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Identifying Novel Therapeutics And Therapeutic Entry Points For The Treatment Of Duchenne Muscular Dystrophy Through Regulation Of Gene Expression

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IDENTIFYING NOVEL THERAPEUTICS AND THERAPEUTIC ENTRY POINTS FOR THE TREATMENT OF DUCHENNE MUSCULAR DYSTROPHY THROUGH REGULATION OF GENE EXPRESSION

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A DISSERTATION

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ABSTRACT

My graduate research has primarily focused on genetic and epigenetic modifiers of Duchenne muscular dystrophy and understanding how those modifiers can be used as novel therapeutic entry points for treatment. A vast array of promising therapeutic strategies is being explored outside the realm of directly targeting dystrophin deficiency. This is primarily due to the lack of successful clinical trials that have demonstrated the ability to benefit a significant number of DMD patients. Here, I have expanded the field's understanding of using the regulation of gene expression as a way to target dystrophin-deficiency associated pathology through, 1) therapeutic treatment of a DMD mouse model using a novel SINE (Selective Inhibitor of Nuclear Export) compound, and 2) characterization of the role of a muscle-enriched microRNA, miR-486, in disease progression. I have demonstrated that inflammation-related DMD pathology can be subdued through indirect inhibition of the expression of inflammation-related genes via SINE compound KPT-350. In addition, I have uncovered novel targets of miR-486, which may be promising and viable targets for therapeutic intervention. Collectively, my studies on these modulators of gene expression have contributed to deepening the field's

understanding of potential avenues for uncovering promising therapeutic targets for DMD pathology.

Keywords: microRNA, Duchenne muscular dystrophy, Genetic modifier, Therapeutics

DEDICATION

This one is for Pop-o.

To my mother, Glenda Hightower, my father, Tim Hightower, and my sisters, Hailey and Kaycee Hightower, this is also for you. This, just like all things I have ever done and will ever do, was possible because of you.

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TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTERS

1 THE PATHOLOGICAL PROGRESSION OF DUCHENNE MUSCULAR DYSTROPHY, CURRENT STANDARDS OF CARE, AND GENE REGULATION AS AN AVENUE FOR PROMISING THERAPEUTICS 1

2 GENETIC MODIFIERS OF DUCHENNE AND FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHIES 20

3 KPT-350 AS A THERAPEUTIC AGENT FOR THE TREATMENT OF INFLAMMATION RELATED SYMPTOMS IN DUCHENNE MUSCULAR DYSTROPHY 49

4 IDENTIFYING NOVEL MICRORNA-486 TARGETS AS POTENTIAL THERAPEUTIC ENTRY POINTS FOR THE TREATMENT OF DUCHENNE MUSCULAR DYSTROPHY 84

5 THE FUNCTIONAL VERSATILITY OF GENETIC AND EPIGENETIC MODIFIERS REVEALS PROMISING TARGETS FOR NOVEL DMD THERAPEUTICS 120

GENERAL LIST OF REFERENCES 134

APPENDIX A: IACUC APPROVAL 145

LIST OF TABLES

Tables *Page*

2. GENETIC MODIFIERS OF DUCHENNE AND FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHIES

1 Genes with Polymorphic Significance in the Clinical Manifestation of Muscular Dystrophies 36

4. IDENTIFYING NOVEL MICRORNA-486 TARGETS AS POTENTIAL THERAPEUTIC ENTRY POINTS FOR THE TREATMENT OF DUCHENNE MUSCULAR DYSTROPHY

1 eCLIP-seq reveals miR-486 target transcripts 115

LIST OF FIGURES

Figure *Page*

2. GENETIC MODIFIERS OF DUCHENNE AND FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHIES

1 Schematic of significant genetic modifiers of Duchenne and Facioscapulohumeral muscular dystrophies and their sub-cellular localization in skeletal muscle 35

3. KPT-350 AS A THERAPEUTIC AGENT FOR THE TREATMENT OF INFLAMMATION RELATED SYMPTOMS IN DUCHENNE MUSCULAR DYSTROPHY

1 KPT-350 short-term dosing reduces percentage of affected DMD zebrafish and improves overall myofiber histology 70

2 KPT-350 long-term treatment extends the lifespan in DMD zebrafish 71

3 KPT-350 treatment improves histological hallmarks of dystrophic pathology 72

4 KPT-350 treatment improves overall locomotion and overall activity in D2-*mdx* mice 73

5 KPT-350 treatment results in decreased inflammatory cytokine expression in D2-mdx mice 74

6 KPT-350 treatment increases overall macrophage populations in D2-mdx mice 75

4. IDENTIFYING NOVEL MICRORNA-486 TARGETS AS POTENTIAL THERAPEUTIC ENTRY POINTS FOR THE TREATMENT OF DUCHENNE MUSCULAR DYSTROPHY

1 MicroRNA-486 skeletal muscle expression decreases in DMD.....104

2 MicroRNA-486 knockout mice demonstrate histological defects in skeletal muscle 106

3 RNA-seq reveals extracellular matrix pathways in miR-486 KO muscle compared to WT 107

4 Targeted chimeric eCLIP-seq reveals direct targets of miR-486.....109

5 Identification and selection of miR-486 knockout mice.....111

6 MicroRNA KO mice demonstrate differences in mass and muscle physiology 112

7 MicroRNA-486 knockout may result in functional and histological cardiac dysfunction.....113

CHAPTER 1

THE PATHOLOGICAL PROGRESSION OF DUCHENNE MUSCULAR DYSTROPHY, CURRENT STANDARDS OF CARE, AND GENE REGULATION AS AN AVENUE FOR PROMISING THERAPEUTICS

The importance of the Dystrophin-Associated Protein Complex in muscular dystrophies and myopathies

The Dystrophin-associated protein complex (DAPC) is essential for the proper function of myofibers. It plays a leading role in structural stability, maintenance, regeneration, and signaling within and between myofibers [1]. The DAPC is made up of several families of proteins, such as dystroglycans, sarcoglycans, syntrophins, and dystrobrevins. Because of their synergistic mechanisms, mutations in many of these proteins and protein families have been implicated in various muscular dystrophies and myopathies, emphasizing the importance of the DAPC as a whole. The functional loss of Dystrophin, as in the case of patients with DMD, results in the total loss of the DAPC and this supports the critical role of Dystrophin in anchoring the actin cytoskeleton to the extracellular matrix [1, 2]. Pathogenic mutations in Dystrophin that do not result in loss-of-function can still result in Becker muscular dystrophy (BMD), a milder form of DMD where partial function of Dystrophin protein occurs [3]. Much of this has been described in detail based on Dystrophin mutation location, where in-frame mutations typically result in BMD versus out-of-frame mutations that typically result in DMD [4-6]. The C-terminus

of Dystrophin binds directly to β-dystroglycan, which links Dystrophin to the laminin-binding region of the α-dystroglycan protein subunit, resulting in the critical anchoring of the actin cytoskeleton to the extracellular matrix. Disruption of the interaction between Dystrophin and β-dystroglycan has been demonstrated to produce a DMD-like phenotype, disruption of the interaction between the dystroglycans and the extracellular matrix is the cause of congenital muscular dystrophy, and disruption in glycosylation of the dystroglycans can result in both congenital muscular dystrophy as well as limb girdle muscular dystrophies (LGMDs) [5, 7-10]. In addition to dystroglycans, five transmembrane proteins known as the sarcoglycans: α, β, γ, δ, and ε have been shown to be essential for anchoring the DAPC cytoskeletal complex to the extracellular matrix [1, 11]. Loss-of-function mutations in any of these sarcoglycans can affect proper sarcolemma localization of the others and result in recessive forms of limb girdle muscular dystrophy. Mutations in the DAPC α-dystrobrevin protein family have been known to cause DMD-like phenotypes and mutations in Laminin-2 can cause severe congenital muscular dystrophy [1, 12, 13].

Overall, mutations or disruption in the localization or glycosylation of the DAPC protein components can result in biological or mechanical dysfunction of myofibers, which highlights the extreme importance of the DAPC to the structural stability and maintenance of skeletal muscle. This is a critical principle in understanding the severity of disruption of the DAPC in patients with DMD who produce no functional Dystrophin protein and highlights the necessity in fully understanding the implications of DAPC disruption in order to grasp the impact of the lack of Dystrophin in dystrophic pathology.

Dystrophin at the DAPC and its structural and nonstructural roles in muscle function

Dystrophin (*DMD*) is the largest gene in the human genome containing 79 exons and comprised of over 2.5 Mb of genomic sequence [14, 15]. The full-length *DMD* mRNA is predominantly expressed in skeletal and cardiac muscle but is also found, in significantly smaller abundance, in the brain [16]. Dystrophin is a large 427-kDa protein product that plays a key role in anchoring the myofiber cytoskeleton to the extracellular matrix in skeletal muscles, with the carboxyl terminus binding to multiple proteins in the Dystrophin-associated protein complex (DAPC) and the amino terminus binding directly to F-actin in the actin cytoskeleton at the sarcolemma [17]. This direct connection provides mechanical stability to the entire myofiber, specifically the integrity of the sarcolemma, especially during muscle contraction [18, 19]. The structural role dystrophin plays to myofibers has shown to be protective, and that when dystrophin expression is altered or reduced, myofibers are particularly susceptible to eccentric contraction-induced injury and death [19-21]. In addition to providing mechanical support during eccentric contraction, dystrophin has shown to be a scaffold for the assembly of many proteins in the DAPC as well as other proteins that are critical for signaling, including nNOS, Calmodulin, and Grb2 [1, 22-24]. Evidence of this scaffolding role has given rise to investigating the role of Dystrophin as a key player in cell signaling, apart from its role as a structural and mechanical necessity, and it has been demonstrated that the absence of Dystrophin disrupts cell-signaling pathways, which contributes to myofiber death in Dystrophin-deficient muscle [24, 25].

Due to the importance of Dystrophin in maintenance of myofiber structural integrity, mechanical force generation, and signaling, it is worth noting that the network of functions affected by the loss of Dystrophin is monumental. This gives rise to the presumption that mutations in other members of this dystrophin-associated network may exist as disease modifiers in patients. Many of these network proteins have been investigated for their role as disease modifiers in pathology and several of them, specifically with regard to the pathological progression of both limb girdle and Duchenne muscular dystrophy, which are discussed in greater detail in chapter 2.

Dystrophin deficiency and disease progression in DMD patients

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy, affecting 1 in 5,000 live male births worldwide [26, 27]. DMD is an X-linked, progressive neuromuscular disorder resulting in muscle loss and reduction of mobility, physical function, and quality of life, resulting in the patients' loss of ambulation by their teen years [28]. Afflicted individuals experience significant respiratory and cardiac complications in the later stages of disease, and most do not live to see their 3rd decade of life [29]. DMD is caused by loss-of-function mutations in the X-linked *DYSTROPHIN (DMD)* gene and the majority of causative mutations are large-multi-exon deletions that typically result in a premature stop codon [30, 31]. As previously discussed, a lack of a functional Dystrophin protein in skeletal muscle results in reduced levels of other DAPC proteins which destabilizes the myofiber membrane [32]. In addition, membrane instability and leakage result in decreased signaling capability of the DAPC further exacerbating myofiber disintegration and death [15]. Over time with continued mechanical stress,

significant myofiber death leads to systemic muscle atrophy and loss of mechanical strength and control [33]. Because of the large structural and signaling network associated with Dystrophin and the DAPC, it is important to delineate primary and secondary disease processes in order to understand how mutations in *DMD* lead to the resulting pathological hallmarks of disease. These primary and secondary disease processes have been outlined and studied extensively, which has helped the field identify targetable pathways for pharmacological development [24, 34].

As an X-linked, genetic disorder, patient mutations are molecularly detectable from birth, however most DMD patients are not diagnosed until 4-5 years of age when their physical capabilities become noticeably different from their peers [35, 36]. Gower's sign, an alternative maneuver to stand up from sitting or lying on the ground, is a clinical hallmark of DMD patients at this age that demonstrates the onset of proximal muscle weakness. Difficulty climbing stairs and toe walking are also early signs of possible onset of proximal weakness. This weakness progresses incrementally throughout childhood years and the median age of ambulation loss in DMD boys is 12-14 years [28]. Multiple studies have shown that by adolescence, a decline in DMD patient physical health coincides with decline of social health, caregiver burnout, and difficulty managing sibling relationships in families with unaffected children [37-39]. At this time of ambulation loss, DMD symptoms including pain and fatigue become increasingly evident and difficult to manage for patients, families, and providers [40]. Self-awareness and understanding of the inevitable nature of disease course also begins to result in significant decreases in mental health status and a respective increase in diagnosis of depression and anxiety in DMD

patients throughout their adolescent years [40-42]. To date, the largest contributor to reported significant decreases in quality of life for DMD patients is the decline in their ambulatory status [43].

One of the most important secondary pathologies that can be used as a biomarker for disease outcome and prognosis of DMD patients is the establishment of fibrosis within the muscle tissue [44]. Endomysial fibrosis is the dysregulated deposition of extracellular matrix resulting from myofiber degeneration [45]. One of the clinical hallmarks of DMD pathology is inflammatory cytokine muscle infiltration due to continual myofiber necrosis and attempted muscle repair [45-47]. Upon myofiber necrosis and acute injury signaling in DMD pathology, neutrophils and macrophages infiltrate the area, which has been shown to be a dysregulated process compared to healthy muscle [47, 48]. This dysregulated inflammatory process results in significant impairment of regeneration and persistent myofiber damage, which ultimately leads to the replacement of regenerating myofibers with aberrant fibrotic tissue deposition [47-49]. With regard to patient prognosis, it has been clearly demonstrated that endomysial fibrosis is strongly correlated with poor motor outcomes in DMD patients [50, 51]. This evidence has led to advancements in therapeutic strategies that attempt to mitigate this inflammation-driven fibrotic response. Additionally, this is the reason why chronic anti-inflammatory agents in particular glucocorticoids are first-line, standard-of-care treatment options for DMD patients.

Current standards of care

There are no curative treatments for DMD. Corticosteroids have been the first-line standard-of-care treatment for DMD patients for several decades [41]. Corticosteroids are primarily prescribed to maintain muscle mass and strength as long as possible, as well alleviate the debilitating side effects of muscle wasting and other comorbidities [52]. However, there are detrimental side effects associated with corticosteroids such as weight gain, bone loss, and metabolic dysfunction in DMD patients that make long-term usage less than desirable. Other pharmacological and non-pharmacological treatments for DMD patients are prescribed to manage cardiac, respiratory, and gastrointestinal symptoms with system-specific care [36, 53, 54]. Recently, multiple strategies have been employed to correct the lack of dystrophin in DMD. One of these strategies is the administration of the gene therapy AAV-μ-dystrophin vector, a smaller, partially functional form of Dystrophin, to prolong the onset of disease severity. This experimental therapeutic has shown positive results in both mouse and dog models of DMD [55-57]. A phase 2 study utilizing AAV-μ-dystrophin as treatment has shown positive results demonstrating reduced fat infiltration in skeletal muscle, a clinical DMD biomarker that results in a progressively negative impact on strength maintenance in DMD patients [58]. Another recently-FDA-approved drug, Eteplirsen/Exondys 51, has shown efficacy in human clinical trials. This novel exon-skipping drug targets exon 51 in the *DMD* gene and allows the "skipping" of commonly mutated exons to form a partially functional, chimeric dystrophin protein that results in a more "Becker-like" muscle pathology [59]. Unfortunately, the specificity of exon-targeting of this drug significantly limits treatment potential of this DMD subset population to approximately 14% of all DMD patients [31]. In addition, long-term Exondys 51 treatment

in DMD patients demonstrated a mere 1-2% increase in dystrophin production compared to untreated controls [59]. The functional benefits of this limited increase in dystrophin production to patients and their quality of life are overall inconclusive to date [60, 61].

There has been a significant effort over the last decade for earlier molecular detection and intervention to extend the amount of time in which quality of life is preserved for DMD patients, although beneficial treatment options for the majority of DMD patients are minimal even if initiated early. Physiotherapy and corticosteroids have been the primary recommended treatments since the 1980s [62, 63]. Multiple studies have shown significant benefits to this combination therapy over time, including longer time to ambulation loss, preserved respiratory function, and the delayed need for surgery due to weakness-induced scoliosis [62, 64-66]. Although the most widely-accepted treatment option, long-term corticosteroid treatment is not without detrimental side effects. Weight gain, immune suppression, bone weakness, and abnormal blood pressure changes are some of the most common adverse corticosteroid side effects experienced by DMD patients [67]. For this reason, several studies have compared the use of various glucocorticoids, such as Prednisone and Deflazacort, and their respective patient's outcomes [65, 68-70]. To date, Deflazacort-treated patients demonstrate the most significant reduction in decline of measured functional abilities such as the 6-minute walk test, rise from supine, and 4-stair climb, however continue to experience negative side effects of long-term treatment [64, 70, 71]. Even with decreased decline of select functional abilities in young patients, long-term risks and benefits of corticosteroid treatment cannot be conclusively determined and patient response variability continues to be a significant challenge in patient care [62, 72,

73]. Collectively, overall inconclusive benefits of long-term treatment, inconsistent patient response to therapy, and variations in resulting provider care continue to demonstrate the significant need for alternative strategies to mitigate secondary pathological symptoms.

Gene regulation as a promising avenue for emerging therapeutics

As studies continue to demonstrate lack of efficacy with regards to directly targeting dystrophin production or muscle growth, it is becoming evident that targeting additional contributors to disease pathology is necessary [74, 75]. One important observation that has contributed to driving this area of research is the fact that most patients exhibit many of the same cardinal hallmarks of DMD, but some demonstrate significant variability in severity and onset of those hallmark symptoms in a disease that was originally thought to have a clear and linear progression [50]. This has given rise to understanding and investigating genetic modifiers of DMD and how naturally occurring gene variants may provide insight into the cause of variability of disease progression between patients. In chapter 2, I discuss several of these gene variants that have been identified as beneficial or detrimental to skeletal muscle function and outcomes in DMD patients and how they have given rise to the potential for novel therapies.

In addition to understanding genetic variants that are playing a role in modulating disease pathology, significant strides have been made in uncovering secondary mechanisms of disease progression. In recent years, identification of dysregulated genes, proteins, and signaling pathways has given rise to the deeper understanding of the

pathological mechanisms that cascade as a result of dystrophin loss, which has created novel entry points for experimental therapeutics. In chapter 3, I discuss the investigation of one of these experimental compounds, a SINE (Selective Inhibitor of Nuclear Export) compound, BIIB100 (previously known as KPT-350), and its beneficial effects mitigating inflammation-related pathology in a DMD mouse model with established disease onset.

One area of research that has gained significant traction and momentum over the last decade with regards to gene regulation is the field of microRNA biology. MicroRNAs (miRNAs) have become of significant interest in the study of gene regulation and secondary signaling pathways in a large number of genetic diseases, partially due to the ability of a single microRNA to target many unique gene transcripts. This creates significant versatility in experimentation and potential therapeutic use for disease. MicroRNAs are small non-coding RNAs, 18-25 nucleotides in length, known to be highly conserved, potent epigenetic regulatory inhibitors of gene expression [76]. MicroRNAs function by binding to complementary sequences on target mRNA transcripts resulting in translational silencing or transcript destabilization and degradation [77, 78]. MicroRNAs have been implicated in many diseases, including cancer, immunological diseases, and neurological diseases [79]. Regarding muscle, there has been extensive evidence showing that select myomiRs, muscle-specific miRNAs, play key roles in myogenic differentiation and development [80-82]. Since this discovery, these and other muscle-specific miRNAs have been coined “dystromiRs” due to their differential expression in dystrophic (dystrophin-deficient) disease that is strongly correlated with disease progression [83, 84]. This suggests there are likely a large number of target gene transcripts that are significantly

dysregulated because of the dysregulation of their target microRNA. As a result, this altered expression may be detrimentally contributing to disease pathology.

In chapter 4, I discuss one particular microRNA, miR-486, and the identification of its target transcripts using a novel method of direct mRNA target detection. MicroRNA-486, a classified "dystromiR," is highly enriched in skeletal and cardiac muscle and was identified by our lab and others as being significantly downregulated in DMD patient muscle [85-87]. It has also been demonstrated to be significantly induced during myoblast differentiation through the direct downregulation of transcription factor, Pax7 [88]. From this, we hypothesized there are likely other novel target transcripts of miR-486 that are overexpressed in DMD pathology as a direct result of a decrease in miR-486 expression in dystrophic muscle that may be detrimentally contributing to disease pathology. Chapter 4 reveals the culmination of my work in understanding skeletal muscle phenotype when miR-486 is ablated in order to determine the extent of the contribution of miR-486 downregulation to the exacerbation of DMD disease pathology and progression.

Modulation of Gene Expression through epigenetic and pharmacologic mechanisms can ameliorate DMD symptoms

Together, the culmination of my graduate work, as outlined in this dissertation, sought to investigate the overarching hypothesis that modulating gene expression through epigenetic and pharmacologic methods aimed at alleviating fibrosis and understanding muscle regeneration would reveal novel therapeutic entry points for the treatment of

Duchenne muscular dystrophy. Here, I performed a set of mechanistic and translational experiments to investigate the relationship between both anti-inflammatory compounds and miR-486 and their effects on dystrophic pathology through two specific aims:

***Specific Aim 1*: KPT-350 administration will ameliorate dystrophic pathologies by reducing inflammation and fibrosis in *mdx* mice.** Test the hypothesis that inhibiting nuclear export of cargo protein by XPO-1 through competitive inhibition by pharmacological compound KPT-350, will directly reduce inflammation and fibrosis symptoms in the dystrophin-deficient D2-*mdx* mouse model.

***Specific Aim 2*: MicroRNA-486 is an epigenetic driver of dystrophic muscle pathologies and disease progression.** Test the hypothesis that miR-486 is a significant epigenetic modifier of dystrophic muscle whose decrease in expression exacerbates dystrophic disease progression via upregulation of miR-486 muscle targets.

Overarching Hypothesis: By uncovering these dystrophin-independent mechanisms of disease progression, modulation, and therapeutic intervention, this work will contribute to propelling the field of alternative treatment strategies forward and help uncover novel methods of targeting secondary pathologies. Cumulatively, this dissertation will explore new avenues of potential therapeutics through the more in-depth understanding of several genetic and epigenetic modifiers of disease pathology addressed by the outlined aims above.

CHAPTER 2

GENETIC MODIFIERS OF DUCHENNE AND FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHIES

by

RYLIE M. HIGHTOWER & MATTHEW S. ALEXANDER

Muscle and Nerve.

Format adapted and errata corrected for dissertation

Abstract

Muscular dystrophy is defined as the progressive wasting of skeletal muscles that is caused by inherited or spontaneous genetic mutations. Next-generation sequencing (NGS) has greatly improved the accuracy and speed of diagnosis for different types of muscular dystrophy. Advancements in depth of coverage, convenience, and overall reduced cost, have led to the identification of genetic modifiers that are responsible for phenotypic variability in affected patients. These genetic modifiers have been postulated to explain key differences in disease phenotypes including age of loss of ambulation, steroid-responsiveness, and the presence or absence of cardiac defects in patients with the same form of muscular dystrophy. Here we review and highlight recent findings on genetic modifiers of Duchenne and Facioscapulohumeral muscular dystrophies based on animal and clinical studies. These genetic modifiers hold great promise to be developed into novel therapeutic targets for the treatment of muscular dystrophies.

