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Potential Drug Treatment for Duchenne Muscular Dystrophy Which Could be Through Upregulation of Lipin1

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POTENTIAL DRUG TREATMENT FOR DUCHENNE MUSCULAR DYSTROPHY WHICH COULD BE THROUGH UPREGULATION OF LIPIN1

A thesis submitted in partial fulfillment

of the requirement for the degree of

Master of Science

By

RAJSI Y. THAKER

M.Sc., Biotechnology, Sardar Patel University, India, 2018

2021

Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

July 28, 2021

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY <u>Rajsi Y Thaker</u> ENTITLED <u>Potential drug treatment for Duchenne muscular dystrophy which could be through upregulation of lipin1</u> BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF <u>Master of Science</u>.

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Abstract

Thaker, Rajsi Y. M.S. Department of Biochemistry and Molecular Biology, Wright State University,2021. Potential drug treatment for Duchenne muscular dystrophy which could be through upregulation of lipin1

Duchenne muscular dystrophy (DMD) is a genetic disorder leading to progressive muscle degeneration and weakness due to mutation in dystrophin gene, which is very important for maintaining muscle membrane integrity. Dystrophin is the largest gene in the human genome therefore more prone to mutation. There is currently no cure for DMD. Our lab recently found that Lipin1 deficient myofibers showed upregulation of necroptosis correlated with the loss of muscle membrane integrity. Our primary approach for ameliorating dystrophic phenotype in DMD is through reduction of necroptosis using drugs which can potentially upregulate Lipin1 expression. In this study, we identified two drugs i.e., dexamethasone which is a glucocorticoid and rosiglitazone which is PPARγ agonist, can elevate Lipin1 mRNA and protein expression levels *in vivo* and *in vitro*. Mdx mice treated with dexamethasone for two weeks and rosiglitazone for one week had elevated Lipin1 expression level and downregulated necroptotic markers including RIPK1, RIPK3, and MLKL. Rosiglitazone treatment in mdx mice also downregulated apoptotic markers including BAX, BAK and cleaved caspase-3. Our future study will identify

whether the effects of dexamethasone and rosiglitazone on the inhibition of necroptotic markers and the improvement of membrane integrity of dystrophic muscles are through the upregulation of Lipin1 expression.

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ACKNOWLEDGEMENT

I would like to express my special thanks of gratitude to my advisor, *Dr. Hongmei Ren*. She was always very helpful when I needed any assistance in my project, and she always brings out best in her students. I have learned many things from her, not only professionally but also in terms of being successful in life. She always encouraged me to work harder and think smarter. She have made a big impact on my life, and I never imagined that I could make so much progress in such a short time, but she always had faith in me and always supported me.

I would also like to thank my committee members, *Dr. Michael Leffak* and *Dr. Weiwen Long* for their expertise and valuable suggestions on my project and for their time to review my thesis. I would like to thank Biochemistry and Molecular Biology department for providing me technical support.

A special thanks to all Ren lab members and it was a great pleasure to work with them. I would like to specially thank *Abdi* and *Abdullah* for all their support and help they have provided me and for always motivating me in difficult times. I am also very thankful for all my friends who have been there for me throughout my time at graduate school. Most importantly I would like to thank my beloved parents who always supported and encouraged me to pursue my goals.

I would also like to thank *Dr. Sanjay Lal,* my former mentor for always believing in me and without whom I could have never imagined pursuing research.

DEDICATIONS

I would like to dedicate this thesis to my Parents,

Yogesh J. Thaker

&

Alpa Y. Thaker

1. Introduction

1.1 Muscular dystrophies (MD).

Skeletal muscles account for 40% of the human body weight. Muscular dystrophies (MD) are a clinically and genetically diverse group of rare muscle disorders that cause progressive weakness and breakdown of skeletal muscles over time (Dalton et al., 2015). There are more than 30 different types of muscular dystrophies which vary in their onset, severity, and muscle groups affected. All muscular dystrophies become more severe with increasing age (Muscular Dystrophy: Hope Through Research NINDS, 2016). Almost all MD are genetically inherited resulting from mutations in one of the thousand genes. These genes are involved in the production of proteins which are crucial for muscle membrane integrity. Sometimes MD can also result from somatic mutations that are not carried by either parent (Lovering et al., 2005; Muscular Dystrophy: Hope Through Research NINDS, 2016). Different gene mutations are involved in causing these various dystrophies. Some examples of these dystrophies include Duchenne and Becker muscular dystrophy; Limb-gridle muscular dystrophy; Distal muscular dystrophy; congenital muscular dystrophy;

Myotonic dystrophy; Emery-Dreifuss; Facioscapulohumeral; Oculopharyngeal etc. (Lovering et al., 2005).

Everyday life of the patients afflicted from muscular dystrophy can become challenging and stressful. The need for assistance to perform their daily life routine becomes increasingly prominent. Regardless of condition, all MD are characterized by declining muscle strength and eventual loss of ambulation, which are likely to reduce independence and self-perception of physical function (Gabriel and Bowling, 2004; Jacques et al., 2018). According to a study, the average annual direct medical costs for a muscular dystrophy patient are more than $80,120 per year; however, costs can increase. Many patients will require household caring that could cost up to more than $58,440 per year. According to the ALS Hope Foundation additionally, invasive ventilation can cost between $150,000 and $330,000 per year (Landfeldt et al., 2014).

There are a variety of diagnostic tests to differentiate various kinds of MD. Muscle weakness from injury, toxic exposure, medications, or other muscle diseases should be ruled out during diagnosis. Some of the tests include:

1. **Blood test**- A blood test can be used to assess the levels of certain substances like creatinine kinase, serum aldolase and myoglobin that are released in the blood circulation. Elevated level of these substances can be an indication of

muscle weakness, injury, or disease. However further testing is likely necessary (Muscular Dystrophy: Hope Through Research NINDS, 2016).

2. **Muscle biopsies**- Muscle biopsies are performed by removing a small piece of muscle tissue with a needle or a small incision to examine the tissue under a microscope for signs of MD which differs from normal muscle. Dystrophic muscles are characterized by centrally located nuclei, muscle fibers appear flattened and narrow, can display inflammation, fibrosis, necrosis and more (Bushby et al., 2010).

3. **Genetic testing**- Genetic testing can be used to look for genes that have been linked to or cause inherited muscle disease. Certain neuromuscular diseases, including Duchenne muscular dystrophy can be confirmed using DNA analysis to determine if there is a mutation within the dystrophin gene (Bushby et al., 2010).

4. **Neurological tests**- Neurological tests are performed to rule out other nervous system disorders which also displays a pattern of muscle weakness and wasting. It also involves assessment of reflexes and coordination that detects contractions. In addition to these tests, heart testing, exercise assessment and MRI are also used (Muscular Dystrophy: Hope Through Research NINDS, 2016).

Of the previously described muscular dystrophies, Duchenne muscular dystrophy (DMD) accounts for approximately 50% of all cases and is considered as the most common type of dystrophy (Muscular Dystrophy: Hope Through Research NINDS, 2016; Lovering et al., 2005).

1.2 Duchenne Muscular Dystrophy (DMD).

In early 1850s, medical journals started reporting cases that described boys who grew progressively weaker and lost their ability to walk with increasing age. In that following decade, French neurologist, Guillaume Duchenne, reported 13 boys displaying the same characteristics and severe form of disease which is now known as Duchenne muscular dystrophy (DMD) (Muscular Dystrophy: Hope Through Research NINDS, 2016).

DMD is a genetic disorder that leads to progressive muscle degeneration and weakness due to mutation in dystrophin gene, which results in the production of a defective protein. The dystrophin gene is the largest gene in the human genome which comprises of 2.4 million base pairs. The sheer size of this gene makes it more prone to mutation (Lovering et al., 2005; Duan et al., 2021). DMD is an X-linked recessive disorder, which means that the mutated dystrophin gene that is responsible for the disorder is located on X chromosome, and it affects mostly males because

they have only one X chromosome (Lovering et al., 2005). DMD affects 1 in 5000 males worldwide and 250,000 individuals in the USA alone (Duan et al., 2021).

Many Patients with DMD fail to receive early treatment as they appear to be normal during the initial stages of life (1-2 years). Noticeable changes begin to appear around three years of age with continues progression of skeletal muscle wasting and weakness (Lovering et al., 2005). Walking problems appear around age of six years. Many individuals show signs such as clumsiness, falling and gait changes as well as difficulties to climb stairs (Ryder et al., 2017). As teenagers their strength starts to fade and everyday life of DMD patients becomes more difficult. Almost at 8-14 years, they are bound to wheelchair as the progression of muscle weakness results in loss of ambulation (Ryder et at., 2017; Lovering et al., 2005; Duan et al., 2021). Once they become wheelchair bound, other related complications involve distorted muscular structure that progresses more rapidly and results in scoliosis that further leads to curving of spine sideways or forward or backward. These complications can lead to some of the orthopaedic problems especially if one shoulder or hip becomes higher than the other. This orthopaedic complication results into secondary complication involving cardiomyopathy in their late teens. At about 14 years of age, children with DMD need cough assistant machines to clear their lungs. Ventilation may also be required at night (Ryder et al., 2017). Other associated complications may include shoulders and arms are held back awkwardly when walking; belly sticks

out; poor balance; thick lower leg muscles and more. The average lifespan of most DMD patients is expected to be around 25 to 30 years; a point where muscles can no longer fight degeneration process (Ryder et al., 2017; Duan et al., 2021).

1.3 Pathogenesis of DMD.

DMD gene encodes muscle specific dystrophin protein which has a molecular weight of 427 KDa. Dystrophin is a rod shaped protein that is present in the cytoplasm. This protein contains four major functional domains: an actin binding domain at N-terminus; a central rod domain that provides flexibility towards mechanical stretch; a cysteine rich domain that interacts with dystroglycan complex; and a C-terminal domain that interacts with other sarcolemmal proteins (Houang et al., 2018). A deficiency or production of dysfunctional dystrophin protein leads to various myopathies referred as muscular dystrophy. DMD and Becker Muscular Dystrophy (BMD) are the most common types, both of which are associated with the mutations of dystrophin (Rumeur, 2015). There are thousands of mutations associated with DMD out of which 60 to 70% mutations comprise deletion mutations, 5 to 15% are duplications and 20% are point mutations, small deletions or insertions (Aartsma-Rus, et al., 2006). Although dystrophin accounts for only 0.002% of total muscle proteins, its absence or dysfunctional form can result in the development of severe and immedicable disorders leading to muscle weakness and wasting (Hoffman et al., 1987). There are a number of animal models to study DMD

such as the MDX mouse model, dystrophin/utrophin double KO mice, golden retriever muscular dystrophy dog model, hypertrophic feline muscular dystrophy cat model, etc. Our study involves the MDX mouse model that contains a non-sense mutation in exon 23 which leads to a short and non-functional dystrophin protein (Blake et al., 2002; Sicinski et al., 1989).

In DMD, the major pathology is loss of muscle membrane integrity and muscle cell death. The current approaches to mitigate the pathology of DMD are focused on strengthening muscle membrane integrity (Dadgar et al., 2014). In our body all the movements are controlled by contracting and relaxing various muscles, even the involuntary ones. A muscle is made up of many long tubular cells known as myocytes which are responsible for contraction (Mukund and Subramaniam, 2020). Dystrophin is an important structural protein that is located near the membrane of myocytes and its main function is to connect the internal cytoskeleton to the extracellular matrix in the muscle. It is also a molecular shock absorber thereby, protecting muscle membrane from shocks with every contraction (Clafin and Brooks, 2008). In normal skeletal muscles, dystrophin amino terminus end binds to F-actin, and its carboxyl terminus end binds to the dystrophin-associated protein complex (DAPC) at the sarcolemma (Nowak and Davies, 2004). Dystroglycans, sarcoglycans, integrins, and caveolin are all components of the DAPC, and

mutations in any of these components cause autosomal inherited muscular dystrophies (Dalkilic and Kunkel, 2003).

In DMD, genetic mutations cause dystrophin to be extremely short, frequently lacking the dystroglycan binding end rendering this protein dysfunctional. In the absence of fully functional dystrophin protein, other membrane proteins diminish. As a result, every time the muscle contracts, small ribs or micro tears form in the muscle membrane because there is no dystrophin to hold the membrane intact. (Straub and Campbell,1997). Small membrane disruptions or micro-tears are thought to cause damage, which in turn can disrupt membrane integrity and result in transient extracellular calcium influx, progressive muscle fiber damage and leakage of intracellular contents from the muscle membrane (Dadgar et al., 2014).

Another pathology in DMD that results due to loss of membrane integrity is, the extracellular calcium influx raises intracellular calcium concentration, which activates calcium release from the sarcoplasmic reticulum (SR) and raises calcium concentration even more. Calcium overload eventually leads to myocyte hyper contracture and cell death (Law et al., 2020). Creatine kinase is another important molecule that diffuses out through the ribs from myocytes and enters the blood, producing a diagnostic marker for DMD (Ozawa and Yoshida, 1999). Muscle repair and regeneration can occur at younger ages, but as patients get older, their ability to regenerate the constantly degenerating myocytes declines, and fat and scar tissues

replace them (Baird et al., 2012). Not only the skeletal muscles, but also the heart muscles (McNally et al., 2015) and diaphragm muscles are affected in DMD (Pennati et al., 2019) with loss of membrane integrity.

1.4 Current treatments in DMD.

Till date there are various available treatments for Duchenne muscular dystrophy but each of them has their own limitations.

1. **Drugs:**

   The major pharmacological treatment for DMD is glucocorticoids, especially FDA-approved prednisone and deflazacort, which have been used for over two decades (Beytia et al., 2012). The mechanism of action of these drugs is through anti-inflammation pathway. These drugs are used in treatment of various diseases caused by an overactive immune system, such as allergies, asthma, autoimmune diseases, and sepsis (Rhen and Cidlowski, 2005). Using these drugs in DMD have reported various benefits by many studies such as increase in muscular strength (Angelini, 2007). Long term use of these drugs helps in maintaining lung and cardiac function (Houde et al., 2008). Another study found that 93% of prednisone-treated patients had no ventricular dysfunction at the age of 12, compared to 53% of untreated patients (Markham et al., 2008). One study has also reported that using these drugs can increase

life expectancy of DMD patients (Beytia et al., 2012). Overall various studies have reported that treating DMD patients with corticosteroids can ease the phenotype of DMD patients to some extent. However, this drug has limitations and side effects and the major one is the reduction in height and weight gain of patients. (Moxley et al., 2010; Markham et al., 2008). Cushingoid facies, acne, hirsutism, arterial hypertension, behavior disorder, delayed puberty, vertebral fractures, immunosuppression, and gastrointestinal problems are some of the other side effects (Beytia et al., 2012). The major setback for these drugs is that they can only be used when the patients are in a plateau phase, i.e., when patients stop making motor progress (Bushby et al., 2010). Both these drugs have adverse effects, prednisone for weight gain and deflazacort for developing cataracts (Manzur et al., 2008). Sometimes these glucocorticoids are combined with immunosuppressants such as ciclosporin-A and azathiprine for treatment of DMD (Griggs et al., 1993; Kirschner et al., 2010). Although these drugs have been used for over two decades, and are known for anti-inflammatory effects, the detailed mechanism of these drugs in rescuing muscle fibers from dying in DMD are still not fully understood.

2. **Gene therapy:**

Dystrophin is the largest known human gene, with 79 exons spread across more than two million base pairs of genomic sequence. Despite current research on the possibility of genetic treatment to directly replace missing dystrophin in patients, there are certain restrictions. The first is the size of the dystrophin gene, and the second is the selection of the vector that can integrate larger amounts of the gene (Mendell et al., 2010; Arechavala-Gomeza et al., 2010; Babbs et al., 2020). Due to these reasons, smaller genes such as micro or mini dystrophin have been developed which can be inserted into the vector. This approach also faces several immunological responses because the vector used in this approach is an AAV (Adeno-virus associated virus) (Mendell et al., 2010; Arechavala-Gomeza et al., 2010). The main limitation of this therapy is that it is difficult to predict how well the corresponding truncated proteins will function in human muscles as none of the micro or mini dystrophin will function as well as the full length dystrophin (Babbs et al., 2020).

