2018

Lipin1 regulates skeletal muscle differentiation through the PKC/HDAC5/MEF2c:MyoD -mediated pathway

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Lipin1 regulates skeletal muscle differentiation through the PKC/HDAC5/MEF2c:MyoD -mediated pathway

A thesis submitted in partial fulfillment
of the requirements for the degree
of Master of Science

By

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B.S. The Ohio Sate University, 2014

2018
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

July 16, 2018

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Abdulrahman M Jama ENTITLED Lipin1 regulates skeletal muscle differentiation through the PKC/HDAC5/MEF2c:MyoD -mediated pathway BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

Jama, Abdulrahman M. M.S. Department of Biochemistry and Molecular Biology, Wright State University, 2018. Lipin1 regulates skeletal muscle differentiation through the PKC/HDAC5/MEF2c:MyoD -mediated pathway

Our previous characterization of global lipin1-deficient (*fld*) mice demonstrated that lipin1 played a novel role in skeletal muscle (SM) regeneration. The clinical relevance of lipin1 has been observed in patients with lipin1 null mutations where they exhibited severe rhabdomyolysis with aggregated and dysfunctional mitochondria. Lipin1 is a key gene that plays an important role in lipid biosynthesis and metabolism. It has dual functions as it contains a phosphatase activity that converts phosphatidic acid (PA) to diacylglycerol (DAG), the penultimate step in triglycerides (TAG) biosynthesis as well as transcriptional co-activator function. In the cytosol and ER, lipin1 carries out its lipid and phospholipid biosynthesis whereas in the nucleus it co-regulates the transcriptional co-activation of genes involved in adipogenesis and fatty acid oxidation. In this study, using cell-type specific Myf5-cre;Lipin1$^{fl/fl}$ conditional knockout mice (Lipin1^{Myf5cKO}) we showed that lipin1 is a major determinant of SM development, termed *myogenesis*. Lipin1 deficiency induced reduced muscle mass. Results from lipin1-deficient myoblasts suggested that lipin1 regulated myoblast differentiation through the protein kinase C (PKC)/histone deacetylase 5 (HDAC5)/Myocyte-specific enhancer factor 2C (MEF2c):MyoD-mediated pathway. Lipin1 deficiency leads to the suppression of PKC isoform activities, as well as the inhibition of their downstream target, class II deacetylase HDAC5 nuclear export, and consequently, the inhibition of MEF2c and MyoD expression in the SM of Lipin1^{Myf5cKO} mice. Inhibition of MyoD induced Pax7 accumulation, which may lead to an increased propensity for satellite cell self-renewal

rather than progression through myogenic differentiation. Our findings provide insights into the signaling circuitry that regulates SM development, and have important implications for developing therapies aimed at treating rhabdomyolysis and muscular dystrophies.

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ACKNOWLEDGEMENT

First, I would like to acknowledge and thank *Dr. Hongmei Ren*, my thesis advisor. Dr. Ren was always there for me whenever I had questions and needed assisting with my projects. Secondly, I would like to thank the Biochemistry and Molecular Biology department for their helpfulness whenever I needed reagents or equipment for experiments.

I would also like to thank *Dr. Helen Chamberlin* from The Ohio State University for her tremendous mentorship and encouragement to pursue graduate studies and giving me the opportunity to work in her lab. Also, thanks to my thesis committee members *Dr. Nicholas Reo* and *Dr. Kwang-Jin Cho* for taking their valuable time to review my thesis.

Lastly, I would like to acknowledge with the outmost sincere gratitude to my family whom have always been there for me. I would have not completed my master's studies without their support and patience.

I. Introduction

i- Myogenesis

Muscles are thought to account for about 40% of the human body. There are three types of muscles including smooth, cardiac and skeletal. Their primary functions are for locomotion and contractile movements of internal organs such as the heart. Both smooth and cardiac muscles differ from skeletal muscles as they function involuntarily and rhythmically. Skeletal muscles on the other hand are anchored to the bones and are primarily responsible for skeletal locomotion in maintaining physical activities and are controlled voluntarily (Iizuka, Machida, & Hirafuji, 2014). During embryonic development, somite cells give rise to mesenchyme stem cells, which in turn give rise to muscle stem cells (Endo, 2015; Sambasivan & Tajbakhsh, 2015). The process of skeletal muscles development also known as *myogenesis* follows a distinct pattern during embryogenesis as well as postnatal in response to injury, termed, *regeneration* (Endo, 2015; Sambasivan & Tajbakhsh, 2015). The regeneration ability of skeletal muscles through muscle stem cells, also known as satellite cells, gives them a unique role in which a cascade of events drive these satellite cells to become mature myofibers and eventually functioning skeletal muscles. Satellite cells exist in a low cycling; mitotically quiescent stage that is ready for committing to myogenesis (Sambasivan & Tajbakhsh, 2015). In healthy muscle, satellite cells reside in a quiescent state where a pool of muscle stem cells is available for muscle repair. Upon injury to muscles, satellite cells exit their proliferative stage and commit to differentiation (Sambasivan & Tajbakhsh, 2015). The

discovery of satellite cells, which were termed as muscle stem cells gave an insight to the process of skeletal muscle development and regeneration. Satellite cells are the precursors to skeletal muscle cells, and have their own unique molecular gene expressions (Danoviz & Yablonka-Reuveni, 2012; Mauro, 1961). Some genes or markers that are important for satellite cells identity include *Pax3* and *Pax7* as well as some myogenic regulatory factors (Sincennes, Brun, & Rudnicki, 2016).

Myogenic regulatory factors (MRFs) are set of genes that are important for the skeletal muscle formation. They govern the process of early muscle stem cells becoming myoblasts to myofiber and subsequently, mature muscle fibers (Asfour, Allouh, & Said, 2018; Sambasivan & Tajbakhsh, 2015). MRFs include *MyoD, Myf5, Myogenin, and Mrf4*. These factors contribute to different stages of muscle development and regeneration (Asfour et al., 2018). MRFs are transcription factors and all have structural similarities in their domains. One of the most important domains across all these factors includes basic Helix-loop-Helix (bHLH) domain. This domain is thought to bind to a specific DNA sequence known as E-box (Asfour et al., 2018; Molkentin, Black, Martin, & Olson, 1995). E-box are ubiquitously found in the promoter of myogenic and non-myogenic genes where they promote or enhance the transcription of these genes. One of the earliest MRFs that is expressed is *myf5*; even as early as in mesoderm stage (Endo, 2015; Maguire, Isaacs, & Pownall, 2012). The expression of these MRFs follows a linear pattern in each stage of myogenesis where *Myf5* is expressed in the earliest muscle stem cell commitment stage as well as *Pax3* and *Pax7* (W. Zhang, Behringer, & Olson, 1995). The transcription factor *MyoD* comes at a later stage than *Myf5* where it acts on myoblasts differentiation (Asfour et al., 2018; Wood, Etemad, Yamamoto, & Goldhamer,

2013). *Myogenin* and *Mrf4* are expressed in the late stage of differentiation and matured myofiber formation (Asfour et al., 2018; Wood et al., 2013).

Another family transcription factors that are involved in myogenesis are Myocyte Enhancing Factor 2 family, MEF2s (Potthoff & Olson, 2007). MEF2 genes are expressed in early mesoderm as well as later stage of myoblast differentiation (Bour et al., 1995). There are many isoforms of MEF2 transcription factors and different isoforms have shown to be uniquely expressed in different tissues. The MEF2 that is mostly present in myogenesis is *MEF2c* isoform (Potthoff & Olson, 2007). This isoform does not have its own myogenic activity and cannot by itself drive the cells to skeletal myogenic differentiation. But with the cooperation of *MyoD*, they both drive and enhance the myogenic differentiation process (Molkentin et al., 1995; D. Z. Wang, Valdez, McAnally, Richardson, & Olson, 2001). MyoD along with other MRFs have unique sites where MEF2 factors bind to enhance myogenesis, these sites are known as MEF2 binding sites, which are located in the promoter regions and close to the E-box sites (Naidu, Ludolph, To, Hinterberger, & Konieczny, 1995).

Next is how and what negatively regulates these factors and myogenesis overall. The intrinsic biological course is to up-regulate sets of genes and processes and to down-regulate them at another time point, when they are not needed. In this case, as aforementioned, the myogenesis process takes place during embryogenesis and in response to injury. When myogenesis is not needed these MRFs and MEFs must be down-regulated (D. Z. Wang et al., 2001). Repressor genes are regulatory factors that down-regulate the expression of genes by binding to a short specific DNA sequence in the promoter regions of other genes (Reynolds, O'Shaughnessy, & Hendrich, 2013). One

of the many ways that DNA transcription is regulated is the process of chromatin remodeling. When DNA is transcribed, it is packaged into histone proteins where they form nucleosomes (Tessarz & Kouzarides, 2014). This makes the cells store all DNA materials as well as control the expression of certain genes. Histone proteins are repressor proteins, which makes the DNA transcriptional complex machinery unable to access the DNA (Bertos, Wang, & Yang, 2001). The histone proteins or chromatins are remodeled based on the need of the cells (Reynolds et al., 2013). Some of the histone remodeling include, methylation, acetylation, phosphorylation, ubiquitylation and sumoylation (Tessarz & Kouzarides, 2014). When histones are methylated, it is considered to be gene repression while acetylation is considered to be gene transcriptional activation as it frees the DNA from the histone proteins, thus making the DNA transcription complex accessible to the DNA for transcription (Bertos et al., 2001; Reynolds et al., 2013; Sincennes et al., 2016).

Histone modification and remodeling plays an important role in gene regulations in myogenesis (Lu, McKinsey, Zhang, & Olson, 2000; McKinsey, Zhang, Lu, & Olson, 2000). It has been evidenced that Histone Deacetylases (HDACs) play an important role in gene transcription. These proteins are shown to inhibit gene transcription by removing acetyl groups from histones, thus making transcription unachievable (Bertos et al., 2001; Sincennes et al., 2016; Tessarz & Kouzarides, 2014). HDACs fall into various classes with distinct tissue expression (Dokmanovic, Clarke, & Marks, 2007). Class I HDACs are ubiquitously expressed as compared to class II (Fischle et al., 1999). One of the tissue-specific expressions of class II HDACs are in skeletal muscle, which plays an important role in myogenesis during embryogenesis and regeneration (McKinsey, Zhang,

Lu, et al., 2000; Sincennes et al., 2016). Through the CaMK (Ca^{2+}/calmodulin-dependent protein kinase) and Protein Kinase D (PKD) signaling pathway, HDAC5, a class II protein binds to 14-3-3 binding protein which are cellular signaling chaperones (McKinsey, Zhang, & Olson, 2000). The binding between HDAC5 and 14-3-3 chaperone proteins is aided by the phosphorylation signal of PKD and CaMK. In a repression scenario, HDAC5 and MEF2c interact, which reduces and diminishes the myogenic differentiation process. This makes HDAC5 reside mostly in the nucleus and carryout its repression activity. Upon PKD and CaMK signaling activation, HDAC5 is exported from the nucleus through phosphorylation manner with the aid of 14-3-3 binding protein (Lu, McKinsey, Nicol, & Olson, 2000; McKinsey, Zhang, Lu, et al., 2000; McKinsey, Zhang, & Olson, 2000).

ii- Rhabdomyolysis/Myoglobinuria

Rhabdomyolysis, often a genetic disorder, is the results of muscle fiber breakdown that leads to toxic release of cellular components into the circulatory system (Zutt, van der Kooi, Linthorst, Wanders, & de Visser, 2014). In the United States alone, it has been reported about 26,000 cases annually (Sauret, Marinides, & Wang, 2002; Zutt et al., 2014). One of the characteristic of rhabdomyolysis is the elevation of serum creatine kinase activity. There are two classes of rhabdomyolysis: acute and severe episodes (Veenstra, Smit, Krediet, & Arisz, 1994). Severe muscular dystrophy phenotypes as well as myoglobinuria, the excessive presence of myoglobin in urine, are often accompanied in patients with severe rhabdomyolysis (Sauret et al., 2002; Veenstra et al., 1994; Zutt et al., 2014). One of the pathology observed in some patients with rhabdomyolysis is an

increased intercellular concentration of calcium. This excess presence of calcium is thought to lead to the activation of cellular proteases and phosphatases, which in turn destruct and teardown myofibers and cytoskeletal membrane proteins (Sauret et al., 2002; Veenstra et al., 1994; Zutt et al., 2014). The cause of rhabdomyolysis cannot be pinned down to one specific underlying genetic disorder although many causes have been identified. Acquired rhabdomyolysis cases are the results of substance abuses (alcohol etc.) accompanied by another causative factor such as lack of exercise or sedentary lifestyle (Sauret et al., 2002; Veenstra et al., 1994), whereas some of inherited cases are attributed to the dysfunction of the terminal glycolysis/gluconeogenesis pathways and lipid metabolism (Zutt et al., 2014; Zutt et al., 2010).