Introduction

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy worldwide with estimates of incidence ranging from 1:3500 to more recent estimates of 1:5000 live male births [1, 2]. The genetic cause of DMD was identified in 1986 as pathogenic loss-of-function mutations in the dystrophin (*DMD*) gene that lead to insufficient, and sometimes undetectable, levels partially-functional, truncated dystrophin protein is produced, typically resulting in a milder clinical pathology [3-6]. DMD patients have severe, progressive skeletal muscle wasting and cardiac defects. Many DMD patients

lose ambulation by their first decade of life [7]. An interesting yet understudied aspect of DMD pathology is that approximately 20–25% of DMD boys develop significant cognitive issues that fall into the Autism spectrum disorder (ASD) scale [7, 8]. One explanation for this variation in DMD boys is the alteration in expression levels of neuronal dystrophin protein isoforms, although it remains unclear as to why some DMD boys develop cognitive impairment and others have normal intelligence [9-13]. As biochemical and molecular techniques for muscular dystrophy diagnostics improved, additional Dystrophin-interacting proteins were identified [14-16]. These proteins are part of a Dystrophin-associated protein complex (DAPC) that bridges the actin cytoskeleton with the extracellular matrix (ECM) [17]. With the advancement in genomic sequencing and coverage of the human genome, improved gene panels shifted muscular dystrophy diagnostic methods from predominantly pathology/histology-based to Sanger sequencing and later whole exome/genome sequencing (WES/WGS) [18-24].

The sequencing of increasingly larger numbers of exomes and genomes from healthy and diseased individuals permits large-scale genetic analyses of modifiers of diseases. The Exome Aggregation Consortium (ExAC) and 100,000 genomes project (UK) have banked large datasets of publically available genomic information [25, 26]. Genomic analyses of children with Mendelian diseases have revealed that genetic modifiers of diseases have an incomplete penetrance of disease symptoms and pathogenicity [27]. Indeed, many predicted loss-of-function (LoF) pathogenic mutations have been identified in healthy children and adults (“human knockouts”), suggesting an incomplete disease penetrance due to protective genetic modifiers [28, 29]. As genomic sequencing has

become more commonplace, the ability to analyze large amounts of data from different populations of healthy and diseased patients has become easier. Large scale databases and registries have been established to better share genomic and medical data among researchers studying muscle diseases [30, 31]. These studies have yielded copious amounts of data on the wide spectrum of phenotypic variation among patients with muscular dystrophies. In conjunction with novel animal model screening platforms, these studies have identified several genetic modifiers of different forms of muscular dystrophy. Whole genomic sequencing among inbred strains of mice, such as the MRL super-healing strain which blocks dystrophic muscle pathology when mated to dystrophin-deficient *mdx* mice, have yielded valuable genetic modifiers of disease pathology [32]. This review will focus on the identification and functional consequences of genetic modifiers in the two most prevalent forms of muscular dystrophy in humans: DMD and Facioscapulohumeral muscular dystrophy (FSHD). We also highlight current advancements in the clinic for these diseases and how genetic modifiers identified from both patient studies and animal models have led to novel therapeutic targets that may affect disease outcomes. These genetic modifiers hold the potential for ameliorating muscular dystrophies, and offer hope for patients with these debilitating diseases.

Genetic Modifiers of Duchenne Muscular Dystrophy

A large number of the Duchenne muscular dystrophy genetic modifiers have been identified in dystrophin-deficient animal models and more recently in genomic analyses of DMD patient cohorts (Table 1). Since the discovery of the first *mdx* mouse arising from a spontaneous mutation in a mouse colony from the United Kingdom, many additional

dystrophin-deficient vertebrate animal models have been generated and phenotypically evaluated [33-36]. *Mdx* mice display progressive skeletal muscle weakness but do not share the same reduced lifespan and early death as observed in DMD patients. This is thought to be due to increased expression of the dystrophin protein analog utrophin, which is expressed during embryonic myogenesis but is silenced during adult myogenesis [37, 38] (Figure 1). An alternative explanation for this phenomenon may be the increased presence in *mdx* muscle of revertant (dystrophin-positive) myofibers, resulting from undefined RNA-splicing or naturally occurring exon-skipping mechanisms [39-41]. Exogenous overexpression of utrophin or compounds that can activate utrophin expression in skeletal muscle remains a promising therapeutic strategy for DMD [42]. Transgenic mice that overexpress utrophin on a dystrophin-deficient background have been shown to rescue both the muscle pathology and motor deficits, thereby preventing muscular dystrophy disease progression [43]. Drug compounds that induce utrophin transcriptional activation in dystrophin-deficient skeletal muscle (SMT C1100; Ezutromid) are in current clinical trials in DMD boys [44]. Ezutromid induces transcription of utrophin mRNA in adult muscle where it is transcriptionally silenced, and thereby results in expression of utrophin protein to compensate for the lack of functional dystrophin protein [45].

Recent studies in *mdx* mice have also implicated additional genetic modifiers of dystrophin-deficiency that are found in inbred mouse strains that may also explain the genetic variation in both DMD mice and humans [46-48]. In humans, the presence of the R577X (rs1815739) null polymorphism in the α-ACTININ-3 (*ACTN3*) gene is associated with better overall muscle endurance [49] Male and female athletic sprinters show a higher

percentage of the 577R allele over non-sprinters and the general population as a whole [50]. In the context of dystrophin-deficiency, the loss of *Actn3* in mice blocks muscle wasting and degeneration in *mdx* mice [51]. A Golden Retriever muscular dystrophy (GRMD) dog was identified from a litter of inbred dogs and found to contain a splice acceptor variant resulting in the retention of intron 6 of the canine dystrophin gene [52, 53]. Interestingly, in a Brazilian colony of GRMD dogs, an "escaper" dog named Ringo was shown to have no detectable levels of dystrophin protein, but a milder clinical phenotype, normal reproductive capabilities, and a normal lifespan [54, 55]. Whole genome sequencing and RNA transcriptome profiling of the muscles from the Golden Retriever muscular dystrophy canine model and the escaper GRMD dog that had a milder phenotype revealed that increased levels of Jagged-1 (*Jag1*) improved dystrophic symptoms in both zebrafish and dog DMD models [56]. With improved genomic editing using clustered regularly interspaced short palindromic repeats (CRISPR) technologies, newer DMD animal models may yield additional genetic modifiers that may lessen or worsen disease symptoms. These dystrophin-deficient models may be beneficial in identifying naturally occurring strain variants that yield additional protective or harmful genetic modifiers of dystrophin deficiency.

Several genetic modifiers of muscular dystrophies have been identified from mouse crosses and are currently being explored for therapeutic targets. Expression levels of two important genetic modifiers (α-7 integrin and laminin-α2) were shown to be elevated in mdx mice and the GRMD dog models of DMD treated with prednisone [57]. Loss of α-7 integrin (*Itga7*) in mice on the *mdx* background worsens muscle weakness and increases

levels of fibrosis [58, 59]. Conversely, transgenic overexpression of α-7 integrin reduces muscle weakness and muscle force deficits in both *laminin-α2* and *mdx/utrn* double mutant mice [60, 61]. A small molecule SU9516 has been shown to rescue muscular dystrophy phenotypes in *mdx* mice due to its ability to transcriptionally activate the α-7 integrin promoter [62]. Other genetic modifiers such as biglycan (*BGN*), sarcospan (*SSPN*), and galectin-1 (*Lgals1*) overexpression have been demonstrated to act as a muscle membrane "glue" to increase muscle myofiber membrane stability and block muscle tearing in *mdx* mice [63-65] (Table 1). Biglycan functions to protect against muscle force loss by acting as a protein "anchor" to stabilize the muscle myofibers and their link to the extracellular matrix (ECM) via an interaction with the dystrophin-glycoprotein complex (DGC) [66, 67]. (Figure 1). In DMD, Biglycan expression levels are slightly elevated as it is capable of interacting and sequestering TGF-β in the ECM [68, 69]. Given the strong role of TGF-β for driving inflammation in DMD, TGF-β antagonists have been effective in blocking some of the dystrophic muscle symptoms associated with DMD [70, 71]. Direct intravenously-administered AAV-mediated overexpression of exogenous human biglycan (*BGN*) was recently shown to ameliorate muscle grip strength deficits and improve overall histology in dystrophin-deficient mice [72]. Similar to biglycan, the protein sarcospan (*SSPN*) also acts as a membrane "glue" to anchor the muscle membrane via an interaction with sarcoglycans, further stabilizing the dystrophin-glycoprotein complex [73]. Transgenic overexpression of sarcospan in *mdx* mice blocks muscle pathology via increasing the levels of utrophin-glycoprotein complex and activates AKT signaling as a compensatory mechanism for the lack of Dystrophin expression [74, 75]. Follow-up transgenic mouse studies demonstrated that high levels of sarcospan were sufficient to

rescue cardiac and pulmonary defects in *mdx* mice [76, 77]. Together these studies strongly support the notion that overexpression (either naturally-occurring genetic variants or artificially engineered) of selected membrane-associated proteins may be beneficial in blocking or ameliorating dystrophic pathology. As with all of these therapeutic compounds resulting from genetic modification or exogenous delivery, it is possible that human DMD patients may harbor protective or pathogenic variants in these genetic factors that may predict disease progression and outcomes. More whole genome studies of both healthy individuals and the DMD population are needed to test this hypothesis.

Genetic Modifiers of DMD Identified From Study of Affected Patient Populations

Case reports of DMD patients have revealed genetic modifiers that might explain differences in clinical severity. In one case report, a DMD patient presented a milder dystrophic clinical pathology, delayed loss of ambulation, and overall short stature due to a growth hormone (GH)-deficiency [78, 79]. A double-blinded controlled study of monozygotic twin DMD boys in which one was administered the growth hormone inhibitor manzindol versus a placebo revealed that the DMD twin receiving growth hormone inhibitor had greater mobility and reduced symptoms compared to his DMD twin on placebo [80]. However, a larger double-blinded studies revealed that manzindol-treated DMD boys showed no significant benefit over placebo-treated individuals [81]. DMD boys with growth hormone deficiency given growth hormone showed no acceleration of dystrophic disease progression in muscle or cardiopulmonary outputs [82, 83]. It has been postulated based on natural history longitudinal studies in DMD boys and dystrophin-deficient animal models that short stature in DMD boys is beneficial for delaying the loss

of ambulation [84]. Mouse models of growth hormone-deficiency such as the *Ames/Dwarf* and Growth hormone receptor (*GHR*) mutant mice have revealed that short stature/growth retardation slows aging via alteration of metabolic responses to insulin/IGF1 signaling [85, 86]. It is likely that the regulation of growth hormone may modulate DMD symptoms and influence phenotypic outcomes. The naturally occurring genetic variants resulting in short-stature/dwarfism in DMD patients have been reported to result in overall milder dystrophic pathology and better outcomes [87].

Due to the robust influence of inflammatory and fibrotic signaling in dystrophic muscle pathology, finding ways to alter these signaling pathways remains a promising strategy as more genetic modifiers have been identified. Recent whole exome analyses of the DMD population collected at various clinic sites have revealed additional genetic modifiers of DMD with ties to the TGFβ signaling pathway. Osteopontin is a secreted extracellular matrix protein thought to be essential for normal osteoclast formation and bone mineralization [88]. Osteopontin protein was shown to be strongly increased in expression levels in the DMD patient muscle biopsies and the serum of *mdx* mice [89]. The *OSTEOPONTIN* (*SPP1*) G allele polymorphism rs28357094 has been shown to be a predictive indicator of loss of ambulation and degree of muscle weakness in DMD patients [90, 91]. Indeed, *Osteopontin* null mice show altered immune signaling and reduced muscle fibrosis in the *mdx* mice via reducing M1 and M2a macrophage populations to the more pro-regenerative M2c macrophage population subset [92].

The *Latent TGFβ Binding Protein 4* (*LTBP4*) gene was initially identified from a screen of gamma-sarcoglycan (*Sgcg*) null mice for cytoprotective single nucleotide polymorphisms (SNPs) between two interbred mouse strains [93]. Studies in DMD patients revealed that four *LTBP4* SNPs (V194I, T787A, T820A, and T1140M; VTTT/IAAM haplotype) were predictive of the age at onset of loss of ambulation and of dilated cardiomyopathy (DCM) onset [94, 95] (Table 1). Follow-up studies in mice and cell culture demonstrated that the IAAM residues bound more latent TGFβ compared to the LTBP4 VTTT protein [96]. Thus, direct inhibition of LTBP4 via neutralizing antibodies or other RNA/protein antagonistic strategies may lead to a novel therapeutic means of blocking inflammation and myofiber breakdown in dystrophin-deficient skeletal muscles. Whole exome analyses of a large DMD cohort demonstrated that a SNP at rs1883832 of the *CD40* gene is predictive of the age at loss of ambulation and further implicated important roles in DMD for both the NFκB and TGFβ signaling pathways [97] (Table 1). CD40 is a cell surface receptor that is predominantly expressed on mature B cells as part of the Tumor Necrosis Factor (TNF) family of proteins [98]. CD40 is required for immunoglobulin class switching and *CD40* human polymorphisms have also been linked to increased susceptibility to immunological diseases [99, 100]. The immune system has long been shown to have an important role in normal muscle growth and regeneration, as well as the progression of muscular dystrophies [101, 102]. The *CD40* rs1883832 SNP is likely to have functional immunological consequences for the progression of DMD symptoms as the dystrophic muscle begins to deteriorate with the advancing of the disease.

Genetic Modifiers of Facioscapulohumeral Muscular Dystrophy

Facioscapulohumeral muscular dystrophy affects 1:8000 individuals worldwide and is caused by a contraction of D4Z4 expansion repeats in the *DUX4* pseudogene resulting in the production of the myotoxic DUX4 protein [103, 104]. DUX4 transcriptional activity is thought to activate pro-apoptotic signaling pathways, immune signaling regulators, and retrotransposons resulting in FSHD disease pathology [105]. One of the more perplexing aspects of FSHD is the presence of the contracted D4Z4 repeat in asymptomatic familial carriers whom show no disease pathology [106, 107]. It has been postulated that single nucleotide polymorphisms (SNPs) affecting DUX4 expression may allow for the permissive state of DUX4 transcriptional activation [108]. Interestingly, mutations in the chromatin-modifying gene structural maintenance of chromosomes flexible hinge domain containing 1 (*SMCHD1*) result in the relaxing of chromatin thereby permitting the expression of the DUX4 protein and affected patients are categorized as FSHD type 2 [109] (Figure 1). Additional FSHD epigenetic regulators including a long non-coding RNA (lncRNA) known as *DBE-T*, have been shown to regulate the expression of DUX4 in skeletal muscles via modulation of transcriptional regulatory complexes [110]. The most interesting aspect of FSHD genetics is that many individuals in FSHD families have been identified as having the FSHD permissive allele, but do not have the phenotypic muscle weakness and thus cannot be diagnosed with FSHD [111]. As more and more FSHD families and patients have their exomes and genomes fully-sequenced, additional genetic modifiers that may be consequential to disease progression and outcome will likely be identified.

Advances in DMD/FSHD Therapeutic Strategies and the Potential Use of Corrective Genome Editing Technologies

The identification of protective variants in human patients offers novel therapeutic entry points for the treatment of muscular dystrophies and muscle diseases in general. Improved adeno-associated viral (AAV) vectors for gene therapy and other biologics have been designed and delivered to patients to overexpress key cytoprotective muscle factors [112]. As next-generation sequencing (NGS) has become cheaper and more accurate, larger-scale genomic analyses of healthy and diseased populations are now routinely being conducted. These studies are important to demonstrate the presence of genetic variations in specific regions of the human genome, and the function of transcriptional regulatory regions. The ENCODE (Encyclopedia of DNA Elements) project lists additional layers of epigenetic (non-DNA-dependent) regulation of gene expression of many common disease-causing genes [113]. Non-coding RNAs (ncRNAs), RNA-splicing factors, and DNA methylation/acetylation factors among other epigenetic elements were shown to be important post-transcriptional regulators of human muscle diseases [114]. The cost of whole genome sequencing has dropped significantly, thus resulting in an increased consideration towards newborn screening of infants for diagnostic and epidemiological purposes [115, 116].

CRISPR genomic editing has emerged as a potential method for correcting small DNA mutations via targeting the specific mutation and replacing it with the corrected sequence [117]. Several recent studies have shown that AAV-mediated CRISPR genomic editing can correct the dystrophin exon 23 mutation in the *mdx* mouse [118-121]. Another recent study demonstrated up to 70% restoration of dystrophin protein expression in the

myogenic area of AAV-CRISPR mediated correction of the *mdx*^{4cv} (exon 53 mutation) mouse [122]. While these therapies may hold promise for direct correction of small *DMD* point mutations or deletions, the majority of DMD patients have large, multi-exon deletions for which CRISPR-mediated genomic corrective editing is not currently feasible [30]. Nevertheless, it may be possible to alter the genomic sequences of patients with muscular dystrophies and insert or remove the functionality of a therapeutic genetic modifier via CRISPR technology.

Recently, the exon-skipping compound eteplirsen (previously referred to as AVI-4658, Sarepta Therapeutics) was conditionally approved by the US Food and Drug Administration (FDA) for the treatment of DMD patients with genetically amenable dystrophin mutations [123]. Eteplirsen functions via bypassing the dystrophin mutation (skipping dystrophin exon 51) resulting in the production of a chimeric, partially-functional dystrophin protein that produces a Becker-like phenotype [124]. In a clinical trial of DMD boys amenable to skipping dystrophin exon 51, it was shown that DMD boys treated with eteplirsen retained ambulation longer than natural history controls with the same mutations [125]. Gene therapies that overexpress a truncated form of dystrophin (micro-dystrophin) have shown efficacy in DMD animal models and are in current clinical trials for DMD [126-128]. The naturally-produced myokine myostatin (also called GDF8) has been shown to be a potent negative regulator of muscle mass in mammals [129]. Subsequently, naturally occurring loss-of-function genetic mutations in the myostatin gene of Belgian Blue cattle was demonstrated to be the direct cause of the doubling of their muscle mass via muscle hypertrophy [130]. Later, human case studies showed that myostatin genetic

variants were responsible for the large, hypertrophic muscles in a young German boy [131]. Genetic loss-of-function mutations of myostatin have been shown to induce muscle hypertrophy, and protect against muscle force deficits in *mdx* mice [132]. Pharmacological blockade of myostatin or the myostatin receptor Activin IIB (ACVRIIB) similarly demonstrated a physiological benefit in protecting against muscle force loss in dystrophic mice [133, 134]. A recent clinical trial involving a soluble form of the human activin receptor type IIB (ACE-031; produced by Acceleron Pharma) given to ambulatory DMD boys showed some benefits over placebo; although the trial was halted due to unforeseen side effects [135]. A similar myostatin/TGF-β pathway inhibitory compound (ACE-081) is currently recruiting for a Phase 2 trial for FSHD patients to alleviate symptoms of muscle weakness (Clinical Trials Identifier: NCT02927080). While these compounds may ameliorate muscle weakness and other dystrophic symptoms, they do not correct the underlying cause of the muscle disease. Many additional strategies towards the treatment of DMD and FSHD are currently under development or in clinical trials and many of these therapeutic targets were originally identified from genomic modifiers of muscular dystrophies [136]. It is likely that no single treatment for DMD or FSHD will fully cure either disease, but combination treatments targeting multiple factors including those identified as genetic modifiers may improve muscle symptoms and extend the lifespans of affected individuals.

As genomic sequencing data from muscular dystrophies becomes more commonplace, the opportunity and ability to identify protective and pathogenic genetic modifiers of muscular dystrophies will increase dramatically. These novel genetic

modifiers may hold biological clues as to why some individuals do not display muscular dystrophy symptoms despite having pathogenic variants. The exciting prospect of exploring these genetic modifiers as therapeutic agents for drug development may lead to novel treatments for treating these debilitating diseases.

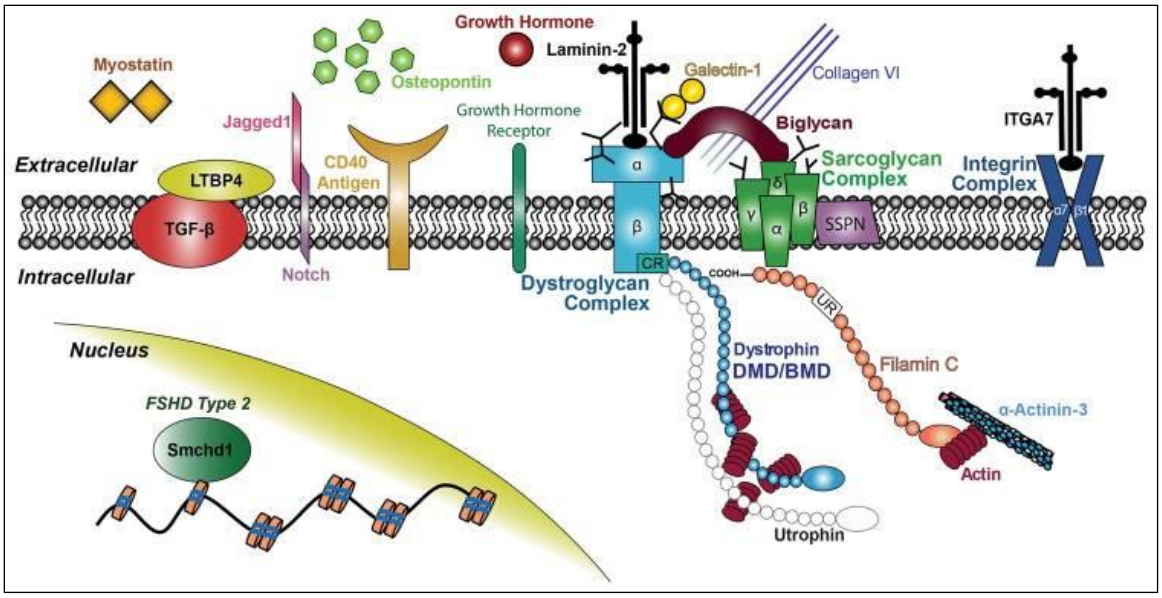

Figure 1. Schematic of significant genetic modifiers of Duchenne and Facioscapulohumeral muscular dystrophies and their sub-cellular localization in skeletal muscle. Dystrophin and the dystrophin-associated protein complex (DAPC) have an important functional role in the transmission of intercellular force to the extracellular matrix (ECM). Of note, Smchd1 is a chromatin-modifier protein that is believed to allow a permissive state for transcriptional activation of the pathogenic DUX4 transcription factor in FSHD Type 2.

Gene Name	Abbreviation	Normal Function	Disease Modification	Modification-Associated Phenotype
Genes with Identified Mutation or Significant Variant Contributing to Pathological Variation				
α-Actinin-3	*ACTN3*	Skeletal muscle-specific actin binding protein	Homozygosity for SNP R577X in DMD patients	Reduced susceptibility to stretch-induced damage, enhanced fatigue recovery, and slowed dystrophic progression
Annexin A6	*ANXA6*	Calcium-dependent membrane and phospholipid binding protein	Exon 11 SNP rs26961431 creates a truncated Annexin A6 protein in mice	ANXA6 truncation results in increased muscle wasting and degeneration in *Sgcg* mutant mice
CD40 Antigen	*CD40*	Transmembrane receptor of the tumor necrosis factor receptor family; involved in T helper cell polarization	Minor allele rs1883832 in the 5′ UTR of CD40 associated with diminished CD40 expression	Differential factor for age of loss of ambulation in DMD patients
Growth Hormone/Growth Hormone Receptor	*GH/GHR*	Regulates normal growth and IGF1 signaling in muscle	Growth hormone-deficiency comorbid with DMD in selected case reports	Case reports of DMD patients with dwarfism/short stature having delayed loss of ambulation
Latent TGFβ Binding Protein 4	*LTBP4*	Regulator of TGFβ and TGFβ signaling	1. IAAM haplotype in DMD patients	1. Reduced inflammation and myofiber breakdown, increased time to loss of ambulation
			2. Recessive T allele at rs10880 and IAAM haplotype in DMD patients	2. Protective against dilated cardiomyopathy (DCM)
Structural maintenance of chromosomes flexible hinge domain containing 1	*SMCHD1*	Chromatin modification; mediates CpG methylation and chromatin relaxation	In FSHD, allelic variants in SMCHD1 result in hypomethylation and inadvertent expression of DUX4; subsequent diagnosis of FSHD2, genotypically distinct from FSHD1.	FSHD type 2 allows for permissive expression of DUX4

Table 1. Genes with Polymorphic Significance in the Clinical Manifestation of Muscular Dystrophies.

