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## ELUCIDATING FUNCTIONAL ROLES FOR MYOGENIN IN ADULT SKELETAL MUSCLE METABOLISM, EXERCISE CAPACITY, AND REGENERATION

Jesse Flynn

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**ELUCIDATING FUNCTIONAL ROLES FOR MYOGENIN IN ADULT SKELETAL  
MUSCLE METABOLISM, EXERCISE CAPACITY, AND REGENERATION**

by

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**ELUCIDATING FUNCTIONAL ROLES FOR MYOGENIN IN ADULT SKELETAL  
MUSCLE METABOLISM, EXERCISE CAPACITY, AND REGENERATION**

**A**

**DISSERTATION**

**Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
And  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment**

**of the Requirements**

**for the Degree of  
DOCTOR OF PHILOSOPHY**

**by**

**Jesse Michael James Flynn, B.S.  
Houston, Texas**

**August, 2010**

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# **ELUCIDATING FUNCTIONAL ROLES FOR MYOGENIN IN ADULT SKELETAL MUSCLE METABOLISM, EXERCISE CAPACITY, AND REGENERATION**

**Publication No.**\_\_\_\_\_

**Jesse Michael James Flynn, Ph. D.**

**Supervisory Professor: William Klein, Ph.D.**

The four basic helix-loop-helix myogenic transcription factors, myogenin, Myf5, MRF4, and MyoD are critical for embryonic skeletal muscle development. Myogenin is necessary for the terminal differentiation of myoblasts into myofibers during embryogenesis, but little is known about the roles played by myogenin in adult skeletal muscle function and metabolism. Furthermore, while metabolism is a well-studied physiological process, how it is regulated at the transcriptional level remains poorly understood. In this study, my aim was to determine the function of myogenin in adult skeletal muscle metabolism, exercise capacity, and regeneration. To investigate this, I utilized a mouse strain harboring the *Myog*<sup>flox</sup> allele and a Cre recombinase transgene, enabling the efficient deletion of myogenin in the adult mouse. *Myog*<sup>flox/flox</sup> mice were stressed physically through involuntary treadmill running and by breeding them with a strain harboring the Duchenne's muscular dystrophy (*DMD*<sup>mdx</sup>) allele. Surprisingly, *Myog*-deleted animals exhibited an enhanced capacity for exercise, running farther and faster

than their wild-type counterparts. Increased lactate production and utilization of glucose as a fuel source indicated that *Myog*-deleted animals exhibited an increased glycolytic flux. Hypoglycemic *Myog*-deleted mice no longer possessed the ability to outrun their wild-type counterparts, implying the ability of these animals to further deplete their glucose reserves confers their enhanced exercise capacity. Moreover, *Myog*-deleted mice exhibited an enhanced response to long-term exercise training. The mice developed a greater proportion of type 1 oxidative muscle fibers, and displayed increased levels of succinate dehydrogenase activity, indicative of increased oxidative metabolism. *Mdx:Myog*-deleted mice exhibited a similar phenotype, outperforming their *mdx* counterparts, although lagging behind wild-type animals. The morphology of muscle tissue from *mdx:Myog*-deleted mice appears to mimic that of *mdx* animals, indicating that myogenin is dispensable for adult skeletal muscle regeneration. Through global gene expression profiling and quantitative (q)RT-PCR, I identified a unique set of putative myogenin-dependent genes involved in regulating metabolic processes. These data suggest myogenin's functions during adulthood are distinctly different than those during embryogenesis, and myogenin acts as a high-level transcription factor regulating metabolic activity in adult skeletal muscle.

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Genes identified to have significantly altered expression in the absence of myogenin

## ABBREVIATIONS

|               |   |
|---------------|---|
| <b>Bdh1</b>   | 3-hydroxybutyrate dehydrogenase-type 1                |
| <b>bHLH</b>   | Basic helix-loop-helix                                |
| <b>CK</b>     | Creatine Kinase                                       |
| <b>DMD</b>    | Duchenne muscular dystrophy                           |
| <b>Dpyd</b>   | dihydropyrimidine dehydrogenase                       |
| <b>F1,6BP</b> | fructose-1,6-bisphosphate                             |
| <b>F6P</b>    | fructose-6-phosphate                                  |
| <b>Fbxo32</b> | F-box protein 32                                      |
| <b>Glut4</b>  | Glucose transporter 4                                 |
| <b>Gys</b>    | glycogen synthase                                     |
| <b>HDAC4</b>  | histone deacetylase 4                                 |
| <b>MRF</b>    | Myogenic Regulatory Factor                            |
| <b>myHC</b>   | Myosin heavy chain                                    |
| <b>nNOS</b>   | neuronal nitric oxide synthase                        |
| <b>Pc</b>     | pyruvate carboxylase                                  |
| <b>PFK</b>    | phosphofructokinase                                   |
| <b>PFK1</b>   | 6-phosphofructo-1-kinase                              |
| <b>PFK2</b>   | phosphofructokinase 2                                 |
| <b>Pfkfb3</b> | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 |
| <b>SDH</b>    | succinate dehydrogenase                               |



|                |  |
|----------------|--|
| <b>TCR</b>     | Transcriptional control region                   |
| <b>Trim63</b>  | Tripartite motif-containing 63                   |
| <b>qRT-PCR</b> | Quantitative Real-Time Polymerase Chain Reaction |

## CHAPTER I:

### **Myogenin regulates adult skeletal muscle metabolism and exercise capacity**

#### **INTRODUCTION:**

The myogenic regulatory factors (MRFs), MyoD [1], Myf5 [2], MRF4 [3-5], and myogenin [6, 7], are basic helix-loop-helix (bHLH) transcription factors best known for regulating skeletal muscle development during the embryonic stages of life [8]. While their functions during adult life are less well known, persistent expression of these factors into adulthood suggests they play a significant role during this time. During embryonic development, Myf5 and MyoD are required for the earlier stages of myoblast determination. In the absence of Myf5, postnatal (P)0 pups exhibit seemingly normal skeletal muscle at birth, but die soon thereafter due to improper rib development [9, 10]. MyoD-null mice are viable and display normal skeletal muscle. In the absence of both Myf5 and MyoD, P0 pups possess no skeletal muscle or skeletal muscle stem cells [11]. Myogenin is required for the latter stages of myoblast differentiation. *Myog*-null mice die perinatally due to severe skeletal muscle deficiency [12]. However, adult myogenin conditional knock-out mice deleted at P1 are viable with normal muscle, although they have 37% less body mass [13]. This suggests that myogenin is dispensable for postnatal development and survival, but plays a role in overall body homeostasis.

Skeletal muscle is composed of a highly complex and organized arrangement of structures designed for contraction and movement. (Figure 1)

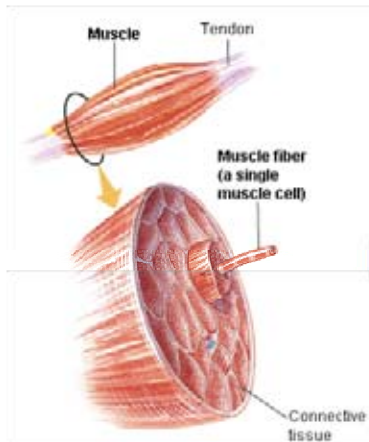
**Figure 1**

Components and structural characteristics of skeletal muscle. (A) Illustration of a whole muscle cross section broken down into myofibers. (B) Illustration of a myofiber cross section broken down into myofibrils. (C) Structure of a sarcomere and the organization of its components. (D) Diagram of proteins comprising the thick and thin filaments of a sarcomere.

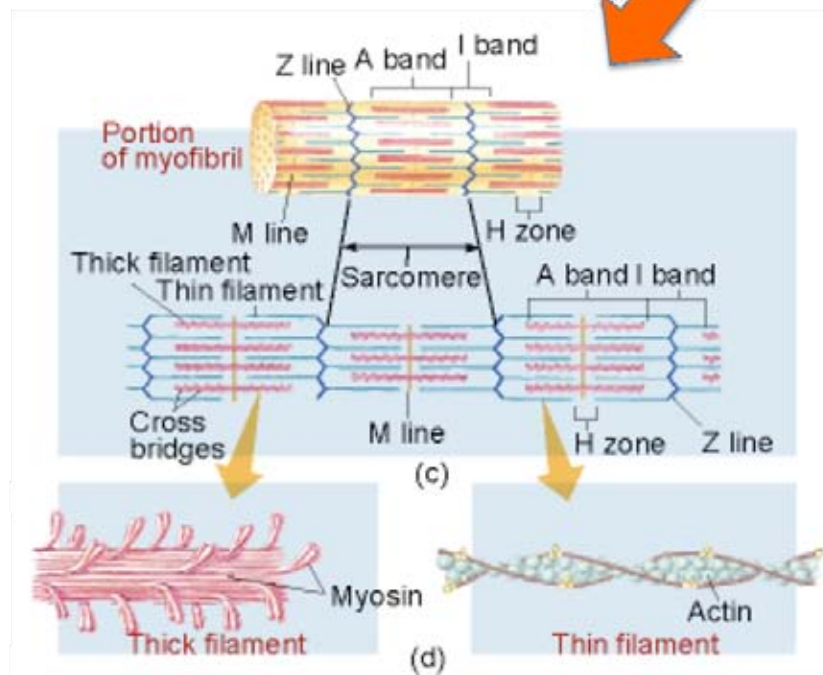
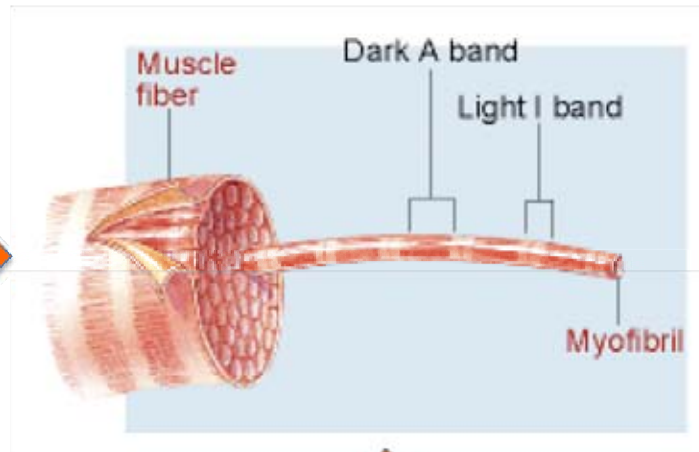
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A.



B.



[for review, see 14]. Muscles are composed of numerous muscle fibers. Each fiber is further broken down to individual myofibrils. Myofibrils contain the functional contraction unit of skeletal muscle, the sarcomere. Sarcomeres are composed of thick myosin filaments in between thin actin filaments and are linked from end to end by the Z line. Myosin filaments contain thick globular heads with an actin-binding site and a Myosin ATPase site. Actin filaments are enwrapped by tropomyosin and troponin. Tropomyosin functions to conceal actin's myosin binding site. Troponin is composed of three polypeptide units which bind actin, tropomyosin, and  $\text{Ca}^{2+}$ . It functions to secure tropomyosin's location over actin's myosin binding site. During contraction,  $\text{Ca}^{2+}$  is released from the sarcoplasmic reticulum surrounding the muscle fibers and binds to troponin. Troponin then undergoes a conformational change causing tropomyosin to shift positions and expose actin's myosin binding site. ATP-bound myosin then binds to actin, and through the dephosphorylation of ATP to ADP, the myosin head shifts toward the center of the sarcomere. This is called the actomyosin crossbridge, or power stroke. The power stroke brings the actin filaments closer together causing contraction. Relaxation is then achieved by the active transport of  $\text{Ca}^{2+}$  back in to the sarcoplasmic reticulum via a  $\text{Ca}^{2+}$  – ATPase pump. This ultimately causes tropomyosin to shift back to its resting position and cover actin's myosin binding sites. This well-developed arrangement of factors provides a foundation through which motion and exercise can be performed.

All vertebrate organisms possess a wide variety of skeletal muscle fibers designed to support the skeletal system that are specialized to maintain body posture as well as perform a myriad of movements and functions [15]. Depending on which myosin heavy chain (myHC) isoform is expressed, they are categorized as types 1, 2A, and 2B [16]. Expression of a particular MyHC isoform dictates the contraction speed for a given fiber [17, 18]. Type 1 fibers, expressing MyHC type 1, contract the slowest while fiber types 2A and 2B contract more rapidly. The endurance of a fiber is largely dependent on the rate by which ATP can be supplied by the tissue during contraction to complete the power stroke cycle [14]. ATP is produced in skeletal muscle via both glycolysis and the citric acid cycle. Glucose is acquired by the intramuscular conversion of glycogen to glucose or can be transported into the muscle from the liver via the blood stream by transporter factors such as Glucose Transporter 4 (Glut4). Glucose is first metabolized anaerobically via glycolysis in the cytoplasm. This process quickly produces 2 net ATP molecules and is the primary source for ATP in the absence of oxygen. Glucose is converted to two pyruvic acid molecules, which then enter the citric acid cycle to undergo oxidative phosphorylation in the mitochondria. This is the primary source for ATP in the presence of oxygen and produces 36 net ATP per glucose molecule.

The rate limiting steps for the production of ATP are regulated by metabolic enzymes. Oxidative enzymes found in Type 1 and 2A fibers supply a slow but constant store of ATP by aerobically metabolizing pyruvic acid derived from glucose furnished by the blood stream. Glycolytic enzymes more prevalent

in type 2B fibers quickly and anaerobically metabolize glucose derived from glycogen supplied by the muscle fiber. This mode of ATP production quickly exhausts glycogen reserves and is only sustainable for short bursts of energy. Thus, oxidative type 1 and 2A fibers are more resistant to fatigue and are required for endurance and skeletal support whereas glycolytic type 2B fibers fatigue more quickly and are utilized for rapid bursts of speed and strength.

Environmental pressures can incite drastic phenotypic alterations in existing mature adult skeletal muscle fibers [19]. Experimentally induced modifications in contraction activity are also known to incite fiber type shifts [20, 21]. These conversions demand tremendous alterations in physical activity for extensive periods of time [22]. As a result, significant modifications in fiber character are often not observed in mammalian exercise training studies [23]. Thus long term studies are necessary for determining experimental effects on skeletal muscle function.

Myogenin has previously been implicated in multiple aspects of mature skeletal muscle function. Denervated adult muscle tissue expresses increased levels of myogenin [24, 25]. However, *Myog*-deleted adult mice are known to be resistant to neurogenic muscle atrophy due to the downregulation of proteolytic factors [26]. These studies provide a link between myogenin and mature muscle fiber turnover. *Myog* mRNA is preferentially expressed in adult slow-twitch (type 1) muscle fibers while *MyoD* transcripts are of greater abundance in fast-twitch (type 2) fibers [27]. Furthermore, the overexpression of myogenin in mature skeletal muscle tissue has been shown to incite shifts in enzymatic activity from

glycolytic to oxidative metabolism [28]. Thus myogenin makes for a good candidate gene to study when investigating the regulatory roles of MRF's in adult exercise and metabolism.

The recent generation of a mouse strain harboring the *Myog*<sup>flox</sup> allele and a Cre recombinase transgene allow for the study of myogenin in the adult mouse. In the present study, *Myog*-deleted adult mice exhibited an enhanced capacity for exercise in response to physical stress and were able to further deplete their glucose stores before reaching exhaustion. Furthermore, these animals displayed an enhanced response to exercise training when given long-term access to running wheels. Global gene expression analysis reveals alterations in the expression of metabolic enzymes in the absence of myogenin. Shifts in metabolic enzyme content may be contributing to the conferment of these observed phenomena.



## **RESULTS:**

### **Tamoxifen injection of adult *Myog*<sup>flox/flox</sup>:Cre+ mice results in efficient deletion of *Myog***

A mouse strain harboring the *Myog*<sup>flox</sup> allele and a Cre recombinase transgene was recently generated in the Klein laboratory [29]. While efficient deletion of *Myog* from the genome of these animals during the embryonic and perinatal stages of life had already been achieved, this had never been attempted during adult life. A single injection of tamoxifen at a dose of 10 mg/40 g of body weight was found to result in a greater than 90% deletion of genomic DNA (Figure 2A). *Myog* transcript levels were determined to be less than 5% of their wild-type counterparts (Figure 2B).

### ***Myog*-deleted adult mice exhibit an enhanced capacity for exercise and oxidative metabolism under low-intensity stress conditions**

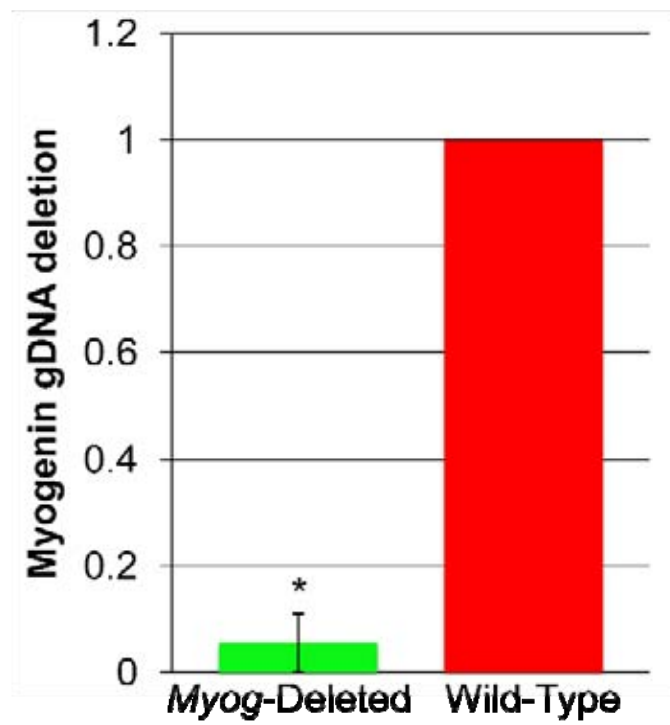
To determine the exercise capacity for *Myog*-deleted mice under low-intensity stress conditions, adult *Myog*-deleted mice were run to exhaustion at a slow, constant speed of 20m/min at 0° incline (Figure 3). These conditions are designed to assess endurance since they primarily employ the use of type 1 slow twitch muscle fibers and oxidative metabolism.

Wild-type mice (tamoxifen-injected *Myog*<sup>flox/flox</sup>) ran on average 4,000 meters before reaching exhaustion whereas their *Myog*-deleted counterparts ran almost 10,000 meters (Figure 4), a significant 2.4 fold difference. Remarkably, some of these animals ran for more than ten hours before reaching exhaustion.

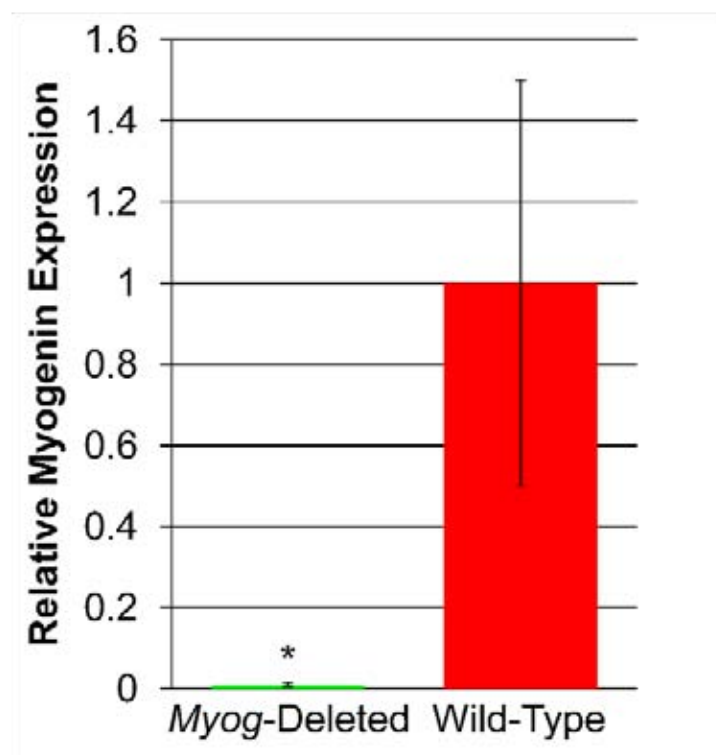
## Figure 2

Efficient deletion of *Myog* in the adult mouse. (A) A single tamoxifen injection at 10mg/40g body weight results in >90% deletion of *Myog* from *Myog*<sup>flox/flox</sup>;Cre+ mice (*Myog*-deleted, n=10; wild-type n=10). Green bar represents *Myog*-deleted values. Red bar represents wild-type control values. Error bars represent one standard deviation (\**P*<0.05). (B) *Myog* transcript levels are also reduced by over 95% in *Myog*<sup>flox/flox</sup>;Cre+ mice. Green bar represents *Myog*-deleted values (n=3). Red bar represents wild-type control values (n=3). Error bars represent one standard deviation (\**P*<0.05).

A.

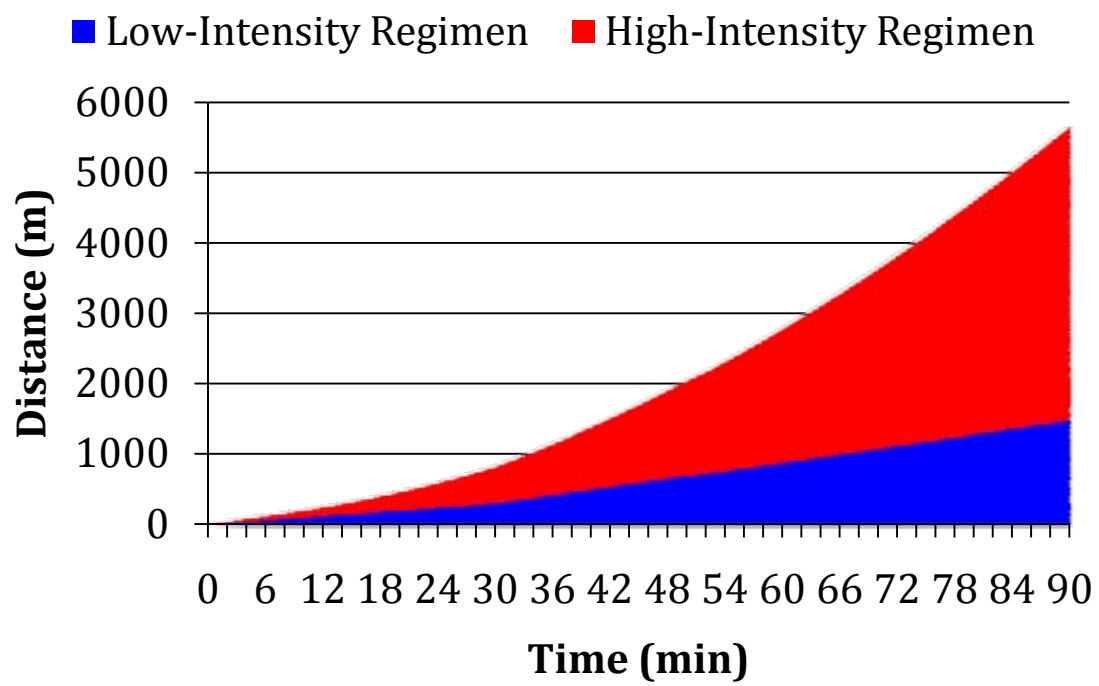


B.



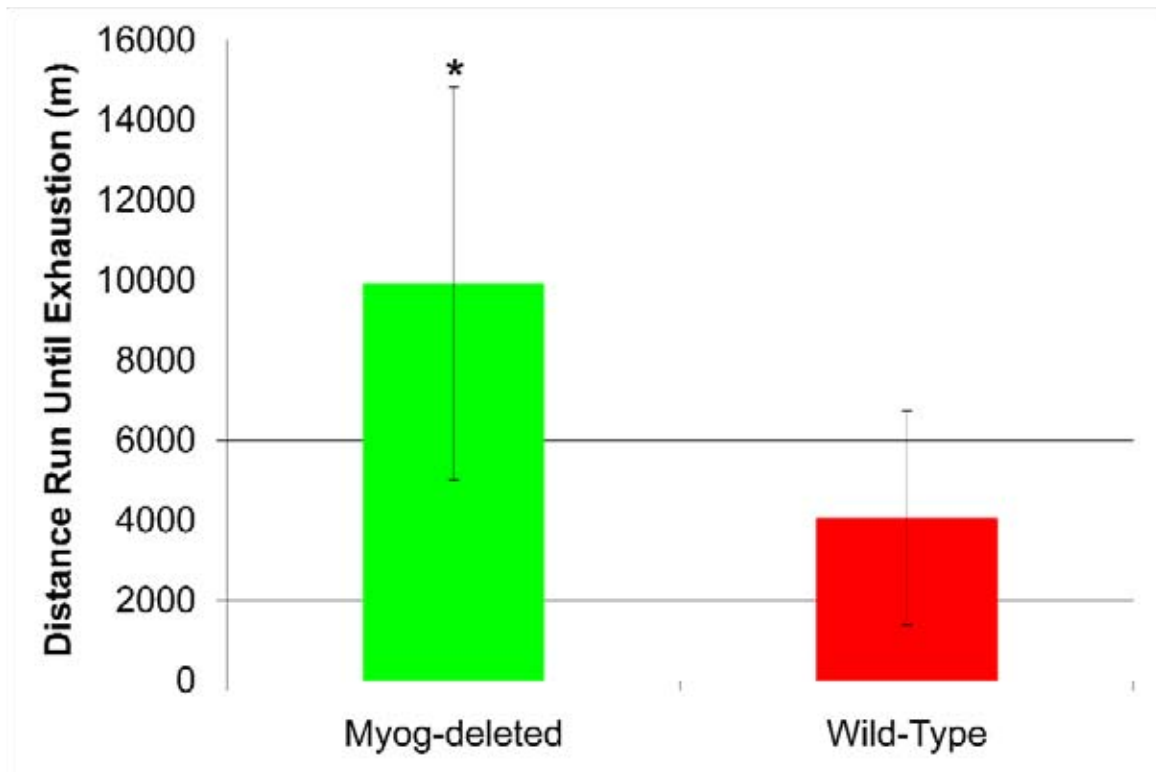
### **Figure 3**

Representation of low- and high-intensity treadmill exercise regimens. The low-intensity regimen (in blue) maintains a constant speed of 20m/min until exhaustion, resulting in the mice running the same distance over any given period of time after the initial warm up period. This regimen is designed to employ primarily oxidative metabolism. The high-intensity regimen (in red) entails increasing the speed of the treadmill by 2m/min every 2 minutes until exhaustion. This results in the mice running ever increasing distances over a given two minute period as time goes on. This regimen is designed to employ glycolytic metabolism.



