended in primary myoblast growth medium consisting of Ham's F-10 (Cellgro) with 20% heat-inactivated fetal bovine serum (FBS) (GIBCO) and 1% penicillin-streptomycin (P/S) (GIBCO). Fresh basic fibroblast growth factor (bFGF) was suspended in DPBS with calcium and magnesium for a working concentration of 25 μg/μl; 2 μl were added for every 10 ml of media for a concentration of 50 μg per 100 cm plate. The resuspended cells were grown and incubated at 37°C in 5% CO_2.

Maintenance of Primary Myoblasts

The media was changed every day with fresh bFGF supplementation. Cells were passaged after reaching 70-80% confluency. Depending on the number of fibroblasts, passages were performed

every 1-5 days with pre-plating. If the total population contained greater than 60% fibroblasts, then pre-plating was performed using a 60 mm ECM coated plate for 10 minutes. If there were a generous number of myoblasts, pre-plating was performed using a 10 cm uncoated polylysine plate for 30 minutes in order to minimize myoblast loss. Myoblasts were enriched within 1-2 weeks and proliferated after enrichment. After proliferation, myoblasts were frozen in 90% FBS and 10% DMSO (Sigma Aldrich) for use at a later date.

Differentiation of Primary Myoblasts

Myoblasts were grown to approximately 90% confluency. Medium was not changed in order to deplete the bFGF concentrations for the two days prior to switching to differentiation medium. Differentiation medium was composed of Dulbecco's Modified Eagle Medium (DMEM; GIBCO), 1% P/S, and 2% equine horse serum (Lonza). Myoblasts were differentiated for 5 days, with medium changed daily.

Genotyping

Since the primary myoblasts were isolated from neonatal mice, polymerase chain reaction (PCR) was required to determine the sex of each mouse. The following oligonucleotide primers were used: YMT FP1, 5'-CTGGAGCTCTACAGTGATGA-3', YMT RP1, 5'-CAGTTACCAATCAACACATCAC-3' to determine the presence of the Y chromosome, and MYO F, 5'-TTACGTCCATCGTGGACAGCAT-3', MYO R, 5'-TGGGCTGGGTGTTAGTCTTAT-3' to confirm the PCR reaction proceeded properly [32]. The PCR conditions were as follows: denaturation at 94°C for 4 min followed by 30 cycles of 45 sec at 94°C, 45 sec at 61°C, 45 sec at 72°C, and 5 min at 72°C.

In addition, PCR was performed to determine the presence of the transgene in pups from transgenic colonies with the following oligonucleotide primers: SPNI1FA, 5'-ACTCCCTGGAATACAGAGGAACT-3', and SPNI2RA, 5'-TACAAGGGGACAGACACTCAGAC-3'. The PCR conditions were as follows: denaturation at 94°C for 2 min followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C.

RESULTS

Isolation of myoblasts is complicated by contamination by rapidly dividing fibroblasts. One goal during preparation of myoblasts is to reduce the relative population of fibroblasts so that they do not exceed that of myoblasts. One to two days after isolating myoblasts, pre-plating can occur using either ECM-coated plates or plain polylysine plates, depending on the number of fibroblasts in culture (Figure 1). It was also highly effective to tap the cell culture dishes for 5 minutes to detach myoblasts at four to five days post-isolation; this method leaves the fibroblasts attached to the dish, while most of the myoblasts detach, leading to greater enrichment of myoblasts.

Every isolation differs depending on the length of time used to dissociate the muscle, as well as the amount of muscle successfully dissected from the mice. Cell isolations may also contain many fibroblasts, which requires subsequent pre-plating in order to decrease the fibroblasts and enrich myoblast growth. Myoblasts become enriched one to two weeks after isolation. During this time period, it is critical to pre-plate the cell populations if many fibroblasts are in culture. Fibroblasts tend to grow much quicker and will overtake the myoblast population if pre-platings are not performed. After two to three weeks, the myoblasts typically increase in number; the myoblasts should then be expanded and frozen for future experimentation.

As the cultures of myoblasts were grown and expanded, one 10 cm plate was used for a differentiation assay in order to ensure the quality of the myoblasts. The ability of the isolated myoblasts to differentiate into myotubes was used to assess the quality of the myoblasts. Primary myoblast growth medium was not changed two days prior to addition of differentiation medium in order to deplete any remaining bFGF. After two to three days in differentiation

medium, elongated myoblasts began fusing into longer, multinucleated myotubes. In total, six lines of primary myoblasts were isolated: wild-type (WT), sarcospan-null (SSPN-null), WT overexpressing SSPN 1.5-fold ($WT^{SSPN1.5}$), WT overexpressing SSPN 3-fold ($WT^{SSPN3.0}$), *mdx*, and *mdx* overexpressing SSPN 3-fold ($mdx^{SSPN3.0}$) (Table 1). Non-transgenic WT and *mdx* myoblasts were isolated as controls for future experimentation.

WT and *mdx* myoblasts were differentiated for 5 days and lysed in ice-cold 1X RIPA buffer. After clarification lysates by centrifugation, samples were run on a 4-20% SDS gel and transferred to nitrocellulose membranes for immunoblotting. Immunoblots were performed for dystrophin and utrophin to ensure appropriate myoblast protein expression. As expected, *mdx* myotubes do not express full-length dystrophin protein when blotted with dystrophin antibody, while utrophin is increased when compared to wild-type myotubes (Figure 1-2). Since the WT myotubes displayed dystrophin and *mdx* myotubes lacked dystrophin, and both lines express utrophin, these cells can provide a close representation of muscle *in vivo*. The isolated myoblasts were expanded and stored in 90% FBS and 10% DMSO at -80°C for future experimentation.

We previously used C2C12 murine cells to examine the role of sarcospan overexpression and knockdown and its effects on cell adhesion. The widely used C2C12 cell line is an immortalized muscle cell line derived from CH3 (wild-type) mice [33]. However, our unpublished studies did not yield clear conclusions for the role of sarcospan in these cells, as the levels of knockdown and overexpression were not consistent. The isolation of primary myoblasts is an important step in the advancement of ongoing research in muscular diseases, as the cells will provide a well-controlled cellular model of over-expression compared to myoblasts engineered to overexpress or knockdown sarcospan.

Myoblasts represent a homogenous population of cells for *in vitro* studies including: the addition of different pharmacological agents impeding protein transport to determine the role of sarcospan in ER/Golgi function and trafficking of membrane complexes and whether this role is what helps ameliorate pathology in *mdx* mice; the addition of different ER stressors to examine whether sarcospan plays a role in the unfolded protein response and how sarcospan may affect *mdx* mice's responses, and immunoblots to determine the effect of over-expression and knockout of sarcospan on cell adhesion and proteins involved in ER/Golgi function. The creation of this cell repository will provide an invaluable resource for future *in vitro* experiments prior to progressing to *in vivo* experiments.

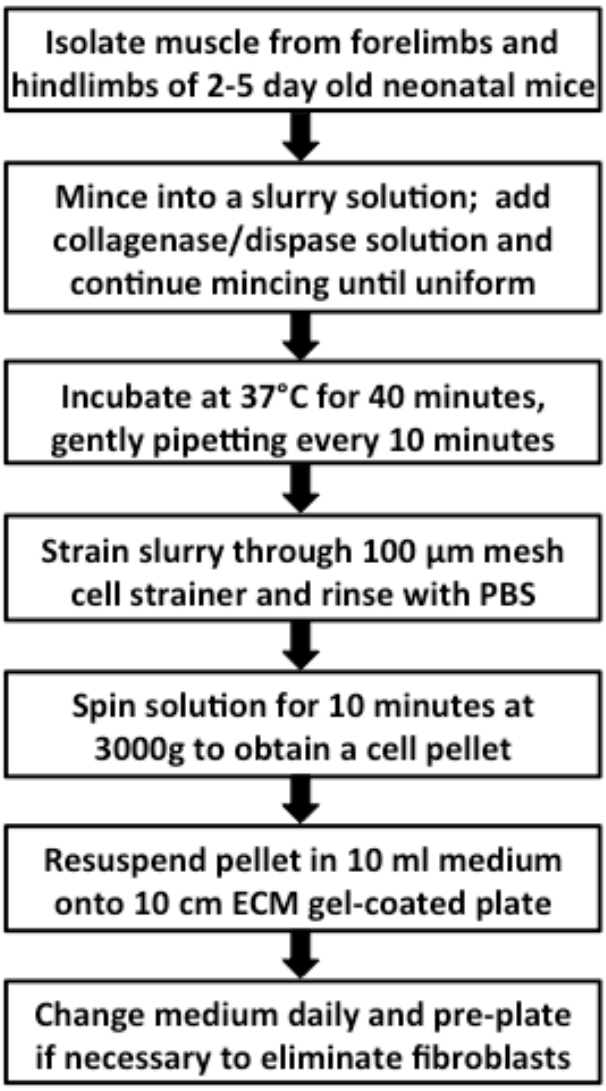

Figure 1-1. Schematic for the isolation of primary myoblasts (with the input of Dr. Carmen Bertoni).

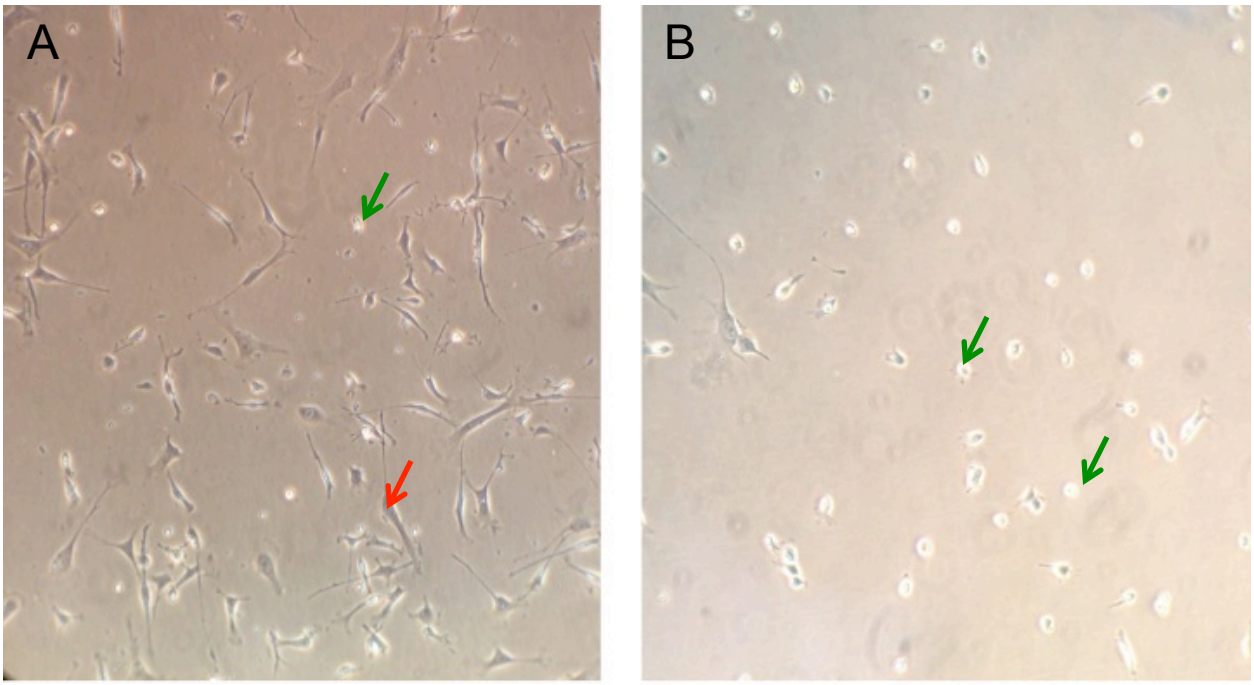

Figure 1-2. Isolation of primary myoblasts. **A:** Cell culture containing both myoblasts and fibroblasts two days after isolation from two-day-old wild-type mice. Note the cells with long, extended processes (red arrow); these are fibroblasts that remained from the isolation. It is necessary to remove fibroblasts early on in culture to prevent proliferation. This can be performed in two ways: by pre-plating on plastic dishes, or by tapping cell culture plate for five minutes against a hard surface. Fibroblasts will attach very quickly but detach very slowly. Also note the primary myoblasts, which are more circular and bright (green arrow). **B:** Figure shows a primary cell culture two weeks post-isolation at 60% confluence. Note that 95% of the cells are bright, round myoblasts (green arrows). Fibroblasts remaining at this point will begin lifting and dying in the next two weeks.

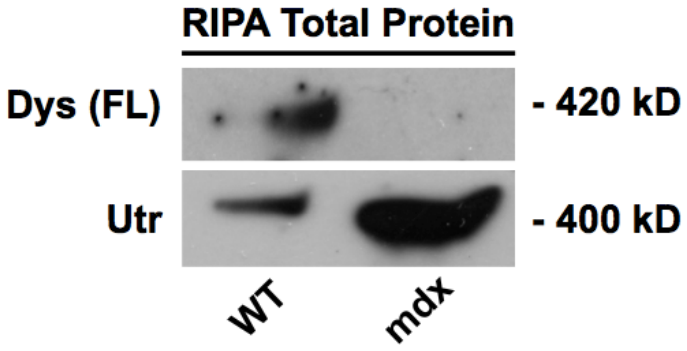

Figure 1-3. Differentiated primary myoblasts express appropriate adhesion complex proteins. Primary myoblasts were differentiated for five days. On day five, differentiated myotubes were solubilized in RIPA buffer, and 47 μg of protein lysate was resolved by SDS-PAGE. Immunoblotting was performed with indicated antibodies—Dys, dystrophin; FL, full-length; Utr, utrophin.

Line
WT males
WT females
mdx males (X^{DMD}/Y)
SSPN-null - males
$SSPN^{3.0}$ - males
$SSPN^{3.0}$ - females
$mdx^{3.0}$ - females
$mdx^{3.0}$ - males

Table 1. Successfully isolated primary myoblasts. Primary myoblasts were isolated as described and frozen in 90% FBS and 10% DMSO at -80°C and transferred to liquid nitrogen as a resource for future experiments involving the role of sarcospan in ER/Golgi function—SSPN, sarcospan; $SSPN^{3.0}$, wild-type mice over-expressing sarcospan 3-fold; *mdx*$^{3.0}$, *mdx* mice over-expressing sarcospan 3-fold.

CHAPTER 2: The Unfolded Protein Response is detected in dystrophin-deficient mouse models and relevant cell culture models

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease caused by mutations in the dystrophin gene, leading to the loss of the dystrophin-glycoprotein complex at the sarcolemma [23]. The *mdx* mouse is one of the most widely used mouse models for DMD. The *mdx* mouse contains a point mutation in exon 23 [34], resulting in a premature termination codon that is predicted to result in a 115 kDa protein [25]. It has been shown that dystrophin mRNA levels are decreased in skeletal muscle, cardiac muscle, and brains of *mdx mice* [35]. However, dystrophin mRNA levels vary widely, depending on the causative mutation in human DMD and Becker muscular dystrophy (BMD) patients [36]. It was previously thought that the 115 kDa dystrophin protein is rapidly degraded after synthesis in *mdx* muscle, but recent studies suggest the possibility of truncated dystrophin being expressed at the sarcolemma [37-39] or retained in the ER/Golgi [31]. These data led our group to further investigate the effects of the accumulation of dystrophin within the ER of whole skeletal muscle as well as myotubes isolated from *mdx* mice.

