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I, Erin R Wissing, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Molecular Genetics, Biochemistry, & Microbiology.

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Uncovering the complexity of muscular dystrophy pathology through disease signaling

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Uncovering the complexity of muscular dystrophy pathology through disease signaling

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By
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Abstract

The muscular dystrophies (MD) are a diverse group of inherited disorders best characterized by muscle wasting. Progressive weakening and wasting of skeletal muscle leads to loss of ambulation in the teen years and premature death in the second or third decade of life. Many of the MDs are caused by mutations in proteins in the dystrophin glycoprotein complex (DGC). In the absence of a functioning DGC, the sarcolemma becomes unstable during contraction and microtears occur allowing an unregulated influx of ions, such as Ca\(^{2+}\), to enter the myofiber leading to the induction of multiple signaling or cellular pathways. Here we present data from three different pathways in skeletal muscle in an effort to shed more light on the complex and multifaceted MDs, and identify targets for future therapeutics in dystrophic patients.

First we show that the potent cyclophilin D (Cyp D) and mitochondrial permeability transition pore (MPTP) inhibitor, Debio-025, can work in combination with prednisone with no adverse effects in \(mdx\) mice. Here we find that treatment of \(mdx\) mice with Debio-025 by oral gavage or subcutaneous injection alone or in combination with prednisone significantly decreases pathology compared to vehicle controls. However, the combination treatment of Debio-025 and prednisone is not additive, indicating that Debio-025 and prednisone could be working through similar pathways. Thus, we found that co-administration with prednisone had no adverse side effects.

The p38 MAPK signal amplification cascade is known to play roles in many cellular processes; however, no definitive link to dystrophy has been described. Here we present data suggesting p38\(\alpha\) as a potent signaling effector which promotes disease progression in MD through a cell death dependent mechanism. To test this hypothesis we crossed p38\(\alpha\) skeletal muscle specific knockdown mice to \(\delta\)-sarcoglycan (\(Sgcd^{-/-}\)) and \(mdx\) mice and found that the loss of p38\(\alpha\) alleviated pathology. Muscle specific over-activation of MKK6-p38 activity was found to produce a severe dystrophic-like muscle wasting pathology, indicating that p38 MAPK is sufficient to cause disease in skeletal muscle due to p38 specific
phosphorylation of the cell death protein Bax (T167). Treatment of Sgcd-/- mice with a p38 inhibitor was also found to reduce disease.

Finally, the ERK1/2 MAPK pathway has been shown to play a role in many cellular processes including skeletal muscle fiber type determination. In dystrophic muscle, the fast fibers are affected first, due to their high contraction speed and force production. In our preliminary studies, we find that skeletal muscle specific over-activation of ERK1/2 MAPK produces a slow fiber-type phenotype in transgenic mice, indicating a role for ERK1/2 in slow twitch fiber preservation. This indicates that ERK1/2 over-activation could be protective in dystrophic mice due to a promotion of slower muscle phenotype.

Our studies have shown in mice that Debio-025 can work in combination with prednisone to decrease pathology, p38α promotes disease pathology, and ERK1/2 over-activation causes a protective fiber type switch. Taken together these studies suggest multiple therapeutic targets that could be used to treat MD.
Dedication

This work is dedicated to my husband Justin and my two beautiful and energetic sons, Logan and Dylan.
Acknowledgements

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List of Abbreviations and Symbols

α – alpha; β – beta; γ – gamma; δ – delta; ε - epsilon
ad - Adenoviral/adenovirus
ALS - Amyotrophic lateral sclerosis
ARC- Apoptosis repressor with a CARD domain
CsA - Cyclosporine A
Cyc c - Cytochrome c
CypD - cyclophilin D
Debio- Debio-025
DKO- Double knockout
DUSP - Dual specificity phosphatase
EBD – Evan’s Blue Dye
EM - Transmission electron microscopy
eMyHC - Embryonic myosin heavy chain
ERK1 - Extracellular signal-regulated kinase 1
ERK2 - Extracellular signal-regulated kinase 2
ERK1/2 – ERK1 and ERK2
ERK5 – Extracellular signal-regulated kinase 5
fl - loxP targeting site
Gastroc. - Gastrocnemius
GFP- Green fluorescent protein
H&E - Hematoxylin and eosin
JNK1 - c-Jun N-terminal kinase 1
JNK2 - c-Jun N-terminal kinase 2
JNK3 - c-Jun N-terminal kinase 3
KO - Knock out
lama2- Laminin-2
MAP3K - MAPK kinase kinase
MAP43K- MAPK kinase kinase kinase
MAPK - Mitogen activated protein kinases
Mapk14 - p38α MAPK
MAPKAPK - MAPK activated protein kinase
MAPKK (MEK or MKK)- MAPK kinase
MEF - Mouse embryonic fibroblasts
CHAPTER 1 – Skeletal Muscle and Muscular Dystrophy Introduction

Skeletal Muscle Structure and Function

Skeletal muscle, one of the most abundant tissues in the human body, is a striated muscle tissue which is made up of individual components termed muscle fibers. Each of these muscle fibers is composed of bundles of long, cylindrical, multinucleated cells composed of actins and myosins repeated as a unit named the sarcomere. (See Figure 1.) The sarcomere is the basic functional unit of the skeletal muscle, and contains all the machinery necessary for muscle contraction. The pattern of repeated sarcomere units is also what gives skeletal muscle its striated appearance. The muscle, as a whole, includes components such as nerves, connective tissue, vascular tissue, and the muscle fibers. The many fibers are held together in bundles by connective tissue (fascicle) and many of these bundles comprise the entire muscle.

Skeletal muscle functions by contracting within the sarcomeric unit. Each sarcomere is composed of thick filaments (myosin), thin filaments (actin), troponin, and tropomyosin which act together to form these contractile units. In muscle contraction the myofilament proteins in the sarcomere, actin and myosin, act to cause tension and are regulated by the proteins troponin and tropomyosin. The contraction begins as the ATP bound to myosin is hydrolyzed by its ATPase into ADP and Pi. As myosin releases the ADP and Pi, it binds to actin and pulls the actin filaments, moving the Z-bands closer together, shortening the sarcomere. (FIGURE 2) Myosin can then bind ATP once again, destabilizing the
connection with actin, allowing the relaxation of the sarcomere and muscle. The contraction process is controlled by the introduction of Ca\(^{2+}\) into the muscle fiber. When the muscle is in a relaxed state, tropomyosin is wrapped around the actin filaments, blocking the myosin binding sites within the sarcomere. However, as Ca\(^{2+}\) becomes present in the muscle, it binds to troponin causing a conformational change of this protein. The change in shape of troponin allows tropomyosin to move deeper into the actin groove in the thin filament, exposing the myosin-actin binding sites, and allowing contraction.

Myogenesis

Skeletal muscle is formed through a process termed myogenesis during embryonic development. Myogenesis is a complex multistep process in which pluripotent mesodermal cells are programmed to commit to the myogenic lineage and give rise to muscle precursor cells, or myoblasts [1,2](Figure3). These pluripotent mesodermal cells, which are derived from the mesoderm in the somites, receive signals from neighboring tissues including Wnts, bone morphogenetic protein 4 (BMP4), sonic hedgehog, and noggin which in turn lead to the expression of transcriptional activators such as Pax 3 and Pax 7 which initiate the expression of myogenic regulatory factors (MRFs) such as MyoD and Myf-5. MyoD and Myf-5 are basic helix-loop-helix
transcription factors and are required for progressive commitment and differentiation of myogenic precursors to embryonic myoblasts. These muscle precursor cells, or myoblasts, are able to proliferate and migrate, but do not yet express muscle specific genes, such as myogenin and MRF4 [2]. A further induction of MyoD, marked by the inhibition of inhibitor of DNA binding (Id), leads to the upregulation of micro RNAs miR-1 and miR-133 which then inhibit factors like serum response factor (SRF) and histone deacetylase 4 (HDAC4). Myoblasts then start to express cell cycle inhibitors such as p38 and p16 causing the myoblasts to exit the cell cycle and allow transcription factors like MEF2 and myogenin to become active [2]. Myogenin and MEF2 family members cooperate in the activation of many muscle specific genes during differentiation of myoblasts that fuse together to form multinucleated myotubes [1]. These newly differentiating myotubes are then innervated by the different types of motor neurons that will determine whether the myotube will differentiate into a slow or fast-twitch fiber [2]. Therefore, myogenesis is a dynamic and tightly-controlled process in which mononucleated myoblasts, withdraw from the cell cycle, differentiate and fuse into multinucleated mature muscle fibers [3].
Skeletal muscle undergoes constant injury due to weight bearing, exercise, and other trauma, and therefore has a way of regenerating or repairing itself. This process of self-repair, or regeneration, is driven by a subset of muscle cells termed satellite cells. Satellite cells are specialized somatic stem cells that are responsible for the maintenance and repair of skeletal muscle [5-8]. These satellite cells, which comprise only 1-6% of the sublaminal nuclei of myofibers, are located between the basal lamina and the muscle fiber plasma membrane, and can remain quiescent for an average of 7 years in adult humans [5-6]. Satellite cells are derived from the Pax3\(^+\)/Pax7\(^+\) progenitor population within the dermomyotome during embryogenesis and, following withdrawal from the cell cycle, position themselves beneath the sarcolemma, quiescent until a muscle injury event [7-8]. Upon muscle injury, satellite cells become mitotically active and proceed into states of programmed proliferation, self-renewal, eventually leading to expression of muscle–specific proteins [5-6]. (FIGURE 4) These activated muscle cells can then migrate to the area of injury and fuse with the damaged muscle fiber, thereby repairing trauma to the existing muscle [5-6].
Fiber Type

Adult skeletal muscle is comprised of a heterogeneous population of specialized myofibers that enable the body to maintain posture and perform a variety of movements [9]. Each muscle has an individualized function, that determines the size, phenotype and fiber composition of the muscle. The heterogeneity of muscles is determined by the speed of contraction and type of energy metabolism of each individual muscle fiber [10]. Skeletal muscle fibers are classified into fiber types, fast or slow, based on the speed of contraction, force development, fatigability, and metabolic functions [11]. Typically, the classification of slow or fast muscle fibers is related to the type of myosin, a structural and functional protein in the sarcomere, expressed by the individual muscle fiber [12]. There are three basic isoforms of myosin heavy chain (MHC) that exist in human skeletal muscle, slow type I and the fast types IIa and IIx/d, while mice express an additional fourth fast isoform of MHC type known as IIb [12]. (See Table 1) Slow twitch, or type I fibers, are characterized by a slower contraction time and low power produced, however, they have high fatigue resistance and therefore are able to maintain contractile activity for longer periods of time [12]. These slow fibers have a high mitochondrial density, express high levels of oxidative enzymes, and use oxidative phosphorylation as their main energy source [10,12]. Fast fiber contraction speeds range from moderately fast (IIa) to very fast (IIx/d/b), and are usually used for short bursts of activity, and have

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIX</th>
<th>Type IIB (mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction Time</td>
<td>Slow</td>
<td>Moderately Fast</td>
<td>Fast</td>
<td>Very Fast</td>
</tr>
<tr>
<td>Size of motor neuron</td>
<td>Small</td>
<td>Medium</td>
<td>Large</td>
<td>Very Large</td>
</tr>
<tr>
<td>Power produced</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
<td>Very High</td>
</tr>
<tr>
<td>Resistance to fatigue</td>
<td>High</td>
<td>Fairly High</td>
<td>Intermediate</td>
<td>Low</td>
</tr>
<tr>
<td>Mitochondrial density</td>
<td>Very High</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Oxidative capacity</td>
<td>High</td>
<td>High</td>
<td>Intermediate</td>
<td>Low</td>
</tr>
<tr>
<td>Glycolytic capacity</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Maximum duration of use</td>
<td>Hours</td>
<td>&gt;30 min</td>
<td>&gt;5 min</td>
<td>&gt;1 min</td>
</tr>
<tr>
<td>Color (appearance)</td>
<td>Red</td>
<td>Light Red</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>Color when stained with Myosin ATPase Stain (alkaline pH)</td>
<td>White</td>
<td>Light Brown</td>
<td>Brown</td>
<td>Brown</td>
</tr>
<tr>
<td>Color when stained with Succinate Dehydrogenase Stain</td>
<td>Dark Blue</td>
<td>Medium Blue</td>
<td>Light Blue/White</td>
<td>White</td>
</tr>
</tbody>
</table>

much lower resistance to fatigue than the slow fibers [12]. These fast twitch fibers utilize more anaerobic metabolism to generate ATP, and therefore have a lower mitochondrial density than slow twitch fibers. These general myosin groups are used to describe the general properties of muscle fibers as determined by different fiber type stains and to describe the basic pattern of fiber type switching, which will be discussed in the following paragraph. In actuality there are many different types of myosin genes which produce a variety of intermediate fibers in different types of skeletal muscle, such as extra ocular muscles which display fiber types including hyper-fast and slow tonic contracting fibers [219].

Diversity of fiber types is necessary for different muscles to perform specialized tasks. For example, the slow type I isoform is expressed more highly in postural muscles, which are used constantly in the body, and therefore must have a lower rate of fatigue [12]. To fulfill these requirements, muscle fibers are characterized by a remarkable plasticity and can respond to physiological and pathological signals and adapt the fiber type composition [9,11-12]. This transformation process in fiber type occurs in response to changes in demands on the muscle, such as increased exercise, aging, atrophy, and disease [10-11]. For example, as a person trains for running a marathon, over time the increase in exercise causes the muscles to undergo a shift in fiber type to a more slow-twitch phenotype, thereby reducing fatigability and increasing the endurance of the runner’s muscles. Muscle fibers can switch within the progressive pathway to their “nearest-neighbor” from slowest to fastest fiber type or vice versa: I↔I/IIa↔IIa/IIx/d↔IIx/d↔IIb↔IIb [10]. Understanding the pathways that govern myofiber remodeling could be very important in several human diseases. For example, in muscular dystrophy, fast twitch muscles appear to be preferentially affected and degenerate at a faster rate, while slow twitch fibers are more likely to be spared [9,13]. Therefore it is important to understand the basis of skeletal muscle plasticity in response to an environmental change.
Muscular Dystrophy

Many of the most severe skeletal muscle diseases are part of a group termed the muscular dystrophies (MD). The MDs are a large group of inherited disorders characterized by progressive muscle weakness, wasting, and, in many cases, premature death [14-17]. The mode of inheritance, age of onset, involvement of particular skeletal muscle type and overall progression have been used to classify the different forms of MD [15]. There are over thirty known and characterized forms of MD, and a majority of these diseases are caused by mutations in genes encoding for proteins of the dystrophin associated glycoprotein complex (DGC), and lead to either partial or complete absence of this complex [14]. The most common and, unfortunately, the most severe form of MD is Duchenne MD (DMD). DMD is caused by mutations in the dystrophin gene, the anchor of the DGC to the cytoskeleton of the muscle, which can cause premature stop codons and the lack of the protein in DMD patients [16,18]. DMD is an X-linked disease and occurs in every 1:3500 live male births [16]. Individuals affected by DMD are typically diagnosed between the ages of 2-5 years of age, and by the time the affected individuals reach 10 years of age, the disease presents itself through loss of ambulation, scoliosis, muscle contractures, severe skeletal muscle necrosis, and early signs of cardiomyopathy [16,20-21]. DMD patients do not typically survive past their twenties due to extensive loss of muscle, respiratory failure, or cardiomyopathy [16,20-21]. DMD is characterized by cyclic degeneration and regeneration of the muscle fibers, until the regeneration process cannot keep up with degeneration of the muscle fibers and the skeletal muscle is replaced by fibrotic and adipose tissue.
Not all MDs progress with the speed and severity of DMD. For example, Becker MD (BMD) is also caused by a mutation in the dystrophin gene; however, in this case a truncated form of the dystrophin protein is still produced leading to a much less severe and more delayed form of MD. In addition, the limb-girdle MDs (LGMD) occur in an autosomal dominant or autosomal recessive pattern and occur at a rate of 1:20,000 individuals [14]. The LGMDs have a much slower progression of disease, and affect the shoulder and hip musculature first. Mutations within the sarcoglycans, a sub-complex of integral membrane proteins in the DGC, cause a subset of autosomal recessive LGMDs [14-15]. The autosomal recessive LGMDs (type 2) usually have a childhood onset and occur with symptoms of elevated creatine kinase (CK) serum levels, limb/girdle weakness, with an overall phenotype similar to DMD. Other MDs are even less common such as Emery-Dreifuss MD (EMD), Facioscapulohomeral MD (FMD), Myotonic Dystrophy (MMD), Oculopharyngeal MD (OPMD), Distal MD (Miyoshi), and Congenital MD (CMD). Thus each type MD has its own pattern of emergence and pathology, and differ in what areas or muscles are affected, disease onset, the effects of the disease on the muscle fibers, and the severity of the dystrophy. (See Table 2)

<table>
<thead>
<tr>
<th>Dystrophy</th>
<th>Inheritance</th>
<th>Protein Missing/Deficient</th>
<th>Age of Onset</th>
<th>Muscles Affected</th>
<th>Heart Affected/Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duchenne (DMD)</td>
<td>X-linked recessive</td>
<td>Dystrophin (Absent)</td>
<td>Early Childhood</td>
<td>Muscles of the hips, legs, shoulders, spine</td>
<td>Cardiomyopathy/Severe muscle weakness and wasting, Death in early 20s</td>
</tr>
<tr>
<td>Becker (BMD)</td>
<td>X-linked recessive</td>
<td>Dystrophin (Partial protein)</td>
<td>Adolescence or adulthood</td>
<td>Similar to DMD</td>
<td>Cardiomyopathy/Similar muscle weakness as DMD but slower progression</td>
</tr>
<tr>
<td>LGMD2C</td>
<td>Autosomal recessive</td>
<td>γ-sarcoglycan</td>
<td>Infancy to early adulthood</td>
<td>Proximal shoulder/pectoral girdle muscles</td>
<td>Cardiac involvement in 2C, 2E, and 2F / some result in a DMD-like phenotype—muscle weakness and wasting</td>
</tr>
<tr>
<td>LGMD2D</td>
<td>Autosomal recessive</td>
<td>α-sarcoglycan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LGMD2E</td>
<td>Autosomal recessive</td>
<td>β-sarcoglycan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal/Miyoshi/myopathy</td>
<td>Autosomal/Recessive</td>
<td>Dysferlin</td>
<td>Late adolescence</td>
<td>Posterior of legs</td>
<td>Slow progression, able to maintain ambulation</td>
</tr>
<tr>
<td>Congenital (CMD)</td>
<td>Autosomal/Recessive</td>
<td>Mucoitin (laminin a2)</td>
<td>At birth</td>
<td>Proximal limb muscles</td>
<td>Contractures, salivary glands, most do not learn to walk</td>
</tr>
<tr>
<td>Emery-Dreifuss (EDMD)</td>
<td>Autosomal Dominant/Recessive</td>
<td>Lamin A/C</td>
<td>Childhood to early teens</td>
<td>Proximal upper extremities and distal lower extremities</td>
<td>Cardiomyopathy/early muscle contractures</td>
</tr>
<tr>
<td>Oculopharyngeal (OPMD)</td>
<td>Autosomal Dominant</td>
<td>Poly-A-binding protein2</td>
<td>Age 40-60 years</td>
<td>Eyelids, throat</td>
<td>Drooping eyelids, aspiration pneumonia</td>
</tr>
<tr>
<td>Facioscapulohemeral (FMD)</td>
<td>Autosomal Dominant</td>
<td>Not identified</td>
<td>Childhood to early adolescence</td>
<td>Face, shoulders, upper extremities (proximal)</td>
<td>Cardiac conduction defects, retinal abnormalities</td>
</tr>
<tr>
<td>Myotonic (MMD)</td>
<td>Autosomal Dominant</td>
<td>Myotitin protein kinase or ZNF9</td>
<td>Infancy (more severe) to adulthood</td>
<td>Distal weakness at first, then proximal later</td>
<td>Cardiac arrhythmias/Myotonia, cataracts (depends on onset)</td>
</tr>
</tbody>
</table>

Table 2. Partial list of the variability of disease and onset in the MDs. Adapted from [19,23]
Many of the proteins affected in the MD disease spectrum are also present in the heart. Therefore, genetic mutations leading to skeletal muscle disease in MDs can also have damaging effects on the cardiac muscle. In some MDs detrimental cardiac effects such as arrhythmias or dilated cardiomyopathy occur, while in others the heart remains unaffected. Individuals with DMD have severe cardiac effects with the absence of dystrophin, including cardiomyopathy; however, patients with other MDs, such as congenital MD, typically do not have any heart anomalies [26]. With the wide ranging effects in the hearts of MD patients, it is very important to treat any cardiac symptoms associated with disease presentation as these heart anomalies are typically the fatal factor for MD patients, along with the respiratory failure common in most dystrophies [15,17,22,26].

Clinically, MDs are diagnosed using a variety of laboratory tests. A measurement of the levels of serum CK in the blood is a simple and inexpensive assay for the most severe forms of MD. CK is found in the muscle fibers; however, upon injury or during severe skeletal muscle wasting, the muscle fiber membrane can rupture, releasing CK into the blood stream. This protein is especially high in DMD patients even during neonatal stages [23]. Other tests, such as electromyography and muscle histology by biopsy are more invasive than a simple CK blood test. Electromyography is a technique that can evaluate and record the electrical activity produced in skeletal muscles. During the test, a small needle electrode is inserted through the skin and into the muscle tissue where the administrator can look for any abnormal spontaneous activity that could indicate muscle damage. Muscle biopsies are a way of examining what is going on structurally within the muscle. Typical pathology presented in the muscles of dystrophy patients include centrally located nuclei and fiber size variability (signs of degenerating and regenerating muscles fibers), an influx of inflammatory cells, an increased amount of serum CK, an increase of fibrotic tissue in the interstitial space between the muscle fibers, and replacement of muscle fibers with adipose tissue [22-23].