#### **Figure 4**

Enhanced exercise capacity in *Myog*-deleted mice during the low-intensity exercise regimen. *Myog*-deleted mice ran 2.4 fold more than the wild-type control group under the low-intensity exercise regimen (*Myog*-deleted, n=4; wild-type n=5). Green bar represents *Myog*-deleted values. Red bar represents wild-type control values. Error bars represent one standard deviation (\* $P<0.05$ ).



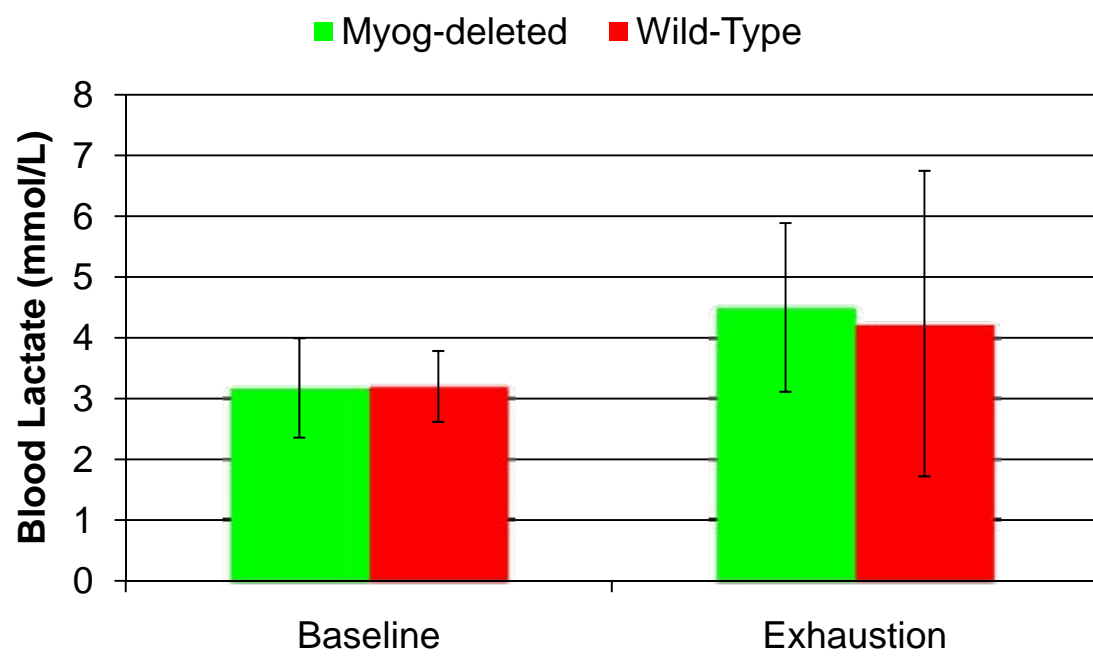
To begin investigating the observed difference in exercise capacity, blood lactate and glucose levels were recorded at baseline and exhaustion to gain insight to the metabolic processes at work. Lactate is a product of glycolysis, and increased lactate at exhaustion is a commonly used indicator of glycolytic metabolism. In contrast, unchanged lactate levels would be expected during the use of oxidative metabolism [30]. Under aerobic endurance conditions such as these, glucose accessed via the blood stream would be the expected fuel source whereas glucose converted from glycogen in the muscle would be primarily utilized during anaerobic stress conditions such as high-intensity sprinting.

Baseline blood lactate levels were virtually identical between the wild-type and *Myog*-deleted groups and increased only slightly at exhaustion (Figure 5). These constant blood lactate levels were consistent with the employment of oxidative metabolism during this aerobic, low-intensity exercise regimen. Blood glucose levels for both wild-type and *Myog*-deleted mice were virtually equal at baseline but were both dramatically decreased at exhaustion (Figure 6). Decreased blood glucose levels are also consistent with the use of oxidative metabolism during this assay. Moreover, blood glucose levels of *Myog*-deleted mice were less than half that of their wild-type counterparts at exhaustion. The data implied that *Myog*-deleted mice were able to further deplete their glucose stores before reaching exhaustion and utilized this as a source of energy. This may give *Myog*-deleted mice a competitive advantage by conferring upon them an enhanced capacity for exercise.



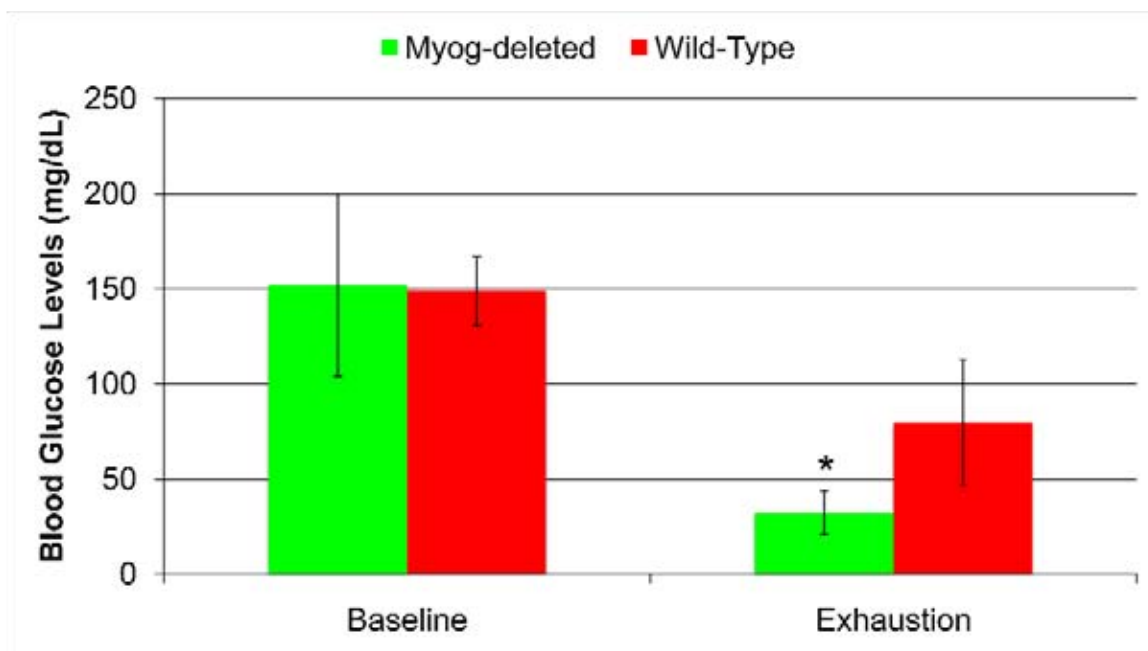
### **Figure 5**

Blood lactate levels in *Myog*-deleted and wild-type mice at baseline and after running to exhaustion under the low-intensity exercise regimen. At baseline, blood lactate levels were virtually equal between the *Myog*-deleted and wild-type groups at approximately 3 mmol/L (*Myog*-deleted, n=4; wild-type n=5). At exhaustion, blood lactate levels for both groups only modestly increased from baseline and were virtually equal between the *Myog*-deleted and wild-type groups at approximately 4.5 mmol/L. Green bars represent *Myog*-deleted values. Red bars represent wild-type control values. Error bars represent one standard deviation.



## Figure 6

Blood glucose levels in *Myog*-deleted and wild-type mice at baseline and after running to exhaustion under the low-intensity exercise regimen. At baseline, blood glucose levels were virtually equal between the *Myog*-deleted and wild-type groups at approximately 150 mg/dL (*Myog*-deleted, n=4; wild-type n=5). At exhaustion, blood glucose values were significantly reduced for both groups. However, *Myog*-deleted values were significantly less than wild-type control values. Green bars represent *Myog*-deleted values. Red bars represent wild-type control values. Error bars represent one standard deviation (\* $P<0.05$ ).



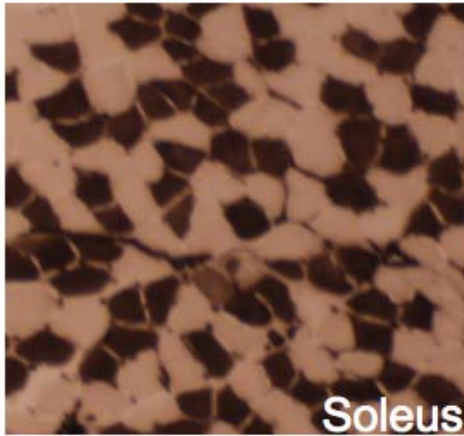
To investigate metabolic and physiological changes in the muscle fibers of *Myog*-deleted mice, analyses were performed to determine fiber type proportions, expression levels of succinate dehydrogenase (SDH), transcript abundance of genes regulating fatty acid metabolism, and metabolic enzyme activity levels.

Fiber typing analysis indicated there was no difference in the proportions of type 1 and type 2 muscle fibers between wild-type and *Myog*-deleted mice (Figure 7). This was expected as these mice had not been exposed to long-term training conditions. Transcript levels for Myosins 1, 2A, and 2B were also determined to be equal between the two groups in the gastrocnemius muscle (Figure 8). SDH is a component of the mitochondrial electron transport chain, and its prevalence is an indicator of overall mitochondrial activity and the aerobic potential of muscle fibers. Assays for SDH revealed equal levels of activity in both groups (Figure 9). No differences were detected in the transcript levels of a panel of genes regulating fatty acid metabolism and Glut4 (Figure 10). Furthermore, assays detecting the enzymatic activities for a panel of both oxidative and glycolytic enzymes determined there to be no differences between the wild-type and *Myog*-deleted groups in either the slow twitch soleus muscle or the fast twitch extensor digitorum longus muscle (Figure 11). Thus, *Myog*-deleted adult mice outran their wild-type counterparts under slow, oxidative conditions but did not display the typical characteristic changes commonly associated with enhanced aerobic exercise capacity.

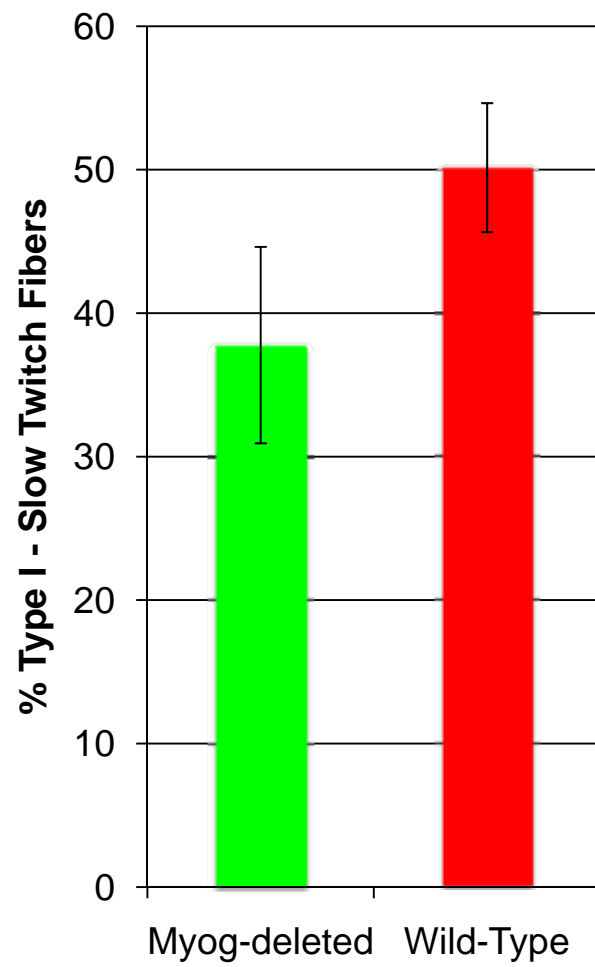
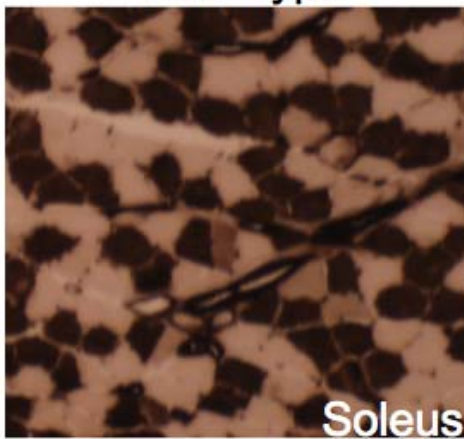
### **Figure 7**

Fiber type proportion in *Myog*-deleted and wild-type mice run to exhaustion under the low-intensity regimen. *Myog*-deleted and wild-type mice did not exhibit a significant difference in the proportions of Type 1 and Type 2 muscle fibers in the soleus muscle (*Myog*-deleted, n=3; wild-type n=3). Green bar represents *Myog*-deleted values. Red bar represents wild-type control values. Error bars represent one standard deviation.

*Myog*-deleted



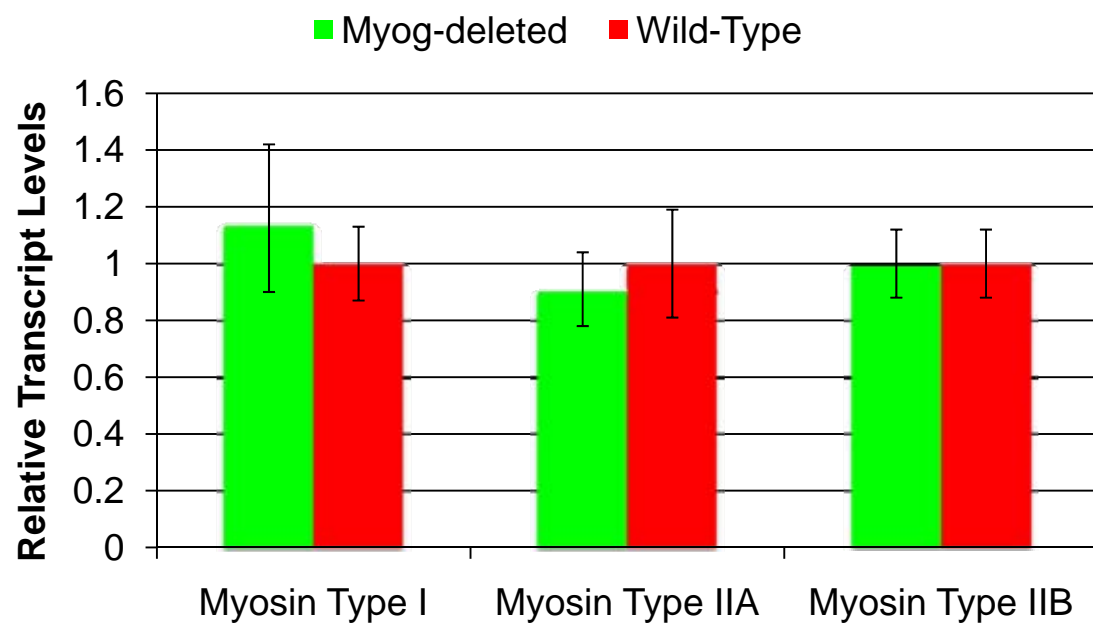
Wild-Type



### **Figure 8**

Transcript expression levels of Myosins Type 1, 2A, and 2B. *Myog*-deleted and wild-type mice did not exhibit a significant difference in the expression of these factors in the Gastrocnemius muscle (*Myog*-deleted, n=3; wild-type n=3). Green bars represent *Myog*-deleted values. Red bars represent wild-type control values. Error bars represent one standard deviation.

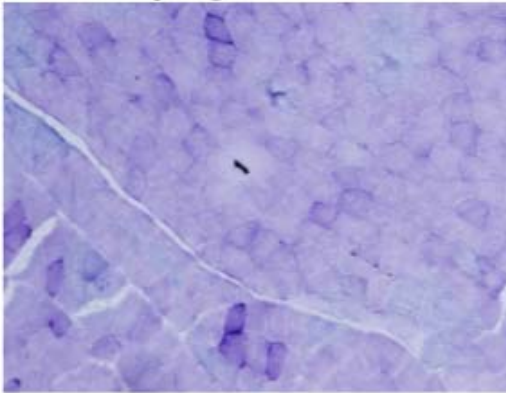




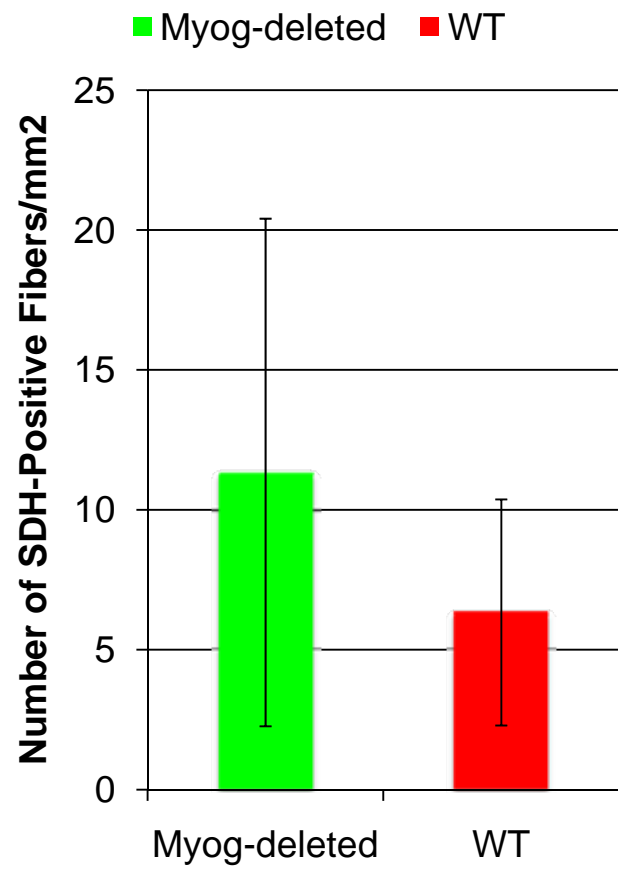
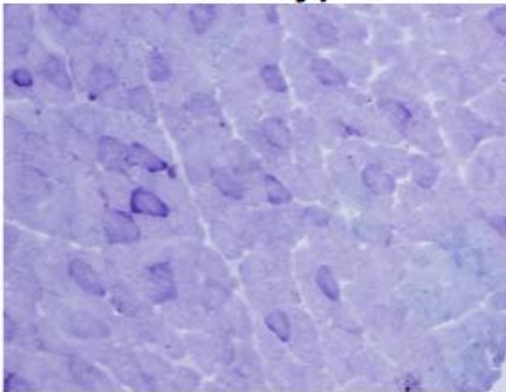
### Figure 9

Succinate dehydrogenase (SDH) activity in *Myog*-deleted and wild-type mice run to exhaustion under the low-intensity regimen. *Myog*-deleted and wild-type mice did not exhibit a significant difference in SDH activity in the Gastrocnemius muscle (*Myog*-deleted, n=3; wild-type n=3). Green bar represents *Myog*-deleted values. Red bar represents wild-type control values. Error bars represent one standard deviation.

**Myog-Deleted**

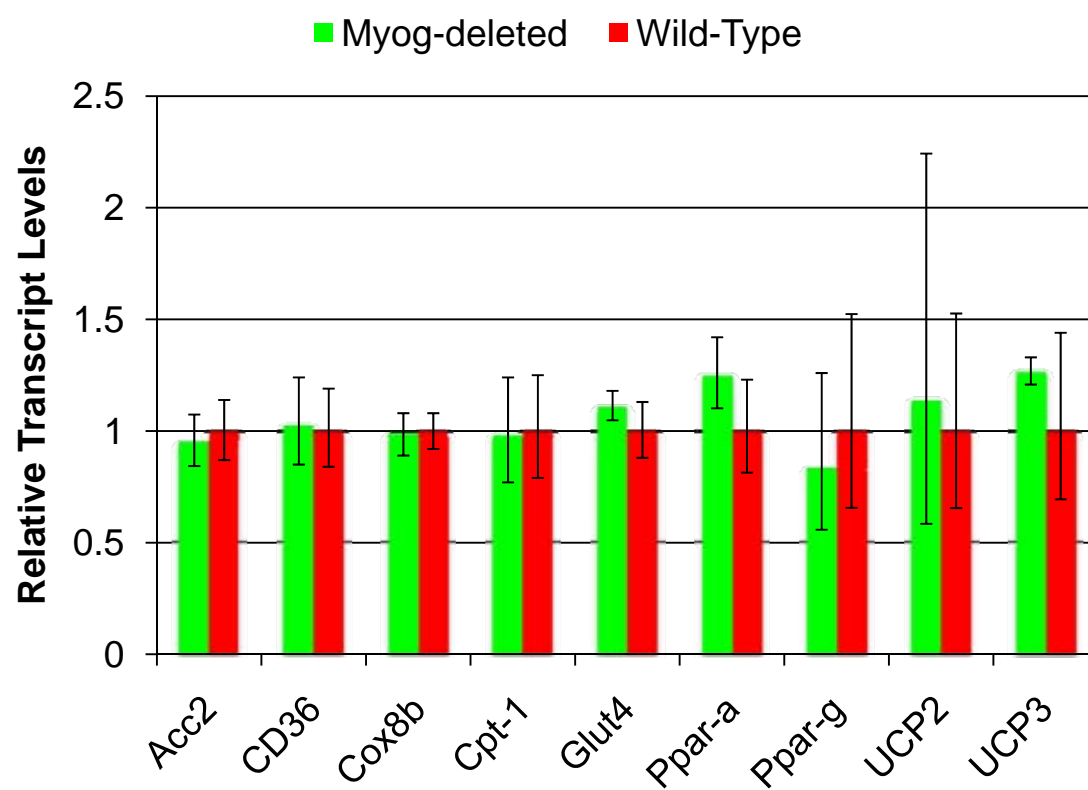


**Wild-Type**



## Figure 10

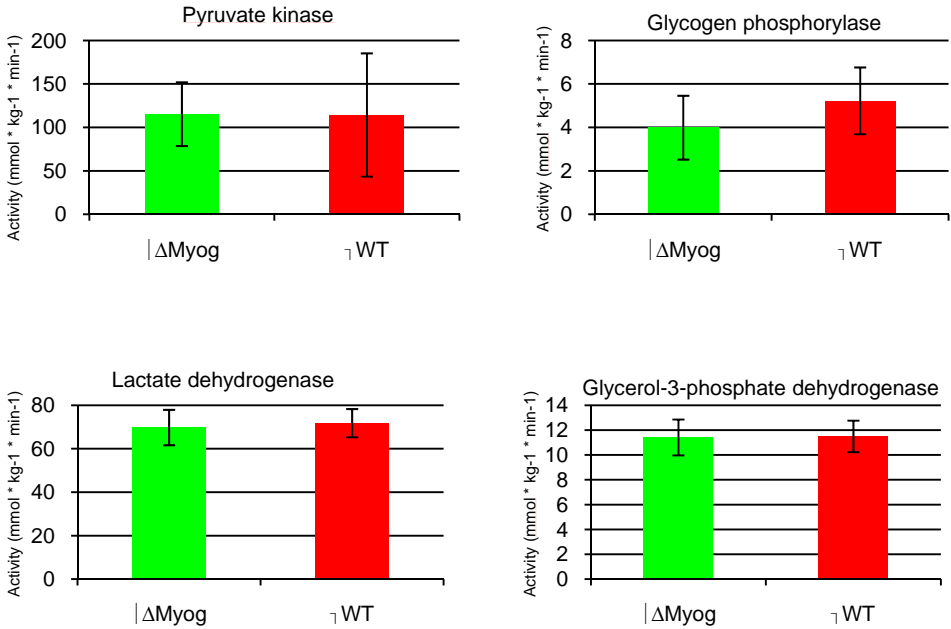
Transcript expression levels of genes regulating fatty acid metabolism. *Myog*-deleted and wild-type mice did not exhibit a significant difference in the expression of these factors in the Gastrocnemius muscle (*Myog*-deleted, n=3; wild-type n=3). Green bars represent *Myog*-deleted values. Red bars represent wild-type control values. Error bars represent one standard deviation. From left to right: *Acc2* - Acetyl-Coenzyme A carboxylase beta, *CD36* - Cluster of Differentiation 36, *Cox8b* - Cytochrome C oxidase subunit 8b, *Cpt-1* - Carnitine Palmitoyltransferase I, *Glut4* - Glucose Transporter Type 4, *Ppar-a* - Peroxisome Proliferator-Activated Receptor Alpha, *Ppar-g* - Peroxisome Proliferator-Activated Receptor Gamma, *UCP2* - Mitochondrial Uncoupling Protein 2, *UCP3* - Mitochondrial Uncoupling Protein 3.



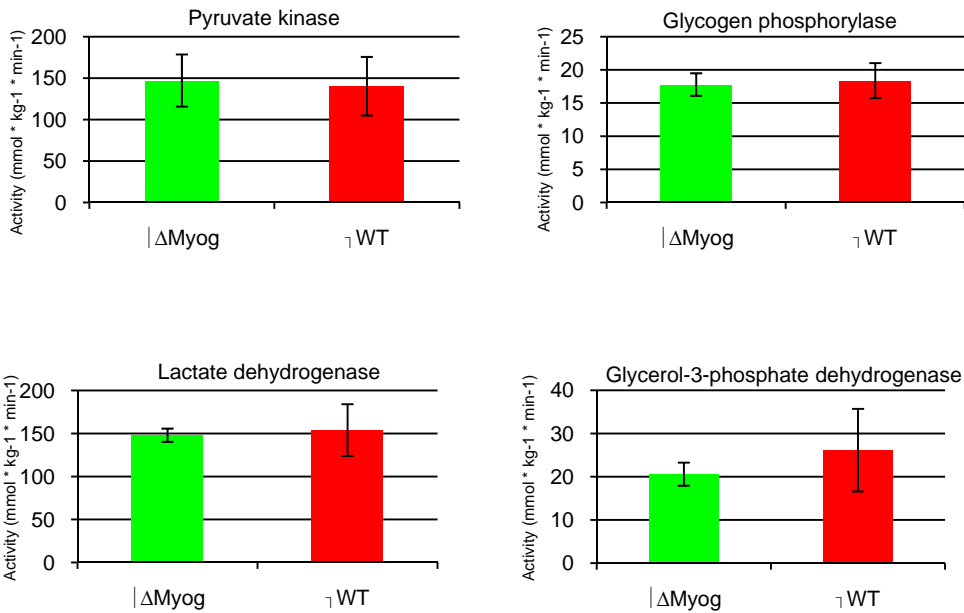
### **Figure 11**

Enzymatic activity levels of factors regulating glycolytic and oxidative metabolism. *Myog*-deleted and wild-type mice did not exhibit any significant differences in enzymatic activity for the factors tested in the Soleus or Extensor Digitorum Longus muscles (*Myog*-deleted, n=3; wild-type n=3). Green bars represent *Myog*-deleted values. Red bars represent wild-type control values. Error bars represent one standard deviation.