The endoplasmic reticulum (ER) is the site of the folding and maturation of most secreted and transmembrane proteins, as well as lipids, sterols, and glycoproteins [40]. Cells continually adjust the protein-folding capacity of the ER in order to maintain the quality of cell-surface and transmembrane proteins as the physiological states or environmental conditions change. The imbalance of protein folding and how the cell responds to the load of extra proteins is termed ER stress. The first experiments detailing the existence of such a response utilized pharmacological

and genetic manipulations that selectively increased ER-resident chaperones [41]. This cellular pathway is termed the unfolded protein response (UPR), and has been implicated in various diseases, including diabetes [42], heart failure [43], Alzheimer's Disease [44], certain dystroglycanopathies [45], and myotonic dystrophy type 1 [46]. The UPR has not been studied extensively in DMD, leading our lab to investigate the effect of the loss of dystrophin and eventual increase in compensatory proteins on the ER folding load.

Proteins are partially translated in the cytoplasm prior to the translocation of the proteins to the ER. Proteins contain signal sequences, which directs the proteins to the calnexin quality control cycle, where calnexin and calreticulin assist in the folding of glycoproteins within the ER [47]. PDI (protein disulfide isomerase) is a 57 kDa protein that catalyzes the formation of disulfide bonds in order to assist in proper protein folding [48]. Proteins that are properly folded and modified are allowed to proceed to the Golgi complex for further modification and packaging. Proteins that are permanently misfolded are led to ER-associated degradation (ERAD) pathways. The accumulation of misfolded proteins leads to the induction of the UPR to combat the perturbation of homeostasis caused by the increased folding load within the ER. One major cellular target of the UPR is glucose-related protein 78 (GRP78), which is also referred to as BiP. BiP is an ER chaperone that binds to unfolded proteins and assists in UPR activation, although the exact mechanism is still debated [49,50].

The UPR is composed of three distinct ER stress transducers—inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) or protein kinase RNA (PKR)-like ER kinase (PERK). Signaling and quality control are initiated and maintained by these three ER transmembrane proteins, although the exact mechanisms of activation and regulation remain unclear. It is possible that the unfolded proteins bind the ER stress transducers, or the

accumulation of unfolded proteins activates the ER chaperone immunoglobulin-binding protein (BiP), which causes the activation of the sensors at the ER membrane. Each sensor contains a phosphorylation domain, which assists in the detection of the protein-folding environment within the ER. IRE1 was the first ER stress transducer discovered in yeast through the use of reporter induced by the UPR [51,52]. The activation of IRE1 causes autophosphorylation and oligomerization, leading to the modification of gene expression. IRE1 activation aims to remodel the proteins expressed in ER-stressed cells to combat the alterations in protein folding load.

PERK is the second arm of the UPR and has a mode of activation is similar to that of IRE1. PERK contains luminal stress-sensing domains. Upon detection of ER stress, oligomerization and autophosphorylation occurs, leading to the direct phosphorylation of eukaryotic translation initiation factor-2 (eIF2α) [53], causing a direct decrease of eIF2α. This leads to a global decrease in translation and subsequent reduction in the folding load of the ER. In addition to translational attenuation, phosphorylated eIF2α also activates the transcription of proteins involved in the UPR; PERK knockout cells have been shown to be defective in the induction of mRNAs and proteins involved in the UPR [54].

ATF6 is a third ER stress transducer that typically exists as a monomer within the ER membrane. ATF6 contains a stress-sensing segment that protrudes into the ER lumen [55]. Upon induction of ER stress, ATF6 is transported to the Golgi and cleaved by resident proteases into an active cleaved form. After cleavage, ATF6 translocates to the nucleus where it activates the expression of UPR target genes, although the specific proteins have yet to be characterized completely.

Our studies aimed to examine the effects of the loss of dystrophin and consequential increase in compensatory proteins on the UPR. We hypothesized that these factors cause an increase in protein folding load within the ER, leading to the accumulation of proteins within the ER/Golgi compartments. This may lead to the induction of the UPR, and perhaps to a perturbation in the response to ER stress within isolated primary myoblasts, myotubes, and whole skeletal muscle from *mdx* mice.

MATERIALS AND METHODS

Dissection and Preparation of Whole Skeletal Muscle

Whole skeletal muscle was dissected from the latissimus dorsi, triceps, pectoralis, and hind limb muscles, excluding the quadriceps, snap frozen in liquid nitrogen and stored at -80°C prior to use. Muscles were ground to a fine powder using a mortar and pestle and added to either 1X RIPA buffer for total protein analysis or 1X homogenization buffer (IMGENEX) for the isolation of the ER/Golgi. Solutions were then dounced 10-20 times each in order to lyse and homogenize the samples and centrifuged accordingly.

Isolation of Primary Myoblasts

Primary myoblasts were isolated as described in Chapter 1.

Cell Viability Assay

2 x 10^5 myoblasts were plated on 60 mm ECM-coated plates and treated with tunicamycin (TM; Sigma Aldrich) for 8 hours at 37°C. Cells were trypsinized (GIBCO) and diluted 1:1 with trypan blue (CellGro); total cells (viable and non-viable) were counted with a hemocytometer. The percentage of viable cells was calculated as the number of non-trypan blue-stained cells divided by the total number of cells.

Statistical Analyses

Statistical analyses were performed with Prism version 6.0c (GraphPad Software). Statistical significance was calculated using ANOVA followed by a post-hoc Tukey's test for the trypan blue tracer assay.

Drug Treatments and Protein Preparation of Myoblasts and Myotubes

Primary myoblasts were grown to confluency in triplicates and differentiated for 5 days prior to the addition of either curcumin (Sigma Aldrich) or tunicamycin (TM) (Sigma Aldrich), as well as DMSO as a vehicle control.

Curcumin was diluted to a 5 mg/ml working concentration in DMSO (Sigma Aldrich) and added to cell culture plates to achieve a final concentration of 5 μg/μl for 24 hours at 37°C. An identical set of cells was treated with DMSO as the vehicle control. TM was diluted to a working concentration of 1 mg/ml in DMSO and added to cell culture plates for a final concentration of 5 μg/μl [56]. An identical set of cells was treated with DMSO as the vehicle control. Cells were treated for 8 hours at 37°C. After TM treatment, cells were dounced, lysed, and solubilized using ice-cold 1X RIPA lysis and extraction buffer (Thermo Fisher Scientific) with 100x protease inhibitor cocktail (IMGENEX). Homogenates were rotated at 4°C for 1 hour and clarified by centrifugation at 4°C for 20 minutes at 15,000 rpm. The supernatant containing the total protein was collected and protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories). Total protein was loaded onto 4-20% SDS-PAGE gels (Thermo Fisher Scientific) for immunoblot analysis.

Immunoblot Analysis

Equal quantities of protein samples were resolved on 4-20% precise protein gels (Thermo Fisher Scientific) using SDS-PAGE and transferred to nitrocellulose membranes (Millipore). 5% blotto (nonfat dry milk (Carnation) in TBS with 0.2% Tween 20 (Thermo Fisher Scientific)) was used to block membranes for 1 hour at room temperature and incubate in primary antibodies overnight

at 4°C. Incubations were performed with the following primary antibodies and dilutions: dystrophin (MANDYS1; 1:50), utrophin (MANCHO3; 1:50), α-DG IIH6 (05-593; 1:1000; Millipore), β-DG (MANDAG2; 1:250), BiP (3177; 1:750; Cell Signaling Technology), calnexin (ab22595; 1:750; Abcam), PDI (3501P; 1:750; Cell Signaling Technology), ERp57 (2881P; 1:750; Cell Signaling Technology), CHOP (2895; 1:250; Cell Signaling Technology), ATF6 (IMG-273; 1:100; IMGENEX), IRE1α (14C10; 1:750; Cell Signaling Technology), phospho-IRE1α (ab104157; 1:1,000; Abcam), phospho-PERK (16F8; 1:500; Cell Signaling Technology), eif2α (sc-11386; 1:750; Santa Cruz Biotechnology), phospho-eif2α (9721; 1:750; Cell Signaling Technology), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; MAB374; 1:10,000; Millipore). Horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare), anti-mouse IgG (GE Healthcare), and anti-mouse IgM (Roche) secondary antibodies were used at 1:2,000 dilution in 5% blotto and incubated at room temperature for 3 hours. Immunoblots were developed using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific).

ER Preparations

ER enrichments from total skeletal muscle and differentiated myoblasts were performed with the Endoplasmic Reticulum enrichment kit according to instructions provided by the manufacturer (IMGENEX). Purified protein samples were quantified using the DC Protein Assay kit. The protein was resolved on 4-20% SDS-PAGE gel.

RESULTS

Proteins involved in the UPR are up-regulated in the ER/Golgi of myotubes from mdx mice

Primary myoblasts were isolated as described in Chapter 1 in order to eliminate contamination from fibroblasts, connective tissue, and other tissues obtained when isolating whole skeletal muscle. Using primary myoblasts eliminates the contamination and effects of other cells such as endothelial cells, smooth muscle cells, immune cells, and fibroblasts, which are commonly found in muscle. ER enrichments were performed in order to obtain greater resolution of the proteins that are more abundant in the ER. Interestingly, the levels in a majority of the chosen resident UPR proteins and chaperones, including calnexin, BiP, PDI, P-IRE1α, eif2α, P-eif2α, and cleaved ATF6, were up-regulated in differentiated *mdx* myoblasts compared to wild-type (Figure 2-1).

Calnexin is a resident ER chaperone with lectin-like properties that facilitates the folding of N-glycosylated proteins [57]. The reason for the increase in calnexin is unclear; however, we hypothesize that the loss of dystrophin may lead to additional stress on the ER protein folding system. The lack of dystrophin in *mdx* mice has been associated with higher protein expression including: filamin 2, a muscle-specific protein that interacts with sarcoglycan [58]; cytoplasmic γ-actin [59]; α7β1 integrin [60]; and utrophin [61]. The up-regulation of these various proteins may place stress on the folding load of the ER, leading to an increased expression of proteins involved in ER quality control, including calnexin, PDI, as well as the ER chaperone, BiP.

Increased levels of one of the main activators of the UPR, IRE1α, suggests the activation of the UPR, although the exact mechanism of activation is currently unknown [62]. The activation of IRE1 leads to the splicing of XBP1, which in turn activates transcription of

chaperones and other UPR proteins. Future experiments should examine the levels of XBP1, which is downstream of IRE1 phosphorylation.

The cause of up-regulation of these resident ER proteins has yet to be elucidated, although we hypothesize that the loss of dystrophin and subsequent accumulation of truncated dystrophin in the ER, as well as the compensatory increase in adhesion complex proteins may contribute to alteration of the homeostasis of the ER and its folding load, leading to the activation of the UPR in order to alleviate cellular stress. In addition, we hypothesize that the up-regulation of these resident ER proteins may lead to a dampened response when isolated *mdx* cells are treated with an ER stressor.

mdx myoblasts treated with tunicamycin display a decreased viability compared to wild-type myoblasts

Since *mdx* myoblasts display an increase in UPR proteins known to assist the cell in compensating for ER stress caused by accumulated or misfolded proteins, *mdx* myoblasts were treated with a known ER stress activator tunicamycin, in order to analyze cell viability after stressful conditions. Tunicamycin inhibits the addition of N-glycans, leading to the accumulation of improperly modified proteins within the ER [63]. The accumulation of improperly modified proteins within the ER is thought to lead to the induction of the UPR and activation of the ER stress transducers [41].

Primary myoblasts isolated from wild-type and *mdx* mice were treated with tunicamycin for 8 hours and incubated at 37°C. A trypan blue tracer assay was performed to assess cell viability after treatment with tunicamycin. The total percentage of viable cells was calculated as the number of unstained cells relative to the total number of stained and unstained cells. Interestingly, *mdx* myoblasts treated with tunicamycin had a significant decrease in viability

when compared to *mdx* myoblasts treated with the vehicle, DMSO (Figure 2-2). These results suggest that *mdx* myoblasts exhibit a deficient response to ER stress; this may be due to the accumulation of adhesion complex proteins within the ER/Golgi, or the accumulation of truncated dystrophin in the ER. These possibilities should be examined in the future.

mdx myoblasts and myotubes display a robust increase in BiP upon addition of the ER stressor, tunicamycin

Since *mdx* myoblasts displayed a trend of decreased viability upon treatment with an ER stressor, we sought to examine the effects of tunicamycin on the UPR within myoblasts and myotubes isolated from wild-type and *mdx* mice. After an 8-hour incubation with 5 μg/ml tunicamycin, total protein lysates were prepared and immunoblots were performed. Although we did not observe a change in calnexin levels, it is apparent that there is a greater up-regulation of BiP upon addition of an ER stressor, tunicamycin (Figure 2-3). The increase in levels of BiP indicates *mdx* myoblasts are more sensitive to the perturbation of ER homeostasis, since BiP is known to be one of the primary targets upon induction of the UPR. Protein disulfide isomerase (PDI) is decreased in *mdx*, while there is a slight increase in phosphorylated eif2α and decrease in eif2α (Figure 2-3), suggesting that ER stress is leading to attenuation of transcription and perhaps up-regulation of other ER stress proteins. ATF6 seems to be unaffected by tunicamycin treatment (Figure 2-3).

After treating primary myoblasts with tunicamycin, we repeated the experiment using primary myotubes. Differentiated myotubes may be a more accurate physiological representation of the ER stress response *in vivo*, since adult muscle is essentially composed of differentiated myotubes. *mdx* primary myotubes displayed a greater increase in BiP compared to

wild-type (Figure 2-4). In addition, the up-regulation of BiP, phosphorylated eif2α, as well as cleavage of ATF6, was more robust in *mdx* myotubes compared to *mdx* myoblasts. These data suggest that myoblasts and myotubes have different responses to ER stress, and differentiated myotubes seem to be more sensitive to ER stress. Differentiated myotubes may have a more robust response in order to balance the perturbation in ER homeostasis. It is also possible that the differentiation of myoblasts into myotubes leads to further increases in the production and folding of compensatory proteins within the ER, which changes how these cells react to ER stress. This also suggests that future studies should be specific in the use of myoblasts or myotubes, as the responses varied when the myoblasts and myotubes were subjected to the same treatment.

Treatment of mdx myotubes with curcumin decreases levels of BiP, P-IRE1α and CHOP, while up-regulating P-PERK and P-eif2α

Experiments in Fukuyama Congenital Muscular Dystrophy (FCMD), which is caused by mutations in *fukutin* [64], have provided insight into the importance of protein processing within the ER/Golgi. The exact role of fukutin remains unclear, but mutations in *fukutin* lead to abnormal glycosylation of α-DG, a reduction in laminin-binding activity [65], and the mislocalization and retention of fukutin in the ER [66]. The information obtained from these studies can be extrapolated to the accumulation of dystrophin and adhesion complex proteins within the ER/Golgi and the UPR. One study analyzed thirteen missense *fukutin* constructs in C2C12 cells and discovered that of those, four were mislocalized to the ER. Using treatments such as curcumin, which is a molecule isolated from the spice turmeric, to improve protein folding, and low temperature culturing conditions, ER retention of fukutin was corrected in the

four mislocalized mutants. This led us to examine the effects of curcumin treatment on the UPR in differentiated *mdx* myotubes and whether it modifies the effects dystrophin accumulation within the ER/Golgi.