There is no known cure for MD, and current therapies only delay the progression of the dystrophic pathology. The standard of care for MD patients include physical therapy (treat contractures
and exercise the muscle), respiratory care (oxygen supplement), and medicating with corticosteroids. These steroids have a variety of functions including regulation of the inflammatory response and metabolism. While the precise mechanisms of action for steroid treatment in the delay of MD are not fully understood, the drug is thought to suppress the immune response, down-regulate JNK signaling, or even up-regulate the dystrophin-like protein utrophin. Prednisone or other glucocorticoid treatment typically results in increase in muscle strength and prolonged ambulation in MD patients; however, chronic treatment with glucocorticoid drugs result in side effects, such as weight gain, bone fragility, mood changes, stunting of growth, and water retention [24-25]. Recent work has been done to establish better dosage guidelines for oral prednisone-like steroids, but the side effects, although lessened, still remain [24,25,26]. However, no current treatments available for MD cure the disease, and only increase the lifespan of the patient for a few years. Therefore, it is critical to better characterize the complex pathways that are affected in MD to assist in the development of better drugs for the treatment of dystrophic disease.

A majority of the MD diseases are caused by mutations within a large protein complex located on the muscle membrane of the fibers. This multi protein complex, termed the DGC, plays a large role in the stabilization of the muscle membrane and can have serious effects on the muscle health if one or more component of this complex is missing.

**Dystrophin Glycoprotein Complex**

The Dystrophin Glycoprotein complex (DGC) is a large oligomeric complex of sarcolemmal (muscle membrane) proteins which connect the cytoskeleton (muscle fibers) to the extracellular matrix, stabilizing the sarcolemma [2]. (Figure 5) Although many of the components are found in other tissues, this large complex of proteins was originally isolated from skeletal muscle membranes [27-30]. Healthy muscles sustain minimal sarcolemmal rupture during the many contractions during daily activity which is due to the stabilization provided by the DGC. The main functions of this complex is to maintain the
rigidity of the sarcolemmal membrane, thereby stabilizing the health and viability of the membrane, preventing contraction induced damage, and transmitting the tension from the contracting muscle fiber to the extracellular matrix. As the muscle contracts, the DGC acts both as an anchor for the sarcolemmal membrane preventing membrane damage, and as a sensor for contractile tension which couples this action to the extracellular matrix and the connecting tendons.

The DGC is known to contain approximately 15 proteins, and each of these proteins functions in the complex by inducing structural support of the membrane, local signaling in the muscle fiber, or both. The main components of the DGC are dystrophin, the sarcoglycans (α, β, γ, and δ), sarcospan, the dystroglycans (α and β), syntrophin, and dystrobrevin. Due to the important stabilizing functions of the DGC, it is not surprising that mutations within this complex are injurious to muscle fibers and lead to muscle disease.

Dystroglycan

The dystroglycans comprise the central protein in the DGC complex. Dystroglycan is produced from one gene and is subsequently cleaved through post-translational modification forming both α-dystroglycan and β-dystroglycan. α-dystroglycan is localized to the external portion of the DGC and binds to the extracellular matrix proteins such as laminin, agrin, and perlican. This protein is linked to the central membrane portion of the DGC through β-dystroglycan, which is a sarcolemmal transmembrane protein.
protein [30-31]. The intracellular domain of β-dystroglycan binds to dystrophin, forming the bridge between the extracellular matrix and the skeletal muscle fiber across the sarcolemma. Dystroglycan gene-deleted mice are embryonic lethal and die before the formation of cardiac or skeletal muscle tissue, indicating an important role in tissues other than striated tissues, such as tissues that adhere to basement membranes (neural tissue) [32]. Dystroglycan chimeric mice, which lack dystroglycan in skeletal muscle and heart, show symptoms of MD indicating that dystroglycan is important in the stability of the sarcolemmal membrane [33]. However, there are no known human dystrophies caused by mutations in dystroglycan; therefore dystroglycan deficient animals are not typically studied in terms of dystrophic disease, but are useful in understanding the role and function of the DGC.

Many of the DGC proteins undergo important post-translational modifications, including the dystroglycans. These modifications have been shown throughout the literature to induce proper localization of many DGC components to the sarcolemmal membrane, and that changes in the localization of any of these proteins can cause muscle pathology [34]. α-dystroglycan is extensively glycosylated, and improper glycosylation of this protein produces progressive muscle pathology as demonstrated in mice with a mutation in glycosyltransferase leading to altered glycosylation in α-dystroglycan; however, the exact mechanism of this disease process has not been fully determined [35-36]. In addition, mutations in two other glucotransferases (LARGE and fukutin) also result in MDs in humans (CMD) [37].

β-dystroglycan not only participates in structural support in the DGC, but also plays a role in local signaling within the sarcolemmal membrane. The β-dystroglycan protein interacts with signaling molecules at the sarcolemmal membrane including caveolin-3, which is a striated muscle specific protein that forms caveolae, or small invagination of the sarcolemmal membrane. While caveolin-3 is not known to be a component of the DGC, mice lacking this protein present with a mild skeletal muscle myopathy, which may be due to improper trafficking of DGC components to the sarcolemmal membrane [38-39]. Caveolin-3 null mice also develop cardiomyopathic symptoms, indicating the importance of this protein.
in striated muscle [38]. Mutations in caveolin-3 are known to occur within humans and produce a mild form of MD, autosomal dominant LGMD-1C MD, which is very similar to the mouse model phenotype of caveolin-3 deletion, therefore confirming the usefulness of this mouse model [40].

**Sarcoglycans and Sarcospan**

The sarcoglycans are among the core components of the DGC, and exist in four main isoforms α, β, γ, and δ. These proteins are single trans-membrane domain proteins and are mainly expressed in muscle. The exact role of the sarcoglycans in the DGC has not fully been elucidated; however, it is widely known that mutations in each of the sarcoglycan genes cause MD in both mice and humans. Mutations in one sarcoglycan gene typically results in total or partial loss of the entire sarcoglycan complex. There are mouse models for the deletion of each of the four main sarcoglycan isoforms. α-Sarcoglycan null mice (Sgca −/−) produce a phenotype similar to human LGMD type 2D, and results in a reduction of β, γ, and δ sarcoglycans at the sarcolemma [41]. However, deletion of β-sarcoglycan (Sgcb −/−) or δ-sarcoglycan (Sgcd −/−) in mice produces a much more severe progressive MD as compared to the Sgca −/− mice [42-43]. It is also of consequence that in the Sgcb −/− and Sgcd −/− mice the more severe phenotype results in a complete loss of the sarcoglycan complex, sarcospan, and the dystroglycans; however, dystrophin can still be detected in normal distributions [44-45]. The discovery of a non-dystrophin binding sarcoglycan complex shed some additional light on the difference between loss of α-sarcoglycan and β/δ-sarcoglycan. This alternative sarcoglycan complex contains δ-sarcoglycan, β-sarcoglycan, and ε-sarcoglycan and therefore the deletion of α-sarcoglycan would have no effect on this complex, but loss of δ or β-sarcoglycan would reduce this complex [43,46]. Contrary to this hypothesis however, the γ-sarcoglycan (Sgcg −/−) null mice develop a mild MD disease and exhibit only a reduction but not a complete loss of the remaining sarcoglycans [45]. Therefore there is a debate as to which sarcoglycan complex γ-sarcoglycan associates.
Sarcospan is another protein that is dependent on proper expression and localization of the sarcoglycans. The sarcospan protein contains a highly hydrophobic core and is oriented with four transmembrane domains with both termini facing the cytoplasm [36]. Deletion of any of the sarcoglycan proteins results in the loss of sarcospan; however, sarcospan null mice have normal expression of DGC components and no dystrophic pathology [47]. Conversely, transgenic skeletal muscle specific over-expression of sarcospan produces a severe MD phenotype, where the mice die between 6-10 weeks of age and display abnormal muscle histopathology [48]. However, these mice do not appear to have any membrane damage, but instead contain non-functional sarcoglycan aggregates [48]. Currently there are no known sarcospan mutations in humans causing a dystrophic disease.

Dystrophin and other binding partners

Dystrophin was first identified in 1986 as the gene mutated in DMD. This gene encodes a 3685 amino acid, 427kDa protein which is transcribed from a 2.4 mega base DNA region with 79 exons. The dystrophin protein contains four distinct domains: 1. N terminal residues that bind filamentous actin, and thereby the cytoskeleton of the muscle, 2. 24 homologous triple helical repeats and four hinge domains which gives dystrophin an elongated, flexible rod shape, 3. a stretch of 400 residues that contains a WW module (binds proline rich motifs), two EF hand modules (Ca$^{2+}$ binding proteins), and two ZZ modules (zinc finger domains), and 4. the C-terminus which anchors dystrophin to the membrane through its interaction with β-dystroglycan [36,49]. Dystrophin is localized to the cytoplasmic face of the muscle cell sarcolemma, and works to physically couple the sarcolemma with the Z-disc of the muscle cell, and is known to be necessary for sarcolemmal membrane integrity [49-53]. In addition, dystrophin interacts with dystrobrevin and syntrophin through its c-terminus, where it provides a platform for local signaling molecule networks.

The discovery of the mdx mouse, which contains a naturally occurring dystrophin point mutation leading to a premature stop codon, was the key event which triggered the studies on the function of
dystrophin [54]. The premature stop codon in the *mdx* mouse results in loss of dystrophin protein, thereby reducing the expression of other DGC members at the sarcolemmal membrane. This loss of dystrophin results in the characteristic MD changes in the muscle of these mice. Muscle from the *mdx* mice presents with a clear dystrophic phenotype, including degenerating fibers associated with fibrosis and decreased muscle function, with the greatest pathology observed in the diaphragm [55-56]. However, even though the mouse has the same lack of functional dystrophin as in the human form of DMD, the progression of the disease is much less severe than in human patients. In contrast to the human form of the disease, *mdx* mice do not develop cardiac anomalies and live close to a normal lifespan [57]. One major difference, and a leading hypothesis for this dichotomy between mouse and human dystrophin loss, is the presence of the utrophin protein in the mouse.

Utrophin is a widely expressed protein with significant homology to dystrophin, and is distributed throughout the sarcolemmal membrane in regenerating muscle. This protein can bind to similar proteins as dystrophin and, therefore, is thought to be able to partially compensate for the loss of dystrophin in the *mdx* mouse [49,59-61]. Continual expression of utrophin in the *mdx* mouse has been shown to partially attenuate the pathological changes caused by the loss of dystrophin; therefore, this suggests a compensatory role for utrophin in the *mdx* mouse [49,59-61]. Mice deficient in both dystrophin and utrophin (*mdx/utrn-/-*) present with a more clinical dystrophic symptoms [60]. These *mdx/utrn-/-* mice show an earlier onset of disease pathology in all muscles, especially in the diaphragm, and die prematurely by 20 weeks of age [60]. Therefore, studies with the *mdx/utrn-/-* mice could provide a more human like assessment of dystrophic disease.

In addition to the role of dystrophin in the support of the sarcolemmal membrane, this protein also plays an active role in facilitating local signaling. Syntrophin and dystrobrevin are two proteins which interact with dystrophin in a location close to the dystrophin protein interaction with β-dystroglycan. Syntrophin contains a plextrin homology domain, which aids in the binding of this protein to signaling molecules such as Grb2, nitric oxide synthase (nNOS), and calmodulin [62-64]. Experiments using a transgenic mouse expressing a mini-dystrophin gene have been used to decipher the contributions
of this protein related to its role in mechanical linkage of the actin cytoskeleton to the core of the DGC versus its role in signaling. This mini-dystrophin gene produced a modified dystrophin protein that possessed the ability to bind dystroglycan and syntrophin allowing signaling; however, the protein lacked the N-terminal domain necessary for the binding to the actin cytoskeleton, removing the mechanical support properties of dystrophin [65]. The expression of this modified dystrophin exacerbated the dystrophic phenotype in the mdx mouse, demonstrating that the mechanical properties of dystrophin are necessary for healthy muscle [65]. This finding was supported by additional experiments that showed that mini dystrophin proteins with at least one actin binding domain were sufficient to reduce the dystrophic phenotype in mdx mice [65]. Mini dystrophin exists in the human population and is found in BMD patients. These patients display a less severe dystrophic disease as compared to DMD patients; therefore, even expression of truncated dystrophin can alleviate some of the dystrophic disease in skeletal muscle.

Dystrobrevin, another important component of the DGC, also binds to syntrophin. This protein is highly homologous to dystrophin, yet lacks the dystrophin rod domain and cannot bind to actin [66]. Dystrobrevin gene-deleted mice display a mild myopathy, even though there is correct localization of other DGC members at the sarcolemma [66]. However, in these knockout mice, there is a lack of nNOS at the sarcolemma, although no direct binding of dystrobrevin to nNOS has been established in the literature [67]. It is hypothesized that the interaction between nNOS and dystrobrevin is facilitated by syntrophin which acts like a docking molecule for nNOS [67]. nNOS is reduced at the sarcolemma of DMD patients, mdx mice, and sarcoglycan null mice possibly suggesting an important role for nNOS in the progression of the dystrophic disease [68]. However, no pathological deficiencies in mdx mice were observed with the lack of nNOS compared to mdx mice with normal levels of nNOS suggesting that nNOS did not affect fiber degeneration in dystrophy [69-70]. On the contrary, over expression of nNOS improves pathology in the mdx mice suggesting that higher levels of nitric oxide are protective, and would most likely work through a free radical scavenging mechanism [69-70]. Therefore, nNOS loss from the sarcolemma due to lack of dystrobrevin in the DGC does not appear to increase susceptibility of fiber degeneration; however,
this loss may reduce the total nitric oxide amount in the skeletal muscle, which could reduce the protective effects in dystrophic muscle.

*Extracellular matrix proteins*

Mutations in extracellular matrix proteins can also result in a MD phenotype similarly to mutations within the core DGC. Deletion of laminin-2, a basement membrane protein, in a mouse model of human CMD, results in a severe MD in which the animal does not survive past 8-10 weeks of age [67]. The loss of laminin-2 not only affects the extracellular matrix of the skeletal muscle, but also affects the mylenation of nerves, which also contributes to the morbidity and mortality of these mice [67]. Collagen VI is another extracellular matrix protein which, when mutated, is known to result in a form of congenital MD in humans [71]. Therefore the DGC provides an essential bridge for the complete connection between the cytoskeleton and extracellular matrix in skeletal muscle, and mutations within this complex produce many of the forms of MD known in humans.
Mouse Models of MD

Mouse models exist for a majority of the known dystrophies. There is a wide variety between these models of dystrophy in the severity of the pathology which is determined by the lifespan of the mouse, the presence of cardiomyopathic effects, and the progression of the skeletal muscle histological and functional alterations. (See Table 3) These histological indices include the presence of central nuclei and fiber size variability (presence of regeneration), increased presence of inflammatory cells, and fibrosis. In normal adult skeletal muscle, the nuclei are located on the periphery of the muscle fibers; however, after satellite cell activation and fusion into the skeletal muscle fibers, the nuclei are centrally located. This provides a distinct quantitative assessment to quantify the amount of regeneration and indirectly determine the amount of degeneration within a muscle. (Figure 6) Regeneration also affects fiber size, as satellite cells fusing together to form new fibers are often smaller in diameter than mature adult fibers. Therefore, there is an observed overall reduction in average fiber area, and also an increase in variability of fiber size in dystrophic muscle as compared to normal, healthy muscle due to the increase in regenerating fibers.

### Table 3. Partial list of mouse MD models [16]

<table>
<thead>
<tr>
<th>Genotype (Protein Absent)</th>
<th>Dystrophy</th>
<th>Lifespan</th>
<th>Skeletal muscle phenotype severity</th>
<th>Cardiomyopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sgca /- (α-sarcoglycan)</td>
<td>LGMD2D</td>
<td>Normal</td>
<td>Moderate</td>
<td>None</td>
</tr>
<tr>
<td>Sgcb /- (β-sarcoglycan)</td>
<td>LGMD2E</td>
<td>Reduced</td>
<td>Severe</td>
<td>Severe</td>
</tr>
<tr>
<td>Sgdd /- (δ-sarcoglycan)</td>
<td>LGMD2F</td>
<td>Reduced</td>
<td>Severe</td>
<td>Severe</td>
</tr>
<tr>
<td>mdx (dystrophin)</td>
<td>DMD</td>
<td>Normal</td>
<td>Mild</td>
<td>Mild</td>
</tr>
<tr>
<td>Utfn-/- (utrophin)</td>
<td>DMD</td>
<td>Normal</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>nNOS (Neuronal NOS)</td>
<td>DMD</td>
<td>4-20 weeks</td>
<td>Severe</td>
<td>Severe</td>
</tr>
<tr>
<td>ColVI -/ (collagen VI)</td>
<td>Ulrich Congenital Dystrophy/Bethlem Myopathy</td>
<td>Reduced</td>
<td>Moderate</td>
<td>?</td>
</tr>
<tr>
<td>Cav-3 (cavolin-3)</td>
<td>Reduced</td>
<td>Mild</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>lamin2a (laminin 2a)</td>
<td>Congenital MD</td>
<td>10 weeks</td>
<td>Severe</td>
<td>?</td>
</tr>
<tr>
<td>DG -/ (dystroglycan)</td>
<td>Embryonic lethal</td>
<td>Not Applicable</td>
<td>Not Applicable</td>
<td>None</td>
</tr>
</tbody>
</table>
| DG chimeric (dystroglycan- skeletal muscle and heart only) | Reduced | Moderate | Severe                            | None

(See Table 3) These histological indices include the presence of central nuclei and fiber size variability (presence of regeneration), increased presence of inflammatory cells, and fibrosis. In normal adult skeletal muscle, the nuclei are located on the periphery of the muscle fibers; however, after satellite cell activation and fusion into the skeletal muscle fibers, the nuclei are centrally located. This provides a distinct quantitative assessment to quantify the amount of regeneration and indirectly determine the amount of degeneration within a muscle. (Figure 6) Regeneration also affects fiber size, as satellite cells fusing together to form new fibers are often smaller in diameter than mature adult fibers. Therefore, there is an observed overall reduction in average fiber area, and also an increase in variability of fiber size in dystrophic muscle as compared to normal, healthy muscle due to the increase in regenerating fibers.
Inflammation is a key aspect to be examined in dystrophic tissue when assessing disease pathology. Inflammation in dystrophic muscle occurs due to the severe degeneration of the skeletal muscle, and typically involves a variety of cell types including macrophages, neutrophils, and cytotoxic T-lymphocytes which invade the tissue to help “clean-up” the degenerating/dying myofibers. This large amount of inflammatory cells eventually induces fibrosis, or the accumulation of excess connective tissue in the interstitial space between muscle fibers, which severely hinders muscle function. Figure 6 depicts the quadriceps muscle of *Sgcd* /- mice which display the discussed pathological indices.

Many of the mutations that are causative in producing a MD phenotype have direct effects on the sarcolemmal stability. Due to this increased instability of the muscle membrane, most MD models can also be evaluated by the amount of membrane rupture measured by the level of the muscle specific protein, CK, in the serum, or the ability of non-membrane permeable dye to enter muscle fibers. CK is a muscle protein which is released into the serum when muscle damage occurs. The level of CK in the serum can be used to assess the amount of muscle damage present in the skeletal muscle. In addition, membrane impermeable dyes, such as Evan’s blue dye (EBD), can also measure the amount of membrane instability and breakage occurring in dystrophic muscles. When a rupture in the membrane of skeletal muscle occurs, EBD can enter into the muscle fiber and bind to albumin producing a fluorescent signal detectable by confocal microscope in sectioned muscle. This can give a direct measurement of the amount of membrane breakage present in a dystrophic mouse muscle.
Current Hypothesis for the Pathology in MD

The MDs are a severe group of disorders mainly affecting skeletal muscle and cardiac muscle. Many types of MDs occur due to mutations within the DGC complex, which, as stated earlier, forms a bridge from the actin cytoskeleton of the muscle fiber to the extracellular matrix, thereby stabilizing the sarcolemmal membrane. When one component of the DGC is missing, the complex cannot form or function normally. This absence of a functioning DGC creates a lack of stability in the sarcolemma, or muscle membrane, which can lead to contraction induced microtears or activation of membrane Ca\textsuperscript{2+} permeable channels, creating a state of Ca\textsuperscript{2+} overload that can render the muscle fibers susceptible to myofiber death, a major phenotype in MD [15,17,72-73].

Muscle degeneration/cell death

The Ca\textsuperscript{2+} overload created by the contraction induced microtears of the muscle membrane can cause many intracellular responses including contractures of the skeletal muscle, swelling of the mitochondria, release of calpain, release of reactive oxygen species (ROS), and induction of cellular signaling pathways, all of which tip the scale of the cell’s fate towards necrosis or cellular death. Cell death is an important process in all tissues, and is a dynamic part of the development and adult homeostasis in organisms. There are two main types of cell death that have been associated with the progressive degeneration of muscle in MD: apoptosis and necrosis.

Apoptosis

Apoptosis, or programmed cell death, is used as a conserved suicide program which is present in all eukaryotic cells. This highly selective form of cell death can result in the removal of a single cell or can involve a large number of cells. Apoptosis, an energy dependent process, can result due to an immune response, DNA damage, cellular stresses, and growth factor deprivation. The goal of this programmed cell death is to minimize damage to all viable tissues and cells, while eliminating any damaged cells. However, misregulation of apoptosis can lead to disease. For example, when a defect in apoptosis is
present within a tissue, this can cause unregulated cell growth and subsequent cancer, while excessive apoptosis can lead to damaging effects such as stroke, heart failure, and neurological disorders.

As cells progress through the apoptotic process, the affected cells undergo distinct changes such as loss of cytoplasm, membrane blebbing, and chromatin condensation [74]. Eventually the cell will fragment into small vesicles that will be taken up, and cleared quickly, by macrophages. In addition, a large inflammatory response is not typically associated with apoptosis because the contents of the cells are not exposed to the surrounding tissue.

Apoptosis can occur through two mechanistic pathways: an intrinsic pathway utilizing the mitochondria, or an extrinsic pathway through cell death receptors. (FIGURE 7) Both of these apoptotic pathways result in the activation of caspases. The caspases are a family of cysteine proteases which when cleaved become active and subsequently cleave other proteins such as structural and DNA repair proteins, eventually leading to death in the cell. The extrinsic pathway, which utilizes cell death receptors, is initiated by binding of a cell death ligand to its specific receptor [75]. Two examples of such ligands are tumor necrosis factor α (TNFα) and Fas, which bind to a receptor causing the activation of caspase 8, and translocation of Bid to the mitochondria (tBid), and the

![Diagram of the two mechanistic pathways leading to apoptosis. Adapted from [80]](image)
release of cytochrome c (Cyt c) [80]. This free Cyt c can then form the apoptosome with Apaf-1 causing the cleavage of procaspase 9, followed by the activation of caspase 3, subsequently resulting in cell death [80].