iii- Lipin proteins

Lipid metabolism is a dynamic process that is important for cellular energy storage, cellular integrity and membrane maintenance. Proteins involved in lipid metabolism play an important role in lipid synthesis and their cellular trafficking (Brose, Betz, & Wegmeyer, 2004; Y. Chen, Rui, Tang, & Hu, 2015). Some of the proteins that are associated in lipid metabolism include the lipin family proteins. This family of proteins consists of three different proteins, lipin1, lipin2 and lipin3 that are expressed in tissue specific and sometimes in an overlapping manner (Figure 1) (Y. Chen et al., 2015; Csaki et al., 2013; Donkor, Sariahmetoglu, Dewald, Brindley, & Reue, 2007). The members of this family have a phosphatidate acid phosphatase (PAP) enzyme activity that catalyzes the conversion or the dephosphorylation of phosphatidic acid (PA) to diacylglycerol (DAG), the penultimate step in synthesis of triglycerides (TAG). In

addition to taking part of energy storage, TAG synthesis, lipin proteins are also involved in fatty acid oxidation through their secondary function. They co-regulate the gene expression of fatty acid oxidation genes (Y. Chen et al., 2015; Csaki et al., 2013; Ren et al., 2010). Structurally, (Figure 2), all lipin family proteins consist of an evolutionarily conserved C-terminal (C-Lip) and N-terminal (N-Lip) domains as well as nuclear localization signal (NLS) (Y. Chen et al., 2015). The Haloacid dehalogenase (HAD) phosphatase motive (DXDXT) is the PAP enzyme and is located in the C-Lip domain as well as the nuclear receptor interacting domain (LXXIL) (Y. Chen et al., 2015; Csaki et al., 2013). Due to its dual activity, lipin1 is trafficked between cellular compartments where it carries its PAP activity in the cytoplasm/ER and the transcription co-regulation in the nucleus (Y. Chen et al., 2015; Harris et al., 2007). Peterson et al (2011) proposed that mTOR1 complex regulates the translocation of lipin1 from the cytoplasm to the nucleus (Peterson et al., 2011). They have shown that when lipin1 is phosphorylated by mTOR1, lipin1 is sequestered in the cytoplasm, where it carries out its PAP activity. On the other hand, when lipin1 is not phosphorylated, it travels to the nucleus where it acts as transcriptional co-activator in genes involved in the fatty acid oxidation pathway as well as adipogenesis (Kim et al., 2013; Peterson et al., 2011).

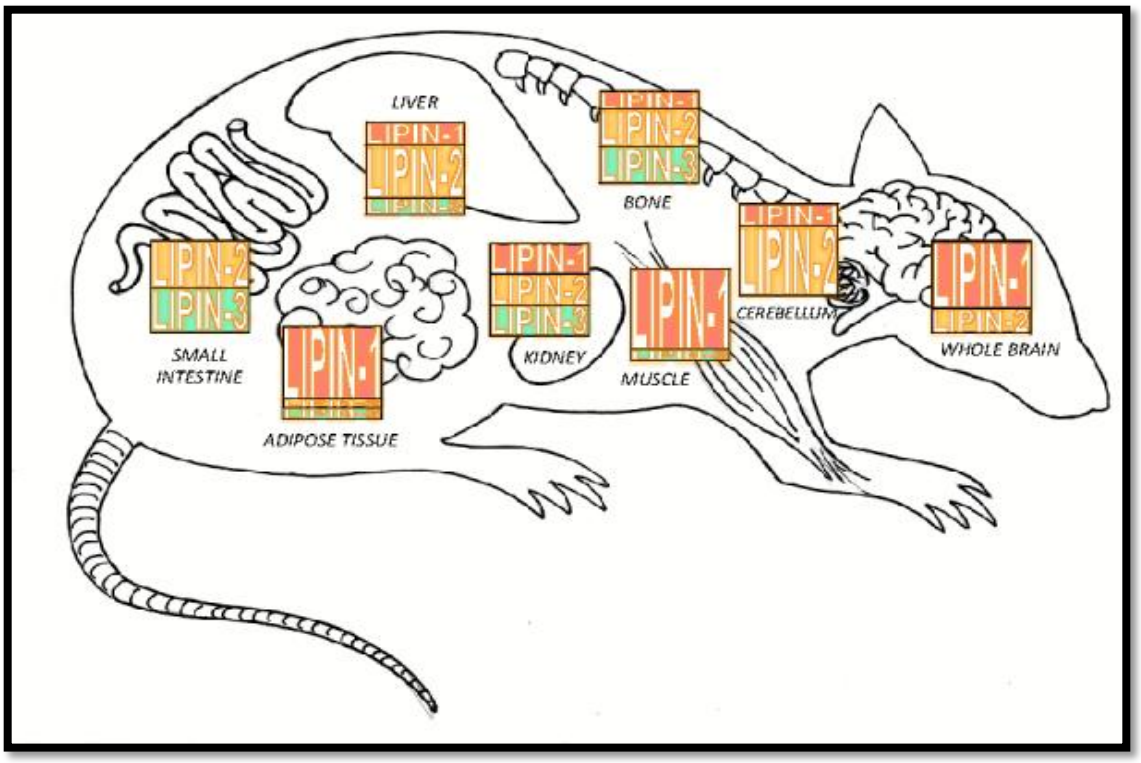

Figure 1: Relative mRNA expression of lipin proteins in different mouse tissue. Lipin1 is predominantly expressed in adipose tissue and skeletal muscles (Csaki et al., 2013).

The lipin family proteins were first identified in a spontaneous mutant mouse strain, which later named fatty liver dystrophy mouse (*fld*). These mice displayed a dysregulated TAG storage (Csaki et al., 2013; Peterfy, Phan, Xu, & Reue, 2001; H. Wang, Airola, & Reue, 2017). Through positional cloning, (Peterfy et al., 2001) lipin1 was identified in mice. From to the null mutation of lipin1 they have characterized these mice to exhibit lipodystrophy, hyper-triglyceridemia, neonatal fatty liver, insulin resistance and peripheral neuropathy (Y. Chen et al., 2015). Lipin1 mutations in humans present similar phenotype as that of mice as they both exhibit smaller and atrophied muscles (Y. Chen et al., 2015; Jiang et al., 2015; Michot et al., 2012). A study carried out

by Michot et al., (2012) has shown that human patients with lipin1 mutations presented severe episodes of rhabdomyolysis. In addition to severe rhabdomyolysis, they also observed lipid droplet accumulation from muscle biopsies (Y. Chen et al., 2015; Michot et al., 2012). When it comes to human patients with lipin1 mutations, the phenotype differs from that of *fld* mice. Human patients do not show lipodystrophy but present severe muscle damage in early childhood (Y. Chen et al., 2015; Sauret et al., 2002).

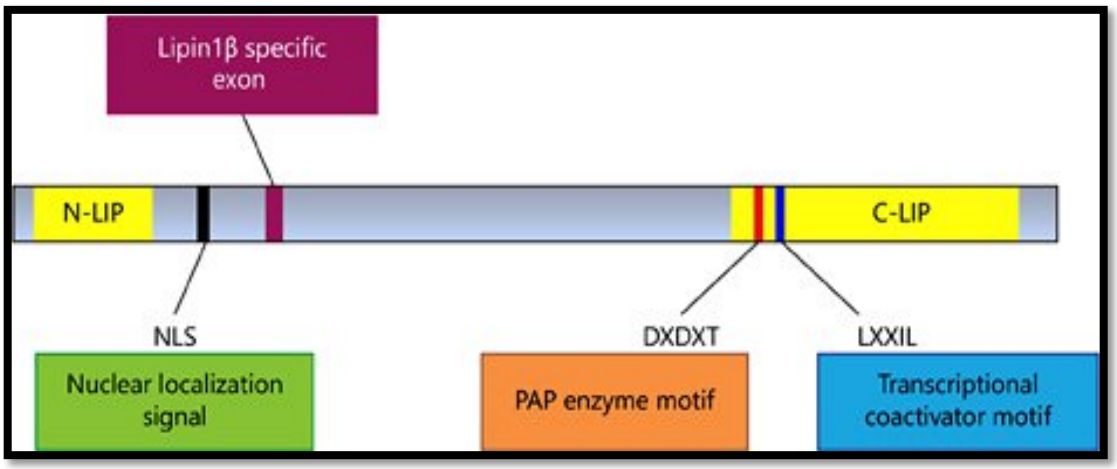

Figure 2*: Lipin1 protein domains(Y. Chen et al., 2015). (NLS = Nuclear Localization Signal. DxDxT = Catalytic motif. LxxIL = Transcription co-activator motif.)*

iv- Lipin1 enzymatic activity

PAP activity of lipin1 is important for its contributive role in phospholipid and TAG metabolism (Ren et al., 2010). TAG is an important molecule in energy storage as well as energy homeostasis. The path to TAG formation is primarily through the Kennedy Pathway, where a sequential acylation of glycerol phosphate takes place (Y. Chen et al., 2015). PA is a precursor for DAG and TAG and therefore dephosphorylated

by lipin proteins through their PAP enzymatic activity, a magnesium dependent (Mg^{2+}) catalytic activity. This means that lipin proteins are specific for PA and not other PA species like lipid phosphate phosphatase, which do not require Mg^{2+} (Y. Chen et al., 2015). Lipin1 is the predominant isoform of lipins, and account for almost all of the PAP activity in skeletal muscle and adipose tissue. This was evidenced in *fld* mice (Y. Chen et al., 2015; Donkor et al., 2007).

v- Lipin1 transcription co-activation activity

Lipin proteins and their lipid metabolism activity lie in their dual function activity (Figure 3). The enzymatic activity of these proteins contribute to the TAG and phospholipid accumulation the cellular system requires (Y. Chen et al., 2015). Their secondary activity is the transcriptional co-activation of proteins involved in fatty acid oxidation as well as genes that are involved in adipocyte differentiation (Y. Chen et al., 2015). Lipin1 co-activates key genes in adipogenesis by interacting with Peroxisome Proliferator –Activated Receptor gamma (PPARγ), which is a master regulator in adipocyte differentiation (Kim et al., 2013). In adipogenesis, lipin1 was found to positively regulate adipocyte maturation and differentiation by interacting, through its Transcripional Activating Domain (TAD), with PPARγ and this interaction has shown to enhance PPARγ activity (Kim et al., 2013). The TAD domain, located at the N-terminal of lipin1, only interacts with PPARγ and not Peroxisome Proliferator-Activating Receptor α (PPARα), which allows the transcriptional co-activation of adipogenesis, seperate from co-activation of genes involved in fatty acid oxidation. The transcription co-activation mechanism of lipin1 protein contributes to TAG and phospholipid build-ups

through maintenance and differentiation, and on the other hand regulating genes that break down fatty acids. Studies using PPARα and PPAR-coactivator-1α (PGC-1α) knockout mice have shown that lipin1 forms a complex with them to activate fatty acid oxidation (Y. Chen et al., 2015; Ren et al., 2010). It is suggested that the neonatal *fld* phenotype is due to the loss of the the co-activator function of lipin1 and inhibition of fatty acid oxidation (Y. Chen et al., 2015; Finck et al., 2006). Although lipin1 is predominately expressed in adipose tissue and skeletal muscle in adult mice, lipin2 predominates the liver and have been shown to be elevated in *fld* mice. This elevation of lipin2 is considered to be a compensatory effect of lipin1 deficiency and this contributes to the hypertriglycemia phenotype in *fld* mice (Finck et al., 2006).

