The Role of Mammalian Mitofusin-2 (Mfn-2) in Lipid Metabolism

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Abstract

The architecture of mitochondria is closely associated with numerous functions for cell signaling, growth and senescence. The overall phenomenon of mitochondrial dynamics, which include fusion and fission events, characterizes the fundamental mechanism governing the cell's bioenergetic needs. Mitofusins are fusogenic proteins that have emerged as key regulators of diverse functions such as respiration, mitochondrial biogenesis, and energy homeostasis. Thus, mitochondrial dynamics and bioenergetics together control the energy demand to supply ratio. Mitofusins (1 and 2) are both intricately associated with mitochondria and mitochondrial associated membranes (MAMs). These organellar communication sites drive and regulate mitochondrial metabolism and energy homeostasis. Although both mitofusin-1 (Mfn-1) and mitofusin 2 (Mfn-2) share some common roles, Mfn-1 is primarily involved in the fusion of the outer mitochondrial membrane, while Mfn-2 primarily affects mitochondrial metabolism by controlling the electron transport chain, fuel oxidation and mitochondrial membrane potential.

The research presented in this thesis centers around the Mfn-2 fusion protein. Specifically, it focuses on the change in mitochondrial morphology and lipid content in the absence of Mfn-2. It was observed that the knockout of Mfn-2 in mouse embryonic stem fibroblasts (MEFs) drastically altered mitochondrial morphology and simultaneously increased lipid droplet size but not number. When cells were provided a further substrate for triglyceride synthesis i.e. oleic acid, the Mfn-2 KO MEFs showed an enhanced capacity to increase the number of lipid droplets compared to WT cells. In Mfn-2 knockout MEF cells, the ability to undergo adipogenesis is enhanced compared to WT MEF cells. Surprisingly we also observed that adipogenesis was induced with control, non-adipogenic media, supplemented with a high concentration (20%) of fetal bovine serum. In conclusion, the data suggest that Mfn-2 is a crucial protein controlling mitochondrial morphology, which has a major role in maintaining cellular homeostasis and lipid metabolism.

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LIST OF ABBREVIATIONS

AAT	Acyl CoA Acyltransferase
ACAT	Acyl CoA:Cholesterol Acyltransferase
ADRP	Adipose Differentiation-Related protein
AIF	Apoptosis Inducing Factor
ATP	Adenosine Triphosphate
C/EBP	CCAAT/Enhancer Binding Protein
cAMP	Cyclic Adenosine Monophosphate
CE	Cholesterol Ester
CMT2A	Charcot-Marie-Tooth Type 2A
CMV	Cytomegalovirus
CoA	Coenzyme A
DG	Diacylglycerol
DGAT	Acyl CoA:Diacylglycerol Acyltransferase
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
Drp1	Dynamin Related Protein 1
E. coli	*Escherichia coli*
ER	Endoplasmic Reticulum
FA	Fatty Acid
FAR	Fatty Acyl CoA Reductase
Fis1	Fission 1 Protein
G3P	Glycerol-3-Phosphate
GPAT	Glycerol Phosphate Acyl CoA Acyltransferase
Grp78	Glucose related protein 78
HDL	High Density Lipoprotein
HEK 293T	Human Embryonic Kidney 293T Cell Line
Hsp70	Heat Shock Protein 70
IDL	Intermediate Density Lipoprotein

IP	Immunoprecipitation
kDa	Kilodalton
LDL	Low Density Lipoprotein
MAM	Mitochondria Associated Membrane
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse Embryonic Fibroblasts
Mfn	Mitofusin
Mfn-1	Mitofusin-1
Mfn-2	Mitofusin-2
MG	Monoacylglycerol
MGAT	Acyl CoA:Monoacylglycerol Acyltransferase
MOM	Mitochondrial Outer Membrane Protein
mtDNA	Mitochondria DNA
NP-40	Nonyl Phenoxypolyethoxylethanol 40
OA	Oleic Acid
OD	Optical Density
Opa1	Optic Atrophy 1
PA	Phosphatidate
PAGE	Polyacrylamide Gel Electrophoresis
PARP1	Poly (ADPribose) Polymerase1
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PFA	Paraformaldehyde
PPAR	Peroxisome Proliferator Activated Receptor
PS	Phosphatidylserine
RAB7	Ras Associated Protein 7
ROS	Reactive Oxygen Species
siRNA	Small Interfering RNA

SE	Sterol Esters
TAG	Triacylglycerol
TMD	Transmembrane Domain
VLDL	Very Low Density Lipoprotein

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1. INTRODUCTION

Obesity is at epidemic proportions in many regions of the world and is thought to result from a variety of factors including stressful lifestyles, poor eating habits, and inactivity. According to Statistics Canada (2010), the percentage of obese people in North America is 25% and in Canada it is 30%. The percentage of overweight people is 60% and 66% in North America and Canada, respectively (Haffner, 2006; Hensrud and Klein, 2006; Francischetti and Genelhu, 2007; Hill *et al.*, 2007). Alarmingly, the rate of obesity in adolescents and children is increasing, suggesting the problem will only worsen over the next decade (Dehghan *et al.*, 2005; Belanger-Ducharme and Tremblay, 2005; Wyatt *et al.*, 2006; Orio *et al.*, 2007). Many obese people develop insulin resistance, which can lead to diabetes mellitus Type 2. Insulin resistance is also an underlying factor for other pathologies such as atherosclerotic coronary heart conditions, hyperlipidemia, and liver malfunction (Tremblay *et al.*, 2002; Katzmarzyk, 2002; Belanger-Ducharme and Tremblay, 2005; Tjepkema, 2005).

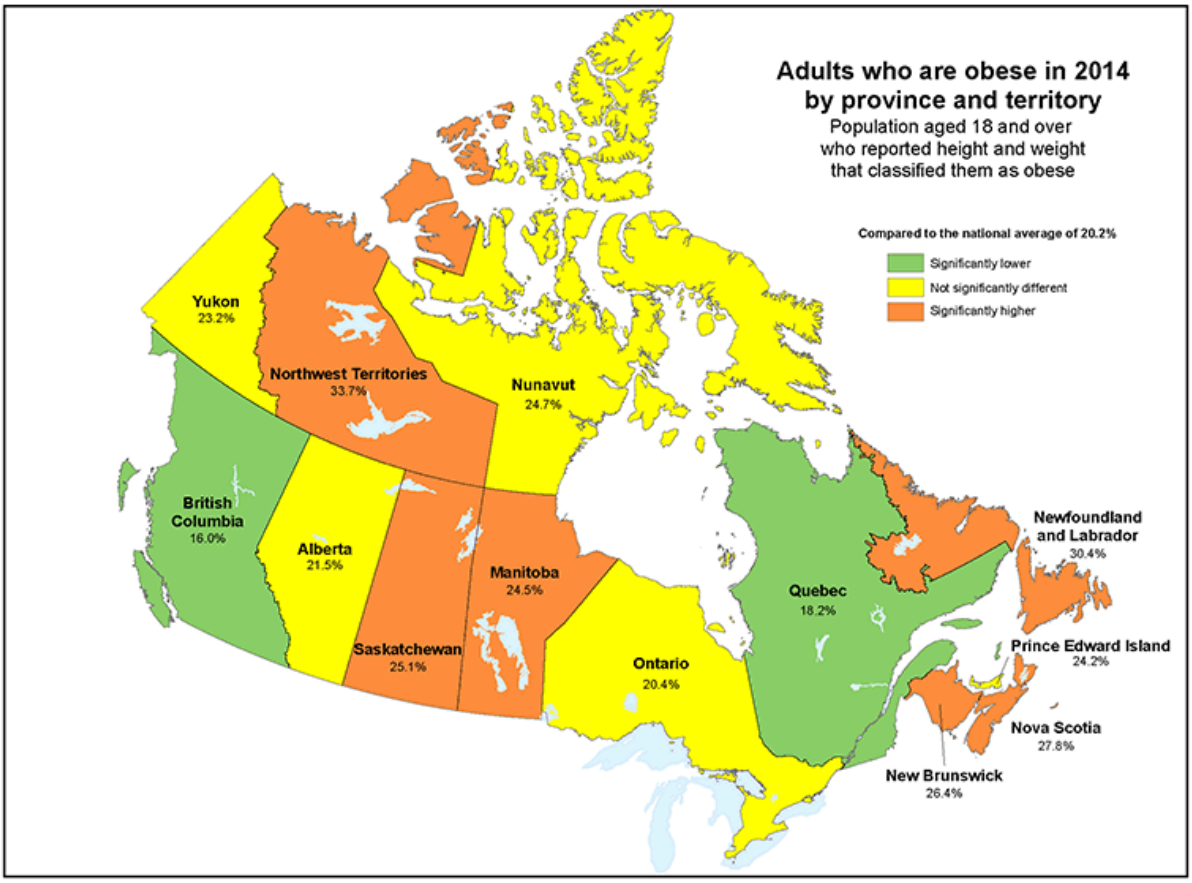
Mitochondria play a crucial role in lipid and energy metabolism. Imbalances in metabolic pathways could cause disruption of cellular energy expenditure, substrate metabolism, and reactive oxygen species disposal, which in turn can lead to type 2 diabetes and obesity (Bournat and Brown, 2010). For example, mitochondrial dysfunction in adipocytes leads to excessive accumulation of lipids, which ultimately leads to obesity and is associated with several pathologies such as hyperlipidemia and atherosclerosis (Santel, 2006). Recent studies suggest fatty liver and its inflammation could cause stomach cancer (Rajala and Scherer, 2003; Berg, and Scherer, 2005; Bugianesi, 2005).

1.1 Obesity: A Canadian and Global Health Issue

Obesity is defined as excessive fat accumulation in the body. A body is considered as overweight if the body mass index (BMI) exceeds 25, while the condition is referred to as clinical obesity if BMI is greater than or equal to 30. According to WHO in a 2014 report (www.who.int/mediacentre/factsheets), the world is experiencing a severe epidemic of obesity and its related disorders, with more than 1.9 billion adults being reported as overweight and 600 million as clinically obese. Moreover, the prevalence of obesity has consistently been increasing and has nearly doubled since 1980. The problem also appears to be starting at an earlier age. For example, worldwide obesity and overweightness in children under the age of 5 reached 42

million in 2013 (de Onis *et al.*, 2010). Two factors that are mainly responsible for the obesity epidemic are increased consumption of energy dense foods (especially with high-fat content) and physical inactivity due to a rise in the desk-bound nature of workplaces. Obesity leads to other health issues, including some common non-communicable medical conditions such as cardiovascular disease, musculoskeletal disorders (especially osteoarthritis) and some forms of cancer, including breast and colon cancer. In 2012, heart disease and stroke were the foremost cause of mortality. Becoming overweight at an early age is strongly associated with an increased risk of premature death or disability, and hypertension in adulthood. Thus, the alarming increase in obesity is leading to a subsequent increase in a variety of non-communicable diseases.

Obesity in Canada is a rising health issue, which is anticipated to exceed smoking as the top reason of preventable mortality (Eisenberg *et al.*, 2011). A recent (2014) health survey done in Canada in adults over the age of 18 suggests that the percentage of obese people is growing rapidly for both men and women (www.statcan.gc.ca). For overweight or obese men, the percentage increased from 57.3% (2003) to 61.8% (2014), and for females, the percentage increased from 41.3% (2003) to 46.2% (2014). Obesity in Canada rose to approximately to 20.2% in 2014, from 15% in 2003. A regional snapshot of the obesity rates as of 2014 is shown in Figure 1.1. The proportion of obese adults was highest in The Northwest Territories at 33.7%. The Canadian provinces or territories, in descending order of obese adults, are as follows: The Northwest Territories (33.7%), Newfoundland and Labrador (30.4%), Nova Scotia (27.8), New Brunswick (26.4%), Saskatchewan (25.1%) and Manitoba (24.5%). These compare to the Canadian average of 20.2%. The report suggests two critical reasons for weight gain; a lack of food security (especially fresh and nutritious food), and a lack of physical activity. It also highlighted that Canadian children (5%) and adults (8%) were facing food security issues, in that they were unable to access sufficient varieties of food, due to affordability. A study revealed that current BMI of Canadians aged 20-39 is that of the people aged 40+ three decades ago (Shields *et al.*, 2010). A similar study was performed in 2010 to quantify the economic burden of obesity. It concluded that the direct Canadian national economic burden of obesity alone was $6 billion in Canada, which was 4.1% of the national health care budget. The WHO along with many other organizations around the globe has determined obesity to be a chronic illness.



Source: Statistics Canada, Canadian Community Health Survey (CCHS), 2014.

Figure 1.1. Canadian Regional Breakdown of Obese Adults in 2014.

Provincial/Territorial breakdown of the percentage of obese adults in the year 2014. Figure was adapted from Statistics Canada, 2014 (www.statcan.gc.ca).

2. LITERATURE REVIEW

2.1 Lipid Droplets

In eukaryotic organisms, the major form of stored energy is triacylglycerol (TAG), which is a neutral lipid. Excessive accumulation of TAG as lipid droplets in adipocytes is the cellular basis for obesity. In most eukaryotes, TAG and sterol esters (SE) are the main components of the lipid droplet core. Apart from TAG and SE, neutral lipids also include free cholesterol, retinol esters, and a small fraction of xenobiotic hydrophobic chemicals like aromatic hydrocarbons (Brown, 2001; Greenberg and Obin, 2008). The core is further enveloped by a monolayer of lipids which are polar in nature (Tauchi-Sato *et al.,* 2002). Lipid droplets also contain some attached and embedded proteins within the envelope (Figure 2.1). Previously, lipid droplets were considered only as a storehouse of energy and inert in cellular functioning. However, a breakthrough study identified a protein named perilipin which precisely localized to the surface of lipid droplets (Greenberg *et al.,* 1991). This research work opened the doors to study lipid droplets as dynamic organelles. A study described a clear genome-wide RNAi screen in *Drosophila melanogaster* to study the dynamic nature of lipid droplets in cellular energy metabolism (Guo *et al.,* 2009).

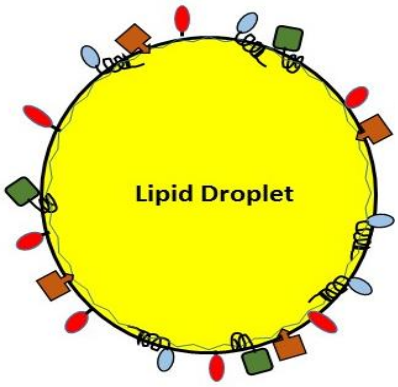


Figure 2.1. The General Architecture of Lipid Droplets.
A spherical lipid droplet containing neutral lipids enveloped by a phospholipid monolayer. Several proteins associated with lipid droplets are either embedded in the phospholipid layer or attached externally to the surface. For example, the well-known and characterized lipid droplet surface proteins are family of perilipin (PLIN). These consists of perilipin 1 (PLIN1), perilipin 2 (ADRP), perilipin 3 (TIP47), perilipin 4 (S3-12) and perilipin 5 (OXPAT/ LSDP5/ MLDP). Apart from PLIN family several other families of proteins from the lipid droplet coat are involved in vesicle docking, membrane trafficking, endocytosis and exocytosis.

Even though the lipid droplet's significance in cellular energy metabolism and pathologies is gaining appreciation, it still remains understudied. The latest functional and proteomic analyses (Bartz *et al.,* 2007; Currie *et al.,* 2014) of lipid droplets suggests a fundamentally new perspective of lipid droplets (Guo *et al.,* 2009; Cohen *et al.,* 2004; Vance, 2003).

Increased levels of TAG and thus lipid droplets are of significant concern. For example, when the lipid droplets increase in hepatocytes, leading to a condition called steatosis, it can lead to fatty liver disease which can give rise to cirrhosis and liver failure (Neuschwander-Tetri, 2007; Saadeh, 2007). It is estimated that about 75% of obese people have fatty liver (Reddy and Rao, 2006). When levels of TAGs are high in the blood, it may lead to atherosclerosis (Rosenbaum *et al.,* 1997). Even in non-adipose tissue, when the levels of TAGs increase, this can lead to insulin resistance and type 2 diabetes (Friedman, 2002; Unger, 2002). Recently the importance of lipid droplets was underscored by the finding that they were found to interact with essential cellular organelles such as endoplasmic reticulum, mitochondria, plasma membrane, and endosomes, and coordinated with these organelles to dissipate metabolic energy for biochemical processes (Guo *et al.,* 2009). The size, number and distribution pattern of lipid droplets in cells strongly associate with several life-threatening pathologies such as myocardial infarction and diabetes (Schaffer, 2003). Therefore, the dynamic nature of lipid droplets and its significance in pathologies is gaining appreciation by the scientific community, and lipid droplets are now considered cellular organelles.

2.1.1 Cellular Functions of Lipid Droplets

Lipid droplets are widespread among eukaryotic cells and have occasionally been observed in prokaryotic cells as well (Alvarez *et al.,* 1996). Adipocytes are the predominant cells that store energy, in the form of one large lipid droplet per cell, which can comprise up to 90% of the cell volume (Frühbeck *et al.,* 2001). Contrary to the established belief that lipid droplets are inert particles, they have been found to be highly dynamic organelles. Lipid droplets buffer the energy fluctuations in and around the cells. For example, regulation of the size and number of lipid droplets may contribute to overall cellular energy homeostasis (Kohlwein *et al.,* 2013). In order to avoid lipotoxicity in times of excess fatty acid availability, the lipid droplets become a storehouse for fatty acids in the form of TAG. When low energy conditions prevail,

lipid droplets can release lipids to be utilized as energy sources (Brasaemle, 2007; Ducharme and Bickel, 2008). The mechanisms involved in these processes remain poorly understood (Listenberger *et al.*, 2003).

Lipid droplets also regulate cellular functions apart from lipid metabolism and energy homeostasis, such as cellular lipid and protein trafficking. They have been shown to serve as a repository for the vast amounts of histones in *Drosophila melanogaster* embryos, thus serving as protein depots during embryogenesis (Cermelli *et al.*, 2006). Moreover, they sequester toxic unfolded proteins such as α-synuclein until they are degraded (Cole and Murphy, 2002). Lipid droplets play a crucial role in the reproduction of various pathogens and are determinants of the onset of the various pathophysiological diseases. For example, hepatitis C virus particles bind to cell membrane lipid droplets during their life cycle (Miyanari *et al.*, 2007), and thus may serve as a potential drug target to block viral reproduction. Also, the cellular status of lipid droplets is known to vary in different pathological conditions. For example, excessive accumulation during obesity can lead to steatosis and type 2 diabetes, while the accumulation of sterol esters in macrophages can lead to atherosclerosis (Brown *et al.*, 1979).

2.1.2 Lipid Droplet Formation

In eukaryotic organisms, the formation of lipid droplets appears to take place in the endoplasmic reticulum (ER). While direct visualization of the initial stages of the lipid droplet formation process are lacking, lines of evidence support a model whereby lipid droplets are derived from the ER. For example, most of the enzymes involved in triacylglycerol or sterol ester synthesis localize to the ER. Moreover, electron microscopy data reveal close apposition between lipid droplets and the ER (Robenek *et al.*, 2005; Londos *et al.*, 1995; Wilfling *et al.*, 2013). In yeast, lipid droplets are found adjacent to the ER membrane (Szymanski *et al.*, 2007), which led to the proposal that lipid droplets derive from the neutral lipid accumulation between the two leaflets of the ER membranes and bud off from the ER, taking with it the outer leaflet of the membrane (Figure 2.2 A) (Ohsaki *et al.*, 2008). Although it is still largely unknown as to which proteins are involved in the budding process and regulation, the key enzymes synthesizing the core components (TAG and cholesterol esters (CE)) of lipid droplets are present in ER membranes. On the other hand, some proteins such as seipin (Boutet *et al.*, 2009) or PAT-protein TIP47 (Bulankina *et al.*, 2009) have been identified as being involved in lipid droplet formation.

Genome-wide screening suggested that there may a group of proteins that work together to regulate lipid droplet formation (Guo *et al.*, 2008; Szymanski *et al.*, 2007). A distinct model for lipid droplet formation was proposed by Ploegh (2007) termed the bicelle model (Figure 2.2 B). In this model, neutral lipids accrue between the ER leaflets and then, instead of budding, nascent lipid droplets are eliminated from the ER membrane, removing phospholipids from both the cytosolic and luminal leaflets. There have been recent efforts to identify whether there are specialized regions in the ER which are responsible for lipid droplet formation. The lipid droplet formation model is based on the fact that neutral lipid synthesizing enzymes such as Diacylglycerol acyltransferase (DGAT) are found in the punctuate pattern across the ER (Shockey *et al.*, 2006). Freeze-fracture studies revealed that lipid droplets never completely detach from the ER and remain surrounded by the ER membrane in an egg-shaped cup (Robenek *et al.*, 2006).

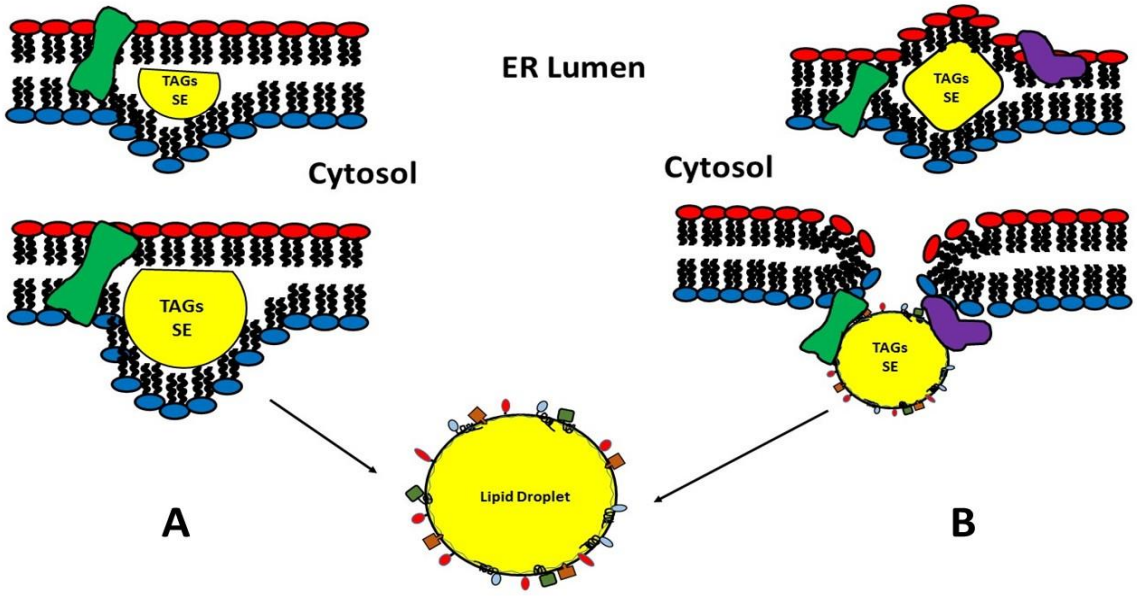


Figure 2.2. Models of Lipid Droplet Formation.
Two models of lipid droplet formation. A) Triglycerides and sterol esters accumulate between two leaflets of the endoplasmic reticulum membrane. After reaching an appropriate size, the lipid droplet buds off the membrane. According to this model (Farese and Walther, 2009), the lipid droplet membrane is an extension of the ER membrane and therefore carries various proteins on its surface. B) This model (Ploegh, 2007) is described in detail (Guo *et al.*, 2009; Czabany *et al.*, 2008) and is known as bilayer excision. As opposed to the first model, the entire lipid droplet buds off from the ER, leaving a temporary hole in the ER membrane. According to this model, the ER membrane might leak some of its content into the cytosol. (Adapted from Guo *et al.*, 2009) TAGs: Triacylglycerol, SE: Sterol Esters.

2.1.3 Lipid Droplet Synthesizing Enzymes

The glycerol phosphate pathway, also known as Kennedy pathway, defines the progressive esterification of fatty acyl coenzyme A (CoA) molecules to a glycerol backbone, generating TAG (Kennedy, 1957). The last important step in this pathway is the conversion of diacylglycerol (DAG) to TAG. This step is accomplished via the establishment of an ester bond between the free hydroxyl group of DAG and a long chain fatty acid (Figure 2.3). The reaction is considered the last step in triglyceride synthesis and thus important for adipose tissue formation. This reaction is catalyzed by the microsomal enzyme DGAT (EC 2.3.1.20). In mammalian cells, diacylglycerol acyltransferase (DGAT) enzymes (DGAT1 and DGAT2) are known to play crucial roles in TG biosynthesis. Two DGAT genes have been identified which code for distinct protein products. In addition, while both DGAT1 and DGAT2 catalyze the same reaction *in vitro*, they are very structurally dissimilar (Cases *et al.*, 1998; Stone *et al.*, 2006).

