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Review

Mitochondrial fatty acid synthesis is an emergent central regulator of mammalian oxidative metabolism

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SUMMARY

Contrary to their well-known functions in nutrient breakdown, mitochondria are also important biosynthetic hubs and express an evolutionarily conserved mitochondrial fatty acid synthesis (mtFAS) pathway. mtFAS builds lipoic acid and longer saturated fatty acids, but its exact products, their ultimate destination in cells, and the cellular significance of the pathway are all active research questions. Moreover, why mitochondria need mtFAS despite their well-defined ability to import fatty acids is still unclear. The identification of patients with inborn errors of metabolism in mtFAS genes has sparked fresh research interest in the pathway. New mammalian models have provided insights into how mtFAS coordinates many aspects of oxidative mitochondrial metabolism and raise questions about its role in diseases such as obesity, diabetes, and heart failure. In this review, we discuss the products of mtFAS, their function, and the consequences of mtFAS impairment across models and in metabolic disease.

INTRODUCTION

Mitochondria are often thought of as predominantly catabolic organelles, breaking down carbon sources for use as fuel through fatty acid oxidation (FAO) and the citric acid (TCA) cycle. Ultimately, these catabolic pathways produce reduced co-factors (NADH and $FADH_2$) that feed electrons to the mitochondrial electron transport chain (ETC), where they drive cellular respiration and ATP production. Alongside these famous energy-producing metabolic processes, mitochondria also harbor a plethora of anabolic biochemical pathways (or steps within larger pathways), including those that build non-essential amino acids, sphingolipids, cholesterol, and steroid hormones. However, one of the most overlooked biosynthetic pathways in mammalian mitochondria is the mitochondrial fatty acid synthesis (mtFAS) pathway.

The mtFAS pathway was first described in the yeasts *Saccharomyces cerevisiae* and *Neurospora crassa*,[1,2] where it was discovered to be required for production of the eight-carbon lipid lipoic acid (LA),[3–5] a required co-factor for several mitochondrial metabolic enzymes involved in the TCA cycle, amino acid catabolism, and purine biosynthesis: pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (OGDH), branched-chain ketoacid dehydrogenase (BCKDH), 2-oxoadipate dehydrogenase (OADH), and the H protein of the glycine cleavage system (GCSH). mtFAS takes place in the mitochondrial matrix and builds fatty acyl chains on a soluble scaffold protein called the mitochondrial acyl carrier protein (ACP) and is highly conserved throughout the eukaryotic kingdom. Unlike the cytosolic fatty acid synthase (FASN), mtFAS is a type II FAS pathway, meaning that the pathway is orthologous to type II bacterial FAS, in which the stepwise addition of 2 carbon units to a growing acyl chain is catalyzed by 6–7 discrete gene products, depending on species (Figure 1).[6] Despite this difference, mtFAS and FASN build *de novo* fatty acids using the same chemistry (Figure 2), with the major difference being that in cytosolic FAS, each enzyme is a domain on the single FASN polypeptide, rather than a discrete enzyme. These enzymes are encoded by nuclear genes and can accommodate chain lengths out to 14–16 carbons *in vitro*.[7]

The first step in mtFAS is the activation of an apo-ACP to holo-ACP via the addition of a 4′-phosphopantetheine (4′-PP) co-factor to a conserved serine residue on ACP by a phosphopantetheinyl transferase (PPT; Figure 1). In yeast, this reaction is catalyzed by Ppt2p, but the mammalian mitochondrial PPT is poorly defined. Studies have suggested that the cytoplasmic PPT aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase (AASDHPPT) can pantheinylate the mitochondrial ACP *in vitro*; however, its requirement for mtFAS activity has not been demonstrated, nor has its mitochondrial localization been described. The 4′-PP co-factor on holo-ACP is absolutely required for subsequent steps, as acyl chains are built on the terminal sulfhydryl group of the 4′-PP.

Once holo-ACP is generated, malonyl-coenzyme A (CoA) ACP transacylase (MCAT) attaches a malonyl side chain. Then, the ketoacyl synthase OXSM (3-oxoacyl-ACP synthase, mitochondrial) condenses the newly generated malonyl-ACP with an even-numbered acyl-ACP, extending the growing acyl chain by two carbons and releasing a molecule of carbon dioxide (Figure 1). In the first round of fatty acyl chain elongation, how the smallest even-numbered acyl-ACP, acetyl-ACP, is generated, particularly in mammals, is not understood. The condensation reaction catalyzed by OXSM creates a keto-acyl

intermediate, and subsequent enzymes in the mtFAS cycle carry out a series of reduction and dehydration reactions to return the newly elongated acyl chain to a fully saturated state, as in cytosolic FAS (Figures 1 and 2). Two of these subsequent enzymes, CBR4/HSD17B8 and MECR, use NADPH to provide the reducing equivalents for catalysis; thus, each cycle of elongation requires two molecules of NADPH (Figure 2).

When an eight-carbon, fully saturated acyl chain has been built on ACP, the cycle reaches a decision point. The octanoyl-ACP can continue through further cycles of elongation, creating long-chain (C14–16) products that will be discussed extensively below, or it can exit mtFAS and proceed through the LA synthesis/protein lipylation pathway. Lipoyl transferase 2 (LIPT2) is the first enzyme in the pathway and catalyzes the transfer of octanoate from ACP to GCSH, which acts as a sort of "new" carrier protein scaffold in addition to its role in the GCSH, producing octanoyl-GCSH. Our current understanding of the pathway supports a model in which lipoic acid synthase (LIAS) then modifies the octanoyl side chain to LA on GCSH, and finally, lipoyl transferase 1 (LIPT1) transfers LA to its other targets, including PDH and OGDH (Figure 3). The intricacies of this mechanism are still an active area of study.[8]

With the discovery that mtFAS is required for LA production, the function of the mtFAS pathway was largely considered to be "solved," despite many observations that the pathway also synthesizes longer fatty acids.[1,3,7,9] Other than a series of papers in the early 2000s that mapped most of the mammalian genes,[7,10–14] the pathway was largely forgotten by the metabolism community over the next 20 years. However, interest in mtFAS has resurged with two major events: the finding that other lipid products of mtFAS have important cellular functions in ETC assembly and iron-sulfur (FeS) cluster biogenesis[15–17] and the discovery of the first humans with disease-causing variants in mtFAS pathway genes.[18] With these discoveries and the advent of CRISPR-Cas9 screening technology, which has enabled previously impossible unbiased screening approaches in mammalian cells, the mtFAS pathway has gained considerable research interest as its importance has emerged in new settings from myoblast differentiation to lipid droplet biology. Over the past few years, innovative studies in mammalian tissue culture systems and new mouse models have begun to shed light on how this pathway functions in mammalian cells, its importance in oxidative mitochondrial metabolism, and its role in both rare genetic diseases and more common metabolic conditions. There is still much to learn.

