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Apoptosis and Necrosis Drive Muscle Fiber Loss in Lipin1 Deficient Skeletal Muscle

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APOPTOSIS AND NECROSIS DRIVE MUSCLE FIBER LOSS IN LIPIN1 DEFICIENT SKELETAL MUSCLES

A thesis submitted in partial fulfillment

of the requirements for the degree

of the Master of Science

By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Sandhya Ramani Sattiraju ENTITLED Apoptosis and necrosis drive muscle fiber loss in lipin1 deficient skeletal muscle BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Sattiraju, Sandhya Ramani M.S. Department of Biochemistry and Molecular biology, Wright State University, 2020. Apoptosis and necrosis drive muscle fiber loss in lipin1 deficient skeletal muscle.

Mutations in lipin1 are suggested to be a common cause of massive rhabdomyolysis episodes in children, however, the molecular mechanism involved in the regulation of myofiber death by lipin1 is not known. In this study, we utilized the skeletal muscle from cell-type-specific lipin1 knockout (Lipin1^{Myf5cKO}) mice to define cell death pathways involved in lipin1 deficient muscles. We observed a significant increase in centrally nucleated fibers and embryonic myosin heavy chain (EMyHC)-positive regenerating fibers in Lipin1^{Myf5cKO} mice compared to wild-type (WT) mice, indicating an increased cycle of degeneration and regeneration in lipin1 deficient muscles. Lipin1 deficient muscles had significantly elevated pro-apoptotic factors (Bax, Bak, and cleaved caspase 9) and necroptotic proteins such as RIPK1, RIPK3, and MLKL compared with WT mice. Moreover, loss of membrane integrity is considered as a hallmark of cell death and we found that Lipin1^{Myf5cKO} mice had significantly higher membrane disruptions as evidenced by increased IgG staining and elevated uptake of Evans Blue Dye (EBD) in muscle fibers. EBD-positive fibers were strongly colocalized with apoptotic or necrotic myofibers, suggesting an association between compromised plasma membrane integrity and cell death pathways. We further show that the absence of lipin1 leads to a significant decrease in the absolute and specific muscle force (normalized to muscle mass). Our work indicates that apoptosis and necroptosis are associated with a loss of membrane integrity in muscle lacking lipin1 and myofiber death may induce an expected decrease in contractile force and a defect in muscle function.

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I. INTRODUCTION

i. Skeletal myopathy

Skeletal myopathies are a group of diseases that are characterized by progressive muscle atrophy and weakness (K. Josiak et al., 2014). The basic characteristics of myopathies are muscle weakening, muscle pain, and, muscular dysfunction. Myopathy patients are usually recognized with continuous inflammation and weakness of the muscle. Myopathies are grouped as hereditary and acquired disorders (Furst D.E. et al., 2012). The hereditary type of myopathies includes muscular dystrophies, congenital myopathies, metabolic myopathies, and mitochondrial myopathies. Muscular dystrophies are caused by an abnormal gene (germline mutations) which interferes with the production of muscle proteins that are essential for the muscle development. Most form of this condition is observed in early childhood. Damaged muscles become progressively weaker. (González-Jamett et al., 2018). Dystrophic patterns i.e. degenerative patterns with necrosis and extensive fibrosis in the muscles are observed and also the central nervous system is involved in muscular dystrophic patients (Koutsoulidou, A et al., 2020). In congenital myopathies, where the patients are born with the underdeveloped skeletal motor system due to which they lack muscle tone and has enhanced muscle weakness. Other myopathies are mitochondrial myopathies which are caused by the defects in the mitochondrial functioning affecting the muscular function (Pfeffer, G. et al., 2011). On the other hand the inflammatory, endocrine, and toxic myopathies are included in the acquired group of myopathies.

During muscle injury conditions ATP starts depleting. As a result, there's an excessive influx of intracellular sodium and calcium ions. The prolonged presence of calcium ions signals the muscles for sustained myofibrillar contractions which depletes the ATP further (Al-Ismaili Z et al., 2011). The increased levels of calcium ion activate the proteases and phospholipases which promotes lysis of cellular membrane and further damage to the ion channels (Huerta-Alardín AL et al., 2011). All these events lead to alternations in myocyte milieu causing inflammation, self-sustained myolytic cascade which causes necrosis and release all the blood components into the muscle environment. These alterations initiate the rhabdomyolysis cascade in patients (Vanholder R et al., 2000).

ii. Rhabdomyolysis

Rhabdomyolysis is a rare condition in which skeletal muscle breaks down, releasing muscle enzymes and electrolytes from inside the muscle cells. Patients who suffer from overwhelming myopathy associated rhabdomyolysis lead to muscle atrophy (muscle loss) and myalgia (muscle pain) (Nance J.R. et al., 2015). The clinical diagnosis of myopathy is made when there are muscle pain and muscle weakness along with elevated levels of CK (Creatinine Kinase). Hence, rhabdomyolysis is known as a severe form of myopathy with muscle breakdown leading to myoglobinuria and finally acute renal failure (Omar MA et al., 2001).

Rhabdomyolysis is a state of injury that can lead to the rapid dissolution of damaged or injured skeletal muscle fibers. Injury of skeletal muscle disrupts the integrity leading to the release of cellular components including myoglobin, creatine kinase into the bloodstream, and extracellular space of the muscle (Huerta-Alardín AL et al., 2011). All these events result in etiology such as muscle pain, muscle weakness, and muscle swelling.

Biochemically, an important symptom of this disease is the darkening of the urine (pigmenturia), known as myoglobinuria. The other evidence is plasma myoglobin increases rapidly and immediately eliminated from the body through renal excretion. This is observed often during post-muscle injury conditions. (P Koskelo et al., 1967). However, all these various initial laboratory evaluations do not necessarily explain the acquired cause of rhabdomyolysis (Nance J.R. et al., 2016). Thus, the researchers anticipate that most of the rhabdomyolysis cases have inherited susceptibility.

Rhabdomyolysis can be caused by a wide range of diseases, which could be classified as sporadic or hereditary/recurrent. According to a recent statistical study depicted that around 26,000 cases were reported annually only in the USA by the National Hospital Discharge Survey. (Melli G et al., 2005). Most of these rhabdomyolysis cases in the adult population are acquired type. This disease is also observed in early childhood which is caused by somatic mutations (Alaygut D. et al., 2017). Clinical etiology in a recurrent type of rhabdomyolysis is different from the acquired type of rhabdomyolysis. In childhood, viral myositis, trauma, collective tissue disorders are responsible for rhabdomyolysis (Torres P.A. et al., 2015). In this disease initiation of the viral infection may leads to a serious condition of muscle degeneration, accounting for one-third of pediatric cases in the USA (Brinley A et al., 2018). Muscle degeneration is one of the clinical manifestations of rhabdomyolysis.

iii. Apoptosis

We discussed in the previous sections about muscle loss and degeneration leading to severe cases of Rhabdomyolysis. These events initiate due to the myoblast death in muscle tissues. (George G.D. et al., 2006). This muscular degeneration can be caused by various cellular processes in which apoptosis is one of the major contributors. Apoptosis is an important process in multicellular organisms to self-destroy when no longer needed or during damage conditions.

It is popularly defined as the process of programmed cell death (Alberts B. et al., 2013). During the initial studies on cell death, regulated and advanced stages were first reported in the larval muscles. Eventually, myobiology researchers concluded that mammalian skeletal muscles also undergo an apoptotic way of cell death. This study leads to breakthroughs in various muscle-related diseases (Sandri M. et al.,1999). The cellular and molecular aspects of myoblasts and myofibers apoptosis and their roles in disease are the present areas of interest in the field of myobiology. This programmed cell death can be triggered in two ways, via intrinsic pathway, which is an endogenous system such as Bax/Bcl2 or via extrinsic system Fas and Fas L involving transmembrane receptors of death receptor family.

iii.1 Intrinsic Apoptosis

The intrinsic pathway is usually observed in all muscle degeneration related diseases. For instance, in DMD disease, in response to an injured environment, impaired regeneration is observed in muscle cells. The aim to study the degeneration and regeneration in muscle cells can be measured by intrinsic pathway markers (Abdel-Salam E. et al., 2009). In this pathway, cells must undergo metabolic stress in order to activate Bcl2 family-related proteins and initiate caspase cascade (Elmore S. et al., 2007). The mitochondria are closely involved in the process of apoptosis as it has two main characteristics. Firstly, the intermembrane space of mitochondria contains distinct pro-apoptotic proteins which can activate the apoptosis leading to cell death upon getting released into the cytosol. Secondly, the mitochondria have fundamental producers of reactive oxygen species which can sometimes have a direct or indirect effect on apoptosis (Adhihetty P.J. et al., 2003).

The composition of mitochondria consists of the outer and inner membrane which separated by intermembrane space. When considered the intrinsic apoptosis in skeletal muscle, up to date limited research was focused on determining the physiological and pathophysiological condition which leads to apoptosis. When muscle tissues and differentiated muscle cells are subjected to experimentation, the intrinsic apoptosis is initiated by a diverse array of non-receptor mediated stimuli that produce intercellular signals on cells activating the mitochondrial events (Elmore S. et al., 2007). In response to the stimulus, alterations in the ratios of pro-apoptotic proteins (Bax and Bak) and anti-apoptotic proteins (Bcl2 and Bclxl) leads to the activation of initiation caspases i.e. caspase 9 (Kale J et al., 2018). The release of cytochrome c from the mitochondria allows the initiation of a caspase cascade. Therefore, once the initiation caspase is activated, all the other caspases such as caspase 3 are cleaved and activated leading to DNA fragmentation. The evidence of apoptosis cell death can be found in numerous skeletal muscle myopathies such as muscular dystrophy and also congenital myopathies. In these muscle degenerative diseases, excessive muscle cell death leads to muscle loss and atrophy (Tews D.S. et al., 1996).

iii.2 Extrinsic apoptosis

Skeletal muscle can also undergo extrinsic stimulus-related apoptosis. This is mainly observed in muscle mass losses during muscular degeneration. The extrinsic pathway develops due to the ligand-mediated stimulation of death receptors located at the plasma membrane of the cell. Extracellular receptors such as cytokine TNF-α and Fas-Ligand (Fas-L) are usually found on the cytotoxic T-lymphocytes and various other immune cells. These ligand binding plays a vital role in the activation of a definite set of proteases which leads to the entire process of Apoptosis (Kumar S. et al.,1999). These commonly occur during the early phase of Apoptosis.