Gene Name	Abbreviation	Normal Function	Disease Modification	Modification-Associated Phenotype
Genes with Protein Expression Differences Contributing to Pathological Variation				
α-7 Integrin	*ITGA7*	Mediates cell-matrix interactions; receptor for basement membrane protein laminin-1	Increased expression in *mdx* mice via transgenic cassette or SU9516 compound	Reduced membrane injury, increased muscle fiber size, decreased levels of fibrosis, extended lifespan
Biglycan	*BGN*	Small leucine-rich repeat proteoglycan; ubiquitous structural ECM protein	Increased expression of Biglycan corresponds with stabilization of Utrophin	Decreased diseased muscle pathology
Galectin-1	*LGALS1*	Laminin-binding protein; mediates cell-matrix interactions	Increased expression in GRMD dogs	Enhanced muscle repair, reduced immune response and apoptosis
Jagged1	*JAG1*	Ligand of the Notch receptor	Increased expression in *mdx* mice	Improved dystrophic histology and physiology
Myostatin	*GDF8*	TGF-β superfamily member	Decreased expression in animals and humans	Doubling of muscle mass via myofiber hypertrophy
Osteopontin	*SPP1*	Diverse matricellular protein	1. Decreased expression in mice and humans	1. Decreased immune signaling and fibrosis, increased muscle regeneration
			2. Dominant G allele at SPP1 rs28357094	2. Protective against dilated cardiomyopathy (DCM)
Sarcospan	*SSPN*	A member of the DAPC, which provides a structural anchor of the myocyte cytoskeleton to the ECM	Transgenic overexpression in *mdx* mice improves muscle function and force.	Increased myofiber membrane stability and reduces muscle tearing
Utrophin	*UTRN*	Autosomal homolog of DMD gene; maintains sarcolemmal integrity during embryonic myogenesis	Overexpression of Utrophin in mice or with Ezutromid compound	Increased lifespan, decreased diseased muscle pathology

Table 1, continued. Genes with Polymorphic Significance in the Clinical Manifestation of Muscular Dystrophies.

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CHAPTER 3

THE SINE COMPOUND KPT-350 BLOCKS DYSTROPHIC PATHOLOGIES IN DMD ZEBRAFISH AND MICE

by

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked muscle wasting disease that is caused by the loss of functional dystrophin protein in cardiac and skeletal muscles. DMD patient muscles become weakened, leading to eventual myofiber breakdown and replacement with fibrotic and adipose tissues. Inflammation drives the pathogenic processes through releasing inflammatory cytokines and other factors that promote skeletal muscle degeneration and contributing to the loss of motor function. Selective inhibitors of nuclear export (SINEs) are a class of compounds that function by inhibiting the nuclear export protein exportin 1 (XPO1). The XPO1 protein is an important regulator of key inflammatory and neurological factors that drive inflammation and neurotoxicity in various neurological and neuromuscular diseases. Here, we demonstrate that SINE compound KPT-350 can ameliorate dystrophic-associated pathologies in the muscles of DMD models of zebrafish and mice. Thus, SINE compounds are a promising novel strategy for blocking dystrophic symptoms and could be used in combinatorial treatments for DMD.

Introduction

Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disease caused by mutations in the *DYSTROPHIN* gene resulting in the lack of production of functional dystrophin protein [1, 2]. DMD affects approximately 1:5,000 live male births worldwide, making it the most common childhood form of muscular dystrophy. Patients with DMD gradually develop muscle weakness, postural instability, cardiac arrhythmias,

respiratory weakness, and loss of ambulation after the first decade of life. The myofiber damage attributed to membrane instability also causes chronic inflammatory responses in dystrophic muscle [3-6]. This inflammatory response is characterized by the infiltration of immune cells that produce inflammatory and fibrotic factors that contribute to the progression of DMD [7]. Isolated DMD patient muscle cells have been shown to express higher levels of collagen and extracellular matrix (ECM) factors compared with heathy muscle cells [8-10]. This progressive increase in endomysial fibrosis is significantly correlated with poor motor outcome and loss of ambulation in DMD patients [11]. There is no cure for DMD, and corticosteroids are the current primary standard-of-care treatment [12, 13]. The functional preservation seen in patients in response to corticosteroid therapy is thought to be a result of their immunosuppressive properties, which reduces the detrimental fibrotic pathology associated with dystrophin deficiency [14-16]. Anti-fibrotic and anti-inflammatory compounds or biologics that target key drivers of inflammation in DMD, such as interleukin-6 (IL-6), transforming growth factor β (TGF-β), tumor necrosis factor alpha (TNF-α), the nuclear factor κB (NF-κB) signaling pathways, or regulatory T cells, have shown therapeutic efficacy in reducing dystrophic symptoms in dystrophin-deficient mice[17, 18]. Regulatory T cells have been shown to block and/or ameliorate dystrophic symptoms in mouse and canine DMD models [7, 17, 19-26].

The nuclear pore functions as a key regulator of intracellular molecules such as proteins, RNA molecules, and ions [27, 28]. The nuclear pore consists of various regulatory proteins called nucleoporins that together form the nuclear pore complex (NPC) [28]. Many of these nucleoporins have direct roles in regulating the transport of key

proteins and RNA macromolecules from the nucleus and the cytoplasm. The NPC is an important regulator of key protein cargos involved in cellular differentiation, immune response, apoptosis, and overall transcriptional and translational machinery [29]. The nuclear protein exportin 1 (XPO1; also called CRM1) has an essential role in protein trafficking that functions as a nucleocytoplasmic regulator of key transcription factors [30, 31]. Recently, direct pharmacological inhibition of XPO1 has been shown to be effective in several types of cancers [32-35]. XPO1 also has been demonstrated to interact with polyglutamine (polyQ) proteins that are produced from expanded nucleotide repeats in disorders such as Huntington's [36, 37]. Some amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) patients also have expansion repeats in the *ATXN2* gene that result in the production of polyQ protein [38]. They have been shown to exhibit defective nuclear export activity, likely regulated by XPO1 function [39-42]. A series of pharmacological inhibitors of XPO1 nuclear export function have been developed initially for cancer therapies[43]. However, recent findings have demonstrated that neurological and neuromuscular disorders containing pathogenic expansion repeats affect XPO1 activity; thus XPO1 could serve as a pharmacological target [44, 45].

KPT-350 is a selective inhibitor of nuclear export (SINE), one member of a series of compounds originally designed by Karyopharm Therapeutics (recently acquired by Biogen and renamed BIIB100) to inhibit XPO1 nuclear export function. Recently, KPT-350 has been shown to block Huntington's disease pathologies via correcting nucleocytoplasmic transport and preventing mutant HTT protein from aggregating around the NPC [44]. KPT-350 and other SINE compounds block inflammation and neurotoxicity

via the regulation of key transcription factors shown to drive these processes such as NF-κB, inhibitor of κB (IκB), FOXO1, FOXP1, and STAT1. Based on these anti-fibrotic and anti-inflammatory effects, we postulated that treatment with KPT-350 might have a beneficial effect in preventing dystrophic muscle pathology in DMD zebrafish and mice by blocking fibrosis and inflammation in their skeletal muscles. We treated dystrophic zebrafish in short- and long-term experiments via immersion of DMD larvae in KPT-350 (or vehicle control) and evaluated their outcomes. In addition, we performed an expanded evaluation of KPT-350 in the DBA2J-*mdx* (D2-*mdx*) DMD mouse model with an emphasis on skeletal muscle histology, serum biomarkers, and overall functional outcomes. These studies were conducted for the pre-clinical evaluation of KPT-350 in two relevant vertebrate DMD models for overall therapeutic efficacy in blocking dystrophic symptoms.

Results

KPT-350 Ameliorates Dystrophic Pathologies and Extends the Lifespan of Dystrophin Mutant Zebrafish

Sapje zebrafish mutants are a well-established model for drug screening and evaluation of muscle pathologies. The *sapje dmd*^{ta222a} mutant zebrafish have myofiber detachment from the sarcolemmal membrane, impaired muscle force production, reduced motility, a decreased lifespan with 95% death by 10 days post fertilization (dpf), and an overall pathology that closely resembles the human disease. Zebrafish are also a powerful model for identifying corrective neuromuscular compounds due to their ability to rapidly uptake small molecules through their gills and skin. Dorsal skeletal muscle birefringence, which assesses muscle fiber integrity via polarized light, is a useful tool for analyzing muscle pathologies as observed in *sapje* homozygous mutants and for rapidly evaluating

the muscle quality of early (4–7 dpf) zebrafish larvae. To test whether KPT-350 might block or ameliorate dystrophic symptoms, we first performed short-term testing in the *sapje* mutant zebrafish. We mated *sapje* heterozygotes and immersed 1 dpf embryos obtained from timed matings in fish water containing either vehicle (0.01% DMSO/fish water), KPT-350 (1, 2.5, or 5 μM), or 5 μM aminophylline for 4 consecutive days while changing the water every other day in a double-blinded (genotype and compound) fashion (Figure 1A). At 5 dpf, the zebrafish larvae were evaluated for muscle birefringence, and we found a reduction in the overall number of the percent affected fish from the expected 25% Mendelian ratio in the KPT-350-treated cohorts, but not in the untreated or vehicle controls (Figure 1B). We also observed a decrease in the percentage of affected fish in the 5 μM aminophylline-treated positive control cohort similar to the ratios of what we previously observed[46]. Evaluation of dorsal muscle birefringence revealed that 1 μM KPT-350-treated fish showed no quantifiable muscle wasting phenotype (Figure 1C). Genotyping of the cohorts revealed that drug-treated cohorts had more *sapje* mutant fish compared with vehicle control. These data suggest that both 1 and 2.5 μM doses of KPT-350 were sufficient to block the dystrophic phenotype in the *sapje* mutant fish in the early stages of dystrophic muscle pathologies.

We next tested whether a long-term KPT-350 treatment drug testing regimen (from 4 to 21 dpf) could minimize dystrophic dorsal muscle pathology (Figure 2A). We used birefringence to screen 4 dpf *sapje* larvae for homozygote mutants (poor muscle fiber integrity) and immersed mutants in water containing either vehicle, KPT-350 (0.1 or 1 μM), or 5 μM aminophylline, previously shown to extend *sapje* mutant survival, as a

positive control[46]. Unaffected wild-type (WT) were assessed as a frame of reference for survival. We observed a significant extension of lifespan with 1 μM KPT-350 treatment (median = 18 days) compared with vehicle treatment (median = 16 days; log rank test) in affected *sapje* homozygote mutants (Figure 2B). These findings in *sapje* mutant zebrafish indicate that KPT-350 treatment may both block and ameliorate dystrophic symptoms and pathologies in a disease-relevant vertebrate model.

Oral Delivery of KPT-350 Blocks Dystrophic Skeletal Muscle Degeneration and Fibrotic Pathologies in D2-mdx Mice

We next pursued expansion of our zebrafish KPT-350 findings in a mouse DMD model. The *mdx* (*DBA/2J*) strain, referred to here as D2-*mdx*, contains the mouse dystrophin exon 23 C>T transition, resulting in a loss of the Dp427m dystrophin protein isoform [47, 48]. This strain exhibits a more severe skeletal muscle phenotype when directly compared with the more commonly used *C57BL6/10ScSnJ mdx* strain, thus making it a more physiologically relevant DMD model for pre-clinical therapeutic evaluation[49, 50]. We evaluated the therapeutic ability of oral KPT-350 given to D2-*mdx* and control WT (*DBA/2J*) mice in the form of peanut butter pellets to ameliorate the dystrophic pathology and improve muscle function. WT and D2-*mdx* mice were treated with either vehicle (0.6% Plasdone PVP K-29/32, 0.6% Poloxamer Pluronic F-68) or KPT-350 (5 mg/kg body weight) three times a week for 8 weeks beginning at 8 weeks of age (Figure 3A). Immediately following euthanasia of animals, muscles were extracted and used for histological and molecular analyses. Histological analysis revealed that KPT-350 treatment significantly reduced dystrophic pathology in D2-*mdx* muscle. First, cross-sectional area (CSA) of the tibialis anterior (TA) was measured, as well as the frequency

of either small (0–999 μm^2), moderate (1,000–1,799 μm^2), or large fibers (>1,800 μm^2) in all cohorts (Figures 3B and 3C). In both WT and D2-*mdx* strains, KPT-350 treatment significantly increased the frequency of large-size fibers with a concomitant decrease in small-size fibers (Figure 3C). This suggests that KPT-350 treatment may be preventing muscle atrophy or degradation in D2-*mdx* muscle. In addition, KPT-350-treated D2-*mdx* muscle had significantly fewer centralized myonuclei and smaller degenerative lesions compared with vehicle-treated D2-*mdx* mice controls (Figures 3D and 3E). Taken together, these data indicate that KPT-350 treatment improves the overall dystrophic phenotype because these treated D2-*mdx* mice presented significantly less muscle atrophy, centralized myonuclei, and overall degeneration, all hallmark characteristics of muscular dystrophy.

KPT-350 Treatment Improves Overall Locomotion and Overall Activity in D2-*mdx* Mice

Open field activity tracking is a validated way of assessing basal ambulatory function in mouse models of skeletal muscle disease [51-53]. Preservation of ambulation is an important component of therapeutic treatment in patients with DMD; therefore, assessing locomotor activity in D2-*mdx* mice can provide insight into preservation of physical function after treatment. In order to determine the functional significance of short-term KPT-350 treatment, we assessed overall locomotor activity and movement velocity in both drug-treated and vehicle-treated D2-*mdx* mice. KPT-350-treated D2-*mdx* mice demonstrated no significant difference in total distance traveled from vehicle-treated D2-*mdx* mice (Figures 4A and 4B). However, KPT-350-treated D2-*mdx* mice showed a significant increase in overall movement velocity compared with vehicle-treated D2-

mdx mice (Figure 4C). This demonstrates that KPT-350 prevents rapid decline of overall locomotor activity, and that KPT-350 treatment results in increased locomotor velocity in D2-*mdx* mice.

KPT-350 Inhibits Inflammatory Cytokines and Improves DMD Serum Biomarkers in D2-*mdx* Mice

Serum cytokines are a valid biomarker for dystrophin-deficient skeletal muscle in dystrophic mice, dogs, and human patient sample s[54-56]. We analyzed whole serum taken from each of the four experimental cohorts via a mouse cytokine array panel to determine whether known biomarkers of dystrophin deficiency were altered in the KPT-350-treated D2-*mdx* mice. Vehicle-treated D2-*mdx* mice showed a significant upregulation of key pro-inflammatory and apoptosis-related cytokines, such as TNF-α, interferon gamma (IFNγ), chemokine (C-X-C motif) ligand 16 (CXCL16), osteopontin (Spp1), IL-1α, IL-1β, IL-2, IL-6, and CD95L, compared with vehicle-treated WT control serum (Figures 5A–5I)[22, 55, 57-59]. Whereas KPT-350 treatment did not induce any changes in WT mice, KPT-350 treatment significantly reduced all of these inflammatory cytokines in D2-*mdx* mice compared with vehicle-treated D2-*mdx* mice (Figures 5A–5I). Moreover, levels of these cytokines were not only reduced, but were restored to WT levels. These cytokines have been established to be pro-inflammatory and detrimental in dystrophic pathology [22, 59, 60]. In addition, we measured IL-10 and IL-15 that have been previously reported to ameliorate the severity of muscular dystrophy in *mdx* mice [61-63]. These cytokines were significantly lowered in D2-*mdx* mice compared with WT mice serum (Figures 5J and 5K). However, KPT-350 treatment significantly increased IL-10 and IL-15 serum levels in D2-*mdx* mice (Figures 5J and 5K). These results demonstrate that

treatment of dystrophin-deficient mice with oral KPT-350 blocks key inflammatory cytokines known to exacerbate dystrophic pathologies, while inducing anti-inflammatory cytokines such as IL-10.

KPT-350 Increases Macrophage Populations in D2-*mdx* Skeletal Muscle

Inflammation-induced muscle damage is a major contributor to the progression and severity of dystrophic pathology. A key component to the inflammatory response is the recruitment of eosinophils and macrophages to damaged muscle. M1-like macrophages are associated with the acute phase of muscle damage. They produce pro-inflammatory cytokines, such as TNF-α, IFNγ, and IL-6, as well as other potentially cytotoxic signals such as nitric oxide (NO) and reactive oxygen species (ROS).[64] In contrast, M2-like macrophages are involved in muscle repair and regeneration, and inhibit the cytotoxic activity of M1-like macrophages in a IL-10-dependent manner [64]. Thus, we used flow cytometry to determine the effect of KPT-350 on these immune cell populations in normal and dystrophic muscle. Single-cell suspensions of TA muscles were interrogated by flow cytometry, and t-distributed stochastic neighbor embedding (TSNE) plots were generated to show the distribution of eosinophils, M1-like macrophages, and M2-like macrophages (Figure 6A). KPT-350 treatment did not affect the immune response in WT mice in any parameters measured (Figures 6B–6G). In D2-*mdx* mice, KPT-350-treated mice had significantly higher frequencies of macrophages compared with vehicle-treated D2-*mdx* mice; however, treatment did not have a significant effect on muscle eosinophilia (Figures 6B–6H). Interestingly, programmed death-ligand 1 (PD-L1), a negative costimulatory molecule that suppresses inflammation, was increased on M1-like

macrophages, and to a lesser extent on M2-like macrophages isolated from KPT-350-treated versus vehicle-treated D2-*mdx* mice (Figures 6F and 6G). In addition, immunohistochemical staining with anti-F4/80 antibodies of TA muscles demonstrated macrophage infiltration in D2-*mdx* muscles (Figure 6H). Collectively, these data show that despite an overall increase in the proportion of macrophages, KPT-350 promotes a functional reprogramming of muscle inflammation, reflected by increased expression of anti-inflammatory cytokines, PD-L1 expression on macrophages, and decreased expression of pro-inflammatory cytokines.

Discussion

Our findings indicated that KPT-350 treatment improved muscle histology and serum biomarkers, and blocked overall dystrophic disease progression in DMD zebrafish and mice, and thus further validates the use of SINE compounds to treat DMD symptoms. The lead SINE compound KPT-330/Selinexor is being evaluated in multiple phase 2 and 3 clinical trials in patients with relapsed or refractory hematological and solid tumor malignancies [65]. These compounds have been shown to block inflammation and reduce neurotoxicity in a rat model of traumatic brain injury (TBI) via regulation of key transcription factors such as FOXP1, FOXO1, and NF-κB[66]. In a mouse model of Huntington's disease, KPT-350 treatment reduced nuclear aggregations of protein plaques generated by aberrant transcription and translation of polyQ sequences [44]. Our findings now demonstrate the therapeutic benefit of the SINE compound, KPT-350, which is known to present the greatest brain penetration among the series of SINE compounds, to block inflammation and overall dystrophic pathologies in DMD zebrafish and mouse models.

In the DMD model of zebrafish, our studies demonstrated the therapeutic efficacy of KPT-350 by the assessment of mutant *sapje* fish and their muscle architecture. KPT-350-treated *sapje* fish had increased muscle integrity and organization as visualized by birefringence and myosin heavy chain (MyHC) staining, respectively. Importantly, long-term dosing with KPT-350 extended the lifespan of the mutant *sapje* fish. These studies provided critical insight into the therapeutic potential of KPT-350 in a DMD animal model.

We next investigated the therapeutic effect of KPT-350 in the mouse DMD model, D2-*mdx* mice. After 8 weeks of treatment, D2-*mdx* mice improved in multiple areas. Histological analyses revealed that KPT-350-treated D2-*mdx* mice had significantly fewer centralized myonuclei and fewer inflammatory areas. Moreover, KPT-350-treated D2-*mdx* showed a higher frequency of large-size fibers with a concomitant decrease in small-size fibers. One potential mechanism for this observation might be that KPT-350 treatment is preventing the degradation of the large type II fibers, which have been shown to be the first to be preferentially degraded in untreated DMD muscle. An alternate explanation is that KPT-350 treatment promotes hypertrophic pathways, because we observed changes in WT muscle in addition to D2-*mdx* muscle. KPT-350 treatment blocks the nuclear export activity of XPO1, which is known to be responsible for the exportation of more than 200 proteins. Some of these are transcription factors, such as FOXO1, and AKT/mechanistic target of rapamycin (mTOR)-related proteins that could influence myofiber metabolism and fiber size[67]. In addition to improved dystrophic muscle histological markers, we saw an increase in KPT-350-treated D2-*mdx* mice activity compared with vehicle-treated

counterparts. Total distance traveled and velocity were increased after KPT-350 treatment, demonstrating that these histological changes are translated into functional changes of the muscle.

In order to elucidate possible mechanisms of KPT-350 actions, we performed a cytokine array for well-known dystrophic cytokines. Most of these cytokines are pro-inflammatory and known to be upregulated in DMD mice and patients [60, 68-71]. Our findings revealed that although there was no difference in serum levels of any cytokines tested after KPT-350 treatment in WT mice, there were dramatic changes in KPT-350-treated D2-*mdx* mice. KPT-350 treatment of D2-*mdx* mice reduced expression levels of multiple pro-inflammatory cytokines to WT control levels. Importantly, previous studies demonstrated that treating dystrophic mice with neutralizing antibodies for some of these cytokines, such as TNF-α and IL-6, blocks the progression of skeletal muscle degeneration [22, 57, 58, 60]. Another study demonstrated that ablation of osteopontin improves dystrophic muscle pathologies and promotes muscle regeneration [55, 59]. Our studies are consistent with these reported results because we also found amelioration of muscle disease pathology accompanied by a marked decrease of these cytokines.

Lastly, we investigated the modulation of innate immune responses in dystrophic muscle. KPT-350 treatment increased the proportion of macrophages in D2-*mdx* mice compared with vehicle-treated D2-*mdx* mice. We further analyzed M1-like and M2-like macrophages that are associated with acute pro-inflammatory responses and muscle regeneration, respectively, and found that both populations were increased. Importantly,

the potentially detrimental effect of expanded M1-like macrophages is likely negated by their increased expression of PD-L1, which inhibits the activation of immune responses and inflammation. Although it is clear that KPT-350 treatment is altering macrophage activation, the exact functional outcome of this regulation is unknown. Some reports suggest that the balance between M1 and M2 macrophages is tightly regulated through macrophage-intrinsic interactions or extrinsically by regulatory T cells [7, 17, 18, 72]. These findings, taken together with the reciprocal regulation of pro-inflammatory and anti-inflammatory cytokines and the increased myofiber CSA, suggest that KPT-350 increases the overall regenerative potential of muscle macrophages. This hypothesis is supported by the improved overall histology observed in KPT-350-treated D2-*mdx* skeletal muscles. However, further investigation is warranted into elucidating the specific roles of inflammation and macrophages to understand the interplay between dystrophic muscle regeneration and the immune response.

