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Exploring the Role of lipin1 in Mitophagy Process Using lipin1 Deficient-EGFP Tagged lC3 Transgenic Mice

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Exploring the role of lipin1 in mitophagy process using lipin1 deficient-EGFP tagged LC3 transgenic mice

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Abdullah Ali Alshudukhi ENTITLED Exploring the role of lipin1 in mitophagy process using lipin1 deficient-EGFP tagged LC3 transgenic mice BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

Alshudukhi, Abdullah Ali. M.S. Department of Biochemistry and Molecular Biology, Wright State University, 2017. Exploring the role of lipin1 in mitophagy process using lipin1 deficient-EGFP tagged LC3 transgenic mice

Lipin1 (phosphatidic acid phosphatase) is a key molecule in the cells with two functions: first, it converts phosphatidic acid into diacylglycerol in the cytosol which in turn makes triglycerides. Second, in nucleus lipin1 acts as a transcriptional factor which regulates the expression of genes involved in the fatty acid oxidation and lipid metabolism. Clinically, *Lpin1* gene mutations have been detected in patients with severe rhabdomyolysis accompanied with aggregated and dysfunctional mitochondria in their type II muscle fiber. Previously, we have observed that mice with lipin1 deficiency had aggregated mitochondria and abnormal autophagosomes formations by electron microscopy. The mechanism underlying the mitochondrial alterations observed in rhabdomyolysis patients is not revealed yet. Therefore, we think there is a link between impaired mitophagy and lipin1 deficiency in skeletal muscle. Mitochondrial quality control is responsible for regulating the turnover of the mitochondria within the cells. Mitochondrial autophagy (mitophagy) is a part of the mitochondrial quality control and considered as a key process within the eukaryotes. Dysregulated mitophagy has been shown to be involved in Parkinson's disease, cardiovascular diseases and neurodegenerative disorders. In this project we want to examine the role of lipin1 deficiency on the impaired mitophagy process in the muscular tissue. We generated a unique mice model called lipin1$^{-/-}$-EGFP-LC3 transgenic mice to study the mitophagy process. This unique model expresses GFPs tagged to autophagosomes in mitophagy.

After analyzing the tissue specimens from the mice we observed increased autophagosomes vacuoles in the lipin1$^{-/-}$ -EGFP-LC3 transgenic mice in the glycolytic muscles fibers compared to the wild type (WT). These autophagosomes represent mitochondrial autophagy. The mitophagy in this context is mediated by Bnip3 (a mitochondrial damage marker) as we found it to be significantly upregulated in the lipin1$^{-/-}$ -EGFP-LC3 transgenic mice. We further treated the mice animals, WT and lipin1$^{-/-}$ -EGFP-LC3 transgenic mice, with chloroquine diphosphate (CQ) which act as autophagy inhibitor and cyclosporine A (CsA), which has been commonly used to improve mitochondrial membrane potential. CQ treatment inhibited the mitophagy and resulted in increased accumulation of LC3 (an autophagy marker) and Bnip3 in the both WT and lipin1$^{-/-}$ -EGFP-LC3 transgenic mice. CsA treatment was effective in increasing the mitochondrial membrane potential which led to decrease expression of the LC3 and Bnip3 in the cytosol of both WT and lipin1$^{-/-}$ -EGFP-LC3 transgenic mice. Lipin1 deficiency upregulated AMP-activated protein kinase (AMPK) in fasting status through a stress produced by nutrients imbalance. AMPK activates FOXO3 (a transcription factor belongs to the forkhead box O-3 family) which has been shown to increase transcription of LC3 and Bnip3 genes. We found that the gene expressions of the LC3 and Bnip3 of lipin1$^{-/-}$ -EGFP-LC3 transgenic mice were slightly increased compared to WT suggesting that the accumulated proteins in the cytoplasm was partially due to increased transcription activity of these molecules. Also, we do not rule out that the increased protein expression in the cytosol is caused by blockage in the mitophagy process as well. Based on these findings, lipin1 deficiency may contribute to the impaired mitophagy in the lipin1$^{-/-}$ -EGFP-LC3 transgenic mice which leads to muscle weakness and atrophy.

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I- Introduction

i- Autophagy

Eukaryotic cells are programmed to function in a concise manner. It keeps the cells performing properly and eliminating any harmful molecules that affect their survival. In the cells there is a system that is responsible for degrading and recycling the cytoplasmic proteins and organelles called autophagy. Autophagy is known as the engulfment process of dysfunctional proteins and defective organelles and subsequently degrade the components by lysosomes and recycle them for the cellular use (Cuervo, 2004). The recycled materials would provide amino acids and energy ready for cellular consumption. Autophagy is in charge of eliminating and breaking down long-lived and impaired proteins as well as organelles. Thus, autophagy is crucial for cellular integrity to ensure a healthy environment within the cytosol and minimize the defects.

Alterations in autophagy function lead to numerous diseases and disorders. Aggregated proteins and increased number of impaired organelles are going to negatively affect the cellular functions leading to cellular damage and death. Impaired autophagy has been linked to the induction of several diseases, for instance cancer, autoimmune diseases, and neurodegenerative diseases (Cuervo, 2004). For instance, Beclin 1, is a mammalian autophagy gene which inhibits cancer induction, was greatly reduced in human breast carcinoma (Liang et al., 1999). Another paper also revealed that genetic mutations occurred in Ultraviolet (UV) radiation resistance–associated gene (UVRAG) (an autophagy inducing gene) reduced the autophagy activity in gastric

carcinoma (Kim et al., 2008). Frameshift mutations in the polyadenine tract were developed in the UVRAG led to microsatellite instability as the paper reported. Autophagy seems to play a critical role in preventing cancer emerging, therefore any defect in autophagy proteins would suppress the defense mechanism and enhances the diseases emerging.

i.1 Important Biomarkers/pathway in autophagy

Balancing between protein biosynthesis and degradation is essential for cellular homeostasis and this task can be done by autophagy. One of the important molecule of autophagy is microtubule-associated protein 1 light chain 3 (LC3). LC3 associates with the phagophore (autophagosome) membrane allowing the cytoplasmic components to be engulfed and degraded (Kabeya et al., 2000). This molecule expressed in the cytosol is in two forms, LC3-I as unbound form in the cytosol. The other form is LC3-II, which is expressed on the autophagosomes membrane, is made by incorporating phosphatidylethanolamine (PE) to LC3-I which allows LC3-II to bind to the autophagosomes membrane (Kabeya et al., 2000). The function of LC3 protein is to interact with other autophagy adaptors which are bound to the defective material to complete the engulfment process. In monitoring autophagy, LC3 has been used as a marker to study the autophagy either by tissue immunostaining or via measuring the protein level of the LC3 (Yoshii & Mizushima, 2017). The amount of the LC3-II proteins represents the abundance of autophagosomes (Kabeya et al., 2000). Thus, using LC3 as a tool to study the autophagy/mitophagy would facilitate the process and provide a precise criteria to evaluate the engulfment process.

There are other autophagy molecules which interact with cellular organelles and are essential for their autophagic engulfment such as p62/SQSTM1. This molecule is also named as the autophagy adaptor and is required in the interaction between LC3 and engulfed cargo. P62 directly interacts with LC3 and facilitates the engulfment and the aggregation of the defective proteins (Pankiv et al., 2007). Once the materials are engulfed, the p62 will be degraded as well as the engulfed components in the autophagosomes by lysosomal enzymes.

Autophagosomes are double membrane structures that are created in the cytosol to engulf defective molecules and organelles and deliver them to lysosomes for degradation (Mizushima, 2004). This process of making autophagosomes required activation and recruitment of several autophagy proteins. To activate the autophagy machinery, the cells first produce a damage-induction signal which suppresses the activity of the mammalian target of rapamycin (mTORC1). The downregulated mTORC1 allows the activation of Unc-51-like kinase 1 (ULK1) complex (an autophagy related molecule) which in turn binds to phosphatidylinositol 3-kinase (PI3K) complex on the Endoplasmic Reticulum (ER) membrane. Once these complexes are translocated to the ER membrane they generate a structure called pre-autophagosomal structure (PAS) with involvement of other molecules like vacuole membrane protein 1 (VMP1) and Atg9. After that, the ER membrane undergoes some changes which resulted in formation of omegasome as a start of the isolation membrane creation. The PI3K recruits its effectors to the ER membrane which leads to increase production of phosphatidylinositol 3-phosphate (PI3P) at that specific site. Generation of local PI3P may help in building the isolation membrane. Lastly, Atg16L recruits LC3 and perform LC3 lipidation by

incorporating phosphatidylethanolamine (PE) which allows LC3 to bind to the autophagosome membrane. Finally, the isolation membrane expanded and then released form the ER membrane to continue the autophagy process. This was a brief explanation of the autophagosome formation as it described in more details in this review (Mizushima, Yoshimori, & Ohsumi, 2011).

ii- Mitophagy

Dysfunctional mitochondria can be cleared through autophagy process. This selective autophagy of mitochondrial is also known as mitophagy. The mitophagy process is responsible for removing damaged mitochondria and delivering them to lysosomes for degradation (Yoshii & Mizushima, 2015). Mitochondrion is one of the vital organelles within the cells. It's involved in several significant and cell's life-dependent biological processes, such as, energy production, metabolism, signaling, apoptosis and cell differentiation. Therefore, due to multiple mitochondrial functions, the mitochondria may develop several types of damage with time during their life (Gomes & Scorrano, 2013). Once this happens, the cells detect intracellular signals that show where the damaged parts are and start to eliminate that from the cytosol. Mitochondrial autophagy (mitophagy) is a mechanism where the cells use to repair or eliminate defects. This occurs through the mitochondrial expression of damage markers on the outer membrane in order to be recognized then engulfed and removed from the cytosol (Yoshii & Mizushima, 2015). Mitochondrial autophagy is essential for mitochondrial network and maintains good members within the cellular community.

The mitophagy is significantly required for the cell's functioning and survival and preventing development of diseases. Misfolded protein, reactive oxygen species (ROS)

species and defective mitochondrial components cause mitochondrial damages. Eventually, these damages would negatively influence the cells and impair their functions. For a larger picture, these mitochondrial abnormalities would affect the organs functions and cause several diseases such as Parkinson's disease, cardiovascular diseases and neurodegenerative disorders (Kanki, Furukawa, & Yamashita, 2015; Redmann, Dodson, Boyer-Guittaut, Darley-Usmar, & Zhang, 2014). This shows how important the mitophagy is in the cells integrity and physiology. Clearing up ROS and defective mitochondria result in maintained mitochondrial functions and cellular homeostasis. The mitochondrial autophagy is capable of recycling the waste products and damaged mitochondria in order to keep the cell's functions and existence.

iii- Biomarkers/Pathways for Mitophagy

The activation of mitophagy is regulated by numerus molecules and proteins. Upon damage to mitochondrion, the organelle expresses proteins and markers on its surface. The role of the damage markers is to decorate defective mitochondria for the recognition by the eliminating system, mitophagy. There are several known markers for damage expressed on the mitochondria. For instance but not limited to, PTEN-induced putative kinase 1 (PINK1), NIP3-like protein X (NIX), Bcl-2/adenovirus E1B 19-kDa-interacting protein 3 (Bnip3), and Parkin (Yoshii & Mizushima, 2015). Basically, these markers get expressed on the outer membrane of the mitochondria and allow their interaction with LC3 directly or via p62 (a general autophagy adaptor) protein to complete the engulfment by the phagophores. Therefore, the mitochondrial damage markers are highly important in the process of recognizing impaired mitochondria and recruiting the autophagy machinery to eliminate them from cytoplasm.

Bnip3 is considered as one of the main mitophagy pathways by which mitochondria get recognized and isolated from the healthy mitochondrial network. Sequestration of mitochondria in a double membrane requires these damage molecules to interact either directly with LC3 or through p62. Bnip3 has the ability to directly bind to LC3 as a mitophagy adaptor (Hanna et al., 2012). This interaction occurs through the LC3 interacting region (LIR) which consists of a WXXL motif in the Bnip3 protein. Thus, Bnip3 is a key molecule in the mitochondrial autophagy since it acts as a damage marker and as a mitophagy adaptor. Another mitophagy marker is Parkin, this molecule translocates from cytoplasm to the dysfunctional mitochondria to tag them for mitophagy. Once Parkin is bound to the mitochondria, it enhances the mitophagic machinery recognition of the dysfunctional organelle (Narendra, Tanaka, Suen, & Youle, 2008). This interaction occurs through recruitment of p62 to facilitate the engulfment by autophagosome (Matsuda et al., 2010). Molecules of mitophagy are either recruited to the mitochondria or expressed from the internal of mitochondria on the surface. The significance of these markers is based on their interactions with autophagic machinery.

iv- Mitochondrial membrane potential and mitochondrial quality control

Alteration in mitochondrial membrane potential leads to initiation of autophagy. One of the important criterion that associates with mitochondrial activity is mitochondrial membrane potential. Mitochondrial membrane potential has been used as a representative of the mitochondrial functionality (Brand & Nicholls, 2011). The H^+ proton gradient across the inner mitochondria membrane creates the membrane potential in the mitochondria. These protons pass the inner membrane through the ATP synthase complex to make ATP via oxidative phosphorylation and provide energy for the cell

(Saraste, 1999). This is one of the major functions of the mitochondria. In case of abnormality, hyperpolarization (increased membrane potential) of the mitochondria produces ROSs which are known to affect the cellular molecules and functions. Increased mitochondrial membrane potential has been linked to disease development such as Alzheimer's, cancer and diabetes (Huttemann et al., 2008). On the other hand, depolarization (decreased membrane potential) of the mitochondrial membrane leads to cellular damage and impaired mitochondrial function, which eventually leads to inability to produce energy to meet cellular needs. Altering physiology of mitochondrial membrane potential has also been associated to negatively influence the mitochondrial performance in particular. Several reports have shown that impaired mitochondrial membrane potential disrupt the fission/fusions events of the mitochondria and furthermore influence mitochondrial quality control and the mitophagy process (Matsuda et al., 2010; Twig et al., 2008).