DISCOVERY OF HUMAN DISEASE-CAUSING GENETIC VARIANTS ELEVATES INTEREST

Human patients with deleterious variants in the genes that encode the LA synthesis pathway (*LIPT2*, *LIAS*, and *LIPT1*) downstream of mtFAS were first described in 2011,[19] followed by many additional reports over the next several years.[20–24] LA deficiency has also been shown in a number of patients with defects in the FeS cluster biosynthesis pathway, as FeS clusters are required for the stability and catalytic activity of the enzyme LIAS.[25] Impairments in the LA synthesis pathway often present with severe neurological symptoms, including seizures, hypotonia, feeding difficulties, and developmental delays.[19]

It was not until 2016 that Heimer et al. described the first human patients with disease-causing variants in the mtFAS pathway itself (*MECR*).[18] The discovery of patients with *MECR* mutations led to the coining of the name mitochondrial enoyl reductase protein associated neurodegeneration (MePAN) syndrome to describe their condition. This report was soon followed by others describing additional variants in *MECR* and the mtFAS gene *MCAT* (Table 1).[26–29] Similar to patients with LA synthesis pathway mutations, MePAN- and other mtFAS-deficient patients often exhibit basal ganglia impairments, hypotonia, developmental delays, and other neurological symptoms. Other patients present with optic neuropathy as the primary symptom.[28,29] Although patients with mtFAS variants present with diverse pathologies, as do those with many other forms of mitochondrial disease, they were all thought to share defective protein lipoylation as a common feature of the underlying molecular pathology. However, in collaboration with Dr. Bryn Webb, we recently described a proband with a novel recessive pathogenic variant in *MCAT* that has intact protein lipoylation. In cells from this patient, defective mitochondrial respiration is driven entirely by ETC assembly defects.[30] This was the first description of a disease-causing mtFAS mutation that has no effect on LA synthesis and raises the question of whether other mtFAS variants may have been overlooked because they lacked a LA defect.

Often, LA and/or mtFAS deficiency are fatal early in life or else cause substantial morbidity; however, these conditions currently have no standard of care.[25,33] LA must be covalently attached to the enzymes that use it for catalysis, and cells lack a scavenging mechanism for exogenous LA.[8] Thus, in our hands and others, LA supplementation is ineffective at rescuing protein lipoylation. The observation that cells cannot scavenge LA implies that although positive effects of supplementation with α-LA (ALA) have been reported in a wide variety of disease settings,[34–38] these are likely a result of some other mechanism, perhaps ALA's ability to act as an antioxidant, rather than through direct effects on mitochondrial enzyme function. The lack of available treatment options has driven renewed research interest in understanding the biological function(s) of mtFAS, as the development of therapies to help these children and their families is particularly challenging because the pathway is so understudied.

mtFAS AND METABOLIC DISEASE

Although mtFAS gene variants clearly drive rare genetic disorders, whether and how the pathway plays a role in more common metabolic diseases such as obesity, diabetes, and heart failure (HF) is not well studied. However, the potential for the dysregulation of mtFAS to play a role is intriguing, given the mitochondrial dysfunction that is prevalent in these disorders. A few reports have suggested a potential role for the mtFAS pathway in diabetes and adipose biology. HTD2 expression and protein lipoylation were found to be decreased in adipocytes from diabetic mice (Figure 4) and knockdown of the enzyme in 3T3-L1 adipocytes impaired mitochondrial function and insulin sensitivity.[39] Another study observed a reduction of OXSM expression in the kidneys of diabetic mice.[40] Cardiac depletion studies in mice have shown that the loss of ACP promotes HF (Figure 4), but whether ACP or mtFAS loss is observed in other models of HF is unclear.[41] More extensive studies are needed to identify