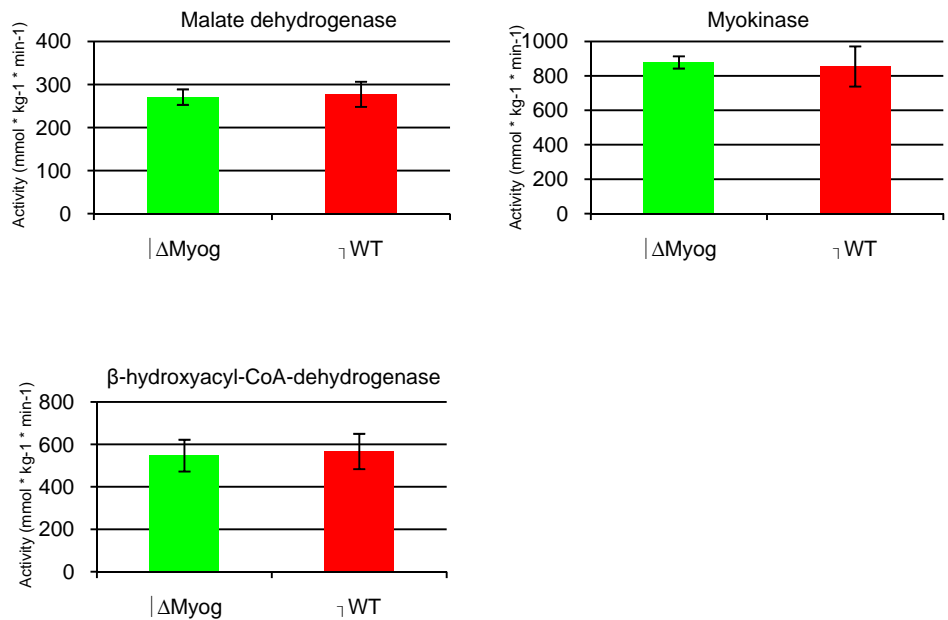
**Glycolytic enzymes activities: Soleus Muscle**



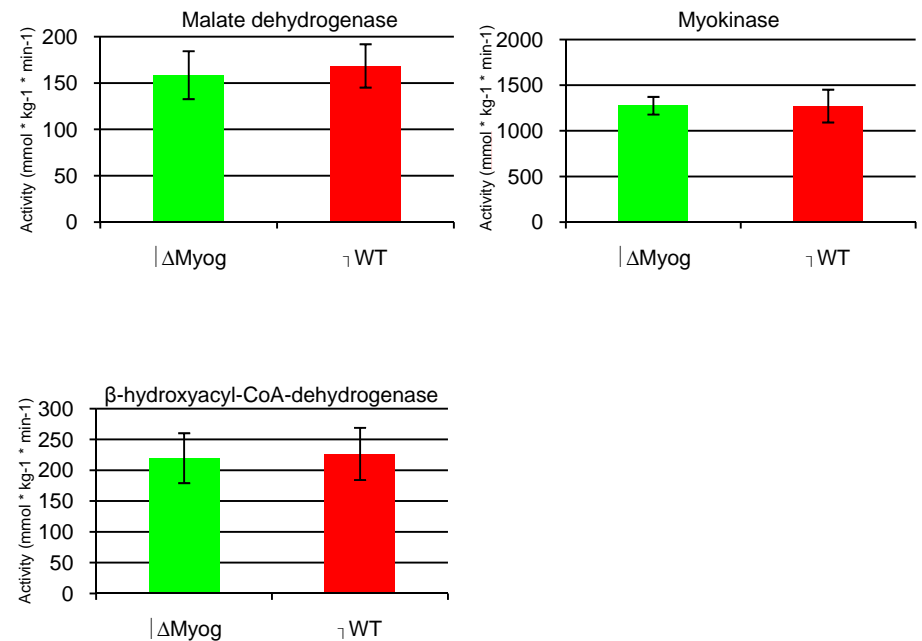
**Glycolytic enzymes activities: extensor digitorum longus (EDL) muscle**



**Oxidative enzymes activities: soleus muscle**



**Oxidative enzymes activities: extensor digitorum longus (EDL) muscle**





### ***Myog*-deleted adult mice exhibit an enhanced capacity for exercise and glycolytic metabolism under high-intensity stress conditions**

To determine the exercise capacity for *Myog*-deleted mice under high-intensity stress conditions, adult animals were run at consistently increasing speeds to exhaustion at 10° incline (Figure 2). These conditions are designed to assess the sprinting aptitude of the mice and employ the use of fast twitch muscle fibers and glycolytic metabolism.

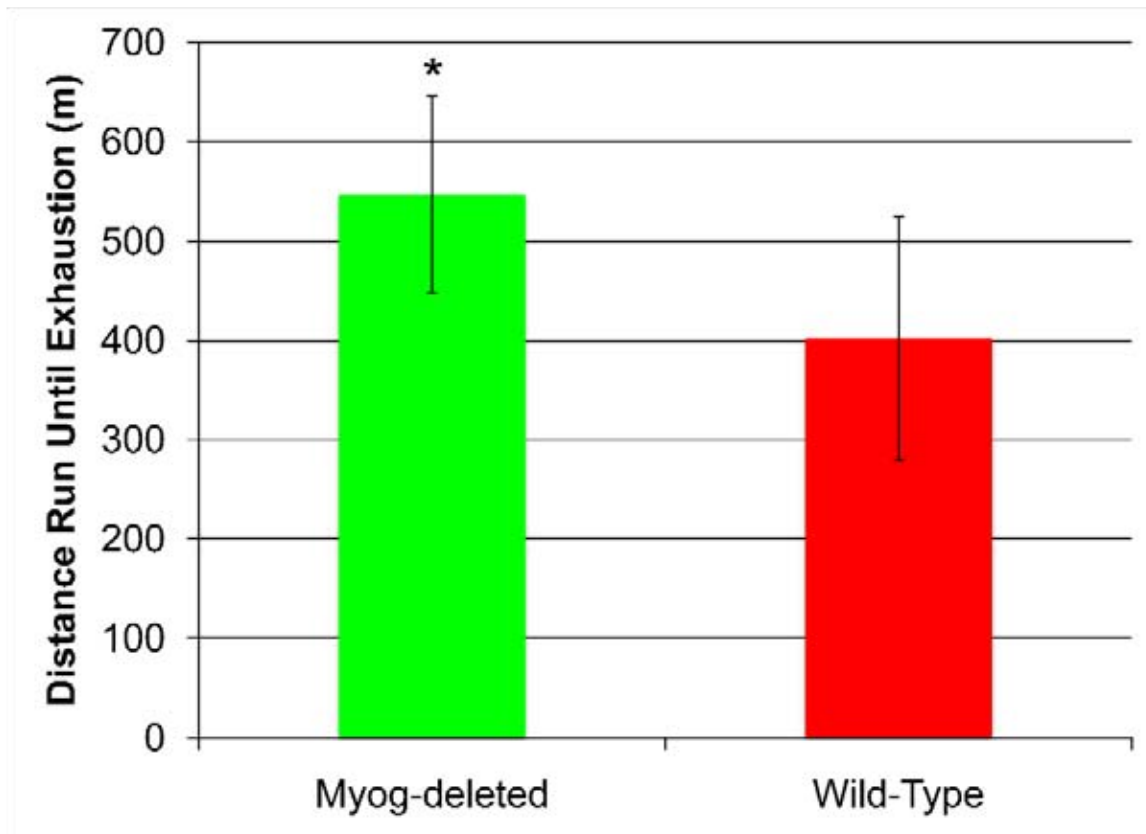
*Myog*-deleted mice ran on average 36% more than their wild-type counterparts 14 days post-deletion (Figure 12). The appearance of the phenotype at this early time point implied the enhanced exercise capacity was due to more sudden changes in fiber composition such as alterations in metabolic enzyme expression levels as opposed to long term modifications such as shifts in fiber type proportions.

At six months post-deletion, *Myog*-deleted mice ran 1.6 fold more than their wild-type counterparts over the course of 12 separate experiments conducted during consecutive days (Figure 13). This indicated the phenotype persists over the long term and suggested the difference in exercise capacity increased over time.

Blood lactate levels increased dramatically in both groups at exhaustion (Figure 14). Increased blood lactate levels were consistent with the use of glycolytic metabolism during this high-intensity exercise regimen. Moreover, *Myog*-deleted levels were 52% higher than that of their wild-type counterparts at exhaustion. However, at the wild-type exhaustion point, blood lactate levels of

## Figure 12

Enhanced exercise capacity in *Myog*-deleted mice during the high-intensity exercise regimen. *Myog*-deleted mice ran 36% more than the wild-type control group under high-intensity exercise conditions at 14 days post-deletion (*Myog*-deleted, n=24; wild-type n=24). Green bar represents *Myog*-deleted values. Red bar represents wild-type control values. Error bars represent one standard deviation (\* $P<0.05$ ).



### Figure 13

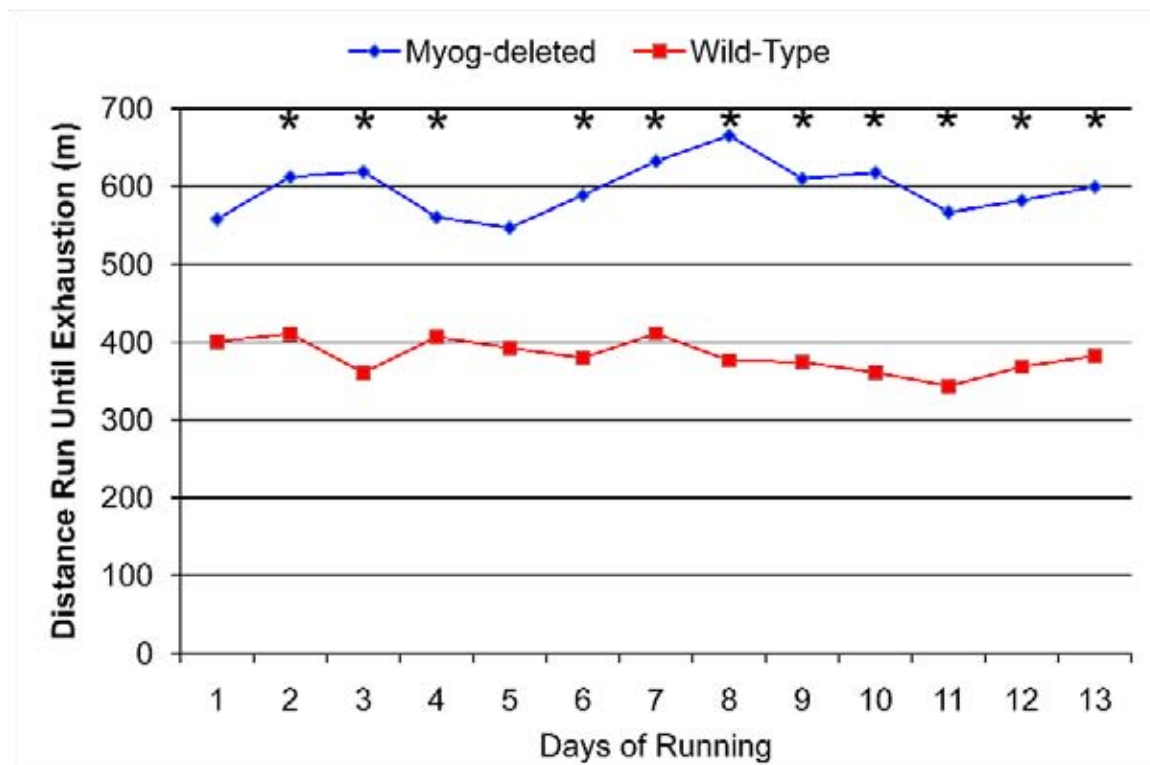
Enhanced exercise capacity in *Myog*-deleted mice during the high-intensity exercise regimen. *Myog*-deleted mice ran an average of 57% more than the wild-type control group under high-intensity exercise conditions at 6 months post-deletion for 12 consecutive days (*Myog*-deleted, n=8; wild-type n=7).

Green bar (A) and green line (B) represent *Myog*-deleted values. Red bar (A) and red line (B) represent wild-type control values. Error bars represent one standard deviation (\* $P < 0.05$ ).

A.

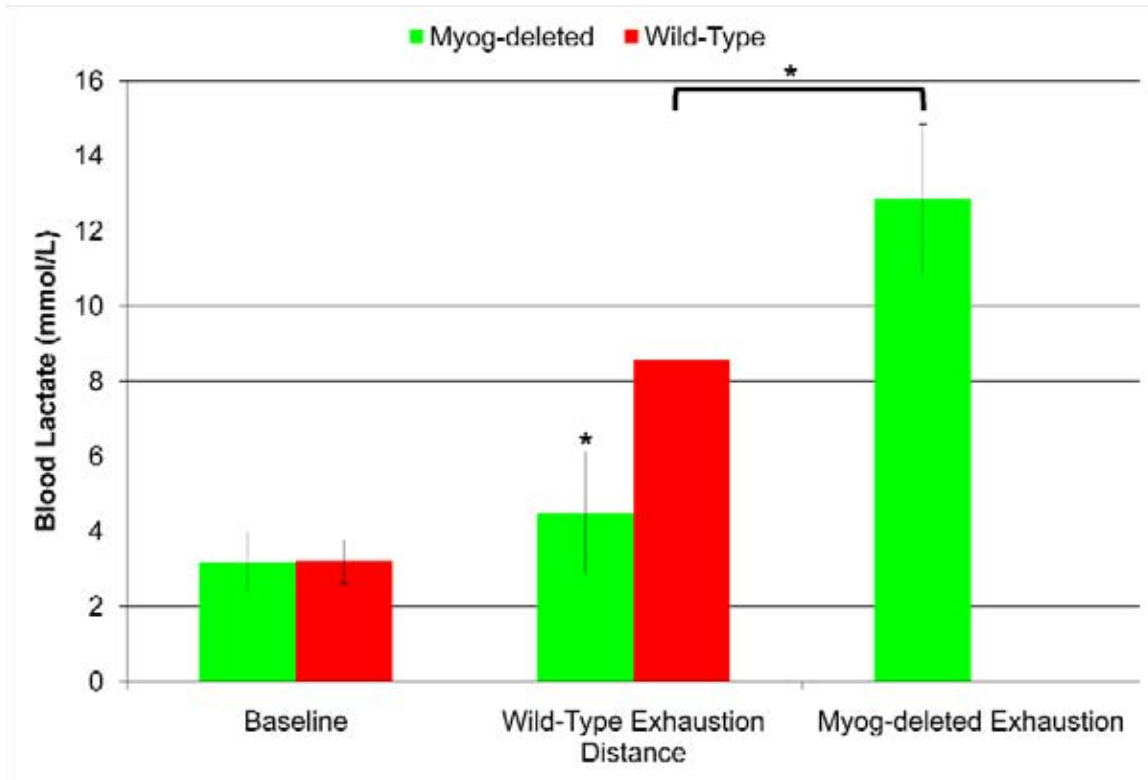


B.



## Figure 14

Blood lactate levels in *Myog*-deleted and wild-type mice at baseline and after running to exhaustion under the high-intensity exercise regimen. Wild-type blood lactate levels at exhaustion were increased by 168% over the baseline values. At the wild-type exhaustion time point, there was no significant increase in the *Myog*-deleted group. *Myog*-deleted blood lactate values at exhaustion had increased by 305% over the baseline values. These levels were also 50% greater than the wild-type blood lactate values at exhaustion (*Myog*-deleted, n=8; wild-type n=7). Green bars represent *Myog*-deleted values. Red bars represent wild-type control values. Error bars represent one standard deviation (\* $P < 0.05$ ).



*Myog*-deleted mice were significantly less than that of the wild-type group. These data suggested that *Myog*-deleted mice did not build up lactate as quickly and possessed a higher lactate concentration threshold before reaching exhaustion. This may be expected as previous studies have shown that lactate production may actually retard muscle acidosis.

Blood glucose levels at exhaustion remained constant for the wild-type group but decreased by 33% for *Myog*-deleted mice (Figure 15). Constant blood glucose levels in the wild-type group suggested the use of glycolytic metabolism during this high-intensity exercise regimen. The decrease in blood glucose for *Myog*-deleted mice indicated that these animals were able to further tap into their glycogen reserves and utilize this as a fuel source to power their glycolytic metabolism. Increased utilization of glucose might be conferring the observed enhanced exercise capacity.

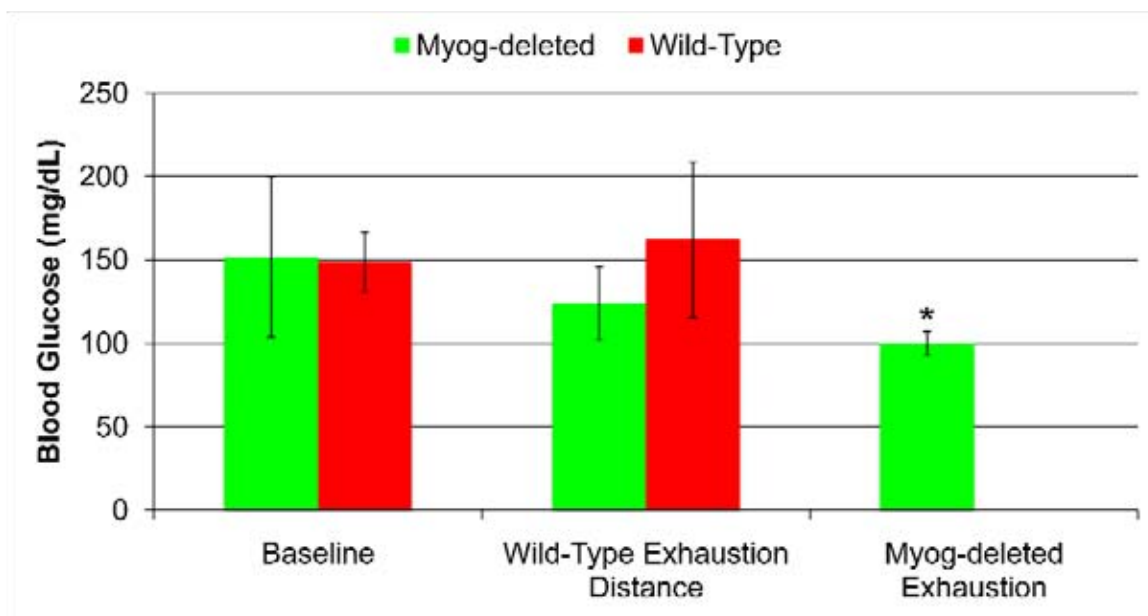
### **Hypoglycemic *Myog*-deleted adult mice no longer exhibit an enhanced capacity for exercise and glycolytic metabolism under high-intensity stress conditions**

To determine if the ability of *Myog*-deleted mice to further deplete their glycogen stores was responsible for the observed enhanced exercise capacity, mice were fasted to deplete their glycogen reserves and run on a daily basis until no difference in running capacity was observed between the *Myog*-deleted and wild-type groups. Baseline and exhaustion blood lactate and glucose levels were then recorded. After 96 hours of fasting, *Myog*-deleted mice reached



### Figure 15

Blood glucose levels in *Myog*-deleted and wild-type mice at baseline and after running to exhaustion under the high-intensity exercise regimen. Wild-type blood glucose levels at exhaustion were unchanged from the baseline values. *Myog*-deleted values at that same time point showed a general downward trend, but the difference was not significant. *Myog*-deleted blood glucose levels at exhaustion were significantly reduced from their baseline values (*Myog*-deleted, n=8; wild-type n=7). Green bars represent *Myog*-deleted values. Red bars represent wild-type control values. Error bars represent one standard deviation (\* $P < 0.05$ ).



exhaustion 10% faster than their wild-type counterparts. Thus, they no longer displayed an enhanced capacity for exercise at this point (Figure 16).

Baseline blood glucose levels of mice fasted for 96 hours were approximately half those of the same mice under normal dietary conditions, indicating that their glycogen stores had been depleted (Figure 17). Blood lactate levels at exhaustion were approximately twice that of baseline for both wild-type and *Myog*-deleted mice, but there was no longer a difference between the two groups (Figure 18). This suggested the glycolytic metabolism of *Myog*-deleted mice was no longer enhanced under hypoglycemic conditions. Blood glucose levels at exhaustion were not significantly altered from the fasting baseline for either group (Figure 17). Furthermore, there was no longer a difference between the two groups. These data implied that because glucose levels were already depleted before exhaustion, *Myog*-deleted mice were no longer able to further access their glycogen stores as a source of additional energy and thus ran the same distance as wild-type mice.

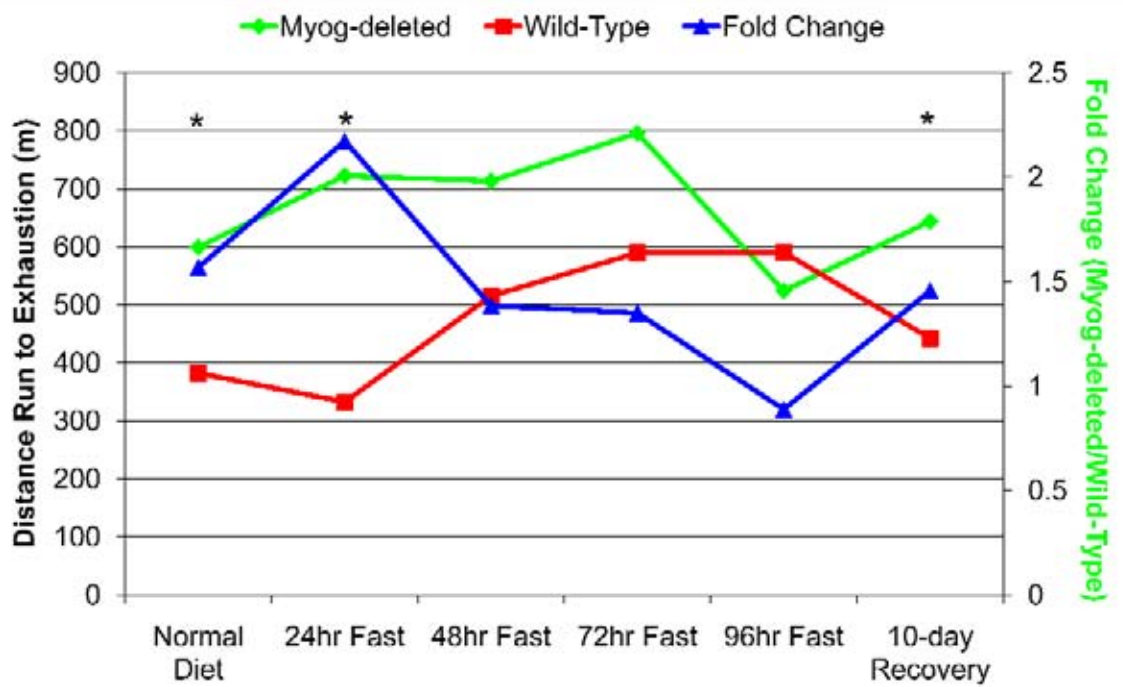
After 96 hours of fasting, normal dietary conditions were restored. After ten days of recovery, by which time glucose levels would be recovered, the *Myog*-deleted group again ran significantly more than their wild-type counterparts showing an enhanced capacity for exercise.

### ***Myog*-deleted mice exhibit an enhanced response to exercise training**

To determine the ability of *Myog*-deleted animals to respond to exercise training, mice were housed in cages with unrestricted access to exercise wheels

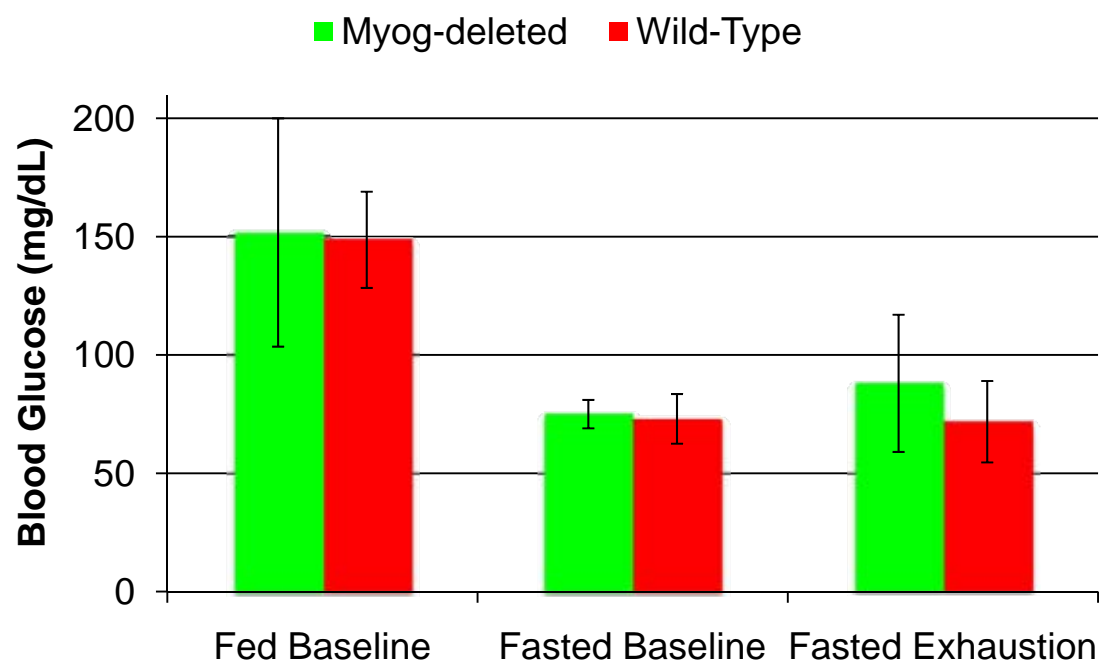
### Figure 16

High intensity running under fasting conditions. After 96 hours of fasting, *Myog*-deleted mice ran slightly less than their wild-type counterparts before reaching exhaustion. Ten days after receiving their food back, *Myog*-deleted mice once again exhibited an enhanced capacity for exercise, running significant more than wild-type mice. Green line represents *Myog*-deleted values. Red line represents wild-type control values. Blue line represents fold change (*Myog*-deleted/Wild-type). Error bars represent one standard deviation (\* $P < 0.05$ ).