The mitochondrial quality control regulates the mitochondrial existence, energy generation and damage repair. The mitochondrial quality control involves fusion and fissions events to control the mitochondrial network and provide more efficient functioning organelles (Romanello & Sandri, 2015). Fusion for instance, allows the fused mitochondria to exchange their metabolites, proteins and mtDNA. Eventually, they increase the amount of energy they produce and increase the performance of the mitochondrial functions in general. In addition, the fusion also offers a great feature where it buffers the damage when two mitochondria fuse together and allow them to repair that damage (Twig et al., 2008). On the contrary, fission does the opposite where it segregates the impaired or defective mitochondria from the network and degrade them

through the mitophagy process (Figure 1). The mitochondrial quality control ensures the balance between fusion and fission in order to maintain a healthy mitochondrial network.

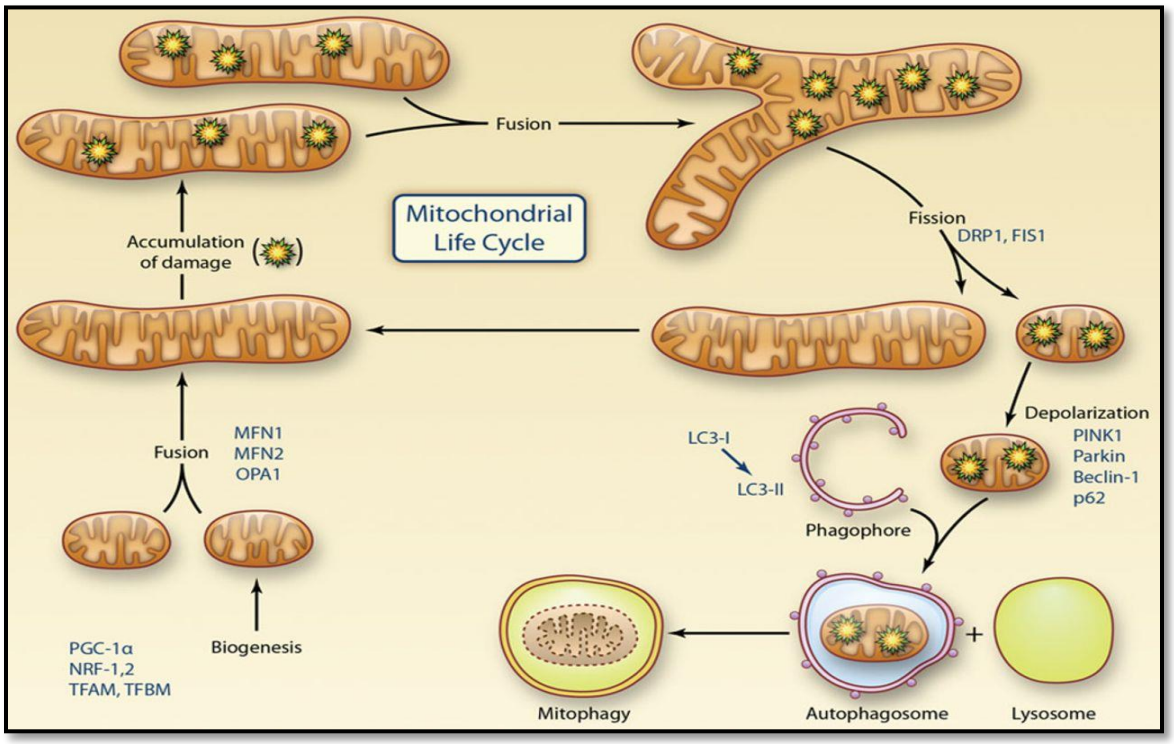


Figure 1 : Schematic diagram for the Mitochondria quality control cycle (Kluge, Fetterman, & Vita, 2013).

Many studies have revealed that alteration in the mitochondrial quality control caused several serious illness. Cai and Tammineni (Cai & Tammineni, 2016) have showed that disturbing the mitochondrial quality control led to defects associated with Alzheimer's disease. Abnormal events in the mitochondrial quality control such as aggregated mitochondria within axons was observed in mice with mutated Mfn2 (Detmer, Vande Velde, Cleveland, & Chan, 2008). Mfn2 is one of the mammalian

mitochondrial fusion-related proteins that is required for the outer mitochondrial membrane fusion process. With many key cellular functions governed by mitochondria, imbalanced mitochondrial quality control has the potential to adversely affect the cellular activities and eventually contributes to diseases onset and progression.

v- Mitochondrial membrane Lipids and mitochondrial quality controls

Mitochondrial membrane lipid composition affects mitophagy. Cardiolipin is one of the unique lipids within the cells associated with mitochondria. It's localized in the inner mitochondrial membrane and contributes to several functions such as energy metabolism and mitophagy. Recently, it has been shown that cardiolipin interacts with LC3 protein to enhance the engulfment of the mitochondria by autophagosomes (Chu et al., 2013). Moreover, cardiolipin involved in mitochondrial membrane remodeling which is required for the membrane fusion process (Cullis, Hope, & Tilcock, 1986). In fusion events, cardiolipin contributes to the regulation of mitochondrial fusion by modifying the morphology of mitochondria (Ban et al., 2017). However, depending on peroxidation cardiolipin can either activate mitophagy or apoptosis in the cells (Ren, Phoon, & Schlame, 2014). Moreover, the mitochondrial membrane lipids contain phospholipids such as phosphatidic acid (PA) and diacylglycerol (DAG). The PA has been shown to regulate the mitochondria aggregation and fusion processes (H. Chen et al., 2003; Ha & Frohman, 2014). The DAG lipid enhances the mitochondrial fission. It has been suggested that DAG may recruit proteins that initiate fission process (Ha & Frohman, 2014). Lipin1 (phosphatidic acid phosphatase) regulates mitochondrial fission and fusion events by converting PA into DAG on the mitochondrial surface and then participates in

the regulation of these events (Ha & Frohman, 2014; Huang et al., 2011). Taken together, lipids of mitochondrial membrane are essential for the organelle activity. Changes in mitochondrial membrane lipid composition can significantly affect the mitophagy and mitochondrial quality control.

v.1 Lipin1

Lipid metabolism is regulated by proteins that govern synthesis, transporting and elimination of lipids. One of the protein groups that regulate lipid metabolism is Lipin proteins. Lipin proteins family are a group of phosphatases that regulate the expressions of several genes that involve in the fatty acid oxidation and lipid metabolism. The lipins also play a significant role in the biosynthesis of the fats and regulate their levels within the cytoplasm (Hu, 2015). This family includes lipin1, lipin2 and lipin3, in which lipin1 is well known and studied (Michot et al., 2012). However, the other two lipins are less known and poorly studied (Y. Chen, Rui, Tang, & Hu, 2015). The lipin1 molecule participates in the synthesis of the phospholipids and triglycerides (TGs). The TGs are the most predominant and largest types of lipids in the cellular system. It is known to be the storage form of the fats in the biological cells. Adipocytes containing TGs are the energy house of the body and readily can be used to provide the body's energetic needs to perform its functions.

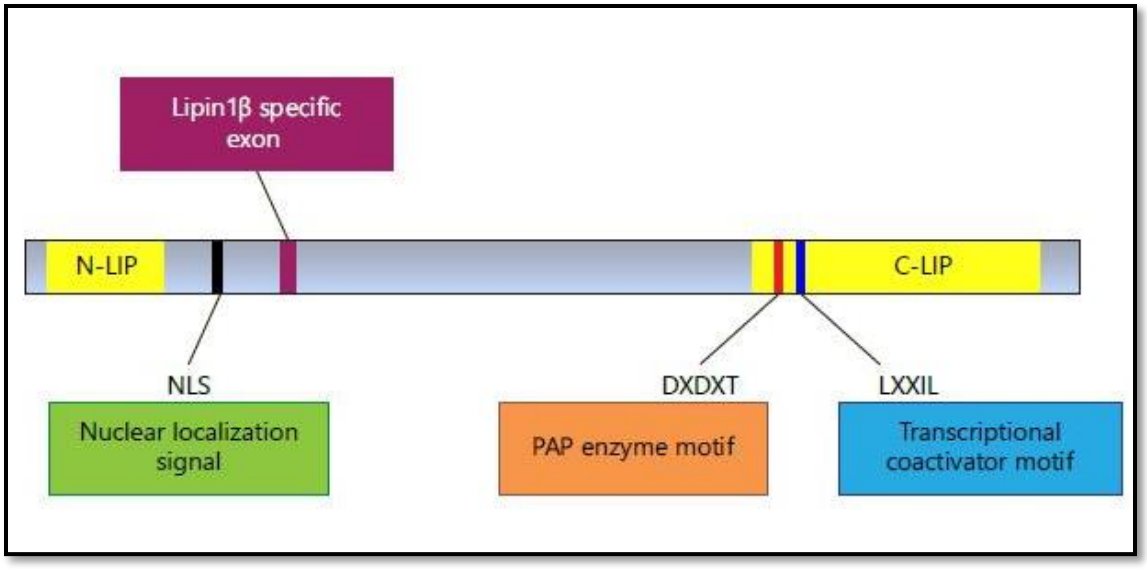


Figure 2: Lipin1 protein structure and motifs (Y. Chen et al., 2015).

Lipin 1 is a vital molecule within the cells with a dual functions. In the cytosol, lipin 1 acts as an enzyme with a dephosphorylation activity (Donkor et al., 2009). It does dephosphorylate phosphatidic acid (PA) to form diglyceride (DAG) which eventually generates triglycerides. Possessing a functional motif the haloacid dehalogenase (HAD)-like phosphatase motif (DXDXT) allows lipin1 to perform this function and bind to its substrates (Figure 2). The other function of lipin1is a nuclear function. Lipin1 molecule enters the nucleus then it acts as a transcriptional factor to facilitate the expression of genes required for the fatty acids oxidation (Figure 3) (Harris et al., 2007). Similar to the enzymatic activity motif, lipin1 has a nuclear receptor interaction motif (LXXIL) required for the binding to the complex involved in the lipogenesis and fatty acid oxidation genes expressions (Harris et al., 2007). Lipin1 contains a nuclear localization signal (NLS) which allows the traffic of the molecule inside the nucleus.

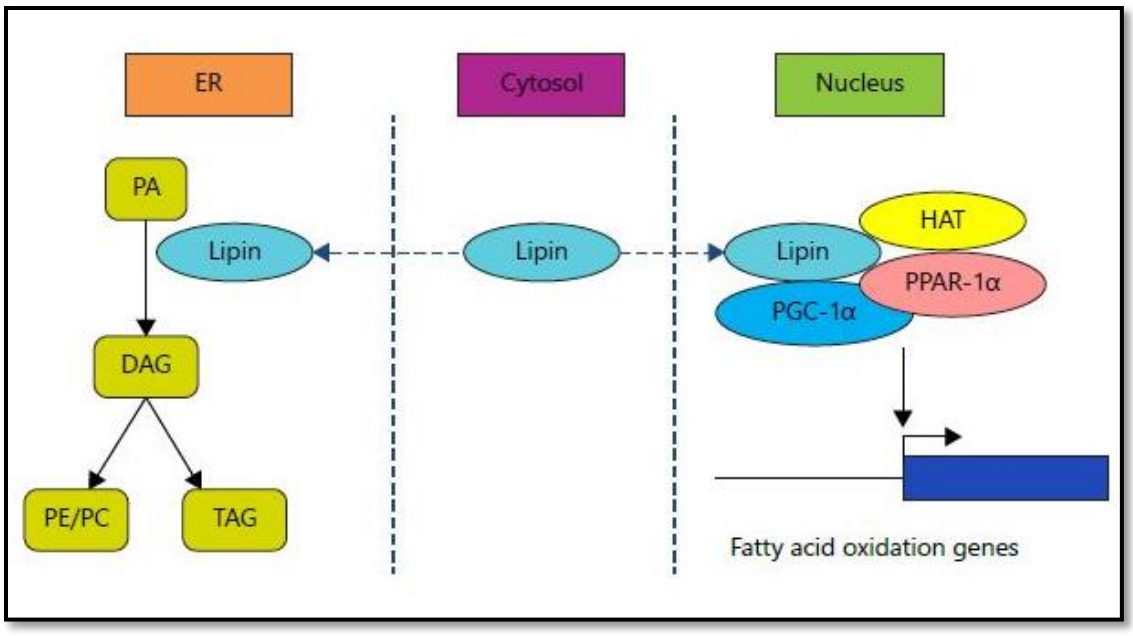


Figure 3: The nuclear and cytoplasmic functions of the Lipin1 protein (Y. Chen et al., 2015).

It has been shown that lipin1 activity is regulated by mTOR pathway. The master molecule, mTOR, phosphorylates lipin1 and promotes its localization in the cytosol (Peterson et al., 2011). Being in the cytosol allows lipin1 to act as enzyme for the conversion of PA into DAG. On the contrary, when mTOR is inactive, lipin1 is dephosphorylated and crosses the nuclear membrane and serves as a transcription factor which then participates in the genetic expression of molecules involved in the lipid metabolism and fatty acid oxidation as is illustrated in Figure 4.