The intrinsic pathway causes cell death through the induced release of apoptotic factors such as Cyt c, apoptosis inducing factor (AIF), and endonuclease G (endoG) from the mitochondria. In this pathway, the mitochondria are extremely important in determining whether a cell will live or die. In this intrinsic pathway, the release of apoptosis factors is caused by the Bcl-2 protein family. This family of apoptotic factors can be divided into pro-apoptotic (Bax, Bak, Bid) and anti-apoptotic (Bcl-2, Bcl-XL) members which either promote or inhibit apoptogen release from the mitochondria. Apoptosis through the intrinsic pathway is caused by an apoptotic signal, most likely due to cellular damage, within the cell. This signal triggers the release of Bax from the pro-survival Bcl-2 in the cytosol, allowing Bax to translocate to the mitochondria and oligomerize with Bak to cause mitochondrial outer membrane permeability (MOMP). (Figure 7) This permeabilization of the outer membrane causes the release of Cyt c into the cytosol, where it can again bind with Apaf-1 to form the apoptosome, cleave procaspase 9, which ultimately leads to the cleavage of caspase 3 and apoptosis [80].

The role of apoptosis in skeletal muscle has not been well defined, and not much has been published on this type of cell death in this striated muscle tissue, especially in MD. It is widely accepted that muscle degeneration in MD is a result of necrotic cell death, and until recently, the impact of apoptosis in the degeneration of dystrophic myofibers was unknown. Early studies of dystrophic muscle identified TUNEL positive nuclei and DNA laddering, both indicators of apoptotic cell death and signs of DNA fragmentation in mdx mouse muscle [76-77]. However, the number of these cells was much too low to be significant, and Bax and Bcl-2 protein levels did not appear to be changed in these dystrophic mice, indicating that apoptosis may not have a prominent role in dystrophic degeneration [78].
Other studies have better clarified apoptotic factor roles in MD. Overexpression of apoptosis repressor with a CARD domain (ARC), a caspase inhibitor, in mdx mice did nothing to alleviate pathology in the dystrophic muscle. However, studies in the laminin-2 null dystrophic mouse model, which is lethal by 2 months of age [67], point to a role of the Bcl-2 family of proteins in the progression of this dystrophy. Deletion of Bax and overexpression of Bcl-2 in the laminin-2 null mouse resulted in an increase in lifespan and the alleviation of the pathology and function in these dystrophic mice; however, deletion of Bax was more effective [79]. This greater effect of Bax on the amelioration of disease in the laminin-2 null mouse may be due to the additional effect on neurons; however, recently Bax has also been linked to the necrotic cell death process, and this type of cell death may also contribute to the more effective rescue of the laminin-2 null mice [79,81]. Another study examined over expression of Bcl-2 in mdx mice, but no improvement in disease pathology was observed; therefore, the effect of over-expression of Bcl-2 may be exclusive to the laminin-2 model of MD [81]. This data could indicate that dystrophies caused by mutations within the core DGC could induce a more necrotic cell death, while dystrophies due to mutations in other extracellular matrix proteins or non-core DGC proteins could have additional apoptotic cell death effects.

**Necrosis**

Necrosis, another significant cell death mechanism, is defined as non-programmed
cell death, and is a result of unexpected cellular damage. During the necrotic process, the damaged cell begins to swell after the interruption of the cellular organization. (See Figure 8) This eventually leads to abnormal cellular metabolism and interruption of ATP production and causes the cell to release its contents. This release of cellular components triggers the inflammatory response, a hallmark of necrosis. Inflammation is a key symptom of DMD patients and a one of the characteristic pathologic indices in dystrophic mice; however, the pathway that leads to necrosis and then the subsequent inflammatory response is not fully understood. A current hypothesis is that necrosis in dystrophic tissues is a result the induction of calpain and ROS, although more general pathways that lead to this type of cell death have not been established as it has been for apoptosis. Recently there has been work establishing a role of Bax and Bak in necrotic cell death through the opening of the mitochondrial membrane permeability transition pore, indicating a role of Bax and Bak in cell death separate from their role in apoptosis [81].

Mitochondrial Permeability Transition

One key event that occurs during necrotic cell death is the opening of the mitochondrial permeability transition pore (MPTP). The effect of the opening of this pore was first observed more than 40 years ago in which mitochondria, in the presence of Ca$^{2+}$, could swell and the extent of swelling could be measured by the decrease in light scattering [83-85]. This swelling event could be diminished by the addition of a Ca$^{2+}$ chelator, suggesting that the swelling may be due to a non-specific permeabilization of the mitochondrial inner membrane [83-86]. As studies continued, evidence that this increased permeability was the result of the opening of a channel came to light, and this channel became known as the MPTP [83,87-89].

One major consequence of the opening of the MPTP is the loss of mitochondrial membrane potential, resulting in the uncoupling of oxidative phosphorylation in the mitochondria, leading to ATP depletion and energetic failure of the mitochondria [83,90-91]. (See Figure 8) In addition, small molecular weight molecules and ions can travel across the membrane, creating a state of equilibrium of
the metabolic gradient between the mitochondria and the cytosol, leading to mitochondrial swelling [83,90-92]. This swelling is caused by the increase level of low molecular weight ions and molecules within the mitochondrial matrix, while original proteins are retained within their compartments, which causes swelling of the matrix compartments since the matrix protein concentration is higher than the cytosol and intermembrane space [83,90-91]. Swelling of the inner membrane space in the mitochondria causes the cristae to unfold, and as the matrix expands, it exerts pressure on the outer membrane that eventually leads to rupture of the mitochondria [83]. This rupture causes the release of Cyt c, and other pro-apoptotic proteins which can subsequently induce cell death [92-97].

A major component of the MPTP has been identified as cyclophilin D through the observation that cycloporine A was a potent inhibitor of the MPTP opening [83,98]. Cyclophilin D is a mitochondrial matrix prolyl cis trans isomerase that directly regulates Ca\textsuperscript{2+} and ROS dependent mitochondrial permeability transition. It has been demonstrated that mice lacking cyclophilin D (Ppif gene-deleted mice) were protected from necrotic death in the heart and brain following ischemic injury, and that isolated mitochondria from these animals had a reduced propensity for Ca\textsuperscript{2+} mediated MPTP opening as reflected by impaired Ca\textsuperscript{2+} overload induced swelling [99-102].

In MD, as discussed previously, the microtears in the sarcolemmal membrane of the skeletal muscle allow the unregulated entry of Ca\textsuperscript{2+} in the muscle fibers. In two models of dystrophic mice, Sgcd -/- and lama-2 null mice, isolated mitochondria swelled in the presence of Ca\textsuperscript{2+}; however, with the deletion of Ppif, mitochondrial swelling and the pathology in the muscle was reduced in these dystrophic models [103]. These results indicate that MPTP opening plays an important role in the dystrophy pathology observed in these models. In this same study, Millay et al. employed the cyclophilin inhibitor Debio-025 in the Sgcd -/- and mdx mice. Debio-025 is a potent cyclophilin inhibitor which inhibits all members of cyclophilins, but has a stronger effect on cyclophilin D and mitochondrial permeability [103]. In this study, Debio-025 was subcutaneously injected into Sgcd -/- mice and mdx mice, and after 6 weeks of treatment, significantly less swelling of mitochondria was observed along with a significant decrease in
Another group, Tiepolo et al, also showed a significant reduction in the dystrophic pathology in \textit{Col6a1/-} myopathic mice, a model of Ulrich Congenital MD and Bethlem Myopathy, after the administration of Debio-025 [104]. These results indicate that Debio-025 could be protective in multiple forms of MD. Debio-025 is currently in clinical trials and has been shown to be effective in the treatment of hepatitis C in humans. Also, this inhibitor is a better treatment option than cyclosporine A which is also a cyclophilin D inhibitor, due to the fact that Debio-025, unlike cyclosporine A, will not suppress the immune system and will not suppress calcineurin, which is important for muscle regeneration in dystrophy [105-107].

As mentioned above, the standard of care in dystrophy patients is the administration of prednisone. While prednisone has been shown to be helpful in patients by allowing the retention of muscle strength and usage for a longer period of time, the beneficial effects may be outweighed by the negative side effects present in treatment with prednisone [22,108-109]. These side effects include excessive weight gain, stunted growth, cataracts, osteoporosis, and hypertension [22,108-110]. However, because it is the standard of care, all clinical trials for dystrophy are required to include prednisone so as to not remove all potential helpful effects in these patients. Therefore, because of the promise showed in these two studies in the possible efficacy in MD treatment with Debio-025, we investigated the possibility of the use of Debio-025 in clinical trials in combination with prednisone. In this study mdx mice were treated for 6 weeks with Debio-025, Debio-025 + prednisone, prednisone, or vehicle by either subcutaneous injection or, in a secondary study, with oral gavage, and the efficacy of the two drugs were tested both individually and in combination. Overall results establish that Debio-025 administration either subcutaneously or orally (gavage) had the same beneficial effect as seen in Millay et al, and that treatment with prednisone did not help or hurt the positive effects of Debio-025 on mdx dystrophic muscle.

This dissertation describes three different pathways that can affect the progression of MD, such as \( \text{Ca}^{2+} \) induced opening of the MPTP, induced signaling pathways, and fiber type switching. We find that these pathways are not the complete pathways leading to dystrophic pathology; rather, it is likely that
many pathways are acting in combination to promote the muscle weakness and wasting pathology characteristic in dystrophic patients. However, we do suggest that blocking these studied pathways could be beneficial to improve muscle pathology and extend quality of life for children with MD.
Debio-025 is more effective than prednisone in reducing muscular pathology in mdx mice

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Abstract

MD results in the progressive wasting and necrosis of skeletal muscle. Glucocorticoids such as prednisone have emerged as a front-line treatment for many forms of this disease. Recently, Debio-025, a cyclophilin inhibitor that desensitizes the mitochondrial permeability pore and subsequent cellular necrosis, was shown to improve pathology in three different mouse models of MD. However it is not known if Debio-025 can work in conjunction with prednisone, or how it compares against prednisone in mitigating disease in dystrophic mouse models. Here we show that Debio-025 reduced the variations in myofiber cross-sectional areas, decreased fibrosis, and decreased infiltration of activated macrophages more efficiently than prednisone. However the use of prednisone and Debio-025 together had no additional effect on these histopathological indexes. Orally administered Debio-025 also reduced CK blood levels and improved grip strength in mdx mice after 6 weeks of treatment, and the combination of Debio-025 with prednisone increased muscle function slightly better than prednisone alone. Thus, our results suggest that Debio-025 is as, effective as or slightly better than, prednisone in mitigating MD in the mdx mouse model of disease.
1. Introduction

The MDs are a group of inherited genetic diseases characterized by progressive muscle weakness and wasting [15]. The most common of these diseases is DMD, an X-linked disorder that occurs in 1 in 3500 male births [15]. Individuals affected by DMD lose the ability to walk between ages 7 and 13, and typically do not survive past their 20’s due to extensive loss of muscle and/or cardiomyopathy [15,18-19]. DMD is caused by mutations in the dystrophin gene, a structural protein involved in the dystrophin–glycoprotein complex (DGC) that connects the underlying contractile elements to the membrane anchored glycoproteins that themselves are affixed to the extracellular matrix [15,17]. The loss of dystrophin causes destabilization of the sarcolemma leading to contraction induced microtears and the influx of ions such as Ca$^{2+}$, as well as the loss of selected proteins from the myofibers [17,1101-114]. Although an area of ongoing controversy, an increase in intracellular Ca$^{2+}$ concentration is thought be a primary effect leading to myofiber necrosis and the secondary replacement with fibrotic and adipose tissue [111-116]. Ca$^{2+}$ overload is known to cause cellular necrosis by directly inducing the opening of the mitochondrial permeability transition pore (MPTP) [91,117]. The MPTP spans the inner and outer membranes of the mitochondria, and when opened for prolonged periods of time, leads to loss of ATP generation, swelling, rupture, and induction of cell death [91,117]. Importantly, MPTP formation can be desensitized by cyclosporine A (CsA) through inhibition of cyclophilin D (CypD), a matrix prolyl cis–trans isomerase that facilitates pore formation due to Ca$^{2+}$ overload or increased reactive oxygen species generation [91,117-119]. Indeed, it was recently shown that Debio-025, a cyclosporine analog that is more potent than CsA in blocking cyclophilin D [15], was capable of reducing muscle pathology in three different mouse models of MD [103,104,120]. Although controversial, CsA treatment itself can have protective effects on muscle pathology in dystrophic animal models and humans [121-124]. However, CsA is not an ideal agent for treating MD since it suppresses the immune system and inhibits calcineurin signaling, which secondarily reduces myotube differentiation and muscle regeneration [125-126]. Thus, non-immunosuppressant analogs of CsA that do not inhibit calcineurin, such as Debio-025, are likely to be far more effective in treating MD.
The corticosteroids prednisone or deflazacort are currently the standard of care for treatment of DMD, which appear to prolong muscle strength and have other minor benefits in reducing disease severity [24,108-109]. However, corticosteroids cause excessive weight gain, stunted growth, cataracts, osteoporosis, and hypertension [22,108-110]. Novel and more effective pharmacologic treatment strategies are desperately needed. Here we investigated the possibility that Debio-025 and prednisone would function more effectively in combination to reduce the overall MD phenotype and pathology in mdx mice. We show that Debio-025 reduced central nucleation, fibrosis, activated macrophage infiltration, and the percentage of the smallest myofibers better than prednisone. However, prednisone and Debio-025 together did not reduce disease better than Debio-025 alone in the mdx mouse model.

2. Materials and methods

2.1. Mice

Male mdx mice (C57BL/10) and male control mice (C57BL/10) were obtained from The Jackson Laboratory (Bar Harbor, ME). mdx and control mice were divided into four treatment groups: vehicle, prednisone, Debio-025 (Debiopharm, Lausanne, Switzerland), and prednisone/Debio-025. We treated mdx and control mice beginning at 6 weeks of age. Debio-025 was administered at 80 mg/kg/day for gavage or 50 mg/kg/day for s.c. injection. Prednisone was given at 1 mg/kg once per day by gavage or subcutaneous injection. All studies lasted for 6 weeks. At the end of the treatment period all mice were sacrificed and muscle weights were recorded. Blood was collected at the end of all studies for analysis of CK levels using a standard assay within the clinical laboratory at Cincinnati Children’s Hospital. All mouse experimentation was approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital.
2.2. Histology

Muscles were fixed in 10% formalin buffered with phosphate overnight, and stored in 70% EtOH until processing. Processed muscles were cut longitudinally at the middle of the muscle and embedded into paraffin. Histological sections (7 µm) were stained with Masson’s trichrome or H&E. Approximately 600 fibers per mouse per muscle were quantified in a blinded manner for central nucleation, myofiber areas, and fibrosis with ImageJ and MetaMorph 6.1 software [127].

2.3. Immunohistochemistry

Paraffin embedded muscle tissue was cut in cross-section at the mid-belly of the muscle (7 µm thickness). Deparafinization of the slides was done following a series of incubation times in xylene, 100% EtOH, 95% EtOH, 70% EtOH, water, and 1X PBS. Antigen retrieval was done by microwave oven treatment in 1X Antigen Retrieval Citra solution (BioGenex, San Francisco, CA). Sections were subsequently blocked in 1% BSA, 0.1% cold water fish gelatin, 0.1% Tween-20, 0.05% sodium azide in 1X PBS. Primary antibody for Mac-3 (BD Pharmingen, Franklin Lakes, NJ) was added at a dilution of 1:50 and incubated overnight in a humidified box at 4 °C. Goat anti-Rat green AlexaFlor secondary antibody (Invitrogen, Tåstrup, Denmark) was added at a dilution of 1:100 and incubated for 1 h at room temperature. Slides were also stained with wheat germ agglutinin-TRITC (Sigma) (1:500) and TO-PRO-3 (Molecular Probes, Carlsbad, CA) (1:1000).

2.4. Grip strength functional assessment

Mice were assessed for average muscle performance by using a grip strength meter (Columbus Instruments, serial number 04513). Each mouse was measured at baseline (time 0) and after 6 weeks of treatment. Mice were allowed to grab a the metal grate that is attached to a force transducer (Digital Force Gauge, DF152) with their front paws only and pulled away from the bar by the tail. Five consecutive pulls were performed for each mouse with a few seconds of rest between each pull.
Normalized grip strength (kg) for each mouse was calculated by dividing absolute peak-force generated by the body weight (kg), averaged across the five pulls.

2.5. Statistical analysis

The results are presented as means ± s.e.m. We used a two way ANOVA for comparisons of all treatment groups for mdx and Wt mice with the Newman–Keuls post hoc test (SigmaPlot). We considered values as significant when P < 0.05.
3. Results

While we have previously shown that Debio-025 can reduce muscle pathology in mdx and Sgcd-/- mice by s.c. injection [16], a number of critical questions remain to be addressed. For example, Debio-025 might have to be administered in combination with prednisone (current standard of care), and it is not clear how these agents would perform together or if one would be superior to the other, nor is it known if oral administration would be equally effective. Here we treated 6 week-old mdx mice for 6 weeks with Debio025 by oral gavage, either alone or in combination with prednisone (Fig. 9A). At the end of the study, mice were sacrificed and the diaphragm, quadriceps, gastrocnemius, tibialis anterior, and soleus were isolated, weighed, and a full histological analysis was performed. As previously reported, mdx mice show pseudohypertrophy with increased muscle weights compared to wildtype (Wt) controls (Fig. 9C–F). Treatment of mdx

![Figure 9](image-url)
mice with Debio-025, or the combination of prednisone/Debio-025 significantly reduced the weight of the gastrocnemius, although reductions in the weights of the quadriceps, tibialis anterior, and soleus only showed a trend (Fig. 9C–F). Prednisone alone also decreased the weight of the gastrocnemius, but none of the other 3 muscles examined (Fig. 9C–F). These results suggest that Debio-025 lessened the pseudohypertrophy in the gastrocnemius of 3-month old mdx mice but that co-treatment with prednisone was of no additive benefit (see discussion).

Extensive histological analysis of the diaphragm revealed substantial improvement in pathology due to Debio-025, prednisone, or Debio-025/prednisone together (Fig. 9B). The histological improvement in disease with these agents was rigorously quantified using multiple endpoints. For example, vehicle-treated mdx muscle showed extensive centrally located nuclei in the diaphragm, quadriceps, and gastrocnemius, a hallmark feature of ongoing degeneration and regeneration of muscle fibers. Treatment of mdx mice with prednisone, Debio-025, and prednisone/Debio-025 together significantly decreased the amount of centrally located nuclei compared with vehicle-treated mdx mice. (Fig. 10A–C). More importantly, Debio-025 significantly decreased the percentage of

Figure 10. Debio-025 reduces pathology in mdx mice better than prednisone. (A–C) Percentage of myofibers containing centrally located nuclei from diaphragm, quadriceps, and gastrocnemius. (D–F) Percentage of myofibers with a cross-sectional area < 100 μm² from diaphragm, quadriceps, and gastrocnemius. The key in A also applies to B–F. *P < 0.05 vs Wt vehicle; P < 0.05 vs mdx vehicle; #P < 0.05 vs mdx prednisone. Error bars represents s.e.m. Six mdx and four Wt mice were analyzed for each panel.
central nuclei compared with prednisone alone in mdx mice, although the combination of prednisone with Debio-025 was not significantly better than Debio-025 alone (Fig. 10A, B and C).

Another index of degenerating and regenerating muscle fibers is an increase in the percentage of smaller myofibers within a given muscle. For example, compared with Wt mice, vehicle-treated mdx mice showed an increase in total fiber numbers with areas less than 100 \( \mu \text{m}^2 \). (Fig. 10D–F). Treatment with prednisone, Debio-025, and prednisone/Debio-025 together reduced this effect, and once again, Debio-025 was significantly more effective than prednisone in all three muscles analyzed, although prednisone alone did not significantly affect the diaphragm (Fig. 10D–F). Consistent with these results, Debio-025, prednisone, and Debio-025/prednisone together significantly reduced the extent of fibrosis in the diaphragm, quadriceps, and gastrocnemius over the 6-week treatment period in mdx mice (Fig. 11A–D). Debio-025 was also superior to prednisone in reducing the extent of muscular fibrosis in histological sections stained with Masson’s trichrome (Fig. 11A–D). These results are also consistent with analysis of macrophage infiltration in dystrophic muscle, which is another hallmark of ongoing muscular degeneration and necrosis. A large significant influx of activated macrophages was observed in Mac-3 antibody-stained histological muscle sections in vehicle-treated mdx mice, compared with essentially none in Wt control.
muscle (Fig. 12A and B). Treatment of *mdx* mice with prednisone showed a marked decrease in macrophage content, which was also significantly reduced with Debio-025 and prednisone/Debio-025 co-treatments (Fig. 12A and B). The same effect was observed with an antibody against CD-45, a marker for neutrophils (Data not shown). Taken together, these data indicate that while prednisone benefits pathology in skeletal muscle of *mdx* mice, Debio-025 treatment is significantly better. However, supplementing Debio-025 with prednisone did not provide additional benefit over Debio-025 alone in reducing skeletal muscle pathology in the *mdx* mouse.

**Figure 12. Debio-025 decreases inflammatory cell infiltration in *mdx* mice.** (A) Mac-3 antibody staining (green) of *mdx* diaphragm treated with vehicle, prednisone (predn.), Debio-025 (debio), or prednisone/Debio-025 together, as well as vehicle-treated Wt diaphragm. The outline of the fibers is shown in red with wheat germ agglutinin-TRITC while nuclei are shown in blue with TO-PRO-3. (B) Quantitation of macrophages per unit area from three different fields each for six *mdx* mice and four Wt mice.
We also investigated other indexes of MD that are more routinely analyzed in the clinic. Blood was collected at the termination of the gavage treatment protocol for assessment of total CK release, which is suggestive of the extent of ongoing muscle degeneration and necrosis. Vehicle and prednisone treated mice showed extremely high blood CK levels compared to wild type controls (Fig. 13A). However, Debio-025 and prednisone/Debio-025 combination treated mice each showed a significant reduction in total blood CK levels (Fig. 13A). We also assessed skeletal muscle function using a grip strength assessment assay, which can reveal the extent of disease in the forelimb musculature. 6-week old wild-type and mdx mice were parsed into the four treatment groups and measured for grip strength before treatment, then again after 6 weeks of drug treatment (Fig. 13B). All mdx treatment groups showed significantly compromised forelimb grip strength compared to wild type mice at the beginning of the study. While vehicle-treated mdx mice continued to show compromised grip strength after six additional weeks, the three drug treatment groups all showed a significant improvement in function, comparable to wild type mice (Fig. 13B). Interestingly, and in contrast to all the histological data, co-treatment with prednisone/Debio-025 slightly but significantly, increased
grip strength better than Debio-025 alone or prednisone alone. These results suggest that
prednisone and Debio-025 each improved muscle function in the *mdx* mice over 6 weeks of treatment.

