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Probing the Effect of Saturated Lipid Stress on Mitochondrial Genome Maintenance

By

Casadora Canary Anne Boone

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

Metabolic Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Assistant Professor Samantha C. Lewis, Chair

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Professor Hei Sook Sul

Spring 2026

Abstract

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Assistant Professor Samantha C. Lewis, Chair

Fatty acid entry into peripheral tissues, particularly saturated fatty acids such as the long chain fatty acid (LCFA) palmitic acid (PA), are linked to tissue damage characterized by organ dysfunction, increased inflammation, and cellular death. Tissue damage of peripheral organs contributes to several metabolic disorders, including non-alcoholic fatty liver disease and Type 2 diabetes mellitus (T2DM). While lipotoxicity has been studied at the organismal level, the mechanisms of lipotoxicity at the cellular, organellar, and molecular levels are not fully understood. Mitochondria are master regulators of cellular energy metabolism and serve as signaling hubs to integrate information from within and outside of the cell to maintain cellular homeostasis. Mitochondria are also the main organelles that conduct beta-oxidation of short, medium, and long-chain fatty acids, and are themselves regulated via lipid-protein interactions at the inner and outer mitochondrial membranes. Mitochondria are unique among organelles in that they harbor a distinct polyploid genome, mitochondrial DNA (mtDNA), that is separate from nuclear DNA, encoding respiratory chain components required for energy production. MtDNA molecules are packaged into nucleoids by the replication and transcription regulating protein TFAM, and nucleoids are located near the inner mitochondrial membrane. Therefore, changes in mitochondrial function and morphology due to lipid stress could impact mtDNA integrity. Although the effects of lipotoxicity on mtDNA maintenance are poorly understood, due to insubstantial investigation, there are some reporting on the possible effects of PA treatment on the mitochondrial genome. For example, mtDNA mutation and depletion have been linked to obesity and metabolic syndrome in humans and mtDNA mutation has resulted from lipotoxic oxidative stress. Despite these findings, the relationship between mtDNA and cellular lipid metabolism remain to be characterized.

Both mtDNA synthesis and lipid droplet biogenesis are coordinated by proteins enriched at ER-mitochondrial contact sites. I used fluorescence microscopy, molecular techniques, and lipidomic analysis, to probe whether lipid droplet biogenesis and mtDNA replication are spatially linked and coordinated. I used Huh7 cells as the model system for this investigation given the relevancy of hepatocytes in lipid metabolism and the development of metabolic dysfunction-associated steatotic liver disease (MASLD, formerly NAFLD). I then induced saturated lipid stress by exposing Huh-7 cells to low-level chronic palmitic acid (PA) exposure. We found that upon PA treatment, mtDNA replication significantly decreases and is not spatially coupled to ER-

mitochondrial contacts needed for lipid droplet biogenesis. Instead, saturated lipid stress caused increased ceramide production, mild ER stress, and reduced mitochondrial function. This could be due to the rerouting of fatty acid trafficking during cellular fatty acid influx, which differed between PA and oleic acid (OA) treatment. Here we propose a model in which saturated lipid stress promotes the production of toxic lipid byproducts that remodels organelles involved in the mtDNA synthesis pathway, whereas OA promotes the neutralization and storage of fatty acids to combat lipotoxicity. By studying the mitochondrial response to saturated lipid excess, this work provides new insight on how mitochondria manages two major functions during stress. Our work demonstrates that mitochondria “shut-off” mtDNA synthesis as a response to nutrient stress during lipid excess.

Prior to this work, it was known that lipotoxicity causes organelle dysfunction that coincides with apoptosis and inflammation. Much of this work was studied to understand the development of metabolic disorders associated with obesity. Investigating the relationship between lipid metabolism and mtDNA maintenance is a highly under-investigated area of research in the field of metabolic biology. Our work begins to characterize the effect of saturated lipid stress on mtDNA replication and organelle remodeling, with the use of the unsaturated fatty acid oleic acid co-treatment as a rescue strategy for lipotoxic insult. However, further studies are required to create a more comprehensive molecular model that connects lipid flux with changes in mtDNA synthesis. These studies would include the use of more defined and dynamic imaging tools to measure contact sites between mitochondria and lipid metabolizing organelles, such as the ER, as well as imaging techniques to assess changes in mitochondria membrane composition that are in close proximity to mtDNA replication sites.

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Chapter 1: Bridging lipid metabolism and mitochondrial genome maintenance

Contents in this chapter are modified from the previously published review article:

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1.1 Introduction

Mitochondria are double membrane-bound, energy producing organelles that arose as a result of an ancient endosymbiosis event [1]. Over millennia, mitochondria have co-opted host cell resources, and in the process, host and endosymbiont metabolism have become inextricably linked [1]. Integration of metabolic pathways at mitochondria relies on their highly organized ultrastructure, consisting of an outer mitochondrial membrane (OMM) porous to metabolites but not to proteins and a structured inner mitochondrial membrane (IMM), separated by an intermembrane space. Within the matrix, mitochondria contain a small polyploid genome, packaged into nucleo-protein structures termed ‘mitochondrial nucleoids’ and distributed throughout dynamic networks [2]. Nucleoids are thought to closely associate with the inner membrane, are limited in their diffusion by the IMM cristae, and are segregated to daughter organelles in a regulated manner by mitochondrial division [3]. The thirteen protein-coding genes in the human mitochondrial genome are subunits of respiratory chain complexes that execute oxidative phosphorylation (OXPHOS) for energy production in the form of adenosine triphosphate (ATP). MtDNA quality control and euploidy are critical to cellular energy production (Figure 1.1B).

Over two thousand proteins are required for mitochondrial biogenesis *in toto*, thus the vast majority of mitochondrial proteins are encoded in the nuclear genome and imported into mitochondria by dedicated, membrane-associated complexes at the outer membrane. Unlike organelles such as cilia or the mitotic spindle, mitochondria cannot be built *de novo* [4-5]. Mitobiogenesis depends on the import of proteins, lipids, and other metabolites to extend the pre-existing network. Thus, mitochondrial mass, form, and function are highly dependent on cellular resources and metabolic signaling [6]. This includes lipids for mitochondrial membrane growth provided by diet or *de novo* synthesis in animals.

Mitochondria play key roles in lipid oxidation and synthesis, and there is growing evidence that physical interactions between mitochondria and other organelles sustain the context-dependent balance necessary for cellular lipid homeostasis, as reviewed elsewhere [4-5]. Dysregulation of lipid homeostasis has damaging effects on mitochondrial form and function, including endoplasmic reticulum (ER) membrane contacts and fission factors that play roles in the mtDNA synthesis pathway. Here, we discuss roles of mitochondria in lipid metabolism and highlight how these pathways are tied to mtDNA maintenance.

1.2 Mitochondrial lipid oxidation and synthesis

Mitochondria can metabolize multiple substrates to power ATP production, including glucose and fatty acids. Glucose, the preferred fuel source, is ultimately converted into acetyl-CoA molecules that channel into the citric acid (TCA) cycle. The TCA cycle provides metabolites to serve as electron carriers that donate electrons to IMM-resident electron transport chain (ETC) complexes with mtDNA-encoded subunits. Four protein complexes at the IMM pump protons to acidify the intermembrane space, generating a gradient that protons are permitted to run down to power ATP synthase complex, driving the phosphorylation of adenosine diphosphate (Figure 1.1, A and B). However, when glucose availability is low, such as during starvation, lipids provide an

alternative fuel source for ATP production. The breakdown of acetyl-CoA supplies the substrate needed for respiration to continue to meet cellular energy demands.

Mitochondria conduct beta-oxidation of short (≤ 6 carbons), medium (8–14 carbons), and long-chain fatty acids (15–21 carbons) and thus play a crucial role in cellular lipid catabolism. Indeed, while peroxisomes partially catabolize long chain and very long chain fatty acids (≥ 22 carbons), their oxidation is completed within mitochondria. Upon entry into the cell, fatty acids may be trafficked directly to specialized lipid droplet (LD) organelles for neutralization and storage, to the ER for phospholipid synthesis and LD biogenesis, or to mitochondria. Mitochondria uptake these lipids from the cytosol via dedicated transporters or regulated trafficking at membrane contact sites with other endomembrane compartments. Stored triacylglycerols are then released from LDs, sites of neutral lipid storage enclosed by a phospholipid monolayer, which are then hydrolyzed into fatty acids by lipases and converted to fatty acyl-CoA by fatty acyl-CoA synthetase. Fatty acyl-CoA molecules must be converted into fatty acyl carnitine by the carnitine palmitoyltransferase 1 (CPT1) on the OMM for uptake by dedicated trans-membrane transport by carnitine acylcarnitine translocases. Within the mitochondrial matrix, carnitine palmitoyltransferase 2 (CPT2) converts the fatty acyl carnitine back into fatty acyl-CoA. These fatty acids are then broken down into two carbon acetyl-CoA units that are shuttled into the TCA cycle for ATP production [7-9].

Mitochondria are sites of lipid catabolism and key players in the de novo synthesis of long chain fatty acids, complementary to dietary lipid intake of short and long chain fatty acids [9-10]. Lipogenesis requires both cytoplasmic and mitochondrial enzymes, all of which are encoded in the nuclear genome that results in the end product palmitic acid (PA) [11-12]. ER-resident enzymes facilitate the elongation and desaturation of PA [13]. The synthesized fatty acids can then be used to form phospholipids that integrate into mitochondrial membranes, which are needed for mitochondrial membrane growth and division. While fatty acid synthesis primarily occurs in the cytosol, one mitochondrial fatty acid synthase has been identified in mammals to create at least two products: octanoate eight carbon chain saturated fat and a long acyl chain of fourteen to sixteen carbons [14-15]. Loss of this mitochondrial pathway can result in reduced protein lipoylation and reduced ETC complex assembly, directly linking mitochondrial lipid synthesis to ATP production [15].

1.3 Mitochondrial regulation of lipid metabolism via membrane contact sites

Mitochondrial lipid metabolism is not an isolated process; other lipid metabolizing organelles, such as the ER and LDs, directly interact with mitochondria for the transfer, anabolism, and catabolism of fatty acids that are transported into the cell. Fatty acids can be accessed for mitochondrial beta-oxidation directly from the cytosol or from LDs through contact sites. Therefore, study of mitochondria, ER, and LDs as a system instead of as isolated components can provide critical insights into mitochondrial lipid metabolism.

1.3.1 Mitochondria and lipid droplet interactions

The role of mitochondria in the oxidation or synthesis of lipids is directed by their contact with LDs, and these interactions are dictated by the nutritional status of the cell along an axis from

starvation to lipid excess. In 2018, Benador et al. reported the identification of two subpopulations of mitochondria with differing lipid metabolizing paradigms: peridroplet mitochondria (PDM) (Figure 1.2B) and cytoplasmic mitochondria (CM) (Figure 1.2A). PDM formed close membrane contact sites with LDs. They found that PDM typically remained adjoined to LDs during density gradient centrifugation. PDM have higher respiratory capacity as measured by oxygen consumption rate, ATP production rate, pyruvate oxidation capacity, as well as OXPHOS protein levels, such as cytochrome c oxidase and ATP synthase, with lower fatty acid oxidation capacity, than CM. These findings led the authors to conclude that PDM function in triglyceride synthesis and transfer to LDs for storage. Investigation by electron microscopy revealed that PDM tend to be longer and tubular than CM, suggesting a link to mitochondrial dynamics. In live laser scanning confocal microscopy, PDM displayed lower rates of fission and fusion over 1 h, measured by a mitochondrial matrix-targeted photoactivatable green fluorescent protein and decreased motility, in time lapse imaging [16-17]. Such heterogeneity among mitochondrial subtypes may allow metabolic flexibility at the cellular and subcellular levels. For example, the relative proportions of PDM and CM mitochondria may be adjusted to meet cell type-specific needs or individual organelles may be trafficked to subcellular target locations to affect local lipid synthesis or breakdown.

Hepatic CM and PDM exhibit distinct proteomic profiles, in which CM associated with ER-mitochondrial contact sites were relatively enriched for proteins associated with fatty acid oxidation, as well as subunits of all five respiratory chain complexes. PDM were relatively enriched with proteins with molecular functions relevant to lipid synthesis and storage, which the authors inferred could promote adaptation to lipid-induced toxicity. CM and PDM functional distinctions can be observed during different nutrient states: starvation promotes fatty acid trafficking to CM for oxidation and energy production, while the fed state promotes fatty acid trafficking to PDM to be esterified and stored in LDs [18]. In contrast to these findings, in myoblasts, mitochondria tethered to LD-demonstrated increased transport and oxidation of fatty acids by interactions with the OMM acyl-CoA synthetase protein Fatp4 during starvation [19]. The extent to which LD-mitochondrial interactions are modulated by nutritional status *in vivo* has been studied in few model systems, but the studies reveal intriguing parallels. In Wistar rats, a model for nonalcoholic fatty liver disease, PDM demonstrated higher beta-oxidation capacity and reduced OXPHOS capacity than CM in rats-fed *ad libitum*; however, in PDM isolated from high-fat diet (HFD)-fed rats, fatty acid oxidation is significantly reduced compared to PDM isolated from *ad libitum* fed rats, with no difference compared to HFD-isolated CM [20]. These contrasting findings suggest that there are differing localization and functions of mitochondrial subpopulations in the context of lipid metabolism, but the factors that determine PDM *versus* CM function may be cell-type-dependent. Fully understanding the contextual differences that influence PDM *versus* CM is still yet to be analyzed, as well as whether these mitochondrial subpopulations differ in their mtDNA genome mutations or abundances.

1.3.2 Mitochondria and ER interactions

ER-mitochondria interactions, also known as mitochondria-associated membranes, were identified as early as the 1950s via electron micrographs of intact endomembranes [21]. ER and mitochondrial interactions are important for metabolite exchange, such as calcium (Ca^{2+}),

phospholipids, and cholesterol [22]. Close contact between mitochondria and ER at mitochondria-associated membranes permits the transfer of phospholipid precursors to mitochondria essential to mitochondrial membrane integrity and the synthesis of mitochondrion-specific lipid species including cardiolipin [22-23]. Mitochondrial membranes are composed of phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, phosphatidylglycerol, phosphatidic acid, and cardiolipin. Most cellular phospholipid synthesis occurs in the ER, with partial synthesis of phosphatidylethanolamine and phosphatidylglycerol occurring in the IMM [23-27]. Disruption of phospholipid composition in either mitochondrial bilayer perturbs mitochondrial fusion and fission dynamics, though the molecular mechanisms remain unclear [28-31]. In contrast, the synthesis of long chain acyl-coenzyme A (LCACA), a substrate of the lipid synthesis pathway, provides a direct connection between lipid metabolism and mitochondrial division. LCACA interacts with the mitochondrial dynamic proteins MIEF1 and MIEF2, which serve as recruiting proteins for the mitochondrial fission mediator dynamin-related protein 1 (Drp1). Increased synthesis of LCACA by long chain fatty acid supplementation, such as with oleic acid, stimulates MIEF1/2 oligomerization and Drp1 recruitment for mitochondrial fission [32]. Fusion and fission are necessary for the even distribution of metabolites and mtDNA between mitochondria. Additional molecular studies that detail how lipid metabolism can regulate mitochondrial dynamics could reveal new insight in the segregation and localization of mitochondrial genomes in coordination with membrane growth.

ER-mitochondria contact sites serve as platforms for mitochondrial fission during growth as well as mtDNA synthesis (Figure 1.2C) [3, 33-35]. Mitochondrial DNA replication and segregation are spatiotemporally coupled to mitochondrial division; thus, mitochondrial membrane growth via lipid synthesis may be spatially linked to mtDNA maintenance; further studies using both mtDNA nucleoid and lipid synthesis markers are needed. The composition and stability of the mitochondrial membranes affects mtDNA integrity, as mtDNA nucleoids are localized near the IMM in membrane domains putatively enriched for cardiolipin and cholesterol [36]. Coupling of mitochondrial membrane growth with division and mtDNA synthesis serves to scale mtDNA nucleoid content to cellular mitochondrial mass [3, 33].

While the repertoire of membrane tethers and transport proteins needed for lipid exchange in mammalian cells is still expanding, one possible protein identified is Mitogaurdin-2 (MIGA2). MIGA2 is an OMM protein that has an FFAT motif (two phenylalanines in an acidic tract), an acidic amino acid region commonly found in lipid-transferring proteins, that interacts with the ER transmembrane VAMP-associated protein B. Additionally, MIGA2 has an LD-targeting domain that contains a hydrophobic pocket where lipids, particularly phospholipids, can be “captured” in this pocket for transport between the ER, mitochondria, and LDs [37-38]. In addition to phospholipid transfer, MIGA2 promotes ER–mitochondrial interactions for lipogenesis and storage of triglycerides in LDs [39]. These findings suggest the possible formation of three way contact between ER, mitochondria, and LDs for the transferring of lipids between the organelles.

1.3.3 Mitochondria, LD, and ER tripartite interactions

Three-way contact sites between ER, mitochondria, and LDs, termed tripartite contacts, are a fairly new area of research in the lipid metabolism field. Two proteins that have been identified

to bring together ER-mitochondria-LD contacts are the oxysterol binding protein and related proteins (ORP). ORP5 and ORP8 are lipid-binding and transfer proteins that associate with ER membranes via a C-terminal transmembrane segment; ORP5/ORP8 are locally enriched at ER-mitochondrial contact sites [40]. As previously discussed, LD biogenesis occurs at the ER; ER-transmembrane acyltransferases synthesize triacylglycerols, which are transferred between the leaflets of the ER membrane bilayer to form a nascent LD. The ER membrane protein seipin will oligomerize to bud off the LD, allowing the LD to fully detach [41]. Interestingly, ORP5 has been reported as necessary to the recruitment of seipin to ER-mitochondria-LD contacts and loss of ORP5/8-compromised LD biogenesis [42]. Another study showing ER-mitochondria-LD tripartite interactions' contribution to lipid homeostasis used serial block face scanning electron microscopy, where images displayed ER "sandwiched" in between mitochondria and LD; these tripartite interactions increased during the fasting state (Figure 1.2D) [18].

An exciting avenue of future research will be to develop and apply tools to visualize, characterize, and manipulate defined subpopulations of membrane contacts *ex vivo* and *in vivo*. Profiling variability among membrane contact site populations at the cellular scale can reveal how the number, cytoplasmic distribution, and/or temporal persistence of contact site classes contribute to organelle remodeling, for example, to tune the pathways of lipid homeostasis to mitochondrial membrane growth and mtDNA replication cues [43-44].

1.4 Lipotoxic impairment of mitochondrial function

1.4.1 Defining lipotoxicity

Lipotoxicity is often defined as the accumulation of fatty acids in nonadipose metabolic tissue that causes cellular dysfunction and cell death [45-46] (Figure 1.3A). Entry of fatty acid into peripheral tissues, particularly saturated fatty acids, such as PA, is linked to tissue damage identified by organ dysfunction, increased inflammation, and cellular death [47]. Many studies of lipotoxicity have employed PA in cell culture or HFD in murine models to induce cellular or tissue damage due to these effects, and studying the effects of other saturated fatty acids as potential inducers of lipotoxicity is yet to be systemically explored [48-50]. Such tissue damage of peripheral organs by lipotoxicity contributes to several metabolic disorders with mitochondrial involvement, including cardiomyopathy, kidney disease, non-alcoholic fatty liver disease, and type 2 diabetes mellitus (T2DM) [47, 51]. Lipotoxicity was first defined in a study which found that elevated triacylglycerol levels in the pancreatic islets of Zucker diabetic fatty rats correlated with an increase in plasma free fatty acids (FFAs) and plasma glucose levels. Moreover, the culture of rat pancreatic islets with FFAs caused a reduction in glucose-stimulated insulin secretion, indicating impaired β -cell function in an excess lipid environment and showing the close relationship between glucose and lipid metabolism at the cellular level [52]. The connection between lipotoxicity and metabolic disease has primarily been explored in murine models fed with an HFD with observations of dysregulated glucose and lipid homeostasis, but the extent to which murine models recapitulate human disease states is still poorly understood [50, 53-54].

At the cellular level, lipotoxicity can cause diacylglycerol and ceramide accumulation, ER stress, activation of inflammatory pathways, increased autophagy, mitochondrial dysfunction via

oxidative stress, and mtDNA damage, such as DNA lesions, which are all signs of cellular dysfunction and the likely impending commitment to cell death [45, 55-57]. The interplay of these effects or whether one class of impairment supersedes others remains poorly understood. As described, mitochondria coordinate lipid metabolism at contact sites with other organelles, and lipotoxicity could plausibly impair these interactions [20, 58-59]. More work is needed to parse the contribution of lipid excess to mitochondrial contact site remodeling and declining cellular health to create mechanistic models of mitochondrial responses to lipotoxicity.

1.4.2 Lipotoxicity induces cell death

PA is a saturated, long chain fatty acid produced during mammalian fatty acid synthesis and is a commonly found saturated fatty acid in the diet [12]. Cells fed excess PA in vitro exhibit reduced cell viability, specifically through activation of apoptosis, which is an extrinsic or intrinsic programmed cell death [60- 63]. Other forms of cell death are induced by excess FFA in specific contexts, including pyroptosis and necroptosis [64-68]. Ferroptosis, an iron-dependent form of programmed cell death, produces lipid-free radicals through the peroxidation of polyunsaturated fatty acids. The accumulation of these lipid peroxides leads to lipid and protein damage [69-71]. The direct or indirect role of mitochondria in these nonapoptotic cell death pathways is as yet unclear.

1.4.3 Lipotoxicity and mitochondrial dysfunction

Cells exposed to a lipotoxic environment demonstrate features of mitochondrial dysfunction, including mitochondrial network fragmentation and reduced mitochondrial membrane potential (Figure 1.3B) [72-74]. Remodeled mitochondrial morphology is associated with changes in nutrient uptake and catabolism in metabolic disease [75]. Consistently, pancreatic islets isolated from Zucker diabetic fatty rats exhibited shortened mitochondria with swollen architecture suggestive of cristae maintenance defects [76]. Genetic induction of mitochondrial fragmentation can, in some cell types, recapitulate functions of increased fatty acid oxidation, suggesting that mitochondrial form plays a physiological, cell type-specific role in lipid catabolism [75]. These findings raise the possibility that the impact of lipotoxicity on mitochondrial function in cells and tissues may be modified by the mitochondrial network pre-lipotoxic insult.

Interestingly, several groups have shown that excess of either saturated or unsaturated fatty acids induces autophagy in cellular models, contributing to elevated mitochondrial turnover in lipotoxic conditions. While PA treatment upregulates canonical autophagy dependent on adenosine monophosphate-activated protein kinase, oleate treatment stimulates a non-canonical autophagy pathway that relies on Golgi signaling (Figure 1.3B) [77]. Given that lipid mobilization is a signal of cellular stress and low glucose availability, induction of autophagy by fatty acid excess may serve a quality control purpose to suppress lipotoxic damage to cells. Whether the strength of autophagic induction differs in starvation versus nutrient excess or obesity is yet to be discovered. There is some evidence to support that autophagy regulates mtDNA copy number as well as the presence of mutated mtDNA; surprisingly, mechanistic studies of mtDNA maintenance and autophagy during starvation or nutrient excess in in vivo animal models lag behind, given that the influence of dietary lipid intake on mitochondrial

membrane composition could provide a means of perturbing both mtDNA nucleoid homeostasis and cristae morphology [78-80].

1.5 Lipid metabolism and mtDNA integrity

1.5.1 mtDNA damage during oxidative stress

Production of reactive oxygen species (ROS) is prominent in mitochondria due to reactions of electrons at ETC complexes with oxygen molecules, producing superoxide anions and hydroxyl radicals [81-82]. Free radicals are molecular species containing atoms with unpaired electrons that can react with nonradical species, including macromolecules—lipids, proteins, nucleic acids, and carbohydrates [81]. While the majority of mtDNA mutations arise via replication errors, mitochondrial nucleoids are located near the IMM and cristae, the location of respiration complexes, and therefore ROS generated by OXPHOS complexes has been proposed to cause oxidative damage to nearby mtDNA [57, 83] (Figure 1.4.3).

In the lipotoxic context, mouse INS-1 cells treated with excess FFAs high in PA had activated nitric oxide synthase and increased nitric oxide levels. Elevated nitric oxide levels damaged mtDNA by generating oxide products, such as nitrous anhydride (N₂O₃), that created mtDNA lesions through deamination reactions [82]. Racheck et al. demonstrated a similar result upon treatment with palmitate with increased superoxide and nitrite levels. This increase in free radicals correlated with increased mtDNA breaks, as well as decreased cellular ATP levels and cell viability [84]. Whether mtDNA lesions may be repairable is controversial. Human 8-oxoguanine DNA glycosylase (OGG1) is a DNA base excision repair enzyme. While the mechanism of mitochondrial base excision repair pathways are not well understood, INS-1 cells overexpressing a transfected mitochondrial targeting sequence vector encoding OGG1 (MTS-OGG1) showed reduced mtDNA damage and apoptosis upon FFA treatment [85]. The relationship between mtDNA damage and lipid metabolism can also be observed at the physiological level: It has been demonstrated that oxidative damage of mtDNA is linked with increased adiposity and obesity onset in mice (Figure 1.3B) [86-87]. Following investigations show that overexpression of OGG1 is a means to regulate adiposity in mice, with OGG1 KO displaying increased fat accumulation [88]. Alternative ways to prevent mtDNA damage during lipotoxic-induced oxidative stress remain elusive.

1.5.2 mtDNA release and activated inflammatory pathways

Mitochondrial DNA can serve as a signaling molecule during cellular stress for the activation of immune pathways such as the inflammasome, toll-like receptor 9, and cGAS-STING [89]. Immune activation occurs downstream of mtDNA efflux from the mitochondrial matrix into the cytosol. The exact mechanism by which mtDNA becomes accessible to the cytosol is not known, but in recent years, the release of mitochondrial nucleic acid into the cytoplasm has been reported during a variety of cellular stress states, typically imminent to cell death. In McArthur et al., mouse embryonic fibroblasts that were treated with an apoptotic-inducing drug led to compromised integrity of the mitochondrial membrane, inducing permeabilization of the mitochondria by the oligomerization of BAK and BAX proteins, which form pores on the outer membrane. They state that the herniating IMM exposes the matrix-housed mtDNA to the innate

immune sensor, cGAS, in the cytosol, activating the cGAS–STING pathway [90]. While the full release of mtDNA into the cytosol was not indicated in this report, the findings were an advance in visualizing mtDNA release through a combination of super-resolution live imaging and electron microscopy. Further investigation is needed to determine the status of the cell and the factors involved that influences its release, as well as if there is a way to prevent mtDNA release for cell death prevention. Live cell imaging to visualize the full release of mtDNA into the cytosol and its interaction with cGAS-STING could enhance our understanding of mtDNA's role in cell death and innate immunity.

One route by which PA treatment may reduce cell viability in culture is mtDNA release from permeabilized mitochondria and the subsequent activation of the innate immune response (Figure 1.4.1) [91-93]. Another report showed that mtDNA release and activation of toll-like receptor 9, another pro-inflammatory marker, led to nonalcoholic steatohepatitis in mice (Figure 1.3B) [94]. mtDNA release may not solely be an apoptotic event, as it may also occur during other forms of cell death, such as pyroptosis or necroptosis [65, 95].

1.5.3 Perturbed lipid storage in primary mitochondrial disease

How does the interrelationship between lipid homeostasis and mtDNA-dependent mitochondrial function contribute to disease? A dedicated mitochondrial replisome consisting exclusively of nuclear-encoded proteins replicates mitochondrial genomes [83]. MtDNA replication defects cause mitochondrial myopathy, peripheral neuropathy, and a starvation-like response in mice even while in a normal nutritional state; this activates alternative energy production pathways, leading to reduced adipocyte size and reduced liver fat content [96]. Several studies have identified LD expansion as a signature of mtDNA depletion and/or deletion in mouse models [97]. Transcriptomic analysis in a mouse model depleted of the mitochondrial helicase suggested progressive impairment of lipid storage; consistently, fluorescence microscopy revealed a dramatic expansion of LDs in astrocytes depleted of the mitochondrial helicase TWINKLE [98]. In a DNA polymerase gamma mutant mouse model, lipidome remodeling was a key feature of metabolic disease progression and correlated with reduced triacylglycerols and hypoglycemia [99]. Consistently, human mitochondrial disease patients with DNA polymerase gamma mutations exhibit enlarged LDs in muscle and in hepatocytes [97, 100]. Taken together, these studies indicate that mtDNA replication defects are linked with disrupted lipid homeostasis either directly or indirectly (Figure 1.4.4).