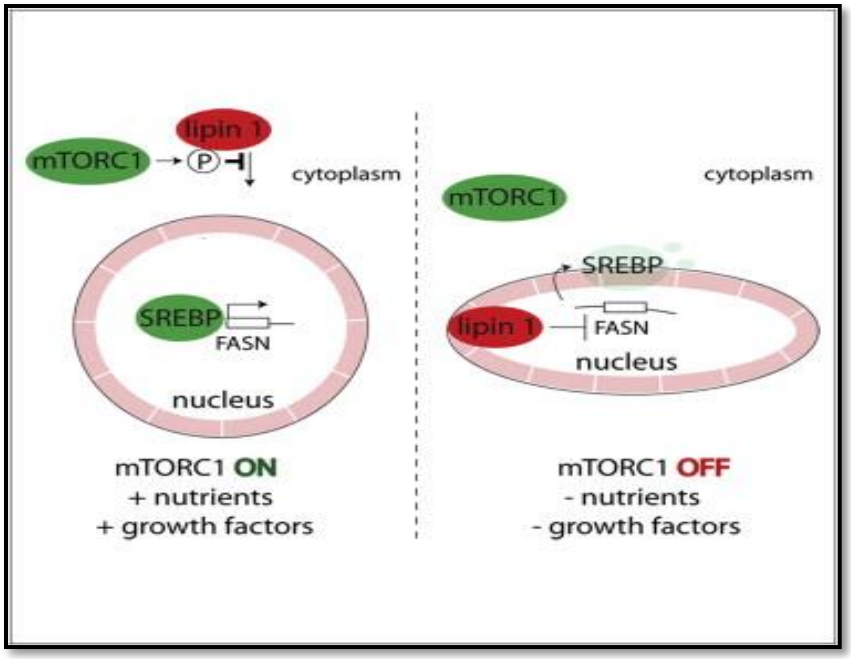


Figure 4: Schematic illustration of lipin1 molecular signaling pathway (Peterson et al., 2011).

Pathologically, lipin1 has been shown to develop genetic polymorphisms that affect glucose hemostasis. Peltonen and colleagues (Suviolahti et al., 2006) have tested some single nucleotide polymorphisms (SNPs) sites within the lipin1 gene and found that lipin1 mutations are involved in regulating insulin expression and lipids phenotypes. These studies revealed a crucial role of lipin1 in the pathogenesis and emerging of life-threatening diseases. Defective lipin1's functions and mutations have been linked to muscular abnormalities such as rhabdomyolysis, which is a breaking down of the muscular tissue leading to releasing its content into the blood stream (Zutt, van der Kooi, Linthorst, Wanders, & de Visser, 2014). A study reported that six deleterious mutations in the lipin1 gene were identified among a group children with severe rhabdomyolysis (Zeharia et al., 2008). A recent clinical study was done by

Michot et al (2012) revealed that patients with severe rhabdomyolysis had autosomal recessive mutations affecting the lipin1 gene. In the previous study, the investigators examined more than 170 patients with muscular symptoms and rhabdomyolysis and found that 18 subjects had two lipin1 mutations with severe rhabdomyolysis. This study also showed that the rhabdomyolysis patients presented mitochondrial aggregations accompanied with altered mitochondrial function in skeletal muscle. In a previous work we generated, fatty liver dystrophy (FLD) mice, which lack lipin1, showed mitochondrial aggregations and abnormal autophagosomes formations in the skeletal muscles. These abnormalities in the mitochondria is suggested to be caused by the defect in the mitochondrial clearance from the cytosol. Therefore, we suggested a role of lipin1 in the impaired mitophagy and mitochondrial function. This project is aimed to study the role of lipin1 in the defective mitophagy.

vi- Approaches to study autophagy/mitophagy

A unique mice model was established by Dr. Ren (2016) to study the role of lipin1 in mitophagy. Fatty liver dystrophy mice lineage (as described in the methodology section) was crossed with a mice model which was generated by Dr. Mizushima's lab (Mizushima & Kuma, 2008) that expresses green fluorescent protein along with Microtubule-associated protein light chain 3 (LC3). So, the new mice model is called Lipin1$^{-/-}$ EFGP-LC3 transgenic mice, which is used in this study to monitor the mitophagy in skeletal muscles. What is unique about these mice is they can express GFP whenever there was LC3 protein expressed on the surface of the autophagosomes. Detecting the GFP signals is used to monitor the mitophagy and its function through use

of muscular tissue sections and visualize them by immunofluorescent microscope (Mizushima & Kuma, 2008).

vii. EDL vs Soleus

In our study we chose the skeletal muscle tissue obtained from mice to investigate the role of lipin1 deficiency on mitophagy. Two types of muscles were collected from the mice in order to be studied and analyzed. The muscles are extensor digitorum longus (EDL) and soleus muscles obtained from the lower limbs of the mice from both genders. EDL is composed of fast-twitch type IIA and type IIB fibers mainly. While, the soleus muscle mostly consists of slow-twitch fibers and type 1 and type IIA (James, Altringham, & Goldspink, 1995). Moreover, the EDL has been described as glycolytic muscle fibers, while the soleus known as the oxidative muscle fibers based on their energy metabolic modes. The properties of these muscles helped us to study the mitophagy and mitochondrial function under different situations and stresses. The EDL specifically, has been shown to present much increase in autophagosomes accumulation with the starvation compared to soleus muscle (Howells, Jordan, & Mathews, 1978; Y. Wang & Pessin, 2013). Therefore, fasting would generate great and significant effects on the EDL muscle tissues and on the cellular metabolic regulation. On the protein degradation level, the EDL muscle has been reported to present a higher rate of the mitophagy process compared to the soleus (Y. Wang & Pessin, 2013; Yamada et al., 2012). EDL was a perfect model to study the mitophagy based on the differences in the muscle atrophy and autophagy activations pathways compared to soleus. For instance, upon fasting the autophagy seemed to be more pronounced in the EDL versus soleus (Howells et al., 1978). Soleus has a higher expression of Peroxisome proliferator-

activated receptor-γ coactivator-1 (PGC1α) which protects the oxidative muscle from atrophy (Yamada et al., 2012). In the oxidative fiber muscles, upregulated PGC1α reduces the activity of FOXO3 and then downregulates the autophagy. On the contrast, several pathways are known to control muscle mass by regulating protein degradation and autophagy in the glycolytic muscle fiber, such as Forkhead box O family (Foxo), transforming growth factor beta (TGFβ) family, autophagy inhibition, and nuclear factor kappaB (NF-κB) (Y. Wang & Pessin, 2013). FOXO3 has been implicated in muscle wasting via induction of autophagy process by increasing the transcription of autophagy-related genes such as LC3 and Bnip3. Knocking out autophagy essential genes, such as ATG5 in the EDL muscle presented a higher reduction in the autophagy process (Raben et al., 2008). Another mechanism which makes the EDL more likely to initiate autophagy during starvation than soleus is due to the role of Fyn protein (a proto-oncogene). Fyn has been shown to suppress autophagy through activation of mTORC1 and inhibition of AMPK. The mechanism underlying the difference between EDL and soleus is thought to be via Fyn activity which has been shown to downregulate Vps34 (an autophagy initiating molecule) (Yamada et al., 2012). During starvation in EDL, Fyn activity is decreased which then leads to upregulation of Vps34 and increased autophagy activity. However, in the soleus Fyn showed no significant change during fasting so Vps34 activity was still inhibited in soleus. Based on this information, the EDL muscle was the main focus to investigate in our study. The muscle fiber types and the physiology of the EDL muscle were the main reasons to select this tissue to understand the role of lipin1 deficiency in defective mitochondrial autophagy.

vii- Hypothesis and specific aims:

Mitophagy is crucial for cells existence and functionality. Dysfunctional mitophagy causes serious illness and disorders. For our knowledge, lipin1 serves as an enzyme converting PA to DAG, also acts as a transcriptional factor regulating genes expression in lipid metabolism. However, defective mitophagy in patients and mice model has been associated with lipin1 deficiency. Based on that, we are hypothesizing that lipin1 may has a role in impaired mitophagy process. Thus, in this project we are aiming to investigate the role of lipin1 deficiency in defective mitochondrial autophagy in skeletal muscles. To accomplish that, the following aims were proposed for this study: 1) To investigate how lipin1 deficiency affects mitophagy process; 2) to visualize the effect of lipin1 deficiency on mitophagy process using Lipin1$^{-/-}$-EGFP-LC3 transgenic mice; 3) to determine whether the improvement of mitochondrial membrane potential could ameliorate Lipin1-associated mitophagy defects.

II- Methods and Materials

i- Generation of Lipin1 -\-- EGFP-LC3 transgenic mice:

To generate the Lipin1 -\-- EGFP-LC3 transgenic mice we crossed Fatty liver Dystrophy (FLD) mice lineage (obtained from the Jackson Laboratory) with the Enhanced Green Fluorescent Protein –Microtubule Light Chain 3 (EGFP-LC3) tagged mice which was generated by Dr. Noboru Mizushima's lab (Mizushima, 2004). The mice genotyping was determined by obtaining mice tail snaps from new-born pups. These tails were digested by 0.5M EDTA solution (pH 8.0), Nuclei Lysis solution and Proteinase K mixture and incubated in water bath at 55C˚overnight. Afterword, the extraction steps were continued using isopropanol to clump and precipitate the DNA. 70% ethanol was used to wash the DNA and increase the DNA yield. Lastly, the extracted DNA was further used for PCR reactions to obtain the genotyping results of the mice using the following primers.

The FLD animals were genotyped by a PCR program which detects the lipin1 mutations, primers used are 5'-CCCTTGAGCACGTTCACA-3', 5'-GGTTGTGGGGACCCTGGA-3' and 5'-GCCTGCTGCAGATGCGTT-3'. The FLD program requires two reverse primers one for the wildtype and the other detects the mutated gene. EGFP gene was detected by a PCR program with the use of these primers 5'-CCTACGGCGTGCAGTGCTTCAGC-3' and 5'-CGGCGAGCTGCACGCTGCGTCCTC-3'. The DNA extraction protocol was obtained from this paper (Laird et al., 1991).

ii- Tissues collection:

Upon establishment of the Lipin1 -\-- EGFP-LC3 transgenic mice, we treated the mice as illustrated in table-1. The treatment included Chloroquine Diphosphate (CD) agent which considered as a mitophagy inhibitor. The other chemical is Cyclosporine A (CsA) which has been used to increase the mitochondrial membrane potential via blocking the mitochondrial permeability transition pore (MPT).

Treatment type	Count of WT	Count of Lipin1 -\-- EGFP-LC3	Injection period	Dosage	Fasting after treatment	Total number of mice
Fasting	3	4	-	-	16hrs	9
CD	4	4	10 days	3.3ul /gram	16hrs	8
CA	3	3	10 days	3.3ul /gram	16hrs	6

Table 1: Lipin1 $^{-/-}$ EGFP-LC3 transgenic mice treatment groups

The age range of these mice was from 8 weeks up to 20 weeks. The animals were euthanized after the treatments and the EDL and Soleus muscle tissues were collected. We applied the Optimal Cutting Temperature (OCT) over each muscle and dipped it in cold Isopentane (−160˚ C) for 10 seconds. After that store the tissues in the -80˚C freezer. For the immunofluorescent staining, we cut the muscles into thin sections between 5-7 u using Cryostat Thermo Scientific Microm HM550 (-20C).

iii- Immunofluorescent staining:

Immunofluorescent staining has been used to evaluate the autophagy in the tissue sections. Tissues were fixed with 4 % Paraformaldehyde for 15 mins at RT.
Then after washing with 1X PBS, they get permeabilized with 0.3% Triton X-100 (ThrrmoFisher, Surfact-Amps X-100, #28314) for 15 mins then washed. Two blocking solutions were utilized in this experiments, first blocked by M.O.M mouse IgG blocking regent (Vector Laboratories, MBK-2213) diluted in PBS for 1 hour at RT. Then slides were washed before adding the second blocking buffer (10 % goat serum+ 1% BSA in PBS) for 1 hour at RT then washed. Following the blocking, the muscle sections were incubated with 1˚Ab overnight at 4˚C. The antibodies diluted in 10 % goat serum+ 1% BSA, i.e. LC3 and Bnip3 as 1:400 dilution factor. On the next day, the secondary antibodies were added after washing the sections, and incubated at RT for 60-90 mins. The secondary antibodies diluted in (10 % goat serum+ 1% BSA in PBS), (Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 555 #21428) by 1:500 dilution factor. Lastly, the slides were washed with PBS+ 0.01% Tween-20 5 x 5 mins, and allowed to dry before adding mounting medium (Vectorshield anti-fade containing DAPI (Vector- H-1200). The Immunofluorescent images were taken by LEICA DMI 6000 B microscope.

iv- Western Blotting:

The protein expression profiles of autophagy and mitophagy markers were measured using immunoblotting. Extracted muscles were homogenized with a mixture

that contains 1X RIPA buffer (20-188 EMD Millipore Sigma) + 0.2% Proteases Inhibitors (SIGMA cOmplete™ Protease Inhibitor Cocktail 04693116001) + 0.2% Phosphatases inhibitors (SIGMA P2850 - Phosphatase Inhibitor Cocktail 1), in which 1ml 1X RIPA + 20 ul of protease inhibitor+ 20ul of Phosphatase inhibitor. Then, tissue homogenization was performed in the ice by adding for each 20mg of tissue 450ul of the homogenization mixture by using manual homogenizers. Then the samples were centrifuged at 12000rpm at 4C˚ for 10 minutes. In order to measure the proteins concentrations, the bicinchoninic acid assay (BCA) assay was utilized. 2mg/ml of bovine serum albumin (BSA) was used as standards (0, 1ul, 2ul, 3ul, 4ul, 5ul and 6ul loaded in 96 well plates). The samples were added by 2ul and duplicated. The amount of samples needed to be resolved by the western blotting was determined by the standard curve from the BCA.