We felt it important to compare the efficacy of gavage vs. s.c. injection. Thus, the entire study
was repeated in *mdx* mice beginning at 6 weeks of age by s.c. injection for 6 weeks with vehicle,
prednisone, Debio-025, and prednisone/Debio-025 together. Once again, we quantified muscle weights
for pseudohypertrophy, central nucleation in histological muscle sections, fiber area variation, and
extent of fibrosis (Fig. 14A–H). The results showed that s.c. injection with Debio-025 was equally
effective to oral administration in reducing muscle pathology in *mdx* mice. While prednisone also
significantly reduced these same indexes of skeletal muscle pathology, Debio-025 was significantly more
effective than prednisone, and once again, the combination of prednisone/Debio-025 together was
identical to Debio-025 alone (Fig. 14A–H). Thus, Debio025 reduced muscle pathology to a greater
extent than prednisone in *mdx* mice within an entirely independent study performed by s.c. injection.
Figure 14. Subcutaneous injection of Debio-025 reduces pathology in mdx mice better than prednisone. (A, B) Muscle weight (MW) normalized to tibia length (TL) for quadriceps and gastrocnemius treated with vehicle, prednisone (1 mg/kg/day), Debio-025 (50 mg/kg/day), or prednisone/Debio-025. (C, D) Percentage of fibers containing centrally located nuclei from quadriceps and gastrocnemius in the indicated treatment groups. (E, F) Percentage of myofibers with surface areas <100 um² from quadriceps and gastrocnemius in the indicated treatment groups. (G, H) Quantification of fibrotic area from trichrome-stained sections of quadriceps and gastrocnemius in the indicated treatment groups. *P < 0.05 vs Wt vehicle; †P < 0.05 vs mdx vehicle; ‡P < 0.05 vs mdx prednisone. The key in A also applies to B–H. The error bars represent s.e.m. Five mdx and three Wt mice were assessed here for each panel.
4. Discussion

The current treatment of choice in the United States for DMD at most specialized care centers is early dosing with prednisone [24,109]. However, corticosteroids are only mildly effective and at the same time cause considerable side effects such as weight gain, osteoporosis, cataracts, hypertension and stunted growth [24,108-110]. Thus, more effective and less toxic pharmacologic agents are desperately needed for treating this disease. We recently identified Debio-025 as an alternative pharmacologic agent in reducing disease severity in two different mouse models of MD [103]. In the current study we extend our previous observations with Debio-025 in three important ways. First, we demonstrated that oral delivery is equally effective to s.c. injection in mitigating skeletal muscle pathology in *mdx* mice. Second, we showed that Debio-025 could be given in combination with prednisone without untoward effect. Third, we showed that Debio-025 is quantitatively more effective than prednisone in reducing skeletal muscle pathology in *mdx* mice.

Unfortunately, we did not observe a synergistic or an additive effect of prednisone/Debio-025 co-treatment in reducing skeletal muscle pathology. We were hopeful that these two agents might act at different molecular levels, so that combinatorial treatment would be even more effective than either one alone in attenuating disease. The exact mechanism of action for corticosteroids in reducing MD disease severity is currently a matter of debate. Prednisone and deflazacort are thought to function primarily as anti-inflammatory agents by reducing immune cell activity and secondary muscle wasting. However, corticosteroids are also known to enhance plasma membrane stability in dystrophic skeletal muscle fibers. This effect could reduce the extent of myofiber degeneration and necrosis, secondarily attenuating the ongoing inflammatory response in skeletal muscle given less cellular debris from dying myofibers [128-130]. This later mechanism of action for corticosteroids could explain why we failed to observe an additive effect on disease reduction between prednisone and Debio-025 together, as we have shown that Debio-025 also functions to reduce myofiber necrosis, resulting in less inflammatory cell recruitment [103]. Debio-025 desensitizes the mitochondria to permeability
transition downstream of increases in Ca^{2+} associated with membrane instability and microtears [91,117]. This desensitization of the MPTP secondarily reduces the propensity of myofibers to undergo necrosis [103]. If prednisone indeed stabilizes the plasma membrane of dystrophic fibers it would lead to less Ca^{2+} influx and less induction of MPTP as a consequence. If true, these results further suggest that combinatorial treatment with prednisone and Debio-025 would not be more protective than either agent alone. However, other drugs that function at a different molecular level could still provide additive benefit with Debio-025, such as anti-fibrotic agents, agents that enhance regeneration, and agents that enhance muscle size and strength.

In the current study, *mdx* mice were treated once daily from 6 to 12 weeks of age. We selected this early time span given the mechanism of action of Debio-025, which simply reduces the initiation rate of myofiber necrosis [103]. We reasoned that such a mechanism of action would be most effective in a preventative manner, rather than employed late in the course of disease when many of the myofibers are lost and replaced with fatty and fibrotic tissue. Thus, agents like Debio-025 are likely to be most effective when employed early, such that disease is prevented rather than reversed. While we could certainly attempt to evaluate this hypothesis more directly by treating much older *mdx* mice with Debio-025, some support is offered by our previous study in 4 week-old *mdx* mice. Millay et al. treated even younger *mdx* mice with Debio-025 for 6 weeks, demonstrating a pronounced prevention of pseudohypertrophy in all skeletal muscles examined [103]. Here we began treatment at 6 weeks of age when the pseudohypertrophy response had already begun, such that this disease manifestation was only significantly reduced in the gastrocnemius, but no other muscles examined. Reutenauer et al. also showed that Debio-025 partially improved the dystrophic phenotype in muscle from 3 week-old *mdx* mice even with only 2 weeks of treatment [120]. These results suggest that Debio-025 might not be overly effective in reversing disease in dystrophic muscle once it begins in earnest, and that treatment should begin as early as possible.

Debio-025 has also shown a beneficial effect in other mouse models of MD. Millay et al. also treated *Sgcd^{−/−}* mice with Debio-025 and reported a similar decrease in dystrophic muscle pathology to
that observed in mdx treated mice. Recently a new study using \textit{Col6a1}^{-/-} mice, a model of human Ullrich congenital MD and Bethlem myopathy, also showed that Debio-025 was effective and could prevent muscle cell death and histopathology [104]. Collectively, these studies suggest a much wider role for Debio-025 in the treatment of Duchenne, limb-girdle, and congenital MDs, further supporting the hypothesis that a mitochondrial-dependent necrotic process is common to diverse dystrophies. A potential next step with Debio-025 would be to employ the Golden Retriever dog model of DMD or human clinical trials.

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CHAPTER 3 – MAPK Family Signaling/p38 MAPK Introduction

MAPK signaling in skeletal muscle

The mitogen activated protein kinases (MAPK) are a family of conserved protein kinases that phosphorylate serine and threonine residues of target proteins to regulate a number of cell activities [131-137]. These kinase pathways create a series of phosphorylation events which creates a signal amplification cascade in which the phosphorylation signal increases as the pathway progresses through its individual series of protein kinases. The MAPK pathways control complex programs such as embryogenesis, differentiation, and cell death [132-134,136]. In addition, these pathways are also involved in short-term changes required for homeostasis and acute hormonal responses [132-134,136]. Each MAPK is activated in response to extracellular stimuli through dual phosphorylation at conserved threonine and tyrosine residues [134-136].

MAPK pathways are composed of three to five tiers of regulation including a MAPK kinase kinase kinase (MAP4K), MAPK kinase kinase (MAP3K), MAPK kinase (MAPKK), MAPK, and MAPK activated protein kinase (MAPKAPK) [137]. The core cascade of the MAPK pathway is composed of a MAPK, a MAPKK (MEK or MKK), and a MAPKKK [131-137]. (Figure 16) MAPKs are activated directly by MAPKKs which are dual specificity protein kinases that generally only recognize certain MAPKs as substrates [132,134]. The MAPKKs (MEKs) are activated by MAPKKKs, which are a structurally diverse group of kinases with less predictable specificities [132,134]. Activated MAPKs are inactivated through dephosphorylation by threonine and/or tyrosine phosphatases, tyrosine phosphatases, and dual specificity phosphatase (DUSPs) [136]. So therefore, as an extracellular stimuli occurs, G protein-coupled receptors and receptor tyrosine kinases

Figure 15. General MAPK signaling pathway.
transduce these stimuli signals into the cytoplasm to effectors, such as Ras, which can then signal to MEKKs and begin the cascade of signal amplification to MEKKs, then to the MAPK, and finally to downstream effectors leading to the appropriate cellular response.

There are four distinct groups of MAPKs: Extracellular signal-regulated kinases (ERK1 and ERK2), p38 MAPK (isoforms α, β, γ, and δ), c-Jun N-terminal kinases (JNK1, JNK2, and JNK3), and ERK5 [135-136,138]. Each of these family members is activated in distinct pathways and transmit signals either independently or coordinately [136]. (Figure 16) Regulation of MAPK activity is important in determining signaling outcomes, and inactivation of these MAPK, plays a pivotal role in various physiological processes [136]. The MAPK pathway output includes the phosphorylation of a wide array of substrates including transcription factors, cytoskeletal elements and other protein kinases. Previously, groups have linked the MAPK pathways to important roles in skeletal muscle regulation including myogenesis, fiber type determination, and a possible role in satellite cell regulation. ERK1/2 have been shown to modulate fiber type within the skeletal muscle, perhaps linking these ERK kinases to preservation or damage of muscle within the MD pathology. A role for MAPK pathway signaling cascades has been suggested in myogenesis; however, different studies have been presented contradicting roles for each MAPK family member, implicating MAPK activation in both positive and negative regulation of myogenic differentiation and its probable role in the progression of MD.

Figure 16. MAPK family signaling pathways. Kinase Mediated Signaling Through MAPKs. p38α cascade is marked in the orange box.
p38 MAPK

There are four isoforms of p38: α, β, γ, and δ. p38α and p38β are ubiquitously expressed, while p38γ and p38δ are expressed in a tissue specific manner [131,139]. p38γ is predominately expressed in skeletal muscle, while p38δ is enriched in the lung, kidney, testis, pancreas, small intestine, and, to a small extent, skeletal muscle [139]. Of the four isoforms, p38α is the best characterized.

The 4 isoforms are part of the typical MAPK cascade which is induced and propagated by phosphorylation. In this MAPK cascade, MAP3Ks phosphorylate and activate (MKK6 or MKK3) which in turn phosphorylate the p38 MAPK isoforms. Numerous MAP3Ks participate in p38 MAPK cascades including ASK1, MEKK1-MEKK4, MLK2 and 3, DLK, Tpl2, TAK1 and Tao1/Tao2 [141]. These large numbers of MAP3Ks phosphorylate and activate one of the two known MAP2Ks, MKK6 or MKK3. All four p38 isoforms can be activated by MKK6, but only p38α and β can also be activated by MKK3 [141]. Once the four isoforms have been activated, these kinases can phosphorylate serine/threonine residues of their target substrates which include transcription factors and additional cytoplasmic protein kinases (MAPK activated protein kinases – MAPKAPK) [139,140,142-143].

p38 MAPK cascades can be induced by a variety of extracellular or intracellular signals, and numerous physical and chemical stresses, including hormones, UV irradiation, ischemia, cytokines, osmotic shock and heat shock [141]. In addition, growth factors, such as erythropoietin, IL-3, IL-2, IL-7, insulin-like growth factor, vascular endothelial growth factor (VEGF), and platelet –derived growth factor, were found to trigger p38α activation in certain cell types [139]. Activation of these p38 isoforms has also been seen due to TGF-β, G-protein coupled receptor agonists, vasoactive peptides, heat shock, cell stretching, and ischemia/reperfusion [139]. While these four p38 MAPK isoforms are activated by many cellular signals, they are also important in many cellular responses.

The p38 MAPK pathway functions in a large number of cellular processes including, cell growth, cell differentiation, cell cycle arrest, cell death and apoptosis, regulation of cytokines, and initiation of
transcription factors 1,131,135,141,144]. The p38 MAPK cascades are also linked to scaffold proteins including JIP2, JIP4, and the recently described oncostatin M (OSM) protein, which interacts with the actin cytoskeleton [141]. One of the best characterized roles of p38 in cellular processes is the role of this protein in cell death.

*p38 MAPK in cell death and disease*

The p38 pathway has long been associated with cell death in multiple cell types. Many groups have demonstrated that the stress activated MAPKs, JNK and p38, were linked to the induction of cell death through multiple cellular signaling pathways, such as TNFα, TGFβ, and Fas [145-147]. In addition, cell culture experiments have shown that activation by an adenoviral MKK6, an upstream kinase of p38 MAPK, is sufficient to activate p38 MAPK and selectively induce cell death in human colon cancer cells [148]. Thus the activation of the p38 MAPK pathway is sufficient to cause cell death in vitro.

The aberrant activation of p38 MAPK has been suggested to be the cause of excess cell death in many disease systems. In Zhou et al, p38 MAPK was found to be over-activated in Myelodyplastic syndromes, a group of clonal stem cell disorders that lead to ineffectual hematopoiesis, or formation of blood cells [149]. In this study it was found that there was an increase in apoptotic cell death in the hematopoietic stem cells driven by an over-activated p38 MAPK, and the inhibition of p38 MAPK, by both genetic deletion and pharmacological inhibitors, decreased the levels of apoptosis and stimulated hematopoiesis in vitro [149]. This indicates a pathologic cell death role in the over-activation of p38 MAPK.

Many studies have also linked p38 MAPK activation to neuronal development, and in fact, the activation of apoptosis has been shown to be crucial during neuronal development, and the misregulation of this cell death can lead to various neurological disorders [72]. Studies have shown that p38/JNK activation are critical for the induction of apoptosis in neuronal cells, such as PC-12 pheochromocytoma cells [72]; therefore, it is plausible that over-activation of these MAPK proteins could be causative in
neurodegenerative disorders where apoptosis is uncontrolled. p38 MAPK has been linked to several of these neurodegenerative disorders in the literature. There is evidence that p38 can phosphorylate the Tau protein involved in Alzheimer’s disease, a neurodegenerative disease which is thought to be caused by formation of plaques in the brain [150-152]. This Tau protein is hypothesized to be an important factor in disease pathogenesis [150-152]. In addition the hyperphosphorylation of Tau, p38 can also be linked to neuronal apoptosis in Alzheimer’s disease through the ASK1-MKK6-p38 MAPK activation pathway, leading to further disease progression [150,153-156].

p38 MAPK has also been linked to disease progression in Parkinson’s disease which is a disease characterized by a progressive loss of dopaminergic neurons, and accumulation of protein deposits, known as Lewy bodies. P38 MAPK has been implicated in the neuroinflammatory response and also neuronal cell death in Parkinson’s through its activation by α-synculin leading to p38 phosphorylation of p53, and subsequent activation of the pro-apoptotic protein Bax [157-162]. This activation of Bax leads to the permeability of the mitochondrial outer membrane and cell death as discussed previously [162]. Lastly, activation of p38 MAPK has been associated with Amyotrophic lateral sclerosis (ALS). In this disease, it has been shown that p38 signaling mediates Fas-dependent apoptosis which causes an upregulation of nitric oxide production in motor neurons. This causes the characteristic progressive neurodegeneration and loss of motor neurons, which in turn result in secondary muscle atrophy, paralysis, and eventually death in affected individuals [163-164]. Thus p38 MAPK has been strongly linked to death signaling pathways through over-activation in multiple disease states.

Recently, multiple roles for p38 MAPK have been described in skeletal muscle, including regulatory roles in myogenesis, fiber type determination, and in the activation of satellite cells during regeneration of damaged muscle tissue.
p38 and Skeletal Muscle Myogenesis

Extracellular signaling molecules regulating skeletal muscle development are largely known; however, the intracellular signaling events involved in this developmental process are largely unknown and poorly understood [1]. The p38 MAPK pathway is most likely one of the major intracellular signaling pathways affecting myogenesis [1]. Previous studies have demonstrated the requirement in vitro and in vivo for p38α in general mouse development and skeletal muscle development outlining many avenues for their involvement of p38 MAPK in the generation of skeletal muscle [1,2,165].

P38 during differentiation

p38 kinase activity has previously been shown to increase over the course of differentiation, and has also been shown to be required for full myoblast differentiation and fusion; therefore, p38 is recognized as a critical regulator of myogenesis [165-166]. The actual mechanisms that regulate p38 MAPK activity in differentiating muscle cells remain unknown; however, these mechanisms appear to be different from responses to stress and cytokines [165].

Sustained activation of p38 is known to occur during differentiation. (20) While p38 α, β, γ, and δ have been shown to be expressed in adult skeletal muscle, p38α and β are specifically activated during myogenic differentiation in cultured cell experiments [140,143, 168]. The phosphorylation and activation of α and β isoforms are gradually induced during the differentiations of myoblasts [1]. However, the expression pattern of p38 phosphorylation during myogenesis is indistinguishable between wild type and myoblasts deficient in p38β, γ, and δ, leading to the conclusion that p38α is the important isoform during skeletal muscle differentiation [143]. Moreover, treatment of myogenic cell lines, including C2C12 cells, with the p38α/β inhibitor, SB203580, prevented myoblast fusion and the induction of differentiation specific genes [1,140]. It was also shown that p38α null myoblasts are deficient in cell cycle arrest, expression of muscle specific proteins, and myotube formation [168]. These myoblasts not only exhibited impaired ability to differentiate and form plurinucleated myotubes, but also presented an enhanced growth
rate in growth media, suggesting p38 deficiency might specifically impact myoblast proliferation [143]. Together these data indicated a requirement for p38α during myoblast differentiation.

Several groups have also examined the effect of hyper activation of p38 by using a constitutively active MKK6 upstream kinase. Ectopic expression of the constitutively active MKK6 is sufficient to override inhibitory factors in proliferating cells and to induce both expression of differentiation markers and the appearance of multinucleated myotubes [165]. This MKK6 effect was also observed in myoblasts growing in high serum, suggesting that p38 activity induces the withdrawal of myoblasts from the cell cycle [1].

While much as been elucidated about the potential role of the p38 isoforms in skeletal muscle in cell culture, in vitro studies are limited to specific stages of myogenesis and are isolated from real developmental context [1]. Recently, multiple groups have described gene-deleted mice for each of the p38 isoforms. Gene-deleted mice of the p38β, γ, and δ isoforms, and double gene-deleted mice of p38γ and δ, show no apparent phenotypes and leads to fertile and viable mice [1,138]. In contrast, the loss of p38α is embryonic lethal [131,135,138,143]. p38α deficient mice die at approximately embryonic day (E)10.5-11.5 due to defective placental development, abnormal yolk sac angiogenesis, massive reductions in the myocardium, malformation of blood vessels in the head region, and insufficient oxygen and nutrient transfer across the placenta [131,135,138,143].

Recently, in an alternative approach, the Muñoz-Canoves group has created a viable p38α depleted mice utilizing an embryo specific Cre-recombinase line (MORE-Cre), in addition to p38β, γ, and δ deficient mice [143]. Again, mice depleted in the p38β, γ, and δ isoforms survived normally into adulthood, but, while the p38α depleted mice survived to term, they died shortly after birth [143]. Histology of the p38α deficient muscle showed that while the structure of the sarcomeres and myofibrils were preserved, the neonatal muscle exhibited increased myoblast proliferation, reduced myofiber growth.
and delayed differentiation of myofibers [143]. This data suggests that p38α is the main p38 isoform that is required during myogenesis for proper muscle formation and maturation.

Finally, studies have also been performed on the p38β, p38γ, and p38δ deficient mice for their ability to maintain muscle regeneration in adulthood [139]. However, no necessary role was found for the three isoforms, which leads to the possibility that the β, γ, and δ isoforms of p38 play redundant roles in skeletal muscle [139]. Therefore, the deletion of p38α is the only deficiency that cannot be compensated for in vivo.

Myogenic control by p38 in skeletal muscle

p38 activities are induced in differentiating myoblasts, and coincide with the induced expression of muscle specific genes such as myogenin, muscle CKs, and myosin heavy chain [143]. Groups have shown that modulating the p38 pathway utilizing chemical inhibitors, or upstream activators, regulates the activity of the promoters of muscle specific marker genes and transcription factors such as myogenin, MyoD, or MLC3F [2]. Previous studies have shown that p38α/β directly phosphorylates several myogenic regulatory proteins such as MEF2 isoforms, E47, and the SWI-SNF chromatin remodeling complex [168]. Thus, there is evidence that p38 not only promotes muscle differentiation and fusion, but is also a critical regulator in myoblast cell cycle exit, to induce the muscle differentiation gene program.

Role of p38 with MyoD/E47

During myogenesis, transcription factors such as MyoD, Myf5, myogenin, and MRF4 are under tight post-translational control by signal transduction pathways, including p38α/β MAPK pathway [168]. MyoD and Myf5 are early transcription factors present in muscle differentiation and are known to be necessary for the myogenic process. While p38 can phosphorylate MyoD in vitro, this action does not affect the role of MyoD in vivo [1]. However, p38 was previously shown to stimulate MyoD activity through an unknown indirect mechanism [169]. Recently it was demonstrated that p38 phosphorylates E47, the bHLH heterodimeric binding partner of MyoD, and that this phosphorylation event is required
for the formation of the functional MyoD/E47 heterodimer in vitro and in vivo [1,169]. This dimer proceeds to initiate muscle specific transcription, as well as chromatin-remodeling SWI/SNF activity and recruitment of RNA polymerase II to muscle specific gene promoters allowing for differentiation and muscle gene transcription [165]. These findings provide evidence for the role of p38 as a key determinant in the regulation of the initiation of muscle differentiation [169].

p38 regulation of MEF2

p38 has also been implicated in muscle development by regulating the MEF2C protein, a transcription factor that acts on many genes encoding muscle structural proteins [138]. p38 activity increases during myogenesis and was shown to phosphorylate and increase the transcriptional activity of specific MEF2 isoforms [166-167]. MEF2A and MEF2C are directly phosphorylated by p38α/β, and this phosphorylation enhances MEF2 dependent gene expression [1,165]. Studies utilizing a MEF2 transgenic reporter mouse treated with the p38α/β inhibitor demonstrated that the blocking of p38α/β isoforms in these mice depleted MEF2 activity and, subsequently, differentiation in the somites [166-167]. Therefore, the p38α/β isoforms play an important role in controlling the MEF2 transcription factors in skeletal muscle myogenesis.

p38 regulation of MRF4

While positive regulation of MyoD/E47 and MEF2 has been described for p38 MAPK, this kinase has also been shown to negatively regulate MRF4. MRF4 is a transcription factor which is involved in the late stages of myogenesis, and has been shown to be phosphorylated by p38 [1,167]. The phosphorylation of MRF4 results in reduced transcriptional activity of muscle specific genes, which is not observed in MRF4 phosphorylation mutants [1]. The MRF4 phosphorylation mutant displayed increased myogenic potential, which suggests that the down regulation of MRF4 activity causes selective silencing of specific genes in the terminal stage of muscle differentiation [1]. When MRF4 is phosphorylated by p38, the muscle is allowed to proceed into the differentiation process of myoblasts fusing to form
myotubes, leading to functional muscle. This negative regulation of MRF4 by p38 allows for the differentiation process to occur by encouraging the cell cycle exit into differentiation for muscle specific cells. Therefore, a role for p38 MAPK has been established throughout the entire myogenic process, demonstrating an important role for this kinase in skeletal muscle generation.