In conclusion, this study demonstrates the novel use of the SINE compound KPT-350 in blocking disease pathology in DMD zebrafish and mouse models. KPT-350 treatment decreased muscle degeneration markers, reduced inflammatory cytokines, and improved overall viability and activity of the animals. Additional experiments into long-term efficacy and systemic effects of SINE compounds in dystrophic mice will be informative toward the advancement of SINE compounds for DMD patients. This study directly contributes to the growing body of knowledge that indicates that reduction of pathologic inflammation and fibrosis can reduce muscle disease symptoms in DMD. Steroid-based anti-inflammatory therapeutics, such as deflazacort and prednisone, have

shown therapeutic efficacy in improving muscle strength and are now considered standard-of-care treatment options for DMD patients[73, 74]. Another steroidal compound in clinical trials, vamorolone, has been shown to decrease inflammation while improving symptoms of cardiomyopathy [75]. With this growing number of therapeutic compounds targeted at preserving muscle function and ambulation, KPT-350 may be a potential candidate considered for pharmacological combinatorial therapy in DMD patients.

Materials and Methods

Zebrafish

WT (AB strain) and *sapje* (*dmd*^{ta222a} backcrossed onto the AB background over 10 generations) were maintained under standard housing and feeding conditions at the University of Alabama at Birmingham (UAB) Aquatics facility under pathogen-free conditions under the protocol number 20320[76]. All adult fish were fed a standard diet of *Artemia salina* (brine shrimp) three times per day under a 14-hour on, 10-hour off light cycle in 3-L tanks with a density of no more than 20 fish per tank. WT and *sapje* mutants were genotyped by PCR as previously described [77].

KPT-350 Zebrafish Dosing Experiments

For short-term dosing experiments, adult *sapje* heterozygotes were mated and their embryos were collected at 1 dpf. Embryos were then randomly sorted into pools of 25 and placed in six-well plates containing either vehicle (0.01% DMSO/fish water), 0.1 μM KPT-350, 1 μM KPT-350, 5 μM KPT-350, or 5 μM aminophylline (Catalog [Cat.] #A1755; Sigma-Aldrich, St. Louis, MO, USA) dissolved in fish water containing 0.01% DMSO

(Cat. #41639; Sigma-Aldrich). A separate initial cohort of 5 μM KPT-350/fish water was found to be toxic to a small percentage of developing fish embryos. For long-term experiments, 4 dpf affected (*sapje*) homozygotes were separated into cohorts of 25 (n = 25 fish per treatment cohort) and given either 0.1 μM KPT-350, 1 μM KPT-350, or 5 μM of aminophylline dissolved in fish water containing 0.01% DMSO. These doses were selected based on comparable doses given for larger juvenile zebrafish evaluated in isolated 1.8-L tanks[46]. Zebrafish genotypes were confirmed at 21 dpf after tricaine (Cat. #A5040; Sigma-Aldrich) euthanization through Sanger sequencing for the A>T transversion at the zebrafish *dystrophin* exon 4 locus as previously described[46]. All experiments were performed in a double-blinded (genotype and drug cohorts) fashion to the experimenter, and all experiments were repeated three times (n = 3 independent experimental replicates). All of the KPT-350 drug used in these experiments was synthesized and provided by Karyopharm Therapeutics (Newton, MA, USA).

Mice

WT (*DBA/2J* strain; stock number 000671) and *mdx* (*DBA/2J* strain; stock number 013141) mice were originally purchased from Jackson Labs (Bar Harbor, ME, USA) and maintained under standard housing and feeding conditions with the UAB Animal Resources Facility under pathogen-free conditions under the animal protocol number 20232. All mice were fed a standard diet of mouse chow (Teklad Global Rodent Diet; 16% protein; Cat. #206S; Envigo, East Millstone, NJ, USA) and had *ad libitum* access to food and water.

KPT-350 Mouse Dosing Experiments

Because KPT-350 was formulated to be taken via oral consumption, we used peanut butter pellets as a means of delivery. The peanut butter pellets that contained either vehicle or KPT-350 compound were made in a manner similar to those previously described [78, 79]. Commercial peanut butter (Jif Creamy; The JM Smucker Company, Orrville, OH, USA) was mixed with either vehicle or 5 mg/kg (mouse body weight) KPT-350. The 5 mg/kg dose was determined based on a study demonstrating XPO-1 occupancy by SINE compounds as well as KPT-350 toxicity studies [39, 80]. The peanut butter-drug mix was then frozen in 1-mm^3 squares in plastic molds (Cat. #106A; Ted Pella, Redding, CA, USA) at −80°C for 4 h prior to dosing. To administer the peanut butter drug pellet, we placed each mouse individually within a red plastic cup (Solo Cup, Lake Forrest, IL, USA) with the bottom removed to observe the mouse and deliver the pellet. A clear plastic lid was placed over the cup to prevent the mouse from escaping. Eight-week-old male mice were used at the start of all dosing experiments. Each mouse was given 15 min to eat the pellet in a contained plastic cylinder with a removed top, and the mice were monitored until the pellet was completely ingested. Peanut butter drug pellets were given to the mice at 8 weeks of age for 8 weeks, three times a week, before physiological testing, final tail vein blood draws, CO_2 euthanasia, and tissue harvest. All of the KPT-350 drug used in these experiments was synthesized and provided by Karyopharm Therapeutics.

Immunofluorescent Stainings

Zebrafish larvae were euthanized by tricaine and decapitated for genotyping of their heads via PCR, and their bodies were placed in glass vials containing in 4%

paraformaldehyde (Alfa Aesar, Ward Hill, MA, USA) overnight at 4°C with gentle rocking. After two 5-min washes in 1× PBS (Boston Bio Products, Ashton, MA, USA), the larvae bodies were then incubated with 0.1% Tween 20/1× PBS (Boston Bio Products) for 5 min three times at room temperature with gentle rocking. The larvae bodies were then incubated with 3% BSA (BSA fraction V; RPI, Mount Prospect, IL, USA)/0.1% Tween 20 for 45 min at room temperature with gentle rocking. The larvae bodies were then incubated in primary monoclonal antisera diluted 1:50 (F-59 concentrate; fast MyHC; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) overnight at 4°C with gentle rocking. The following day, the larvae bodies were then washed twice in 0.1% Tween 20 for 5 min each, before being incubated with secondary antisera (goat anti-mouse IgG H+L cross-absorbed Alexa Fluor plus 488 secondary antibody; Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:200 in 3% BSA/0.1% Tween 20 for 45 min at room temperature with gentle rocking. The larvae bodies were then washed three times in 0.1% Tween 20, before being mounted on frosted coverslides (Fisher Scientific) and VECTORSHIELD AntiFade Mounting Media (Vector Laboratories, Burlingame, CA, USA) with coverslips for imaging. Slides were imaged with a Nikon TE2000-U inverted fluorescent microscope (Nikon Instruments, Melville, NY, USA) using OpenLab software version 3.1.5 (Improvision/Perkin Elmer, Waltham, MA, USA). The images were later modified in Adobe Photoshop Creative Cloud version 2018 (Adobe Systems, San Jose, CA, USA) for clarity and resolution.

Histochemical Stainings and Analyses

Mouse skeletal muscles were immersed in 10% neutral-buffered formalin (Cat. #HT501128; Sigma-Aldrich, St. Louis, MO, USA) overnight before being embedded in paraffin blocks (Cat. #P3683; Sigma-Aldrich). Paraffin blocks were later transversely sectioned from top to bottom on a Leica RM2125 microtome (Leica Microsystems, Buffalo Grove, IL, USA) at a thickness between 5 and 10 μm following a published protocol[81]. Slides with sections were stained with either H&E (Sigma-Aldrich) or Masson trichrome (Sigma-Aldrich) following the manufacturer's guidelines. Slides were then imaged on an Omax Trinocular Metallurgical Microscope (Microscopenet.com, Kitchener, ON, Canada), and images were enhanced for clarity in Adobe Photoshop Creative Cloud version 2018.

Mouse Cytokine Array Profiling

The mouse cytokine array (Mouse Cytokine Antibody Array 6) was obtained commercially (RayBiotech, Peachtree Corners, GA, USA) and used on 10 μL of whole serum obtained via cardiac puncture at the conclusion of the experiment. Peripheral blood from each mouse was collected at the time of tissue harvest and was immediately stored at −80°C until use for analysis. For all procedures, the manufacturer's protocol was followed for analyses. Whole blood was thawed on ice, centrifuged at 1,000 × *g* for 5 min, and diluted 1:1,000 in PBS. Serum supernatant was used for cytokine array incubation. After antibody incubation, spot intensities of cytokine array were assessed using a Bio-Rad (Hercules, CA, USA) ChemiDoc XRS imaging system. For all quantification analyses, the

cytokine signal intensities normalized to the WT vehicle control group. Array blots were quantified using the ImageJ (Fiji platform) software [82].

Mouse Activity Tracking

Twenty-four hours prior to experiment termination and tissue harvest, mice were analyzed for overall locomotive activity using the ActiTrack software platform (Harvard Apparatus, Holliston, MA, USA) with isolated individual chambers adapted from a previously described protocol[83]. Mice were adapted to the room and open-field chambers 1 day prior to activity, and were given a 5-min additional adaptation period prior to activity recording. Mouse activity was recorded for 6 min with no external stimulation. EthoVision XT Version 12 (Noldus, Leesburg, VA, USA) was used to analyze all activity measurements.

Immunological Profiling Using Flow Cytometry

Single-cell suspensions from pooled quadriceps and TA muscles were generated as previously described [84]. The interrogation of live macrophage populations was performed via staining with Zombie NIR viability dye (Cat. #423105; BioLegend, San Diego, CA, USA), CD11b (PerCP-Cy5, Cat. #101228; BioLegend), F4/80 (PE, Cat. #123110; BioLegend), Siglec-F (BV421, Cat# 56268; BD Biosciences, San Jose, CA, USA), Ly6C (FITC, Cat. #128006; BioLegend), CD206 (Alexa Fluor 647, Cat. #141711; BioLegend). Cells were analyzed using a BD FACSAria Fusion flow cytometer (BD

Biosciences), and data were analyzed using FlowJo software (FlowJo, version 10; FlowJo, Ashland, OR, USA).

Statistical Analyses

For the Kaplan-Meier survival plot (Figure 2B), a log rank test was performed to determine statistical significance. For all other graphs, unless otherwise stated, two-way ANOVA with a Tukey honestly significant difference (HSD) correction was used for comparisons among groups and determination of statistical significance, with an *a priori* hypothesis of *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Study Protocol Approvals

All zebrafish studies were carried out under the approval of the UAB Institutional Animal Care and Use Committee under protocol number 20320. All mouse studies were carried out with the approval of the UAB Institutional Animal Care and Use Committee under protocol number 20323. All protocols were submitted and managed by M.S.A.

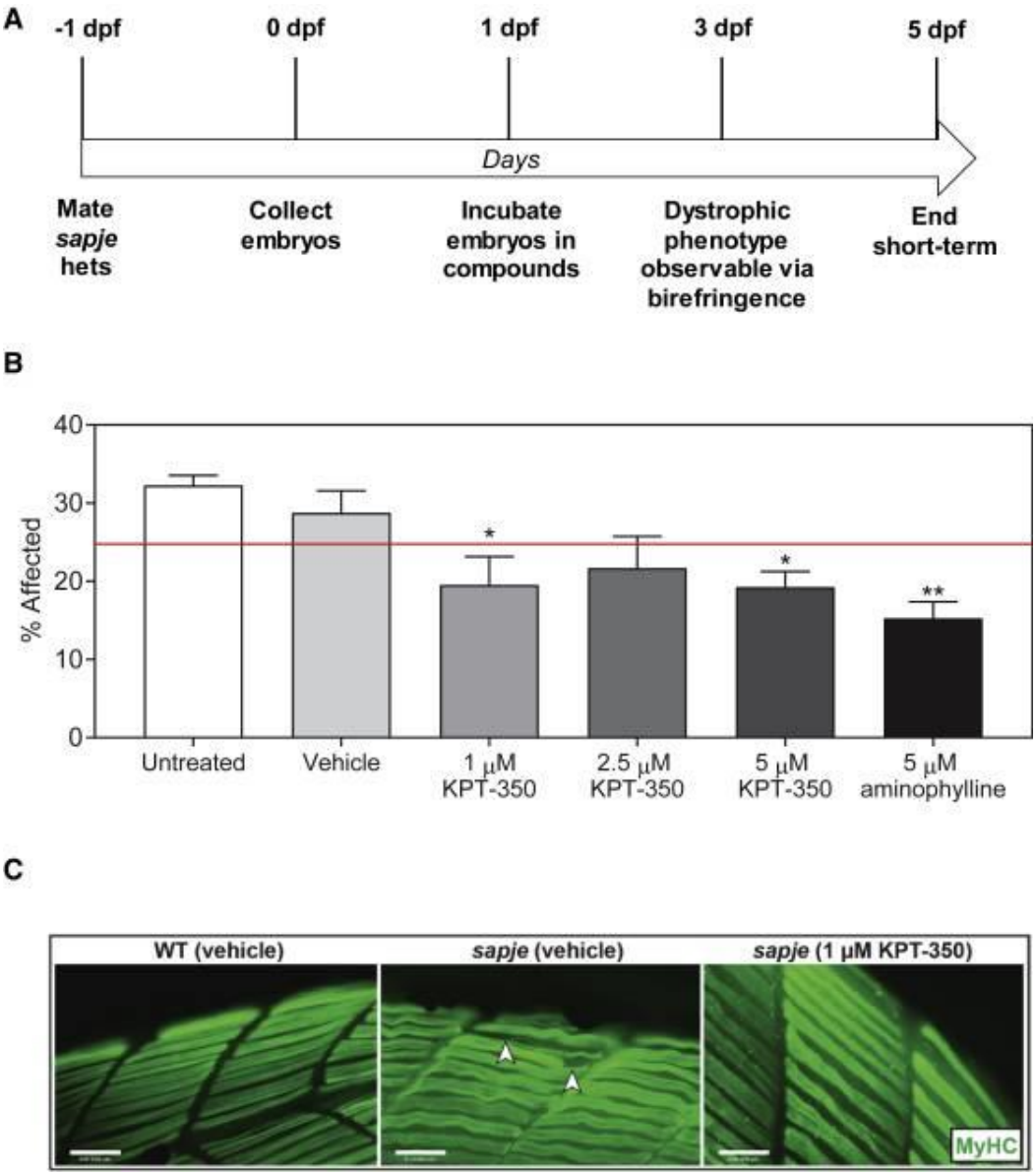


Figure 1: KPT-350 Short-Term Dosing Reduces Percentage of Affected DMD Zebrafish and Improves Overall Myofiber Histology. (A) Schematic for short-term KPT-350 treatment. (B) Summary graph of total percent of affected embryos from paired sapje heterozygous matings after vehicle treatment, KPT-350 treatment (0.1 μM, 1 μM, 5 μM), or 5 μM aminophylline (positive control) (n = 20 fish per cohort; experiments were repeated three times independently). The red line demarcates 25% expected Mendelian ratio of affected *sapje* homozygote fish. (C) Immunofluorescent staining of fast myosin heavy chain in WT + vehicle, *sapje* + vehicle, and *sapje* + 1 μM KPT-350 treatment. White arrows designate disrupted muscle fiber. The SEM is shown for the averaged experimental results. For each experiment, n = 20 fish per cohort were used, and the experiment was repeated four times independently in a double-blinded fashion.

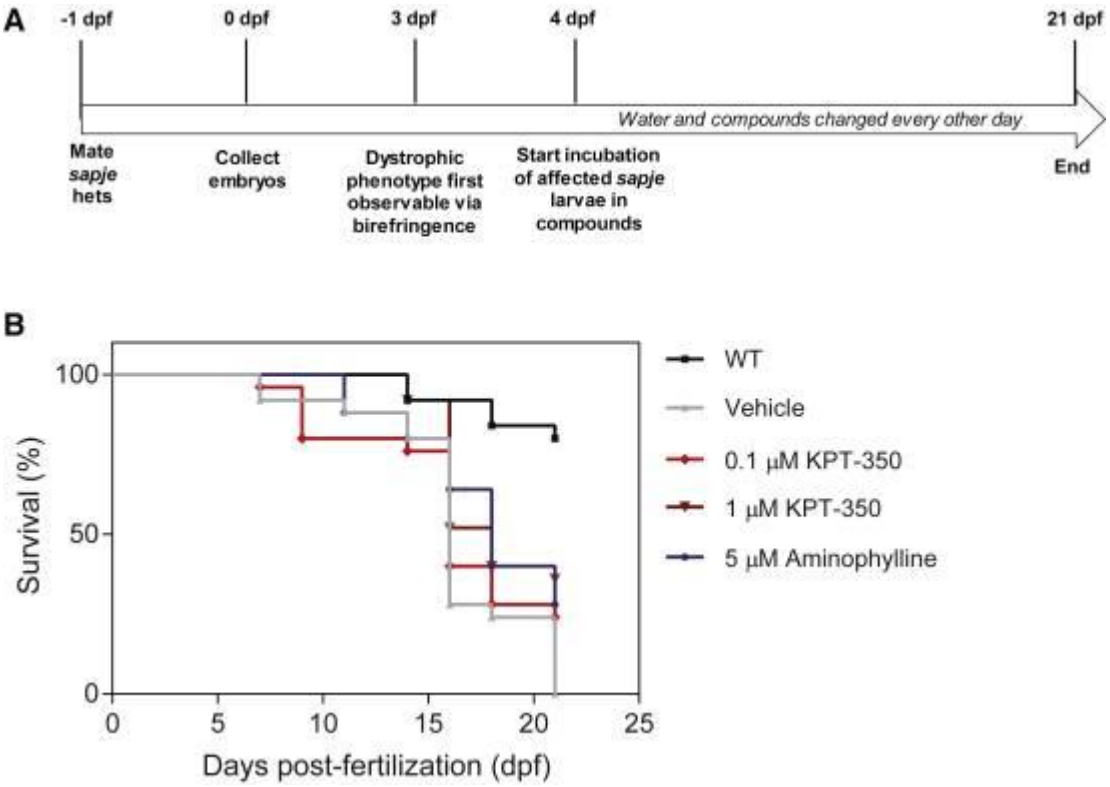


Figure 2: KPT-350 Long-Term Treatment Extends the Lifespan in DMD Zebrafish. (A) Schematic for long-term KPT-350 treatment. (B) Survival plot of long-term experiment for WT (untreated), *sapje* mutant vehicle (0.01% DMSO/fish water), *sapje* mutant 0.1 and 1.0 μM KPT-350-treated, and 5 μM aminophylline (positive control)-treated cohorts from 4 to 21 dpf. 1 μM KPT-350 treatment significantly extended lifespan in *sapje* homozygote mutants compared with vehicle treatment. For each experiment, n = 20 fish per cohort were used, and the experiment was repeated three times independently in a double-blinded fashion. Log rank test was used to determine statistical significance.

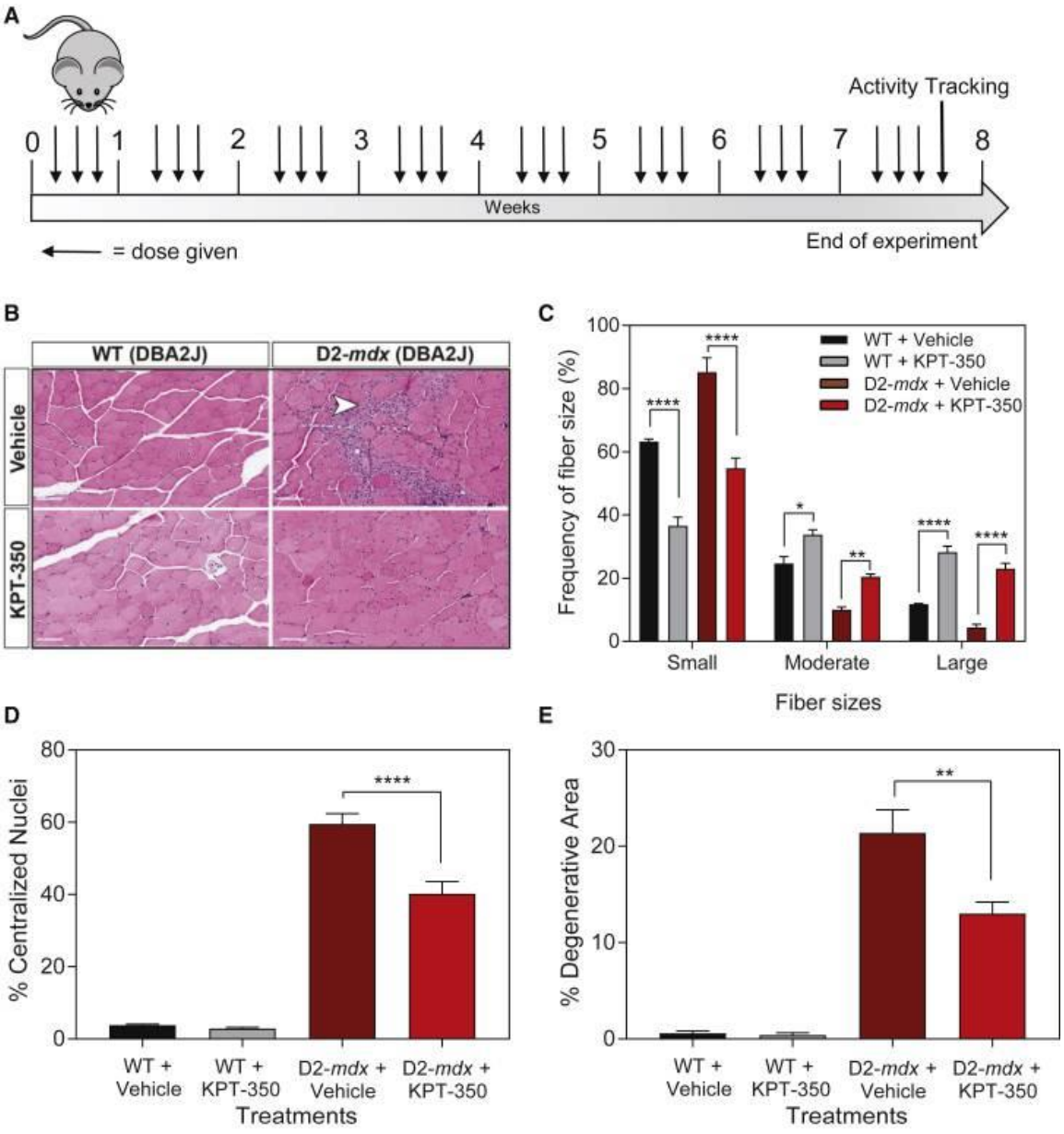


Figure 3: KPT-350 Treatment Improves Histological Hallmarks of Dystrophic Pathology. (A) WT and D2-*mdx* mice were given oral KPT-350 at 5 mg/kg three times a week for 8 weeks beginning at 8 weeks of age. (B) Tibialis anterior muscles were sectioned and stained in H&E (representative images shown, original magnification ×20, scale bars: 100 μm). KPT-350 treatment significantly increased the frequency of large-size fibers with a concomitant decrease in small-size fibers in both WT and D2-*mdx* cohorts (C). In addition, KPT-350-treated D2-*mdx* mice had significantly fewer centralized myonuclei (D) and less inflammation (E) than the vehicle-treated D2-*mdx* mice. Mean ± SEM. n = 5. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, two-way ANOVA.