Curcumin is a spice derived from turmeric that targets the NF-κB pathway [67] and has been used to treat dystrophic muscle. The activation of NF-κB, which is involved in the modulation of immune responses and regulation of myogenesis, is increased in DMD patient muscle [68]. Intraperitoneal administration of curcumin inhibits NF-κB activation and reduces dystrophic pathology in *mdx* mice [69]. However, a different study reports that curcumin treatment is unable to inhibit the NF-κB pathway and does not improve specific force in the diaphragm muscle of *mdx* mice [70]. Additionally, the effect of curcumin on the UPR has yet to be seen in *mdx* cells; thus we sought to examine the consequences of curcumin treatment on the UPR in differentiated wild-type and *mdx* primary myoblasts.

Wild-type and *mdx* myoblasts were differentiated into myotubes in 5 days. The cultures were then treated with 5 μg/μl curcumin for 24 hours. Surprisingly, these levels of curcumin may have been toxic, as some myotubes were observed lifting off the cell culture plates in both wild-type and *mdx* cultures, while those treated with the vehicle control, DMSO, were unaffected and remained attached. RIPA buffer was added to obtain total protein lysates from the treated cells. Interestingly, we observed a marked decrease in BiP, cleaved ATF6, P-IRE1α, and CHOP upon curcumin treatment (Figure 2-5). However, levels of proteins in the PERK pathway, including phospho-PERK and phospho-eif2α, were increased. This suggests that while certain UPR proteins, such as ATF6 and BiP, are down-regulated with curcumin treatment, other pathways, such as the PERK pathway, are up-regulated in an attempt to restore homeostasis. It is possible that as BiP is down-regulated, more proteins accumulate and are not directed to the

ER-associated degradation pathway. This may lead to the activation of pathways such as PERK in order to attenuate further transcription and translation.

ER/Golgi enrichments from 6-week old mdx mice display an increase in UPR markers compared to wild-type

After examining the UPR-related proteins in myoblasts and myotubes, we investigated whether there are changes *in vivo* in 6-week old *mdx* mice. ER/Golgi enrichments were performed on whole skeletal muscle isolated from 6-week old wild-type and *mdx* mice. Interestingly, the ER enrichments from *mdx* mice displayed an up-regulation in the initial ER chaperones calnexin, BiP, and PDI (Figure 2-6). Perhaps the onset of pathology in these animals may lead to an increased protein-folding load, leading to higher chaperone levels.

The three arms of the UPR—IRE1α, PERK and ATF6 pathways—all displayed increased levels in the ER/Golgi (Figure 2-6). However, P-eif2α, which is downstream of PERK, maintains the same levels. Further downstream of PERK is CHOP, which is involved in regulation of cell viability via the activation of ERO1α, which promotes oxidizing conditions in the ER and can lead to cell death [71]. In addition, CHOP null fibroblasts display a slower induction of the UPR and dampened protein synthesis [71]. The increased level of CHOP in the ER/Golgi of *mdx* mice suggests the muscle has already activated the UPR, and may be promoting cell death via CHOP. Further studies should examine the downstream consequences of the activation of CHOP.

The overall increase in the protein levels of the chosen UPR markers may indicate an accumulation of unfolded or misfolded proteins in *mdx* mice, which leads to a perturbation of homeostasis within the ER. The loss of dystrophin in *mdx* mice leads to a compensatory increase

in the UGC and integrins. This in turn may lead to the activation of either pro-survival or pro-apoptotic pathways. Future studies examining these downstream pathways may provide better insight on the effect of ER homeostasis on *mdx* disease progression, or vice versa.

Progression of pathology leads to changes in the expression of ER stress markers and an increase in α-DG in the ER/Golgi

Since our previous experiment displayed an increase in ER stress markers in ER/Golgi enrichments in 6 week old mice, we hypothesized that there may be an increase in the same ER stress markers as pathology progresses. ER/Golgi enrichments were performed on whole skeletal muscle isolated from 3-week and 10-week old wild-type and *mdx* mice in order to examine the how the expression of ER stress proteins changed before and after the onset of pathology. Interestingly, *mdx* mice display an increase in UPR proteins when compared to wild-type mice at 3-weeks of age (Figure 2-7). However, upon aging and the progression of pathology, wild-type mice decrease the expression of many of these proteins, including ATF6, CHOP, PDI, and eif2α, while other proteins are increased, including phospho-eif2α, BiP, *mdx* mice seem to show an increase in many proteins, including calnexin, BiP, and ATF6.

Previous experiments in our lab have shown that dystrophin is retained within the ER/Golgi. Thus, we sought to examine whether other DGC/UGC proteins are also retained in the ER/Golgi of *mdx* mice. ER/Golgi enrichments were performed on whole skeletal muscle isolated from 3-week and 10-week old wild-type and *mdx* mice. Whole skeletal muscle was dissected from the latissimus dorsi, triceps, pectorialis, and hind limb muscles, excluding the quadriceps. A decrease in the expression of utrophin within the ER is observed as the mice age

and pathology progresses. This result is expected since utrophin was found to be present at the sarcolemma during fetal development and declined upon birth [72].

Interestingly, we found an increase in the abundance of α-DG within the ER/Golgi in 10-week old *mdx* mice compared with the 3-week old *mdx* mice, while the wild-type levels of α-DG within the ER/Golgi remained the same with the progression of pathology (Figure 2-8). As disease and the cycle of degeneration and regeneration progresses, the damage to the muscle may lead to an increase in the production of DGC and UGC proteins in order to compensate for this physiological stress. The increased production of DGC and UGC proteins may lead to the increase in α-DG that is observed in the ER/Golgi. The increase in α-DG may also lead to an increase in calnexin, which is a lectin protein that binds glycosylated proteins with the ER, which may disrupt homeostasis within the ER/Golgi. The cellular effects of increased α-DG may vary and future experiments should examine whether this stress leads to translational attenuation or apoptosis.

DISCUSSION

In our current study, we demonstrate that the UPR is activated within *mdx* myoblasts, myotubes, and 3- and 6- week old *mdx* mice when compared to wild-type. The UPR is induced upon accumulation of unfolded or misfolded proteins. If homeostasis is not restored, the UPR induces the activation of apoptotic pathways in order to remove stressed cells that are producing the misfolded proteins [73]. It was previously shown that apoptosis is the precursor to necrosis in dystrophin-deficient muscle [74]. We observed the UPR to occur very early on prior to pathogenesis in myoblasts differentiated for 5 days, as evidenced by the up-regulation of many of the related ER stress proteins in the ER/Golgi enrichments.

Isolated *mdx* myoblasts and myotubes also display an increased sensitivity to the UPR. Treatment with the known ER stressor, tunicamycin, led to a greater increase in BiP and the PERK pathway in *mdx* when compared to wild-type. This may be due a greater folding load in *mdx* mice due to the lack of dystrophin, which leads to a diminished capacity to deal with perturbations in ER homeostasis. The lack of dystrophin in *mdx* mice and subsequent production and accumulation of compensatory proteins within the cell and ER/Golgi may cause a more robust response to cellular stress in order to ameliorate protein accumulation more quickly and efficiently.

In addition, we observed an increase in UPR proteins in the total protein of 3-week and 6-week old *mdx* mice compared to wild-type, although the levels of these proteins in the ER decreases from 3-week to 10-week old. It is possible that as pathology progresses, *mdx* mice are eventually unable to maintain high levels of UPR proteins to combat ER stress, leading to activation of apoptotic pathways and eventually necrosis, which occurs at high levels in *mdx* mice.

However, curcumin did not decrease the expression of UPR proteins in *mdx* myotubes, although the compound did alter cells' ER stress response. In fact, myotubes were observed to detach from the plates, indicating some toxicity at concentrations of 5 μg/ml. Further studies using different concentrations may lend more insight into how curcumin affects differentiation in *mdx* myotubes. Curcumin down-regulated BiP, but simultaneously up-regulated the PERK pathway. Although conclusions are unable to be drawn from this study, these data suggest that treatments with curcumin should be analyzed more closely in order to optimize the dosage, since myotubes began detaching from the cell culture plates after 24 hours of treatment.

Interestingly, we also observed an increase in the accumulation of α-DG with the progression of pathology. The physiological consequences are unclear, but the accumulation may lead to an increase in UPR. If *mdx* mice are unable to tolerate increased protein levels within the ER and protect the cells through the UPR, then this could lead to increased apoptosis and necrosis. More experiments need to be performed in the future to elucidate the exact consequences and pathways activated in the ER through the loss of dystrophin. This data will be helpful in identifying new pharmacological agents that can target the UPR in order to prevent premature apoptosis and necrosis of cells with ER that are overloaded with unfolded or misfolded proteins.

In addition, the accumulation of α-DG in the ER compartments suggests that perhaps proteins are accumulating within the ER, and the cells cannot compete with the rate of transport to the cell surface as *mdx* pathology progresses, or there is a deficiency in the protein transport system. Future studies examining the role of ER chaperones and sarcospan in the amelioration of pathology may be helpful in elucidating the role of the UPR in *mdx* mice.

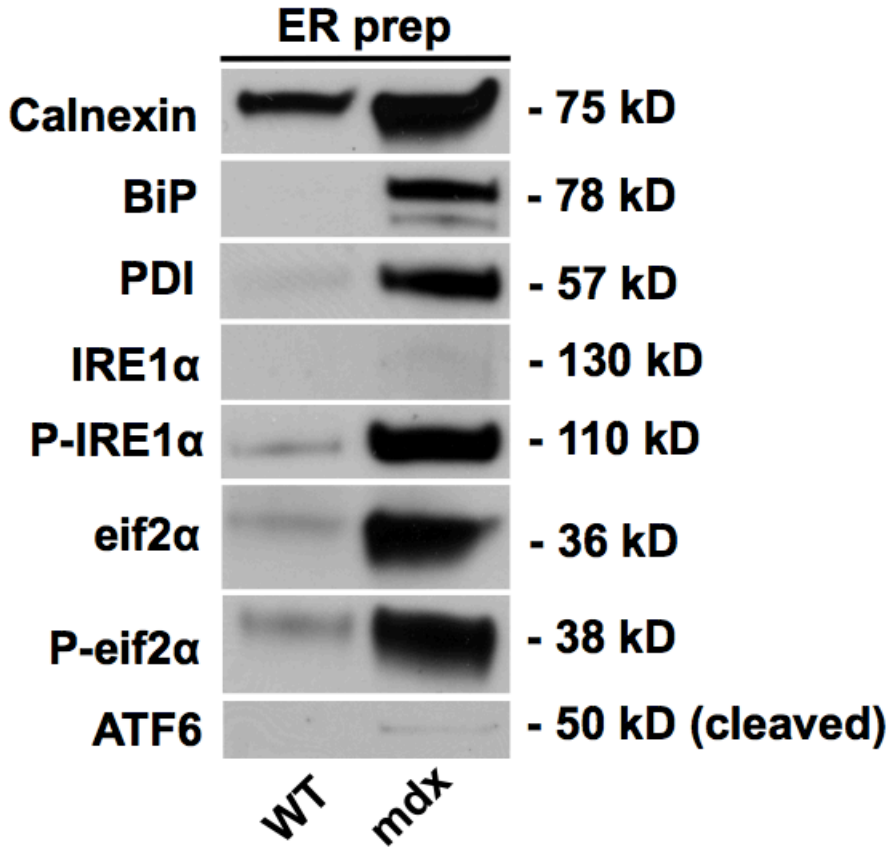

Figure 2-1. Chaperones and ER stress proteins are up-regulated in differentiated myoblasts from *mdx* mice. Primary myoblasts were isolated and purified from 2-5 day old neonatal mice. The primary myoblasts were grown to 100% confluency for differentiation into myotubles. 19 µg samples were analyzed by immunoblotting against calnexin; BiP, binding immunoglobulin protein; PDI, protein disulfide-isomerase; IRE1α, inositol requiring enzyme 1α; P, phospho; eif2α, eukaryotic initiation factor 2α; ATF6, activating transcription factor 6.

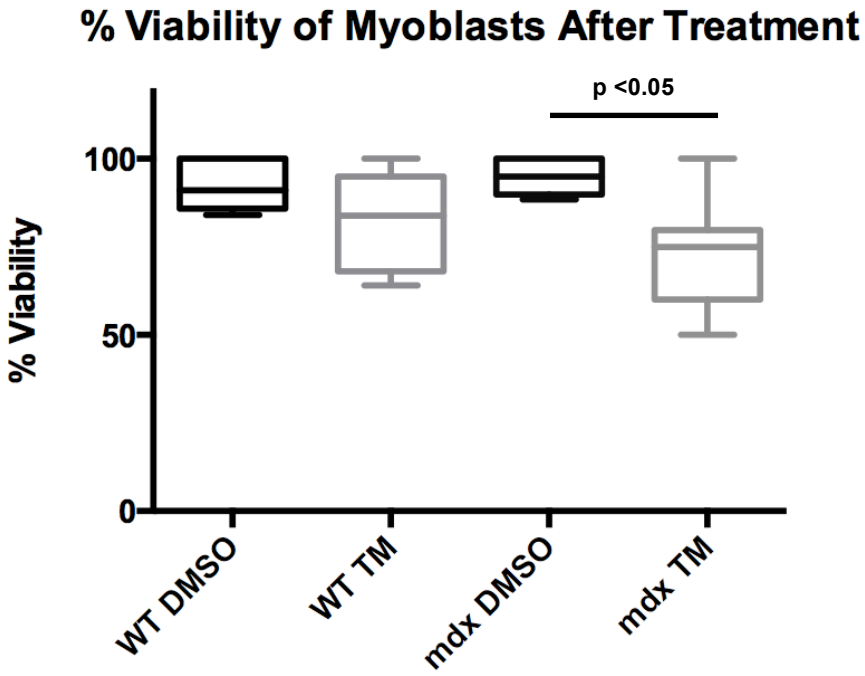

Figure 2-2. *mdx* myoblasts are less viable upon treatment with tunicamycin compared to wild-type. Primary myoblasts were grown to 70% confluency (n=9 for each treatment) and treated with 5 µg/ml TM. Cells were incubated for 8 hours, washed with 1X PBS, trypsinized, and resuspended with 1 ml primary myoblast growth medium and viability was analyzed after treatment with trypan blue. Statistical significance was calculated using ANOVA followed by a post-hoc Tukey's test and the percentage of viable *mdx* cells treated with DMSO and tunicamycin was found to be significant with $p<0.05$. DMSO, dimethyl sulfoxide; TM, tunicamycin.