Another gene therapy developed is replacing dystrophin with a surrogate protein such as utrophin which is a structural paralogue of dystrophin. Early in human and mouse development, utrophin is found at the muscle membrane alongside dystrophin. Dystrophin and utrophin bind to a similar complex of proteins at the muscle membrane but differ in the isoform of their binding

protein. Replacing dystrophin by utrophin can strengthen muscle membrane integrity and improve dystrophic phenotype to some extent (Babbs et al., 2020). Utrophin can be re-expressed at the sarcolemma of regenerating fibers in damaged muscle which was evidenced by the re-expression of developmental myosin (Guiraud and Davies, 2017; Guiraud et al., 2019), but this therapy also has limitations as administration of utrophin requires use of AAV virus as vector that can result in immunological responses (Babbs et al., 2020).

3. **Exon Skipping:**

Dystrophin gene has thousands of mutations which will differ in different patient. The TREAT-NMD DMD global database reports 7,149 DMD mutations, comprising of majority of mutations that clusters near two hot spots, one near the 5′ end and others clustered around exon 51 (Bladen et al., 2015). The reading frame rule which states that mutations in exons which delete sections of the gene, leaving the reading frame intact, would result in a truncated, partially functional protein whose expression resulted in milder disease (Guiraud et al., 2015). This reading frame rule is used to explain the observed genotype/phenotype relationships and has been used to transform DMD into a milder form like Becker muscular dystrophy by exon skipping therapies that uses modified RNA molecule referred to as an antisense

oligonucleotide (AONs), AONs can bind to specific pre-mRNA sites, masking and excluding this exon from the splicing process (Pichavant et al., 2011). Initially, these AONs were used for skipping of exon 51 but then this is only applicable to 13-15% of the DMD populations (Aartsma-Rus et al., 2017).

Recent studies show that using 2'-O-methyl-phosphorotioates (2'OMeAO) and phosphorodiamidate morpholino oligomer (PMOs), which are negatively charged back bone of AONs, on mdx mice at exon 23, showed the presence of dystrophin in many skeletal muscle fibers, but not in the heart and using PMO on mdx mice and in DMD patients at exon 51 showed dystrophin levels restored only by 22-32% in skeletal muscles (Lu et al., 2005; Gait et al., 2019). The limitation of exon skipping is that, depending on the size and location of the mutation, different exons need to be skipped and it could only be applied to a specific type of mutation. Also, the effect of exon skipping only lasts for 2-3 months and requires repeated administrations. (Aartsma-Rus et al., 2017; Gait et al., 2019).

Other therapies include the use of aminoglycosides, myostatin, and vitamin D supplement (Beytia et al., 2012). Despite the fact that the molecular causes of DMD have been known for several years, currently there is no cure for this disorder.

1.5 Muscle fiber death and wasting in DMD.

Morphological criteria are frequently used to classify different types of cell death, which are classified as apoptotic, necrotic, autophagic, or associated with mitotic catastrophe (Galluzzi et al., 2007). Necrosis is involved in a variety of physiological and pathological processes. Recently, a new type of necrosis has been identified as necroptosis. Necroptosis has been linked to immune system regulation, cancer development, and cellular responses to a variety of stresses (Wu et al., 2012). The current scenario holds that necroptosis is the major cell death pathway in DMD (Morgan et al., 2018). However, involvement of apoptosis has also been reported by Serdaroglu et al., (2002), Sandri et al., (2001). The comprehensive understanding of the mechanism leading from the absence of dystrophin to the muscular degeneration by muscle cell death pathway is still lacking.

i. **Necroptotic cell death:**

The general mechanism of necroptosis is that RIPK1 and RIPK3 (Receptor-Interacting Protein Kinase 1 and 3) interact with each other, resulting in the formation of a functional heterodimer complex that promotes oligomerization of MLKL (Mixed-Lineage Kinase-domain-Like pseudokinase) by phosphorylating it. The oligomeric form of MLKL translocate from the cytosol to the plasma membrane, resulting in the formation of the pore and an inflammatory response. (Dhuriya and Sharma, 2018) (Figure 1 (A)).

A recent study done by Morgan et al., (2018) reported that necroptosis is the major cell death pathway associated with skeletal muscle pathology in dystrophin deficient muscles. Although they observed RIPK1, RIPK3 and MLKL gene upregulation in mdx muscle fibers, they discovered strong RIPK3 immunoreactivity which was associated with mdx muscle fibers. They also showed that TNFα alone can initiate necroptosis if RIPK3 is overexpressed. Overall, this study demonstrates that skeletal muscle tissue can undergo a programmed form of necrosis that is necroptosis, and this is the first study to show a strong involvement of RIPK3 out of other necroptotic markers RIPK1 and MLKL to be associated with muscle death in mdx mice as it shows a strong immunoreactivity within mdx muscle as well as in human DMD muscle biopsies.

ii. **Apoptotic cell death:**

The apoptotic mechanisms are extremely complex and sophisticated, involving an energy-dependent cascade of molecular events. According to a current research, there are two major apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Each pathway activates its own initiator caspase (8,9,10) which activates executioner caspase-3. Cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies, and finally phagocytosis of the

apoptotic bodies by adjacent parenchymal cells, neoplastic cells, or macrophages are all cytomorphological features of the execution pathway (Elmore, 2007) (Figure 1 (B)). Several studies have shown the upregulation of proapoptotic markers like BAK, and BAX associated in DMD (Serdaroglu et al., 2002). One study showed involvement of caspase 3 expression that correlates with skeletal muscle death via apoptotic pathway in DMD (Sandri et al., 2001). It is still a debatable topic that which cell death pathway drives myofiber death in DMD.

A
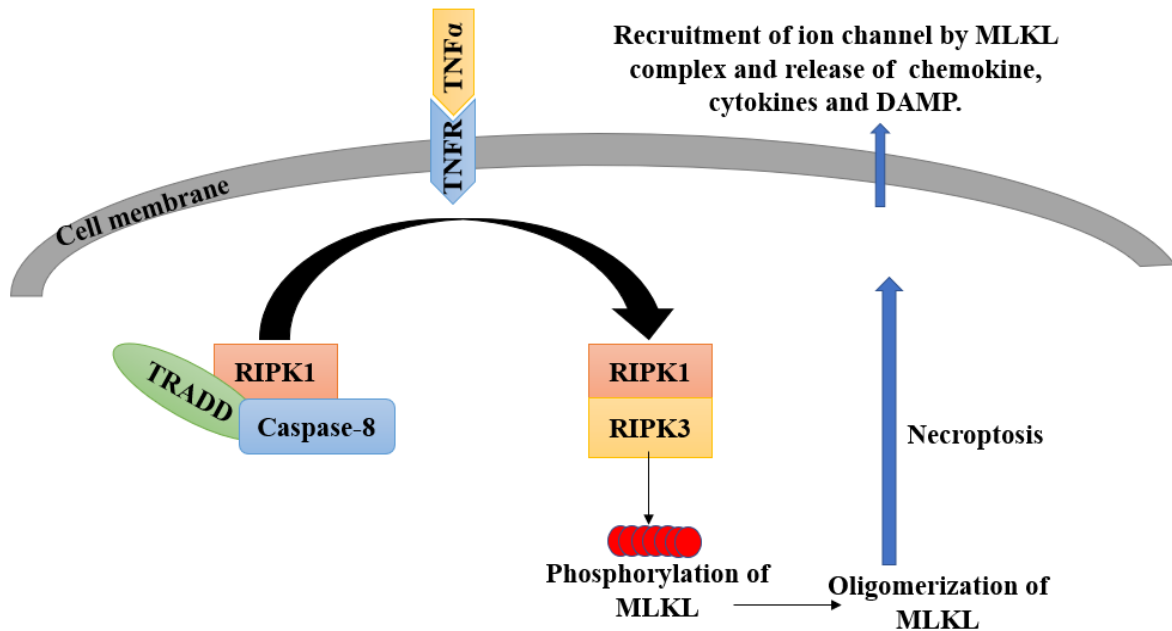
B
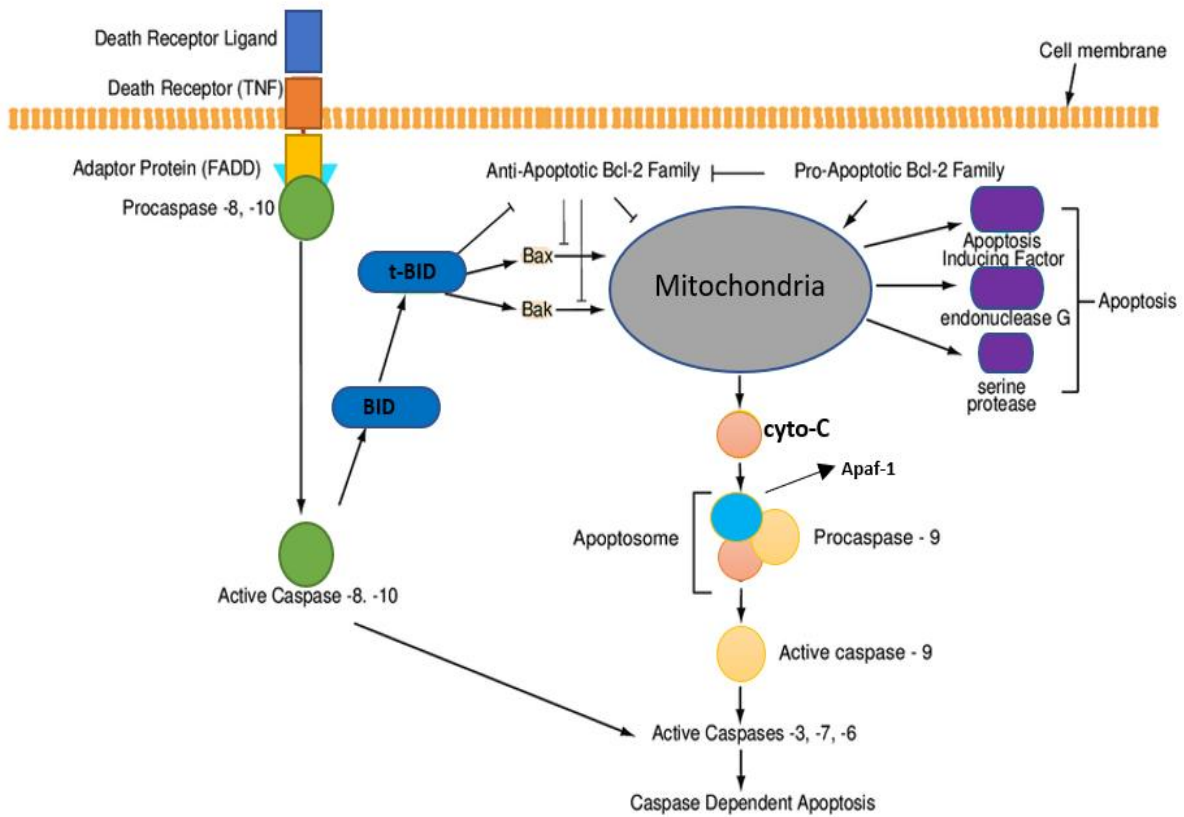
Figure 1: Muscle fiber death in DMD. (A) Adapted schematic diagram of molecular mechanism initiating necroptosis (Dhuriya and Sharma, 2018), (B) Adapted schematic diagram of molecular mechanism initiating apoptosis (Guerin et al., 2006).

1.6 The Lipin Protein Family.

The regulation of lipid metabolism is critical for preserving systemic energy homeostasis, cellular integrity, and membrane maintenance. Obesity and adipose-deficiency conditions, such as lipodystrophy, are two extremes of lipid metabolic dysfunction that have been linked to changes in the expression or function of distinct sets of genes associated with inappropriate lipid accumulation in tissues (Phan and Reue, 2005; Chen et al., 2015). Lipin family proteins are evolutionarily conserved proteins with roles in lipid metabolism and disease. This protein family consists of three distinct proteins, Lipin1, Lipin2, and Lipin3, which are expressed in tissue-specific and sometimes overlapping manner (Chen et al., 2015). It has been identified that out of these three proteins, Lipin1 deficiency in mice leads to lipodystrophy, hyper- triglyceridemia, neonatal fatty liver, insulin resistance and peripheral neuropathy (Chen et al., 2015). Also in humans, Lipin1 mutations leads to similar phenotypes as described in mice and displays smaller and atrophied muscles (Chen et al., 2015; Jiang et al., 2015). Lipin1 expression is higher in skeletal muscles relative to other Lipin family proteins, and Lipin1 is responsible for all PAP (Phosphatidic Acid Phosphatase) action in skeletal muscles (Donkor et al., 2007; Michot et al., 2012; Jiang et al., 2015). Not only in TAG (Triacylglycerol)

metabolism but recent study shows that Lipin1 is also critical in skeletal muscle differentiation and development (Jiang et al., 2015, Jama et al., 2018).

The N-terminal (N-LIP) and C-terminal (C-LIP) domains are found in all members of the Lipin protein family and are evolutionarily conserved. The C-LIP domain comprises two important protein functional motifs that are responsible for the dual activity of Lipin1. First, the DXDXT motif is essential for PAP (Phosphatidate Acid Phosphatase) enzymatic activity that converts phosphatidate (PA) to diacylglycerol (DAG) during the biosynthesis of triglycerides (TAG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (Carman and Han, 2009; Reue and Brindley, 2008). The PAP (Phosphatidate Acid Phosphatase) activity of Lipin1 is significant because it helps with phospholipid and TAG (Triacylglycerol) metabolism (Ren et al., 2010). Second, the LXXIL motif is required for Lipin1 to bind with nuclear receptors and function as a transcriptional regulator. The Lipin1 unique exon is derived from the Lipin1 gene's alternative splicing (Chen et al., 2015). Lipin1 has been shown to localize to the nucleus in adipocytes and hepatocytes, and protein phosphorylation may influence subcellular localization acting as transcriptional coactivator (Reue and Dwyer, 2009) (figure 2 (A)).

Overall, it has been discovered that Lipin has two functions: it is a PAP enzyme that is essential for lipid synthesis, and it is also a transcriptional coactivator that stimulates fatty acid oxidation. Lipin proteins go from the cytosol to the endoplasmic

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reticulum (ER) and catalyze the conversion of PA to DAG, a major substrate for the synthesis of TAG, PE, and PC. Lipin1 also functions as a transcriptional coactivator in the nucleus, interacting with PGC-1α, PPARγ, and other factors including histone acetyltransferase (HAT) to promote the expression of genes involved in fatty acid oxidation (Chen et al., 2015). Lipin1 interacts with Peroxisome Proliferator Activated Receptor gamma (PPARγ), a master regulator of adipocyte differentiation, to co-activate critical genes in adipogenesis (Kim et al., 2013). (Figure 2 (B)).

A

Lipin1β specific exon

N-LIP   C-LIP

NLS: Nuclear
localization signal

DXDXT: PAP enzyme
motif

LXXIL:
Transcriptional
coactivator motif

B

ER | Cytosol | Nucleus

PAP → Lipin ↔ Lipin ↔ Lipin

PAP → DAG → PE/PC, TAG

HAT, PGC-1α, PPAR-1α

Regulation of genes involved in adipocyte differentiation and FA oxidation
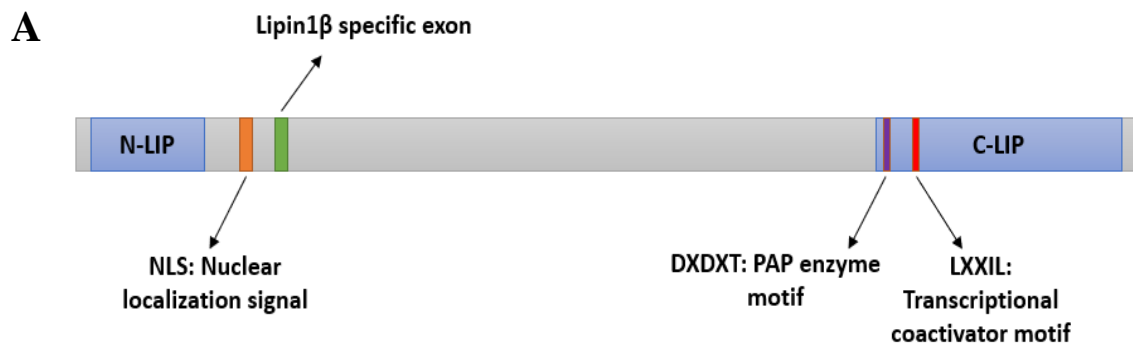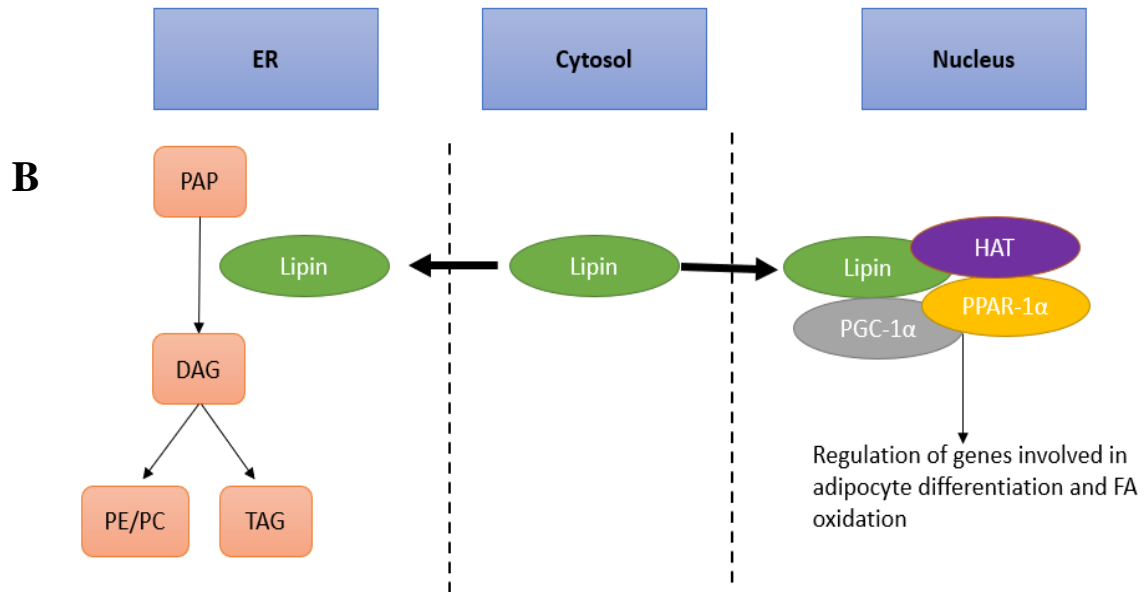
Figure 2: The Lipin Protein Family. (A) Adapted domain structure and functional motifs of Lipin (Chen et al.,2015), (B) Adapted diagram of the dual functions of Lipin (Chen et al., 2015).