### **Figure 17**

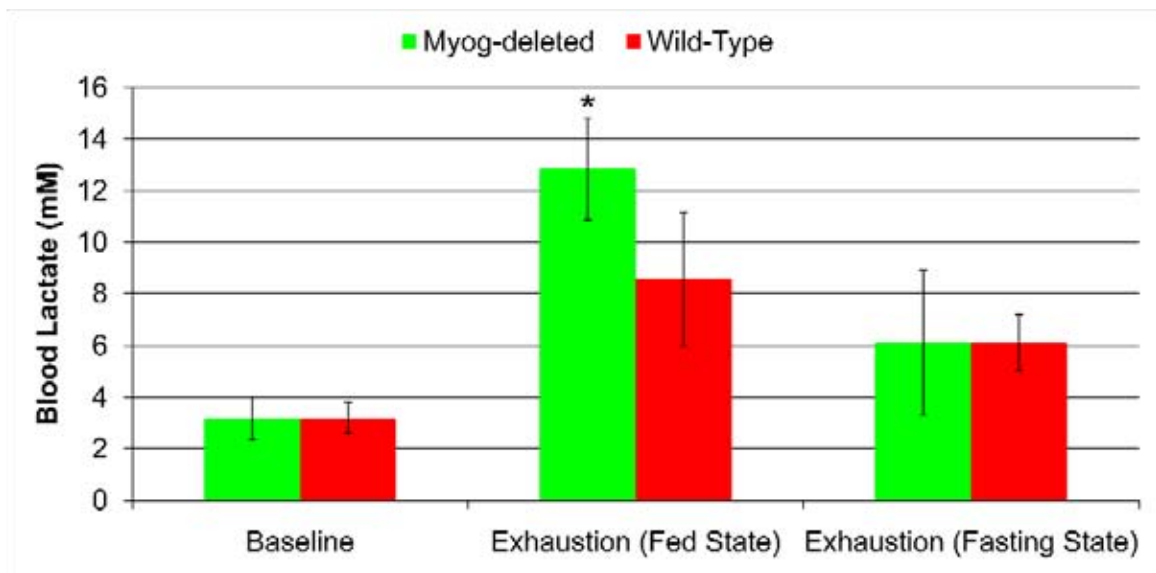
Blood glucose levels in *Myog*-deleted and wild-type mice under fasting conditions at baseline and after running to exhaustion under the high-intensity exercise regimen. *Myog*-deleted and wild-type blood glucose levels under fasting conditions at baseline were approximately half that of the fed baseline levels, indicating these animals were hypoglycemic. At exhaustion, there was no significant decrease in blood glucose for either group, and there was no difference between the two groups. Green bars represent *Myog*-deleted values. Red bars represent wild-type control values. Error bars represent one standard deviation.



### Figure 18

Blood lactate levels in *Myog*-deleted and wild-type mice under fasting conditions at baseline and after running to exhaustion under the high-intensity exercise regimen. At exhaustion, blood lactate levels in *Myog*-deleted and wild-type mice were both approximately twice that of baseline values, but there was no longer a difference between the two groups. Green bars represent *Myog*-deleted values. Red bars represent wild-type control values. Error bars represent one standard deviation (\* $P < 0.05$ ).





outfitted with a mechanism allowing for the application of a loaded resistance for a period of six months and then subjected to the high-intensity exercise regimen. Adaptation to exercise is a long-term process and is generally accompanied by fiber type changes, increased mitochondrial content, and an improved capillary to fiber ratio [31].

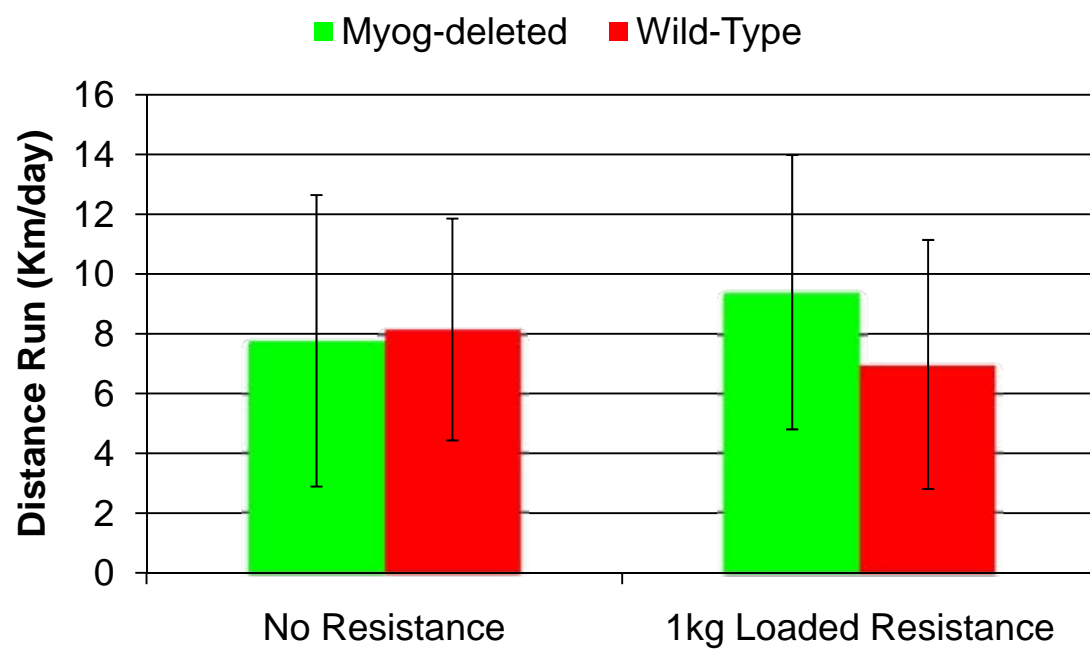
Under resistance-free conditions on the running wheels, wild-type and *Myog*-deleted mice ran virtually equal distances (Figure 19). When a 1 kg loaded resistance was added to the wheels, *Myog*-deleted mice ran on average 32% farther than their wild-type counterparts for 19 consecutive days. While compelling, this trend toward increased voluntary running was not statistically significant.

Trained wild-type mice, those housed in exercise cages for the previous six months, ran 1.8 fold farther to exhaustion under the high-intensity exercise regimen than untrained mice, while trained *Myog*-deleted mice ran 2.2 fold farther to exhaustion than their untrained counterparts (Figure 20). Thus, the exercise capacity of trained *Myog*-deleted mice increased by an additional 21% as compared to wild-type animals. This implied myogenin indirectly regulated the pathways controlling long-term metabolic and physiological processes in the muscle fibers.

To investigate the metabolic and physiological changes as a result of training in the muscle fibers of these animals, histological analyses were performed to determine fiber type proportions and overall SDH activity. Both wild-type and *Myog*-deleted groups displayed increases in type 1 muscle fibers

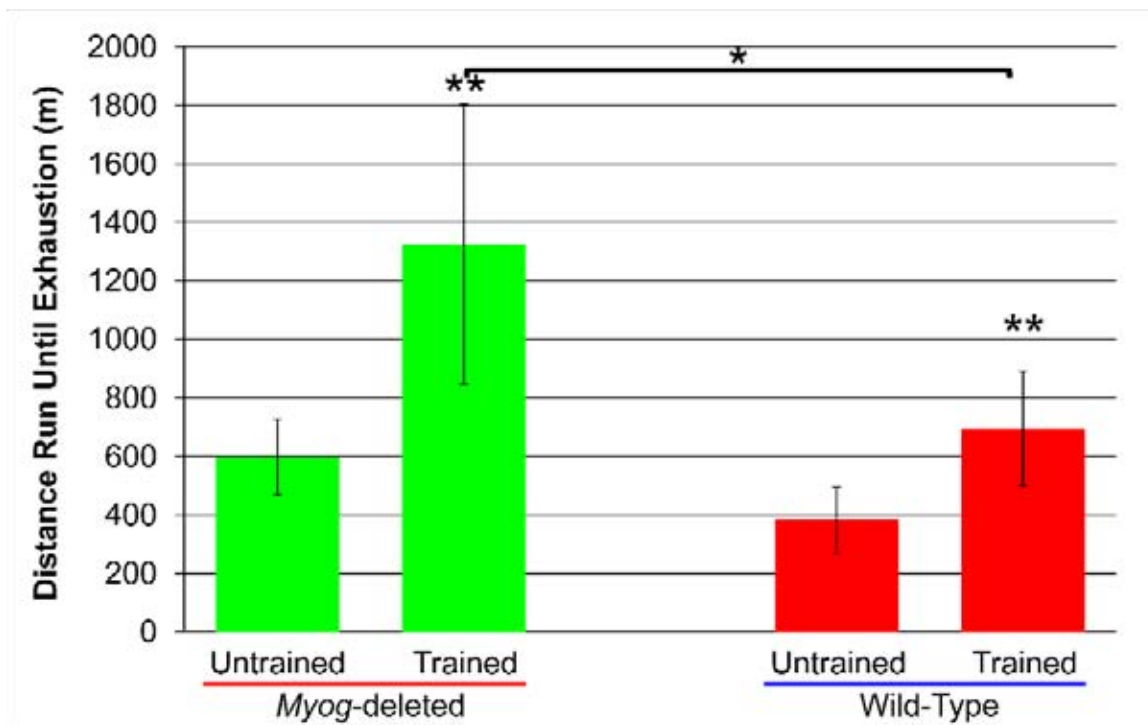
### Figure 19

Voluntary wheel running under no resistance and 1kg loaded resistance conditions. Under no resistance conditions at 4 to 5 months post-deletion, wild-type and *Myog*-deleted mice exhibited no differences in voluntary running over the course of 18 consecutive days of running. Under a 1kg loaded resistance at 5 to 6 months post-deletion, *Myog*-deleted mice exhibited a nonstatistical trend toward increased running as compared to the wild-type group over 18 consecutive days of running. Green bars represent *Myog*-deleted values (n=11). Red bars represent wild-type control values (n=11). Error bars represent one standard deviation (\* $P < 0.05$ ).



## Figure 20

Enhanced response to exercise training in *Myog*-deleted mice. After 6 months of exercise training on voluntary running wheels, both wild-type and *Myog*-deleted mice exhibited increased running capacities under the high-intensity exercise regimen. When making a comparison between the untrained and trained values for each genotype, *Myog*-deleted mice displayed significantly greater gains in running capacity over the wild-type group (Untrained *Myog*-deleted, n=8; Trained *Myog*-deleted, n=11; Untrained wild-type, n=7; Trained wild-type, n=10). Green bars represent *Myog*-deleted values (n=11). Red bars represent wild-type control values (n=11). Error bars represent one standard deviation (\* $P<0.05$ ).



after training (Figure 21), but this gain was greater in the *Myog*-deleted animals, causing the difference between the wild-type and *Myog*-deleted groups after training to be statistically significant. This shift in fiber type proportions suggested the loss of myogenin promoted gradual changes in MyHC isoform expression and fiber contraction speed. Determination of SDH activity revealed significantly more positive fibers in the gastrocnemius for both the wild-type and *Myog*-deleted groups after training (Figure 22). However, this gain was greater in the *Myog*-deleted animals, causing the difference between the wild-type and *Myog*-deleted groups after training to be statistically significant. Further increased SDH levels suggested that these fibers possessed an increase in number and distribution of mitochondria and had greater overall oxidative potential.

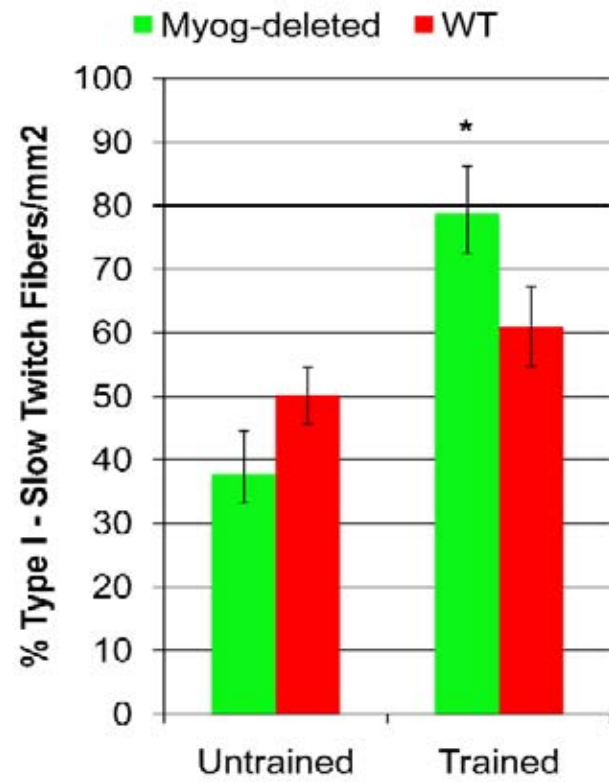
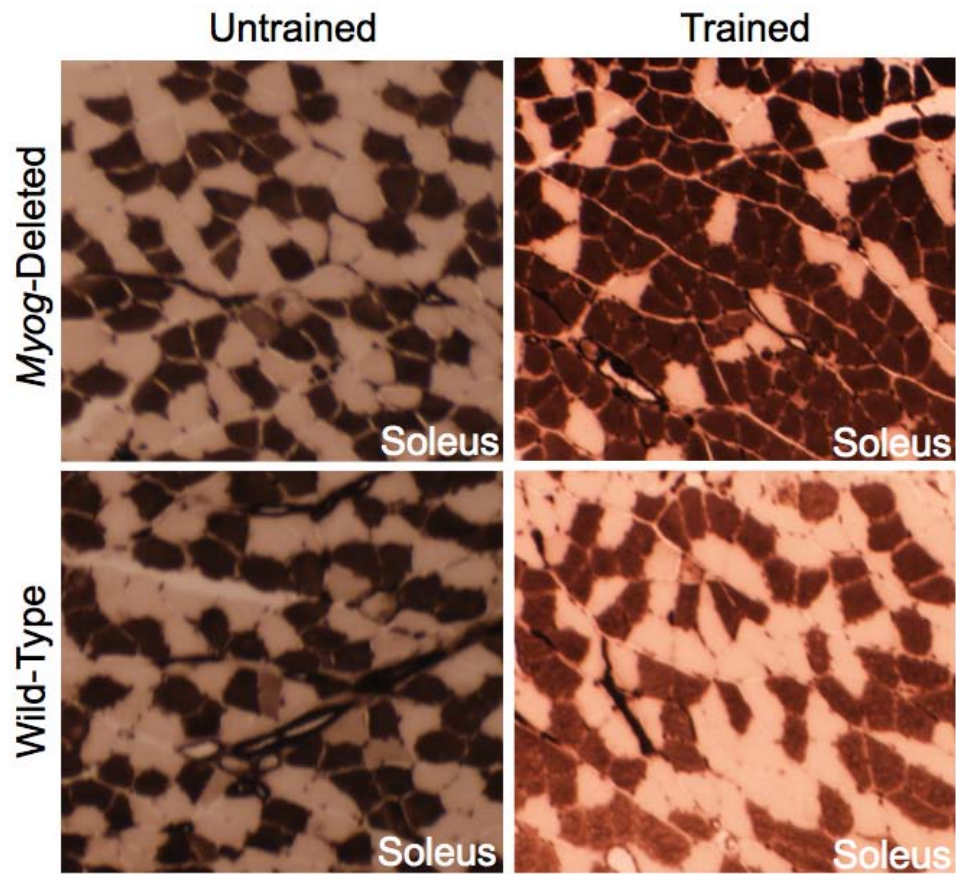
### **Myogenin may regulate genes controlling metabolic processes**

To begin establishing a basis for elucidating the mechanisms driving enhanced exercise capacity, putative myogenin gene targets were identified in adult skeletal muscle via global gene expression profiling followed by qRT-PCR. Hybridization with adult wild-type and *Myog*-deleted probes identified multiple genes with altered expression in the absence of myogenin (Table 1). Genes regulating metabolic processes including 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*Pfkfb3*), pyruvate carboxylase (*Pc*), dihydropyrimidine dehydrogenase (*Dpyd*), and 3-hydroxybutyrate

### **Figure 21**

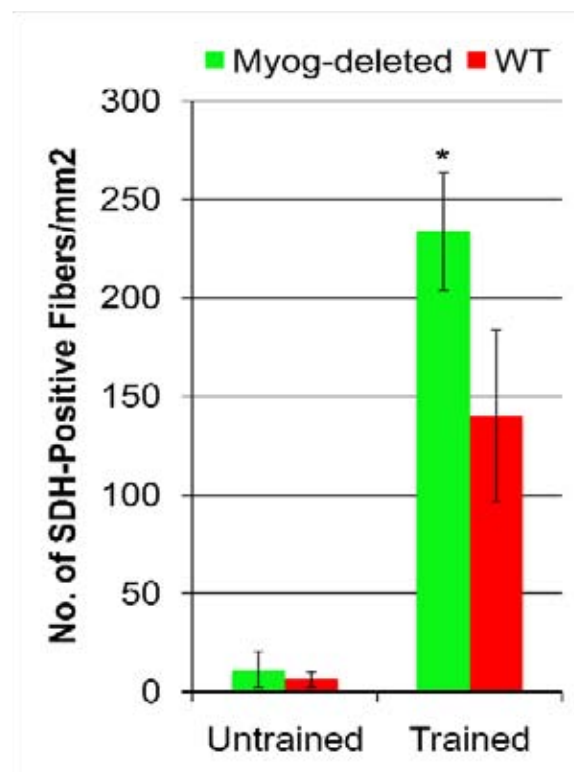
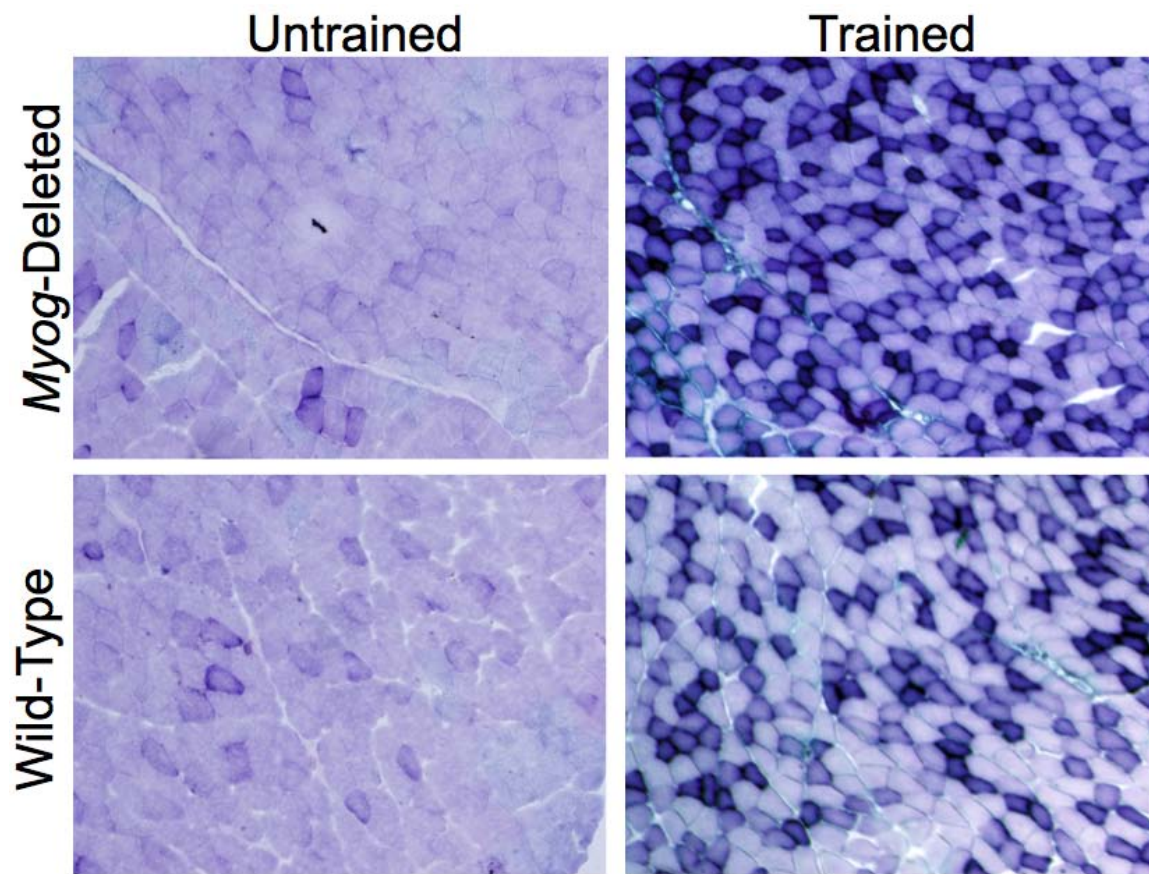
Increased proportion of type 1 muscle fibers in hindlimb muscle of trained *Myog*-deleted mice. Untrained wild-type and *Myog*-deleted mice exhibited no significant differences in type 1 muscle fibers (dark fibers). After 6 months of continuous training in voluntary running wheels, trained *Myog*-deleted mice displayed significantly more type 1 muscle fibers over the trained wild-type group. Green bars represent *Myog*-deleted values (n=3). Red bars represent wild-type control values (n=3). Error bars represent one standard deviation (\* $P<0.05$ ).





## Figure 22

Increased proportion of succinate dehydrogenase (SDH) positive fibers in hindlimb muscle of trained *Myog*-deleted mice. Untrained wild-type and *Myog*-deleted mice exhibited no significant differences in SDH positive muscle fibers. After 6 months of continuous training in voluntary running wheels, trained *Myog*-deleted mice displayed significantly more SDH positive muscle fibers over the trained wild-type group. Green bars represent *Myog*-deleted values (n=3). Red bars represent wild-type control values (n=3). Error bars represent one standard deviation (\* $P < 0.05$ ).



**Table 1**

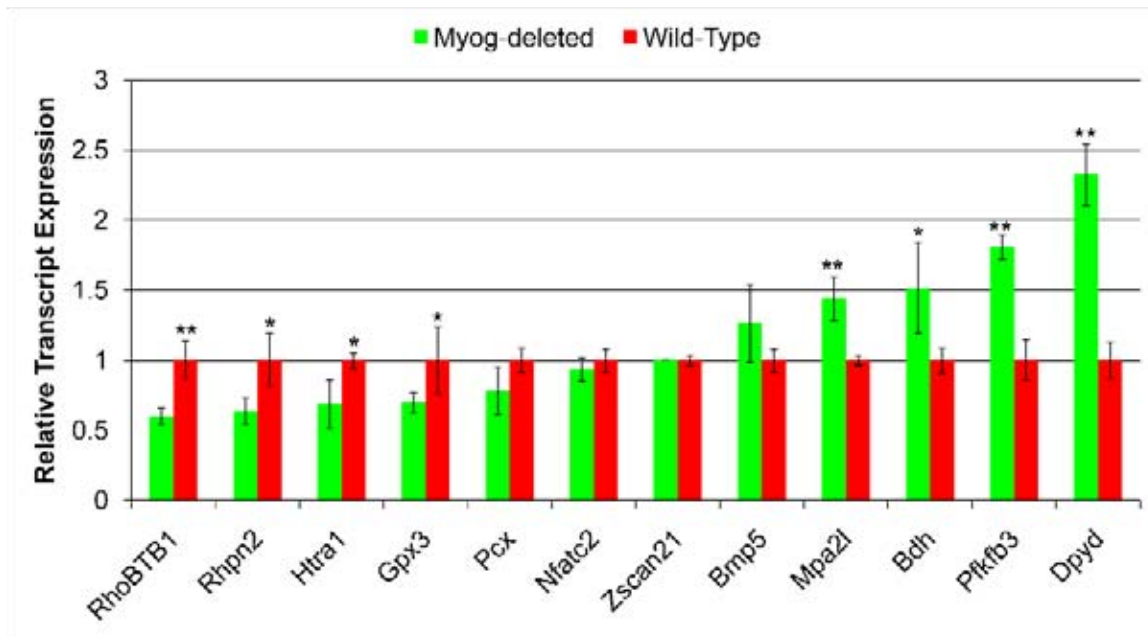
Genes identified to have significantly altered expression in the absence of myogenin. Global gene expression profiling via microarray analysis identified numerous genes involved in metabolism and signal transduction (Myog-deleted n=3, wild-type n=3). Alterations of 1.4 fold or more were considered to be significant.

| <b>Fold change</b> | <b>Regulation</b> | <b>Gene Symbol</b> | <b>Gene Title</b>                                      | <b>Biological Process</b>  |
|--------------------|-------------------|--------------------|--|--|
| 2.4                | down              | Rfx7               | regulatory factor X, 7                                 | regulation of transcription, DNA-dependent   |
| 1.8                | down              | Rhobtb1            | Rho-related BTB domain containing 1                    | small GTPase mediated signal transduction  |
| 1.8                | down              | Rhpn2              | rhophilin, Rho GTPase binding protein 2                | signal transduction  |
| 1.7                | down              | Myog               | myogenin   | regulation of transcription, DNA-dependent   |
| 1.6                | down              | Htra1              | HtrA serine peptidase 1                                | regulation of cell growth /// proteolysis /// negative regulation of transforming growth factor beta receptor signaling pathway /// negative regulation of BMP signaling pathway   |
| 1.6                | down              | Gpx3               | glutathione peroxidase 3                               | glutathione metabolic process /// response to oxidative stress /// hydrogen peroxide catabolic process /// protein homotetramerization /// protein homotetramerization /// oxidation reduction                               |
| 1.6                | down              | Zscan21            | zinc finger and SCAN domain containing 21              | transcription /// regulation of transcription, DNA-dependent /// multicellular organismal development /// spermatogenesis /// cell differentiation /// positive regulation of transcription, DNA-dependent /// oogenesis     |
| 1.5                | down              | Pcx                | pyruvate carboxylase                                   | gluconeogenesis /// oxaloacetate metabolic process /// metabolic process /// lipid biosynthetic process  |
| 1.4                | up                | Bmp5               | bone morphogenetic protein 5                           | skeletal system development /// ossification /// multicellular organismal development /// pattern specification process /// cell differentiation /// growth /// cartilage development  |
| 1.6                | up                | Pfkfb3             | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 | fructose metabolic process /// fructose 2,6-bisphosphate metabolic process /// metabolic process   |
| 1.8                | up                | Mpa2l              | macrophage activation 2 like                           | immune response  |
| 1.9                | up                | Dpyd               | dihydropyrimidine dehydrogenase                        | 'de novo' pyrimidine base biosynthetic process /// pyrimidine base catabolic process /// uracil catabolic process /// thymidine catabolic process /// UMP biosynthetic process /// metabolic process /// oxidation reduction |
| 2.0                | up                | Bdh1               | 3-hydroxybutyrate dehydrogenase, type 1                | metabolic process /// oxidation reduction  |

dehydrogenase-type 1 (Bdh1) were shown to be significantly altered in *Myog*-deleted mice. These changes in gene expression levels were verified by qRT-PCR (Figure 23). In the absence of myogenin, this assay confirmed that the expression levels of multiple genes regulating metabolic processes in glycolysis were altered. The gene expression profiling analysis will provide a starting point from which to determine the underlying basis for enhanced exercise capacity in *Myog*-deleted mice.