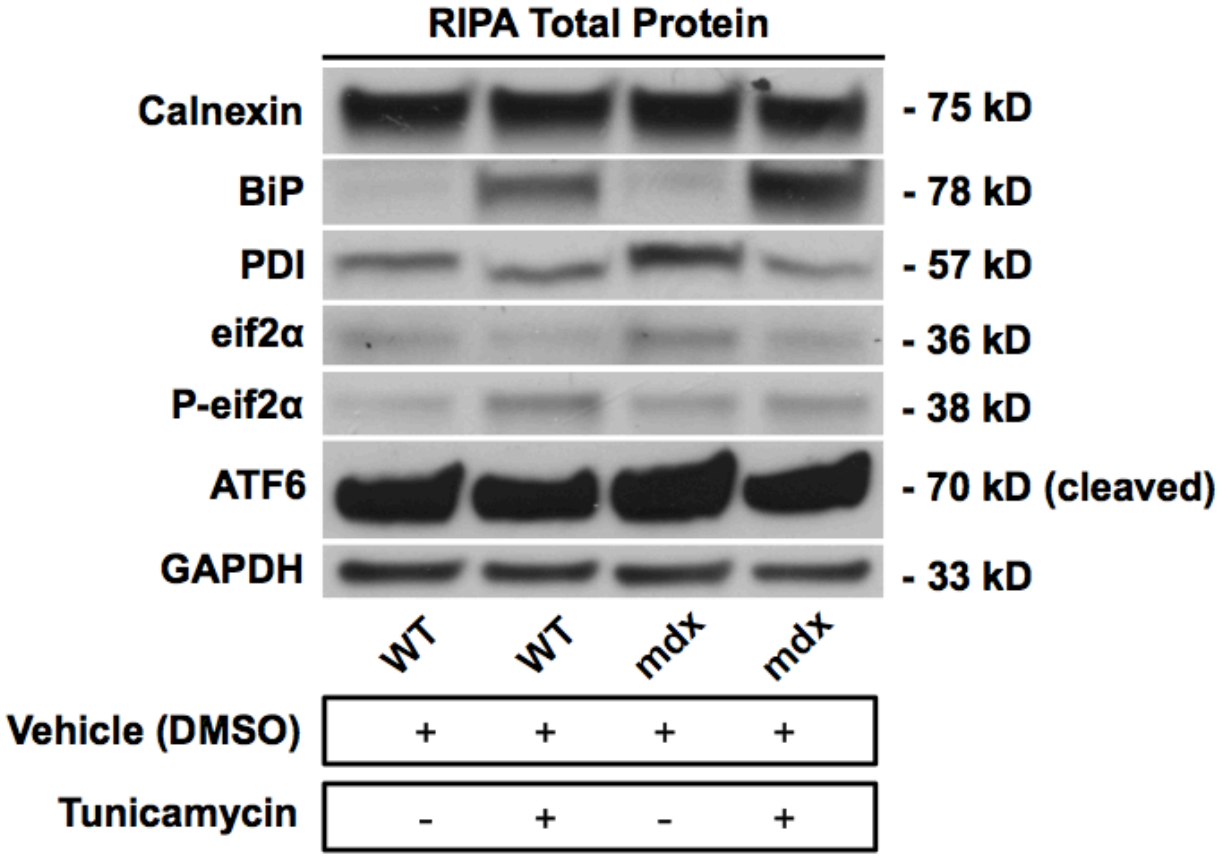

Figure 2-3. Tunicamycin treatment upregulates BiP in *mdx* myoblasts. Primary myoblasts were grown to 70% confluency and treated with 5 μg/ml TM for 8 hours and lysed in 1X RIPA buffer. 25 μl of tunicamycin dissolved in a 10 μg/ml stock was added to each plate for a final concentration of 5 μg/ml. After treatment, cells were washed with PBS and lysed in 1X RIPA buffer. 50 μg of protein was loaded into each well and samples were analyzed by immunoblot against the following antibodies—Calnexin; BiP, binding immunoglobulin protein; PDI, protein disulfide-isomerase; IRE1α, inositol requiring enzyme 1α; P, phospho; eif2α, eukaryotic initiation factor 2α; ATF6, activating transcription factor 6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

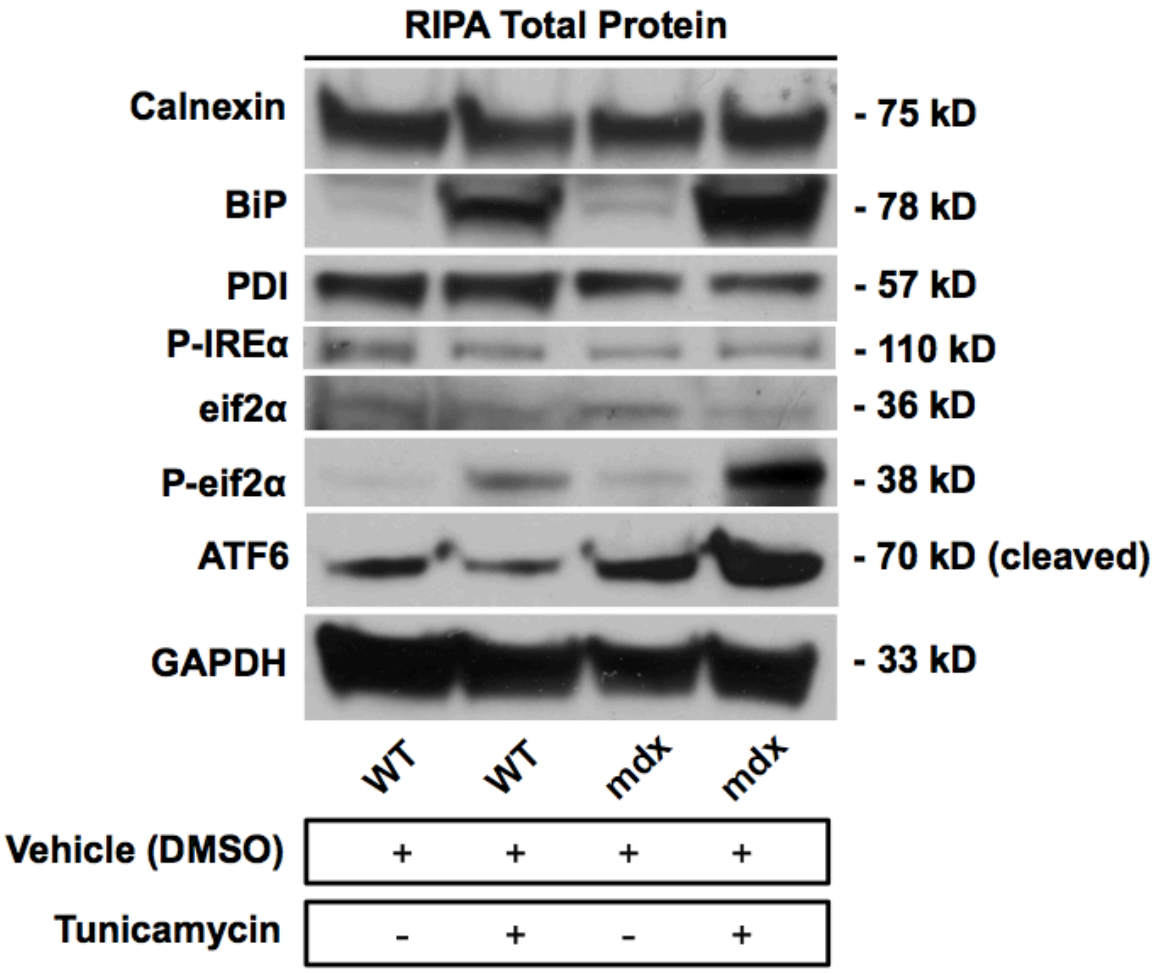

Figure 2-4. Tunicamycin treatment causes a greater upregulation of BiP in differentiated *mdx* myoblasts compared to wild-type. Primary myoblasts were grown to confluency and differentiated for 5 days into primary myotubes. On day 5, the primary myotubes were treated with 5 μg/ml TM for 8 hours and then lysed in 1X RIPA buffer. 50 μg of protein was loaded into each well and samples were analyzed by immunoblotting as shown. Calnexin; BiP, binding immunoglobulin protein; PDI, protein disulfide-isomerase; IRE1α, inositol requiring enzyme 1α; P, phospho; eif2α, eukaryotic initiation factor 2α; ATF6, activating transcription factor 6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

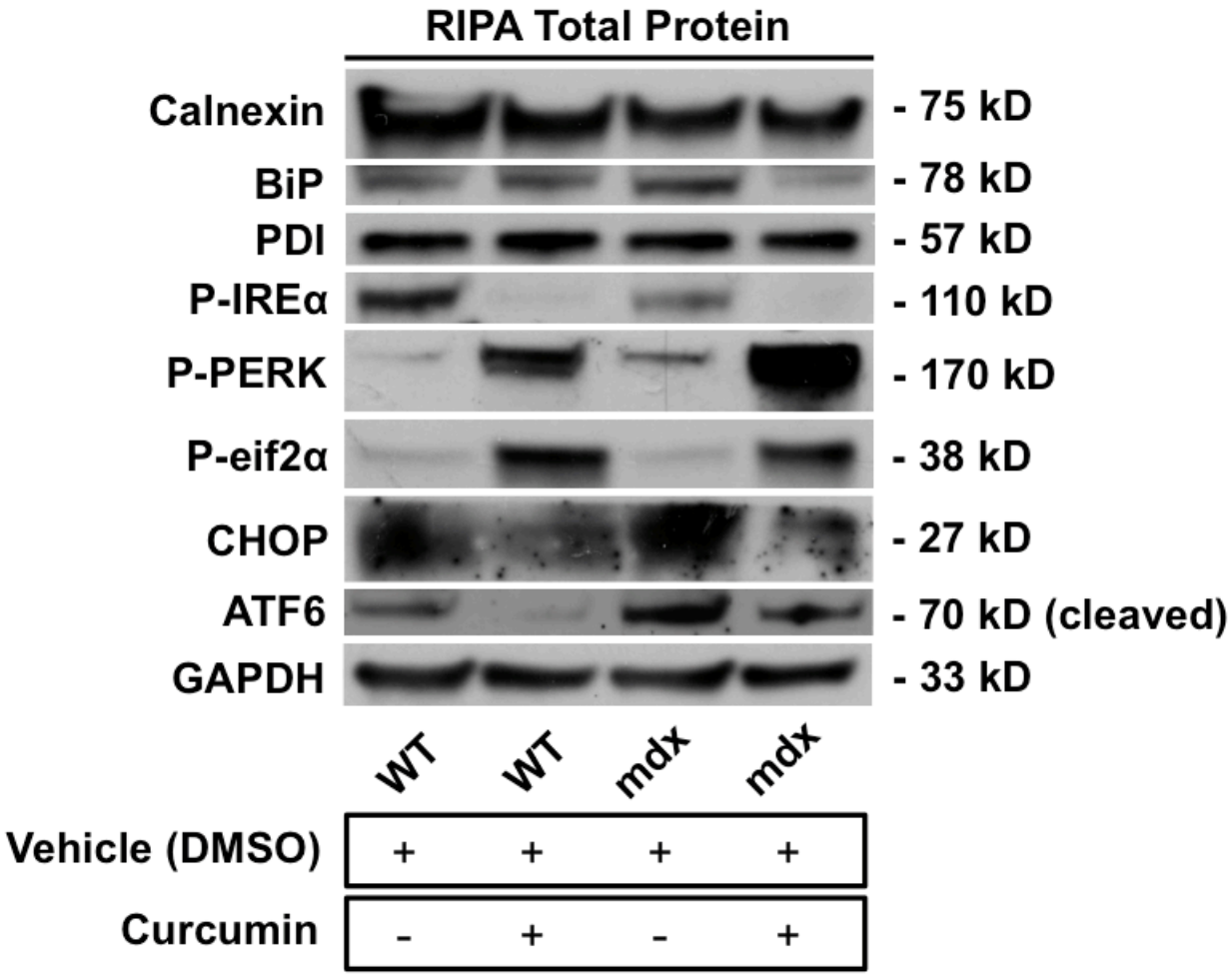

Figure 2-5. Treatment of *mdx* and wild-type cells with curcumin alters expression of UPR proteins. Cells were grown to 100% confluency and differentiated for 5 days. On day 5, curcumin was added to the cell culture dishes for a final concentration of 5 μg/ml. Cells were incubated for 24 hours at 37°C and solubilized using 1X RIPA buffer. 50 μg protein samples were resolved by SDS-PAGE and subjected to immunoblotting with the indicated antibodies. Calnexin; BiP, binding immunoglobulin protein; PDI, protein disulfide-isomerase; IRE1α, inositol requiring enzyme 1α; P, phospho; eif2α, eukaryotic initiation factor 2α; ATF6, activating transcription factor 6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

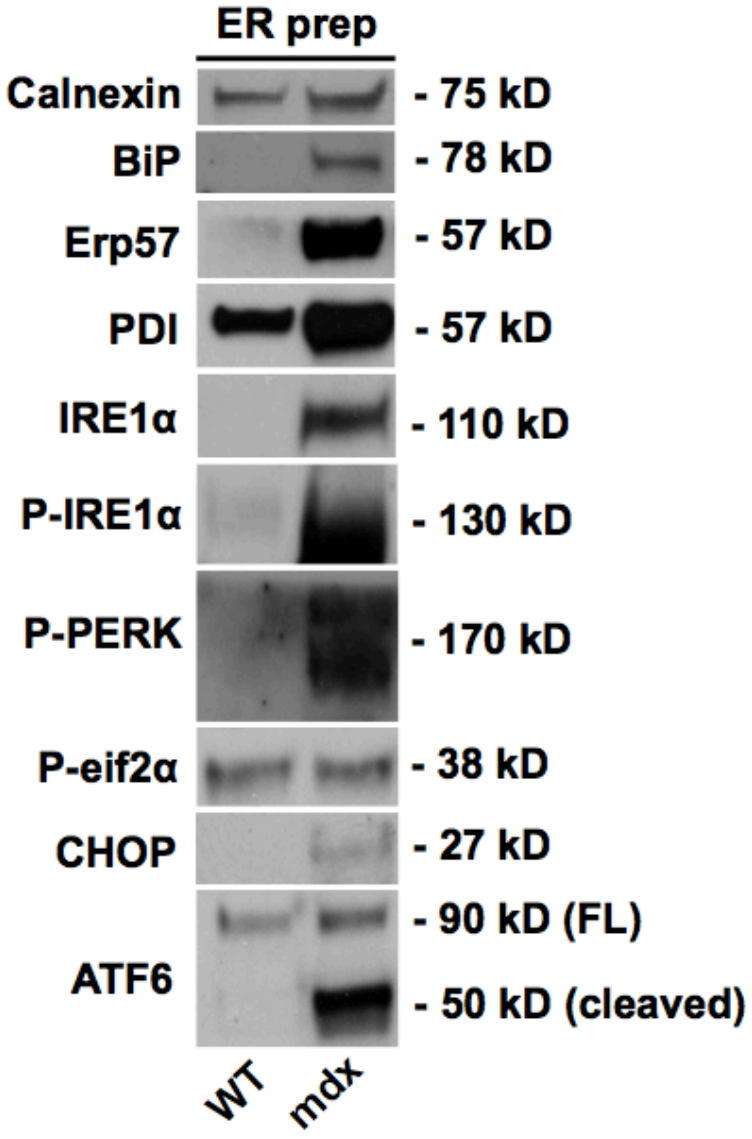

Figure 2-6. Chaperones and ER Stress proteins are up-regulated in 6 week old *mdx* mice. The membranes of endoplasmic reticulum from were enriched from total skeletal muscle obtained from 6 week old WT and *mdx* mice using a kit from IMGENEX. 50 μg samples were resolved by SDS-PAGE and analyzed by immunoblotting as shown. Calnexin; BiP, binding immunoglobulin protein; Erp57, endoplasmic reticulum stress protein 57; PDI, protein disulfide-isomerase; IRE1α, inositol requiring enzyme 1α; P, phospho; eif2α, eukaryotic initiation factor 2α; CHOP, C/EBP homology protein; ATF6, activating transcription factor 6; FL, full-length.