1.7 Lipin1 roles in DMD.

Till date it has been discovered that Lipin1 plays an important role in TAG metabolism, adipocyte differentiation and also its role has been critical for skeletal muscle differentiation and development. In human patients, Lipin1 deficiency contributes to severe episodes of rhabdomyolysis, a condition characterized by

breakdown of damaged skeletal muscle fibers which in turn leads leakage of muscle contents into circulation and muscle biopsies of rhabdomyolysis patients has shown accumulation of lipid droplets (Michot et al., 2012; Zhang, 2012; Chavez et al., 2016; Torres et al., 2015,). In addition, recent studies have also discovered Lipin1 roles in pathology of DMD. There was one study performed in which they investigated several gene expression profiles in early phase of DMD and they discovered that Lipin1 was one of the differentially regulated genes and was grouped into differentially regulated muscle genes during early phases of DMD (Pescatori et al., 2007). An earlier study reported that Lipin1 deficiency leads to dysregulated sarcoplasmic reticulum calcium uptake (Paran et al., 2015). The key reason to study Lipin1 in DMD is that deficiency of Lipin1 leads to an upregulation of necroptosis through loss of membrane integrity as suggested by Sattiraju et al., (2020) and loss of membrane integrity is the major pathology in DMD. This all leads us to investigate more in first, what is the mode of muscle cell death pathway in DMD, second, whether Lipin1 deficiency can contribute toward muscle death and weakness in DMD and third, whether upregulation of Lipin1 in DMD can rescue muscle fiber from dying.

Since, there is no cure for DMD, and we now know that Lipin1 is critical to maintain membrane integrity, which is the main pathology that occurs in DMD, our primary goal for this study is to identify drugs that can upregulate Lipin1 expression,

consequently, and rescue dying muscle fibers. With this approach we tested two drugs:

Dexamethasone:

Dexamethasone is a glucocorticoid medication. It is used to treat many inflammatory and autoimmune disorders such as rheumatoid arthritis and bronchospasm. It has also been used to treat skin diseases, allergies, asthma, chronic obstructive lung disease. Dexamethasone has anti-inflammatory and immunosuppressant effects (Umeki et al., 2015).

Dexamethasone enters cell membrane and binds to the glucocorticoid receptors in cytoplasm and then this complex enters into nucleus and alters expression of targeted genes.

Rosiglitazone:

Rosiglitazone is a thiazolidinedione-class antidiabetic medication. It operates as an insulin sensitizer by attaching to the PPAR receptor in fat cells and increasing the cells insulin sensitivity. They lower blood glucose, fatty acid, and insulin levels. They work by binding to receptors stimulated by the peroxisome proliferator (PPARs). PPARs are transcription factors residing in the nucleus that are activated by ligands such as thiazolidinediones. Thiazolidinediones enter cells, bind to nuclear receptors, and change gene expression. The several PPARs include PPARα,

PPARβ/δ, and PPARγ. Thiazolidinediones bind to PPARγ (Nissen and Wolski, 2007).

1.8 Hypothesis & Aims.

Loss of membrane integrity is the major pathology in DMD. Also, it has been identified that necroptosis is the major pathway leading to muscle death in DMD. So, one of the approaches in ameliorating dystrophic phenotype in DMD, is through reduction of necroptosis which eventually strengthens muscle membrane integrity. Recent study suggests that Lipin1 is critical to maintain muscle membrane integrity and deficiency of Lipin1 leads to upregulation of necroptosis in muscles. Our primary goal in this study is to ameliorate dystrophic phenotype in mdx mice through reduction of necroptosis using drugs which can potentially upregulate Lipin1 expression. A study reported by Zhang et al., (2008) suggested that dexamethasone can upregulate Lipin1 gene expression in adipose tissue and a study reported by Yao-Borengasser et al., (2006) and Festuccia et al., (2009) suggested that Lipin1 expression is positively correlated with insulin sensitivity and Lipin1 expression in adipose tissue is induce by insulin sensitizing compounds such as thiazolidinediones that activates PPARγ and one such agonist of PPARγ is rosiglitazone. Therefore, we hypothesize that dexamethasone and/or rosiglitazone could be potentially used therapeutically in treating patients with DMD which could be through upregulation of Lipin1. With that we came up with three aims:

1. Test the hypothesis that dexamethasone can ameliorate dystrophic phenotype of mdx mice which could be through upregulation of lipin1.

2. Test the hypothesis that rosiglitazone can inhibit cell death pathways in mdx mice which could be through upregulation of lipin1.

3. Test the hypothesis that dexamethasone can strengthen membrane integrity in mdx mice which could be through upregulation of lipin1.

2. Materials and Methods

2.1 Cell culture and C2C12 myoblast differentiation.

Mouse C2C12 myoblast cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM)(Gibco), supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco) and 1% Penicillin-Streptomycin (Gibco) under humidified air containing 5% CO_2 at 37°C. The cells were split and passaged every two days to prevent them from reaching 100% confluency. For differentiation, cells were passage into 6-well plate containing DMEM. After cells reached 100% confluency, media was replaced by differentiation medium containing DMEM supplemented with 2% Horse serum (Gibco) and 1% Penicillin-Streptomycin (Gibco) and allowed cells to differentiate for 4 days changing fresh media every 2 days. The differentiating myoblasts were then subjected for dexamethasone and rosiglitazone treatment.

2.2 C2C12 myoblast differentiation and drug treatment.

Dexamethasone (Thermo Fisher Scientific) was prepared at different concentrations of 5µM, 10µM, 15µM, 25µM, and 50µM in differentiation media and added to the respective wells of 6-well plate of differentiating C2C12 myoblasts on beginning of day 4 of differentiation. After day 4, treated cells were harvested and used for Western blot and mRNA analysis. For different time point experiment, 10µM

concentration of dexamethasone was prepared in differentiation media and added to differentiating C2C12 myoblast on beginning of day 3 of differentiation and treated for 6 hours, 12 hours, 24 hours, and 48 hours (Sheng-Han et al., 2017). After 48 hours of treatment, cells were harvested and used for Western blot and mRNA analysis.

Rosiglitazone (TCI AMERICA) was prepared at 10µM of concentration in differentiation media and added to the differentiating C2C12 myoblast on beginning of day 3 of differentiation and treated for different time points, 6hours, 12hours, 24hours, and 48 hours. After 48 hours of treatment, cells were harvested and used for Western blot and mRNA analysis.

2.3 Animals and drug treatment.

C57B1/6J (Wild type BL6), C57BL/10ScSnJ (Wild type BL10, stock No: 000476), and C57BL/10 ScSn-Dmd [mdx]/J (mdx, stock No: 001801) mice were purchased from The Jackson Laboratories, (Bar Harbor, ME, USA). The genetic cause of DMD are mutations of the dystrophin gene on the X chromosome. Only male mice were used in this study. Experiments were performed on 2-4 months old mice. These mice had free access to drinking water and regular chow, unless mentioned. All animal experiments were performed under guidelines and regulations approved by Animal

Care and Use Committee of Wright State University. Mice were randomly divided into two groups of control and treated mice.

Dexamethasone Treatment:

Six B10 wild type mice and six mdx mice were used. There were four groups which consisted of wild type non-treated, wild type treated, mdx non-treated and mdx treated mice groups. Three mice were used in each group. For treated group, dexamethasone was provided in the drinking water such that each mouse received the drug concentration of 3mg/Kg daily (Baehr et al., 2011). Dexamethasone treated water was provided every other day and water consumption was measured. The non-treated group received regular water. Both the groups received regular chow. The duration of the treatment was 14 days and on day 14 mice were euthanized for tissue collection. Gastrocnemius muscle tissues were collected for Western blot and immunostaining (figure 3 (A)).

Rosiglitazone Treatment:

Six B10 wild type mice and six mdx mice were used. There were four groups which consisted of wild type non-treated, wild type treated, mdx non-treated and mdx treated mice groups. Three mice were used in each group. Rosiglitazone was injected intraperitoneally (i.p.) once daily at a dose of 10mg/Kg for each mouse (Carmona et al., 2005). Each group received regular water and regular chow. The duration of

treatment was 7 days. On day 7, mice were euthanized for tissue collection. Gastrocnemius muscle tissues were collected for Western blot and immunostaining (figure 3 (B)).

A

B10 Control   B10 Treated

14 Days

Dexamethasone
(Water Treatment)

Water for the mice was changed every other day.

After 14 days mice were euthanized, and gastrocnemius muscle were collected.

14 Days

MDX Control   MDX Treated

Concentration=3mg/kg (Daily)

Tissues were used to perform Western blots.

Tissues were used to perform several staining.

B

B10 Control   B10 Treated

7 Days

Rosiglitazone (i.p. injection)

Each mice received regular water and regular chow.

After 7 days mice were euthanized, and gastrocnemius muscle were collected.

7 Days

MDX Control   MDX Treated

Concentration=10mg/kg (Daily)

Tissues were used to perform Western blots.

Tissues were used to perform several staining.

Figure 3: Schematic diagram for the drug treatment in mice. (A) Dexamethasone at concentration of 3 mg/kg given via drinking water for 14 days to the treated mice groups consisting of B10 wild type and mdx mice, (B) Rosiglitazone at concentration of 10 mg/kg given via i.p. injections for 7 days to the treated mice groups consisting of B10 wild type and mdx mice.

2.4 Western blotting.

C2C12 myoblast cells were washed in ice cold 1X PBS (Gibco) and lysed using cocktail containing RIPA buffer (20-188 EMD Millipore Sigma), 0.2% Protease Inhibitors (SIGMA Complete™ Protease Inhibitor Cocktail 04693116001) and 0.2% Phosphatases inhibitors (SIGMA P2850 - Phosphatase Inhibitor Cocktail 1). After lysing, cells were collected into fresh 1.5 ml eppendorf tube and sonicated for 10 seconds and then centrifuged at 14,000 rpm for 10 mins at 4°C. Pellet was discarded, and supernatant was collected into a fresh 1.5 ml eppendorf tube. Muscle tissues were homogenized using glass homogenizer with cocktail containing RIPA buffer (20-188 EMD Millipore Sigma), 0.2% Protease Inhibitors (SIGMA Complete™ Protease Inhibitor Cocktail 04693116001) and 0.2% Phosphatases inhibitors (SIGMA P2850 - Phosphatase Inhibitor Cocktail 1). Once lysed, samples were centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was collected

into new 1.5ml eppendorf tube. The Bicinchoninic acid assay also known as BCA assay was carried out to measure protein concentration in each sample. 2mg/ml BSA was used as standard in following concentration of 0, 1µl, 2µl, 3µl, 4µl, 5µl, 6µl loaded in 96 well plate. Samples were used in the range of 1-4µl. duplicates were prepared for both standard and sample. Plate was incubated at 37°C for 30mins. Protein absorbance was measured using SynergyH1 microplate reader with the wavelength at 567. The standard curve was generated and used to calculate the protein amount to be loaded.

Equal amount of protein was loaded and separated using 7.5%-15% SDS-PAGE (40%Acrylamide Solution 37.5:1 Bio-Rad Cat. 1610158). Samples were prepared for running by mixing the calculated amount of protein sample to be loaded (10µg-20µg), RIPA and 4X loading dye was added to every sample. Samples were then heated in boiling water (100°C) for 5 mins, and then allowed to cool down followed by spinning them down. Then the samples and the protein ladder (#26616 Thermo Fisher Scientific) were loaded in SDS gel in 1X running buffer with initial voltage at 90 v and then increased to 120v after the samples have crossed the stacking gel. After the run was completed, transfer was done using PVDF membrane (Immun-Blot PVDF Membranes for protein blotting. (0.2µm) (BIO-RAD). Membrane was soaked in methanol for 1-2 mins, filter papers and sponges were soaked in 1X transfer buffer. Sandwich for transfer was prepared in order by placing sponge, filter

paper, gel, membrane, filter paper and sponge. Air bubbles were removed before placing the sandwich in the transfer tank. The blot was on the cathode side and the gel was on the anode side. The whole cassette was placed in the transfer tank with ice block in the tank and allowed to transfer for 2 hours at 150V with 0.30mA current. After transfer, the membranes were blocked using 5% nonfat milk solution for 1 hour at room temperature with rocking. Afterwards, the membranes were incubated with primary antibodies diluted in 1% BSA TBST solution (dilution 1:1000), overnight at 4°C with rocking. Next day, membranes were washed with TBST (1x TBS buffer + 0.1% Tween20, Fisher Cat#: 175476) and incubated with secondary antibodies (dilution 1:5000) for 1 hour at room temperature with rocking. The secondary antibodies were diluted in 1% BSA TBST. Membranes were again washed with TBST and visualized using Chemiluminescence kit from Pierce™ ECL Western Blotting Substrate (catalog # 32106). The instrument used for the visualization was Amersham Imager 600 (GE life sciences). Antibodies that were used was from cell signaling technology include: lipin1 (#14906), Bax (#2772), Bak (#12105), cleaved caspase 9 (ccp-9, #9508), cleaved caspase 3 (ccp-3, #9664), RIPK3 (#95702), RIPK1 (#3493T), MLKL (#37705S), GC Receptor (#12041S), pMLKL (#74921S), PERK (#5683S), PPARγ (#2435S), p-NFκB (#3039S) Goat anti-mouse IgG-HRP(#7076S) and goat anti-rabbit IgG-HRP (#7074S) secondary antibodies were used for detection. GAPDH (#5174S) antibody was used as a

loading control. Western blots were quantified by densitometry using NIH Image J software and all values were normalized to a loading control.

2.5 RNA extraction and Quantitative Real-Time PCR.

For C2C12 cells and muscle tissue RNA extraction, 1 ml of Trizol reagent (Invitrogen) was used. After cells/tissue were lysed with Trizol, for every 1 ml Trizol used, 200 µl of chloroform was added and the tubes were vigorously shaken for 15 seconds. Samples were then incubated for 2-3 minutes at room temperature followed by centrifugation at 12,000 g for 15 minutes at 4˚C. The colorless upper aqueous phase was carefully transferred to a new tube and mixed with 500 µl of isopropanol. After that, samples were incubated at room temperature for 10 minutes with rocking followed by centrifugation at 12,000 g for 10 minutes at 4˚C. The supernatant was carefully discarded. The pellet was then washed with 70% ethanol to remove the residual isopropanol and was centrifuged at 7,500 g for 10 minutes at 4˚C. The ethanol was carefully removed by pipetting. The pellet was resuspended with RNase-free water. RNA quality and concentration was measured by using 21 NanoDrop 2000 (Thermo Fisher Scientific). For cDNA synthesis, 1µg of total RNA was used for reverse transcription with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) reaction was performed in a QuantStudio Real-Time PCR System (Thermo Fisher

Scientific) system using SYBR®Green Real-time PCR Master Mix (Bio-Rad). For qPCR analysis, The Ct ($2^{-\Delta\Delta Ct}$) method was used. For list of primers used, see Table 1. All primers used was from Integrated DNA Technologies.