1.5.4 mtDNA mutations and metabolic disease

Quality control of the polyploidy mitochondrial genome is necessary for overall mitochondrial and respiratory function, as mtDNA mutations cause myriad heritable musculoskeletal and neurodegenerative disorders and are also closely linked to other metabolic diseases including diabetes and glucose intolerance [101-102]. Maternally Inherited Diabetes and Deafness is caused by an mtDNA mutation in the mt-tRNA^{Leu} gene, which leads to decreased OXPHOS function and ATP production. This, ultimately, reduces insulin secretion and promotes beta cell apoptosis. In addition to hyperglycemia and hearing loss, other clinical features include macular dystrophy, cardiac failure, and short stature [103-105]. Kearns-Sayre is an mtDNA-mutation disorder identified by an mtDNA deletion that can range from 2000 to 7000 bp [106-107]. While

the key features of this disease are ophthalmoplegia, bilateral pigmentary retinopathy, cardiomyopathy, and cognitive impairment, there are cases where individuals with this disorder also develop diabetes, requiring insulin injection for hyperglycemia [108-110]. There is a clear connection between glucose and lipid homeostasis in type 2 diabetes mellitus, which raises the possibility that mtDNA-linked diabetes may be associated with systemic lipid dysregulation (Figure 1.4.2).

Clinical manifestations of mtDNA-disease indicate that mtDNA integrity is closely tied to lipid homeostasis, but cellular models for many of these diseases remain to be developed. Nonalcoholic fatty liver disease, which is commonly associated with obesity and an HFD, can progress into steatohepatitis (NASH) or liver failure. A mutation in the mt-CYB gene, encoding for the OXPHOS protein cytochrome b, was associated with NAFLD progression into NASH [111]. Another lipid-based disease caused by mtDNA mutation is lipomatosis, an abnormal overgrowth of adipose tissue in typically lean body regions. Mitochondrial mt-tRNA^{Lys} mutations are linked to lipomatosis [112-116]. Another mutation in the mt-tRNA^{Leu} gene was identified to cause hyperglycemia associated with lipoma development and polyneuropathy, which the authors speculate could be a possible link between mitochondrial diabetes and disrupted systemic lipid metabolism [117]. While certain mitochondrial DNA alleles are correlated with higher rates of obesity, mechanistic studies that could identify mtDNA variants associated with obesity in otherwise isogenic animal models are needed [118-124].

1.6 Conclusion

Mitochondrial metabolism is central to cellular homeostasis, yet these organelles do not perform their myriad bioenergetic, biosynthetic, and catabolic functions in isolation. Mitochondria must interact with other cellular organelles and cytosolic metabolite signaling pathways for efficient lipogenesis or fatty acid oxidation. Studies in the model invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster* will be useful in this regard because they provide a foundation for understanding how mitochondrial form and function are linked in homeostasis and can reveal fundamental cellular mechanisms shared among animals. Future effort to perform *in vivo* imaging of live animal models will be essential to dissect this relationship across different tissues and cell types. New sensors and probes that can reveal the genesis and fates of lipid species and their contributions to lipotoxic phenotypes via dynamic tracking over time in live cells and animals are needed.

Looking forward, discovering methods to repair or reverse lipotoxic insult to mitochondria may lead to treatments for damaged tissues. An endogenous response that could improve lipotoxic conditions of the cell is the sequestration and neutralization of excess lipids into LDs for storage. A proposed concept is that LD sequestration and neutralization of the cytosolic FFA pool and toxic lipid by-products, such as ceramides and diacylglycerols, could reduce altered lipid composition of the ER membrane, preventing activation of ER stress pathways [41]. Mitochondrial fatty acid overload could also be modulated via the inhibition of import proteins [125]. Combining sequestration of fatty acids into LDs and partial inhibition of fatty acid import into mitochondria during high-fat intake could mitigate cellular lipotoxicity. A nutritional means to mitigate lipotoxic impairment of mitochondria in cells could be the use of mono- and polyunsaturated fatty acids, which has been reported to ameliorate the effects of PA treatments and

HFDs [126-133]. Finally, inhibiting mitochondrial transcription led to improved weight management and reduced liver fat content in HFD-fed mice, supporting a connection between mtDNA-encoded gene expression and lipid metabolism at the organismal level [134]. Further investigation of how modulation of the mitochondrial central dogma may integrate with lipid metabolism could lead to advancements in drug treatments for both lipid-based disorders and primary mitochondrial disease.

While the harmful effects of excess fatty acids on mitochondria are coming into focus, how mitochondrial and cellular stress responses to lipid dysregulation are induced and regulated is less clear. Even more so, it is unclear if links between lipid dysregulation and mtDNA maintenance are direct or indirect. Potentially fruitful avenues of future research include the following: (1) dissecting the role of membrane contact sites on mtDNA maintenance during excess lipid exposure (Figure 1.4.5), (2) determining whether altered lipid composition of the mitochondrial membrane and impaired membrane integrity affects maintenance of the mitochondrial genome, (3) investigating if induction of autophagy due to lipid excess could lead to breakdown of mitochondria that may indirectly impact mtDNA levels and localization in the cell. There is strong evidence to support that mtDNA replication and integrity are mechanistically linked to cellular lipid homeostasis, but how mtDNA maintenance may be impacted during compromised nutritional states, such as starvation and lipid excess, is poorly understood. This is particularly in the context of postmitotic peripheral tissues. Unraveling these mysteries may uncover novel therapeutic approaches to primary mitochondrial disease, metabolic syndromes, and even obesity.

1.7 Focus of this work

The cellular and molecular mechanisms that bridge fatty acid excess and metabolic disease onset have not been fully elucidated. Lipotoxic damage to mitochondria has been suggested to be a factor for cell death and peripheral tissue damage. Mitochondria, alone and in concert with other lipid regulating organelles (i.e., ER and LD), support fatty acid metabolism during lipid flux. Mitochondrial contact with ER is also known to promote mitochondrial division and mtDNA synthesis at division sites. Due to the importance of mitochondrial contact sites in both lipid metabolism and mtDNA maintenance, the overall research goal is to understand if these two processes are spatially linked. Currently, the research focus is to identify the mechanisms that connect influx of the saturated fatty acid palmitic acid (PA) with alterations of mitochondrial dynamics and mtDNA maintenance, using laser scanning confocal microscopy and molecular biology techniques.

1.8 Figures

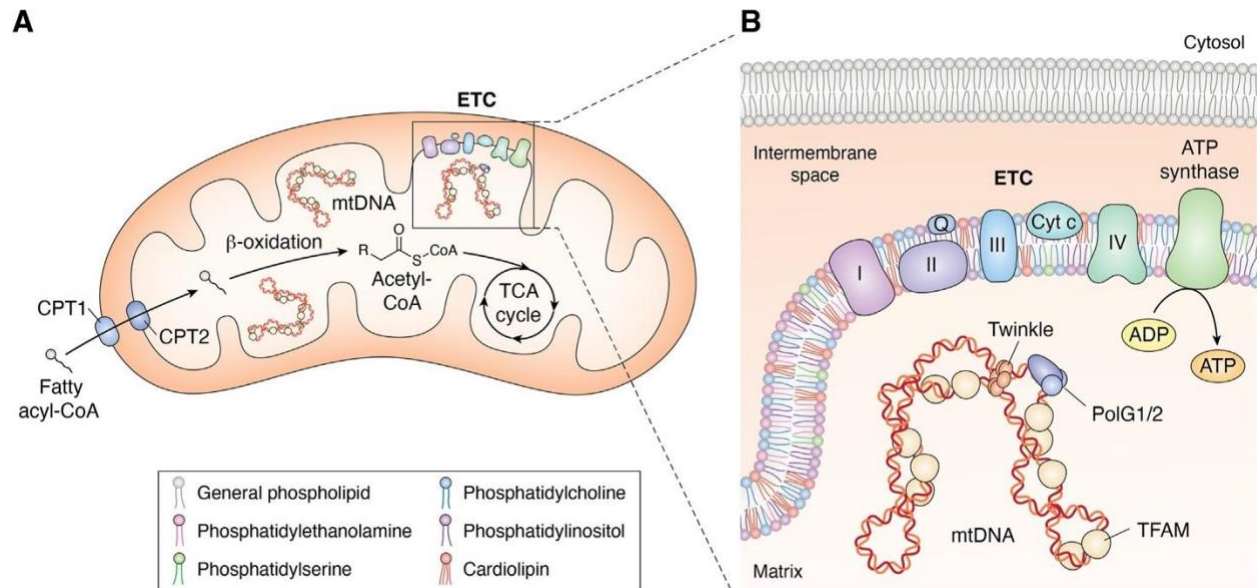


Figure 1.1. Mitochondrial ultrastructure and genome localization. *A*, Mitochondrial β -oxidation and respiration are displayed. Cytosolic fatty acyl-CoA molecules are converted into acylcarnitine by CPT1 at the outer mitochondrial membrane (OMM) and reconverted into acyl-CoA by CPT2 at the inner mitochondrial membrane (IMM) upon entering the mitochondrial matrix. Acyl-CoA is broken down into acetyl-CoA that can be channeled into the TCA cycle for respiration. *B*, The OMM and IMM are distinct in their structures and displayed are phospholipids that make up the IMM, with cardiolipin being unique to the IMM. The mitochondrial genome (mtDNA) is considered to be localized near the IMM, which is also the location of electron transport chain complexes (complexes I, II, III, IV, coenzyme Q, cytochrome c, and ATP synthase), whose functions result in ATP production. mtDNA is compacted into a protein–nucleic acid complex called the nucleoid, with TFAM being necessary for its compaction. Mitochondrial replication is facilitated by nuclear-encoded PolG1 bound to processivity factor PolG2, and Twinkle is the helicase that unwinds mtDNA for replication. ATP, adenosine triphosphate; CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine palmitoyltransferase 2; Cyt C, Cytochrome C; ETC, electron transport chain; PolG1/2, polymerase gamma 1 and 2; Q, coenzyme Q; TCA, Tricarboxylic acid cycle; TFAM, mitochondrial transcription factor A.

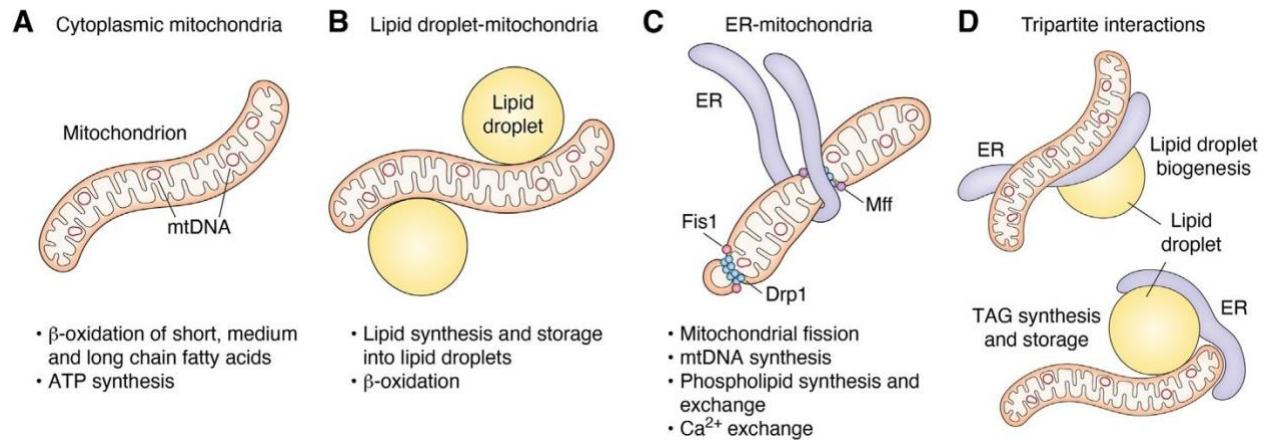


Figure 1.2. Functional organization of mitochondrial contact sites. *A*, In the context of lipid metabolism, cytoplasmic mitochondria breaks down cytosolic fatty acids during β -oxidation to produce acetyl-CoA molecules that are channeled into the TCA cycle for respiration. *B*, Mitochondrial-lipid droplet interactions may function in both lipid synthesis, for lipid storage in droplets, as well as oxidation for energy production, depending on the state of the cell. *C*, ER-mitochondria interactions are necessary for mitochondrial fission, and ER-mitochondrial division sites mark locations for mtDNA synthesis and segregation into daughter mitochondria. Drp1 and mid-zone receptor Mff facilitates the process of mitochondrial division at ER-mitochondria contact sites, and smaller division sites can occur at the periphery of the mitochondria without the need for ER-mitochondrial contact, facilitated by Drp1 and peripheral receptor Fis1. *D*, Tripartite interactions can include both a conformation where the ER is “sandwiched” between the mitochondria and budding lipid droplet during lipid droplet biogenesis (top) and a conformation of the ER wrapping around the lipid droplet and mitochondria for TAG synthesis and storage. Drp1, Dynamin-related protein 1; Fis1, Fission Protein 1; Mff, mitochondrial fission factor; TAG, triacylglycerol.

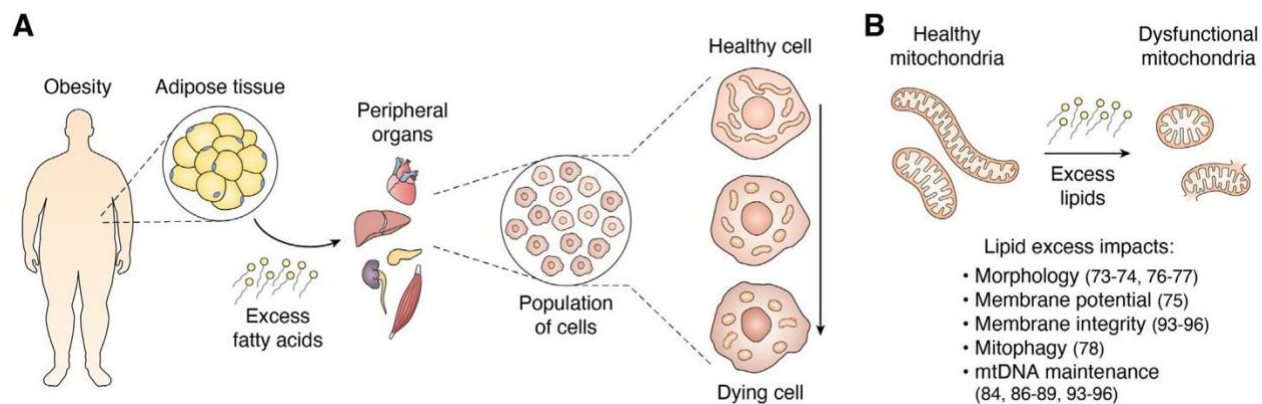


Figure 1.3. Lipotoxicity and mitochondrial dysfunction across biological scales. *A*, During obesity, there is an accumulation of excess fat in adipose tissue. Fatty acids released into circulation from adipose tissue can enter into peripheral organs, such as the (heart, liver, skeletal muscle, kidney, and pancreas). Periphery tissue damage resulting from fat accumulation is termed lipotoxicity, where excess FAs cause cellular dysfunction. A population of cells in periphery tissue can gradually transition from healthy to dying during chronic excess lipid exposure. *B*, Mitochondria can become dysfunctional during these conditions due to changes in their morphology and function, as well as the maintenance of their genomes.

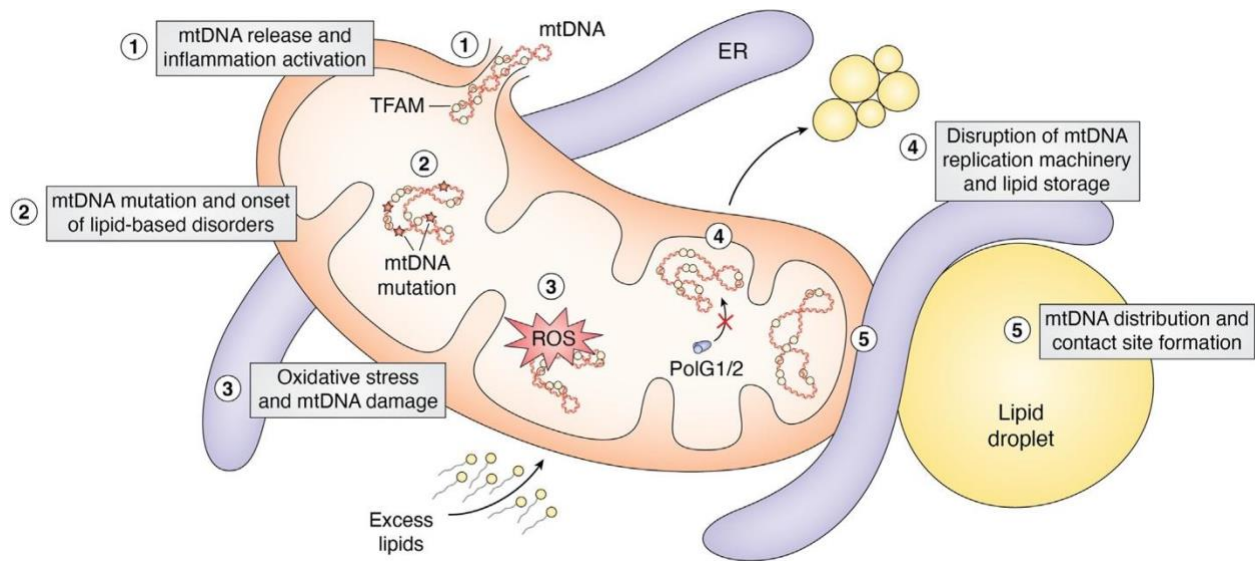


Figure 1.4. Lipid excess is linked to mtDNA maintenance. Displayed are five ways that an excess lipid environment could impact mtDNA maintenance: (1) mtDNA release from the mitochondrial matrix into the cytosol may activate inflammatory pathways; (2) mtDNA mutations can cause metabolic, and more specifically, lipid-based disorders; (3) oxidative stress by reactive oxygen species (ROS) can result in mtDNA damage, such as DNA lesions; (4) disruption of mtDNA replication machinery can lead to perturbed lipid storage; (5) mtDNA distribution across the mitochondrion and in relation to contact sites, such as tripartite contact sites, may affect lipid metabolism.

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Chapter 2: Saturated lipid stress attenuates mitochondrial genome synthesis in human cells

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2.1 Abstract

Fatty acids are trafficked between organelles to support membrane biogenesis and act as signaling molecules to rewire cellular metabolism in response to starvation, overnutrition, and environmental cues. Mitochondria are key cellular energy converters that harbor their own multi-copy genome critical to metabolic control. In homeostasis, mitochondrial DNA (mtDNA) synthesis is coupled to mitochondrial membrane expansion and division at sites of contact with the endoplasmic reticulum (ER). Here, we provide evidence from cultured hepatocytes that mtDNA synthesis and lipid droplet biogenesis occur at spatially and functionally distinct ER-mitochondria membrane contact sites. We find that, during saturated lipid stress, cells pause mtDNA synthesis and mitochondrial network expansion secondary to rerouted fatty acid trafficking through the ER and lipid droplet biogenesis, coincident with a defect in soluble protein import to the ER lumen. The relative composition of fatty acid pools available to cells is critical, as monounsaturated fatty acid supplementation rescued both ER proteostasis and mtDNA synthesis, even in the presence of excess saturated fat. We propose that shutoff of mtDNA synthesis conserves mtDNA-to-mitochondrial network scaling until cells can regain ER homeostasis.

2.2 Introduction

Mitochondria play a central role in lipid metabolism through beta-oxidation of fatty acids for ATP production, as well as through interactions with other lipid metabolizing organelles including the endoplasmic reticulum, lipid droplets, peroxisomes, and lysosomes [1-3]. Lipid droplets (LDs) are the major sites of neutral lipid storage in cells. Membrane contact sites between mitochondria, LDs, and the endoplasmic reticulum (ER) define a subset of peri-droplet mitochondria that preferentially participate in ATP production via fatty acid beta-oxidation [4-7]. Cellular nutrient and energy demand modulate fatty acid flux into and out of the LD compartment and thus the balance between lipid storage and usage, as well as the abundance of peri-droplet mitochondria [2, 8-9].

Beyond energy conversion, fatty acids are trafficked between organelles to support membrane biogenesis and act as signaling molecules that are needed to rewire cellular metabolism in starvation, overnutrition, and in response to environmental cues [1, 10-17]. These signaling events, and subsequent execution of metabolic programs, depend on membrane contact sites between mitochondria, LDs, and the ER. Disruption of lipid homeostasis, particularly excess cellular uptake of saturated fatty acids, remodels lipid trafficking through LDs and ER, impinging organellar biogenesis and protein quality control functions [18-20]. This lipotoxic insult activates cellular stress responses that monitor cytoplasmic protein quality control, including the ER unfolded protein response (ER-UPR) and the broader integrated stress response (ISR) [10, 21-24].

Lipotoxicity also leads to mitochondrial dysfunction, although the mechanisms by which saturated lipid stress influences mitobiogenesis are not clear [7-8; 25-26]. Mitochondria harbor their own genome, mitochondrial DNA (mtDNA) which encodes essential subunits of the electron transport chain complexes I, III, IV, and V [27]. MtDNA is maintained by dedicated proteins that constitute the mitochondrial replisome, minimally consisting of a DNA polymerase

(POLG1 and POLG2), single-stranded binding protein (SSBP1), and helicase (TWINKLE) [27-29]. In humans, deleterious mtDNA mutations are linked to increased lipid deposits in the skeletal muscle and liver [25, 30]. In mice, mtDNA replication defects have been associated with an overall decrease in lipid production that correlates with peripheral neuropathy [31]. Moreover, both saturated fatty acid treatment in cell culture and obesity in mice are associated with an increased load of mtDNA lesions and mitochondrial membrane herniation, leading to the release of pro-inflammatory mtDNA into the cytosol [32-38]. This suggests that mtDNA synthesis and integrity are coupled to lipid metabolism by largely unknown crosstalk machineries.

Lipids move between ER, LDs, and mitochondria via membrane contact sites (MCS) that serve as platforms for lipid and ion transfer, lipid droplet biogenesis, mitochondrial fission, mtDNA synthesis, and mtDNA nucleoid partitioning in proliferating cells [39-46]. The abundance and size of ER-mitochondria MCS are responsive to nutrient availability, ISR activation, as well as the progression of mtDNA synthesis [47-52]. Whether these disparate functions occur simultaneously at all ER-mitochondria MCS, or are distributed amongst spatially or regulated classes of such, is not well understood.

Here we investigate how two well-characterized MCS functions, lipid droplet biogenesis and mtDNA synthesis, are spatially organized in cells and the consequences of organellar reorganization during lipid stress. We find that the subset of mitochondria engaged in mtDNA synthesis are distinct from those participating in tripartite interactions for LD biogenesis. During stress induced by excess saturated fatty acid, recruitment of the mtDNA polymerase subunit POLG2 to mtDNA is impaired and mtDNA synthesis is attenuated, concurrent with ER dilation. ER morphology, POLG2 recruitment, and mtDNA synthesis are rescued by rebalancing fatty acid pools via supplementation with a monounsaturated fatty acid. We propose that endomembrane remodeling in lipid stress promotes enhanced lipid neutralization and storage at the cost of mtDNA synthesis; thus, prioritizing metabolic adaptation over mitochondrial biogenesis.

2.3 Results

2.3.1 MtDNA synthesis and LD maintenance occur at distinct ER-mitochondria contacts

To identify the positioning of lipid droplets relative to sites of mtDNA synthesis at ER-mitochondria contacts, we transiently transfected human hepatoma (Huh7) cells labeled with BODIPY-665/676 with three fluorescent reporter proteins: the ER marker KDEL- mRuby, mitochondrial matrix-targeted BFP, and tagged mtDNA polymerase subunit POLG2-GFP. Airyscan fluorescence microscopy revealed POLG2-GFP foci in a subset of mitochondria, that were enriched at points of ER-mitochondria colocalization, as previously shown in other immortalized mammalian cell lines (Figure 2.1A, top right) [43]. On average, each cell contained 120 POLG2-GFP foci that were sparsely distributed among the mitochondrial population, where they often colocalized with, or were adjacent to, ER tubules engaged in mitochondrial constriction (Figure 2.1A-B). We observed Bodipy-labeled LDs at tripartite intersections with ER and a subset of mitochondria throughout the cytoplasm (Figure 2.1A, lower right). To determine whether sites of contact with LDs were spatially linked to sites of mtDNA synthesis,

we segmented regions of colocalization between ER and mitochondria marked by POLG2- GFP and quantified the number that also intersected with LDs (Figure 2.1C). Fewer than 20% of ER-mitochondria contacts were associated also with LDs, indicating that ER- mitochondria-LD tripartite contacts are distinct from sites of mtDNA replication.

To validate these observations we employed a genetically-encoded reporter for LD biogenesis, GFP-tagged Seipin. Seipin forms homo-oligomeric complexes that support ER-LD contact site maintenance and the trafficking of neutral, esterified lipids into nascent LDs [4; 51]. We marked active mtDNA nucleoids in the same cells using an mKate-tagged allele of SSBP1, the mitochondria-specific single-stranded binding protein required for mtDNA synthesis and transcription (Figure 2-S1). Seipin-GFP formed fluorescent rings around a subset of spherical, perinuclear LDs, most of which were distal to mitochondria marked by SSBP1-mKate. These results are consistent with a model in which subcellular compartmentalization maintains a subpopulation of mitochondria engaged in mtDNA synthesis distinct from another subpopulation associated with LDs in Huh7 cells.

Given that both mitochondria and LDs make functionally essential contacts with the ER, we considered that ER remodeling to support the biogenesis of one organelle interaction partner could involve a reciprocally altered interaction with the other. The lipid composition of mitochondrial membranes informs mitochondrial division by the mitochondrial division dynamin Drp1 via its receptors, tuning mitochondrial fission [43, 44]. Mitochondrial morphology and fatty acid oxidation (FAO) rates are intimately linked, with greater FAO leading to fragmentation of the mitochondrial network [53]. Moreover, a high fat diet entrains mtDNA depletion, suggesting that mtDNA maintenance is functionally linked to lipid homeostasis across different scales from organelles, to cells, to tissues and the whole organism [Reviewed in 25]. To address this hypothesis, we tested whether saturated lipid stress induced via excess of the saturated fatty acid palmitate (PA), would impact mtDNA synthesis at ER-mitochondria contacts. PA is a long-chain saturated fatty acid linked to perturbed mtDNA integrity in humans with metabolic disease, as well as to liver lipotoxicity, steatosis, and systemic inflammation [19, 38, 54-55].

We pulse-labeled cells expressing POLG2-GFP with the synthetic thymidine analog 5- ethynyl-2-deoxyuridine (EdU), then fixed and stained them to detect EdU- AlexaFluor647, the endogenous mitochondrial outer membrane protein Tom20, or GFP, by immunofluorescence (Figure 2.1D-G). In cells treated with only the DMSO vehicle, distinct POLG2-GFP puncta colocalized with EdU foci in tubular mitochondrial networks consistent with the phenotype of the marker in live cells (Figure 2.1D). In contrast, POLG2-GFP was diffusely localized within mitochondria in cells cultured in PA- supplemented medium, (Figure 2.1E) in which only a minority of POLG2-GFP puncta colocalized precisely with EdU (Figure 2.1E, cyan circle). When POLG2 puncta were present, they often did not colocalize with EdU (Figure 2.1E, white circle). In parallel, mitochondria were fragmented upon exposure to this nutrient excess, consistent with previous reports [56-58]. These observations indicate that the recruitment of at least one component of the mtDNA replisome to mtDNA at ER-mitochondria contacts is perturbed in cells fed excess PA (Figure 2.1F). Thus, we sought to quantify mitochondrial function, mtDNA copy number and mtDNA synthesis systematically in our lipid stress model.