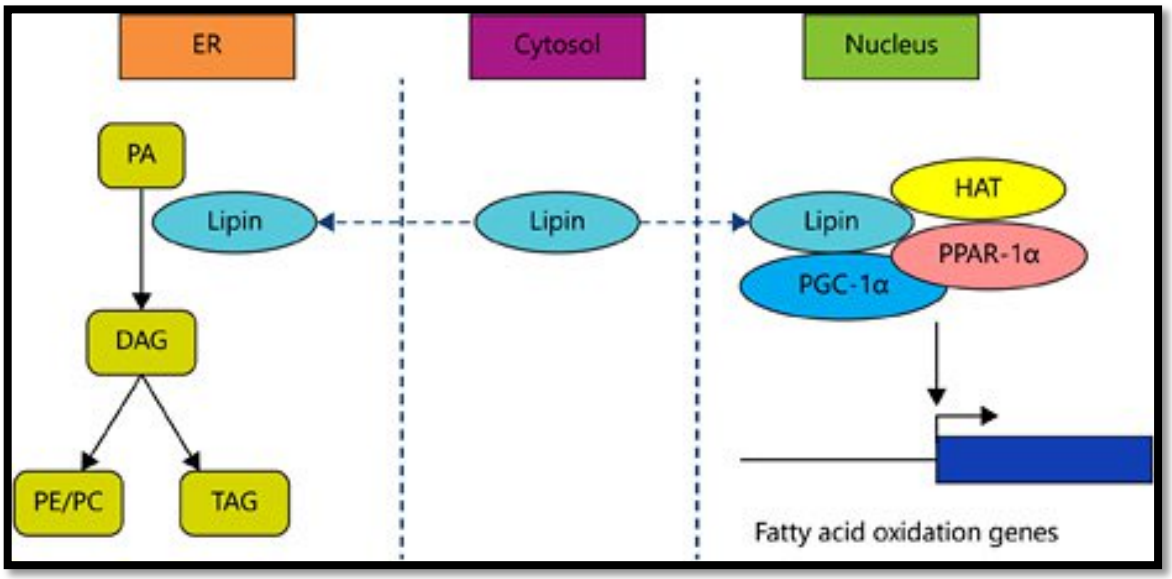

Figure 3*: Lipin proteins and their dual function in ER for enzymatic activity, converting PA to DAG and Nucleus for transcriptional co-activation of fatty acid oxidation genes (Y. Chen et al., 2015).*

vi- Phospholipids and secondary signaling

One of the main features of lipin1 is its contribution to TAG accumulation through dephosphorylation of PA to DAG and eventually TAG synthesis (Qi, Sun, & Yang, 2017). DAG is an important molecule as a precursor for TAG synthesis as well as a lipid secondary signaling molecule (Figure 4). The secondary signaling activity of DAG plays a central role in a variety of biological signaling pathways (Newton, 2001; Q. J. Wang, 2006). DAG is mostly anchored to the plasma membrane where it carries both activities of TAG synthesis and secondary signaling activity that leads to the recruitment of Protein Kinase C (PKC) (Eichmann & Lass, 2015). PKC consists of many isoforms and some of them interact with DAG and tumor-promoting phorbol esters, DAG analogs, which leads to their activation (Newton, 2001). One of the characteristics those isoforms have is the presence of C1 domain, a cysteine rich domain (Newton, 2001). This domain is important for the interaction between DAG and PKCs. When PKCs get activated and sequestered to the plasma membrane from the cytoplasm, they are phosphorylated, leading to downstream activation of other kinases that are required for cellular differentiation and cell growth (Eichmann & Lass, 2015; Newton, 2001; Q. J. Wang, 2006). As stated, PKC consists of a variety of isoforms or isozsymes. They are put into four different classes: Conventional (α, γ, βI & βII), novel (δ, ε, η, & θ) and atypical (ξ & ι/λ) as well as PKCμ and υ. The latter isoforms are sometime classified as their own group of protein kinases called Protein Kinase D or PKD (Newton, 2001). Conventional and novel isoforms are activated by DAG as they contain C1 domains (Q. J. Wang, 2006).

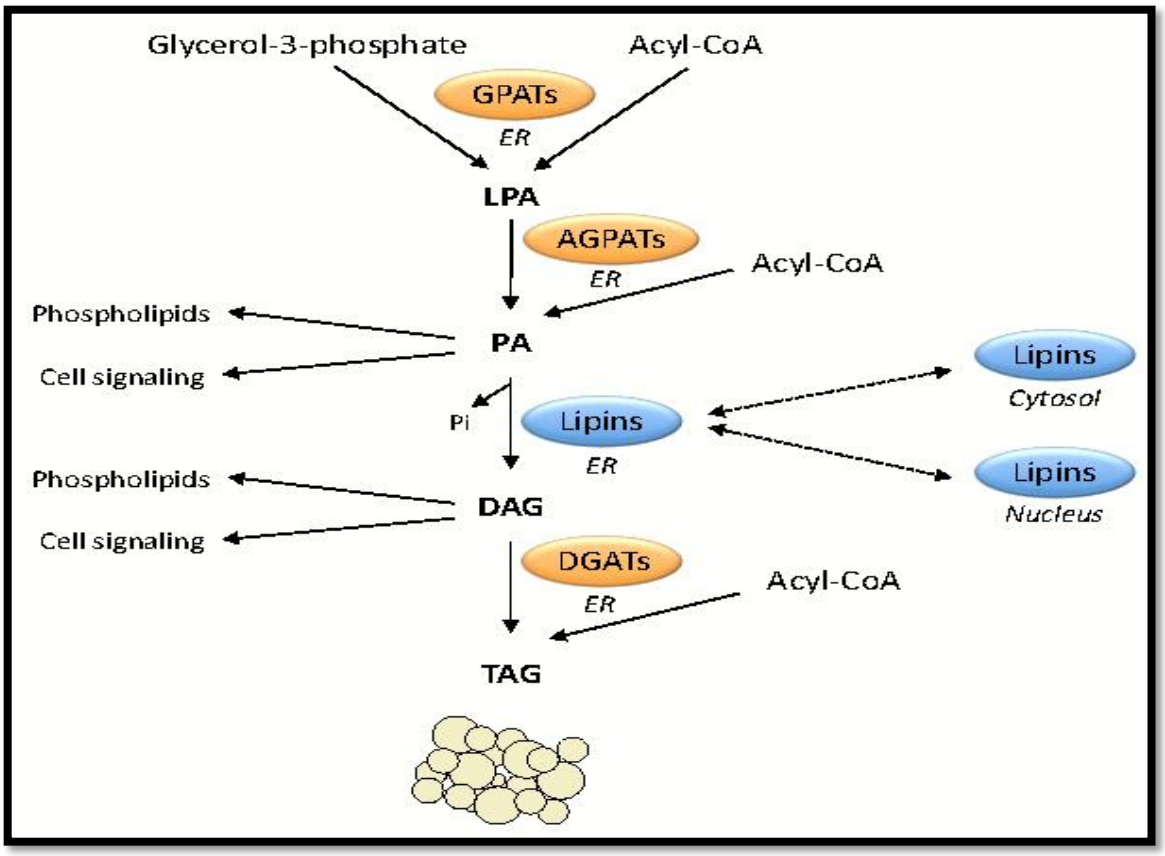

Figure 4*: Glycerol phosphate pathway for glycerolipid synthesis*

vii- Lipin1 in skeletal muscles

As aforementioned, lipin1 expression is the highest in the skeletal muscles compared to other lipin family proteins and all PAP activity in skeletal muscles is through lipin1 (Donkor et al., 2007; Jiang et al., 2015; Michot et al., 2012). Patients with lipin1 mutations that result missense, nonsense and frame shift show severe phenotype of rhabdomyolysis and myoglobinuria. The underlying molecular mechanisms of what contributes this pathology to these mutations are not clearly understood. In a study done by Jiang et al (Jiang et al., 2015) in *fld* mice has shown that lipin1 is important for skeletal muscle differentiation. They used barium chloride to induce muscle injury and monitored the muscle regeneration process in *fld* and WT mice. Upon injury to muscles,

they have shown that these mice had impaired skeletal muscle regeneration compared to WT mice. They have concluded that lipin1 is important for skeletal muscle regeneration (Jiang et al., 2015).

viii- Hypothesis and Aims

Recent human studies have suggested that children with homozygous null mutations in the LPIN1 gene suffer from rhabdomyolysis. Despite the well-known roles of lipin1 in lipid biosynthesis and transcriptional regulation, the pathogenic mechanisms leading to rhabdomyolysis remains unknown.

Through the work of Jiang et al (Jiang et al., 2015), it is established that lipin1 is an important gene for skeletal muscle regeneration upon injury in *fld* mice. These mice are lipin1 global knockout, meaning the mice are completely lipin1 deficient in every tissue. Systemic factors due to global lipin1 deficiency may also contribute to skeletal muscle phenotype of fld mice. Therefore, we hypothesize that lipin1 regulates skeletal muscle during development. With that we came with three aims: 1) Generating mice with lipin1 conditional knockout in *myf5* expressing progenitor cell. Myf5 gene, as mentioned earlier, is part of the myogenic regulatory factors and the earliest MRFs to be expressed (Bentzinger, Wang, & Rudnicki, 2012; Endo, 2015; Wood et al., 2013). In this study, we used this unique mouse model to explore the role of lipin1 in skeletal muscle to provide new pathophysiologic insights into the role of lipin1 in rhabdomyolysis. 2) Determine the role of lipin1 in skeletal muscle differentiation using myoblasts. 3) Identify the impact of lipin1 deficiency on muscle satellite cells.

II. Materials and Methods

i- Generating Lipin1^{Myf5cKO} mice and Genotyping

To generating Lipin1^{Myf5cKO,}, lipin1$^{flox/flox}$ mice (Nadra et al., 2008) were crossed with myf5-Cre mice that were purchased from Jackson laboratory (Stock No: 007893). Homozygous lipin1 deficiency in skeletal muscle and their lipin1-positive littermates (wild-type, WT) were used for this study. For genotyping, mice tails from newborns were collected. In ice, 120µl of EDTA solution (pH 8.0) and 500µl of Nuclei Lysis Solution were combined. 600µl of EDTA + Nuclei solution were added per tail followed by 8µl of 20mg/ml Proteinase K. The mixture and tail were incubated at 55˚C water bath overnight. 3µl of RNase were then added to the digested tails followed by inverting and incubating 15-30 minutes at 37˚C. In a room temperature, 200µl of Protein Precipitation Solution were then added followed by a vigorous vortexing at high speed for 20 seconds. The samples were then chilled on ice for 5 minutes. After ice incubation, the samples were centrifuged for 4 minutes at 13,000-16,000 x g to precipitate proteins. The supernatant containing the DNA were carefully removed and transferred to a clean 1.5ml microcentrifuge. 600µl of Isopropanol were then added to precipitate the DNA followed by 5 minutes of centrifugation at 13,000-16,000 x g at room temperature. 70% ethanol was then used to wash DNA for several times and centrifuged for 1 minute at 13,000-16,000 g at room temperature. Carefully, the ethanol was aspirated. The DNA pellet was

then rehydrated with 50µl of dH_2O. DNA was stored at 4˚C for short periods or at 80˚C if not needed right away.

Two PCR programs were used to identify the correct transgenic mice. One PCR program was to identify the presence of positive *Cre*$^+$ allele. The other PCR program was to identify the flox/flox allele or lipin1 mutation. In both programs, DreamTaq DNA polymerase (Thermo fisher cat#: EP0702) was used. After the PCR reaction, DNA was separated 1% Agarose (Alfa Aesar Cat#: J66369) gel electrophoresis. The PCR primers used to detect Cre$^+$ transgenic mice were one forward primer and two reverse primers. One reverse primer was to detect the wild-type allele and the other one for the mutant allele. The primers are: Forward 5'-CGTAGACGCCTGAAGAAGGTCAACCA-3', reverse primers: 5'-CACATTAGAAAACCTGCCAACACC-3' and 5'-ACGAAGTTATTAGGTCCCTCGAC-3'. For the flox/flox allele to detect the lipin1 mutant mice, the primers used are: Forward 5'-ATAAGCGGCCGCCTGAGCACGTTCACACATAC-3' and reverse, 5'-GTCGTCGACCTCTTCTCACACTCTGCCCA-3'.

ii- Tissue Collection and Cutting

Once genotyping was completed, transgenic mice aged 8 to 20 weeks were euthanized and the skeletal muscles, Gastrocnemius (GAS) and Tibialis Anterior (TA), were collected. Tissue for Immunohistochemistry and H&E staining were applied with Optimal Cutting Temperature (OCT) and quickly frozen by dipping in an Isopentane (-160˚C) for 10 seconds. The tissues were then stored at -80˚C freezer. For the H&E staining, the muscle tissue were cut into thin section about 7-15 microns using Thermo

Fisher cryostat micron HM550 at a temperature set -20˚C. For muscle tissues designated for western blotting and RNA, they were quickly frozen using liquid nitrogen.

iii- Cell culture and C2C12 myoblasts Differentiation

Mouse C2C12 myoblast cells were cultured cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco) and was supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1% penicillin-streptomycin (P/S) (Invitrogen) under humidified air containing 5% CO2 at 37 °C. The cells were split and passaged every two days to avoid 100% confluency. For differentiation, C2C12 cells were passaged into 6 well plates with full media. Next day, top three well cells were transfected with 0.4µl of shLpin1 adenovirus and 12µl of 8mg/ml polybrene. The bottom, the control, were transfected with either shLacz or no virus at all. Following day, the media was changed and let the cells recover from transfection. Monitering for 100% confluency in the next day or so, Day zero (DT0) were harvested. The remaing cells were then given differentiation media by replacing growth medium with differentiation medium consisting of Dulbecco's modified Eagle's medium supplemented with 2% horse medium (Gibco) and 1% penicillin-streptomycin (invitrogen). The cells were given fresh media every two days. Day 4 (DT4) and Day 6 (DT6) were subsequently harvested. The differentiated myotubes were then analyzed using western blot or mRNA analysis.

iv- Immunofluorescence

C2C12 cells were plated in a 6 well plate and were differentiated. At day 6, the media was removed from the cells and washed with 3X 1xPBS. Cells were then fixed

using 4% paraformaldehyde made with PBS for 20 minutes at room temperature. The residual paraformaldehyde (32% solution EMS cat#: 15714-s lot# 121010) was removed by washing the monolayer 2X with 1xPBS. The cells were then permeabilized by incubating them with 0.1% Triton X-100 made in PBS for 15 minutes at room temperature. 3x wash with 1xPBS followed. Cells were then blocked using 1% BSA (Thermo fisher lot: 155423) made with PBS for 1 hour at room temperature. The cells were then incubated with the Mf20 monoclonal antibody (DSHB) to detect MHC-expressing cells overnight at 4°C and subsequently with an Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary antibody (Invitrogen) for 1 h at room temperature. Next, cells were incubated with DAPI for 10 minutes followed by 3x with PBS, 5 minutes each. The plates were then let dry and images were taken using an inverted microscope Olympus IX70 equipped with a Leica DFC7000T camera.

