University of Nevada, Reno

The Role of Galectin-1 in Duchenne Muscular Dystrophy Disease Progression

A thesis submitted in partial fulfillment
of the requirements for the degree of

Bachelor of Science in Biology and the Honors Program

by

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UNIVERSITY OF NEVADA RENO **THE HONORS PROGRAM**

We recommend that the thesis
prepared under our supervision by

JORDAN J. TICE

entitled

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requirements for the degree of

BACHELOR OF SCIENCE, BIOLOGY

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Dean Burkin, PhD, Thesis Advisor

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Pam Van Ry, PhD, Thesis Advisor

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Tamara Valentine, PhD, Director, **Honors Program**

Abstract

Duchenne Muscular Dystrophy (DMD) is a genetic disorder caused by mutations in the dystrophin gene and affects 1 out of 5,000 male children (Heller et al.). Patients who are diagnosed with this life-threatening disease usually suffer from respiratory failure due to irreversible effects on the diaphragm resulting in premature death. *Mdx* mice have the same dysfunctional dystrophin gene as patients and are the standard mouse model used for DMD. No cure for this devastating disease exists. The Burkin Lab at the University of Nevada, Reno has shown that *mdx* mice treated with recombinant Galectin-1 protein exhibit improved skeletal muscle viability and pathology. However, the mechanisms of these improvements have yet to be elucidated. **This study has provided evidence that Galectin-1 plays a role in the mechanism and pathology of DMD for potential uses as a protein treatment therapy in patients afflicted with the disease.**

Acknowledgments

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Introduction

Disease Background

Muscular dystrophies constitute a group of various genetic mutations that affect different muscle types. These mutations in the genome lead to damaged or truncated (incomplete, shortened) protein complexes in the muscle fibers. Dystrophies are caused by the loss of or damage to the dystrophin protein in the extracellular matrix leading to progressive muscle wasting (Rogers, Baumann and Otis; Grady et al.). With dystrophin absent, muscle fibers no longer have the 'glue' to hold them together and are prone to muscle contraction induced injury (van den Bergen et al.).

Dystrophies are vast in severity, phenotype, and age of onset. Typical symptoms of muscular dystrophies include muscle weakness, delayed motor development, decreased life expectancy, and respiratory failure (Gawlik and Durbeej). Specific symptoms of the other dystrophies are beyond the scope of this paper (for review, see (Bulfield et al.; Gussoni et al.)). Nine types of dystrophy are known and include Becker muscular dystrophy (BMD), Merosin-deficient congenital muscular dystrophy (MDCMD), Duchenne muscular dystrophy (DMD), Emery-Dreifuss muscular dystrophy (EDMD), and Limb-Girdle muscular dystrophy (LGMD). Perhaps two most common of these dystrophies are DMD and its less severe counterpart BMD. In BMD, the dystrophin gene is typically truncated or mutated whereas in DMD, the gene is completely absent (van den Bergen et al.). However, BMD symptom presentation is widely variable, and many patients experience symptoms as severe as those of DMD (van den Bergen et al.). Duchenne Muscular Dystrophy (DMD) is an X-linked disorder that affects the skeletal and cardiac muscle of 1:5,000 male children (Heller et al.). DMD is caused by a mutation in

the dystrophin gene. Dystrophin is a protein that helps bind integral membrane proteins to the actin cytoskeleton (Heller et al.).

The *mdx* mouse is a model organism for DMD, with the same mutation and similar histology (Burkin and Kaufman; Burkin et al.). Currently, a cure does not exist for DMD and treatments are limited. It has been recently shown that treatments with Galectin-1 (Gal1) in *mdx* mice have reduced muscle pathology and improved muscle function (Van Ry et al.). These improvements were a result of the actions of Gal1 on increasing muscle stability and integrity.

The main characteristic of muscular dystrophies results from a deoxyribonucleic acid (DNA) mutation where an insertion, deletion, duplication, translocation, or base-pair switching occurred on a locus (section, location) of the X-chromosome (a sex determining chromosome; XX females, XY males) (Hoffman and Kunkel). Based on the known sources of common dystrophies, research scientists speculate that the wide variety of severity and symptoms of muscular dystrophies is due in part to the size of the gene on the X-chromosome. The gene that undergoes mutations in light of a muscular dystrophy diagnosis is more than 10 times larger than the known characteristic of any other gene (Burmeister and Lehrach; Monaco et al.; Van Ommen et al.; Kenwrick et al.). Therefore, it is speculated that cause of the diseases is from the large diversity from where a gene could possibly be mutated.

Muscles of the mammalian body function to provide a source of movement, heat, and support, which help maintain homeostasis. All three types of muscle tissues, skeletal, cardiac, and smooth muscle, share similar characteristics of contraction processes. All muscle groups contain actin and myosin fibers that interact in similar mechanisms to

produce a contraction. In skeletal muscles, when the brain processes a motor signal to contract a muscle, a signal from the brain travels along the spinal cord to the peripheral nerves ultimately reaching a neuromuscular junction. Neurotransmitters, such as acetylcholine, are released to trigger the muscle cell. The instant efflux of acetylcholine activates the sarcolemma to release calcium ions (Ca^{2+}) thereby causing the production of adenosine triphosphate (ATP). Furthermore, ATP activates the actin and myosin microfilaments to slide antiparallel to each other causing a decrease in the length of the overall muscle equating to a contraction.

Several components of the extracellular matrix, such as scaffolding molecules, that serve as structural and biochemical support, are vital to proper contraction and extension of muscles (Figure 1). The utrophin glycoprotein complex (UGC), dystrophin glycoprotein complex (DGC), and α7β1 integrin are essential for stabilization of the cytoskeleton in muscle fibers (Grady et al.; Gautam et al.). UGC and DGC, along with the dystroglycan protein, are all vital in the process of stimulating the neuromuscular junction (Blake et al.). Dystrophin is an extracellular protein that is the most widespread membrane-spanning protein that is found in between cytoskeletal transmembrane components (Le Rumeur, Winder and Hubert). The homolog to dystrophin, utrophin, has been shown to complement dystrophin in normal muscle function (Deconinck et al.). Utrophin gene has also been found to code for multiple exons and have been proposed to evolved from the same ancestral protein (Blake et al.). Lastly, α7β1 integrin is a laminin (another extracellular protein) receptor molecule in cardiac and skeletal muscle tissue that parallels the DGC activity and also communicates with the cytoskeleton's actin filaments (Burkin et al.).