The caspases can behave in a cascade-like fashion which occurs in the mid-phase of extrinsic apoptosis (Nicholson D.W. et al., 1999). The cell response to the binding of Fas-L binds to the Fas receptors. This binding sequesters the procaspase8 to the cellular membrane by FADD (Fas Associated Death Domains), an adaptor protein that is closely bound to the Fas receptors (Baker S.J. et al., 1998). Accumulation of caspase-8 near the DISC (Death-inducing signaling complex) at the plasma membrane leads to the activation of caspase-8. Activated caspase-8 can directly cleave and simultaneously activate caspase-3 exerting its apoptotic effects on the structures of the cell membrane. These caspase activations are responsible for the cytosolic biochemical breakdown and nuclear targets leading to distinct morphological features of apoptosis (Hood D.A et al., 2003).

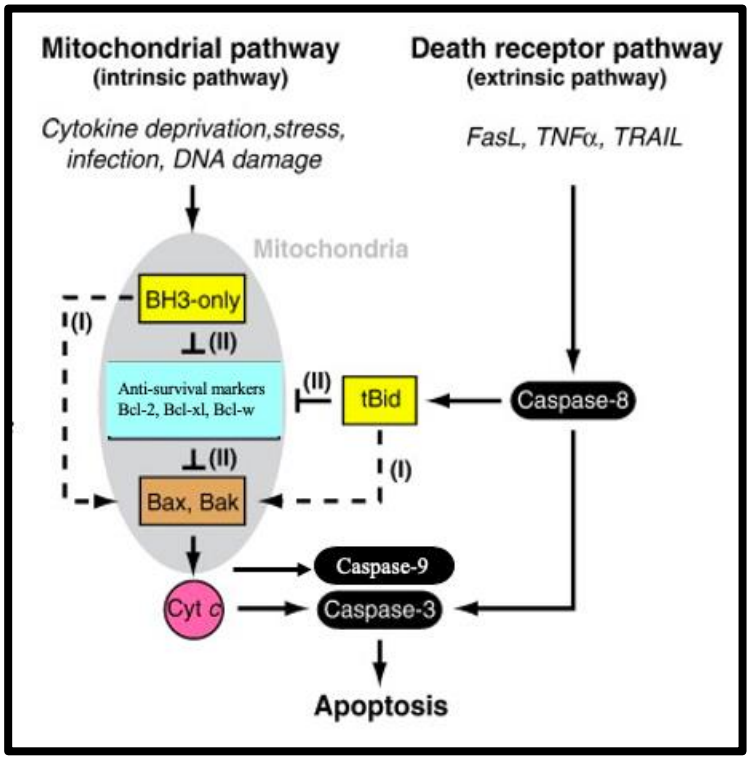


Figure 1: Apoptotic cell death pathway in skeletal muscle. (Westphal D. et al., 2011)

iv. Necrosis

Necrosis is the highly effective and rapid form of cell death in which essentially every part of the cell starts disintegrating. It is especially characterized by deregulation in the cell ion homeostasis which leads to cell swelling, a characterized dilation is observed in mitochondria along with endoplasmic reticulum (Mattson M.P. et al., 2012). Many skeletal muscle diseases such as Muscular dystrophy, myopathy, and others are estimated to occur due to necrosis way of cell death (Lopes Ferreira M. et al., 2001). Degeneration of myofibers is multifactorial but the myofibers are susceptible to the mechanical stress, cytotoxic factors induced and more importantly these various factors cause an inflammatory process which leads to excessive muscle loss (Rosenberg A.S. et al., 2015). Historically, necrosis is known to be an unregulated means of cell death, but recent studies have shown that necrosis can genetically be controlled; which is termed as 'Necroptosis' (Degterev A. et al., 2005). This Necroptosis is a caspase-independent cell death pathway, but it insignificantly overlaps with the extrinsic apoptotic pathway (Chen Q. et al., 2018). Few proteins are common in both pathways such as Tumor necrosis factor- α (TNFα) which is an inflammatory cytokine, but it also acts as a strong pro-necrosis factor in myofibers. TNFα involves two members of the receptor-interacting protein (RIP) family of kinases which form complex to ultimately stimulate the inflammation proteins in the cell. Hence, necroptosis/ necrosis muscle fibers show huge characteristic inflammation (Morgan J. E. et al., 2018). The ubiquitinated links are attached to prevent caspase 8 or RIPK3 interaction with the RIPK1 to induce death signals and consequently lead the cell survival, all this is termed as Complex I. Fas-associated death domain (FADD) and pro-caspase 8 interact which forms a Death inducing signaling cascade (DISC).

This complex when dissociated from the plasma membrane forms a Complex II (Linkermann A. et al., 2013). The activity of Caspase-8 increases with the proximity to complex II. Hence, the important role of active caspase 8 is to prevent necroptotic activity. This can be effectively achieved when RIPK1and RIPK3 is cleaved to form a necroptosome. This necroptosome consists of RIPK1 and RIPK3 along with a Mixed linage kinase-like domain (MLKL) (Jevnikar A.M. et al., 2013). The recruitment of MLKL is finally leading to the disruption of the membrane and resulting in irreparable damage to the muscle cells (Reigger J. et al., 2019).

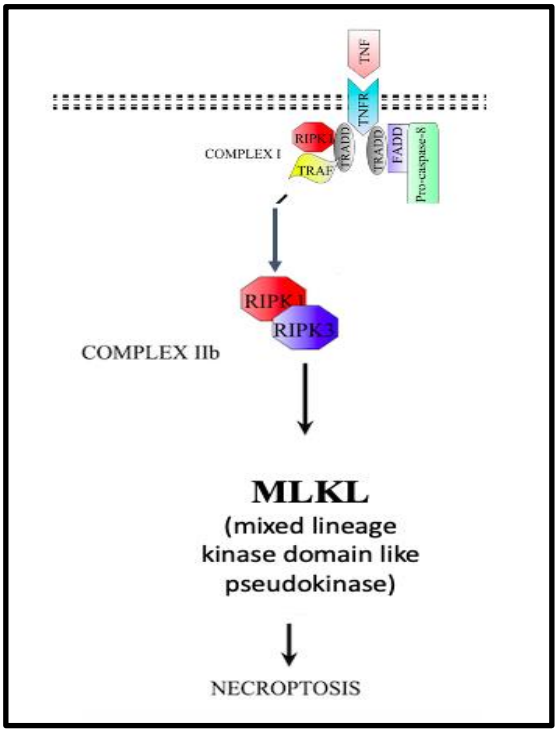


Figure 2:Necroptotic (Necro-apoptotic) pathways observed in skeletal muscle (Escobar M.L. et al., 2015)

The apoptosis is known to be the popular form of programmed cell death (PCD) which is regulated by distinct Caspase cascade. When this programmed cell death signaling is dysregulated the cell death pathway shift to Necroptosis which can major defects in the developmental stages. This necroptosis pathway regulated by a few specific Receptor

interacting protein kinases 1 and RIPK3. These kinases act as important signaling interactions as they promote the recruitment of MLKL. Tumor necrosis factor (TNF-1)- ligation induces signaling via the NF-κB pathway which involves the polyubiquitination of RIPK1. RIPK1 loses its default pro-survival function to promote cell death function (Linkermann A. et al., 2014). The pathway in which cells induce cell death RIPK1 and RIPK3 interact with each other via RIP homotypic interaction motifs (RHIMs) which is present in both kinases.

These interactions between RIPs form 'necrosome' which an oligomeric cytosolic complex in which reciprocal phosphorylation of RIPK1 and RIPK3 leads to recruitment and activation of MLKL (Orozco S. L. et al., 2018). The activated MLKL is translocated to and gets disrupted in the plasma membrane. Loss of membrane integrity during necroptosis results in the release of cellular contents leading to inflammatory responses in the muscles (Zhang X. et al., 2019).

v. Lipin1

In the human body, triacylglycerols (TAG) are the long-term storage sites in adipose tissue, and they provide a huge source of fatty acids. Fatty acids undergo oxidation reactions to provide energy to the skeletal muscles (Greenberg A.S. et al., 2011). Lipin1 is an intracellular protein that controls metabolism by acting at different regulatory levels (Harris T.E. et al., 2011). Lipin1 is an Mg^{2+} - dependent phosphatidic acid phosphatase enzyme (PAP1). This is a very essential enzyme used for lipid biosynthesis and adipocyte differentiation. This lipin protein family consists of three members, lipin1, lipin2, and lipin3.

Out of all these lipin proteins, lipin1 is the most prominent protein as it is expressed in both adipose tissue and skeletal muscle. (Carman G.M. et al., 2009) they discovered that lipin1 has two crucial molecular functions. Firstly, lipin1 acts as a phosphatide acid phosphatase activity (PAP1) enzyme which catalyzes the phosphatide (PA) to diacylglycerol (DAG) particularly during triglycerides (TGs), phosphocholine (PC) and phosphatidylethanolamine biosynthesis

(Carman G.M. et al., 2009). Secondly, lipin1 is expressed in various tissues but the most prominent expression is observed in adipose tissue, skeletal muscle and testis (Reue K. et al., 2009). Lipin1 nuclear function may be related to transcription coactivation (Finck et al., 2006) which helps in activating hepatic fatty acid oxidation genes during fasting conditions. Lipin1 precisely interacts with Peroxisome Proliferator Activator Receptor α (PPARα) and PPARγ coactivator 1α (PGC1α). These combined forms a complex and modulates the fatty acid oxidation expression.