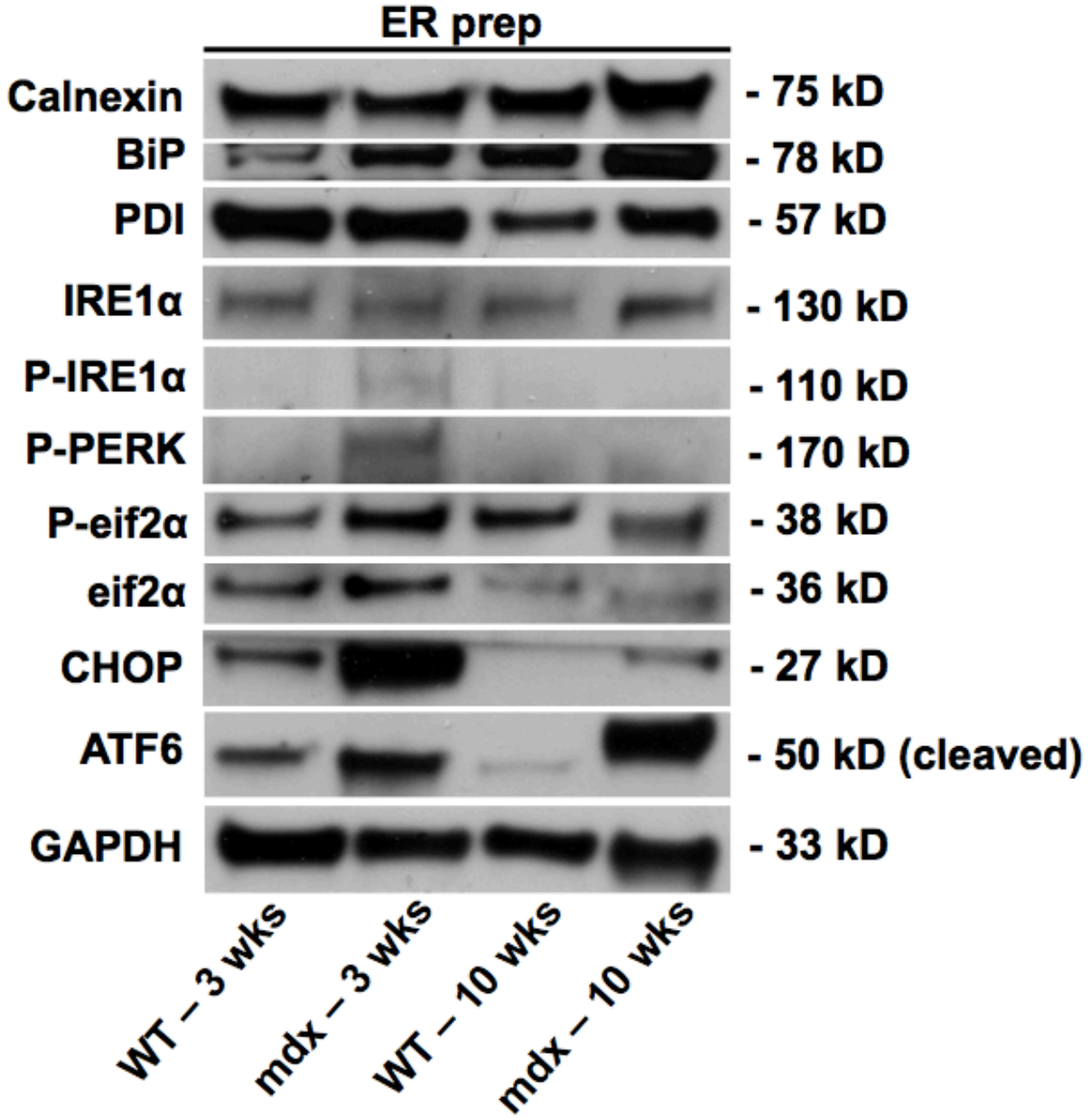

Figure 2-7. Chaperones and UPR proteins change in expression as pathology progresses with age. The membranes of endoplasmic reticulum from were enriched from total skeletal muscle obtained from 6-week old WT and *mdx* mice using a kit from IMGENEX. 38 μg protein samples were resolved by SDS-PAGE and analyzed by immunoblotting as shown. Calnexin; BiP, binding immunoglobulin protein; PDI, protein disulfide-isomerase; IRE1α, inositol requiring enzyme 1α; P, phospho; eif2α, eukaryotic initiation factor 2α; CHOP, C/EBP homology protein; ATF6, activating transcription factor 6; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

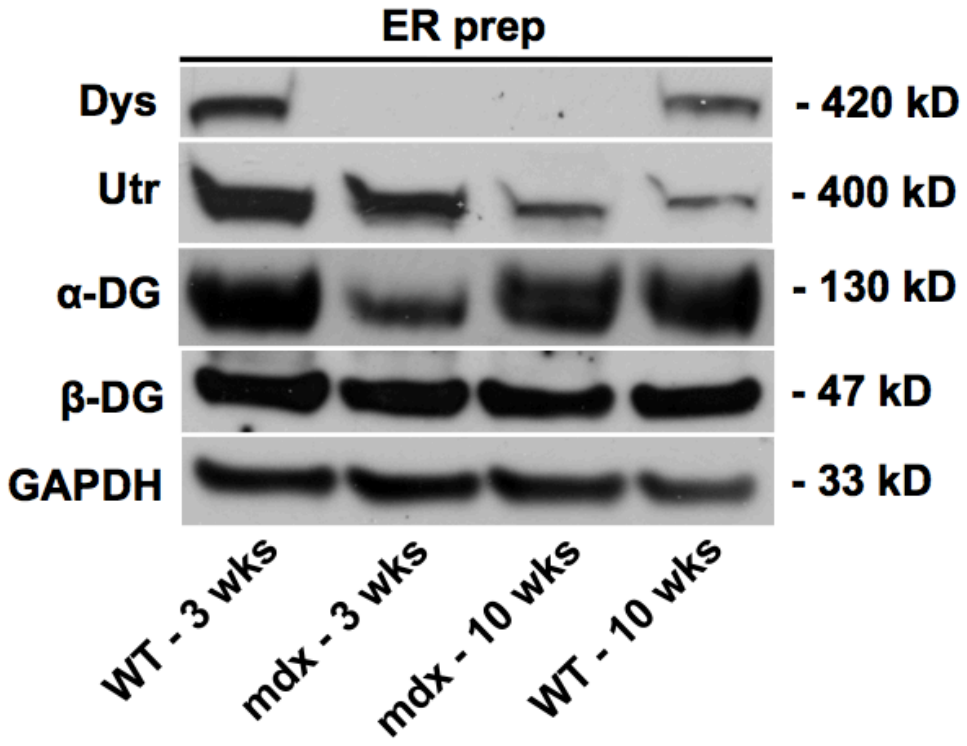

Figure 2-8. There is an increase in the retention of α-dystroglycan as pathology progresses in *mdx* mice. Whole skeletal muscle was obtained from 3 week old wild-type and *mdx* mice. ER enrichments were performed according to manufacturer's instructions (IMGENEX). 45 µg of protein was resolved using SDS-PAGE and analyzed using immunoblotting as shown. Dys, dystrophin; Utr, utrophin; α-DG, α-dystroglycan; β-DG, β-dystroglycan; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

CHAPTER 3: The potential of sarcospan in adhesion complex replacement therapies for the treatment of muscular dystrophy

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease caused by mutations in the dystrophin gene leading to a loss of the dystrophin-glycoprotein complex (DGC) at the sarcolemma [23]. DMD occurs in approximately 1 in 3,500 male children, leading to respiratory or cardiac failure in the second decade of life. In 1986, the gene encoding dystrophin was identified, and mutations in the *dystrophin* gene were determined to be responsible for DMD [3,4]. While dystrophin was identified over 25 years ago, there is still no cure for the disease [3]. The N-terminus of dystrophin binds directly to filamentous actin (F-actin) in the cytoplasm of the myofiber and the C-terminus of dystrophin is associated with a group of proteins including neuronal nitric oxide synthase (nNOS), α-syntrophin, α-dystrobrevin, and β-dystroglycan (DG) [5,6]. Dystrobrevin binds to the intermediate filament protein syncoilin, which provides a connection between desmin and the DGC that is thought to be important for maintaining mechanical strength and structural organization necessary for muscle contractions [7]. nNOS requires binding to both dystrophin and syntrophin for sarcolemmal localization and produces nitric oxide, which stimulates blood flow necessary to meet the metabolic demands of healthy muscle [8-12]. The DGC functions to stabilize the sarcolemma during muscle contractions by providing a critical connection between the extracellular matrix (ECM) and the intracellular actin cytoskeleton [13]. α-DG, a peripheral membrane protein, provides the connection between the transmembrane β-DG and laminin-211 in the ECM [14,15]. The central mucin domain of α-DG is heavily glycosylated and the extent of glycosylation

determines the affinity of DGC attachment to the ECM [6,14-19]. The sarcoglycan-sarcospan (SG-SSPN) subcomplex stabilizes α-DG's association with β-DG at the cell surface [20-22]. Loss of the DGC in DMD renders the sarcolemma susceptible to membrane ruptures, which initiates the cycles of myofiber degeneration and regeneration characteristic of DMD [13,16,23,24].

Many forms of muscular dystrophy result from a loss of muscle cell attachment to its surrounding ECM. In addition to the DGC, two adhesion complexes span the sarcolemma and facilitate this connection: the utrophin-glycoprotein complex (UGC) and α7β1 integrin. The UGC is homologous to the DGC, where utrophin replaces dystrophin [75,76]. α7β1 integrin is the main heterodimeric integrin expressed in adult skeletal muscle [77-79]. Mutations in genes encoding the protein components of the DGC/UGC, α7β1 integrin, and ECM cause various forms of muscular dystrophy, which are classified based on the severity of disease and the muscle groups predominantly affected. Autosomal recessive limb-girdle muscular dystrophies (AR-LGMDs) encompass a wide array of genetic disorders and disease onset typically occurs between 10-30 years of age. Mutations in α-, β-, γ-, and δ-SG and mutations in glycosyltransferases resulting in the hypoglycosylation of α-DG cause AR-LGMD subtypes 2C-2F, 2K, and 2N-2O respectively [80-89]. Congenital muscular dystrophies (CMDs) are an autosomal recessive disease characterized by severe muscular dystrophy with onset occurring within the first year of life. Various forms of CMD are also caused by hypoglycosylation of α-DG due to mutations in glycosyltransferases as well as mutations in laminin α2, collagen 6, and α7 integrin [90-94]. Notably, patient mutations in SSPN and β1D integrin have not been identified [95-97].

There are many strategies currently being investigated for the treatment of muscular dystrophy. Several therapeutic approaches focus on adenoviral delivery of gene replacement therapy or exon skipping to produce a shortened, but functional dystrophin protein. This review will focus on therapeutic strategies to replace the DGC with compensatory adhesion complexes, UGC and α7β1 integrin. In particular, this review will discuss the potential of SSPN as a therapeutic target to upregulate both the UGC and α7β1 integrin. SSPN is a tetraspanin-like protein that was discovered as a core-component of the DGC [98]. Although SSPN has been shown to ameliorate the pathology of the *mdx* mouse model of DMD [31,99], many questions remain to be answered before SSPN-related therapies are suitable for patients.

Restoring myofiber function with compensatory adhesion complexes

The adhesion complexes responsible for stabilizing the myofiber membrane have distinct biochemical and functional properties, including sarcolemmal localization and ECM/actin connections in normal muscle. In muscle, the DGC is the most widely expressed adhesion complex, as it is found at all regions of the sarcolemma including the neuromuscular and myotendinous junctions (NMJs and MTJs) [4,100-103]. The UGC, a homologous complex to the DGC, is restricted to NMJ and MTJ regions of the sarcolemma, where utrophin replaces dystrophin [104,105]. Although utrophin and dystrophin are structurally homologous, they bind to F-actin through distinct sites [106,107] and only dystrophin contains nNOS binding sites [108]. It is thought that muscle ischemia occurs in DMD because nNOS is not anchored to the sarcolemmal membrane [108]. The UGC also differs from the DGC in the glycosylation of α-DG, which is a critical determinant of the binding specificity for ECM ligands [31,109-111]. α7β1 integrins, which are also highly enriched within NMJ and MTJ structures and expressed at

low levels at non-junctional regions of the sarcolemma, are the predominant integrin heterodimers expressed in adult muscle [77-79]. Integrins bind F-actin through a complex of proteins including integrin-linked kinase (ILK), PINCH, and α/β-parvin [112-114]. The ILK, PINCH, and parvin protein complex has been implicated in facilitating cell signaling through Akt/PKB, GSK3β/β-catenin, JNK, and α-Pix/Rac1 pathways [112,113,115]. ILK interacts specifically with β1 integrin and deletion of ILK causes muscular dystrophy that resembles the α7 integrin deficient mouse model [116]. Laminin-211 binds α7β1 integrin through laminin-type G domains (LG) 1-3, whereas binding to α-DG is mediated through LG1-3 and 4-5 [117-121]. Given that many differences exist between the three adhesion complexes, the extent to which UGC- and integrin-based therapeutics for DMD fully replace DGC function remains an unanswered question.

The concept of the UGC and integrins acting as compensatory adhesion complexes and their potential as therapeutic targets to replace the DGC arose from studies in the *mdx* mouse model of DMD. Muscle pathology in *mdx* muscle is less severe than that observed in boys with DMD and the *mdx* mouse maintains a fairly normal lifespan [26]. It was hypothesized that increased abundance of the UGC and α7β1 integrin at extra-junctional regions of the sarcolemma in *mdx* muscle may partially compensate for the loss of the DGC, resulting in moderate dystrophic pathology without affecting lifespan, which is in stark contrast with the severe dystrophic pathology and premature lethality observed in the DMD population. To experimentally address this hypothesis, *mdx*:utrophin-null and *mdx*:α7 integrin-null double knockout mice were created. The additional loss of utrophin or α7 integrin in *mdx* mice exacerbated dystrophic symptoms to more closely resemble DMD. Furthermore, lifespan was shortened to 20- and 4-weeks, respectively for utrophin-deficient and α7 integrin-deficient *mdx*

mice [122-125]. Significantly, over-expression of either utrophin or β1D integrin in *mdx* muscle prevented muscular dystrophy, demonstrating that the UGC and integrins are capable of replacing the DGC in the mouse model of DMD [126,127]. Mental retardation and brain abnormalities have been detected in about 30% of patients with DMD [128]. Utrophin and its smaller isoforms are expressed in the blood vessels in all brain regions, walls of the lateral ventricle, cortex, subiculum, thalamus, brain stem nuclei, superior colliculus, and deep cerebellar nuclei [129]. Although utrophin stabilizes the sarcolemma in *mdx* mice, utrophin was not up-regulated in a compensatory fashion in the brain of *mdx* mice [129]. Arginine butyrate alleviated *mdx* muscle disease through utrophin upregulation and increased all utrophin isoforms in the brain of *mdx* mice, but did not rescue cognitive deficits observed in behavioral assays including exploration, emotional reactivity, and spatial and fear memories [129]. These results suggest that utrophin cannot fully compensate for the loss of dystrophin in all tissues. The ability of α7β1 integrin to ameliorate cognitive deficits in *mdx* mice has not been tested.