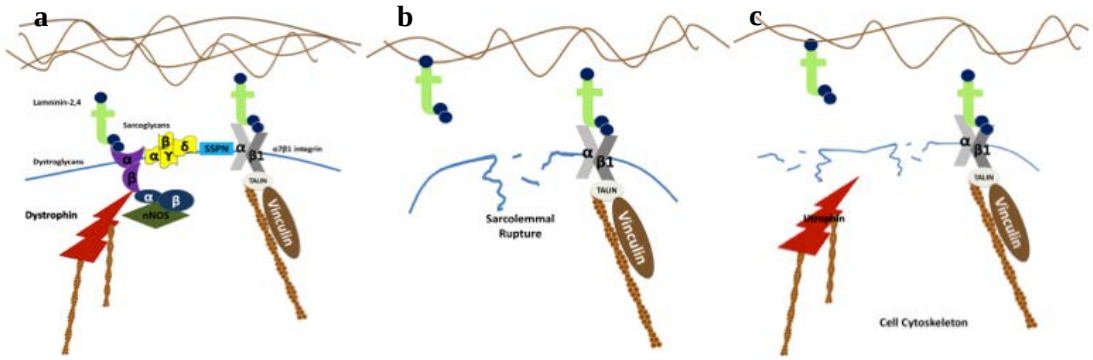

Figure 1: (a) Normal muscle tissue, (b) *mdx* muscle, and (c) *Gal1*$^{-/-}$*mdx* muscle. Normal muscle tissue is composed of protein complexes that hold the adjacent fibers together (a). When most of those complexes are absent, as in the dystrophic mouse, sarcolemmal ruptures (membrane damage) will occur (b).

While the muscles of the *mdx* mouse model and DMD patients, alike, go through cycles of repair and regeneration, the extracellular complexes are absent and therefore cause fibrosis (formation of muscle scar tissue) and fatty infiltration (Figure 1). The increase in fatty infiltration and build up of non-functional fibrotic tissue causes the physical appearance of hypertrophied (pseudohypertrophy) muscles (Figure 1). Gal1 protein in the cytosol (intracellular) has been shown to control the inflammatory response and acts as a mediator in homeostasis (Almkvist and Karlsson).

Skeletal muscle tissue undergoes natural cycles of microtearing from use resulting in hypertrophy. This increase in volume of a muscle fiber occurs from initiating cycles of repair and regeneration. After intense physical activity and depletion of the satellite pool via eccentric contractions, the muscles are left in an injury-induced state (Rogers, Baumann and Otis; Pratt et al.). In a normal, healthy person following injury, regeneration of muscle should be prompted when DNA transcription and protein translation within the nuclei is triggered and leads to muscle hypertrophy (Rogers, Baumann and Otis). When the protein

complexes—DGC, UGC, and α7β1 integrin—are not coded for, the muscle remodeling process does not occur. The damage triggers an immune response leading to an increase in fibrosis and inflammation between the muscle fibers—the trademarks of muscular dystrophy diseases (Gibertini et al.; Ishizaki et al.).

Duchenne Muscular Dystrophy (DMD) is a genetic disorder caused by mutations in the dystrophin gene. Patients who present with this life-threatening disease usually suffer from respiratory failure due to irreversible effects on the diaphragm resulting in premature death. *Mdx* mice have the same dysfunctional dystrophin gene as patients and they are the standard mouse model used for DMD. Thus far, a cure for DMD has not been found. The Burkin Lab at the University of Nevada, Reno School of Medicine has shown that *mdx* mice treated with recombinant Gal1 protein exhibit improved skeletal muscle viability and pathology. However, the mechanisms of these improvements have yet to be elucidated.

Galectin proteins

Gal1 is either monomeric or dimeric and has an affinity for β-galactoside proteins. It has a carbohydrate recognition domain, which modulates many cellular processes such as cell adhesion, immune response, and inflammation response (Camby et al.). Gal1 binds to glycoproteins on the surface of the cell and interacts with extracellular matrix proteins. Gal1 has also been shown to affect differentiation of myoblasts *in vitro* and is involved in muscle regeneration (Goldring et al.). Previous work in the Burkin Lab at the University of Nevada, Reno has shown that *mdx* mice treated with Gal1 have increased muscle strength and activity compared to saline treated control animals (Van Ry et al.). These preliminary results indicate that Gal1 could be used as a therapeutic treatment for DMD. Gal1's role in sarcolemmal stabilization, improved muscle repair, increased angiogenesis

and actions of cardiac function leaves the mechanism of action for the improvement seen in this disease model unclear. This project will characterize the role Gal1 plays in the extracellular matrix stabilization of muscle when used as a treatment of DMD.

As Gal1 has been shown to play a role in proliferation of cells, it is expected to see decreased differentiation and proliferation in the skeletal muscles of the double-knockout (*mdx/Gal1*$^{-/-}$) mouse. Exercise induced injury and the aging process cause damage to the muscle tissue with decreased repair and regeneration. By knocking out the Gal1 protein, the vital role within the muscle fibers will be identified. Characterization of the mouse model will help the scientific community to further understand the role of Gal1 in the extracellular matrix of muscle cells.

mdx Mouse Model

The *mdx* transgenic mouse model has been used as the primary source for studying the affects of treatment therapies for DMD. The mouse is characterized by a genotype lacking the dystrophin extracellular protein in its sarcolemma (Le Rumeur, Winder and Hubert; Heller et al.; Burkin et al.). The weight of the *mdx* mouse is increased to due pseudo-hypertrophy due to the fibrotic buildup. For this reason, centrally located nuclei (CLN) are a definitive marker for presenting fiber damage in the tissues. In addition to muscle weakness and damage, the *mdx* mouse also models decreased bone health (Novotny et al.). Many patients who are on bed rest due in part to muscle damage often experience symptoms related to degenerative bone diseases. While young boys experience more severe symptoms, the *mdx* mouse is similar to the wild type (non-diseased) mouse in its apparent ability to remain active and bear weight on their hind legs.

The *mdx* mouse is typically used because Gal1 protein has been shown in several research studies to play a role in cell growth and repair in areas of muscle tissue, epithelial cells, immune cells, and interstitial fluids (Case et al.; Sakaguchi et al.; Hughes). Compared to a normal, wild type mouse, the *mdx* mouse exhibits increased atrophy in the muscle fibers. In humans, the calf muscles typically appear enlarged and hypertrophied. However, this illusion is caused by inflammation and fluid increase within the sarcolemma and myofibrils.

The objective of the present study was to test the hypothesis that Gal1 served as a major disease modifier in DMD. The following ensued to test the hypothesis: (a) bred dystrophin deficient mice (*mdx* mice) with Gal1 null mice ($Gal1^{-/-}$ mice) to produce $Gal1^{-/-}$*mdx* (double knockout) mice. This novel mouse model will allow for the determination of the role that Gal1 plays in disease progression in DMD. (b) Record physiological measurements of muscle strength using a weekly grip strength assay. (c) Body weight averages will be recorded for purpose of normalizing physiological measurements. (d) Track activity using Opto-Varimex 4 program. (e) Assess muscle strength using *in vivo* muscle contraction. (f) Evaluate muscle strength using *in vitro* muscle contraction by employing isometric, eccentric, and fatigue muscle strength protocols. (g) Quantify markers of DMD disease pathology including centrally located nuclei, minimum Feret's diameter, fibrosis and levels of key skeletal muscle stabilizing proteins. Several components of the extracellular matrix, such as scaffolding molecules that serve as structural and biochemical support, are vital to proper contraction and extension of muscles. This study has characterized the role of Gal1 in the extracellular matrix stabilization of muscle when used as a treatment of DMD.