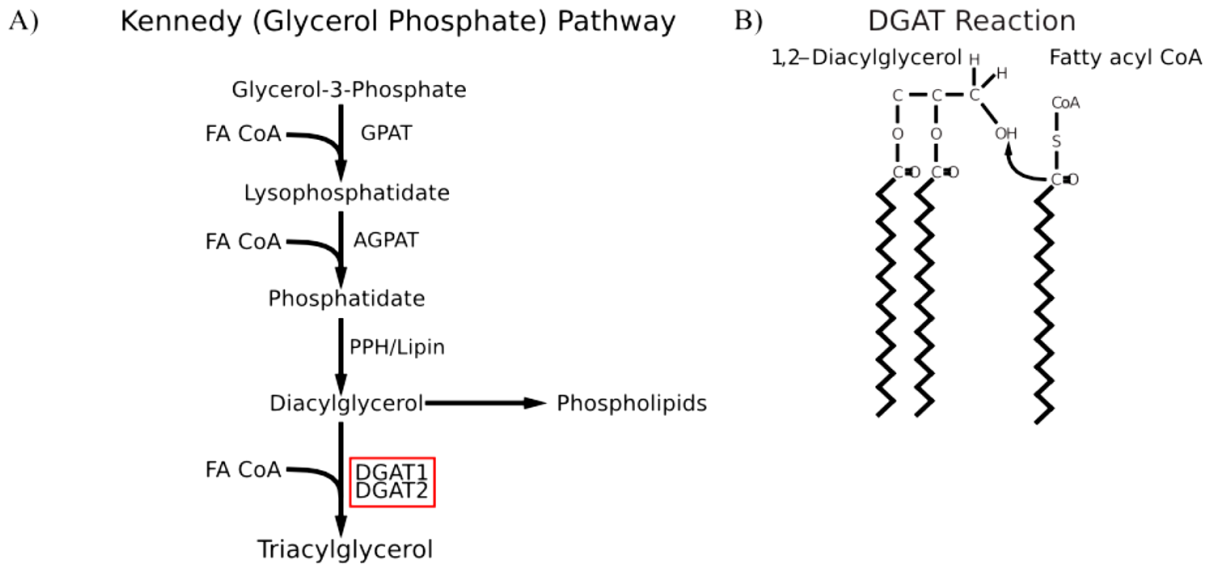


Figure 2.3. Triglycerol Formation.

A) DGAT is responsible for catalyzing the crucial last step in the glycerol phosphate pathway where diacylglycerol is acylated to form triacylglycerol. B) Mechanism of DGAT catalyzing the formation of an ester bond between a long chain fatty acid and the free hydroxyl group of diacylglycerol (Figure from Gibellini and Smith, 2010).

DGAT1 is a member of a large family of membrane-bound O-acyltransferases (MBOAT) that includes ACATs. DGAT2 belongs to the DGAT2/acyl CoA:monoacylglycerol acyltransferase (MGAT) family that includes several monoacylglycerol acyltransferases and a wax synthase (Cases *et al.*, 1998; Farese *et al.*, 2000; Hofmann, 2000; Buhman *et al.*, 2001; Cases *et al.*, 2001; Yen *et al.*, 2002; Cao *et al.*, 2003; Cheng *et al.*, 2003; Yen and Farese, 2003; Turkish and Sturley, 2007). DGAT1 and DGAT2 are highly expressed in white adipose tissue, and play a role in TAG metabolism. They are expressed to a lesser extent in the mammary gland, liver, skeletal muscle and small intestine. (Cases *et al.*, 1998, 2001; Kuerschner *et al.*, 2008; Shockey *et al.*, 2006). DGAT1 appears to be a major modulator of energy homeostasis while DGAT2 is responsible for the bulk synthesis of TAG. Despite the fact that both DGATs catalyze the same reaction and demonstrate similar localization to the ER, these enzymes have separate roles in lipid metabolism. DGAT1 knockout mice are viable but have only 50% of the adipose mass of wild-type mice, whereas DGAT2 knockout mice die shortly after birth (Oelkers *et al.*, 2002; Sorger and Daum, 2002; Stone *et al.*, 2004).

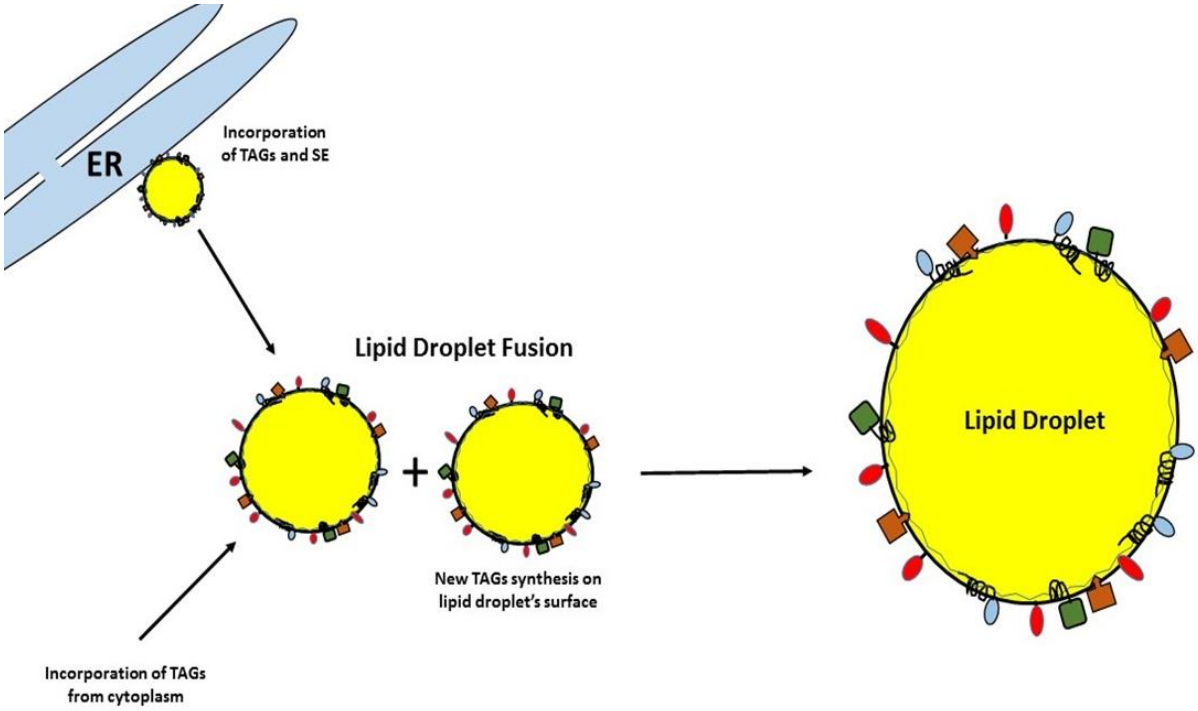


Figure 2.4. Models of Lipid Droplet Formation and Expansion.

DGAT and ACAT enzymes fill nascent lipid droplets with neutral lipids. Lipid droplets can be attached to or detached from the ER. They also grow by the fusion process.

During adipogenesis, localized budding of lipid droplets occurs at several loci throughout the cell. These lipid droplets grow by expansion or fusion (coalescence) into a single larger droplet (Figure 2.4) (Song *et al.,* 2002). When neutral lipids accumulate within the endoplasmic reticulum lipid bilayer, they form nascent lipid droplets (Robenek *et al.,* 2004; Wolins *et al.,* 2006). During lipid droplet biosynthesis, nascent lipid droplets could remain attached to the ER, where they are filled with TAGs by DGAT or they could separate from the ER (Figure 2.4). Enzymes involved in neutral lipid synthesis are found in the endoplasmic reticulum. DGAT2 is known to interact with lipid droplets and also associate with the MAM (Kuerschner *et al.,* 2008; Stone *et al.,* 2009). Several enzymes involved in lipid biosynthesis, including DGAT2, are enriched in mitochondria-associated membranes (MAM), which are specialized domains of the ER that are in physical contact with mitochondria. In eukaryotic cells, the majority of membrane phospholipids are synthesized on ER membranes, which are subsequently trafficked to other cellular organelles by unknown mechanisms (Voelker, 2003; 2009).

2.1.4 Lipid Droplet Breakdown

The process of lipid droplet breakdown (lipolysis) is better understood relative to lipid droplet biogenesis. Lipolysis is induced under conditions of high-energy need or when there is metabolite need for membrane synthesis. Several hormones are known to induce lipolysis, such as glucagon, ghrelin, cortisol, testosterone, epinephrine and norepinephrine. However catecholamines are the most potent stimulator through β-adrenergic receptors. These hormones trigger G protein-coupled receptors on the cell surface, which further stimulate signal transduction by activating adenylate cyclase, resulting in increased production of cAMP, which activates PKA. PKA activation is a crucial step since it regulates several other proteins involved in the process of lipolysis. Perilipin, an important regulator of basal and induced lipolysis, is one of those proteins that is phosphorylated by PKA. Under basal conditions, perilipin protects the surface of lipid droplets by localizing to the lipid droplet surface and shielding the droplet's surface from lipases (Figure 2.5). However, once the lipolysis starts, perilipin helps to gather the required machinery on lipid droplets in a phosphorylation-dependent manner (Brasaemle, 2007; Tansey *et al.,* 2001). In the basal state, perilipin binds to comparative gene identification-58 (CGI-58) protein product. CGI-58 is a coactivator of adipose triglyceride lipase (ATGL). Upon phosphorylation, perilipin induces the release of CGI-58, which then forms a complex with

ATGL. This process activates ATGL, which catalyzes the cleavage of the first fatty acyl chain from TG, thus initiating TG hydrolysis on the surface of lipid droplets (Lass *et al.*, 2006; Zimmermann *et al.*, 2009).

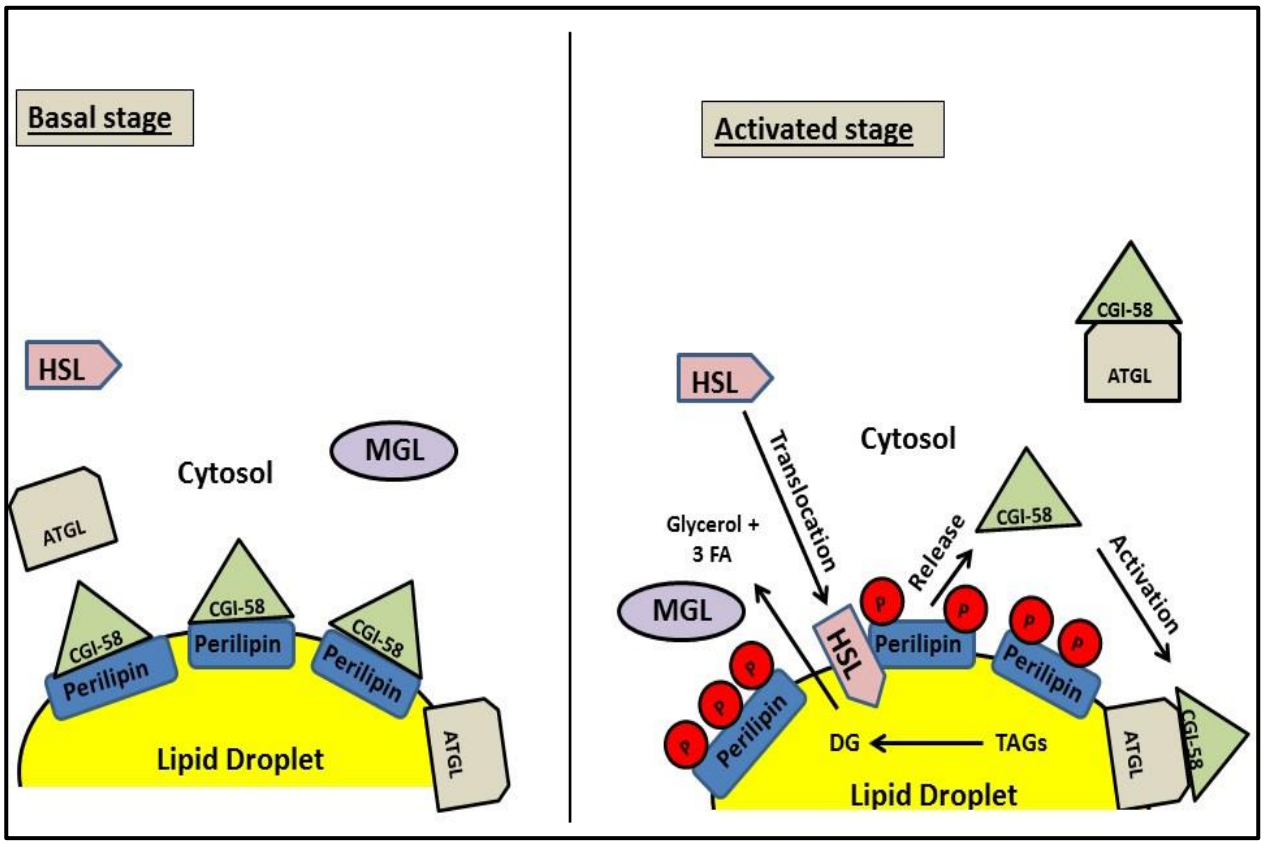


Figure 2.5. Regulation of Lipolysis.

In the basal state, neither HSL nor ATGL are bound to lipid droplets, which maintains the overall low lipase activity by low functional ATGL and HSL. When CGI-58 is bound to perilipin, this complex prevents activation of ATGL by unbound CGI-58. In the activated state, perilipin is phosphorylated, and this phosphorylated perilipin does not bind CGI-58, thus releasing CGI-58, which further activates ATGL and HSL. ATGL hydrolyzes one acyl chain from triglyceride, producing a diglyceride. This diglyceride acts as a substrate for HSL. HSL hydrolyzes another acyl chain, producing a monoacylglycerol and free fatty acid. Finally, monoacylglycerol hydrolase converts monoacylglycerol into glycerol and free fatty acid (Adapted from Frühbeck *et al.*, 2014).

Hormone-sensitive lipase (HSL) is another important target for PKA phosphorylation. HSL removes the second fatty acyl chain from the TAG backbone. Upon phosphorylation, HSL is targeted to lipid droplets, where it interacts with phosphorylated perilipin (Sztalryd *et al.*, 2003). Monoacylglycerol lipase removes the last acyl chain. The released glycerol can be further exported to the liver for further metabolism (Fredrikson *et al.,* 1986). The released free fatty acyl chains are used as a substrate for β-oxidation to generate energy in the form of adenosine-5'-triphosphate (ATP) in mitochondria or peroxisomes.

Lipid droplets are often located in proximity to several organelles, particularly the ER (Ozeki *et al.,* 2005; Turro *et al.,* 2006), endosomes (Liu *et al.,* 2007), mitochondria (Sturmey *et al.,* 2006) and peroxisomes (Binns *et al.,* 2006; Schrader, 2001). In each instance, a functional connection exists between lipid droplets and these organelles. However, only peroxisomes and mitochondria can use fatty acids released from hydrolyzed triglycerides to produce ATP by β-oxidation. The close apposition between lipid droplets and mitochondria has been well documented (Sturmey *et al.,* 2006). It was suggested that the regions of direct membrane contact between lipid droplets and these organelles facilitate the direct transfer of fatty acyl chains into mitochondria and peroxisomes (Murphy *et al.,* 2009). The molecular mechanism of breakdown of neutral lipids in the lipid droplet core by lipases is unknown. Two different theories have been put forward. The first theory suggests the possibility of catalytic sites of lipases getting access to lipid droplet core (Farese and Walther, 2009). The second theory suggests that due to the surface composition of lipid droplet's phospholipid, a small segment of neutral lipids might constantly be getting exposed to the catalytic site of lipases, which are bound to the droplet.

Lipid droplet fragmentation, where larger lipid droplet breaks down into smaller ones during lipolysis, may be one of the ways lipase getting access to lipid droplet's interior since it would result in a broader surface area and more contact sites. In one study, fragmentation of lipid droplets occurred only in cells that were treated with β-receptors agonists (Brasaemle *et al.,* 2004). A novel pathway for lipid droplets degradation was discovered in hepatocytes (Singh *et al.,* 2009). Under starvation conditions, lipid droplets are degraded by a process known as macroautophagy. Autophagosomes partially or wholly sequester lipid droplets and hydrolyze them in lysosomes for energy purposes. It is yet to be understood how macroautophagy works to maintain lipid droplet homeostasis and whether this process is preserved among different cell types.

2.2 Mitochondria

2.2.1 Role of Mitochondrial Dynamics in the Cell

The mitochondrion is a semi-autonomous organelle because it contains circular DNA and ribosomes for protein synthesis, whereas its division is dependent on signaling from nuclear DNA. Mitochondria play a significant role in the production of ATP by oxidative phosphorylation. They are involved in many catabolic and anabolic pathways, such as the β-oxidation of fatty acids, the oxidation of acetyl CoA through the TCA cycle, and the biosynthesis of essential biomolecules (such as phospholipids). Research using yeast genetic screens has revealed new proteins involved in regulating the size, shape, dynamics and energy flow in mitochondria (Dimmer *et al.,* 2002; Scott *et al.,* 2003). Mitochondria are dynamic in shape. They adopt a variety of shapes from small, rounded up spheres to elongated interconnected tubules. Mitochondria achieve these distinct shapes and sizes by the continual processes of mitochondrial fission and mitochondrial fusion (Figure 2.6). The diverse functions of mitochondria are attributed in part to their morphology, which in turn is regulated by the activities of the molecular machines involved in fusion and fission. This dynamic behavior helps the cell to survive and respond quickly to its continuously changing physiological and biochemical conditions (Nakada *et al.,* 2002). A shift toward fusion favors the formation of the interconnected mitochondrial network, which is critical for metabolically active cells, as they channel the dissipation of energy (Skulachev 2001). On the contrary, a shift towards fission produces numerous mitochondrial fragments which are often seen as numerous short rods or small spheres, a characteristic of dormant or sluggish cells (Collins *et al.,* 2002). For example, when embryonic stem cells undergo differentiation, the mitochondrial network becomes elongated (Chung *et al.,* 2007). During formation of the synapses in neurons, mitochondrial division is increased to establish a new mitochondria in protruding neurons (Li *et al.,* 2004). Moreover, whenever an apoptotic signal is triggered in a cell, the mitochondrial network undergoes fragmentation to facilitate remodeling of cristae to release cytochrome c (Youle and Karbowski, 2005).

A particular group of proteins mediate the processes of mitochondrial fusion and fission (Hales and Fuller, 1997; Chen *et al.,* 2003). Whenever this dynamic is disturbed, it affects cellular functions since this dynamic mitochondrial morphology is involved in several cellular processes

such as apoptosis (Frank *et al.*, 2001; Karbowski and Youle, 2003; Bossy-Wezel *et al.*, 2003). For example, when mitochondria lack the ability to fuse normally, respiratory capacity is reduced, and as a result cell growth slows. It has been demonstrated that a mouse with a complete deficiency in mitochondrial fusion either by deleting Mfn-1 or Mfn-2 is embryonically lethal (Chen *et al.*, 2003, 2005). Charcot-Marie-Tooth syndrome, a neurodegenerative disease in humans, is linked to a mutation in the Mfn-2 gene which codes for a mitochondrial fusion protein. Conversely, when the optic atrophy 1 (OPA1) gene encoding a mitochondrial fusion protein is mutated, autosomal dominant optic atrophy ensues (Alexander *et al.*, 2000; Zuchner, 2004). Thus, both mitochondrial fusion and fission proteins play a critical role in energy metabolism. It has been shown that silencing Mfn-2 in fibroblast cells leads to a reduction in oxygen consumption and glucose oxidation (Kawalec *et al.*, 2015). Meanwhile, in L6E9 muscle cells, decreased rates of pyruvate or palmitate oxidation were associated with Mfn-2 repression (Bach *et al.*, 2003, 2005). Although the role of mitochondrial dynamics in cellular processes and energy metabolism is well established, its role in lipid metabolism and lipid droplet dynamics remains to be elucidated.

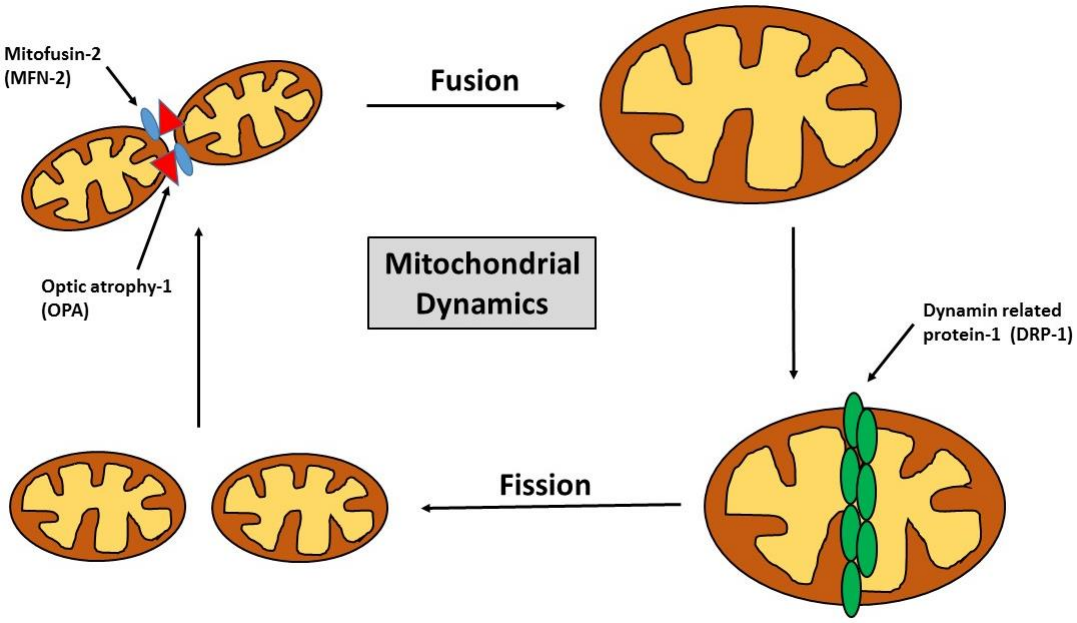


Figure 2.6. Mitochondrial Dynamics of Fusion and Fission.

Mfn-2 and Opa1 are mitochondrial fusion proteins located on the mitochondrial outer and inner membrane respectively. These proteins assist in fusing two mitochondria. Drp1 is another protein that is involved in the mitochondrial fission process.

2.2.2 Mitochondrial Fission

Fission is the separation of an elongated mitochondrion from 3-30 μm into two or more segments, some as small as 0.5-0.7 μm. Fission is a continuous process and is essential for various cellular processes. It ensures that mitochondria are distributed to regions of the cell in which energy consumption is considerable (Chang and Reynolds, 2006). Fission also facilitates the allocation of mitochondria in the mitotic and meiotic cells, as newer mitochondria rapidly divide and easily translocate into the new daughter cells (Jagasia *et al.,* 2005; Youle and Karbowski, 2005). Mitochondrial fission is carried out by an assembly of proteins. The most important of these are Fis1 and Drp1 (dynamin-related protein 1) (Karbowski *et al.,* 2004). Drp1 is a cytosolic protein. However, a mini-reserve is contained in punctate spots on mitochondrial strands, and a subsection of these spots indicate future sites of fission (Smirnova *et al.,* 2001). Inhibition of Drp1 by RNAi leads to extended length and interconnectivity of mitochondrial strands (Lee *et al.,* 2004, Smirnova *et al.,* 2001). Fis1 is another crucial factor of the mammalian mitochondrial fission machinery. Studies have provided evidence showing that increased levels of Fis1 promote mitochondrial fragmentation while decreased levels of both Fis1 and Drp1 promote elongation (James *et al.,* 2003; Stojanovski *et al.,* 2004; Santel and Fuller, 2001). Fis1 is small protein (16 kDa) that is evenly distributed on the outer surface of mitochondria. Fis1 is embedded in the outer membrane of mitochondria via a C-terminal transmembrane domain. The bulk of protein extends out into the cytosol, with this domain consisting of six antiparallel helices that forms a helical bundle (Dohm *et al.,* 2004; Suzuki *et al.,* 2005).

The mechanism whereby mitochondrial fission occurs is poorly understood in eukaryotes, but in yeast, the fission assembly complexes are well-studied (Griffin *et al.,* 2005; Tieu and Nunnari, 2000; Zhang and Chan, 2007). Fis1 and Drp1 are essential components of the mitochondrial fission machinery. Drp1 is present on OMM as well as in cytosol. As mentioned above, a subset of the sites on the mitochondria where Drp1 resides become actual fission sites. Drp1 interacts with Fis1 via another factor called Mdv1 (Ingerman *et al.,* 2005; Okamoto and Shaw, 2005). Interaction of Fis1 and Drp1 and other fission assembly factors activate fission (James *et al.,* 2003; Yoon *et al.,* 2003). Dnm, a homolog of Drp1, covers and constricts mitochondria, thereby carrying out the process of fission (Roux *et al.,* 2006). Association of Drp1 with other fission machinery leads to constriction at fission sites on mitochondria, which eventually produces two separate organelles. Four Drp1 receptors proteins are found on the

mitochondrial surface; mitochondrial fission factor (MFF), mitochondrial fission 1 (Fis1), and Mitochondrial dynamics proteins 49kDa (MID49) and 51kDa (MID51) (Mishra and Chan, 2014). MFF, MID49, and MID51 have key roles in fission, while Fis1 play a lesser role (Loson *et al.*, 2013, Otera *et al.*, 2010).