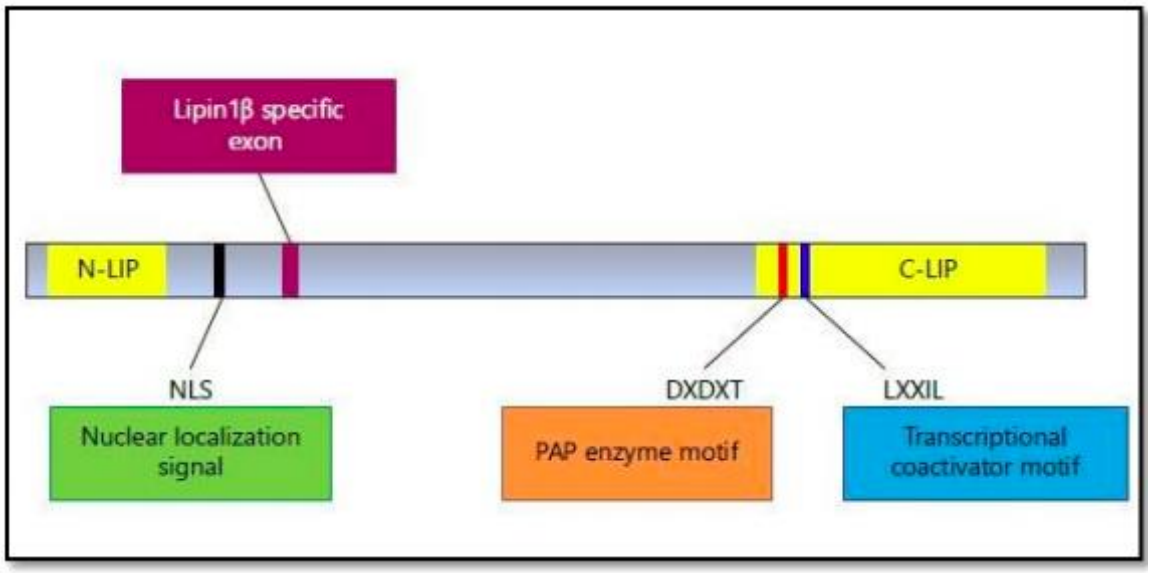


Figure 3:Representation of classical roles in lipin1(Y. Chen et al, 2015)

Figure 3 depicts the structural representation of the lipin1 protein. All the lipin1 protein family has two evolutionarily conserved regions. These regions are N-LIP (N-terminal) and C-LIP (C-terminal) of the lipin gene. Both terminals had different motifs that express very specific functions of the lipin gene. N-LIP has NLS (Nuclear Localization Signal) the domain which is arginine and lysine-rich region. This motif helps lipin to translocate to the nucleus and this subcellular localization can be influenced by protein phosphorylation.

On the other hand, C-LIP has two protein functional motifs; one is a haloacid dehalogenase (HAD)-like phosphatase motif DXDXT, this helps in enzymatic activity of the lipin1 i.e. phosphatidate phosphatase activity. Another motif is LXXIL which is a nuclear receptor interaction motif regulating the transcription coactivator activity of the nuclear lipin. (Y. Chen et al., 2015).

The lipin1 family was first identified in a spontaneous mutant mouse strain, which was later named as fatty liver dystrophy(*fld*). These mutated mice showed a dysregulated fatty acid synthesis (Csaki et al., 2013; Peterfy et al., 2001; Wang H. et al., 2017). Through positional cloning (Peterfy et al., 2001) lipin1 was identified in mice. In a subset of individuals mutations in these mice characterize to exhibit lipodystrophy, hypertriglyceridemia, neonatal fatty liver, insulin resistance, and peripheral neuropathy (Y. Chen et al., 2015). Humans also exhibit similar phenotypes for the same lipin1 mutations as observed in mice and they exhibit smaller & atrophied muscle (Michot et al., 2012; Jiang et al., 2015). According to a recent study, human patients with lipin1 mutations tend to show severe cases of rhabdomyolysis (Michot C. et al., 2012). Along with rhabdomyolysis they also observed excessive accumulation of lipid droplets in the skeletal muscles (Michot C. et al., 2012; Chen Y. et al., 2015). However, the current understanding of lipin1 doesn't explain the phenotypes seen in these rhabdomyolysis patients. Our lab aims to research on different roles of lipin1 in various muscle degenerative diseases.

vi. Hypothesis and aims

Recent studies have explained that deficiency of lipin1 leads to severe episodes of rhabdomyolysis (Michot C. et al., 2012). Our laboratory has already found that lipin1 deficiency leads to increased centrally nucleation in skeletal muscle (Jama A. et al., 2018). Therefore, we hypothesize that lipin1 deficiency promotes muscle degeneration through apoptosis and necrosis. With this ideology, we came up with three aims: 1) whether lipin1 deficiency leads to apoptosis and necroptosis in skeletal muscles. This degeneration is explained by aim 2) whether deficiency affects membrane integrity and muscle function. As mentioned in earlier chapters, muscle injury is the initiation of degradation in myofibers leading to myofiber death. In which necroptosis and apoptosis contribute to major degeneration of muscle fibers. 3) To determine whether myofiber death is associated with plasma membrane disruption.

II. MATERIALS AND METHODS

i. Animal

Generation of Lipin1 ^{Myf5cKO} knockout mice

To generate Lipin1 ^{Myf5cKO}, we crossed Lipin1$^{flox/flox}$ (Nadra et al., 2008) with myf-Cre mice. These myf-Cre mice were acquired from Jackson's Laboratory (Stock no: 007893). This study was performed using Lipin1 wildtype mice and skeletal muscle-specific lipin1 deficient knockout mice. To check the genotype information of these mice, mice tails were collected from respective pups. Experiments were performed on 2-4-months-old mice. These mice had free access to drinking water and regular chow. All animal experiments were performed under the relevant guidelines and regulations approved by the Animal Care and Use Committee of Wright State University.

ii. Tissue collection and cutting

Optimal Cutting Temperature (OCT) was used to immerse the entire muscle and later they were dipped in -160 °C isopentane for 20 seconds. To maintain the temperature of the fixed tissue, they are put on dry ice for a few seconds before storing them in the -80 °C freezer. These fixed tissues are used for various staining when they are cut at a thickness of 7μm using the Cryostat Thermo Scientific Microm HM550. This machine is usually maintained at -20°C.

iii. Western blotting

This was used to analyze the different biomarkers of apoptosis and necrosis. Muscle tissues were isolated and snap-frozen in liquid nitrogen for subsequent homogenization or lysed in RIPA buffer (10 mM Tris-HCL pH 7.4, 30 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) supplemented with proteinase inhibitors (SIGMA complete ™ Protease Inhibitor Cocktail 04691166001) and phosphatase inhibitors (SIGMA P2850 -Phosphate Cocktail1) before the use. We used 1ml 1X RIPA buffer + 0.2% Protease + 0.2% Phosphatase. We used tissue samples for the experiments. Frozen tissue samples were thawed at room temperature by letting them on ice for about 10mins. About 20- 50 mg of tissue was added to 450μl of homogenizing mixture for each sample. Samples were subjected to centrifugation at 12000rpm at 4°C for 10mins. Protein concentration was measured using bicinchoninic acid (BCA) assay. Bovine serum albumin (BSA) of concentration 2mg/ml was used as standard (0, 2μl, 4μl, 6μl, 10μl, 12μl were loaded in a 96well plate). 3ul-5ul of the sample was added to the plate. Each standard and sample was duplicated for precision. Assay plates were incubated in a 37°C incubator for 20-30mins. Later these plates were analyzed for protein absorbance at a wavelength of 567 using Synergy H1 microplate reader. The BCA assay standard curve provided the concentration values for the samples to-be added for western blotting.

Gels were prepared using (30% acrylamide solution; 37:5:1 BioRad Cat: 1610158) separated by 7.5–15% SDS-PAGE to separate the protein based on their polypeptide chain length. The desired amount of protein (20 μg - 60 μg) was measured before running the samples and adjusted the volumes using RIPA buffer and 4X loading dye. Muscle lysates were determined for concentration in each sample and equal amounts of proteins were used, boiled at 95°C for 5 min in 1 × SDS sample buffer.

Thenceforth, the protein ladder (Fisher CatNo:26616) (Bio-Rad; Cat No:161-0373) and protein samples were loaded on the gel-filled with 1X running buffer. The power was set to a voltage of 100V and eventually changed to 120V with 0.3 A current for the proteins to enter into the stacking gel. Later, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using a Mini Trans-Blot Cell System (Bio-Rad, Hercules, CA, USA) which were soaked in methanol for 1-3 mins and then transferred into transfer buffer. Filter papers and sponges were all soaked in the 1X transfer buffer. Once gels are finished running, they were removed from the plates and placed on the filter paper and then PVDF membrane, finally closed them with sponges around them. To transfer the proteins, the membranes were stacked into the gel holder cassette of the transfer system. These cassettes are transferred into the transferring box equipment, set at voltage 150V and 0.3 A current for a span of 2 hrs submerged in a box of ice. The membranes are separated from the cassettes and subjected to blocking to avoid the extra unspecific binding. A blocking buffer is prepared using a 1% casein buffer and 1X TBS solution in a 1:9 ratio. These blots are cut according to the desired biomarker size and incubated for about 1hr. Later these blots are incubated with the primary antibodies overnight at 4°C. Primary antibodies used include lipin1 (#14906; Cell Signaling Technology, Beverly, MA, USA), Bax (#2772), Bak (#12105), Bcl-2 (#3498), Bcl-xl (#2764), cleavedcaspase9(ccp9, #9508), cleavedcaspase3(ccp3, #9664), RIPK3 (#95702), RIPK1 (#3493T), and MLKL (#37705S). These different primary antibodies are diluted using antibody solution (10% casein and 0.1% Tween 20). After overnight incubation, these blots are washed (3X) with TBST solution (1X TBS and 0.1% Tween 20, Fisher CatNo:175476). Then the same blots are probed with secondary antibodies for 1h at 25°C. After the incubation, blots are washed again with TBST solution.