Results

Duchenne muscular dystrophy is characterized by increased muscle weakness and decreased muscle repair and regeneration. The overall body mass of the mice resulted was as hypothesized (Figure 2). The *Gal1*$^{-/-}$*/mdx* mouse consistently had the greatest body mass while the C57/Black10 mice were the lowest in overall body mass (Figure 2).

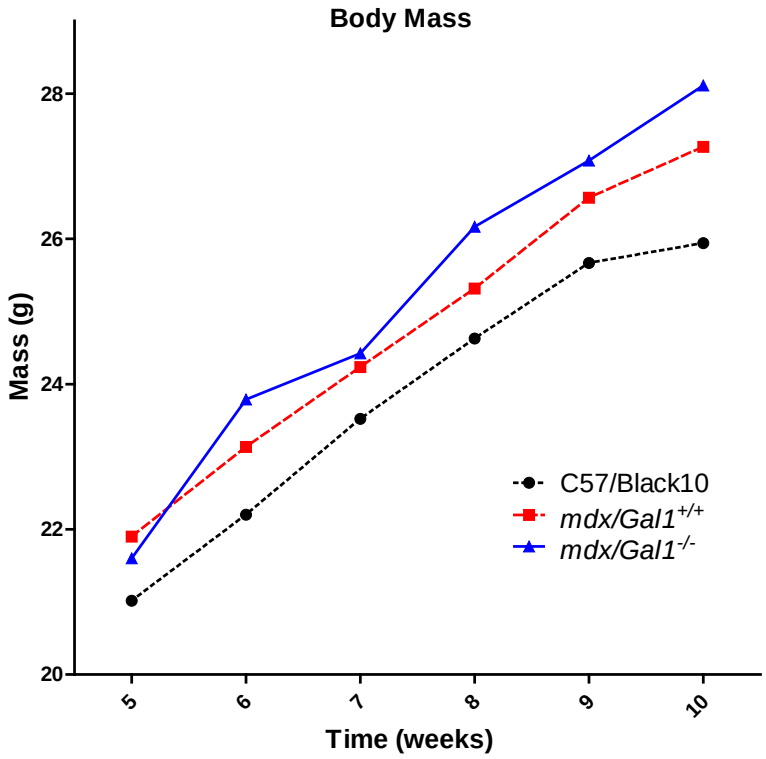

Figure 2: The wildtype mice (C57/Black10) had the lowest body weight compared to the dystrophic mice (*mdx*) and the *mdx/Gal1*$^{-/-}$. While the *mdx/Gal1*$^{-/-}$ were lower than *mdx* mice during the first week of testing (week 5), they surpassed *mdx* mice in mass by week 6.

Grip strength analysis using the tensometer showed that dystrophic *mdx* mice were weaker than the *Gal1*$^{-/-}$*/mdx.* Analysis with an ordinary 2-ANOVA confirmed that grip strength analysis was significant throughout each week (p=0.0003; 13.76% variation)

(Figure 3). Force measurements of isometric contractions in the EDL showed that the Gal1 protein plays a significant role in the physiology of muscle contractions (Figure 5). Compared to the *mdx* mouse, *Gal1-/-mdx* mouse had a much greater number of centrally located nuclei further emphasizing the support that Gal1 plays in the cell (Figure 4, 5).

Force contraction data was consistent throughout showing lower or decreased force in the muscles (Figures 5-10). A significant difference was shown in isometric tetanic force data between the *mdx* mouse, *Gal1-/-mdx* mouse (Figure 5). Stiffness was measured in an eccentric stretch analysis showing that recovery in the *Gal1-/-mdx* was slower, if it occurred at all (Figure 8). If the muscle was exercised to exhaustion, it remained limp and did not recovery fully.

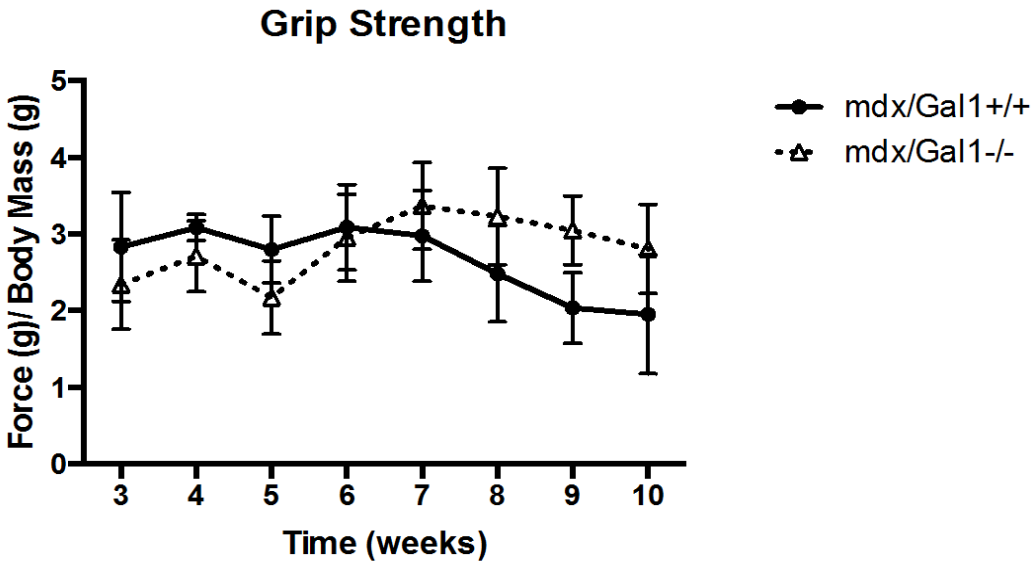

Figure 3: Grip strength analysis. Results of force tensometer between the dystrophic *mdx* mice (n≥3) and *mdx/Gal-/-* mice (n≥3).