*Role of p38 MAPK in fiber type determination*

While exercise and use of the muscle is known to determine the fiber type of a muscle cell, recent data has suggested that p38 MAPK could also play a role in fiber type determination [9,170]. p38 MAPK has been reported to control the myosin heavy chain II\(d/x\) (MyHCl\(d/x\)) promoter activity in myotubes, a fast twitch fiber promoter [9]. It has also been reported that the pharmacological inhibition of p38\(\alpha\) and p38\(\beta\) MAPKs resulted in the down regulation of fast adult MyHCl\(d/x\) gene activity [171]. This study demonstrated that the inhibition of these two p38 MAPKs are involved in the down-regulation of the MyHCl\(d/x\) promoter activity during increased Ca\(^{2+}\), a stimulus know to mediate fast-to-slow fiber type transformation in myotubes [171]. Constitutively activated MKK6, an upstream kinase of p38, is also reported to promote the fast MyHC expression in the presence of forced MyoD expression [9]. Taken together, these studies show that p38 MAPK is involved in the transcriptional activation to preserve fast promoter activity in skeletal muscle cells [9,170].

In many types of MD, the muscle of the patients tends to go through a shift from fast to slow twitch fibers. Because of the weakness in the DGC, contraction induced tears occur more often in fast twitch fibers which contract with greater force and speed. By switching the fast fibers to slow fibers, the skeletal muscle is attempting to preserve as much functional muscle as possible [9,13]. Therefore, the presence of p38 in dystrophic muscle could increase the muscle damage by promoting the fast fiber type in muscle cells, and it is possible that a lack of p38 would help to preserve muscle fibers longer during the disease.
**Satellite cells**

Regeneration of adult skeletal muscle depends on satellite cells that activate, proliferate, and fuse to form new myofibers, which grow actively to replace the damaged tissue [139]. Active MAPKs are present in proliferating satellite cells, and are localized to the nucleus in MM14 cells, a satellite cell line, suggesting that these kinases may function before cell differentiation [6]. The inhibition of p38 MAPK activity prevents the activation and proliferation of these cultured satellite cells on intact myofibers, indicating a role of p38 MAPK in satellite cell maintenance and regeneration in skeletal muscle [6]. The introduction of MyoD is an important step in the activation of satellite cells. Under p38 inhibitor conditions, there was a lack of MyoD induction within the MM14 cells, impeding satellite cell activation and differentiation [6]. The inhibition of p38 MAPK in either MM14 cultured cell or endogenous satellite cells induces a reversible quiescent state whereby the cells are unresponsive to external stimuli, similar to that observed for normal adult satellite cells in uninjured muscle tissues [6]. A recent report has demonstrated the requirement for p38α/β in the activation of quiescent satellite cells although the mechanism underlying this effect remains unknown [165]. These studies indicate an influential role of p38α/β in the maintenance and activation of satellite cells in injured or uninjured skeletal muscle.

All p38 isoforms are expressed during the regeneration process in adult muscle tissue after induced injury [139]. However, in the MM14 satellite cell line, inhibition of p38α/β by the p38 inhibitor SB203580 resulted in a failure to differentiate and proliferate [6]. Therefore the authors predict that the satellite cell quiescent state is maintained by the inhibition of p38α/β MAPKs [6]. The literature further suggests that the activation of the skeletal muscle satellite cells is associated with the activation of p38α/β MAPKs and suggests that these MAPKs function as a molecular switch determining the activation state of the satellite cell [6,168].

Recently, studies have indicated that p38α is the main regulator in the induction and maintenance of satellite cells [139]. Muscles from knockout animals in p38β, γ and δ were injured using cardiototoxic
injection [139]. The authors observed no major differences for any of the parameters analyzed post-injury in regenerating muscle of these null p38β, γ, or δ compared to age-matched control mice [139]. The results suggest potential redundancies and/or compensatory mechanisms among these isoforms in adult regenerating muscle, or that p38α alone could sustain regeneration in muscle lacking the other isoforms [139]. Retroviral delivery of p38γ could not effect differentiation and fusion of p38α deficient myoblasts, while similar delivery of p38α fully rescued both myogenic functions [139]. Thus, the results of this study indicate that, despite achieving similar activation levels during satellite cell differentiation, p38α and γ play distinct roles in myogenesis, where p38α can control the expression and replace p38γ, however p38γ neither regulates p38α expression nor can compensate for its absence during myogenesis [139].

**Evidence of a role of p38 MAPK in MD**

Indications of a role of p38 MAPK in MD are present in the literature; however, no direct role has been established for p38 in the progression of the disease. A majority of the data relating p38 activation to MD has to do with the phosphorylation of p38 during exercise or muscle stimulation. While data create questions about p38 involvement in MD, no concrete conclusions can be made. Recently a more direct link has been established in regards to p38 signaling in mouse MD which will be described in subsequent sections.

*Exercise and MD*

Exercise is a common model in research to examine the effects of mechanical stress on skeletal muscle. The exercise creates damage within the muscle fibers which is then repaired by satellite cell activation, fusion, and incorporation into the damaged muscle as healthy fibers. In MD models, the injury is harder to repair due to the already weakened state of the muscle. Therefore, a more pronounced phenotype can be presented. Several groups have recently examined p38 signaling in muscle under different mechanical stress conditions including running and electrical stimulation. The levels of p38 can
indicate the propensity for this kinase pathway to signal more during baseline muscle activity or stressful stimulation, which could help to elucidate the role of this kinase family in muscle disease.

Recent muscle work has shown an increase of p38 activation during muscle mechanical stress in normal wild type mice. Studies have indicated an increase of p38 following cycling exercise, high-force eccentric contractions, electrical stimulation, and prolonged exercise [142]. This exercise induced increase in p38 MAPK phosphorylation appears to be physiologically relevant because it is associated with an induction of its main downstream targets [142]. This data suggests that during injury there is an increase in p38 signaling, which could implicate a role for this kinase during injury or regeneration of injured muscle.

Total protein levels of phosphorylated p38 at baseline and during exercise have also been examined in mouse MD models; however, a clear consensus of the phosphorylation status has not been established. Conflicting reports have indicated either an increase in p38 at baseline in the mdx mouse model of MD, or no change in this same model [144,171]. In exercise models, p38 phosphorylation was shown to not be increased by exercise in C57BL/10 wild type muscles. When muscles were compared between wild type exercised and non-exercised mice, there was no observed change in phosphorylation of p38 [144]. However, the level of p38 phosphorylation in exercised mdx mice was significantly increased compared to non-exercised mdx mice and wild type controls [144]. This increase of p38 activation within the exercised mdx and a small increase in the non-exercised mdx mice, suggests a role of p38 in muscle pathology during MD. Because there was no detected increase within the wild type muscle under either condition, it is likely that activated p38 has a pathological role within the mdx mouse muscle.

**p38 expression levels during mouse MD**

No direct role of p38 MAPK in the pathogenesis of MD has been established. A few recent studies have addressed a basic expression status of the p38 MAPK during dystrophic disease in mice. Deletion of dual specific phosphatase-1 (DUSP1), a negative regulator of p38 MAPK signaling,
exacerbated the dystrophic pathology in *mdx* mice by impacting regeneration [172]. This study suggests a pathogenic role for p38 in the *mdx* mouse, as deregulation of this p38 kinase increases the pathological indices present in the muscle [172]. However, this same group showed that mice lacking DUSP10, another negative regulator of p38 MAPK, showed improved muscle pathology in the *mdx* background, suggesting, instead, that p38 signaling was protective [173]. This dichotomy could be partially explained, however, since previous studies have shown differential control of p38 activity and targets due to different dual specific phosphatases. For example Auger-Messier et al, [174] showed that de-regulation of p38 MAPK by the deletion of DUSP1,4 is deleterious in the heart following trans-aortic constriction; however, deletion of DUSP10 is protective, again insinuating multiple levels of control and signaling of p38 MAPK [174]. In addition in vitro, phospho-p38 was induced by two-fold after oxidative stress in *mdx* cultured myotubes versus wild type myotubes; however, treatment with the p38 inhibitor SB203580 improved the survival of the *mdx* myofibers after oxidative stress [175]. This indicates a role of p38 signaling in the progression of the myotubes into oxidative cell death [175]. Thus the role that this kinase might play in affecting MD remains unclear.

**p38 MAPK conclusions**

The role of p38 MAPK within skeletal muscle has been described mainly within the parameters of myogenesis. However, the direct role of p38 isoforms in muscle disease has not yet been elucidated. While data point toward a necessary role for p38α/β in the initiation of satellite cells during muscle injury, the actual role of these kinases during muscle regeneration has not been clearly defined. The current data point toward a role of p38 MAPK in muscle development, regeneration, and disease; therefore, we hypothesize that p38 is a critical signaling effector in the progression of MD. p38 has a long history of being linked to cell death in many cell types, and because cell death/necrosis is such a prominent feature of MD, we believe that p38 could play a role in the initiation or progression of myofiber death during dystrophic disease.
CHAPTER 4

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p38α MAPK signaling underlies muscular dystrophy and myofiber death through a Bax-dependent mechanism

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Abstract
MDs are a diverse group of genetic muscle diseases that lead to muscle wasting and in most cases, premature death. Cytokines and inflammatory factors are released during the disease process where they promote deleterious signaling events that directly participate in myofiber death. Here we show that p38α, a kinase in the greater mitogen-activated protein kinase (MAPK) signaling network, serves as a nodal regulator of disease signaling in dystrophic muscle. Deletion of Mapk14 (p38α encoding gene) in the skeletal muscle of mdx (lacking dystrophin) or Sgcd (δ-sarcoglycan encoding gene) null mice using the muscle-specific Myl1-cre knock-in allele resulted in a significant reduction in pathology at early and late ages. We also generated MAPK kinase 6 (MKK6) muscle-specific transgenic mice to produce constitutive p38 activation in skeletal muscle to model neuroendocrine signaling, which resulted in severe myofiber necrosis and many hallmarks of MD. Mechanistically, we show that p38α directly induces myofiber death through a mitochondrial-dependent pathway involving direct phosphorylation and activation of the pro-death Bcl-2 family member Bax. Indeed, muscle-specific deletion of Bax, but not the apoptosis regulatory gene Tp53, significantly reduced dystrophic pathology in the muscles of MKK6 transgenic mice. Moreover, use of a p38 MAPK pharmacologic inhibitor reduced dystrophic disease in Sgcd−/− mice, collectively suggesting a novel therapeutic approach.
Introduction

The MDs are a large cadre of inherited disorders that are characterized by progressive muscle weakness and wasting and, in many cases, premature death [15]. The MDs are generally caused by mutations in proteins in the DGC, an oligomeric protein complex that connects the cytoskeleton and contractile elements within the myofiber to the extracellular matrix, thus stabilizing the sarcolemmal membrane [15,17,176]. Deficiencies in this complex creates instability of the sarcolemmal membrane that leads to contraction induced microtears or activation of membrane Ca\textsuperscript{2+} permeable channels, creating a state of Ca\textsuperscript{2+} overload that can lead to myofiber death [17,112,176]. Unregulated influx of Ca\textsuperscript{2+} in combination with increased inflammatory signaling through G-protein coupled receptors and receptor tyrosine kinases on the myofiber sarcolemmal membrane, stimulates intracellular signaling that can be detrimental and initiate myofiber death [73]. These signaling events lead to activation of the mitogen-activated protein kinase (MAPK) family of proteins, which includes p38 MAPK, a known regulator of death in other cell types [72,149,145-147]. Given that myofiber death and muscle wasting is an integral part of the pathology of MD, we hypothesized that p38 MAPK could play a pathogenic role.

p38 MAPK signaling has been shown to affect some aspects of skeletal muscle development and maturation in the mouse. Specifically, the p38\(\alpha\) isoform, which is the most prevalent isoform expressed in skeletal muscle, can affect myoblast fusion to form myotubes [3,139,143,165]. Very little is known of p38’s direct role in the pathogenesis of MD, although it was shown to be upregulated in exercise trained mdx mice, a genetic model of DMD, but unchanged in wildtype (Wt) exercise trained mice [144]. Further, deletion of dual specificity phosphatase-1 (Dusp1), a negative regulator of p38 MAPK and c-Jun N-terminal kinase (JNK) signaling, exacerbated the dystrophic pathology in mdx mice by impacting regeneration, suggesting a pathologic role for p38 in the mdx mouse [172]. However, mice lacking Dusp10, another negative regulator of p38 MAPK and JNK, showed improved muscle pathology in the mdx background suggesting that p38 signaling was protective [173]. In vitro, phospho-p38 was induced by 2-fold after oxidative stress in mdx cultured myotubes vs. Wt, while the p38 inhibitor SB203580 improved the survival of mdx myofibers after oxidative stress [175]. Thus, there is little direct
understanding of the role that this kinase might play in affecting MD. Here we show that p38 has an important role in directly programming myofiber death in mouse models of MD through at least one prominent mechanism involving the pro-death effector Bax.
**Results**

**Muscle-specific deletion of Mapk14 (p38α) reduces pathology in dystrophic mice**

To understand the role of p38 signaling in MD we first examined the activation of the p38 MAPK pathway in two mouse models of MD: *Sgcd<sup>−/−</sup>* mice, a model of LGMD type -2F, and *mdx* mice (which

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**Figure 17. Baseline expression of p38 MAPK in dystrophic and knockdown mice.** (A,B) Western blot for the indicated proteins from 3 month-old *Sgcd<sup>−/−</sup>* or *mdx* dystrophic quadriceps tissue. Fold increase in phospho-p38 (pp38) and phospho-MK2 are shown. (C) Western blot for the indicated proteins from 6 week-old quadriceps tissue of *Myl1-cre, Mapk14<sup>fl/fl</sup>*, and *Mapk14<sup>fl/fl</sup>-Myl1-cre* mice. (D) Representative baseline histological sections stained with H&E from quadriceps of 6 week-old *Myl1-cre, Mapk14<sup>fl/fl</sup>* and *Mapk14<sup>fl/fl</sup>-Myl1-cre* mice. Scale bars are 100 mm. (E) Percentage of myofibers containing central nuclei in the quadriceps muscle of 3 and 6 month-old *Myl1-cre, Mapk14<sup>fl/fl</sup>* and *Mapk14<sup>fl/fl</sup>-Myl1-cre* mice, quantified from histological sections. (F) Percentage of fibrotic area in the quadriceps muscle of 3 and 6 month-old *Myl1-cre, Mapk14<sup>fl/fl</sup>* and *Mapk14<sup>fl/fl</sup>-Myl1-cre* mice, quantified from histological sections. (G) Average time spent running on a treadmill of 3 month-old *Myl1-cre, Mapk14<sup>fl/fl</sup>* and *Mapk14<sup>fl/fl</sup>-Myl1-cre* mice. (H) Average amount of CK found in the serum of 3 and 6 month-old *Myl1-cre, Mapk14<sup>fl/fl</sup>* and *Mapk14<sup>fl/fl</sup>-Myl1-cre* mice. (I) Quantitation of type I fibers in the quadriceps of *Mapk14<sup>fl/fl</sup>* and *Mapk14<sup>fl/fl</sup>-Myl1-cre* mice at 6 months of age. Four mice each were analyzed with greater than 1000 fibers per mouse assayed. (J) Fiber area distribution quantified from H&E-stained histological sections from the quadriceps in the indicated genotypes of mice at 6 months of age. At least 600 fibers were counted total from 5 separate mice each. *P< 0.05 versus Wt or Mapk14<sup>fl/fl</sup>. Number of animals analyzed in each group is shown in the bars of each figure panel.
lack dystrophin), a model of human DMD. At 3 months of age, diseased skeletal muscle from Sgcd−/− mice showed significant hyperphosphorylation of p38 and its downstream target MK2 by western blotting (Figure 17A). A significant increase in phospho-p38 was also observed in skeletal muscle from mdx mice (Figure 17B), collectively suggesting enhanced activation of the p38 MAPK pathway in muscular dystrophy.

To directly evaluate the role that p38α might play in MD we instituted a muscle-specific loss-of-function approach in the skeletal muscle of mice using a Mapk14-loxP targeted allele (fl) in combination with the muscle-specific Myl1-Cre knock-in allele. In a different line of mice, previous genetic targeting of Mapk14 only resulted in a partial loss-of-function phenotype that produced viable mice with smaller skeletal muscle myofibers [3], although if targeted correctly, complete germline deletion of Mapk14 is embryonic lethal [177-178]. The loxP-targeted Mapk14 line that we employed showed no effect on protein expression when homozygous, and we observed very efficient deletion of the gene product in skeletal muscle in the presence of the Myl1-cre allele, with a slight upregulation in total MKK6 protein (Figure 17C). No deletion was observed in other tissues such as heart, liver and lung (data not shown). Gross histological examination of Mapk14fl/fl-Myl1-cre crossed mice showed relatively normal overall muscle histology at 3 and 6 months of age, although some central nucleation of myofibers was observed (Figure 17D,E). Central nuclei in myofibers can be due to increased activity of satellite cells, which may or may not be due to associated regeneration from myofiber loss. However, no other pathological indexes of disease were observed, such as fibrosis, increase in serum CK levels that indicated myofiber breakdown, or a reduction in running performance/endurance of the mice (Figure 17F, G and H), suggesting that loss of Mapk14 was not directly pathological. Embryonic myosin heavy chain re-expression is also typically observed when new fibers are generated to replace dying fibers, but such expression was not observed in Mapk14fl/fl-Myl1-cre mice (data not shown), again suggesting that loss of p38α from skeletal muscle was not pathologic but likely was enhancing satellite cell activity or otherwise affecting the movement of central nuclei to the periphery of myofibers as they mature. Finally, as previously reported we observed a preponderance of smaller fibers in skeletal muscle lacking Mapk14 [3], as well as an increase in type I
fibers that are part of the slow fiber-type program (Figure 17I and J). Thus, while deletion of Mapk14 from skeletal muscle did not produce pathology, it did slightly impact the developmental status of the tissue with smaller fibers and a 2-fold increase in slow fibers.

To examine its role in pathology, skeletal muscle-specific targeting of Mapk14 was performed in the Sgcd−/− dystrophic background. Gross histological examination of quadriceps muscle sections showed...
a significant reduction in pathological indices in the Mapk14fl/fl-Myl1-cre Sgcd−/− mice compared with Sgcd−/− only control mice (Figure 18A and Figure 19A). Diaphragm showed a similar level of protection associated with the loss of the Mapk14 gene at 3 and 6 months of age (Figure 19B and C). Quadriceps and diaphragm muscle from Sgcd−/− mice showed the characteristic increase in central nucleation of myofibers, suggesting regeneration due to continual degeneration, while this index was significantly reduced in Sgcd−/− with muscle-specific deletion of Mapk14 (Figure 18B and Figure 19B). A marked reduction in interstitial fibrosis was also observed in the quadriceps and diaphragm muscle of Mapk14fl/fl-Myl1-cre Sgcd−/− mice compared with Sgcd−/− mice at both 3 and 6 months of age (Figure 18C and Figure 19C). Consistent with these histological features, Mapk14fl/fl-Myl1-cre Sgcd−/− mice performed significantly better with a forced treadmill running protocol compared with Sgcd−/− mice, suggesting improved muscle performance or endurance (Figure 18D). Mice with muscle-specific targeting of the Mapk14 gene in the Sgcd−/−
background also showed a dramatic reduction in serum CK levels at 3 months of age and far less inflammation and macrophage recruitment to the quadriceps and diaphragm compared with Sgcd\(^{-/-}\) mice, again demonstrating significantly less disease associated with loss of p38\(\alpha\) (Figure 18E, F and G). However, instability of the myofiber sarcolemma in the quadriceps, as examined by Evan’s blue dye (EBD) uptake in mice, still showed equally prominent leakiness between Mapk14\(^{fl/fl}\) Mapk14\(^{fl/fl}\)-Myl1-c re Sgcd\(^{-/-}\) mice and Sgcd\(^{-/-}\) mice, indicating that the underlying membrane defect due to the loss of δ-sarcoglycan was still present, suggesting that the loss of p38\(\alpha\) protected myofibers from degeneration (Figure 18H and Figure 20). No EBD positive fibers were observed in control mice containing either Myl1-c re or Mapk14\(^{fl/fl}\)-Myl1-c re alleles (Figure 20 and data not shown). Thus, loss of p38\(\alpha\) expression protected skeletal muscle from degeneration in the Sgcd\(^{-/-}\) genetic background.

**Figure 20.** Visualization of EBD positive myofibers in Sgcd\(^{-/-}\) mice with and without Mapk14. Representative Evan’s blue dye (EBD) uptake in the fibers from the quadriceps as visualized in histological sections for red fluorescence, which marks all the myofibers that are either dying or have membrane ruptures. Data from 3 month-old Mapk14\(^{fl/fl}\), Mapk14\(^{fl/fl}\)-Myl1-c re, Mapk14\(^{-/-}\) Sgcd\(^{-/-}\) and Mapk14\(^{fl/fl}\)-Myl1-c re Sgcd\(^{-/-}\) mice are shown. Loss of Mapk14 did not reduce the leakiness of the myofiber membranes in Sgcd\(^{-/-}\) mice.
We also crossed the Mapk14fl/fl-Myl1-cre alleles into the mdx genetic background to determine if loss of p38α could similarly reduce muscle pathology in a second dystrophic mouse model due to loss of dystrophin.