2.3.2 Lipid accumulation model to probe effects on mitochondrial function

We established a lipotoxicity model in which to examine reporters of organelle morphometrics, mtDNA synthesis, and fatty acid trafficking in cells subject to an accumulation of excess PA. Palmitate cannot cross mitochondrial membranes directly but must be trans-esterified to a neutral lipid prior to mobilization into droplets or mitochondrial uptake via the carnitine palmitoyltransferase (CPT1) shuttle [59]. Huh7 cells cultured in 0.1-0.3 mM PA for 24 h exhibited reduced Mitotracker fluorescence intensity consistent with impaired mitochondrial inner membrane potential and accumulated LDs, as shown by Bodipy 493/503 staining (Figure 2.2A-C). Mitochondrial network size was also reduced in PA-treated cells relative to non-treated and DMSO vehicle-treated controls (Figure 2.2D). We validated the reduction in membrane potential by quantifying the fluorescence intensity of tetramethyl rhodamine ester (TMRE), which was also significantly reduced (Figure 2.2E-F).

Despite these changes, cell viability remained high, indicating that our treatment regime was sufficient to influence cell metabolism without crossing a threshold of dysfunction that would instigate a commitment to cell death (Figure 2.2G). Cells cultured in PA exhibited small yet statistically significant changes in the oxygen consumption rate (Figure 2.2H-I) but not in the proportion of ATP generated by oxidative phosphorylation versus glycolysis, or lactate efflux (Figure 2.2J-K). Thus, PA treatment led to an expansion of cellular lipid stores accompanied by measurable impacts on mitochondrial network organization without confounding effects from bioenergetic dysfunction.

2.3.3 Saturated lipid stress attenuates mtDNA synthesis

We next sought to determine the impact of cell culture in the presence of excess saturated fatty acid on mtDNA homeostasis using direct metabolic labeling of DNA synthesis. Given the altered POLG2 recruitment to nucleoids in PA-treated cells, we considered that mtDNA might be depleted from mitochondrial networks due to decreased replication efficiency. Alternatively, we hypothesized that, if overall mitochondrial network growth were abrogated, suppression of mtDNA synthesis could serve to maintain the scaling of mitochondrial genome copies to network volume until the lipid stress were relieved.

To test our hypothesis, we quantified the number of mitochondrial DNA and mitochondrial EdU foci in cells cultured in the presence of pharmacologic inhibitors of diacylglycerol acetyltransferase enzymes DGAT1 and DGAT2 for 48 h, with or without the 24 hour 0.3 mM PA treatment. Once long-chain saturated fatty acids enter cells, they are esterified in the ER then accumulate as neutral lipids in nascent LDs that then lens and bud from the ER membrane bilayer into the cytosol. LD lensing and budding are stabilized by DGAT1 and DGAT2, respectively. If the efficient neutralization and storage of free fatty acids were a requirement for mtDNA replication, then DGAT1/DGAT2 inhibition should exacerbate the effect of PA on mtDNA synthesis.

We found that acute PA exposure alone had no effect on mtDNA/dsDNA foci or their density in mitochondrial networks normalized for network size (Figure 2.3A-B), which was validated by quantitative PCR of mtDNA copy number per cell (Figure 2-S2A). In contrast, we observed a

significant simultaneous decrease in the proportion of mitochondrial EdU foci in the same cells (Figure 2.3C). Strikingly, the density of EdU foci, but not dsDNA foci, in mitochondrial networks was significantly decreased, indicating that during saturated lipid stress cells maintain their mtDNA pool in a quiescent state (Figure 2.3D).

In the same experiment we used pharmacological manipulation of fatty acid trafficking between ER, LDs, and mitochondria to determine which FA routes were relevant to EdU labeling of mtDNA in our model. Combinatorial DGAT1 and DGAT2 inhibition was sufficient to reduce mitochondrial DNA synthesis, and further exacerbated the attenuation induced by PA treatment alone (Figure 2.3C), demonstrating that inhibition of TAG biosynthesis is sufficient to perturb mtDNA.

These effects were concurrent with a significant reduction in LDs per cell, demonstrating the effectiveness of DGAT1/2 inhibition (Figure 2-S2B-C). In contrast, direct inhibition of mitochondrial fatty acid uptake using etomoxir, a small molecule inhibitor of CPT1, had no impact on mitochondrial dsDNA foci abundance or EdU labeling (Figure 2-S2D-F). These results demonstrate that mtDNA synthesis is not co-regulated with mitochondrial fatty acid uptake, while suggesting that the accumulation of free fatty acids in the ER when they can't be trafficked into LDs is more relevant. These findings led us to wonder whether lipid accumulation in the ER may impinge on mtDNA synthesis via altered ER function and/or MCS with mitochondria.

2.3.4 Decreased mtDNA synthesis correlates with rerouted FA trafficking through the ER

High fat diet causes ER dilation and MCS remodeling in mouse models as well as human liver tissue [20, 47, 50]. Moreover, recent work indicates that the quantity of stable ER-mitochondria contacts in cultured cells is tuned to the level of integrated stress response (ISR) activation during the ER unfolded protein stress response (ER-UPR) [60]. Thus, we asked whether excess saturated fatty acids impinge on ER proteostasis.

We first assessed the abundance of ER stress marker proteins CHOP, XBP1s, and GRP78 after 24 h cell culture in PA by immunoblot (Figure 2.4A). We observed weak induction of XBP1s and CHOP in cells supplemented with 0.3 mM PA, yet no change in GRP78 protein levels, consistent with mild ER-UPR activation. We next assessed ER protein import competence by comparing the localization of fluorescent reporter constructs targeted to the ER membrane versus the ER lumen via transient transfection with GFP-SEC61b and KDEL-mRuby, respectively. We predicted that ER dysfunction would result in reduced import of soluble proteins to the ER lumen, specifically. Consistently, GFP-Sec61b and KDEL-mRuby were robustly colocalized in control cells (Pearson's $R = 0.90$), yet KDEL-mRuby fluorescence was largely cytoplasmic in cells cultured in excess palmitate (Pearson's $R = 0.13$) (Figure 2.4B). These data validate that saturated lipid stress compromises ER function.

To monitor the intracellular fates of exogenous lipids and how they may be rerouted during nutrient excess, we labeled cells with the fluorescent fatty acid analogue Bodipy 558/568 C12 (Red C12) in complete media with or without PA supplementation (Figure 2.4C). In control cells transiently transfected with GFP-Sec61b and mito-BFP, and labeled with Bodipy Far Red, Red

C12 colocalized with punctate LDs (Figure 2.4D). In contrast, PA-treated cells exhibited a greater proportion of cellular Red C12 fluorescence colocalized with ER and mitochondria. Concurrent with Red C12 accumulation in non-LD organelles, we noted Red C12- and Bodipy Far Red-enriched domains enclosed by whorls of ER membrane in contact with mitochondria within PA-treated cells (Figure 2.4D, yellow arrow), suggestive of nascent LDs trapped in the ER. Cellular uptake of Red C12 was consistent across replicates (Figure 2.4E), and thus we quantified the density of Red C12 fluorescence in each organelle upon culture in DMSO versus PA (Figure 2.4F-H). These analyses revealed a 3-fold increase in Red C12 density colocalized with ER upon culture in palmitate, whereas mitochondrial and LD Red C12 density was increased 1.1 to 1.5-fold. Consistent with the impaired mtDNA synthesis in DGAT1/2i cells, these results indicate that impaired fatty acid transfer from ER to LD is likely to contribute to mitochondrial dysfunction as lipids accumulate in ER and mitochondria.

2.3.5 Rebalanced fatty acid ratios rescue mtDNA synthesis

We next considered whether ER function and attenuated mtDNA synthesis could be rescued by rebalancing the ratio of saturated to unsaturated fatty acids available to cells. The monounsaturated fatty acid oleic acid (OA) exerts cytoprotective effects against PA-induced cell death and ER stress in multiple mammalian hepatocarcinoma cell lines and in skeletal muscle cells via increased triacylglycerol (TAG) flux and mitochondrial beta-oxidation [55; 61; 62]. Thus, we asked whether supplementation of cell culture medium with 0.3 mM OA was sufficient to rescue ER luminal protein import, mtDNA synthesis, or both. We reasoned that, if mtDNA synthesis is attenuated downstream of ER dysfunction, rescue of ER protein import by addition of oleic acid should also rescue mtDNA replication.

We transiently transfected Huh7 cells with KDEL-mRuby, mitochondrial matrix-targeted BFP, and POLG2-GFP then incubated them with PA, OA, or both simultaneously. As we had previously observed, mitochondrial POLG2-GFP signals were diffuse in cells treated with 0.3 mM PA alone, suggestive of decreased recruitment of the replisome machinery to mtDNA nucleoids (Figure 2.5A). Treatment with OA alone had no discernible impact on POLG2-GFP foci as compared to no-treatment or DMSO controls. In contrast, distinct POLG2-GFP puncta were present in cells treated with PA and OA simultaneously, despite the fact that mitochondria remained fragmented in some cells (Figure 2.5A, Figure 2-S3). Strikingly, the KDEL-mRuby import to the ER was completely rescued by simultaneous culture in OA and PA (Figure 2.5A, see lower left panel).

Consistent with our previous observation of the minor effects of PA supplementation on mitochondrial respiration and cell viability, analysis of ER and mitochondrial mass by western blot detection of endogenous Calreticulin and Tom20 revealed no differences between cells treated with DMSO, PA, OA, or OA and PA simultaneously (Figure 2-S4A), indicating that KDEL import was rescued without significant change in ER mass per cell. Further, mitochondrial network size (Figure 2-S4B) and mtDNA synthesis (Figure 2-S4C-E) were unaffected by inhibition of the ISR using the potent small molecule ISRIB, either alone or in combination with PA treatment. ISRIB blocks eIF2a-dependent adaptation to the ER-UPR, thus, these data indicate that our observations are largely independent of the suppression or activation of cytosolic protein translation in stress [60]. Taken together, these results are consistent with the

hypothesis that ER dysfunction during lipotoxic insult disrupts interactions with mitochondria and, as a consequence, mtDNA synthesis.

Finally, we validated that rebalancing saturated and unsaturated fatty acids in the cell culture medium rescued mtDNA labeling by EdU. We quantified the number of mitochondrial double-stranded DNA foci and EdU foci per cell in samples cultured in PA, OA, or both. Cell culture in excess OA alone had no impact on the number of mitochondrial dsDNA foci per cell or EdU labeling; the density of replicating mtDNAs in mitochondria was also unchanged (Figure 2.5B-D). However, concomitant supplementation with excess PA and OA rescued EdU labeling of mtDNA (Figure 2.5D). The proportion of mtDNA nucleoids engaged in replication was not significantly different in OA+PA-treated cells from untreated or DMSO-treated cells (Figure 2.5E, Figure 2-S5).

In sum, these findings indicate that the relative composition of the fatty acid pools available to cells rather than their absolute amounts influences the proportion of mtDNA nucleoids engaged in DNA synthesis, which is determined by the overall function of the ER and its interplay with mitochondria in response to saturated lipid stress.

2.4 Discussion

Cell homeostasis requires coordination between multiple processes in response to intrinsic and extrinsic cues. Given the spatial distribution of biochemical functions into distinct organelles, MCSs between them serve as hubs for such coordination. Here, we provide evidence that mtDNA synthesis and LD biogenesis occur at spatially and functionally distinct intersections with the ER, with potential implications for understanding pathological lipotoxicity (Figure 6).

We posit that peri-droplet mitochondria are a distinct subpopulation excluded from mtDNA synthesis and mitochondrial biogenesis. Peri-droplet mitochondria are well-characterized players in FA trafficking and are critical to cells successfully making the metabolic shift to beta-oxidation when substrates suitable for breakdown by oxidative phosphorylation are limiting [17, 53, 55, 63]. Our findings are consistent with a model in which peri-droplet mitochondria predominantly supply ATP and reducing equivalents for LD biogenesis, while a separate mitochondrial population dedicated to catabolism is sustained by ongoing mtDNA synthesis and biogenesis in proliferating cells. Peri-droplet mitochondria must at some point originate from the proliferating pool of mitochondria. Previous work demonstrated that mitochondrial network morphology interacts with CPT1 activity to shape fatty acid utilization [53]. Thus, it is likely that mitochondrial specialization to serve the needs of LD metabolism is coupled to division from the proliferating mitochondrial pool.

Primary mitochondrial defects induce the upregulation of mitochondrial biogenesis via the mitochondrial UPR [10, 60]. In contrast, we found that a lipotoxic insult, specifically an imbalance between saturated and unsaturated FAs, suppressed mtDNA synthesis and mitochondrial biogenesis. FA imbalance undoubtedly disturbs membrane biogenesis and seems to have its most dramatic effect on the architecture and function of the ER, to which the arrest of mtDNA synthesis is likely secondary [22-24]. However, we cannot rule out that FA imbalance may also alter the properties of the mitochondrial membranes, directly affecting mtDNA

synthesis by altering the properties or composition of the replisome, e.g., via the recruitment of proteins like POLG2 to membrane microdomains. Since growth rate and the catabolic functions of mitochondria were barely impacted in our study, and mtDNA copy number also remained at or near its starting value, the longer term effect of fatty acid imbalance on mtDNA is hard to predict. One possibility is that the partial arrest of mtDNA synthesis heralds a more general interruption of cell growth and proliferation until lipid stress is alleviated. Another possibility is that the stress induces a repurposing of mitochondria from catabolic to anabolic functions, increasing the pool of mitochondria dedicated to supporting LD biogenesis at the expense of those that continue to undergo proliferation.

While we observed a significant decrease in mitochondrial membrane potential in cells supplied with excess FA, there were minimal effects on mitochondrial respiratory capacity or lactate efflux, indicating that cells were still able to meet their energy demands. A limitation of this study is that all experiments were conducted with cells cultured in the presence of glucose [24]. While we found that the proportion of ATP generated by OXPHOS versus glycolysis did not significantly change upon PA treatment, alternative pathways for energy production via glycolysis or peroxisomal beta-oxidation presumably remain intact in the cells. Whether our observations are specific to liver-derived cells or represent a common paradigm among eukaryotic cells remains to be explored. LDs are found widely, but can be especially abundant in the liver, e.g. in cases of non-alcoholic fatty liver disease [21, 24]. Moreover, cultured hepatocarcinoma cells are distinct from the bulk of hepatocytes in that they undergo rapid proliferation. Whether mtDNA synthesis and biogenesis are affected by saturated fatty acids in the same way in post-mitotic cells remains to be tested.

Cell culture in excess saturated FA has been linked to decreased endomembrane fluidity [64]. Thus, one possibility is that the addition of monounsaturated FA restores fluidity to an extent sufficient to neutralize and traffic the free FA pool out of the ER, into LDs. In parallel, it is plausible that there exists a threshold of membrane fluidity required for the formation and stabilization of MCS between organelles. If indeed saturated lipid stress reduces ER, but not mitochondrial membrane fluidity in cultured cells, this could plausibly impact the recruitment of MCS proteins to these sites and thus MCS functions, including mtDNA synthesis.

A subset of ER-mitochondria MCS coordinate mitochondrial membrane growth and division with mtDNA synthesis, which occurs on the mtDNA copies adjacent to these sites [25, 27, 43]. Our results raise the intriguing possibility that mtDNA replisome proteins depend on the local lipid microenvironment at these contacts for their recruitment, assembly, or processivity. Recent work suggests that lipid modifications on the outer mitochondrial membrane are signals for local enrichment of receptors for the mitochondrial division dynamin, DRP1 [65]. Thus, differential composition of a lipid platform at ER-mitochondria MCS dedicated to mtDNA synthesis versus LD biogenesis could be a mechanism to specify MCS classes with dedicated functions and to coordinate the recruitment of the relevant proteins to these sites across both mitochondrial membranes.

Dysregulated mtDNA copy number is a signature of a high-fat diet, both in humans and in animal models [66-67]. Therefore, a fuller understanding of how lipid stress impacts mtDNA metabolism provides a promising route to the identification of new mitochondrial biomarkers

and therapeutic targets, whether in overnutrition, malnutrition, starvation, or various forms of metabolic disease.

2.5 Materials and Methods

Cell culture and fatty acid treatment

Huh7 human hepatoma cells were obtained from the University of California, Berkeley Cell Culture Facility (Research Resource Identifier SCR_017924). Cells were maintained in low glucose culture media, prepared with Dulbecco's Modified Eagle Medium (DMEM) lacking glutamine, phenol red, sodium pyruvate, and HEPES (Gibco Cat. No. A1443001), supplemented with 10% dialyzed fetal bovine serum (Gibco Cat. No. A3382001), 1% L-glutamine (100X) (Gibco Cat. No. 25030081), 1% penicillin/streptomycin (Gibco Cat. No. 15140122), and 5 mM [+] D-glucose (Gibco Cat. No. A2494001). Cells were maintained at 37 °C in a humidified 5% CO₂ chamber, on Falcon tissue culture dishes (Corning Cat. No. 353003), or 6 well, flat bottomed tissue culture plates (GenClone Cat. No. 25105). For imaging, cells were cultured in glass-bottom 35 mm dishes (Mattek) in 2 mL of complete medium. The long chain fatty acids palmitic acid (Sigma-Aldrich Cat. No. P0500) or oleic acid (Cole-Parmer Cat. No. C2110000-500C) were made up as 1 mL stock solutions at concentrations of 0.1 M and 0.3 M respectively. Fatty acid solutions were prepared by dissolving lyophilized fatty acid in dimethyl sulfoxide (DMSO). To increase solubility, the solution was warmed to 37 °C and vortexed for 1 minute or until all lyophilized material was visibly dissolved. Stock solutions of fatty acids in DMSO were stored at -20 °C. Cells were treated with a 1:1000 dilution of palmitic or oleic acid in complete culture medium for 24 h in 5% CO₂ at 37 °C with final concentrations, as described in the main text.

Plasmids and Transient Transfection

Cells were plated onto glass-bottom dishes 24 h prior to transient transfection. Transfections were conducted for 4-5 h in serum-free medium (Opti-MEM; Gibco Cat. No. 31985070) using Lipofectamine 2000 (Invitrogen Cat. No. 1168027). Following the 4-5 h incubation in transfection mixture, cells were treated with fatty acid, as described above, for 24 h, followed by live imaging. Plasmids and resource identifiers are reported in Table S1.

General Airyscan fluorescence microscopy

All imaging was performed with an inverted 63x/1.4 NA oil objective using the laser scanning confocal Zeiss LSM 980 microscope with Airyscan 2. Images were taken with laser lines 405, 588, 561, and 639 and captured using bidirectional scanning, with a scan speed of 0.5-2 seconds.

Live cell imaging

Live imaging was performed at 37 °C in a humidified chamber with environmental control set to 5% CO₂. Vital dyes were used at the following final concentrations: MitoTracker Deep Red (50 nM; Invitrogen Cat. No. M22426), BODIPY 493/503 (0.67 µM; Invitrogen Cat. No. D3922), BODIPY 665/676 (50 ng/mL; Invitrogen Cat. No. B3932), BODIPY 558/568 C12 (5 µM;

Thermo Scientific Cat. No. D3835), and Tetramethylrhodamine, ethyl ester (TMRE; 0.2 μ M; Invitrogen Cat. No. T669). Cells were incubated in complete medium at 5% CO₂ and 37 °C for 12-15 minutes to allow staining, with the exception of BODIPY dyes. For BODIPY 665/676 and BODIPY 558/568 C12, cells are treated with the dye for 24 h concurrent with fatty acid treatment. Reagents and pharmacologics were dissolved in DMSO and applied to cells in complete medium as indicated in Table S2.

Immunofluorescence

Cells were plated onto glass-bottom 35 mm dishes (Mattek) 2-4 days before fixation, with a final confluency of 40-60%. Staining with BODIPY 493/503 was completed prior to fixation, with a final concentration of 3.8 μ M with a 20-25 minute incubation.

4% Paraformaldehyde fixation

Cells were fixed with 2 mL of pre-warmed (37 °C) 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 20-30 minutes at room temperature, protected from light. The fixation reaction was quenched by adding 1 M glycine at a 1:100 dilution directly to the PFA in the imaging dish and incubated at room temp for 5 minutes. After quenching, samples were gently washed with PBS. Cells were then permeabilized in 2 mL of 0.1% TritonX-100 diluted in PBS for 10 minutes. Following permeabilization, cells were washed with 2 mLs of blocking buffer (1X TBS, pH 7.6, 0.1% Tween-20, 1% bovine serum albumin) for 10 minutes at room temperature, gently shaking. EdU labeling and immunolabeling followed blocking.

Immunolabeling

After a 10 minute wash in blocking buffer, the solution was aspirated and 2 mLs of fresh blocking buffer added to the sample. Primary antibodies were diluted 1:1000 in this solution, and incubated either at room temperature for 1 h, or at 4 °C overnight. Samples were washed with 2 mLs blocking buffer for 10 minutes, and then a 1:2000 dilution of secondary antibody in blocking buffer was added for a 1 h incubation. A final wash in blocking buffer and imaging conditions were as described above. Primary and secondary antibodies are reported in Table S3.

EdU labeling of mitochondrial DNA

This protocol was adapted from a previously reported method [69]. 5-ethynyl-2' - deoxyuridine (EdU) labeling was performed using the Click-iT™ Plus EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647 dye (Invitrogen Cat. No. C10640), prepared according to manufacturer instructions. Click-iT® EdU buffer additive was used at a final concentration exactly ten-fold the manufacturer recommendation. We reserved 1 mL of conditioned medium from each sample. Then 1 μ L of 10 mM EdU stock diluted in 0.5 mL conditioned medium was mixed thoroughly and added dropwise to cells. Cells were incubated in 5% CO₂ at 37 °C for 30-45 minutes for pulse labeling, as described in the main text and figure legends. After the EdU pulse, the labeling medium was replaced with the 1 mL of reserved conditioned medium and samples were incubated in 5% CO₂ at 37 °C for 10 minutes. Samples were fixed as described above. Post-permeabilization, sites of EdU incorporation were rendered fluorescent by copper click reaction.

Following the click reaction, 1.5 mLs of blocking buffer was added for a brief wash, then aspirated.

General Image quantification

2D and 3D image processing and maximum intensity projections of z-stack images were performed using Zeiss ZEN Blue software version 3.7 (Carl Zeiss). Quantification of features within Airyscan processed .czi files were analyzed as follows, using FIJI and Zeiss Arivis. Airyscan processed .czi files were converted to .sis files to be used on Zeiss Arivis Pro 4.2.1 image analysis software. Colocalization analysis of GFP-Sec61b and KDEL-mRuby in Figure 4 was performed in Fiji using the 'coloc2' plugin with bisection threshold regression.

Fiji analysis of Mitochondrial area, dsDNA foci count, and EdU foci count

Airyscan processed .czi files were z-projected at maximum intensity. Trainable Weka Segmentation was used to create a classifier to detect mitochondria (Tom20 signal), mtDNA foci, and EdU foci. A region of interest mask for mitochondria was then created to discard any signal outside of segmented mitochondria. The number of mtDNA or EdU foci segmented within the mitochondrial mask were then quantified. Several images were used to create the classifiers using a macro code written in FIJI, and a custom macro was also created to quantify mitochondrial area, and mtDNA and EdU foci count.

TMRE, Mitotracker, and BODIPY Segmentation

Machine Learning Segmentation was used for the detection of faint to bright TMRE or Mitotracker labeled mitochondria. A denoising parameter was applied to the BODIPY 493/503 channel to smooth lipid droplets, using the discrete gaussian method. Machine Learning Segmentation was applied to the BODIPY channel for classification of lipid droplets. After segmentation of lipid droplets, a watershed function was used to separate individual droplets that may be touching. A size-based object filter was used to remove objects smaller than a surface voxel area of $0.25 \mu\text{m}^2$ for both the TMRE and BODIPY channels. Mean and maximum signal intensity, surface voxel area, or roundness was measured from the segmented and filtered objects. Images were run as a batch analysis, and values were then exported as .xlsx files.

POLG2-GFP foci analysis

For the segmentation of mito-BFP expressing mitochondria, a shape detection function was used for the mito-BFP channel to identify bright filaments that are $0.25 - 1 \mu\text{m}$ in length. An intensity threshold segmenter was used to classify Mito-BFP expressing mitochondria, and a size-based object filter was used to remove segmented mitochondria smaller than $0.1 \mu\text{m}^3$. The blob finder segmentation tool was used to classify POLG2-GFP, with a split sensitivity of 50% set to identify GFP puncta with an average diameter of $0.4 \mu\text{m}$. A size-based object filter was used to remove segmented POLG2-GFP smaller than $0.04 \mu\text{m}^3$, and a signal intensity object filter was applied to remove segmented POLG2-GFP with below-threshold signal intensity. The compartmentalization function allowed for the quantification of POLG2-GFP that are inside or that intersects with segmented mitochondria. Volume and surface voxel area was measured from

the segmented and filtered objects. Images were run as a batch analysis, and values were then exported as .xlsx files.

Red C12 fatty acid lipid transfer assay

We adapted a previously reported method to monitor fatty acid trafficking in cells [68]. We defined regions of interest (ROI) delineating each respective organelle and applied an object mask filter to exclude extra-cellular signals. Images were converted to .sis files for the remainder of the analysis. In Zeiss Arivis, the blob finder segmentation tool was used to segment BODIPY 665/676 stained cells. An intensity threshold segmenter was used to classify mitochondria, and a size-based object filter was used to remove segmented mitochondria smaller than $0.1 \mu\text{m}^3$. Machine Learning Segmentation was used for the segmentation of the ER channel. Using the object math function, segmented mitochondria and LDs that intersected with segmented ER were subtracted from the ER. The signal intensity of the Red C12 channel was then measured within the segmented ER, mitochondria, and LDs, as well as within the entire ROI of the cell to get a whole cell measurement of Red C12 intensity. After batch analysis, values were then exported as .xlsx files. To determine the ratio of Red C12 within the organelles, named the Red C12 colocalization index, the signal intensity of Red C12 in each of the three organelles was divided by the whole cell RedC12 intensity value. The proportion of cytosolic Red C12 was determined by subtracting the sum of the organelle Red C12 ratios from 1. The density of Red C12 in each organelle was determined by dividing the Red C12 colocalization index for each organelle by organelle surface voxel area.

Tripartite MCS Intersection analysis

Similar to the classification methods described above, ER, POLG2 foci, mitochondria, and lipid droplets were segmented and filtered. An object math feature was applied to determine all intersection points between segmented ER, mitochondria, and POLG2 (three-way intersection) per cell. After determining these three-way intersections, another object math feature was applied to identify points where the three-way intersections overlap with lipid droplet signal. Images were run as a batch analysis, and values were then exported as .xlsx to determine the ratio of three-way to four-way intersections.

Western blotting

Cells were cultured and treated with fatty acids for 24 h in 6 well, flat bottomed tissue culture plates (GenClone Cat. No. 25105) at 40-60% confluency. Cells were trypsinized (Gibco Cat. No. 25200056), collected, and spun down at $800 \times g$ for 2 minutes. The supernatant was aspirated, and cells were resuspended in lysis buffer (1:10 dilution of 10X TBS, 0.5% SDS dissolved in ultrapure distilled water, 0.05% TritonX-100, 5 mM EDTA, and ultrapure distilled water). Loading dye was prepared with 2x Laemmli Sample Buffer and 2-mercaptoethanol (BME), according to manufacturer instructions. A 1:2 dilution of cell lysate and loading dye was boiled for 5 minutes at 90°C , and then spun down at $21,300 \times g$ for 5 minutes. Samples were loaded into a SDS-PAGE gel (Invitrogen Cat. No. XP04205BOX) for electrophoresis separation. The gel was then placed in coomassie blue staining dye (Bio Rad Laboratories Cat. No. 1610786) and destained with deionized water for protein level quantification to ensure that the same amount of total protein was loaded per well. After quantifying overall protein concentration between

samples, samples were prepared with loading dye (made with 4x Laemmli Sample Buffer and BME, according to manufacturer instructions) similar as before, and ran on a second SDS-PAGE gel. Proteins were transferred to a nitrocellulose gel using the iBlot NC mini-stack (Invitrogen Cat. No. IB23002). The membrane was placed in blocking buffer (see immunofluorescence protocol) and a 1:5000 dilution of primary antibody, protected from light, and incubated at 4 °C overnight. After incubation in primary antibody, the membrane was washed with blocking buffer for 10 minutes, followed by incubation in a 1:5000 dilution of secondary antibody in blocking buffer for 1 h at room temperature. The membrane was washed with blocking buffer for 10 minutes, replaced with fresh blocking buffer, and imaged on a Licor Odyssey Clx Imager.