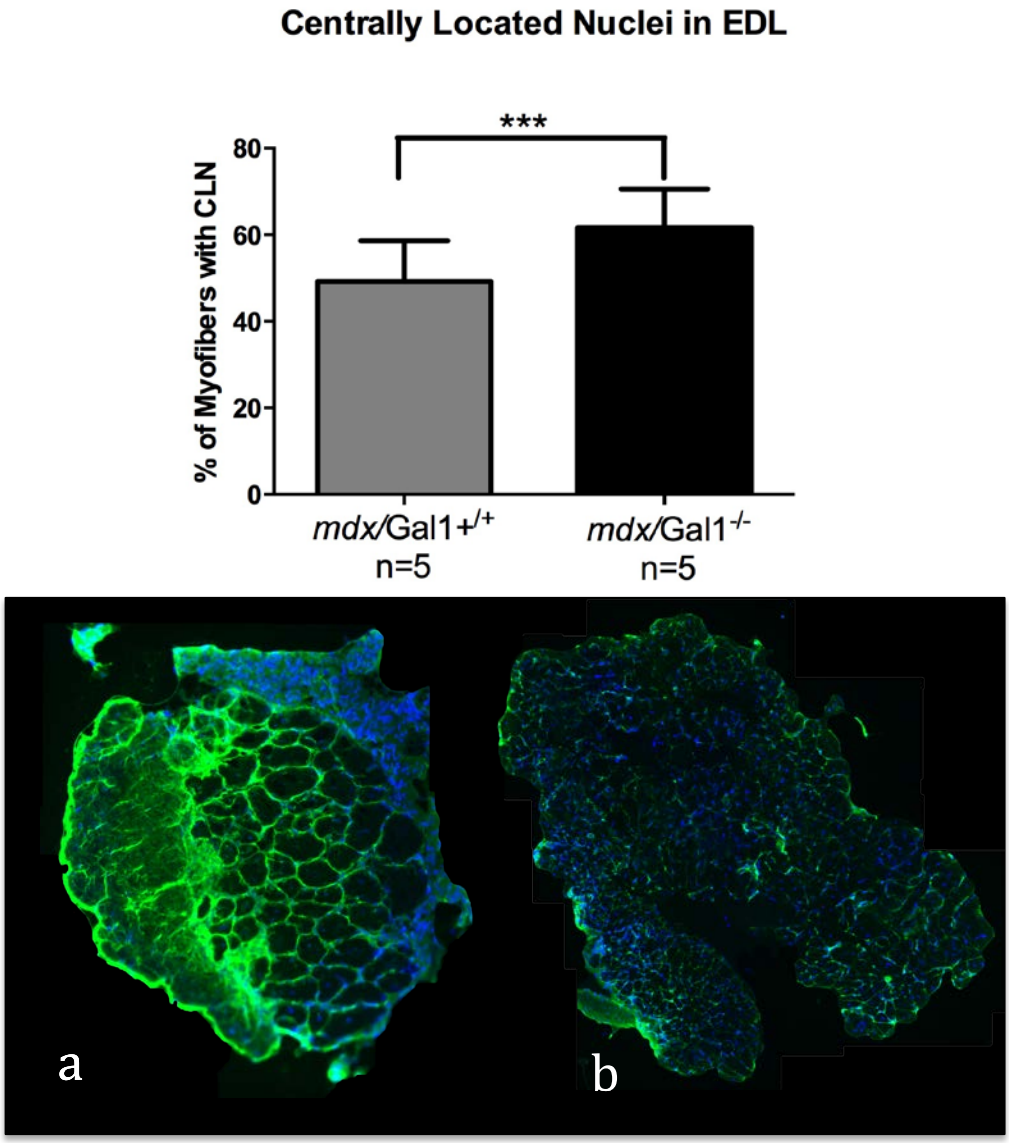

Figure 4: Wheat germ agglutinin immunofluorescence stain of (a) *mdx* and (b) *Gal1-/-mdx* EDL tissue cryosections imaged at 20X magnification analyzed with centrally located nuclei counts. Increased centrally located nuclei in the EDL indicate reduced muscle pathology and stimulation of repair cycle. FITC (green) fluoresces the myofibril membranes and DAPI (blue) highlights the nuclei. Centrally located nuclei (fluoresced by DAPI) signifies damage in the muscle fibrils ([a] *mdx*,n=5; [b] *Gal1-/-mdx*, n=5).

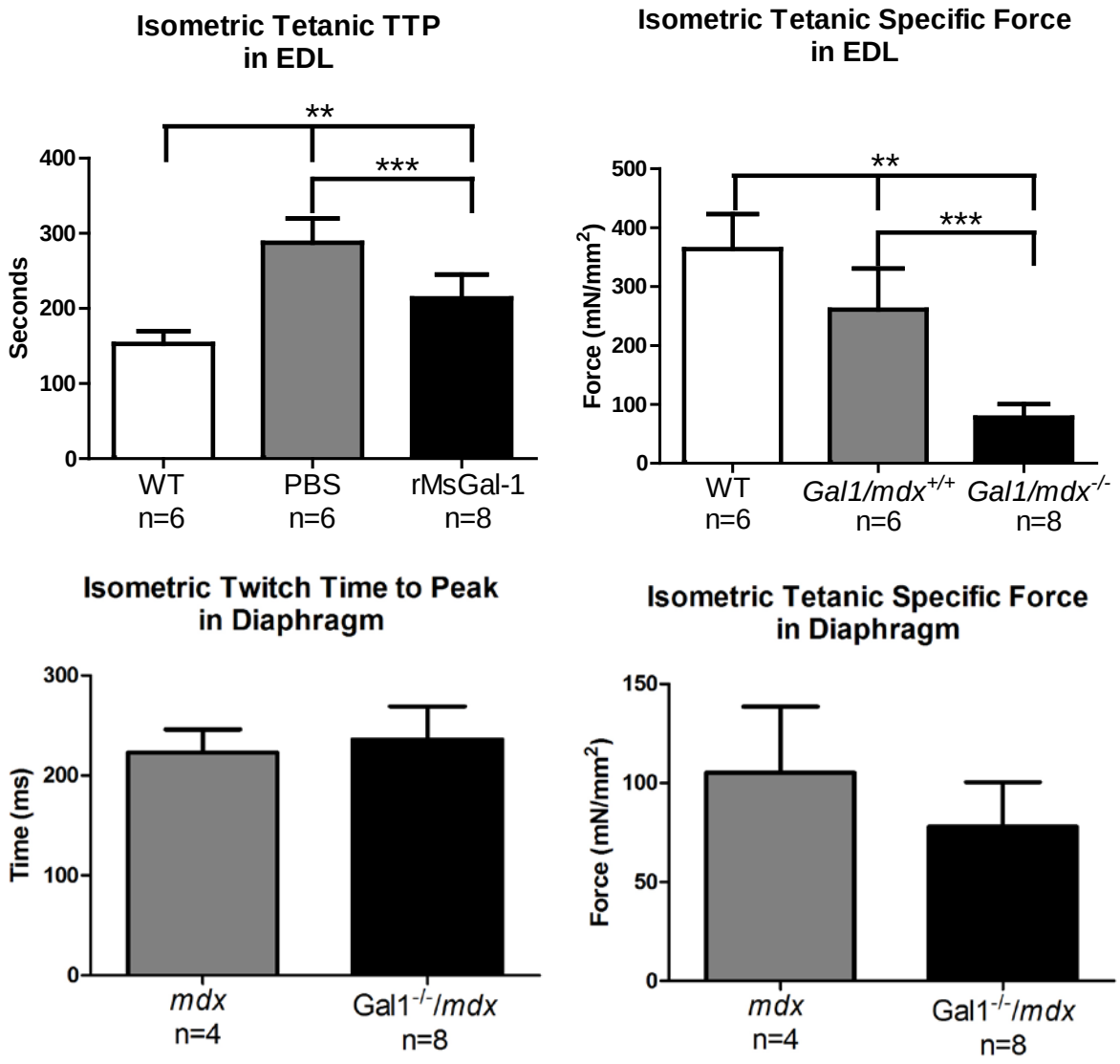

Figure 5: Tetanic force measurements in the diaphragm and EDL normalized by body mass. Analysis using a t-test resulted in significance in isometric tetanic specific force of the EDL ($t=7.021$, $df=12$, $p<0.0001$).