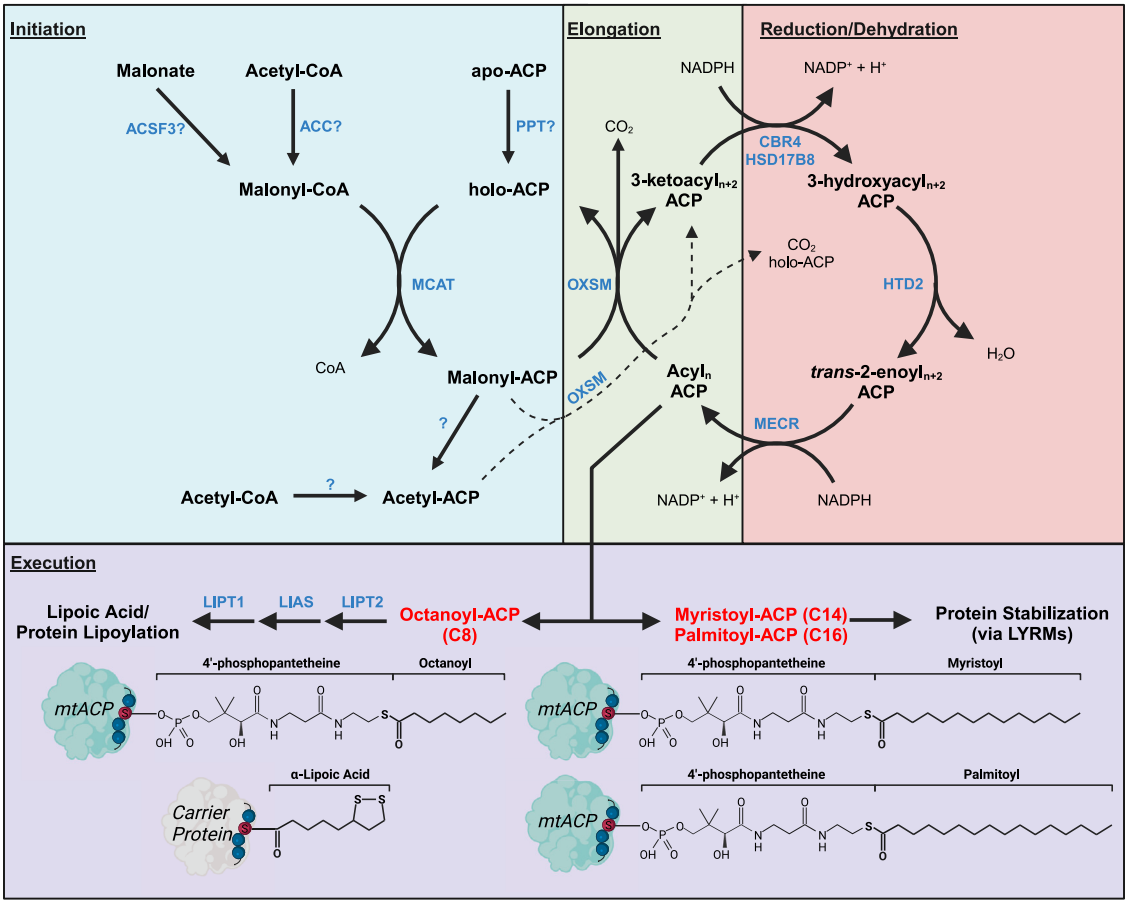

Figure 1. The mitochondrial fatty acid synthesis (mtFAS) pathway

The synthesis of fatty acids within mitochondria relies upon the sequential addition of two-carbon units to a growing acyl chain that is covalently attached to the acyl carrier protein (ACP). Mitochondrial malonyl-CoA, the committed substrate for mtFAS, enters the pathway via the enzyme MCAT, which transfers a malonyl group from coenzyme A (CoA) to ACP. This malonyl-ACP is condensed with an even-numbered acyl chain already in the cycle by OXSM, releasing CO_2, and extending the growing acyl chain by 2 carbons. Where this even-numbered chain comes from in the first cycle (acetyl-ACP, dashed line) is not well defined. The subsequent enzymes in the cycle (CBR4/HSD17B8, HTD2, MECR) catalyze a series of reduction and dehydration reactions that return the nascent acyl chain to a fully saturated state. Once the growing acyl chains reach 8, 14, or 16 carbons, they leave the pathway to execute their functions in cellular physiology. Blue text indicates enzymes and red text indicates mtFAS products.

whether altered activity of the mtFAS pathway might be pathogenic in mitochondrial dysfunction in these disease settings, but these studies are challenging in part due to low throughput, as the best indicator of mtFAS activity remains immunoblotting for protein lipoylation.

mtFAS IS AN ESSENTIAL PATHWAY IN MAMMALIAN CELLS

Guided by genetic and biochemical studies in yeast, early work in mammalian systems was largely focused on LA synthesis and ACP and showed that these pathways are coupled as they are in single-celled eukaryotes.[42] Intriguingly, the gene that encodes ACP is named *NDUFAB1* because it is also a stable subunit of ETC complex I,[43] but it was unclear until recently how this was functionally related to its role in mtFAS. Thus, much of the early work examining the consequences of mtFAS impairment was viewed through the lens of compromised LA synthesis as the major mechanistic functional outcome. For example, knockdown of *NDUFAB1* kills HEK293T cells in culture,[44] an effect that the authors largely attributed to loss of LA synthesis. The DepMap tool developed by the Broad Institute shows how widely applicable this finding is, with *NDUFAB1* being classified as a common essential gene among cancer cell lines in the database, and several other genes in the pathway are "strongly selective" (*OXSM*, *MECR*, *LIAS*, and *LIPT1*).[45] Our own experience supports this notion, as we failed to isolate any viable knockout CRISPR clones for the mtFAS genes *MCAT*, *OXSM*, or *MECR* in the C2C12 mouse skeletal myoblast cell line.[15] The essentiality of mtFAS and LA synthesis is not limited to *in vitro* systems, as complete knock out of *MECR*, *LIAS*, and *LIPT1* have all been shown to cause embryonic lethality in mice.[24,46,47] *MECR* mutation also causes developmental lethality in *Drosophila*.[48] Notably, none of the patients with disease-causing variants in mtFAS genes harbor two alleles that are predicted to cause complete loss of function (such as two early nonsense mutations, for example), supporting the idea that total loss of the pathway is likely also not viable in humans.

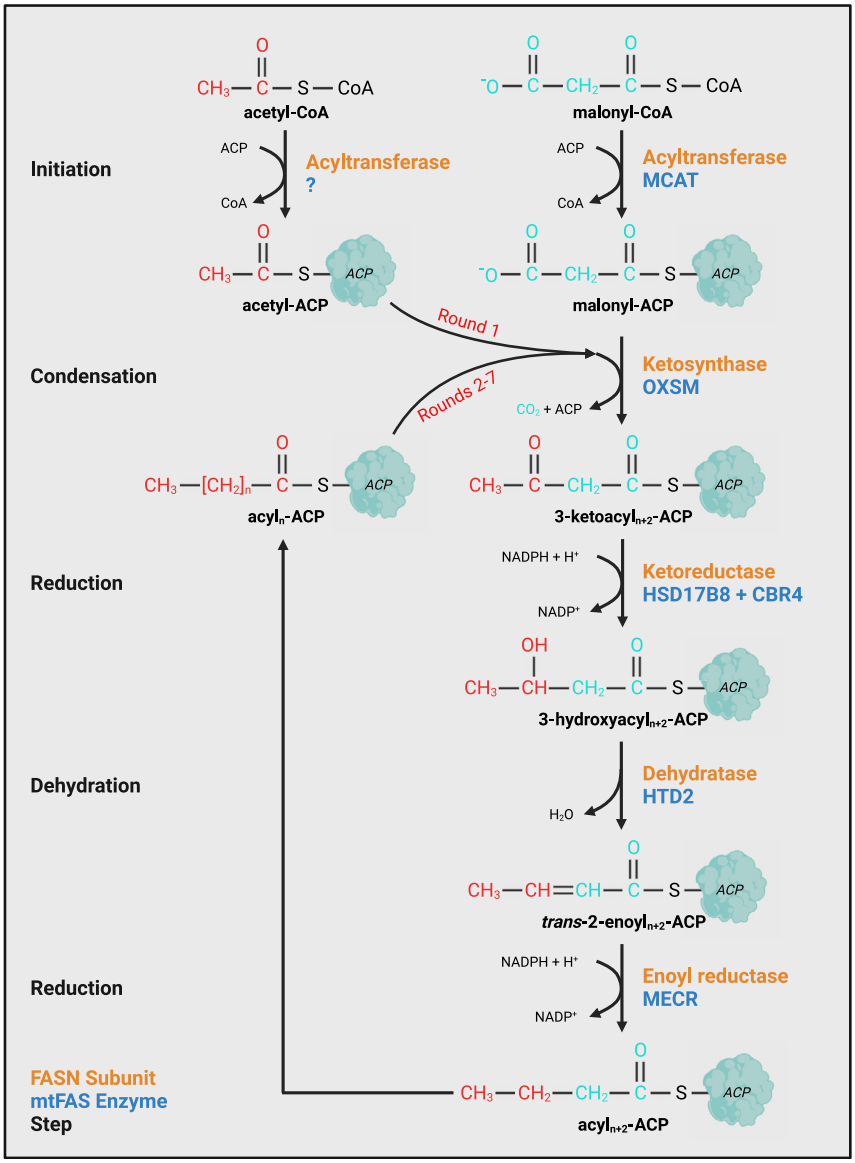

Figure 2. mtFAS mirrors cytosolic FAS

The enzymatic steps in mtFAS and cytosolic FAS (catalyzed by FASN) are identical, although mtFAS utilizes individual enzymes for each step (blue text), whereas cytoplasmic FAS uses different domains encoded on a single polypeptide (FASN, orange text). The two carbons added during each round of elongation are always derived from malonate and are combined with acetyl-ACP in the first round, producing a four-carbon acyl chain that becomes the substrate for future rounds. Multiple rounds of this cycle produce a single product in cytoplasmic FAS (palmitoyl-ACP), whereas mtFAS produces octanoyl-ACP and longer chain products (myristoyl-ACP and palmitoyl-ACP).