The lysates were ran in the acrylamide gel (30% acrylamide Solution 37.5:1 Biorad Cat. 1610158) in order to separate the proteins. 12.5 % and 7.5 % of the acrylamide gels were used in our experiments according to the proteins sizes. The protein samples were mixed with 10ul of 4 x loading dye and boiled for 5 mins. After boiling the samples, they were spun and loaded into the acrylamide gels. The power box was set by voltage 120V once it goes through the stacking gel. For transferring the proteins, first, the PVDF membranes are cut and soaked in methanol for 1 minute then moved to transfer buffer. To transfer the proteins form the gel into the membrane the gel and the membrane were stacked in a gel holder cassette of the transfer system. Then, transferred them into the transfer box and sat up the power at 0.30 A and 150 V for 2 hours with ice surrounding the transfer box. To get rid of the unspecific binding casein solution was

utilized and diluted in 1X PBS with 1:9 ratio to block the membranes. Blocking of the membrane was for 60 minutes at the room temperature. Most of our protein antibodies we obtained them form Cell Signaling Technology. We used LC3B (D11) XP® Rabbit mAb #3868, Parkin Antibody #2132, Phospho-PKD/PKCμ (Ser744/748) Antibody #2054, Phospho-p70 S6 Kinase (Thr389) Antibody #9205, p70 S6 Kinase Antibody #9202, Akt (pan) (C67E7) Rabbit mAb #4691, Phospho-Akt (Thr308) (D25E6) XP® Rabbit mAb #13038. From Abcam Anti-GAPDH antibody [EPR16891] (Rb) (ab181602), Anti-BNIP3 antibody (Rb) (ab38621), Anti-SQSTM1 / p62 antibody [EPR4844] (Rb) (ab109012), PKC mu Polyclonal Antibody (Rb) PA5-38387 Invitrogen. These antibodies were diluted in 10% casein + 0.1 % Tween 20. Blots were incubated with the antibodies for overnight at 4C◦. For secondary antibodies, Anti-Rabbit IgG (H+L), HRP Conjugate #W4018, Anti-Mouse IgG (H+L), HRP Conjugate #W4028. The secondary antibodies were incubated for 1 hour at the RT. These antibodies were diluted in 10% casein + 0.1 % Tween 20 + 0.1% 10% SDS. To visualize the blots, a chemiluminescent kit was used from Pierce™ ECL Western Blotting Substrate catalog # 32106. Visualization of the blots was done by the Amersham Imager 600 instrument.

v- Quantitative PCR:

The mRNA expression was evaluated for some of the mitophagy proteins to assess the transcription activity of the markers during different scenarios in the mutant mice. The extraction of the RNA was performed using a modified version of the Invitrogen protocol which involves TRIzol. Muscles were disrupted and homogenized by

TRIzol and pestle homogenizer. 1ml of TRIzol was added to each 20-30 mg of muscle mass. After the tissue disruption, Chloroform was added to the lysate to separate the RNA layer from the proteins phase. Isopropanol and 70% Ethanol were further used to clump the RNA and help in precipitation of RNA and increase the RNA yield. The complete extraction protocol is available online (https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf). All the primers used in this study were purchased from Integrated DNA Technologies (IDT) as follow: LC3 primers 5'-TCGTTGTGCCTTTATTAGTGCATC-3' and 5'-CACTGCTCTGTCTTGTGTAGGTTG-3'. Bnip3 primers 5'-TTGGGGCATTTTACTAACCTT-3' and 5'-TGCAGGTGACTGGTGGTACTAA-3'. Lipin1 primers 5'-CAGCCTGGTAGATTGCCAGA-3' and 5'-GCAGCCTGTGGCAATTCA-3'. P62 primers 5'-GCTCCAGTTTCCTGGTGGAC-3' and 5'-GCACCTGTCTGAGGGCTTCT-3'. The qPCR experiments were done using QuantStudio™ 7 Flex Real-Time PCR System from TheromFisher Scientific Company.

vi- Experimental design:

In Figure 5 we are summarizing the experiments that we used in our project. Beginning from establishing the mice model then injecting the mice with CD and CA. Then mice were euthanized and muscles were collected. Following different tissue homogenizations, proteins or RNA were extracted in order to perform western blotting or qPCR analysis. Also, the muscle were cut into thin sections in order to visualize the autophagosomes and stain with antibodies against some molecules.

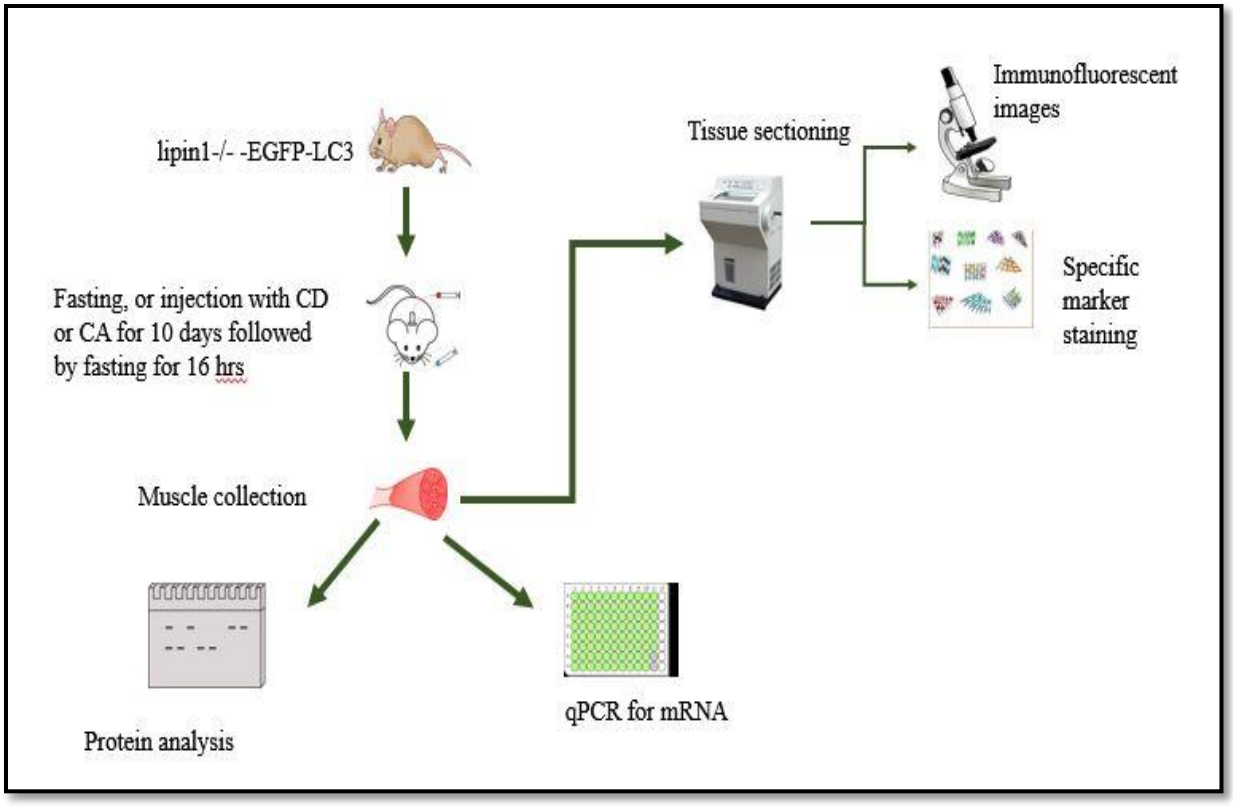


Figure 5: Schematic illustration of the experimental design for our project

III- Data and results

Aim 1: To investigate how lipin1 deficiency affects mitophagy process.

1- The novel lipin1 deficient EGFP-LC3 transgenic mice model expresses endogenous GFP.

The main goal for establishing the lipin1 deficient EGFP-LC3 transgenic mice model is to provide a reliable tool to investigate the mitochondrial autophagy in vivo. Therefore, we want to examine the muscle tissue of the new mice model to check whether they can express endogenous GFPs or not. After we analyzed the tissue samples from the lipin1 $^{-/-}$ EGFP-LC3 transgenic mice, we were able to visualize the GFP punctates in the EDL muscle using immunofluorescent microscope (Figure 6). These GFP dots represent the autophagic vacuoles formed in the cells as it has been shown before (Kabeya et al., 2000). Since these mice are genetically modified to express GFP along with expressed LC3 protein we did not stain the cryosections with any antibody against LC3. These sections were only stained with DAPI and fixed with paraformaldehyde. All the wild type (WT) muscles we used in this study they express GFP protein tagged to LC3 protein. At the basal level, WT EDL muscle showed a basal level of the autophagosomes created in the cytoplasm as the image shows in (Figure 6, upper panel). However, after fasting (16 hours) the number of the phagophores in the WT was higher due to an increased autophagy/mitophagy rate to provide energy to the cells (Kuma et al., 2004). Fasting is commonly used to physiologically trigger the autophagy/mitophagy process in the cells without the need for any chemical agents

(Alirezaei et al., 2010; Levine & Kroemer, 2008). Comparing the lipin1 deficient EGFP-LC3 mice muscles to the WT, in basal status the GFP punctates were higher and bigger in size versus the WT basal (Figure 6, lower panel). In contrast, when the mutant mice were starved (16 hours) the number and the size of the GFPs were dramatically higher than the fasted WT animals. We suspect that the increased number of the autophagosomes in the lipin1 deficient mice was due to a defect in the mitophagy. The mitophagy process is initiated; however, a blockage in the pathway led to accumulated autophagosomes in the cytosol. Therefore, lipin1 deficient mice had an accumulated autophagosomes in the cytoplasm of the EDL muscle.

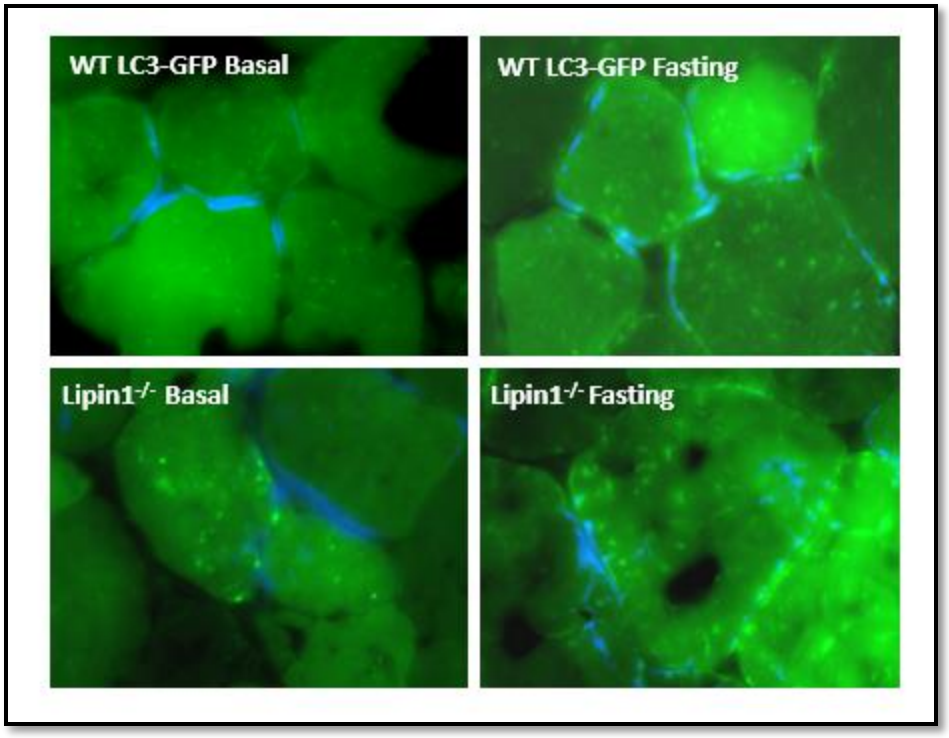


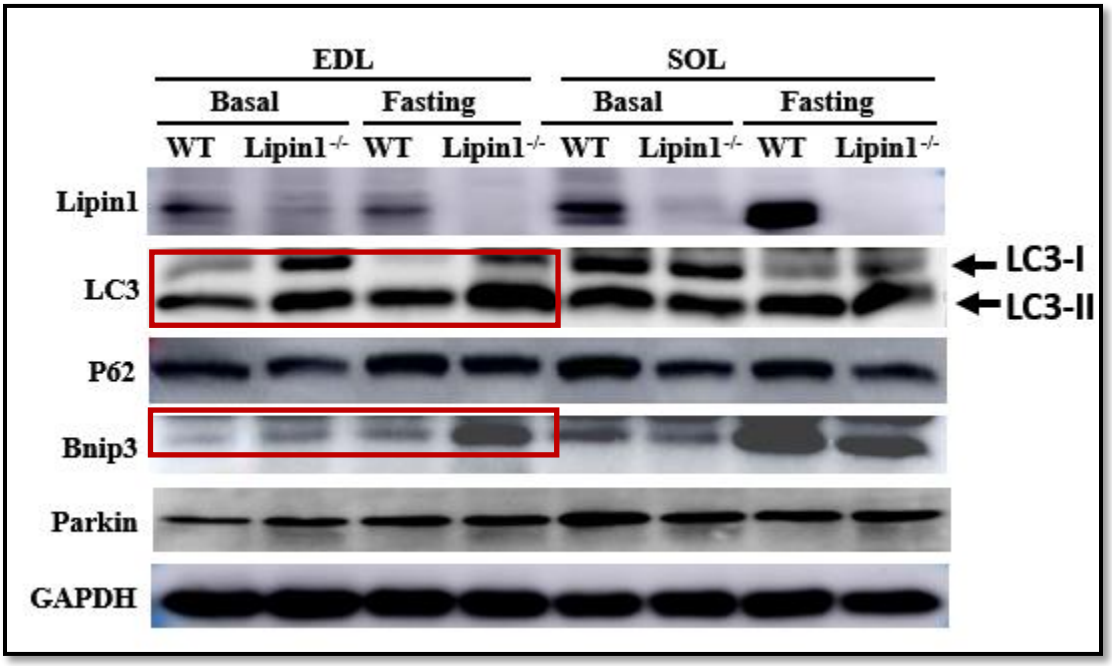
Figure 6: The novel lipin1 deficient EGFP-LC3 transgenic mice model expresses endogenous GFP. These immunofluorescent images obtained from EDL muscles from WT and the lipin1 deficient EGFP-LC3 mice and examined by the immunofluorescent microscope. The sections were only fixed and stained with DAPI. At the top, the sections belong to the WT mice with two conditions, basal and fasting (16 hours). Upon 16 hours fasting, the WT muscle showed an increased autophagosomes formation compared to the basal level. In the bottom the lipin1 -/- EGFP-LC3 EDL muscles also in two conditions, basal and fasting. The count of the GFP dots was higher in the mutant mice compared to the WT at the basal. However, a greater rise was observed in the fasting lipin1 -/- EGFP-LC3 EDL muscles in comparison to the fasted WT mice. The magnification of these images is 100X.