Table 1: Primer sequences for RT-qPCR.

Primer	Forward 5'→3'	Reverse 5'→3'
LIPIN1	CCTTCTATGCTGCTTTTGGGAACC	GTGATCGACCACTTCGCAGAGC
PPARγ	GCCCTTTGGTGACTTTATGGA	GTAGGTTCTGTTGGACGACG
GAPDH	CCAATGTGTCCGTCGTGGATCT	GTTGAAGTCGCAGGAGACAACC

2.6 Immunohistochemistry and Immunostaining.

B10 and MDX mice from both the groups treated and non-treated were euthanized and gastrocnemius muscles were dissected and fixed frozen in liquid nitrogen using chilled methylbutane. 10 µm muscle sections were prepared using a cryostat and stored at -20°C until subjected to staining. Following staining procedures were carried out:

Hematoxylin and Eosin (H&E) Staining: H&E staining Kit (Vector Laboratories, Inc.) was used to perform H&E staining as per the manufacturer's instruction. The ratio of total myofibers with centrally located nuclei in each field was counted and the percentage of central nucleation was determined. Tissue sections were rehydrated for 1 minute and then hematoxylin was added to completely cover tissue section for 5 minutes, slides were then rinsed in 2 changes of D/W of 15 seconds each to remove excess stain and the bluing reagent was applied and slides were incubated for 10-15 seconds and then the slides were rinsed in 2 changes of D/W of 15 seconds. Slides were then dipped in 90% ethanol for 10 seconds. Eosin Y solution was applied and incubated for 2-3 minutes and then slide was rinsed in 90% ethanol for 10 seconds and slide was dehydrated in 3 changes using 100% ethanol for 1-2 minutes.

IgG staining: This staining is used to determine muscle membrane integrity. The abnormal presence of blood proteins within myofibers represents convenient markers for myonecrosis in situ. Staining these proteins with IgG has been used as methods to detect membrane integrity. The borders of muscle fiber are stained with Laminin. Slides were removed from -20°C and thawed for 20 minutes at room temperature. Sections were rehydrated with 1X PBS for 5 minutes and blocked with 5% BSA for 45 minutes at room temperature with covering the slides. Then sections were incubated with primary antibody (dilution 1:10) that was diluted in 1% BSA

solution at 37°C for 1 hour. Sections were washed with 1X PBS, 3 times for 5 minutes and then sections were incubated with secondary antibody (dilution- 1:5000) in 1% BSA solution. Sections were washed with 1X PBS, 3 times for 5 minutes. Sections were allowed to air dry for 15 minutes and mounted with mounting medium with DAPI. The antibodies used was Laminin - Primary antibody (Ab11575, Abcam) and IgG- secondary antibody (Invitrogen).

2.7 Statistical Analysis.

All the statistical data are presented as mean $\pm$ SD number (n) of independent experiments. Statistical significance was calculated using a two-tailed Student's t test. $p<0.05$ is considered statistically significant.

3. Results

Aim 1: Test the hypothesis that dexamethasone can ameliorate dystrophic phenotype of mdx mice which could be through upregulation of Lipin1.

3.1 Dexamethasone increases Lipin1 expression levels in cell culture system.

We investigated the effects of dexamethasone on the expression levels of Lipin1 in C2C12 myoblasts cells. We used two different approaches, first, we used different concentrations of the drug i.e., 5 µM, 10 µM, 15 µM, 25 µM, and 50 µM to treat the differentiating myoblasts cells on day 3 of differentiation and after 24 hours that was on day 4 of differentiation, cells were harvested for protein and mRNA expression analysis. Second, we used 10µM of dexamethasone to treat differentiating myoblasts cells on day 2 of differentiation for different time points for 6 hours, 12 hours, 24 hours, and 48 hours, and on day 4 of differentiation, cells were harvested for protein and mRNA expression analysis. Through Western blot analysis, we found that Lipin1 protein expression levels were increased with increasing concentration of dexamethasone compared to non-treated cells (figure 4 (A)) and we found that Lipin1 protein expression levels were increased gradually at 12 hours and 24 hours

after treating differentiating myoblasts cells with 10μM dexamethasone compared to non-treated cells (figure 4 (C)). RT-qPCR was performed for relative mRNA expression analysis of Lipin1, and we found that relative mRNA expression of Lipin1 was increased with increasing concentration of drug compared to non-treated cells (figure 4 (B)). Also, we found that relative mRNA expression of Lipin1 was increased at 24 hours after treating differentiating myoblasts cells with 10μM dexamethasone compared to non-treated cells (figure 4 (D)).
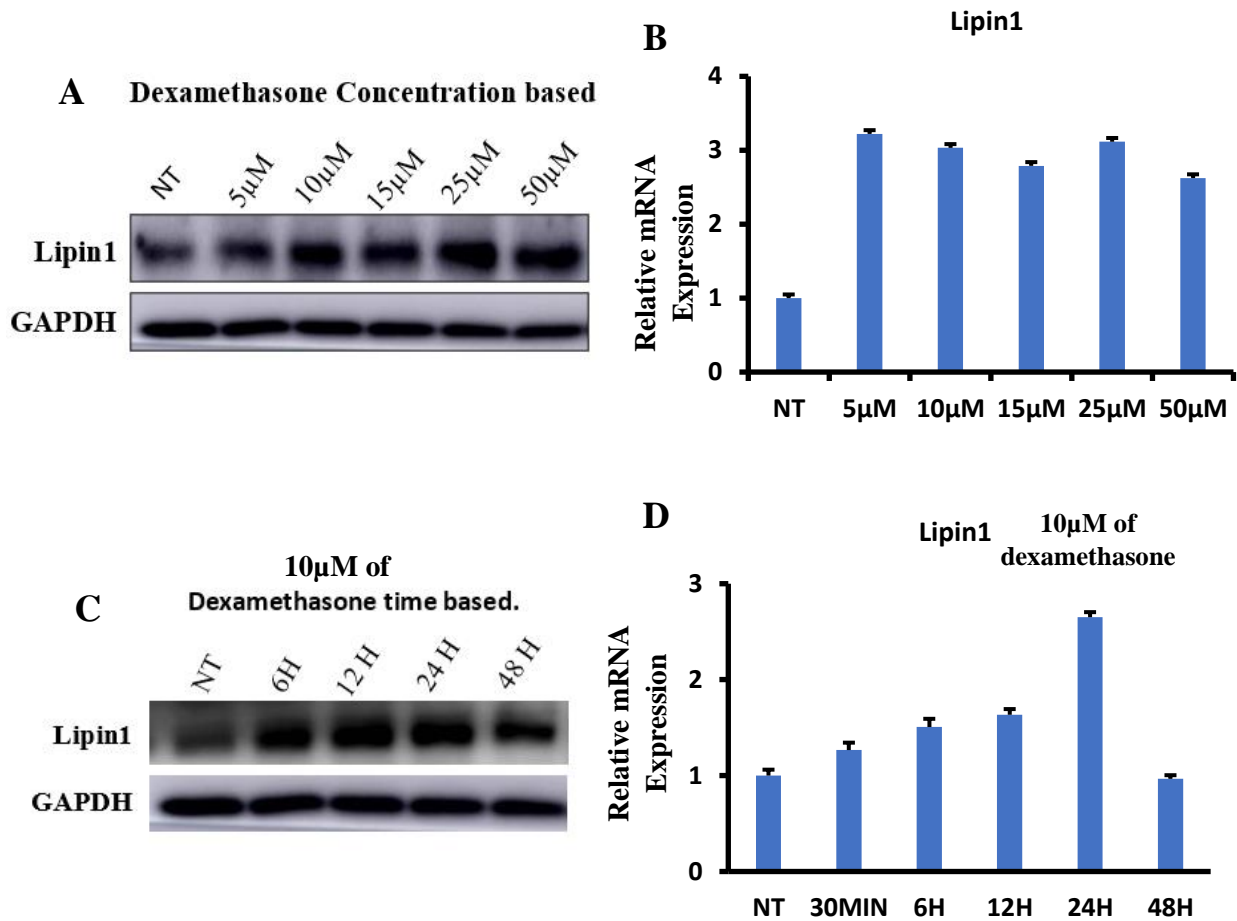
**Figure 4: Dexamethasone increases Lipin1 expression levels in cell culture system.** (A) Western blot, (B) mRNA expression of Lipin1 in C2C12 differentiated myoblasts treated with different concentrations of dexamethasone for 24 hours, (C) Western blot, (D) mRNA expression of Lipin1 in C2C12 differentiated myoblasts treated with 10μM of dexamethasone for different periods of time. (NT)- non-treated.

3.2 The effect of Dexamethasone on muscle morphology of mdx mice.

After we found that Lipin1 protein and mRNA expression levels were increased in C2C12 myoblasts upon dexamethasone treatment, we then investigated the effects of the dexamethasone on muscle morphology in B10 wild type mice and mdx mice which is the mouse model for DMD. We divided mice in two groups: non-treated and treated mice with dexamethasone. Each group consisted of six B10 wild type mice and six mdx mice, out of which three were control mice and three were treated mice. Dexamethasone was given via drinking water at a concentration of 3mg/kg daily. All mice were feed chow diet. The drinking water that contained dexamethasone for treatment group was changed every other day for 14 days. At day 14, mice were euthanized, and gastrocnemius muscles were collected for Western blot analysis and immunostaining (figure 3 (A)).

To start with, we investigated the effect of dexamethasone on the muscle morphology of mdx mice. We performed H&E staining to examine the degeneration and regeneration in B10 wild type mice and mdx mice before and after the treatment. The morphology of mdx gastrocnemius muscle fiber showed inflammatory cell infiltration with macrophages or lymphocytes and displayed centrally nucleated muscle fiber (arrowheads figure 5 (A) MDX basal). The morphology of B10 wild type gastrocnemius muscle fibers after treatment was similar to non-treated B10 wild type muscle fibers, which did not show any inflammatory cell infiltration with

macrophages or lymphocytes or centrally nucleated muscle fiber (figure 5 (A) B10 basal and B10 DEX). Interestingly, we found that upon treatment with dexamethasone, mdx gastrocnemius muscle fibers showed decrease inflammatory cellular infiltration with macrophages or lymphocytes (figure 5 (A) MDX DEX). Quantitative analysis of the H&E stained mdx muscle fiber showed increased central nucleation of about 50% compared to the B10 wild type gastrocnemius muscle fibers (figure 5 (B)), indicating increased muscle degeneration and regeneration in mdx mice. After treatment of mdx mice with dexamethasone, we observed improvement on the muscle morphology as they displayed less inflammatory cellular infiltration with macrophages or lymphocytes (figure 5 (A)), but we did not observe any decrease of centrally nucleated muscle fibers (figure 5 (B)).

A

WT BASAL   WT DEX

MDX BASAL   MDX DEX

B

Basal   Treated
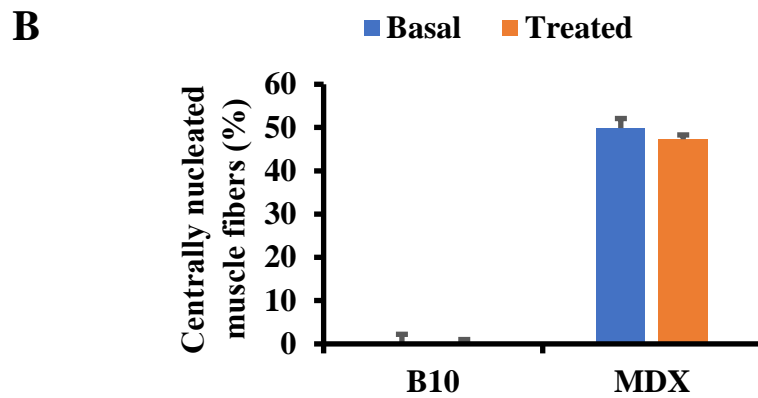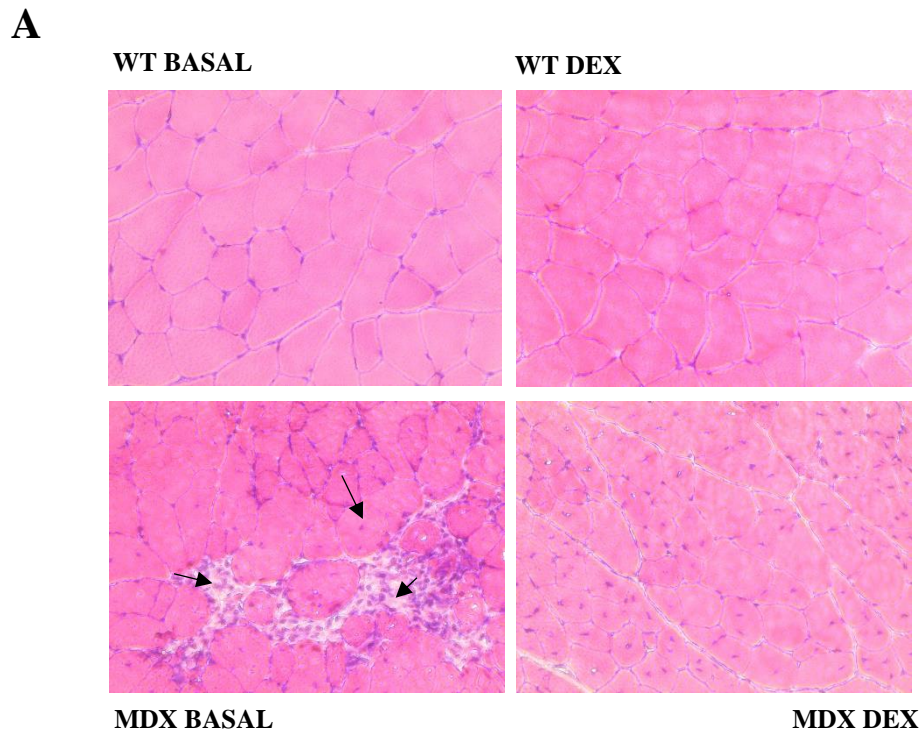
Figure 5: The effect of Dexamethasone on muscle morphology of mdx mice. (A) H&E staining of Gastrocnemius muscle tissue sections, arrow heads indicate inflammatory cell infiltration with macrophages or lymphocytes and displayed centrally nucleated muscle fiber, (B) Quantification of centrally nucleated muscle fibers from WT (Wild Type) non-treated (NT) and treated with dexamethasone mice and MDX non-treated (NT) and treated with dexamethasone mice. (3mg/kg). (n=3 mice/group).