DNA isolation

After fatty acid treatment as described above, trypsinized cells were resuspended in 100 μ L lysis buffer and kept on ice. A 1 μ L aliquot of RNase A (Thermo Scientific Cat. No. EN0531) was added to the lysed cells for a 5 minute incubation on ice, followed by the addition of 1 μ L Proteinase K (Thermo Scientific Cat. No. EO0491) for an additional 5 minutes. The lysate was then diluted with 600 μ L of nuclease free water and phenol-chloroform extraction was performed followed by ethanol precipitation and air-drying. DNA samples were then resuspended in 20 μ Ls of nuclease free water, vortexed/flicked to ensure even mixing and nano-dropped to measure the concentration of DNA. Samples were then used directly for quantitative PCR or kept at -20 °C.

Quantitative PCR measurement of mtDNA copy number

Primer sequences are reported in Table S4. To prepare samples for qPCR, CutSmart buffer (New England BioLabs Cat. No. B7204S) was added to the DNA at a 1:10 dilution (e.g. 2 μ L in 18 μ L of DNA sample). BAMHI-HF digestion enzyme was added at 1 μ L (New England BioLabs Cat. No. R3136S), and tubes were incubated at 37 °C for 40 minutes. The concentration of DNA used was 50 ng/ μ L, and the samples were diluted with nuclease free water as needed. qPCR primers were designed for the mitochondrial gene *rnr2* and the nuclear gene *b2m*. To prepare the qPCR reaction mix the following reagents were used: 0.1 μ M of forward and reverse primers, 1:10 dilution of isolated DNA, 1:2 dilution of SsoAdvanced Universal SYBR® Green Supermix (BioRad Cat. No. 1725270), and nuclease free water. As an example, one reaction included: 0.2 μ L each of forward and reverse mtDNA and nuclear primers, 10 μ L of SYBR green qPCR master mix, and 7.6 μ L of nuclease free water. After the reaction mixture was prepared, samples were pipetted into 96 well PCR plates with 2 μ L of isolated DNA and 18 μ L of reaction mix, for 20 μ L of solution per well. Each sample was run in triplicate. Plates were sealed, spun down 5 minutes, and the qPCR was run on a Biorad CFX Opus 96 with the following cycling conditions: denaturation for 15 seconds at 95 °C and annealing/extension for 45 seconds at 58 °C, for a total of 40 cycles. Mitochondrial genome copy number was calculated from qPCR data by averaging the Ct values of the mtDNA technical replicates and the nuclear DNA (nDNA) technical replicates. The average mtDNA Ct value was normalized to the nuclear Ct value (Δ Ct) of the negative control (no treatment sample). All other Δ Ct values were normalized to this value ($\Delta\Delta$ Ct). Finally, mtDNA copy number was indicated as the fold change of the $\Delta\Delta$ Ct.

Seahorse XF respirometry analysis

All respirometry was conducted in a Seahorse XF96 or XFe96 Analyzer (Agilent Technologies). All experiments were conducted at 37°C and at pH 7.4. The placement of treatment groups on the XF plate was randomized across biological replicates as best as possible to avoid biased results. Respiration was measured in medium containing 8 mM glucose, 2 mM glutamine, 2 mM pyruvate, and 5 mM HEPES. Cells were plated at 2×10^4 cells/well and allowed to adhere for 24 hours prior to treatment. Respiration was measured in response to oligomycin (1 μ M), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (0.75 nM or 1.5 μ M), and rotenone (0.2 μ M) with antimycin A (1 μ M). Calculations of respiratory parameters were made according to standard protocols [70–71]. Briefly, ATP-linked respiration was calculated by subtracting the oxygen consumption rate insensitive to rotenone and antimycin A from the measurements after injection of oligomycin. Maximal respiration was calculated by subtracting the oxygen consumption rate insensitive to rotenone and antimycin A from the maximum rate obtained after injection of FCCP. Lactate efflux rates and ATP production rates were calculated as previously described [72].

Cell viability and normalization

When normalizing respirometry experiments and metabolite quantification to cell number, cells were fixed immediately upon completion of the assay with 2% (w/v) formaldehyde for 20 min at room temperature and kept refrigerated between 1-14 days until assessment. On the day prior to cell counting, cells were stained with Hoechst (Thermo Fisher #33342) at 10 ng/mL overnight at room temperature. Cell counts were obtained using the Operetta High Content Imaging System (Perkin Elmer).

Statistical analyses

All data collection, organization, and calculations were performed with Microsoft Excel. Data was plotted and statistically analyzed using Prism version 10.1.1. Plots display the mean values $\pm$ standard deviation. Statistical parameters, such as cell count and number of biological replicates (N) can be found in the figure legends. For experiments involving 2 groups, statistical significance was determined using a two-tailed unpaired t-test. For experiments involving 3 or more groups, statistical significance was determined using one-way ANOVA and Dunnett's multiple comparisons test. For the RedC12 colocalization index in Figure 4, two-way ANOVA and Tukey's multiple comparisons test was applied. P-values < 0.05 were considered significant.

Table S1. Plasmids

Plasmid	Source and Reference
Mito-BFP	mito-BFP was a gift from Gia Voeltz (Addgene plasmid # 49151 ; http://n2t.net/addgene:49151 ; RRID:Addgene_49151) https://pubmed.ncbi.nlm.nih.gov/21885730/
Mito-mCherry	mCherry-Mito-7 was a gift from Michael Davidson (Addgene plasmid # 55102 ; http://n2t.net/addgene:55102 ; RRID:Addgene_55102) https://www.ncbi.nlm.nih.gov/pubmed/18228502

Plasmid	Source and Reference
KDEL-mRuby	KDEL-mRuby was a gift from Joerg Wiedenmann https://pubmed.ncbi.nlm.nih.gov/19194514/
KDEL-BFP	BFP-KDEL was a gift from Gia Voeltz (Addgene plasmid # 49150 ; http://n2t.net/addgene:49150 ; RRID:Addgene_49150) https://pubmed.ncbi.nlm.nih.gov/21885730/
POLG2-GFP	POLG2-GFP was a gift from Bill Copeland https://pubmed.ncbi.nlm.nih.gov/26123486/
GFP-SEC61b	GFP-SEC61b was a gift from Christine Mayr (Addgene plasmid # 121159 ; http://n2t.net/addgene:121159 ; RRID:Addgene_121159)
Seipin-GFP	Seipin-turboGFP was ordered from Origene. The plasmid was cloned from human mRNA NCBI accession NM_032667, human BSCL2/Seipin product catalog number RG203625
SSBP1-mKate	SSBP1-mKate was custom synthesized by Origene based on NCBI accession NM_003143, human SSBP1 mRNA transcript variant 5

Table S2. Chemicals and Pharmacologies

Reagent/Resource	Reference Source	Identifier/Catalog No.
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (30 minutes, 20 μ M)	Cayman Chemical Company	Cat# 15218
T863 (DGAT1 inhibitor) (48 hours, 20 μ M)	MedChemExpress	Cat# HY-32219
PF-06424439 (DGAT2 inhibitor) (48 hours, 10 μ M)	MedChemExpress	Cat# HY-108341A
Etomoxir (2 hours, 40 μ M)	Thomas Scientific	Cat# C986W52
Tunicamycin (2 hours, 12 μ M)	Cell Signaling Technology	Cat# 12819S
ISRIB (24 hours, 0.2 μ M)	MedChemExpress	Cat# HY-12495A
Oligomycin	Sigma-Aldrich	Cat# 75351
Rotenone	Sigma-Aldrich	Cat# R8875
Antimycin A	Sigma-Aldrich	Cat# A8674
FCCP	Sigma-Aldrich	Cat# C2920
Glucose	Sigma-Aldrich	Cat# G8769
Sodium Pyruvate	Sigma-Aldrich	Cat# P5280
L-Glutamine	Sigma-Aldrich	Cat# G3126
HEPES buffer	Sigma-Aldrich	Cat# 83264
DMEM Powder	Sigma-Aldrich	Cat# 5030
Sodium Chloride	Sigma-Aldrich	Cat# S7653
Phenol Red	Sigma-Aldrich	Cat# P0290

Reagent/Resource	Reference Source	Identifier/Catalog No.
Water suitable for cell culture	Sigma-Aldrich	Cat# W3500
Dulbecco's phosphate-buffered saline (DPBS)	Fisher Scientific	Cat# 14-190-250
16% Paraformaldehyde	Fisher Scientific	Cat# 50-980-487
Hoescht 33342	Fisher Scientific	Cat# H3570

Table S3. Primary and Secondary antibodies

Description	Source	Catalog No.
TOM20 Polyclonal Antibody	Proteintech	11802-1-AP
Anti-dsDNA antibody [35I9 DNA] - BSA and Azide free	abcam	ab27156
Calreticulin Polyclonal Antibody	Invitrogen	PA5-34786
GRP78 Polyclonal Antibody	Invitrogen	PA1-014A
CHOP Polyclonal Antibody	Proteintech	15204-1-AP
XBP-1s (E9V3E) Rabbit mAb	Cell Signaling Technology	40435S
β -Actin (13E5) Rabbit mAb	Cell Signaling Technology	4970
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 405	Invitrogen	A31553
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 488	Invitrogen	A32790
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 680	Invitrogen	A32802
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ Plus 800	Invitrogen	A32730

Table S4. Primers for quantitative PCR of mtDNA copy number

Gene	Forward Primer	Reverse Primer
<u>NM_004048.4</u> Homo sapiens beta-2-microglobulin (B2M) mRNA	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
<u>NC_012920.1:1671-3229</u> Homo sapiens mt-Rnr2	AGACTTCACCAGTCAAAGCGA	ACATCGAGGTCGTAAACCCT

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UC LEADS: Leadership Excellence through Advanced Degrees (B.D & A.D.)

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2.7 Figures

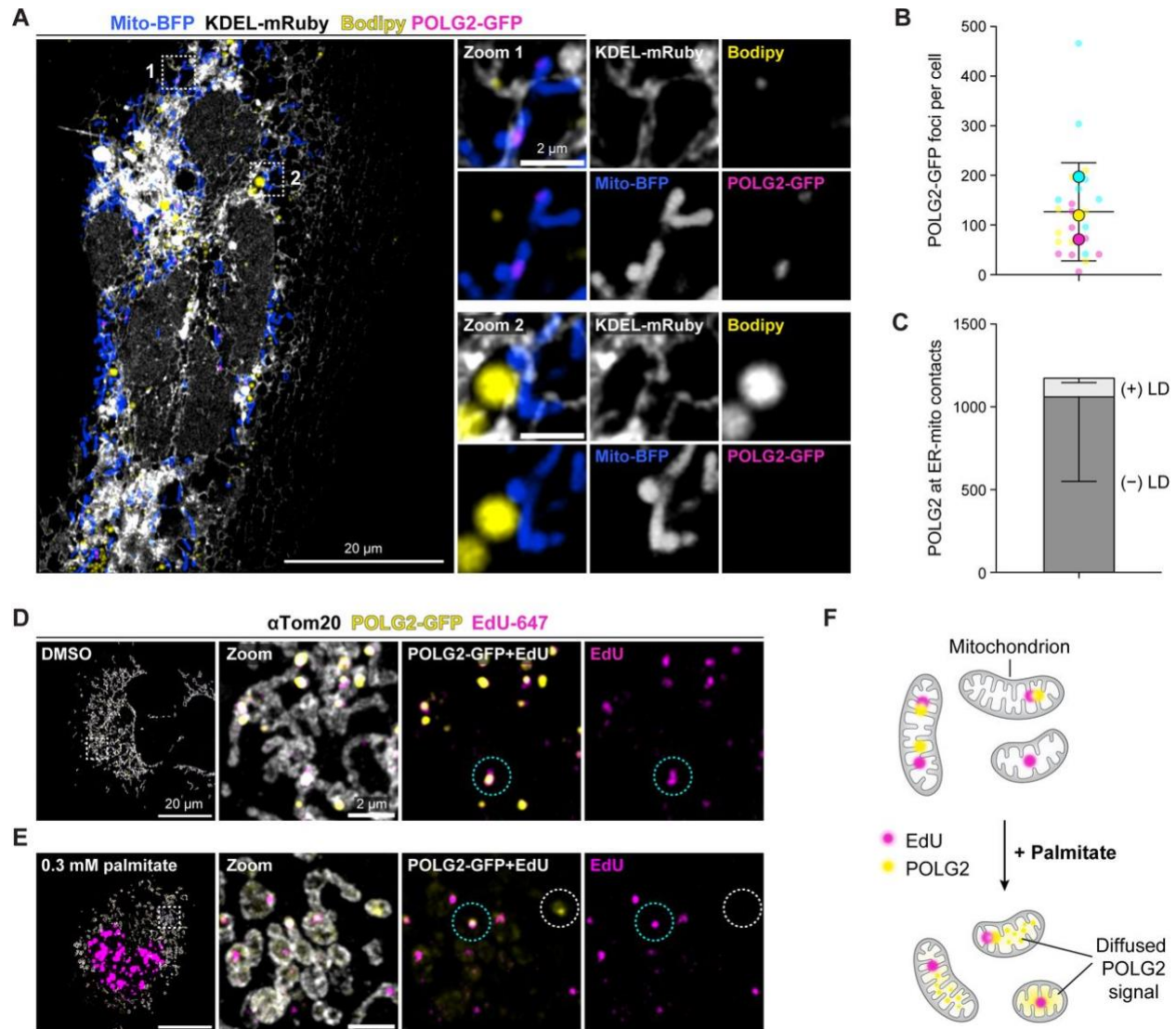


Figure 2.1. Mitochondrial genome synthesis and lipid droplet maintenance occur at distinct ER-mitochondria contacts. (A) Huh-7 cell transiently transfected with markers KDEL-mRuby (gray), mito-BFP (blue), POLG2-GFP (magenta), and stained with the far red neutral lipid staining dye BODIPY 665/676 (yellow). Scale bar 20 μ m. Zoom 1, ER-mitochondria colocalization at replicating mtDNA nucleoid. Zoom 2, Tripartite contact between ER-mitochondria-lipid droplet. Scale bar 2 μ m. (B) Quantification of POLG2-GFP foci per cell, N=3,167 foci from 25 cells across 3 replicates. (C) Quantification of the number of POLG2-GFP positive ER-mitochondrial intersections with or without lipid droplets association. N=3,529 intersections from 25 cells across 3 replicates. (D-E) Fluorescent labeling of nascent DNA in mitochondria of Huh-7 cells (EdU-AlexaFluor647, magenta) transiently transfected with POLG2-GFP (yellow), and immunolabeled with anti-Tom20 (gray), then treated for 24 hours with DMSO (D) or 0.3 mM PA (E). Cyan circles indicate colocalization between POLG2-GFP and EdU; white circles denote GFP signal without EdU-AlexaFluor647. N = 37 cells (DMSO), or 34 cells (0.3 mM PA) from 3

biological replicates. Scale bars, 10 μm and 2 μm . (F) Schematic comparison of mitochondrial EdU and POLG2-GFP signal in control versus PA-treated cells.

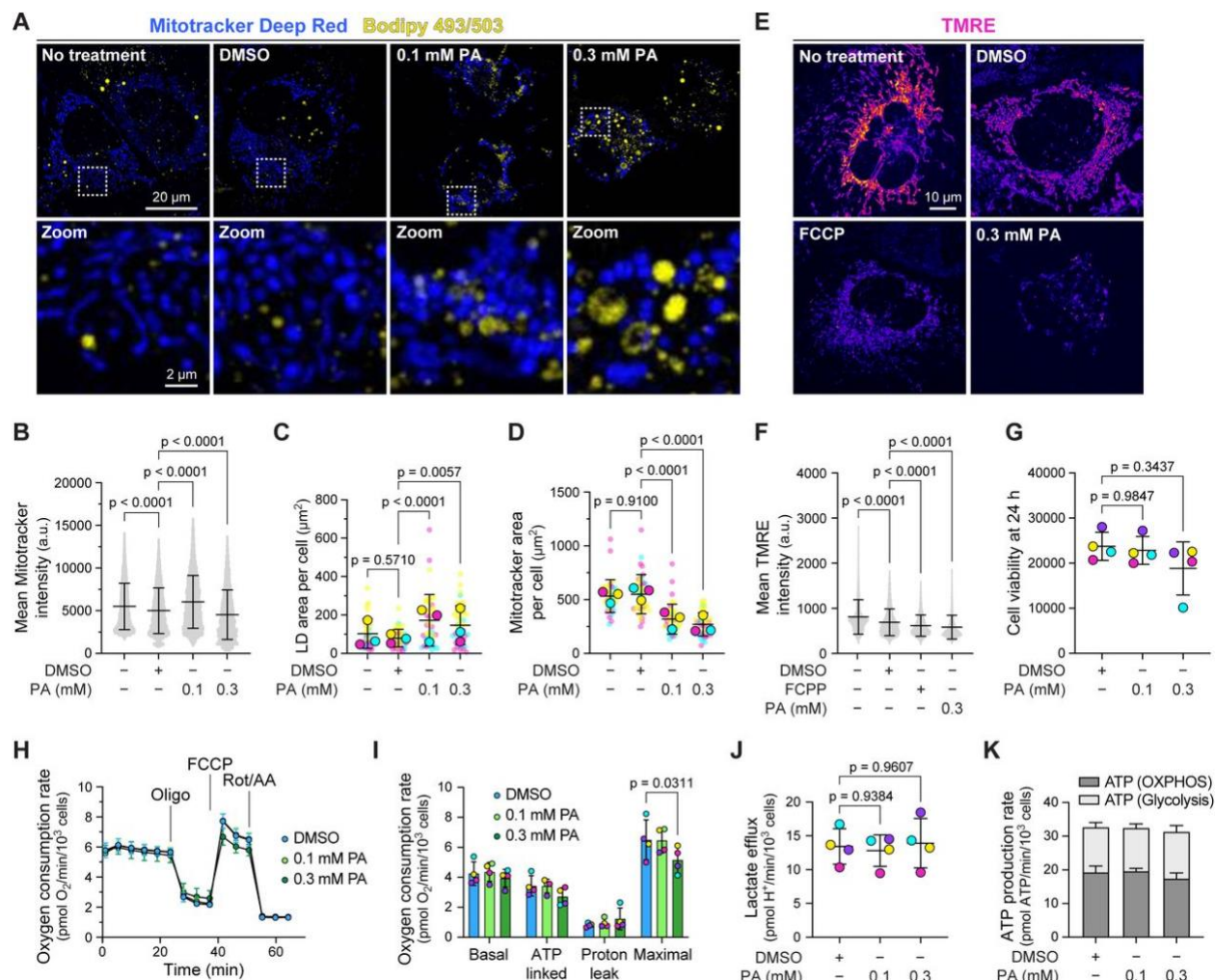


Figure 2.2 Lipid accumulation model to probe effects on mitochondrial function. (A) Untreated Huh-7 cells versus DMSO vehicle, or palmitate and stained with BODIPY 493/503 (yellow), and Mitotracker Deep Red (blue). Scale bars, 10 μm . (B) Mean Mitotracker Deep Red fluorescence intensity in segmented mitochondria, in the presence or absence of palmitate, quantified from cells in (A). $N = 54$ cells (NT), 52 cells (DMSO), 64 cells (0.1 mM PA), 47 cells (0.3 mM PA) cells, from 3 biological replicates. (C) Lipid droplet area (μm^2) per cell, quantified from cells in (A). One-way ANOVA (Dunnett's multiple comparisons test). (D) Segmented mitochondrial area (μm^2) per cell, quantified from cells in (A). (E) Untreated Huh-7 cells versus DMSO vehicle, FCCP positive control, or palmitate and stained with TMRE (fire LUT) and BODIPY 493/503. Scale bar, 10 μm . (F) Mean TMRE fluorescence intensity in segmented mitochondria, quantified from cells in (E). $N = 34$ cells (NT), 32 cells (DMSO), 25 cells (FCCP), and 26 cells (0.3 mM PA), from 3 biological replicates. (G) Mean $\pm$ SD of cell count in DMSO versus PA after 24 h. $N = 94976$ (DMSO), 91345 (0.1 mM PA), 75341 (0.3 mM PA) cells from 4 biological replicates. (H) Representative respirometry trace of cells treated as in (G), $N = 4$ biological replicates. (I) Aggregate Basal, ATP-linked, Proton leak, and Maximal respiration in intact Huh7, as in (G), $N = 4$ biological replicates. (J) Lactate efflux rate from respirometry experiments in (H-I), one-way ANOVA (Dunnett's multiple comparisons test). (K) ATP production rates calculated for Huh7 cells, $N = 4$ biological replicates.

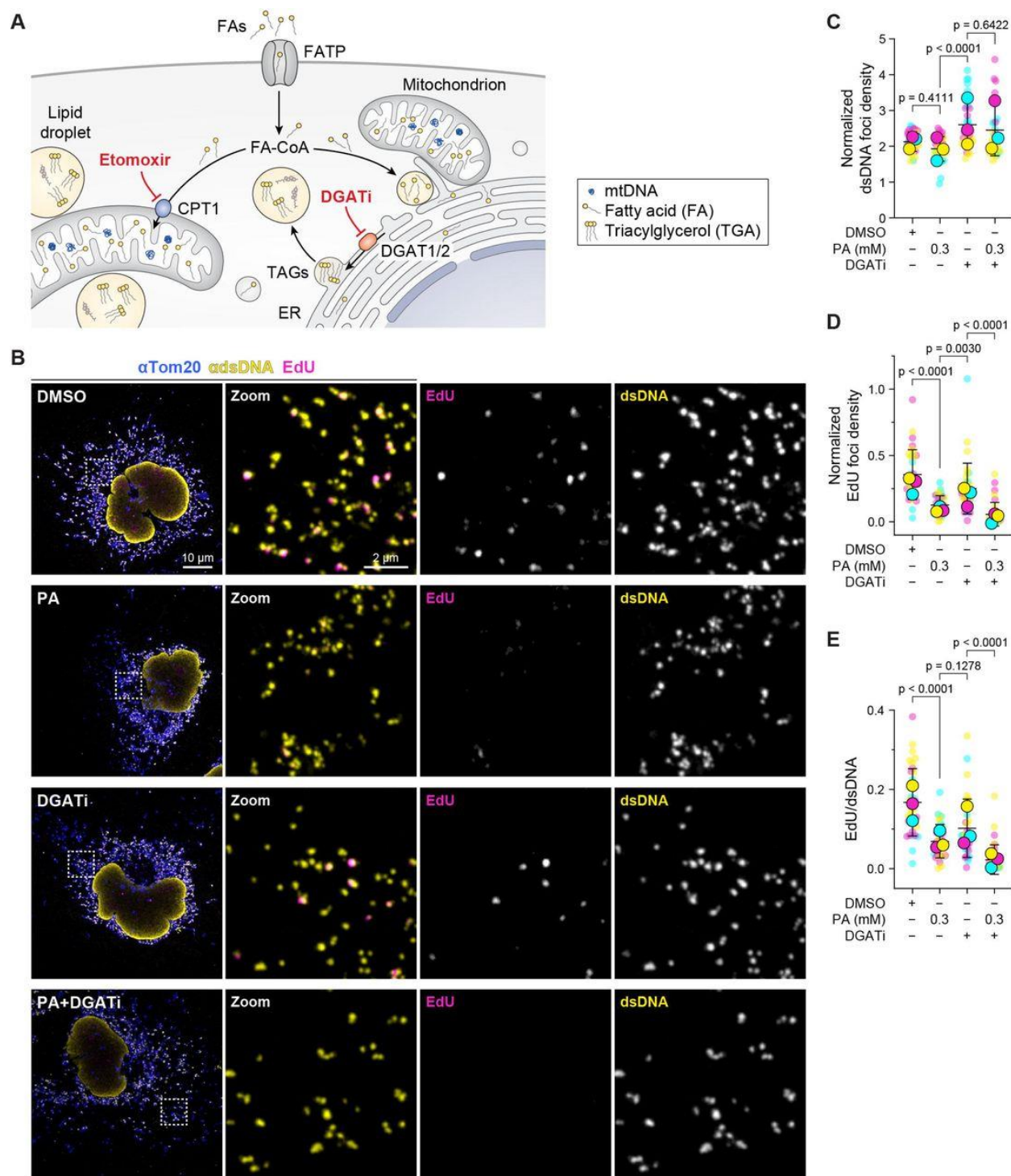


Figure 2.3 Saturated lipid stress attenuates mtDNA synthesis. (A) Schematic of fatty acid trafficking in cells upon treatment with inhibitors of DGAT1/2 (DGATi) or mitochondrial fatty acid uptake by CPT1 (Etomoxir). (B) Huh-7 cells treated with DMSO vehicle, T863/PF-06424439 (DGATi), 0.3 mM PA or both, then pulse-labeled with EdU-AlexaFluor647 (magenta) and immunolabeled with anti-Tom20 (blue) and anti-dsDNA (yellow). Scale bar, 10 μ m. (C) Quantification of mitochondrial dsDNA foci per cell, normalized to segmented mitochondrial

area. (D) Quantification of mitochondrial EdU-647 foci per cell, normalized to segmented mitochondrial area. (E) Calculation of the proportion of mtDNA nucleoids engaged in DNA synthesis on a per-cell basis, from (C-D). One-way ANOVA (Dunnett's multiple comparisons test). Cyan, magenta and yellow data points represent individual cells from each replicate. For (C-E), N=32 cells (NT), 34 cells (DMSO), 36 cells (0.3 mM PA), 36 cells (DGATi), and 36 cells (DGATi + 0.3 mM PA), from 3 biological replicates.