As with the Sgcd−/− mice, we observed a marked decrease in the gross histopathology from quadriceps sections of Mapk14fl/fl-Myl1-cre mdx mice as compared with mdx only mice (Figure 21A and Figure 22A). Central nucleation and fibrosis in the mdx background was also significantly reduced with muscle-specific deletion of Mapk14 in the quadriceps and the diaphragm at 3 and 6 months of age (Figure 21B and C, and Figure 22B and C). Muscle performance/endurance as measured by treadmill running was also improved in the Mapk14fl/fl-Myl1-cre mdx mice compared with mdx only mice (Figure 21D, controls shown in Figure 18D), and serum CK levels were significantly reduced at both 3 and 6 months of age by deletion of the Mapk14 gene (Figure 21E). Finally, the number of activated macrophages in quadriceps of Mapk14fl/fl-Myl1-cre mdx mice was also significantly reduced compared with mdx only (Figure 21F). Hence genetic deletion of Mapk14 in the mdx background, which models the most common form of human MD,
was similarly protective to the data generated in the Sgcd+/− background.

Over-activation of p38α causes severe muscle wasting pathology in mice

To determine if p38 activation was itself sufficient to induce a pathologic response in skeletal muscle we created a skeletal muscle-specific transgenic mouse model expressing a constitutively active form of MAPK kinase 6 (MKK6) under the control of the skeletal α-actin (SKA) promoter (MKK6 Tg, Figure 23A). Examination of skeletal muscle protein extracts from these transgenic mice showed a
7.8-fold increase in p38 phosphorylation status suggesting greater activity (Figure 23B). Prominent histopathology was observed in all skeletal muscles examined from MKK6 Tg mice at 3 weeks, 3 months
and 6 months of age (Figure 23C and Figure 24, and data not shown). Myofiber necrosis was so profound that muscle weights from the transgenic mice were severely reduced compared to Wt controls at 3 weeks, 3 months, and 6 months (Figure 23D and data not shown). A closer examination of the muscle histology of the MKK6 Tg mice showed large amounts of centrally nucleated myofibers, fibrosis, fatty tissue replacement and activated macrophage infiltration in the quadriceps and diaphragm at 3-4 weeks, 3 months, and 6 months of age, indicating degenerating and regenerating muscle fibers due to the MKK6
transgene (Figure 23E, F, G and H, and Figure 24B and C). Analysis of embryonic myosin heavy chain protein expression indeed showed that despite the high levels of myofiber necrosis, newly formed regenerating fibers were present (Figure 25). The data also show that regeneration was not induced by loss of *Mapk14* and hence not the reason for mild levels of persistent central nucleation shown in Figure 1D and E. Finally, ultrastructural analysis by transmission electron microscopy in skeletal muscle sections showed prominent mitochondrial swelling and rupture as early as 1 week after birth, and by 2 weeks prominent necrotic fibers throughout the muscle were observed (Figure 23I). Taken together, these data show that p38 activation in skeletal muscle is fully capable of inducing myofiber necrosis and a fulminant MD-like phenotype.

While MKK6 is functionally dedicated to p38 regulation, it was of concern that overexpression of this activated mutant protein might be inducing a MD-like phenotype independent of its regulation of p38α, or that another p38 isoform (β, γ, or δ) could be involved. To address this issue we crossed MKK6 Tg mice with the muscle-specific *Mapk14* deleted mice to determine if the MKK6 Tg phenotype was solely due to p38α downstream signaling. Remarkably, *Mapk14*<sup>fl/fl</sup>-Myl1<sup>-cre</sup> MKK6 Tg mice showed a near complete rescue in the MD-like disease state observed in MKK6 Tg mice at 1 and 6 months of age (Figure 26A, B, C and D). Histological assessment from quadriceps of *Mapk14*<sup>fl/fl</sup>-Myl1<sup>-cre</sup> MKK6 Tg mice showed near complete resolution of myofiber necrosis, irregular size distribution, inflammation, central myofiber nucleation and fibrosis that typifies the MKK6 Tg phenotype.

**Figure 25.** Visualization of newly formed myofibers due to MKK6-p38 induction or deletion of *Mapk14*. Representative immuno-stained histological sections for embryonic myosin heavy chain (eMyHC) expression in green from quadriceps of MKK6 Tg mice versus mice lacking *Mapk14* (*Mapk14*<sup>fl/fl</sup>-Myl1<sup>-cre</sup>). Staining of muscle from *Mapk14*<sup>fl/fl</sup> mice is shown as a control. The data show that loss of *Mapk14* does not result in newly regenerating myofibers, despite the increase in central nucleation shown in Figure 1, but that fiber loss due to MKK6 activity produces substantial new myofibers. Blue staining is with TO-PRO to show nuclei.
Therefore, these data show that the muscle pathology induced by the MKK6 transgene is entirely due to signaling through p38α.

p38 induces myofiber necrosis and skeletal muscle disease through Bax

The most prominent disease feature in MKK6 Tg mice was the necrosis of myofibers and extreme loss of muscle mass, even at a very early age. Thus, we hypothesized that p38α was somehow
directly regulating myofiber death in the context of MD, possibly by phosphorylating one more known cell death effectors. To this end we first surveyed an array of known cell death effector proteins for changes in phosphorylation status from skeletal muscle of MKK6 Tg mice using the phos-tag electrophoresis system with subsequent western blotting (Figure 27A). The most striking change was in
the migration of Bax, suggesting that it was prominently phosphorylated by MKK6-p38 in vivo, while no migration changes were noted in other known death-affecting proteins (Figure 27A). However, we did observe increased protein content for Bcl-2, Bid, Bak, Bax, Arc and Xiap in muscle from MKK6 Tg mice at 3 weeks of age suggesting alterations in cell death signaling that were likely secondary and/or compensatory (Figure 27A and B). The pro-apoptotic protein Bax was previously shown to be phosphorylated by p38 at threonine 167, which increased the cell death promoting activity of this protein in cell culture [179-181]. Use of a Bax-T167 phosphorylation-specific antibody confirmed its enhanced phosphorylation status in MKK6 transgenic muscle (Figure 27A and B). Acute infection of C2C12 myotubes in culture with a recombinant adenovirus (Ad) expressing the activated MKK6 mutant (AdMKK6) showed high levels of Bax-167 phosphorylation with almost no baseline phosphorylation in control uninfected cultures (Figure 27C).

To investigate the potential mechanistic effects of p38-mediated phosphorylation of Bax in regulating cell death we employed mouse embryonic fibroblasts (MEFs) deleted for Bax and Bak1 (DKO), and compared them against Wt MEFs [182]. These MEFs were first infected with AdMKK6, no virus or a control AdGFP (green fluorescent protein) and sorted by FACs for propidium iodide (PI) uptake to quantify cell death. Compared with Wt MEFs, DKO MEFs were resistant to cell death induced by AdMKK6, suggesting that p38 activation-induced killing required Bax/Bak (Figure 27D). However, to more directly implicate Bax, and specifically the phosphorylation of threonine 167 in Bax, we reconstituted DKO MEFs with Wt Bax or a mutant form of Bax that cannot be phosphorylated at T167 (Figure 27E). The data show that reconstitution with Wt Bax restored AdMKK6-induced killing in the DKO MEFs, but not with the AdBaxT167A mutant (Figure 27E). Western blotting confirmed expression from each of these adenoviruses, and that AdBax and AdBaxT167A were expressed at comparable levels in the MEFs (Figure 28A). These results indicate that phosphorylation of Bax T167 is required to mediate the pro-cell death effects of MKK6-p38 signaling.
To investigate whether this mechanism holds true in vivo, we crossed the MKK6 transgene into the *Bax* ^fl/fl^ genetic background. Previous work has shown that the deletion of *Bax* in the *Lama2*-deficient mouse background was sufficient to reduce the severity of dystrophic disease and extend the lifespan of these mice [79,82]. Analysis of histopathology and quantitation of disease showed that deletion of *Bak1* did not alleviate dystrophic disease due to the MKK6 transgene, while deletion of *Bax* or the combined deletion of *Bax/Bak1* did produce dramatic improvement in skeletal muscle pathology with less central nucleation, fibrosis, inflammation and fatty replacement (Figure 27F, G and H, and data not shown). Transmission electron microscopy confirmed these results and showed swollen mitochondria with sarcomeric disarray in skeletal muscle from MKK6 Tg mice but deletion of *Bax* or double deletion of *Bak1/Bax* in the MKK6 Tg background noticeably improved mitochondrial morphology and sarcomeric structure, as well as a reduction in myofibers with ongoing necrosis (Figure 28B). The MKK6
transgene also lead to dramatic phosphorylation of Bax at T167 in muscle tissue in vivo, which was essentially absent when Mapk14 was deleted (Figure 28C). Assessment of muscle function by treadmill running was also consistent with the histopathology, as deletion of Bak1 did not improve the poor performance/endurance of MKK6 Tg mice, while \( \text{Bax}^{\text{fl/fl}-\text{Myl1-cre}} \) MKK6 Tg mice and \( \text{Bax}^{\text{fl/fl}-\text{Myl1-cre}} \text{ Bak1}^{-/-} \) MKK6 Tg mice were significantly improved (Figure 27I). These data suggest that loss of Bax or the combined disruption of Bax/Bak1 reduced myofiber necrosis due to MKK6-p38α signaling in skeletal muscle, suggesting that one important mechanism whereby p38 activation contributes to MD is through regulation of Bax. As a control for this entire approach, we also deleted the \( \text{Tp53} \) gene, which encodes the tumor suppressor/apoptotic regulator p53 protein (Figure 29). However, the loss of p53, which typically lessens apoptotic cell death, was not protective in the MKK6 transgenic background, and in fact, appeared to worsen muscle disease leading to greater muscle weight loss (Figure 29). Thus, the protection from MKK6-p38 driven muscle disease due to loss of Bax is likely a more proximal mechanistic effect.

The results to this point suggested that a p38 pharmacologic inhibitor might have a therapeutic effect in models of MD. Hence we instituted a drug treatment protocol...
in Sgcd−/− mice at 2 dosages of SB731445, 50 and 12.5 mg/kg/day. This inhibitor was previously shown to be highly specific for p38 MAPK inhibition and to have good pharmacokinetics in vivo [183]. The treatment dosages used here resulted in blood levels of approximately 200-350 ng/mL of drug. Mice were treated from 3-12 weeks of age with inhibitor-laden chow or vehicle chow (Figure 30A). Treatment at both the high and low dose of SB731445 resulted in significantly less central nucleation of myofibers with less fibrosis in both the quadriceps and diaphragm (Figure 30B and C). Treatment with both dosages also reduced total CK levels at 12 weeks of age, as well as enhanced running time on the treadmill, with lower levels of tissue macrophage infiltration (Figure 30D, E and F). Thus, pharmacologic inhibition of p38 over 9 weeks significantly reduced dystrophic disease manifestation in juvenile Sgcd−/− mice.
Figure 30. Pharmacological inhibition of p38 in Sgcd<sup>−/−</sup> mice. (A) Schematic of the SB731445 treatment regimen in Wt or Sgcd<sup>−/−</sup> mice with formulated chow at 50 mg/kg/day or 12.5 mg/kg/day versus vehicle chow. (B) Percentage of fibers containing central nuclei from histological sections taken from quadriceps and diaphragm after 9 weeks of treatment with vehicle or inhibitor in Sgcd<sup>−/−</sup> or Wt mice. (C) Percentage of fibrotic area in histological sections taken from quadriceps and diaphragm after 9 weeks of treatment with vehicle or inhibitor in the indicated mice. (D) CK as units per liter found in the serum of vehicle, 50mg/kg/day inhibitor, or 12.5 mg/kg/day inhibitor treated Sgcd<sup>−/−</sup> or Wt mice. (E) Average time spent running on the treadmill in 9 week treated Wt or Sgcd<sup>−/−</sup> given vehicle or inhibitor. (F) Representative immunohistochemical images for activated macrophages with Mac-3 antibody (green) from histological sections of the quadriceps in 9 week treated Vehicle and inhibitor treated Sgcd<sup>−/−</sup> mice. The red staining is for membranes with WGA-TRITC. Magnification is 200X. *P< 0.05 versus Wt Vehicle treated mice; #P< 0.05 versus Sgcd<sup>−/−</sup> vehicle treated mice. The number of mice used in each experiment is shown within the bars of each panel.
Discussion

p38 MAPK is a widely recognized activator of apoptosis in diverse cell types, but it has not been previously associated with myofiber death in MD. Our current work shows that p38 MAPK signaling is upregulated in 2 distinct mouse models of MD, indicating a possible role in over-activation of this MAPK family member in the progression of dystrophic disease. Loss of myofibers in MD is probably not due to apoptosis proper, although effectors of apoptosis appear to be involved [103,184-189]. Dying myofibers show a necrotic phenotype, and, while the term necrosis implies an unregulated default process, there are mounting data that many forms of necrosis are regulated [190]. We have previously shown that Bax/Bak can regulate cellular necrosis through a direct effect on the outer mitochondrial membrane that is distinct from its mode of releasing Cyt c in apoptosis [81]. Bax/Bak impart a change in permeability of the outer mitochondrial membrane that permits necrosis through the mitochondrial permeability transition pore (MPTP), which is a regulated phenomenon that can directly lead to necrosis [81,83]. Other studies by our group and others have shown that MPTP-dependent necrosis of myofibers is a prominent mechanism underlying the degeneration of skeletal muscle in MD [103,104]. Thus, it would appear that loss of myofibers in MD is a regulated process and the effectors underlying it could be targeted therapeutically. Indeed, Bax-dependent regulation of mitochondrial-driven cell death would appear to be a more nodal control point, although it is not clear how one would antagonize Bax or Bak. As stated earlier, the full pathogenesis of MD in Lama2 gene-deleted mice was reduced by deletion of Bax, although overexpression of Bcl-2, which inhibits the activity of Bax/Bak did not reduce pathogenesis in mdx mice [79-82]. Here we showed that p38 signaling directly activates Bax by phosphorylation at T167 in skeletal muscle to enhance its cell death promoting functions. Loss of Bax, but not Bak1, antagonized the extent of skeletal muscle necrosis and ensuing histopathology observed in MKK6 transgenic mice, while loss of a different pro-death factor, p53, had no effect suggesting a more proximal disease effect through Bax.

However, it is also possible that MKK6-p38 influence the death of myofibers in MD through other effectors, as deletion of Bax/Bak1 did not fully rescue skeletal muscle disease in MKK6 transgenic mice. Moreover, inhibition of p38 may be protective to skeletal muscle independent of myofiber death,
possibly affecting the differentiation status of skeletal muscle [165,191], or even by reducing the fibrotic response mediated by surrounding fibroblasts [1]. Indeed, data in myotube cultures and in hypomorphic Mapk14 gene-deleted mice (using the epiblast-specific MORE-cre allele) would suggest that p38 inhibitors might antagonize regeneration of myofibers in MD, which should exacerbate disease [3,165]. However, the Myl1-cre knock-in allele that was employed to delete Mapk14 is not active in satellite cells thus, in our model, p38 loss should not directly affect regeneration of skeletal muscle and the protection demonstrated in the loss p38 loss by the data presented here is most likely due to decreased myofiber necrosis without effects from satellite cells. Taking into account all of these considerations, while pharmacologic p38 inhibitors may suggest an attractive new treatment to mitigate dystrophic pathology, it might not be mechanistically straightforward given antagonistic effects on satellite cells and regeneration. However, it is also possible that a p38 inhibitor would be mostly protective by reducing myofiber necrosis, necessitating less need for regeneration in the first place, as well as by possibly reducing the inflammatory response in muscle during ongoing disease. Indeed, our results with SB731445 in Sgcd−/− mice from 3-12 weeks of age suggest that p38 inhibitors, which are in clinical trials for various inflammatory-based disease indications, could be employed to treat MD patients [191-193]. But some caution is warranted because while we did observe prominent protection in Sgcd−/− mice with SB731445, parallel studies in mdx mice and TO-2 hamsters showed mixed results with some pathologic indexes being reduced, but others not (data not shown). Additional studies are clearly needed to determine if a properly designed and dosed p38 pharmacologic inhibitor might represent a bonefide therapeutic strategy to employ in MD.
Materials and Methods

Mice

Mapk14-loxP-targeted mice were described previously and are in the C57BL/6 background [195]. Sgcd−/− were described previously and are also in the C57BL/6 background [44]. Bax-loxP-targeted and Bak1 null mice (C567BL/6/SV129 background) were obtained from Jackson Laboratories as were mdx (C57BL/10) mice and control mice (C57BL/10). Mice expressing cre recombinase under the control of the myosin light chain 1f (Myl1) genomic locus (knock-in) were provided by Steven Burden (Skirball Institute, NYU) and were previously described and are also in the C57BL/6 background [196]. Tp53 null mice were obtained from Jackson Laboratories (C57BL/6/SV129 background). Constitutively active MKK6 transgenic mice (FVBN background) were generated by subcloning a constitutively active human MKK6 into the pcDNA3.1 vector driven by the human α-skeletal actin promoter [197]. Only littermates were compared with the MKK6 Tg was crossed into either the Mapk14 or Bax/Bak1 targeted backgrounds. Mice were sacrificed by Isoflurane inhalation followed by cervical dislocation. The quadriceps muscle was excised and either snap frozen or placed into 10% phosphate buffered formalin for later analysis. Serum was collected for CK analysis. SB731445 was formulated in mouse chow (Research Diets) at 2 dosages to effectively treat mice at approximately 12.5 or 50 mg/kg/day over 9 weeks. All mouse experimentation was approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital. Both male and female mice were used. No human subjects or materials were used.

Western Blot Analysis

Protein extracts were prepared from the quadriceps muscle by homogenization in cell lysis buffer (10 mM Tris-HCL (pH 7.5), 150 mM NaCl, 4% Glycerol, 0.5 M NaMetabisulfite, 1% Triton X-100, 0.1% NaDeoxycholate, 0.05% SDS) supplemented with dithiothreitol (1 mM) , protease inhibitors (Roche), and phosphatase inhibitors (Roche). Protein extracts were run on SDS-PAGE or Phos-tag gels AAL-107 (Wako Pure Chemical Industries), transferred to a PVDF membrane and immunodetected as specified by the manufacturer (Amersham Biosciences). Antibodies used in this study were: phospho-p38
(Cell Signaling, 1:1000), p38α (Cell Signaling, 1:1000), phospho-MK2 (amino acid 334, cell signaling, 1:800), MK2 (Cell Signaling, 1:800), MKK6 (Cell Signaling, 1:1000), MKK3 (Cell Signaling, 1:1000), Bax (Santa Cruz, 1:1000), Bak (Millipore, 1:500), Bid (Cell Signaling, 1:800), Bel-2 (Santa Cruz, 1:500) Bcl-XL (Cell Signaling, 1:800), phosphor-Bax (Abcam, 1:100), ARC (Caymen Chemical, 1:1000), Xiap (Cell Signaling, 1:1000), α-tubulin (Santa Cruz, 1:2500), and Gapdh (Fitzgerald Industries, 1:2500).

**Pathological Indices**

Paraffin-embedded histological sections (5 µm) of skeletal muscle were prepared and stained with Hematoxylin and Eosin, or Masson’s trichrome. Three pictures of each quadriceps or diaphragm muscle per mouse were taken and the entire field of view was counted per mouse for analysis of central nucleation with ImageJ software [127]. Percentage of fibrosis in three pictures for each muscle/mouse was analyzed using Metamorph 7.1 software [127]. Masson’s trichrome stains were also used to determine percentage of adipose tissue replacement ImageJ software [127]. We analyzed the quadriceps from Bak1−/−, Baxββ-Myl1-cre, Baxββ-Myl1-cre Bak1−/−, MKK6 Tg, Bak1−/− MKK6 Tg, Baxββ-Myl1-cre MKK6 Tg, and Baxββ-Myl1-cre Bak1−/− MKK6 Tg mice for ultrastructural alterations by transmission electron microscopy as described previously [127].

**Muscle Functional Assessment**

Mice were subjected to forced treadmill running utilizing a ramping speed protocol as previously described [198]. The time spent on the treadmill before exhaustion or the time taken to complete the protocol was recorded as “average maximum time running.”

**Immunohistochemistry**

Slides were stained for Mac-3 and Embryonic Myosin Heavy Chain (eMyHC) as previously described [199,200].
Evan’s Blue Eye Uptake

Mice were exercised using the ramping speed protocol at a 15° downward incline. EBD was injected (10 mg/ml in PBS) intraperitoneally (0.1 ml per 10 g body weight) twenty-four hours after training. The following day, the mice were subjected once more to the ramping speed protocol on the downward incline, and following completion of the protocol were euthanized. Quadriceps muscle and diaphragm were embedded in Optimal Cutting Temperature Compound (Tissue-Tek) and frozen in liquid nitrogen. Tissue sections were prepared (5 µm) and analyzed by fluorescent microscopy.

Adenoviruses

The MKK6 adenovirus was previously described [201]. Adenoviruses for the inducible expression of wild-type Bax and Bax T167A mutant were generated using with AdenoX system (Clontech).

Cell Death Assay

SV40 wild-type and Bax/Bak1 double knock-out (DKO) MEFs were cultured in IMDM media (Fisher Scientific) supplemented with 10% bovine growth serum, 1% penicillin/streptomycin (Invitrogen), and 1% MEM Non-essential Amino Acids (Invitrogen). Cells were infected with adenovirus for MKK6, inducible Bax Wt, or inducible Bax T167A. Bax expression was induced with the addition 200 µM doxycycline. Forty eight-hours post infection, cells were collected and stained (Biovisions) the proportion of live vs. dying cells was determined by flow cytometry (BD LSRII).