v- DNA transfection and Immunofluorescence

In a 6 well plate, COS7 cells were seeded onto glass coverslips. Upon 80-90% confluency, cells were transfected with GFP-tagged HDAC5 (Addgene # 32211) with/without HA-tagged lipin1, HA-tagged Lipin D712A mutant or HA-tagged PKCu (Addgene #10808) plasmids using transfection reagent polyethylenimine (PEI). 6 hours post transfection; the media was the replenished with fresh full media (DMEM+10%FBS+1%P/S). After 24 hours post transfection, the media was removed and cells were fixed using 4% paraformaldehyde for 15 minutes at room temperature. Fixation was followed by washes, 2x washes with PBS, and then permeabilization with 0.1% Triton X-100. After washes, the cells were blocked with 1% BSA (Thermo Fisher)

in PBS for 1 hour. The cells were then incubated with Rabbit anti HA primary antibody (1:1000) in 1% BSA in PBS overnight at 4˚C. The cells were then subsequently incubated with secondary antibody, Alexa Flour Goat anti-Rabbit 555 with 1% BSA blocking reagent. Cells were then labeled with DAPI for nuclei staining. Co-localization of HDAC5 and Lipin1, Lipin1D712A or PKCu was examined using inverted microscope Olympus IX70 equipped with a Leica DFC7000T camera.

vi- Immunohistochemistry

GAS and TA muscles from both wild type and Lipin1^{Myf5cKO} were cryosectioned-using cryostat that was cut into 7-15µ. The cryosections biopsies on microscope slides (Thermo fisher Cat#: 22-230-900) were then let air dried for 1 hour at room temperature. The sections were then fixed with 4% paraformaldehyde, followed by 3x 3 minutes washes. Heat activated antigen retrieval was performed by placing the slides in 10mM citrate buffer (pH 6.5) and then put the coplin jar in boiling water at 92˚C for 20 minutes using pressure cooker. The slides were then let cool at room temperature for 1 hour with the citrate buffer. Liquid blocker was then used to draw the section boundaries. Endogenous peroxidases were then blocked with 3% H_2O_2 for 7 minutes at room temperature following 3x 3 minute washes. The slides were then washed 3x 3 minutes. Sections were then blocked using M.O.M (Vectorlabs MKB-2213) in PBS for 1 hour at room temperature, followed by another blocking with 1% blocking reagent (Invitrogen TSA kit lot: 1856764) for another 1 hour at room temperature. Pax7 (DSHB) and Rabbit laminin (Abcam11575) primary antibodies in 1% blocking reagent were incubated overnight at 4˚C, 1:5 and 1:200, respectively. Next day, the sections were washed with

PBS 3x 5 minute at room temperature, followed by incubating with secondary Goat anti mouse Biotinylated (Jackson Immune research cat#: 115-065-205) in 1% blocking reagent (1:1000) at room temperature. After 3x 5 minute washes, the sections were incubated with SA-HRP (Invitrogen TSA kit) in PBS for 1 hour at room temperature. Sections were then incubated with Alexa flour 488 (1:200) (Invitrogen) and Goat anti-Rabbit 555 (1:250) in amplification diluent (Invitrogen) for 1 hour at room temperature. After another set of washes, sections were incubated with DAPI (1:2000) in PBS for 10 minutes at room temperature. The sections/slides were then let dry and mounted with Vectasheild Mounting media (Vector labs, H-1000). Slides were then viewed using inverted microscope Olympus IX70 equipped with a Leica DFC7000T camera.

vii- RNA extraction and Quantitative real-time PCR

For C2C12 cells and muscle tissue RNA extraction, 1ml of Trizol reagent (Invitrogen) was used. After cells/tissue were lysed with Trizol, 200µl of chloroform was added and vigorously shaken for 15 seconds. Samples were then incubated for 2-3 minutes at room temperature followed by centrifugation 12,000 g for 15 minutes at 4˚C. The colorless upper aqueous phase was carefully taken to a new tube and mixed with 500µl of isopropanol. Samples were then 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 then carefully removed. 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 then carefully removed by pipetting. The pellet was then resuspended with RNase-free water. RNA quality and concentration was measured by using

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.

Primer	Forward 5'---->3'	Reverse 5'---->3'
Lipin-1	CCTTCTATGCTGCTTTTGGGAACC	GTGATCGACCACTTCGCAGAGC
Myf5	TGTATCCCCTCACCAGAGGAT	GGCTGTAATAGTTCTCCACCTGTT
MyoD	GCAGGCTCTGCTGCGCGACC	TGCAGTCGATCTCTCAAAGCACC
MEF2c	TCTGTCTGGCTTCAACACTG	TGGTGGTACGGTCTCTAGGA
Gapdh	CCAATGTGTCCGTCGTGGATCT	GTTGAAGTCGCAGGAGACAACC

Table 1: Primer sequences used for qPCR 1

viii- MicroRNA analysis

Total RNA was isolated from GAS muscle of Lipin^{Myf5cKO} and control mice using TRIzol reagent (Invitrogen). For miRNA RT-PCR, cDNA from 200 ng of total RNA was synthesized using Taqman microRNA reverse transcription kit (Applied Biosystems). Real time PCR of Hsa-miR-1 (Applied Biosystems) was carried out with Taqman Universal master mix II following the manufacturer's instructions (Applied Biosystems). U6 SnRNA (Applied Biosystems) was used as an internal control for microRNA analysis.

ix- Western blotting

For protein expression analysis, muscle tissues were homogenized 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 with homogenizer, samples were centrifuged at 12,000 rpm for 10 minutes. The supernatant was collected and aliquoted in new 1.5ml microcentrifuge tubes. Bicinchoninic acid assay (BCA) was carried out for protein concentration analysis for each sample. About 2mg/ml of BSA was used as standards (0 for blank, 1µl, 2µl, 3µl, 4µl, 5µl and 6µl) and loaded in 96 well plates. 1-3µl of samples was then loaded. Each standards and samples were duplicated. Plates were then incubated at 37˚C for 20-30 minutes followed by protein absorbance measurement using SynergyH1 microplate reader with a wavelength at 567. The standard curve was used to calculate the sample concentrations and water was water was added to adjust the volumes.

Acrylamide gel (30% acrylamide Solution 37.5:1 BioRad Cat. 1610158) was used to separate proteins using gels (7.5%-15% gels). Samples were prepared for running after the desired amount of protein for running was calculated (20µg-60µg). After readjusting with dH_2O, 4x-loading dye was added to each sample. Samples were then heated in boiling (95˚C) for 5 minutes followed by spin down. Afterwards, the samples and protein ladders (Fisher Cat#: 26616) (BioRad Cat#: 161-0373) were loaded into the SDS gel in running buffer with the voltage set to 100v and increasing it to 120v once it goes through the stacking gel. For transferring, PVDF membranes were soaked into methanol for 1-3 minutes and then into a transfer buffer. Sponges, soaked in transfer buffer were then

placed on the black place followed by filter paper also soaked in transfer buffer. The SDS gels, after the running was completed, were removed from the plates. Gels were then placed on the filter paper and the sponged followed by the PVDF membrane. Using a glass pipette, the bubbles were carefully removed. Another set of filter paper and a sponge soaked transfer buffer was placed. The transfer system was set up so that the black plate of the sandwich is close to the black side of the system. The system was then filled with transfer buffer. To avoid an increase in in temperature during the transfer, the system was place an ice container surrounding it. The proteins were transferred to the membrane at 0.30A for 2 hours. After transfer, membranes were blocked using 1% casein buffer for 1 hour at room temperature. Afterwards, the membranes were incubated with primary antibodies made with 1% casein buffer overnight at 4˚C with rocking. Next day, memberanes were washed with TBST (1x TBS buffer + 0.1% Tween20, Fisher Cat#: 175476) and incubated with secondary antibodies for 1 hour at room tempereture with rocking. The scondary antibodies were diluted in 1.0% casein + 0.1 % Tween 20 + 0.1% 10% SDS buffer. The blots were visualized using Chemiluminescense kit from Pierce™ ECL Western Blotting Substrate (catalog # 32106). The instrument used for the visualization is Amersham Imager 600 (GE life sciences). Antibodies that were used include lipin1 (Cell signaling Technology, Cat#: 14906, phospho-PKCμ 744/748 (Cell signaling Technology, Cat#: 2054,), MyoD (Abcam Cat#: 16148), MEF2c (Cell signaling Technology Cat #: 5030) phospho-PKCα (Santa Cruz Biotechnology cat#: sc-377565), phospho-PKCθ (Santa Cruz Biotechnology cat#: 271922) and Pax7 (DHSB and Abcam cat#: 34360), along with goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP

seondary antibodies (Promega, USA). For loading control primary antibodies, GAPDH (Abcam cat#: 181602) and Beta-actin (Sigma cat#: 1978).

Statistical analysis. In each graph, unless noted otherwise, data represent mean ± SD of (n)-number of independent experiments. Statistical significance was calculated by a two-tailed Student t-test. P values < 0.05 were considered statistically significant.

III. Data and Results

1. Lipin1 knockout in Myf5-expressing precursors leads to suppression of skeletal muscle development.

The aim of generating mice that lack lipin1 in skeletal muscle progenitor precursors was to investigate the role of lipin1 in skeletal muscles development. The loss of lipin1 in skeletal muscles did not cause severe ectopic phenotypes although an altered fat deposition was observed (Figure 5A). Some phenotypes that are observed in *fld* mice such as hind-limb clasping reflexes, tremors, or an unsteady gait were absent from these conditional knockout Lipin1^{Myf5cKO} mice. On the other hand when lipin1 was knocked out in the skeletal muscle progenitors, the muscles of these mice were developmentally suppressed. About 24% muscle mass reduction was observed in both Tibialis anterior (TA) and gastrocnemius (GAS) of Lipin1^{Myf5cKO} mice (Fig 5A-C) compared to their wild type cohorts.

Next we measured the protein expression of key genes that are important for myogenesis in Lipin1^{Myf5cKO} mice. First, we measured the lipin1 protein expression and found that it has been completely abolished in both the TA and GAS muscles as expected (Figure 6A). Once we established that lipin1 expression is depleted in these muscles with observed phenotype, in muscle mass change, we sought to further investigate key markers for myogenesis. Myf5, MyoD and MEF2c protein expression were analyzed. The expression levels of Myf5 were not changed in both TA and GAS even though lipin1 was depleted in Myf5 expressing progenitors. On the other hand, MyoD and MEF2c their

expression were reduced by 48% and 60%, respectively (Figure 6B). This change in MyoD and MEF2c and no change in Myf5 protein levels further illustrates that Myf5 is expressed earlier than both MyoD and MEF2c and that lipin1 deficiency had no impact on Myf5 protein expression. In addition to measuring protein expression, we also analyzed the mRNA levels of these genes. Lipin1 was reduced in the mRNA level as well as MyoD and MEF2c. But there was no change in Myf5 mRNA levels, consistent with the protein expression levels (Figure 6C).

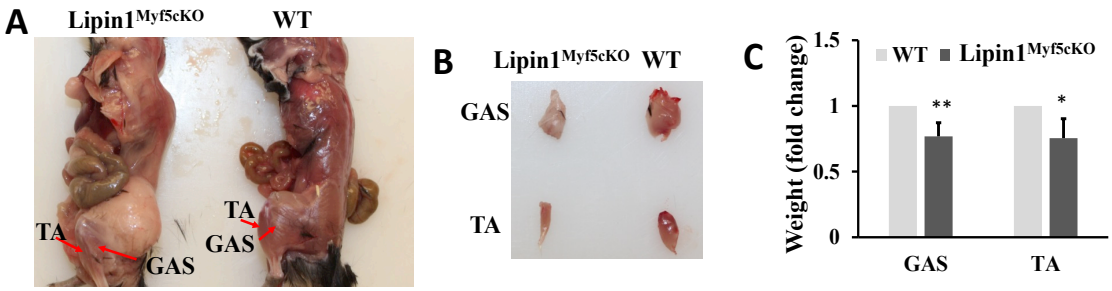

Figure 5*: Lipin1 deficiency in Myf5-expressing cells results in reduced skeletal muscle mass.* (A) Appearance of Tibialis anterior (TA) and gastrocnemius (GAS) muscles in WT and Lipin1^{Myf5cKO} mice at 3-month-old of age (TA and GAS were indicated by arrows). Isolated TA and GAS muscle (B) and the muscle weight (C) in WT and Lipin1^{Myf5cKO} mice. (WT vs. Lipin1^{Myf5cKO}, *p<0.05; **p<0.01 Student's t-test).