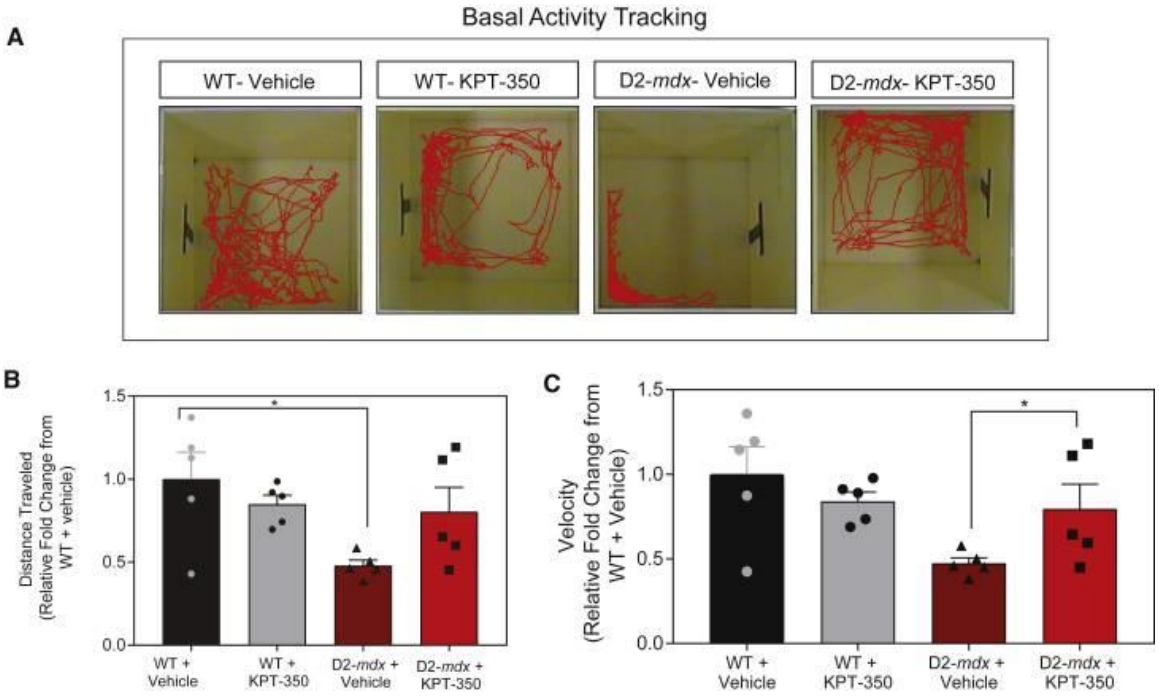


Figure 4: KPT-350 Treatment Improves Overall Locomotion and Overall Activity in D2-*mdx* Mice. (A) Representative locomotor activity tracings from WT and D2-*mdx* mice treated with KPT-350 or vehicle. Red tracings designate route of physical movement for each mouse taken 1–2 days prior to the experimental end (14-week-old mice). (B) Summary graph demonstrating quantified locomotor activity tracings of distance traveled by each WT and D2-*mdx* mouse treated with KPT-350 or vehicle. (C) Summary graph demonstrating quantified locomotor velocity of WT and D2-*mdx* mice treated with KPT-350 or vehicle.

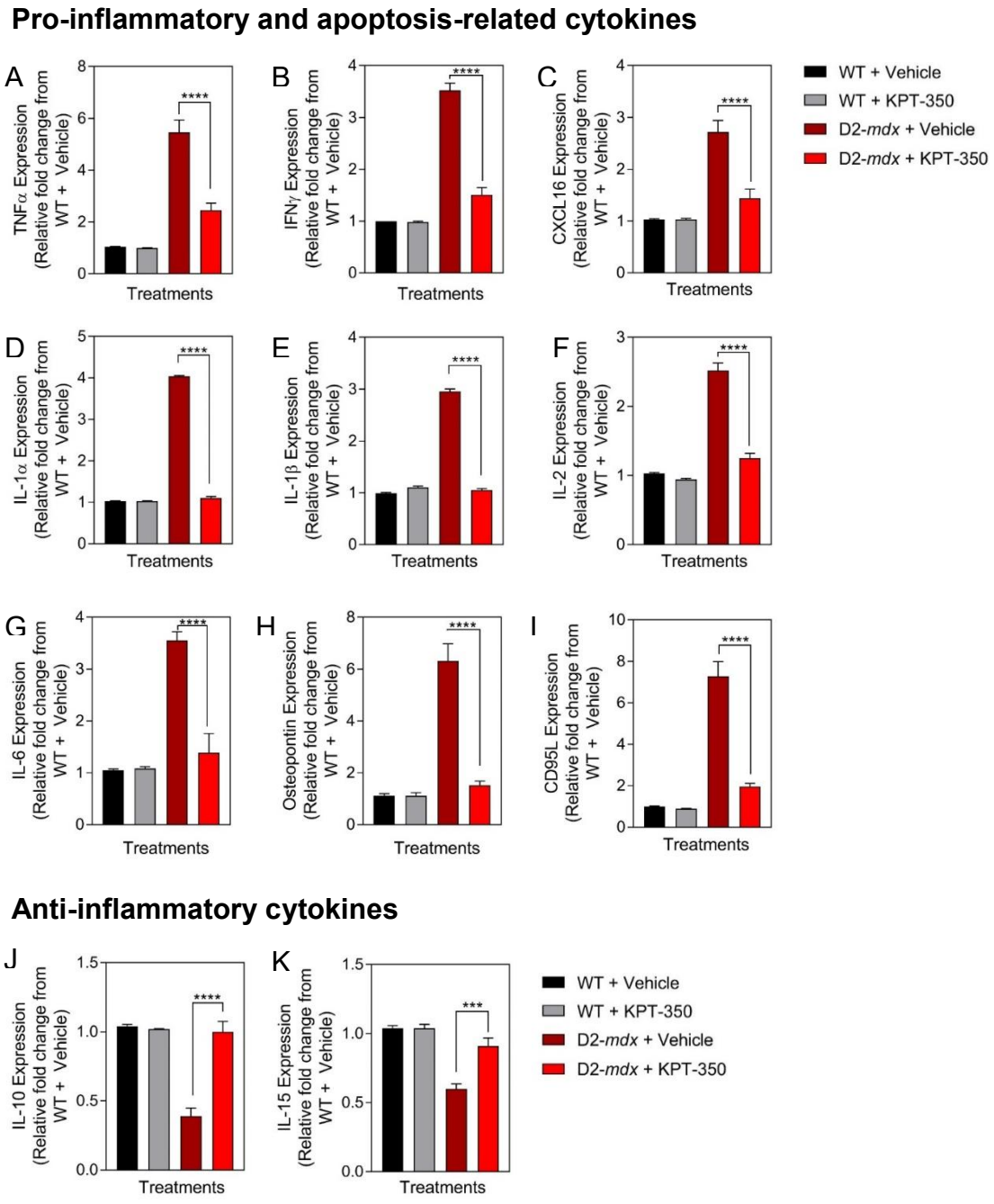
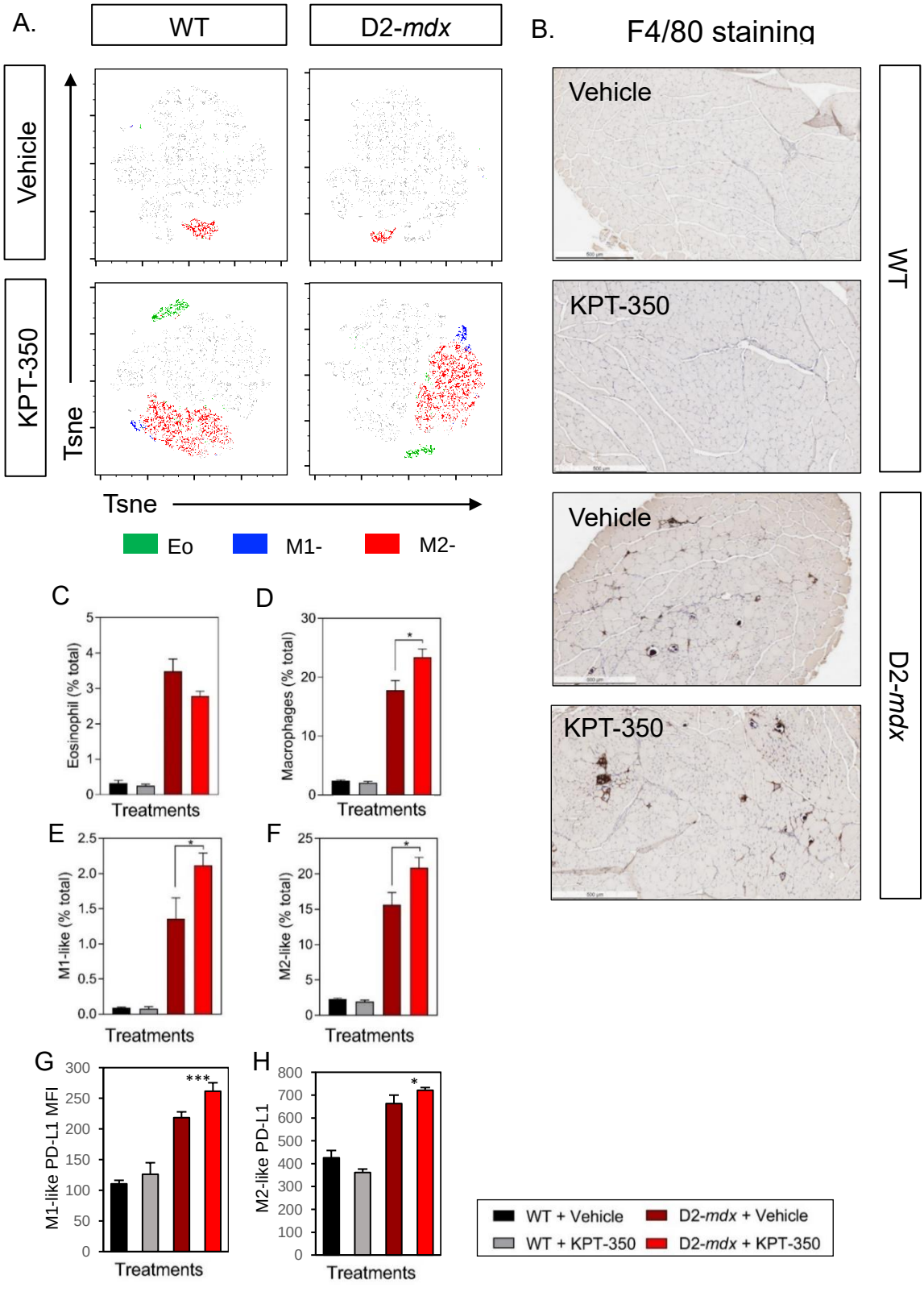


Figure 5: KPT-350 Treatment Results in Decreased Inflammatory Cytokine Expression in D2-*mdx* Mice. (A–I) WT and D2-*mdx* mice were given oral KPT-350 three times a week for 8 weeks. Terminal blood serum was collected and assayed for pro-inflammatory cytokines, which are upregulated in D2-*mdx* mice. KPT-350 treatment significantly reduced various pro-inflammatory and apoptosis-related cytokines. In addition, KPT-350 treatment significantly increased the expression of anti-inflammatory cytokines that are reduced in vehicle-treated D2-*mdx* mice (J and K). Mean ± SEM. n = 5. ***p < 0.001, ****p < 0.0001, two-way ANOVA with a Tukey correction.


A.
WT
D2-mdx
Vehicle
KPT-350
Tsne
Tsne
Eo
M1-
M2-
B.
F4/80 staining
Vehicle
KPT-350
Vehicle
KPT-350
WT
D2-mdx
C
Eosinophil (% total)
Treatments
D
Macrophages (% total)
Treatments
E
M1-like (% total)
Treatments
F
M2-like (% total)
Treatments
G
M1-like PD-L1 MFI
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Treatments
H
M2-like PD-L1
*
Treatments
WT + Vehicle
WT + KPT-350
D2-mdx + Vehicle
D2-mdx + KPT-350

Figure 6: KPT-350 Treatment Increases Overall Macrophage Populations in D2-*mdx* mice. (A) TSNE plots of flow cytometry data gated on live cells of WT and D2-*mdx* mice treated with vehicle or KPT-350. For each color, green represents eosinophils ($CD11b^+F4/80^+Siglec\text{-}F^+$), blue represents M1-like macrophages ($CD11b^+F4/80^+Siglec\text{-}F^-Ly6c^+CD206^-$), and red represents M2-like macrophages ($CD11b^+F4/80^+Siglec\text{-}F^-Ly6c^-CD206^+$) populations. Eight-week-old WT and D2-*mdx* male mice were given oral KPT-350 three times a week for 8 weeks. Single-cell suspensions were prepared from quadriceps and tibialis anterior muscles for analysis via flow cytometry. (B–E) Quantification of flow cytometry analysis showing the frequency (of total cells) of eosinophils (B), total macrophages (C), M1-like (D), or M2-like (E) macrophages. (F and G) Mean fluorescence intensity (MFI) of PD-L1 in M1-like (F) or M2-like (G) macrophages. (H) F4/80 staining in the tibialis anterior muscle of the four experimental cohorts. Scale bars, 500 μm. Mean ± SEM. n = 5. *$p < 0.05$, two-way ANOVA with a Tukey correction

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CHAPTER 4

MICRORNA-486 IS AN EPIGENETIC MODULATOR OF DUCHENNE MUSCULAR DYSTROPHY PATHOLOGY

by

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Abstract

Duchenne muscular dystrophy (DMD) is an X-linked progressive muscle disorder resulting in muscle weakness and cardiomyopathy. MicroRNAs have shown to play a significant role in muscle development, metabolism, and disease processes. We demonstrated that miR-486 expression is reduced in DMD muscles and is dysregulated in dystrophic muscles. *miR-486* knockout mice developed disruption of muscle architecture, decreased physical activity, increased fibrosis, and metabolic defects that were exacerbated on the dystrophic *mdx*^{5cv} background. We integrated RNA-seq and chimeric eCLIP-seq data to identify direct *in vivo* targets of miR-486 and associated deregulated signatures in skeletal muscle. Together, our studies identify miR-486 as a strong driver of muscle remodeling in DMD and highlight chimeric eCLIP-sequencing as a useful tool to identify direct *in vivo* microRNA target transcripts.

Main

Skeletal muscle is a remarkable organ that has a high amount of plasticity due to intrinsic regenerative capabilities in response to exercise, traumatic injury, and disease. In Duchenne muscular dystrophy (DMD), the lack of a functional dystrophin protein causes skeletal muscle weakness, cardiac arrhythmia, respiratory failure, and eventual patient death. Recent progress has been shown in the development *DYSTROPHIN* gene restoration approaches, such as in the case of EXONDYS 51 (Eteplirsen), which can be used by a select group of DMD patients that are amenable to exon-skipping of exon 51 to restore the *DYSTROPHIN* reading frame[1]. Other *DYSTROPHIN* gene-independent strategies involve the overexpression of a micro-dystrophin (μDYS) via adeno-associated viral

(AAV) vectors which are under clinical development[2]. The culmination of all of these strategies center on improvement of dystrophic pathologies in skeletal and heart muscle, but do not address the systemic affects that occur due to dystrophin protein loss.

In DMD, several microRNAs are dysregulated in expression levels including a group of muscle-enriched microRNAs called myomiRs[3-5]. In DMD patients, microRNAs can be utilized as biomarkers that are dysregulated in serum from DMD patients, referred to as "dystromiRs"[6, 7]. When used in combination with a therapeutic treatment, such as exon-skipping PMOs, these dystromiRs can be restored to normal levels making them attractive candidates for marking dystrophic disease progression[8]. In response to glucocorticoid treatment, a subset of NFκB-dependent microRNAs that drive chronic muscle inflammation were shown to improve in response to the steroid treatment[9, 10]. MicroRNA-486 is a mammalian-specific, muscle-enriched microRNA that is embedded within the *ANKYRIN1* (*ANK1*) locus[11, 12]. Previously, we demonstrated that miR-486 regulates DOCK3/PTEN/AKT signaling pathways in skeletal muscle, and muscle-specific miR-486 overexpression improves dystrophic symptoms in dystrophin-deficient (mdx^{5cv}) mice[13]. While the reduction of miR-486 expression in dystrophic muscle is known, the suitability of miR-486 as a DMD biomarker, the mechanism for miR-486 dysregulation in DMD muscle, and the functional consequences of the loss of miR-486 in the heart and muscle remain to be elucidated.

Here, we determined that mir-486 KO mice exhibit both histological and functional deficits in skeletal muscle. Additional examination of miR-486's effects on DMD disease

progression were performed and we revealed that the ablation of miR-486 enhances disease progression in the *mdx*^{5cv} mouse model, an established DMD genetic model. Lastly, we performed microRNA-mRNA chimeric enhanced cross-linking immunoprecipitation sequencing (chimeric eCLIP-seq) which identified direct *in vivo* miR-486 targets and deregulated pathways with roles in dystrophic muscle pathogenesis. Together, our studies reveal the extent of miR-486-driven DMD pathological remodeling and opens the door for potential therapeutic targets.

First, we aimed to assess endogenous miR-486 expression levels in normal and DMD patient skeletal muscles to understand expression in human disease specifically regarding ambulatory status, an important biomarker of disease progression[14, 15]. MicroRNA-486 expression in ambulatory DMD quadricep muscles was significantly lower than healthy quadricep muscle (**Figure 1A**). MicroRNA-486 expression in DMD non-ambulatory muscle was decreased even further compared to both healthy muscle and ambulatory DMD muscle (**Figure 1A**). Skeletal muscle biopsies from Becker muscular dystrophy (BMD) patients showed slightly decreased miR-486 expression demonstrating that the decrease in miR-486 expression as seen at two stages of DMD disease progression is specific to pathology as it relates to the loss of a functional dystrophin protein **(Figure 1A).** To confirm this pattern in a DMD mouse model, we assessed miR-486 expression levels in three different muscle groups across five DMD disease-relevant time points (1, 3, 6, 9, and 12 months-old) in both *mdx*^{5cv} and wild type mice. The *mdx*^{5cv} mice demonstrated significantly decreased expression of miR-486 in the tibialis anterior (TA), soleus, and diaphragm compared to WT and this decrease in expression remains consistent throughout

lifespan (**Figure 1B**). MyoD and SRF are important transcriptional activators in skeletal muscle that drive myogenic differentiation of satellite cells into mature myofibers during differentiation and repair[16, 17]. Consequently, myogenic differentiation and muscle fiber repair mechanisms are impaired in DMD pathology[18, 19]. We sought to determine if MyoD and SRF signaling played a role in miR-486 expression regulation by testing MyoD and SRF binding of the miR-486 host gene, *ANKRYIN1* (*ANK1*) using chromatin immunoprecipitation (ChIP), specifically the muscle-enriched *ANK1-5* promoter. Both MyoD and SRF binding at the human *ANK1-5* promoter were decreased in DMD patient myoblasts and myotubes **(Figure 1C).** This result led us to investigate the expression levels of other MyoD and SRF-related myogenic factors across the lifespan of mdx^{5cv} mice as dystrophic disease pathology progressed. Quantitative PCR of MyoD and SRF-signaling factors were decreased in correlation with dystrophic disease progression in the mdx^{5cv} mouse muscles **(Figure 1D)**.

As miR-486 expression is significantly decreased in DMD, we wanted to define the direct role of miR-486 in muscle. MiRNA-486 KO mice were generated by CRISPR-targeting of the 128 base pair (bp) pri-miRNA-486 stem loop resulting in an 85 bp deletion of the stem loop sequencing without disrupting the *ANK1* gene locus **(Supplemental Figures 1A and 1B)**. Confirmation of miR-486 KO was further validated via northern blot, in which miR-486 was undetectable in both miR-486 KO TA (tibialis anterior) and cardiac tissue **(Supplemental Figure 1C).** Western blot then was performed to ensure host ANK1-5 protein isoform was unperturbed, which allowed us to conclude that any phenotypes we observed or tested in these animals were due to knockout of miR-486 alone and not due to

disrupted host gene expression or function **(Supplemental Figure 1D).** We next evaluated the role of miR-486 in normal myogenic differentiation by measuring myogenic fusion in the *miR-486* KO primary myoblasts. The *miR-486* KO myoblasts showed decreased myogenic fusion compared to WT controls (**Figures 1E and 1F**).

To understand the effects of complete ablation of miR-486 in skeletal muscle, we examined histological hallmarks of dystrophic pathology in miR-486 KO TA muscles compared to WT controls (**Figure 2A**). Histopathological analysis of the *miR-486* KO mice revealed a decreased overall cross-sectional area, a significant increase in centralized myonuclei, and increased fibrosis that was exacerbated on the dystrophic *mdx*^{5cv} background (**Figures 2B-2D**). We sought to understand if these disruptions were playing a larger role in basic whole body composition or locomotion in the miR-486 KO mice. Interestingly, miR-486 ablation did not alter fat mass, lean mass, or overall total body mass (**Supplemental Figures 2A-C**). No change was observed in peak tetanic muscle force in the miR-486 KO mice (**Supplemental Figure 2D**). Levels of basal locomotor activity were significantly decreased in the *miR-486* KO males, as measured by open field activity tracking **(Supplemental Figure 2E**). DMD-associated dilated cardiomyopathy is a significant cardinal hallmark of advanced DMD pathology[20]. We also sought to understand if decreased miR-486 expression plays a role in this pathological process by assessing cardiac function in the miR-486 KO mice. Histopathological analysis of the miR-486 KO mice showed an increased amount of fibrosis compared to WT controls (**Supplemental Figures 3A and 3B**). Echocardiograms on the adult *miR-486* KO mice revealed a decreased fractional shortening, ejection fraction, and several other parameters

that were exacerbated in the miR-486 KO:*mdx*^{5cv} (dKO) mice **(Supplemental Figures 3C-3M).**

Since miR-486 has been named a myomiR because of its enriched expression in skeletal muscle, we sought to understand the relationship between miR-486 ablation and the expression of 4 dystromiRs, miR-1, -133a, -133b, and -206**.** Interestingly, only miR-133 expression was significantly decreased in 6 month miR-486 KO TA muscle compared to WT **(Figure 3A-D).** To understand the effects of miR-486 ablation on the overall skeletal muscle transcriptome, we then performed bulk RNA sequencing on whole TA muscles from WT and miR-486 KO mice to compare directly and indirectly dysregulated transcripts. We sought to identify transcripts both directly and indirectly dysregulated as a result of miR-486 ablation. RNA-seq revealed 96 transcripts significantly increased in expression and 190 transcripts significantly decreased in expression in miR-486 KO TA muscle compared to WT **(Figure 3E).** In order to identify potential pathways that may be preferentially targeted by miR-486, a pathway enrichment analysis was performed on the 96 transcripts significantly increased in expression and the 190 transcripts significantly decreased in expression using the online tool, g:Profiler. The top 5 enriched pathways identified by g:Profiler were extracellular matrix, collagen-containing extracellular matrix, regulation of multicellular organismal processes, extracellular region, and vasculature development as shown by the g:GOSt multi-query Manhattan plot **(Figure 3F).** The top 10 transcripts increased in expression and top 10 transcripts decreased in expression in miR-486 KO compared to WT are outlined in **Figure 3G-H.**

Given that miR-486 is a potent biomarker of DMD disease progression and requirement for normal skeletal and cardiac muscle function, we sought to identify its direct mRNA targets. To complement the RNA-seq data that we used to bioinformatically predict miR-486 targeted pathways, we employed chimeric enhanced cross-linking immunoprecipitation (eCLIP-seq) to identify direct *in vivo* miR-486 muscle targets that may influence muscle growth and function. Two biological replicates of both WT and miR-486 TA muscle were isolated from 6 month male mice and used to immunoprecipitate Argonaute-2 (Ago2)-microRNA complexes and their target RNA transcripts[21]. MiR-486-specific sequencing adaptors were then ligated to the microRNA-mRNA hybrid molecules called "chimeras". De-crosslinking was performed and the miR-486-bound transcripts were then amplified into a cDNA library (**Figure 4A**). The library was directly sequenced to reveal miR-486 bound *in vivo* transcripts using miR-486 KO TA muscle as a control. The chimeric eCLIP-seq revealed 18 differentially expressed transcripts that demonstrated reverse complementarity to the miR-486-specific 8-mer (AUGUACUG) binding motif, which partially overlapped the known conserved miR-486 8-mer seed site (UCCUGUAC) (**Figure 4A**). The eCLIP-seq peak tracks demonstrate the chromosomal location of the 18 differentially expressed transcripts throughout the mouse genome **(Figure 4B).** The sequencing peaks of one of the identified genes, Mt2, are highlighted as an example using the sequencing visualization tool IGV (Integrated Genome Viewer) **(Figure 4B).** Chimeric eCLIP-seq revealed miR-486 binding to many 3'UTRs but also, interestingly, to a large numbers of coding sequences (CDS) in muscle (**Figures 4C and 4D**). The top 10 peaks identified through eCLIP-seq analysis are listed in **Figure 4E** and the full list of identified peaks is outlined in **Table 1.** We then performed functional

enrichment analysis using the g:Profiler tool set to identify key gene ontology (GO) pathways of miR-486 muscle chimeric eCLIP-seq targets[22]. The g:Profiler analysis revealed significant enrichment of miR-486 binding to contractile fiber, myofibril, sarcomere, and other muscle-associated targets as shown by the g:GOSt multi-query Manhattan plot (**Figure 4F**). We then performed quantitative PCR on the top 10 targets identified through eCLIP-seq analysis and found that two of those transcripts, Mt2 and Auh, contain at least 50% sequence complementarity to the miR-486 seed site and are significantly overexpressed in miR-486 KO mouse TA muscle compared to WT controls **(Figure 4A and 4G).**

Our findings demonstrate that miR-486 drives pathogenic remodeling of dystrophic muscle by targeting key muscle structural, metabolic, and extracellular matrix remodeling factors. Indeed, miR-486 is required for normal skeletal and cardiac muscle growth and function through modulation of these factors as well. Loss of miR-486 expression has profound consequences on mouse skeletal and cardiac muscle architecture, fibrotic depositions, centralized myonuclei, and overall muscle performance. In cardiac muscle, miR-486 ablation resulted in significant pathological remodeling of the heart and overall decreased cardiac output. The dynamic nature of miR-486 expression and its role in both ECM remodeling and metabolic regulation suggests its requirement in muscle during times of stress and disease. Recently miR-486 has been shown have decreased levels in circulating exosomes isolated in healthy and sedentary individuals following a bout of exercise, and thereby regulating IGF1-signaling[23]. Interestingly, from that same study,

miR-486 expression was higher at baseline expression compared to sedentary individuals which suggests higher miR-486 expression with improved muscle health.