Statistical Analysis

The results are presented as means ± s.e.m. We used the student’s two-tailed t-test to calculate significance. Values were considered significant if P < 0.05.
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Abbreviations: CK, serum creatine kinase; DGC, dystrophin glycoprotein complex; DKO, double knock-out; DUSP, dual specificity phosphatase; EBD, Evan’s blue dye; fl, loxP targeting site; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MEFs, mouse embryonic fibroblasts; SKA, skeletal α-actin; Wt, wildtype,
CHAPTER 5 – ERK1/2 MAPK Introduction and Preliminary Data

ERK 1 and ERK2 MAPK Introduction

ERK1 and ERK2 were the first MAPKs to be identified and cloned from vertebrates [142]. As mentioned previously, ERK1 and ERK2 are part of a three-kinase phospho-relay module [134]. This signaling cascade is initiated by activation of small G-proteins which transmit the signal further by recruiting the small MAP3Ks to the plasma membrane where they become activated and begin the three kinase phospho-relay [137]. The activated MAP3Ks transmit the cascade signal further by phosphorylating and activating the MAPKK proteins MEK1 and MEK2, the upstream kinases of ERK1 and ERK2 [137]. (See Figure 31) Activated MEK 1 and MEK2 act as dual specificity kinases and phosphorylate tyrosine and threonine residues on ERK1 and ERK2, activating these MAPKs [137-138,141,144]. Upon dual phosphorylation, both ERKs become a potent protein serine/threonine kinase, which phosphorylates downstream substrates on the defined consensus site Pro-Xaa-Ser/Thr-Pro [137]. There is also evidence that the ERK MAPKs seem to participate in multiple feedback loops that are important for the reduction of their activity at later stages after stimulation, including the inhibiton phosphorylation of the upstream proteins, such as the upstream exchange factor SOS, Rafs, and MEKs [137].
The ERK1/2 proteins are widely expressed, activated by a large number of extracellular stimuli, and have been linked to many cellular processes. ERK 1 and ERK2 have been shown to regulate proliferation, differentiation, and meiosis in cells [134,141,144,202]. The activation of ERK1/2 has also been shown to be induced by mitogenic stimuli such as growth factors, cytokines, and phorbol esters, which are known to activate a variety of receptors and G proteins [134,141,144,202].

Upon stimulation, the ERKs have been demonstrated to phosphorylate a large number of substrates, some of which localize to the cytoplasm of the cell to be phosphorylated, while others are phosphorylated in the nucleus [137]. These substrates, upon stimulation, phosphorylate and activate a series of transcription factors such as ELK1, cFos, p53, Ets1/2, and c-Jun [137]. Deregulation of ERK1/2 MAP kinase pathway has been associated with various pathologic conditions including proliferative disorders and developmental abnormalities [202]. Temporal regulation of this pathway is critical for determining the signal output of the cascade.

The in vivo functions of ERK1 and ERK2 have been studied in mouse models. ERK1 null mice are viable, fertile, and of normal size [138,142]. Further characterization of this null mouse model shows a modest T-cell defect, decreased adiposity, and protection against obesity and insulin resistance [138,164]. Therefore, the ERK1 null mice seem to be a model of gain of function rather than pathogenic.

The ERK2 deficient mice, however, die early in development at approximately E6.5 [138]. These mice fail to produce mesoderm, and present abnormal placental development. The ERK2 deficient mice also show increased apoptosis, but no modification in proliferation, insinuating a defect in differentiation [131,138]. Due to the embryonic death of the ERK2 deficient mice, it is clear that ERK1 cannot compensate for ERK2 in vivo [202].

To determine if ERK1 and ERK2 are playing different roles within development, ERK2 levels were examined in ERK1 null mouse embryonic fibroblasts (MEFs) [202]. ERK2 protein levels were found to be unperturbed, but the activation of ERK2, measured by phosphorylation was found to be
elevated and more sustained in the ERK1 null MEFs compared with wild type controls [202]. The ERK1 KO MEFs seemed to proliferate faster than control cells, while ERK2 deficient cells proliferated poorly [202]. Therefore, the ERK2 MAPK appears to mediate the proliferative signal in these cells, while ERK1 has some type of inhibitor effect [202]. A role in cardiac hypertrophy has also been assigned to ERK1/2 signaling. Kehat et al showed the transition of cardiac growth from eccentric to concentric hypertrophy through the depletion of ERK1/2 in the heart or the over-activation of the ERK1/2 pathway through a MEK1 transgenic mouse respectively [203,204]. Therefore, there are multiple roles established for ERK1/2 in cells in different cell types.

While roles of ERK1 and ERK2 have been addressed in many tissue types, the role of these kinases has not been well described in skeletal muscle. Data relating to ERK1/2 signaling in skeletal muscle is mainly found in fiber type determination and in levels of activity during exercise in wild type and mdx MD mice. However, with little data in either of these two areas, a clear picture of ERK1/2 signaling within the pathogenesis of MD, or skeletal muscle in general, is lacking.

**ERK1/2 signaling in fiber type Determination**

The ERK signaling pathway has been shown to play a role in fiber type determination in skeletal muscle. In a study by Murgia et al. in 2000, Ras was found to be involved in nerve-activity-dependent regulation of muscle genes [205]. Transfection of constitutively active Ras into soleus muscle after injury induced the expression of MyHC-slow protein, while dominant negative Ras prevented the activation of the MyHC-slow gene [205]. This MyHC switch was shown to be induced by RasS35 which selectively stimulates the MAPK (ERK) pathway, and only RasS35 was able to induce MyHC-slow in a large number of fibers in regenerating muscle after injury [205]. These findings were further supported by the observation that a constitutively active MAPK kinase mutant induced numerous MyHC-slow-positive fibers in denervated muscle [205]. However, this study implies that RasS35 is a selective kinase of the ERK pathway, while truthfully Ras can phosphorylate many targets, including other MAPKs. Therefore
this study is not conclusive for the role of ERK MAPK in fiber type determination. In addition, the constitutively active MAPK kinase mutant was never defined specifically as either MEK1 or MEK2, the upstream kinases of ERK1/2; therefore, we cannot definitively conclude that this active MAPK kinase mutant is activating ERK1/2.

Other studies have elucidated an intricate role of ERK1/2 in slow twitch fiber type determination. Higginson et al. investigated the role of the Ras-Raf-MEK1/2-ERK1/2 pathway in this muscle fiber-specific gene expression using primary rat myotubes [206]. It was discovered that after treatment with U0126, a MEK1/2 specific inhibitor, there was a significant increase in mRNA levels of MHCIIIX and MHCIIIB transcripts, and a significant decrease in slow myosin mRNA expression as compared in untreated controls [206]. These results suggest that the MEK1/2 pathway is involved in downregulation of the IIX and IIB isoforms of MHC and in the upregulation of the slow myosin isoforms, indicating the ERK1/2 pathway preserves the formation of slow twitch myofibers [206].

However, Shi/Gerrard et al found the opposite, in which ERK1/2 was more activated in fast twitch muscles, and that a decrease in ERK activity caused an increase in slow twitch fibers [10]. They further showed that pharmacological blocking of ERK1/2 pathway further increased the slow-twitch fiber type-specific reporter activity and repressed fast twitch reporters in vitro [10]. Expression of ERK1/2 in skeletal muscle slow and fast twitch fibers has been actively assessed in the literature. In slow twitch muscle, ERK1 and ERK2 are phosphorylated at similar levels; however in fast twitch muscle, ERK2 is preferentially activated [10]. This higher level of ERK2 phosphorylation would indicate a role of this MAPK in the maintenance of the fast fiber phenotype [10]. In addition to these findings, the overexpression of constitutively active ERK2 in cell culture induced MyHCIIb reporter by 4.8 fold in soleus muscle, a primarily slow twitch muscle; however, the constitutively active ERK2 had no effect on the slow reporter activity in vivo, where it had an effect on the slow reporter activity in cell culture experiments [10]. In summary, there are mixed opinions on whether the ERK pathway drives the slow or
fast twitch fiber type, but there is evidence for a role of these MAPK family members in fiber type determination.

As stated earlier, the first muscle fibers affected in dystrophy are the fast fibers due to faster and more frequent contractions in fast fiber type fibers [9,13]. It is possible that a change in ERK1/2 activation could be used in dystrophic muscles to alleviate the pathology within diseased muscles, once the determination has been made on which fiber type ERK1/2 preferentially activate in vivo.

**Role of ERK1/2 in Exercise and MD**

Several studies in the MAPK field have demonstrated that the ERK pathway can be activated by contractile muscle activity [14]. Data has shown that ERK1/2 activity increases after running, cycling exercise, or high-force contractions [142]. In previous studies, ERK1/2 activity increased at the end of all forms of exercise, separate from any endocrine or paracrine influence, suggesting that the activation of this MAPK signaling pathway may be a component of general responses to exercise [142].

The activation of ERK1/2, JNK, and p38 signaling cascades have been widely studied in skeletal muscle contraction during exercise and may be involved in the regulation of mechanically induced gene expression [10]. Studies by Ikeda et al. used the C57BL/10 mdx mice to examine the phosphorylation and protein levels of ERK1/2 in exercised wild type (Wt) and dystrophic mice. The study found that the ratio of phosphorylated ERK1/2 to ERK1/2 total in Wt mice was significantly higher after the mice were subjected to exercise [144]. The phosphorylation in the exercised mdx mice was significantly higher than either the exercised Wt (4 fold) or the non-exercised mdx mice (2 fold) [144]. Therefore, this study concludes that treadmill exercise increases the phosphorylation of ERK1/2 in mdx muscles compared with both non-exercised and exercised Wt muscle [144]. It has been shown in multiple studies that ERK1/2 protein levels are increased in dystrophic tissue, whereas the relative phosphorylation status of this protein remains at control levels; therefore, ERK1/2 activation may also be necessary for developing the dystrophic phenotype, but may not be sufficient [171].
ERK1 and ERK2 Conclusions and Rationale

The role of ERK1/2 kinase pathway within skeletal muscle remains poorly understood and under studied. However, there are promising indications of a role of these two MAPKs within the pathological conditions of MD. Roles have been suggested for ERK1/2 activation or inhibition in fiber type switching, and studies utilizing exercised dystrophic and Wt muscle also point towards ERK1/2 and a possible role in muscle injury and recovery; however, the mechanism has not been elucidated. Therefore we have begun studies to clarify the role of ERK1/2 in fiber type switching by the over activation of ERK1/2 MAPK utilizing constitutively active MEK1 transgenic mouse. We find that overstimulation of the ERK1/2 pathway induces a fiber type switch to a slower phenotype.

Constitutive activation of ERK Causes a switch to a slower twitch muscle

To understand the role of ERK1/2 activation in skeletal muscle in vivo, we created a transgenic mouse in which the skeletal α-actin promoter drives a constitutively active MEK1 MAPKK. (Figure 32A) We examined two lines of MEK1 Tg constitutively active mice, the high expressing line (13.9) and the low expressing line (17.8) as depicted by the western blot in Figure 32B. An increase in phospho-ERK was noted in the 17.8 line; however, pERK1/2 levels seemed to decrease in the high line as compared to Wt baseline. This may be due to the high activation levels of ERK1/2 by the 13.9 transgene, and therefore, a feedback loop may be downregulating phosphorylation of ERK1/2. Gross histological analysis of sections of Wt versus the high line (13.9) and low line (17.8) showed mostly normal muscle organization, with slightly less organization noted in the 13.9 high transgenic line. (Figure 32C) After closer examination, we found that the muscle weights of the MEK1 13.9 high line were significantly smaller.
than both the 17.8 low line and the Wt at 6 months of age. (Figure 32D) In addition, quantitation of the central nuclei and fiber area distribution in these transgenic lines showed an increase in the percent of nuclei in the middle of the muscle fibers, and a large decrease in the average size of the muscle fibers in both the high and low lines of the MEK1 Tg mice. (Figure 32E and F) The increase in central nucleation is interesting, mainly because the gross histology sections did not look pathologic, with the exception of the high line. Further characterization of this phenotype will be necessary before determining if this increase in central nucleation is indeed pathologic.
One interesting finding in these MEK1 Tg mice was the color of the muscle of the mice. While the Wt had the normal white/light pink coloration of the muscles, the 17.8 MEK1 line had a slightly deeper pink color, and the muscles of the 13.9 MEK1 line appeared very red. (Figure 33) This color change of the muscle could be indicative of a fiber type switch change, since slower twitch muscles, such as the soleus, tend to appear redder in color due to the more oxidative state in these muscles. To address this question we performed two separate fiber type stains: the Myosin ATPase stain, which labels fibers as fast twitch and slow twitch, and a Succinate Dehydrogenase stain which labels fibers by levels of non-oxidative and oxidative fibers. The Myosin ATPase stain utilizes the enzymatic reaction of ATP hydrolysis by myosin in the muscles, under specific pH conditions, to determine the specific myosin due to the rate of ATP hydrolysis. The Succinate Dehydrogenase stain measures the amount of succinate dehydrogenase activity, indicating the amount of mitochondria and the level of oxidative capacity of the fiber. Fast twitch fibers tend to have fewer mitochondria, and therefore less succinate dehydrogenase activity, leading to a “less” oxidative phenotype. Slow twitch fibers contain more mitochondria, and therefore express a higher level of succinate dehydrogenase activity. This leads to a much more oxidative fiber. Gross histological analysis of slides stained with either slides showed a visual difference in Type I fibers, especially in the MEK1 13.9 line as compared to Wt muscles. (Figure 34A) In the Myosin ATPase stain, we observed greater numbers of “white” fibers (Type I fibers in alkaline stain) and fewer numbers of the very dark fibers (Type II fibers), and greater numbers of dark blue fibers (Type I) in the Succinate Dehydrogenase stained sections. After careful quantitation, using the Myosin ATPase stain, there were significantly more

![Image](image_url)
Type I fibers in the MEK1 13.9 Tg mice compared with the Wt controls. (Figure 34B) In addition, utilizing the Succinate Dehydrogenase stain, we found greater numbers of slow twitch fibers in both MEK1 Tg lines in comparison with the Wt controls. This data indicates that over-activation of the ERK1/2 pathway could lead to an increase in slow/more oxidative muscles and indicate a role in the preservation of the slow twitch phenotype in mice.
Finally, to begin to form a link with ERK1/2 activity in dystrophy, we performed baseline western blots comparing Wt mice to Sgcd -/- mice and blotting for ERK1/2 pathway members. We see an increase in pMEK1 levels as compared to Wt baseline levels (3.27 fold increase) (Figure 35). It also appears that there is an increase in pERK1/2 levels in the Sgcd-/- mice compared with the Wt mice; however, when the signal levels for pERK1 and pERK2 are normalized to the total ERK1/2 levels, we actually find a reduction in both phosphorylation levels in the Sgcd-/- mice compared to the Wt mouse (3.06 and 3.36 fold reduction respectively. While this data somewhat contradicts the literature, our preliminary data collectively suggests that ERK1/2 actual phosphorylation levels may be lower in this model of the Sgcd -/- dystrophic mouse.

Discussion

The ERK1/2 family of MAPK proteins has been characterized throughout the years as kinases which promote growth, differentiation, and cell survival. The link between ERK1/2 expression and the preservation of a certain fiber type has been studied but there is some dissention in the field [10,205,206]. Along these lines, ERK1/2 expression in dystrophy has been alluded to; however, no direct link in pathology or any beneficial effect of ERK1/2 signaling has been established. In our preliminary study, we find that over activation of ERK1/2 MAPK increases the slow twitch fiber type in the quadriceps muscle, while creating a mild central nucleation which has yet to be determined as pathologic. It has been suggested previously in the literature that slow twitch muscles are less likely to have contraction induced damage, and are spared longer during dystrophy as compared to fast twitch fibers [9,13]. Therefore, the upregulation of the slow twitch fiber type by over activation of ERK1/2 in the MEK1 Tg mice could be protective against muscle damage and loss in MD. A cross of these Tg mice with the Sgcd-/- and mdx
mice would be a necessary cross to determine if this increase of Type I fibers is indeed protective against myofiber damage, and prolong the health of the muscle for a longer period of time.

Furthermore, analysis of the loss of ERK1, ERK2 or both in skeletal muscle could also clarify the role of these kinases in fiber type switching. Potentially, because we see an induction of slow twitch fibers in the MEK1 transgenic mice with constitutive activation of ERK1/2, we could see a decrease in slow twitch fibers following the deletion of ERK1 and/or ERK2. If so, we would then hypothesize that the loss of ERK1/2 in dystrophic skeletal muscle could increase the propensity for damage, therefore inducing a more degenerative state. We saw a significant decrease in total ERK1/2 activity in the Sgcd-/- mice in our preliminary western blot indicating, that during this disease state, the ERK1/2 pathway is down regulated suggesting that inducing this pathway in dystrophy could be protective. More careful western blot experimentation will be necessary to determine if this preliminary trend holds true for the Sgcd -/- mouse and other dystrophic mouse models, such as mdx mice.

Further analysis of the pathways affected by this over activation of ERK1/2 through the MEK1 Tg or deletion of the ERK1/2 MAPK in mice will be a necessary and interesting expansion of this study. While it has been shown that ERK1/2 can affect fiber type determination, it is not evident what pathways are affected by the ERK1/2 MAPKs to cause this switch in myosin expression and oxidative state. Once identified, these pathways could indicate specific targets to induce a slow twitch phenotype in dystrophic models and potentially increasing myofiber longevity. In conclusion, the ERK1/2 MAPK pathway appears to play a role in the preservation of the slow twitch fiber type in skeletal muscle, and could be a potential induction target in the treatment of MD.
**Materials and Methods**

**Mice**

Constitutively active MEK1 transgenic mice (FVBN background) were generated by subcloning a constitutively active human MEK1 into the pcDNA3.1 vector driven by the human α-skeletal actin promoter [197]. Mice were sacrificed by Isoflurane inhalation followed by cervical dislocation. The quadriceps muscle was excised and either snap frozen or placed into 10% phosphate buffered formalin for later analysis. All mouse experimentation was approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital. Both male and female mice were used. No human subjects or materials were used.

**Western Blot Analysis**

Protein extracts were prepared from the quadriceps muscle by homogenization in cell lysis buffer (10 mM Tris-HCL (pH 7.5), 150 mM NaCl, 4% Glycerol, 0.5 M NaMetabisulfite, 1% Triton X-100, 0.1% NaDeoxycholate, 0.05% SDS) supplemented with dithiothreitol (1mM), protease inhibitors (Roche), and phosphatase inhibitors (Roche). Protein extracts were run on SDS-PAGE or Phos-tag gels AAL-107 (Wako Pure Chemical Industries), transferred to a PVDF membrane and immunodetected as specified by the manufacturer (Amersham Biosciences). Antibodies used in this study were: phospho-ERK1/2 (Cell Signaling, 1:1000), ERK1/2 (Cell Signaling, 1:1000), phosphor-MEK1 (Cell signaling, 1:1000), MEK1 (Cell signaling, 1:1000).

**Pathological Indices**

Paraffin-embedded histological sections (5 µm) of skeletal muscle were prepared and stained with Hematoxylin and Eosin, or Masson’s trichrome. Three pictures of each quadriceps or diaphragm muscle per mouse were taken and the entire field of view was counted per mouse for analysis of central nucleation with ImageJ software [127].
Fiber type staining

To stain for Myosin ATPase activity we used the protocol previously described [218]. Succinate DH stain was performed by the histology core at Cincinnati Children’s Hospital.

Statistical Analysis

The results are presented as means ± s.e.m. We used the student’s two-tailed t-test to calculate significance. Values were considered significant if P < 0.05.
CHAPTER 6 – Discussion and Future Directions

MD is a severe skeletal muscle disease which results in muscle breakdown and decreased lifespan in many cases. Many MDs are caused by mutations in proteins within the DGC, which maintains a connection between the skeletal muscle fibers and sarcolemmal membrane during muscle contraction [18,20,30,36]. In the absence of a functional DGC, tears that occur in the muscle membrane can lead to an unregulated flux of Ca$^{2+}$ which can trigger the initiation of multiple cell death pathways. This suggests that a non-functional DGC could be more of a weak link aiding in the cell death of muscle fibers. Pathways up-regulated following the Ca$^{2+}$ influx include the opening of the MPTP, or the activation of intracellular signaling pathways, such as the p38 MAPK cascade [103]. There is clear evidence for a role of the MPTP in MD [103,104]; however, the direct role of p38 in the progression of MD has not been previously addressed. Furthermore, studies have shown that fast fiber type myofibers are affected earlier in the dystrophic disease progression, while slow twitch fibers are initially more resistant to disease. ERK1/2 MAPK have been linked to the fiber type determination; however, the current literature is contradictory and no clear role for this pathway has been established at baseline or during disease.

Debio-025 and the alleviation of the dystrophic phenotype

Previous work from Millay et al. has shown the efficacy of MPTP inhibition through the deletion of the MPTP regulator cyclophilin D, to alleviate dystrophic disease in the Sgcd -/-, mdx, and lama2 -/- mice [103]. Debio-025, a potent cyclophilin inhibitor, also produced a significant reduction of disease in the Sgcd -/- and mdx models of dystrophy [103]. Because of the positive effect seen in dystrophic mice, it was hypothesized that Debio-025 may be a promising pharmacological treatment for dystrophic patients. However, since prednisone is the current gold standard of treatment in dystrophy, the combined effect of Debio-025 in conjunction with prednisone must be tested in order to assess the potential of Debio-025 for a clinical trial. To test the efficacy of combined Debio-025 and prednisone treatment, we conducted two separate drug trials in the mdx model of DMD, where Debio-025 and prednisone were given either by
subcutaneous injection or by oral gavage. Debio-025 and Debio-025/prednisone treated mice showed improvements in grip strength, gross histology, fibrosis, central nucleation, and observed fewer activated macrophages in the skeletal muscle compared to vehicle or prednisone only treated mice. Surprisingly, the combined treatment of Debio-025 and prednisone did not have any additive effect over the Debio-025 alone treatment, indicating that these two drugs possibly inhibit similar dystrophic pathways. Prednisone is a known inhibitor of the inflammatory response, which is typically secondary to the necrotic cell death response in dystrophic muscle [24,108,109]. It is possible that the blocking of the MPTP opening through the inhibition of cyclophilin D by Debio-025 blunts the necrotic cell death in the muscle fibers, thereby lessening the subsequent inflammatory response needed to clear out the necrotic fibers. Due to this decrease in inflammatory response as a result of Debio-025, the requirement of the inhibitory effect of prednisone is diminished suggesting no additional benefit of prednisone treatment. This data suggests that treatment with both prednisone and Debio-025 would be feasible in clinical trials, and it is likely that these two drugs could work conjointly and without diminishing any beneficial effect in the dystrophic patients.

Future studies with Debio-025 may be necessary before a clinical trial would be feasible. A good next step would be testing this drug in a large animal model such as the golden retriever dog model of DMD, which resembles more closely pathophysiology of human disease. Also treatment beginning in later stages of the disease in mice would be valuable to show if Debio-025 could alleviate disease pathology if administered after disease onset. Typically children with dystrophy are not diagnosed with the disease until symptoms have become pronounced, and so it is more than likely that any treatment with Debio-025 would only begin once muscle wasting has already commenced. Finally, it would be prudent to do an extended trial of Debio-025 in animal models of dystrophy. Children with this disease would need to be on this drug for long term treatment since this drug will not be a cure for the dystrophic disease; therefore, it is important to discern if any toxic effects occur with extended use of Debio-025. Debio-025 has already been tested in stage II clinical trials for treatment of hepatitis C in adults with
minimal toxicity [208]. However, more stringent standards of toxicity are typically employed in clinical trials involving children, so effective dose and adverse side effects will need to be determined for this age group.