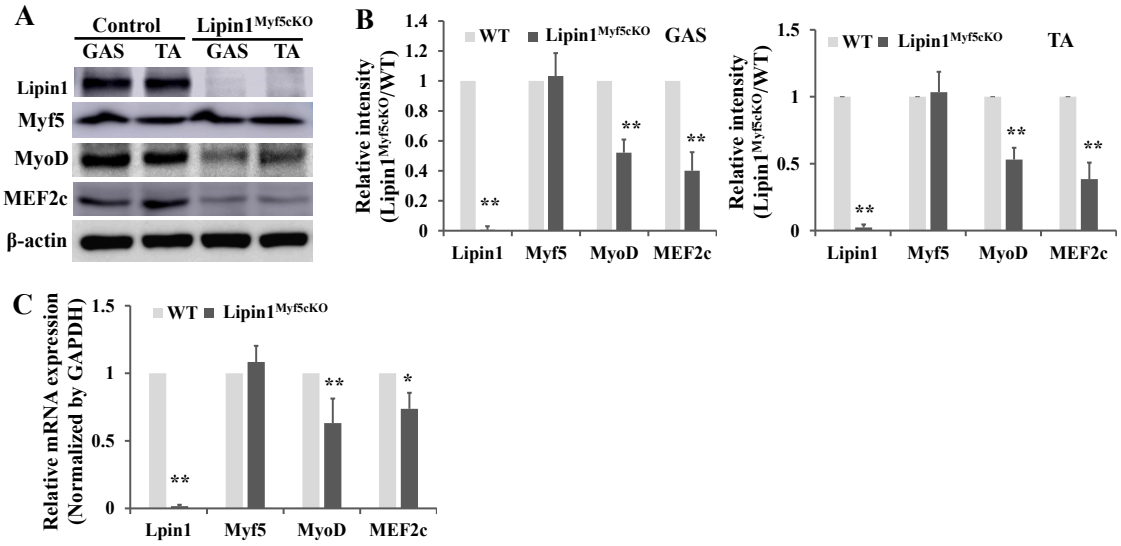

Figure 6*: Lipin1 depletion in Myf5-expressing progenitor cells inhibit MEF2 and MyoD expression, but did not alter Myf5 expression*. Western blot (A), the densitometry graphs (B), and mRNA expression (C) of lipin1, myogenic regulatory factors (Myf5 and MyoD) and of myogenic enhancer factor (MEF2c) in GAS and TA muscles of 3-month-old WT and Lipin1^{Myf5cKO} mice. Unless otherwise indicated, data are from one representative (n = 3) of at least three independent experiments. (*p<0.05; **p<0.01 Student's t-test).

2. Lipin1 deficiency leads to reduction in PKC activation.

Phosphatidic acid phosphatase (PAP) activity of lipin1 is important for converting phosphatidic acid (PA) to diacylglycerol (DAG). This makes lipin1 an important molecule for DAG synthesis (Newton, 2001; Q. J. Wang, 2006). Studies have shown that lipin1 overexpression leads to an increase in cellular DAG contents while a depletion of lipin1 leads to the reduction in DAG accumulation (Ren et al., 2010). DAG is an important molecule in both Triglyceride (TAG) synthesis but as well as functioning as a secondary signaling molecule. DAG is shown to recruit certain protein kinases to the plasma membranes where it leads to their activation (Newton, 2001). Some of these proteins include Protein Kinase C μ/PKD as well as other PKC isoforms (Brose et al., 2004; Eichmann & Lass, 2015; Q. J. Wang, 2006). With this in mind, we sought to measure the protein expression of PKCμ and other PKC isoforms such as α and θ. It has been shown that PKCμ is activated when it is phosphorylated at residues Serine 744 and 748. When we measured the phospho-PKCμ at these sites in TA and GAS in Lipin1^{Myf5cKO} mice, the results showed a decrease in their activation. A western blot analysis showed that phopho-PKCμ at Serine 744 and 748 was reduced by about 70% compared to the wild-type suggesting a decrease in the activation. PKCα phosphorylated at Serine 657 was also reduced by 63% in both TA and GAS muscle of Lipin1^{Myf5cKO} mice. PKCθ, phosphorylated at Threonine 538 was reduced by about 40% in the TA muscle (Figure 7). The total levels of PKC were not changed. This led us to conclude that lipin1 deficiency leads to the reduction of PKC activation through DAG in the skeletal muscles. As aforementioned, PKC activation leads to downstream signaling cascade that are important for cellular differentiation and cell growth. This also makes PKC proteins

important for myogenesis. So far we saw the reduction in myogenic factors and the diminished activation of PKCs. This led us to further look into signaling mechanism and relationship between PKC, MyoD and MEF2c. Brunelli et al (Brunelli, Relaix, Baesso, Buckingham, & Cossu, 2007) has implicated PKCμ in MyoD expression but a detailed mechanism of action is not well understood.

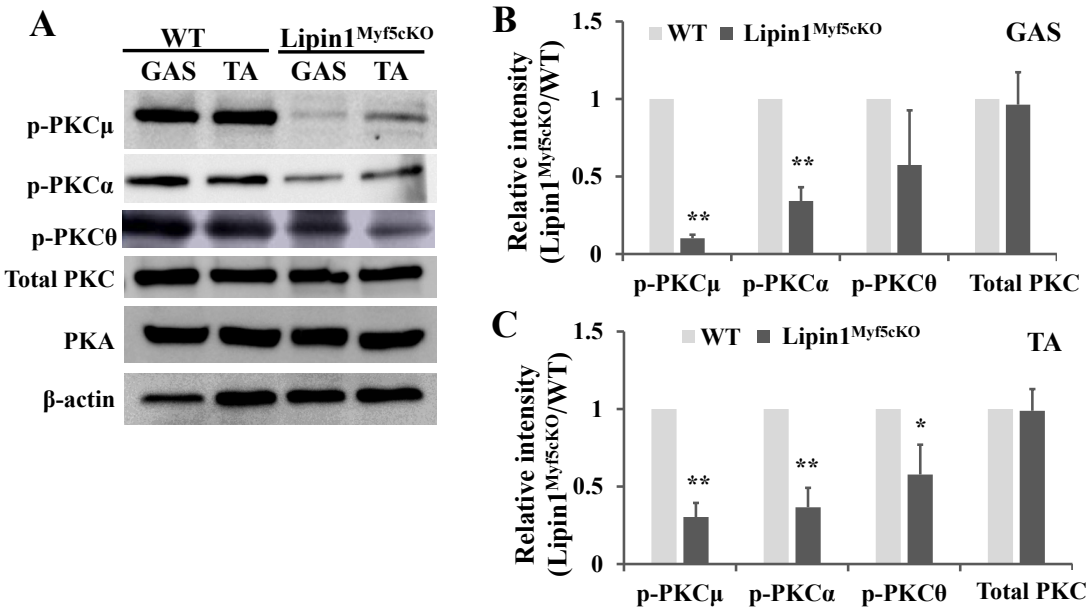

Figure 7*: Lipin1 deficiency diminishes PKC activation.* *A Representative immunoblots* (A) and densitometry graphs of lipin1 and different protein kinase expression and phosphorylation in GAS (B) and TA (C) muscles in WT and Lipin1^{Myf5cKO} mice at 3-month-old.

3. Lipin1 is required for myoblast differentiation in cell culture.

To explore the mechanism, we used C2C12 cells for *in vitro* studies where we knockdown lipin1 and differentiate them to form myotubes, which eventually fuse together and form myofibers. Our aim here was to clarify if lipin1is important for myoblast differentiation. We knocked down lipin1 using adenovirus driven shRNA. The cells were then differentiated for 6 days. Myotube formation was examined using Mf20 immunostaining to detect myosin heavy chain (MHC)-positive myotubes. We found that lipin1 knockdown in C2C12 cells inhibited myoblast differentiation and impacted myotube formation compared to the wild type or shLacZ treated cells (Figure 8A). The shRNA treated C2C12 has a reduced fusion index compared to the wild type. Fusion index is calculated as the percentage of nuclei contained in myosin-positive myotubes. The index for the shRNA treated was reduced to 30% from 49% of that of the control (Figure 8B). This experiment was consistent with the pervious work done by Jiang et al (Jiang et al., 2015) that lipin1 is required for myoblast differentiation.

Molecularly, we wanted to see if the same proteins that are down regulated in the tissue were also down regulated in these shRNA treated C2C12 cells. In this approach, we knockdown lipin1 in C2C12 cells and differentiated them at different time points: Day 0 (before differentiation), Day 3 (3 days post-differentiation) and Day 6 (6 days post-differentiation). These cells were then harvested for protein analysis using western blot. We analyzed the expression levels of myogenic factors and Protein Kinase C. First, the expression levels of Myf5 were not altered, consistent with tissue data. Next, MyoD and MEF2c were measured, which showed a dramatic reduction at Day 6 post-differentiation. A reduction of 75% of MyoD compared to the wild type was observed.

Lastly, PKCμ activation was analyzed by measuring phospo-PKCμ. Consistent with the tissue data, PKCμ activation was reduced dramatically, a 60% reduction at Day 6 compared to the wild type (Figure 8C, D).

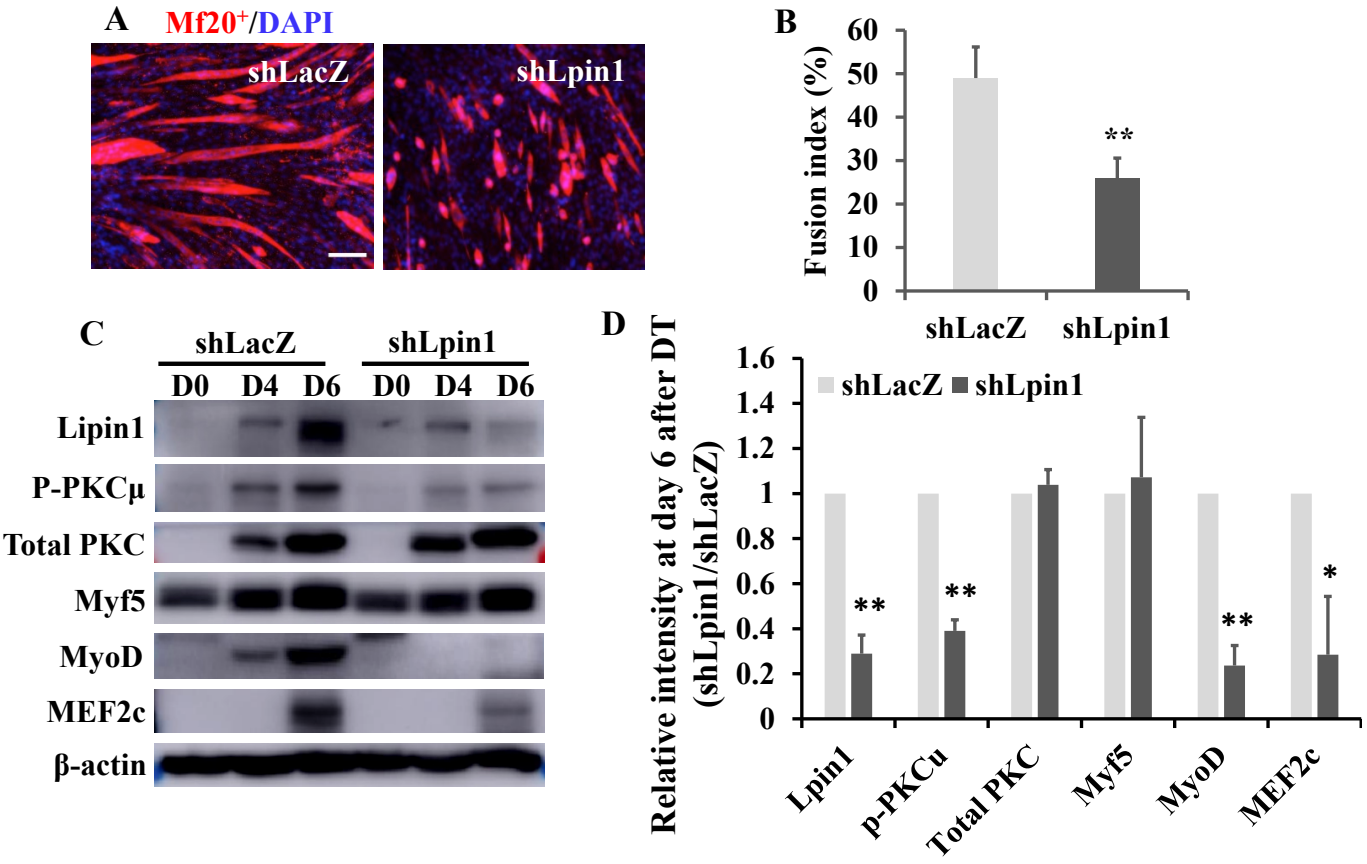

Figure 8*: Lipin1 is required for myoblast differentiation in cell culture.C2C12 myoblasts were infected with adenovirus to knockdown lipin1 (shLpin1) or control shLacZ before differentiation.* (A) Cells were fixed and stained for $Mf20^{+}$ and DAPI (Scale bars, 50μm). (B) Fusion index was determined by counting the percentage of nuclei within $Mf20^{+}$ myotubes over total nuclei within five randomly selected fields per sample. Protein expression levels (C) and densitometric analysis of PKC (D), myogenic differentiation markers and MEF2c in differentiated myoblasts. *p<0.05; **p<0.01.