There is growing evidence that restoration of dystrophin via exon-skipping compounds can restore the transcriptomic properties of dystrophic muscle, including miRNAs, to that of normal or at least Becker-like muscle[24]. Our combinatorial RNA-sequencing and eCLIP-seq of skeletal muscles from miR-486 KO mice identified a network of miR-486 muscle targets including coding and non-coding regions of muscle targets that play key roles in dystrophin-deficient muscle pathology. MicroRNA profiling studies demonstrated that miR-486 levels increased in the right ventricles of hypoplastic left heart syndrome (HLHS) patients, and miR-486 levels were elevated in response to cyclic stretch[25]. Other studies demonstrate that miR-486 levels are modulated during development, stress, and disease states via modulation of key cellular growth pathways through direct regulation of FOXO, SMAD, PI3K, and AKT[12, 13, 25, 26]. These growth pathways were also shown to be dysregulated in dystrophic skeletal muscles, and a potential site of therapeutic modulation via miR-486 transgenic overexpression in skeletal muscle to restore these signaling pathways[12, 13].

Additional questions remain as to what myogenic cell populations and at what stages of normal and dystrophic development, miR-486 dysregulation occurs. Several studies have identified miR-486 expression as enriched in quiescent muscle satellite cells, along with other muscle-enriched myomiRs prior to activation[27, 28]. As activation of muscle satellite cells occurs along with myoblast fusion, miR-486 expression increases

which may represent the switch from MyoD-driven miR-486 expression to SRF-driven expression that we show at the *miR-486/ANK1-5* locus. While miR-486 expression was shown to regulate Pax7 in quiescent mouse muscle satellite cells, there are likely additional miR-486 targets in MSCs as well as other non-muscle cell types. Furthermore, many of the miR-486 mRNA targets we identified are also expressed in non-muscle cell types, additional levels of post-transcriptional regulation may yield novel tissue-specific miR-486 targets.

MicroRNA-486 overexpression may be a valid means of restoration of key dysregulated signaling pathways in dystrophic muscle that affect growth and muscle function. There is growing evidence that the collagens and the ECM remodeling that occurs in DMD serves as a biomarker for disease progression and a therapeutic target[29-32]. Additional evidence demonstrating the restoration of microRNAs in exon-skipped *mdx* mice, suggests that miR-486 expression profiling may be useful in evaluating dystrophic replacement and restoration strategies in muscle[8, 33-35]. Given our previous work demonstrating that miR-486 transgenic overexpression in a dystrophic mouse model can ameliorate dystrophic pathologies, strategies to either induce miR-486 expression levels or direct miR-486 overexpression might be explored for therapeutic disease modulation.

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Author Contributions:

R.M.H., A.S., A.L.R., M.A.L., D.A.S., M.M.B, G.C.R, L.I., E.C.L., M.X, and M.S.A. all performed experiments or analysis of data. M.A.L., M.M.B., J.S.D., D.J., E.C.L., M.X., and M.S.A. provided unique reagents. R.M.H., A.L.R., and M.S.A. analyzed all the data and wrote the manuscript. All authors approved of the manuscript prior to submission.

Declarations of Interest

All authors declare no conflicts of interest.

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Methods

Mice:

The miR-486 KO mice were generated as previously described[36]. Mice were initially maintained on the *FVB/NJ* (Jackson Labs; #001800) strain and were later backcrossed onto the *C57BL/6J* (Jackson Labs; #000664) strain for more than six generations to ensure isogenicity. All analyses were performed on the same strain background. The *mdx*^{5cv} (Jackson Labs; #002379) were also kept on the *C57BL/6J* strain, and homozygote mutant females were mated to the *miR-486* KO males to generate *miR-486 +/-*:*mdx*^{5cv} mice and later *miR-486 KO*:*mdx*^{5cv} (DKO) mice. Unless otherwise stated, only 6-month-old male mice were used in all experimental conditions. All mouse strains were maintained under standard housing and feeding conditions with the University of Alabama at Birmingham Animal Resources Facility under pathogen-free, sterile conditions and all protocols were approved under the animal protocol number 21393.

Chromatin Immunoprecipitation (ChIP):

Chromatin immunoprecipitation was performed on normal human and DMD myoblasts and myotubes in a protocol previously described[37]. Genomic DNA was isolated from

myoblast (70% confluency in growth medium) and myotube (4 days differentiation in low serum differentiation medium) and ChIP was performed using the Simple ChIP Enzymatic Chromatin IP Kit (Cat# 9003; Cell Signaling Technology; Danvers, MA) following the manufacturer's protocols. For the MyoD ChIP assays, a rabbit polyclonal (MyoD clone M-318; Cat# sc-760; Santa Cruz Biotechnology; Dallas, TX) was used to immunoprecipitate MyoD bound to the mouse *miR-486* locus. For SRF ChIP assays, a rabbit polyclonal (SRF clone G-20; Cat# sc-335; Santa Cruz Biotechnology; Dallas, TX) was used to immunoprecipitate SRF bound to the mouse *miR-486* locus. Myoblasts were seeded at 4 $x10^5$ cells/15 cm dish in 10 dishes per cohort. The myoblast fraction consisted of normal human myoblasts grown to approximately 70% confluency. For the myotube fraction (five 15 cm dishes), after reaching 90% confluency, myoblasts were differentiated into myotubes by serum withdrawal (2% FBS) for 4 days. Cells were harvested following three washes in ice-cold 1 X DPBS, were crosslinked with 1% formaldehyde (Cat# F8775; Sigma-Aldrich; St. Louis, MO) for one hour at room temperature, and then chromatin was sheared using by incubating the DNA pellets with micrococcal nuclease for 20 min at 37^{o}C. Ten percent of chromatin was removed for input controls, while the approximately 200 mg of total chromatin was used to purify DNA fragments following immunoprecipitation with 5 mg of either MyoD or SRF antibodies. The following morning, the samples were incubated with 30 ml of ChIP Grade Protein G magnetic beads (Cat# 9006S; Cell Signaling Technology; Danvers, MA) for 2 hours at 4^{o}C under gentle rotation. Following several stringent washes, the chromatin was eluted off the columns in 1 mM Tris-HCL pH 8.0 (Cat# 15568025; ThermoFisher; Waltham, MA) and purified on DNA-binding columns (Cat# D4029; Zymo Research; Tustin, CA) before a final elution in sterile water. MyoD

and SRF ChIP-seq annotation of ANK1-5 was completed using the publicly available UCSC Genome Browser track data hubs annotation resource available at genome.ucsc.edu. For the MyoD TF binding site at the human *ANK1-5* locus the following primers were used Fwd: 5'-GAGCTCCAAGACTGAGGACTGGAC-3' and Rev: 5'-CAGGGAGGATGGAGATCAGAGCC-3'. The non-specific MyoD site negative control primers next to the human *ANK1-5* locus used were Fwd: 5'-CTGCACGTCAGCCTCCCAAAG-3' and Rev: 5'- ACTGGGATCCTCCAGGGGCC-3'. The MyoD TF positive control primers used were Fwd: 5'-CAGTGAACAATGGTGCTTGG-3' and Rev: 5'- TTCCACATTCACGCAGAGAG-3'. For the SRF TF binding site at the human *ANK1-5* locus the following primers were used Fwd: 5'-ACAGTAGGTGAGTTGCAGGGTTAG-3' and Rev: 5'-GGGCTCAGGGACAGTCAAGTGAGC-3'. The non-specific SRF site negative control primers next to the human *ANK1-5* locus used were Fwd: 5'-GAAACACGGAGCAGCCTGGC-3' and Rev: 5'-ATGAGGATCGACTGTACATGC-3'. The SRF TF positive control primers used were Fwd: 5'-TGGTTGGATAACAGAGGCAGA-3' and Rev: 5'-GCTTCTGTTGTGGCGTCTTT-3'.

Western blots:

Protein lysates from cell and tissues were lysed and homogenized (tissues) in Mammalian Protein Extraction Reagent (M-Per) lysis buffer (Cat# 78501; ThermoFisher; Waltham, MA) supplemented with cOmplete protease inhibitor tablets (Cat# 1183617000; MilliporeSigma; Burlington, MA). Approximately 50 µg of lysate were electrophoretically

resolved on 4-20% Novex Tris-glycine gradient gels (Cat# XV04200PK20; ThermoFisher; Waltham, MA). Proteins were then transferred to 0.2 µm PVDF membranes (Cat# 88520; ThermoFisher; Waltham, MA), and incubated overnight at 4^{o}C with gentle rocking in primary antisera diluted 1:1000 in 5% bovine serum albumin (Cat# A30075; RPI Corp; Mount Prospect, IL)/1xTBS-Tween (Cat#IBB-581X; BostonBioProducts; Ashland, MA). Membranes were washed three times in 1xTBS-Tween for 5 minutes each, and then incubated in secondary antisera (1:2000 dilution) for 1 hour at room temperature. Membranes were then washed four times in 1x TBS-Tween for 15 minutes each before the addition of Novex Chemoluminescent Substrate Reagent Kit (Cat# WP20005; ThermoFisher; Waltham, MA). Membranes were exposed onto PerfectFilm audioradiography film (Cat#B581; GenHunter; Nashville, TN) and developed on a Typhoon Variable Mode Imager (Amersham Pharmacia; Little Chalfont, United Kingdom). Western blot densitometry was performed using open-source ImageJ software.

Muscle Histochemical Staining:

Mice were perfused through the cardiac apex with Cardioplegia Solution A (Cardioplegia Solution A Data Sheet, Baxter Healthcare LTD, Baxter International, NZ). Hearts were then dissected, the left ventricle isolated, and the basal and apical thirds removed, leaving only the middle third of the left ventricle (the “donut”) remaining. This left ventricular transverse section was drop-fixed in 10% neutral buffered formalin (Cat#HT501128; MilliporeSigma; Burlington, MA) overnight at 4°C and held in 70% ethanol for 24-48 hours before being processed as previously described [38]. After heart dissection, the remaining skeletal muscles were collected for use. Mouse skeletal muscle tissues were

cryo-frozen by covering the tissue Optimum Cutting Temperature (OCT) solution (FisherScientific; Hampton, NH; Cat#23-730-571) and completely submerging the tissues in a liquid nitrogen-chilled Isopentane (Cat#AC397221000; FisherScientific; Hampton, NH) bath as unfixed tissues. Blocks were later cut on a cryostat and 7-10 µm thick sections were placed on SuperFrost Plus slides (Cat#FT4981GLPLUS; ThermoFisher; Waltham, MA). Hematoxylin and eosin (H&E) staining was performed as previously described[12]. Mouse skeletal muscle tissues were cryo-frozen and sectioned as described above. Masson's Trichrome staining was performed on frozen sections using the Masson's Trichrome stain kit (Cat# 25088-100; Polysciences; Warrington, PA) and the following protocol: "Histopathology in Masson's Trichrome stained muscle sections," published by the TREAT-NMD Neuromuscular Disease Network resource center which follows a published protocol[39]. Mouse hearts were perfusion fixed in 10% neutral buffered formalin (Cat#HT501128; MilliporeSigma; Burlington, MA) overnight at 4°C and held in 70% ethanol for 24-48 hours before being processed as previously described [38].

Muscle Contractility:

Peak tetanic force of ex-vivo isolated EDL muscle was measured in WT, miR-486 KO, and *mdx*^{5cv} mice at 6 months of age using physiological preparations as previously described [13, 40]. EDL muscles were dissected from anesthetized mice, cut into small bundles, pinned at their approximate in vivo length to a Sylgard base in a small petri dish, and permeabilized with a relaxing solution containing 0.1% Triton X-100 (3 hours at 4°C). The skinning solution was then replaced with a relaxing solution containing 50% glycerol and the bundles stored at -20◦C for up to 4 weeks. Single fiber segments were

isolated from the bundles and mounted between a high-speed position motor (Aurora Scientific, model 308) and an isometric force transducer (Aurora Scientific model 403) using 10-0 monofilament. The fiber segment was extended to a sarcomere spacing of 2.6 μm and studied by rapidly exchanging relaxing and activating solutions at 15◦C. A slack test was used to assess fiber unloaded shortening velocity (V0). The greatest force attained during the test, measured as the difference between unloaded force and peak active force, was taken as fiber force. Differences between genotypes were evaluated by an ANOVA.

RNA-sequencing and data analyses:

Adult 6-month-old male TA muscles from WT and *miR-486* KO mice were snap frozen in liquid nitrogen. Muscle samples were mechanically homogenized and total RNA was extracted using a miRVana Isolation Kit (Cat#AM1560; ThermoFisher; Waltham, MA) following the manufacturer's protocol. The total RNA was amplified using the Sure Select Stranded RNA-Seq kit (Agilent Technologies; Santa Clara, CA) using standard protocols. A ribominus kit (Cat# K155002; ThermoFisher; Waltham, MA) was used to deplete large ribosomal RNAs. All biological replicates contained a minimum of 35.7 million reads with an average number of 39.6 million reads across the replicates. The FASTQ files were uploaded to the UAB High Performance Computer cluster for bioinformatics analysis with the following custom pipeline built in the Snakemake workflow system (v5.2.2)[41]: first, quality and control of the reads were assessed using FastQC, and trimming of the Illumina adapters and bases with quality scores of less than 20 were performed with Trim_Galore! (v0.4.5). Following trimming, the transcripts were

quasi-mapped and quantified with Salmon[42] (v0.12.0, with `--gencode` flag for index generation and `-l ISR`, `--gcBias` and `--validateMappings` flags for quasi-mapping) to the mm10 mouse transcriptome from Gencode release 21. The average quasi-mapping rate was 70.4% and the logs of reports were summarized and visualized using MultiQC[43] (v1.6). The quantification results were imported into a local RStudio session (R version 3.5.3) and the package "tximport"[44] (v1.10.0) was utilized for gene-level summarization. Differential expression analysis was conducted with DESeq2[45] package (v1.22.1). Following count normalization, principal component analysis (PCA) was performed, and genes were defined as differentially expressed genes (DEGs) if they passed a statistical cutoff containing an adjusted p-value <0.05 (Benjamini-Hochberg False Discovery Rate (FDR) method) and if they contained an absolute $\log_2$ fold change >=1. Functional annotation enrichment analysis was performed in the NIH Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8) by separately submitting upregulated and downregulated DEGs. A p-value <0.05 cutoff was applied to identify gene ontology (GO) terms. The FASTQ files of the current study have been uploaded to NCBI's Gene Expression Omnibus under accession number GSE155787.

Chimeric eCLIP-Sequencing:

For the chimeric CLIP-sequencing we used adult 6-month-old male TA muscles from WT and *miR-486* KO mice (n = 4 muscles for each cohort) that were snap frozen in liquid nitrogen and chimeric eCLIP-sequencing was performed with assistance by Eclipse BioInnovations (Eclipse BioInnovations; San Diego, CA) in a previously established

protocol[46]. A detailed description of the chimeric CLIP-seq procedures including Ago2 immunoprecipitation, library amplification, and other quality control analyses can be found in the **Supplemental Figures and Methods.**

Real time quantitative PCR (rt-qPCR):

Total RNA was extracted from muscle tissue using the miRVana (Cat# AM1560; ThermoFisher; Waltham, MA) kit following the manufacturer's protocol. One microgram of total RNA was reverse transcribed using a Taqman Reverse Transcription kit following the manufacturer's protocol (Cat# N8080234; Applied Biosystems; Foster City, CA). For microRNA fractions, 50 nanograms of total small RNA was used for all reverse transcription reactions. Taqman assay probes were purchased from Applied Biosystems corresponding to the individual genes. Quantitative PCR (qPCR) Taqman reactions were performed using Taqman Universal PCR Master Mix (Applied Biosystems; Cat# 4304437). Samples were run on the Fluidigm Biomark HD system (Fluidigm Corp.; San Francisco, CA) in 96.96 Dynamic Array plates. Relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method[47].

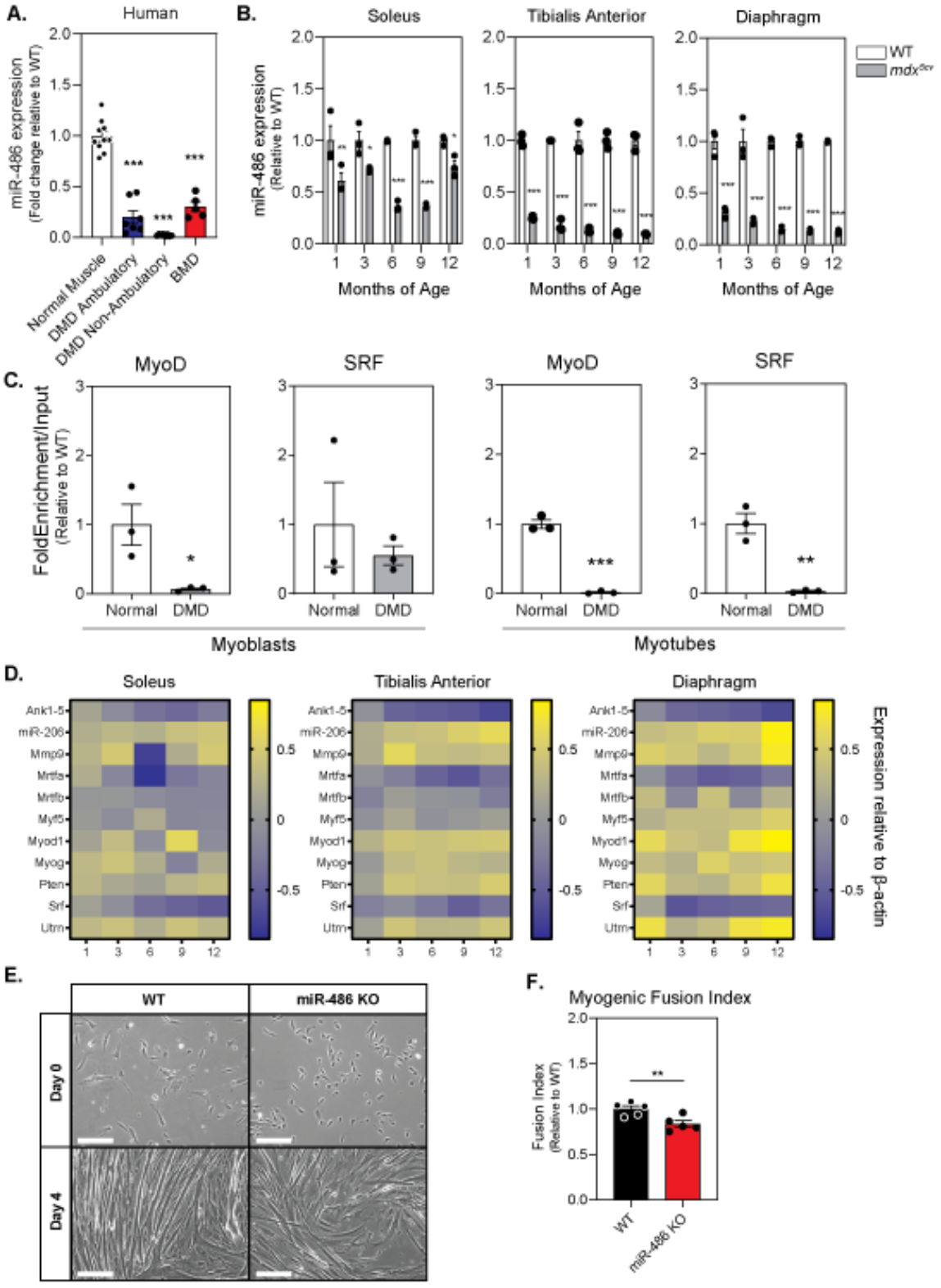

A.
Human
miR-486 expression
(Fold change relative to WT)
Normal Muscle
DMD Ambulatory
DMD Non-Ambulatory
BMD
B.
Soleus
Tibialis Anterior
Diaphragm
miR-486 expression
(Relative to WT)
Months of Age
WT
mdx5cv
C.
MyoD
SRF
FoldEnrichment/Input
(Relative to WT)
Normal
DMD
Myoblasts
Myotubes
D.
Soleus
Tibialis Anterior
Diaphragm
Ank1-5
miR-206
Mmp9
Mrtfa
Mrtfb
Myf5
Myod1
Myog
Pten
Srf
Utrn
Expression relative to β-actin
E.
WT
miR-486 KO
Day 0
Day 4
F.
Myogenic Fusion Index
Fusion Index
(Relative to WT)
WT
miR-486 KO

Figure 1. MicroRNA-486 skeletal muscle expression decreases in DMD.
A. Quantitative PCR reveals decreased expression of miR-486 in dystrophic human skeletal muscle compared to control human skeletal muscle and Becker muscular dystrophy (BMD) muscle. **B.** Quantitative PCR reveals decreasing expression of miR-486 in the *mdx*^{5cv} dystrophic mouse model skeletal muscle at 1, 3, 6, 9, & 12 months of age compared to WT control muscle. **C.** ChIP reveals myogenic factors MyoD and SRF demonstrate decreased binding at the promoter of ANK1-5 in isolated *mdx*^{5cv} myoblasts and differentiated myotubes compared to WT controls. **D.** Heat maps demonstrate changes in expression of myogenic factors in *mdx*^{5cv} tibialis anterior, soleus, and diaphragm muscles over 1, 3, 6, 9, and 12 months of age compared to WT control muscle. Yellow indicates an increase in expression and blue indicates a decrease in expression relative to WT control muscle. **E.** Phase contrast reveals disrupted myoblast fusion and differentiation. Photomicrographs show differentiated myotubes after 4 days of culturing from primary isolated satellite cells. WT and microRNA-486-/- satellite cells were isolated from <p10 pups and cultured for 4 days. 10X, scale bar = 200µm. **F.** Myogenic fusion index calculated from day 4 myotube culture images depicted in panel E. Percentage fusion calculated by dividing the number of nuclei within multinucleated myofibers by the total number of nuclei.

