THE ROLE OF ACYL-COA THIOESTERASE 1 IN HEPATIC LIPID METABOLISM

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Dedication

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Abstract

The liver is an essential organ for maintaining homeostasis and is vital for storage, synthesis, oxidation, and recirculation of lipid in fed and fasted states. In response to fasting, fatty acids (FAs) flux from adipose tissue to the liver and are converted to acyl-CoAs for incorporation into complex lipids or transportation into the mitochondria for oxidation. The latter process is orchestrated by a group of proteins that are transcriptional targets of peroxisome proliferator activated receptor α (PPARα). However, little is known about hepatic acyl-CoA thioesterase 1 (ACOT1), a member of the broader acyl-CoA thioesterase family that catalyzes the conversion of acyl-CoAs back to FAs and coenzyme A. Thus, this research is aimed to understand the role of ACOT1 in fasting lipid metabolism. To investigate its physiological importance, we employed adenovirus-mediated knockdown, overexpression in tissue culture, as well as generation of a whole-body *Acot1* knockout mouse line. Our results show that ACOT1 preferentially hydrolyzes acyl-CoA molecules that are destine for mitochondrial β-oxidation. As such, acute *Acot1* knockdown results in reduced liver triglyceride (TG) and enhanced FA oxidation *in vivo* and *in vitro*. Increased FA oxidation correlated to greater hepatic glucose production and storage. Additionally, we determined that ACOT1 regulates PPARα by providing FA ligands. As such, supplementation with a PPARα synthetic ligand rescues the *Acot1* knockdown phenotype. Furthermore, *Acot1* overexpression increases

PPARα activity only when ACOT1 is catalytically active. Together these data suggest that ACOT1 regulates PPARα through its hydrolysis product. We also discovered that ACOT1 translocates to the nucleus during prolonged fasting, potentially to provide a local pool of FAs to activate PPARα. Thus, acute *Acot1* knockdown solicits enhanced FA oxidation, yet reduces PPARα target gene expression. Complications of this disconnect between metabolism and gene expression was evident by increased oxidative stress and inflammation, often seen in fibrotic and cirrhotic stages of non-alcoholic fatty liver disease (NAFLD), when *Acot1* was knocked down. To further our investigation of ACOT1, we compared whole-body *Acot1* knockout mice to their wild type littermates. We demonstrate that *Acot1* knockout lead reduces adiposity, by decreasing adipocyte size and increasing adipocyte number. *Acot1* knockout also reduced hepatic TG, providing protection from oxidative stress and inflammation that precedes TG accumulation. However, *Acot1* knockout reduced glucose tolerance suggesting impaired glucose homeostasis. These results suggest *Acot1* knockout impaired lipid storage potential, increasing lipid intermediates, and contributing to glucose intolerance. Taken together, hepatic ACOT1 regulates FA oxidation and protects from oxidative stress and inflammation, whereas whole-body ACOT1 contributes to lipid storage.

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Chapter 1

The Role of Hepatic Thioesterase Activity in Fatty Acid Metabolism

Mallory Franklin Wrote This Chapter In It's Entirety

Hepatic Metabolism

The liver is an essential organ for whole-body energy homeostasis during feeding and fasting. Hepatic metabolism orchestrates a delicate balance between energy storage and energy production dependent on the nutritional state (1). In the fed state, the liver is important for storing glucose as glycogen and synthesizing fatty acids (FAs) to be used at a later time. In the fasted state, the liver ensures adequate maintenance of blood glucose and provides ketones as an energy source for the brain and other organs (1).

The liver is the primary organ responsible for the oxidation, synthesis, and distribution of lipids. Hepatic lipids can be stored as TG in lipid droplets oxidized through β-oxidation or secreted as very low-density lipoprotein (VLDL) (2). In the fed state, hepatic lipids are derived from the uptake of chylomicron remnants or are synthesized via *de novo lipogenesis* (DNL), the process of converting excess carbohydrates into FAs. DNL is orchestrated largely through two transcription factors, sterol response element binding protein 1c and carbohydrate response element binding protein. Insulin increases expression of sterol response element binding protein 1c, while glucose stimulates carbohydrate response element binding protein, increasing the transcription of glycolytic and lipogenic genes essential for DNL. During DNL, citrate from the tricarboxylic acid cycle is shuttled to the cytosol and serves as two carbon building blocks for new FAs. The initial step of FA synthesis is achieved by acetyl-CoA carboxylase forming malonyl-CoA (3). Newly synthesized FAs, primarily palmitic acid, can be elongated or

desaturated and esterified to a glycerol backbone to form TG. TG can then be stored in lipid droplets or used for VLDL maturation and secretion. VLDL then enters circulation and distributes lipid to adipose depots for storage or other peripheral tissues for utilization (2).