2- Lipin1 deficiency induces inhibition of autophagy and mitophagy in the Lipin $^{-/-}$ EGFP-LC3 transgenic mice.

As shown in Figure 6, the mitophagy pattern was increased in the Lipin $^{-/-}$ EGFP-LC3 transgenic mice compared to the WT based on the immunofluorescent analysis. We wanted to confirm that the increased GFPs dots in the cytoplasm were a result of increased mitophagy activity. Thus, we examined the protein expression profiles of the mitophagy and autophagy markers LC3, p62, (Mizushima, 2015) Bnip3 and Parkin in the WT and Lipin $^{-/-}$ EGFP-LC3 transgenic mice. We obtained the lysates from EDL and soleus muscles from the WT and Lipin $^{-/-}$ EGFP-LC3 transgenic mice collected from two conditions, basal and fasting (16 hours) (Figure 7, panel A). Lipin1 was only expressed in the WT muscles and totally knocked out in the lipin1$^{-/-}$ EGFP-LC3 mice. This confirmed that the genetic lipin1 knockout was successful in the lipin1$^{-/-}$ EGFP-LC3 mice. The autophagic molecule LC3 shows two bands in the western blot, the upper one is LC3-I and the lower one is LC3-II. The difference here is that LC3-I has been reported to migrates lower than the LC3-II as it indicated by the arrows (Figure, 7) (W. Wang, Chen, Billiar, Stang, & Gao, 2013). At the basal level, the autophagic marker LC3II (the lower band) expression was raised in the mutant versus the WT. On the other hand, in response to fasting LC3II protein expression was increased by two fold compared to the basal and the rest of the conditions in the EDL muscle of the mutant mice. Upon stressing the cells with serum starvation, autophagy was upregulated to balance the energy by recycling some of the cytoplasmic content. The mitochondrial damage markers were examined too. Bnip3 of the EDL muscle was upregulated in the Lipin1$^{-/-}$ EGFP-LC3 at the basal level.

Moreover, Bnip3 was further raised in response to nutrient withdrawal in the mutant as the expression profile revealed (Figure 7, A). Upregulation of Bnip3 protein suggests that the defective mitophagy in the EDL of the lipin1 deficient EGFP-LC3 mice was mediated by Bnip3 in this context. Similar to a previous report (Howells et al., 1978) the EDL muscle presented a greater response for fasting in comparison to the soleus (check introduction, EDL versus soleus). The autophagic signaling pathways are sensitive to food withdrawal in the EDL. The other mitochondrial damage marker, Parkin, didn't show significant changes in all treatments and conditions. Put together, lipin1 deficiency markedly activates the mitophagy in the skeletal muscle of the Lipin $^{-/-}$ EGFP-LC3 transgenic mice as the expression of major autophagy and mitophagy markers were elevated.

A


EDL
SOL
Basal
Fasting
Basal
Fasting
WT
Lipin1-/-
WT
Lipin1-/-
WT
Lipin1-/-
WT
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Lipin1
LC3
P62
Bnip3
Parkin
GAPDH
LC3-I
LC3-II

B

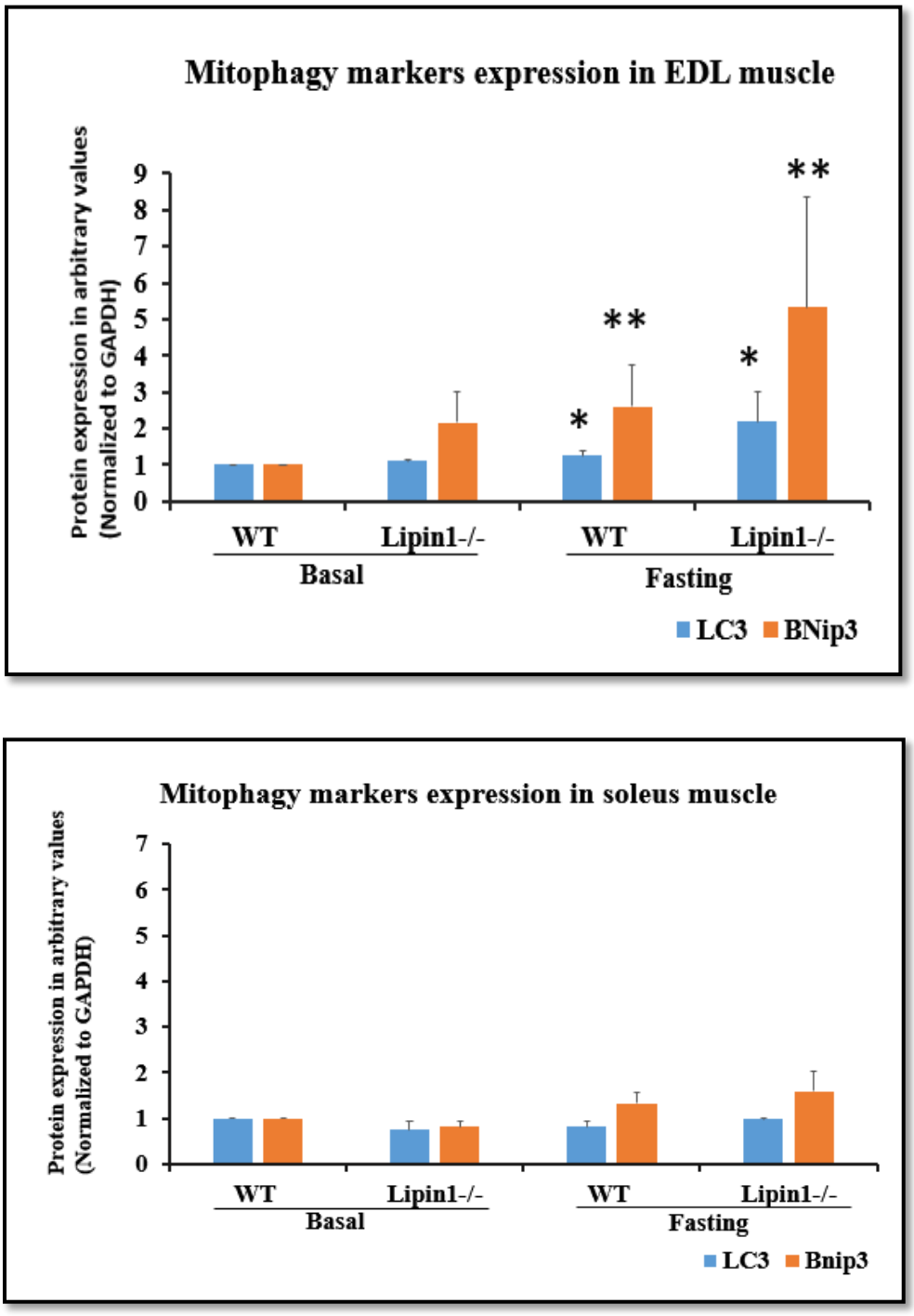


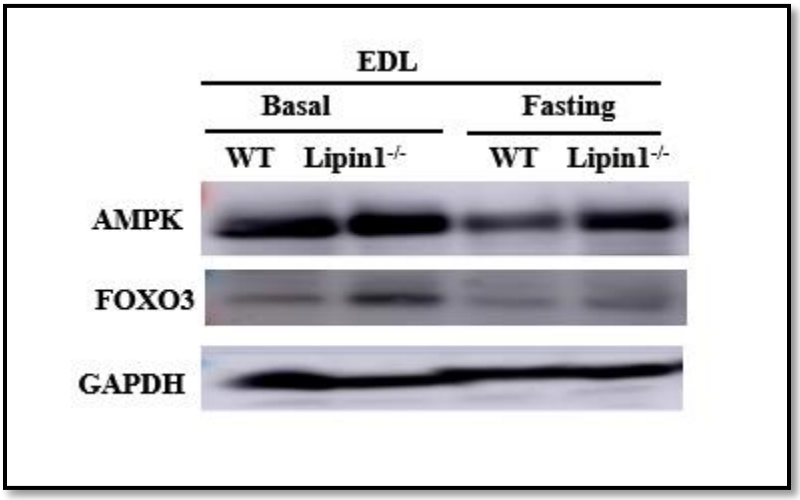
Figure 7: Lipin1 deficiency upregulates autophagy and mitophagy markers in the EDL and soleus muscles of the Lipin $^{-/-}$ EGFP-LC3 transgenic mice. **(A)** Representative

immunoblots of key proteins involved in autophagy and mitophagy from EDL and SOL muscles in WT and lipin1$^{-/-}$ EGFP-LC3 transgenic mice. From each mouse type, WT and lipin1$^{-/-}$ EGFP-LC3, we divided each type into basal and fasting (16h). Then mice were sacrificed and muscle tissue was collected for western blotting analysis. The protein lysates were separated on 12.5% SDS-PAGE and then immunoblotted with antibodies against the mitophagy markers. (B) The first chart belongs to the EDL muscle, the second chart belongs to soleus muscle. The basal WT was used as a control. The expression of the major markers was normalized to GAPDH. Error bars represent the standard deviation (SD). Comparing fasting to basal condition significant changes observed in the EDL with ($*$) p-value ≤ 0.07, ($**$) p-value ≤ 0.08, but no significant changes in soleus. GAPDH was used as a loading control.

3- Lipin1 deficiency induces upregulation of AMPK in the Lipin$^{-/-}$ -EGFP-LC3 transgenic mice.

To check how lipin1 deficiency contributes to the mitophagy alteration we assessed the several mitophagy activation signaling pathways and examined the protein expression of potential molecules that may have a role in the defective mitophagy. Based on literature, the expression level of FOXO3 (belongs to the forkhead family of transcription factors) has been shown to be regulated by the AMP-activated protein kinase (AMPK) (Greer et al., 2007). FOXO3 has been shown to regulate the expression of LC3 and Bnip3 (Mammucari, 2007). Then we looked at the foxo3 activation by measuring the phosphorylated FOXO3 (s413) expression level in the lipin1$^{-/-}$ EGFP-LC3 mice. The AMPK and p-FOXO3 were upregulated in the mutant mice during fasting status. The FOXO3 expression was higher than the control condition (Figure 8). We suggest that Lipin1 deficiency induced lipid or nutrient repression, which in turn activates AMPK, and increase its activity then upregulates FOXO3. The activated FOXO3 will then increase the transcription of autophagy/mitophagy markers, LC3 and Bnip3. We didn't run statistics for this data to check the significance.

(A)



(B)

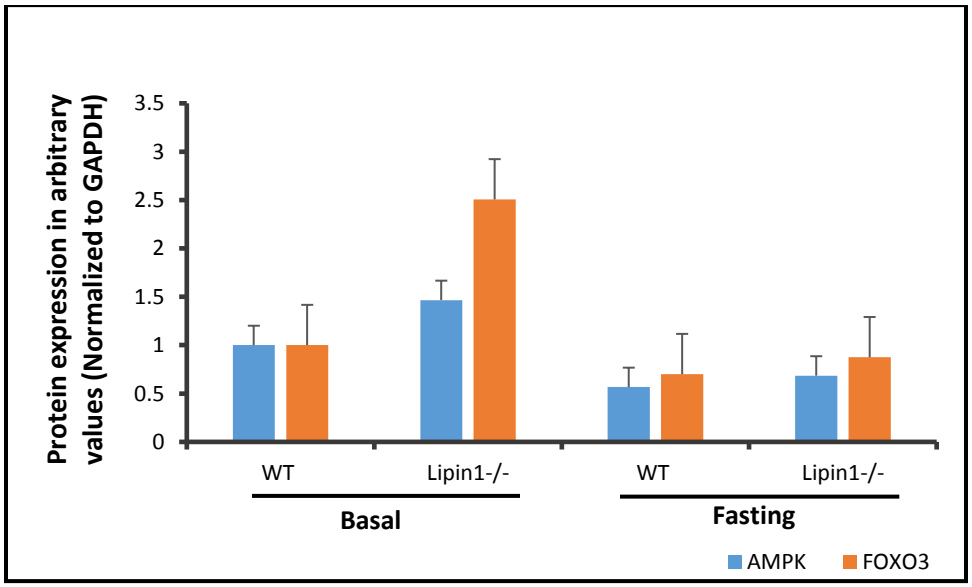


Figure 8: Lipin1 deficiency induces upregulation of AMPK in the Lipin$^{-/-}$ -EGFP-LC3 transgenic mice: (A) Western blotting of the potential proteins involved in dysfunctional

mitophagy in the lipin1$^{-/-}$ -EGFP-LC3 transgenic mice. WT and lipin1$^{-/-}$ -EGFP-LC3 transgenic mice were restricted from food for 16 hours followed by muscle tissue collection. The EDL was homogenized and protein lysate was collected for resolving by 7.5% SDS-PAGE gel. The separated proteins were probed with AMPK and p-FOXO3 (s413) antibodies. The AMPK and FOXO3 were activated in the lipin1$^{-/-}$ -EGFP-LC3 transgenic mice. (B) The expression of the AMPK and FOXO3 was normalized to GAPDH. The error bars represent the SD. GAPDH was used as a loading control.