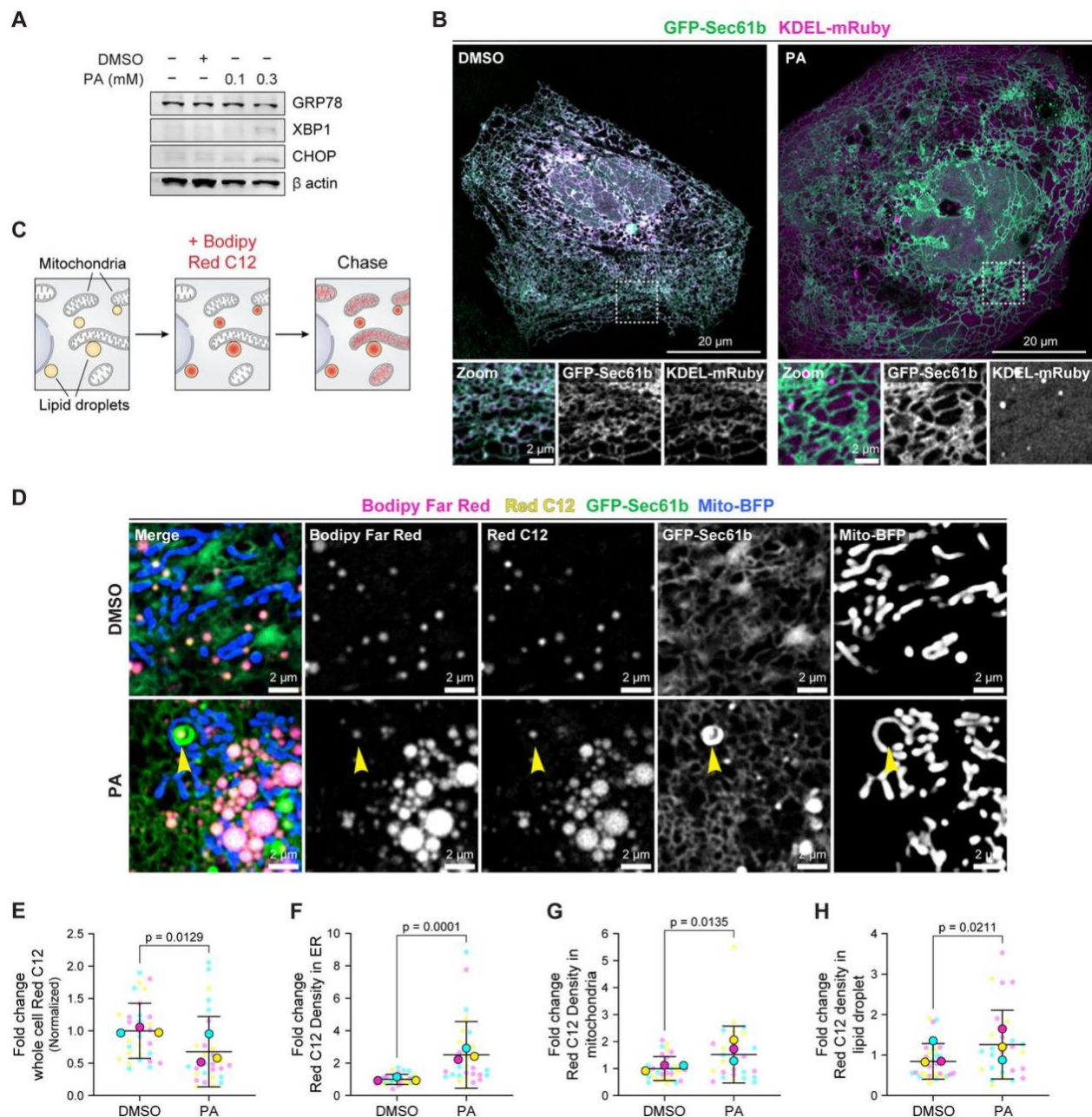


Figure 2.4 Impaired mtDNA synthesis is correlated with rerouted FA trafficking. (A) Untreated Huh7 cells versus DMSO or PA, analyzed by immunoblot for ER stress marker protein abundance relative to beta-actin. (B) Huh-7 cells transiently transfected with KDEL-mRuby and GFP-Sec61b, treated with DMSO (left) or palmitate (right). Scale bars, 20 μ m; 2 μ m. (C) Schematic of BODIPY 558/568 (Red C12) loading into cells in culture. (D) Huh-7 cells transiently transfected with Mito-BFP and GFP-Sec61b, labeled with Bodipy Far Red and Red C12, treated with DMSO (top row) or palmitate (bottom row). Arrow indicates Colocalized Bodipy Far Red and Red C12 within a bulb of ER membrane. Scale bar, 2 μ m. (E) Quantification of total Red C12 fluorescence per cell/uptake in cells from panel D. N = 32 cells (DMSO), 28 cells (PA) from 3 replicates. Quantification of Red C12 fluorescence density within

segmented ER (F), mitochondria (G), and lipid droplets (H) in cells labeled as in D. Significance determined via two-tailed unpaired t-test.

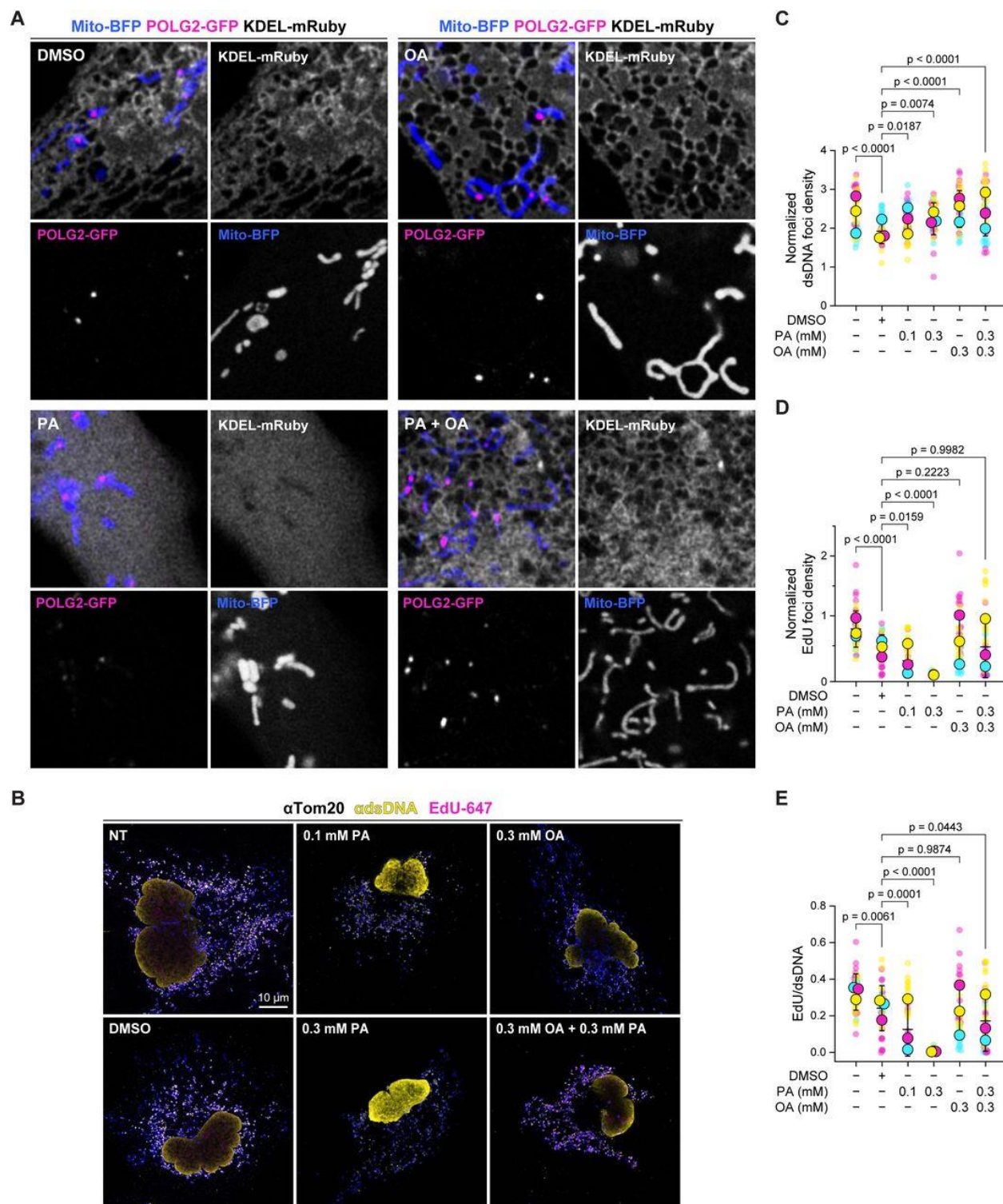


Figure 2.5 Rebalanced fatty acid ratios rescue mtDNA synthesis. (A) Huh-7 cells transiently transfected with Mito-BFP, POLG2-GFP, and KDEL-mRuby and treated with DMSO, oleic acid (OA), palmitate (PA), or both (OA+PA). (B) Huh-7 cells treated with DMSO vehicle, OA, PA, or both, then pulse-labeled with EdU-AlexaFluor647 (magenta) and immunolabeled with anti-Tom20 (blue) and anti-dsDNA (yellow). Scale bar, 10 μ m. (C) Quantification of mitochondrial

dsDNA foci per cell normalized to segmented mitochondrial area reported by Tom20 (μm^2), data from (B). (D) Quantification of mitochondrial EdU-647 foci per cell normalized to segmented mitochondrial area, data from (B). (E) Calculation of the proportion of mtDNA nucleoids engaged in DNA synthesis on a per-cell basis, from (B), one-way ANOVA (Dunnett's multiple comparisons test). Cyan, magenta and yellow data points represent individual cells from each replicate. For (C-E), N = 50 cells (NT), 55 cells (DMSO), 61 cells (0.1 mM PA), 52 cells (0.3 mM PA), 52 cells (0.3 mM OA), 49 cells (0.3 mM OA+PA), from 3 biological replicates.

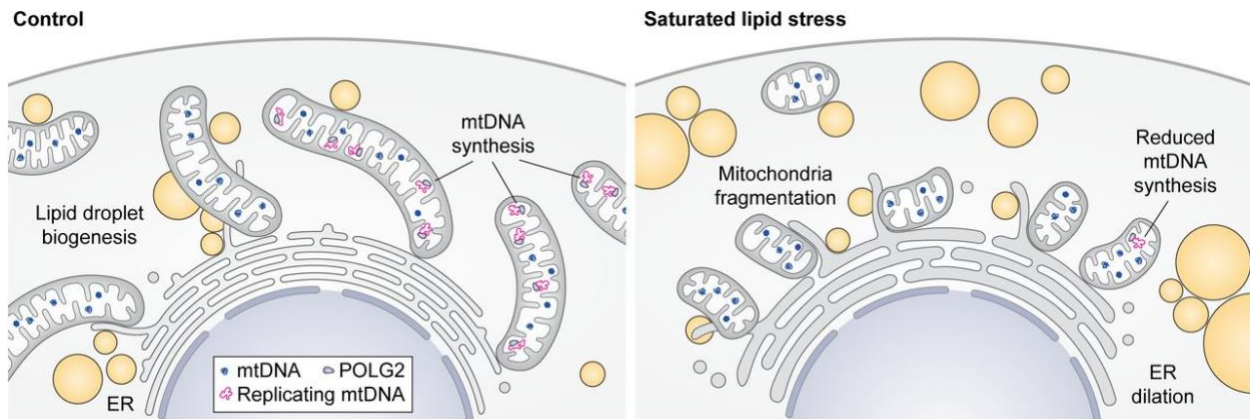


Figure 2.6 Saturated lipid stress attenuates mtDNA synthesis downstream of ER dysfunction.

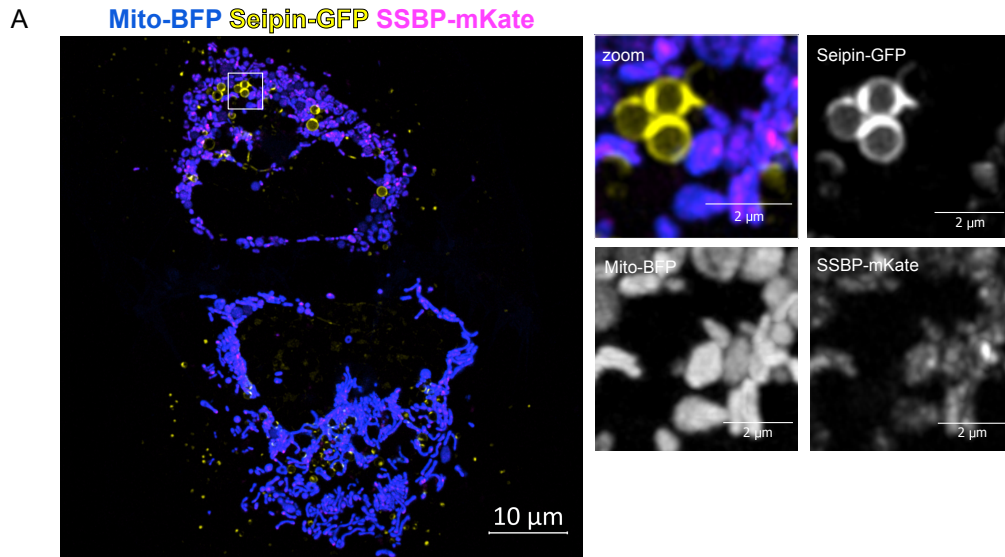
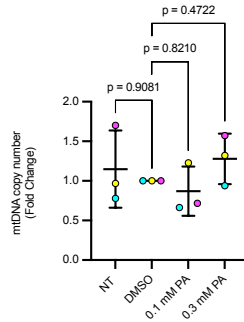
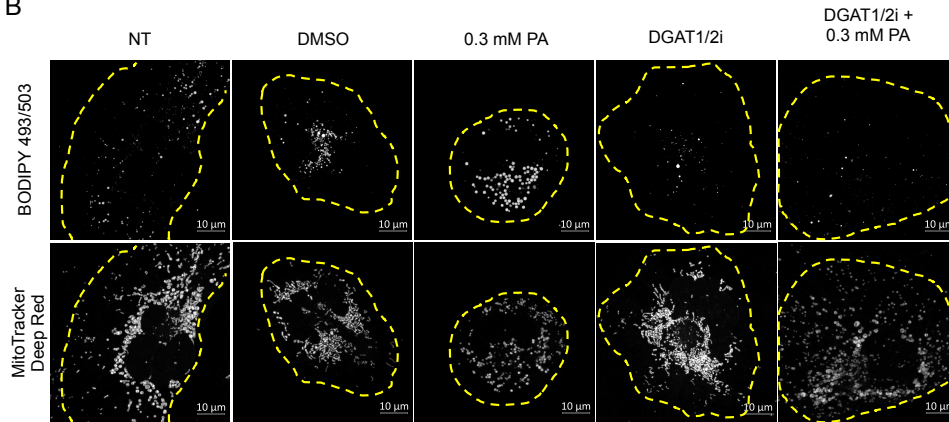


Figure 2-S1 SSBP1 localization relative to lipid droplet biogenesis marked by Seipin-GFP. (A) Huh-7 cell transiently transfected with mito-BFP (blue), and SSBP1-mKate (magenta), and seipin-GFP (yellow) imaged by laser scanning confocal microscopy. Scale bars: 10 μm (whole cell). N = 26 cells were imaged from 2 biological replicates.

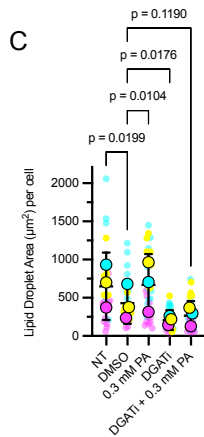
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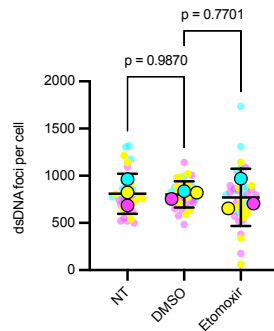
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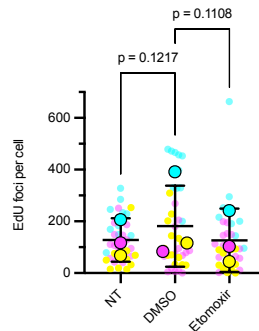
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D



E



F

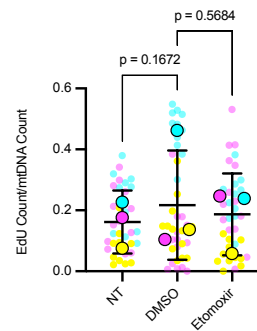


Figure 2-S2 Inhibition of fatty acid routing to LDs, but not routing to mitochondria, decreases EdU labeling of mitochondrial DNA. (A) Fold change in mtDNA measured by quantitative PCR normalized to nuclear DNA and DMSO vehicle. (B) Representative fluorescence micrographs of Huh-7 cells stained with BODIPY493/503 and MitoTracker Deep Red and treated as in B-D. (C) Lipid droplet area (μm²) per cell as segmented from Bodipy 493/503 fluorescence in DMSO vehicle, PA, DGATi, or PA+DGATi. For B-C, N = 32 cells (NT), 34 cells (DMSO), 36 cells (0.3 mM PA), 36 cells (DGATi), and 36 cells (DGATi + 0.3 mM PA), from 3 biological replicates. (D) Quantification of the number of dsDNA foci per cell DMSO versus etomoxir. (E) Quantification of the number of EdU foci per cell DMSO versus etomoxir. (F) Calculation of the proportion of mtDNA nucleoids engaged in DNA synthesis on a

per-cell basis. For D-F, N = 40 cells (NT), 40 cells (DMSO), 38 cells (Etomoxir) cells from 3 biological replicates. Statistical analysis by one-way ANOVA (Dunnett's multiple comparisons test).

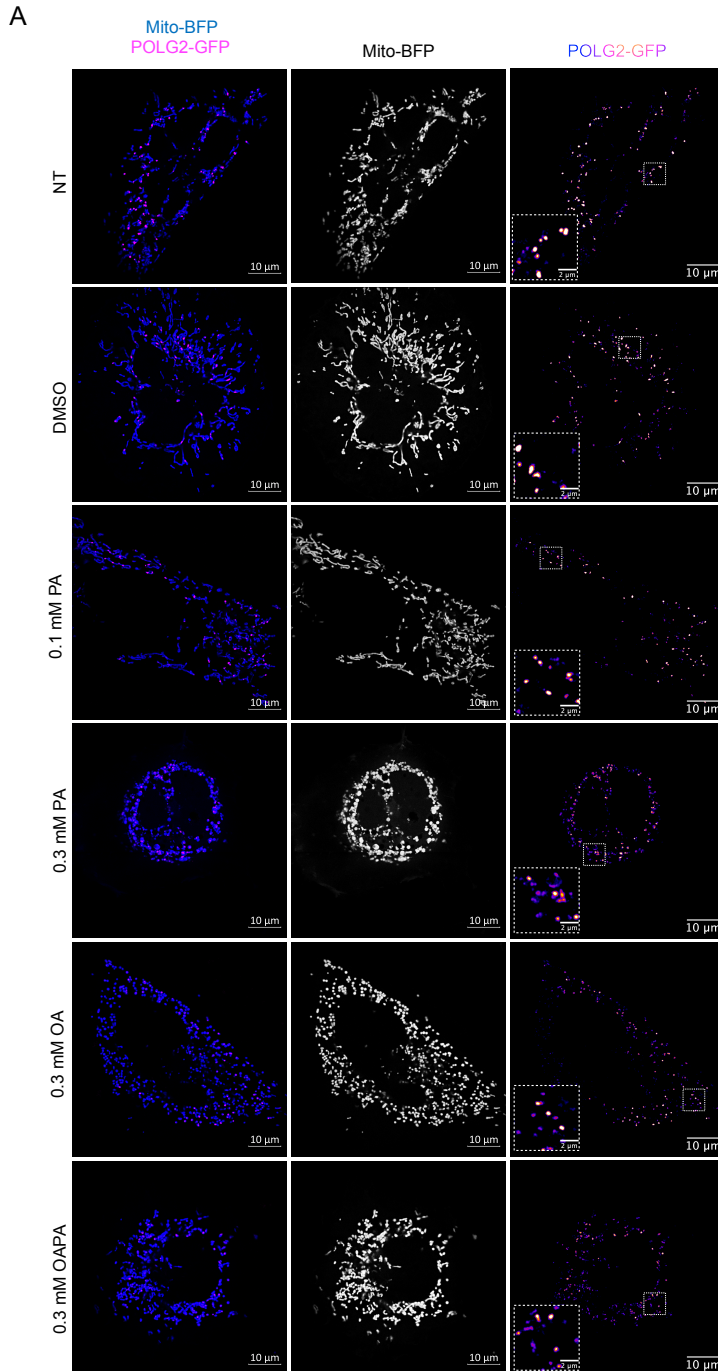
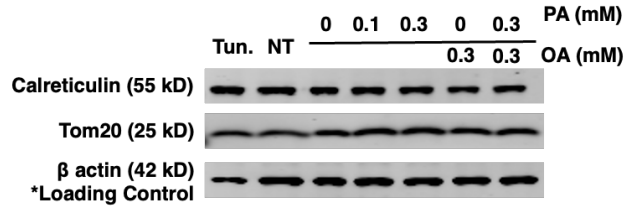
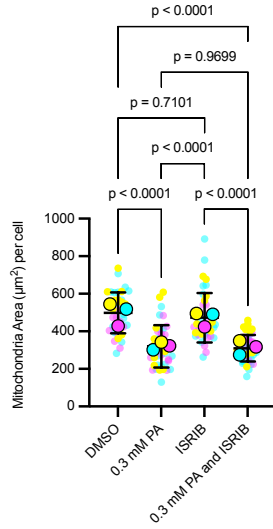


Figure 2-S3 POLG2-GFP foci in cells treated with PA, OA, or both. (A) Huh-7 cells transiently transfected with mito-BFP and POLG2-GFP then treated for 24 hours: Non-treated (NT), DMSO (vehicle), 0.1 mM PA, 0.3 mM PA, 0.3 mM OA, and 0.3 mM OAPA. Scale bars, 10 μ m. N = 35 (NT), 30 (DMSO), 30 (0.1 mM PA), 30 (0.3 mM PA), 33 (0.3 mM OA), 30 (0.3 mM OAPA) cells were imaged from 3 biological replicates. The mito-BFP channel is shown in greyscale and POLG2-GFP channel with a ‘fire’ LUT.

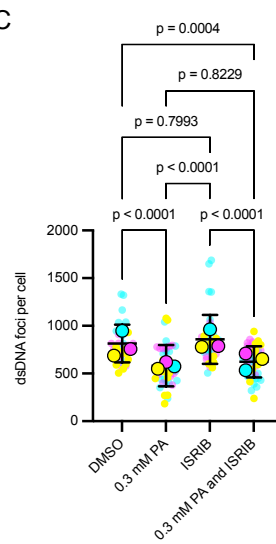
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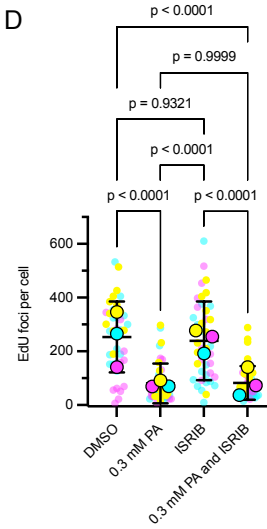
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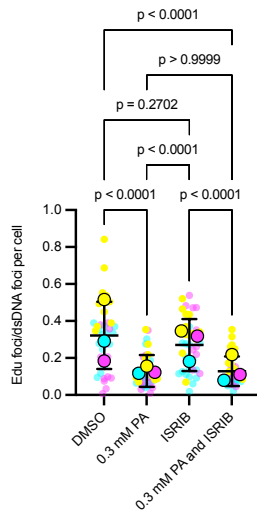


Figure 2-S4. ISRIB does not modify the effect of saturated fatty acids on mtDNA synthesis. (A) Immunoblot of Calreticulin, Tom20, and β -actin in Huh-7 cells treated for 24 hours as follows: Tunicamycin positive control (Tun.), No treatment negative control (NT), DMSO (vehicle), 0.3 mM PA, 0.2 μM ISRIB, and 0.2 μM ISRIB combined with 0.3 mM PA.

Representative of 2 biological replicates. (B) Quantification of mitochondrial area (μm^2) in ISRIB- and PA-treated cells. (C) Quantification of the number of dsDNA foci per cell. (D) Quantification of the number of EdU foci per cell. (E) Quantification of the number of EdU foci per mitochondrion divided by the number dsDNA foci per mitochondrion. For B-E, N = 39 (DMSO), 41 (0.3 mM PA) cells, 40 (ISRIB), 42 (ISRIB and 0.3 mM PA combined) from 3 biological replicates. Statistical analysis by one-way ANOVA (Dunnett's multiple comparisons test).

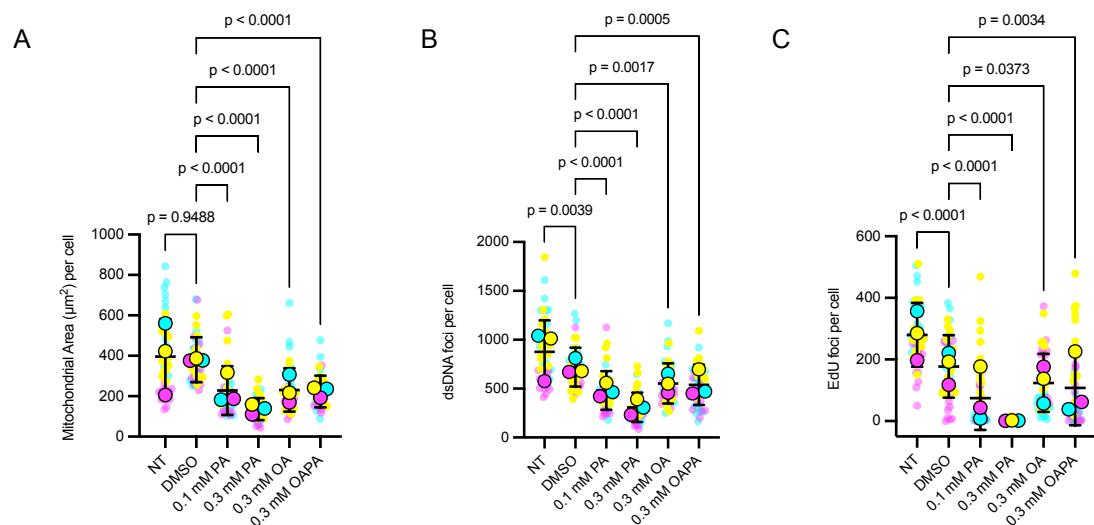


Figure 2-S5 Mitochondrial parameters in cells treated with oleic acid. (A) Quantification of mitochondrial area (μm^2) in OA- and PA-treated cells. (B) Quantification of mitochondrial dsDNA foci per cell. (C) Quantification of mitochondrial EdU-647 foci per cell. Same dataset as shown in Figure 5C-E, without normalization to mitochondrial network area. For (C-E), N = 50 cells (NT), 55 cells (DMSO), 61 cells (0.1 mM PA), 52 cells (0.3 mM PA), 52 cells (0.3 mM OA), 49 cells (0.3 mM OA+PA), from 3 biological replicates.

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Chapter 3: Perspectives, Questions, and Conclusion

3.1 Perspectives

Exploring alternative conceptual models to probe the impacts of lipotoxicity on mitochondrial function and mtDNA maintenance will further clarify how mitochondria respond to nutrient stress and advance our understanding of mitochondrial regulation and metabolism. This chapter proposes several potential contenders for future research and reflects on the significance and limitations of this body of work.

3.1.1 Mitochondrial morphology and dynamics in response to lipid stress

Mitochondrial form and function are interconnected. Its double membrane, cristae folding, and matrix make mitochondria powerful respiratory and metabolizing organelles. Mitochondrial morphology can differ between cell types, ranging from a “string-like” appearance, such as in human fibroblast cells, to a more condensed “bean-like” shape, such as in hepatocytes. These morphological differences exist to meet the metabolic demands across cell types. Morphological changes can also influence how mitochondria interact with other organelles. Cellular stress conditions drive mitochondrial remodeling, such as hyperfusion [1], cristae disruption [2-4], pearling [5] outer membrane permeabilization [6-7] fragmentation [8-9], and membrane fluidity changes [10]. Current studies have not determined if these structural changes are fully reversible.

In lipotoxic environments, mitochondria exhibit increased fragmentation [11-12]. Our preliminary results demonstrate that HepG2 and Huh-7 cell mitochondria are round and swollen after treatment upon toxic PA treatment (Figure A1). Mitochondrial fragmentation in lipotoxic conditions is also reported to occur during metabolic disease. In the fed state, obese mice liver tissue have more spherically shaped mitochondria compared to fasted mice [13-14]. Pancreatic islets isolated from Zucker diabetic fatty (ZDF) rats have “short and swollen” mitochondria [15], and this morphology is similarly observed during palmitate treated cardiac tissue in rats and T2DM patient islet cells [16-17]. Dysregulation of fusion and fission dynamics may explain mitochondrial remodeling during nutritional stress. Mitochondrial fusion and fission is mediated by several proteins: Dynamin-related protein 1 (Drp1), a GTPase, forms an oligomer ring to constrict mitochondria for division. Drp1 is assisted by receptors, such as midzone receptor mitochondrial fission factor (Mff) and peripheral receptor fission 1 protein (Fis1). The outer and inner membrane proteins Mitofusin (Mfn1/2) and Opa1 control fusion [18]. Research supports that lipotoxicity-induced fragmentation results from decreased Mfn1/2 or increased Drp1 activity [19-20]. Altered mitochondrial structure under lipid stress correlates with decreased membrane potential, increased ROS production, and decreased fatty acid oxidation [19, 21-22]. Since we observe both mitochondrial fragmentation and declined mitochondrial function, a plausible future direction is to measure the gene expression and protein levels of fusion and fission regulators under lipotoxic stress. Changes in mitochondrial dynamics may drive cellular dysfunction and metabolic disease onset.