Since total loss of mtFAS is not viable in animal models, some labs shifted to studying ACP knockout in specific tissue settings (Figure 4). Skeletal muscle knockout of ACP disrupts glucose homeostasis and insulin signaling and results in death at postnatal day 5.[49] Cardiac depletion of ACP results in defective bioenergetics, elevated ROS, progressive cardiomyopathy, HF, and death.[41] In both models, the metabolic impairments that drove pathology at the whole tissue level corresponded to defective protein lipoylation, along with a loss of ETC complex abundance—inferred to result from loss of ACP as a structural subunit of complex I. However, because these studies were performed in ACP knockouts, it was not possible to discern the extent to which these specific phenotypes resulted from loss of mtFAS activity.

Only a few studies have looked at the effects of losing mtFAS (rather than ACP) in whole animal models (Figure 4). MECR knockout is lethal during mouse development due to placental defects.[47] Specific knockout of MCAT in Purkinje cells recapitulates many of the phenotypes seen in mtFAS-deficient patients, indicating that this may be the target cell population in human mtFAS deficiency.[50] Stuart Smith's group studied the effects of knocking out mtFAS after development by crossing mice with a floxed allele of MCAT with an inducible whole-body Cre. Similar to the ACP-depletion models discussed above, loss of MCAT led to loss of protein lipoylation, decreases in ETC complex abundance, and overt metabolic insufficiency characterized by weight loss despite increased food consumption, reduced muscle strength, hypothermia, and shortened lifespan.[51] These effects were still interpreted to result from loss of LA synthesis and TCA cycle function.

LIMITATIONS OF AVAILABLE TOOLS

The development of new *in vivo* mtFAS knockout mouse and *Drosophila* models over the past several years has contributed significantly to our understanding of mtFAS function in various tissue settings; however, this understanding is far from complete. Because total loss of mtFAS and LA synthesis is embryonically lethal, interrogation of the effects of mtFAS loss in specific cell types/tissues must be accomplished by crossing into tissue-specific, inducible Cre models. This process is slow, and there are many tissues, such as immune cells, that have yet to be examined.

Moreover, there are still technical limitations in our ability to monitor mtFAS activity. As noted above, the best indicator of mtFAS activity remains immunoblotting for protein lipoylation. With the demonstration of patient variants with mtFAS product preferences[30] and outstanding questions surrounding pathway inputs and flux, more comprehensive assays to measure mtFAS activity in a quantitative and product-specific way are necessary. For example, there is currently no method to distinguish mammalian mtFAS products by mass spectrometry. Every

Table 1. Reported pathology-causing variants in mitochondrial fatty acid synthesis genes

	Affected gene	Patient(s)	Inheritance	Mutation(s)	Protein alt.	Phenotype	Earliest onset	Paper
NM_016011.3	MECR	6 males, 1 female	AR	c.695G>A	G232E	dystonia, optic atrophy in 7/8, abnormal brain MRI	15 months –6.5 years	Heimer et al.[18]
				c.855T>G	Y285*			
				c.830+2_830+3insT	Unknown			
				c.854A>G	Y285C			
				c.772C>T	R258W			
				c.247_250del	Asn83Hisfs*4			
	MECR	2 males	AR	c.830+2_830+3insT	Unknown	hypotonia, spasticity	6 months	Frésard et al.[31]
				c.−39G>C	Unknown			
	MECR	1 female	AR	c.772C >T	R258W	dystonia, optic atrophy, abnormal brain MRI	3.5 years	Gorukmez et al.[26]
				c.1009C >T	R337*			
	MECR	1 male	AR	c.910G>T, homozygous	D304Y	dystonia, abnormal brain MRI	7.6 years	Liu et al.[32]
	MECR	2 females	AR	c.772C>T, homozygous	R258W	acute optic atrophy	5 years	Fiorini et al.[29]
NM_173467.4	MCAT	2 males	AR	c.C634T	L81R	optic atrophy	8 years	Li et al.[27]
				c.T242G	R212W			
	MCAT	1 female	AR	c.424-2A>G	Unknown	acute optic atrophy	20 years	Gerber et al.[28]
				c.1039G>A	E347K			
	MCAT	1 male	AR	c.812T>C, homozygous	T271I	hypotonia, nystagmus, abnormal brain MRI	premature birth	Webb et al.[30]

known mtFAS product exists attached to a protein and is therefore undetectable by mass spectrometry after traditional lipid and metabolite extraction methods. A quantification method for mtACP acylation in plants[52] presents an exciting opportunity for the future study of mtFAS products using mass spectrometry. This method takes advantage of the sequence surrounding the 4′-PP attachment site, which is unique to mtACP. Cleavage of the peptide backbone with aspartate (D) endoproteinase created unique mass identifiers corresponding to a short peptide, 4′-PP modification, and various acyl chains on *Camelina Sativa* mtACP.[52] This method is attractive for identifying mtACP-associated acyl chains as no other AspN cleavage product of a 4-phosphopantethenylated protein would yield the same peptide as would be expected from mtACP, including the cytoplasmic acyl carrier subunit of FASN.[53] However, this method has yet to be adapted for use in mammalian systems.

MOLECULAR CONSEQUENCES OF mtFAS IMPAIRMENT BEYOND LA

Across model systems, studies examining the consequences of mtFAS impairment had often observed decreases in ETC complex abundance, but the mechanism behind this observation had not been rigorously defined. As mentioned above, some studies attributed ETC the loss to lack of ACP as a structural subunit of complex I, especially when ACP itself was targeted. Others postulated that when ACP was still expressed, the effect(s) of mtFAS loss on the ETC was indirect, a downstream consequence of lack of LA and reduced TCA cycle function. J.K. Hiltunen, A.J. Kastaniotis, and colleagues insightfully suggested that the acyl chain on ACP provided cells with a means to "sense" acetyl-CoA levels in the mitochondrial matrix but lacked a molecular mechanism for how this would occur.[54,55] These interpretations were complicated by experiments showing that mitochondrial translation is affected in yeast mtFAS mutants via an effect on RNase P,[56] providing yet another possibility for how lack of ACP acylation might affect ETC complex abundance.