Protein bands were detected by using Chemiluminescence kit from Pierce^{TM} ECL western blotting substrate (catalog #32106). The instrument used to visualize in Amersham Imager 600 (GE Healthcare Life Sciences, Little Chalfont, UK). Goat anti-mouse IgG-HRP (#w402B; Promega, Madison, WI, USA) and goat anti-rabbit IgG-HRP (#w401B; Promega) secondary antibodies were used for detection. GAPDH (Ab181602; Abcam) antibody was used as a loading control. Western blots were quantified by densitometry using NIH Image J software and all values were normalized to loading control.

iv. Histological and Immunohistochemical analysis

Lipin1^{Myf5cKO} and WT mice for each group were euthanized for histological or immunohistochemical analysis. Immediately following sacrifice, the gastrocnemius muscles were dissected and frozen in 2-methyl butane chilled to a slurry on liquid nitrogen. Sections of frozen tissue were prepared at 7-15 μm using a cryostat and stored these cryosection biopsies on microscope slides (Thermofisher Cat No: 22-230-900) at −20°C until stained. Hematoxylin and eosin staining were used for the analysis of mean myofiber cross-sectional area and central nucleation, a hallmark of muscle degeneration/regeneration cycles accordingly to the manufacturer's protocol (Vector Laboratories, H3502). The ratio of myofibers with centrally located nuclei to the total myofibers in the field was counted. For immunostainings, sections were blocked with 5% BSA-PBS and incubated with the primary antibody. Negative controls were performed by omitting the primary antibody. Primary antibodies used in this study are laminin (Ab 11575, Abcam), Bax (#14796; Cell Signaling Technology), cleaved caspase 3 (#9664), and RIPK3 (#95702). The primary antibody was incubated in humid chambers at 37°C for 1h, the slides were rinsed in PBS and incubated in goat anti-rabbit (Alexa Fluor 488) secondary antibody (1:1000; Invitrogen) for 1 h.

After a rinse in PBS, the slides were cover slipped with Vectorshield anti-fade mounting medium with DAPI. Images were obtained using an inverted microscope (IX70; Olympus, Tokyo, Japan) equipped with a DFC7000T camera (Leica Microsystems, Wetzlar, Germany).

v. TUNEL staining

Frozen muscle sections were deparaffinized, rehydrated, fixed with 4% paraformaldehyde, and permeabilized using 0.1% Triton X-100 and 0.1% sodium citrate buffer. The permeabilized sections were incubated for 1 hour in TUNEL (terminal deoxynucleotidyl transferase dUTP nick end-labeling) reaction mixture from an *in-situ* cell death detection kit (Roche, Indianapolis IN, USA), washed, and incubated with Vector shield anti-fade mounting medium with DAPI (Vector Laboratories, H-1200). The protocol was followed as described in Sigma Aldrich *in-situ* cell death detection kit.

vi. Evans blue dye (EBD) assay

Evans Blue staining is an azo dye which detects the presence of serum albumin in cell or tissue. Blood proteins such as serum albumin leak into the damaged tissues making the dye detect those tissue stains red under fluorescence. To confirm the damaged muscle fibers both wildtype and lipin1 deficient muscle fibers were injected with Evans Blue Dye solution(10 mg/ml stock in sterile saline, 0.1 ml/10 g body weight) (intra-peritoneal) and euthanized 24 hours later. The skeletal muscles were dissected and snap-frozen isopentane cooled optimal cutting temperature (OCT) embedding media (Tissue-Tek, Sakura-Americas, Torrance CA, USA).

Frozen muscle OCT blocks were cryo-sectioned at 5-7 µm thickness and stained with laminin antibody before analyzed by fluorescence microscopy. The EBD stained damaged muscle fibers are observed in red fluorescence as shown below in the Fig4.

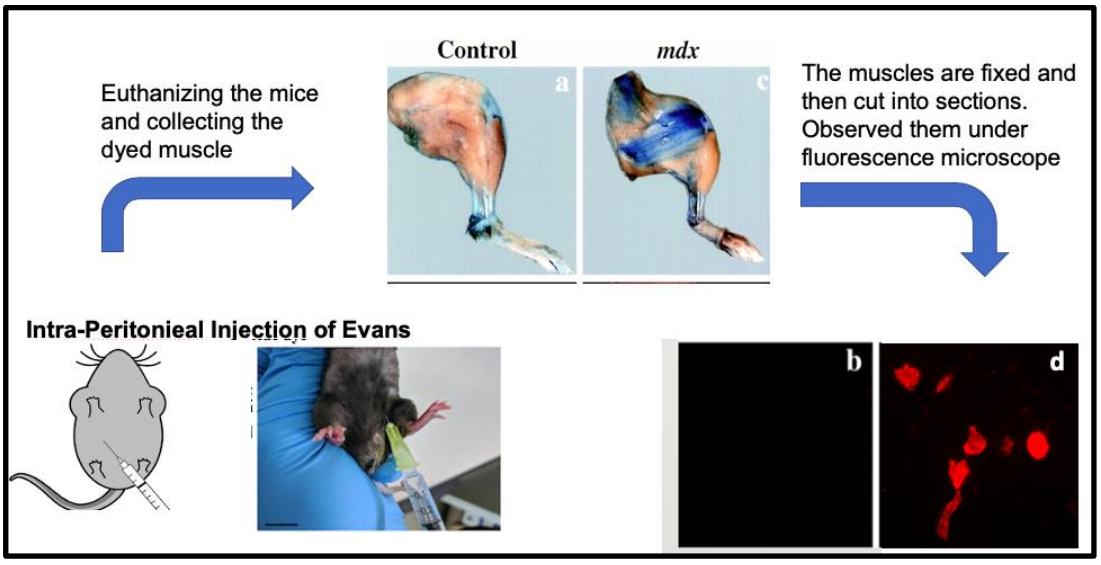


Figure 4: A new procedure to detect the membrane damage especially in the Lipin1^{Myf5cKO} mice. (Fantin, A. et al., 2018).

Statistical analysis: All the graphs and data discussed are provided as the mean ± SE number (*n*) of independent experiments. Statistical significance was calculated using a two-tailed Student's *t-test*. Force frequency was measured by the Boltzmann equation and paired t-test using OriginPro 2019 or 2020 (OriginLab Corp., Northampton, MA).

III. DATA AND RESULTS

1. Lipin1 deficient skeletal muscle indicates a loss of muscle function.

To check the muscle function in the lipin1 deficient mice, our lab collaborated with Dr. Andrew Voss's lab to analyze the contractile force in both WT and Lipin1^{Myf5cKO} muscle fibers. His lab primarily worked on measuring the *in vivo* isometric force to the planar flexor (including medial and lateral gastrocnemius). They calculated the average WT and Lipin1^{Myf5cKO} with the response to a single twitch (action potential) and maximum tetanic force (measured for 0-100 Hz). They observed lower masses in Lipin1^{Myf5cKO} planar flexor muscles as a sign of muscle degradation. When the average twitch and 100Hz frequency was measured in WT and Lipin1^{Myf5cKO} were significantly lowered in Lipin1^{Myf5cKO} as compared to WT mice. A group of 13 wild type (WT) and 7 lipin1 deficient knockout (Lipin1^{Myf5cKO}) mice were used for the experiment. As a result of muscle degeneration, a marked reduction of total muscle contractile force was observed in Lipin1^{Myf5cKO} muscle fiber.

2. Lipin1 deficiency induces the degeneration of muscle fiber.

In order to understand what causes the physiological defects in lipin1 deficient muscle. We assessed the muscle morphology in Lipin1^{Myf5cKO} muscles by performing Hematoxylin and Eosin (H&E) histology (Figure 5A) to examine the degeneration of Lipin1^{Myf5cKO} muscle fibers. We observed degenerated myofibers in Lipin1^{Myf5cKO} muscles, displayed characteristics such as pale muscle fibers with cytoplasmic disruptions, regional hyper contraction, cellular infiltration and my phagocytosis (white arrows, figure 5A).

This staining also depicted central nucleation muscle fibers in Lipin1^{Myf5cKO} muscle (white stars, Figure 5A). Quantitative analysis of the H&E stained muscle showed that 37.46% of the Lipin1^{Myf5cKO} but no WT gastrocnemius fibers had central nuclei (Figure 5B, $p = 1.0x10^{-4}$, n=5 mice/group), indicating an increase in skeletal muscular degeneration in Lipin1^{Myf5cKO}. Finally, figure 5C shows the fiber size distribution. The shift in the bar graph is likely to explain the increase in the regeneration of myofibers in the following result.