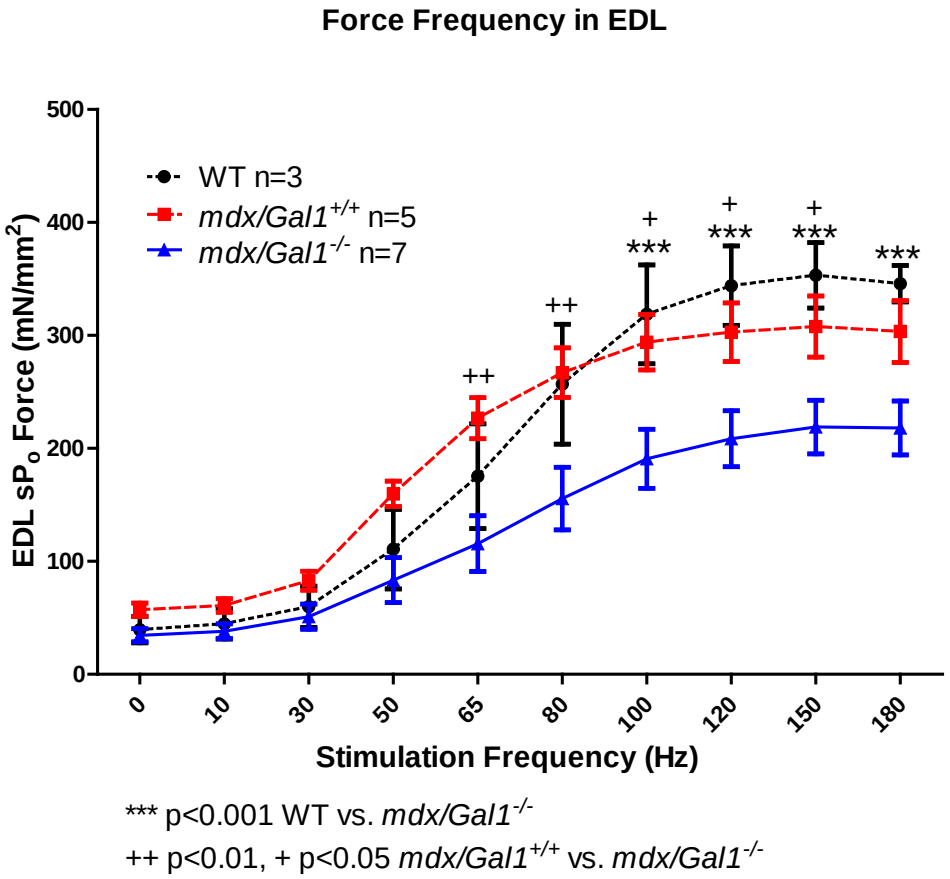

Figure 6: Isometric tetanic force measurements. The loss of the dystrophin protein complex along with the Gal1 protein showed significantly decreased force in the *mdx/Gal1*$^{-/-}$ mouse. The wild type mice show a steady increase in muscle force with increased stimulation frequency peaking at 150 Hz.

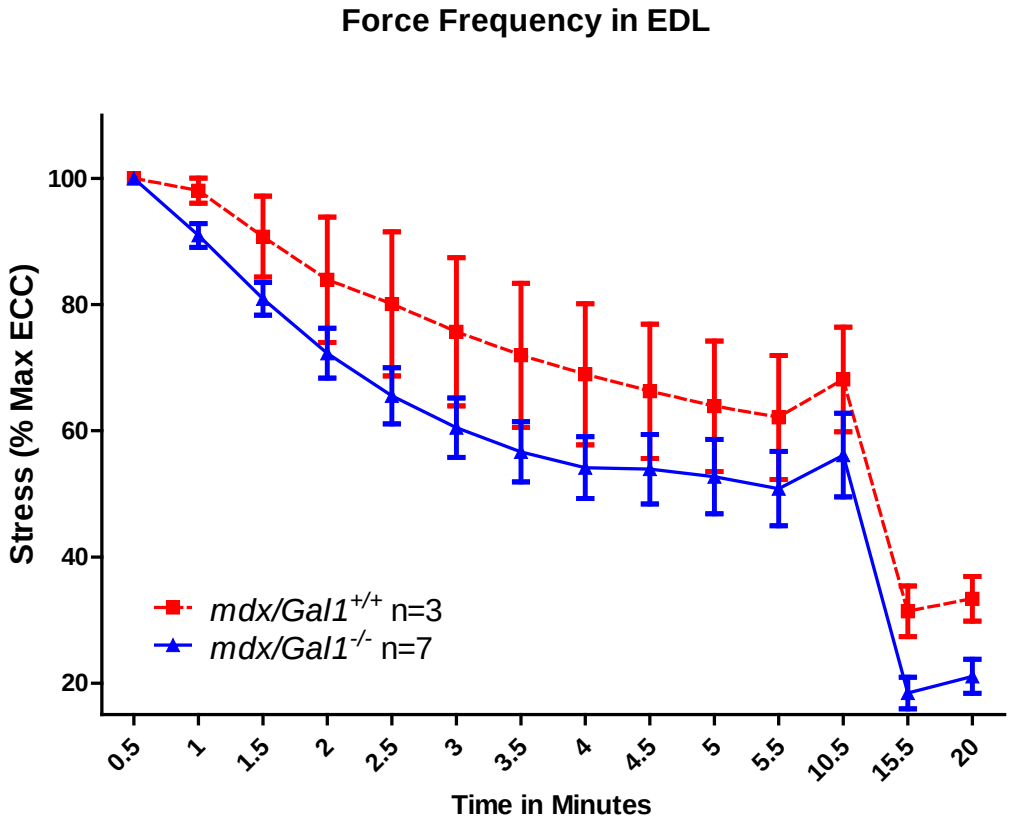

Figure 7: Eccentric stretch maximum contraction. Maximum stress was sustained until 10.5 minutes and rapidly decreased falling below 20% of the maximum. Both muscle groups had the same amount of stress applied. Percentage of maximum stress was normalized based on the measured length of the EDL muscle.