3.3 Dexamethasone increases Lipin1 protein expression levels in wild type and mdx mice.

Recent study showed that Lipin1 deficient myofibers resulted in loss of muscle membrane integrity (Sattiraju et al., 2020) and loss of membrane integrity is the major pathology in DMD (Dadgar et al., 2014). So, we investigated the effect of dexamethasone on Lipin1 protein expression levels in B10 wild type mice and mdx mice. As dexamethasone is an agonist of glucocorticoids, we also measured glucocorticoid receptor protein expression levels and we found that there was no change in expression of glucocorticoid receptor protein levels in either of the treated mice groups (figure 6 (A)). This was consistent with the data reported for glucocorticoid receptor protein expression levels after dexamethasone treatment by Umeki et al., (2015). Interestingly, we found that in mdx mice, Lipin1 protein expression levels were downregulated by 0.5 fold with a *p* value of 0.001 compared to B10 wild type mice. This data suggested that Lipin1 protein expression was significantly downregulated in mdx mice nearly by half the levels as compared to B10 wild type mice. After dexamethasone treatment, through Western blot analysis, we found that Lipin1 protein expression levels were upregulated in B10 wild type mice treated with dexamethasone by 2.2 fold with a *p* value of 0.0001 compared to non-treated B10 wild type mice. It was interesting to discover that dexamethasone upregulated Lipin1 protein expression levels in treated mdx mice by 2.0 fold with a

p value of 0.04 compared to non-treated mdx mice (figure 6 (A)). The densitometry

revealed significant upregulation of Lipin1 protein expression levels in B10 wild

type mice and mdx mice treated with dexamethasone (figure 6 (B)).
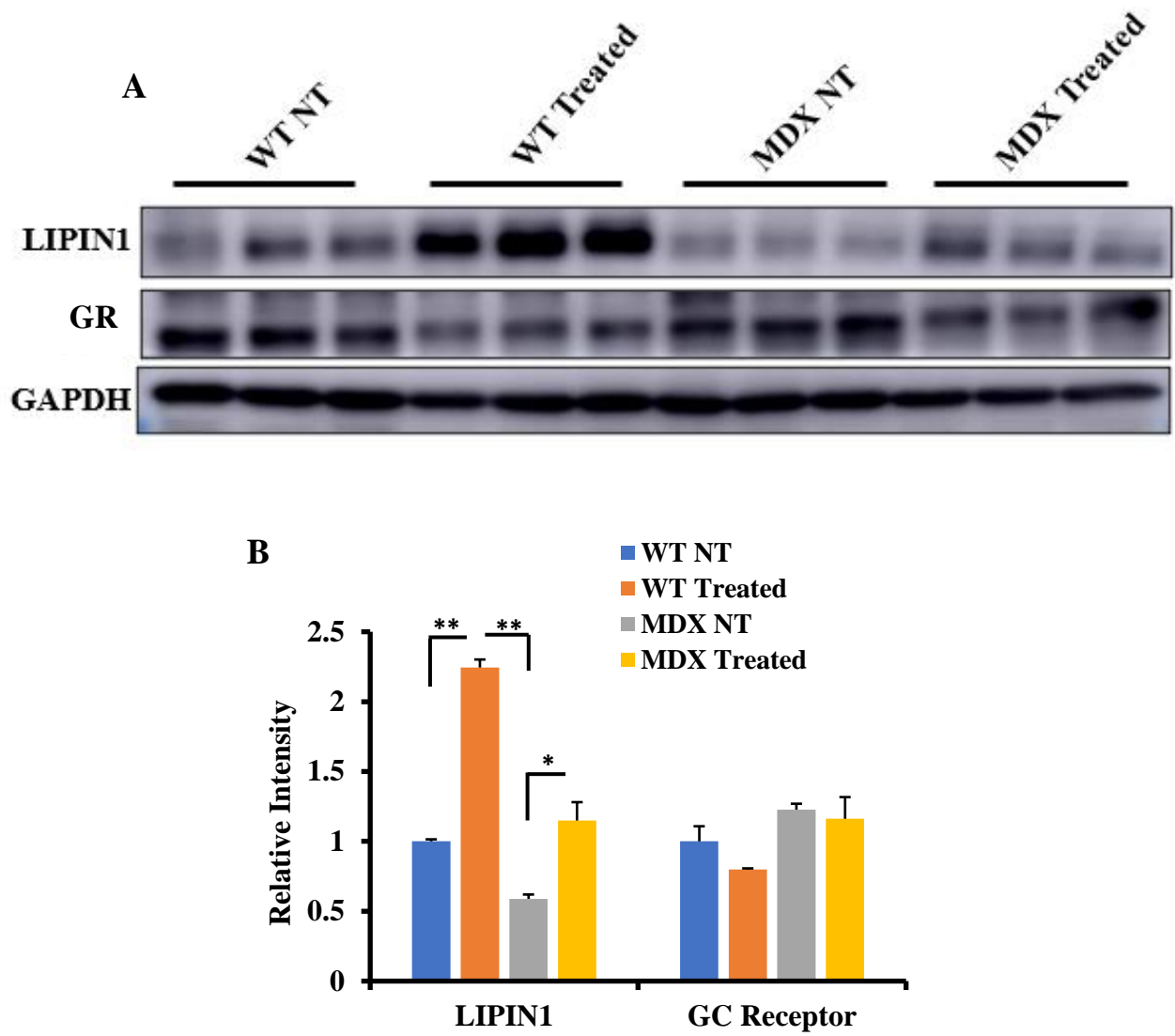
Figure 6: Dexamethasone increases Lipin1 protein expression levels in wild type and mdx mice. (A) Western blot, (B) the densitometry of Lipin1 and Glucocorticoid receptor (GR) in gastrocnemius muscles of WT(Wild Type) and MDX non-treated (NT) and treated mice with dexamethasone (3mg/kg) (n=3 mice/group) (* $p<0.05$; ** $p<0.01$ Student's t-test)

3.4 Dexamethasone treated mice shows decreased expression of necroptotic markers.

After we observed that dexamethasone elevated Lipin1 protein expression levels in mdx mice, we further measured necroptotic markers before and after dexamethasone treatment in B10 wild type mice and mdx mice. Recent study has showed that necroptosis involving RIPK1, RIPK3, and MLKL is the major death pathway contributing to muscle cell death and wasting (Morgan et al., 2018). So, we investigated the effects of dexamethasone on the protein expression levels of necroptosis markers, RIPK1, RIPK3, MLKL, and p-MLKL. Through Western blot analysis, we found elevation of necroptosis markers including RIPK1, RIPK3, MLKL and pMLKL in mdx mice compared to the B10 wild type mice (figure 7 (A)). RIPK3 was elevated by 2.5 fold with a p value of 0.005, MLKL was elevated by 2.0 fold with a p value of 0.02, and RIPK1 was elevated by 5.6 fold with no significant p value in mdx mice compared to the B10 wild type mice (figure 7 (B)). This data suggested that there was an activation of necroptosis in mdx mice. After dexamethasone treatment, we were interested to see if the drug was able to rescue mdx muscle fibers from dying. We found that these necroptosis markers were not elevated in the treated B10 wild type mice after dexamethasone treatment compared to non-treated B10 wild type mice. Interestingly, we discovered that in treated mdx mice with dexamethasone, resulted in reduction of protein expression levels of

necroptosis markers including RIPK3 and MLKL (figure 7 (A)). RIPK3 was reduced to 75% with a p value of 0.02 and MLKL was reduced to 52% with a p value of 0.004 in treated mdx mice compared to non-treated mdx mice (figure 7 (B)). The densitometry showed that dexamethasone significantly upregulated Lipin1 in mdx mice. Also, there was significant reduction in RIPK3 and MLKL in treated mdx mice with dexamethasone compared to non-treated mdx mice (figure 7 (B)). In addition, we discovered an intriguing negative correlation between Lipin1 with RIPK3 and MLKL protein expression levels in mdx mice before and after dexamethasone treatment. We found that in non-treated mdx mice, when Lipin1 was downregulated, there was significant upregulation of RIPK3 and MLKL. Upon dexamethasone treatment in mdx mice, when Lipin1 was significantly increased, we found that there was significant downregulation of RIPK3 and MLKL. This data suggested that there might be a negative correlation between Lipin1 and necroptosis markers expression pattern (figure 7 (C)).

A

B

C

Dexamethasone Treatment

Dexamethasone Treatment

48
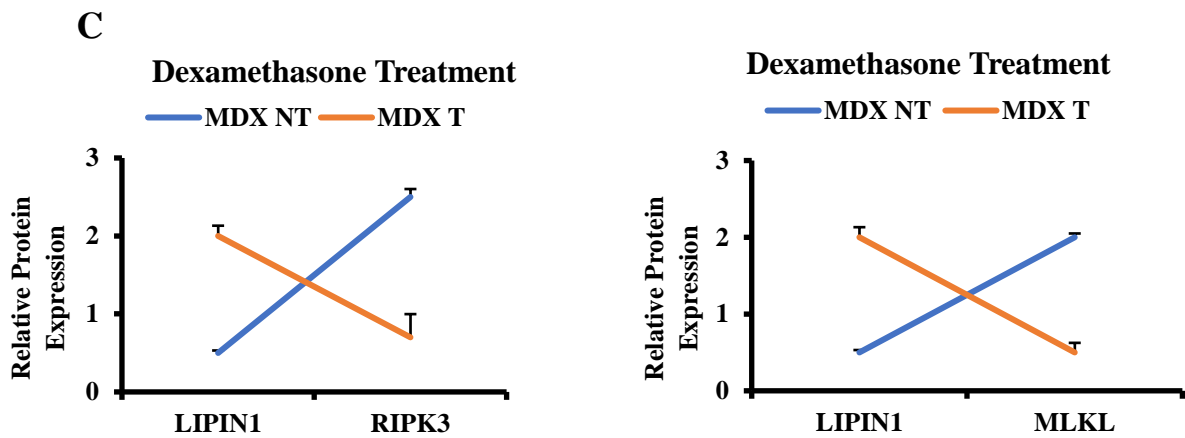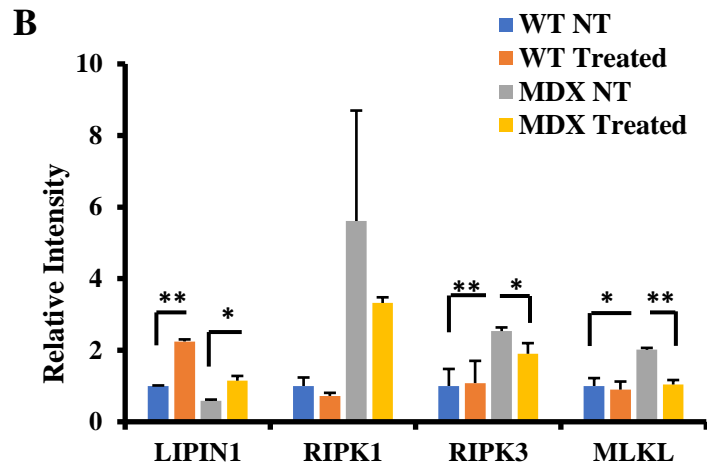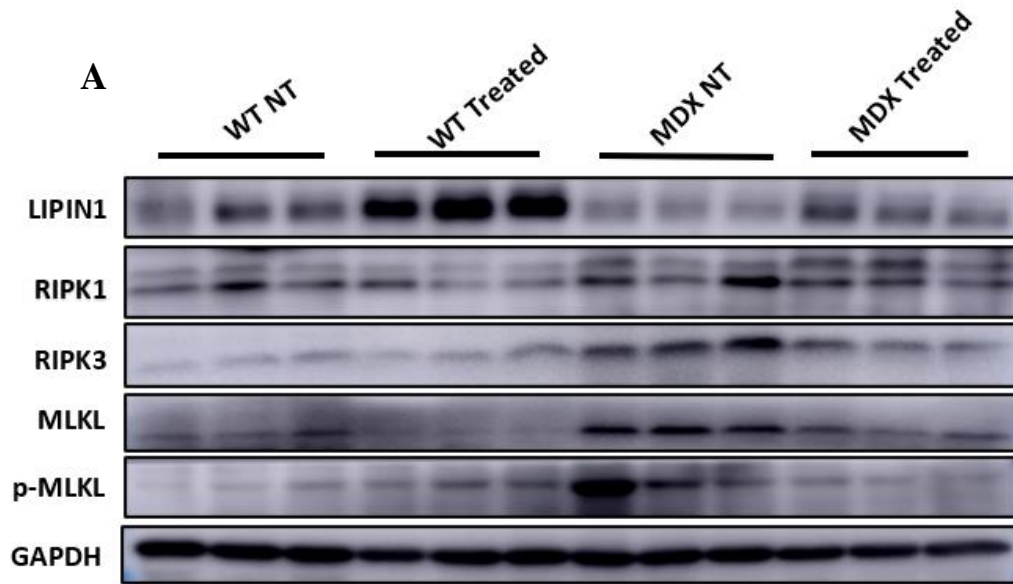
Figure 7: Dexamethasone treated mice shows decreased expression of necroptotic markers. (A) Western blot, (B) the densitometry for Lipin1 and necroptosis markers, RIPK1, RIPK3, MLKL, and p-MLKL in gastrocnemius muscles of WT (Wild Type) and MDX non-treated (NT) and treated mice with dexamethasone (3mg/kg), (n=3 mice/group), (C) Representative graph showing correlation of Lipin1 with RIPK3 and MLKL in mdx mice before and after dexamethasone treatment (n=3 mice/group) (NT)-non-treated, (T)-Treated. RIPK1: Receptor-Interacting serine/threonine Protein Kinase. MLKL: Mixed-Lineage Kinase-domain Like pseudokinase (* $p<0.05$; ** $p<0.01$ Student's t-test).

3.5 Dexamethasone treated mice do not show decreased expression of apoptotic markers.

We also determined the effect of dexamethasone on protein expression levels of pro-apoptotic markers BAK and BAX. Through Western blot analysis, we found elevation of proapoptotic markers BAK and BAX in mdx mice compared to B10 wild type mice (figure 8 (A)). BAK was elevated by 1.5 fold with a *p* value of 0.006 and BAX was elevated by 1.9 fold with no significant *p* value in mdx mice compared to B10 wild type mice (figure 8 (B)). Dexamethasone treatment did not elevate BAK and BAX in treated B10 wild type mice compared to non-treated B10 wild type mice. However, we did not find any reduction in proapoptotic markers BAK and BAX in mdx mice after dexamethasone treatment (figure 8 (A)). The densitometry showed that BAK was significantly elevated in mdx mice compared to the B10 wild type mice, but these markers were not reduced in mdx mice upon treatment with dexamethasone (figure 8 (B)).
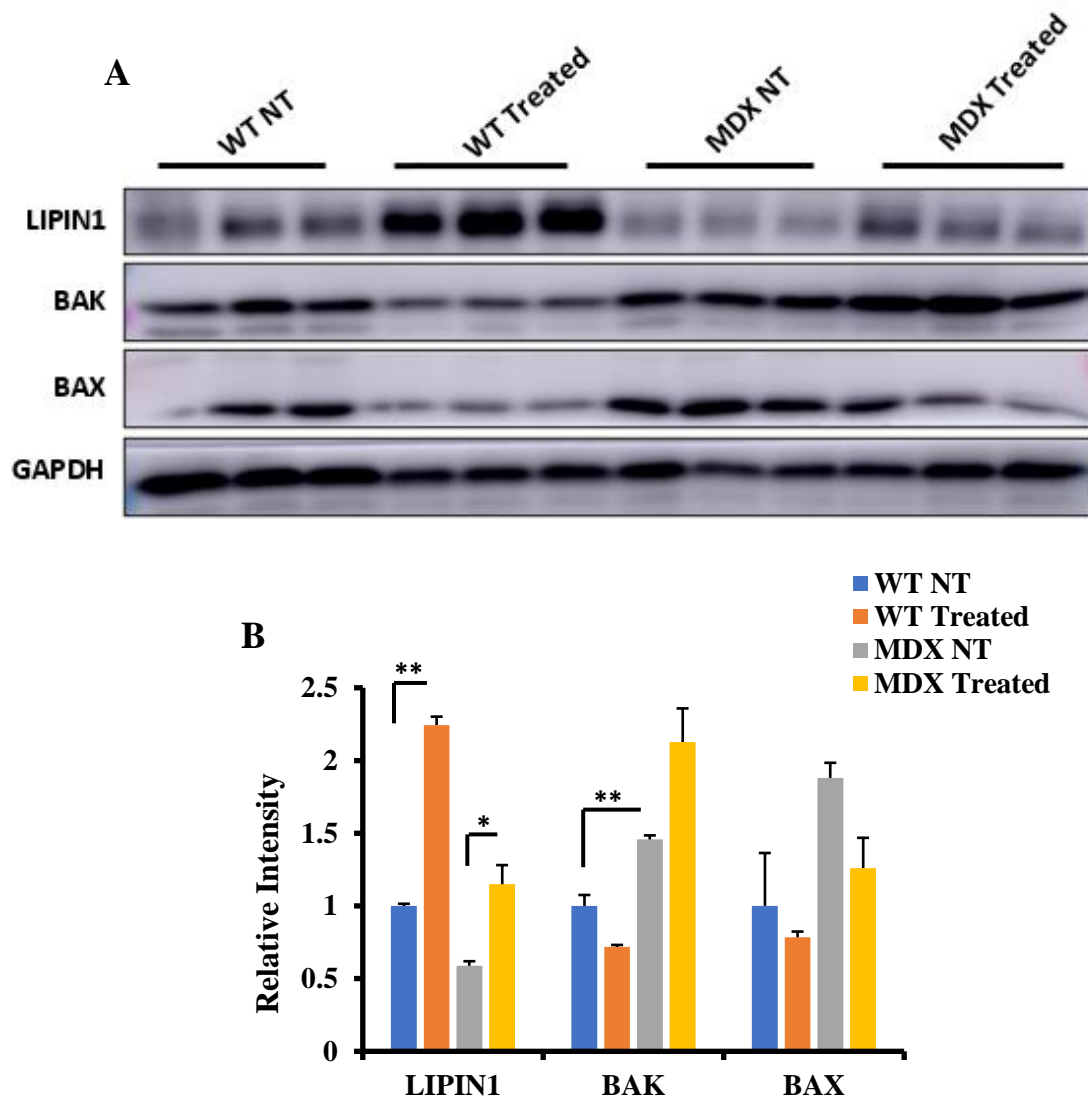
Figure 8: Dexamethasone treated mice do not show decreased expression of apoptotic markers. (A) Western blot, (B) the densitometry for Lipin1 and pro-apoptotic markers, BAK and BAX in gastrocnemius muscles of WT (Wild Type) and MDX non-treated (NT) and treated mice with dexamethasone (3mg/kg) (n=3 mice/group) (* $p<0.05$; ** $p<0.01$ Student's t-test).