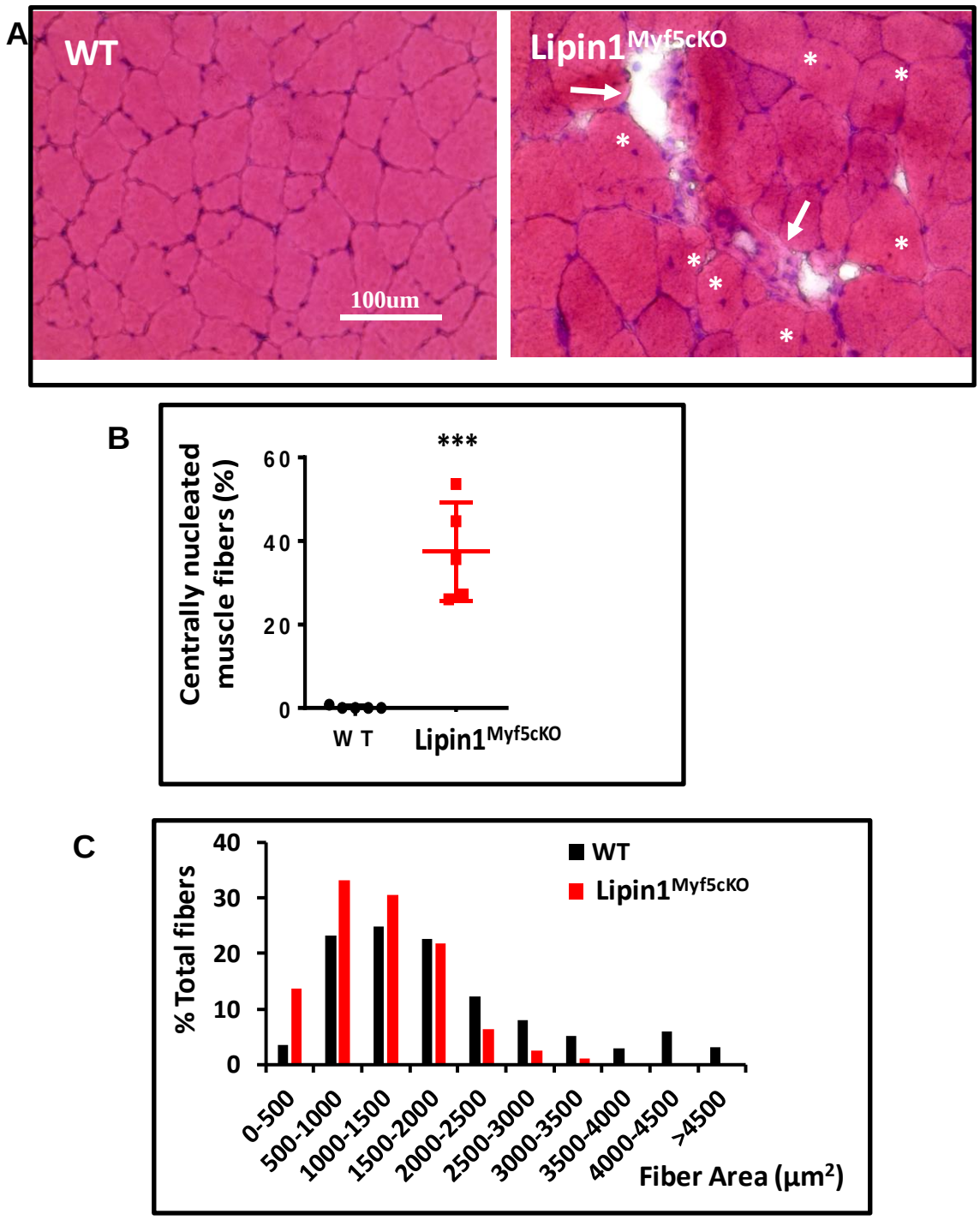


Figure 5: Enhanced muscle degeneration is observed in Lipin1^{MyfcKO}

H&E staining is performed on the gastrocnemius muscles of WT and lipin1^{Myf5cKO}. Figure 5A) shows the myofiber necrosis marked with arrows and asterisks in lipin1^{Myf5cKO} and WT doesn't show any such attributes.

Figure 5B) is the quantification for central nucleation present in both where n=5 mice/group. And finally, figure 5C) depicts the muscle fiber size of the gastrocnemius muscle fibers. The wildtype (WT) is shown in black and the Lipin1^{Myf5cKO} (KO) is shown in red. Scale bar = 100 µm. ***$p < 0.005$

3. Lipin1 deficiency leads to regeneration in Lipin1^{Myf5cKO} muscle fibers.

Regeneration in skeletal muscle is a sign of injury occurring in lipin1 deficient skeletal muscle. Degeneration and regeneration are a continuous process when the muscle in undergoing injury or damaged conditions. To examine whether lipin1 deficient leads to regeneration in skeletal muscle. We performed immunostaining for embryonic myosin heavy chain (EMyHC, green), laminin (red), and nuclei (blue) (Figure 6A) staining on WT and Lipin1^{Myf5cKO} muscle sections. WT muscle fibers don't show any kind of green areas (EMyHC positive fibers) whereas Lipin1^{Myf5cKO} muscle fibers show about 2.16% of expressed embryonic myosin which is consistent with the increased fiber regeneration in Lipin1^{Myf5cKO} muscle in the previous result section. Figure 6B shows the quantification analysis of expressed embryonic myosin in WT and Lipin1^{Myf5cKO} muscle fibers (p = 5.45x10^{-6}, n=7 mice/group).

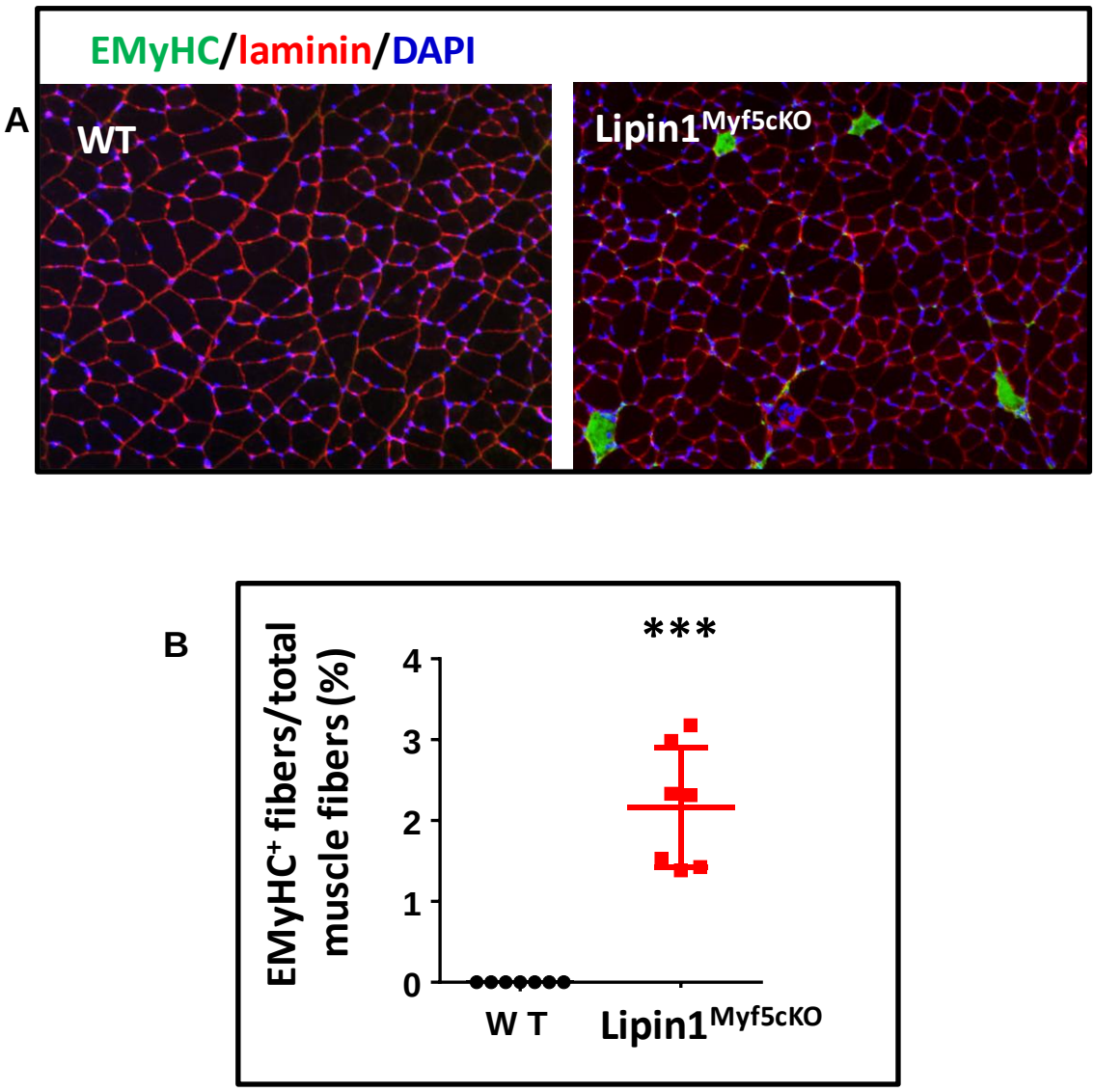


Figure 6: Enhanced regeneration in Lipin1^{Myf5cKO} muscle fibers.

Immunostaining for Embryonic myosin (EMyHC) in WT and Lipin1^{Myf5cKO} gastrocnemius muscle fibers. Figure 6A) depicts the fibers stained with green (EMyHC positive fibers) and figure 6B) shows the number of EMyHC-stained fibers vs total fiber number in the muscles. (n=7 mice/group). Scale bar = 100 μm. ***p>0.005.

4. Lipin1 deficiency drives intrinsic apoptosis in Lipin1^{Myf5cKO} muscle fibers.

To determine whether muscle degeneration in Lipin1^{Myf5cKO} mice was due to the induction of apoptotic or necrotic way of death, we first evaluated the protein expression of western blots and supported those results with TUNEL-staining data. As shown in 7A and 7B, western blotting was performed to analyze pro-apoptotic protein markers like Bax and Bak which shows a considerable increase (205% and 330% with $p = 0.02$ respectively, n=3 mice/group) in gastrocnemius in Lipin1^{Myf5cKO} muscle when compared to WT muscle. We also checked the executioner apoptotic protein marker i.e. cleaved caspase 9. There was a significant increase (231%, p = 0.007) of cleaved caspase 9 in Lipin1^{Myf5cKO} muscle compared with WT controls. The expression of the anti-apoptotic protein such as Bcl-2 was also observed and compared to WT controls. The expression of the Bcl-2 was increased not significantly different in Lipin1^{Myf5cKO} muscle samples as compared to WT muscle samples (p=0.07), indicating a protective response that may not be a direct consequence of lipin1 deficiency. Consequently, we detected apoptosis through DNA fragmentation by performing TUNEL staining in the muscle sections. We detected no TUNEL positive in WT muscle fibers but Lipin1^{Myf5cKO} muscle sections showed positive to TUNEL staining (figure 7C). In figure 7D, we quantitatively measured apoptotic nuclei revealed that frequently of TUNEL-positive myonuclei increased to 1.41% in Lipin1^{Myf5cKO} mice (p=0.0003, n=3 mice/group). Moreover, we noticed that apoptosis was present both in interstitial cells and myofibers in lipin1 deficient mice.