### Figure 23

Transcript expression levels of genes identified to be altered in the absence of myogenin. qRT-PCR confirms the altered expression of genes in *Myog*-deleted mice identified during global gene expression profiling via microarray analysis. Green bars represent *Myog*-deleted values (n=3). Red bars represent wild-type control values (n=3). Error bars represent one standard deviation (\* $P < 0.05$ ; \*\* $P < .01$ ).





## DISCUSSION:

The role of myogenin in regulating embryonic skeletal muscle differentiation has been thoroughly investigated. The results presented here identify new functions for myogenin in response to exercise stress and demonstrate that *Myog*-deleted adult mice possess an enhanced capacity for exercise soon after deletion. These mice display increased levels of lactate build-up at exhaustion and are able to further tap into glycogen reserves for energy production that wild-type mice are unable to access. The data suggest that the loss of myogenin abruptly alters metabolic processes in adult mice, thus conferring their enhanced capacity for exercise. When given access to a means of training over time, these shifts in metabolic processes translate into the acceleration of changes in muscle fibers to induce rapid shifts in fiber type and mitochondrial activity. The results provide evidence indicating that myogenin regulates metabolic homeostasis during adult life and may negatively regulate exercise performance. These findings are particularly striking, because myogenin is known to be vital for muscle growth and development during embryonic and postnatal life. Furthermore, the results are consistent with findings in the literature suggesting that the myogenin:MyoD expression ratio regulates fiber phenotype [27]. *MyoD* transcripts have been shown to selectively accumulate in fast twitch muscle fibers. In *Myog*-deleted mice, this ratio is presumably tipped in favor of MyoD, suggesting the development of fast-twitch characteristics in previously slow-twitch fibers.

The exercise stress experiments and blood tests performed here are the first to use *Myog*-deleted mice to identify functions for myogenin in adult exercise and metabolism. This physiological approach identified a metabolic regulatory role for myogenin during exercise. Under high-intensity exercise conditions, *Myog*-deleted mice exhibited increased lactate levels at exhaustion. There is much debate regarding lactate production as the cause of muscle fatigue and acidosis. Long held views state that a virtually instantaneous and complete ionization of lactic acid is caused by the low pKa of the carboxylic acid functional group within lactic acid [32-38]. Thus the decrease in pH and discharge of a ( $H^+$ ) proton from lactic acid to finally create lactate is defined as lactic acidosis [39]. Compelling evidence to the contrary indicates that metabolic acidosis is primarily brought about via alternative mechanisms such as ATP hydrolysis or increases in the glyceraldehyde-3-phosphate dehydrogenase reaction rate in glycolysis [40-44]. Furthermore, studies strongly suggest that lactate production and removal from the muscle actually retards acidosis by consuming  $H^+$  protons [45] and concomitantly transporting  $H^+$  across the sarcolemma [30, 46]. Lactate circulating through the blood can also be taken up and metabolized by other organs such as the kidneys, skeletal and cardiac muscle, and liver. Thus increased lactate levels may be contributing to the enhanced exercise capacity of *Myog*-deleted mice by slowing acidosis and muscle fatigue. Furthermore, this impediment to acidosis may be enabling *Myog*-deleted mice to metabolize and deplete additional glucose stores before reaching exhaustion significantly later than wild-type animals.

*Myog*-deleted mice also ran farther during the low-intensity exercise regimen and exhibited significantly decreased glucose levels at exhaustion, but did not display increased lactate levels. One possible explanation for this would be a difference in the expression levels of glucose transporter proteins, but no such differences were detected in the global gene expression analysis or subsequent GLUT4 transcript detection by qRT-PCR. However, the activity and arrangement of these proteins at the cell membrane have yet to be determined and could influence the uptake of glucose. Alternatively, increased lactate production may still be occurring but the mechanisms for clearing lactate from the blood in *Myog*-deleted animals may have been able to compensate.

The long-term effects of *Myog*-deficiency in the adult have yet to be elucidated, but extensive voluntary training has uncovered a role in adaptation to continuous exercise. After six months of open access to running wheels, *Myog*-deleted mice responded to exercise training 21% better than wild-type mice. The glucose and lactate advantages proposed above may be slowing the onset of fatigue during exercise, allowing *Myog*-deleted mice to run farther and for extended periods of time. Over the course of months, even a mild enhancement may translate into significant additional gains in exercise capacity. The loss of myogenin dramatically shifts fiber type proportions from type 2 to type 1 in response to long-term training. This shift in fiber type proportions suggests that *Myog*-deficiency may promote gradual changes in MyHC isoform expression and fiber contraction speed. Furthermore, significant increases in SDH abundance far beyond the increases seen in wild-type mice suggest an

enhancement in oxidative metabolism possibly through increases in mitochondrial count and distribution.

These results are also consistent with previous data suggesting alterations in glycolytic metabolism can be uncoupled from changes in MyHC fiber type [28]. *Myog*-deleted animals displaying an enhanced exercise capacity under the high-intensity regimen two weeks post-deletion did not exhibit a shift in fiber type proportions. Previous enzyme studies utilizing transgenic mice engineered to overexpress myogenin reported a shift in enzyme activity from glycolytic to oxidative metabolism, but no differences in fiber type proportions were observed. While metabolic enzyme activities determined here in wild-type and *Myog*-deleted mice did not reveal shifts in activity, the panel tested did not comprehensively cover the large number of enzymes involved in regulating metabolism.

Previous studies have shown myogenin regulates distinctly different pathways during various stages of life. During embryonic development, many genes encoding structural and contractile components are strongly downregulated in the absence of myogenin [47], whereas expression levels of factors regulating cell fusion, signaling, and adhesion are altered in adult skeletal muscle stem cells (satellite cells) [13]. Global gene expression analysis performed here in adult skeletal muscle now identifies potential roles for myogenin in the regulation of metabolic processes. Pc catalyzes the initial reaction of glycolysis of pyruvate to oxaloacetate, and when the liver detects excess lactate levels, Pc activity increases [for review, see 48]. Human Pc

deficiencies often result in increased lactate levels and/or high lactate:pyruvate ratios [49-51]. Thus, reduced expression of Pc may be contributing to increased lactate levels at exhaustion in *Myog*-deleted mice.

PFKFB3 was significantly upregulated in *Myog*-deleted mice. PFKFB3 encodes PFK2, which has been shown to regulate the activity of PFK1 and ultimately drive a rate-limiting reaction during glycolysis, the conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6BP) via PFK-1 [for review, see 52]. It is expressed in skeletal muscle, activated by hypoxic stimuli, and exhibits an approximately 400:1 kinase:phosphatase ratio to enhance glycolytic flux [53-55]. Myogenin has been shown to repress genes regulating glycolysis and upregulate genes involved in oxidative metabolism. Myogenin is known to activate HDAC4, a repressor of PFK-1 [24]. In the absence of myogenin, HDAC4 levels may decrease, deregulating PFKFB3 and increasing glycolytic activity.

The results presented here suggest that reduced myogenin expression would be advantageous to mice in the wild. Enhanced exercise capacity would presumably enable these animals to better escape from predators or become superior hunters. Thus the question remains as to why the expression of myogenin persists throughout adulthood. There are multiple possible explanations. *Myog*-deleted mice derived from laboratory stocks have been removed from the wild for many generations. They have not been subjected to natural environmental pressures and selection processes. Thus, it is possible that *Myog*-deleted mice do not display the same survival characteristics as mice

in the wild. Also, the results imply that *Myog*-deleted mice are able to further deplete their glucose stores before reaching exhaustion. This may adversely enable them to easily become hypoglycemic, which can lead to unconsciousness, neuroglycopenia, and potentially death. In wild-type mice, myogenin may act to restrain skeletal muscle activity in favor of glucose conservation. Furthermore, consistent muscle mass is achieved by a balance between the rates of protein synthesis and degradation. Recent studies performed in collaboration with the Olson laboratory at The University of Texas Southwestern Medical Center in Dallas indicate that myogenin is an essential activator of muscle atrophy. Through its role in the activation of E3 ubiquitin ligases, myogenin may act to maintain constant muscle mass by promoting muscle proteolysis.

The mechanism by which myogenin regulates different pathways during embryogenesis, postnatal development, and adult life have yet to be elucidated. Previous studies have focused on embryonic and postnatal development, when skeletal muscle is undergoing dramatic periods of stem cell proliferation and differentiation. Conversely, adult skeletal muscle is kept under a much less dynamic state of maintenance. As the requirements necessary to sustain muscle homeostasis change from one developmental time point to another, so may the functions of myogenin change to maintain a viable equilibrium. *Myog*-deleted adult mice displayed an enhanced capacity for exercise under both low-intensity and high-intensity running regimens. Myogenin may be regulating pathways that maintain adult muscle fiber character. It may act as a master

sensor of muscle stress to alter fiber metabolic properties in response to changing environmental conditions.

This research lends itself to a number of possible future directions. At the transcriptional level, elucidating the mechanisms conferring differential gene regulation by myogenin in embryonic versus adult skeletal muscle may provide a basis for establishing how this is achieved in other families of closely related factors. Furthermore, the unexpected functions for myogenin identified here may lead to more translational research projects with the ultimate goal of generating clinical applications. Blocking the expression of myogenin may provide useful to the sports medicine industry when developing strategies to aid in muscle repair and regeneration. Moreover, this research questions the role of myogenin in muscle wasting disorders. The functions for myogenin during periods of constant muscle regeneration may be best uncovered by downregulating its expression in mice harboring the Duchenne's muscular dystrophy (DMD<sup>mdx</sup>) allele.

## CHAPTER II:

### **Myogenin regulates exercise capacity but is dispensable for muscle regeneration in adult *mdx* mice**

#### **INTRODUCTION:**

Muscular Dystrophy is the most prevalent childhood muscle disorder found in humans, affecting 1 in 3500 newborn males [56]. Duchenne's muscular dystrophy (DMD) is a chronic muscle wasting disease leading to progressive muscle weakness and atrophy. The disease is caused by mutations within the dystrophin gene, a critical structural component of the muscle cell membrane [57]. Physiological symptoms of this disorder become apparent within the first few years of life, and muscle strength quickly deteriorates by puberty and usually results in early mortality. Common diagnoses and signs of DMD include increased blood creatine kinase concentration, increased rates of myofiber regeneration, centrally located myofiber nuclei, mitochondrial swelling, and ultimately myofiber necrosis [58]. Animal models of DMD include the *mdx* mouse, which has been studied extensively to better understand human DMD [59-65].

DMD is associated with muscle metabolism dysfunction and muscle weakness. Neuronal nitric oxide synthase (nNOS) is a dystrophin-associated protein found near the muscle sarcolemma, and its expression is greatly reduced in *mdx* mice [66]. Recently, others have shown that the loss of interaction between nNOS and PFK contributes to glycolytic dysfunction and increased fatigability [67]. The forced expression of nNOS in *mdx* mice



enhances muscle endurance and glycogen metabolism. These experiments provide important insights into why *mdx* mice and humans with DMD experience progressive muscle weakness associated with muscle wasting and atrophy.

Mammalian muscle fibers are broadly divided into several subtypes based upon their expression of different myosin isoforms: type 1, type 2A, and type 2B (type 2X in humans) [68]. Types 1 and 2A are primarily oxidative in nature and confer increased exercise endurance with a high resistance to muscle fatigue during aerobic exercise. Type 2B fibers are glycolytic in nature and allow for anaerobic sprinting or short bursts of strength but fatigue more quickly. Type 1 fibers are commonly referred to as slow twitch whereas type 2 fibers are labeled as fast twitch. Glucose is a major energy source for all muscle metabolism, but glycolytic fibers are particularly dependent on glucose for energy production. Glucose stored as glycogen within skeletal muscle fibers is utilized to quickly meet the demands of high-intensity exercise. Fatty acids and glucose acquired from the liver via the blood stream provides the major source of energy that is utilized during low-intensity exercise. Lactate is a by-product of anaerobic metabolism and its blood concentration rises coincident with an increase in exercise intensity [69].

The maintenance and regeneration of skeletal muscle are orchestrated by the four basic helix-loop-helix Myogenic Regulatory Factors (MRFs): MyoD, Myf5, MRF4, and myogenin. Myogenin is required for embryonic skeletal muscle development and for achieving normal body size after birth [12, 13, 29]. Myogenin is primarily expressed in slow-twitch/oxidative myofibers and is

sufficient to induce a shift from glycolytic to oxidative metabolism when overexpressed [27, 28]. Recently, myogenin was discovered to transcriptionally activate the E3 ubiquitin ligases Fbxo32 and Trim63 following muscle denervation [26]. The loss of myogenin during adult life confers a resistance to denervation-induced muscle atrophy. Moreover, as described in chapter 1, myogenin plays a role in regulating metabolism and exercise endurance during adult life.

In this chapter, I address whether the loss of myogenin in adult *mdx* mice alters *mdx* muscle regeneration and performance. Surprisingly, the absence of myogenin appeared to have no adverse effects on muscle regeneration. Moreover, the exercise capacity of *mdx:Myog*-deleted adult mice was significantly improved over the *mdx* control group, nearly to the level of wild-type mice. This favorable improvement in exercise endurance was associated with significant changes in nNOS and Fbxo32 expression, implicating a potential mechanism for the improvement in muscle fatigue resistance. The results indicate that further investigation into the manipulation of *Myog* in mammals with DMD will be a worthy avenue for the development of new treatment options for this disease.

## RESULTS:

### ***Myog* is not required for survival in adult *mdx* mice**

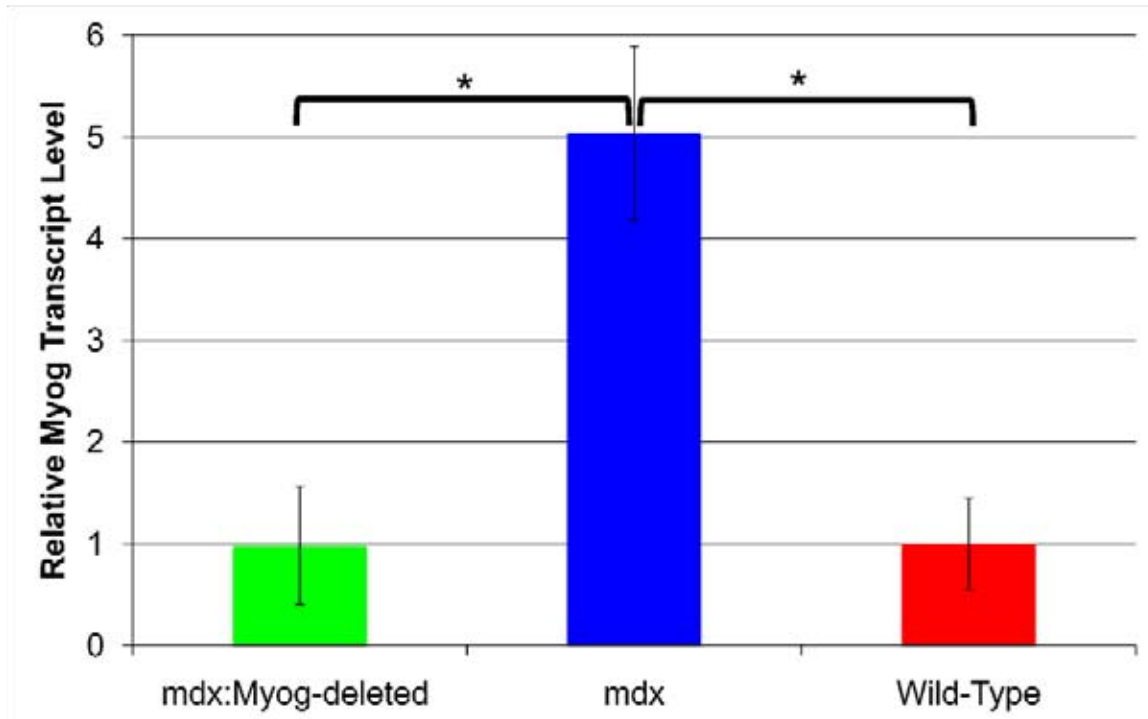
To determine the role of myogenin in adult skeletal muscle maintenance and regeneration, *Myog* conditional knockout mice harboring a tamoxifen-inducible Cre recombinase transgene [29] were interbred with the DMD<sup>*mdx*</sup> mouse strain. Wild-type (tamoxifen-injected *Myog*<sup>flox/flox</sup>) and *mdx* animals in the same mixed genetic background were also generated as controls. Cre-expressing adult animals 8-12 weeks old were then rendered *Myog*-deficient via a single injection of tamoxifen. Determination of transcript expression showed that *Myog* transcript levels in *mdx* mice were 5-fold higher than their wild-type counterparts (Figure 24). Moreover, *mdx:Myog*-deleted mice expressed equal levels of *Myog* transcripts compared to wild-type. This is expected as *mdx* mice are in a constant state of regeneration, when myogenin levels are highly upregulated. *Mdx:Myog*-deleted mice exhibited seemingly normal behavior and displayed no overt adverse phenotypic effects. The results indicated that *Myog* is not required for survival in adult *mdx* mice.

### ***Mdx:Myog*-deleted mice exhibit an enhanced capacity for exercise over their *mdx* counterparts**

Based on the results described in chapter 1, I reasoned that the loss of myogenin may confer an enhanced capacity for exercise in *mdx* mice. *Mdx*, *mdx:Myog*-deleted, and wild-type mice were run on a treadmill to exhaustion under a high-intensity exercise regimen to determine their exercise capacities.

## Figure 24

Reduced abundance of *Myog* transcripts in tamoxifen-injected *mdx:Myog<sup>flox/flox</sup>*; Cre+ mice. *Myog* transcript levels in adult *mdx* mice were increased by 5 fold over wild-type levels. *Myog* transcript expression in *mdx:Myog<sup>flox/flox</sup>*; Cre+ mice treated with a single IP injection of tamoxifen at a dose of 10mg/40g body weight was approximately equal to that of the wild-type control group. Green bar represents *mdx:Myog*-deleted values (n=3). Blue bar represents *mdx* values (n=3). Red bar represents wild-type control values (n=3). Error bars represent one standard deviation (\* $P < 0.05$ ).



Over the course of 3 consecutive days of running, *mdx:Myog*-deleted mice ran on average 11% less than the wild-type group, but 37% farther than their *mdx* counterparts before reaching exhaustion (Figure 25). The results suggested that the absence of *Myog* conferred a significant positive effect on exercise endurance in *mdx* mice.

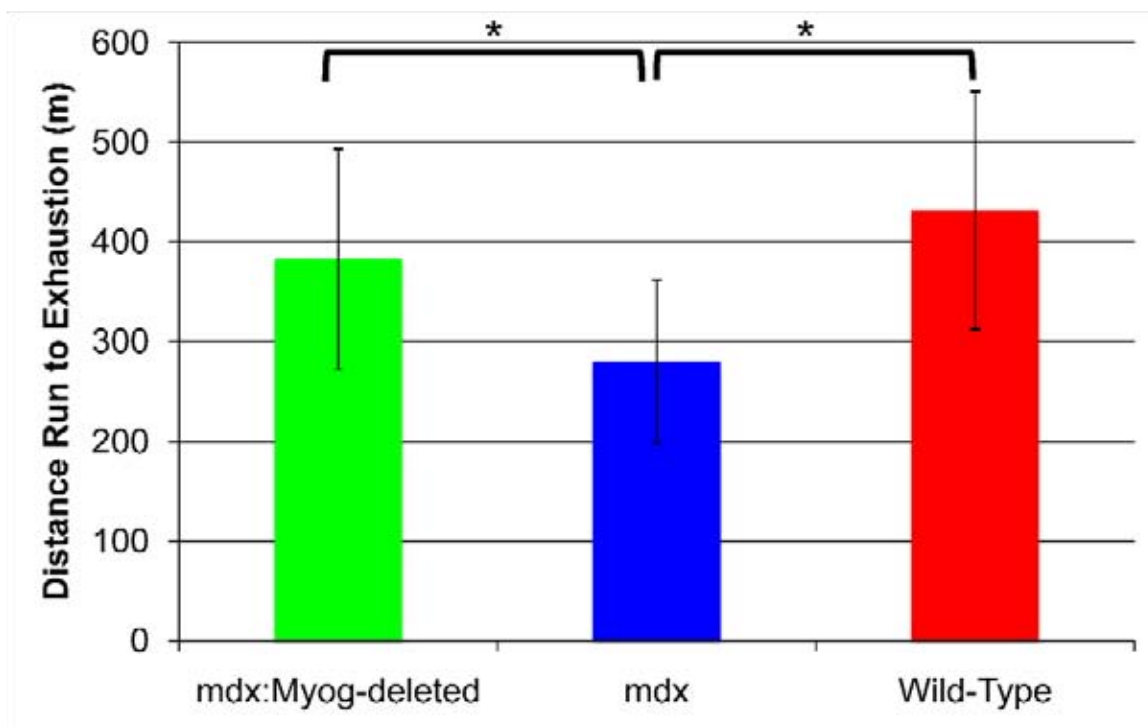
### **The absence of myogenin alters blood metabolite concentrations during exhaustive exercise**

Deletion of *Myog* enhances exercise endurance and is associated with alterations in blood metabolite concentrations. To investigate whether these phenomena also accompanied increased exercise capacity in *mdx:Myog*-deleted mice, their metabolic profiles were determined. Pre-exercise blood glucose concentrations were approximately equal between wild-type, *mdx*, and *mdx:Myog*-deleted groups. Moreover, these concentrations were virtually unchanged at exhaustion when compared to pre-exercise concentrations, and when compared between groups (Figure 26). These constant blood glucose levels indicate the use of glycolytic metabolism during this high-intensity exercise.

Lactate is a product of glycolysis, and increased blood lactate at exhaustion is a commonly used indicator of glycolytic metabolism. Virtually unchanged lactate levels would be expected during the use of oxidative metabolism. Blood lactate levels at exhaustion were significantly increased for all three test groups. However, the concentration of blood lactate in *mdx:Myog*-

### Figure 25

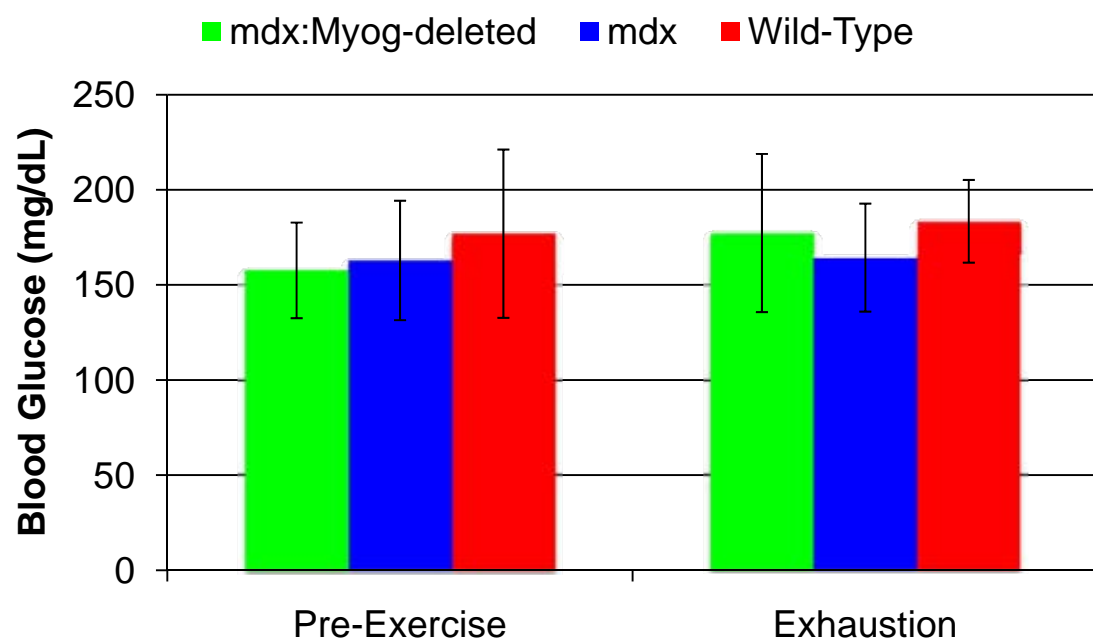
Enhanced exercise capacity in *mdx:Myog*-deleted mice during the high-intensity exercise regimen. *mdx:Myog*-deleted mice ran on average over the course of 3 consecutive days of running 37% more than the *mdx* control group and 11% less than the wild-type mice under high-intensity exercise conditions (*mdx:Myog*-deleted, n=14; *mdx*, n=14; wild-type n=14). Green bar represents *mdx:Myog*-deleted values. Blue bar represents *mdx* values. Red bar represents wild-type control values. Error bars represent one standard deviation (\* $P < 0.05$ ).





### Figure 26

Blood glucose levels in *mdx*, *mdx:Myog*-deleted, and wild-type mice after running to exhaustion under high-intensity exercise conditions. Baseline blood glucose levels were virtually equal for all three test groups at between 158 to 177 mg/dL (*Myog*-deleted, n=14; wild-type n=14). At exhaustion, blood glucose values remained virtually unchanged for all groups. Green bars represent *mdx:Myog*-deleted values. Blue bars represent *mdx* values. Red bars represent wild-type control values. Error bars represent one standard deviation.



deleted mice was 14% greater than wild-type and 51% greater than *mdx* animals (Figure 27). The results suggested that *mdx:Myog*-deleted mice possessed a higher lactate concentration threshold before reaching exhaustion.