2.2.3 Mitochondrial Fusion

In the process of mitochondrial fusion, first the outer membranes of two mitochondria are fused followed by the inner membrane, and eventually their internal contents are combined (Ishihara *et al.*, 2004). Mfn-1, Mfn-2, and Opa1 are membrane GTPases involved in fusion. Mfn-1 and Mfn-2 are present on the mitochondrial outer membrane (Knott *et al.*, 2008; Rojo *et al.*, 2002), while Opa1 is found in the intermembrane space (Alexander *et al.*, 2000; Delettre *et al.*, 2000, 2001; Olichon *et al.*, 2002). Mitochondrial fusion facilitates the revival of mitochondria, as the mixing of contents allow replacement of damaged components (Chan, 2006; Chen *et al.*, 2005; Ono *et al.*, 2001). Elongation of mitochondria has been shown to increase the life span of yeast and fungus (Scheckhuber *et al.*, 2007). These studies have suggested that mitochondrial size may have a functional role in senescence.

2.2.4 Mitochondrial Fusion Proteins/Machinery

The Soluble NEM Sensitive Adaptor Receptor (SNARE) family of proteins are involved in most intracellular fusion events. During fusion, Rab GTPase and its effector protein along with fusogenic lipids like phosphatidic acid interact in a systematic fashion to mediate the initial segregation and tethering of different specific compartments and membranes of vesicles (Sollner *et al.*, 1993; Bonifacino and Glick, 2004).

This process is highly regulated, which ensures that the correct membranes fuse with each other. Until recently, none of these Rab GTPases or its effector proteins were known to play a role in mitochondrial fusion. A superfamily of lipid-modifying enzymes called phospholipase D (PLD) has a mitochondrial member, which is found to play a role in mitochondrial fusion. Mitochondrial PLD tethers to the mitochondrial membrane by hydrolyzing cardiolipin to form a fusogenic lipid, phosphatidic acid (Choi *et al.*, 2006). Mammalian mitochondrial fusion is regulated by three essential GTPases (Table 1.1) (Figure

2.7) that are highly conserved; Mfn-1, Mfn-2 and Opa1 (Legros *et al.*, 2002; Ishihara *et al.*, 2003).

Recently, Cao *et al.*, (2017) crystallized an Mfn-1 protein construct that contained the GTPase domain and a four-helix bundle. The crystal structure of this internally-modified Mfn-1 revealed structural details and a possible mechanistic model for how Mfn-1 mediates mitochondrial tethering. Of particular interest was the observation that disruption of the GTPase domain eliminated the fusogenic activity of Mfn-1, presumably because it prevents dimerization of Mfn-1 which is mediated by the GTPase domain. While a crystal structure for Mfn-2 is not yet available, it is proposed that Mfn-2 will have similar mechanism of action given the overall amino acid sequence similarity shared by the two Mfn isoforms.

Table 2.1. Mitochondrial Fusion Proteins

Protein	**Location**	**Function**
Mfn-1 /Fzo1p/Fzo	OMM	GTPase from the dynamin family, with two heptad repeats, required for mitochondrial fusion and tethering.
Mfn-2 /HSG/dMfn	OMM	GTPase from dynamin family, 62% same identity to Mfn-1, exhibit regulatory properties. If mutated causes CMT2A
Mgm1p/Opa1	IMM	Dynamin-like GTPase, eight different splice variants are found in humans. Possess a coiled-coil domain. These are required for inner membrane fusion and cristae structure.

OMM-outer mitochondrial membrane, IMM-inner mitochondrial membrane.

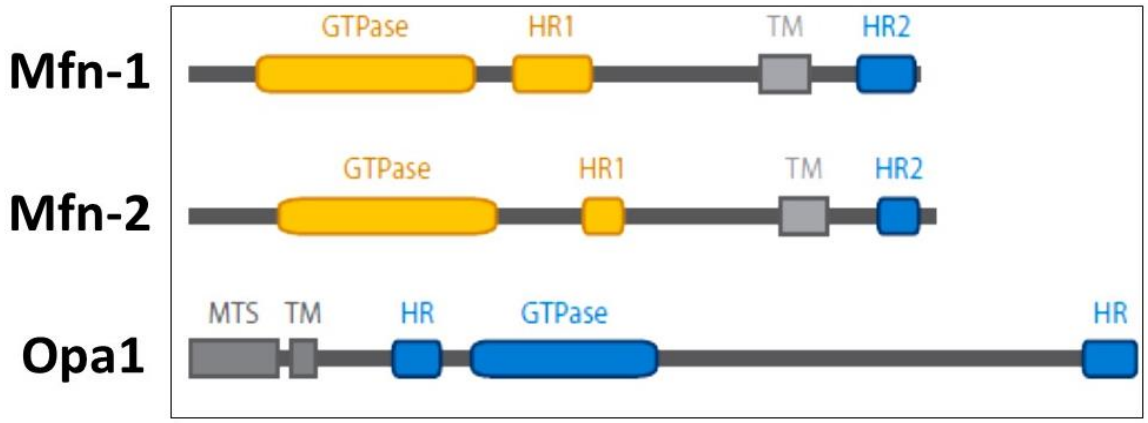


Figure 2.7. Three large GTPases involved in mitochondrial fusion.

Mfn-1 and Mfn-2 participate in the fusion of the mitochondrial outer membrane, whereas Opa1 participates in the fusion of the inner mitochondrial membrane. HR- heptad repeat, TM- transmembrane segment, MTS- mitochondrial targeting sequence.

2.3 Endoplasmic Reticulum-Mitochondrial Connections

The compartmentalization of eukaryotic cells allows segregation of membrane-bound organelles and their respective functions within the cytoplasm. The endoplasmic reticulum is the largest membrane-bound organelle and carries out or participates in many cellular functions. It is the site where luminal, secretory and membrane proteins are synthesized as well as translocated to the secretory and endocytic compartments. It functions as an intracellular storage site for calcium and also has lipid biosynthetic enzymes (Elbaz and Schuldiner, 2011). It has become evident through high-resolution microscopy that the ER forms contacts with other cytoplasmic organelles (Friedman and Voeltz, 2011). Specific proteins on each organelle localize to these contact sites to perform their specific function. Mitochondria and ER have well-characterized contact sites. Various roles for ER-mitochondria junctions have been identified, such as regulating lipid synthesis, calcium signaling, and mitochondrial biogenesis. Studies examining intracellular trafficking of various biomolecules are revealing the dynamic nature of these connections.

2.3.1 Mitochondria Associated Membrane

It is well-known that mitochondria and ER are in close apposition to each other (Franke and Kartenbeck, 1971). Due to improvements in differential centrifugation and electron

microscopy, it has been shown that the ER makes its contact with mitochondria through specialized regions termed mitochondria-associated membrane or MAM (Pickett *et al.,* 1980; Meier *et al.,* 1981). MAMs are the specialized membrane domains of ER, which are attached to mitochondria. They are involved in autophagosome formation, lipid transport, Ca^{2+} homeostasis, as well as other processes (Rizzuto *et al.,* 1993, 1998). Studies have confirmed that MAMs are enriched in lipid synthesizing enzymes, which suggests a possible mechanism of lipid transport from ER to mitochondria (Vance, 2014; Cui *et al.,* 1993; Rusinol *et al.,* 1994; Shiao *et al.,* 1995, 1998; Stone and Vance, 2000; Stone *et al.,* 2009).

2.3.2 Structure of the ER–Mitochondria Contacts

Electron and fluorescence microscopy is commonly used to study MAMs. It should be noted that these contact sites do not involve membrane fusion, thereby maintaining their individual organelle identities. The contact site between the ER and mitochondria was found to be 10-30 nm wide (Csordás *et al.,* 2006 and Friedman *et al.,* 2011). This suggests that the two organelles are tethered together by proteins which are located on opposite membranes. The evidence that ribosomes are extruded from the ER at contact sites supports the hypothesis that the contact sites are specialized regions (Csordás *et al.,* 2006 and Friedman *et al.,* 2011).

Different contact regions in a given organelle may have different structural features. In some cases, ER tubules circumscribe almost entirely around the mitochondrial membrane (Friedman *et al.,* 2011). Contact sites may also be stable structures, allowing two organelles to stay tethered to each other even as they travel along the cytoskeleton (Friedman *et al.,* 2010). Live cell imaging has shown that the two organelles can be trafficked in a coordinated fashion without any noticeable disruption in their contact points (Friedman *et al.,* 2010). This steadfastness of the tight association between these organelles despite their dynamics suggests that their association is vital for the normal functioning of the cell. Numerous functions that transpire at these contact sites are extensively studied to understand whether the various functions are carried out at specialized contact loci/domains or whether they occur synergistically through a common domain.

2.3.3 Roles of ER–Mitochondria Interactions

Contact sites among the ER and mitochondria provide a means to coordinate the activities and functions of the two organelles. It has become clear that these contacts can allow regulation of one organelle by the other, as well as concerted regulation of cell biological processes through bidirectional trafficking of factors between the two organelles. The four primary functions that have been characterized for ER–mitochondria contacts include control of lipid biosynthesis, mitochondrial division, Ca^{2+} signaling and coordinated dynamics of the two organelles.

2.3.3.1 Lipid Exchange During Biosynthesis

The majority of the enzymes involved in lipid biosynthesis are located either on the ER membrane or on the mitochondrial membrane. In certain cases, the enzymes required for single phospholipid synthesis are also localized on either of these organelles. Mitochondria-associated membrane (MAM) is a fraction of the ER which is attached to mitochondria and which can be isolated biochemically. This fraction is enriched in enzymes including phosphatidylserine (PS) synthase which is involved in lipid synthesis (Vance, 1990; Stone and Vance, 2000; Voelker 2000). At the ER-mitochondria interface, the biosynthesis of two of the cell's most abundant phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) is coordinated by largely uncharacterized molecular complexes (Van Meer *et al.,* 2008). PS is first synthesized by enzymes on the ER, then translocated to the mitochondrial outer membrane (OMM), and then transferred again to the inner mitochondrial membrane (IMM) where it is converted to PE (Osman *et al.,* 2011). PE must be translocated from the OMM to the ER in order for it to be converted to PC. The exchange of lipid between the two membranes is bidirectional and extensive, even if the mechanism for the exchange and the factors involved in lipid transport remain unclear in mammalian cells (Iwasawa *et al.,* 2011; Kornmann, 2013; Simmen *et al.,* 2005; Szabadkai *et al.,* 2006).

It is fascinating to contemplate how biosynthesis and lipid transfer between these two membranes could be regulated to maintain the steady-state ratios of phospholipids found in each of these organelles. In a yeast screen, an ER-mitochondria joining complex was identified which might coordinate phospholipid synthesis between the two membranes (Kornmann *et al.,* 2009). The aim of the synthetic screen was to identify mutants that displayed disrupted ER-mitochondria joining and whose phenotype might be complemented with an artificial tether. A four-member

complex was identified, which consisted of the ER–mitochondria encounter structure (ERMES), which consists of maintenance of mitochondrial morphology protein 1 (Mmm1), and mitochondrial distribution and morphology protein 10 (Mdm10), Mdm12 and Mdm34. On the mitochondria, all the four components are colocalized at punctate structures (Kornmann 2009, 2011). Mmm1, Mdm12, and Mdm34 belong to a group of seven yeast proteins which share a synaptotagmin-like mitochondrial-lipid binding protein (SMP) domain, which may be necessary for their localization at the ER–mitochondria junction (Toulmay and Prinz, 2012). It is predicted that the SMP domain belongs to the tubular lipid-binding protein superfamily (TULIP). TULIP family members demonstrate lipid-binding activity, and some members are known to be involved in lipid trafficking (Kopec *et al.,* 2010). It was also observed that there is a lower rate of PS conversion to PC in yeast strains that have mutations in components of the ERMES complex. This observation indicates that ERMES might also be essential for coupling at sites of lipid exchange (Kornmann *et al.,* 2009). Conversely, it has been reported that there is no substantial effect of ERMES components deletion on PS to PE conversion (Nguyen *et al.,* 2012). Work in the future will be required to determine how many functions are performed by the ERMES at the ER-mitochondria contacts. A homologue of ERMES has not yet been identified in mammalian cells.

2.3.3.2 ER control of mitochondrial biogenesis

No alteration in the ER-mitochondria contact occurs even when the mitochondria undergoes fission and fusion. Dynamin-related protein 1 (Drp1) in vertebrates drives mitochondrial division. The mitochondrial membrane recruits Drp1 which is a cytoplasmic protein, where it circumscribes the OMM as a helical oligomer. Drp1 hydrolyses GTP, thereby starting the fission. This development causes a conformational change in the oligomer, which allows it to clench the membrane and trigger fission (Ingerman *et al.,* 2005; Bleazard *et al.,* 1999; Labrousse *et al.,* 1999; Smirnova *et al.,* 2001). There arises a question related to the factor which recruits this division machinery from the cytosol to a particular location along the mitochondrial membrane. In yeast, Dnm1 is recruited from the cytosol to the mitochondrial membrane with the help of mitochondria fission-1 protein (Fis1) and mitochondrial division protein 1 (Mdv1) (Tieu *et al.,* 2002; Mozdy *et al.,* 2000; Tieu and Nunnari, 2000). While vertebrate orthologues of Fis1 exist, an orthologue for Mdv1 has yet to be identified (Otera *et al.,* 2010). Drp1 is dependent on

OMM protein mitochondrial fission factor (MFF) for its recruitment to the mitochondrial membrane (Otera *et al.*, 2010; Gandre-babbe and van der Bliek, 2008). A conserved complex on the OMM has not been identified which recruits Drp1 to fission sites. The localization of Drp1 is correlated with the sites of ER tubule contact with the mitochondrial membrane (Friedman, 2011). The ER tubules in vertebrate cells circumscribe the mitochondrial membrane at constriction sites which are marked by Drp1 and by the cofactor MFF (Friedman, 2011). Drp1 or MFF depletion does not disrupt ER-mitochondria contacts, suggesting that the fission machinery recruitment is independent of contacts (Friedman, 2011). This contact is also retained after fission occurs.

The site of division machinery recruitment is not only marked by the ER but also marks the position where mitochondria constrict for a long time interval before division occurs (Friedman, 2011). It has been proposed that a protein other than a dynamin family member must first constrict mitochondria since the mean mitochondrial diameter far exceeds the diameter of the helix which is formed by Drp1 (Ingerman *et al.*, 2005; Gandre-babbe and van der Bliek, 2008; Legesse-Miller *et al.*, 2003). The ER might drive initial constrictions of mitochondria before the division machinery is recruited. This phenomenon is demonstrated when the ER contacts circumscribe mitochondrial constriction sites. Normal fission of mitochondria is prevented which results in an elongated morphology, and is likely due to depletion of Drp1 or MFF. However, the mitochondria are still constricted at the positions where the mitochondrial membrane is circumscribed by the ER tubules (Friedman, 2011). Even before MFF and Drp1 recruitment, the ER is located at mitochondrial constrictions.

There is no establishment of a causal relationship between ER contact and mitochondrial constrictions. One possibility is that the ER simply associates with these sites without causing the mitochondrial constriction. The mitochondrial surface might be probed by the ER until it finds a region with the proper amount of membrane curvature which indicates a constriction (Figure 2.8). Alternatively, the ER might physically wrap around and squeeze the mitochondria at contact sites to promote constriction. This seems reasonable, as lipid biosynthesis also occurs at ER-mitochondria contact sites, and the domains of lipid asymmetry generated at these contacts could change the shape of the OMM and IMM in a way that drives constriction. Despite the mechanism used, it is evident that protein complexes localized to the OMM at the ER-

mitochondria interface must be required to recruit the factors that regulate mitochondrial division.

There is a possibility that mitochondrial fusion is also influenced by contact with the ER. In mammalian cells, two mitochondria are joined to direct their fusion by both Mfn-1 and Mfn-2 proteins on OMM (Chen *et al.*, 2003). The contact between mitochondria and ER (De Brito and Scorrano, 2008) is tethered by Mfn-2. Mouse embryonic fibroblasts lacking Mfn-2 show disrupted ER-mitochondria contact which can be rescued by ectopically expressing Mfn-2 (De Brito and Scorrano, 2008). Whether Mfn-2 promotes tethering of ER-mitochondria is unclear, although Mfn-2 depletion does not affect tethering of ER at constriction sites in mammalian cells (Friedman *et al.*, 2011). Considering that both mitochondrial fusion and ER-mitochondria tethering is affected by the Mfn-2, there is a possibility that ER contact is also required for fusion.

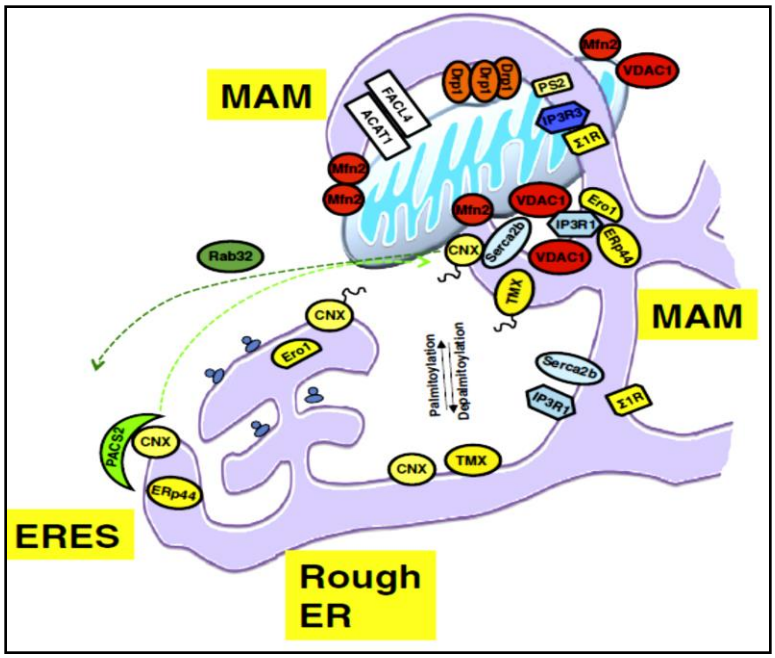


Figure 2.8. Diagram of MAM-Enriched Proteins in the Mammalian Cell.

A schematic representation of MAMs and related proteins in a mammalian cell and their interplay. The MAM is the center for lipid metabolism (white color; represented as a top portion of MAM), mitochondrial fission driven by protein Drp1 (orange color; represented as the Drp1 oligomers) and several ER-chaperones and oxidoreductases (yellow color; indicated at the base of MAM). Palmitoylation plays a major role in the enrichment of MAM proteins and cytosolic sorting proteins (green color; represented as PACS-2 & Rab32 protein). ER-Mito or MAM tethering is maintained and involves a variety of proteins like Mfn-2, Grp75, and VDAC1. Proteins such as SERCA2b, IP3R1 and IP3R3 are found on the MAM (blue color) and are involved in calcium handling (Figure was adapted from Raturi and Simmen, 2013). ERES: ER-mito encounter structure, MAM: Mitochondria Associated Membrane.

2.4 ER-Mitochondria Contacts: Function of the Junction

2.4.1 Mitofusin

The mitofusins in yeast and metazoans are similar in domain structure and in GTPase activity. They have two transmembrane regions embedded in the outer mitochondrial membrane, with most of the protein present in the cytosol and a short loop in the intermembrane space (Fritz *et al.,* 2001; Rojo *et al.,* 2002). Mfn-1 and Mfn-2 are two isoforms of mammalian mitofusin proteins which share similar structural motifs with other members of conserved mitochondrial GTPases, including an N-terminal domain that has a highly conserved GTPase and a bipartite transmembrane domain which anchors these proteins to the outer membrane of mitochondria. The carboxy terminus has a conserved domain known as a heptad repeat which interacts with 'mitofusin heptad repeat' of adjacent or approaching mitochondria for initial docking and fusion mechanism. Thus, Mfn-2 is a critical GTPase for the formation of mitochondrial-ER contacts and forms homo- and heterodimers to tighten the contact between the organelles (de Brito and Scorrano, 2008).

Research on mitochondrial dynamics has gained momentum in recent years, as mitochondrial dynamics has been found to be involved in many important biological processes including apoptosis and aging (Detmer and Chan, 2007). The foundation of mitochondrial dynamics machinery involves three GTPases; Opa1, Mfn-1, Mfn-2 and their interacting factors (Okamoto and Shaw, 2005; Hoppins *et al.,* 2007). Mitofusins are involved in mitochondrial outer membrane fusion as well as in the tethering of mitochondria with the ER (Szabadkai, 2006; de Brito and Scorrano, 2008). This spatial arrangement of mitochondria and ER facilitates the intake of Ca^{2+} released from the ER into mitochondria (Rizzuto *et al.,* 1993, 1998). This particular juxtaposition of mitochondria and ER has been shown to be essential for lipid synthesis. The molecular mechanism underlying this interaction and lipid biosynthesis and regulation is not clear. It is important to understand better this interaction, as it might play a crucial role in lipid synthesis and storage of lipid droplets. Also, it plays a role in controlling Ca^{2+} homeostasis in mitochondria and apoptotic signaling (Ferri and Kroemer, 2001). Several diseases like heart failure, obesity, neuropathies and cancer appear to be linked to mitochondrial dynamics (Zorzano *et al.,* 2009).

Mfn-1 and Mfn-2 are from a family of high-molecular-mass transmembrane GTPases. These proteins have homologues in diverse organisms from yeast to humans. They are present

in the outer mitochondrial membrane. These proteins have large N-terminal and relatively short C-terminal domains (Rojo *et al.*, 2002; Santel and Fuller, 2001). Both proteins have a coiled-coil domain near the C-terminal end facing the cytoplasm, two transmembrane domains, and a GTPase domain near the N-terminus. Mfn-2 is 757 amino acids long compared to 741 amino acids long for Mfn-1. Human Mfn-2 protein is 62% identical and 77% similar to Mfn-1 protein.

The levels of both Mfn-1 and Mfn-2 vary between tissues. Mfn-1 is predominantly expressed in liver, heart, adrenal glands, pancreas and testis (Santel and Fuller, 2001; Bach *et al.*, 2003), whereas Mfn-2 is predominantly expressed in skeletal muscle, heart, brain, adrenal glands and brown adipose tissue (Santel and Fuller, 2001; Bach *et al.*, 2003). There are many cell types, for example MEFs and HeLa cells, that express both Mfn-1 and Mfn-2 proteins (Bach *et al.*, 2003; Chen *et al*., 2003; Santel *et al.*, 2003). In mammalian cells, both proteins co-exist, which raises the question as to whether they have distinct roles or have some functional redundancy.

2.4.2 Function of Mitofusin-1 and Mitofusin-2 in Mitochondrial Fusion

Functional mutagenesis studies of mitofusins have demonstrated that these proteins are responsible for regulating mitochondrial fusion in mammalian cells. Overexpression of Mfn-1 results in changes in some cell types accompanied by grapelike perinuclear clusters of mitochondria (Santel *et al.*, 2003). The mitochondrial morphology was observed to be altered due to overexpression of Mfn-2 in cultured cells, specially resulting in the generation of reticular structures and extensive perinuclear clustering (Rojo *et al.*, 2002; Santel and Fuller, 2001; Bach *et al.*, 2003; Pich *et al*., 2005). MEF cells lacking Mfn-1 or Mfn-2 display different types of fragmented mitochondria (Chen *et al*., 2003; 2005).

Loss of Mfn-1 results in either very short mitochondrial tubules or minuscule uniform spheres with diameters similar to that of normal tubules. In contrast, loss of Mfn-2 in cells results in mitochondrial spheres with varying sizes (Chen *et al*., 2003; 2005). These findings suggest that the cells that contain only Mfn-1 retain more fusion activity as compared to cells that contain only Mfn-2. Hence, it can be concluded that Mfn-1 plays a larger role in mediating GTP-dependent tethering of mitochondria compared to Mfn-2. Consistent with this, the GTPase activity of Mfn-1 is greater than that of Mfn-2 (Ishihara *et al*., 2004).

Mfn-1 and Mfn-2 are necessary for the process of embryonic development, and it has been observed that mice deficient in either of the genes die in mid-gestation. Mfn-2 silenced

embryos have a striking reduction in placental trophoblast giant cells, which causes a severe and specific interruption of the giant cell layer, whereas placental trophoblastic giant cell layer of Mfn-1 deficient cells are normal (Chen *et al*., 2003). These findings point towards different biological roles for these two proteins.