High-throughput proteomics analysis of mtFAS-deficient cells provided insights into the mechanism behind ETC regulation by the pathway, showing that ACP physically interacts with a family of late-stage ETC assembly factors known as the LYRM proteins for their conserved leucine-tyrosine-arginine motif.[15–17,57] We showed that the LYRM proteins require an acyl chain on ACP for binding—when ACP is not acylated, such as in a mtFAS mutant, the LYRM proteins are destabilized and/or unable to perform their complex assembly function, and ETC abundance declines.[15,17] Indeed, the two subunits of ACP bind complex I via LYRM protein subunits, and early data were clear that the majority of ACP in complex I was acylated,[43,58] but the importance of these data had not been appreciated. The ACP-LYRM-binding mechanism explained the concurrent loss of the other ETC complexes in addition to complex I, is conserved from yeast to mammals, and also provides the mechanism linking acetyl-CoA (the substrate of mtFAS) to ETC assembly and function (Figure 3).[15,17]

In support of a direct binding ETC assembly mechanism, we also found that *LIPT1* mutant cells had no defects in abundance or assembly of ETC complexes I–IV, indicating that the loss of ETC complexes in mtFAS mutants is not downstream of impaired TCA cycle function.[15] Others have also found that protein interactions with the 4′-PP co-factor and acyl chains on ACP

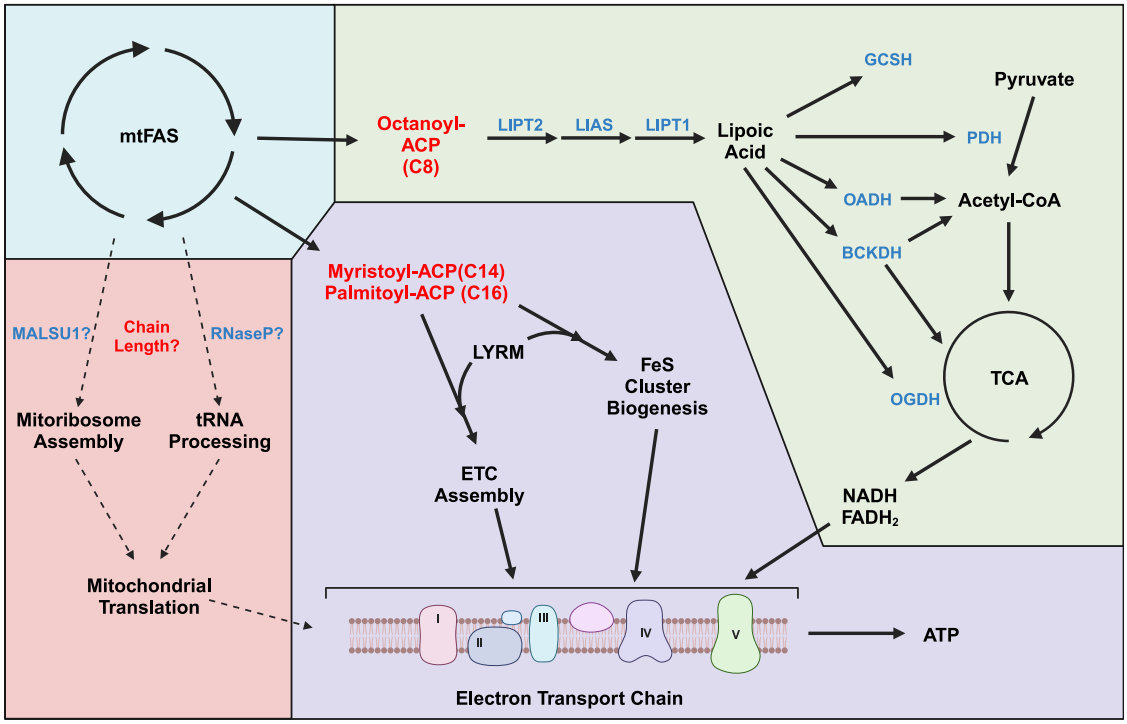

Figure 3. Functional roles of mtFAS

mtFAS products contribute to the maintenance of oxidative metabolism through a variety of mechanisms. Acyl-ACPs of 8 carbons in length are converted to lipoic acid that is used in the lipoylation of numerous enzymes involved in maintenance of TCA function and the production of reduced cofactors that deliver electrons to the ETC. Longer chain acyl-ACPs interact with members of the LYRM family of proteins that support the assembly of ETC complexes and the production of FeS clusters. Evidence suggests mtFAS-derived acyl-ACPs of unknown length also regulate the production of ETC proteins via modulation of mitochondrial translation, in yeast via RNase P, and possibly in mammalian cells via MALSU1. Blue text indicates enzymes, and red text indicates mtFAS products. GCSH, glycine cleavage system protein H; PDH, pyruvate dehydrogenase; OADH, 2-oxoadipate dehydrogenase; BCKDH, branched chain keto acid dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase.

are integral to its function, lending further strength to this model.[59] A recent study by Tanvir Rahman et al. demonstrated definitively that the longer products of the mtFAS pathway are required for ETC assembly, using a cleverly engineered mutant of the mtFAS enzyme MECR.[60] This engineered point mutant limits mtFAS product acyl chain length to ~8–10 carbons by blocking the cavity in MECR that the fatty acid fits into during catalysis. Cells expressing the mutant are thus able to efficiently synthesize octanoate and LA but cannot make longer chain products and still exhibit profound ETC assembly defects.[60]

Our recent patient case report described a disease-causing variant in *MCAT* in which protein lipoylation is intact, but ETC assembly is compromised.[30] This finding is interesting because the enzymatic function of MCAT is to attach malonyl-CoA to ACP as carbon enters the pathway to initiate a new cycle of fatty acid elongation (Figure 1)—thus, in contrast to the Autio lab's engineered *MECR* variant, it is less clear how *MCAT* mutation would affect one product of mtFAS (long-chain products that support ETC assembly) over another (octanoate and downstream LA). One intriguing possibility is that partial MCAT impairment creates limited substrate entering the pathway and that under these conditions, kinetics of the enzymes might favor the shunting of octanoate toward lipoylation over further elongation. Understanding this decision point and its control will become increasingly important as we enhance our understanding of the different cellular roles played by varied mtFAS products.

LA, its precursor octanoate, and the long-chain products of mtFAS that support ETC assembly all exist covalently bound to ACP and/or are transferred to their target enzymes by lipoyltransferases (LIPT2 and LIPT1). Whether mtFAS products are ever released as free fatty acids or contribute to lipid pools beyond these covalent modifications is still an open research question. To our knowledge, there is no annotated mitochondrial thioesterase (TE) enzyme that participates in the mtFAS pathway in any animal species. Early studies in *S. cerevisiae* found defects in some lipids, but none that are not also altered in other petite yeast mutants (yeast mutants that have mitochondrial respiratory defects for reasons other than mtFAS), suggesting that the identified changes in lipid abundance are not likely due to loss of direct products of the mtFAS pathway but rather an indirect result of lost ETC function.[61] Dr. Deborah Murdock's group undertook the first lipidomics analysis of mtFAS-deficient and overexpressing cells and similarly concluded that mtFAS does not contribute significantly to the fatty acid components of mitochondrial lipids.[62] We undertook stable isotope labeling into lipids in mtFAS-deficient cells and also came to a similar conclusion.[15] However, the Murdock group did find decreased levels of ceramides and their precursors upon ACP knockdown.[62] In opposition to this result, a recent study showed that MECR loss corresponded to increased levels of ceramides in both *Drosophila* and *MECR*-deficient patient fibroblasts.[48] In this new study, reducing levels of ceramides rescued some of the

phenotypes of MECR loss, suggesting an intriguing hypothetical mode of treatment for MECR deficiency. What factors contribute to the conflict between these findings and earlier studies remains to be elucidated.