4. Inhibition of PKCμ activity suppresses myoblast differentiation through inhibiting MyoD and MEF2c expression in vitro.

After establishing that lipin1 knockdown in C2C12 exhibited down-regulated myogenic regulatory factors as well as diminished activation of PKCμ, we sought whether diminishing PKCμ activity with an inhibitor would result in a reduction of myogenesis and myogenic factors. In this approach, we used CID755673, a selective inhibitor for PKCμ, to selectively inhibit PKCμ activity. C2C12 were differentiated at Day 0, Day 4 and Day 6. The PKCμ inhibitor was added to the media before and after differentiation. Cells were then harvested for immunostaining and protein expression using western blot. Similar to lipin1 knockdown, when C2C12 cells were treated with CID755673 inhibitor, the myotube formation and differentiation was reduced as evidenced by the MHC-positive myotubes detected by Mf20 Immunostaining (Figure 9A). In addition, the fusion index was reduced from 50% to 31% for the CID755673 treated (Figure 9B). This confirms that PKCμ activation is important for myotube formation and C2C12 differentiation. Next, we measured the protein expression of phospho-PKCμ Ser744/748, MyoD and MEF2c. Their protein expression was also reduced by 70%, 75% and 63%, respectively, at Day 6 (Figure 9C, D).

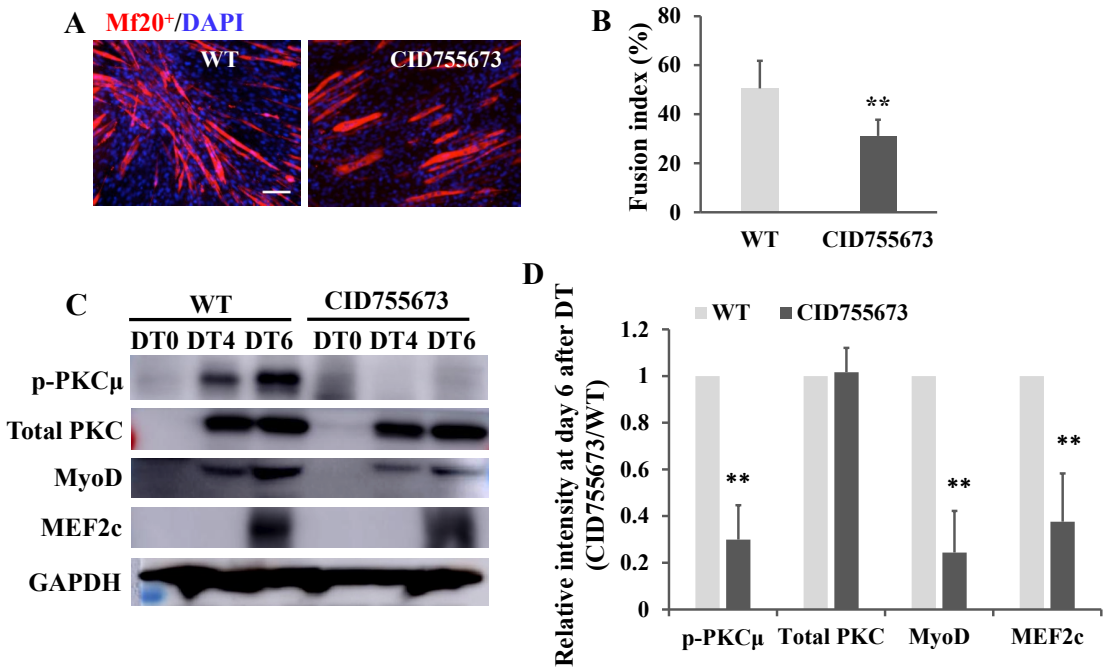

Figure 9*: Inhibition of PKCμ activity suppresses myoblast differentiation through inhibiting MyoD and MEF2c expression in vitro*.C2C12 myoblasts were treated with a PKCμ inhibitor, CID 755673, for 6 days, and subjected to myoblast differentiation. 6 days after differentiation, (A) cells were fixed and stained with Mf20 and DAPI. (Scale bars, 50μm) (B) Fusion index was calculated. (C) Cell lysate was harvested, and protein expression was measured by western blot. (D) Protein expression levels in C were quantified by densitometric analysis and normalized to β-actin. **p<0.01.

5. Lipin1 deficiency decreases MEF2 and MyoD by inhibiting the nuclear export of HDAC5.

The story has so far been that lipin1 deficiency leads to the inactivation of PKCμ through the loss of DAG accumulation. However, how does the diminished activity of PKCμ lead to decreased MyoD and MEF2c is not fully clear. It has been suggested that MyoD and MEF2c interact with each other through its DNA binding and dimerization motifs, which in turn activate myogenesis process (Black, Molkentin, & Olson, 1998; Kaushal, Schneider, Nadal-Ginard, & Mahdavi, 1994; Molkentin et al., 1995). It has also been shown that MEF2c proteins interact with Class II Histone Deacetylase 5, HDAC5. This interaction is thought to result in the repression of MEF2c protein transcriptional activity, which is important for myogenic gene expression (Bertos et al., 2001; Lu, McKinsey, Nicol, et al., 2000). In addition, the signaling cascade chaperone proteins, the 14-3-3 binding proteins are thought to aid the interaction between MEF2c and HDAC5 through PKCμ and CaMK signaling cascades (Bertos et al., 2001; McKinsey, Zhang, & Olson, 2000). For full cooperative activity of MEF2c and MyoD, the HDAC5 protein must be dissociated from MEF2c and exported from the nucleus to the cytoplasm (McKinsey, Zhang, Lu, et al., 2000; McKinsey, Zhang, & Olson, 2000). With this in mind, we sought to measure HDAC5 activity through its phosphorylated form at Ser 259. When HDAC5 is phosphorylated, it indicates that the signaling cascade through PKCμ is active and that MEF2c repression is relieved. When we measured the protein expression levels of phospho-HDAC5 in skeletal muscles of Lipin1^{Myf5cKO} mice, we saw a substantial reduction in its phosphorylation, indicating that the upstream signaling cascade, PKCμ, is lost or reduced (Figure 10A). To further solidify what we are observing in the tissue is consistent with cell culture, we measured the phospho-HDAC5

in the differentiated C2C12 cells treated with CID755673, a PKCμ inhibitor. As shown in Figure 10B, the phospho-HDAC5 is reduced in these differentiated cells at Day 6 treated with the inhibitor. In both tissues and cells, the total HDAC5 protein levels were not changed. These experiments suggest that PKCμ is important for translocating HDAC5 from the nucleus through phosphorylation, which leads to the transcriptional function of MEF2c and MyoD to advance.

Next to delineate the subcellular localization of HDAC5 we found that, through overexpression, HDAC5 is exclusively localized in the nucleus (Figure 10C). Since HDAC5 is a transcription repressor protein, it always localizes in the nucleus. However, when we co-overexpressed lipin1 with HDAC5, it induced the nuclear export of HDAC5. To further clarify which activity of lipin1 was responsible for the HDAC5 nuclear export, we co-overexpressed HDAC5 with lipin1 that was mutated in its catalytic activity site, D712A. Interestingly, this did not induce HDAC5 nuclear export; further illustrating that lipin1 catalytic activity is important for HDAC5 nuclear export and further transcriptional de-repression of genes; in this case myogenic genes. Additionally, we co-overexpressed HDAC5 with PKCμ, which also led to the nuclear export of HDAC5. To determine if DAG is responsible for the PKCμ activation, we used Phorbol 12-myristate 13-acetate (PMA), a DAG analogue. Cells were overexpressed with HDAC5 and then treated with PMA for 30 minutes. HDAC5 subcellular localizations were then evaluated. We observed that PMA treatment drove the subcellular localization of HDAC5 from the nucleus to the cytoplasm, same as PKCμ and lipin1 co-overexpression (Figure 10D).

Throughout these experiment, we concluded that a signaling cascade exists in lipin1, DAG, PKCμ and HDAC5. Consequently, we believe that the activation of PKCμ

through lipin1/DAG and subsequent HDAC5 nuclear export that results in the dissociation of MEF2c-HDAC5 complex, leads to the activation of MEF2c and myogenic regulatory factors. Our overall conclusion is that lipin1 deficiency hinders skeletal muscle differentiation by inhibiting MEF2 and MyoD through PKCα and PKCμ activity and that it failed to induce HDAC5 nuclear export and release the inhibitory function of HDAC5 to MEF2c (Fig 10E).

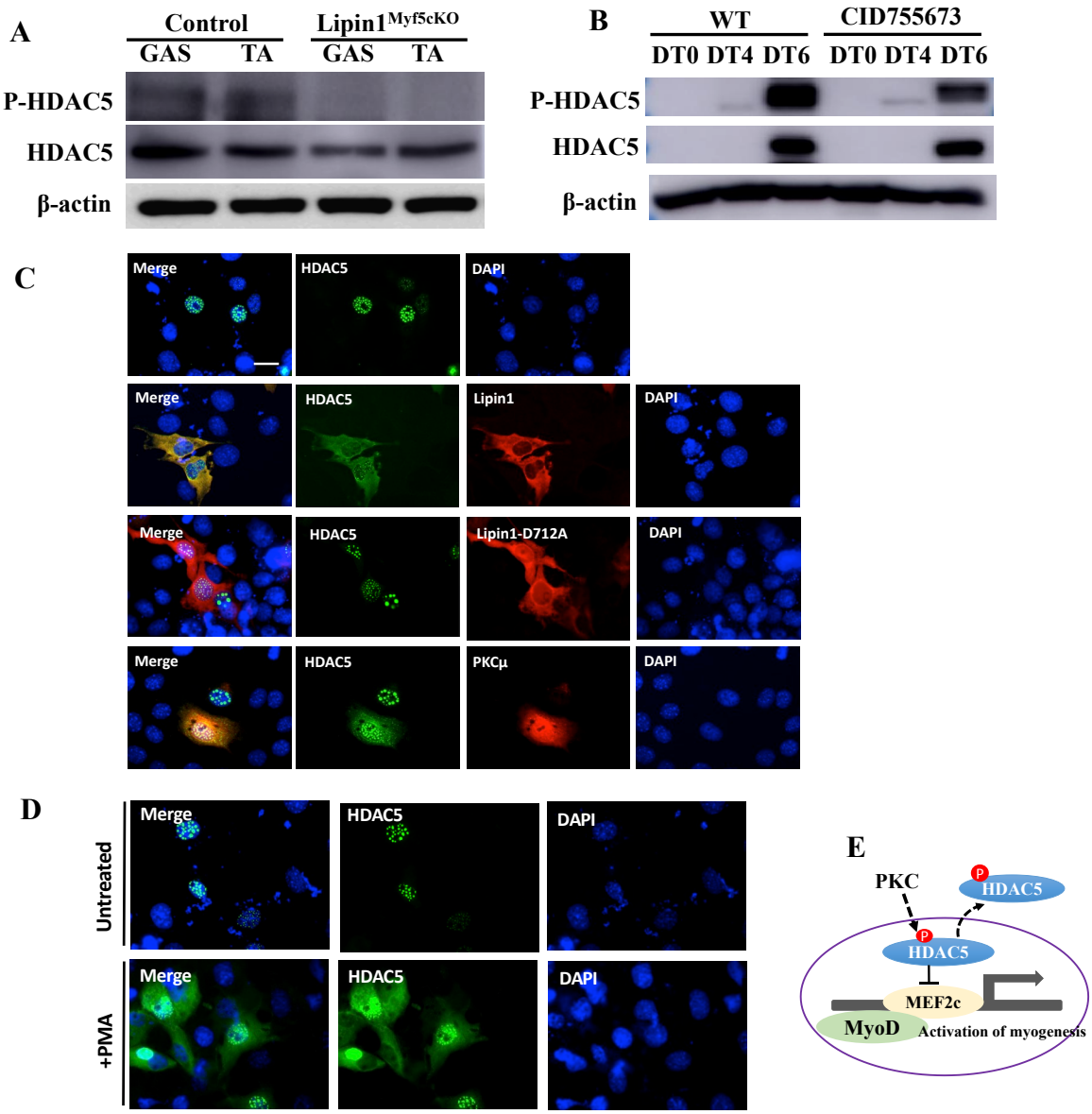

Figure 10*: Lipin1 regulates HDAC5 nuclear export through activation of PKCμ.* (A) Western blot of HDAC5 and phosphorylated HDAC5 in the GAS and TA muscle of WT and Lipin1^{Myf5cKO} mice. (B) C2C12 myoblasts were treated with CID 755673 for 6 days, and subjected to myoblast differentiation. 6 days after differentiation, cell lysate was

harvested; the protein expression levels of HDAC5 and phosphorylated HDAC5 were measured by western blot. (C) Cos7 cells were transfected with either GFP-tagged HDAC5 or along with HA-tagged lipin1, catalytic mutant lipin1-D712A or HA-tagged PKCμ. Cells were fixed 24 h after transfection, and subcellular localization of lipin1 and HDAC5 were examined by anti-HA (red), anti-GFP (green) antibodies. Nuclei were detected by DAPI (blue). Co-localization of HDAC5 and lipin or PKCμ were determined. (D) Cos7 cells transfected with HDAC5 treated with/without PKC agonist, PMA, for 30min. Subcellular localization of HDAC5 was examined by immunostaining. (Scale bars, 50μm) (E) Model of PKCμ regulation of HDAC5 subcellular localization.