3.6 Dexamethasone treated mice shows decreased expression of ER stress markers.

So far, we observed that dexamethasone could downregulate necroptosis markers but not the apoptosis markers. Studies reported by Pauly et al., (2017) and Rashid et al., (2019) showed that there was an activation of ER stress pathway in DMD so, we also investigated the effects of dexamethasone on ER stress markers, PERK and GRP78 in dystrophic muscles. Through Western blot analysis, we found elevation of ER stress markers PERK and GRP78 in mdx mice compared to B10 wild type mice (figure 9 (A)). PERK was elevated by 3.5 fold with a p value of 0.0005 and GRP78 was elevated by 2.2 fold with a p value of 0.001 in mdx mice compared to B10 wild type mice (figure 9 (B)). We noticed that after the mice were treated with dexamethasone, PERK and GRP78 protein expression levels were not elevated in treated B10 wild type mice compared to non-treated B10 wild type mice. Interestingly, we discovered that dexamethasone treatment reduced ER stress markers PERK and GRP78 in treated mdx mice compared to non-treated mdx mice (figure 9 (A)). PERK was reduced to 48% with a p value of 0.002 and GRP78 was reduced to 68% with a p value of 0.05 in treated mdx mice compared to non-treated mdx mice (figure 9 (B)). The densitometry showed significant reduction of PERK and GRP78 in treated mdx mice compared to non-treated mdx mice with dexamethasone (figure 9 (B)).
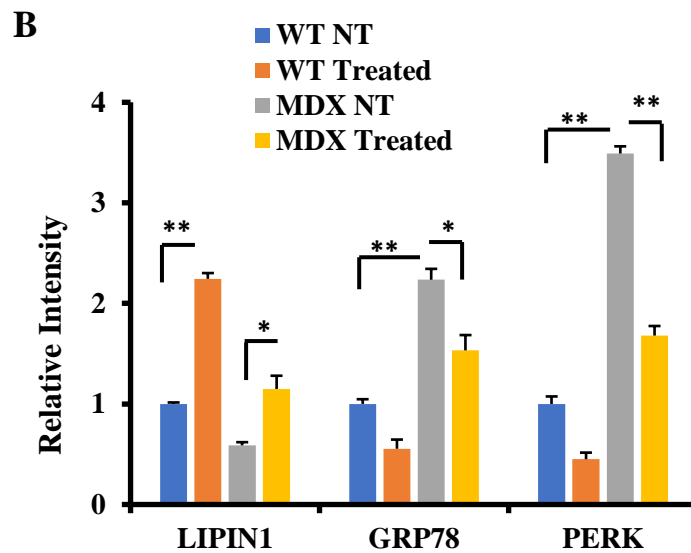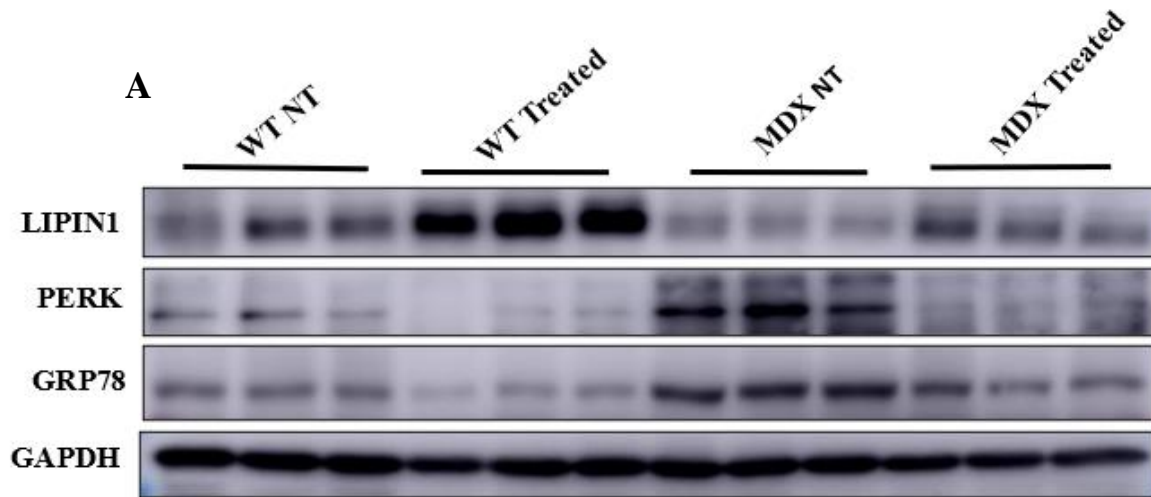
Figure 9: Dexamethasone treated mice shows decrease expression of ER stress markers. (A) Western blot, (B) the densitometry for Lipin1 and ER stress markers, GRP78 and PERK in gastrocnemius muscles of WT (Wild Type) and MDX non-treated (NT) and treated mice with dexamethasone (3mg/kg) (n=3 mice/group) (* p<0.05; ** p<0.01 Student's t-test).
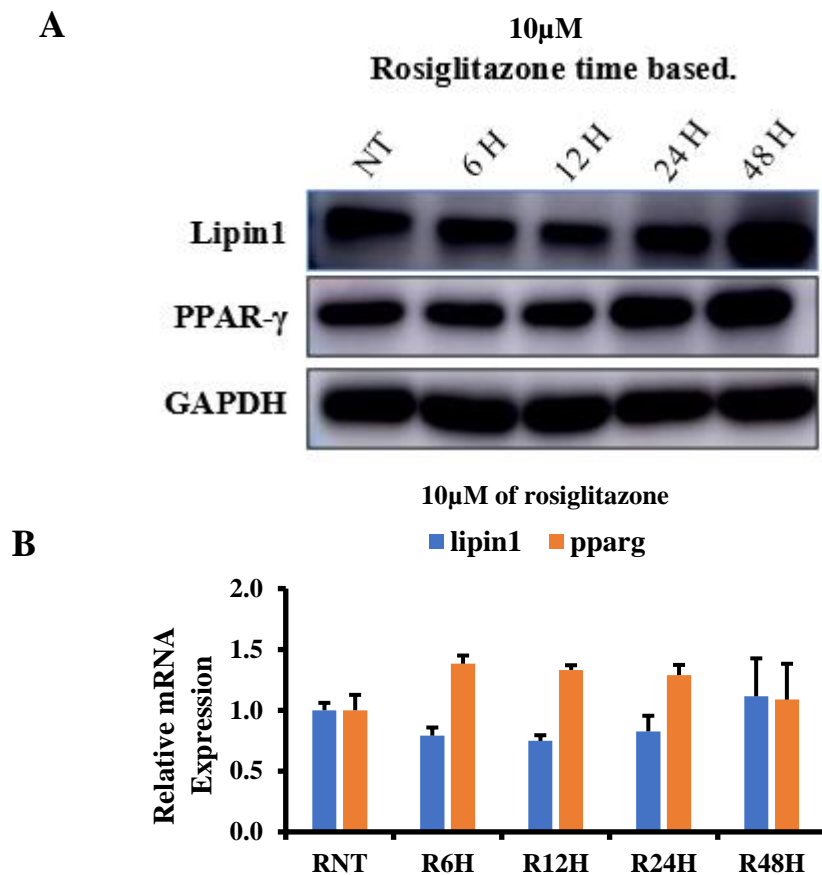
Aim 2: Test the hypothesis that rosiglitazone can inhibit cell death pathways in mdx mice which could be through upregulation of Lipin1.

3.7 Rosiglitazone increases Lipin1 expression in cell culture system.

We used another drug rosiglitazone which is an agonist of peroxisome proliferator-activated receptor gamma (PPARγ). We investigated the effects of rosiglitazone on the expression levels of Lipin1 in C2C12 myoblasts. We used 10µM of rosiglitazone to treat differentiating myoblasts cells on day 2 of differentiation for different time points for 6 hours, 12 hours, 24 hours, and 48 hours and on day 4 of differentiation, cells were harvested for protein and mRNA expression analysis. Through Western blot analysis we found that Lipin1 protein expression levels were gradually increased as the duration of treatment was increased compared to non-treated cells. The maximum Lipin1 protein expression levels increased were observed at 48 hours after the treatment compared to non-treated cells. Similar expression pattern was observed for PPARγ protein expression levels as rosiglitazone was the agonist of PPARγ. Rosiglitazone treatment increased the expression of PPARγ protein levels as the duration of treatment was increased. The maximum increase for PPARγ protein expression levels were observed at 24 hours and 48 hours after the treatment compared to non -treated cells (figure 10 (A)). RT-qPCR was performed for relative

mRNA expression analysis of Lipin1 and PPARγ and we found that relative mRNA

expression of Lipin1 was increased at 48 hours compared to non-treated cells. The

relative mRNA expression of PPARγ was gradually increased at 6 hours, 12 hours

and 48 hours after the treatment compared to non-treated cells. (figure 10 (B)).

A

10μM
Rosiglitazone time based.

NT   6H   12H   24H   48H

Lipin1

PPAR-γ

GAPDH

10μM of rosiglitazone

■ lipin1   ■ pparg

**B**

Relative mRNA Expression

RNT   R6H   R12H   R24H   R48H

Figure 10: Rosiglitazone increases Lipin1 expression in cell culture system. (A) Western blot, (B) mRNA expression of Lipin1 and PPARγ in C2C12 differentiated myoblasts treated with 10μM of rosiglitazone for different time points. (R)-Rosiglitazone, (NT)- non-treated.

3.8 Rosiglitazone increases Lipin1 protein expression levels in wild type and mdx mice.

After we observed that Lipin1 protein and mRNA expression levels were increased in C2C12 myoblasts upon rosiglitazone treatment, we then investigated the effects of rosiglitazone on Lipin1 protein expression levels in B10 wild type mice and mdx mice. We divided mice in two groups: non-treated and treated with rosiglitazone. Each group consisted of six B10 wild type mice and six mdx mice out of which three were control mice and three were treated mice. Rosiglitazone was injected intraperitoneally (i.p.) at a concentration of 10mg/kg once daily for 7 days (Carmona et al., 2005). All the mice were feed chow diet and given regular water. At day 7, mice were euthanized, and Gastrocnemius muscles were collected for Western blot analysis and immunostaining (figure 3 (B)).

To start with, we investigated the effect of rosiglitazone on Lipin1 protein expression levels in B10 wild type mice and mdx mice. Through Western blot analysis, we found that in mdx mice, Lipin1 protein expression levels were downregulated by 0.5 fold with a p value of 0.06 compared to B10 wild type mice. This data suggested that Lipin1 protein expression was downregulated in mdx mice nearly half the levels as compared to B10 wild type mice. After rosiglitazone treatment, interestingly, we found that Lipin1 protein expression levels were upregulated in treated B10 wild type mice by 1.7 fold with a p value of 0.002 compared to non-treated B10 wild type

mice. It was interesting to discover that rosiglitazone upregulated Lipin1 protein expression levels in treated mdx mice by 2.2 fold with a p value of 0.01 compared to non-treated mdx mice (figure 11 (A)). As rosiglitazone is a PPARγ agonist, we also measured PPARγ protein expression levels in B10 wild type mice and mdx mice. We found that in mdx mice PPARγ protein expression levels were similar as B10 wild type mice. After rosiglitazone treatment, we found that PPARγ protein expression levels were increased in treated B10 wild type mice by 1.3 fold with a p value of 0.02 compared to non-treated B10 wild type mice. However, we did not find any upregulation of PPARγ protein expression levels in treated mdx mice compared to non-treated mdx mice. (figure 11 (A)). The densitometry revealed significant elevation of Lipin1 protein expression levels in B10 wild type mice and mdx mice after rosiglitazone treatment (figure 11 (B)). We did not find any significant change in total PPARγ protein expression levels across the B10 wild type mice and mdx mice groups. This was consistent with the data reported for total PPARγ protein expression after rosiglitazone treatment by Choi et al., (2010).
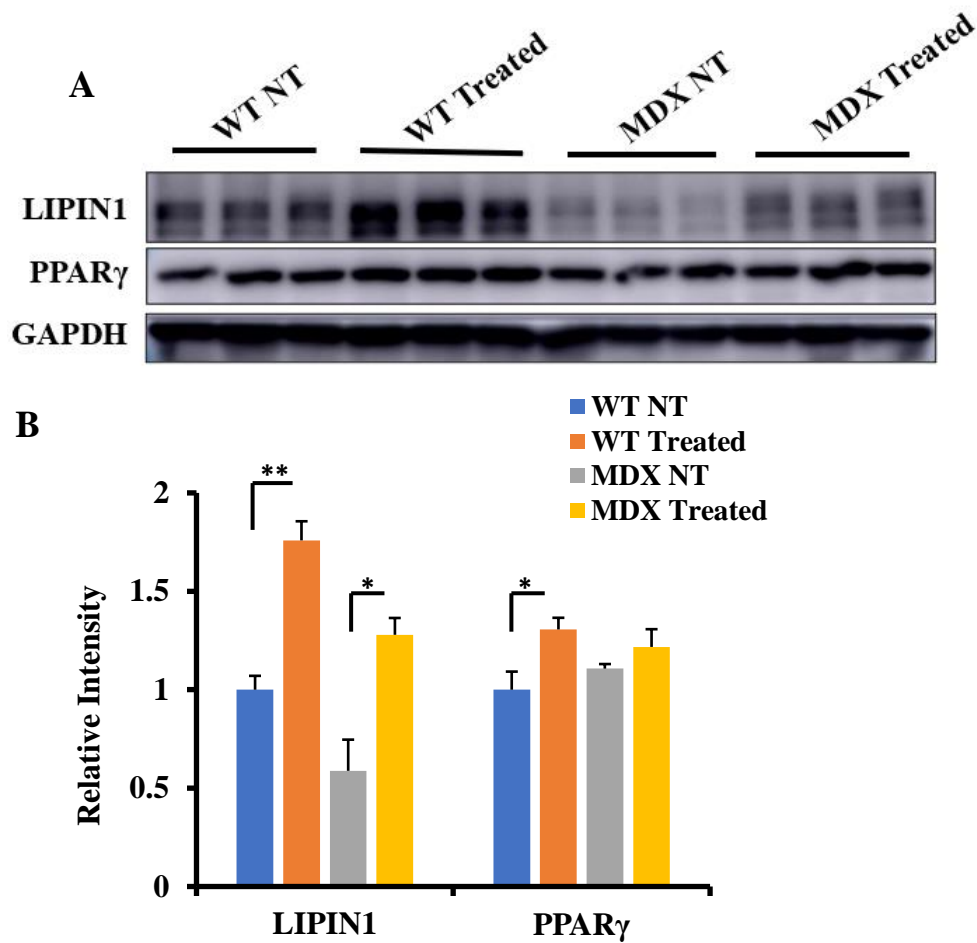
Figure 11: Rosiglitazone increases Lipin1 protein expression levels in wild type and mdx mice. (A)Western blot, (B) the densitometry of Lipin1 and PPARγ in gastrocnemius muscles of WT (Wild Type) and MDX non-treated (NT) and treated mice with rosiglitazone (10mg/kg) (n=3 mice/group) (* $p<0.05$; ** $p<0.01$ Student's t-test).