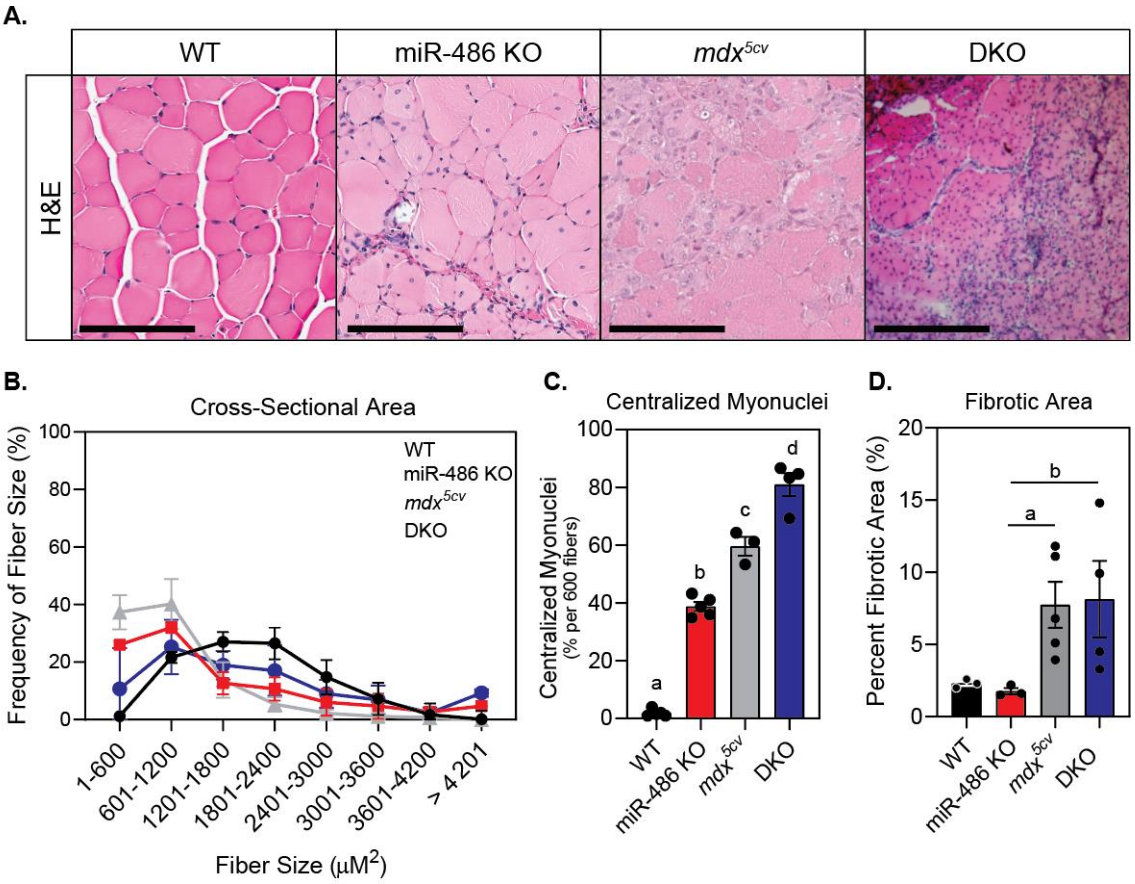
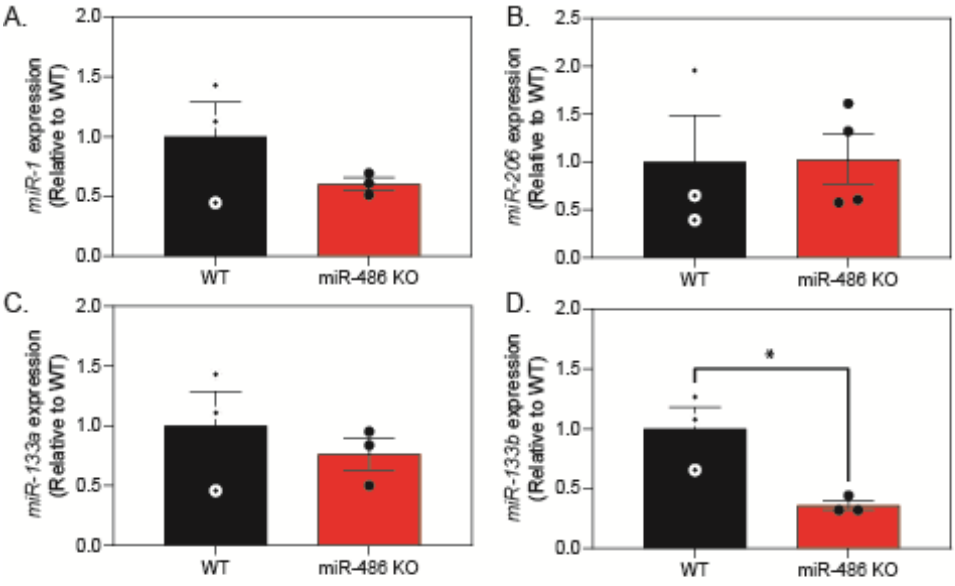
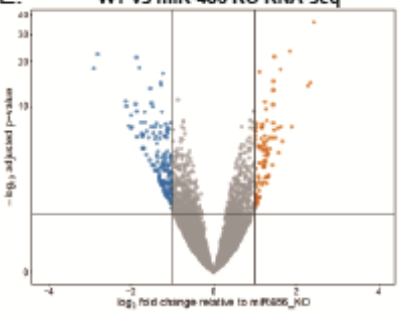


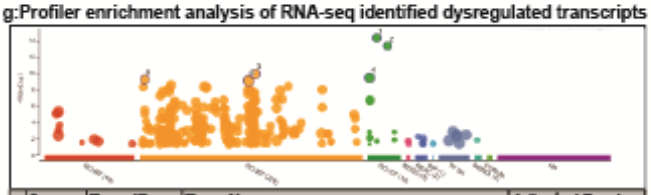
Figure 2. MicroRNA-486 knockout mice demonstrate histological defects in skeletal muscle. **A** H&E staining of transverse sections of TA muscles at 6 months of age. Scale bars = 200 μM. **B.** Cross-sectional area of myofibers in TA muscles were measured using ImageJ based on H&E staining. 600 fibers from five mice of each genotype were counted. **C.** Centralized myonuclei in WT and miR-486 KO TA muscle at 6 months of age were counted using ImageJ. 600 fibers from 5 mice of each genotype were counted. Means with different letters are significantly different (Tukey's HSD, $p < 0.05$). **D.** Fibrotic area was quantified as a percentage of total area using ImageJ. 5 mice of each genotype were counted. *p=<0.05.



E. WT vs miR-486 KO RNA-seq



F. g:Profiler enrichment analysis of RNA-seq identified dysregulated transcripts



	Source	Term ID	Term Name	Adjusted P-value
1	GO:CC	GO:0031012	Extracellular matrix	4.76E-15
2	GO:CC	GO:0062023	Collagen-containing extracellular matrix	4.59E-14
3	GO:BP	GO:0051239	Regulation of multicellular organismal process	1.17E-10
4	GO:CC	GO:0005576	Extracellular region	3.51E-10
5	GO:BP	GO:0001944	Vasculature development	5.67E-10

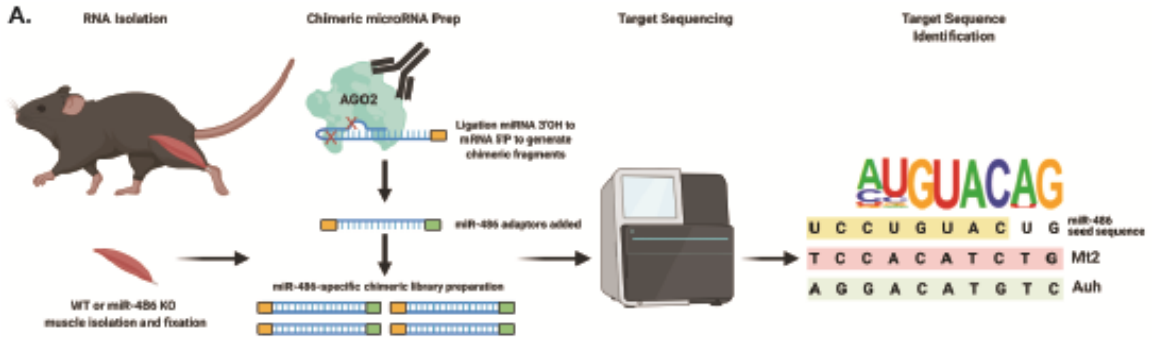
G. Top 10 transcripts increased in expression in miR-486 KO compared to WT as identified by RNA-seq

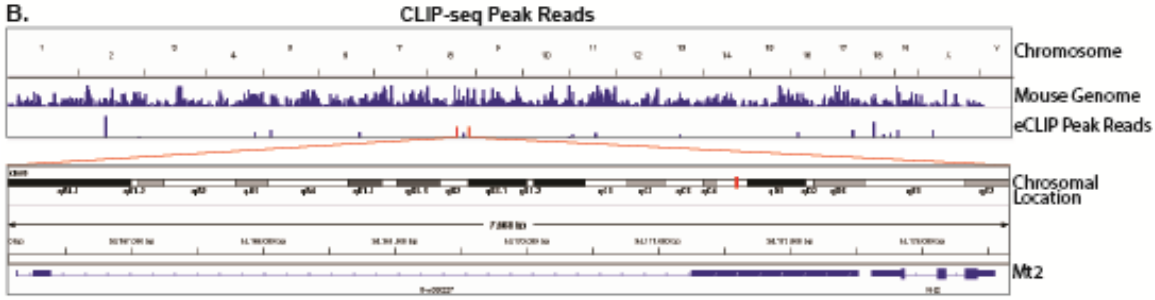
	Gene Symbol	Gene Description	Log2 Fold Change (KO vs WT)	Adjusted p-value
1	Rasd2	RABD family, member 2	2.43	8.27E-37
2	H2-Q8, H2-Q6	c("histocompatibility 2, Q region locus 8", "histocompatibility 2, Q region locus 6")	2.34	3.77E-15
3	Unc13c	unc-13 homolog C (C. elegans)	2.29	1.82E-14
4	H2-Q7	histocompatibility 2, Q region locus 7	1.88	6.82E-08
5	Ppp1r3c	protein phosphatase 1, regulatory (inhibitor) subunit 3C	1.84	3.54E-24
6	Cuzd1	CUB and zona pellucida-like domains 1	1.63	6.59E-08
7	Idh2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	1.62	1.79E-18
8	Cyp2c44	cytochrome P450, family 2, subfamily c, polypeptide 44	1.57	2.51E-05
9	Cda	cytidine deaminase	1.49	1.05E-06
10	Klf15	Kruppel-like factor 15	1.47	3.2E-22

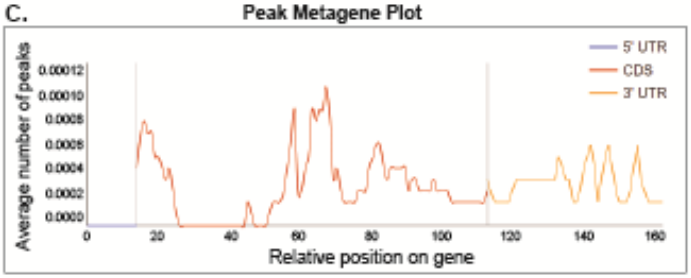
H. Top 10 transcripts decreased in expression in miR-486 KO compared to WT as identified by RNA-seq

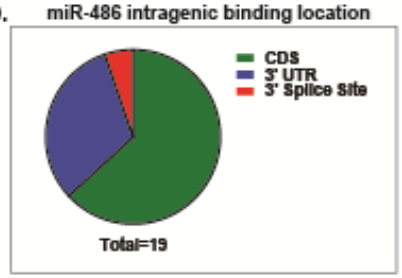
	Gene Symbol	Gene Description	Log2 Fold Change (KO vs WT)	Adjusted p-value
1	Ces2c	carboxylesterase 2C	-2.90	1.11E-18
2	Actc1	actin, alpha, cardiac muscle 1	-2.81	4.95E-23
3	Egr1	early growth response 1	-2.12	1.66E-11
4	Myl2	myosin, light polypeptide 2, regulatory, cardiac, slow	-2.11	9.82E-11
5	Gys2	glycogen synthase 2	-2.00	2.46E-09
6	Gadl1	glutamate decarboxylase-like 1	-1.89	4.62E-11
7	Smox	spermine oxidase	-1.87	6.00E-22
8	Arsl	arylsulfatase I	-1.86	6.32E-10
9	Col1a1	collagen, type I, alpha 1	-1.80	7.21E-19
10	Otog	otogelin	-1.76	1.79E-08

Figure 3. RNA-seq reveals extracellular matrix pathways in miR-486 KO muscle compared to WT. A-D. Quantitative PCR reveals differential expression of myomiRs in 6-month-old miR-486 KO TA muscle compared to WT. **E.** Volcano plot demonstrating the fold change and significance of differential gene expression in 6-month-old miR-486 KO TA muscle compared to WT controls. N=5 mice/cohort were used for comparative analysis. **F.** g:Profiler enrichment analysis of 85 transcripts increased in expression and 159 transcripts decreased in expression based on ≥ 2.0 $\log_2$ fold change of WT vs miR-486 KO RNA-seq analysis. The top 5 pathway hits are listed below the graph. **G.** Table of top 10 transcripts increased in expression in 6-month-old miR-486 KO mouse TA muscle compared to WT as identified by RNA-seq. **H.** Table of top 10 transcripts decreased in expression in 6-month-old miR-486 KO mouse TA muscle compared to WT as identified by RNA-seq.



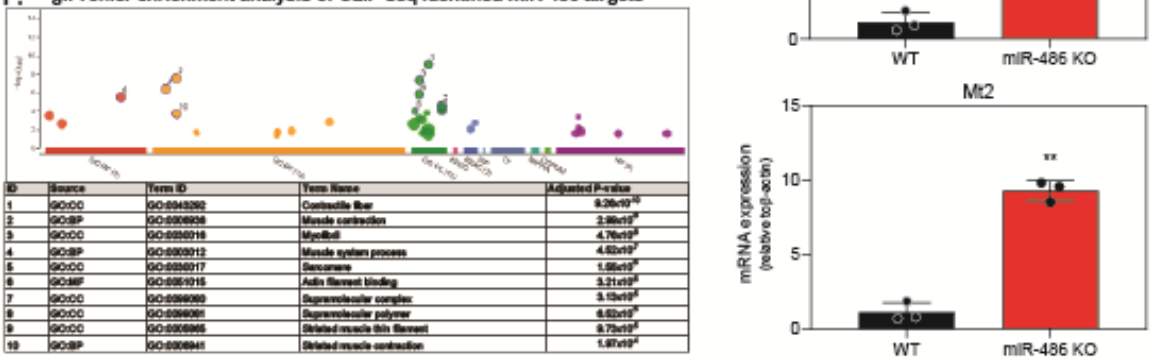






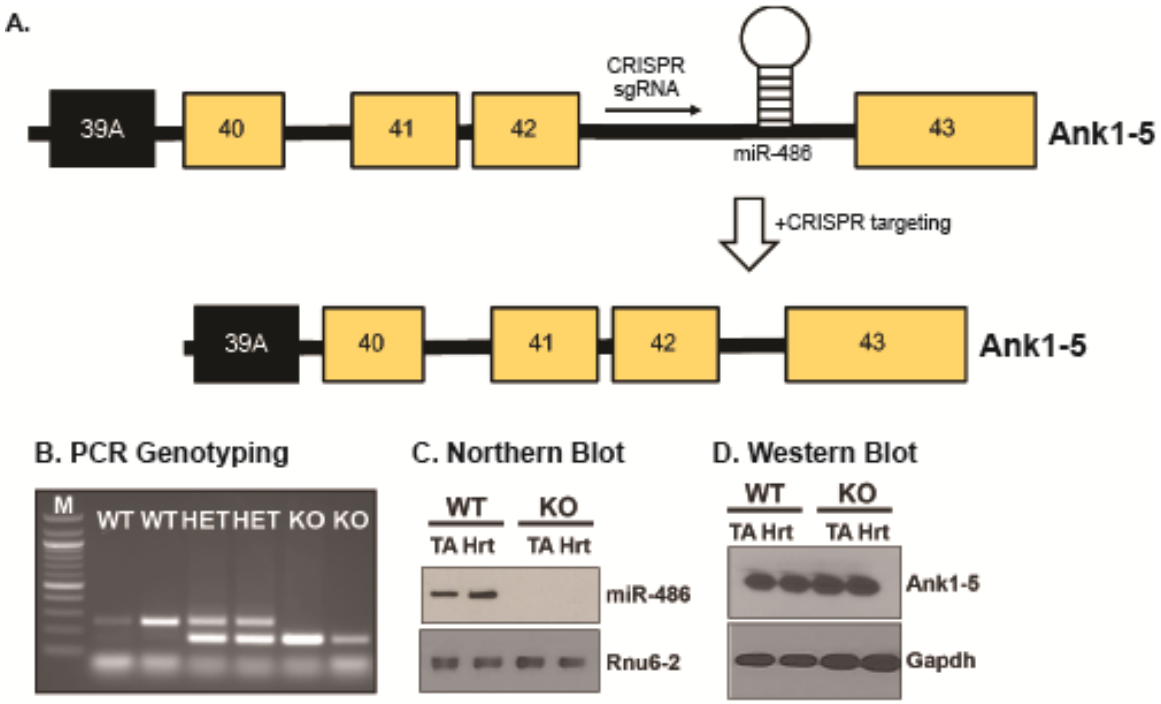
E. Top 10 peaks identified via CLIP-seq

	Gene Symbol	Gene Description	Chromosome	Log2 Fold Change	Strand	miR-486 binding location
1	Eifkp1	Eukaryotic translation initiation factor 4G	chr10	10.84	+	3' splice site
2	Lamp2	Lysosomal associated membrane protein 2	chrX	10.65	-	CDS
3	Bhlhe40	Basic helix-loop-helix family member e40	chr6	10.35	+	3'UTR
4	Auh	AU RNA Binding Methylglutaconyl-CoA Hydratase	chr13	10.02	-	3'UTR
5	Mt2	Metallothionein 2	chr8	9.94	+	CDS
6	Atp5a1	ATP synthase subunit alpha 1	chr10	9.49	+	CDS
7	Myom1	Myomesin 1	chr17	8.87	+	CDS
8	Naca	Nascent polypeptide-associated complex subunit alpha	chr10	8.79	+	CDS
9	Bin1	Myc box-dependent interacting protein 1	chr18	8.59	+	CDS
10	Camk2a	Calcium/calmodulin dependent protein kinase II alpha	chr18	8.59	+	3'UTR

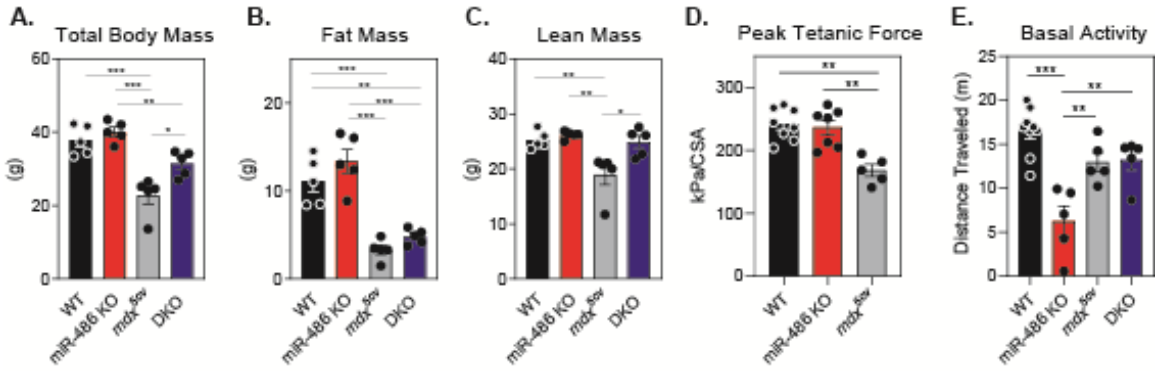


ID	Source	Term ID	Term Name	Adjusted P-value
1	GO:CC	GO:0043292	Contractile fiber	9.26×10^{-10}
2	GO:BP	GO:0006936	Muscle contraction	2.99×10^{-9}
3	GO:CC	GO:0030016	Myofibril	4.76×10^{-9}
4	GO:BP	GO:0003012	Muscle system process	4.62×10^{-7}
5	GO:CC	GO:0030017	Sarcomere	1.05×10^{-6}
6	GO:MF	GO:0051015	Actin filament binding	3.21×10^{-6}
7	GO:CC	GO:0099080	Supramolecular complex	3.10×10^{-5}
8	GO:CC	GO:0099081	Supramolecular polymer	6.52×10^{-5}
9	GO:CC	GO:0005865	Striated muscle thin filament	9.70×10^{-5}
10	GO:BP	GO:0006941	Striated muscle contraction	1.67×10^{-4}

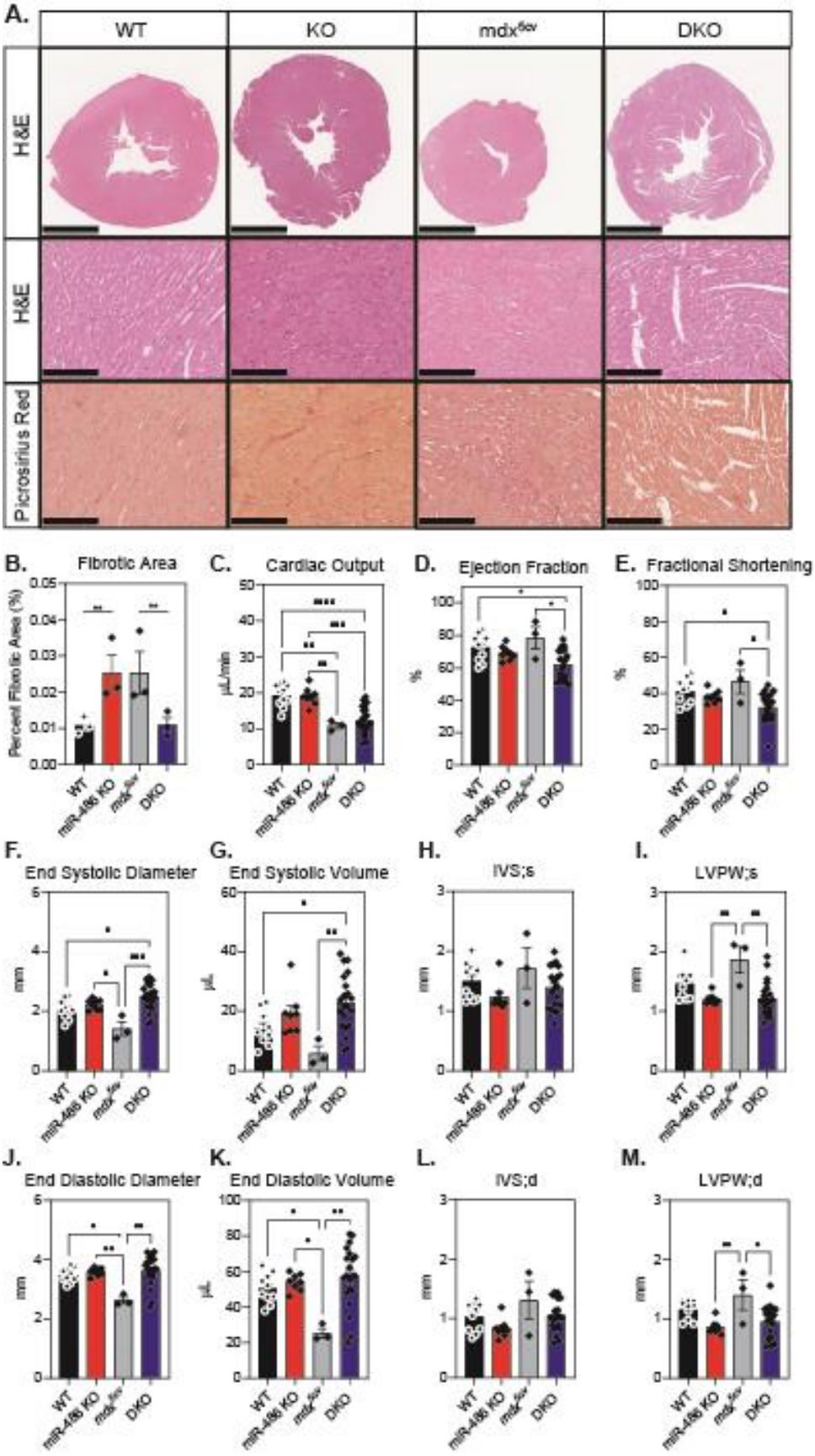
Figure 4. Targeted chimeric dCLIP-seq reveals direct targets of miR-486. A. Schematic demonstrating the workflow for the novel chimeric eCLIP-seq technique to identify miR-486 *in vivo* skeletal muscle target transcripts. TA muscle was harvested from six-month-old WT and *miR-486 KO* male mice and total RNA isolation was completed. The Ago2-miR-486 complex bound to target RNA transcripts was isolated and then sequencing was performed to map the reads of transcripts bound to the Ago2-miR-486 complex. **B.** Chromosomal location of a single top "hit", Mt2, as identified by eCLIP-seq as a direct target of miR-486. Peaks generated using Integrative Genome Viewer. **C.** Metagene plot demonstrating overall miR-486 binding location by relative position on target gene. **D.** Pie chart demonstrating the proportion of miR-486 gene targets and the respective intragenic binding location of miR-486. **E.** Table outlining the top 10 transcripts that were identified as direct targets of miR-486 via CLIP-seq. **F.** g:Profiler enrichment analysis graph demonstrates the most significant cellular pathways associated with the 18 direct miR-486 targets identified via CLIP-seq. The pathway ID number in the table correlates with the numbered dots in the accompanying graph above. **G.** quantitative PCR of Auh and Mt2 in miR-486 KO TA muscle compared to WT. Data points are individual biological replicates, n=3/cohort and error is presented as SEM. **p=≤0.01. mRNA levels are normalized to β-actin and miR-486 KO levels are shown as relative to WT.