6. Lipin1 deficiency affects miR-1 expression and Pax7 transcriptional activity in Lipin1^{Myf5cKO} mice.

Pax7 is an important marker for satellite cells. The function of these cells is to serve as myogenic stem cells that are readily available for differentiation after muscle injury (Endo, 2015; Sambasivan & Tajbakhsh, 2015). Some studies have shown that Pax7 acts upstream of other myogenic factors such as MyoD (Sambasivan & Tajbakhsh, 2015; von Maltzahn, Jones, Parks, & Rudnicki, 2013; Wood et al., 2013). Although there was no change in the Myf5 gene and protein expressions in Lipin1^{Myf5cKO} mice, we observed an increase of Pax7 protein levels, up to two folds, in their skeletal muscles (Figure 11A, B). In addition to protein, immunohistochemistry against pax7 was done. The pax7 expressing satellite cells in the Lipin1^{Myf5cKO} mice were increased two fold compared to the wild type (Figure 11C, D). It has been reported that pax7 inhibits satellite cells to differentiate and keeps satellite cells in their quiescent stage (von Maltzahn et al., 2013). Liu et al (Liu et al., 2007) has reported that microRNAs (MiR) play an important role in myogenesis where they are positively regulated by MEF2c expression. These MiRs are thought to regulate differentiation process tightly. Another study has proposed that pax7 is inhibited by these MiRs (J. F. Chen et al., 2010). Some of these MiRs include miR-1. After observing the increase of pax7 protein levels in Lipin1^{Myf5cKO} mice, we wanted to see if the miR-1 was impacted. We measure the miR-1 levels and observed a decrease in Lipin1^{Myf5cKO} mice compared to the wild type. This led us to believe that the reduction of MEF2c contributed to the reduction in miR-1, a negative regulator of pax7, thus increasing the pax7 levels (Figure 11E).

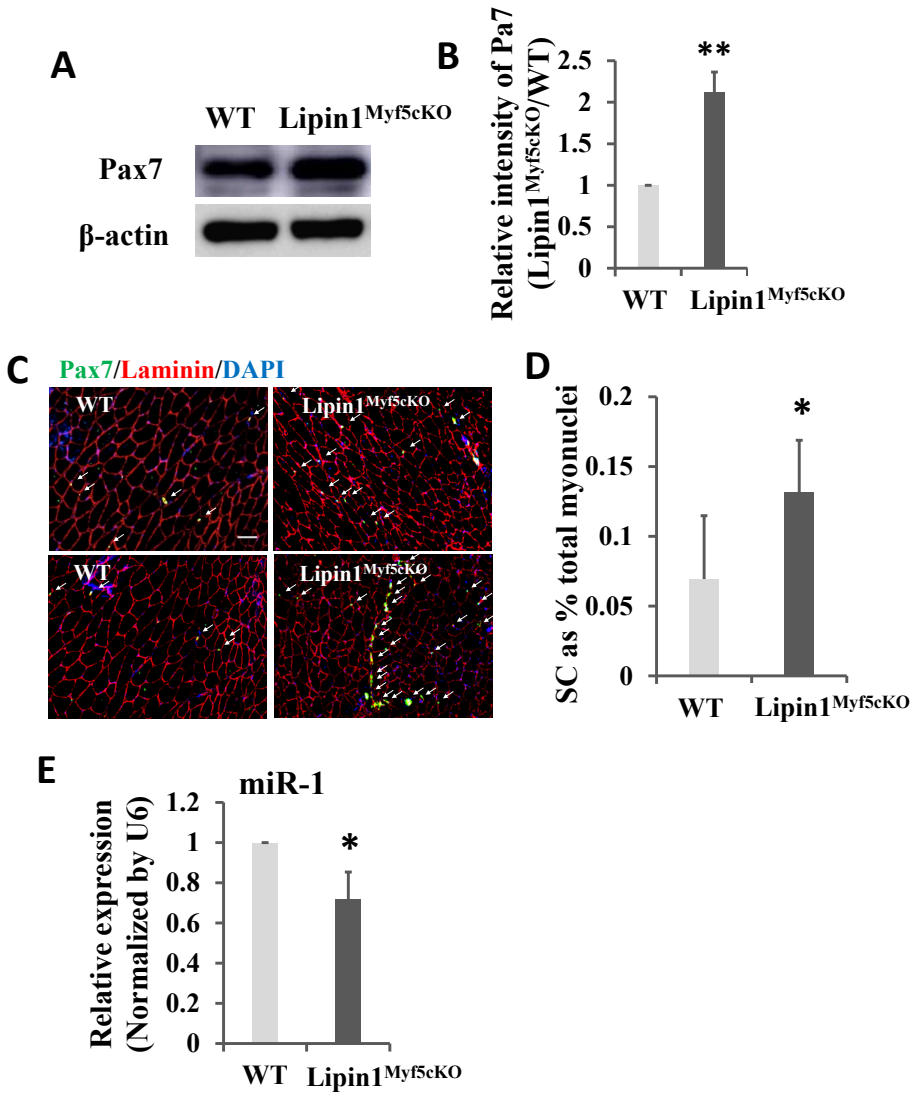

Figure 11*: Suppression of MEF2c and MyoD induces accumulation of Pax7.* Western blot (A) and densitometric analysis (B) of Pax7 expression in GAS muscle of 3-month-old WT and lipin1^{Myf5cKO} mice. (C) Immunostaining of Pax7 (green) and laminin (red) on transverse sections of GAS muscle of 3-month-old WT and lipin1^{Myf5cKO} mice. Scale bar = 100μm. (D) Quantification of the number of Pax7-positive cells divided by the total number of nuclei in a given microscopic field. For each muscle section, at least 6

different fields were quantified and averaged. Data are mean ± s.e.m. (E) RT-PCR analysis of miR-1 expression in GAS of WT and Lipin1^{Myf5cKO} mice. *p<0.05; **P<0.01.

IV. Discussion

Our study suggests that lipin1 plays an important role in skeletal muscles development (Figure 12). We showed that lipin1 deficiency leads to the reduction of skeletal muscle mass through the loss of PKCμ and PKCα signaling activity, which regulates HDAC5-cytoplasmic and nuclear shuttling. When HDAC5 is phosphorylated at Ser259 by PKCμ or PKCα, it is exported from the nucleus, releasing its inhibitory function in myogenic genes such as MyoD and MEF2c; this in turn leads to the activation of myoblast differentiation and skeletal muscle development.

Our *in vitro* experiments found that the absence of lipin1 in myoblasts leads to an inhibition of myoblast differentiation. This reduction was due to the loss of PAP activity of lipin1, which subsequently is needed for DAG and TAG synthesis. Our results also suggested that PKCμ and PKCα were reduced in their activation through the loss of phosphorylation. This led us to believe that PKC activation was important for myoblast differentiation and becoming mature myotubes. We believe that the reduction in activation of PKCμ and PKCα were due to the loss of the DAG. It has been shown that DAG, a secondary signaling molecule, leads to the activation of protein kinases such as PKCμ and PKCα isoforms (Q. J. Wang, 2006). A study by Kleger, et al (2011), have showed that the depletion of PKCμ by shRNA in C2C12 myoblast led to the inhibition of myoblast differentiation while an ectopic expression of PKCμ showed enhanced myoblast differentiation (Kleger et al., 2011). In addition, we found that lipin1 deficiency leads to the reduction of MyoD and MEF2c expression and that their decrease in

expression is due to the upstream loss of PKCμ activation. We believe that myoblast differentiation is regulated by PKCμ activity through the activation of myogenic factors; MyoD and MEF2c by showing that PKCμ inhibition resulted in stunted myoblast differentiation and reduction in MyoD and MEF2c.

The cooperative activity between MyoD and MEF2c transcription factors are thought to promote the expression of myogenic factors that are needed to shift myoblasts from an undifferentiated myoblasts to differentiated myoblasts that become myotubes (Lu, McKinsey, Zhang, et al., 2000). Our study suggests that lipin1 deficiency leads to the reduction of MyoD and MEF2c expression in both *in vivo* and *in vitro* and that their expressions are dependent on each other. Previous studies have suggested that MyoD and MEF2c interact with each other during myogenesis through their DNA binding domains (Potthoff & Olson, 2007; Tapscott, 2005). Since MyoD and MEF2c are transcription factors, it is possible that they both positively regulate each other's expression. For clarification on their impact on each other, further studies need to be carried on their role in myogenesis.

In our study, we showed that lipin1 regulates MyoD and MEF2c expression through relieve of HDAC5 suppression from the nucleus and export to the cytoplasm. We showed that lipin1 catalytic activity is important in this myoblast differentiation and skeletal muscle development. Here, lipin1 activity leads to the phosphorylation of HDAC5 and resulting in its trafficking from the nucleus to the cytoplasm. For further confirmation if the catalytic activity of lipin1 is the ultimate regulator of HDAC5 nucleocytoplasmic trafficking, we deployed lipin1 inactive mutant for catalytic activity, D712A. It showed that lipin1 mutant did not lead to the nuclear export of HDAC5,

meaning that the catalytic activity of lipin1 is required for the HDAC5 nucleocytoplasmic trafficking. Furthermore, when we co-overexpressed PKCμ and HDAC5, instead of HDAC5 only, we observed that HDAC5 nuclear export in PKCμ co-overexpression compared to the HDAC5 alone. Our study also showed that treatment of PMA, a DAG agonist, led to the nuclear export of HDAC5, showing that DAG secondary signaling activity is responsible for the HDAC5 trafficking.

In our findings, we propose that lipin1 regulates the transcription de-repression of myogenic factors by regulating HDAC5 nucleocytoplasmic trafficking through DAG/PKC pathway. In early-undifferentiated myoblasts, HDAC5 is bound to MEF2c through the HDAC-interacting domain to repress the MEF2c activity to block myoblast differentiation. During myoblast differentiation and skeletal muscle development, MEF2c is relieved from repression by signaling cascade that leads to the phosphorylation of HDAC5 and its translocation to the cytoplasm, thus allowing the MEF2c to be activated.

In addition to the reduced expression of myogenic factors such as MyoD and MEF2c, we observed an increase of Pax7 expression in Lipin1^{Myf5cKO} mice at the protein level. This increase of Pax7 due to lipin1 deficiency leads us to conclude that the reduction of MyoD and MEF2c factors play a role. Pax7 expression is induced in satellite cells and this observed increase of Pax7 is propelling these cells for self-renewal instead of progressing to the next stage of becoming myoblasts and eventual myogenic differentiation. Some studies suggest that myogenic factors such as MyoD regulate the expression of Pax7 (Sincennes et al., 2016; Wood et al., 2013). This phenotype is consistent with a previous study (P. Zhang, Verity, & Reue, 2014) in that lipin1 deficient *fld* mice showed necrosis as well as persistent muscle fiber regeneration.

In summary, our study has identified that lipin1 plays an important role in regulating skeletal muscle development through the activation of PKCμ and PKCα isoforms that leads to the signaling cascade that activates the myogenic factors that are essential for myogenesis by relieving the repressive activity of HDAC5.

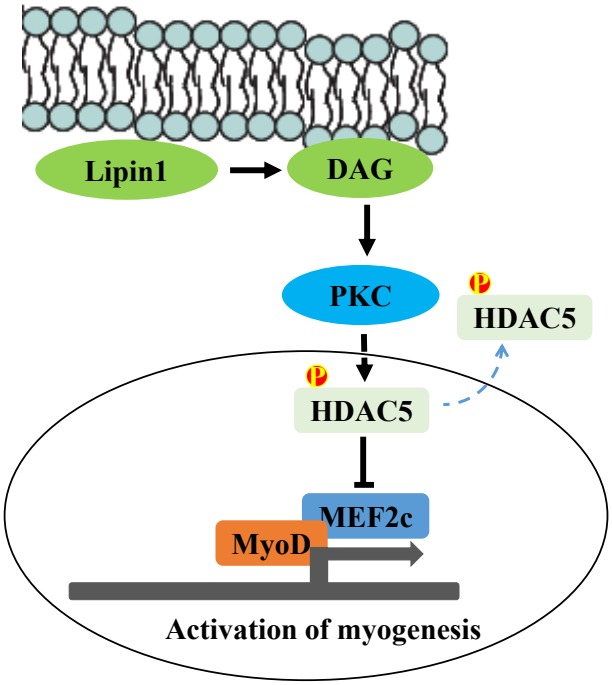

Figure 12*: Lipin1 is critical for embryonic SM differentiation.*

V. References

Asfour, H. A., Allouh, M. Z., & Said, R. S. (2018). Myogenic regulatory factors: The orchestrators of myogenesis after 30 years of discovery. *Exp Biol Med (Maywood), 243*(2), 118-128. doi:10.1177/1535370217749494

Bentzinger, C. F., Wang, Y. X., & Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harb Perspect Biol, 4*(2). doi:10.1101/cshperspect.a008342

Bertos, N. R., Wang, A. H., & Yang, X. J. (2001). Class II histone deacetylases: structure, function, and regulation. *Biochem Cell Biol, 79*(3), 243-252.