Although the UGC and α7β1 integrin do not fully replace the DGC, they remain excellent candidates for protein replacement therapies. The packaging limit of adeno-associated virus (AAV) capsids prevents the delivery of the most ideal protein for the treatment of DMD, the entire *dystrophin* gene [130]. Additionally, recent evidence that the expression of AAV delivered mini-dystrophin is circumvented by an immune response to dystrophin in patient clinical trials [131], strengthens the importance of UGC and α7β1 integrin protein replacement therapies. A thorough investigation of the biochemical, structural, and functional differences between the UGC, α7β1 integrin, and the DGC may lead to the future development of combinatorial therapies designed to replace the DGC. The first goal is to determine if UGC- and α7β1 integrin-based therapies are safe and beneficial to patients with DMD. To date, only three

therapies designed to replace the DGC with the UGC or integrins are near clinical trials. Two therapies designed to upregulate utrophin are currently in or near Phase 1 clinical trials: BMN195 (Summit plc) and biglycan (Trivorsan Pharmaceuticals). BMN195, a 5-(ethylsulfonyl)-2-(naphthalene-2-yl) benzodoxazole, was identified in a screen for small molecules, which up-regulated utrophin mRNA 25% in human myoblasts, increased utrophin protein levels two-fold in DMD patient cells, and demonstrated efficacy in treatment of *mdx* mice [132]. Specifically, BMN195 reduced regeneration, inflammation, serum CK levels, and fibrosis, and prevented membrane damage due to eccentric contractions in treated *mdx* mice [132]. Although BMN195 was shown to be safe at all doses in a Phase 1 clinical trial by BioMarin Pharmaceuticals, it did not achieve plasma concentrations, even at the highest doses, required to increase utrophin expression[1]. Summit plc has since reformulated BMN195 to allow for better absorption and initiated Phase 1 clinical trials. The second utrophin upregulation therapy, biglycan, is an extracellular protein that is highly expressed, similar to utrophin, in regenerating and developing muscle [133,134]. Injection of recombinant human biglycan protein improves muscle pathology in the *mdx* mouse by increasing cell surface expression of utrophin and γ-SG [135]. Furthermore, the inability of biglycan to ameliorate dystrophic pathology of the utrophin-deficient *mdx* mouse demonstrates the requirement of utrophin for biglycan therapies [135]. Biglycan is currently being developed for Phase 1 clinical trials by Trivorsan Pharmaceuticals. The only therapeutic designed to upregulate α7 integrin near Phase 1 clinical trials is laminin-111 protein. Laminin-111 is an ECM protein present in cardiac and skeletal muscles during embryonic development that is replaced by laminin-211 in adult muscle [120,121]. Injection of Engelbreth-Holt-Swarm-derived purified natural mouse laminin-111

[1] http://investors.bmrn.com/releasedetail.cfm?ReleaseID=703221

protein improves dystrophic pathology in the *mdx* and laminin α2-deficient dy^W mouse models [136-139] through the upregulation of both utrophin and integrins at the sarcolemma. Prothelia is currently developing laminin-111 for clinical trials and a Phase 1 trial will occur upon the completion of preclinical trials. Laminin-111 has the potential for therapeutic use in merosin-deficient congenital muscular dystrophy (MDC1A), DMD, and LGMD2I (mutations in fukutin-related protein (FKRP)). The outcome of these clinical trials may illuminate whether utrophin and integrin therapeutics will alleviate the symptoms of DMD (Figure 1).

Although introduction of recombinant laminin-111 protein was initially discovered to increase α7β1 integrin protein levels, it has also been shown to increase the levels of utrophin in *mdx* muscle [139]. Given that neither the over-expression of utrophin or α7β1 integrin fully rescue dystrophic symptoms, it would be interesting to determine whether the upregulation of both adhesion complexes would be more beneficial than targeting a single complex alone. Experiments to directly test UGC and integrin association have not been performed and numerous murine genetic experiments are often complicated with compensatory actions of other proteins. Migration of the three adhesion complexes in the same sucrose gradient fractions after biochemical isolation of DG bound proteins using succinylated wheat germ agglutinin (sWGA) lectin raises the interesting question of whether integrins and the UGC/DGC physically interact at the sarcolemma [140,141]. If laminin-111 is successful in Phase 1 trials, the potential benefit of targeting both the UGC and α7β1 integrin may be addressed in DMD patients. Similar to laminin-111, Adam12 and SSPN also secondarily upregulate both the UGC and integrins at extra-junctional regions of the sarcolemma when over-expressed in *mdx* mice [31,99,142] (Figure 1). Adam12 is an active metalloproteinase that is expressed during muscle development

and regeneration. The mechanism by which it promotes cell adhesion through the stabilization of the UGC and α7β1 integrin is currently unknown [143-145].

Recent studies have implicated a role for SSPN in facilitating interactions between the UGC and α7β1 integrin. SSPN, a tetraspanin-like protein, functions with the SGs to stabilize the association of α-DG with β-DG in the DGC [20,21,98]. Over-expression of threefold levels of SSPN ameliorates *mdx* muscle by increasing the levels of the UGC, α7β1 integrin, and laminin-binding to α-DG [31,99]. Interestingly, the increase in laminin-binding to α-DG was not observed with 1.5-fold levels of SSPN over-expression in *mdx* mice, demonstrating that there is a minimum threshold of SSPN expression required for restoring normal ECM binding to DG [31]. To determine if laminin-binding to α-DG is required for SSPN-mediated amelioration of dystrophic symptoms, threefold SSPN transgenic mice were crossed with LARGE^{myd} mice. LARGE, like-acetylglucosaminyltransferase, is a glycosyltransferase responsible for elongating O-mannose glycans in the mucin domain of α-DG and mutations in *LARGE* abolish laminin-binding to α-DG [146]. Over-expression of SSPN does not ameliorate muscular dystrophy in the LARGE^{myd} mouse model of hypoglycosylated α-DG (MDC1D) despite increasing levels of the UGC at the sarcolemma, demonstrating that functionally glycosylated α-DG capable of binding to laminin is a requisite for SSPN-mediated amelioration of dystrophic pathology [31]. Recent genetic studies demonstrated that the combined loss of SSPN and α7 integrin results in extensive muscle pathology and decreased specific force production possibly due to decreased protein levels of the DGC and UGC in 4.5 month old diaphragm muscle [140]. These studies suggest that SSPN and integrins genetically interact and affect protein abundance of the DGC/UGC, but do not establish a direct interaction between these adhesion complexes. Investigation of the dependence of SSPN-mediated amelioration of *mdx* mice on α7 integrin and utrophin would

clarify whether both adhesion complexes are essential for SSPN-based therapeutics. Interestingly, α7 integrin over-expression in *mdx*:utrophin-null mice causes a 10% reduction in regeneration, a 3-fold increase in mean survival age, and reduced severity of joint contractures [147]. These results suggest that α7 integrin reduces the severity of muscular dystrophy in *mdx* mice partially independent of utrophin, although the muscle pathology was not significantly reduced. It is possible that utrophin is required for the full rescue effect of α7 integrin in *mdx* muscle or that higher than 2-fold levels of α7 integrin are needed to rescue *mdx* muscle independently of utrophin. The ability of utrophin alone to rescue *mdx*:α7 integrin-null muscle has not been tested. Thus, additional evidence is needed to establish a direct SSPN-mediated interaction between the UGC and α7β1 integrin at the sarcolemma while the benefit of upregulating both adhesion complexes for the treatment of DMD remains undetermined.

Glycosylation of α-DG and amelioration of muscular dystrophy

Appropriate glycosylation of sarcolemmal proteins is integral for proper muscle function. Over fifty percent of all known CMDs result from hypoglycosylation of α-DG and therefore are known as dystroglycanopathies [148]. Mutations in seven genes are known to cause aberrant glycosylation of α-DG. These gene products include: protein O-mannosyl transferase-1 and -2 (POMT1 and POMT2), protein O-linked mannose β-1,2-N-acetylglucosaminyltransferase (POMGnT1), LARGE, fukutin, FKRP, and Isoprenoid Synthase Domain Containing (ISPD) [64,88,89,91,149-156]. POMT1/2 act collaboratively to add the initiating mannose to serine or threonine residues of the mucin domain of α-DG [157,158]. Classical mannose glycans are then elongated by the addition of a β-1,2-*N*-acetylglucosamine residue [89,149]. On laminin binding glycan structures, LARGE creates the unique moiety required for binding of α-DG to laminin as

well as other ECM components (Figure 2) [91,159,160]. Binding of α-DG to laminin has been shown to be dependent upon the xylosyl and glucuronyl transferase activities of LARGE [161]. Furthermore, knockout of LARGE activity causes severe effects in functional glycosylation [162] and decreases in α-DG molecular weight [163]. Precise enzymatic functions of fukutin, FKRP and ISPD remain elusive; however, mutations in these genes lead to hypoglycosylation of α-DG and subsequent CMD phenotypes [64,150,152,153,156,164].

The dystroglycanopathies represent a diverse group of muscular dystrophies with broad phenotypic severities and wide ranging causative gene mutations. Walker-Warburg syndrome (WWS) cases result from the largest group of genetic mutations and can be attributed to mutations in POMT1, POMT2, LARGE, fukutin, FKRP, ISPD, and β-1,3-*N*-acetylglucosaminyltransferase (B3GNT1) [152,156,165,166]. Patients presenting with muscle-eye-brain disease (MEB) represent a similarly heterogeneous population of phenotypic severities and genetic mutations. MEB has been reported to result from mutations in POMGnT1, fukutin and FKRP [89,166,167]. Limb-girdle muscular dystrophy types 2K, N, M, I, and O result from mutations in POMT1, POMT2, fukutin, FKRP, and POMGnT1, respectively [80,166-168]. Congenital muscular dystrophy types 1C and 1D result from mutations in FKRP and LARGE, respectively [91,169]. Furthermore, an insertion of a retrotransposon in the *fukutin* gene is known to cause Fukuyama congenital muscular dystrophy (FCMD) [64]. The genetic basis for the various dystroglycanopathies is varied and the resulting phenotypes are over-lapping, which reinforces the need for a thorough understanding of the connection between specific genetic mutations and resulting biochemical dysfunctions that underlie or cause muscular dystrophy.

While DMD results from mutations in dystrophin, it is noteworthy that significant changes in glycosylation have been reported in the mouse model for DMD. The lectin *Wisteria*

floribunda agglutinin (WFA) recognizes terminal β1,4 GalNAc residues of α-DG in skeletal muscle [111,170] and binding is normally restricted to the NMJ and MTJ in wild-type myofibers [110]. However, WFA binds to the extrasynaptic sarcolemma in addition to the NMJ on *mdx* muscle cryosections [31,109]. This redistribution of WFA staining to the extrasynaptic sarcolemma is concomitant with the redistribution of utrophin and the associated UGC in *mdx* mouse muscle.

Over-expression of both full length and truncated utrophin rescues the dystrophic phenotype in *mdx* mice [127,171-174]. As WFA preferentially binds to α-DG in the UGC, increasing GalNAc modification of α-DG has been one approach to increase extrasynaptic utilization of utrophin as a potential therapy for DMD. The over-expression of terminal β-GalNAc glycosyltransferase *galgt2* in wild-type mice has been shown to increase levels of UGC proteins, laminin binding and reactivity of α-DG to WFA [110,111]. Similar results were obtained from *galgt2* over-expression in *mdx* mice, where rescue of laminin-binding and the dystrophic phenotype were additionally noted [110,111]. Interestingly, transgenic over-expression of SSPN also increases the WFA reactivity of α-DG in *mdx* and LARGE^{myd} muscle while loss of SSPN reduces UGC levels and the reactivity of α-DG with WFA [31]. Importantly, no mitigation of dystrophic pathology or rescue of laminin-binding is observed in LARGE^{myd} mice over-expressing SSPN [31]. These studies are significant as they demonstrate that laminin-binding is required for SSPN-mediated amelioration of dystrophic pathology and that the glycans detected by increasing WFA reactivity are distinct from the structure created by LARGE activity. It has been proposed that SSPN over-expression mediates changes in glycosylation via increased galgt2 activity [31]; however, this mechanism requires further validation through the creation of galgt2-deficient *mdx* SSPN transgenic mice. Interestingly, over-expression of *galgt2* has also

been demonstrated to be effective in ameliorating the dystrophic phenotype in mouse models of congenital muscular dystrophy 1A (dy^W) and α-SG-deficient LGMD 2D [175,176]. It is noteworthy, however, that the over-expression of a neuronal homolog *galgt1*, which adds terminal β-GalNAc residues distinctly to ganglioside glycolipids, caused muscle pathology including decreased myofiber diameter and increased central nucleation in wild-type mice [177]. These stark differences resulting from the over-expression of either *galgt2* or *galgt1* demonstrate the tissue and acceptor substrate specificity of glycosylation and reinforce the importance of thoroughly evaluating the effects of manipulating glycosylation.

While genetic over-expression of a glycosyltransferase provides one potential therapeutic approach for manipulating glycosylation of sarcolemmal glycoproteins, pharmacologic approaches also provide potential therapeutics. Using high throughput screening, the small molecule lobeline, was identified as a pharmacological treatment that altered C2C12 glycosylation *in vitro* [178]. Compounds from the Prestwick library of about 1200 FDA approved small molecules were added to myoblasts in differentiation media and changes in glycosylation were measured after two days of treatment [178]. Specifically, binding of terminal β-GalNAc modifications as detected by WFA binding increased in C2C12 cells as well as isolated wildtype and *mdx* myoblasts following treatment with lobeline during differentiation [178]. Lobeline treatment increased abundance of UGC proteins and laminin-binding in wild-type and *mdx* primary cell cultures [178]; these results are similar to changes resulting from *galgt2* over-expression. It is noteworthy that the increase in WFA binding following lobeline treatment was dependent upon complex N-glycans and not O-mannose glycans such as those required for laminin-binding [178]. Furthermore, DMNJ inhibition of complex N-glycans necessary for WFA binding caused a decrease in laminin-binding as detected by laminin overlay

[178]. While lobeline was first FDA approved for smoking cessation, *in vitro* studies have shown that lobeline may potentially act as a protein folding chaperone or nicotinic antagonist through the dopamine or vesicular monoamine transporter [179-182]. In the C2C12 studies, the molecular mechanism by which lobeline increased WFA binding *in vitro* was not determined. While the ability of lobeline to alter muscle glycosylation *in vivo* remains to be demonstrated, these studies represent strong proof of principle for the pharmacologic manipulation of sarcolemmal protein glycosylation as a potential therapeutic.

Protein aggregation and amelioration in muscular dystrophies

The *mdx* mouse contains a point mutation in exon 23 [34], resulting in a premature termination codon that is predicted to result in a 115 kDa protein [25]. It has previously been shown that dystrophin mRNA levels are decreased in skeletal muscle, cardiac muscle, and brains of three *mdx* mouse strains [35]. However, dystrophin mRNA levels vary widely, depending on the causative mutation in human DMD and Becker muscular dystrophy (BMD) patients [36]. It was previously thought that the 115 kDa dystrophin protein is rapidly degraded after synthesis in *mdx* muscle, but studies suggest that truncated dystrophin may be expressed at the sarcolemma [37,38] or retained in the ER/Golgi [31].