Beyond metabolism, mitochondrial morphology regulates the synthesis, maintenance, and segregation of mtDNA [23-26]. Mitochondrial nucleoids are located near cristae junctions of the IMM [27-28]. Several studies report that mtDNA replication and distribution is closely associated with mitochondrial dynamics. For example, as described in Chapters 1 and 2, mtDNA replication and segregation is spatially linked to sites of mitochondrial division [29]. Likewise,

genetic perturbations of mitochondrial fusion and fission proteins impede mtDNA replication and the even distribution of nucleoids across the mitochondrial network [28, 30-32]. How mitochondrial morphology affects mtDNA integrity during fatty acid excess remains an open question. To address this question, mtDNA copy number and distribution, as well as the abundance and localization of replication factors (e.g., POLG, TFAM, TWINKLE), can be assessed following genetic manipulation of fission and fusion proteins. I hypothesize that increased mitochondrial fragmentation reduces mtDNA replication and the abundance of mtDNA replication factors, consistent with a prior reporting linking mitochondrial fragmentation with reduced mtDNA synthesis [33-34]. Addressing this question may clarify the relationship between mtDNA maintenance and mitochondrial dynamics during nutrient stress.

Given the role of the ER in mtDNA distribution during mitobiogenesis, remodeling the ER could restore mtDNA replication decline during lipotoxic stress. Several reports support that lipotoxicity directly influences ER morphology. Liver tissue sections derived from obese mice and high fat diet fed mice demonstrated ER reorganization and decreased ER mass compared to wild type mice [13]. Furthermore, research supports that the ratio of sheet to tubule ER structure differs between lean and obese states [35]. Further examination is necessary to determine if there is a direct link between altered ER morphology and function with declining mtDNA synthesis during lipotoxicity. I performed a preliminary experiment overexpressing the ER tubule structural protein Rtn4 and quantifying EdU labeled mtDNA (as in Figure 2.3B-E). In wild-type Cos-7 cells, Rtn4 overexpression is known to have no effect on the quantity of replicating nucleoids [29]. Since mitochondrial division is facilitated by mitochondrial contact with tubular ER, we wanted to assess if overexpressing Rtn4 would enhance mtDNA replication during lipotoxic stress, as a recovery mechanism. Upon PA treatment, Rtn4 overexpression had no significant effect on mtDNA replication (Figure A2), indicating that ER tubule formation is not correlated with mtDNA replication in normal or stress conditions. Instead, lipotoxicity may drive ER remodeling by altering membrane composition. PA exposure causes ER membrane dilation or expansion via increased saturated phospholipid content in ER membranes [36-37]. PA incorporation into phospholipids at the ER is also associated with ER stress. PA treatment increased protein abundance of the ER stress-mediated apoptosis factor C/EBP homologous protein (CHOP) [37]. In our work, we also observed a mild increase in CHOP levels upon PA exposure (Figure 2.4A). There are three main ER stress pathways, including the PERK-eIF2 α -ATF4, IRE1-XBP1, and ATF6 pathways [38]. Lipotoxic stress is reported to trigger all three ER stress pathways [39-41]. CHOP is a key factor in the PERK-eIF2 α -ATF4 pathway, working as a transcription factor to enhance the expression of apoptotic inducing genes. Activation of this ER stress pathways might connect observations of PA-induced organelle dysfunction with cell death.

3.1.2 mtDNA release and inflammation onset during lipotoxic stress

MtDNA release and inflammation onset are well established features of cellular dysfunction and cell death [42]. Lipotoxicity may promote mtDNA release by increasing ROS that damage mitochondrial membranes. It is well supported that PA treatment increases ROS levels. It has been proposed that enhanced ROS production during PA exposure could result from increased fatty acid oxidation, upregulation of NADPH oxidase, or ER stress (as a by-product) [43]. Increasing levels of ROS triggers cellular oxidative stress. The state of oxidative stress induces permeabilization of the mitochondrial membrane by the oligomerization of BCL-2

Antagonist/Killer (BAK) and BCL-2 associated X (BAX) proteins that form a pore on the outer mitochondrial membrane. Outer membrane pore formation releases cytochrome c that activates apoptosome formation and caspase cleavage to trigger apoptosis [44]. In addition to cytochrome c, mtDNA is released [16, 39, 45-47]. A possible mechanism for mtDNA release into the cytosol could be by trafficking mtDNA into endosomes for disposal during replication stress. These endosomes can rupture, releasing mtDNA into the cytosol [48]. Cytosolic mtDNA activate inflammatory pathways, such as the cGAS-STING pathway, and mtDNA release is described to occur during lipotoxicity, as measured by qPCR [42, 49-51].

Since previous research suggests that lipotoxic conditions promote mtDNA release, I examined if mtDNA is preferentially released into the cytosol upon PA treatment. I imaged labeled Tom20 and dsDNA by indirect immunofluorescence to determine the localization of mtDNA relative to the OMM boundary in HEPG2 and Huh-7 cells treated with 0.9 mM and 0.3 mM PA, respectively, for 24 hours. (Figure A3). DsDNA signal was observed that did not colocalize with the Tom20 marker, implying mtDNA release to the cytoplasm. However, DNA fluorescence in situ hybridization (DNA-FISH) is needed to verify cytosolic mtDNA during PA treatment. It remains unclear if mtDNA release and inflammation onset is beneficial or harmful to the cell overall. The cGAS-STING pathway recognizes foreign, cytosolic DNA, triggering interferon production and antiviral innate immunity. This response can promote cell death or senescence to protect tissues. However, prolonged activation, such as during mtDNA release under cellular stress, drives chronic inflammation, which contributes to tissue damage and organ failure in metabolic disease [52-54]. This raises a broader question: Is inflammation a response to, or cause of, tissue damage during lipotoxicity? If chronic inflammation results from mtDNA release during lipotoxic stress, it may exacerbate cellular and tissue damage.

Although the effects of lipotoxicity on mtDNA integrity are still poorly understood, there is evidence in the literature that PA treatment has an effect on mitochondrial genome integrity [55-56]. PA treatment is associated with a decrease in mtDNA copy number, although this finding is not consistent, as some studies report no change [57-58]. PA treatment may increase ROS levels that can damage DNA. Treating rat insulinoma cells (INS-1) with a combined oleate and palmitate (OA/PA) treatment increased nitric oxide (NO) levels and mtDNA lesions. This report argued that NO reacts with molecular oxygen to produce N_2O_3 that deaminates mtDNA bases and creates mtDNA lesions [55]. MtDNA lesions were identified by ligation-mediated PCR (LM-PCR), which identifies DNA lesions and DNA methylation patterns across short base-pair (bp) sequences. This group overexpressed a mitochondrially targeted DNA base excision repair enzyme, human 8-oxoguanine DNA glycosylase, in INS-1 cells, which prevented mtDNA lesion formation during lipotoxic stress [56]. Although this work identified mtDNA deamination as a response to PA-induced ROS production, their approach for identifying mtDNA lesions did not account for other forms of damage. MtDNA lesions represent a minor fraction of damage compared to replication errors (deletions) or DNA breaks [59]. Further approaches, such as long range qPCR, can be used to identify mtDNA deletions and strand breaks following lipotoxic treatment [60-61].

3.1.3 Potential recovery mechanisms following lipotoxic mitochondrial damage

There is ample evidence that lipid excess can induce mitochondrial dysfunction, but whether this dysfunction is reversible remains unclear. If it is possible to reverse organelle dysfunction after cellular stress, cell death and tissue damage that coincides with metabolic disease onset can be prevented. Identifying therapies to repair organelle damage after lipotoxic stress can create avenues for clinical translation by promoting increased cellular health and tissue repair. Potential intervention strategies include: (1) removal of damaged mitochondria by autophagy (2) neutralization and storage of potentially harmful lipid species (3) use of unsaturated fatty acids to mitigate saturated lipid stress (as described in Chapter 2) (4) enhanced fatty acid oxidation by peroxisomes.

3.1.3.1 Autophagy during nutrient stress

Autophagy is the cells' "waste management" system to degrade damaged cellular sub-compartments and recycle macromolecules. Autophagosomes fuse with lysosomes, which houses protease enzymes in an acidic environment to allow cargo degradation. Mitophagy is the selective degradation of damaged mitochondria. The canonical mechanism of mitophagy is the Pink1-Parkin pathway. Damaged mitochondria accumulate Pink1, a damage-sensing kinase that is normally degraded in healthy mitochondria, on the OMM due to impaired protein import. Pink1 recruits Parkin, an E3 ubiquitin ligase, that ubiquitinates OMM proteins. This initiates autophagosome formation around the damaged mitochondrion and its subsequent lysosomal degradation [62-63]. There are also Parkin-independent mitophagy pathways, some being lipid-based, such as cardiolipin-mediated and ceramide-mediated mitophagy [64-66]. These pathways might connect lipotoxicity with mitophagy induction. Across species, autophagy is widely reported during starvation to replenish macromolecules needed for survival [67-71]. However, nutrient excess by PA treatment can also induce autophagy [72-75]. Lipotoxicity-induced mitophagy may be required to degrade damaged mitochondria and restore mitochondrial quality control. We proposed that autophagy and lysosomal degradation would increase during lipotoxic treatment, based on reduced mitochondrial area following PA exposure (Figures 2.2D and 2-S5A). I performed live cell imaging of lysosomes upon PA treatment. PA treated Huh-7 cells had fewer lysosomes compared to control cells. Additionally, treating cells with an autophagy inhibiting drug (SAR405) had no restorative effect on mitochondrial density (Figure A4). Together, these results opposed our hypothesis and suggest that reduced mitochondrial area is not caused by increased autophagy. Instead, impaired mitochondrial biogenesis during PA-induced stress may be the underlying cause for this observation. By conducting RNA-seq, we could assess if mitochondrial biogenesis promoting factors (e.g., PGC-1 α) are downregulated in PA treated samples. If we could induce mitophagy during lipid stress to target the removal of damaged mitochondria, this may restore mitochondrial function under lipotoxic conditions.

3.1.3.2 Neutralization and storage of excess lipids

Sequestration and neutralization of excess lipids into LDs could improve lipotoxic damage in cells. Olzmann and Carvalho postulate that sequestration and neutralization of cytosolic free fatty acids and their toxic lipid by-products, such as ceramides, may prevent ER membrane

remodeling and ER stress [76]. Our data showed that inhibiting lipid storage by way of DGAT inhibitors further exacerbated decreased mtDNA replication during PA treatment (Figure 2.3B-E). Likewise, lipid droplets near sites of mtDNA replication were twice as large than other droplets on average in both normal and lipid stress conditions (Figure A5). Lipidomics data from our work show elevated levels of ceramides in PA treated samples. However, co-treatment with OA increased triacylglyceride levels, a lipid species typically stored in droplets (Figure A6). Preventing LD accumulation by DGAT inhibition in astrocytes caused lipotoxicity-induced cell death, and this study proposed a model that LD formation promotes cell survival during lipotoxicity [77]. In this work, I propose that OA promotes fatty acid neutralization and sequestration, protecting cells during lipotoxicity by preventing ceramide accumulation, further discussed in “Reflections and Limitations”. LD production may also prevent mitochondrial fatty acid “overload”. Inhibiting mitochondrial fatty acid overload by knocking out mitochondrial fatty acid import proteins improved glucose homeostasis in high fat diet fed mice [78]. The caveat of inhibiting mitochondrial fatty acid import is that there is an accumulation of cytosolic fatty acids that could damage organelles. Therefore, combining increased sequestration of fatty acids into LDs with partial inhibition of mitochondrial fatty acid import could be a stronger repair mechanism for lipotoxicity.

3.1.3.3 Unsaturated fatty acids in mitigating lipotoxic stress

Lipotoxic impairment of mitochondria could be improved by exogenous treatments with unsaturated fatty acids, as demonstrated in Chapter 2. Excess PA causes organelle damage and decreased cell viability. Because of this, most lipotoxicity research uses PA or diets high in saturated fat to induce cell and tissue damage [79-81]. This raises a question of whether mono- or poly-unsaturated fatty acids (MUFAs and PUFAs, respectively) could prevent or reverse the impact of PA-induced lipotoxicity. Multiple studies, including our research, demonstrate that oleic acid reduces the harmful effects of PA [57-58, 82-83]. Poly-unsaturated FAs have received little attention as a repair agent for mitochondrial dysfunction, but there are a few reports on PUFAs preventing lipotoxicity overall [84-87]. More experimentation would need to be done to study the effects of pro- and anti-inflammatory PUFA treatment on mitochondrial function and if PUFA treatment may prevent mitochondrial damage during lipotoxicity when co-treated with PA. Using methods from Chapter 2, I would measure mitochondrial membrane potential and ER stress protein abundance under PUFA and PA co-treatment. If organelle function improves, I would then assess mtDNA synthesis using EdU labeling and qPCR.

3.1.3.4 Mitochondria and peroxisome interactions

Interactions between mitochondria and peroxisomes (mito-pex) support mitochondrial function and health [88-89]. In yeast, it was demonstrated that mito-pex tethering through Pex34 and Fzo1 contributes to fatty acid oxidation [90]. At the organismal level, impairing mito-pex interactions causes disease onset, such as Zellweger syndrome, a disorder with abnormal mitochondrial function and morphology in muscle tissue [91-92]. Data suggests that there are tripartite interactions between mitochondria, peroxisomes, and ER (mito-pex-ER). Knocking out the gene encoding Pex16, an ER-resident protein needed for peroxisome biogenesis, alters mitochondrial function, morphology, and genome maintenance [93-95]. In yeast, peroxisomes are localized at ER-mitochondrial junctions at sites of mitochondrial acetyl-CoA synthesis [96]. Within mice

liver tissue, electron microscopy reveals co-wrapping of the ER around mitochondria and peroxisomes in response to lipid flux changes, termed “peroxisome-wrapER-mitochondria” complexes [97]. Another study reported that mice with peroxisome deletion demonstrated changes in inner mitochondrial membrane morphology, reduced mitochondrial membrane potential, and reduced OXPHOS complex activity. This coincided with increased smooth ER and lipid droplet formation [98]. These reports reveal a promising research area that focuses on the role of peroxisomal contact sites in maintaining lipid metabolism. Compromising these contact sites may cause lipid dysregulation. Peroxisomal activity was reported to exacerbate PA-induced lipotoxicity by producing excess ether lipids, indicating that peroxisomal activity may further promote lipotoxic stress [99]. Our preliminary results show increased abundance of small peroxisomes in PA treated cells compared to control cells (Figure A7). Future studies should define the functional role of these small peroxisomes, and whether modulating peroxisomal biogenesis rescues lipotoxic stress.

3.2 Reflection and limitations

3.2.1 Evaluation and significance of this work

Lipotoxic stress causes mitochondrial and ER dysfunction and induced cell death. In this work, we investigated how lipotoxicity affects mitochondrial genome replication. At the beginning stage of this project, I proposed four initial questions: (1) Does fatty acid treatment change mitochondrial morphology, and do these changes impact mtDNA replication? (2) Do we observe any mtDNA mutations/deletions? (3) Is there an increase in ROS production, and does this alter mtDNA integrity? (4) Can lipotoxic damage of mitochondria/mtDNA be repaired? I hypothesized that lipotoxicity alters mitochondrial morphology (increases fragmentation) and increases ROS levels that directly impair mtDNA replication. This damage can be repaired by co-treatment with unsaturated fatty acids. These initial questions guided the early direction of this project. However, the project shifted from this initial framework.

ER-mitochondrial contact sites regulate both mtDNA synthesis and lipid metabolism. I thus examined how excess saturated fatty acids remodel these contact sites. By focusing our attention on contact site remodeling, we could create a comprehensive model that directly links mtDNA replication and lipid metabolism. We specifically wanted to determine if sites of lipid droplet biogenesis were spatially linked to mtDNA synthesis. We hypothesized that because ER-mitochondrial contact sites coordinate both mtDNA synthesis and LD biogenesis, lipotoxic remodeling of these contacts may compromise mtDNA replication by prioritizing lipid neutralization and storage to limit toxic lipid accumulation in the cytosol. As presented in Chapter 2, PA treatment in Huh-7 cells caused mitochondrial dysfunction and reduced mtDNA replication, which was further declined by inhibiting fatty acid storage in lipid droplets. These changes coincided with increased fatty acid trafficking to the ER, altered ER morphology, and mild ER stress. These results were mitigated by OA co-treatment. We also show that ER-mitochondrial contact sites involved in lipid droplet biogenesis and mtDNA synthesis are spatially distinct and remodeling these sites does not drive decreased mtDNA replication. Collectively, we conclude that mtDNA replication decline during saturated lipid stress is consequence of organelle dysfunction and remodeling due to fatty acid re-routing and is prevented by increased lipid storage in droplets. This work provides novel insight on the

relationship between lipid stress and mtDNA replication and reinforces established effects of lipotoxicity in liver cells.

3.2.2 Connection to prior work in the field

This dissertation work, as far as we know, is the first to utilize robust fluorescence microscopy and image analysis to investigate mtDNA replication upon fatty acid treatment. Prior work in the field has focused on lipid utilization, lipid transfer at contact sites, and lipotoxicity induced mitochondrial dysfunction (Chapter 1). There are limited reports that address a direct connection between lipid biology and the mitochondrial genome. These studies report increased rate of mtDNA mutations during lipotoxic oxidative stress, which are linked to lipid based disorders, mtDNA release during lipotoxic insult, and perturbed lipid storage (Chapter 1).

This work shows a decrease in mtDNA replication upon low-level, chronic palmitic acid treatment using fluorescence labeling techniques. Previous studies suggest that patients with lipid-based metabolic diseases display reduced mtDNA copy number in their peripheral tissues [100-103]. We measured mtDNA copy number in PA treated cell lysate, but there were no significant changes (Figure 2-S2A). We concluded that while total mtDNA levels in cell lysate is not changing, palmitic acid is primarily affecting replicating mtDNA. Therefore, our work demonstrates that lipid stress causes mtDNA replication to “shut-off”, revealing a new discovery in the field. While our work did not support that contact site remodeling decreases mtDNA replication during lipid stress, we observed overall organelle remodeling. PA is known to cause ER stress and mitochondrial dysfunction. Our work showed reduced mitochondrial membrane potential and ATP synthesis (Figure 2.2F, Figure A8). Additionally, PA treatment caused induction of mild ER stress that correlated with reduced ER protein import and abnormal morphology (Figure 2.4, Figure 2.5A). These changes support prior work in the lipotoxicity field that excess saturated lipids leads to overall organelle dysfunction. Despite these observations, we are still left to understand what is mechanistically reducing mtDNA synthesis during lipid stress. Perhaps, it is not contact site remodeling but changes in fatty acid trafficking and utilization that impairs mtDNA synthesis.

Using the fatty acid trafficking dye Red C12, I assessed whether fatty acids are re-routed differently between mitochondria, ER, and LDs during lipotoxic stress. Previous reports have used Red C12 during nutrient starvation, showing increased fatty acid trafficking to mitochondria for β -oxidation and energy production [104]. To the best of our knowledge, Red C12 has not been used to monitor lipid trafficking during nutrient excess, indicating a new, beneficial use of this imaging tool. I hypothesized that if PA treatment increases fatty acid trafficking to mitochondria, mitochondria may perform increased beta-oxidation and scale back on mtDNA synthesis. However, if PA treatment increases fatty acid trafficking to the ER, there may be increased lipid droplet production or ceramide synthesis since both processes occur at the ER. My results showed that palmitic acid caused mild (though non-significant) Red C12 enrichment at the ER (Figure A9), while oleic acid treatment significantly increased RedC12 enrichment at the ER and mitochondria (Figure A10). To fully understand the implications of this fatty acid re-routing, lipidomics were performed (Figure A6). Lipidomics revealed increased ceramide production upon PA treatment, and increased TAG production upon OA treatment. As discussed in Section 3.1.3.2, ceramide production is a known cause of organelle dysfunction,

while TAGs are typically stored in lipid droplets [76-77, 105-107]. Based on this data, I propose a model that PA promotes fatty acid rerouting to the ER for ceramide production. This increase in ceramide production is causing mitochondrial and ER dysfunction that indirectly suppresses mtDNA synthesis. In contrast, OA promotes lipid neutralization and sequestration, which protects against organelle damage and mtDNA synthesis decline. To our knowledge, there are no reports demonstrating a direct relationship between mtDNA replication and ceramide levels. Elevated ceramide levels have been shown to promote OMM permeabilization and mtDNA release, activating inflammatory response [108-110]. If ceramides also directly impair mtDNA replication, this could show a direct connection between mitochondrial membrane damage and genome maintenance, an area of current investigation [111-112]. Future studies should test if increasing ceramide levels impairs mtDNA replication by facilitating OMM pore formation [113]. If confirmed, ceramides could serve as a biomarker for mtDNA synthesis abnormalities, supporting their emerging use as a biomarker for metabolic diseases [114-116].

3.2.3 Limitations of this work

Up to this point, the effect of lipotoxicity on mtDNA replication is an underexplored area of research. Although few studies directly contradict our findings, there were some results that did not align with previous reports. Our data did not display a significant reduction in cell viability upon PA treatment. Numerous papers strongly support that excess palmitic acid treatment causes cell death [16, 36, 117-118]. We conducted several different types of cell viability experiments (some data shown, Figure A11), and none of them demonstrated a significant or consistent reduction in viability. However, upon fluorescence imaging, we qualitatively observed smaller and less healthy appearing cells during PA treatment, particularly at the 0.3 mM PA concentration. PA treated cells form a heterogeneous population containing both healthy and non-healthy cells. This heterogeneity can increase variance and reduce statistical significance when analyzed. For example, I have observed variation in lipid droplet area within PA treated samples. In one experiment we observed a mild increase in LD area with PA (Figure 2.2C), while in another experiment there was no significant difference in LD area (Figure A9C). Additionally, experiments utilizing whole cell lysate, such as qPCR, western blotting, cell viability assays, and respirometry demonstrated mild or non-significant results compared to control samples. Fluorescence-Activated Cell Sorting (FACS) is a future method we could use to separate healthy and non-healthy cells in a PA treated population, reducing variability, and refining our analysis. Fluorescently labeled ceramides, such as NBD-C6-ceramide, could distinguish between healthy vs non-healthy cells, with non-healthy cells displaying higher levels of ceramide fluorescence. We could also use a lipid droplet stain to separate cells to determine whether lipid droplet abundance positively correlates with cell viability.

Whether the method of fatty acid delivery to cells influenced organelle biogenesis or function was an unknown faced in this project. I used two hepatoma cell lines for this work, HepG2 (preliminary work) and Huh-7 (as reported in Chapter 2). These cells were maintained in low glucose (5mM) Dulbecco's Modified Eagle Medium (DMEM). It is recommended to use fatty acid concentrations between 0.5 – 2.0 mM with BSA to mimic lipotoxic conditions in vivo [79]. I created PA treatments with the addition of fatty acid free BSA in low glucose DMEM [119]. Surprisingly, mitochondria in cells treated with the BSA control harbored mitochondria with low membrane potential, implying compromised mitochondrial health despite no decline in cell viability. To avoid this confounding variable, I treated cells with PA directly dissolved in DMSO

without BSA conjugation. Without BSA conjugation, PA at concentrations ≥ 0.3 mM was not fully soluble in cell culture media. Therefore, experiments using 0.3 mM PA were not exposed to fully solubilized PA. The lack of BSA conjugation and full PA solubility could also explain the variability observed between samples. To address this, we performed additional experiments, using a lower concentration of BSA for PA delivery, specifically measuring mitochondrial TMRE intensity and ER protein import. These results were similar to those observed with DMSO-delivered PA (Figure 2.2E-F vs. Figure A12A-B; Figure 2.4B and A12D vs. Figure A12C and E).

A further limitation of this study is the imaging and analysis of ER-mitochondrial contact sites. We used some of the latest image analysis software to rigorously measure the ratio of ER-mitochondrial contact sites in relation to LDs and the mitochondrial DNA polymerase accessory subunit protein POLG2. Comparable to Figure 2.1A-C, I performed a more in-depth analysis of these contacts using vehicle and BSA-conjugated PA treated cells (Figure A13-A14). We quantified the spatial proximity of these organelle interactions, but we could not determine if these interactions were actual tethered contacts. The ER is an expansive organelle that touches most, if not all, other organelles in the cell. Thus, having an accurate tool to define tethered contacts would refine our interaction analysis. We used the Split GFP-based Contact Site Sensor (SPLICS), which is a fluorescence imaging tool that labels ER-mitochondrial contact sites (Figure A15) [120]. Our data showed relatively similar SPLICS foci count across samples, supporting our current view that ER-mitochondrial contacts are not driving the lipotoxic outcomes that we observed. In addition, SPLICS forms permanent contacts between organelles that are irreversible. ER-mitochondrial contacts in a healthy cell state are extremely dynamic. Permanent ER-mitochondrial contacts may confound interpretations of cellular responses to lipid stress. We attempted to use a recently developed contact site imaging tool called Contact Fluorescent Protein (Contact-FP) that is reversible [121]. We were not successful in imaging ER-mitochondrial contact sites with this tool without creating very apparent morphological abnormalities (data not shown). We have considered using a proximity ligation assay, but this experiment would be limited to fixed-cell imaging, and the dynamic nature of ER-mitochondria contact sites would not be visualized. Having a contact-site imaging that is reversible and suitable for live cell imaging would be ideal to re-evaluate contacts upon fatty acid treatment. Using time-lapse imaging, I would track the rate of contact site assembly and disassembly of contact sites under control and PA conditions. While the number of contact sites may not differ significantly across conditions, their dynamics may differ. Aberrant ER-mitochondrial contact formation near sites of mtDNA replication during PA treatment may suggest impaired mitochondrial division and subsequent mtDNA synthesis.

3.2.4 Open questions and future directions

I focused this work on the impact of lipid excess on mtDNA replication, but mitochondrial maintenance is defined as the synthesis, transcription, distribution, mutation, and localization of mtDNA in the cell. This project did not address how saturated lipid stress affects mtDNA transcription or protein translation (mitochondrial central dogma). MtDNA replication is closely interconnected with mtDNA transcription due to similar regulating factors, such as the mtDNA compacting factor transcription factor A (TFAM) and mitochondrial RNA polymerase (POLRMT) [122-123]. Since we observe a stark decrease in mtDNA replication upon PA

treatment, I would hypothesize that mtDNA transcription would decrease as well. A reduction in mtDNA transcription may lead to reduced mitochondrial translation, as well. I could use RNA fluorescence in situ hybridization (RNA-FISH) and HPG-labeling to quantify sites of active transcription and translation in cells treated with fatty acids.

In Chapter 1, I address how cytoplasmic mitochondria (CM) and peridroplet mitochondria (PDM) are two functionally different mitochondrial subpopulations. We measured the ratio of peridroplet and cytoplasmic mitochondria containing POLG2, with no observable differences (Figure A13-A14). As described in “Limitations of this work”, a more robust analysis of these mitochondrial subpopulations should be performed with better contact site labeling tools. A feasible, preliminary experiment I could perform would be designing a proximity ligation assay to quantify the proximity between PDM and EdU labeled mtDNA. This would allow me to assess if mitochondrial-lipid droplet contacts and mtDNA replication are spatially linked.

In addition to organelle heterogeneity, I would like to investigate how cellular heterogeneity responds to lipid stress. Do large-scale cell population changes influence the activation of cellular stress pathways? For example, within a single dish of PA-treated cells, do distinct clusters of cells respond differently to treatment, possibly through local cell-to-cell signaling? What determines whether a cell will be healthy or non-healthy after treatment? An experiment that may partially address these questions is to measure if lipid droplet abundance correlates with increased mtDNA synthesis. As proposed in the “Perspectives” section of this chapter, lipid storage into droplets may prevent the lipotoxic effects of excess cytosolic fatty acids. Given that we observed further reduced mtDNA replication after lipid storage inhibition (Figure 2.3), I hypothesize that cells with more lipid droplets have increased mtDNA synthesis compared to cells with fewer droplets. Co-labeling of lipid droplets and mtDNA replication sites can be quantified and their correlation analyzed to test this. If a positive correlation is observed, this would indicate that lipid sequestration supports mitochondrial homeostasis, and that mtDNA replication is coupled to cellular lipid availability.