### **Myog is not required for muscle regeneration in the adult mouse**

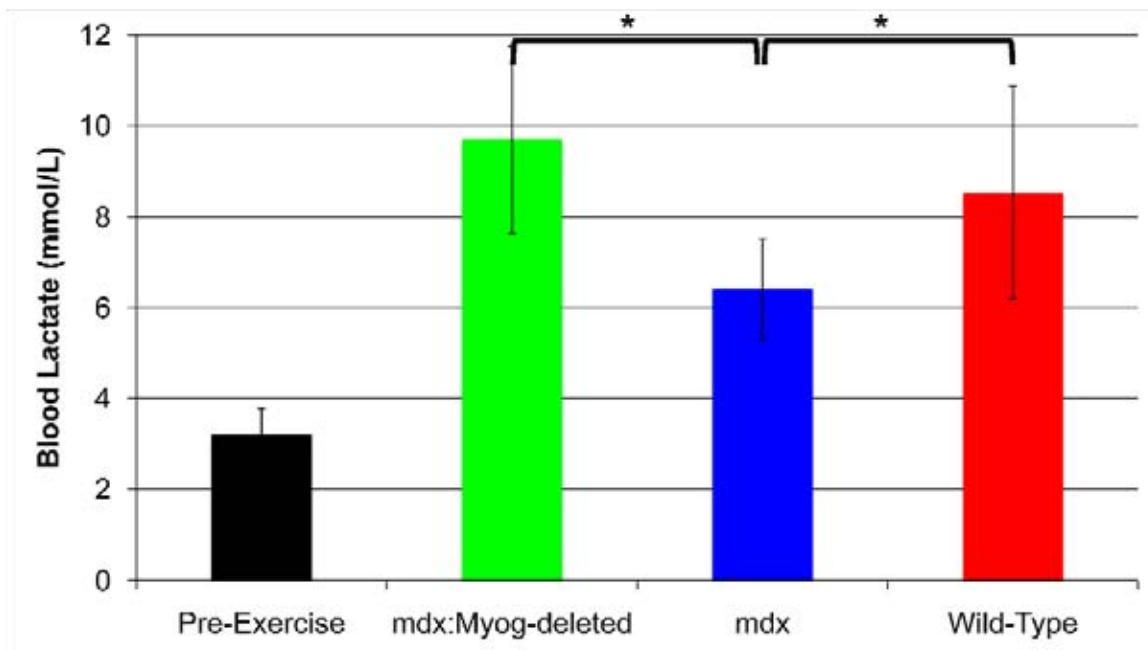
Histological analyses revealed the muscle morphology of *mdx:Myog*-deleted mice to be similar if not identical to that of *mdx* mice. Conventional signs of muscle regeneration were observed in both groups including the presence of myofibers of widely varying sizes containing centrally located nuclei (Figure 28A). Van Gieson staining for collagen and elastin also revealed similar patterns of mild fibrosis in *mdx* and *mdx:Myog*-deleted mice (Figure 28B). To determine the impact of the absence of myogenin on the dystrophin-glycoprotein complex, blood CK activity was determined. Both *mdx* and *mdx:Myog*-deleted mice show dramatically elevated CK levels compared to wild-type control mice (Figure 29). These results demonstrated that myogenin's absence in adult *mdx* mice did not reverse the pathological effects of muscular dystrophy in skeletal muscle. Furthermore, the absence of *Myog* in *mdx* mice did not exacerbate muscle dystrophy, indicating that *Myog* is dispensable for muscle regeneration.

### **Myofiber characteristics of *mdx:Myog*-deleted adult mice**

The deletion of *Myog* in *mdx* mice might alter muscle properties such as fiber type proportion and glycogen content. qRT-PCR was performed to determine transcript levels for myosin type 1, type 2A, and type 2B isoforms in the

### Figure 27

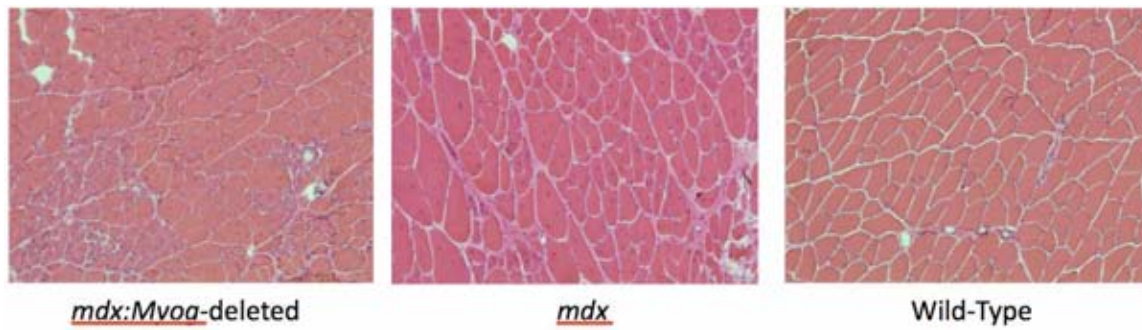
Increased blood lactate levels in *mdx*, *mdx:Myog*-deleted, and wild-type mice after running to exhaustion under high-intensity exercise conditions. Blood lactate levels were significantly elevated for all three test groups at exhaustion. Wild-type and *mdx:Myog*-deleted values were significantly greater than those of the *mdx* group. Green bar represents *mdx:Myog*-deleted values (n=12). Blue bar represents *mdx* values (n=11). Red bar represents wild-type control values (n=10). Error bars represent one standard deviation (\* $P < 0.05$ ).



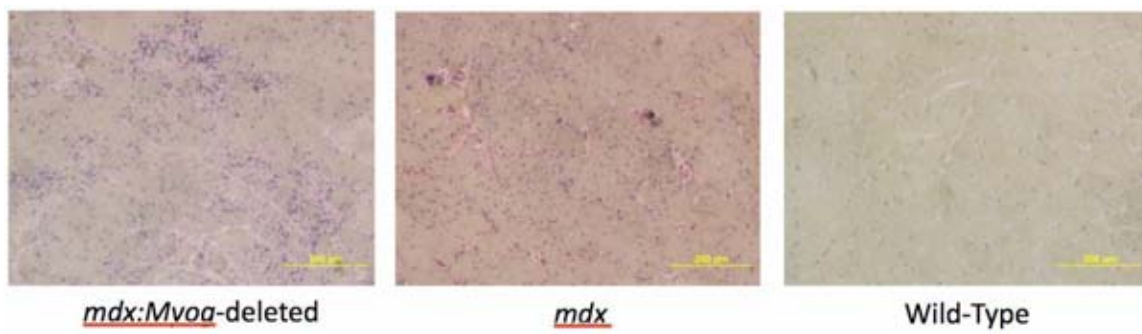
### **Figure 28**

Histological evaluation of *mdx*, *mdx:Myog*-deleted, and wild-type hindlimb muscle. (A) Hematoxylin and eosin stained *mdx* and *mdx:Myog*-deleted muscle sections both displayed classic characteristics of regeneration including wide variations in fiber caliber and centrally located nuclei that were not observed in wild-type samples. (B) Staining for collagen and elastin reveals similar levels of fibrosis in *mdx* and *mdx:Myog*-deleted muscle sections that are not observed in wild-type samples.

A



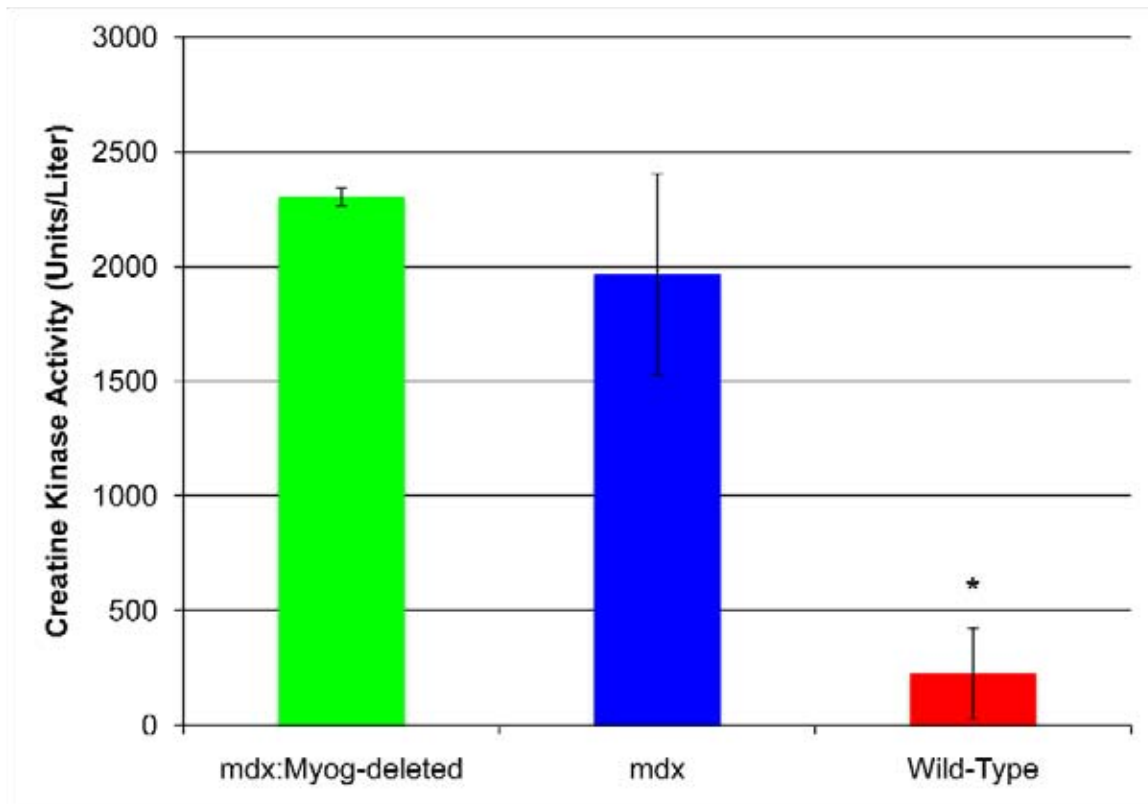
B



### Figure 29

Increased concentration of blood creatine kinase in *mdx* and *mdx:Myog*-deleted mice. Blood creatine kinase levels were significantly elevated for both *mdx* and *mdx:Myog*-deleted mice compared to the wild-type control group, but there was no significant difference between the *mdx* and *mdx:Myog*-deleted groups. Green bar represents *mdx:Myog*-deleted values (n=9). Blue bar represents *mdx* values (n=10). Red bar represents wild-type control values (n=6). Error bars represent one standard deviation (\* $P < 0.05$ ).





gastrocnemius muscles of *mdx*, *mdx:Myog*-deleted, and wild-type control mice. No significant changes in the expression of these transcripts between *mdx* and *mdx:Myog*-deleted mice were detected (Figure 30).

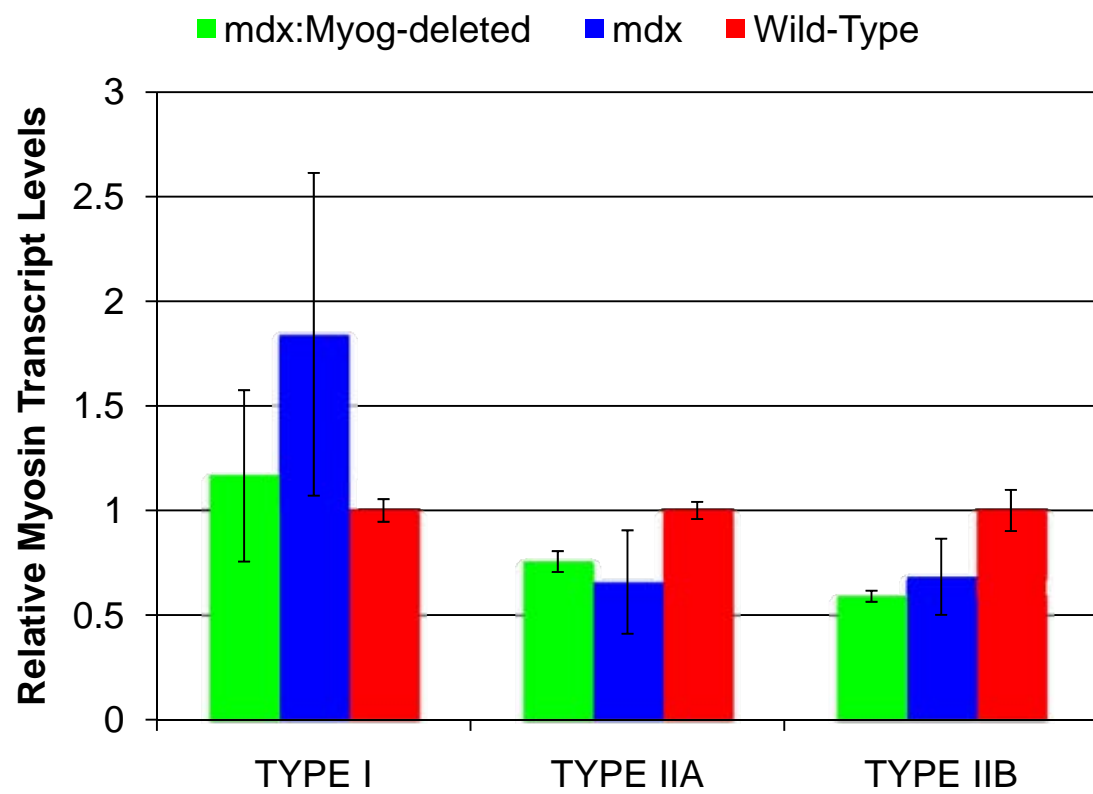
Glycogen staining of muscle sections revealed a dramatic increase in glycogen content in *mdx:Myog*-deleted muscle compared to *mdx* and wild-type muscle samples (Figure 31A). However, attempts to quantify the data using whole muscle homogenates with a glycogen biochemical assay were unable to confirm the significance of this observation (Figure 31B). The results indicated that the deletion of *Myog* from *mdx* mice did not alter myosin isoform distribution but may have increased glycogen abundance.

### **Expression levels of genes regulating muscle fatigue, metabolism, and proteolysis are altered in *mdx:Myog*-deleted adult mice**

To begin elucidating the mechanisms driving the observed enhanced exercise capacity of *mdx:Myog*-deleted mice over their *mdx* counterparts, putative myogenin target genes were identified in adult skeletal muscle. Transcript levels of genes regulating muscle development, metabolic processes and muscle proteolysis such as nNOS, Pfk, Hdac4, Trim63, Fbxo32, glycogen synthase, and muscle glycogen phosphorylase were measured. Of these genes, the expression of nNOS and Fbxo32 were significantly altered in the absence of myogenin (Figure 32). These changes in expression may contribute to the observed enhanced exercise capacity in *mdx:Myog*-deleted mice over the *mdx*

### Figure 30

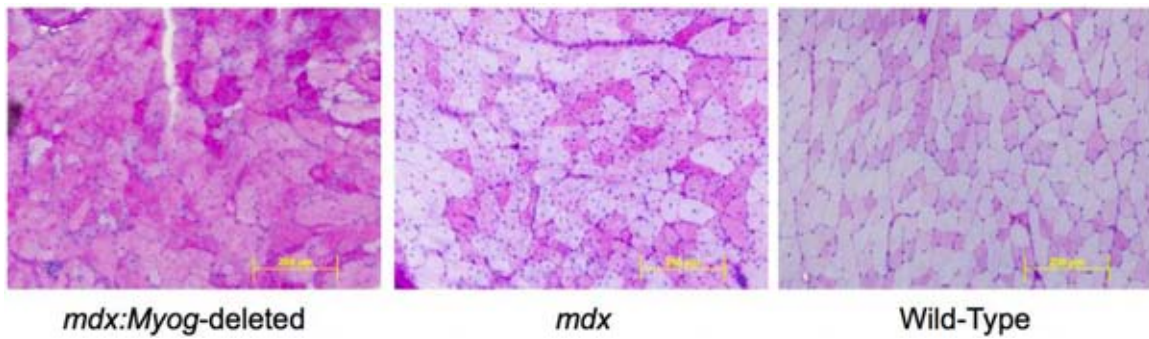
Myosin expression levels in *mdx*, *mdx:Myog*-deleted, and wild-type hindlimb muscle. No significant changes in transcript expression values, as assessed by qRT-PCR, for myosins type 1, 2A, and 2B were observed between the *mdx* and *mdx:Myog*-deleted groups. Slight but significant alterations in myosins types 2A and 2B were detected between wild-type and *mdx:Myog*-deleted mice. Green bars represent *mdx:Myog*-deleted values (n=3). Blue bars represent *mdx* values (n=3). Red bars represent wild-type control values (n=3). Error bars represent standard error of the mean.



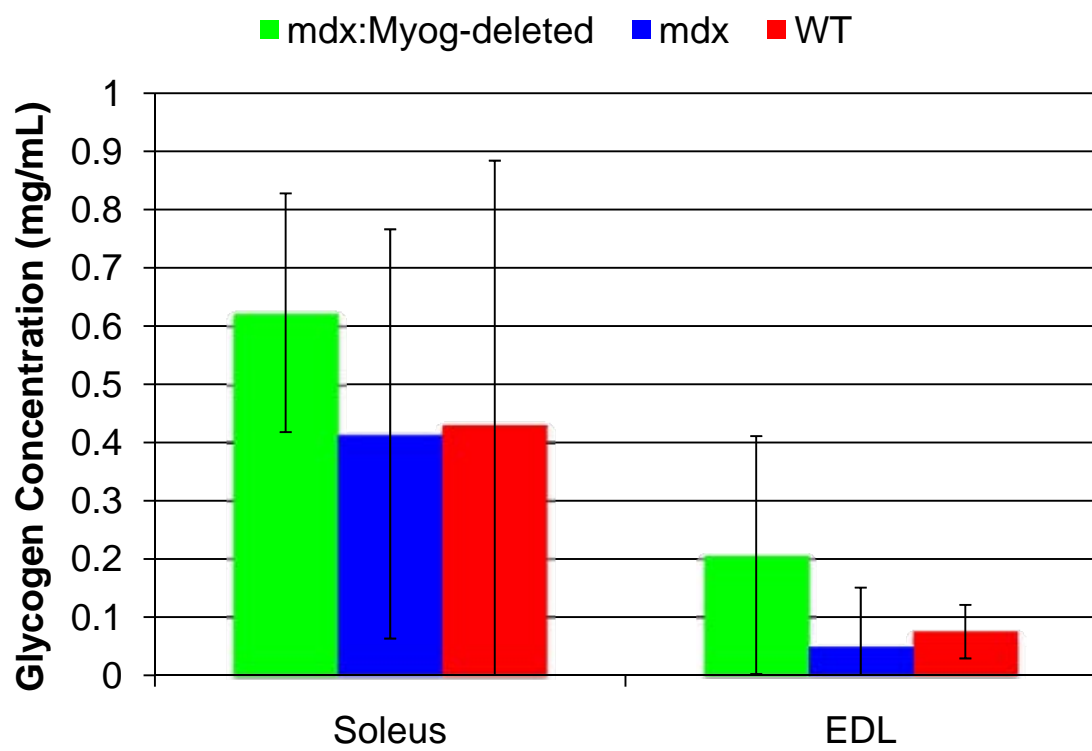
### Figure 31

Glycogen content in wild-type and *Myog*-deleted hindlimb muscle. (A) PAS staining for glycogen in the gastrocnemius reveals similar levels of glycogen in *mdx* and wild-type samples, but suggests the *mdx:Myog*-deleted sections may harbor increased glycogen content. (B) Biochemical data suggests a nonstatistical trend toward increased glycogen content in the soleus and EDL muscles of *mdx:Myog*-deleted mice. Green bars represent *mdx:Myog*-deleted values (n=3). Blue bars represent *mdx* values (n=3). Red bars represent wild-type control values (n=3). Error bars represent standard error of the mean (\* $P < 0.05$ ).

A.

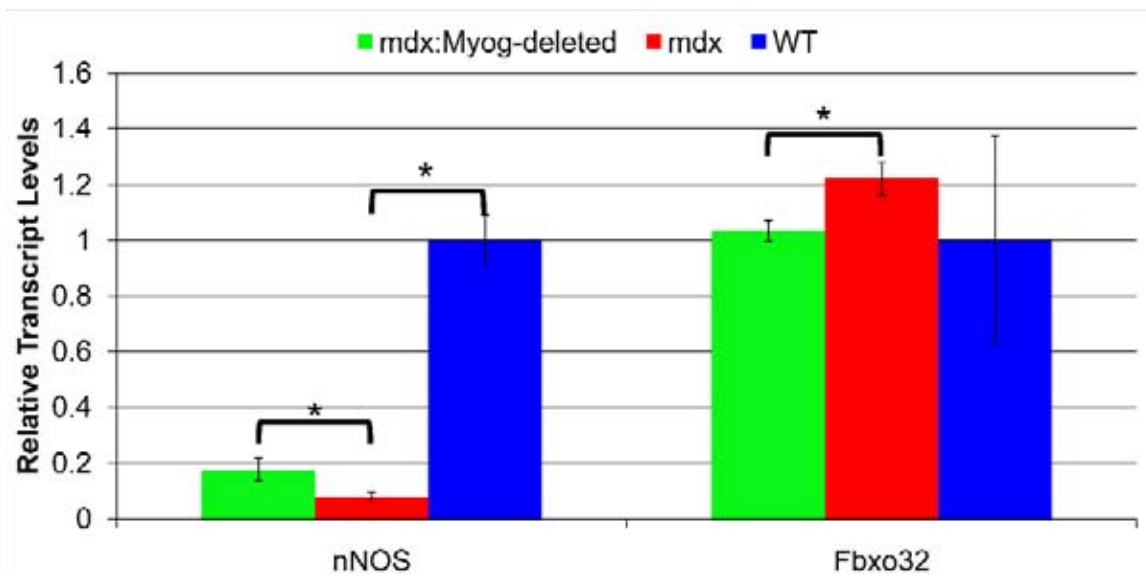


B.



### Figure 32

Altered expression of nNOS and Fbxo32 in *mdx*, *mdx:Myog*-deleted, and wild-type hindlimb muscle. As verified by qRT-PCR, transcript levels of nNOS were dramatically reduced in *mdx* mice. Expression of nNOS was increased by 2.2 fold in *mdx:Myog*-deleted mice over their *mdx* counterparts. Fbxo32 expression was determined to be significantly increased in *mdx* mice over the *mdx:Myog*-deleted group and showed a trend toward increased expression over wild-type mice. Green bars represent *mdx:Myog*-deleted values (n=3). Blue bars represent *mdx* values (n=3). Red bars represent wild-type control values (n=3). Error bars represent one standard deviation (\* $P < 0.05$ ).





group and could provide insight into the mechanisms by which myogenin exerts its effects.

## DISCUSSION:

Myogenin is an essential transcription factor involved in the development of muscle fibers during embryogenesis [12, 29, 70]. Myogenin's role in the maintenance and regeneration of adult skeletal muscle has been long speculated but not tested in model systems where muscle regeneration is genetically compromised. In this study, essential coding and regulatory regions of the *Myog* locus were conditionally deleted from *mdx* adult mice. Efficient deletion of *Myog* was achieved after tamoxifen-induced Cre-mediated recombination. *Mdx:Myog*-deleted mice exhibited no remarkable differences in their behavior or mobility when compared to wild-type controls. These results are consistent with previous studies indicating that *Myog* is dispensable during adult life [13, 29].

Given that *Myog* is activated upon muscle injury to help facilitate muscle repair and regeneration, and *mdx* mice exist in a constant state of muscle regeneration [71], I expected that *Myog* transcripts would be upregulated in muscle from *mdx* mice. *Myog* transcript levels were dramatically increased in *mdx* mice while *mdx:Myog*-deleted mice exhibited levels much closer to wild-type controls. As skeletal muscle stem cells are activated to repair and fuse with existing muscle fibers, myogenin expression increases in the latter stages of differentiation. These compensatory events in *mdx* mice result in muscle hypertrophy characterized by increases in muscle mass, fiber count, centrally located nuclei, and distribution of the fiber calibers in *mdx* mice [60, 72, 73]. The results described in this chapter confirm previously published studies indicating

that myogenin expression is elevated in *mdx* mice [74]. The efficient deletion of *Myog* and its subsequent downregulation in *mdx* mice resulted in no noticeable adverse effects and indicates that *Myog* is not required for survival or normal muscle function in these animals.

Since dystrophic muscle exists in a constant state of damage repair [62, 63, 65, 75], I hypothesized that vigorously exercised *mdx* mice would exhibit a dramatic reduction in exercise capacity compared to wild-type mice. Under high-intensity exercise conditions, *mdx* mice ran 35% less than wild-type controls. *Mdx:Myog*-deleted mice ran 36% farther than the *mdx* control group, nearly equaling the distances achieved by wild-type control mice. These data agree with previous findings described in chapter I and indicate that *mdx* mice, like wild-type mice, benefit from the loss of myogenin and exhibit similar gains in exercise capacity following *Myog*-deletion.

Results described in chapter I indicated that the deletion of *Myog* enhanced exercise endurance by altering skeletal muscle metabolism. The metabolic changes were evidenced by alterations in blood glucose and lactate concentrations following exercise. While blood glucose was significantly depleted below wild-type levels in *Myog*-deleted mice following high-intensity exercise, *mdx:Myog*-deleted mice exhibited blood glucose levels equal to that of the wild-type and *mdx* control groups. This result is expected as the high-intensity exercise regimen is designed to employ the use of glycolytic metabolism, which primarily utilizes muscle glycogen as a fuel source. *Mdx:Myog*-deleted mice often reach exhaustion before metabolizing significant

amounts of liver glycogen, thus leaving blood glucose concentrations at normal levels. The lack of observable blood glucose depletion in *mdx:Myog*-deleted mice suggests that the inherent muscle weakness of *mdx* mice limits exercise endurance, thus preventing their blood glucose levels from dropping below those of wild-type mice. Blood lactate concentrations were significantly elevated in *mdx:Myog*-deleted mice compared to the *mdx* control group following high-intensity exercise. These results suggest that the loss of myogenin in *mdx* mice confers an increased lactate threshold before reaching exhaustion.