OTHER ROLES FOR mtFAS IN MAMMALIAN CELLS

Several papers have implicated mtFAS LA and/or ACP in mitochondrial processes beyond ETC assembly (Figure 3). A well-characterized LYRM protein, LYRM4, is a core subunit of the FeS cluster biogenesis machinery, interacts with ACP, and requires acylation for stability and function in mammalian cells.[15,16] Downstream of mtFAS, LA has been shown to play an important role in cuproptosis, as copper directly binds to lipoylated proteins, causing their aggregation and FeS cluster protein loss, and ultimately leads to cell death.[63] New CRISPR screening data have also implied another role for mtFAS pathway in lipid droplet biology,[64] showing that disruption of mtFAS enzymes increased the expression of PLIN2, a positive regulator of lipid droplets, but the exact mechanisms and physiological implications of these findings remain to be seen.

A few papers also suggest a potential role for mtFAS and/or ACP in mitochondrial ribosome (mitoribosome) assembly (Figure 3).[57,65] Structural analyses found ACP bound to a mitoribosome assembly intermediate via MALSU1, another protein with an LYR motif.[65] Interestingly, in the structure, unlike others that have been published of ACP bound to LYRM proteins,[66,67] ACP is not acylated. ACP and MALSU1 are bound to the large mitoribosomal subunit in such a way that they would preclude the small subunit from binding. To our knowledge, the role of mtFAS in mammalian mitoribosome assembly and/or mitochondrial translation has not been studied. These data raise the interesting possibility of a potential variation on the ACP-LYRM binding model in which the acylation of ACP releases binding to MALSU1 and allows the assembly of mitoribosomes to continue. This is distinct from the RNase P mechanism through which mtFAS regulates mitochondrial translation in yeast; RNase P is functionally but not structurally conserved in mammalian cells.[68] It is intriguing that mammalian cells may have evolved a completely separate mechanism through which to regulate mitochondrial translation, highlighting the potential importance of this putative regulatory relationship. Underscoring this idea is the observation that in humans, the cDNA for the mtFAS enzyme HTD2 is encoded on the same transcript as RPP14, a component of human RNase P, providing another unexpected link between these two pathways.[13]

Recently, a genome-wide association study suggested a potential role for mtFAS in the maintenance of mtDNA copy number.[69] Although the underlying mechanism(s) by which mtFAS might affect the levels of mtDNA are undescribed, this is yet another potential mechanism through which mtFAS might regulate levels of mitochondrially encoded proteins. However, we did not see an effect of impaired mtFAS on the abundance of mitochondrially encoded proteins in the unbiased quantitative proteomics analyses we performed in MCAT-, OXSM-, and MECR-deficient cells[15]; however, it is worth noting that our mutants are hypomorphs and not total nulls, and we did not quantify mtDNA copy number or assay mitochondrial translation directly. The possibility remains that these functions of mtFAS are preserved in incomplete loss-of-function models and would need to be defined in a mammalian model in which total knockout of mtFAS is not incompatible with growth.

TOWARD THE GOAL OF ACTIVATING mtFAS: UNDERSTANDING REGULATION

Whether mtFAS function is impaired in diabetes, HF, or other pathological settings, activation of mtFAS could prove to be a promising therapeutic strategy. Driving mitochondrial respiration has quickly become a focus for therapy in many of these common metabolically relevant disease settings, as mounting evidence suggests that restoration of oxidative mitochondrial metabolism combats pathology.[70–75] Augmenting mitochondrial function via mtFAS may hold particular therapeutic promise, as the pathway coordinately regulates so many central mitochondrial functions (TCA cycle, ETC assembly, FeS cluster biogenesis, and perhaps others). Depending on their mechanism of action, strategies that rescue mtFAS dysfunction could hold promise for patients with rare mtFAS deficiencies as well.

Overexpression of ACP has provided promising evidence that suggests increased mtFAS pathway activity is protective against metabolic disease. For instance, mice overexpressing ACP are protected from obesity and insulin resistance when fed a high-fat diet.[49] Similarly, overexpression of ACP in the heart shields mice against ischemia-reperfusion injury.[41] However, in contrast, overexpression of *MECR* leads to cardiac dysfunction,[76] implying that careful attention must be paid to which step in the pathway is targeted, and more research is needed to understand the ramifications of activating specific mtFAS enzymes. Moreover, absent efficient and cost-effective approaches for gene therapy, developing strategies to drive mtFAS in disease settings will first require an understanding of the pathway's regulation, an area that has still hardly been touched by research (Figure 5).

A crucial question in the many factors that likely regulate mtFAS pathway activity is where the carbon that fuels the pathway comes from. Malonyl-CoA is the committed substrate for both cytoplasmic FAS and mtFAS, and in fact, the generation of malonyl-CoA is the rate-limiting step in cytoplasmic FAS. Cytoplasmic malonyl-CoA is produced from acetyl-CoA by two acetyl-CoA carboxylase enzymes (ACC1 and ACC2), which are highly regulated by AMP-activated protein kinase and other mechanisms. Thus, the parallel step in mitochondria is a prime candidate in the regulation of mtFAS activity. Unfortunately, however, the source of mitochondrial malonyl-CoA in mammalian cells is unclear and highly disputed in the literature. Mitochondria do not import malonyl-CoA; therefore, there must be an intramitochondrial enzymatic source. In yeast, this source is Hfa1, a mitochondrially localized ACC that is required for mtFAS.[78] Experiments in yeast lacking the mitochondrial pyruvate carrier (MPC) showed that depletion of mitochondrial acetyl-CoA through limiting pyruvate entry into the mitochondrial matrix blocks mtFAS function via substrate limitation. MPC knockout yeast show loss of protein lipoylation, ETC assembly, and other endpoints downstream of mtFAS activity.[17] These data support a model in which mitochondrial malonyl-CoA is derived from mitochondrial acetyl-CoA and raise the exciting possibility that the pathway may be nutritionally regulated.