This work could be further elevated by evaluating how PA treatment affects mtDNA synthesis in other cell types, such as pancreatic islet cells, as well as primary hepatocytes from healthy and obese patients with metabolic disease. Several questions I would like to ask include: Is the mitochondrial response to palmitic acid treatment universal across peripheral tissue cell types? How might mtDNA synthesis differ between cells with large lipid pools (e.g., adipocytes) vs. cells with minimal lipid storage (e.g., fibroblasts)? Performing work across various cell and tissue types would increase the scope of this research by making it more biomedically relevant. This would broaden the audience to include both basic scientific and clinical researchers.

3.3 Conclusion

Lipotoxicity has detrimental effects at the physiological and cellular levels, leading to the onset of metabolic diseases. The systems that define lipotoxicity at the cellular and molecular level still remain to be fully characterized. It is well-established that toxic levels of saturated fatty acids, such as palmitic acid, can lead to mitochondrial dysfunction, ER stress, and the onset of inflammatory pathways. However, whether saturated lipid excess impacts the mitochondrial genome in mammalian cells is a relatively unexplored area. Because mitochondria facilitates

lipid metabolism and mtDNA encodes electron transport chain components, defining how saturated lipid stress affects mtDNA maintenance may clarify the link between mitochondrial function and nutritional stress.

This work provides a framework for understanding how lipid metabolism and mtDNA maintenance are connected. Chapter 1 gives a detailed review of lipid metabolism in mitochondria, mitochondrial interactions with lipid metabolizing organelles, and current knowledge that links mtDNA maintenance with lipid stress. By reviewing this prior work, we were motivated to study how lipotoxicity affects mtDNA replication. In Chapter 2, we used fluorescence microscopy and molecular techniques to examine how palmitic acid treatment alters mtDNA replication in Huh7 cells. We found that fatty acid re-routing and organelle dysfunction during lipid stress suppressed mtDNA synthesis. Oleic acid co-treatment restored organelle function and mtDNA synthesis. I proposed that this is due to the neutralization and storage of excess fatty acids into lipid droplets that is typical of oleic acid treatment, preventing the synthesis of toxic lipid species (i.e., ceramides).

Knowledge gaps remain in understanding how PA suppresses mtDNA replication in mammalian cells. Further research is needed to find therapeutic strategies that combat lipotoxic mitochondrial damage. For example, increasing lipid storage by DGAT1/2 overexpression or inhibiting ceramide production may restore mtDNA synthesis. Chapter 3 discusses research directions to explore the mechanism underlying mtDNA reduction during lipid stress, including studies of mitochondrial morphology, dynamics, and mtDNA release. This chapter also highlights potential strategies to recover lipotoxic damage, and reflects on the relevance, limitations, and future directions of this work.

Altogether, this dissertation defines a link between lipotoxicity and mtDNA maintenance. Because mitochondria regulate both lipid metabolism and their own genome, a connection between these two processes is likely. Using a lipid stress model, our work demonstrated that lipid dysregulation and mtDNA replication are interconnected through homeostatic changes in mitochondria and the ER. Lipid sequestration and storage mitigates lipotoxic effects and supports cellular function. Further work is needed to define a more streamlined mechanism bridging lipid regulation to mtDNA synthesis. This dissertation provides a foundation for studying mitochondrial function and mtDNA maintenance during nutritional stress.

3.4 References

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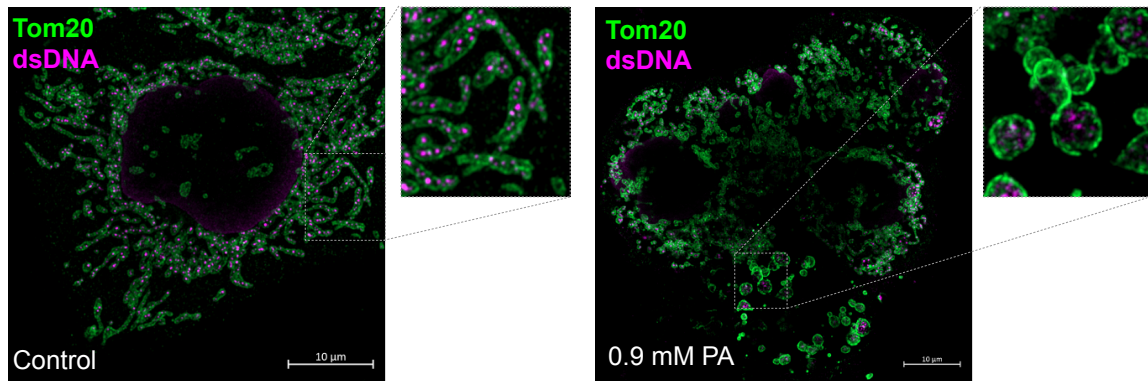
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Appendix

A



B

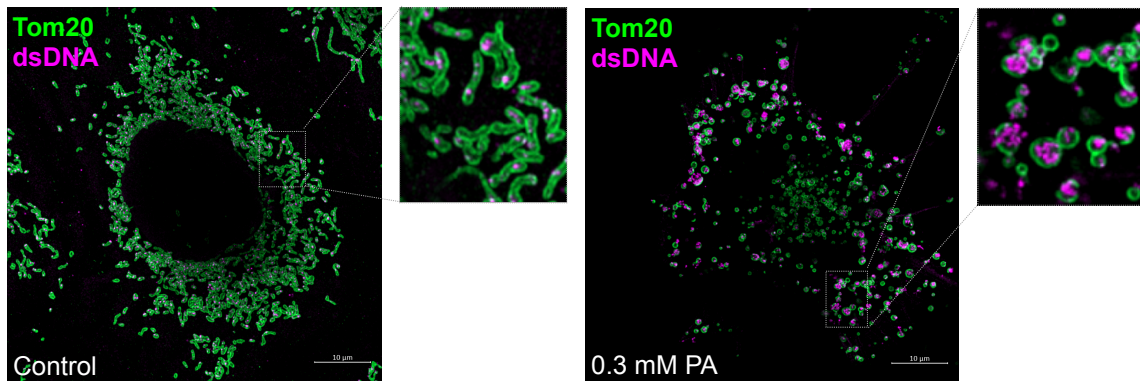


Figure A1. Saturated lipid stress alters mitochondrial morphology. (A) HepG2 cells treated with BSA vehicle control or 0.9 mM PA for 24 h. (B) Huh-7 cells treated with DMSO control or 0.3 mM PA for 24 h. Cells were immunolabeled with anti-Tom20 (green) and anti-dsDNA (magenta). Scale bar, 10 μm . Zoomed regions display mitochondrial morphological changes, with mitochondria appearing rounder and more fragmented in the PA-treated cells.

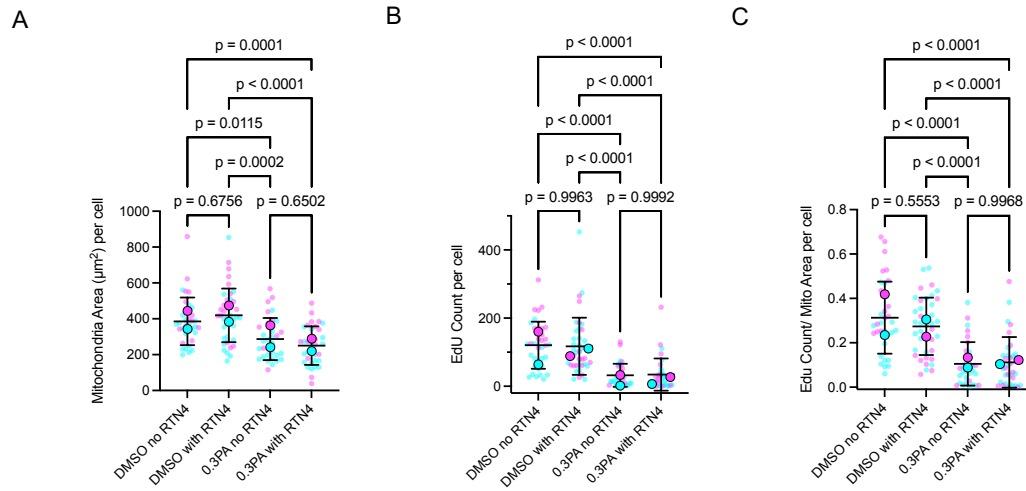


Figure A2. ER tubule structural protein Rtn4A overexpression does not alter mtDNA replication in PA treated cells. Huh-7 cells were treated with DMSO vehicle or 0.3 mM PA, with or without Rtn4-GFP overexpression, pulse-labeled with EdU-AlexaFluor647, and immunolabeled with anti-Tom20. (A) Quantification of mitochondrial area (μm^2) (B) Quantification of mitochondrial EdU-647 foci per cell, normalized to segmented mitochondrial area. This data represents two biological replicates.

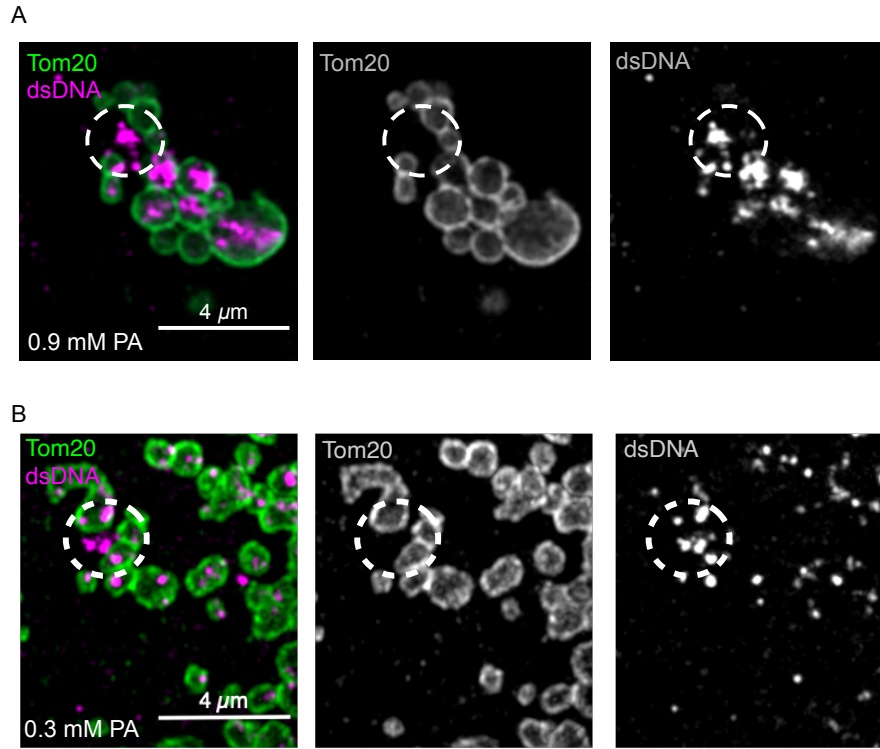


Figure A3. Examples of non-colocalized mtDNA with Tom20 in PA-treated cells. (A) HepG2 cells treated with 0.9 mM PA for 24 h. (B) Huh-7 cells treated with 0.3 mM PA for 24 h. Cells were immunolabeled with anti-Tom20 (green) and anti-dsDNA (magenta). Scale bar, 4 μ m. White dotted circles highlights DNA signal excluded from the Tom20 signal.

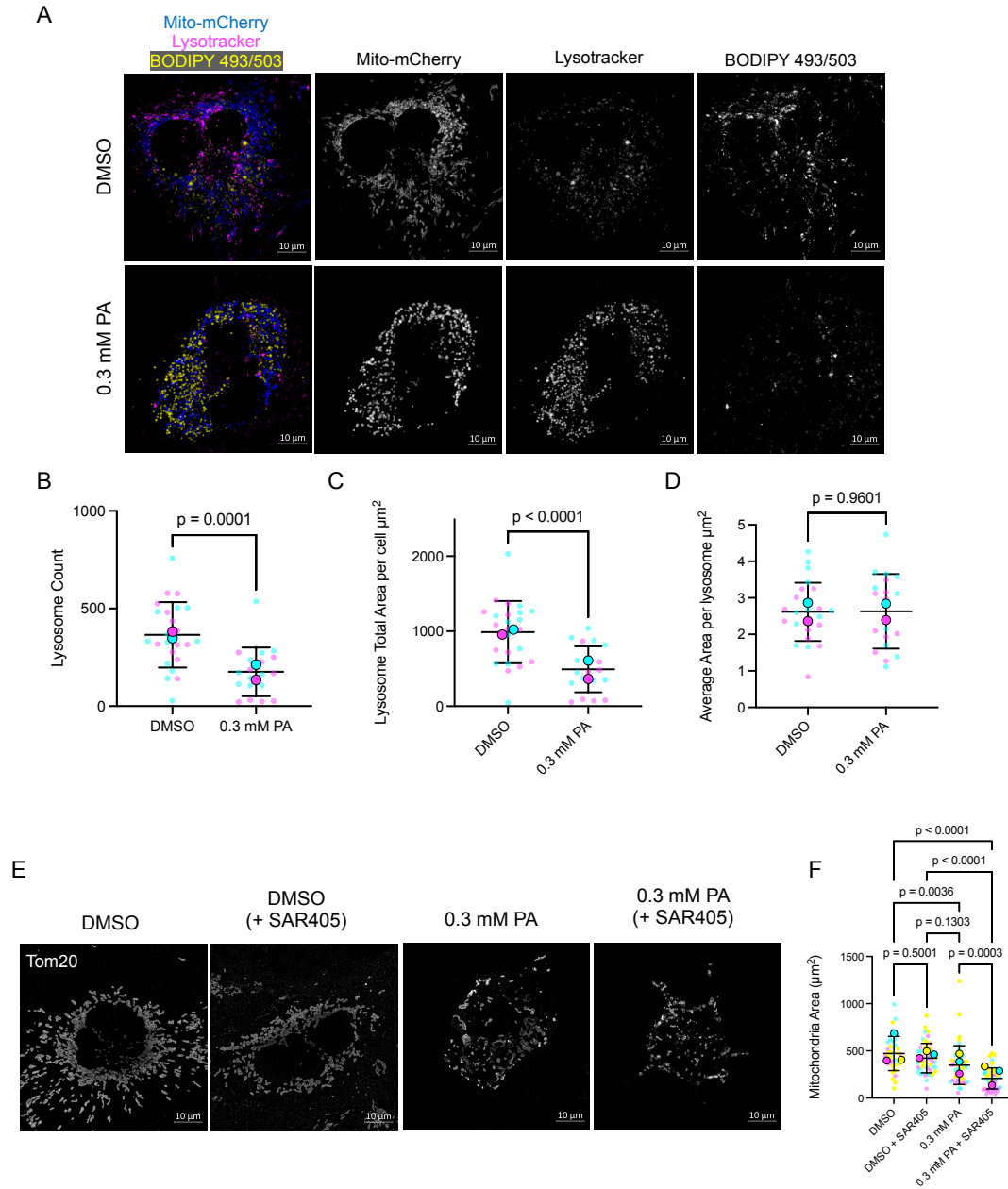


Figure A4. Palmitic acid-induced mitochondrial area decrease is not due to autophagy. (A) Images are live Huh-7 cells that were treated for 24 hours with DMSO (vehicle) or 0.3 mM PA. Cells are expressing mito-mCherry (blue) and were stained with Lysotracker (magenta), and BODIPY 493/503 (yellow). (B) Quantification of lysosome count per cell. (C) Quantification of lysosome total sum area per cell (μm^2). (D) Quantification of the average lysosome area per segmented lysosome. Scale bars: 10 μm . 2 biological replicates. (E) Images are fixed Huh-7 cells that were treated for 24 hours as follows: DMSO (vehicle), DMSO with SAR405 (1 μM), 0.3 mM PA, and 0.3 mM PA with SAR405. Cells were immunolabeled with an antibody against Tom20 (gray). Scale bars, 10 μm . (F) Quantification of mitochondrial area (μm^2) per cell. 3 biological replicates. Significance determined via two-tailed unpaired t-test (B-D) and one-way ANOVA, Dunnett's multiple comparisons test (F).

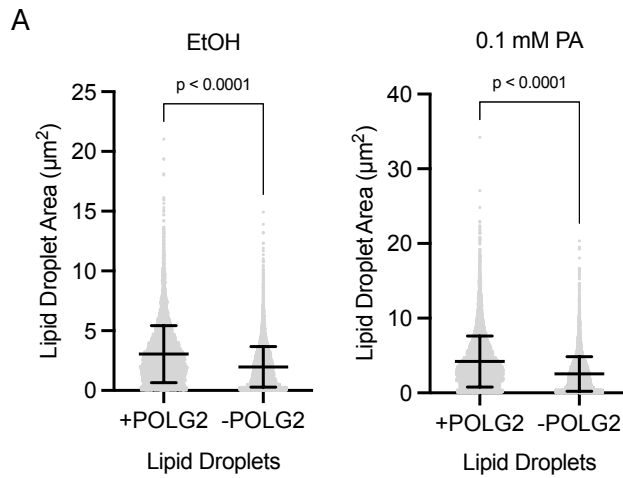


Figure A5. Larger lipid droplets are located near sites of mtDNA replication. Huh-7 cells were treated with vehicle control (EtOH) or 0.1 mM PA conjugated to BSA. This data set is the same as that shown in Figure A13-A14. Huh-7 cells were transiently transfected with markers KDEL-mRuby, mito-BFP, POLG2-GFP, and stained with the far red neutral lipid staining dye BODIPY 665/676. (A) Quantification of lipid droplet (μm^2) area near or distant from sites of POLG2-GFP foci. This data represents three biological replicates.

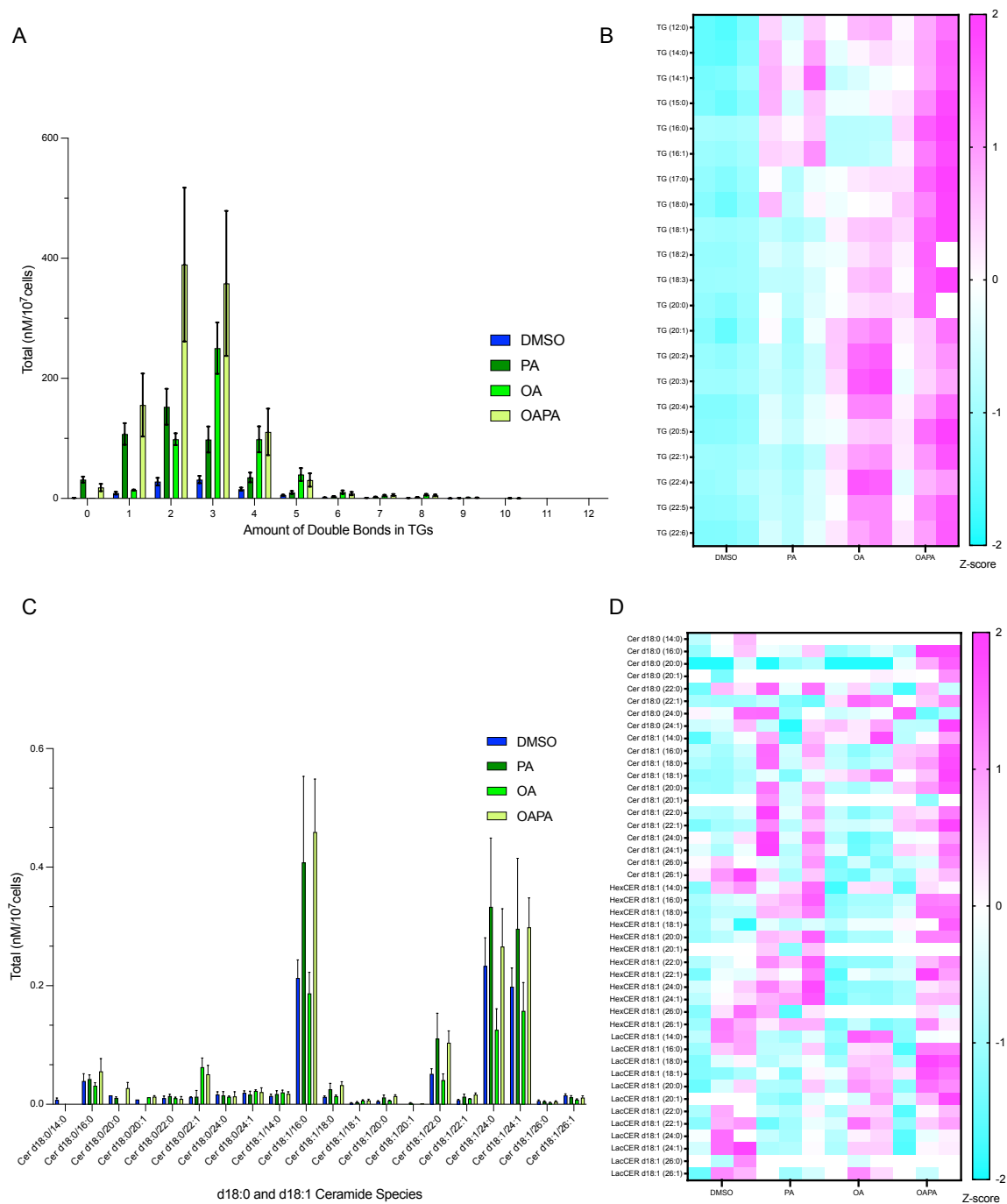


Figure A6. Lipidomic analysis of triglyceride (TG) and ceramide levels during lipid excess. Huh-7 cells were treated with vehicle control (DMSO) or 0.3 mM palmitic acid, oleic acid, or both (OAPA). (A) Total TG levels (nM/10⁷ cells) organized by the amount of double bonds in the TG species. (B) Heatmap of TG abundance (z-score) across conditions. (C) Total d18:0 and d18:1 ceramide species (nM/10⁷ cells). (D) Heatmap of ceramide abundance (z-score) across conditions. Heatmap rows represent individual species and columns represent treatment conditions across three biological replicates.

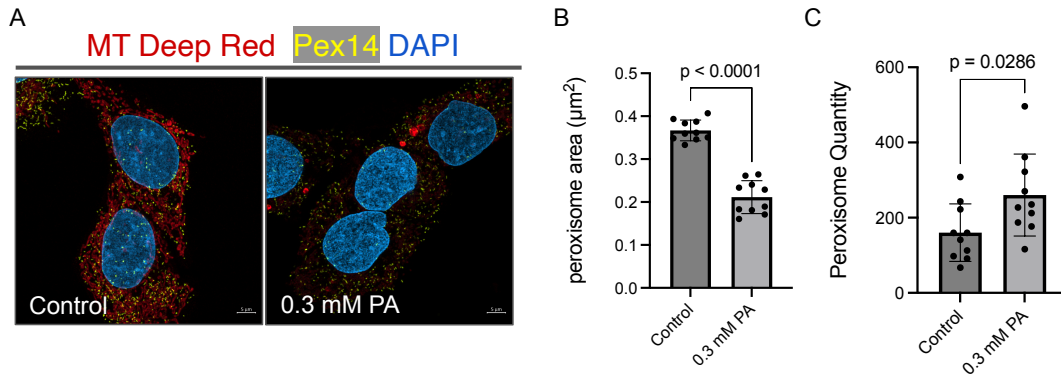


Figure A7. Saturated lipid stress increases the presence of smaller peroxisomes. (A) HepG2 cells were treated with BSA vehicle control or 0.3 mM PA for 24 h. Cells were stained with MitoTracker (MT) Deep Red (red) and DAPI (blue) and were immunolabeled with anti-Pex14, a peroxisome resident protein (yellow). Scale bar, 5 μm . (B) Quantification of peroxisome area per image (μm^2). (C) Quantification of peroxisome quantity per image. This data represents one biological replicate. Statistical test is two-tailed, unpaired t-test.

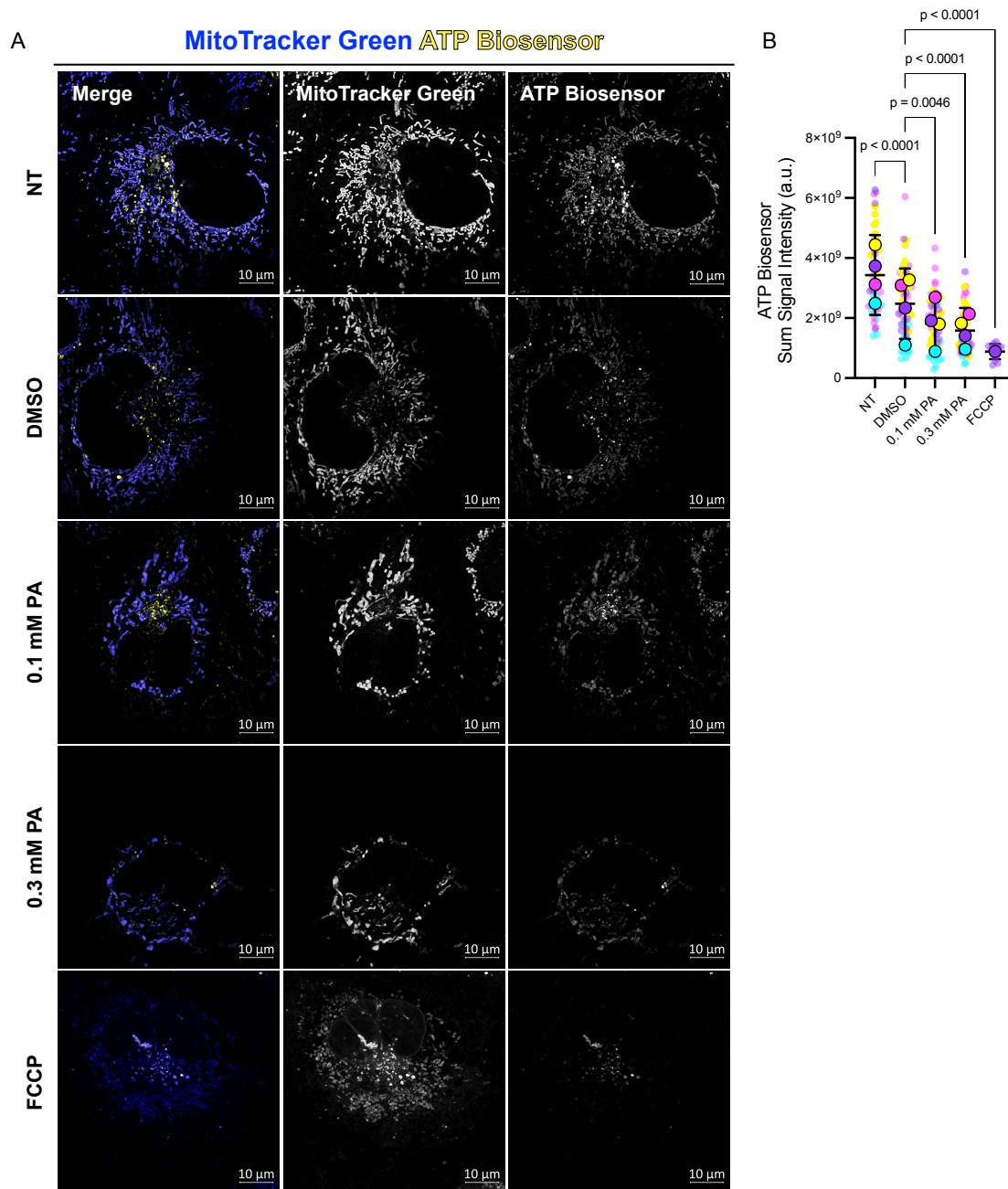


Figure A8. Palmitic acid treatment reduces mitochondrial ATP production. (A) Images are live Huh-7 cells in untreated, DMSO vehicle, FCCP positive control, or palmitate-treated samples. Cells were stained with MitoTracker Green (blue) and ATP Biosensor (yellow). Scale bar, 10 μ m. (B) Quantification of ATP biosensor sum signal intensity within the segmented mitochondrial network. 4 biological replicates (only one with FCCP). Significance determined via one-way ANOVA, Dunnett's multiple comparisons test.