As discussed in chapter I, lactate production may actually retard muscle acidosis. The conversion of pyruvate to lactate by lactate dehydrogenase also generates NAD<sup>+</sup>. This aids in sustaining the NAD<sup>+</sup>/NADH cytosolic redox potential which promotes substrate flux through glycolysis and ultimately enables the glycolytic production of ATP [30]. Furthermore, this reaction consumes an additional proton from the cytosol and is thus alkalizing to the muscles. The discharge of lactate from the muscle is facilitated by monocarboxylate transporter [32, 76-79], a known symport for proton removal from muscle fibers. The transport of lactate out of the muscle by this mechanism is stoichiometrically accompanied by the removal of protons. In fact, published data suggests the rate of proton removal outpaces the rate of lactate removal in active muscle [80]. Thus, increased blood lactate levels may contribute to the observed increase in exercise capacity.

*Mdx* mice exhibit defects in muscle regeneration due to the absence of dystrophin at the sarcolemma [81]. These defects in muscle regeneration are

readily apparent after bouts of intense exercise and generally result in chronic muscle weakness. As *mdx:Myog*-deleted mice exhibited enhanced exercise endurance, I determined whether their muscles displayed signs of improved muscle function. No remarkable changes were detected in the muscle pathology of *mdx:Myog*-deleted mice when compared to the *mdx* control group. Characteristic signs of muscle regeneration such as centrally located nuclei and wide variations in myofiber diameter were observed in both *mdx:Myog*-deleted and *mdx* mice. *Mdx* muscles are also particularly prone to fibrosis due to the increased production of connective tissue that is coincident with defective muscle regeneration and muscle atrophy [58]. No differences were detected in the abundance of fibrotic tissue from the muscles of *mdx:Myog*-deleted mice compared to the *mdx* control group. Elevated blood CK levels resulting from the leakage of CK through the damaged sarcolemma are also characteristic of dystrophic muscle [82]. Dramatically elevated blood CK levels were observed in both *mdx:Myog*-deleted mice and the *mdx* control group when compared to wild-type. Given that CK levels remained elevated in *mdx:Myog*-deleted mice, the sarcolemmal defects normally associated with dystrophin deficiency are likely unrepaired in these animals. Although *mdx:Myog*-deleted mice display enhanced exercise endurance, the pathology of these mice resembles that of the *mdx* control group.

Surprisingly, *mdx* mice did not display an increased penetrance of muscular dystrophy following the deletion of *Myog*. The mice displayed no overt adverse phenotypic characteristics with seemingly normal activity levels. This is

unexpected since *mdx:MyoD(-/-)* mice are known to exhibit a severe dorsal-ventral curvature of the spine, become progressively less active, and incur premature death [83]. This suggests that the loss of myogenin neither enhances nor impairs muscle regeneration and is thus dispensable for these processes.

In mammalian muscle, enhanced exercise endurance is typically associated with increases in type 1 and type 2A muscle fibers as well as increases in muscle glycogen content [84]. To determine whether a change in muscle fiber type or glycogen content was conferring the observed enhanced exercise capacity in *mdx:Myog*-deleted mice, histological, biochemical, and gene expression analyses were performed. Selected hindlimb muscles from *mdx:Myog*-deleted mice displayed no significant differences in myosin isoform expression when compared to the *mdx* control mice. The staining of muscle for glycogen abundance suggested there was increased glycogen content in *mdx:Myog*-deleted samples. Biochemical analyses of whole muscle glycogen content also revealed a trend toward increased glycogen in *mdx:Myog*-deleted muscle. The results indicated that myofibers of *mdx:Myog*-deleted mice may contain more glycogen than their *mdx* counterparts, but display many other characteristics similar to that of *mdx* mice.

Dystrophin deficiency confers a complex pathophysiology in *mdx* mice due to the secondary loss of other dystrophin-glycoprotein complex factors [67]. In fact, 80-90% of all dystrophin-glycoprotein associated factors are absent in *mdx* mice [66, 85]. These proteins are synthesized as normal, but without dystrophin they are unable to incorporate into the sarcolemma properly and are

thus targeted for degradation [86]. One of these factors is nNOS. Mice deficient for nNOS are unable to oppose vasoconstriction resulting in ischemia and increased fatiguability [87, 88]. Furthermore, nitric oxide has been shown to regulate neuromuscular transmission [89] as well as enhance mitochondrial biogenesis [90] and GLUT4 production and transport [91], suggesting that reduced nitric oxide production could contribute to the early onset of fatigue [92].

Recently, Wehling-Henricks et al. showed that a loss of interaction between nNOS and PFK contributes to glycolysis defects and increased fatigability in *mdx* mice [67]. Given these results, I determined whether nNOS and PFK expression would be affected by the deletion of myogenin from *mdx* mice. The results confirmed a dramatic decrease in nNOS expression in *mdx* muscle compared to wild-type and detected a 2.2-fold increase in nNOS expression in *mdx:Myog*-deleted muscle compared to *mdx* samples; PFK expression remained unchanged. The increase in nNOS expression in *mdx:Myog*-deleted mice could contribute to their reduced fatigability and may help explain their enhanced exercise endurance compared to the *mdx* control group.

Recently, Moresi et al. showed that *Myog*-deleted mice are resistant to denervation-induced muscle atrophy [26]. Myogenin was found to activate Trim63 and Fbxo32, E3 ubiquitin ligases involved in muscle proteolysis and atrophy. Trim63 and Fbxo32 are normally upregulated in various models of muscle wasting, and a deficiency of these factors protects against muscle atrophy. The loss of myogenin was associated with preserved muscle mass

following denervation and diminished expression of Trim63 and Fbxo32. Their results suggest the involvement of myogenin in a molecular pathway responsible for muscle wasting disorders and prompted us to determine Trim63 and Fbxo32 expression levels in *mdx* mice lacking myogenin. Although no changes in Trim63 expression were observed, a significant reduction in Fbxo32 expression in *mdx:Myog*-deleted mice was detected. This result is consistent with the study described above and suggests that the loss of myogenin may downregulate Fbxo32 activity. The reduction in Fbxo32 expression may help protect against the inherent and chronic muscle atrophy found in dystrophic muscle.

In humans, DMD is a chronic muscle disease leading to progressive muscle weakness and muscle wasting. The *mdx* mouse model described here reliably demonstrates that the exercise capacity of *mdx* mice is considerably less than that of wild-type control mice. The observed decrease in exercise capacity of *mdx* mice is similar to the muscle weakness typically observed in humans with DMD and is likely caused by similar mechanisms. When *Myog* was deleted from *mdx* mice, significant increases in exercise endurance were observed that are associated with changes in the expression of nNOS and Fbxo32. While *mdx:Myog*-deleted mice exhibited an enhanced capacity for exercise, no differences were detected in the histopathology of their muscles compared to the *mdx* control group. Thus, the loss of myogenin from *mdx* mice may rescue their inherent glycolytic dysfunction and muscle weakness, allowing for increased muscle endurance.



Currently, there are no effective treatments to stop the progression or reverse the effects of DMD. Therapies and treatments for this disease, such as corticosteroids and orthotics, are limited and only serve to delay the onset of progressive muscle weakness and eventually death. New therapeutic strategies aim to treat DMD at the molecular level, such as gene replacement therapy and exon skipping. Our results suggest that reducing *Myog* expression in *mdx* mice could contribute to the development of new therapeutic strategies for human DMD. Future directions could include the development of an RNAi delivery system targeting *Myog* transcripts in adult *mdx* mice. If similar enhancements in exercise capacity are observed in those animals, a more translational research approach may be warranted to apply this strategy to larger animals with the ultimate goal of developing therapies for humans with DMD.

## **APPENDIX:**

### **Elucidating the mechanisms conferring selective target gene activation by myogenin**

#### **INTRODUCTION:**

Developmental diversity is regarded, in part, to be the evolutionary result of individual transcription factor family members taking on specific roles in regulating selective gene targets [93]. Little is known about how closely related transcription factors distinguish their respective target genes. Elucidating mechanisms that confer this selectivity will provide insight into how this is achieved in transcription factor families composed of closely related members. As gene duplications are a major feature of the human genome, understanding the mechanisms by which this functional specialization is obtained is especially pertinent [94].

As discussed in chapters 1 and 2, the roles of the myogenic regulatory factors (MRFs), MyoD [1], Myf5 [2], MRF4 [3-5], and myogenin [6, 7], have been well established in embryonic skeletal muscle development. Of special interest to this investigation are myogenin and MyoD. The functions of these two factors during embryogenesis are markedly different, yet they share nearly indistinguishable basic helix-loop-helix DNA binding sequences and remarkable homology in other domains [95]. The transcriptional mechanisms enabling MyoD and myogenin to selectively activate specific sets of target genes are still largely unknown. MyoD and myogenin both bind to sequence consensus sites called E-boxes (CANNTG) found in the transcriptional control regions (TCRs) of

muscle-specific genes. MyoD regulates the initial stages of limb skeletal muscle development during the early determination of myoblasts, embryonic skeletal muscle stem cells. The expression of MyoD also persists into the differentiation stages of myogenesis. Myogenin is upregulated in the latter stages of myogenesis and is required for the terminal differentiation of myoblasts into myofibers [96, 97].

Myogenin selectively activates target genes such that MyoD cannot compensate for the loss of myogenin. Although myogenin and MyoD share significant sequence homology, in *Myog*-null myoblasts, MyoD cannot activate the transcription of many myogenin target genes. *Myog*-null mice exhibit skeletal muscle precursor cells expressing MyoD, but possess few or no functional myofibers and die pernatally due to a severe skeletal muscle deficiency [12, 70, 98, 99]. Thus myogenin activates its own unique set of target genes. Discerning how this mode of selectivity is achieved is an important step in obtaining a comprehensive understanding of the myogenic regulatory network. Extensive knowledge of the myogenic regulatory network makes the skeletal muscle gene regulatory system a valuable model for determining how closely related transcription factors regulate selective clusters of target genes [100, 101].

Both chromatin modifications and cofactors have been shown to play important roles in regulating skeletal muscle-specific gene activation. For example, histones H3 and H4 are acetylated following the recruitment of the histone acetyltransferase p300 to the promoter of muscle-specific genes [96].

Cofactors such as E12/47 and MEF2C are critical for synergistically activating muscle-specific genes [102-104], and E-boxes and MEF2 binding sites are often found in very close proximity [105]. Elements such as these may play a role in conferring target gene selectivity.

Recent investigations have attempted to explain how myogenin and MyoD may utilize their functional differences to work in concert during the activation of late-expressing genes in myogenesis [96, 106, 107]. MyoD is known to bind at the TCR's of muscle-specific genes and activate the expression of early-expressing factors [108-110]. Its expression also persists into the differentiation stages of myogenesis where it binds to and initiates chromatin remodeling in late-expressing genes through the recruitment of factors such as HAT3/4 and BRG1, although it is thought to be insufficient for the activation of these factors [106, 108]. Myogenin is highly upregulated in the latter differentiation phases of myogenesis and activates the transcription of late-expressing genes. However, myogenin appears to be incapable of binding to the TCRs of many muscle-specific genes in the absence of MyoD. Ohkawa et al speculate that myogenin replaces MyoD at the E-box to initiate transcription in late-expressing genes whereas others hypothesize MyoD is required to first bind and initiate the remodeling of chromatin in order to allow the concomitant binding of myogenin at the TCR and activate transcription [106, 108-110]. These models discerning MyoD- from myogenin-mediated gene activation fail to address the mechanisms by which binding to gene regulatory regions early in myogenesis differs from binding to regulatory regions late in myogenesis.

Myogenin and MyoD share significant sequence similarities, yet they function in different capacities. The mechanisms conferring the specific activation of early- or late- expressing genes in myogenesis have yet to be identified. I hypothesize that myogenin acts as a selective activator of transcription through a regulatory network composed of both chromatin modifications and cofactors that are distinct from those employed by MyoD, thereby allowing it to activate specific sets of target genes.

In this appendix, I describe the creation of a Tet-On *Myog*-inducible cell culture system. This system allows for the activation and deactivation of *Myog* in myoblasts under either proliferating or differentiating conditions. Once established, these cells will be used as a tool to elucidate the mechanisms responsible for conferring target gene specificity.

## **Results**

*Myog*-null myoblasts were isolated and characterized from the tongue tissue of *Myog*<sup>-/-</sup> E15.5 mouse embryos. Embryos were isolated and tongue tissue was dissected. Tissue samples were then finely minced, treated with collagenase, and grown in culture. Cells from separate embryos were pooled after genotyping confirmed the absence of myogenin. Pre-platings were performed to remove fibroblasts and other contaminating cells. Genotyping performed after pooling and pre-plating indicated both the absence of the wild type allele and the presence of a neomycin cassette (as a result of generating the knockout mouse) (Figure 33). Immunofluorescence using primary antibodies against markers for skeletal muscle stem cells indicated that the cells expressed these markers (Figure 34). When placed in differentiation media for 24 hours, cells began to elongate and fuse together, classic signs of differentiation (Figure 35). This analysis verified that the isolated cells were embryonic skeletal muscle stem cells.

The Retro-X Tet-On Advanced Inducible Expression System from Clontech ([www.clontech.com](http://www.clontech.com)) was employed to generate *Myog*-null myoblasts stably infected with an inducible form of myogenin. The system requires the generation of a double-stable cell line. First, the cells must be stably infected with the vector coding for the tetracycline-controlled transactivator (pRetroX-Tet-On Advanced). The cells are then infected with the vector containing the gene of interest (pRetroX-Tight-Pur).

**Figure 33**

Genotype verification of isolated *Myog*-null cells. Putative myoblasts isolated from *Myog*-null E15.5 tongue tissue exhibit both the absence of the wild type allele (A) and the presence of a neomycin cassette (B) (as a result of generating the knockout mouse).

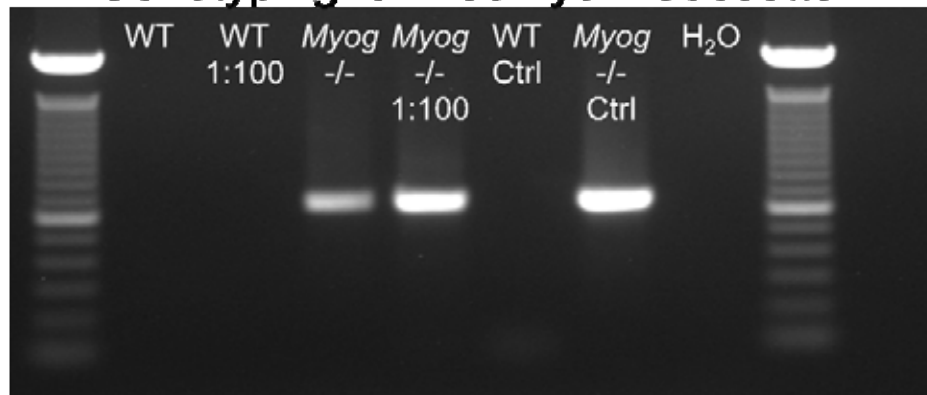
A.

### Genotyping for Wild-Type Exon 1



B.

### Genotyping for Neomycin Cassette

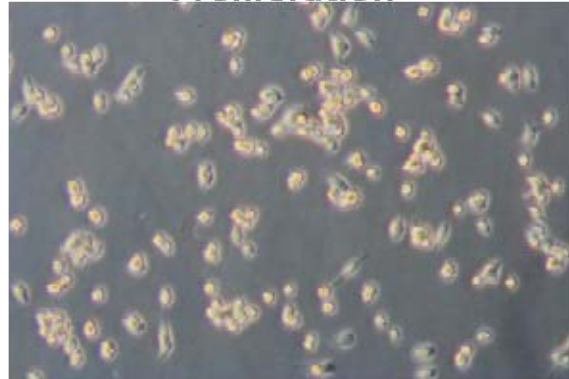




### **Figure 34**

Differentiation of putative myoblasts into myotubes. Putative myoblasts isolated from *Myog*-null E15.5 tongue tissue proliferate continually in high-concentration fetal bovine serum (FBS) media. When switched to low-concentration horse serum media for 24 hours, cells cease proliferation and begin to differentiate, elongating and fusing together to form myotubes.

**Proliferation**



**24hrs Differentiation**

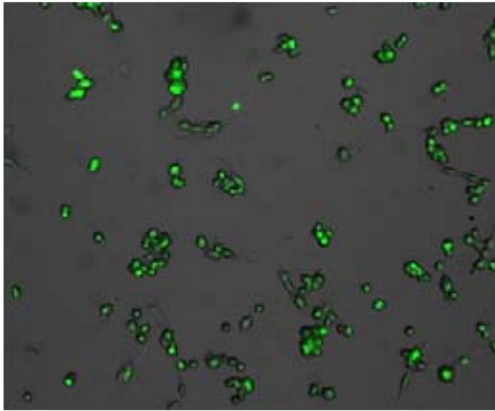


### **Figure 35**

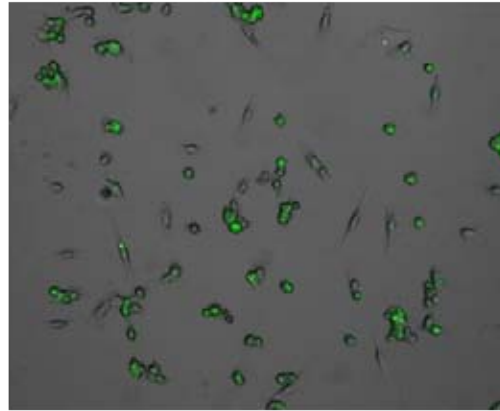
Verification of expression of skeletal muscle stem cell markers.

Immunofluorescence using primary antibodies against markers for skeletal muscle stem cells (MyoD, C-met, Pax3, M-cadherin, and Desmin) indicated the isolated cells do express these markers.

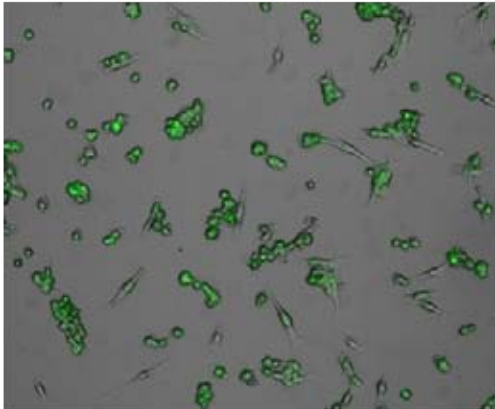
**MyoD**



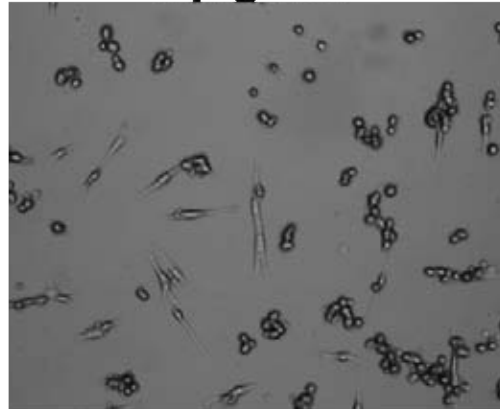
**Pax3**



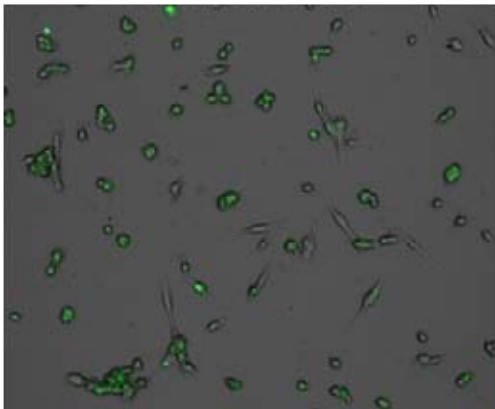
**C-met**



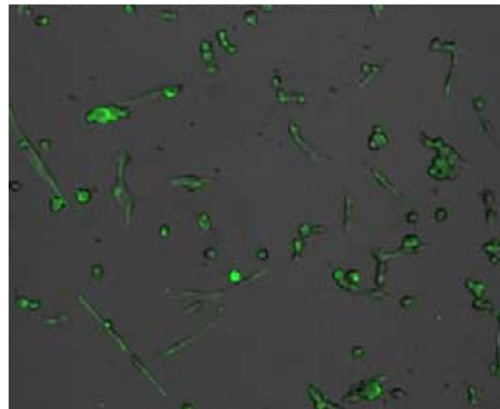
**myogenin**



**M-Cadherin**



**Desmin**



The pRetroX-Tet-On Advanced vector utilizes a neomycin cassette so that stably infected cells can be selected for using G418 in drug selection. Because *Myog*-null myoblasts already expressed a neomycin cassette as a result of generating the null line, the vector was engineered with an alternate method for drug selection. The neomycin cassette was excised and a blasticidin cassette from another vector was subcloned in its place (Figure 36).

*Myog* cDNA followed by an IRES-GFP cassette was subcloned into the pRetroX-Tight-Pur vector (Figure 37). This was achieved by generating a *Myog*-GFP topovector, removing the *Myog*-GFP sequence, and ligating into the linear pRetroX-Tight-Pur vector to create the final pRetroX-Tight-Pur-*Myog*-GFP vector. This vector was later sequenced to verify the orientation and the absence of mutations.

Both vectors described above were transiently transfected into Eco-Pack 293 packaging cells to generate retrovirus stocks. Virus-containing medium was collected and the viral titer was determined by colony formation.

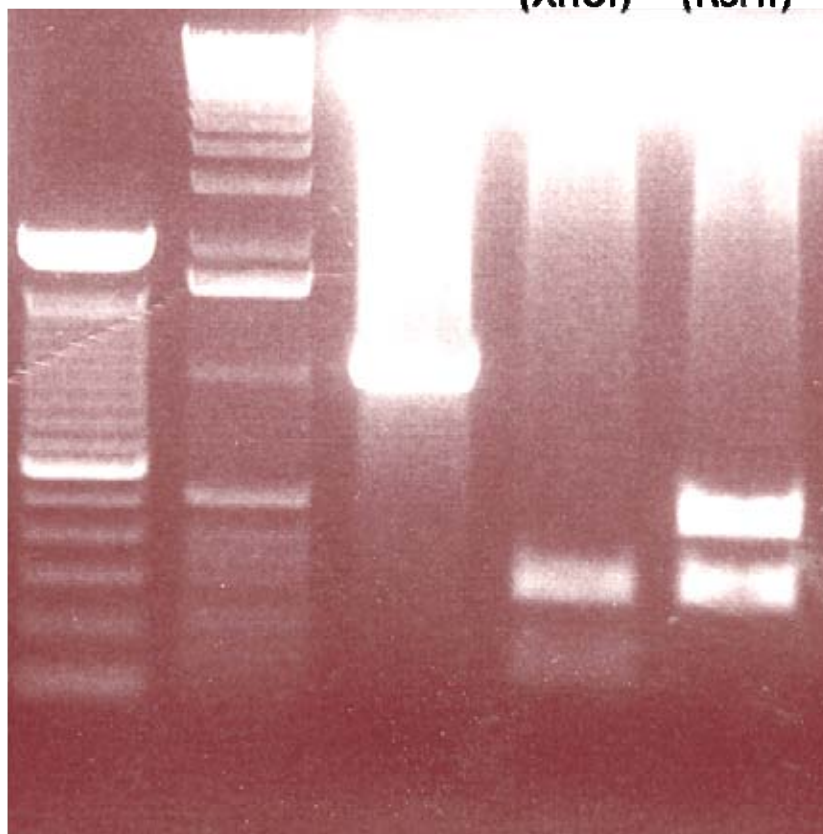
A death curve for the *Myog*-null myoblasts with varying concentrations of blasticidin & puromycin was performed to determine the minimal concentrations necessary to eradicate all the cells. These concentrations were then used for drug selection.

A single-stable RetroX-Tet-On Advanced cell line was then generated by infecting *Myog*-null myoblasts with the RetroX-Tet-On Advanced vector. The infection was allowed to proceed for 24 hours. Blasticidin was then added to the plates and 40 colonies were isolated using cloning discs.

**Figure 36**

Verification of replacement of Neomycin cassette for Blasticidin cassette in the pRetroX-Tet-On Advanced vector. The neomycin cassette was excised and subcloned in its place was a blasticidin cassette. Restriction digestions indicate the insertions were of the expected sizes.

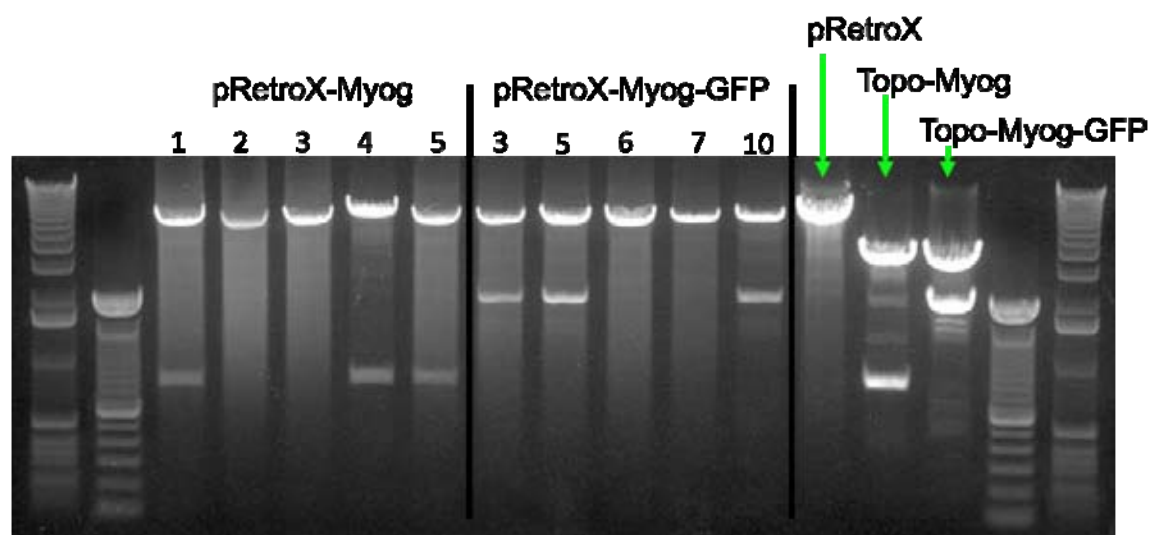
| 100bp<br>ladder | 1kb<br>ladder | pRetro<br>+neo | pRetro<br>+Blast<br>(XhoI) | pRetro<br>+Blast<br>(RsrII) |
|-----------------|---------------|----------------|----------------------------|-----------------------------|
|-----------------|---------------|----------------|----------------------------|-----------------------------|



### **Figure 37**

Verification of *Myog*-GFP cassette insertion into the pRetroX-Tight vector. *Myog* cDNA followed by an IRES-GFP cassette was subcloned into the pRetroX-Tight-Pur vector. Restriction digestions indicate the insertions were of the expected sizes.