3.9 Rosiglitazone treated mice shows decreased expression of necroptotic markers.

After we observed that rosiglitazone elevated Lipin1 protein expression levels in mdx mice, we further measured necroptotic markers before and after rosiglitazone treatment in B10 wild type mice and mdx mice. We investigated the effects of rosiglitazone on the protein expression levels of necroptosis markers, RIPK1, RIPK3, and MLKL. Through Western blot analysis, interestingly, we found elevation of necroptosis markers including RIPK1, RIPK3 and MLKL in mdx mice compared to B10 wild type mice (figure 12 (A)). RIPK1 was elevated by 6.2 fold with a *p* value of 0.004, RIPK3 was elevated by 24.1 fold with a *p* value of 0.008 and MLKL was elevated by 8.4 fold with a *p* value of 0.03 in mdx mice compared to B10 wild type mice (figure 12 (B)). This data suggested that there was an activation of necroptosis in mdx mice. After rosiglitazone treatment, we found that necroptosis markers were not elevated in treated B10 wild type mice compared to non-treated B10 wild type mice. It was very fascinating to discover that in mdx mice rosiglitazone treatment resulted in reduction of protein expression levels of necroptosis markers including RIPK1 and RIPK3 in treated mdx mice compared to non-treated mdx mice (figure 12 (A)). RIPK1 was reduced to 30% with a *p* value of 0.008, RIPK3 was reduced to 40% with a *p* value of 0.03 (figure 12 (B&C)). The densitometry showed that there was significant downregulation of necroptosis

markers, RIPK1 and RIPK3 after rosiglitazone treatment in mdx mice compared to non-treated mdx mice (figure 12 (B & C)). In addition, we discovered an intriguing negative relationship of Lipin1 with RIPK1 and RIPK3 protein expression levels in mdx mice before and after rosiglitazone treatment. We found that in non-treated mdx mice when Lipin1 was downregulated, there was significant upregulation of RIPK1 and RIPK3. Upon rosiglitazone treatment in mdx mice, when Lipin1 was significantly upregulated, we found that there was significant downregulation of RIPK1 and RIPK3. This data suggested there might be a negative correlation between Lipin1 and necroptosis markers expression pattern (figure 12 (D)).

D
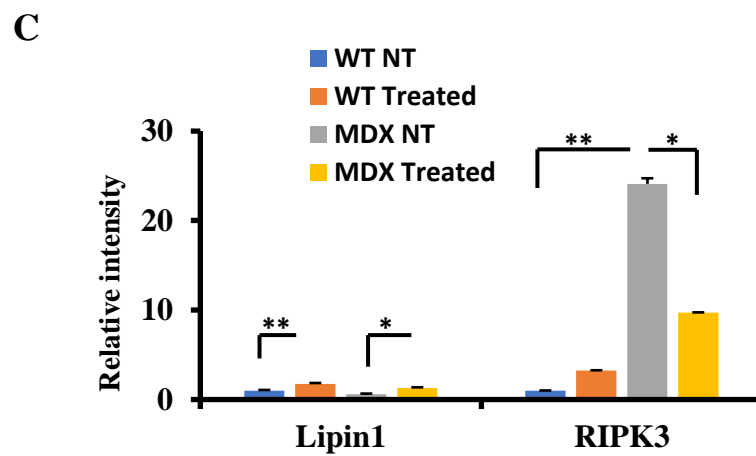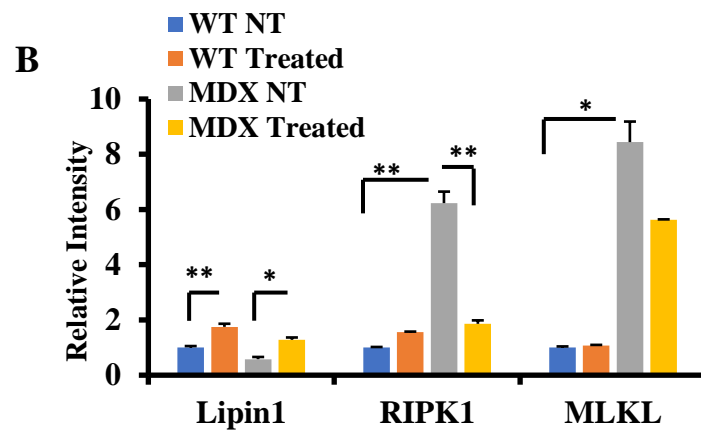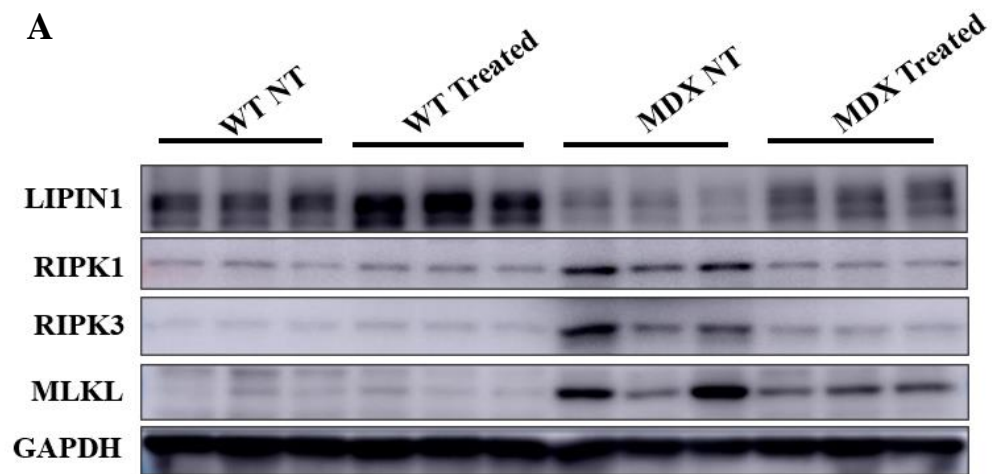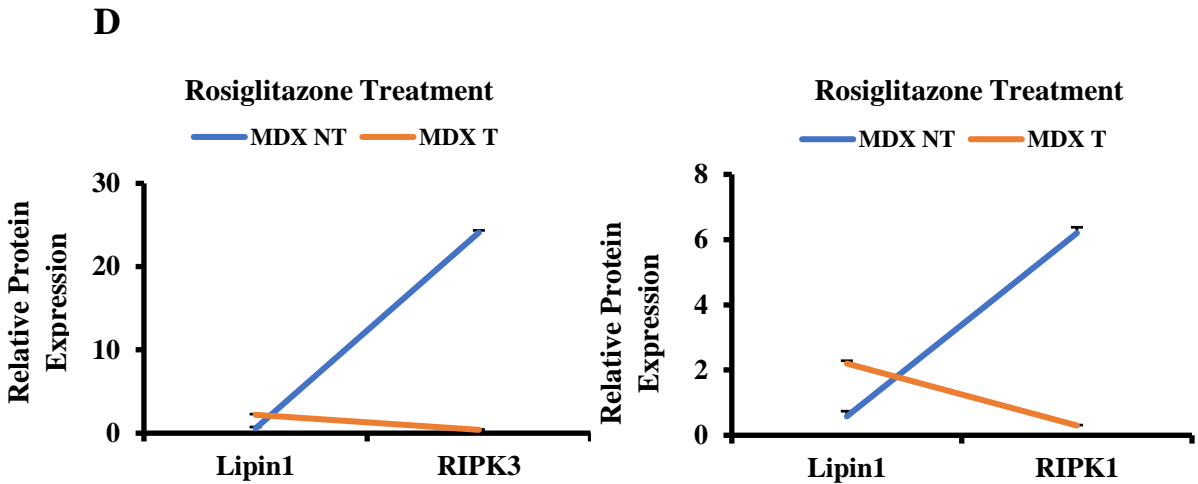
Figure 12: Rosiglitazone treated mice shows decreased expression of necroptotic markers. (A) Western blot, (B&C) the densitometry for Lipin1 and necroptosis markers, RIPK1, RIPK3, MLKL in gastrocnemius muscles of WT (Wild Type) and MDX non-treated (NT) and treated mice with rosiglitazone (10mg/kg), (D) Representative graph showing correlation of Lipin1 with RIPK1 and RIPK3 in mdx mice before and after rosiglitazone treatment (n=3 mice/group) (NT)-non-treated, (T)-Treated. RIPK: receptor-interacting serine/threonine-protein kinase. MLKL: mixed-lineage kinase-domain-like pseudokinase (* $p<0.05$; ** $p<0.01$ Student's t-test).

3.10 Rosiglitazone treated mice shows decreased expression of apoptotic markers.

We also determined the effect of rosiglitazone on protein expression levels of pro-apoptotic markers BAK, BAX, cleaved caspase 3 and Cleaved caspase 9. Through Western blot analysis, we found an elevation of pro-apoptotic markers BAK, BAX and cleaved caspase 3 in mdx mice compared to B10 wild type mice (figure 13 (A)). BAK was elevated by 3.4 fold with a p value of 0.006, BAX was elevated by 7.1 fold with a p value of 0.00002 and cleaved caspase 3 was elevated by 4.0 fold with a p value of 0.02 in mdx mice compared to B10 wild type mice (figure 13 (B)). After rosiglitazone treatment, we found that pro-apoptotic markers were not elevated in B10 wild type treated mice compared to non-treated B10 wild mice. Interestingly, we discovered that rosiglitazone treatment further reduced BAK, BAX AND cleaved caspase 3 in treated mdx mice compared to non-treated mdx mice (figure 13 (A)). BAK was reduced to 53% with a p value of 0.02, BAX was reduced to 61% with a p value of 0.005 and cleaved caspase 3 was reduced to 38% with a p value of 0.04 in treated mdx mice compared to non-treated mdx mice (figure 13 (B)). The densitometry showed that there was significant reduction in BAX, BAK and Cleaved caspase 3 in treated mdx mice compared to non-treated mdx mice (figure 13 (B)).
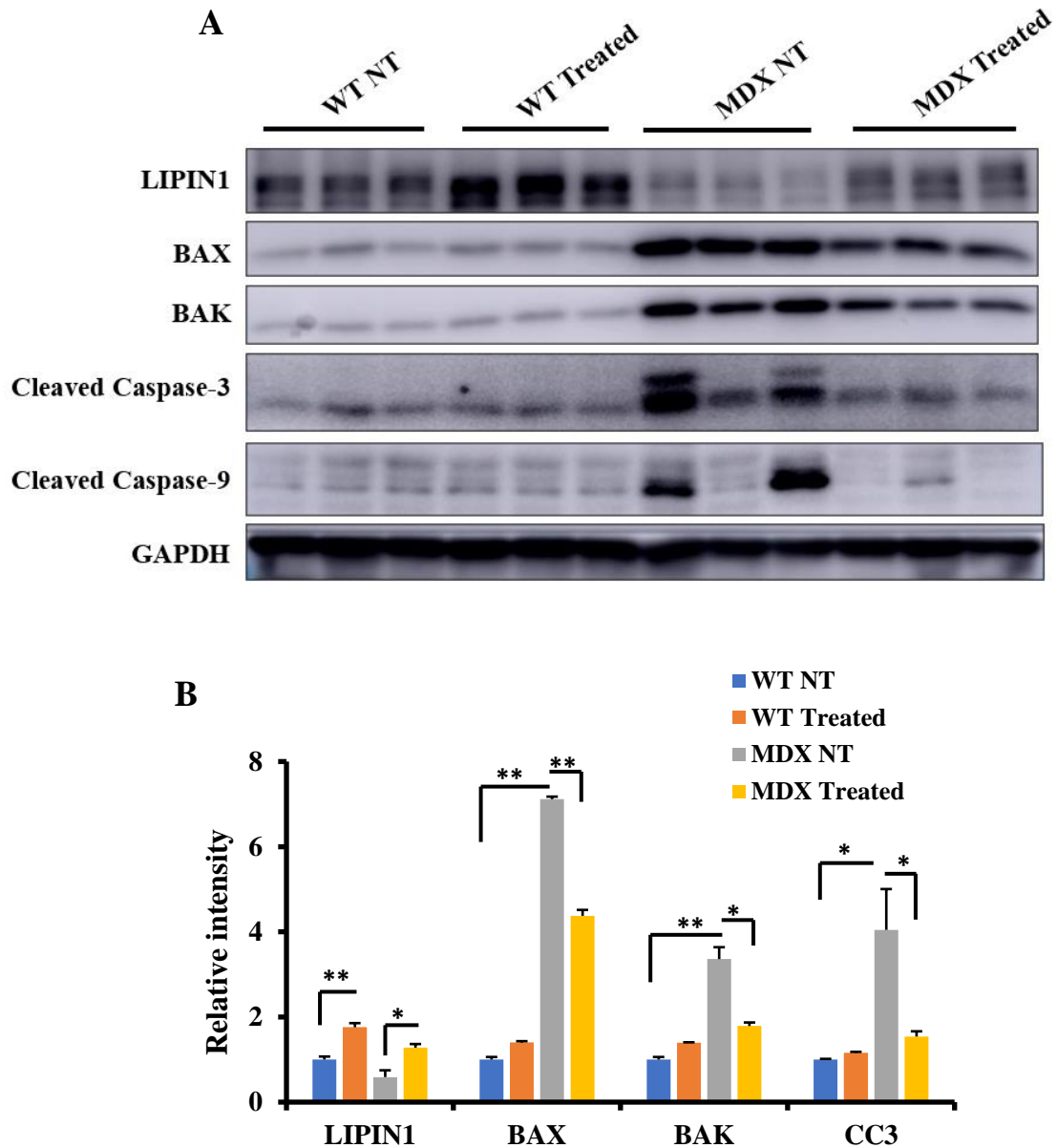
Figure 13: Rosiglitazone treated mice shows decreased expression of apoptotic markers. (A) Western blot, (B) the densitometry for Lipin1 and pro-apoptotic markers, BAK and BAX, cleaved caspase-3 (CC3) in gastrocnemius muscles of WT (Wild Type) and MDX non-treated (NT) and treated mice with rosiglitazone (10mg/kg) (n=3 mice/group) (* $p<0.05$; ** $p<0.01$ Student's t-test).

3.11 Rosiglitazone treated mice shows decrease expression of ER stress markers.

So far, we observed that rosiglitazone could downregulate necroptosis markers as well as apoptosis markers. We also investigated the effects of rosiglitazone on ER stress markers, PERK and GRP78 and inflammation marker p-NFκB in dystrophic muscles. Through Western blot analysis, we found an elevation of ER stress markers PERK and GRP78 in mdx mice compared to B10 wild type mice (figure 14 (A)). PERK was elevated by 4.1 fold with a p value of 0.0002 and GRP78 was elevated by 1.5 fold with a p value of 0.03 in mdx mice compared to B10 wild type mice (figure 14 (B)). We also investigated inflammation marker in mdx mice and found that there was an upregulation of p-NFκB by 2 fold with a p value of 0.01 in mdx mice compared to the B10 wild type mice (figure 14 (A)). After rosiglitazone treatment, we found that, PERK and GRP78 protein expression levels were not elevated in treated B10 wild type mice compared to non-treated B10 wild type mice. Interestingly, we discovered that rosiglitazone treatment downregulated PERK and GRP78 in treated mdx mice compared to non-treated mdx mice (figure 14 (A)). PERK was reduced to 50% with a p value of 0.005 and GRP78 was reduced to 83% with no significant p value in treated mdx mice compared to non-treated mdx mice (figure 14 (B)). Also, we noticed that the rosiglitazone treatment did not elevated p-NFκB protein expression levels in treated B10 wild type mice compared to non-