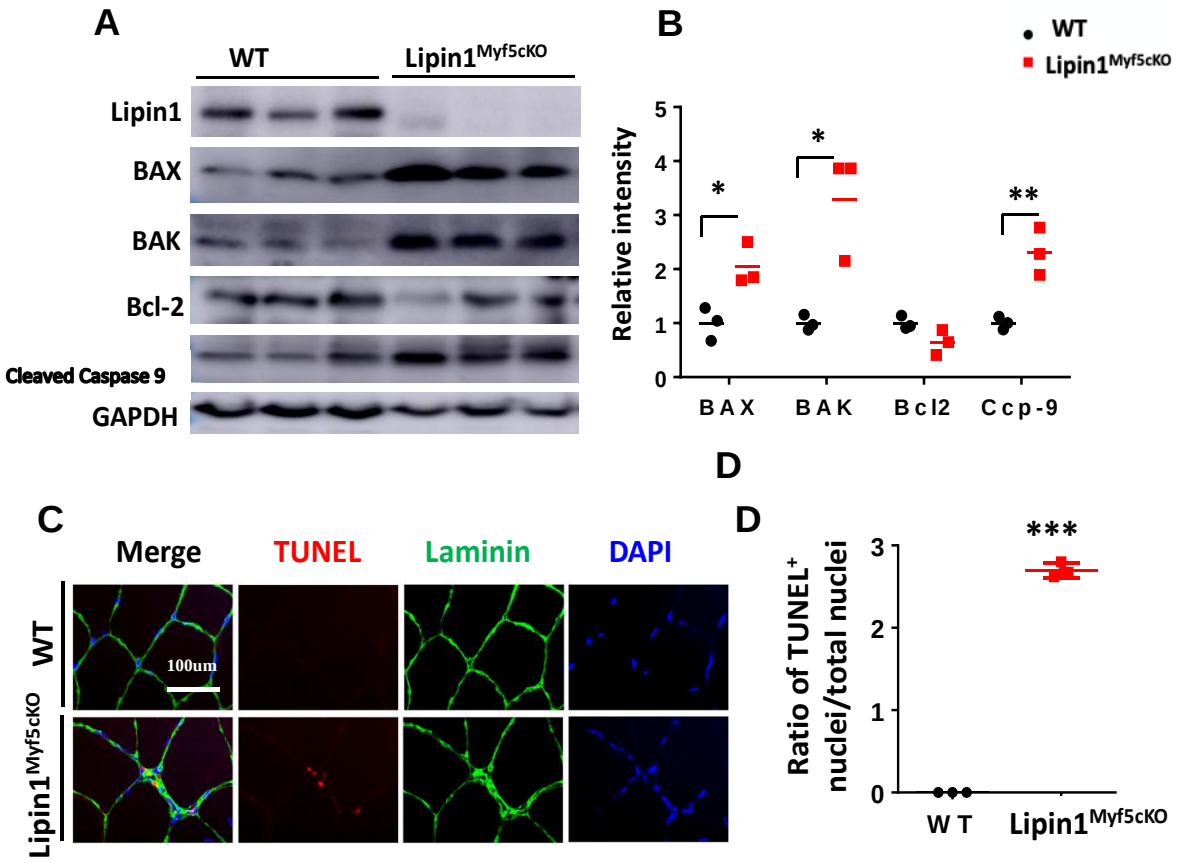


Figure 7:Western blot analysis of apoptosis-related proteins and TUNEL assay in gastrocnemius from WT and Lipin1^{Myf5cKO}.

Figure 7A), Western blot assays of pro-apoptotic (Bax and Bak), anti-apoptotic marker (Bcl-2), and apoptotic executioner marker (Cleaved Caspase 9) in gastrocnemius of WT and Lipin1^{Myf5cKO} mice. We used GAPDH as the loading control and confirmed the expression of Lipin1 in the WT and Lipin1^{Myf5cKO} muscle samples. Figure 7B) explains the densitometry of western blots performed on the apoptosis markers which is presented as mean ± SD from three individual experiments.

Figure 7C) is the illustration of the immunofluorescence assessment of TUNEL staining in the gastrocnemius of WT and Lipin1^{Myf5cKO} mice. Those muscle sections were also simultaneously stained with Laminin (green) and DAPI (blue) for the muscle membrane and the nuclei. Finally, figure D) shows the quantification of TUNEL positive cells (n=3 mice/group). *$p < 0.05$; **$p<0.01$, ***$p<0.005$, Lipin1^{Myf5cKO} versus WT measured by t-test.

5. Lipin1 deficiency induces muscle necroptosis in skeletal muscles.

We also examined the other type of cell death i.e. necrosis in skeletal muscles. We aimed to study if the major contributor to cell death is through necroptosis (programmed necrosis)? Also, if this kind of cell death can occur in the lipin1 deficient skeletal muscle. To evaluate this process of necroptosis, we performed western blotting for proteins belonging to the necroptosis machinery i.e. RIPK1, MLKL, and RIPK3. The enhanced expression of these proteins shows strong evidence of necroptosis in lipin1 deficient skeletal muscles. We performed these experiments on the gastrocnemius muscle tissue of the WT and Lipin1^{Myf5cKO} mice. These necroptosis proteins MLKL, RIPK1 and RIPK3 show a significant increase by 130% ($p=0.047$), 724% ($p=0.018$) and 496% ($p=0.018$) respectively when compared to the WT muscle samples. These experiments were repeated to increase the significance of the data produced (Figure 8A & 8B) (Considered n=3 mice/group).

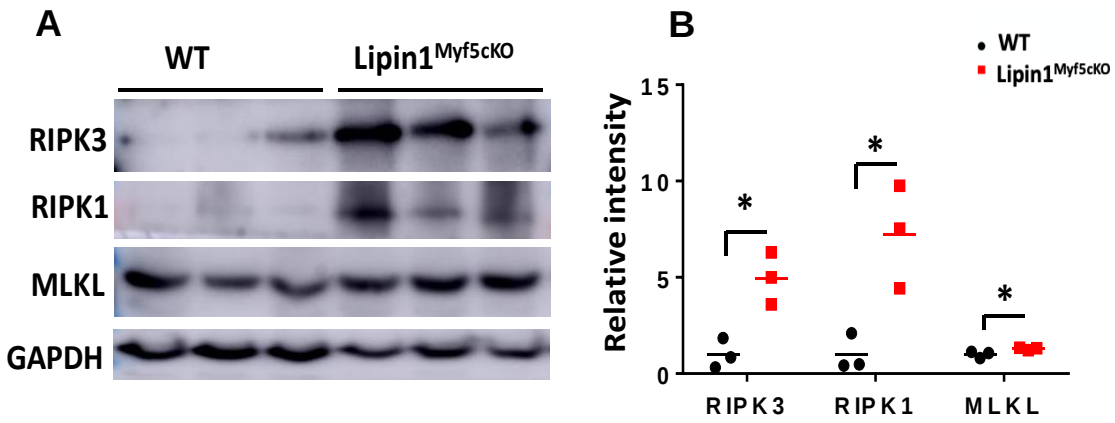


Figure 8:Western blot analysis was performed to understand the necroptosis markers in gastrocnemius muscle of WT and Lipin1^{Myf5cKO}

The above figure 8A) shows the western blot data of necroptosis markers i.e. RIPK3, MLKL, and RIPK1 in WT and Lipin1^{Myf5cKO} muscle samples. Figure 8B) depicts the quantification analysis of this western blot. (n= 3 mice/group) (*p > 0.05)

The WT group was shown in Black and Lipin1^{Myf5cKO} (KO) was shown in red.

6. Plasma membrane breakdown in Lipin1^{MyfcKO} muscle.

The loss of plasma membrane integrity is the depicts the presence of muscle degeneration in the lipin1 deficient skeletal muscle. To identify these damaged skeletal muscle fibers, we stained them with the membrane-impermeable marker IgG. As shown in Figure 9A and 9B, gastrocnemius muscles from Lipin1^{Myf5cKO} mice demonstrated a 3.65% of total muscle fibers were IgG-positive compared to zero in WT muscle (p = 7.7x10^{-8}, n=6 mice/group), suggesting the loss of membrane integrity.