2.4.3 Mitochondrial Metabolism is Regulated by Mitofusin-2 Protein.

The process of mitochondrial oxidation is regulated by Mfn-2 (Bach *et al.*, 2003; Pich *et al*., 2005) therefore Mfn-2 loss-of-function results in reduced oxygen consumption, decreased mitochondrial membrane potential as well as oxidation of glucose, pyruvate and fatty acids, without change in mitochondrial mass (Bach *et al.*, 2003; Pich *et al*., 2005). In contrast, Mfn-2 gain-of-function induces glucose oxidation and increases mitochondrial membrane potential (Pich *et al*., 2005). Surprisingly, overexpression of an Mfn-2 truncated mutant lacking the transmembrane domains and the C-terminal tail enhances glucose oxidation, as well as mitochondrial membrane potential but has no effect on mitochondrial morphology (Pich *et al.*, 2005). Therefore, it was suggested that mitochondrial oxidation and mitochondrial fusion are regulated by different mechanisms that involve Mfn-2. The role of Mfn-1 in mitochondrial metabolism is still under investigation. Specific subunits of the electron transport chain undergo significant alterations during experiments with Mfn-2 'loss-of-function' and 'gain-of-function' (Pich *et al*., 2005). Nuclear-encoded electron transport chain subunits I, II, III and V are repressed in Mfn-2 loss-of-function experiments, whereas subunits of electron transport chain complexes I, IV and V are induced in Mfn-2 gain-of-function conditions. Therefore, mitofusin-2 is critical in regulating mitochondrial metabolism. Interestingly, obesity and diabetes type 2 are characterized by defective mitochondrial oxidation and Mfn-2 gene expression inhibition (Bach *et al.*, 2003; 2005). Conversely, it has been found that weight loss and exercise lead to an increase in Mfn-2 gene expression and enhanced mitochondrial oxidation (Cartoni *et al*., 2005; Mingrone *et al*., 2005).

2.4.4 Mitofusin-2 Dysfunction in Neurological and Biochemical Diseases

An impairment in mitochondrial functioning can have catastrophic consequences and is present in a number of human diseases such as obesity, diabetes, neurodegenerative disorders such as Parkinson's disease and Alzheimer disease, and a variety of cancers (Schon and Manfredi, 2003). Neuronal functions are affected primarily due to flaws in mitochondrial fusion

and fission dynamics. Nerve cells need high amounts of energy and solely depend on mitochondria for ATP. Thus, neurons are very sensitive to mitochondrial functioning and flaws in mitochondrial function lead to neurodegenerative diseases (Martens and McMohan, 2008). Interestingly, through investigations, a connection between Charcot-Marie-Tooth neuropathy type 2A (CMT2A) and human Mfn-2 gene mutations have been revealed, signifying the important role of mitofusins proteins in at least some neurological diseases (Zuchner *et al.,* 2004; Kijima *et al.,* 2005; Lawson *et al.,* 2005).

Studies in rats have clearly defined the role of Mfn-2 in maintaining a mitochondrial membrane potential and energy metabolism. When Mfn-2 was repressed, it reduced oxidation of glucose, produced mitochondrial proton leakiness, and disrupted mitochondrial morphology and thus cellular respiration. The authors also showed how suppressed expression of Mfn-2 led to distorted mitochondria, which might be an important factor in obesity (Bach *et al.,* 2003).

2.4.5 Transcriptional Control of Adipocyte Formation

Cardiovascular disease and type 2 diabetes are more likely to be developed in obese individuals than their lean counterparts. The increase in the adiposity in these individuals results from an increase in both adipocyte number and individual fat cell size. In some people, the disproportionate increase in visceral adipose depots is linked to the development of certain metabolic disorders. Eventually, if there is a way to understand the mechanism of adipose formation, this will provide valuable information to help in the fight against the growing cases of obesity in the world. Scientists in the last few years have begun to define the transcriptional events regulating preadipocyte differentiation and adipocyte functions. An elaborate network of transcriptional factors drives the differentiation of preadipocytes and adipocyte. Transcriptional factors coordinate expression of hundreds of genes which are responsible for establishing the phenotype of mature fat cells. PPARγ and C/EBPα are the two principal adipogenic factors at the center of this network. These factors initiate the differentiation process. It is considered that PPARγ is the master regulator of adipogenesis. Studies performed in preadipocyte cell lines as well as mesenchyme-derived precursor cells have provided much of the information regarding this complex network and the importance of PPARγ and C/EBPα. Green and associates have significantly advanced our understanding of the molecular mechanism controlling adipogenesis through their studies in 3T3-L1 and 3T3-F422A preadipocytes cell lines (Green and Kehinde,

1975, 1976). Even though committed to the lineage of the adipocyte, 3T3-L1 preadipocytes exert similar characteristics to other 3T3 fibroblasts. The confluent 3T3-L1 preadipocytes differentiate due to exposure to the adipogenic inducers like fetal bovine serum (FBS), dexamethasone, IBMX, and insulin. The activation of an adipogenic program by this drug cocktail happens via two well-defined phases. Clonal expansion is a phase often referred to when stimulated cells immediately re-enter the cell cycle and progress through at least two cell divisions. During this time, PPARγ and C/EBPα expression function together to express specific adipogenic transcription factors as well as cell cycle regulators. Subsequently, the cells undergo terminal differentiation which is manifested by lipid droplet production. Many studies performed in both mouse and human tissue has supported the validity of this 3T3-L1 system as an appropriate model of adipocyte formation in animals (Ruiz-Ojeda *et al.,* 2016).

Adipogenesis is attenuated by a number of factors which serve as molecular switch to function in controlling the progenitor's fate, either positively or negatively. Both *in vivo* and *in vitro* studies have provided evidence that supports the role of PPARγ as the master regulator of adipogenesis, whereas C/EBPα lacking cells are capable of adipocyte differentiation (El-Jack *et al.,* 1999; Wu *et al.,* 1999). Spiegelman and collaborators have demonstrated the critical role of PPARγ in regulating adipogenesis. They elucidated expression of adipose fatty acid binding protein FABP4 through transcription factors. A series of 'gain-of-function' studies were performed, where PPARγ in non-adipogenic mouse fibroblasts was ectopically expressed. They were able to show that PPARγ alone can initiate the entire adipogenic program, and gave rise to fat cells which can perform many functions of mature adipocytes (Tontonoz *et al.,* 1994).

The PPARγ knockout cells failed to develop into adipocytes (in animal model), whereas wild type derived cells had normal adipose depots (Barak *et al.,* 1999). Therefore, it was difficult to evaluate the impact of PPARγ absence on its role in adipose tissue. The tetraploid embryo strategy (Barak *et al.,* 1999) generated only one mouse, which died soon after birth but allowed the researchers to observe that PPARγ deficiency resulted in the absence of adipose tissue in these animals. A separate study where PPARγ expression was knocked down in mice resulted in severe lipodystrophy (Koutnikova *et al.,* 2003).

A number of studies have also established C/EBPα as a principal player in adipogenesis. Feytag and co-workers showed that the ectopic expression of C/EBPα induced adipogenesis in a variety of fibroblastic cells (Freytag *et al.,* 1994). Establishment of C/EBPα knockout mice

proved difficult since they died shortly after birth due to insufficient glucose supply (Wang *et al.*, 1995). In the absence of PPARγ, C/EBPα is incapable of driving the adipogenic program, whereas C/EBPα-deficient MEFs can differentiate into adipocytes via PPARγ (Rosen *et al.,* 2002). During terminal adipogenesis, it appears that C/EBPα does provide a critical function of maintaining expression of PPARγ (Wu *et al.,* 1999).

2.4.6 MAM in Health and Disease

It has been proposed that MAM is a site which is affected in most of the neurodegenerative diseases (Schon and Area-Gomez, 2010). The role of MAM in diabetes is an emerging field of research (Sebastian *et al.,* 2012). There are many proteins from MAM associated with disease states such as cancer (Pinton *et al.,* 2011). Thus, the study of MAM has enormous potential for advancing our understanding of human diseases and development of new therapeutic approaches.

Neuronal cells, in particular axons and dendrites, need vast amounts of energy for their activities. Thus, they mostly rely on mitochondria for energy. Thus, the mitochondria are a very crucial for neuronal survival (Kann and Kovacs, 2007; Knott and Bossy-Wetzel, 2008; Knott *et al.,* 2008) and their role in neuronal survival depends on the distribution of smooth or rough ER throughout axons and dendrites (Ramirez and Couve, 2011). Similar to this finding, a number of studies have been able to attribute neuronal defects to mutations in a number of ER structural proteins. For example, mutations in Mfn-2 can lead to a peripheral neuropathy called Charcot-Marie-Tooth type 2A disease, wherein the longest neurons die (Zuchner *et al.,* 2004; Zuchner and Vance, 2006; Cartoni and Martinou, 2009). Along with the potential involvement of the MAM, it has been suggested that a defect in mitochondrial transport leads to the disease (Cartoni and Martinou, 2009). This defect could develop from an inability of Mfn-2 mutated neurons either to form the MAM or to interact with kinesin (Zhao *et al.,* 2001).

Reticulon-4, also called neurite outgrowth inhibitor (Nogo), promotes ER tubulation but may influence the calcium-dependent apposition between the ER and mitochondria (Yang and Strittmatter, 2007; Sutendra *et al.,* 2011). Such a function could potentially be assigned to many enzymes that regulate ER tubulation, such as receptor expression enhancing protein 1 (REEP1) that regulates ER network formation, and mitochondrial membrane dynamics (Park *et al.,* 2010; Goizet *et al.,* 2011). Interestingly, REEP1 is a gene associated with hereditary spastic paraplegia,

which is reminiscent of the well-known role of the microtubule severing protein spastin in both the connection of ER tubules to the cytoskeleton (Park *et al.,* 2010; Moss *et al.,* 2011) and the trafficking of mitochondria along axons (McDermott *et al.,* 2003; Kasher *et al.,* 2009).

The dynamic membrane contacts at the MAM may require the calcium-dependent Ras GTPases Miro-1 and Miro-2 that promote mitochondrial clustering when active by forming a link between mitochondria and microtubules and kinesin heavy chain (KIF5) (Fransson *et al.,* 2006; Liu and Hajnoczky, 2009). These calcium-dependent interactions may provide a mechanism behind the ability of mitochondria to move towards calcium puffs released by IP3Rs (Giacomello *et al.,* 2010). The Miro protein complex also interacts with Mfn-2, which is also essential for the transport of mitochondria along the axons (Misko *et al.,* 2010).

Another neurodegenerative disease associated with MAM is familial Alzheimer's disease. Mutations in presenilin-1 and presenilin-2 can lead to reduced ER calcium content and increased calcium transfer from ER–mitochondria when compared to wild type (Schon and Area-Gomez, 2010; Zampese *et al.,* 2011). There are many other consequences due to the increased ER–mitochondria calcium transport in cells that results from mutation in presenilin-2. The consequences can include markedly increased apoptosis susceptibility.

2.5 Hypothesis

Given the role of Mfn-2 in mitochondrial fusion and in the interaction of ER and mitochondria, as well as the importance of ER-mitochondrial contacts in lipid metabolism, it is hypothesized that Mfn-2 knockout MEFs will show altered mitochondrial dynamics and impaired TAG synthesis and storage.

2.6 Rationale and Specific Objectives

Our research group is interested in understanding the link between mitochondrial dynamics, ER-mitochondria interactions and lipid metabolism. Several studies have revealed that ablation of Mfn-2 protein disrupts ER-mitochondria connection and impairs Ca^{2+} signaling (de Brito *et al.*, 2008; Filadi *et al.*, 2015). Ca^{2+} homeostasis and signaling is crucial for proper functioning of the Kreb's cycle in the mitochondrial matrix. Lipid droplets are closely associated with the mitochondrial network to provide fatty acids for β-oxidation. Thus, by ablating Mfn-2 expression, ER-mitochondria interactions will be disrupted and are expected to result in alterations in Ca^{2+} signaling and lipid metabolism. The specific objectives are:

- To assess mitochondrial morphology in MEF WT and Mfn-2 KO cells.
- To assess lipid droplet morphology (size and number) in MEF WT and Mfn-2 KO cells.
- To assess lipid droplet morphology (size and number) in MEF WT and Mfn-2 KO cells in response to oleic acid and/or insulin.
- To assess whether Mfn-2 is required for the differentiation of MEFs into adipocytes.

3. MATERIALS AND METHODS

3.1 Reagents

Analytical grade or higher reagents were used for all experiments. Names of reagents, suppliers, and addresses of suppliers are listed in Table 3.1.

Table 3.1. List of Reagents and Suppliers

Cell Culture Reagents	**Supplier/Address**
Dulbecco's Modified Eagle's Medium – High Glucose with L-Glutamine and Sodium Pyruvate (SH30243.01)	Fisher /Ottawa, Ontario, Canada
Antibiotic-Antimycotic (100 X) (Penicillin and streptomycin)	Invitrogen/Burlington, Ontario, Canada
Trypsin 0.25%	Thermo Fisher Scientific/ Grand Island, USA
CHAPS	Bio Basic/Markham, Ontario, Canada
IBMX	Sigma-Aldrich/Oakville, Ontario, Canada
Dexamethasone	Sigma-Aldrich/Oakville, Ontario, Canada
Insulin solution from bovine pancreas	Sigma-Aldrich/Oakville, Ontario, Canada
Troglitazone	Sigma-Aldrich/Oakville, Ontario, Canada

Reagents for Confocal Imaging	**Supplier/Address**
Oleic Acid	Sigma-Aldrich/Oakville, Ontario, Canada
BODIPY	Sigma-Aldrich/Oakville, Ontario, Canada
Anti-Hsp70 (JG1)	Invitrogen /Burlington, Ontario, Canada

ProLong Diamond Antifade Mountant	Invitrogen /Burlington, Ontario, Canada
Alexa Fluor^{R} 594 antibody	Invitrogen /Burlington, Ontario, Canada

Protein Analysis Reagents	**Supplier/Address**
30% Acrylamide/Bis Solution (29:1)	Bio-Rad/Hercules, California, USA
PageRuler^{TM} Prestained Protein Ladder Plus (SM1811, SM0671)	Fermentas/Burlington, Ontario, Canada
ImmobilonTM Western, Chemiluminescent HRP Substrate (WBKLS0100)	Millipore/ Etobicoke, Ontario, Canada
cOmplete Ultra, EDTA-free Protease Inhibitor Tablets	Roche /Mississauga, Ontario, Canada
Oil Red O solution (0.5% in isopropanol) cat# O1391	Sigma-Aldrich/Oakville, Ontario, Canada

List of Antibodies used	**Supplier/Address**
Anti-Mfn-1	Abcam, Toronto, Ontario, Canada
Anti-Mfn-2	Abcam, Toronto, Ontario, Canada
Anti-PPARγ	Santa Cruz, Dallas, Texas USA
Anti-FABP (C-15): sc-18661	Santa Cruz, Dallas, Texas USA
Anti-Calnexin	Abcam, Toronto, Ontario, Canada
Anti-C/EBPα	Santa Cruz, Dallas, Texas USA
Anti-Hsp70 (JG1)	Invitrogen /Burlington, Ontario, Canada
Anti-ADRP	Thermo Fisher Scientific/ Grand Island, USA

3.2 Cell lines

3.2.1 Mouse Embryo Fibroblasts (MEF)

MEF (CF-1) (ATCC® SCRC-1040™) cells from *Mus musculus* (mouse embryos) were used for establishing MEF cell lines. These cells have the capacity to synthesize collagen and extracellular matrix, making them adherent in nature. Wild-type (WT) and Mfn-2 protein knockout (Mfn-2 KO) MEFs were obtained from American Type Culture Collection (ATCC) Manassas, Virginia, USA.

3.2.2 3T3-L1

3T3-L1 (ATCC® CL-173™) cells were obtained from *Mus musculus* (mouse embryos). This cell line was developed by two scientists, George Todaro and Howard Green from Department of Medicine, New York University. Abbreviation 3T3 is derived from '**3'**-day '**T'**ransfer, with rigid inoculum density of '**3'** x 10^5 cells. These cells were immortalized and were stable after 20-25 generations in culture. A specific version of 3T3 is abbreviated as 3T3-L1 (Green and Meuth, 1974).

3.3 Mammalian Cell Culture

Mammalian tissue culture and other adipogenesis experiments were performed inside a biosafety level 2 cabinet. MEF WT and Mfn-2 KO MEFs were passaged in 1:10 dilutions. Both WT and Mfn-2 KO cells were passaged by washing a 90-100% confluent cell culture plate with 1X phosphate buffered saline (PBS) followed by addition of 1 mL of porcine-derived pancreatic trypsin (0.25%). The cells were incubated at 37°C, 5% CO_2 for 2-5 minutes to allow the cells to detach from the surface of the culture plate. Appropriate amounts of pre-warmed (37°C) medium consisting of 10% fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) – high glucose with L-glutamine and sodium pyruvate, were added to the trypsinized cells, to inactive the trypsin. The cells were resuspended in various amounts of medium depending on the downstream experiments. Generally, 10 mL of medium was used for culturing cells in 100 mm plates and 2.5 mL in 6-well plates. Antibiotic-antimycotic (penicillin and streptomycin stock 100 X) was used at 10 µL/mL concentration in all culturing experiments.

3.3.1 Adipogenesis

Many diverse actions contribute to the differentiation of a stem cell into the adipocyte lineage. This includes the synchronization of an intricate network of transcription factors, co-factors and signaling intermediates from several pathways (Rosen and MacDougald, 2006). A review (Scott *et al.,* 2011) on alternative methods on adipogenic induction and standard protocol was published recently. Cells were grown to 100% confluence and day 2 post-confluent cells was considered day 0 of the protocol. On day 0, cells were treated with cocktail media, which consisted of troglitazone (10 µM), dexamethasone (1 µM), insulin (10 µg/mL) and 3-isobutyl 1-methylxanthine (IBMX- 500 µM) in DMEM + 20% FBS with Antibiotic-Antimycotic. A total volume of 7 mL of cocktail media was added to the 100mm plate and 2 mL to 35 mm plates. The volume of medium per plate was reduced since adipogenesis is enhanced in the presence of a thin medium layer (Sheng *et al.,* 2013).

For adipogenesis, cells were grown in cocktail media on day 0 until day 4. From day 4 to day 8 cells were treated with maintenance media consisting of troglitazone (10 µM) and insulin (10 µg/mL) in DMEM + 20% FBS. On day 10 cells were harvested as described in section 3.4.1. For control experiments, cells were treated with DMEM + 20% FBS from day 0 of the experiment until harvest (day 10).

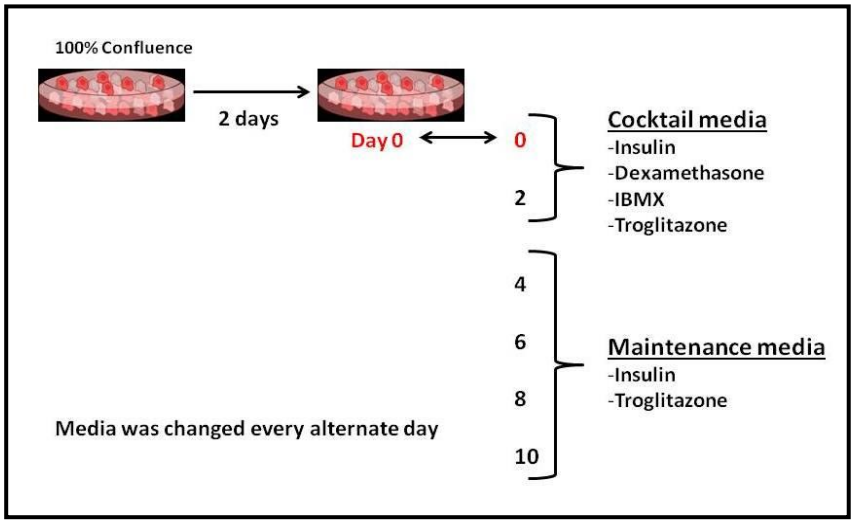


Figure 3.1. Flowchart of adipogenesis

Cells were grown in tissue culture plate to 100% confluence. Two days post-confluence was considered as day 0 of the experiment. Cells were treated with cocktail media consisting of adipogenic drugs (troglitazone, dexamethasone, insulin and IBMX in DMEM + FBS) on day 0 and 2, from day 4 until day 8 cells were treated with maintenance media (troglitazone and insulin in DMEM + FBS). Cells were harvested at day 10.

3.4 Mammalian Cell Culture Protein analysis

3.4.1 Preparation of Protein Extracts from Cultured Cells

Eight mL of 1X PBS (room temperature) was used to wash cultured cells twice before harvesting. Approximately 200 to 500 µL of lysis buffer was added to the washed cell layer for 100 mm plates, depending on cell density or thickness of cell layer. For Western blotting the cells were lysed in RIPA buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, 5% (v/v) glycerol, 0.1% sodium deoxycholate, 0.1% SDS (w/v), 1% (v/v) Triton X-100, supplemented with 1X cOmplete® protease inhibitor (Roche). The cells were then incubated at 4°C for 15-30 minutes with gentle agitation. After incubation, cells were dislodged using a scraper and collected into a microcentrifuge tube. The cells were further subjected to fine needle passage 20-25 times, using a 25-gauge needle and mixed using a vortex intermittently for proper mixing and disruption of cell membranes. Microcentrifuge tubes with cell lysate were centrifuged at 1000 x g for 10 minutes to pellet the cell debris. The supernatant was collected in a separate microcentrifuge tube for further use. Cells from adipogenesis experiments, especially from day 6 and 10 were treated differently after a centrifugation step. Since these cells have large amount of lipid droplets, a fat layer forms on top of the microcentrifuge tube. Therefore, supernatant below fat layer and above the cell pellet was used for experiments

3.4.2 Protein Quantification of Cellular Extracts

The prepared cell extracts were quantified for total protein concentration using the Bradford assay. 1 µL of initial cell lysate was added to a mixture of solution (3:1 Bradford assay reagent and double distilled water). The color change due to the presence of proteins with Bradford reagent was read on a Bio-Rad SmartSpec™ Plus spectrophotometer at 595 nm. The absorbance values obtained were compared to a standard curve derived from known BSA protein samples. An appropriate amount of ddH_2O was added to each sample to adjust the final protein concentration of the sample to 1 µg/µL.

3.4.3 SDS Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Mini Gel Tank system (from Life Technologies) and the Blot® system (from ThermoFischer). 1x MOPS (3-(N-morpholino) propanesulfonic acid) buffer from Bolt® was used as running buffer. Ten well precast Bolt™ 4-12% Bis-Tris Plus Gels were used for efficient separation of proteins.

These Bis-Tris gels have a pH of 6.4 that reduces protein modifications resulting in clear and sharp bands. To estimate the molecular weights of the sample, 10-170 kDa PageRuler^{TM} Prestained Protein Ladder Plus was simultaneously resolved along with samples.

The protein samples were mixed with SDS loading buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue, and 1% β-mercaptoethanol) and boiled for 5 minutes in a heat block to denature protein samples before loading on the gel. Protein samples (25 μg) were loaded onto the gel, and the gel was run at 150 V until the dye front reached the bottom of the gel.

3.4.4 Western Blotting

For Western blotting 25 μg of protein was loaded per well. After SDS-PAGE electrophoresis, the gel was removed and incubated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at RT for 10-20 minutes before transfer. For transfer, a Bio-Rad Mini Trans-Blot transfer cell or a Bio-Rad Criterion Blotter (midi format, plate electrodes) was used. The wet transfer method was used to transfer the proteins from the gel to the Immun-Blot^{R} polyvinylidene difluoride (PVDF) membrane with 0.22 micrometers pore size (Bio-Rad). The transfers were performed in transfer buffer at 70 V for 2 hours or 25 V overnight at 4°C.

When the wet transfer was complete, the membrane was blocked using 5% (W/V) milk in PBST (1X PBS, 0.05% Tween-20) for 1 hour with gentle agitation at RT. The membrane was subsequently probed with primary antibody for 1 hour with gentle agitation at RT or overnight at 4°C. The primary antibody was diluted in 5% milk in PBST (see Table 3.1). The membrane was washed for 10 min with PBST three times. The membrane was then incubated with a secondary HRP-conjugated antibody. Incubation was performed for 1 hour with gentle agitation at RT followed by a 3X washing with PBST for 10 min each. The PVDF membrane was developed using Immobilon^{TM} Western, Chemiluminescent HRP Substrate (WBKLS0100) for 5 minutes and autoradiography film in a dark room. For a full list of antibodies used and dilutions see Table 3.1.