A 70-80 kDa dystrophin reactive to a C-terminal antibody was found in a group of Japanese Spitz dogs displaying progressive Duchenne-like muscular dystrophy [38]. These dogs displayed exercise intolerance, an abnormal gait, and pain upon handling of muscles beginning at 10 to 12 weeks of age. The symptoms worsened over time, suggesting that the 70-80 kDa dystrophin was unable to restore sarcolemmal stability. In a separate study, immunohistochemical assays revealed expression of truncated dystrophin in myotubes isolated

from three 12-week old aborted fetuses at risk for DMD, suggesting that mutant dystrophin is synthesized in human DMD patients [37]. Recently, the truncated form of dystrophin was detected within the ER/Golgi compartments of *mdx* mice [31], suggesting that the 115 kDa dystrophin may be retained in the ER/Golgi compartments.

The mutation in *mdx* mice leads to the loss of the β-DG binding domain, leaving only the actin binding domains in the dystrophin fragment [25]. The N-terminus of dystrophin binds directly to F-actin in the cytoplasm of the myofiber, while the C-terminus of dystrophin contains cysteine-rich domains that bind the C-terminus of β-DG [5,183]. Recent studies utilizing truncated dystrophin have provided more insight into which segments of the protein are required for sarcolemmal stability. One study utilized rAAV6-microdystrophin to evaluate the role of the actin-binding domains in sarcolemmal stabilization [184]. Four months after injection into the tibialis anterior, muscles treated with micro-dystrophins lacking actin-binding domains displayed decreased specific force and a decreased ability to protect against contraction-induced injury [184], indicating the importance of an intact actin-binding domain in maintaining sarcolemmal stability.

Other studies have analyzed different isoforms of dystrophin. The dystrophin isoform Dp116 is expressed in Schwann cells within the peripheral nervous system [185]. This isoform lacks the actin-binding domains while retaining the complete dystroglycan-binding domain [185]. Although expression of Dp116 in *mdx*:utrophin-null mice increased muscle mass, life span, and maximal force, the specific force and histopathology was not improved [185]. It is hypothesized that Dp116, or the dystroglycan binding domain, attempts to stabilize the DGC at the sarcolemma through other interactions with the cytoskeleton, although specifics have yet to be elucidated [185]. The expression of a non-muscle isoform of dystrophin, Dp71, which lacks

N-terminal actin-binding domains and spectrin-like repeats, restores the DGC in muscle, suggesting that the β-DG binding domain is required for assembly of the DGC [186]. Thus, it would be interesting to examine other mice with C-terminal mutations in dystrophin. Such experiments would assess the ability of truncated dystrophin to be incorporated and expressed with the DGC at the sarcolemma and restore functionality of dystrophic muscle. This provides an exciting opportunity to examine ways to express fragmented dystrophin at the sarcolemma to improve dystrophic pathology. Other studies involving protein retention within cellular compartments in different muscular dystrophies may provide insights into the therapeutic translation of these findings.

Studies in FCMD, which is caused by mutations in *fukutin [64]*, have provided insight into the importance of protein processing within the ER/Golgi. The exact role of fukutin remains unclear, but mutations in *fukutin* lead to abnormal glycosylation of α-DG [65], a reduction in laminin binding activity [65], and the mislocalization and retention of fukutin in the ER [187]. A recent study analyzed thirteen missense *fukutin* constructs in C2C12 cells and discovered that four were mislocalized to the ER. To understand whether mutant fukutin glycosylates α-DG, site-directed mutagenesis was used to generate mutants for transfection into fukutin-null mouse embryonic stem cells [66]. The expression of the four missense fukutin mutants restored α-DG reactivity against the IIH6C4 antibody and laminin-binding to α-DG, suggesting α-DG is functionally glycosylated with the expression of mutant fukutin [66]. Using treatments to improve protein folding, including curcumin, a molecule isolated from the spice turmeric, and low temperature culturing conditions, ER retention of fukutin was corrected in the four mutants shown to be mislocalized in the ER. In order to elucidate the reasons for aberrant fukutin trafficking, brefeldin A was used to examine anterograde transport and nocodazole was used to

examine retrograde transport [66]. After incubation with brefeldin A, the fukutin mutants remain accumulated in the ER [66]. The results suggest that while mutant fukutin proteins are able to be processed in the ER, they are unable to be transported to the Golgi through the anterograde pathway, providing valuable insight into how the protein trafficking process is affected upon the mislocalization of a mutant protein. Thus, it is possible that truncated dystrophin may be retained within the ER/Golgi compartments and is not transported to the cell surface in *mdx* muscle. It will be important for future studies to utilize a similar strategy to elucidate the effects of 115 kDa dystrophin on retrograde and anterograde transport in *mdx* cells. Additionally, treatment with pharmacological agents, such as curcumin or exposure to low temperature, may potentially alleviate accumulation of truncated dystrophin in the ER/Golgi compartments and improve the transport of DGC and UGC components to the sarcolemmal membrane.

Curcumin has also been used to target the NF-κB pathway in dystrophic muscle [67]. It has been shown that DMD patients have increased activation of NF-κB, which is involved in the modulation of immune responses and regulation of myogenesis [68]. Intraperitoneal administration of curcumin inhibits NF-κB activation and reduces dystrophic pathology in *mdx* mice [69]. However, a different study reports that curcumin treatment does not improve specific force in the diaphragm muscle of *mdx* mice [70]. Future studies examining the effect of curcumin on the accumulation of truncated dystrophin in the ER/Golgi will be important in determining what role, if any, it plays in pathogenesis.

Protein mislocalization has also been implicated in LGMDs 2C-F pathologies caused by mutations in the SG genes [188]. The severity of LGMD 2C-F pathology and cellular fate of the affected SG varies according to the mutation [188]. According to data compiled from the Leiden University mutation database, the most common mutations are α-p.R77C, β-p.S114F, and γ-

p.C283Y, which cause a mild to severe phenotype [188]. These data led to an examination of the noted point mutations and their effect on intracellular fate and expression of the SGs at the cell surface. Following treatment with kifunensine, an α-mannosidase I inhibitor that prevents ER-associated degradation [189], the expression of sarcoglycans with mild mutations thought to cause the least amount of structural modification was restored at the cell surface. Future studies manipulating ER quality control in wild-type and *mdx* cells might provide more information on how changes in the ER can affect DGC and UGC expression at the cell surface.

Heat shock proteins and proteasome inhibition in the treatment of muscular dystrophy

Heat shock proteins (Hsp) are induced to combat cellular stress when organisms are under environmental strains such as heat, or during disease and infection [190]. Analysis of skeletal muscle from young DMD patients revealed an induction of Hsp72 and Hsp65 in hypercontracted fibers and Hsp90 in regenerating muscle [191]. Hsp72 is also increased at the mRNA level following electrical stimulation for tetanic contractions in isolated single skeletal muscle fibers from *Xenopus laevis* [192] and after exercise in normal Wistar rats [193], suggesting a role for Hsp72 in protection against muscle stress. Treatment of *mdx* and utrophin-deficient *mdx* mice with BGP-15, a pharmacologic inducer of Hsp72, showed that an increase in Hsp72 expression improved SERCA function and dystrophic pathology, decreased kyphosis, and ultimately extended lifespan [194]. Targeting Hsp72 to improve protein folding and quality control during cellular stress and disease progression provides a possible avenue to improve muscle function while prolonging the lifespan of patients with muscular dystrophy.

Ubiquitin is an important component in proteasome regulation and can direct proteins towards degradation [195]. Ubiquitin was also found to be elevated in hypercontracted,

regenerating, and necrotic myofibers in the skeletal muscle of young DMD patients [191]. The increase in ubiquitin protein may suggest misregulation of the ubiquitin-proteasome pathway as well as an increase in protein degradation in DMD patients, which may contribute to the pathogenesis of DMD. Recent studies attempting to ameliorate dystrophic pathology using proteasome inhibitors have produced mixed results. Velcade and MLN273, two FDA-approved proteasome inhibitors, were injected into the gastrocnemius muscle of *mdx* mice [196,197]. A truncated 97 kDa dystrophin product, as well as an increase in α-DG, β-DG, and α-SG, were detected using immunoblotting and immunohistochemistry 24 hours post treatment [196,197]. However, physiological functionality was not examined and experiments such as grip strength and force production were not performed. This information is critical for the pursuit of such pharmacological agents as treatments for DMD (Fig. 3A).

Although some drugs such as Velcade and MLN273 have shown promising results in the attenuation of dystrophic pathology in *mdx* mice, other studies have described conflicting results. In a separate study, long-term administration of a different proteasome inhibitor, MG-132, failed to restore dystrophin expression in *mdx* muscle and ultimately increased susceptibility to contraction-induced damage [198]. The effects of proteasome inhibitors on grip strength, force production, and DGC protein expression need to be analyzed more rigorously to better determine their efficacy in improving muscle function and dystrophic pathology.

A possible function for sarcospan as a chaperone protein

New data suggests a role for SSPN in protein trafficking to the sarcolemma. A recent study isolated ER/Golgi membranes from *mdx* muscle and immunoblots revealed an increase in α-DG and utrophin compared to wild-type muscle [31]. This suggests a possible compensatory

mechanism is at play, whereby the system attempts to utilize utrophin upon the loss of dystrophin (Fig. 3B). Interestingly, SSPN transgenic *mdx* mice revealed decreased levels of WFA reactive α-DG and utrophin in ER/Golgi preparations, while immunofluorescence assays showed an increased abundance of these proteins at the sarcolemma [31]. SSPN over-expression increased WFA binding 1.8-fold per α-DG molecule as detected by WFA overlay [31]. Increased extrasynaptic binding of WFA may be the result of general chaperone trafficking of glycoprotein complexes by SSPN. However, the increase in WFA-reactive glycosylation per α-DG protein demonstrates a role for SSPN in specific changes in glycosylation of α-DG. This modification in α-DG glycosylation may result from downstream transcriptional effects of increased Akt signaling [31] or could additionally be a more direct result of chaperone activities of SSPN in trafficking. SSPN might act directly as a chaperone to stabilize α-DG during ER/Golgi trafficking and addition of glycans resulting in increased glycan modification per α-DG molecule. Importantly, over-expression of SSPN drives changes in glycosylation of α-DG, which aid in the amelioration of *mdx* pathology [31]. These studies highlight a promising role for SSPN as a therapeutic and it would be interesting to examine SSPN over-expression in dystroglycanopathy mouse models where direct manipulation of glycosylation ameliorates dystrophic pathology [175,176]. SSPN may possess chaperone-like functions, and the over-expression of SSPN may improve overall protein folding and quality control as well as transport to the cell surface.

How to achieve forced over-expression of sarcospan

Membrane proteins are synthesized by ribosomes on the ER and disulfide bonds are synthesized and rearranged in the ER lumen [199]. Since SSPN is an integral membrane protein

with disulfide bonds requiring processing through the ER, it most likely cannot be administered systemically. The small size of SSPN makes it an excellent gene to be delivered through AAV. AAV delivery of α-SG in α-SG-deficient patients and γ-SG in γ-SG-deficient patients resulted in no adverse events, demonstrating that intramuscular AAV delivery is likely to be safe in adult patients [200,201]. The only patients that did not express SG following AAV delivery had pre-existing immunity to the AAV serotype used, demonstrating the need for pre-screening of AAV serotypes [200,201]. However, there are many challenges to be overcome before systemic delivery of AAV is feasible. Systemic delivery of therapeutics will be required for amelioration of fatal dystrophic symptoms in the diaphragm and heart muscles. Surprisingly, a T-cell mediated immune reaction against dystrophin prevented the expression of mini-dystrophin following AAV delivery in DMD patients [131]. The precise reason for the varied T-cell immune responses (against mini-dystrophin and against self revertant dystrophin) in the 6 trial patients is unknown. These results warrant caution in future AAV mini/micro-dystrophin and exon skipping trials. Importantly, immune responses should not be a problem in secondary approaches to upregulate utrophin or α7β1 integrin for protein replacement therapeutics. A drug screen for compounds that upregulate SSPN offers an additional approach to AAV-mediated SSPN therapy. A similar approach led to the discovery of BMN195 for utrophin upregulation and is currently in Phase 1 clinical trials.

Unanswered questions for sarcospan-based therapeutics

Over-expression of SSPN under the human skeletal actin promoter results in a 60% reduction in regeneration in the *mdx* mouse model of DMD by replacing the DGC with the UGC and α7β1 integrin [31,99]. As with most therapeutic targets, there are many questions that

remain to be answered for SSPN-based therapeutics. For SSPN to become a viable therapeutic for the treatment of DMD, the following questions remain to be addressed:

1) *Does SSPN delivery with AAV prevent dystrophic pathology in the mdx mouse model?* This is an important question that needs to be answered, as it will determine the delivery system used for SSPN-based therapeutics. It would also be useful to further determine the feasibility of AAV delivery of SSPN in the golden retriever model of DMD (GRMD), as the dog model provides a more realistic clinical model for systemic delivery.

2) *Does SSPN eliminate/reduce dystrophic pathology in the heart and diaphragm?* The human skeletal actin promoter is not highly expressed in the diaphragm or heart muscles. Since death occurs from respiratory and/or cardiac failure in DMD patients, an ideal therapeutic target should prevent dystrophic pathology in the heart and diaphragm. This question can be addressed with systemic delivery of AAV6-SSPN in the *mdx* mouse model. AAV6 has been shown to infect the heart and the diaphragm [202,203].

3) *Can SSPN reverse or prevent dystrophic pathology after the onset of dystrophy?* The human skeletal actin promoter is turned on early in muscle development. SSPN is likely preventing the onset of dystrophic pathology rather than reversing pathology. Boys with DMD are often diagnosed with the disease well after the onset of dystrophic pathology, so effective treatments should reverse or halt pathology already in progress. To determine if SSPN is effective after the onset of disease, a murine inducible transgenic system could be engineered to turn SSPN

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

4) *Is widespread expression of SSPN safe?* The promoter used in murine studies of SSPN over-expression in the *mdx* mouse model restricted SSPN expression to striated skeletal muscle [204]. An effective treatment in patients with DMD would require systemic delivery of SSPN in order to target the heart and diaphragm. It is important to determine whether long-term systemic delivery of SSPN is safe. This question can also be addressed with systemic delivery of AAV-SSPN in *mdx* mice. Since AAV infection of particular tissues depends on the AAV serotype, it would be important to test SSPN delivery with the serotypes approved for use in patients.

5) *Does SSPN upregulate the UGC and integrins in human myoblasts?* Many recent treatment strategies have been tested in DMD myoblasts, including BMN195 and dantrolene [132,205]. The ability to demonstrate that a therapy acts through similar targets in human cells offers a nice proof-of-principle for the approach being tested.

6) *Which patient populations will benefit from SSPN-based therapeutics?* It is important when developing therapies for muscular dystrophy to determine how many different muscle diseases will benefit from the treatment. This will allow for the design and recruitment of appropriate patient populations for clinical trials. SSPN is effective in ameliorating pathology in the *mdx* model of DMD [31,99]. SSPN did not reduce dystrophic pathology in the LARGE^{myd} model of hypoglycosylation of α-DG (MDC1D) [31]. It would be interesting to determine if SSPN can ameliorate various limb-girdle and congenital muscular dystrophies.