4- Moderate elevation in the gene transcription activity of the mitophagy markers of the lipin1-/- -EGFP-LC3 mice:

The protein expression of the mitophagy markers were elevated in the lipin1$^{-/-}$ -EGFP-LC3 animals (Figure 7). So, we want to measure the transcriptional activity of the autophagy/mitophagy markers to test whether the increased expression in the cytosol was due to increased gene transcription or not. The EDL muscle was collected from WT and lipin1$^{-/-}$ -EGFP-LC3 from two different conditions, basal and fasting (16 hours). Then, the mRNA was extracted from the tissue and then we generated cDNA library. qPCR was performed to obtain the quantitative analysis of the gene expression of Lipin1, LC3, p62 and Bnip3 (Figure 9). The lipin1 gene transcription was only detected in the WT animals, and the mutant ones had no activity. In the fasted status, LC3 and p62 of the lipin1$^{-/-}$ -EGFP-LC3 revealed a slight increase in the gene transcription activity compared to starved WT. For the mitophagy marker, Bnip3, of the lipin1$^{-/-}$ -EGFP-LC3 mice we detected a slight increase of the gene transcription compared to the fasted WT. These data suggest that the increase of the protein expressions in the cytosol of lipin1$^{-/-}$ -EGFP-LC3 mice was to some extent due to increase transcription activity.

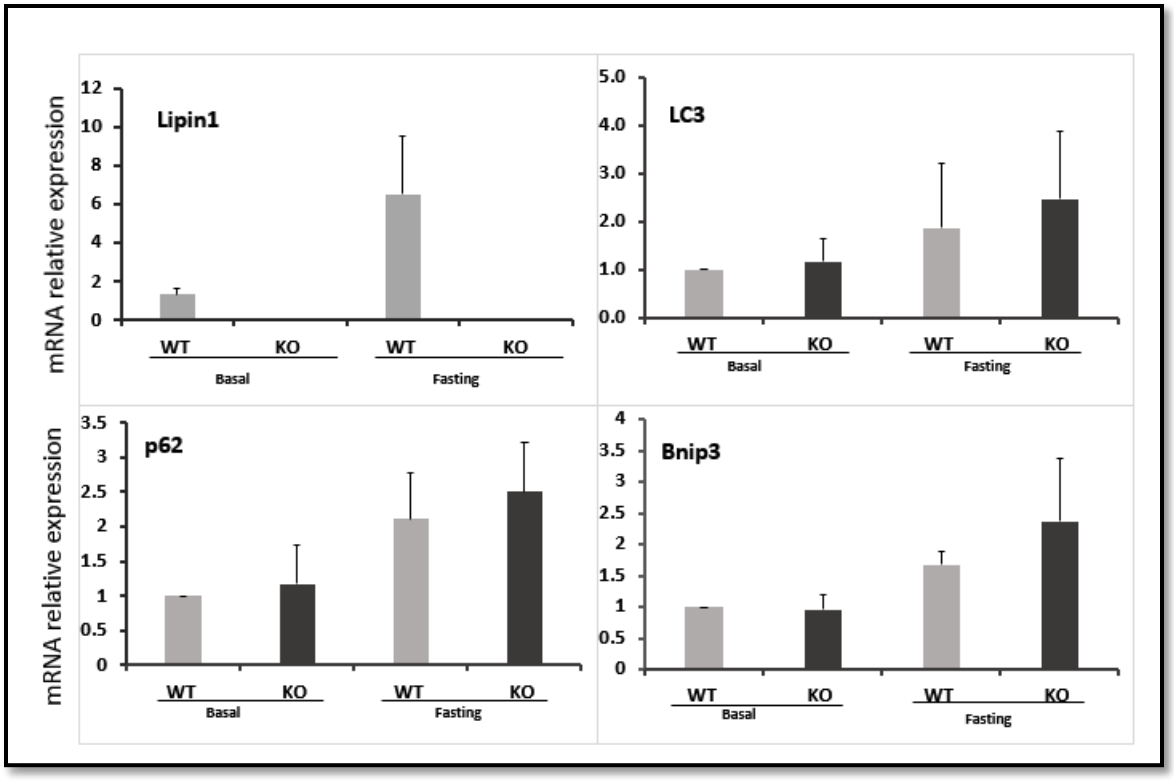


Figure 9: Moderate elevation in the gene transcription activity of the mitophagy markers of the lipin1$^{-/-}$ -EGFP-LC3 mice: The mRNA transcription level of the mitophagy markers were evaluated in the EDL muscle of the WT and lipin1$^{-/-}$ -EGFP-LC3 mice. The WT and lipin1$^{-/-}$ -EGFP-LC3 mice each was divided into two groups basal and fasting (16 hours). Then, the EDL muscle were harvested and digested to extract the RNA. cDNA library was generated and qPCR result were obtained from the different groups mice. We examined the gene transcription level of the Lipin1, LC3, p62 and Bnip3. Error bars represent the SD.

Aim 2: To visualize the effect of lipin1 deficiency on mitophagy process using Lipin1$^{-/-}$ -EGFP-LC3 transgenic mice.

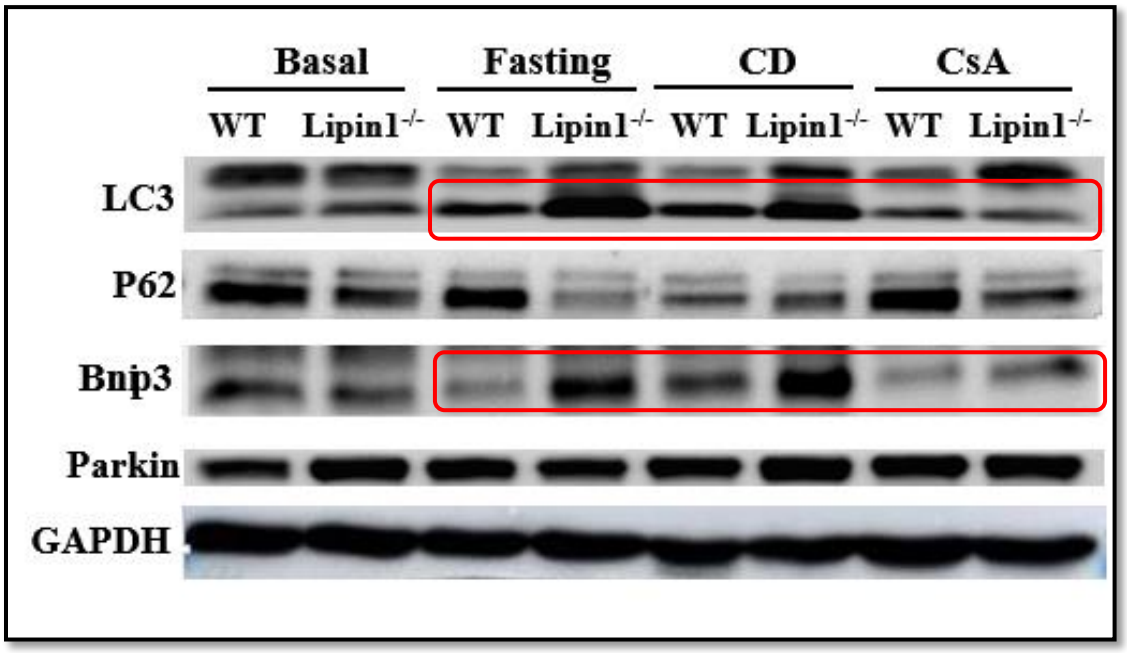
Aim 3: To determine whether the improvement of mitochondrial membrane potential could ameliorate Lipin1-associated mitophagy defects.

5- Cyclosporine A ameliorated lipin1 deficient induced mitophagic defect in the EDL muscle:

In Figure 7 the mitophagy markers were increased in the lipin1$^{-/-}$ -EGFP-LC3 transgenic mice; therefore, we want to treat the mice either to inhibit mitophagy, or improve mitochondrial function and reduce mitophagy activity. Thus, we injected the WT and Lipin1$^{-/-}$ -EGFP-LC3 transgenic mice with Chloroquine diphosphate (CD) and cyclosporine A (CsA) then collected the EDL muscles. The CD considered as a mitophagy blocker where the mitophagy process is initiated but is not completed (Yoon et al., 2010). The CsA has been shown to improve mitochondrial membrane potential via preventing the mitochondria permeability transition (MPT) (Fournier, Ducet, & Crevat, 1987). The animals were injected for 10 days followed with 16 hours of fasting (as described in methods). The collected EDL muscles were used for western blotting experiment (Figure 10). We once more looked at the protein expressions of autophagy/mitophagy markers by resolving them with 12.5% SDS-PAGE gel. In fasting status, similar to what we observed in figure 7, LC3 was upregulated in lipin1$^{-/-}$ -EGFP-LC3 mice compared to WT. Same scenario happened to Bnip3, and its expression was increased in the lipin1$^{-/-}$ -EGFP-LC3 samples versus the WT. The upregulation of these markers was due to fasting, which induces the autophagy machinery to provide energy to the cell. The next group is the CD treated animals. Since the CD inhibits mitophagy we

saw similar expression of LC3 in both WT and lipin1$^{-/-}$ -EGFP-LC3 due to blocking the clearance process in both groups as it is highlighted in a red box (Figure 10). In the case of Bnip3, the mitochondrial damage marker, the protein was upregulated only in the lipin$^{-/-}$ -EGFP-LC3 mice as the blots show (Figure 10). This result suggested that the defective mitophagy in lipin1$^{-/-}$ -EGFP-LC3 animals was mediated by the Bnip3 pathway. On the other hand, Parkin didn't show any significant response in this context. To test whether improving mitochondrial function may facilitate the impaired mitophagy, the mice were injected with CsA for 10 days followed by 16 hours of fasting. The autophagy markers' expressions were evaluated by western blotting (Figure 10). In response to CsA treatment LC3 showed a basal level similar to the WT basal condition, which revealed an effective CsA treatment on ameliorating mitophagy process. The downregulation of LC3 expression was detected in both WT and lipin1$^{-/-}$ -EGFP-LC3. Moreover, the Bnip3 was downregulated in the WT and lipin1$^{-/-}$ -EGFP-LC3 mice. These results suggest that CsA treatment rescued the defect in the mitophagy which resulted from lipin1 deficiency.

(A)


Basal
Fasting
CD
CsA
WT
Lipin1-/-
WT
Lipin1-/-
WT
Lipin1-/-
WT
Lipin1-/-
LC3
P62
Bnip3
Parkin
GAPDH

(B)

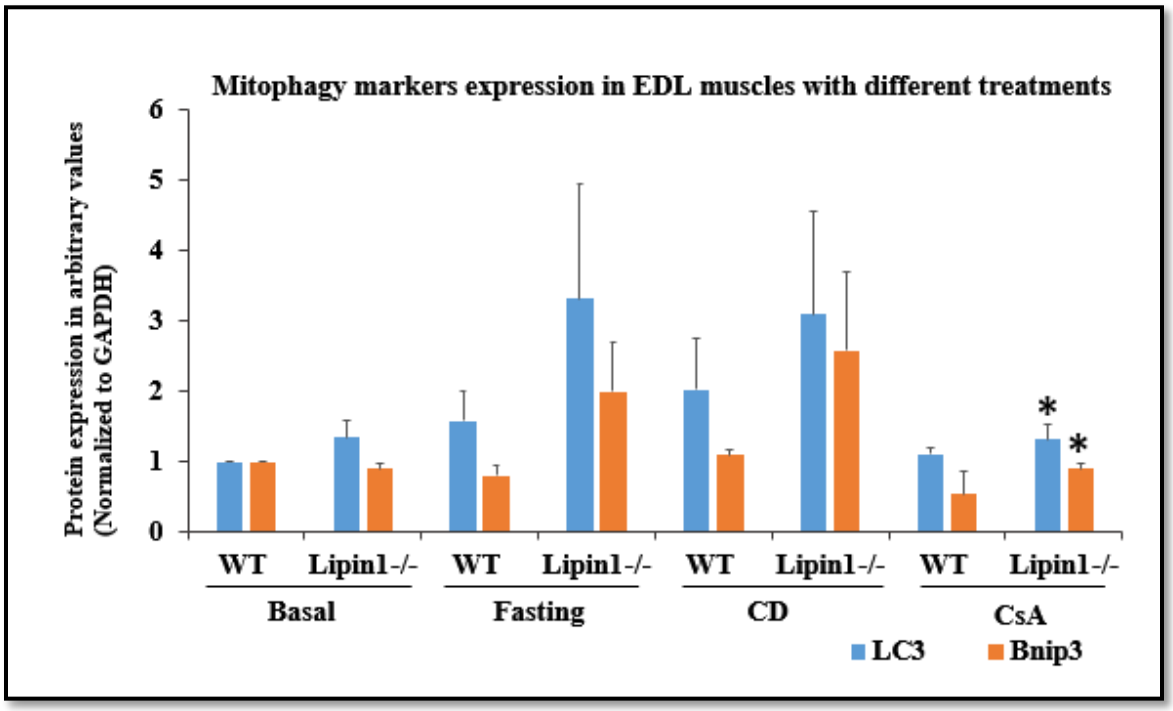

Mitophagy markers expression in EDL muscles with different treatments
Protein expression in arbitrary values
(Normalized to GAPDH)
6
5
4
3
2
1
0
WT
Lipin1-/-
WT
Lipin1-/-
WT
Lipin1-/-
WT
Lipin1-/-
Basal
Fasting
CD
CsA
*
*
LC3
Bnip3

Figure 10: Cyclosporine A treatment ameliorated the defective mitophagy due to lipin1 deficiency in EDL muscle of the WT and the lipin1$^{-/-}$ -EGFP-LC3 mice. **(A)** These western blots show several autophagy/ mitophagy markers. The EDL muscles were collected from four different groups of conditions: basal, fasting, CD treated and CsA treated mice groups, as it appears in the top of the blots. In the fasting group, the mice were exposed for 16 hours of fasting then euthanized. In the CD treated group, the animal were injected with CD for 10 days followed by 16 hours of fasting then mice were sacrificed. The mice of the CsA group were injected with CsA for 10 days followed by 16 hours of fasting then mice were euthanized. Each group contained two subgroups, WT and lipin1$^{-/-}$ -EGFP-LC3 mice. The protein lysates were separated using 12.5% SDS-PAGE gel and blotted with antibodies against mitophagy markers LC3, p62, Bnip3 and Parkin. **(B)** The expression of the major markers was normalized to GAPDH. The basal WT was used as a control. The error bars represent the SD. Significant changes with $p \leq 0.05$ are indicated with an asterisk compared to WT in fasting state. GAPDH was used as a loading control.