Black, B. L., Molkentin, J. D., & Olson, E. N. (1998). Multiple roles for the MyoD basic region in transmission of transcriptional activation signals and interaction with MEF2. *Mol Cell Biol, 18*(1), 69-77.

Bour, B. A., O'Brien, M. A., Lockwood, W. L., Goldstein, E. S., Bodmer, R., Taghert, P. H., . . . Nguyen, H. T. (1995). Drosophila MEF2, a transcription factor that is essential for myogenesis. *Genes Dev, 9*(6), 730-741.

Brose, N., Betz, A., & Wegmeyer, H. (2004). Divergent and convergent signaling by the diacylglycerol second messenger pathway in mammals. *Curr Opin Neurobiol, 14*(3), 328-340. doi:10.1016/j.conb.2004.05.006

Brunelli, S., Relaix, F., Baesso, S., Buckingham, M., & Cossu, G. (2007). Beta catenin-independent activation of MyoD in presomitic mesoderm requires PKC and depends on Pax3 transcriptional activity. *Dev Biol, 304*(2), 604-614. doi:10.1016/j.ydbio.2007.01.006

Chen, J. F., Tao, Y., Li, J., Deng, Z., Yan, Z., Xiao, X., & Wang, D. Z. (2010). microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. *J Cell Biol, 190*(5), 867-879. doi:10.1083/jcb.200911036

Chen, Y., Rui, B. B., Tang, L. Y., & Hu, C. M. (2015). Lipin family proteins--key regulators in lipid metabolism. *Ann Nutr Metab, 66*(1), 10-18. doi:10.1159/000368661

Csaki, L. S., Dwyer, J. R., Fong, L. G., Tontonoz, P., Young, S. G., & Reue, K. (2013). Lipins, lipinopathies, and the modulation of cellular lipid storage and signaling. *Prog Lipid Res, 52*(3), 305-316. doi:10.1016/j.plipres.2013.04.001

Danoviz, M. E., & Yablonka-Reuveni, Z. (2012). Skeletal muscle satellite cells: background and methods for isolation and analysis in a primary culture system. *Methods Mol Biol, 798*, 21-52. doi:10.1007/978-1-61779-343-1_2

Dokmanovic, M., Clarke, C., & Marks, P. A. (2007). Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res, 5*(10), 981-989. doi:10.1158/1541-7786.MCR-07-0324

Donkor, J., Sariahmetoglu, M., Dewald, J., Brindley, D. N., & Reue, K. (2007). Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns. *J Biol Chem, 282*(6), 3450-3457. doi:10.1074/jbc.M610745200

Eichmann, T. O., & Lass, A. (2015). DAG tales: the multiple faces of diacylglycerol--stereochemistry, metabolism, and signaling. *Cell Mol Life Sci, 72*(20), 3931-3952. doi:10.1007/s00018-015-1982-3

Endo, T. (2015). Molecular mechanisms of skeletal muscle development, regeneration, and osteogenic conversion. *Bone, 80*, 2-13. doi:10.1016/j.bone.2015.02.028

Finck, B. N., Gropler, M. C., Chen, Z., Leone, T. C., Croce, M. A., Harris, T. E., . . . Kelly, D. P. (2006). Lipin 1 is an inducible amplifier of the hepatic PGC-1alpha/PPARalpha regulatory pathway. *Cell Metab, 4*(3), 199-210. doi:10.1016/j.cmet.2006.08.005

Fischle, W., Emiliani, S., Hendzel, M. J., Nagase, T., Nomura, N., Voelter, W., & Verdin, E. (1999). A new family of human histone deacetylases related to Saccharomyces cerevisiae HDA1p. *J Biol Chem, 274*(17), 11713-11720.

Harris, T. E., Huffman, T. A., Chi, A., Shabanowitz, J., Hunt, D. F., Kumar, A., & Lawrence, J. C., Jr. (2007). Insulin controls subcellular localization and multisite phosphorylation of the phosphatidic acid phosphatase, lipin 1. *J Biol Chem, 282*(1), 277-286. doi:10.1074/jbc.M609537200

Iizuka, K., Machida, T., & Hirafuji, M. (2014). Skeletal muscle is an endocrine organ. *J Pharmacol Sci, 125*(2), 125-131.

Jiang, W., Zhu, J., Zhuang, X., Zhang, X., Luo, T., Esser, K. A., & Ren, H. (2015). Lipin1 Regulates Skeletal Muscle Differentiation through Extracellular Signal-regulated Kinase (ERK) Activation and Cyclin D Complex-regulated Cell Cycle Withdrawal. *J Biol Chem, 290*(39), 23646-23655. doi:10.1074/jbc.M115.686519

Kaushal, S., Schneider, J. W., Nadal-Ginard, B., & Mahdavi, V. (1994). Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. *Science, 266*(5188), 1236-1240.

Kim, H. E., Bae, E., Jeong, D. Y., Kim, M. J., Jin, W. J., Park, S. W., . . . Kim, K. S. (2013). Lipin1 regulates PPARgamma transcriptional activity. *Biochem J, 453*(1), 49-60. doi:10.1042/BJ20121598

Kleger, A., Loebnitz, C., Pusapati, G. V., Armacki, M., Muller, M., Tumpel, S., . . . Seufferlein, T. (2011). Protein kinase D2 is an essential regulator of murine myoblast differentiation. *PLoS One, 6*(1), e14599. doi:10.1371/journal.pone.0014599

Liu, N., Williams, A. H., Kim, Y., McAnally, J., Bezprozvannaya, S., Sutherland, L. B., . . . Olson, E. N. (2007). An intragenic MEF2-dependent enhancer directs muscle-specific expression of microRNAs 1 and 133. *Proc Natl Acad Sci U S A, 104*(52), 20844-20849. doi:10.1073/pnas.0710558105

Lu, J., McKinsey, T. A., Nicol, R. L., & Olson, E. N. (2000). Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. *Proc Natl Acad Sci U S A, 97*(8), 4070-4075. doi:10.1073/pnas.080064097

Lu, J., McKinsey, T. A., Zhang, C. L., & Olson, E. N. (2000). Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol Cell, 6*(2), 233-244.

Maguire, R. J., Isaacs, H. V., & Pownall, M. E. (2012). Early transcriptional targets of MyoD link myogenesis and somitogenesis. *Dev Biol, 371*(2), 256-268. doi:10.1016/j.ydbio.2012.08.027

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol, 9*, 493-495.

McKinsey, T. A., Zhang, C. L., Lu, J., & Olson, E. N. (2000). Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature, 408*(6808), 106-111. doi:10.1038/35040593

McKinsey, T. A., Zhang, C. L., & Olson, E. N. (2000). Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. *Proc Natl Acad Sci U S A, 97*(26), 14400-14405. doi:10.1073/pnas.260501497

Michot, C., Hubert, L., Romero, N. B., Gouda, A., Mamoune, A., Mathew, S., . . . de Lonlay, P. (2012). Study of LPIN1, LPIN2 and LPIN3 in rhabdomyolysis and exercise-induced myalgia. *J Inherit Metab Dis, 35*(6), 1119-1128. doi:10.1007/s10545-012-9461-6

Molkentin, J. D., Black, B. L., Martin, J. F., & Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell, 83*(7), 1125-1136.

Nadra, K., de Preux Charles, A. S., Medard, J. J., Hendriks, W. T., Han, G. S., Gres, S., . . . Chrast, R. (2008). Phosphatidic acid mediates demyelination in Lpin1 mutant mice. *Genes Dev, 22*(12), 1647-1661. doi:10.1101/gad.1638008

Naidu, P. S., Ludolph, D. C., To, R. Q., Hinterberger, T. J., & Konieczny, S. F. (1995). Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. *Mol Cell Biol, 15*(5), 2707-2718.

Newton, A. C. (2001). Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev, 101*(8), 2353-2364.

Peterfy, M., Phan, J., Xu, P., & Reue, K. (2001). Lipodystrophy in the fld mouse results from mutation of a new gene encoding a nuclear protein, lipin. *Nat Genet, 27*(1), 121-124. doi:10.1038/83685

Peterson, T. R., Sengupta, S. S., Harris, T. E., Carmack, A. E., Kang, S. A., Balderas, E., . . . Sabatini, D. M. (2011). mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. *Cell, 146*(3), 408-420. doi:10.1016/j.cell.2011.06.034

Potthoff, M. J., & Olson, E. N. (2007). MEF2: a central regulator of diverse developmental programs. *Development, 134*(23), 4131-4140. doi:10.1242/dev.008367

Qi, Y., Sun, L., & Yang, H. (2017). Lipid droplet growth and adipocyte development: mechanistically distinct processes connected by phospholipids. *Biochim Biophys Acta, 1862*(10 Pt B), 1273-1283. doi:10.1016/j.bbalip.2017.06.016

Ren, H., Federico, L., Huang, H., Sunkara, M., Drennan, T., Frohman, M. A., . . . Morris, A. J. (2010). A phosphatidic acid binding/nuclear localization motif determines lipin1 function in lipid metabolism and adipogenesis. *Mol Biol Cell, 21*(18), 3171-3181. doi:10.1091/mbc.E10-01-0073

Reynolds, N., O'Shaughnessy, A., & Hendrich, B. (2013). Transcriptional repressors: multifaceted regulators of gene expression. *Development, 140*(3), 505-512. doi:10.1242/dev.083105

Sambasivan, R., & Tajbakhsh, S. (2015). Adult skeletal muscle stem cells. *Results Probl Cell Differ, 56*, 191-213. doi:10.1007/978-3-662-44608-9_9

Sauret, J. M., Marinides, G., & Wang, G. K. (2002). Rhabdomyolysis. *Am Fam Physician, 65*(5), 907-912.

Sincennes, M. C., Brun, C. E., & Rudnicki, M. A. (2016). Concise Review: Epigenetic Regulation of Myogenesis in Health and Disease. *Stem Cells Transl Med, 5*(3), 282-290. doi:10.5966/sctm.2015-0266

Tapscott, S. J. (2005). The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. *Development, 132*(12), 2685-2695. doi:10.1242/dev.01874

Tessarz, P., & Kouzarides, T. (2014). Histone core modifications regulating nucleosome structure and dynamics. *Nat Rev Mol Cell Biol, 15*(11), 703-708. doi:10.1038/nrm3890

Veenstra, J., Smit, W. M., Krediet, R. T., & Arisz, L. (1994). Relationship between elevated creatine phosphokinase and the clinical spectrum of rhabdomyolysis. *Nephrol Dial Transplant, 9*(6), 637-641.

von Maltzahn, J., Jones, A. E., Parks, R. J., & Rudnicki, M. A. (2013). Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. *Proc Natl Acad Sci U S A, 110*(41), 16474-16479. doi:10.1073/pnas.1307680110

Wang, D. Z., Valdez, M. R., McAnally, J., Richardson, J., & Olson, E. N. (2001). The Mef2c gene is a direct transcriptional target of myogenic bHLH and MEF2 proteins during skeletal muscle development. *Development, 128*(22), 4623-4633.

Wang, H., Airola, M. V., & Reue, K. (2017). How lipid droplets "TAG" along: Glycerolipid synthetic enzymes and lipid storage. *Biochim Biophys Acta, 1862*(10 Pt B), 1131-1145. doi:10.1016/j.bbalip.2017.06.010

Wang, Q. J. (2006). PKD at the crossroads of DAG and PKC signaling. *Trends Pharmacol Sci, 27*(6), 317-323. doi:10.1016/j.tips.2006.04.003

Wood, W. M., Etemad, S., Yamamoto, M., & Goldhamer, D. J. (2013). MyoD-expressing progenitors are essential for skeletal myogenesis and satellite cell development. *Dev Biol, 384*(1), 114-127. doi:10.1016/j.ydbio.2013.09.012

Zhang, P., Verity, M. A., & Reue, K. (2014). Lipin-1 regulates autophagy clearance and intersects with statin drug effects in skeletal muscle. *Cell Metab, 20*(2), 267-279. doi:10.1016/j.cmet.2014.05.003

Zhang, W., Behringer, R. R., & Olson, E. N. (1995). Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. *Genes Dev, 9*(11), 1388-1399.

Zutt, R., van der Kooi, A. J., Linthorst, G. E., Wanders, R. J., & de Visser, M. (2014). Rhabdomyolysis: review of the literature. *Neuromuscul Disord, 24*(8), 651-659. doi:10.1016/j.nmd.2014.05.005

Zutt, R., van der Kooi, A. J., Linthorst, G. E., Wanders, R. J., Verschuuren, J. J., & de Visser, M. (2010). [Recurrent rhabdomyolysis: screening for underlying disease]. *Ned Tijdschr Geneeskd, 154*, A2290.