treated B10 wild type mice. However, rosiglitazone did not reduce p-NFκB protein expression levels in treated mdx mice compared to non-treated mdx mice (figure 14 (A)). The densitometry showed significant reduction of PERK in treated mdx mice compared to non-treated mdx mice with rosiglitazone after Lipin1 was significantly increased in treated mdx mice (figure 14 (B)).
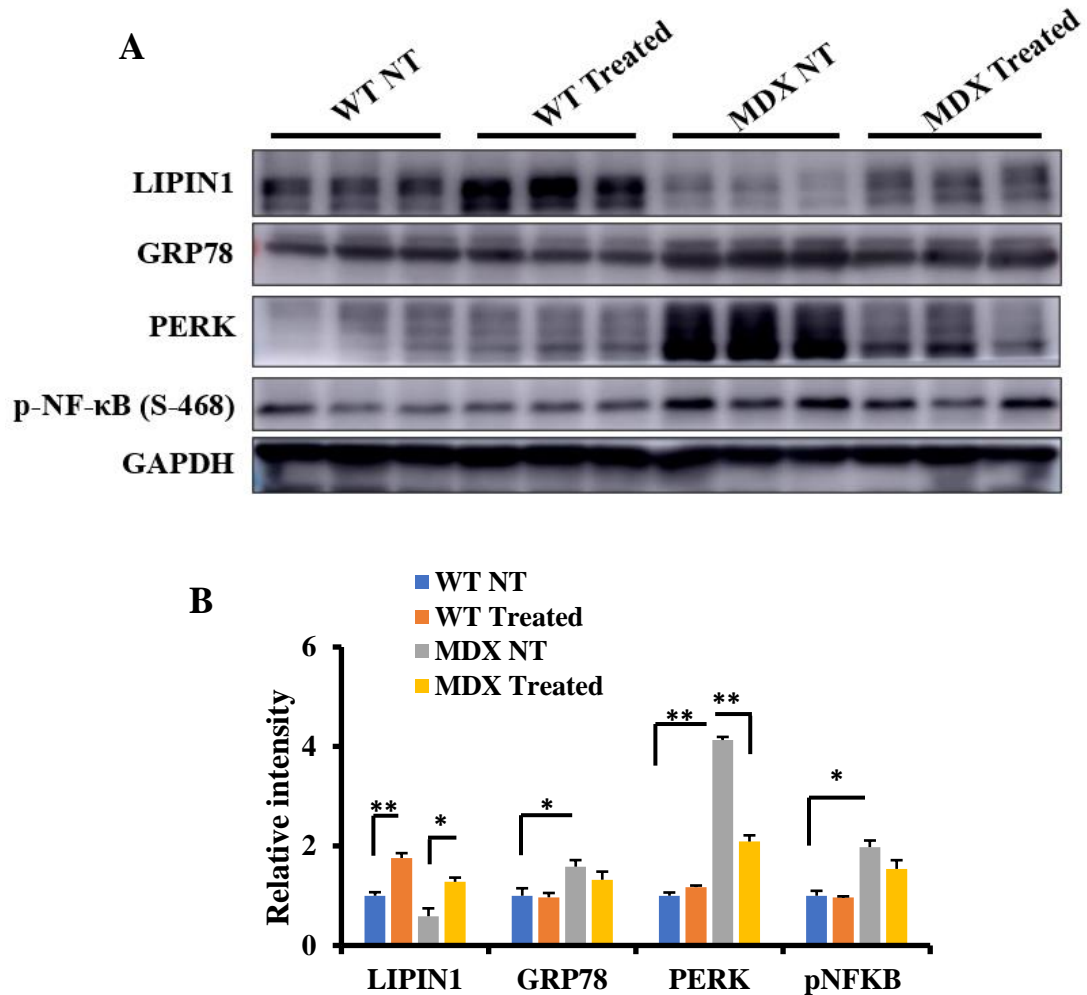
Figure 14: Rosiglitazone treated mice shows decrease expression of ER stress markers. (A) Western blot, (B) the densitometry for Lipin1 and ER stress markers, GRP78 and PERK and inflammation marker p-NFκB in gastrocnemius muscles of WT (Wild Type) and MDX non-treated (NT) and treated mice with rosiglitazone (10mg/kg), (n=3 mice/group) (* p<0.05; ** p<0.01 Student's t-test).

Aim 3: Test the hypothesis that dexamethasone can strengthen membrane integrity in mdx mice which could be through upregulation of Lipin1.

3.12 Dexamethasone effects on muscle membrane integrity in mdx mice.

DMD is caused by a lack of the cytoskeletal protein dystrophin, which is required for the structural integrity of the muscle cell membrane (Houang et al., 2018; Lapidos et al., 2004; Blake et al., 2002; Vianello et al., 2014). So, we investigated effects of dexamethasone on muscle membrane integrity of mdx mice before and after the treatment. The B10 wild type mice and the mdx mice were subjected to dexamethasone treatment via drinking water at concentration of 3mg/kg for 14 days and on day 14, mice were euthanized, and gastrocnemius muscles were dissected and fixed frozen in liquid nitrogen using chilled methylbutane. 10 µm muscle sections were prepared using a cryostat and were then subjected to immunostaining with membrane-impermeable marker, IgG to detect any damage in muscle membrane integrity. Laminin is represented in red immunofluorescence which stained the muscle membrane borders and IgG is represented in green immunofluorescence which stained the damaged muscle fibers. The B10 wild type mice muscle tissue sections did not show any IgG positive muscle fiber before and

after dexamethasone treatment. On the other hand, non-treated mdx muscle tissue sections showed increased IgG positive muscle fibers of about 4% with green fluorescence compared to the B10 wild type mice tissue sections which had 0% IgG positive muscle fibers that might suggest loss of membrane integrity in mdx mice. Treated mdx mice tissue sections with dexamethasone showed IgG staining with 2.5% IgG positive muscle fibers which were 1.5% less compared to non-treated mdx mice. However, we did not find any significant p value due to higher variation between individual mdx mice and there were only 3 mice used but, including more number of mice in future might give a p value (figure 15 (A&B)).

A

IgG/**Laminin**

WT BASAL   WT DEX
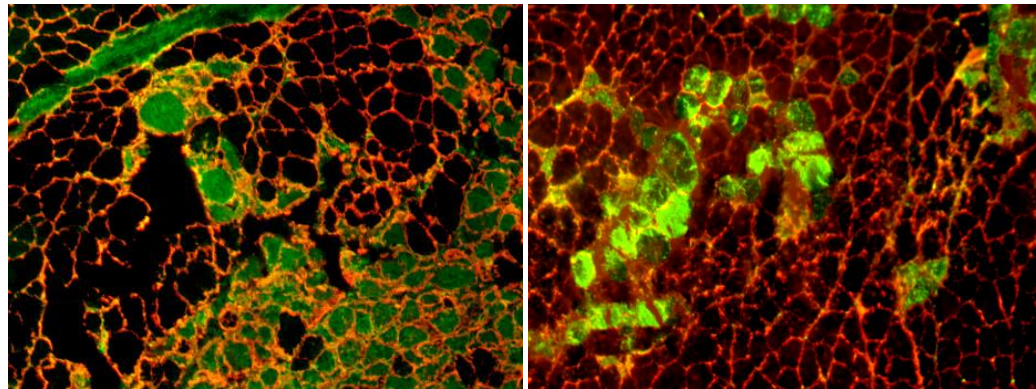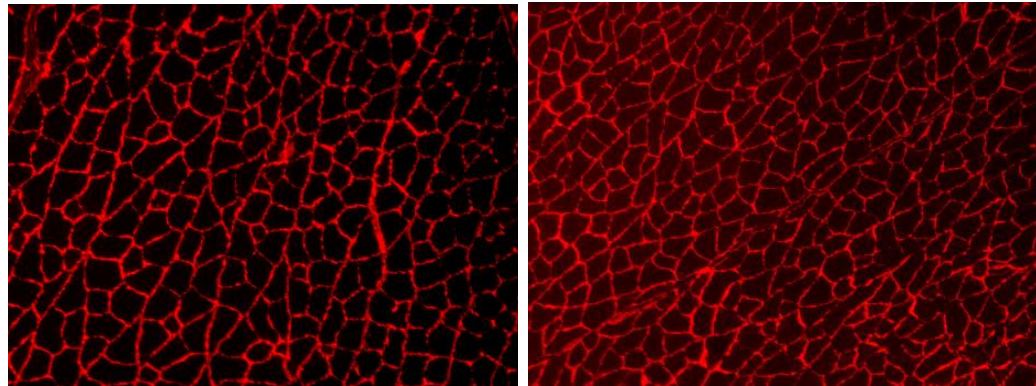
MDX BASAL   MDX DEX
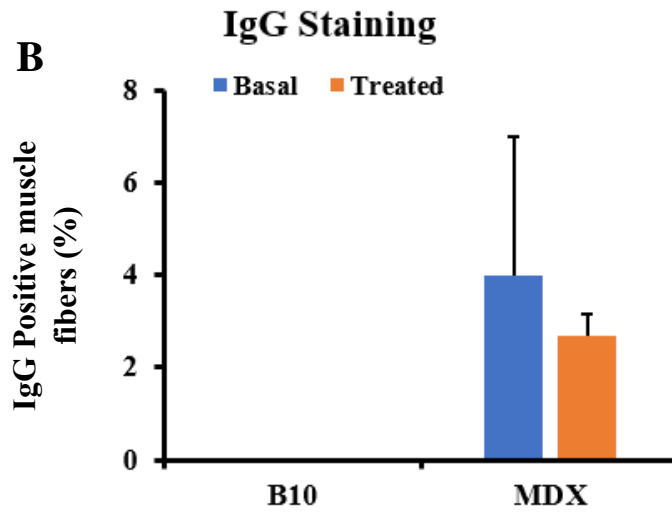
Figure 15: Dexamethasone treatment improves muscle membrane integrity in

mdx mice. (A) Gastrocnemius muscle sections from WT (Wild Type) and mdx

71

non-treated (NT) and treated mice with dexamethasone (3mg/kg). (n=3mice/group) were immunostained with goat anti-mouse IgG and laminin to detect permeable/damaged fibers, (B) Quantification (%) of IgG-positive fibers (n = 3 mice/group).

4. Discussion

Our study suggests that dexamethasone and rosiglitazone can ameliorate dystrophic phenotype in mdx mice. We showed that dexamethasone can downregulate necroptosis in mdx mice. We showed that rosiglitazone can downregulate necroptosis and apoptosis in mdx mice. We also showed that both these drugs can elevate Lipin1 protein expression levels.

Our study showed that dexamethasone and rosiglitazone can downregulate RIPK3 in mdx mice which is the key molecule involved in necroptosis. This is consistent with study reported by Morgan et al., (2018), who provides the first evidence of the involvement of necroptotic muscle cell death in diseases affecting muscles, especially RIPK3 playing a key role in degenerative process in dystrophin deficient muscles in mice and humans. RIPK3 have been suggested to play an important role in induction of necroptosis, fibrosis, and inflammation, which can be triggered by excess TNFα activation, Toll-like receptors, and T-cell receptors. (Jouan-Lanhouet et al., 2014; Newton, 2015; Shlomovitz et al., 2017; Morgan et al., 2018). Eliminating RIPK1, MLKL, and especially RIPK3 can result in reduction of necroptosis, inflammation and fibrotic tissue deposition which results from degeneration/regeneration episodes in mdx mice and improves myofiber survival and muscle function (Morgan et al., 2018).

Not only necroptosis, but apoptosis has also been suggested to drive muscle cell death in patients with DMD (Tidball et al., 1995; Sandri and Carraro, 1999; Serdaroglu et al., 2002). Our study showed that rosiglitazone can downregulate BAK, BAX and cleaved caspase 3 in mdx mice. BAX has been shown to be increased in the blood of DMD patients (Abdel-Salam et al., 2008). BAX and caspase 3 expression has shown to correlate with the skeletal muscle apoptosis in DMD, suggesting apoptosis via mitochondria mediated release of cytochrome *c* (Sandri et al., 2001). However, it has been suggested that caspase 3 also have non-apoptotic roles as it has shown to be involved in myogenic progenitor differentiation and fusion that was reported by Fernando et al., (2002). Caspase 8 activation is required to drive the muscle fiber fate towards apoptosis and not toward necroptosis (Dhuriya and Sharma, 2018), however the study reported by Morgan et al., (2018) showed that there is no cleaved caspase 3 positive myofibers when necroptotic pathway was inhibited in mdx mice, suggesting that inhibiting necroptosis does not generate a switch from necrotic toward apoptotic death pathway in mdx muscles. However, both apoptosis and necroptosis can coexist in the muscles of the patients with neuromuscular disorders that also includes DMD (Sciorati et al., 2016). So as of now, the muscle cell death pathways especially in DMD, whether it is through necroptosis or apoptosis is still the area of debate. However, identification of drugs that can effectively inhibit muscle apoptosis could lead to development of

therapeutic strategies in the treatment of DMD and our data suggests that reduction of BAK, BAX and cleaved caspase 3 by rosiglitazone can ameliorate dystrophic phenotype in mdx mice.

It has been known that in DMD, the major pathology that occurs is the loss of muscle membrane integrity due to dysfunctional dystrophin protein which is the part of a large membrane-spanning complex of glycoproteins and act as molecular shock absorber and dysfunctional dystrophin leads to muscle cell death (Clafin and Brooks, 2008; Nowak and Davies, 2004; Dalkilic and Kunkel, 2003; Straub and Campbell, 1997; Dadgar et al., 2014; Lynch et al., 2000). Biological membranes comprise of various lipids, including phospholipids, sphingolipids, glycolipids, and sterols, that are important for maintaining membrane structure, fluidity, and stability (Dawaliby et al., 2016; Li et al., 2006). One of the therapeutic approaches in DMD is to stabilize the impaired muscle membrane integrity by identifying proteins that have similar functions as dystrophin and can strengthen membrane integrity. One of the studies reported by Babbs et al., (2020), shows that by replacing dystrophin with utrophin might help to strengthen membrane integrity as it can bind similar proteins as of dystrophin, but it has limitations as the utrophin gene delivery is through AAV virus that can generate immunological responses in the patients. Another protein, Lipin1 has phosphatidate phosphatase (PAP) activity which is important for synthesizing DAG which is a precursor for PC and PE which are important phospholipids in

membrane protein trafficking (Schuler et al., 2016; Testerink et al., 2009). Lipin1 deficient myofibers contributes to necroptosis and loss of membrane integrity through altering membrane phospholipid contents suggested by Sattiraju et al., (2020). In addition, Lipin1 has shown to be an important protein in the skeletal muscle development by enhancing MEF2c activity which further regulate various sarcolemmal membrane structural genes that are important for membrane integrity (Jama et al., 2018; Blais et al., 2005; Potthoff et al., 2007).

Our study showed that in mdx mice Lipin1 protein levels were downregulated which falls in line with Lipin1 deficiency contributing to loss of membrane integrity suggested by Sattiraju et al., (2020). Our *in vitro* experiments suggest upregulation of Lipin1 protein and mRNA levels in C2C12 myoblasts cells upon dexamethasone and rosiglitazone treatment. Our study showed that these drugs also elevate Lipin1 protein levels in mdx mice.

It has been suggested that synthetic glucocorticoid, dexamethasone can upregulate Lipin1 gene expression through the molecular mechanism which involves, binding of dexamethasone with glucocorticoid receptors in cytosol and then this complex enters the nucleus where it binds directly to the Lipin1 5' flanking sequence. The sequence that was present in Lipin1 upstream region between -421 and -285 revealed the presence of an imperfect palindromic 15 mer sequence that was similar to the

sequence of glucocorticoid response element (GRE) that was located at -311 to -297, upregulating Lipin1 gene expression (Zhang et al., 2008).

Studies have reported that Lipin1 expression is positively correlated with insulin sensitivity, as Lipin1 expression in adipose tissue is induce by insulin sensitizing compounds such as thiazolidinediones that activates PPARγ, however, the mechanism by which Lipin1 expression is induce in adipose tissue upon thiazolidinediones administration is yet not clear (Yao-Borengasser et al., 2006; Festuccia et al., 2009). We believe that since rosiglitazone improves insulin sensitivity, it might be involved in upregulation of Lipin1 through improving insulin signaling pathway. However, mechanism behind upregulation of Lipin1 via rosiglitazone still needs to be unrevealed, but we think that in mdx mice rosiglitazone treatment can improve insulin sensitivity and downstream effectors of insulin signaling pathway such as AKT activation can lead to inhibition of mTOR complex (O'Reilly et al., 2006), which in turn will allow Lipin1 to enter the nucleus that was initially inhibited by mTOR complex (Peterson et al., 2011) and thus rosiglitazone can potentially upregulate Lipin1 protein expression.

In addition, recent study has reported protective role of PPARγ in inhibition of apoptosis and necroptosis in septic cardiac dysfunction in rats by activation of PPARγ protein levels in rosiglitazone treated rats (Peng et al., 2017). However, in our study we do not observe upregulation of total PPARγ protein levels upon

rosiglitazone treatment in mdx mice. This was consistent with the data reported for total PPARγ protein expression after rosiglitazone treatment by Choi et al., (2010). It might be possible that the phosphorylated form of PPARγ is affected and so we cannot exclude the possibility of inhibition of RIPK3 and RIPK1 via PPARγ pathway. So, more work is needed to be done to further confirm the mechanism of rosiglitazone on inhibition of RIPK3 and RIPK1 in mdx mice.

To summarize our study, we identified two drugs, dexamethasone, and rosiglitazone, can elevate lipin1 mRNA and protein expression levels in mdx mice and can downregulate RIPK1, RIPK3 and MLKL. Rosiglitazone can also downregulate apoptosis markers including BAK, BAX and cleaved caspase 3 in mdx mice. Dexamethasone might strengthen membrane integrity but including more mice number in future can help to get a significance. Our future study will identify whether the effects of dexamethasone and rosiglitazone on the inhibition of necroptotic markers in dystrophic muscles are through the upregulation of Lipin1 expression.

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