6- Impaired mitophagy was recovered as a response to CsA treatment in the EDL muscular tissue of the lipin1-/- -EGFP-LC3 mice:

The impaired mitophagy process was ameliorated upon treatment with CsA according to the protein expression profiles of the lipin1$^{-/-}$EGFP-LC3 mice (Figure 10). So, we want to confirm that by examining the EDL tissue with an immunofluorescent microscope and looking for GFPs dot which represent the LC3 protein that is related to autophagosomes' abundance (Figure 6). We obtained cryosections from the EDL muscles from different treated groups of mice (as details shown in Figure 10) to examine the autophagosomes' formation in the lipin1$^{-/-}$ -EGFP-LC3 mice (Figure 11). Those sections were only stained with DAPI and fixed with paraformaldehyde. Upon fasting for 16 hours, the GFP dots were increased in the WT (Figure 11 top panel) which is an indication of increased autophagy rate to compensate for the nutrient deficient status. In the lipin1$^{-/-}$EGFP-LC3 mice (Figure 11 lower panel) the GFP punctates were increased in both number and size compared to the WT. In the case of CD treatment, since the mitophagy was blocked, both WT and lipin1$^{-/-}$ -EGFP-LC3 mice showed almost similar increased pattern of GFP dots with a little bit of increase in the mutant mice. Interestingly, the CsA agent was able to decrease the number of GFP dots in the lipin1$^{-/-}$ -EGFP-LC3 mice EDL tissue suggesting that improving mitochondrial membrane potential rescued the autophagy defect caused by lipin1 deficiency. This suggests that ameliorating mitochondrial function would result in improved mitophagy process and reduced the autophagosomes formation in the cytoplasm.

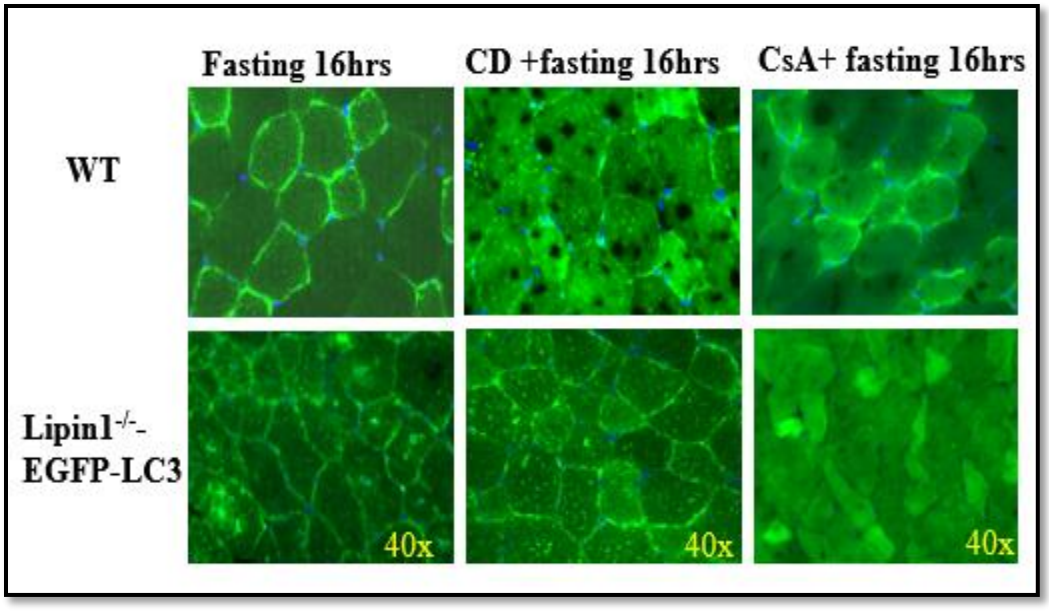


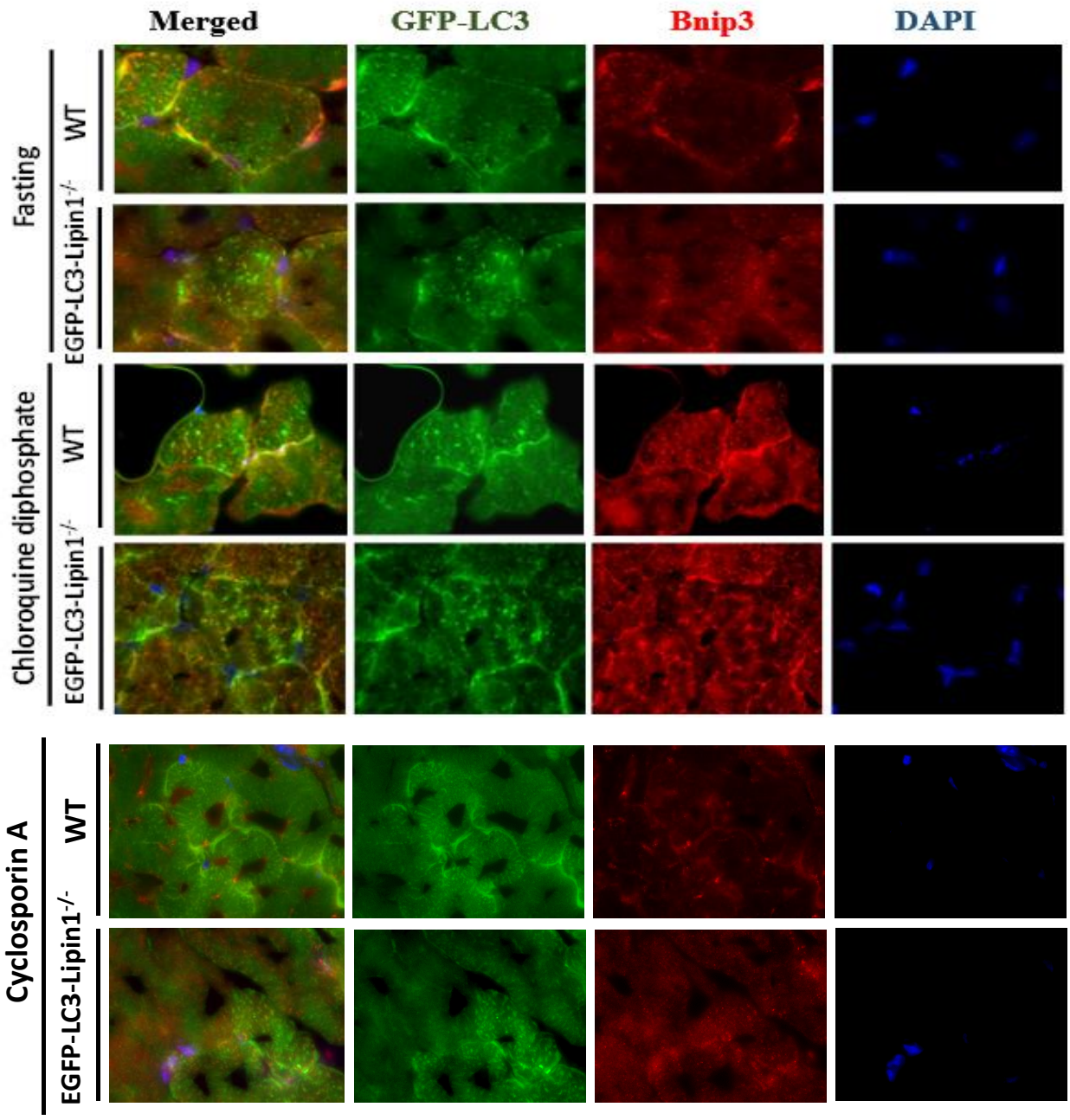
***Figure 11: Impaired mitophagy was recovered as a response to CsA treatment in the EDL muscular tissue of the lipin1$^{-/-}$ -EGFP-LC3 mice*:** These immunofluorescent images are collected from different treatments of the EDL muscles of WT and lipin1$^{-/-}$ -EGFP-LC3 mice. These sections are only stained with DAPI and fixed by 4% formaldehyde. The mice were divided into 3 groups: fasting, CD and CsA treated mice. Each group involved WT and lipin1$^{-/-}$ -EGFP-LC3 subjects. In the fasting group, the animals were only restricted from food for 16 hours but they had access to water, then mice were euthanized and tissue was collected. For the CD (mitophagy inhibitor) injected group, WT and lipin1$^{-/-}$ -EGFP-LC3 mice were injected for 10 days followed with fasting for 16 hours then EDL was harvested from both. For both WT and lipin1$^{-/-}$ -EGFP-LC3 mice the count of GFP punctates was almost similar. The last two groups of mice were injected with CsA for 10 days and followed by fasting (16 hours) then muscle tissue was collected from both. In the two examined groups of animals both showed lower numbers of GFPs expressed in the cytoplasm. The magnification of these images is 40X.

7- The defective mitophagy caused by lipin1 deficiency is mediated by Bnip3 pathway:

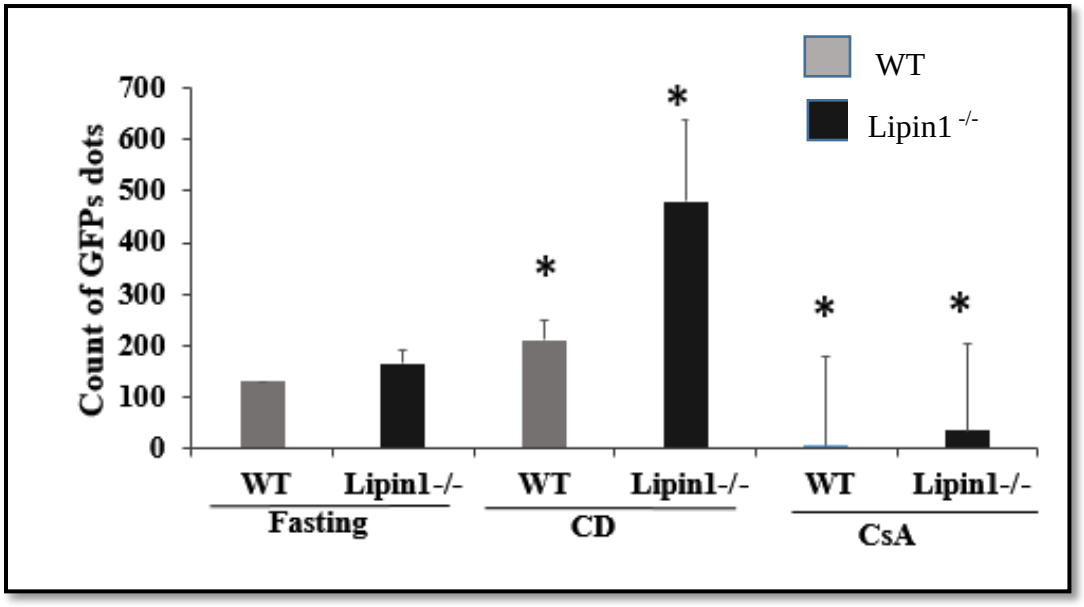
As the immunoblotting data (Figure 10) revealed, Bnip3 (the mitochondrial damage marker) showed significant accumulation in the lipin1$^{-/-}$ -EGFP-LC3 mice. Thus, we wanted to confirm that observation and also see whether Bnip3 interacts and co-localizes with LC3 protein or not with immunofluorescent staining. So, the EDL muscles of WT and lipin1$^{-/-}$ -EGFP-LC3 mice were immunostained with an antibody against Bnip3 protein and visualized by an immunofluorescent microscope to examine the co-localization of the LC3-GFP and Bnip3. Indeed, we found that Bnip3 proteins were co-localized with some of the LC3 proteins in the cytosol (Figure 12, panel A). The cryosections which have been stained with Bnip3 antibody are obtained from the same groups used in Figure 11. In the fasting group, the WT showed a high number of GFPs with around 35% co-localization of Bnip3. In the lipin1$^{-/-}$ -EGFP-LC3 mice, the number of GFPs and co-localizations were higher than the WT (Figure 12, panel A). The increase of GFPs was a result of the impaired mitophagy. When the mitophagy was inhibited by CD, the two groups of mice presented increased number of GFPs with more than 40% co-localized Bnip3. The Bnip3 co-localization was higher than the WT as the quantification analysis revealed (Figure 12, panel B). Interestingly, when the mice were treated with CsA, the GFPs and Bnip3 cytosolic expression dramatically decreased in both WT and lipin1$^{-/-}$ -EGFP-LC3 mice (Figure 12, panel A). The decrease in the GFPs is a representative of a reduction in autophagosomes abundance in the CsA treated animals. This is also consistent with the western blotting data (Figure 10) where the LC3 and Bnip3 expression reduced in response to CsA treatment. Since CsA increases

mitochondrial membrane potential, it is possible that the CsA restored the defect in mitophagy caused by lipin1 deficiency and decreased the autophagosomes population which are required to eliminate the dysfunctional mitochondria. These observations are consistent with proteins expression profiles of LC3 and Bnip3 (Figure 10) suggesting a direct interaction and co-localization of Bnip3 with LC3. The number of GFPs were counted in the fasting, CsA and CD groups as is shown in (Figure 12, panel B). The GFPs and Bnip3 counting included 10 different fields from each group (Figure 12, panel B top chart) and then the Bnip3 was normalized to total GFPs as it shown in (Figure 12, panel B lower chart).