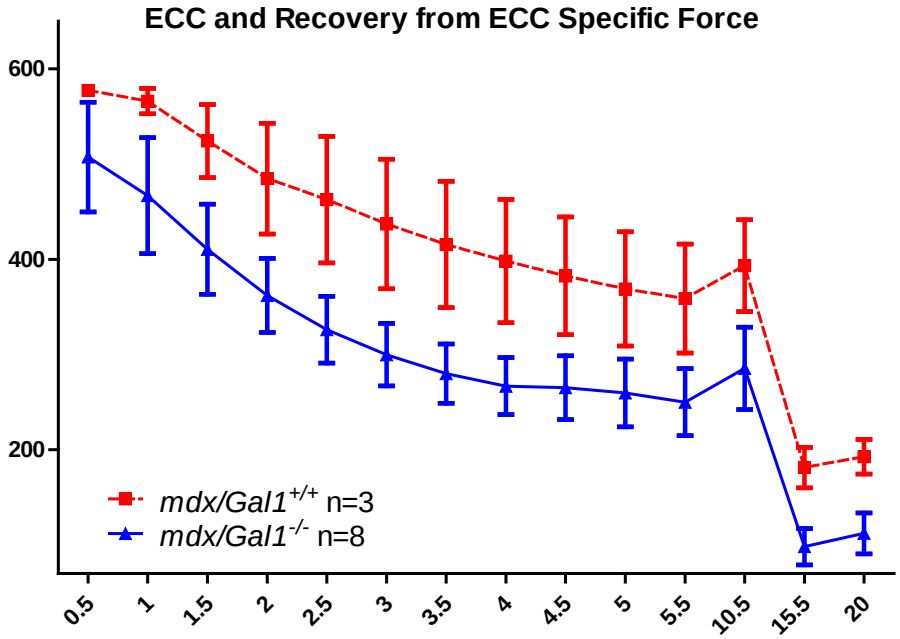

Figure 8: Eccentric stretch maximum contraction specific force. Maximum stress normalized with the weight and length of the muscle to determine specific force in the EDL muscle.

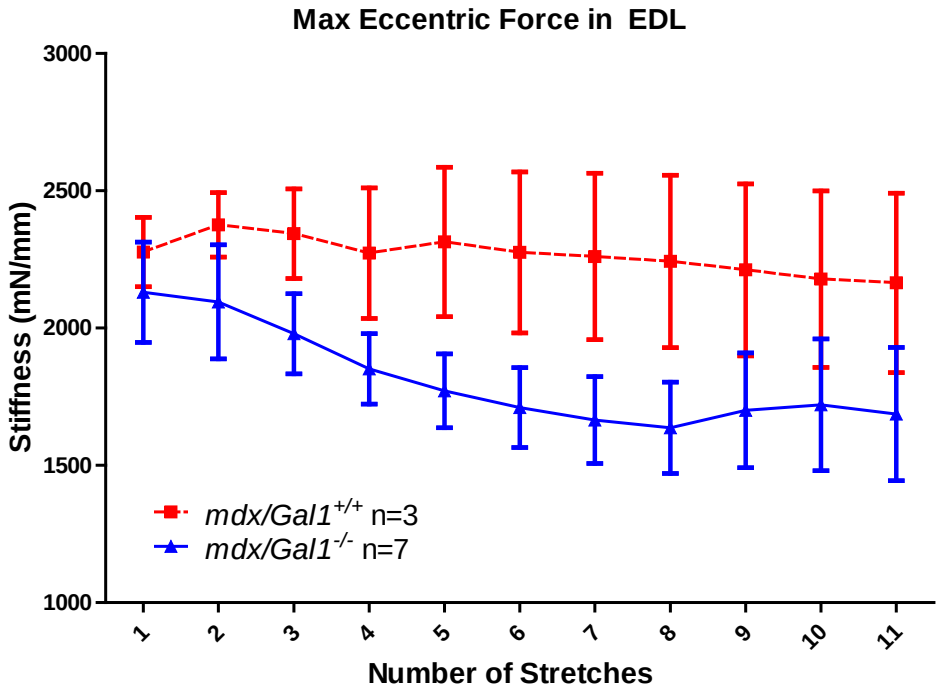

Figure 9: Maximum eccentric stretch force in EDL muscle. The increased number of stretches in the double knockout mouse resulted in a decrease of muscle stiffness. The dystrophic *mdx* mouse muscle showed an opposite effect with a less drastic decrease in muscle strength and stiffness with increase number of stretches.

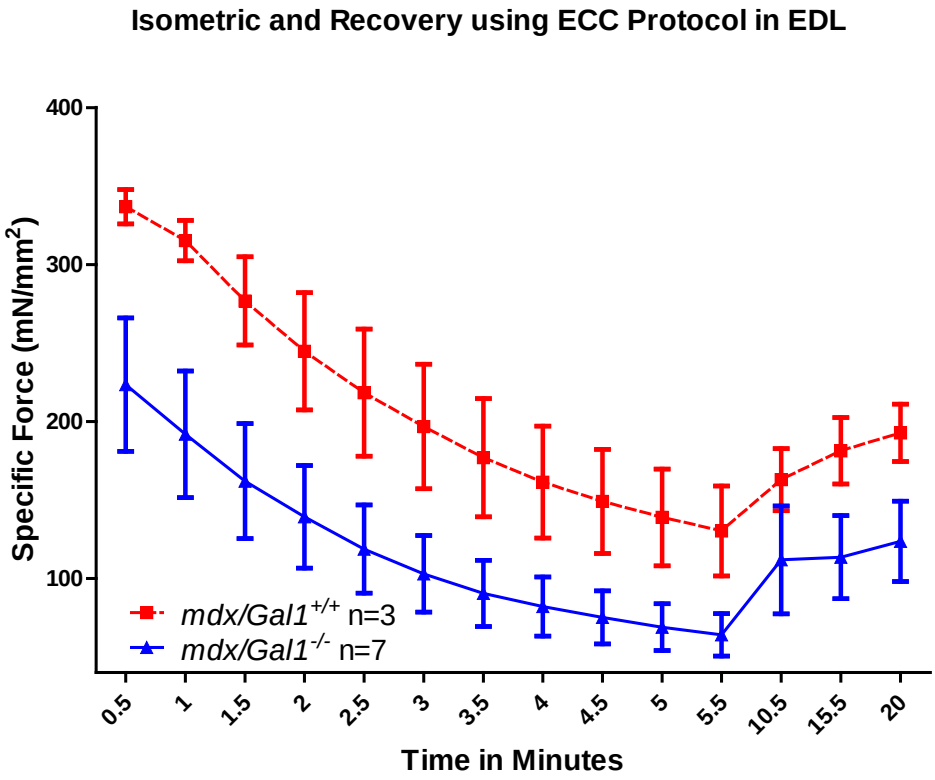

Figure 10: Isometric and recovery of the EDL muscle. The double knockout mice remained lower than the *mdx* mice in specific force during twitch contractions.