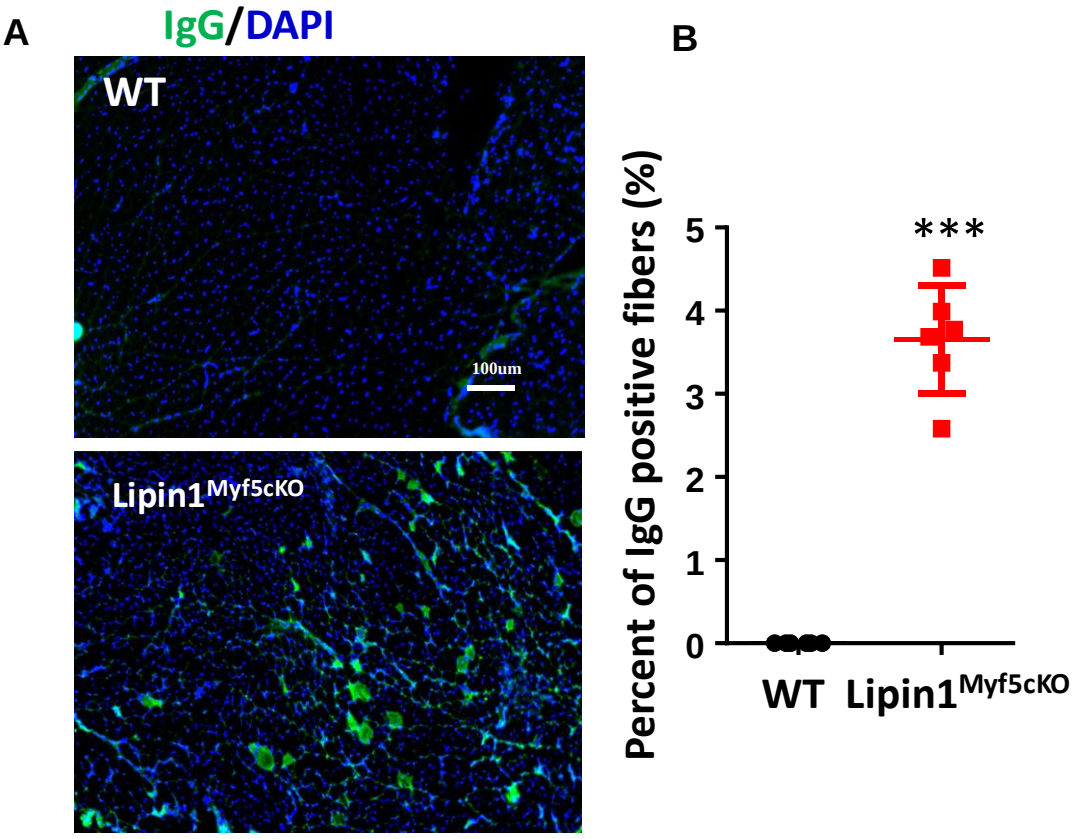


Figure 9: Membrane disruption was detected using an IgG staining method in gastrocnemius sections of Lipin1^{Myf5cKO} mice

The myofiber damage in Lipin1^{Myf5cKO} mice was detected by IgG staining. Figure 9A) gastrocnemius muscle sections from WT and Lipin1^{Myf5cKO} mice were immunostained with goat anti-mouse IgG to detect permeable/ damage fibers. 9B) Quantification (in %) of IgG- positive fibers (n=6 mice/group). Scale bar = 200 μm. ***p>0.005.

7. To observe plasma membrane damage in Lipin1^{MyfcKO} muscle by Evans blue dye (EBD) staining.

The loss of plasma membrane integrity is a hallmark of necrotic cell death. To identify these damaged skeletal muscle fibers, we performed Evans blue dye staining in WT and Lipin1^{Myf5cKO} muscle fibers. To confirm that these muscle fibers were indeed damaged, we injected EBD into the Lipin1^{Myf5cKO} and WT mice. As shown in Figure 10A and 10B, 3.62% of EBD-positive fibers were detected in gastrocnemius of Lipin1^{Myf5cKO} mice, but not in WT mice ($p = 5.42x10^{-7}$, n=5 mice /group).

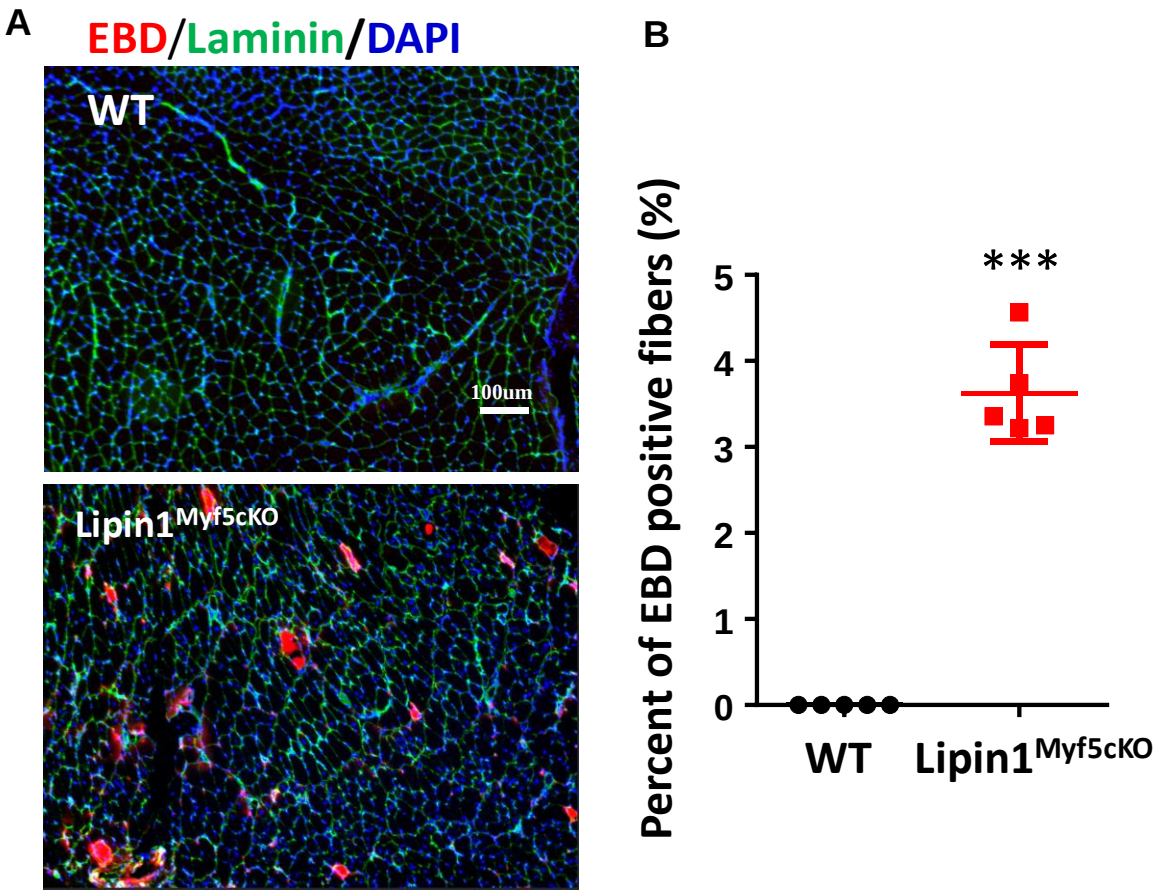


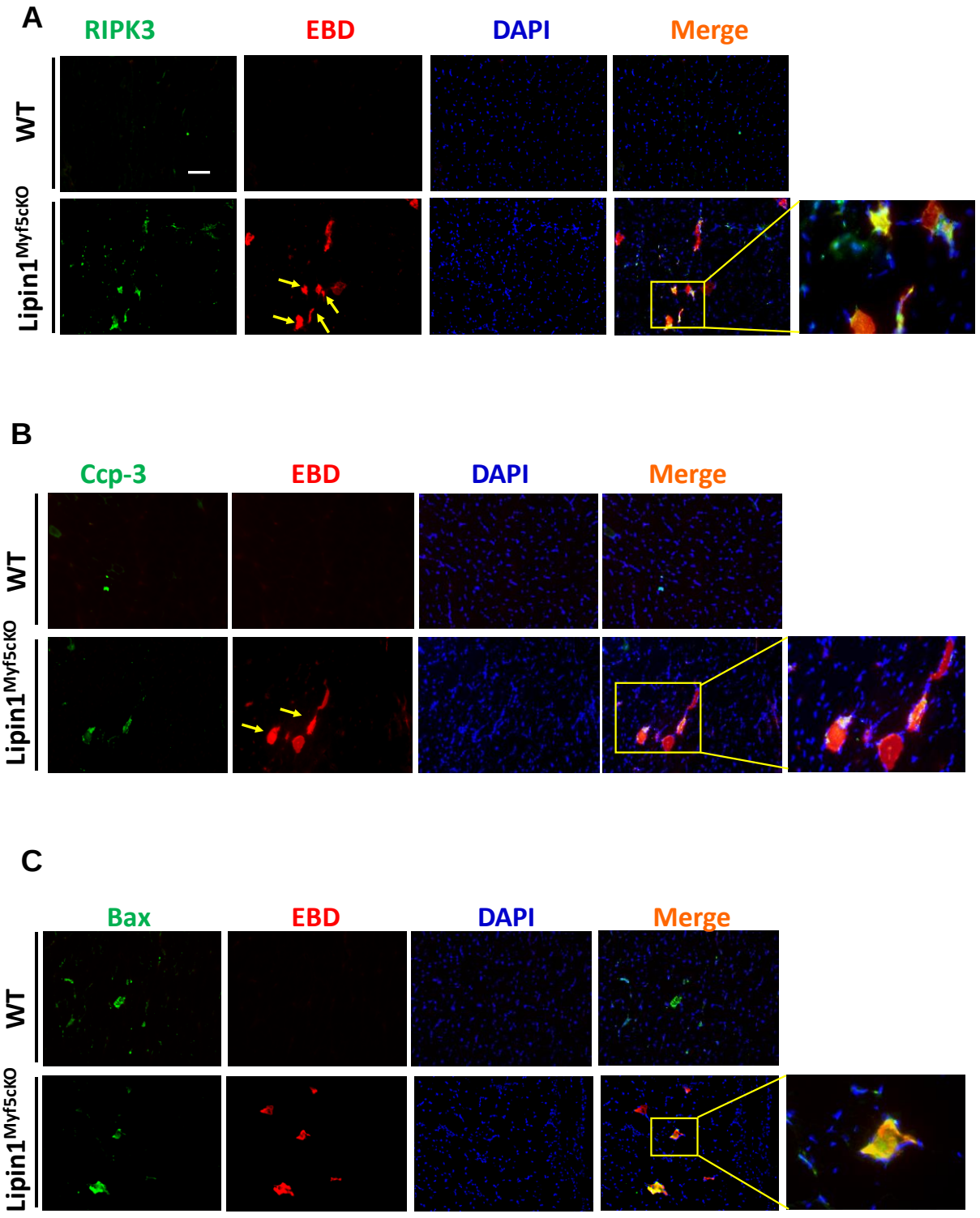
Figure 10: Evans blue dye (EBD) staining performed in gastrocnemius muscle of Lipin1^{Myf5cKO} mice

The membrane damage can also be detected in EBD stained WT and Lipin1^{Myf5cKO} muscle samples. Figure 10A) shows the representative images of EBD positive staining in both wildtype muscle fibers (WT) and lipin1 deficient skeletal muscle fibers (Lipin1^{Myf5cKO}). Figure 10B) explains the quantification percentage of EBD-positive necrotic fiber in Lipin1^{Myf5cKO} mice (n=5 mice/group).