Supplemental Figure 1. Identification and selection of miR-486 knockout mice. A Schematic demonstrating sgRNA targeting sites at the ANK1 locus. Black boxes indicate alternative exon 39a, which is the starting exon for ANK1-5. **B** PCR genotyping reveals an 85 bp deletion in intron 42 of ANK1. Deletion contains the entire stem loop of the mature miR-486 sequence. **C** Northern blot confirms no miR-486 detected in miR-486 KO tibialis anterior (TA) or heart muscle. **D** Western blot confirms no change in expression of host gene ANK1-5, which is critical to evaluating phenotypes as a direct result of miR-486 deletion.



Supplemental Figure 2. MicroRNA-486 KO mice demonstrate differences in mass distribution and muscle physiology. A. Total body mass shows overall weight of mice, measured in grams. *p≤0.05, **p≤0.01, ***p≤0.001. **B-C.** Fat & lean masses measured using quantitative magnetic resonance (QMR) demonstrating differences in mass distribution between genotype cohorts. **p≤0.01, ***p≤0.001. **D.** Ex-vivo isolated muscle physiology reveals differences in peak tetanic force relative to myofiber cross-sectional area between genotype cohorts. **p≤0.01. **E.** Locomotor activity, measured over 6 minutes using an open-field test, reveals differences in overall basal activity between genotype cohorts. **p≤0.01, ***p≤0.001.


A.
WT
KO
mdx5cv
DKO
H&E
H&E
Picrosirius Red
B.
Fibrotic Area
Percent Fibrotic Area (%)
C.
Cardiac Output
µL/min
D.
Ejection Fraction
%
E.
Fractional Shortening
%
F.
End Systolic Diameter
mm
G.
End Systolic Volume
µL
H.
IVS;s
mm
I.
LVPW;s
mm
J.
End Diastolic Diameter
mm
K.
End Diastolic Volume
µL
L.
IVS;d
mm
M.
LVPW;d
mm
WT
miR-486 KO
mdx5cv
DKO

Supplemental Figure 3. MicroRNA-486 knockout may result in functional and histological cardiac dysfunction. A. Top row: representative hematoxylin and eosin stained transverse sections of isolated left ventricle (LV). Scale bar = 2mm. Middle row: representative hematoxylin and eosin stained left ventricular myocardial sections. Scale bar = 200µM. Bottom row: representative picrosirius red stained images of left ventricular myocardial sections. Scale bar = 200 µM. Dark red areas indicate fibrotic tissue. **B.** Fibrotic area calculated from picrosirius red stained sections using ImageJ (5 images from 3 animals per genotype). **p≤0.01. **C-M.** Cardiac function parameters were obtained using VisualSonics small animal echocardiogram equipment and analyzed using VevoLab software. 8-20 mice per genotype were analyzed. Data is represented as mean ± SEM. *p≤0.05, **p≤0.01.

A. Full list of peaks identified via CLIP-seq

Chromosome	Log2 Fold Change	Strand	Gene Symbol	Gene Name	miR-486 binding location
chr16	10.84	+	Eif4g1	Eukaryotic translation initiation factor 4G	3' splice site
chrX	10.53	-	Lamp2	Lysosomal associated membrane protein 2	CDS
chr6	10.25	+	Bhlhe40	Basic helix-loop-helix family member e40	3' UTR
chr13	10.02	-	Auh	AU RNA binding methylglutaconyl-CoA Hydratase	3' UTR
chr8	9.94	+	Mt2	Metallothionein 2	CDS
chr18	9.48	+	Atp5a1	AtP synthase subunit alpha 1	CDS
chr17	8.87	+	Myom1	Myomesin 1	CDS
chr10	8.79	+	Naca	Nascent polypeptide-associated complex subunit alpha	CDS
chr18	8.59	+	Bin1	Myc box-dependent interacting protein 1	CDS
chr18	8.59	+	Camk2a	Calcium/calmodulin dependent protein kinase II alpha	3' UTR
chr5	8.52	-	Phtf2	Putative homeodomain transcription factor 2	3' UTR
chr2	8.52	-	Ttn	Titin	CDS
chr4	8.39	+	Trim63	Tripartite motif containing 63	3' UTR
chr19	8.30	+	Pygm	Glycogen phosphorylase muscle associated	CDS
chr11	6.62	-	Pgam2	Phosphoglycerate mutase 2	CDS
chr11	5.66	+	Myh4	Myosin heavy chain 4	CDS
chr19	5.57	-	Actn3	Alpha-actinin-3	CDS
chr2	4.79	-	Tnnc2	Troponin C2	CDS

Table 1. eCLIP-seq reveals miR-486 target transcripts. A. Table outlining 18 targets of miR-486 identified by eCLIP-seq. Targets are listed in descending order of log_2 fold change. Targets were identified in 6-month WT TA muscle compared to miR-486 KO TA muscle. N=2 mice/cohort. “miR-486 binding location” refers to the intragenic region of the mRNA target where miR-486 binds.

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CHAPTER 5

THE FUNCTIONAL VERSATILITY OF GENETIC AND EPIGENETIC MODIFIERS REVEALS PROMISING TARGETS FOR NOVEL DMD THERAPEUTICS

The field of genetic and epigenetic modifiers provides novel avenues for therapeutic entry points for DMD

Over the course of the last several decades, there has been significant effort in the area of pharmacological development of DMD therapeutics [1-4]. Because of the vast array of clinical phenotypes that are strategically targeted, categories of experimental therapeutic agents range widely, including those aimed at Dystrophin protein restoration, anti-inflammation, Myostatin inhibition, and modulation of Utrophin (a Dystrophin analog) expression [1, 2, 4-9]. Although a significant number of compounds have undergone clinical trials, the results from these studies have been disappointing and have resulted in few approved new standard-of-care treatment options for DMD patients [10-13]. One notable exception to this was the 2017 FDA approval of the corticosteroid Deflazacort. Although originally approved for medical use in Europe in 1985, it was not approved in the United States until over 30 years later for the treatment of DMD patients 5 years and older [14, 15]. Approval of Deflazacort was a positive step in providing an additional corticosteroid for patients who may be unresponsive to other options; however, the use of corticosteroids remains highly controversial with regard to risks-vs-benefits of long-term treatment [16, 17]. The combination of a multitude of failed clinical trials and mildly efficacious corticosteroids leaves a severe lack of viable treatment options for patients and

demonstrates the importance of utilizing different multidisciplinary approaches to identifying therapeutic entry points outside of agent categories that have demonstrated lack of efficacy.

Our lab, as well as others, has demonstrated that differential expression of epigenetic regulators of gene expression can influence disease pathology and functional outcomes in DMD animal models [18-25]. Several of these epigenetic regulatory mechanisms, including histone acetylation and deacetylation, methylation modulation, and microRNA expression, are being investigated to further understand secondary pathologies that are contributing to disease progression independent of Dystrophin expression [25-28]. Investigation of the epigenetic regulation of these secondary pathologies provides experimental benefit because of the multitude of potential therapeutic entry points in a single mechanistic pathway. Continuing to uncover both genetic and epigenetic mechanisms for these disease-driven changes in gene expression is crucial to the advancement of therapeutic development for the treatment of DMD primarily because successfully targeting and restoring functional full-length Dystrophin remains unachievable to date.

Epigenetic regulation of gene expression by microRNAs, specifically miR-486, is a promising avenue for therapeutic development

MicroRNAs are short, 18-24 nucleotide non-coding RNAs that function most predominantly as potent inhibitors of gene expression by binding to target mRNA transcripts containing complementary sequences [29]. MicroRNAs, also commonly referred to as miRs, have grown in popularity with regard to understanding mechanisms of

gene expression modulation in over 50 animal genomes [30]. MicroRNAs are involved in nearly every cellular process and are critical for normal development and homeostasis of both cells and whole organisms[31]. MicroRNAs have become a prominent field of study because of their ability to target up to thousands of genes [32-34]. In addition, it has been demonstrated that microRNAs can preferentially target specific gene families and pathways [33]. This characteristic provides a significant benefit with regard to therapeutic development because secondary disease pathways may be targeted broadly at multiple steps within those pathways for identifying one or more optimal therapeutic entry points.

A family of muscle-enriched microRNAs, known as myomiRs, have been investigated due to their correlation with severity and progression of muscle diseases [35-38]. This myomiR family includes miR-1, miR-133a/b, miR-206, miR-208a/b, miR-486, and miR-499[39]. Despite a myriad of studies that demonstrate this correlation between myomiR expression and disease progression, the field is lacking in a deeper understanding of how directly modulating expression of these microRNAs might contribute to disease pathology. It has been shown previously that decreased expression of miR-1, -133, and -206 are associated with inhibited muscle regeneration and that overexpressing them in dystrophic muscle can partially restore muscle regeneration capacity and delay disease progression [40-43]. Interestingly, although these miRs demonstrate decreased expression in skeletal muscle, the inverse is seen in serum of DMD patients, where serum levels of these miRs is significantly increased compared to healthy controls [44]. This discrepancy in myomiR expression based on the serum or tissues being investigated and the inherent tissue-specific expression of families of microRNAs reveals the need for studies that dive

deeper in understanding patterns of expression changes in disease pathology how those expression changes may be therapeutically relevant.

Contributing to this effort previous data from our lab has shown that transgenic muscle-specific overexpression of miR-486 in the dystrophic *mdx*^{5cv} mouse model can improve histological and functional deficits observed in this DMD model [18, 19]. In chapter 4, I further elucidate the role of miR-486 in skeletal muscle by demonstrating that its global ablation in mice can significantly disrupt muscle histology, as evidenced by abnormal myofiber cross-sectional area, abundant centralized myonuclei, and altered distribution of myofiber types. Each of these phenotypes is a hallmark of myofiber degeneration, demonstrating that ablation of miR-486 likely plays a role in maintaining architectural homeostasis of skeletal muscle. In addition to these results, I argue that understanding the direct downstream effects of miR-486 ablation can lead us to understanding how modulating miR-486 expression may be a novel therapeutic strategy. To demonstrate the importance of this, I undertook two separate sequencing strategies in order to identify novel target pathways directly modulated by miR-486 expression in skeletal muscle: 1) Traditional RNA-sequencing and, 2) chimeric eCLIP-sequencing (enhanced cross-linking immunoprecipitation sequencing). This miR-486 study is novel in the use of chimeric eCLIP technology pioneered by Eclipse Bioinnovations, Inc (San Diego, CA), which identifies direct mRNA targets of miR-486 by selectively isolating mRNAs bound by miR-486 and sequencing the miRNA-mRNA bound products. Utilizing this two-method sequencing approach was critical to understanding both direct targets of miR-486 *in vivo* as well as understanding the potential biological relevance of those targets.

Much is still left to uncover with regard to miR-486 and its direct role in modulating dystrophic pathology in different muscle groups, at different age time points, and in characterizing transient overexpression to identify therapeutic potential. First, experiments need to be completed to validate the eCLIP-identified miR-486 target transcripts. This should be done in both WT, miR-486 KO, and *mdx*^{5cv} skeletal muscle. I predict that the subsequent protein products from the miR-486 mRNA targets will be overexpressed in miR-486 KO muscle as well as in *mdx*^{5cv} muscle compared to WT. This is due to the fact that if miR-486 expression is decreased in disease pathology which is further exacerbating disease progression, it is the overabundance of proteins from the miR-486 mRNA targets that are likely contributing to that exacerbation. Once those proteins are identified, experiments can be done both in cell culture and in animal models to investigate the downregulation of those proteins and determine if a potential pathological benefit of that downregulation for DMD exists. Likely, the overexpression of multiple proteins will be found to be contributing to DMD disease exacerbation and future experiments will need to be done to elucidate their pathological roles in tandem as well as individually.

Several promising patterns were seen after analyzing the eCLIP and RNA-seq datasets that may give a foundation for identifying and characterizing these potentially pathological proteins. Gene ontology profiling of all transcripts both increased and decreased in expression in miR-486 KO muscle compared to WT, identified via RNA-seq, revealed that three of the top five pathways of were related to the extracellular matrix. This was a key finding because we know extracellular matrix remodeling during inflammation

and fibrosis is an established pathological process in DMD [45, 46]. I have emphasized the importance of anti-inflammatory therapeutics in modulation of disease and if extracellular matrix components are significantly dysregulated, this is an area of needed continued study with high therapeutic relevance. This is also an interesting finding because of the significant disruption seen in the skeletal muscle histology, demonstrated in chapter 4 (Figure 2A). Although an increase in fibrotic tissue is not seen in the 6-month miR-486 KO TA muscle sections, it does not discount the role that dysregulated extracellular matrix remodeling could play in producing the phenotype seen in the miR-486 KO TA muscle. Further investigation of specific ECM components, specifically those already known to be involved in dystrophic pathology, would shed light on the overall role of the overall effects of miR-486 ablation on extracellular matrix and skeletal muscle architecture.

Two experimental considerations will be important in fully characterizing many of the discussed phenotypes and the potential for therapeutic intervention: age and muscle fiber type specificity. It has been well established that fast-twitch, glycolytic type 2 fibers preferentially degenerate faster than other muscle fiber types in dystrophic pathology [47]. Because of this, it is important to be able to characterize fiber type specific effects of phenotypes seen as a result of miR-486 KO or modulation of its target transcripts and their subsequent proteins. Future experiments aimed at investigating those potentially pathologically overexpressed proteins as a result of decreased miR-486 expression should be completed in both highly oxidative and highly glycolytic muscle groups to determine the correlation between fiber type and phenotypic severity of those overexpressed proteins. I predict that any proteins that are identified as pathologically overexpressed in both miR-

486 KO and *mdx*^{5cv} will be significantly overexpressed in highly glycolytic muscle groups, like the TA compared to more predominantly oxidative muscle groups, like the soleus.

In addition to delineating the correlation between miR-486 expression, its direct targets, and previously established disease pathology, understanding these pathological patterns over multiple age groups will be important for future investigation of therapeutic potential of miR-486. All experiments discussed in chapter 4 were completed on 6-month male mice in order to ensure characterization of a robust phenotype after established disease. However, investigating the progression of phenotype and expression of miR-486 targets at multiple age time-points (1, 2, & 12 months) will allow for identification of important time-relevant changes in phenotype or to see patterns of progression over time. Especially with regard to investigating miR-486 as a potential therapeutic, understanding when miR-486 overexpression is most beneficial for phenotype rescue will be key for future preclinical studies.

Targeting inflammation without glucocorticoids presents the beginning of a necessary direction for treatment development

As previously described, the risks and benefits to long-term use of corticosteroids for patients with DMD are controversial and fail to demonstrate efficacy across the entire affected patient population [48-52]. However, mitigating inflammation and fibrosis has remained the primary therapeutic strategy in dystrophic disease mitigation because patients who do respond positively to treatment experience a delayed time to ambulation loss, reduced or delayed need for scoliosis surgery, and preserved respiratory function [53]. This demonstrates the need to develop therapeutics targeted at modulating inflammation and

fibrosis that maintain a mechanism of action different from traditional corticosteroids. To contribute to this effort, I undertook a study investigating the efficacy of a novel experimental anti-inflammatory drug, KPT-350 (now renamed "BIIB100" after rights were obtained by Biogen Inc.). KPT-350 is classified as a SINE (Selective Inhibitors of Nuclear Export) compound [54]. The mechanism behind SINE compounds and their anti-inflammatory properties lies in their inhibitory binding of XPO1 (CRM1), resulting in a nuclear accumulation of I_KB, which binds NF_KB and prevents the transcription of inflammation-related genes [55-57]. The results of this study, discussed further in chapter 3, demonstrate that inhibiting NF_KB mediated transcription of genes involved in inflammation and fibrosis can ameliorate histological evidence of myofiber degeneration. This evidence includes reduced centralized myonuclei, restoration of fiber size distribution, and decreased total fibrotic area. Dystrophic mice treated with KPT-350 demonstrated a significant increase in basal locomotor activity compared to untreated controls. Regarding inflammation and fibrosis, KPT-350 treatment resulted in a reduction of pro-inflammatory and fibrotic cytokines like TNF-α, interferon-δ, and interleukins. These results taken together are positive in the amelioration of dystrophic symptoms from a histological and anti-inflammatory standpoint and demonstrate clinical relevance similar to currently prescribed corticosteroids [58]. Future investigation of this compound should be aimed at addressing long-term treatment in a dystrophic mouse model for the identification of negative side effects that mirror those induced by corticosteroids. Overall, this work contributes to the field of advancing therapeutic strategies for the mitigation of dystrophic pathologies through pharmacologically modulating gene expression of factors involved in secondary DMD disease pathways.

In addition to elucidating the role of non-glucocorticoid anti-inflammatory compounds, our efforts with testing KPT-350 provided an additional perspective worth investigating: the potential role of targeting nucleocytoplasmic transport as an avenue for anti-inflammatory therapeutic development. To date, much of the work uncovering the mechanisms of nucleocytoplasmic transport as a therapeutic strategy has been within the field of cancer and chemotherapeutic development [59-61]. The key targetable factor in many of these studies, as it was in our KPT-350 study, is the cargo export protein, XPO1. XPO1 controls the cytoplasmic translocation from the nucleus of many RNAs and proteins critical to various cellular functions [62]. In many cancers, XPO1 has found to be overexpressed which often leads to excess nucleocytoplasmic transport [63]. In addition to increased nucleocytoplasmic transport, overexpression of XPO1 has been associated with drug resistance and is inversely correlated with disease prognosis in several cancers [64-66]. This evidence has led to an abundance of studies investigating both the possibility of improving cancer prognosis by decreasing XPO1 expression and inhibiting XPO1 nucleocytoplasmic transport [67]. In addition to cancer, a few neurodegenerative and neuromuscular diseases have been showed to result from inhibited nucleocytoplasmic transport due to toxic protein aggregation in the cytoplasm. For example, both Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) are contain the abnormal deposition and aggregation of the RNA binding protein TDP43 in the cytoplasm [68, 69]. This pathological characteristic provided an opportunity to investigate the possible therapeutic benefit of reducing nucleocytoplasmic transport by inhibiting XPO1. It was found that two different compounds, KPT-350 (described extensively in chapter 3)

and KPT-335 both moderately reduced cytoplasmic TDP43 aggregation, which increased cell survival in neuronal cultures [69]. In addition, this particular study reported that these SINE compounds provided a neuroprotective benefit, independent of XPO1 inhibition [69]. Other studies initially demonstrated similar neuroprotective and pathologically beneficial effects with *in vivo* XPO1 inhibition, like the reduction of cortical hyperexcitability in a mouse model of traumatic brain injury [70]. In 2019, the FDA approved the first SINE compound, trade name Selinexor, for the treatment of relapsed or refractory multiple myeloma and has since been approved for large B-cell lymphoma. Although a positive step for therapeutic options for patients, off-target effects have been less than encouraging. Some studies have revealed significant systemic toxicity in addition to minimal efficiency in both human and animal models of disease which has prevented and even halted certain clinical trials [71]. In addition, thrombocytopenia, natremia, anorexia, nausea, diarrhea, and emesis have all been reported as significant adverse events for many Multiple Myeloma patients and 18% of patients report discontinuing treatment because of the severity of those side effects [72]. Overall, this demonstrates that although targeting nucleocytoplasmic transport does have data to support its potential positive effects, a lot more investigation is needed in order to make human disease treatment a reality.

Final Thoughts

As the field of therapeutic strategies for DMD treatment advances, we will be left with yet more questions about off-target effects of pharmacological treatment, risk versus benefit of resulting side effects, drug development and clinical trial costs, and efficacy

standards that have yet to be clinically determined. MicroRNAs are a promising avenue for investigation into identifying cascading secondary pathologies that can be targeted for treatment with a single compound. Additionally, developing anti-inflammatory therapeutics that provide an alternative mechanism from corticosteroids could have an extraordinary impact on patient quality of life. Realistically, specific and strategic combinatorial therapies need to be investigated as standard-of-care treatment options due to single-therapy treatments having provided little evidence of long-term benefit across the entire DMD patient population. This shines light on the fact that DMD research needs to be included in the emerging field of precision medicine. By identifying patients' inherited or *de novo* genetic modifiers of disease, understanding their microRNA profiles as biomarkers for disease progression and potential as therapeutic compounds, and effectively measuring efficacy in response to anti-inflammatory treatment, a therapeutic cocktail could be crafted for individual patients. This multi-pronged axis could be utilized to drive a multidisciplinary approach in identifying how to give DMD patients the best treatment options and the most enriched quality of life for as long as possible.

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APPENDIX A

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 10-Aug-2018

TO: Alexander, Matthew

FROM:

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 10-Aug-2018.

Protocol PI:	Alexander, Matthew
Title:	A miR-486/DOCK3 signaling axis modulates dystrophin-deficient pathology
Sponsor:	National Institute of Child Health and Human Development/NIH/DHHS
Animal Project Number (APN):	IACUC-21393

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 09-Aug-2021.

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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