**p38α is an important player in the progression of MD**

p38 MAPK is a widespread effector for numerous cellular responses in many tissues. The role in cell death pathways of p38 has been thoroughly demonstrated; however, a direct role in dystrophy has yet to be identified. Previous studies have noted increased activation of p38 after exercise or in dystrophic muscle, and have shown that modulation of the levels of p38 inhibition by DUSP 1 or DUSP10 in skeletal muscle can affect the dystrophic phenotype in *mdx* mice either exacerbating or alleviating disease [172,173]. Therefore, because of the well-established role of p38 MAPK in cell death, and the preliminary suggestion of a role of this kinase in disease, we hypothesized that p38α MAPK plays a significant role in the progression of MD.

Genetic ablation using the p38α floxed mouse crossed with the myosin light chain 1F (MLC) Cre recombinase knock in mouse, produced a mild non-pathological phenotype at baseline which was reminiscent of the phenotype of delayed maturation previously found by Munoz-Canoves [3,165]. Therefore, because no pathologic response from skeletal muscle lacking p38α was observed, we crossed these mice with the *Sgcd* −/− and *mdx* mouse models of MD.

Following the ablation of p38α in the *Sgcd* −/− and *mdx* mice, we observed a significant reduction of all measured pathological indices including central nuclei, fibrosis, activated macrophages, serum CK, and the double deficient mice gained the ability to run for longer periods of time. These data indicate that the loss of p38α alleviates the pathology in the dystrophic muscle and that p38α could play a role in the pathologic progression of dystrophic disease. We questioned whether the loss of p38α was somehow protecting or assisting in membrane stability in the dystrophic mouse muscle, and that this stabilization was the source of the decrease in pathology. However, following injection with Evan’s blue dye (EBD)
and forced treadmill running, we observed the same number of EBD positive fibers in the quadriceps muscle in both $Sgcd^{-/-}$ and $p38fl/fl$, $Sgcd^{-/-}$, MLC Cre mice, indicating that the loss of $p38\alpha$ did not affect membrane permeability. Therefore, we hypothesized that the decrease in visible pathology in the $p38fl/fl$, $Sgcd^{-/-}$, MLC Cre mice was due to an event downstream of the initial dystrophic injury, such as cell death.

To further investigate a role of $p38$ MAPK in MD we wanted to determine if over-activation of $p38$ MAPK was sufficient to cause a dystrophic like disease. To do this, we created a skeletal muscle specific $p38$ MAPK over-activation model utilizing the human skeletal alpha actin (SKA) promoter driving a constitutively active MKK6. This model of $p38$ over-activation produced a severe muscle wasting phenotype characterized by large amounts of central nucleation, fibrosis, adipose tissue replacement of myofibers, and infiltration of activated macrophages. The muscle wasting was so profound, the muscles of the MKK6 Tg mice had less than half of the muscle mass of Wt littermates prior to 3 months of age. These data indicate that over-activation of $p38$ MAPK in skeletal muscle is sufficient to cause a severe dystrophic like pathology.

The main pathological phenotype of the MKK6 Tg mice was the immense level of muscle wasting and fiber drop out, which points towards a mechanism of cell death for $p38$ MAPK over-activation. A role of $p38$ in cell death has been firmly established in the literature for many different cell types; therefore, it is reasonable to hypothesize that $p38$ MAPK could be triggering part of the cell death response in dystrophic muscle [72,149,145-147]. Recently other groups have shown a direct phosphorylation link between $p38\alpha$ MAPK and the pro death Bel-2 family protein Bax on residue T167 [179-181]. Indeed in MKK6 Tg muscle tissue and in cells with overactive $p38$ MAPK, we find an increased amount of phosphorylated Bax at residue T167. A cell death assay utilizing Bax and Bak double knock out MEFs with Wt Bax or T167A mutant Bax adenovirally reconstituted in these cells further showed that the phosphorylation of Bax at T167 is important for $p38$ driven cell death. It would be interesting to see if this T167 phosphorylation dependent mechanism holds true in vivo, by making a
mouse with a skeletal muscle specific T167A mutation, to further elucidate the full ramifications of Bax phosphorylation driven cell death by p38 MAPK.

Our mechanistic data demonstrating the phosphorylation and activation of Bax, a known cell death pathway initiator, by p38 confirm the hypothesis that p38 MAPK is involved in the progression of dystrophic pathology through mediating cell death signaling. However after the genetic deletion of Bax and/or Bak in the MKK6 Tg mouse, we only observed a partial rescue of the severe muscle wasting pathology indicating that there are other p38 driven pathways involved in the MKK6 Tg phenotype. The deletion of p53, a well-known cell death protein, did not improve the muscle wasting histopathology in the MKK6 tg, and actually decreased the muscle mass even further in the main hind limb muscles. Therefore, the Bax partial rescue of the MKK6 Tg mouse is not through the classical p53 pathway, and there must be other cell death proteins activated by p38.

One possible additional target for p38 is Bim. Bim is part of the classical apoptosis pathway which typically include Bax and Bak, and literature has shown a role in p38 MAPK phosphorylation inducing apoptosis through Bim [215]. However, this pathway includes activation of the apoptotic cell death pathway, and in dystrophy, necrotic cell death appears to be the predominant form of death in the myofibers. It is possible that, in a similar fashion to Bax and Bak, the Bim cell death effector may also participate in a more programmed necrotic cell death pathway that has yet to be defined. Therefore, analysis of other known cell death effectors activated by p38 MAPK would aid in determining the full mechanism of the p38 driven muscle wasting in the MKK6 Tg mouse.

The effect of the deletion of Bax and/or Bak in the dystrophic models would also be of interest to further elucidate the p38α mechanism in the progression of dystrophy. Previous literature, as mentioned earlier, has shown alleviation of disease with the deletion of Bax in the lama2-/- mice, indicating a promising effect in other dystrophic mouse models [79,82]. Analysis of the deletion of Bax and/or Bak in
the Sgcd−/− or mdx mice could establish a stronger link between the p38 phosphorylation of Bax and myofiber death in dystrophic tissue.

Again, our mechanistic studies regarding the loss and over-activation of p38 MAPK in dystrophic muscle suggest that p38 activity is partially responsible for the progression of the muscle wasting phenotype observed in the Sgcd−/− and mdx dystrophic mice. The strong pathology and muscle loss in the MKK6 Tg mice and its subsequent rescue by the deletion of p38α MAPK, and partial rescue with the loss of Bax, indicates that the fiber degeneration in this transgenic mouse is almost exclusively p38α focused, and that a major component of the pathway includes increased p38 driven cell death. No differences were detected in the amount of membrane damage after exercise between Sgcd−/− and p38fl, Sgcd−/−, MLC Cre mice indicating the primary injury still occurs in these mice, and the partial rescue due to the loss of p38α functions downstream of this initial injury. Taken together, our data suggest that loss of p38α in dystrophic skeletal muscle inhibits the cell death response which is secondary to the initial dystrophic injury.

In addition, it is likely that the increase in muscle function in the dystrophic p38α deficient mice is due to the retention of muscle mass resulting from significantly decreased muscle fiber death. The MKK6 Tg mice are an enhanced model of dystrophic muscle death, and so we observe greater loss of function in the skeletal muscle due to lack of muscle fibers/muscle mass. The loss of p38α in the MKK6 Tg prevents any upregulation of p38α induced activity, avoiding the initiation of death in these mice. This suggests that the death and loss of muscle fibers are the more important in the loss of function in these muscles, and not the initial cellular injury such as membrane microtears.

Overall our mechanistic results from the MKK6 Tg mouse and p38floxed mouse crossed with the dystrophic mouse models strongly indicate that p38 MAPK over-activation leads to myofiber death in skeletal muscle, while p38 deletion confers protection from degeneration and cell death. To translate our genetic data regarding protective effects of p38 deletion in dystrophic mice into a potential treatment, we
treated Sgcd-/- mice with a p38α/β inhibitor, SB731445, at both a high and low dose. Both doses of SB731445 showed improvements in the dystrophic muscle pathology, including central nucleation, fibrosis, CK, and exhaustion times during running as compared to vehicle controls. These results are exciting in our efforts to translate the genetic inhibition of p38 MAPK activity into a possible treatment for MD. However treatment of mdx mice and TO-2 hamster (δ-sarcoglycan null hamsters) showed mixed results. Therefore, more in-depth studies may need to be done before a clinical trial could be feasible.

MD is a highly devastating disease that mainly affects the skeletal muscle, but also has a contribution from other cell types such as inflammatory cells, fibrotic/collagen tissue, and satellite cells also factor into progression of the disease. In our studies, the deletion of p38α is skeletal muscle specific due to the use of the MLC1f Cre recombinase; therefore, p38α is still expressed in all other tissue and cell types. p38 has also been strongly linked to the inflammatory response signaling in the literature [147]. In our model, however, we do not affect the levels of p38 in the inflammatory cells, so we cannot measure the effect of a loss of p38α in the dystrophic inflammatory response. Conversely, treatment of dystrophic mice or dystrophic patients with a p38 inhibitor would affect p38 activity levels in all tissues and cell types. While loss of the inflammatory response in dystrophic mice has not lead to a robust rescue of the disease, it would still be of interest to investigate a combined effect of the decrease of p38 activity in the combination of both dystrophic muscle and the inflammatory response using an immune cell specific cre recombinase, such as LysM cre (macrophages) to see if any additional benefit could be achieved [213].

The replacement of skeletal muscle fibers with fibrotic tissue is another main phenotype of the dystrophic disease. As myofibers are lost, the area of myofiber dropout can be filled with fibrotic tissue, which can lead to the worsening of muscle function due to muscle stiffening and reduction of contractile fibers. p38 MAPK has been shown to affect fibrosis in the literature [140,146,216]; however, in our study, once again, p38α is not deleted in the fibroblasts. Therefore our observation of less pathology is not due to a direct defect in fibrosis. However, we did observe an increase in TGFβ transcript in the MKK6 tg mouse which was slightly decreased after the deletion of p38α, indicating that even during a myofiber
specific over-expression of p38 activity, fibrotic signaling could still be affected. This decrease could be due to the reduction of the cell death in the MKK6 Tg tissue after the depletion of p38α, but it would still be of interest to examine the role of p38α during the fibrotic response in dystrophic tissue.

As stated earlier, p38α and p38β have been linked to the re-activation of quiescent satellite cells [6]. Satellite cells are activated upon injury, proliferate, and then differentiate to form new myocytes which can then fuse to the existing myofibers. These satellite cells are exhausted during myopathic diseases such as MD. It is hypothesized that this loss of the satellite cell population is due to a finite number of satellite cells in the muscle cell population from birth, which lose the ability to self-renew, or satellite cell death after activation and therefore lack of self-repopulation [210]. The ability of satellite cells to re-activate from quiescence could also have an effect on the regenerative capacity of the skeletal muscle. The over-activation of p38 MAPK could lead to over-activation of satellite cells, causing the exhaustion of the pool of regenerative satellite cells to occur earlier than normal in the dystrophic tissue. However, deletion of p38α in skeletal muscle cells could also be problematic, due to an inhibitory effect on the satellite cells ability to re-enter the cell cycle. This could eventually further exacerbate disease with either change in p38α expression. However, in our study, the utilization of the MLC-1f cre recombinase to delete p38α is skeletal muscle specific and does not remove the expression of this MAPK in satellite cells; therefore, the regenerative property of the satellite cells following activation remains intact. A subsequent study examining the loss of total p38α in all skeletal muscle cells, including satellite cells, will be important to address the effect of the loss of p38α on satellite cells during injury or dystrophy. Pharmacologic inhibition of p38 could also have a direct effect on the regenerative capacity of the skeletal muscle in the satellite cells. Therefore, a comprehensive dose response curve examining the effect of each dose of the inhibitor on the ability of satellite cells to exit quiescence would be of great importance prior to any clinical trial.

In the search for the mechanism underlying the beneficial effect of the deletion of p38α in dystrophic mouse models, we made a number of observations on how the loss of p38α changes other
aspects of the skeletal muscle, such as fiber size and fiber type. At baseline, we observed, on average, smaller myofibers following the deletion of p38α in hind limb muscle. This finding was consistent with previously reported data from the Muñoz-Canoves group, which showed the deletion of p38α could delay the maturation of a myofiber [3,165]. However, we also observed an increase in the number of Type I (slow twitch) fibers, as measured by a succinate dehydrogenase stain. This finding is consistent with previous reports that p38 has the ability to stimulate the fast fiber type when active, and so it is reasonable, that the loss of p38α could stimulate the slow fiber type [170]. This data, in combination with the increased switch to smaller fibers, could also point to another survival mechanism in the myofibers of p38α depleted dystrophic fibers. Smaller fibers, and slow twitch fibers, do not produce as strong a contractile force as larger, or fast twitch, fibers; therefore, these p38α depleted fibers may have less sarcolemmal damage after contraction, which could decrease the propensity for myofiber death. It would be interesting to further investigate this alternative mechanism, and determine if this hypothesized decrease of force production could be another way the loss of p38α could protect against dystrophic myofiber loss.

Overall, our results indicate the pharmacologic inhibition of p38α could slow the progression of MD. The observation that Mapk14, Sgcd -/-, MLC Cre and Mapk14, mdx, MLC Cre mice still exhibit significant disease compared to controls suggests that treatment with a p38 inhibitor would not cure dystrophy. Many pathways are involved in the dystrophic disease progression and the p38α MAPK pathway is only one of these that play a significant role. Other factors that could be involved include Ca2+ induced cell death (calpain), reactive oxygen species, other cell death signaling mechanisms, and other effects due to sarcolemmal tears. Nonetheless, our results indicate a significant improvement in dystrophic mice after the genetic and pharmacologic inhibition of p38α MAPK, and the experiments presented illustrate a central role of skeletal muscle p38α in the cell death aspect of dystrophic disease progression.
**ERK1/2 play a role in driving the slow twitch muscle phenotype**

The ERK1/2 pathway historically has been characterized as a pro-growth, differentiation inducing, and pro-survival pathway [134,141,144,202]. These MAPKs are widely expressed and linked to a variety of cellular processes; however, there is no definitive role for ERK1/2 established in skeletal muscle. Previous studies have linked ERK1/2 to the generation of distinct fiber type in skeletal muscle, and have shown the activity of these two kinases to be induced by exercise [9,142,144,205,206]. Our preliminary data of the MEK1 Tg model of ERK1/2 over activation, shows a significant increase in the slow twitch fiber phenotype in skeletal muscle, which could indicate an increase in endurance during exercise training, and less force generated during muscle contraction. Therefore, we could hypothesize that ERK1/2 play a role in the preservation of the slow twitch phenotype in mouse muscle, and that due to the properties of slow twitch muscle, it is possible that over activation of ERK1/2 could be protective in MD. However, it is unclear if one or both of the ERK MAPKs is the activator of this phenotype. Previous results have shown that ERK2 can preferentially activate the fast fiber type; therefore, it is possible that the increase in slow twitch fibers in the MEK1 Tg mice is due, specifically, to over activation of ERK1 MAPK. Crossing the ERK1/2 deleted mice, both as single and double deletions, to the MEK1 Tg mice could clarify the difference between the two ERK MAPKs in this system, and determine which kinase is the activator of this slow phenotype.

In addition, another way to test the preference of ERK1/2 in fiber type determination is to utilize muscle overload approach. This surgical-based approach consists of the removal of the gastrocnemius and soleus muscles from the mouse lower hind limb. This procedure results in an overload stimulus on the remaining plantaris muscle, which is forced to compensate for the functional load of the ablated gastrocnemius and soleus muscles. The plantaris has been shown previously to hypertrophy greater than 100% following 8 weeks of this functional overload, and that there is a significant increase in the percentage of slow fibers after 8-12 weeks post operation [211]. Again, this technique will help to clarify the role of ERK1/2 in fiber type determination in skeletal muscle. We would expect the MEK1 17.8 to be
“primed” to switch to a slow phenotype, and therefore would take less time to show a slow twitch switch. Deletion of ERK1 and/or ERK2 will demonstrate the need for one or both of these MAPK proteins in the promotion of the slow twitch fiber phenotype seen in the MEK1 Tg mice. We would expect that if one or both of these kinases were involved in the mechanistic switch to slow fibers, the deletion of ERK1 or/and ERK2 should prevent the effects of the muscle overload surgery.

The mechanism of action of the ERK1/2 pathway in determining fiber type has not been delineated. It would be of interest to identify what downstream targets of ERK1/2 are involved in myofiber switching. Previous research has identified p300/Nfatc1 as targets for ERK1/2 and has shown that the ERK1/2 mediated phosphorylation of p300 enhances Nfatc1 transactivation by acetylation which is essential for the Ca\(^{2+}\) induced slow myosin expression [11]. Identification of other downstream targets could be found using a systematic screen of known slow myosin proteins and ERK1/2 downstream targets utilizing the phos-tag gel blot system in MEK1 17.8, ERK1/2 deleted mice, and Wt mice to identify changes in phosphorylation patterns for the target proteins. The phos-tag gel electrophoresis system utilizes a special acrylamide that contains a tag that will bind to phosphorylated proteins, thereby slowing the migration. This slowing of the migration produces a visible shift in phosphorylated proteins as compared to non-phosphorylated proteins on the acrylamide gel using a total antibody. The phos-tag system allows us to screen for any possible difference in phosphorylation status without the use of phospho-specific antibodies. Understanding this signaling pathway promoting the slow twitch fiber type could be useful in developing targeting schemes to induce a slower twitch phenotype, possibly to use for the treatment of MD.

As stated earlier, previous studies have indicated that the fast twitch fibers are preferentially affected first in dystrophy [9,13]. This could indicate that an increase in slow fibers in dystrophic tissue could reduce the effects of this disease. We would hypothesize that the loss of ERK1/2 would exacerbate disease, while the over activation of these kinases (MEK1 Tg) would lead to a protection of the skeletal muscle in dystrophic mice. Previous studies have indicated that an increase in slower fiber type can
reduce disease. Studies examining the deletion of PGC-1α in the *mdx* mouse show that these mice undergo a fast to slow fiber type shift which was protective against muscle contraction induced damage [212]. Another study examining the treatment of dystrophic *mdx* mice with Wnt7a also showed a shift from the fast to slow phenotypes within the myofibers of these muscles, and that these mice displayed a significant increase in muscle strength and reduced contractile damage [213]. Together these two studies show the potential that our MEK1 Tg mouse model of ERK1/2 over activation has in lessening the dystrophic phenotype. However, recent literature has cited a pathologic role for ERK1/2 in EMD[214].

EMD is caused by mutations within the laminin A/C gene which leads to slowly progressive muscle weakness and wasting, and cardiomyopathy. In this study, ERK1/2 was found to be up regulated prior to the appearance of pathology, and treatment with an inhibitor for MEK1/2 improved muscle pathology and function [214]. This effect may not be the same for the *Sgcd* -/- or *mdx* mouse due to the fact that laminin is connected with the extracellular matrix, and not a necessary part of the sarcolemmal “core” of the DGC. Therefore, it will be interesting to examine the effect of loss and over activation of ERK1/2 activity in dystrophic tissue.

In addition to a probable role in fiber type determination, ERK1 and ERK2 have been characterized as pro-survival proteins; however, no anti-death roles have been defined for these kinases in skeletal muscle. Recent studies have shown that ERK1/2 phosphorylation of Bim-EL promotes the degradation of this pro-apoptotic protein, thus protecting cell from apoptosis [217]. It would be interesting to see if the increase or decrease of ERK1/2 in the dystrophic background has any anti-degenerative/cell death effects. While apoptosis does not seem to be the central cell death mechanism in dystrophy, it is possible that Bim, like Bax and Bak, could participate in a more programmed type of necrotic cell death yet to be defined [81,184-187].

Overall our preliminary results indicate that ERK1 and ERK2 play a role in skeletal muscle by acting within the fiber type determination signaling pathway, and that it is possible that over activation of these two MAPK proteins could alleviate the dystrophic phenotype. However, much is needed to be done
to clarify how this pathway is working to promote the slow twitch phenotype, and if this increase in slow twitch fibers by ERK1/2 is protective in different mouse models of MD.

Concluding Remarks

MD is a severe disease of the skeletal muscle which, due to muscle weakness and wasting, affects the quality of life and, in many cases, leads to premature death of affected individuals. The MDs encompass a wide array of mutations affecting many proteins and signaling pathways thus making treatment of these diseases difficult. In this dissertation, we demonstrate the roles of two individual pathways in the progression of MD including the mitochondrial permeability transition pore and p38α MAPK, and suggest a potential role for ERK1/2 MAPK. While we show that treatment with Debio-025, a MPTP inhibitor, and SB731445, a p38α/β inhibitor both significantly reduce disease pathology in dystrophic mouse models, neither of these two drugs could fully rescue the fulminant dystrophic biology. Additionally, if our preliminary data holds true for the over activation of ERK1/2 protection through the increase in slow twitch fibers, this switch will most likely not sustain the protective effect over the long term, as slow twitch fibers will still eventually be affected by the loss of membrane stability.

While the only true cure for the dystrophies would be replacement of the mutated gene sequence, it is still important to find treatments that can prevent the progression of the dystrophic disease and prolong muscle function. Debio-025 and SB731445 both significantly decrease pathology in dystrophic mice and increase muscle function, suggesting promise for each of these drugs in the treatment of dystrophic patients. As with all disease treatments, timely drug administration will provide the strongest effect, thus early disease diagnosis is critical. Because Debio-025 and SB731445 have demonstrated low toxicity in short term clinical trials in adult disease, we have high hopes that these pharmaceutical approaches can be translated for use in children. Therefore, we believe our proposed treatments could offer prolonged ambulation and quality of life for children with MD.


References


84. CROFTS AR, CHAPPELL JB. Calcium ion accumulation and volume changes of isolated liver mitochondria. reversal of calcium ion-induced swelling. *Biochem J.* 1965;95:387-392.


118. Halestrap AP, Davidson AM. Inhibition of Ca2(+)-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. *Biochem J.* 1990;268(1):153-160.