3.5 Laser Scanning Confocal Microscopy

Biological research has evolved with advances in confocal microscopy. Studies on mitochondrial network and lipid morphology require the ability to obtain excellent quality high-resolution images on fixed cells. Leica TCS SP5 confocal microscope was used to image fixed

WT and Mfn-2 KO MEF cells. Cells were stained for lipid droplets with BODIPY 493/503 and for mitochondria with anti-Hsp70, a mitochondrial marker. This microscope allows SuperZ stage recording of large 3D Z-stacks of MEF cells. Images were obtained with high speed, to minimize bleaching of the signal.

3.5.1 Treatment of Cells with Oleic Acid and Insulin

MEF-WT and Mfn-2KO cells were grown in 6-well plates with a glass coverslip. Both cell lines were cultured at 37˚C with 5% CO_2 in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. When cells were at 30-40% confluence, that point was considered as 0 h. Cells were fed with the above media containing either insulin (5 µg/mL), oleic acid (18:1) (0.5 mM /mL) or both and incubated for an additional 13 h. Cells were fixed at this point and prepared for immunostaining as described in section 3.5.2. The fixed cells were co-stained with Alexa594 and BODIPY 493/503 and images were obtained as outlined in section 3.5.2.

3.5.2 Immunofluorescence with BODIPY and Anti-Hsp70

Cells were fixed using 4% PFA in 1X PBS for 15 minutes, then gently washed twice with 1X PBS. They were then permeabilized with 0.2% Triton X-100 for 5 minutes, and then washed with 1X PBS twice. Coverslips were then incubated with anti-Hsp70 (dilution 1:200) followed by secondary goat anti-mouse 594 (dilution 1:200) for 1 hour each. Lipid droplets were stained with BODIPY 493/503 (dilution 1:100). Coverslips were washed with 1X PBS and mounted on slides with ProLong Diamond Antifade Mountant. Confocal images were obtained as Z-stacks and analyzed using ImageJ software to quantify size and number of lipid droplets in cells.

3.5.3 Image Acquisition

US National Institute of Health released an image analysis tool 'ImageJ' which provides various options for analyzing particle size scenarios. Images obtained by ImageJ can be analyzed manually or automatically. For analyzing lipid droplets in this research, automatic particle sizing method was employed.

Procedure for Automatic Particle Sizing

The automatic particle sizing method can be used for image analysis only if the particles, lipid droplets in this case, are spherical, well-dispersed in the plane and in high-contrast compared

to the background. The green fluorescent lipid droplets meet these requirements and thus automatic particle analysis was used.

I. Convert Image to Monochrome: From option 'Image' on the software, one can view 'type' as an option and convert the image into an 8-bit, 16-bit or 32-bit image. For particle analysis, the images were converted to 8-bit. ImageJ software was set to read the image in grayscale and thus considered only brightness as the factor analyzed.

II. Setting a Threshold Limit: The threshold limit for a given parameter was set on ImageJ software to read exactly what comprises a particle in a particular image. The path was Image→Adjust→Threshold.

The window of brightness/darkness is more or less representative of the lipid droplets in the image. For example, if the lipid droplets are dark, and the background is white, we define pixel brightness/darkness to be read by the software as lipid droplets (Figure 3.2). The software is very sensitive to counting grainy background pixels, but this can be minimized to a great extent by this threshold setting. For the threshold setting, the software has two options; manual threshold setting, and automatic threshold setting. After defining the window of brightness/darkness, ImageJ will convert the greyscale image into black and white, where every lipid droplets or point on the image is either a dark particle representing lipid droplet or white/clear background.

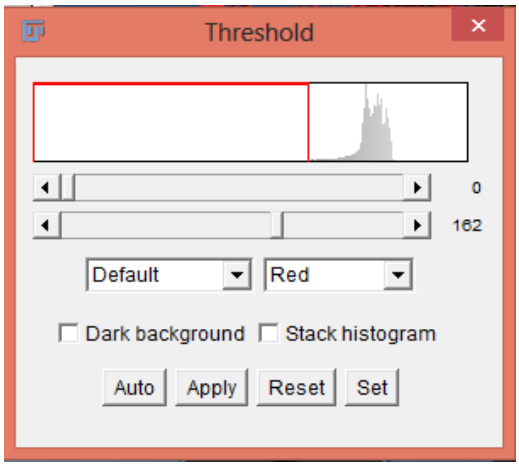


Figure 3.2. Threshold Panel in ImageJ

The top slider with value 0 controls minimum brightness, whereas the bottom slider controls maximum brightness. By adjusting both sliders, one can fix a defined "window of brightness/darkness."

III. Watershed Separation

When an image is converted to a binary image, the software can read and distinguish between black and gray pixels based on the intensity (Figure 3.3). The edge of the particle has gray pixels which are measured and considered as ultimately eroded points. In that area where two points are touching each other with gray pixels, a watershed line is drawn to separate them as individual particles.

In binary images, particles overlapping can be easily separated using the watershed option. On the software menu the command can be found: Process → Binary → Watershed.

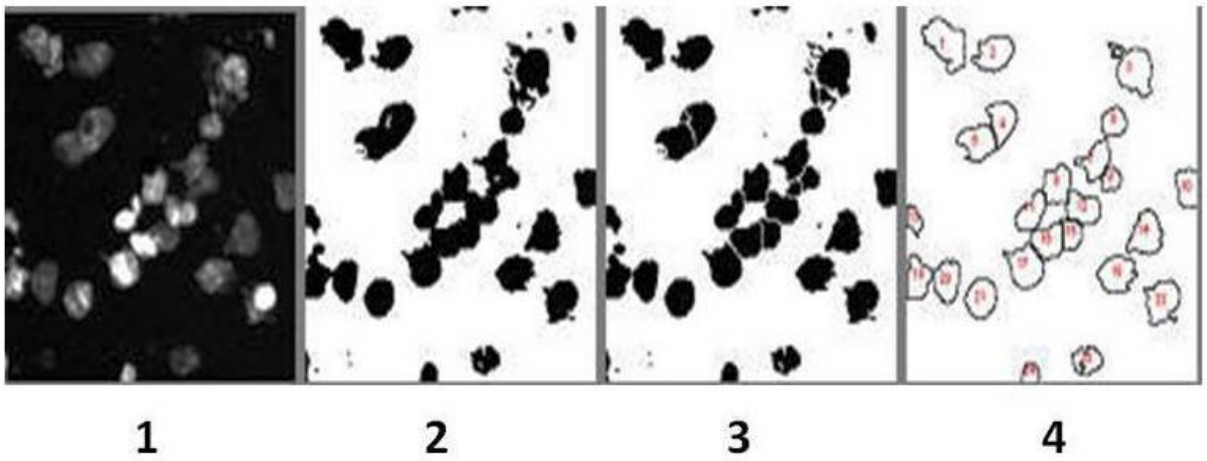


Figure 3.3. Flowchart of Watershed Option in ImageJ

The first panel is the actual image which could be 16-bit or 32-bit, which is converted to a binary image of 8-bit in second panel. Particles are connected to each other at several loci/points. If they are not separated, the software will read it as a single point to a group of connected particles. Therefore, the option watershed is used as shown in the third panel, where a line is drawn across the gray pixel area, and particles are separated. The fourth panel shows the outlined particles.

IV. Analysis of Particles

For analyzing the particles in the binary image, the menu command Analyze → Analyze particles was used. This provides information about each particle in the picture (Figure 3.4). The minimum and maximum size of lipid droplets can be set, as can the maximum pixel area size to exclude any object that is not a lipid droplet or any other contaminant/object that is of no interest in the image. Many objects like a line or cylindrical shape can be detected by the software. Therefore, roundness values are set between 0 and 1.0 as this helps to exclude unwanted objects from the readings.

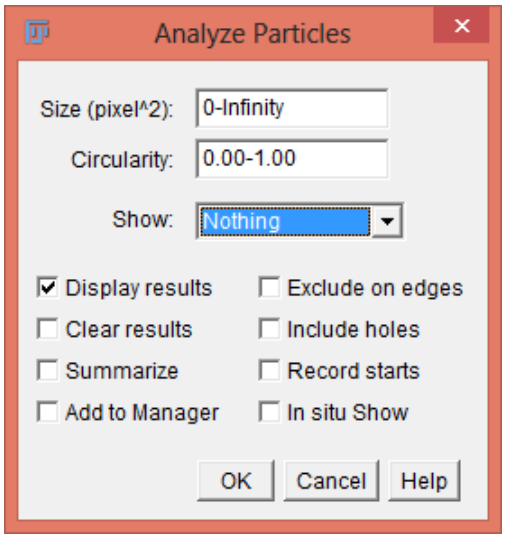


Figure 3.4. Particle Analysis Panel in ImageJ

The 'Show' drop-down menu has various options including - Nothing, Bare Outlines, Ellipses, Masks, Count Masks, Overlay Outlines, etc., Other options on panels include Add to Manager, Exclude on edges, Include holes, Record starts, etc. Option to show was selected → 'Outlines' option to help display the detected objects in the image. We also chose to 'Display results', 'Summarize', and Record starts.

3.5.4 Analysis of Lipid Droplets Size and Number by ImageJ Software

Each cell was analyzed from the image using ImageJ software. Data was obtained and analyzed. This analysis produced an output file for each individual lipid droplet in a single cell. For purposes of analysis, the average size of lipid droplet per cell was copied to Excel, from which the average of all analyzed cells was calculated and graphed.

3.6 Oil Red O Staining

Oil Red O (ORO) staining was performed for quantifying and imaging of lipids. ORO is a diazon dye which is hydrophobic and lipid soluble, with a maximum absorption wavelength of 518 nm. ORO stains not only neutral lipids but also cholesteryl esters in fixed cells. ORO does not stain biological membranes. The theory behind ORO staining is that the dye is minimally soluble in the solvent. By adding this dye to water, its solubility is reduced further. ORO stain mixed with water was prepared fresh because the dye separates out after a few hours. The lipophilic dye therefore quickly moves out of the water and strongly associates with the lipids in cells or tissue sections.

From the cell culture plate, the media was aspirated and washed with 1X PBS. 4% PFA in PBS was used to fix cells for 15 min. PFA was aspirated out, and the cell layer washed 3X with PBS to remove any traces of PFA. 60% Isopropanol was added to plates to remove traces of PBS from the cell layer. ORO stain was added to the culture plates, and cells were incubated for 30 min. ORO stain was aspirated out, and plates were rinsed with 70% ethanol to remove excess stain. Ultrapure water was added to the plate and images were obtained.

3.7 Quantification of Neutral Triglycerides

Quantification of TAGs was performed using a TAGs quantification kit from Abcam (Cat # ab65336). This assay is colorimetric and therefore provides a sensitive and easy protocol for quantification of TAGs. The principle behind this assay is that the triglycerides are broken down into free fatty acids and glycerol. The glycerol component is further oxidized to produce a product which readily reacts with a probe in the kit, which develops color. This product has a maximum absorption wavelength of 570 nm. The kit is very sensitive and can detect a minimum of 2 micromoles to 10 micromoles of triglyceride. This kit detects monoglycerides and diglycerides as well.

A fresh set of standards was prepared before performing each experiment by diluting the concentrated standard in assay buffer Triglyceride standards ranging from 0 nmol to 10 nmol were prepared. Cells were washed with PBS and re-suspended in 5% NP-40 in ddH_2O in microcentrifuge tubes. These tubes were placed in a heat block, and the temperature was gradually increased to 90°C. The samples, which had turned cloudy, were removed from the heat block and cooled at room temperature. These steps was repeated twice, and tubes were centrifuged for 2 minutes at high speed to remove cell debris or insoluble material. Samples were

diluted so that the concentration fell within the range of the standards. Lipase was added to each well containing either the triglyceride standard or samples, and then incubated at room temperature for 20 minutes which allowed the lipase to digest the tri/mono/diglycerides into glycerol and fatty acids. A reaction mix was then added to each well that consisted of triglyceride assay buffer, triglyceride probe, and triglyceride enzyme mix in 23:1:1 ratio. This master mix was prepared for all standards and samples. Reaction mix (50 μL) was added to each well and the 96-well plate was incubated at room temperature for 60 minutes, protected from light by covering with a paper towel. The absorbance of each well was measured at 570 nm.

4. RESULTS

4.1 Validation of Lack of Mfn-2 Expression in Mfn-2 KO MEFs.

In order to validate the lack of Mfn-2 expression in the knockout cell line, Western analysis was performed to assess Mfn-2 protein levels in WT MEFs and in the knockout line. As shown in Figure 4.1, Mfn-2 expression was only observed in WT MEFs.

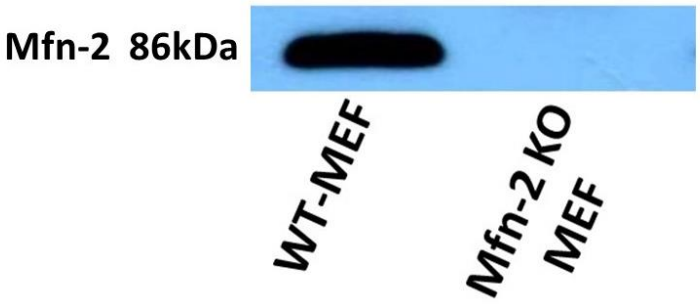


Figure 4.1. Immunoblot of cell lines for Mfn-2

WT and Mfn-2 KO MEF Cells were grown in DMEM + 10% FBS until 100% confluence. Cells were harvested, cell lysates prepared, and Western blotting performed.

4.2 Mitofusin-2 Protein is Essential for Maintaining Normal Mitochondrial Morphology

To investigate the role of Mfn-2 in normal mitochondrial morphology, an immunofluorescence experiment was conducted in WT and Mfn-2 KO MEF cells to visualize Hsp70 since this protein is a mitochondrial marker. As shown in Figure 4.2 (WT-A), the mitochondrial network in WT cells had the appearance of wiry strands or long threads that randomly extended in various directions in the cell. Even though mitochondrial morphology in WT MEFs predominantly consisted of tubular and longer mitochondrial strands, a small percentage of cells had shorter mitochondrial strands on the periphery as shown in Figure 4.2 (WT-B).

In Mfn-2 KO cells, the mitochondrial morphology in Mfn-2 KO MEF cells was distinctly altered. Most cells showed a fragmented pattern of much shorter threads (Figure 4.2, Mfn-2 A), although a few presented longer threads (Figure 4.2, Mfn-2 B). This experiment confirmed the role of Mfn-2 in maintaining the length of mitochondrial strands and overall morphology.

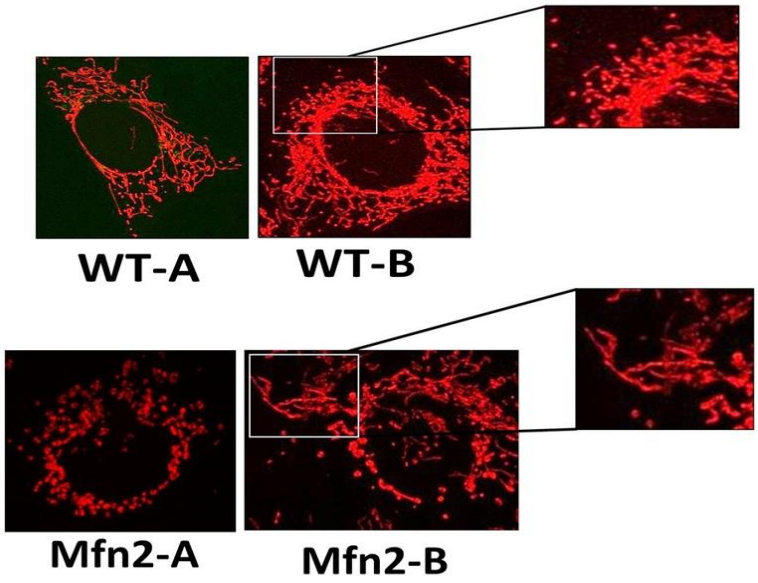


Figure 4.2. Knockout of Mfn-2 Results in Altered Mitochondrial Morphology.

WT and Mfn-2 KO MEF cells were grown on coverslips, fixed with paraformaldehyde and permeabilized with Triton-X 100. To view mitochondrial morphology, cells were incubated with anti-Hsp70 a mitochondria-specific marker. WT-A and B, and Mfn-2 A and B, are representative of the two predominant morphologies that presented in WT and Mfn-2 cells, respectively, with WT-A being by far the most frequently observed. The insets are magnified views of the region indicated by the box.

4.3 Absence of Mitofusin-2 Protein Increases Size of Lipid Droplets

Because the structural tether between the mitochondria and the ER is essential for lipid trafficking, it was felt likely that knockout of Mfn-2 might significantly interfere with overall lipid metabolism. To investigate this, the number and size of lipid droplets were assessed in WT and Mfn-2 KO MEF cells.

As shown in Figure 4.3, Mfn-2 KO cells showed the characteristic mitochondrial fragmentation pattern. Staining with BODIPY, a neutral lipid dye, allowed visualization of the lipid droplets. The droplets appeared to be localized in close proximity to mitochondria (merged panel), consistent with previous observations by Sturmey *et al.*, (2006). Subsequent analysis of lipid droplets in 30-50 cells indicated that the number of droplets showed little difference between

WT and Mfn-2 KO MEFs, averaging 43 ± 3 and 40 ± 2 per cell, respectively (Figure 4.5, control group). However, lipid droplet size was significantly enlarged in Mfn-2 KO cells, averaging 0.38 μm^2 compared to 0.2 μm^2 in WT cells (Figure 4.6, control group).

4.4 Absence of Mfn-2 Protein Increases Triglyceride Content in MEF Cells

Our results from earlier experiments indicated that Mfn-2 KO MEF cells had larger lipid droplets compared to WT cells. This is likely to result in increased total triglyceride in the Mfn-2 KO cells. It was decided that this be confirmed by assessing total neutral lipid. As expected, we found that Mfn-2 KO MEFs had almost twice the amount of triglycerides compared to WT MEF (Figure 4.4).

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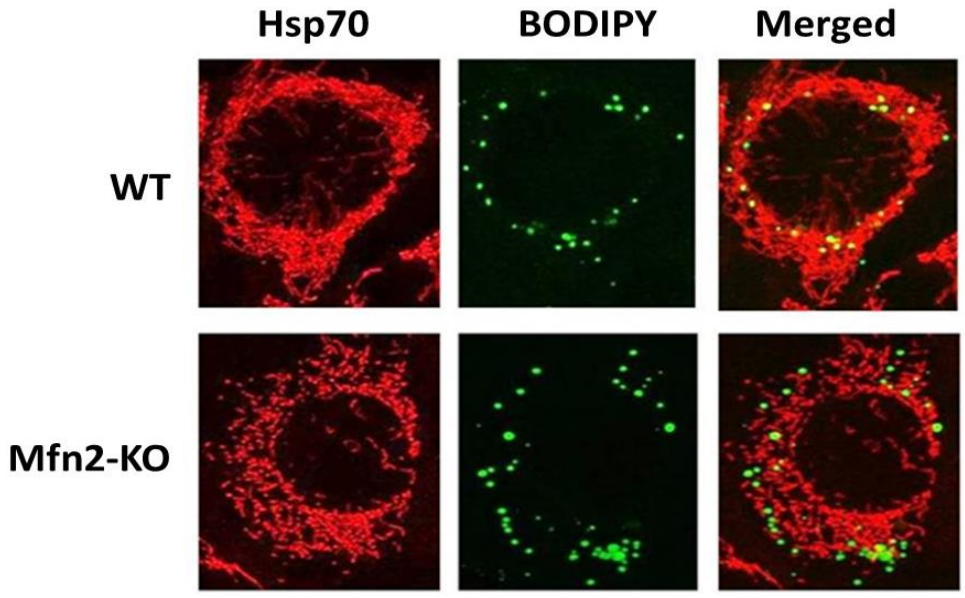

Figure 4.3. Lipid droplets localize closely to mitochondria.

WT and Mfn-2 KO cells were grown in DMEM + 10% FBS on coverslips and stained with anti-Hsp70 and BODIPY to stain mitochondria (in red) and lipid droplets (in green), respectively. The merged panel shows the relative position of mitochondrial strands and lipid droplets.

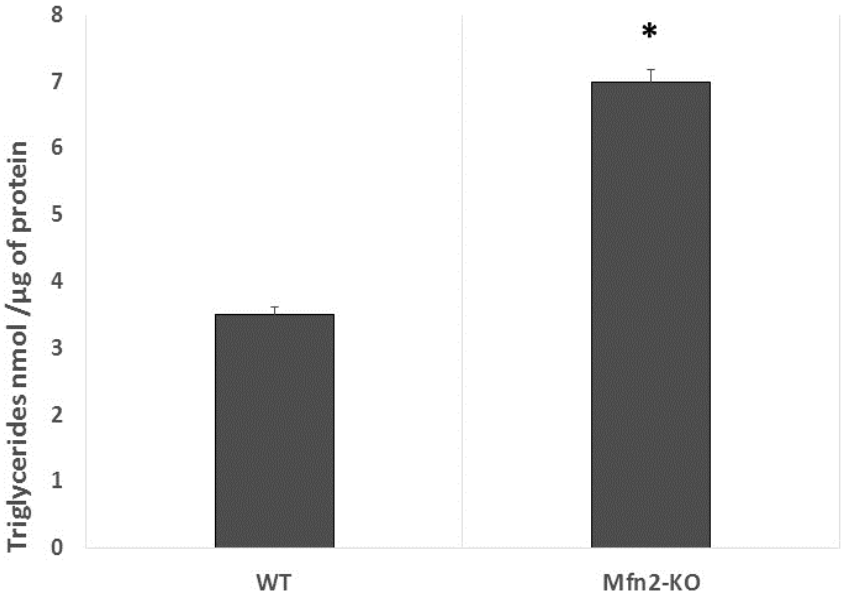

Figure 4.4. Mfn-2 KO MEFs show increased triglycerides relative to WT MEFs.

WT and Mfn-2 KO MEFs were grown to 30-40% confluency. Cells were harvested, and protein concentration was measured. Neutral lipid was then assessed as described in the Materials and Methods section. The results shown are averages ± SEM of three experiments. ANOVA single factor was used to analyze statistical significance (*) with p values ≤ 0.05, between WT and Mfn-2 KO cells.

4.5 The Effect of Insulin and Oleic Acid on Lipid Droplet Morphology

The positive effect of insulin and fatty acids on lipids formation and induction of lipid droplet formation is well known (Griffin and Sul 2004). Therefore, the effects of both insulin and oleic acid on lipid droplet morphology were examined in WT and Mfn-2 KO MEFs.

Treatment with insulin for 13 h had no significant effect on lipid droplet number in either WT or Mfn-2 KO MEFs (Figure 4.5). Conversely, oleic acid (OA) treatment resulted in a marked increase in the number of lipid droplets, approximately doubling in WT cells and tripling in the Mfn-2 KO cells. Treatment of cells with both insulin and OA led to a decrease in droplet number compared to OA treatment alone, with a larger decrease seen in Mfn-2 KO cells compared to WT (Figure 4.5).

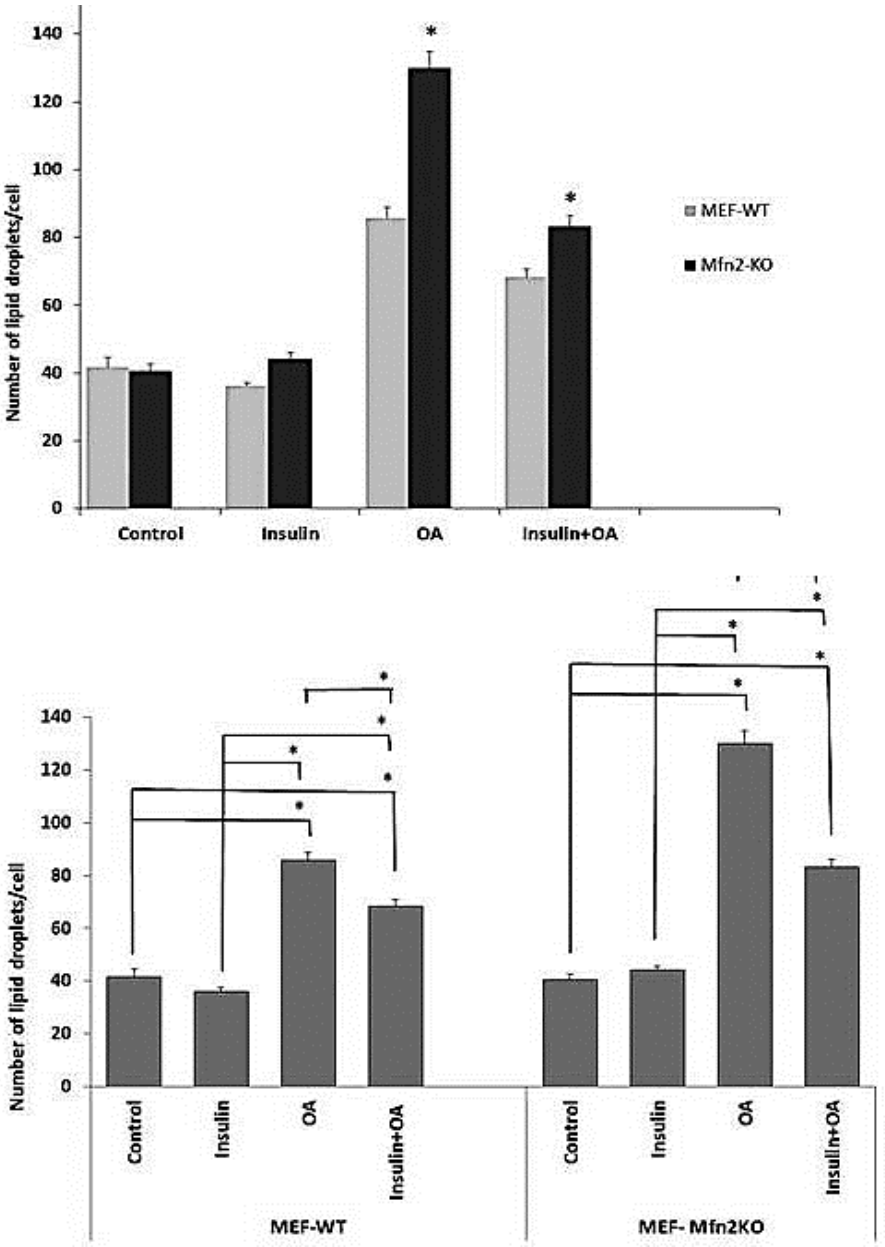

Figure 4.5. Effect of Insulin and OA on Lipid droplet number in WT and Mfn-2 KO MEFs.