Discussion

In this study we have produced mice that lack both dystrophin and Gal1 proteins and examined muscle pathology and function. The results have shown that Gal1 serves as a major modifier of disease progression the *mdx* mouse model of DMD and that the loss of Gal1 in *mdx* muscle increases muscle damage and the requirement for muscle repair. *Mdx* mice lacking Gal1 exhibit reduced muscle contractility and the *Gal-1*$^{-/-}$*mdx* mouse serves as novel model to study muscle disease progression. Since patients who are affected with DMD do not primarily suffer from skeletal muscle damage, the *mdx* mouse may not be as effective of a model for treatment testing. The development and characterization of the

Gal-1$^{-/-}$*mdx* mouse model may be used to develop new therapeutics for the treatment of DMD.

The increased severity with the lack of both dystrophin and Gal1 proteins has shown a significant difference in the force and strength analysis as compared to the current *mdx* mouse model. Since patients who suffer from the disease have a more severe phenotype than the current *mdx* model, studies for protein treatment therapeutics would show improved significance as compared to patients.

Materials and Methods

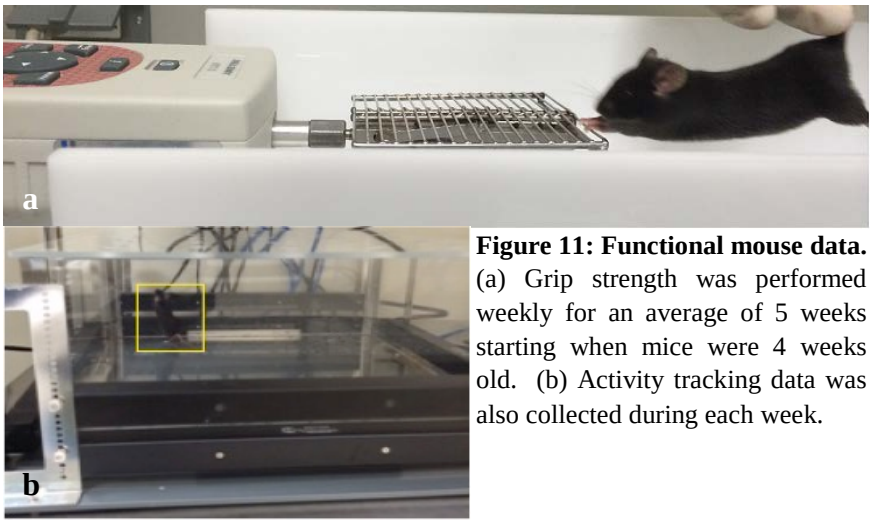

Figure 11: Functional mouse data. (a) Grip strength was performed weekly for an average of 5 weeks starting when mice were 4 weeks old. (b) Activity tracking data was also collected during each week.

Grip Strength and Open Field Activity Test

To measure activity levels, mice were placed in an open field, Plexiglas box with an open-air lid. Parameters such as distance traveled, resting time, and vertical sensor brakes (standing on hind legs) were measured using the Opto-Varimex-4 System with Auto-track software. Each test lasted for 30 minutes and was performed every week beginning at 4-5 weeks of age. Grip strength was assessed using a tensometer (Figure 2a). Mice were held by the tail (appropriate way to handle a mouse) and allowed to grab on to the bar most proximal to the force reader. The mouse was pulled along the bars and the tensometer measured with how much force the mouse resisted the force of the researcher.

Muscle Contraction

Muscle analysis was utilized for *in vivo* and *in vitro* analysis using the Aurora Scientific Dynamic Muscle Control Software. In order to study *in vivo* muscle

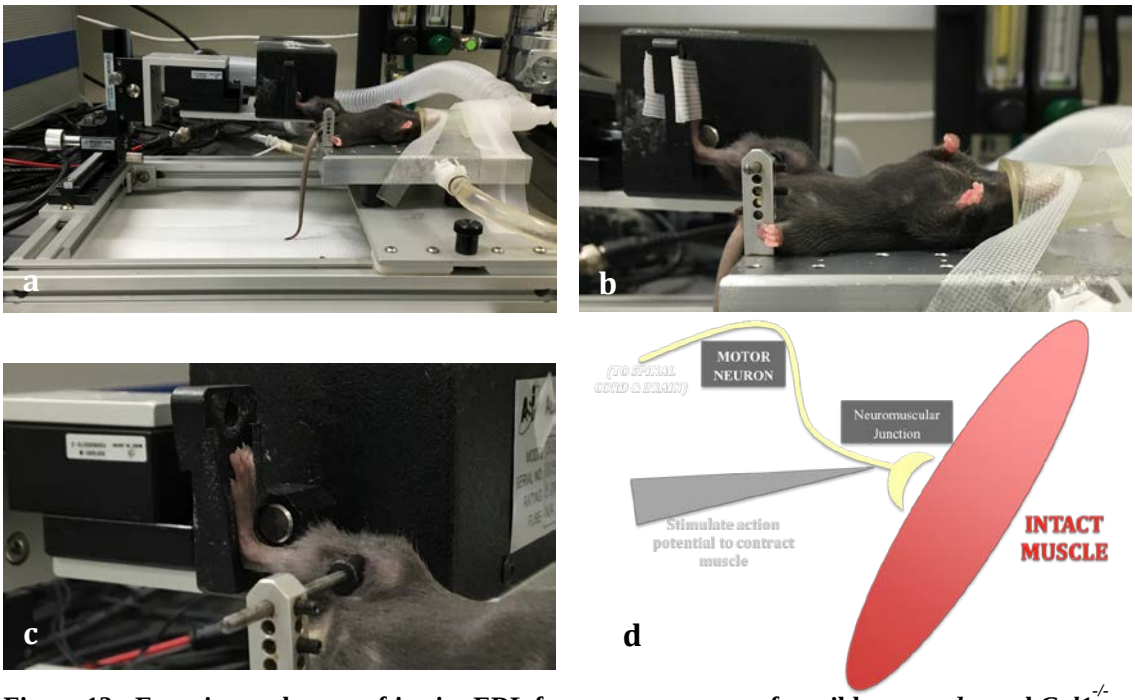

Figure 12: Experimental setup of *in vivo* EDL force measurements for wild type, *mdx*, and *Gal1*$^{-/-}$ *mdx* mice. (a,b,c) Mice were anesthetized while their right was placed on the pedal. (d) Contraction of the foot and lower leg was stimulated in time increments.

contractility of skeletal muscles, ten *mdx*, *Gal1-/- mdx*, and wild type mice were tested at 6, 8, and 10 weeks of age. Isometric twitch, tetanus, and force frequency and eccentric stretch relations of the right crural muscle group (tibialis anterior [TA], extensor digitorum longus [EDL], and extensor hallucis longus [EHL]) were gathered. The protocol was adapted from previous studies (Lowe et al.). For a short time, the mice were anesthetized using 1.5% isoflurane in 400 milliliters (mL) O_2 per minute and placed on a temperature controlled, water-heated platform to help maintain their core body temperature (35°C- 37°C).