Sadly, mammalian cells do not express an Hfa1 ortholog, and the source of mitochondrial malonyl-CoA is murkier. Acyl-CoA

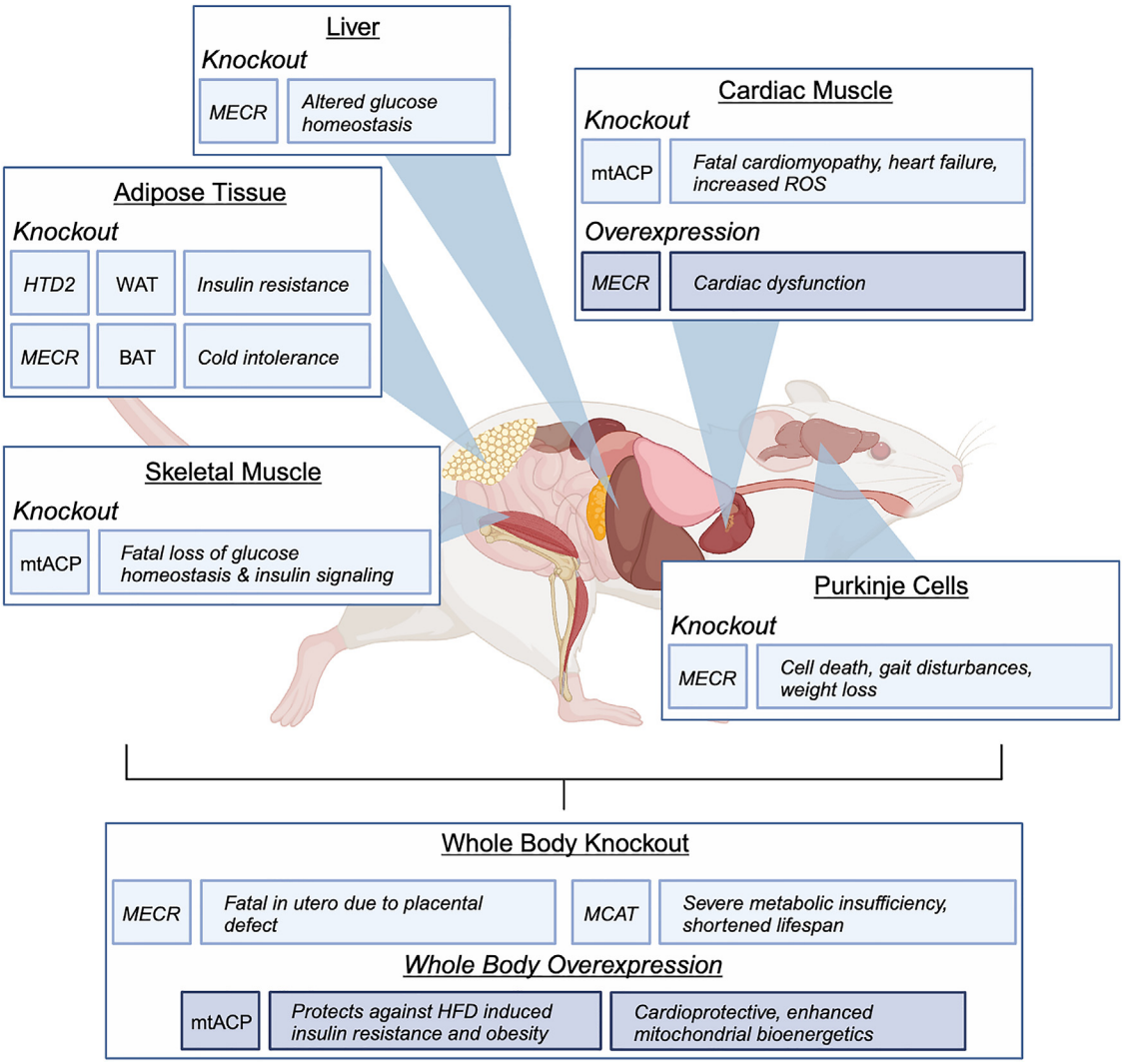

Figure 4. Reported tissue-specific mtFAS phenotypes in mouse

Emerging studies using novel genetic models have begun to provide insights into the role of mtFAS pathway genes in various physiological settings. Clockwise from top right: cardiac muscle: cardiac-specific (Mlc2v-Cre) knockout of ACP results in fatal cardiac abnormalities.[41] In contrast, overexpression of mtFAS enzyme MECR in cardiac muscle cells resulted in cardiac dysfunction.[76] Purkinje cells: knockout of MECR in Purkinje cells (Pcp2-Cre) results in cell death and associated symptoms of motor abnormalities, recapitulating patient phenotypes. Whole-body weight loss is also observed.[50] Skeletal muscle: skeletal muscle-specific knockout (Mlc1f-Cre) of ACP is fatal at postnatal day 5 and results in dysregulation of whole-body glucose homeostasis. Adipose tissue: adipose-specific knockouts of HTD2 and MECR result in insulin resistance in white adipose tissue (Adipoq-Cre)[39] and cold intolerance in brown adipose tissue (Ucp1-Cre).[77] Liver: liver-specific knockout of MECR (albumin-Cre) in mice results in altered glucose homeostasis.[77] Bottom: whole body knockouts: knockouts of mtFAS machinery have severe symptoms that are incompatible with life.[47,51] In contrast, overexpression of mtACP (pan-tissue) protects against high-fat diet (HFD)-induced metabolic abnormalities and is cardio-protective against ischemia-reperfusion injury.[41,49]

synthetase family member 3 (ACSF3), a mitochondrial enzyme that catalyzes the conversion of free malonate to malonyl-CoA, is often annotated as the enzyme that supplies malonyl-CoA for mtFAS in mammalian cells.[79] However, studies have found that ACSF3 is not required for protein lipoylation and instead postulate that the enzyme is primarily involved in malonate detoxification.[80] The finding that loss of ACSF3 has no effect on protein lipoylation implies that either ACSF3 does not fuel mtFAS *in vivo* or at least that there is an alternate source that is able to compensate for mtFAS loss. One possibility is that ACSF3 and a mitochondrially localized isoform of ACC1 may both produce mitochondrial malonyl-CoA in tandem (Figure 1).[81] The relative contributions of each enzyme, and whether the pathways can compensate for one another, are unclear. Understanding the roles of ACSF3 and mitochondrial ACC1, and their requirement for mtFAS activity, will be the first step toward understanding the metabolic regulation of mtFAS activity and developing strategies to tune mtFAS function.