Top panel: WT and Mfn-2 KO MEFs were grown in DMEM + 10% FBS to 30-40% confluency, and then treated with insulin and/or oleic acid (OA) for 13 h. Cells were fixed and stained with BODIPY. Confocal microscopy was used to image the fixed cells, and ImageJ software was used to analyze the number of lipid droplets. *Bottom panel*: the data shown in the top panel is displayed differently, comparing the effect of insulin and oleic acid treatments within each cell type. The results shown are averages ± SEM of three experiments. ANOVA single factor was used to analyze statistical significance (*) with p values ≤ 0.05, between WT and Mfn-2 KO cells (*Top panel*). ANOVA single factor was used to analyze statistical significance (*) with p values ≤ 0.05 between treatment within each cell type (*Bottom panel*).

Lipid droplet size in response to insulin and OA was also assessed. The average size of lipid droplets was larger in Mfn-2 KO MEFs compared to WT under all treatment conditions (Figure 4.6). Insulin treatment alone had no significant effect on droplet size in either cell line, similar to the lack of effect on droplet number. OA treatment increased lipid droplet size by approximately 70% in both WT and Mfn-2 KO MEFs, while the combined treatment with both insulin and oleic acid resulted in only a slight, statistically insignificant increase in droplet size compared to oleic acid treatment alone. The overall conclusion from these experiments is that the absence of Mfn-2 leads to an increase in lipid droplet size but not number, although when provided additional substrate for triglyceride synthesis i.e. oleic acid, the Mfn-2 KO MEFs show an enhanced capacity to increase the number of lipid droplets compared to WT cells.

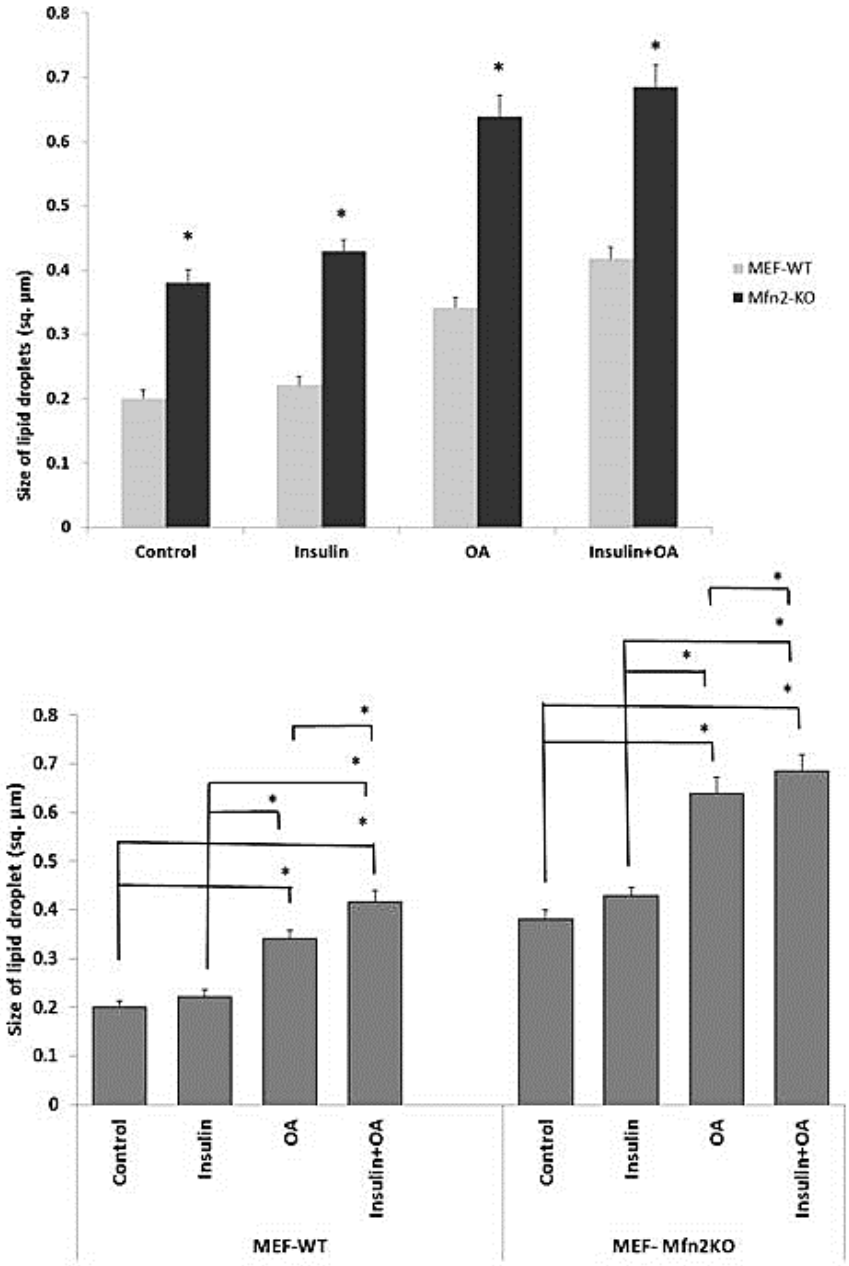

Figure 4.6. Effect of Insulin and OA on lipid droplet size in WT and Mfn-2 KO MEFs.

Top panel: WT and Mfn-2 KO MEFs were grown in DMEM + 10% FBS to 30-40% confluency and then treated with insulin and/or oleic acid for 13 h. Cells were fixed and stained with BODIPY. Confocal microscopy was used to image fixed cells, and ImageJ software was used to analyze lipid droplets. *Bottom panel*: the data shown in the top panel is displayed differently, comparing the effect of insulin and oleic acid treatments within each cell line. The results shown are averages ± SEM of three experiments. ANOVA single factor was used to analyze statistical significance (*) with p values ≤ 0.05 between WT and Mfn-2 KO cells (*Top panel*). ANOVA single factor was used to analyze statistical significance (*) with p values ≤ 0.05 between treatment within each cell type (*Bottom panel*).

4.6 Role of Mfn-2 in Adipogenesis

MEFs are multipotent stem cells that can be induced to differentiate into adipocytes, making them useful for the molecular aspects of adipogenesis. Given that Mfn-1 and Mfn-2 are involved in fusion of outer mitochondrial membrane and lipid trafficking across ER and mitochondria and that the absence of Mfn-2 results in enhanced lipid accumulation (see above), it was decided to investigate the role of Mfn-2 in adipogenesis using the knockout cell line.

Cells were cultured to 100% confluency, further cultured for an additional two days, and then treated with control or adipogenic media for 10 days. The expression of adipogenic marker genes coding for C/EBPα, FABP, and PPARγ were assessed by Western blot analysis in WT MEFs (Figure 4.7, upper panel). Interestingly, all three of the marker proteins assessed increased in expression over the 10 days, regardless of whether they were cultured in control or adipogenic media. The main difference observed was in the expression pattern of C/EBPα, which was induced by day 6 in adipogenic media followed by a reduction at day 10, while in control media, induction of C/EBPα was not observed until day 10. It should be noted that the doublet band present has been noted by others and is likely due to posttranslational modifications (Khanna-Gupta, 2008, Trivedi *et al.*, 2008).

In Mfn-2 KO cells, a similar induction of adipogenic markers was observed even with control media, although C/EBPα was strongly induced by day 6 and no detectable FABP expression was observed at day 0 (Figure 4.7, lower panel). Moreover, when cultured in adipogenic media, C/EBPα expression was induced slightly on day 6 but more pronounced on day 10, thus presenting a different response than WT MEFs. These data suggested that adipogenesis could be induced in MEFs by culturing them at 100% confluency and that there is no need to culture them in an adipogenic medium. Moreover, there appeared to be little impact on the adipogenic capability in Mfn-2 KO MEFs relative to WT cells, except for some minor changes in the pattern of C/EBPα induction.

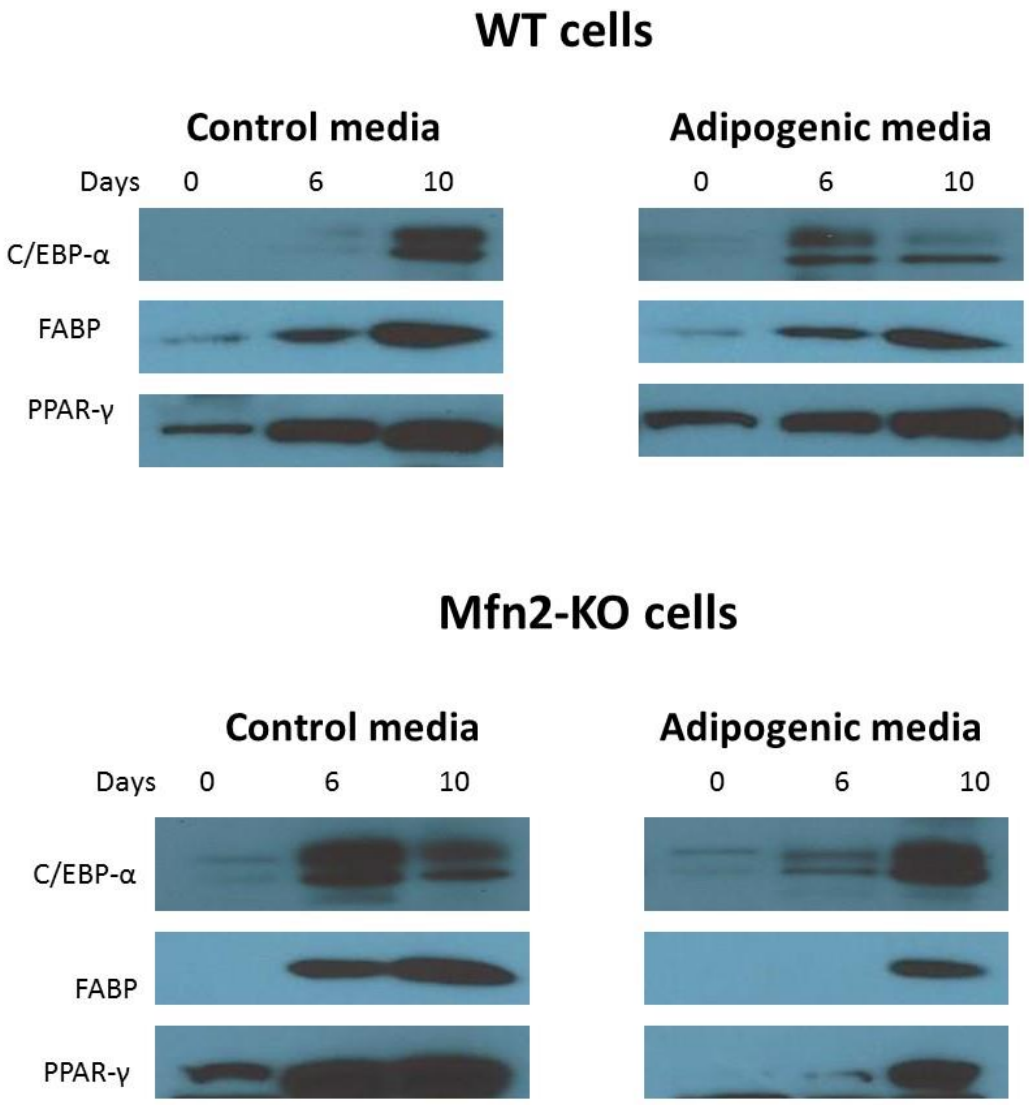

Figure 4.7. Induction of Adipogenic Marker Proteins in WT and Mfn-2 KO MEFs.

Cells were treated with control or adipogenic media as described in section 3.3.1. Cells were harvested on day 0, 6 and 10 of the experiment, cell lysates prepared, and Western blotting performed. Experiments were performed three times.

The expression profile of several relevant proteins including positive and negative regulators involved in adipogenesis was next examined in WT MEFs. Mfn-1 expression was fairly constant in cells incubated in control media. While an induction of Mfn-1 from day 0 through day 10 was observed when cells were cultured in adipogenic media, the level reached at 10 days was similar in cells treated with either control or adipogenic media. Mfn-2 expression was similar in both groups and unchanged by culturing in either media. It was noted that the apparent molecular weight of Mfn-2 was slightly greater in the day 0 sample in each treatment group.

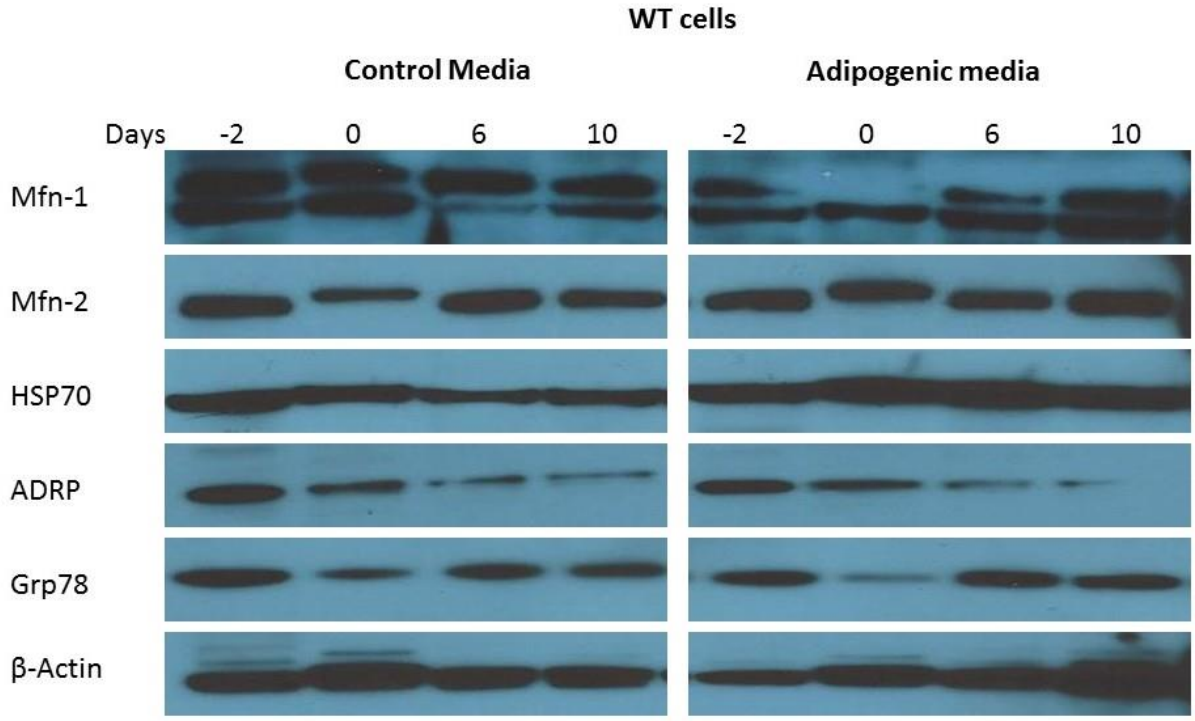

Figure 4.8. Expression profile of select proteins in WT MEF cells treated with control or adipogenic media.

WT MEFs were cultured in control or adipogenic media for the number of days indicated, and then cell lysates were prepared for Western blot analysis for the proteins indicated. β-actin was assessed as a loading control. Day -2 marks the day when cells reached 100% confluency, and day 0 marks the day when the cells were fed control or adipogenic media. Experiments were performed three times.

Hsp70 was assessed primarily as a control since previous studies have shown that this protein remains constant during adipogenesis induced in 3T3-L1 cells (Baldini *et al.*, 1995). The data in Figure 4.8 indicated that a steady expression of Hsp70 is observed in MEFs regardless of whether cells were cultured in control or adipogenic media. ADRP has been shown to associate with lipid droplets and to be highly expressed in preadipocytes but to gradually decrease in expression as adipocyte maturation occurs (Brasaemle *et al.*, 1997). This same expression pattern was observed in WT MEFs, with highest expression seen at day -2 which decreased with further culturing of confluent cells in either control or adipogenic media (Figure 4.8). These data provide further support that adipogenesis occurs in confluent MEFs even without the addition of adipogenic media.

Grp78 is glucose related protein of 78 kDa and is also known by other names as Binding immunoglobulin protein (BiP) or heat shock 70 kDa protein 5 (HSPA5). Grp78 is a chaperone that binds to the newly synthesized proteins in ER lumen and assists them in protein folding. It is also responsible for transport of misfolded proteins for degradation by the proteasome (Lee, 2005). It is a protein involved in maintaining endoplasmic reticulum homeostasis and structure (Hendershot, 2004; Lee *et al.*, 2005) and has been found recently to be essential for adipogenesis (Zhu *et al.*, 2013). Moreover, Grp78 expression was found to be undetectable in 3T3-L1 preadipocytes but to increase significantly upon induction of adipogenesis (Zhu *et al.*, 2013). In Figure 4.8, it is shown that Grp78 levels increased from day 0 to day 10 in both treatment groups. Somewhat surprisingly, levels at day -2, when cells first reached confluency, were as high as after 10 days of culturing in either media. In Mfn-2 KO cells, the expression of all of the proteins assessed (Mfn-1, Hsp70, Grp78, β-actin) remained relatively constant throughout the culturing time period regardless of which media the cells were treated with (Figure 4.7).

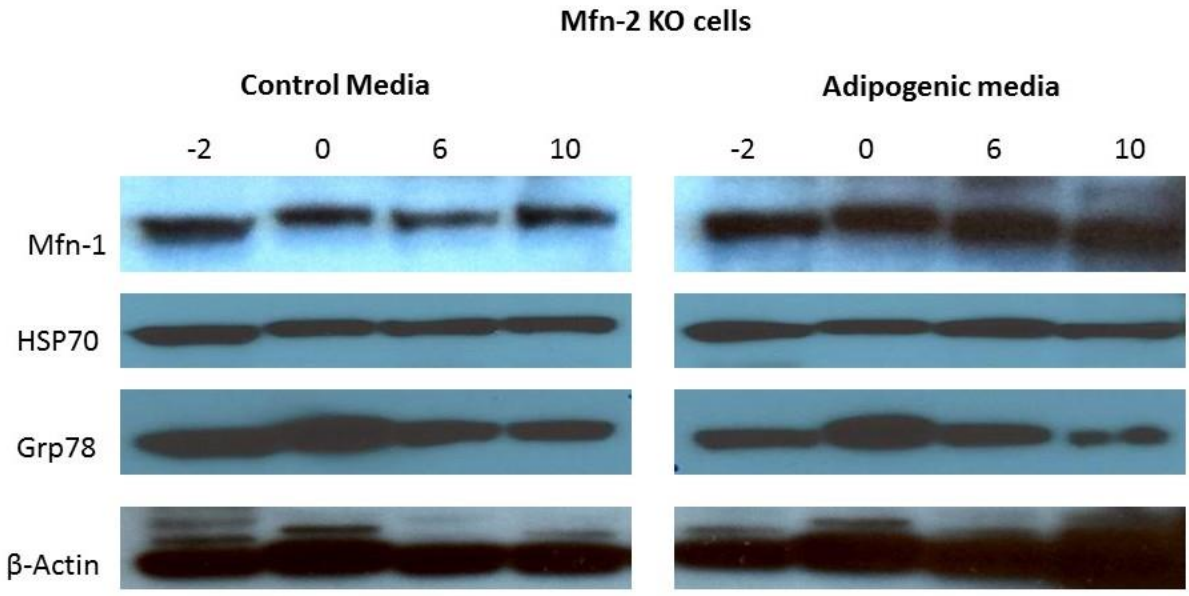

Figure 4.9. Expression profile of select proteins in Mfn-2 KO MEF cells treated with control or adipogenic media.

Mfn-2 KO MEFs were cultured in control or adipogenic media for the number of days indicated, and then cell lysates were prepared for Western blot analysis for the proteins indicated. β-actin was assessed as a loading control. Day -2 marks the day when cells reached 100% confluency, and day 0 marks the day when the cells were fed control or adipogenic media. Experiments were performed three times.

We next investigated lipid accumulation in WT and Mfn-2 KO MEFs after induction of adipogenesis using Oil Red O staining. The intensity of staining was similar in WT and Mfn-2 KO MEFs on day 0, suggesting similar lipid content (Figure 4.10 A). After 5 or 10 days of culturing in control media, both WT and Mfn-2 KO cells showed an increase in lipid content (Figure 4.10, B and C). Surprisingly, WT and Mfn-2 KO cells cultured for 5 or 10 days in adipogenic media showed lower intensity of red color relative to control media. This finding raised a question as to whether adipogenesis had actually been induced with adipogenic media.

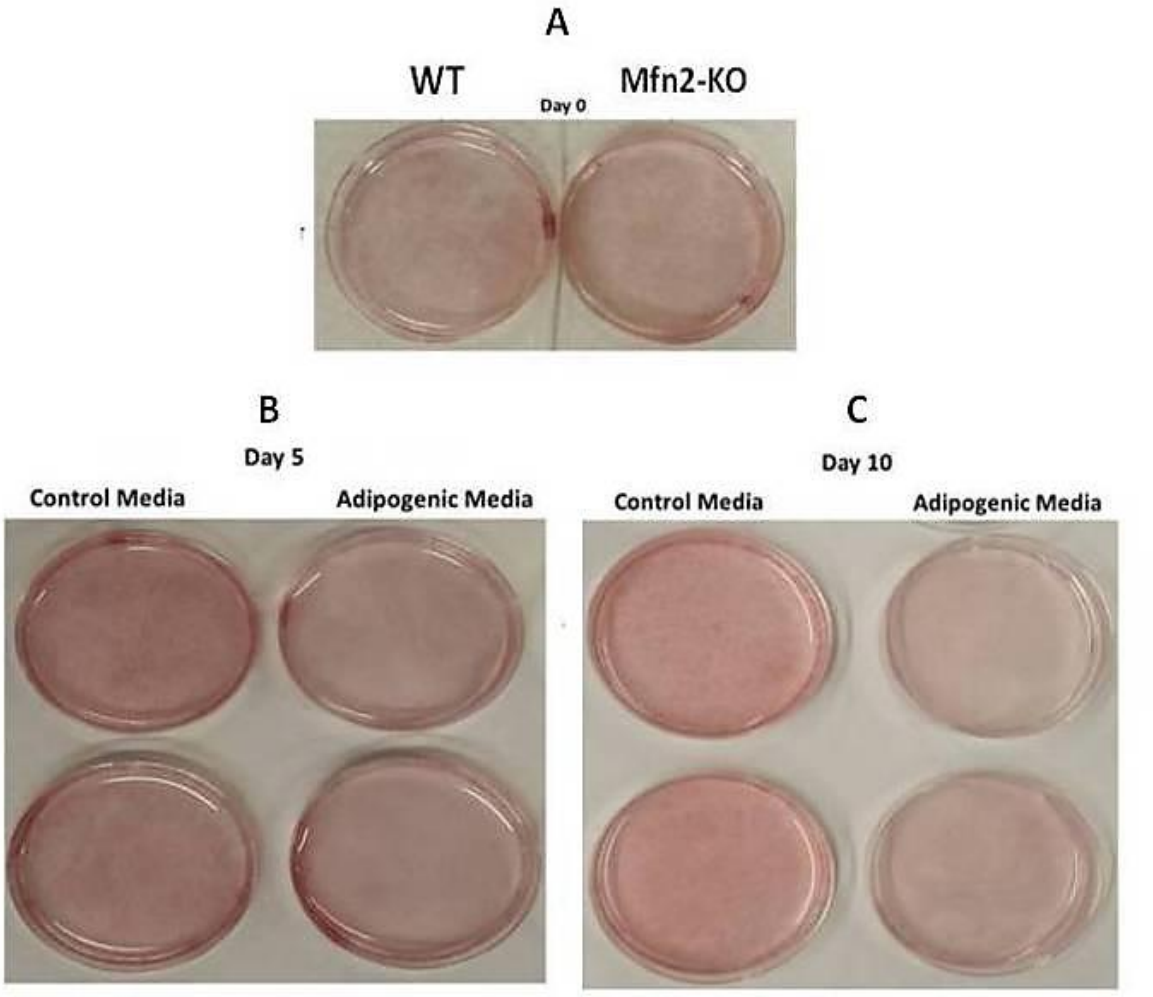

Figure 4.10. Oil Red O Staining in MEFs Undergoing Adipogenesis.