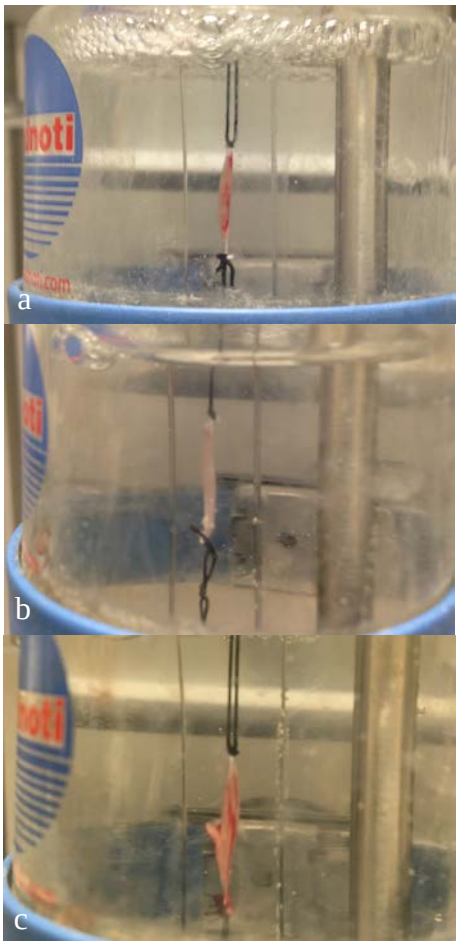

Figure 13: *In vitro* muscle analysis of the extensor digitorum longus (EDL). (a) Wild type, (b) *mdx*, (c) *Gal1*$^{-/-}$*mdx*. Leg muscles were isolated and immersed in a physiological salt solution (PSS), oxygenated bath. Isometric tetanic and twitch force measurements were performed for muscle analysis. (c) The *Gal1*$^{-/-}$*mdx* EDL tore during eccentric stretch analysis. Since patients who present with this life-threatening disease usually suffer from respiratory failure due to irreversible effects on the diaphragm, *in vitro* analysis was also performed on the diaphragm.

The right knee was secured with a clamp while the right foot was posited in a foot pedal (Figure 3). The pedal was attached to the Aurora Scientific Dynamic Muscle 300B servomotor (Aurora, Ontario, Canada) (Figure 3). A sterilized probe was inserted into the skin on the lateral side of the knee in order to stimulate flexion of the muscles via the common peroneal nerve (Figure 3). The stimulations and probe positioning were optimized by 5-10 isometric contractions (200 millisecond [ms] train of 0.1 ms pulses at 300 Hertz [Hz]). Stimulation tests occurred at 80% of full strength. After the optimization, 3 twitch and 3 tetanus isometric contractions ensued, each 30 seconds apart. Following, isometric torque contractions (200 millisecond [ms] train of 0.1 ms pulses at 300 Hertz [Hz]) were performed to measure torque (20-300 Hz) as a function of force frequency. After each test, the muscle's mass was recorded and then embedded in a 30% sucrose/OCT solution for cryoprotection.

In vitro muscle contractility studies were examined to understand the contractile force and mechanical properties of 10 EDL muscles and 5 diaphragm strips of male mice at 10 weeks of age. The EDLs and diaphragms were removed under deeply anesthetized mice

and the muscles were prepared for undergoing contraction analysis following a previously described protocol (Wolff et al.). To prepare muscles for contraction testing, they were hung up and immersed in an oxygenated, physiologic salt solution (PSS, pH 7.6, 30°C) bath (Figure 4). The hanger was connected to a computer-controlled, dual mode Aurora Scientific 300B servomotor. Each test per muscle consisted of 3 twitch, 3 tetanus, a 10-step isometric force frequency sequence, and a set of eccentric stretch analysis sequence with 10 minute resting periods between each set of tests.

Between the final tetanic analyses and the isometric force frequency analyses, the muscle length (L_0) was measured. The recorded L_0 measurements were used to optimize the eccentric stretch analyses. A baseline of 1.00 L_0 was used for each stretch measurement analysis. The eccentric stretch consisted of instantaneously stretching the muscles without stimulation (stretch rate ~50 L_0/s) and then returning to baseline. After resting for 2 minutes at the baseline, each muscle underwent eccentric contractions where the muscle was stimulated with 80 Hz and stretched to 1.10 L_0 while alternating between 0.5 L_0/s and 2.0 L_0/s. The stretches were repeated 6 times, once every 4 minutes. After the active stretches, the tissues were placed in Procion orange dye for 30 minutes and then washed in two PSS bathes for 5 minutes each. Muscles were blotted, weighed, and embedded in 30% sucrose/OCT for sectioning later on.

The *mdx* and Gal1 double knockout mouse was as a preclinical model to determine the role that Gal1 plays in disease pathology in patients with Duchene Muscular Dystrophy. The Burkin Lab has a colony of *mdx* mice and has obtained Gal1 knock out mice. These mice have been set up as breeders in anticipation of receiving the Honors Undergraduate Research Award. Genotyping was performed to determine which mice are negative for

Gal1 (*Gal1*$^{-/-}$) and dystrophin (*mdx*/*Gal1*$^{-/-}$). The mice were tested weekly for grip strength using a tensometer and activity via the Opto-Varimex-4 System with Auto-track software for a 30 minute time period. The instrument is able to calculate distance traveled, resting time (no movement) and vertical movement (standing on back legs). Muscle strength was measured every two weeks starting at 6 weeks of age on the lower leg muscles involved with planter flexion. As an end point measure, the extensor digitorum longus was assessed for isometric strength and fatigue as previously described (Van Ry et al.). Activity and muscle strength are outcome measures that have a strong correlation to health-related quality of life measures in patients and are widely accepted outcome measures for determining physiological changes in disease state.

Histological stains of major skeletal muscles such as Oregon Green-488 labeled wheat germ agglutinin (WGA) and Sirius red provided further evidence of changes in disease state. WGA and FITC immunofluorescence stain outline the muscle fiber. The percentage of centrally located nuclei in mouse muscle tissue indicates the extent of damage that has occurred in the skeletal muscle evaluated. The Sirius red stain was used to detect fibrosis in the muscle. Physiological and histopathology techniques were be completed in order to identify if Gal1 plays a role in the mechanism and pathology of DMD protein and if it is necessary for therapeutic treatment.

Already, we have identified the negative adverse effects of Gal1's absence in the muscle tissue. Reproducibility needs to be increased before we can confidently determine the significance of the results. Muscle strength analysis and activity data has been collected thus far. Staining and analysis of muscle tissue has begun and histopathology will follow.

The results of the tests have provided twitch and tetanus force data including stress, muscle force divided by cross sectional area, whole muscle characteristics (Call et al.). These properties offer an understanding of the degree of damage within the dystrophic and wild type muscle fibers and the level of recycle and regeneration taking place.

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