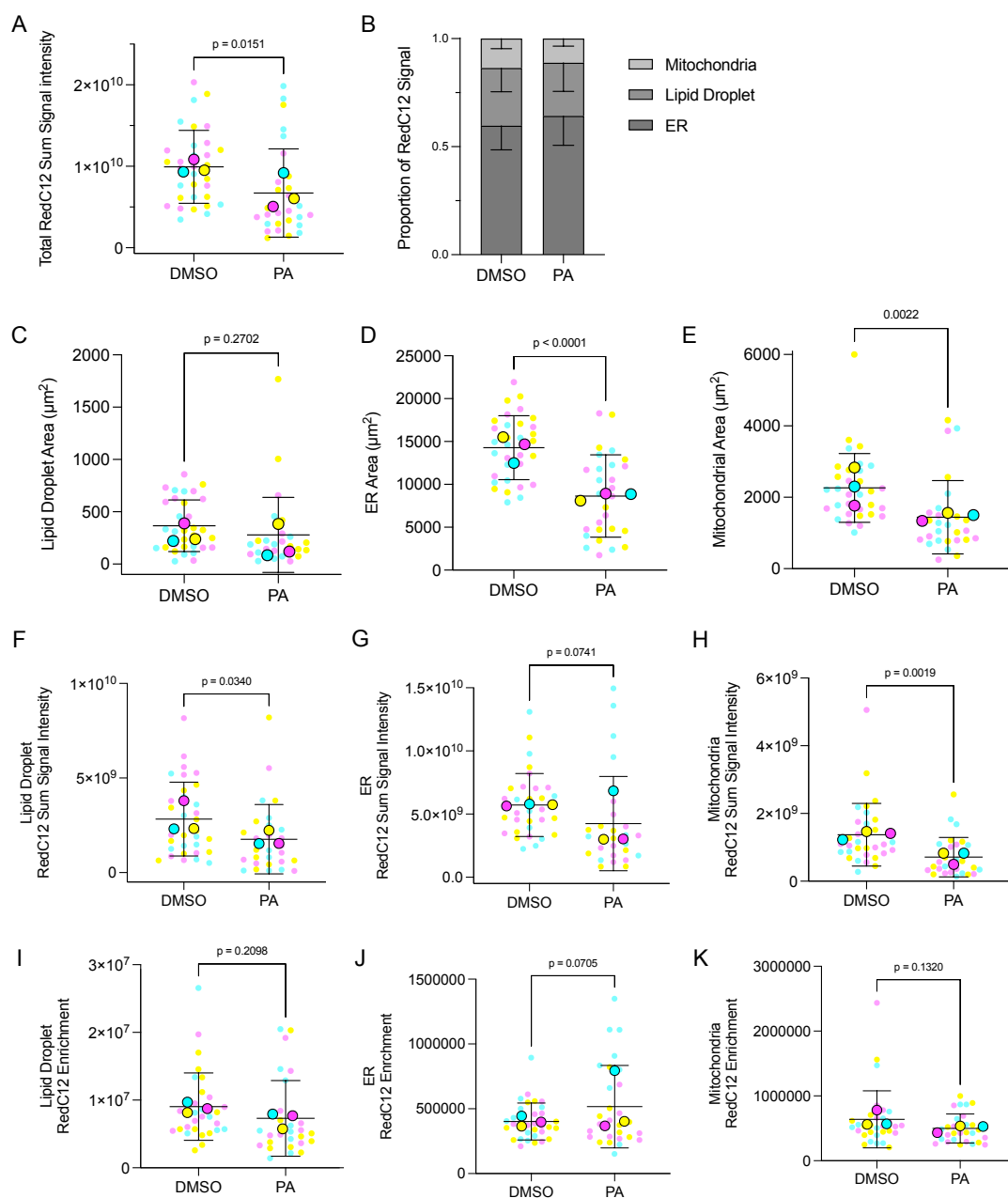


Figure A9. Palmitic acid causes mild fatty acid trafficking to the ER. This data set is the same as that shown in Figure 2.4D. Huh-7 cells treated with DMSO vehicle or 0.3 mM PA were transiently transfected with Mito-BFP and GFP-Sec61b, labeled with Bodipy Far Red and Red C12. (A) Total Red C12 fluorescence, quantified as the sum of the LD, ER, and mitochondrial RedC12 sum signal intensities per cell (F-H). (C-E) Quantification of LD, ER, and mitochondria area per cell (μm^2). (I-J) Quantification LD, ER, and mitochondria Red C12 enrichment, quantified as the RedC12 sum signal intensity divided by organelle area. 3 biological replicates. Significance determined via two-tailed, unpaired t-test.

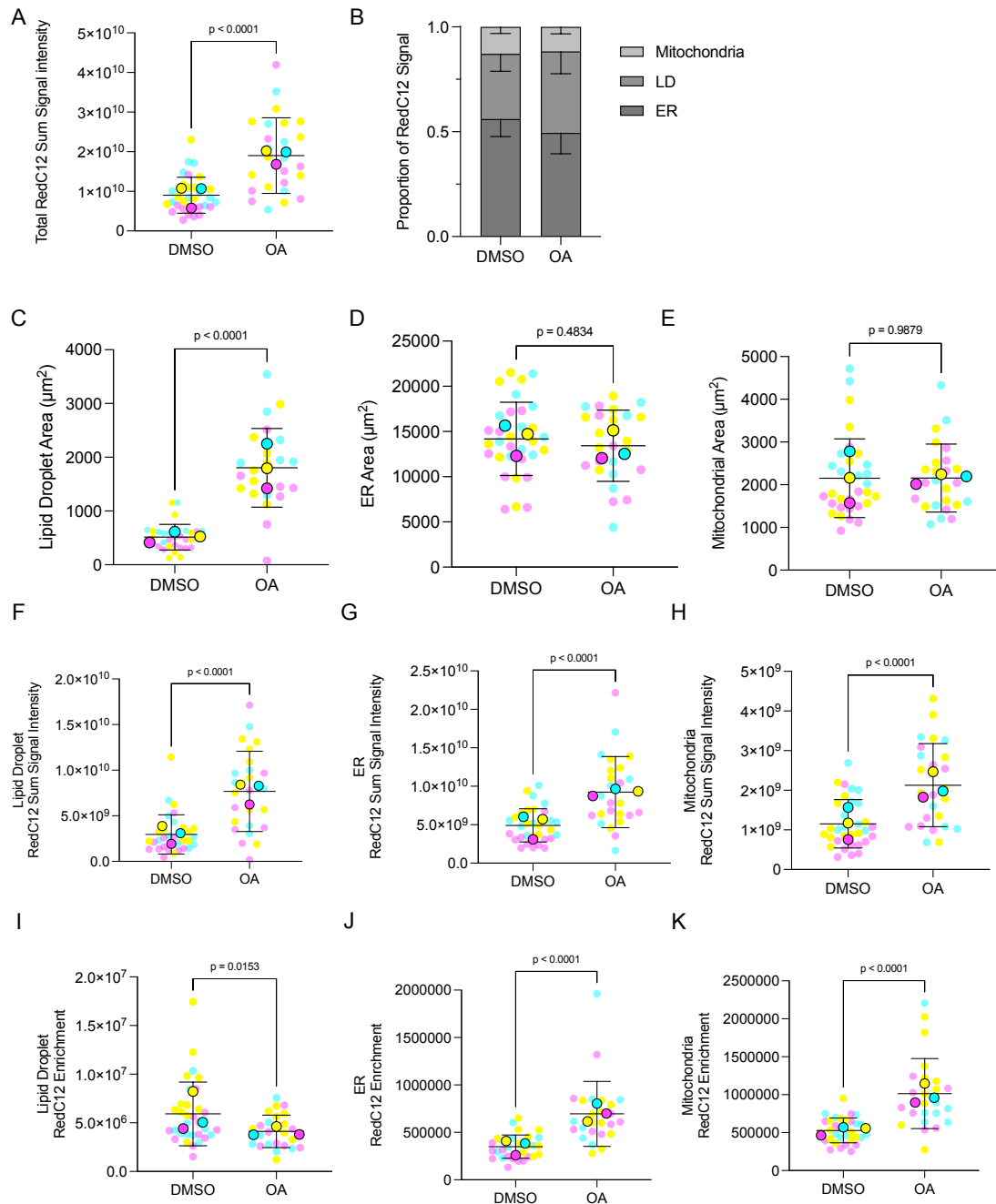


Figure A10. Oleic acid causes enhanced fatty acid trafficking to the ER and mitochondria. Huh-7 cells treated with DMSO vehicle or 0.3 mM OA were transiently transfected with Mito-BFP and GFP-Sec61b, labeled with Bodipy Far Red and Red C12. (A) Total Red C12 fluorescence, quantified as the sum of the LD, ER, and mitochondrial RedC12 sum signal intensities per cell (F-H). (C-E) Quantification of LD, ER, and mitochondria area per cell (μm^2). (I-J) Quantification LD, ER, and mitochondria Red C12 enrichment, quantified as the RedC12 sum signal intensity divided by organelle area. 3 biological replicates. Significance determined via two-tailed, unpaired t-test.

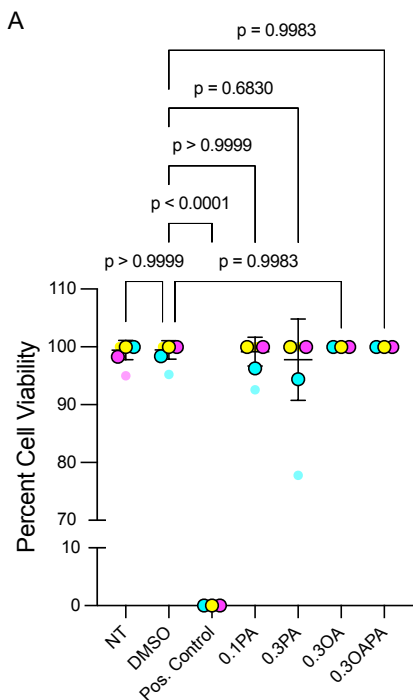


Figure A11. Low level, chronic palmitic acid treatment does not reduce cell viability. Untreated Huh-7 cells versus DMSO vehicle, PA, OA, and combined PA+OA treated cells were stained with dead-cell indicator SYTOX Green and nuclear stain Hoescht. The presence of SYTOX green signal signifies cell death. Live cell images were taken as 5x5 tiles. (A) Quantification of the percent cell viability, calculated as the number of sytox labeled nuclei divided by the number of hoescht labeled nuclei per tiled image. 3 biological replicates. Significance determined via one-way ANOVA, Dunnett's multiple comparisons test.

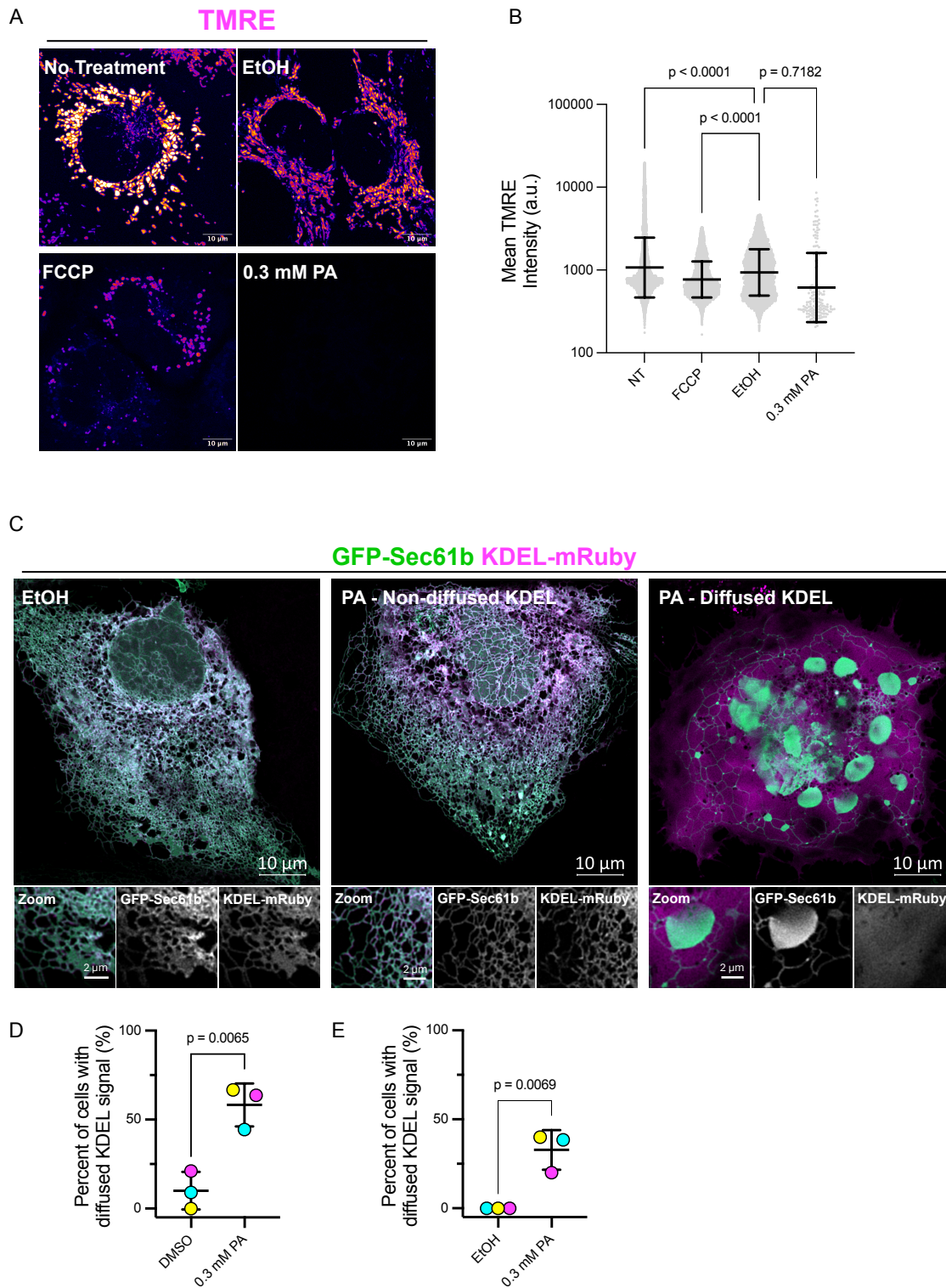


Figure A12. Key experiments comparing of BSA-conjugated PA to DMSO delivered PA treatment. (A) Untreated Huh-7 cells versus DMSO vehicle, FCCP positive control, or palmitate and stained with TMRE (fire LUT) and BODIPY 493/503 (not shown). Scale bar, 10 μ m. (B)

Mean TMRE fluorescence intensity in segmented mitochondria. (C) Huh-7 cells transiently transfected with KDEL-mRuby and GFP-Sec61b, treated with DMSO or palmitate, with (far right panel) or without (middle panel) diffused KDEL signal. Scale bars, 10 μm ; 2 μm zooms. (D) Quantification of the percent of cells with diffused KDEL signal in DMSO vehicle versus 0.3 mM PA treated cells. This quantification is performed on the data represented in Figure 2.4B. (E) Quantification of the percent of cells with diffused KDEL signal in EtOH vehicle versus BSA-conjugated 0.3 mM PA treated cells. Statistical significance determined by one-way ANOVA, Dunnett's multiple comparisons test (B) and two-tail, unpaired t-test (D-E).

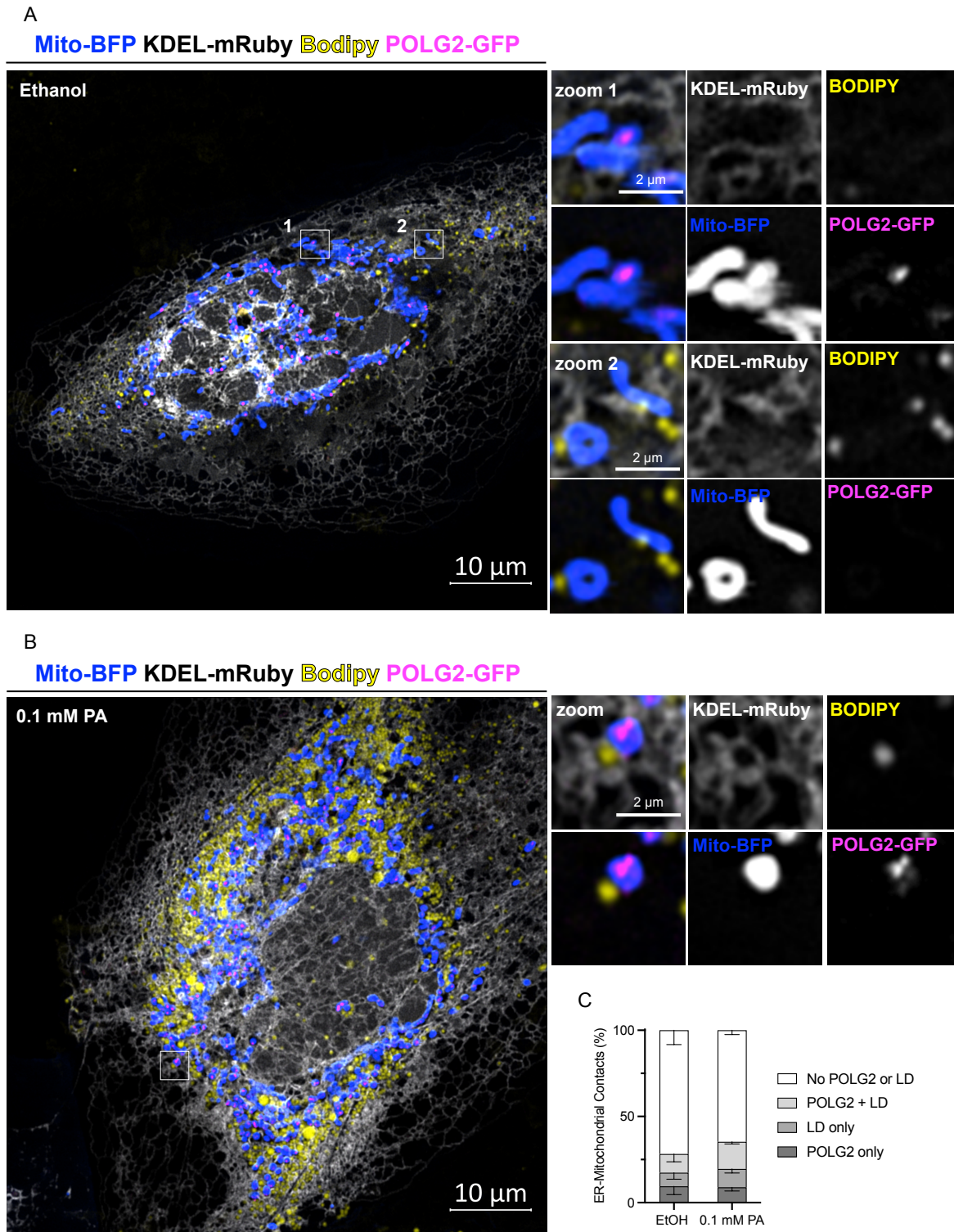


Figure A13. Mitochondrial genome synthesis and lipid droplet maintenance do not preferentially co-occur at the same ER-mitochondrial contact sites. (A-B) Ethanol vehicle treated Huh-7 cells were transiently transfected with markers KDEL-mRuby (gray), mito-BFP (blue), POLG2-GFP

(magenta), and stained with the far red neutral lipid staining dye BODIPY 665/676 (yellow). Scale bar 10 μm . Zoom 1, ER-mitochondria colocalization at replicating mtDNA nucleoid. Zoom 2, Tripartite contact between ER-mitochondria-lipid droplet. Scale bar 2 μm . (B) 0.1 mM BSA-conjugated cell labeled with the same fluorescent markers. Zoom, ER-mitochondria-LD tripartite interaction proximal to replicating mtDNA nucleoid. (C) The percent of ER-mitochondrial contacts with no POLG2 or LD, LD only, POLG2 only, or both. 3 biological replicates.

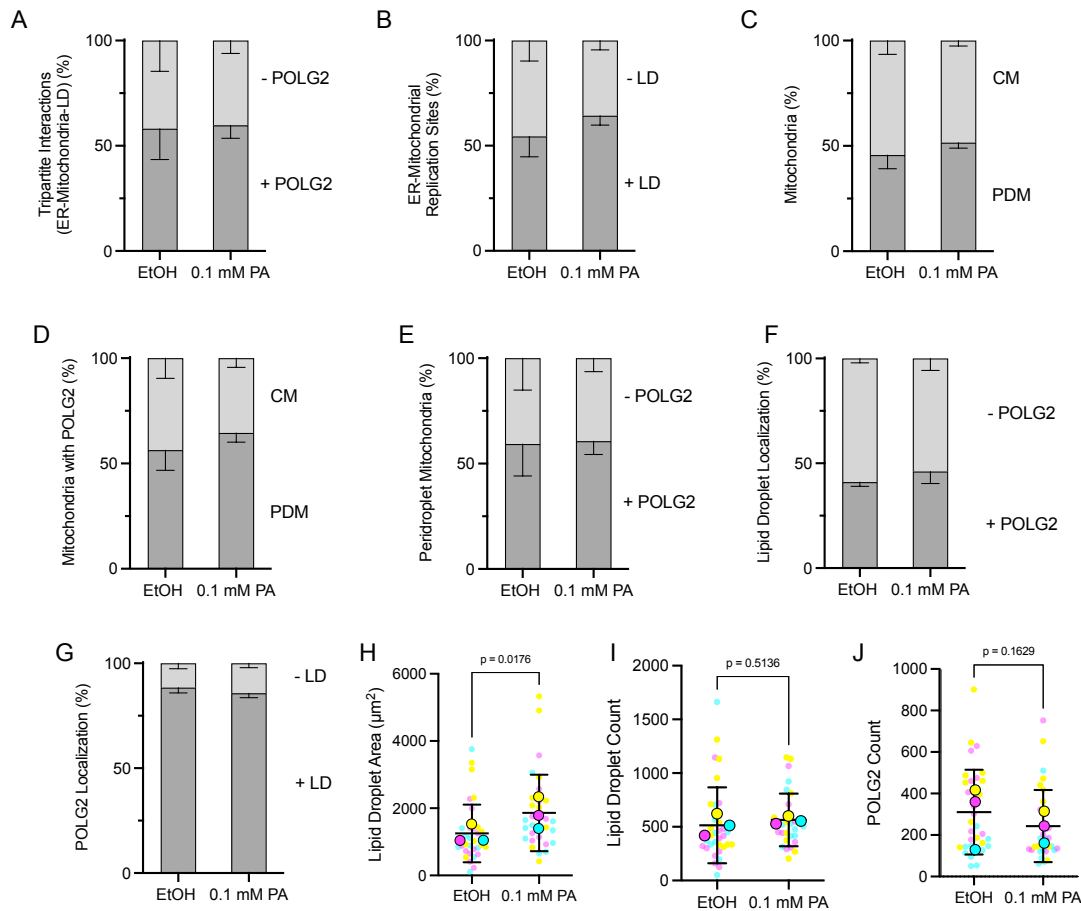
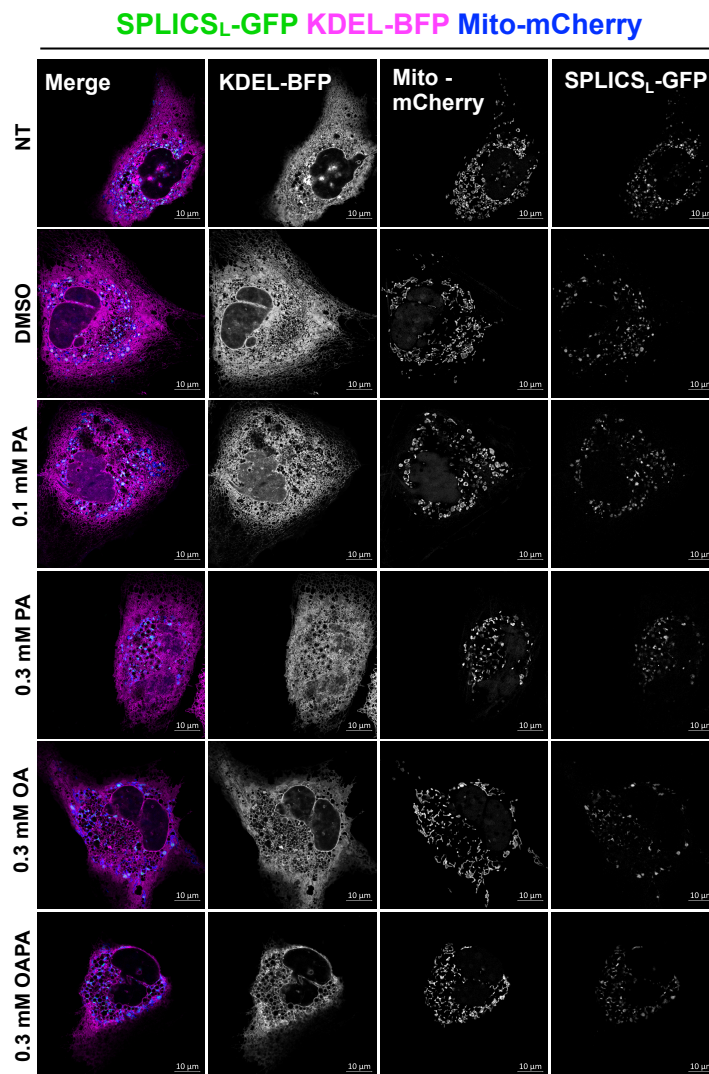


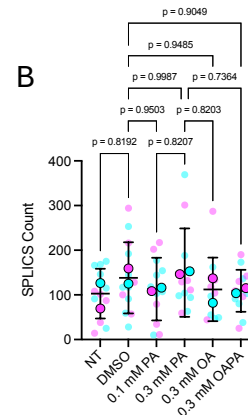
Figure A14. In-depth analysis of ER, mitochondrial, LD, and POLG2 interactions.

Quantification was performed on the data represented in Figure A13. (A) Percentage of ER-mitochondrial-LD interactions with or without POLG2 signal. (B) Percentage of ER-mitochondrial replication sites proximal to LDs. (C) Percentage of cytoplasmic mitochondria (CM) versus peridroplet mitochondria (PDM). (D) Percentage of CM vs. PDM with POLG2. (E) Percentage of PDM with or without POLG2. (F) Percentage of lipid droplets near or distant from POLG2 signal. (G) Percentage of POLG2 near or distant from lipid droplets. (H) Lipid droplet area (μm^2) in vehicle- versus palmitate-treated samples. (I) Lipid droplet count in vehicle- versus palmitate-treated samples. (J) POLG2 count in vehicle- versus palmitate-treated samples. Significance determined via two-tail, un-paired t-test (H-J).

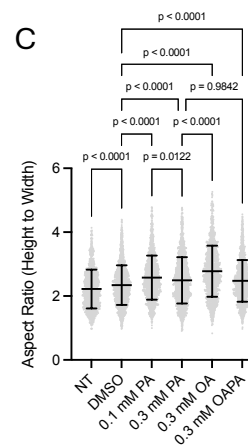
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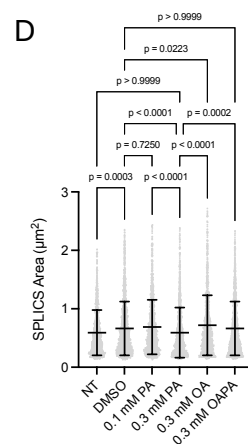


Figure A15. Lipotoxicity does not alter contact site formation. (A) Untreated Huh-7 versus DMSO (vehicle), palmitic acid, oleic acid, and combined OA+PA were transiently transfected with KDEL-BFP (magenta), mito-mCherry (blue), and SPLICS_L-GFP (green), a split-green fluorescent protein that measures long (L) contact sites of 40-50 nm. Merged images are on the far left and individual channels are shown in grayscale. Scale bars, 10 μm. (C) Quantification of SPLICS count per cell. (C) Quantification of aspect ratio (height to width) of SPLICS_L foci. (D) Quantification of SPLICS area (μm²). 2 biological replicates. Significance determined via one-way ANOVA, Dunnett's multiple comparisons test.