Adipogenesis was induced in WT and Mfn-2 KO MEFs as described in section 3.3.1. Day 0 was two days post 100% confluency and when cells were fed control or adipogenic media. Cells were fixed on day 0, 5, and 10 (A,B and C, respectively) and Oil Red O staining was performed.

In the adipogenesis experiment, fresh media was refed every other day. It was noted that for cells fed the adipogenic media, there was significant cell detachment which reduced the cell density on the plate and likely contributed to the reduced Oil Red O staining seen in the adipogenic media group shown in Figure 4.10. This reduced cell density was confirmed by phase contrast microscopy on day 4 and visualization of the total cell pellet on day 10 (Figure 4.11). Examination of WT and Mfn-2 KO cells by high-resolution microscopy (Figure 4.12) indicated that both appeared to have undergone differentiation when cultured for 10 days in control or adipogenic media.

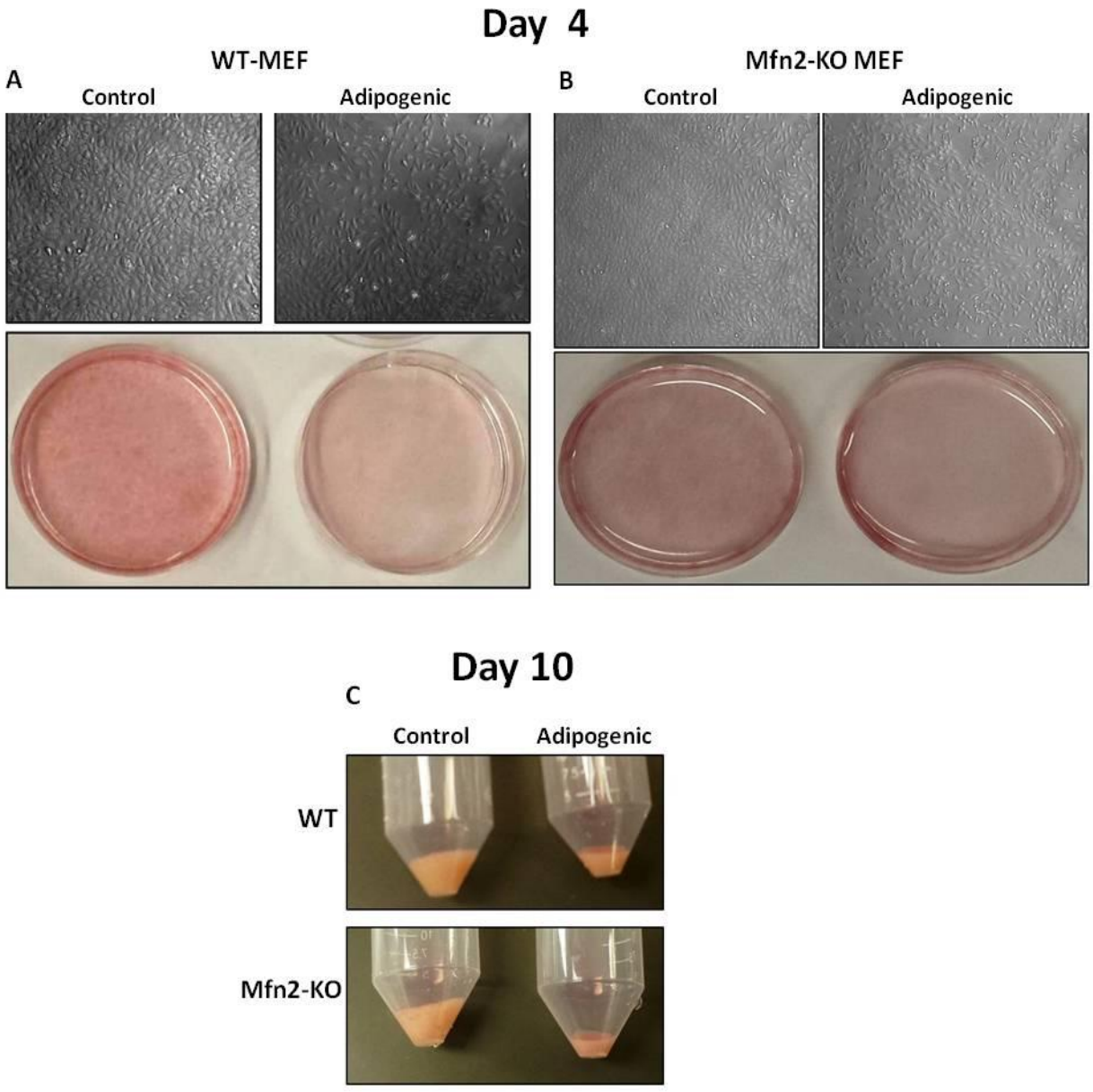

Figure 4.11. Adipogenic media enhances cell detachment.

Adipogenesis was induced in WT and Mfn-2 KO MEF cells as previously described. *Panel A (WT) and Panel B (Mfn-2 KO)*: Cells were fixed on day 4 for oil red O staining (*lower*), and images obtained by high-resolution (60X) microscopy (upper). *Panel C*: On day 10 cells cultured in either control or adipogenic media from three 100 mm plate were harvested into a Falcon tube and subjected to centrifugation.

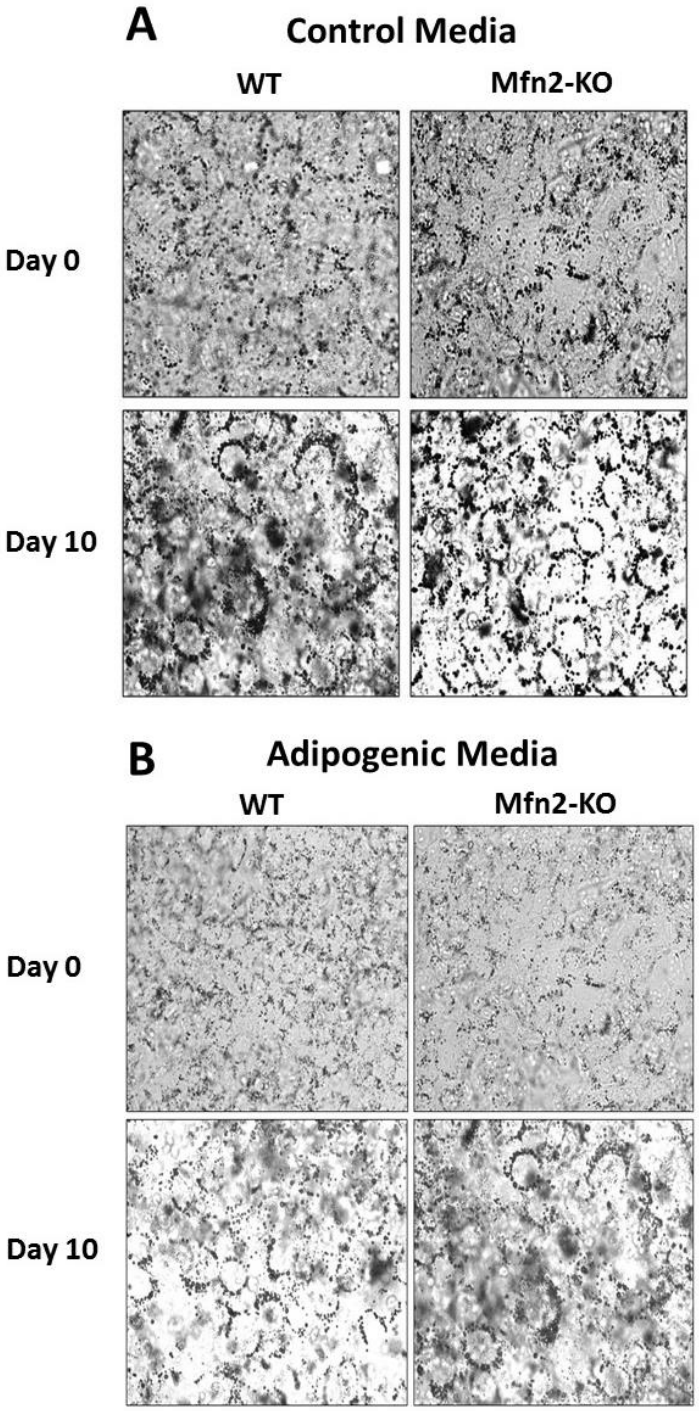

Figure 4.12. Oil Red O staining of WT and Mfn-2 KO MEFs in Control and Adipogenic media.

Adipocyte differentiation was induced as described in the Materials and Methods section. Cells were stained with Oil Red O, and images obtained by high-resolution microscopy at 100X magnification. (A-Control media and B-Adipogenic media)

4.7 Comparison of Adipogenesis in MEFs and 3T3-L1 Cells

Given the uncertainty as to whether adipogenesis was actually induced in MEFs when cultured in adipogenic media, it was decided to compare MEFs with 3T3-L1s, the gold-standard (pre-adipocyte) cell line used in adipogenic studies.

Initially, the effect of culturing the cell lines in adipogenic cocktail on lipid droplet number and size were assessed by immunostaining with BODIPY (Figure 4.13). As shown in panels A and B, in WT and Mfn-2 KO MEFs, the lipid droplet number and size increased after 10 days of culturing in adipogenic media. 3T3-L1 cells showed a similar response with respect to droplet size, although the number of droplets did not appear to increase (panel C). Figure 4.14 shows the average lipid droplet number and size, respectively, from the analysis of 30-50 cells from each cell line. MEFs (WT and Mfn-2 KO) after 10 days of adipogenesis had diverse size of lipid droplets, while in 3T3-L1 cells after 10 days of adipogenesis, most lipid droplets where of uniform size (Figure 4.13).

In western blotting experiments surprisingly all treatments and control experiments with insulin, oleic acid had higher number and size of lipid droplets in Mfn-2 KO cells. All markers in differentiation of Mfn-2 KO MEF were expressed either early in adipogenesis process or expressed at a higher level as compared to WT MEFs, but number and size of lipid droplets in immunofluorescence experiment Mfn-2 KO MEF had less average number and size of lipid droplets.

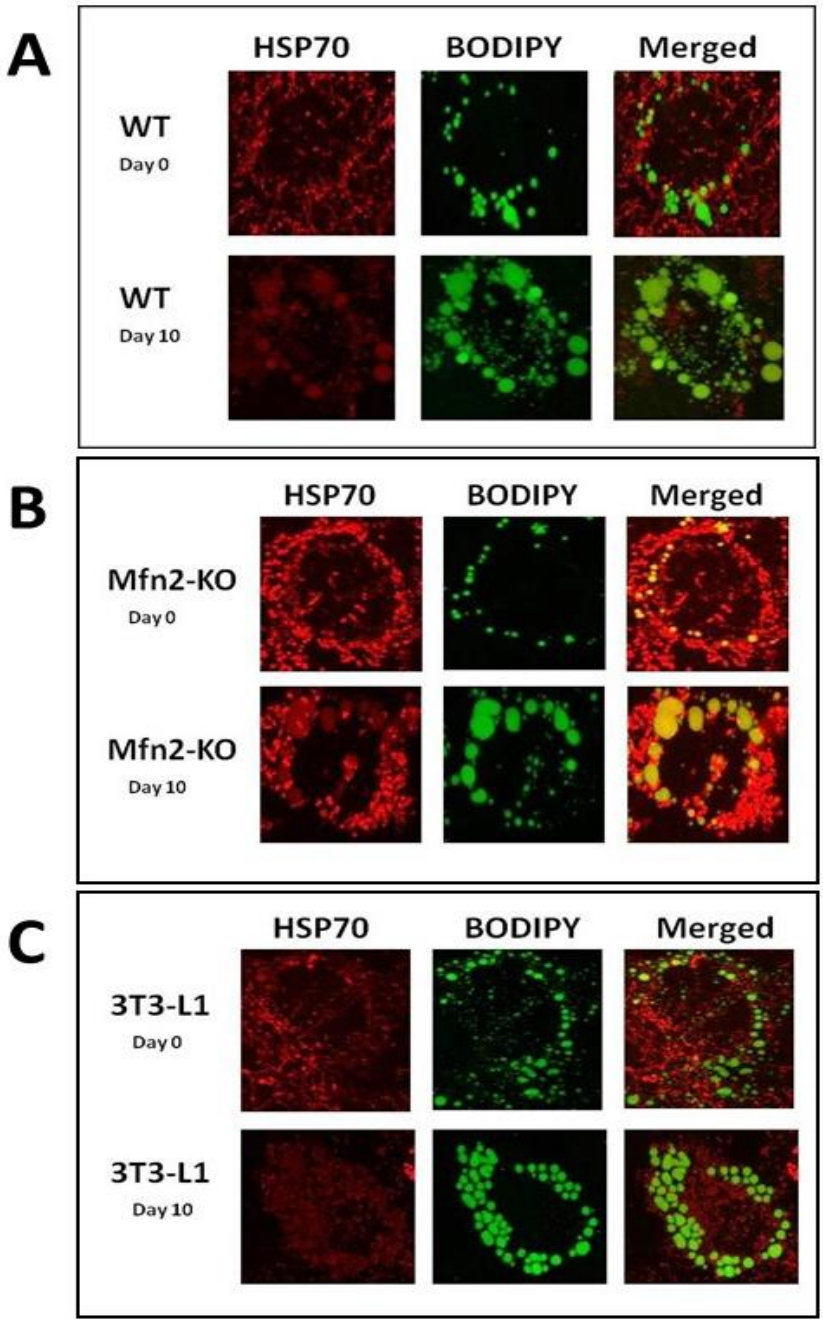

Figure 4.13. Comparison of adipogenesis in MEFs and 3T3-L1 cells.

MEFs (WT & Mfn-2 KO) and 3T3-L1 cells were grown on coverslip in 6-welled plate until 100% confluence was reached. Cellular differentiation was induced, 2-day post 100% confluent MEFs (considered as day 0). Cells were fed with adipogenic media. Cells were fixed on day 0 and 10 for immunofluorescence. Cells were incubated with anti-Hsp70 to stain mitochondria and BODIPY 493/503 to stain lipid droplets. Images were obtained and analyzed using ImageJ software for particles analysis. Total of 30-50 cells were analyzed from each group. Lipid droplet's number and size (μm^2) were analyzed. Data was statistically significant (*) with p-value ≤ 0.05.

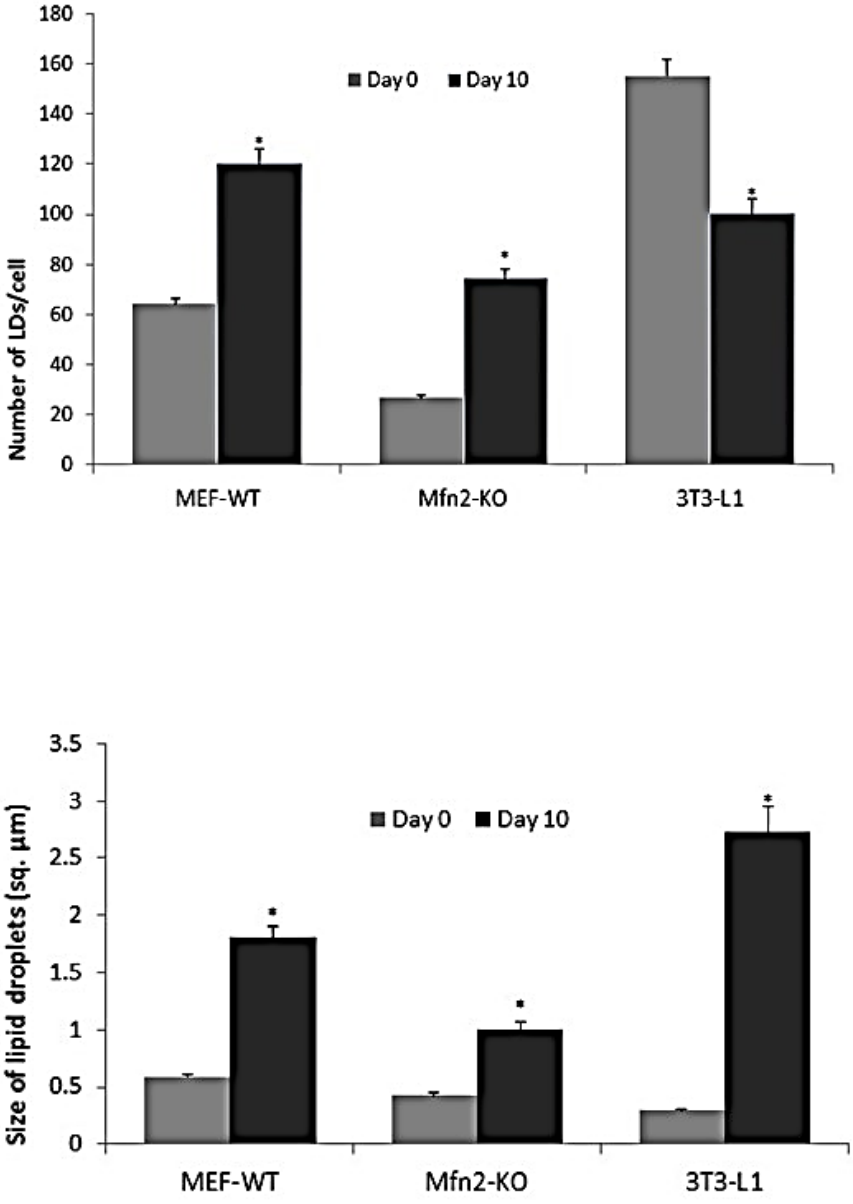

Figure 4.14. Effect of adipogenesis on lipid droplet number and size in MEFs (WT, Mfn-2 KO) and 3T3-L1 cell lines.

Adipogenesis experiment was carried in WT and Mfn-2 KO MEFs and 3T3-L1 cells. Cells were grown on coverslip to 100% confluency and treated with adipogenic media. Cells were fixed and permeabilized on day 0 and 10 and then incubated with BODIPY 493/503 (1:100) to stain lipid droplets. Images were obtained with a confocal microscope and analyzed using ImageJ software for particles analysis. A total of 30-50 cells were analyzed from each group. Statistically significant (*) with P-value ≤ 0.05.

5. DISCUSSION

5.1 Mfn-2 is Crucial for Maintaining Mitochondrial Morphology

The size, shape, and number of mitochondria vary in different cell types. For example, there is a single mitochondrion in retina cell whereas there are hundreds of mitochondria in hepatocytes (Tandler and Hoppel, 1986; Ogawa *et al.*, 2003). Mitochondrial cristae are also extremely diverse and can form different shapes depending on the pathophysiological state of the cell (Scheffler, 1999). These are evidence that mitochondrial dynamics is complex and consists of continuous fission and fusion process which varies according to cellular physiological events (Bereiter-Hahn and Voth, 1994; Chan, 2006). Several studies in the last two decades (Frank *et al.*, 2001; Karbowski, and Youle, 2003; Bossy-Wetzel *et al.*, 2003; Olichon *et al.*, 2003; Sugioka *et al.*, 2004; Arakaki *et al.*, 2006) established that mitochondrial fusion and fission is important for cell survival and apoptosis. Various proteins such as Mfn-1, Mfn-2, Opa1, Fis1, and Drp1 are involved in mediating and regulating mitochondrial dynamics. One study suggested that sustaining mitochondrial morphology is crucial to normal cell function, based on the observation that deletion of mitochondrial fusion GTPase (Mfn-1 or Mfn-2) in mammalian cell causes low respiratory capacity, ultimately leading to sluggish cell growth (Chen *et al.*, 2005).

Bereiter-Hahn and Voth (1994) proposed the term "mitochondrial dynamics" and that it encompassed fusion, fission, motility and frequent shape changes, ranging from punctate structures to wiry networks. Distribution of mitochondria inside a cell can be strikingly different. Mitochondria are often crowded at sites of high-energy demand. In this thesis, imaging of WT and Mfn-2 KO MEF cells revealed highly tubular and fragmented mitochondria, respectively. The mitochondrial network in WT cells displayed the wiry strands or long threads that arbitrarily stretched in several directions along the cell's length. Although WT MEFs predominantly consisted of tubular and longer mitochondrial strands, some cells had punctate or shorter mitochondrial strands on the periphery. In Mfn-2 KO cells, the mitochondrial morphology was markedly different, with the predominant feature being a fragmented pattern of much shorter threads. Other studies using complementary approaches are supportive of this conclusion. Chen (2005) showed that if mitochondria were compromised for fusion activity, the mitochondrial network appeared more fragmented, and cells lacked normal respiration capacity. When mitochondrial fusion activity is lost in mice, the fetus dies in early stages of development

(Chen, 2003). A study showed that overexpression of Mfn-2 resulted in significant changes in mitochondrial morphology (Huang *et al.,* 2007). A study reported visible abnormalities in mitochondrial morphology of cardiac myocytes after knockout of Mfn-2, including a significant deviation in mitochondrial size (Chen *et al.,* 2007). Most of the mitochondria was abnormally enlarged. Similar observations were made on Mfn-2 knockout in other mouse models (Chen *et al.,* 2003, 2007). Chen *et al.,* (2010) deleted one *Mfn-1* allele and two *Mfn-2* alleles from mouse skeletal muscle and observed formation of remarkably large mitochondria, although the total mitochondrial mass in cardiac myocytes was approximately the same. In the current study, we also observed that Mfn-2 KO MEF cells had fragmented mitochondrial morphology throughout different confluency states. However, WT MEFs cells had a wiry network which gradually became fragmented, especially in the post-confluence state. Similar observations were made by Kita *et al.,* (2009) in 3T3-L1 cells. Thus, the role of Mfn-2 in maintaining the length of mitochondrial strands and overall morphology was established. Though mitochondrial morphology is distorted in Mfn-2 KO MEFs, it does not affect total mitochondrial mass in a cell (Chen *et al.,* 2003, 2007).

5.2 Lipid Droplets are Often Co-localize with the Mitochondrial Network

In our study, confocal microscopy confirmed the close association of lipid droplets with mitochondria. Several other studies with different cell types have reported close association of lipid droplets with mitochondrial network (Shaw *et al.,* 2008; Sturmey *et al.,* 2006; Tarnopolsky *et al.,* 2007). Tarnopolsky *et al.,* (2007) provided evidence suggesting that this association between lipid droplet and mitochondria could be an efficient way to transfer fatty acids destined for β-oxidation between these organelles. There is also evidence that the interaction of mitochondria with lipid droplets might vary according to the cell type, and that the release of fatty acids from lipid droplets might vary in cell types depending on whether lipolysis or lipophagy occurs. For example, hepatocytes, which have low levels of lipase, engage in lipophagy instead of lipolysis (Singh *et al.,* 2009; Walther and Farese, 2012).

Broad exchange of lipids and their precursors continuously occurs between the mitochondrial and ER membranes. Recent research on lipid transfer proteins and membrane-tethering complexes provides insight into the mechanisms of these transport processes, which are fundamental for mitochondrial dynamics and cellular homeostasis (Tatsuta *et al.,* 2013). The spatial arrangement of mitochondria and ER facilitates both lipid metabolism and the uptake of

Ca^{2+} released from the ER into the mitochondria (Rizzuto *et al.,* 1992, 1993, 1998; Voelker, 2000, 2005). Some studies suggested that since Mfn-2 tethers the ER and mitochondria. Disruption of Mfn-2 may lead to the following consequences (Chen *et al.,* 2003; Area-Gomez *et al.,* 2012). First, it can increase the distance between two organelles. Second, it can suppress phospholipid transport between the ER. Third, it results in decreased calcium metabolism in the mitochondria. However, a recent report (Filadi *et al.,* 2015) on Mfn-2 disruption suggests that Mfn-2 maintains the distance between ER and mitochondria. They suggested that disrupting Mfn-2 brings both organelles too close, causing abnormal calcium metabolism and toxicity. Thus, we know that the lipid droplets are synthesized in ER, and interact closely with mitochondria. This association of ER, mitochondria and lipid droplets is disturbed in Mfn-2 KO cells. Silencing of Mfn-2 thus disrupts tether between ER and mitochondria, affecting overall lipid and calcium metabolism.