The viability of the RetroX-Tet-On Advanced clones was tested by infecting them with a luciferase reporter vector that is activated by the tetracycline-controlled transactivator encoded by the RetroX-Tet-On Advanced vector. Each clone was then treated with 500 ng/ml of Doxycycline (Dox) and the fold induction was determined. Clones exhibiting 20- to 50-fold induction were chosen to generate double-stable lines (Figure 38).

To accomplish this, single-stable *Myog*-null myoblasts were infected with the pRetroX-Tight-Pur-*Myog*-GFP vector to generate double-stable lines. The infection was allowed to proceed for 24 hours. Puromycin was added to the plates and colonies were isolated using cloning discs.

Double-stable cell lines were then screened by qRT-PCR for the presence of *Myog* transcripts in the presence and absence of Dox at 500 ng/ml. Multiple lines exhibiting significant induction in the presence of Dox and little to no induction in the absence of Dox (Figure 39) were frozen in liquid nitrogen. Cell line 11 was chosen as the primary line for further study.

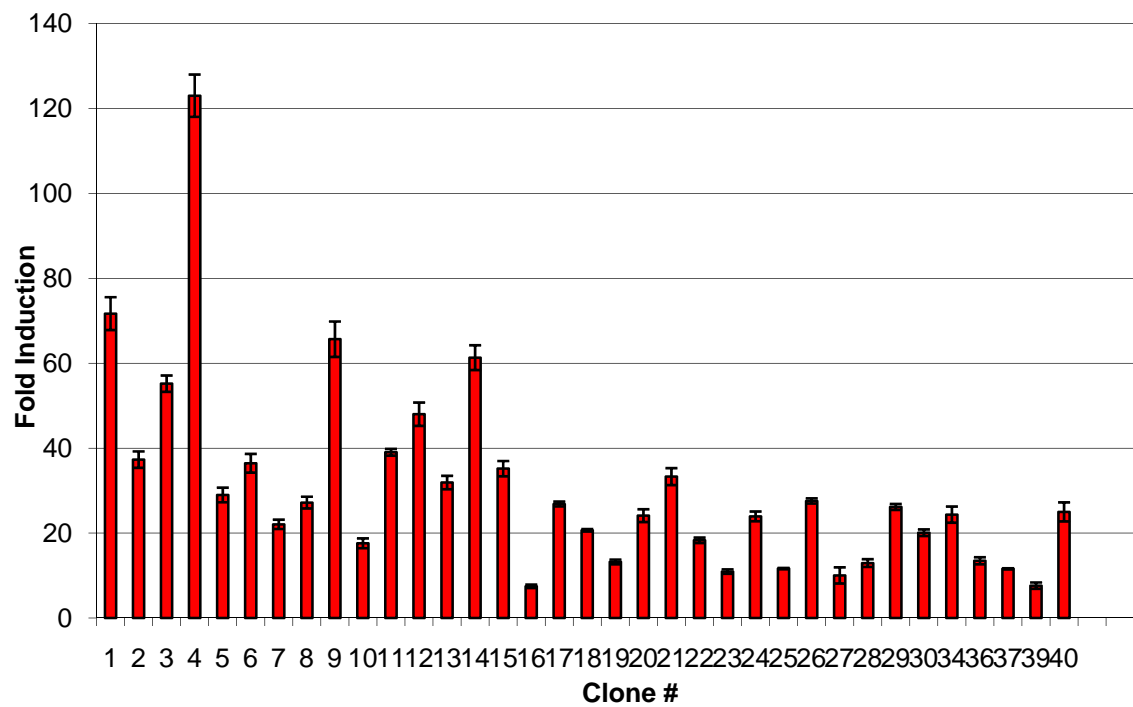
Since the pRetroX-Tight-Pur vector contained *Myog* cDNA followed by an IRES-GFP cassette, induced double-stable *Myog*-null myoblasts expressed GFP (Figure 40). Furthermore, double immunostaining showed the presence of myogenin in the nucleus and GFP in the cytoplasm of the same cells (Figure 41).

To more accurately reflect the endogenous expression of myogenin that is observed in wild-type differentiating myoblasts, I attempted to determine the concentration of Dox required to induced *Myog* expression to wild-type levels in

**Figure 38**

Luciferase activity of selected single stable myoblast colonies. Subpopulations of cells infected with the RetroX-Tet-On Advanced vector and a luciferase reporter vector that is activated by the tetracycline-controlled transactivator encoded by the RetroX-Tet-On Advanced vector displayed varying levels of reporter activity. Clones exhibiting 20- to 50-fold induction were chosen to continue making double-stable lines with.

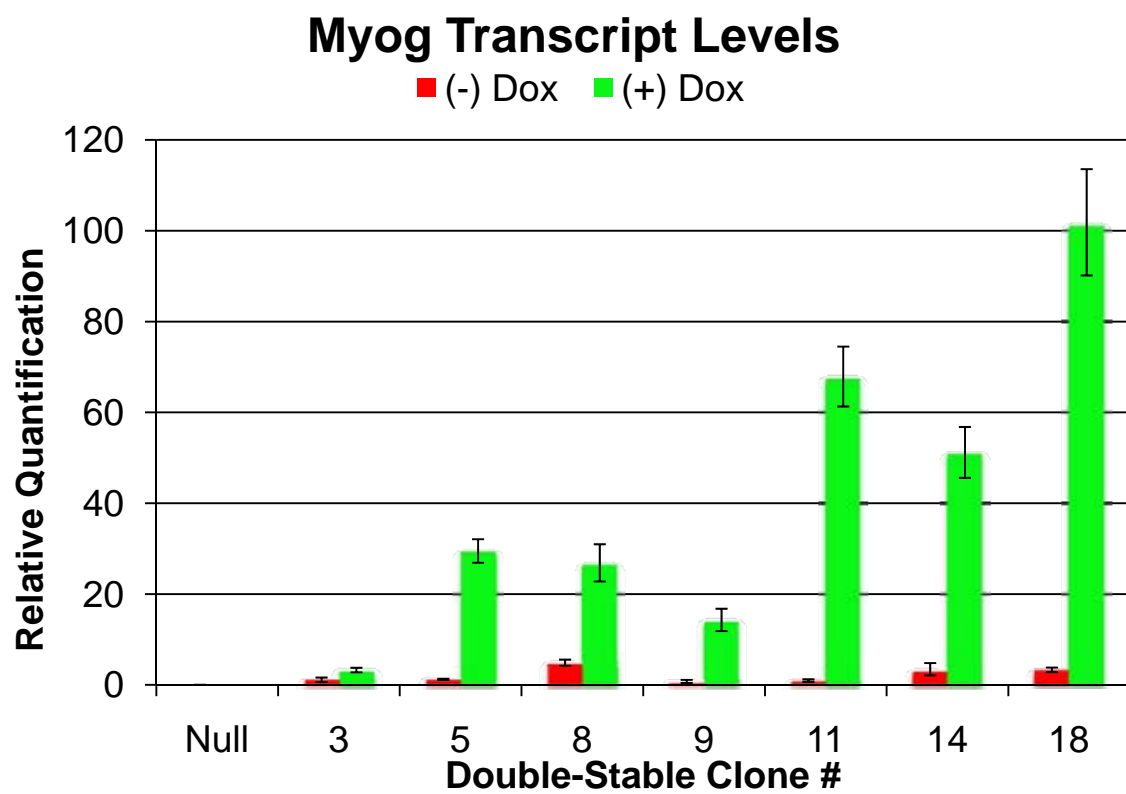
### Single Stable Myoblast Luciferase Assay



### **Figure 39**

Doxycyclin induction of *Myog* expression in double-stable myoblasts.

Subpopulations of cells infected with the RetroX-Tet-On Advanced vector and pRetroX-Tight-Pur-Myog vector displayed varying levels of *Myog* activation in the presence of Doxycyclin. Clones exhibiting high *Myog* expression in the presence of Dox and little to no *Myog* expression in the absence of Dox were chosen for further experiments.



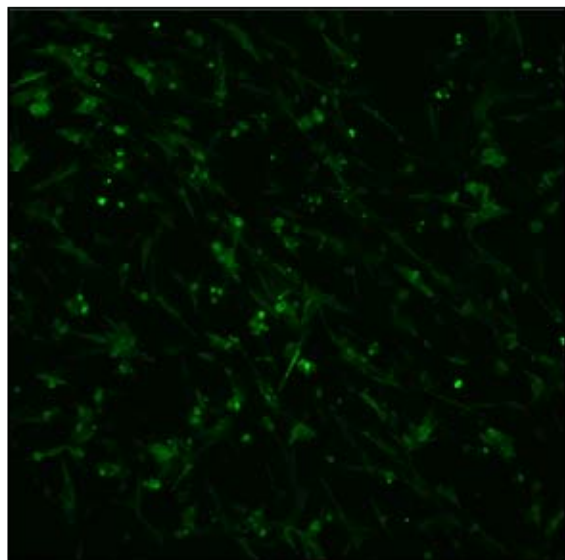
**Figure 40**

GFP reporter expression in double-stable myoblasts in the presence and absence of Dox. Double-stable myoblasts fluoresce green only in the presence of Dox indicating the induction of myogenin.

**(-) DOX**



**(+) DOX – 48hrs**





**Figure 41**

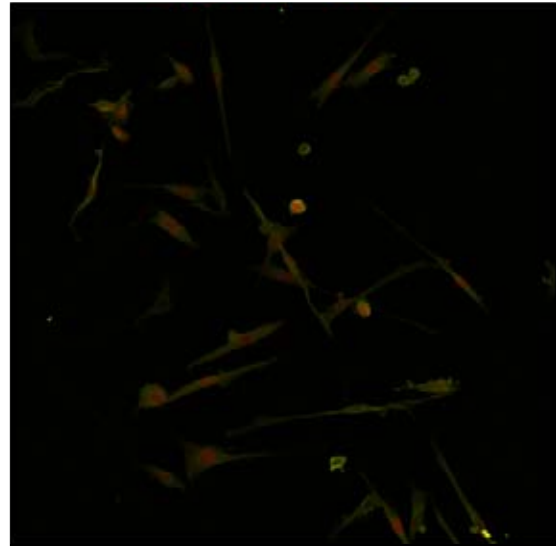
Expression of myogenin and GFP in the same cells in the presence of Dox.

Immunofluorescence indicates the nuclear expression of myogenin and the cytoplasmic expression of GFP in the same cells only in the presence of Dox.

(-) DOX



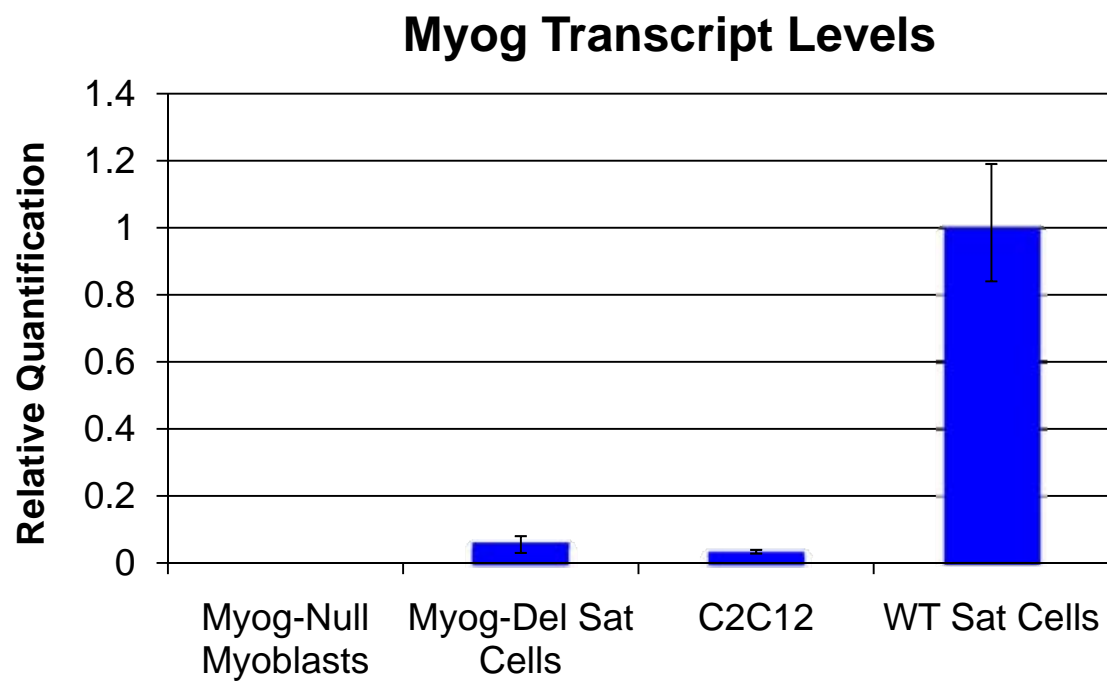
(+) DOX – 48hrs  
myogenin GFP



double-stable myoblasts. To accomplish this, a primary culture of wild-type embryonic myoblasts was required. However, numerous attempts to isolate and establish a wild-type line were unsuccessful due to technical difficulties such as bacterial or yeast contaminations, unsuccessful preplatings due to an overabundance of fibroblasts, and unviable populations of myoblasts either dying or not proliferating. In attempts to circumvent these issues, I determined whether an established line of commercially available C2C12 myoblasts would be an acceptable substitute. qRT-PCR was used to determine the *Myog* transcript levels in C2C12 myoblasts compared to other control myogenic cell lines (Figure 42). C2C12 myoblasts possessed fewer *Myog* transcripts than either wild-type satellite cells (adult skeletal muscle stem cells) or *Myog*-deleted satellite cells. Furthermore, they had little more *Myog* than *Myog*-null myoblasts. I therefore concluded that C2C12 myoblasts were an unacceptable alternative for wild-type myoblasts.

**Figure 42**

Expression of *Myog* in various populations of skeletal muscle stem cells. *Myog* expression levels in C2C12 myoblasts are similar to that of *Myog*-deleted satellite cells or *Myog*-null myoblasts and are far less than that of wild-type satellite cells.



## **Future Directions**

To proceed with this project, several control experiments are necessary. The concentration of Dox required to recapitulate endogenous wild-type myogenin levels must be determined. In addition, the expression patterns of genes previously determined to be either embryonic-specific [47] or adult-specific [13] must be determined in the presence of myogenin to demonstrate that they recapitulate the gene activity patterns observed in the wild-type scenario. If these experiments are successfully completed, this system will provide a unique opportunity to identify the associated cofactors and chromatin marks in the presence and absence of myogenin using chromatin immunoprecipitation and MALDI-TOF mass spectrometry. With these approaches, we can begin elucidating the mechanisms conferring myogenin's selective target gene activation.

## MATERIALS AND METHODS

### Generation of *Myog*-deleted Mice

Mice used for this research were homozygous for the *Myog*<sup>flox</sup> allele and harbored the CAGGCre-ER transgene [29, 111]. These animals consisted of a C57BL/6 and 129 mixed background. Efficient deletion of *Myog* genomic DNA in ~10 week old mice was achieved by a single injection of tamoxifen dissolved in corn oil at a concentration of 10mg/40g body weight. All experimental procedures described below followed the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the The University of Texas M.D. Anderson Cancer Center. In chapter 1, wild-type mice are defined as tamoxifen-injected *Myog*<sup>flox/flox</sup> animals not harboring the Cre-recombinase gene. In chapter 2, wild-type mice are defined as tamoxifen-injected mdx:*Myog*<sup>flox/flox</sup> animals not harboring the Cre-recombinase gene.

### Maximal Exercise Capacity

*Involuntary Treadmill Running:* Treadmill exercise regimes we derived from previously published protocols [112]. Adult wild-type and *Myog*-deleted mice were run on a rodent treadmill (Columbus Instruments Animal Treadmill: Exer 3/6). This treadmill is equipped with a rear electrical stimulus grid set to deliver 0.2 mA, an uncomfortable but not physically harmful shock. Mice were determined to have reached the point of exhaustion when they made contact

with the grid for a period of 10 seconds. The stimulus was then shut off in the lane of the exhausted mouse.

The low-intensity exercise regime consisted of mice warming up for a period of 30 minutes at 10 m/min (0° incline). The speed was then increased to 20m/min (0° incline), and mice were allowed to run to exhaustion. Exercise duration and distance were recorded at exhaustion.

The high-intensity exercise regime consisted of mice warming up for a period of 10 minutes at 10m/min on a 10° incline. The speed was then increased by 2 m/min every 2 minutes while maintaining a 10° incline until exhaustion. Exercise duration, distance, and maximum speed were recorded at exhaustion.

*Voluntary Wheel Running:* Cages containing exercise wheels were built in-house. Exercise wheels were suspended in the center of the cage and held in place by wires fastened to the cage top and base. One mouse was housed in each cage. Running wheels were connected to sensors recording time, average speed, maximum speed, and distance ran since the last reset.

### **Assay for Blood Glucose and Blood Lactate**

Blood glucose concentration was determined by using a Precision Xtra glucometer (Abbott) at rest and within 30 seconds of reaching exhaustion.

Blood lactate concentration was determined by using a Lactate Pro blood lactate test meter (Arkray) at rest and within 30 seconds of reaching exhaustion.



## Histological Assays

Hind-limb muscles were dissected and snap frozen in LN<sub>2</sub>-cooled isopentane. Ten to 12 µm fresh-frozen sections were then cut on a Cryostat.

*Fiber Typing ATP Staining Protocol:* A protocol from Washington University's Neuromuscular Disease Center (<http://neuromuscular.wustl.edu/pathol/histol/atp.htm>) was utilized to perform this assay. Sections were incubated for 5 min in pre-incubation solution pH 4.6 (5.0 mL Barbitol Acetate Solution [1.47 g sodium barbitol, 0.97 g sodium acetate, 50 mL final volume in H<sub>2</sub>O], 10 mL 0.1N HCl, 4.0 mL H<sub>2</sub>O), rinsed with H<sub>2</sub>O, and then incubated for 25 min in ATP solution H 9.4 (60 mg ATP powder, 6.0 mL of 0.1 M sodium barbitol, 21.0 mL of H<sub>2</sub>O, 3.0 mL of 0.18 M Calcium Chloride). Sections were washed with 3 exchanges of 1% calcium chloride for 10 min total, 2% cobalt chloride for 10 min, and 4 exchanges of 1:20 solution of 0.1M sodium barbitol, 5 exchanges of H<sub>2</sub>O. Sections were then exposed to 2% ammonium sulfide for 20-30 seconds, washed with 5 exchanges of H<sub>2</sub>O, dehydrated in ascending alcohols, cleared with xylene, and mounted with canada balsam.

*Succinate Dehydrogenase Enzyme Stain:* A modified protocol [113] from the University of Nottingham Medical School was utilized for this assay (<http://www.nottingham.ac.uk/pathology/protocols/sdh.html>). Sections were incubated for 5 minutes in medium containing 0.2 mL of 500 mM sodium succinate, 0.7 mg phenazine methosulphate, and 2.0 mL NBT Solution (6.5 mg KCN, 185 mg EDTA, 100 mg Nitroblue tetrazolium, 100 mL 100mM phosphate buffer pH 7.6 {12 mL of Solution A [1.36g KH<sub>2</sub>PO<sub>4</sub> in 100mL H<sub>2</sub>O] and 88 mL of

Solution B [1.42g  $\text{Na}_2\text{HPO}_4$ /100mL  $\text{H}_2\text{O}$ ]). Sections were then washed, fixed for 15 minutes in formaline, dehydrated in ascending alcohols, cleared with xylene, and mounted.

*Glycogen Stain:* The Periodic Acid-Schiff Staining System from Sigma-Aldrich was utilized for this assay. Sections were hydrated with deionized water, fixed with 10% neutral buffered formalin for 30 minutes, and then incubated in Periodic Acid Solution for 5 minutes at room temperature. Sections were then rinsed in distilled water and incubated in Schiff's Reagent for 15 minutes at room temperature. Slides were then washed in running tap water for 5 minutes and counterstained in Hematoxylin Solution for 90 seconds. Slides were again washed in running tap water, dehydrated in ascending alcohols, clear with 2 exchanges of Xylene, and mounted.

*Fibrosis stain for Collagen and Elastin:* Muscle sections were hydrated with deionized water and then fixed for 30 minutes in neutral buffered formalin. Slides were then incubated in Working Elastic Stain Solution for 10 minutes (20 mL Hematoxylin Solution, 3 mL Ferric Chloride Solution, 8mL Weigert's Iodine Solution, 5 mL Deionized water). Slides were then washed in deionized water and incubated in Working Ferric Chloride Solution for 1 minute (3mL Ferric Chloride Solution, 37 mL deionized water). Sections were then washed in tap water followed by rinsing in 95% alcohol. Slides were then rinsed in deionized water, incubated in Van Gieson Solution for 5 minutes, rinsed in 95% alcohol, dehydrated in ascending alcohols, cleared with 2 exchanges of Xylene, and mounted.

## **Affymetrix Gene Microarray Hybridization and Analysis**

Hindlimb muscles of trained wild-type and *Myog*-deleted mice were treated with Trizol and used to isolate RNA from. Methods for this assay have been described previously [13]. Hybridization probes were prepared from 100 ng of total RNA from each sample. Three samples from each group tested were then utilized in standard Affymetrix protocols to yield biotin labeled cRNA fragments hybridized to Mouse Genome 430 2.0 GeneChips (Affymetrix, Santa Clara, CA). Affymetrix GCOS software with Robust Multi-chip Averaging normalization was employed to scan hybridized genechips [114]. For statistical analysis, two-tailed pooled t-tests were conducted with the minimum significance value set to  $P < 0.05$ . Genes exhibiting expression changes greater than 1.4 fold were retained for additional experimentation. The microarray data discussed in this dissertation are MIAME compliant have been deposited in NCBI's Gene Expression Omnibus [115] and are accessible through GEO Series accession number GSE22046, (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22046>).

## **RNA Isolation and qRT-PCR**

RNA was isolated from hind-limb skeletal muscle of trained mice using the Qiagen RNeasy Plus kit protocol. Reverse transcription was performed as previously described [47]. TaqMan Gene Expression Assays (Applied Biosystems) were utilized to determine cDNA abundance for each gene assayed.

## Enzymatic Analysis

Enzymatic analysis was performed on Soleus, Extensor Digitorum Longus (EDL), and Plantaris muscles from wild-type and *Myog*-deleted mice. An abbreviated protocol includes the following steps: fast- and slow-twitch muscles were isolated and snap frozen in LN<sub>2</sub>. Muscle was homogenized in stabilizing medium at a 1:100 dilution based on wet weight. Stabilizing medium consists of 50% glycerol, 20 mM phosphate buffer, pH 7.4, 5 mM beta-mercaptoethanol, 0.5 mM EDTA, 0.02% BSA [28].

Assays performed include: Pyruvate Kinase (PK, EC 2.7.1.40), Hexokinase (HK, EC 2.7.1.1), Lactic Dehydrogenase (LDH, EC 1.1.1.27), Glycerophosphate Dehydrogenase (GPDH, EC 1.1.1.8), Glycogen Phosphorylase (PHRL, EC 2.4.1.1), Fructose-6-Phosphate Kinase (PFK, EC 2.7.1.11), Adenylate Kinase (AK, EC 2.7.4.3),  $\beta$ -Hydroxybutyrate Dehydrogenase (BDH, EC 1.1.1.30), Carnitine Acetyltransferase (CAT, EC 2.3.1.7), Pyruvate Carboxylase (PC, EC 6.4.1.1), Citrate Synthase (Cit-Syn, (EC 4.1.3.7),  $\beta$ -Hydroxyacyl-CoA Dehydrogenase (BOAC, EC 1.1.1.35), and Malic Dehydrogenase (MDH, EC 1.1.1.37).

Cuvettes were prepared with the buffer and substrates required for the particular assay and monitored with a spectrophotometer at  $A_{340\text{nm}}$  until constant. Diluted homogenates were then added to each cuvette in duplicate and monitored with continuous spectrophotometric rate determination, recording the change in  $A_{340\text{nm}}$  for approximately 10 minutes at 25°C. The change in  $A_{340\text{nm}}$ /minute was determined using the maximum linear rate for both the test

and the blank. Enzyme activities are given as  $\text{mmol}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ . Detailed protocol information is available from the Sigma Aldrich Enzyme Explorer Assay Library (<http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer.html>).

### **Biochemical determination of glycogen content**

Soleus and extensor digitorum longus (EDL) muscles were harvested from wild-type, *mdx*, and *mdx:Myog*-deleted mice. An abbreviated protocol followed from MBL International Corporation ([www.mblintl.com](http://www.mblintl.com)) includes the following steps: muscles were isolated and snap frozen in liquid nitrogen. Muscle was homogenized in  $\text{dH}_2\text{O}$ , boiled for 5 minutes, and centrifuged at 13,000rpm for 5min. Glucoamylase hydrolysis enzyme mix was then added to the supernatants. A master mix of development buffer, development enzyme mix, and OxiRed probe were then added to the samples and left to incubate at room temperature protected from light for 30 minutes. Samples were then measured colorimetrically using a spectrophotometer at a wavelength of 570nm. Non-hydrolysis control readings were then subtracted from the experimental raw values, and glycogen concentration was determined by applying the final values to a standard curve.

### **Statistical Analysis**

Statistical analyses were performed using 2-tailed pooled student's *t*-tests in Microsoft Excel 2008. A confidence level of  $p<0.05$  was considered to be

statistically significant. Error bars represent one standard deviation from the mean.

*Increased Training Response:* Thanks to the Department of Biostatistics for their help in the statistical analysis of this data. A general linear model was fitted to evaluate the effects of marker (wild-type vs. *Myog*-deleted), training status, and their interaction on dependent variable distance. Analysis indicates that when comparing to marker wild-type group, the difference in distance between the training and non-training groups is significantly different.

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**Vita**

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