CONCLUSIONS

In the *mdx* model of DMD, the SSPN transgene ameliorates dystrophic pathology by increasing the UGC and α7β1 integrin extra-synaptic sarcolemma, the synapse specific GalNAc glycosylation of α-DG, and the transport of utrophin and α-DG from the ER/Golgi to the cell surface [31,99,206]. These studies raise important questions about the mechanisms by which SSPN over-expression accomplishes these observed effects. Biochemical evidence that SSPN is a component of both the DGC and UGC complexes and genetic analysis of mice lacking both SSPN and α7 integrin have led to the proposal that SSPN stabilizes the UGC and integrins through direct interactions at the sarcolemma [31,98,140,206]. More experiments are needed to address the requirement of utrophin and α7 integrin for SSPN-mediated amelioration of *mdx* muscle, as well as the possibility that the UGC and α7β1 integrin function synergistically at the sarcolemma. Additionally, SSPN may act either directly or indirectly as a chaperone protein to facilitate the efficient assembly and export of the UGC to the cell surface [31]; however, more studies are needed to elucidate the role of SSPN in the ER/Golgi. Over-expression of constitutively active Akt signaling has been shown to be beneficial in *mdx* muscle by increasing the UGC and integrins at the sarcolemma, reducing membrane damage, and improving the force generating capacity of muscle [207,208]. SSPN-mediated amelioration of *mdx* muscle results in a similar increase in active Akt signaling and downstream muscle growth pathways [31]. Many therapeutic approaches for DMD concentrate on a single target. Although experimental strategies for the treatment of DMD have been developed for over 25 years, steroids remain the only approved drugs to slow the progression of the disease. Thus, the best treatment strategy remains an open question. SSPN is unique because it incorporates several beneficial therapeutic targets into a single protein that is small and easily packaged in AAV delivery systems. SSPN is

also ubiquitously expressed in other tissues throughout the body so systemic delivery and immune response should not be an issue [98]. We are currently experimentally addressing the unanswered questions for SSPN therapeutics.

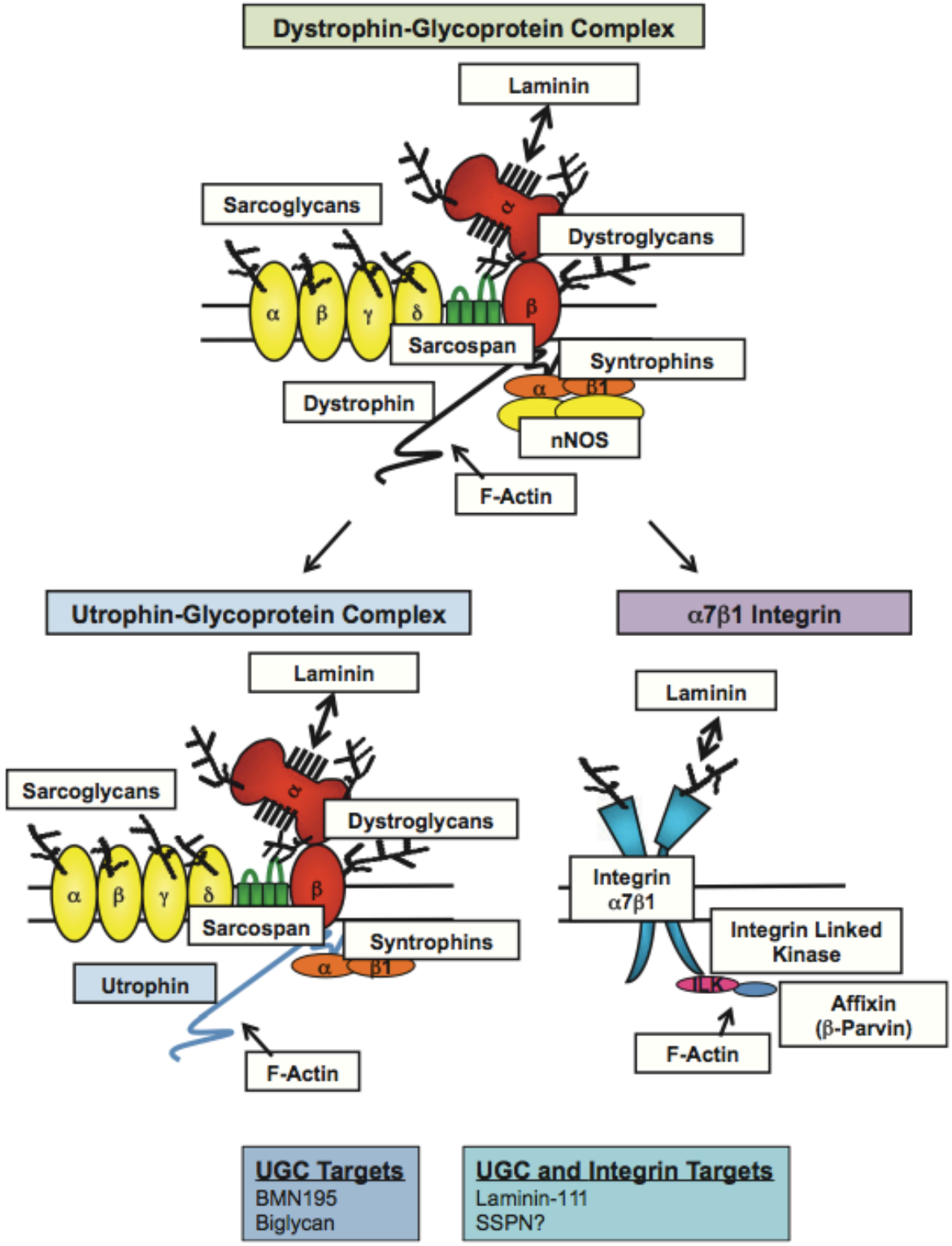
Dystrophin-Glycoprotein Complex
Laminin
Sarcoglycans
Dystroglycans
α
β
γ
δ
β
Sarcospan
Syntrophins
α
β1
Dystrophin
nNOS
F-Actin
Utrophin-Glycoprotein Complex
α7β1 Integrin
Laminin
Sarcoglycans
Dystroglycans
α
β
γ
δ
β
Sarcospan
Syntrophins
α
β1
Utrophin
F-Actin
Laminin
Integrin
α7β1
Integrin Linked
Kinase
ILK
Affixin
(β-Parvin)
F-Actin
UGC Targets
BMN195
Biglycan
UGC and Integrin Targets
Laminin-111
SSPN?

Figure 3-1. UGC- and α7β1 integrin-mediated replacement therapy for the DGC in DMD. The DGC, UGC and α7β1 integrins function to prevent contraction-induced damage of the sarcolemma by maintaining connections between the actin cytoskeleton and ECM. The DGC is composed of dystrophin, the dystroglycans (α- and β-DG), the sarcoglycans (α-, β-, γ- and δ-SG), sarcospan (SSPN) and the syntrophins (α- and β-subunits). Neuronal nitric oxide synthase (nNOS) requires dystrophin and syntrophin to be anchored to the sarcolemmal membrane, where it is thought to function in preventing functional muscle ischemia. The UGC is homologous to the DGC, where utrophin replaces dystrophin. However, many differences exist between the UGC and DGC, including the glycosylation of a-DG, the domains in which actin binding occurs, and the lack of nNOS-binding sites on utrophin. α7β1 integrin differs from the UGC/DGC in the globular domains by which laminin binds and the presence of adaptor proteins that facilitate actin binding, including ILK and β-parvin. BMN195 and biglycan are two utrophin upregulation therapeutics that are near/in clinical trials and laminin-111 is the only dual (utrophin and integrin) target therapy that is near clinical trials. AAV delivery of SSPN should be considered as an additional dual target therapy.

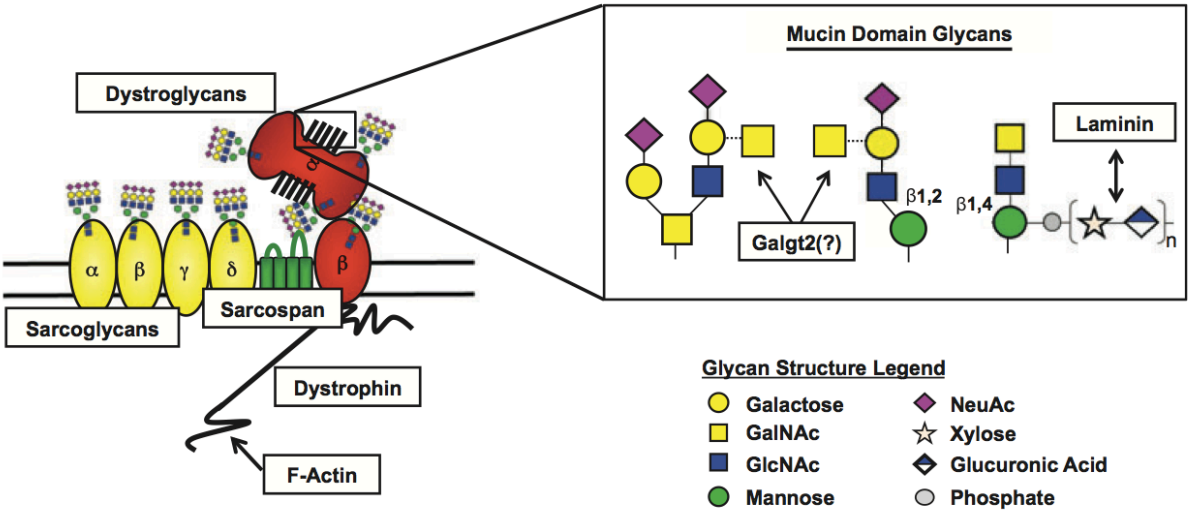

Figure 3-2. Glycosylation of the DGC. Known and putative sites of glycosylation of DGC component proteins are depicted. Inset of laminin- binding glycan is provided along with potential site of Galgt2 modification. Colored symbols used to represent glycan structures are in accordance with the guidelines outlined by the Consortium of Functional Glycomics.

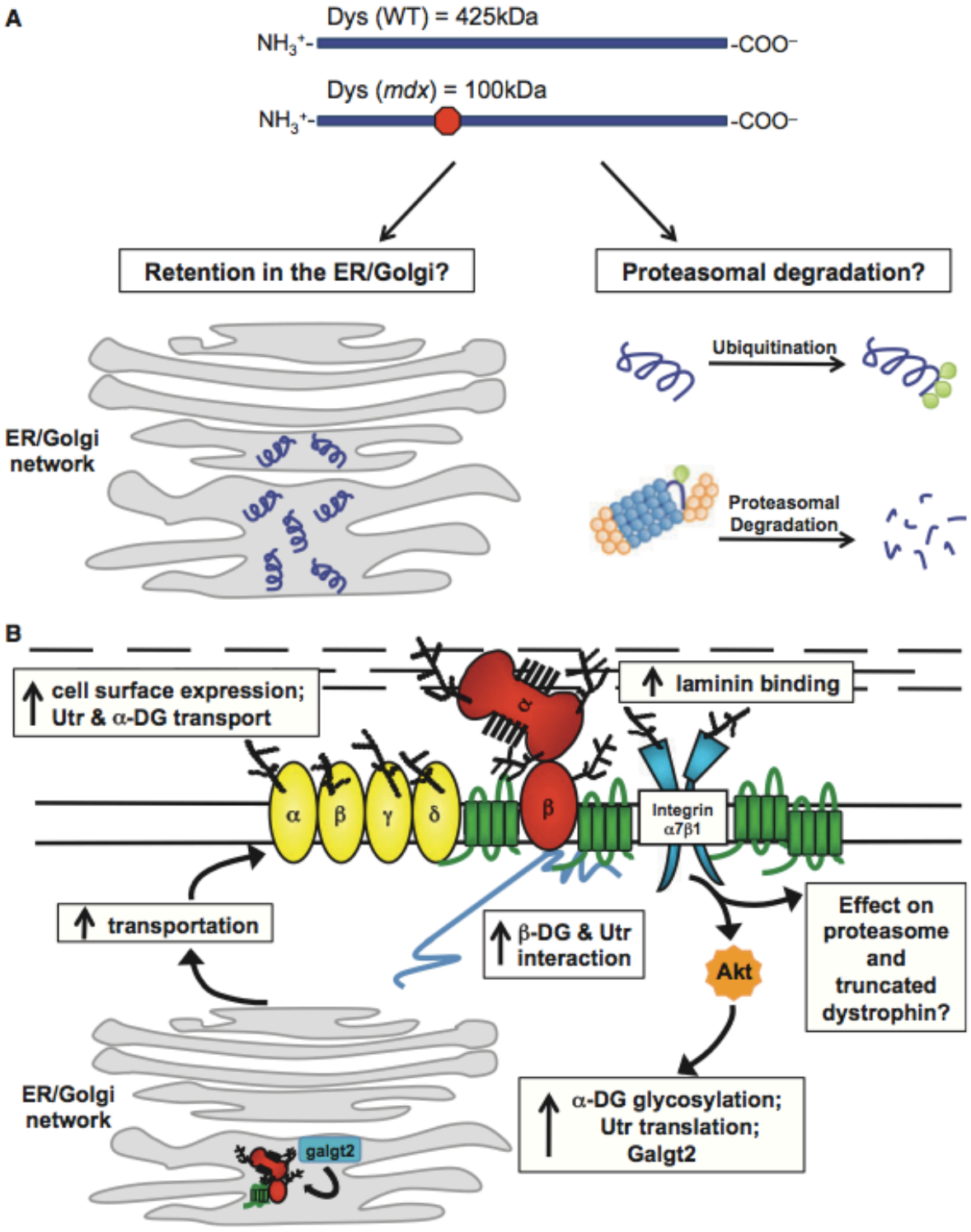
A
Dys (WT) = 425kDa
NH3+-
-COO−
Dys (mdx) = 100kDa
NH3+-
-COO−
Retention in the ER/Golgi?
Proteasomal degradation?
ER/Golgi network
Ubiquitination
Proteasomal Degradation
B
cell surface expression; Utr & α-DG transport
laminin binding
α
β
γ
δ
β
Integrin α7β1
transportation
β-DG & Utr interaction
Akt
Effect on proteasome and truncated dystrophin?
ER/Golgi network
galgt2
α-DG glycosylation; Utr translation; Galgt2

Figure 3-3. Effects of SSPN overexpression in mdx mice on the cell surface protein expression and protein processing and possible outcomes of truncated dystrophin within the cell. (A) Wild-type mice express full-length dystrophin, which is 425 kDa. The mdx mutation leads to a premature stop codon that results in a truncated dystrophin of 100 kDa. Dystrophin is depicted in purple, the proteasome is blue and orange, and ubiquitin is green. Truncated dystrophin can be retained in the ER/Golgi. Alternatively, truncated dystrophin is ubiquitinated and sent to the proteasomal degradation pathway. **(B)** The overexpression of SSPN in mdx muscle leads to molecular events resulting in the restoration of laminin binding and rescue of mdx pathology. SSPN activates Akt, which leads to an increase in utrophin and integrins. Galgt2, one enzyme responsible for GalNAc modification of α-DG, is also increased in isolated ER/Golgi membranes. SSPN also improves utrophin-DG transportation to the sarcolemma while simultaneously restoring laminin-binding and membrane stability. SSPN's effect on the trafficking of truncated dystrophin and proteasomal degradation is still unknown. DGs (red), SGs (yellow), SSPN (green), integrins (blue) and Akt (orange) are shown. Utrophin (Utr) is depicted in gray.

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