5.3 Mfn-2 Tether between ER-Mitochondria is Involved in Energy Homeostasis.

Mitofusins are involved in mitochondrial fusion as well as in tethering mitochondria with the ER (Rizzuto, 2006; de Brito and Scorrano, 2008). De Brito and Scorrano (2008) discovered an important structural component of the ER-mitochondria tether. This study established the role of Mfn-2 in providing the physical foundation for ER-mitochondria communication and assisting in several biochemical processes such as Ca^{2+} signaling and lipid trafficking (Scharwey *et al.,* 2013). Silencing of Mfn-2 in HeLa and MEF cells not only loosens ER-mitochondria interactions but also disrupts ER-mitochondria morphology. Thus, genetic and biochemical evidence suggested a model in which Mfn-2 bridges the ER and mitochondria. These bridges could form a homotypic or heterotypic tether with both Mfn-1 and Mfn-2.

Mitochondria are at the hub of several crucial cellular mechanisms (Ferri and Kroemer, 2001; Rizzuto and Pozzan, 2006). To accomplish these responsibilities, mitochondria are functionally and spatially placed in a complex grid of interconnected ER, often in close contact (Rizzuto and Pozzan, 2006). These membrane contact sites between ER and mitochondria dictate mitochondrial Ca^{2+} uptake from the ER, eventually affecting cellular metabolism (Rizzuto *et al.,* 1992, 1993, 1998). Likewise, these membrane contact sites are important for the biosynthesis of mitochondrial lipids, which transpires at MAM patches of ER (Vance, 1990). The fine molecular architecture of the ER-mitochondria tether is unknown; however, it is probably proteinaceous (Csordás *et al.,* 2006). A multifunctional sorting protein (PACS2) is

enriched in ER and indirectly controls ER and mitochondria juxtaposition through BAP31 (Simmen *et al.*, 2005). Correspondingly, the dynamin-related GTPase Drp1 is essential for ER-mitochondria fission and can even alter tethering by perinuclear clumping of mitochondria (Pitts *et al.*, 1999; Szabadkai *et al.*, 2004). Additional GTPases include Opa1, which is involved in apoptosis and mitochondrial fusion (Cipolat *et al.*, 2004; Freeza *et al.*, 2006), and Mfn-1 and Mfn-2 which are essential for mitochondrial fusion (Koshiba *et al.*, 2004). Mfn-2 is also crucial not only for neuronal differentiation but also embryonic development (Chen and Chan, 2006; Chen *et al.*, 2003; Chen *et al.*, 2007). A peripheral neuropathy, Charcot-Marie-Tooth (CMT) type 2A, is characterized by axonal degeneration and is caused by mutations in Mfn-2 (Zuchner and Vance, 2006).

There is a growing body of evidence that supports the hypothesis that the Mfn-2 mediated tethering of ER and mitochondria plays a significant role in energy homeostasis. For example, Bach *et al.*, (2003) showed that the silencing of Mfn-2 led to reduced oxygen consumption and glucose oxidation in fibroblasts. In L6E9 rat skeletal muscle cell, decreased oxidation of palmitate and pyruvate was observed after knockdown of Mfn-2 expression (Bach *et al.*, 2003, 2005). A study in 3T3-L1 preadipocyte cells (Kita *et al.*, 2009) found that when mitochondrial fission was silenced (Fis1 and Drp1), mitochondrial fusion activity increased and cellular TAG content was decreased. In contrast when mitochondrial fusion activity was silenced (Mfn-2 and Opa1), mitochondrial fission increased, ultimately leading to a fragmented mitochondrial morphology and increased TAG content. Mice lacking Drp1 die shortly after birth due to developmental anomalies, especially in the forebrain (Ishihara *et al.*, 2009). *Drp1* and *Fis1* null MEF cells showed elongated and highly interconnected mitochondrial morphology (Losón *et al.*, 2013). Whereas Mfn-2 and Opa1 knockout MEFs have highly fragmented mitochondrial morphology (Chen *et al.*, 2003, 2005; Song *et al.*, 2009)

Insulin and fatty acids have positive growth effects on lipogenesis and induction of lipid droplet formation (Griffin and Sul, 2004). In this study, we investigated the changes in lipid droplet number and size in WT and Mfn-2 KO MEF cells. Mfn-2 KO cells in control and treatment (insulin, oleic acid, and the combination of both) had either a higher number or bigger size of lipid droplets as compared to WT cells. Mfn-2 KO cells treated with oleic acid accumulated three times the number of lipid droplets as compared to WT which doubled their number. When cells are under starvation, the mitochondria shift to new fuel i.e. TAG from lipid

droplets (Berg *et al.,* 2002). Thus, fatty acids drive oxidative phosphorylation and are used to produce ATP, affecting the overall number and size of lipid droplets (Finn and Dice, 2006; Kerner and Hoppel, 2000). Several studies on lipid trafficking (Herms *et al.,* 2013; Kassan *et al.,* 2013; Wang *et al.,* 2010) have suggested that incorporation of exogenously added fatty acids in cell culture media leads to formation of TAGs, which ultimately forms new lipid droplets or increase size of existing lipid droplets. Lipid droplet number and size are also affected by two distinct processes, lipolysis and lipophagy (Singh *et al.,* 2009; Zechner *et al.,* 2012). A recent study in Mfn-1 KO MEFs on fatty acid trafficking in starved cells revealed that cells with fragmented mitochondria are unable to oxidize fatty acids at the same rate as WT MEFs (Rambold *et al.,* 2015). Our studies further confirm that these extra fatty acids which are not oxidized at normal rate accumulate within a cell. These fatty acids could cause toxicity and thus they are cycled back to existing lipid droplets. Absorption of these fatty acids in lipid droplets further leads to an increase in either lipid droplet number or size in the fragmented mitochondrial cell. Lipid droplets consists primarily of triglycerides (Bartz *et al.,* 2007), therefore in the current study we analyzed triglyceride content in WT and Mfn-2 KO cells. Total lipid was twice in the Mfn-2 knockout cells compared to WT cells, even though they had approximately the same number of lipid droplets. Thus our data indicated that in Mfn-2 KO MEF cells, the increased triglycerides were stored in existing lipid droplets, expanding their size instead of increasing the number of droplets in the cell.

5.4 Loss of Mfn-2 Induces Adipogenesis

Lipid droplets are storage depots of excess energy and are found in all organisms and in most cell types (Wan *et al.,* 2007; Fujimoto *et al.,* 2008). The lipid droplet core is composed of neutral TAGs (Cheng *et al.,* 2009) and it is enveloped by a monolayer of phospholipids embedded with various set of proteins (Fujimoto and Ohsaki, 2006; Walther and Farese, 2009; Meex *et al.,* 2009). Abnormalities in lipid droplet formation and regulation could lead to atherosclerosis (Paul *et al.,* 2008; Fujimoto *et al.,* 2008). Lipid droplet formation and turnover is crucial for cell survival, however it is unclear whether regulation of these mechanisms are brought about through internal (cellular lipid availability) or external molecular signaling (Digel *et al.,* 2010). Several studies (Nakamura *et al.,* 2005; Andersson *et al.,* 2006) suggested that lipid droplet generation, growth and localization is dependent on the ERK pathway and phospholipase D, and it is well-

established that lipid droplet formation signaling is stimulated in response to fatty acid exposure. A study (Rohwedder *et al.*, 2014) established in Huh-7 cells that addition of exogenous fatty acids in cell culture media induces detectable lipid droplets. Fatty acids are the fundamental units of the structural cellular membranes and functional units of lipid metabolism (Dowhan, 1997; Nohturfft and Zhang, 2009; Walther and Farese, 2012). Elevated levels of free fatty acids in serum contribute towards the pathogenesis of metabolic abnormalities, whereas adipocyte cells have exceptional capacity to stock extra free fatty acids in lipid droplets as TAGs. Excess accumulation of TAGs in skeletal muscle leads to insulin resistance (Shulman, 2000). Adipogenesis is accomplished by a number of factors which serves as a molecular switch to function in controlling the progenitor's fate, either positively or negatively. Both *in vivo* and *in vitro* studies provide evidence that supports the role of PPARγ as the master regulator of adipogenesis (El-Jack *et al.*, 1999; Wu *et al.*, 1999). Our study determined that the ER-mitochondria tether mediated by the mitofusins had a role in adipogenesis. WT and Mfn-2 KO MEFs were differentiated by incubating 48 hours post confluent cells with an adipogenic cocktail media. After several days, WT and Mfn-2 KO MEFs showed huge cytosolic lipid droplets, characteristic of adipocytes (Figure 4.13). Several markers characterizing adipogenesis were analyzed by Western blotting to confirm that the adipogenesis was induced in both WT and Mfn-2 KO MEFs (Figure 4.7, 4.8, 4.9). However, our results indicate a slight different induction of adipogenesis in Mfn-2 KO cells as compared to WT. Therefore, it appears that Mfn-2 is not required for adipogenesis, but it could have some role in regulation of adipogenesis and needs to be explored. Mesenchymal stem cells from bone marrow accumulated greater quantity of lipids upon exposure to a combination of dexamethasone, IBMX, insulin and indomethacin (Pittenger *et al.*, 1999). During adipogenesis there is an increase in mitochondrial metabolism and ROS activity (Wang *et al.*, 2015), however, it is unclear whether high mitochondrial metabolism is necessary for adipogenesis. mTORC1 stimulates ROS generation during adipogenesis and antioxidants targeting mitochondria prevent adipogenesis (Tormos *et al.*, 2011). PI3-kinase signaling and downstream effector mTORC1 are well-known regulators of PPARγ-dependent adipogenesis and lipogenesis (Laplante and Sabatini, 2009). Oxygen consumption by mitochondria and overall ROS levels are augmented during adipogenesis (Imhoff and Hansenn, 2010; Wilson-Fritch *et al.*, 2003). Tormos *et al.*, (2011) observed a gradual increase in the oxygen consumption rate, accompanied by increased ROS levels during adipogenesis. One

probable reason for slightly early induction of adipogenesis in Mfn-2 KO MEFs may be low respiratory capacity and low oxygen consumption.

5.5 Adipogenesis is Strongly Induced in Presence of High Concentration of FBS.

Adipocyte physiology and adipogenesis have been extensively studied *in vivo* and *in vitro.* Two common cell culture models for understanding adipogenesis are 3T3-L1 and OP9 (Green and Kehinde, 1974; Nakano *et al.,* 1994). The most frequently used protocol for inducing adipogenesis involves incubating 48 h post confluent monolayer cells with adipogenic cocktail media (IBMX, dexamethasone and insulin plus 10% FBS) for another 48 h. After 48 h of cocktail media, cells are grown in media with insulin for another 48-72 h. Adipogenesis using 10% FBS was performed, however limited differentiation was observed (data not shown). 3T3-L1 and OP9 cell lines easily differentiate into adipocytes after 6-7 days of treatment. However, several studies on variations in adipogenesis efficiency and low yields have been reported (Mehra *et al.,* 2007; Wolins *et al.,* 2006; Zebisch *et al.,* 2012). Some studies have observed high variation in Oil Red O staining, indicating variation in lipid accumulation, mostly when cells are incubated in different culture plates or media. One probable reason for this variation could be different oxygen content since low-oxygen content in media inhibits adipogenesis (Pettersen *et al.,* 2005; Sheng *et al.,* 2014; Yun *et al.,* 2002).

Our studies further revealed that in order for MEF cells to undergo adipogenesis, culture media requires (20%) of fetal bovine serum, instead of 10%. In our study on adipogenesis, we also noticed an early and strong induction of cell differentiation in MEF cells with DMEM media + 20% fetal bovine serum, as compared to the traditional method of using synthetic modulators like IBMX, dexamethasone, and troglitazone. In Figure 4.7 and 4.10, we observed strong induction of FABP and PPARγ on day 6, and Oil Red O staining indicated accumulation of more lipids in the control media. The complex cocktail of growth factors in serum might have a stimulatory effect on cell growth, allowing them to achieve greater density before attaining the resting state. Fat accumulation might then occur much more rapidly and to a greater degree than in traditional adipogenic media. A study was published (Green and Meuth, 1974) on pre-adipocyte cell line 3T3-L1 which revealed that a high concentration (20 - 30%) of calf serum increased the rapidity and degree of the fat accumulation. Even though differentiated cells easily detach from culture plate surface, we observed (Figure 4.11) that cells cultured in adipogenic media easily detached from culture plate surface as compared to cells in control media. Several

studies have observed similar cell detachment in adipogenic media (Barnes *et al.*, 2003; Gregoire *et al.*, 1998).

5.6 Model showing the Relationship between Mitochondrial Morphology and Lipid Droplet Size

Based on data presented in this thesis, a model describing the relationship between Mfn-2, mitochondrial morphology and lipid metabolism is proposed (Figure 5.1). In cells expressing Mfn-2 (WT, upper panel), both fission and fusion processes are unimpaired. Most of the mitochondria adopt a wiry or tubular network appearance, and there are extensive, well-developed contacts between the ER and mitochondria that are mediated by Mfn-1 and 2. Respiration and β-oxidation are also unimpaired. Lipid metabolism is well-controlled, and includes the esterification of some free fatty acids into TAGs by the triacylglycerol synthetase complex (not shown) located on the ER membrane. These TAGs are stored in lipid droplets (yellow circles).

In the absence of Mfn-2, the fusion process is impaired. The resulting imbalance between the fission and fusion processes leaves most of the mitochondria in the fragmented state. Moreover, the contacts between the fragmented mitochondria and the ER are impaired, which affect overall lipid metabolism. Exacerbating this is the fact that fragmented mitochondria show impaired β-oxidation of fatty acids (Zanna *et al.*, 2008; Schönfeld *et al.*, 2010). This results in an accumulation of fatty acids, and in order to avoid fatty acid toxicity, the cell increases TAG synthesis. The increased TAGs, which are stored in lipid droplets, result in larger lipid droplets size rather than number of droplets.

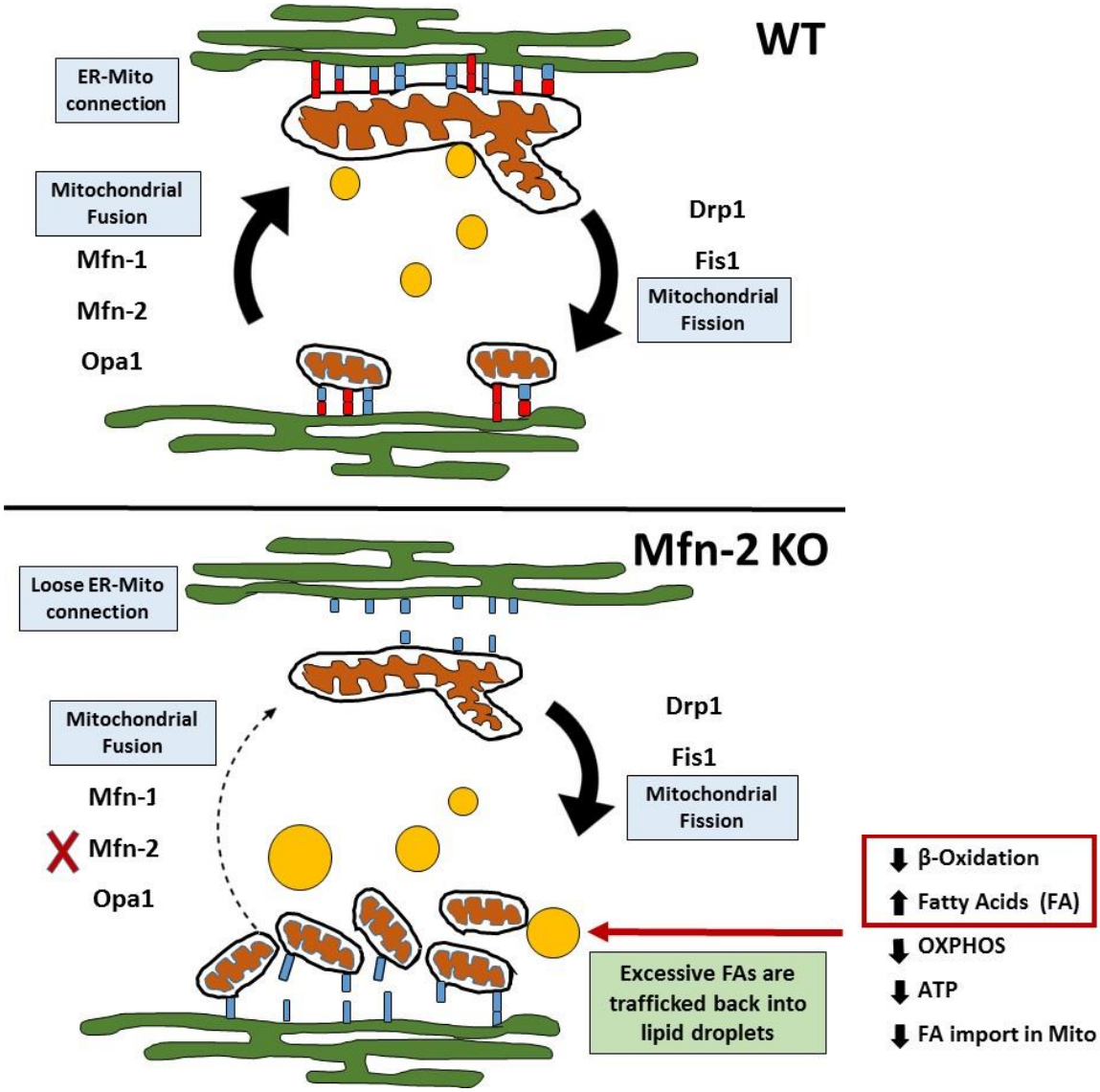

Figure 5.1. Schematic showing mitochondrial dynamics and the relationship between mitochondrial morphology and lipid droplet size.

Upper Panel (WT MEF): Mitochondria is in physical contact with ER via Mfn-1 and Mfn-2 homodimer and heterodimers. Fission proteins such as Drp1 and Fis1 along with other fission factors mediate mitochondrial fission, giving rise to smaller fragmented mitochondria. Conversely, mitochondrial fusion proteins such as Mfn-1, Mfn-2 and Opa1 are involved in mitochondrial fusion, giving rise to a wiry or tubular network of mitochondria. In either form mitochondria are continuously interacting with the ER. In normal cells, mitochondrial fusion and fission are in equilibrium. *Lower Panel* (Mfn-2 KO MEF): In the absence of Mfn-2, cells display primarily fragmented mitochondria, with low O_2 consumption, causing slow respiration, thus low ATP production and slowed β-oxidation. This leads to increased free fatty acid pool, which are esterified and trafficked back into existing lipid droplet as TAGs. This re-trafficking of FA cause increase in lipid droplet size. ATP: adenosine triphosphate, FA: Fatty Acids, ER: Endoplasmic Reticulum, TAGs: triglycerols.

The study described in this dissertation helps to establish a crucial role for Mfn-2 in maintaining mitochondrial morphology and lipid metabolism. It was shown that in the absence of Mfn-2, mitochondrial morphology is abnormal and appears fragmented. Mitochondrial morphology and lipid droplets are associated with various pathologies, therefore to understand the relationship between mitochondrial morphology and lipid metabolism is crucial. We found in Mfn-2 KO MEFs, lipid droplets size is almost double, likely, due to slower β-oxidation. We further demonstrated that in absence of Mfn-2, adipogenesis proceeds slightly differently and with increased efficiency. We also predict that the extra accumulated TAGs in Mfn-2 KO MEFs could act as an inducing factor for adipogenesis. A study revealed exogenously added FAs could induce accumulation of TAGs in preadipocytes (Xie *et al.,* 2006) and adipogenesis (Oster *et al.,* 2010). Several transcription factors can influence adipogenesis. This phenomenon is clinically important because impaired adipogenesis may cause dysfunctional adipocyte, which can lead to potential risk of metabolic diseases. We also compared adipogenesis in two different culture media, one with high concentration of FBS and other adipogenic media with cocktail of synthetic drugs (insulin, IBMX, dexamethasone and troglitazone). We found that culture media with a high concentration of FBS was able to induce adipogenesis strongly as compared to adipogenic media. Our studies also found adipogenic media consisting of synthetic drugs leads to cell detachment, when compared to culture media with high FBS. This experiment revealed that the study of adipogenesis in high FBS mimics better *in vivo* conditions, as mouse do not have synthetic adipogenic inducers. Apart from that use of synthetic cocktail drugs is costly and are also associated with other experimental problems such as cell detachment. Therefore, our version of adipogenesis-protocol is more economical and mimics better *in vivo* conditions. However, cells detached from adipogenic media were not analyzed for TAG content or adipogenic markers, therefore we are unable to compare both media composition for differentiation efficiency. Further experimental testing is needed to confirm standard media composition.

The studies presented here focus on mitochondrial morphology and lipid droplets (size & number). Further studies to test the model proposed are required. Mfn-2 regulates the distance between ER and mitochondria, and thus could also affect phospholipid biosynthesis and trafficking between organelles. Lipid droplets consists mainly of triacylglycerol, however levels of phospholipids are closely connected with lipid droplet formation and size. Thus, studying

mitofusins and metabolism is crucial. In summary, we know Mfn-2, a mitochondrial protein plays crucial role in regulating mitochondrial morphology and lipid metabolism. Thus, cellular silencing of Mfn-2 not only reduces the mitochondrial membrane potential but also the rate of glucose oxidation and suppresses proton leak. These observations propose that Mfn-2 plays a crucial role in developing obesity because silencing Mfn-2 gene in cell may account for impaired energy expenditure and decreased oxidative capacity, which is associated with obesity. Therefore, these finding offers us some understanding of the relationship between mitofusins and metabolism, which could be further exploited for the development of better drugs to fight against obesity and related problems.

6. FUTURE DIRECTIONS

While this study furthers our understanding of the relationship between the mitochondria-ER tether and lipid metabolism, there is much work needed to fully understand the role of Mfn-1 and 2 in mitochondrial dynamics and lipid metabolism. The following section proposes areas of future research to advance this understanding.

6.1 Examine Effects of Overexpression of Mitofusins (Mfn-1 And Mfn-2) Protein on Lipid Metabolism

Changes in the levels of mitochondrial fusion and fission proteins allow mitochondria to react to various cellular environments, such as nutritional stress (acute and chronic), that ultimately affect lipid metabolism (Putti *et al.,* 2015). Though a few mitofusin knockdown studies have been conducted, the role of these proteins in regulating lipid metabolism using overexpression approaches has not been performed. Studies examining the effects of overexpression of Mfn-1 and -2 on mitochondrial dynamics and lipid metabolism are critical experiments to perform to advance our understanding.

6.2 Identify Individual Roles of Mfn-1 and Mfn-2 in Maintaining Mitochondrial Morphology and Lipid Metabolism

Mfn-1 and Mfn-2 are both essential for mitochondrial fusion and maintenance of mitochondrial morphology. However, the molecular mechanisms of the GTPase-dependent reaction, as well as the functional division of the two Mfn proteins, are unknown. Identifying individual roles of mitofusin proteins in mitochondrial dynamics and lipid metabolism would be of great interest. As mentioned above, this study was limited to Mfn-2 KO MEFs; therefore, further investigation of the Mfn-1 KO and Mfn-1and-2 KO MEFs would be of great value in understanding the role of mitofusins in lipid metabolism.

6.3 Study Time Course Effect of Insulin and Oleic Acid on Lipid Accumulation in Mfn-2 KO

In the current study, we treated WT and Mfn-2 KO MEFs with insulin, oleic acid and the combination of both for 13 h. Some studies suggested that exposure to fatty acids induces lipid droplet formation through an acute fatty-acid-receptor-mediated pathway as well as through a mechanism involving lipid uptake which would be anticipated to have longer time-frame (Rohwedder *et al.,* 2014). Thus a time-course study ranging from 0 to 96 hour may provide us

with more information on changing mitochondrial morphology and lipid accumulation at a different time interval in fusion compromised MEFs.

6.4 Study Role of Mfn-1 and Mfn-2 in Adipogenesis

The current study detected a slightly different pattern of adipogenesis in Mfn-2 KO MEF cell when compared to WT. It would be interesting to conduct a quantitative study, to understand and quantify expression of adipogenesis markers in WT and Mfn-2 MEF cells. This detailed information on adipogenesis markers provide new information regarding the adipogenesis process.

6.5 Study Effect of Different Media Composition on Adipogenesis

Our studies demonstrated that WT and Mfn-2 KO MEFs were able to undergo adipogenesis in control media containing a high concentration of FBS. Results also suggested that as high concentration of FBS was able to induce adipogenesis strongly and early without any cell detachment, as compared to adipogenic media. Thus a quantitative study with varying concentration of FBS or other adipogenic inducers might provide us with better and cheaper methods for inducing adipogenesis.

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