Scale bar = 200 μm. ***p>0.005.

8. **To understand reduced muscle function as a result of myofiber's death and disrupted muscle membrane.**

To determine whether loss of membrane integrity could be induced in necroptotic or apoptotic myofibers, we examined the co-localization of RIPK3 and cleaved caspase-3 (ccp-3) with damaged EBD positive myofibers by using a common immunoblotting technique (Figure 11). We found an intense colocalization between EBD-positive staining with RIPK3 (Figure 11A), cleaved caspase-3 (Figure 11B) and Bax (Figure 11C) in muscles from Lipin1^{Myf5cKO} mice. Approximately, 80.94% of RIPK3-positive muscle fibers and 81% of Cleaved Caspase-3-positive myofibers were-EBD positive (Figure 11D, n=4 mice/group). Interestingly, among the Bax-positive myofibers, 95.4% myofibers were EBD-positive suggesting that membrane damage may lead to the initiation of apoptosis. These staining data showed a strong association between the loss of membrane integrity and necrotic/apoptotic cell death and supported the implication that membrane disruption may lead to necroptotic/apoptotic myofibers.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RIPK3
EBD
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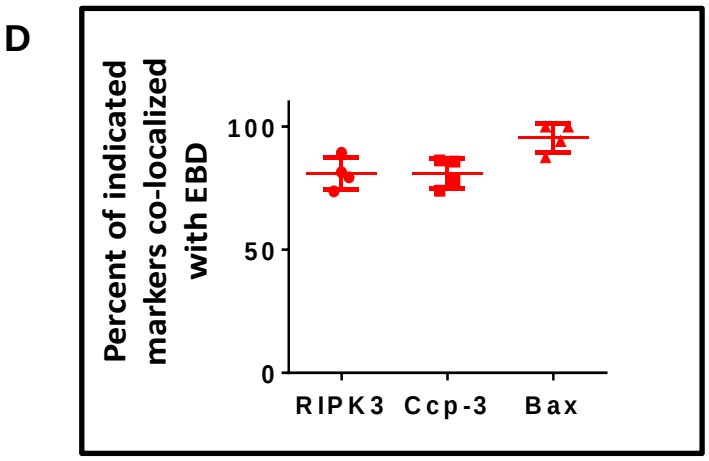

Figure 11:Colocalization of Evans blue dye staining with the RIPK3 (necroptosis marker), BAX (Pro-apoptotic marker) and Cleaved-Caspase3(executioner apoptotic marker) in gastrocnemius muscle fibers of the Lipin1^{Myf5cKO} mice

Gastrocnemius muscles from WT and Lipin1^{Myf5cKO} mice. These muscles were collected from mice one-day post-injection of EBD, and muscle sections of the same were immunostained with either primary antibodies against 11 A) RIPK3, 11 B) cleaved caspase3(Ccp-3) or 11 C) Bax. 11 D) Comparison of percentages that are co-localized of EBD with RIPK3, cleaved caspase 3, or Bax in WT and Lipin1^{Myf5cKO} muscles, these were examined and quantified (n=4 mice/group). Scale bar = 100 µm.

IV. DISCUSSION

Our data in this study showed that deficiency in lipin1 induces caspase-dependent intrinsic apoptosis and also induce RIPK3-dependent necroptosis in skeletal muscles. The present study showed that lipin1 deficiency can also lead to impaired membrane integrity and reduced contractile force. The underlying cell death signaling events are enhanced by lipin1 deficiency in skeletal muscles. Disrupted membrane integrity and cell death can cause muscle degenerative diseases such as rhabdomyolysis, muscle dystrophy and various other myopathies. This understanding about cell death events can help us develop new treatment methods for these muscle disorders. The muscle degeneration and regeneration were consequential in Lipin1^{Myf5cKO} muscle samples having 37% of its fibers to be centrally nucleated and 2% were expressing positive to embryonic myosin heavy chain staining. Any healthy muscle fiber contains nucleation around the periphery of the muscle fiber, but due to damage or injury muscle nuclear arrangement starts deforming. This event causes the disposition of the nuclei in muscle fiber. This suggest that Lipin1^{Myf5cKO} muscle undergoes a recurrent cycle of degeneration and regeneration. This particular pathology was also shown in a H&E staining of Lipin1^{Myf5cKO} muscle samples by Jama A. et al 2018 leading to muscle degeneration.

Our studies provide the first evidence that apoptotic and necroptotic cell death co-exists in lipin1 deficient skeletal muscle acquired from Lipin1^{Myf5cKO} mice. In respect to skeletal muscles, there are three major cell death pathways: apoptosis, autophagic cell death, and necrosis.

(Zhang et al, 2014; Abdullah A. et al, 2018) proved that lipin1 deficiency in mice is associated with a blockade in autophagic flux, a deregulated mitophagy, and a marked accumulation of abnormal mitochondria and autophagic vacuoles in skeletal muscles. In this present study, we observed that the elevations of TUNEL-positive nuclei and apoptotic DNA fragmentation in lipin1 deficient muscles.

We observed enhanced intrinsic apoptosis by performing western blot to test pro-apoptotic markers BAX and BAK, and activation of the cleaved caspase 9 in WT and lipin1 deficient muscle samples. Our data also indicated that there is upregulation of necroptosis expression of RIPK1, MLKL, and more importantly RIPK in lipin1 deficient skeletal muscles. This demonstrates that lipin1 deficiency does induce necroptosis activation in skeletal muscles. (George G. Schweitzer et al, 2019). On the contrary, apoptosis and necrosis can coexist in the degenerating/ regenerating muscles, including those of patients with neuromuscular disorders, such as inflammatory myopathies, dystrophies, metabolic and mitochondrial myopathies and drug-induced myopathies (Sciorati C, 2016). Our data have also showed loss of membrane integrity is strongly associated with necroptosis and apoptosis. It has been proposed that the initial events in the muscle cell necrosis in a variety of pathological conditions including Duchenne Muscular Dystrophy (DMD) are the focal breakdown of the plasmalemma (Nadra K et al, 2008) (Mokri B, 1998). Membrane rupture allows the influx of calcium and leads to necrotic muscle fiber death. Our data showed that approximately 81% of RIPK3 myofibers were EBD-positive, suggesting the disruption of membrane integrity occurs relatively early and may lead to necroptosis. Lipin1 is an

Mg^{2+}-dependent phosphatide phosphatase (PAP1) enzyme catalyzing the dephosphorization of phosphatidic acid, yielding diacylglycerol which are lipid precursors used for the synthesis for the major membrane phospholipids

phosphatidylethanolamine and phosphatidylcholine. phosphatidylethanolamine and phosphatidylcholine are the most important lipoproteins for maintaining the membrane structure, fluidity, and stability (Li Z et al, 2006). These proteins tend to affect membrane protein trafficking (Shuler MH et al, 2016) (Testerink N. 2008). Lipin1 deficiency may affect membrane integrity by altering membrane phospholipid contents and affecting membrane protein expression. Moreover, it is generally accepted that loss of membrane integrity occurs late in apoptosis (Nelson J et al, 2011) (Rogers C et al, 2017). Interestingly, we observed pro-apoptotic marker Bax-positive muscle fibers highly colocalized with EBD- positive muscle fibers. It suggests that loss of membrane integrity may contribute to the early stages of apoptosis. This is consistent with the previous study that increased membrane permeability which may lead to the induction of calcium influx and eventually trigger apoptosis (Matsuda R et al, 1995). Therefore, plasma membrane changes during apoptosis and programmed necrosis are more complicated than previously expected. We understand that lipin1-deficient muscles, depending on the extent of cell membrane damage, the cellular response may be adaptive and where possible, homeostasis is restored. Cell death may undergo necrosis or apoptosis, which depends on the severity of muscle damage. As a consequence of muscle degeneration, a marked reduction of total muscle force was observed. When isometric forces were corrected for the muscle mass (the specific force, N/g), there was still a significant difference in the force between lipin1 deficient and control mice suggesting that a defect in Lipin1^{Myf5cKO} muscle in addition to the atrophy conditions.

Certainly, previous studies have reported that sarcolemma disruption has been implicated that reduction of muscle force after muscle injury is a common event to occur. Lovering and De Deyne identified a complementary correlation between maximal tetanic tension and EBD-positive muscle fibers suggesting that membrane integrity plays a critical role in the maintenance of muscle contractile force (Lovering RM and De deyne PG, 2004). Enhanced apoptosis and necroptosis could change contractile properties and leads to reduced contractile force production.

We examined the limitation of the current study and identify areas for future research. Despite revealing that lipin1 plays an important role in protecting against myofiber degeneration and maintain membrane integrity. Inadequate membrane repair has been implicated to participate in the pathogenesis of muscular dystrophies and cardiomyopathies. (Han R et al, 2007), (Lammerding J et al, 2007). Our future direction would be to examine whether lipin1 deficiency leads to defective membrane repair. Furthermore, we have determined lipin1 deficiency induces coexisted myocyte apoptosis and necrosis. This enable further investigation into whether myocyte apoptosis and necrosis are two separate processes or a continuum of events and the conditions the cells choose between different types of cellular deaths or survival.

In brief, we found that loss of plasma membrane integrity due to lipin1 deficiency in skeletal muscle fibers may play a primary role in the death of the muscle fibers and the course of related muscle disorders. We demonstrate that muscle loss from atrophy and degeneration induced by lipin1 deficiency results in impaired force production. We propose that lipin1-mediated muscle degeneration and higher muscle membrane vulnerability are some of the potential mechanisms that lead to muscle weakness. Our data provided a novel point for an understanding of the mechanism that regulates muscle cell death, which could be utilized for the effective treatment of related muscle diseases.

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