Beyond substrate-level regulation, mtFAS enzymes are likely regulated on many other levels, including transcriptionally. One recent study using CRISPR screening to identify Myc-regulated genes required for cancer cell growth observed that the growth of several Myc-dependent cancer cell lines relies on intact mtFAS genes.[82] The authors found that *MCAT*, *MECR*,

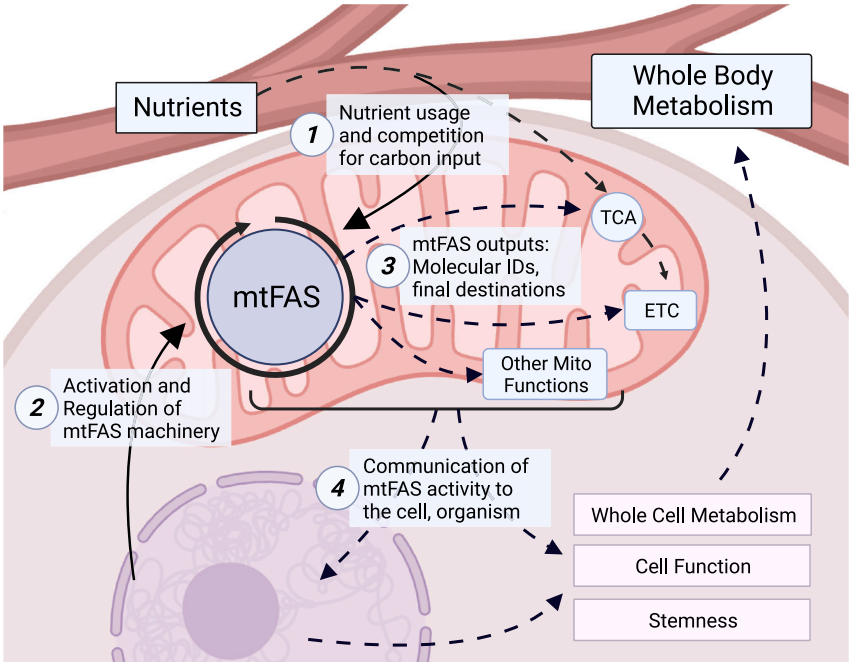

Figure 5. Looking forward: The role of mtFAS in mammalian cells

Although recent studies have provided fundamental insights into mtFAS function in cells, there are still many unknowns. Areas of particular interest and importance for the field include the following: (1) the nutrient sources (glucose, fatty acids, etc.) that contribute to mtFAS acyl chains. Although malonyl-CoA is required for chain elongation, both malonate and acetyl-CoA have been proposed as sources of malonyl-CoA. How mtFAS competes with other carbon uses (i.e., TCA) in the mitochondria is also unknown. (2) The activation and regulation of mtFAS machinery. The enzyme responsible for activation of mtACP (via 4′-phosphopantetheinylation) is unannotated, and other post-translational modifications of mtFAS machinery have not yet been investigated. Little is known about how potential modifications, protein turnover, and translation of mtFAS enzymes influence pathway activity. (3) Outputs of mtFAS. Known actions of mtFAS acyl chains include octanoate for LA synthesis and acylated ACP for ACP-LYRM interactions. The decision of whether octanoate exits the cycle for LA synthesis or undergoes continued elongation is a crucial control point, but how this decision is controlled is undescribed, and the relative proportions of each of these product pools are unknown. Moreover, for long-chain fatty acid products of mtFAS, their molecular identity (i.e., chain length), how they are removed from ACP (a thioesterase?), and ultimate destinations (i.e., incorporation into specific mitochondrial lipids? Degradation?) are undefined. (4) Communication of mtFAS activity to the cell. Many models are beginning to show large-scale effects of mtFAS dysfunction on cellular phenotypes that include transcriptional rewiring. We do not understand the mechanism whereby mtFAS activity influences whole-cell metabolism and gene expression. Whole-cell metabolism and gene expression in turn influence cell function, stemness, and could have implications for whole body metabolism. How mtFAS functions change in specific cell types has limited characterization as well.

OXSM, and *HSD17B8* were all within 50 kbp from the sites of Myc-binding motifs, suggesting that their expression is likely Myc regulated. Lending support to this claim, guides targeting the Myc binding motif closest to the *MECR* gene drop out of the Myc-motif-targeting CRISPR library screen as well, implying that Myc binding to this area is required for cancer cell growth and providing strong evidence to support the idea that mtFAS genes are transcriptionally regulated by Myc in mammalian cells.[82]

CRISPR screening data have also identified an interesting interplay between oxygen/hypoxia and mtFAS genes. In a genome-wide CRISPR growth screen of K562 cells, enzymes involved in mtFAS, FeS cluster biogenesis, and mitochondrial pyruvate metabolism were essential in normoxia but not in 1% oxygen tension (hypoxia).[83] The authors of these results postulate that hypoxia creates an environment where loss of LA is tolerated, since it is otherwise essential in mammals.[8] This finding may have important implications for treating patients with mtFAS disorders. Follow-up studies found that hypoxia acts through HIF-independent mechanisms to activate FeS cluster biogenesis and protein lipoylation; however, these effects appear to be through direct effects on the FeS cluster assembly (ISCU) complex, rather than mtFAS, as they occur on reconstituted ISCU complex *in vitro*.[84,85]

Whether other mechanisms regulate mtFAS function and what those mechanisms may be remain to be defined. For example, nothing is known about whether ACP or any mtFAS enzyme(s) are post-translationally modified, what those modifications are, and whether they might influence pathway activity. Moreover, as mentioned above, data in yeast provide evidence that mtFAS activity and its downstream effects on protein lipoylation and ETC assembly depend on matrix acetyl-CoA levels.[17] Whether and how changes in nutrient source and abundance affect mtFAS activity and downstream sequelae remains to be demonstrated.

LOOKING FORWARD

Ultimately, many fundamental questions concerning the action of mtFAS in cells remain unanswered and, in many cases, completely unexplored (Figure 5). Understanding the full importance of the mtFAS pathway in mammalian oxidative metabolism and cell biology will also require resolving the molecular identity of the full complement of mtFAS lipid products and their ultimate destination(s) in cells. We now appreciate that mtFAS has two major outputs—octanoate for LA synthesis and longer acyl chains that promote ACP-LYRM protein interactions. The general ratio of these two products is not known, nor are the factors that control the decision point between octanoate exiting the mtFAS cycle for LA synthesis versus continuing through further cycles of elongation. One way that these two pools of product might be stratified is by the enzymology of mtFAS components. *In vitro* studies using the yeast ortholog of OXSM describe a biphasic substrate specificity for six-carbon- or twelve-carbon-chain lengths, generating octanoyl-ACP and myristoyl-ACP most readily.[86]

Moreover, like octanoate, which is subsequently converted to LA, the longer acyl chain outputs of mtFAS likely go somewhere in cells, but their ultimate destination remains a mystery. Unlike FASN, which encodes a TE domain, there is no annotated TE enzyme that plays a role in mtFAS. We also know that the

pathway does not significantly contribute to major cellular lipid stores such as phospholipids and triglycerides.[15] Whether there might be a more specific contribution of mtFAS products to specialized lipids, such as sphingolipids or cardiolipins, is still an intriguing possibility, especially given the emerging reports discussed above that find alterations in ceramides and other bioactive signaling lipids. Whether these effects are direct or secondary to changes in oxidative metabolism downstream of mtFAS function remains to be seen. Regardless, describing how acylated ACP is turned over, whether and how these longer fatty acid products are incorporated into mitochondrial lipids, and if so, the identities and functions of those lipid products, will all also be crucial steps in developing a full, accurate picture of mtFAS function in cells. As unbiased genetic screens lead more and more researchers to the pathway, answering these fundamental questions about the role(s) of mtFAS in mammalian biology and mitochondrial metabolism is of paramount importance.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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