A


Merged
GFP-LC3
Bnip3
DAPI
Fasting
WT
EGFP-LC3-Lipin1-/-
Chloroquine diphosphate
WT
EGFP-LC3-Lipin1-/-
Cyclosporin A
WT
EGFP-LC3-Lipin1-/-

B



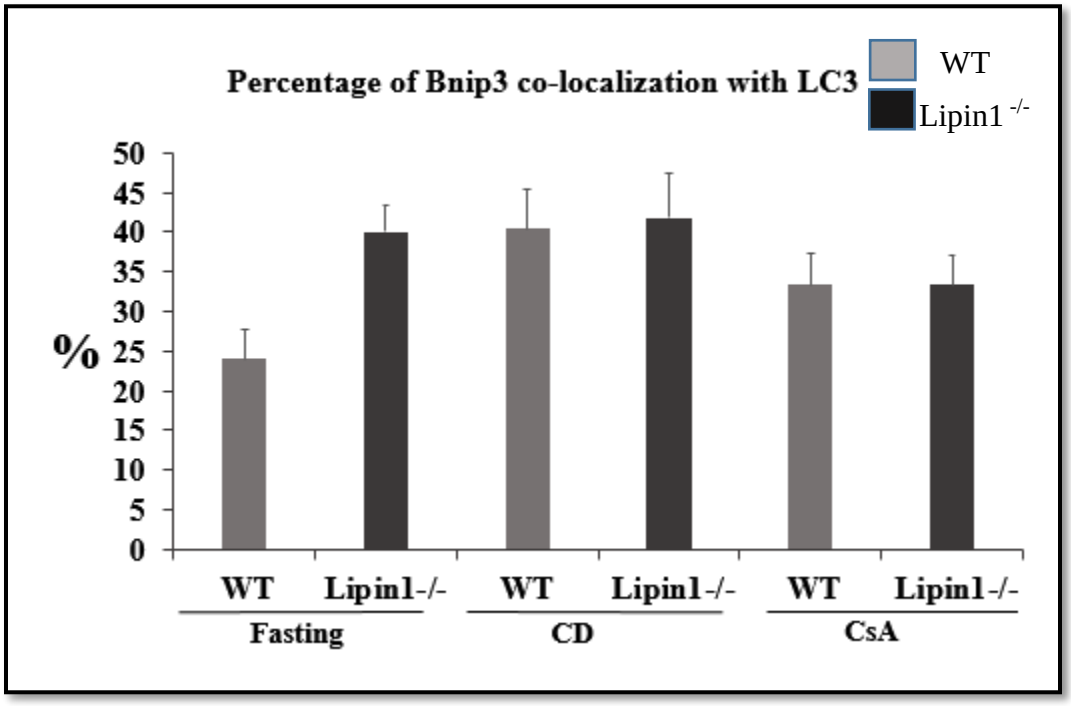


Figure 12: The defective mitophagy caused by lipin1 deficiency is mediated by Bnip3 pathway: The EDL muscle sections of the WT and lipin1$^{-/-}$ -EGFP-LC3 mice were only immunostained with Bnip3 antibody to examine its co-localization with GFP-LC3 protein. **(A)** In the fasted group, WT and mutant mice were fasted for 16 hours then

sacrificed. EDL muscle was collected and cut in to thin sections (5-7 u) and then immunostained with Bnip3 antibody. The lipin1$^{-/-}$ -EGFP-LC3 mice presented a much higher number of GFPs and Bnip3 co-localized together compared to the WT (the top two panels). In the CD treated group, animals were injected with CD for 10 days, then followed by starvation 16 hours. The EDL was collected and cut into thin sections and stained with only Bnip3 antibody. Due to the effect of the drug both WT and lipin1$^{-/-}$ -EGFP-LC3 muscles showed increased number of GFPs and Bnip3 with more GFPs abundance in the mutant animals than WT. In contrast, CsA treated mice were also injected for 10 days with CsA and then fasted for 16 hours. Then we did similar procedure followed with the CD group. CsA Treatment was effective in ameliorating the mitochondrial dysfunction and therefore decreased the GFPs and Bnip3 expressions in both WT and lipin1$^{-/-}$ -EGFP-LC3 as it shown in the lower two panels. **(B)** The number of the GFPs were quantified from 10 different fields for each group (the top chart). The ratio of Bnip3 co-localized to GFP-LC3 was also calculated as it shows in the lower chart. The error bars represent SD. Significant changes with p-value < 0.002 compared to fasting state.

IV- Discussion

Mitochondrial clearance (mitophagy) is one of the fundamental processes within eukaryotic cells. Its role is to assess mitochondrial population integrity and eliminate dysfunctional ones from the cytosol (Yoshii & Mizushima, 2015). Several serious illnesses have been associated with defective mitophagy such as Parkinson's disease, cardiovascular diseases and neurodegenerative disorders (Kanki et al., 2015; Redmann et al., 2014). Phosphatidic acid phosphatase, also called lipin1, is involved in phospholipids' and triglycerides' metabolism and also acts as a transcriptional co-activator regulating the fatty acid oxidation and lipogenesis (Y. Chen et al., 2015). Lipin1 has been found to be mutated in muscular tissue obtained from patients with severe rhabdomyolysis (Zeharia et al., 2008). These patients also exhibited with aggregated and dysfunctional mitochondria in their type II muscle fibers. From a previous work we have done, electron microscope imaging revealed that mice with lipin1 knockout had aggregated mitochondrial accompanied with abnormal autophagosomes formations in the glycolytic muscle sections. We thought that there is a link between impaired mitophagy and mitochondrial functions, and lipin1 deficiency in skeletal muscle. Taken together, lipin1 deficiency may be involved in promoting dysfunctional mitochondria and defective mitophagy in skeletal muscles, which in turn leads to muscle atrophy.

The need to find approaches to study the mitophagy and mitochondrial function in vivo is necessary in terms of providing more specific and reliable tools. So, we generated

a mice model to examine the mitochondrial autophagy in skeletal muscles. The unique model is called lipin1$^{-/-}$ -EGFP-LC3 transgenic mice (see methods), which expresses green fluorescent protein (GFP) along with microtubule-associated light chain 3(LC3) protein, which decorates the autophagosome surface. This mice model allowed us to visualize autophagic vacuoles by looking for GFPs in the cytosol **(Figure 6)** of the muscle cryosections, as has been used commonly (Mizushima & Kuma, 2008).

Two different muscles fibers where the dysregulated mitophagy has been examined in our study, glycolytic fibers, which is a major component in Extensor Digitorum Longus (EDL) and oxidative fiber, such as soleus. Based on the difference in the muscle atrophy and autophagy activations pathways in these two different muscles, we looked at different responses of the EDL and soleus muscles obtained from lipin1$^{-/-}$ -EGFP-LC3 transgenic mice during starvation. Upon fasting the autophagy seemed to be more pronounced in the EDL versus soleus (Howells et al., 1978). Soleus has a higher expression of PGC1α which protects the oxidative muscle from atrophy by inhibiting activity of FOXO3 (Yamada et al., 2012). In contrast to the oxidative fibers, PGC1α is reduced in the glycolytic fibers which leads to increased activity of FOXO3 and then upregulates autophagy. Several pathways are known to influence the glycolytic muscle fiber during fasting and control muscle mass by regulating protein degradation and autophagy, such as Forkhead box O family (Foxo), transforming growth factor beta (TGFβ) family, autophagy inhibition, and nuclear factor kappaB (NF-κB) (Y. Wang & Pessin, 2013). FOXO3 and autophagy gene mutations have been implicated in altering muscle mass and autophagy (see introduction). Another paper started that the EDL is more vulnerable to initiates mitophagy than soleus by inhibiting autophagy-related

molecules (Yamada et al., 2012). This feature is very valuable for us as it facilitates the study of mitophagy in skeletal muscles and monitors its regulation and activation.

Not only autophagy markers found to present a significant changes in EDL, also mitochondrial damage markers were upregulated too in the lipin1$^{-/-}$ -EGFP-LC3 transgenic mice such as Bnip3 (Figure, 7). Several mitochondrial damage markers have been established in literature, we have looked at major pathways and their markers in a previous study and we reported that only Bnip3 was increased in the lipin1 deficient mice. We also looked at the expression profile of Parkin (a mitochondrial damage marker) and we found no significant response was observed in lipin1$^{-/-}$ -EGFP-LC3 transgenic mice. Therefore, we speculate that the defective mitophagy seen in this new mice model was mediated by Bnip3 pathway. The role of Bnip3 has been controversial among investigators (Vasagiri & Kutala, 2014). Many studies found that Bnip3 was activated in hypoxic condition and then upregulates autophagy (Guo et al., 2001). Other studies revealed that Bnip3 overexpression contributed to the mitochondrial dysfunction by increasing the mitochondrial permeability transition (MPT) pore opening, also leads to mitochondrial fragmentation/fission (Kubasiak, Hernandez, Bishopric, & Webster, 2002; Landes et al., 2010; Vande Velde et al., 2000). Bnip3 role in activation mitophagy can be elucidated by the following potential mechanism (Ney, 2015); 1) Bnip3 causes mitochondrial dysfunctional via increased production of ROS, which then activates autophagy (Scherz-Shouval & Elazar, 2011). 2) Bnip3 can suppress mTOR by binding to Rheb (an activator of mTOR) and upregulates the autophagy (Li et al., 2007). The first mechanism is consistent with our results especially during fasting where we observed a dramatic elevated expression of Bnip3 in the lipin1$^{-/-}$ -EGFP-LC3 transgenic mice, which

leads to dysfunctional mitophagy. We may suggest that increased Bnip3 expression leads to elevated ROS which in turn causes a mitochondrial damage.

Altered mitochondrial membrane potential leads to induction of damage signals and then activates mitophagy. When mitochondria are depolarized, fission and fusion events are disrupted, which eventually affects the mitochondrial function and they are not able to produce enough ATPs to meet cellular demands (Matsuda et al., 2010; Twig et al., 2008). This leads to activate mitophagy and eliminate these damaged organelles. Promoting the mitochondrial membrane potential by some drugs would enhance the performance of dysfunctional mitochondria. We treated the WT and lipin1$^{-/-}$ -EGFP-LC3 transgenic mice with a mitophagy inhibitor Chloroquine diphosphate (CQ) and with an agent which increases the mitochondrial membrane potential, Cyclosporine A (CsA), then monitor the mitophagy rate in these two groups (De Palma et al., 2014; Merlini et al., 2008; Muratsubaki et al., 2017). CQ inhibited the mitophagy in EDL muscle of WT and lipin1$^{-/-}$ -EGFP-LC3 transgenic mice. LC3 and Bnip3 were both upregulated in WT and lipin1$^{-/-}$ -EGFP-LC3 transgenic mice as we detected that in western blotting and immunofluorescent imaging (Figures, 10 and 12). On the other hand, mice injected with CsA, their EDL demonstrated a dramatic decrease in autophagy markers, which might be due to improved mitochondrial function and subsequently the mitophagy was decreased in both WT and lipin1$^{-/-}$ -EGFP-LC3 transgenic mice as we analyzed the samples with immunoblotting and immunostaining (Figures 10 and 12) . The enhanced mitochondrial function with CsA treatment (Bunger, 2013) was robust in the skeletal muscle of the mice with lipin1 deficiency suggesting a new therapeutic approach for patients with muscle atrophy.

The autophagy marker, LC3, requires molecules to act as adaptors to facilitate the interaction between LC3 and engulfed material. One of the common autophagy adaptors is p62/SQSTM1 which is essential for engulfment of the cytoplasmic components by the autophagosomes (Pankiv et al., 2007). In mitochondrial autophagy, some of the major mitochondrial damage markers don't required p62 binding to mitochondria to be engulfed, they rather interact with LC3 directly such as Bnip3. Bnip3 possess an LC3 interacting region (LIR) which allows the molecule to interact with LC3 then facilitates the engulfment (Hanna et al., 2012). We found that protein expression of Bnip3 was increased in the mutant mice (Figure, 10). We further examine whether Bnip3 co-localized with LC3 or not by immunofluorescent staining. The Immunofluorescent imaging revealed that Bnip3 co- localized with LC3 in the EDL muscle of the lipin1$^{-/-}$ - EGFP-LC3 transgenic mice suggesting a direct interaction of Bnip3 with LC3 which is consistent with published studies in literature(Figure, 12) (Hanna et al., 2012).

One potential mechanism where lipin1 deficiency may play a role in defective mitophagy is through altering the mitochondrial lipid composition. As mentioned before, lipin1 converts PA into DAG on the mitochondrial outer membrane. DAG and PA have been shown to be essential for fission and fusion processes (H. Chen et al., 2003; Ha & Frohman, 2014; Huang et al., 2011). Since, lipin1 function is impaired in the lipin1$^{-/-}$ - EGFP-LC3 transgenic mice, this led to abnormal lipids composition of mitochondria and affects the fission/fusion steps then subsequently causes dysfunctional mitophagy.

To further elucidate the role of lipin1 deficiency on impaired mitophagy we looked at the major mitophagy activation pathways. From literature we found that during food depletion regulation of LC3 and Bnip3 genes transcription was through FOXO3

(Mammucari et al., 2007). During fasting, AMPK upregulates FOXO3 (Bujak et al., 2015) and allow its translocation to the nucleus to activate the transcription of the autophagy/mitophagy molecules (Figure, 8). Based on that, we suggest that lipin1 deficiency induced nutrients imbalance which in turn activates AMPK and subsequently leads to increase expression of LC3 and Bnip3 genes. This continuous mitophagy activation results in breaking down the muscular tissue and eventually causes muscle atrophy. So, we attributed the increased LC3 and Bnip3 in the cytosol in the lipin1$^{-/-}$ - EGFP-LC3 transgenic mice was partially due to FOXO3 activation. However, we don't rule out the possibility that the accumulated proteins in the cytoplasm was due to mitophagy blockage.

In conclusion, our study suggests the mechanism by which lipin1 deficiency contributes to the skeletal muscle dysregulated mitophagy and atrophy. Moreover, we demonstrated that the transcriptional factor FOXO3 was upregulated in the lipin1$^{-/-}$ - EGFP-LC3 transgenic mice which can be used a therapeutic approach to reduce mitophagy rate in the skeletal muscle. For future studies, we want to examine 1) how lipin1 deficiency leads to protein degradation; 2) identify whether lipin1 plays any role in mitophagy/lysosomal degradation process; 3) assess mitochondrial membrane lipid composition to understand the effect of lipin1 deficiency.

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