Conversely, during fasting, glucagon inhibits sterol response element binding protein 1c and subsequent FA synthesis (4). β-adrenergic stimulation induces lipolysis in adipose tissues, resulting in the release of non-esterified fatty acids (NEFAs) into circulation (5). Serum NEFAs are readily taken up by the liver through fatty acid transport proteins and cluster of differentiation 36 protein (6). These FAs are then converted to acyl-CoA molecules by a family of acyl-CoA synthetases (ACSL) (7) and are transported into the mitochondria through carnitine transfer protein 1α (CPT-1α), where they serve as a source of energy through β-oxidation (8). CPT-1α activity is inhibited by malonyl-CoA produced by DNL (3), which ensures that FAs are not being synthesized and oxidized simultaneously. FAs can be completely oxidized to CO_2 or partially oxidized to ketone bodies that re-enter circulation (2). Importantly, hepatic FAs regulate their own metabolism by serving as signaling molecules that activate transcription factors necessary for these oxidative pathways (2).

The liver also serves as a regulator of whole-body glucose homeostasis. In the fed state, the liver clears 30-40% of glucose coming from the digestive system (9), converting most to glycogen for storage. In mice, the liver can store up to 90mg of glycogen per gram of liver tissue (10). Once capacity is reached

excess glucose is converted to FAs through DNL as described above (3). During fasting, the liver maintains blood glucose through glycogenolysis and gluconeogenesis (1). Glycogenolysis is the breakdown of glycogen while gluconeogenesis is the synthesis of glucose from glycerol, pyruvate, lactate, or branch chained amino acids (1). During initial fasting, glycogen is the primary source of blood glucose, while gluconeogenesis is the primary source in prolonged fasting. In mice, glycogen stores are depleted by 50% after 8 hours of fasting and by 12 hours are depleted by 80%. Reciprocally, gluconeogenesis and ketogenesis increase during 12 to 16 hours of fasting to compensate for glycogen loss (10). Human studies indicate a longer tolerance to fasting. Glycogen stores reach 50% after 22 hours of fasting and by 42 hours are roughly 15%. Gluconeogenesis accounts for 50% of blood glucose at 22 hours of fasting and nearly all blood glucose by 42 hours (11,12). By 4 hours of fasting plasma NEFA levels begin to increase due to adipose lipolysis, promoting fatty acid oxidation in the liver and providing an energy source for gluconeogenesis. The presence of FAs inhibit glycogenolysis (13,14) while enhanced FA oxidation can increase gluconeogenesis (15). These studies suggest that FA oxidation is important for the activation of gluconeogenesis during fasting.

Non-Alcoholic Fatty Liver Disease

Lipid imbalance can lead to hepatic lipid accumulation (6,16) which defines a disease known as non-alcoholic fatty liver disease (NAFLD). Imbalance can occur due to increased intake of dietary lipid, increased synthesis of FAs via DNL, or increased uptake of NEFA, as well as a reduction in VLDL secretion or reduced FA oxidation. These imbalances increase hepatic TG storage (16–18). In clinical studies, patients with increased hepatic lipid had TG stores that were derived from NEFA (60%), DNL (25%), and dietary lipid (15%) (19), suggesting that the major contributor to hepatic steatosis is NEFAs derived from adipose tissue lipolysis.

NAFLD is an inclusive title for a spectrum of liver disease states that includes liver steatosis, non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (16). NAFLD is tightly correlated to a number of metabolic diseases including obesity, Type 2 Diabetes, and Cardiovascular Disease (20–23). Recent evidence suggests that one third of all Americans have NAFLD (23) and the prevalence and severity of the disease increases amongst overweight and obese populations (24). The progression of NAFLD is one of the leading causes of liver failure in the United States (20–22). Simple liver steatosis is considered a benign form of liver disease and lipid sequestered as non-toxic TG is an innate protective mechanism (25). Progressive states of NAFLD are marked by increased lipid intermediates that cause oxidative stress, mitochondrial dysfunction, and increase inflammation that lead to fibrosis,

cirrhosis, and insulin resistance (26,27). Classically, NALFD has been described by a two-hit hypothesis, in which the initial hit is hepatic steatosis, while the subsequent hit is the inflammation that progresses the disease (28). Recently, *Tilg et al.* have suggested a multiple hit model in which a sequential or co-occurrence of lipid accumulation, mitochondrial dysfunction, inflammatory response, and ER stress propagates the progression of simple steatosis to NASH (25). Therefore the exact mechanism by which NAFLD progresses is not well defined by one pathway, because each pathway is able to propagate the next. As such, it is unclear which condition appears first when progressing from steatosis to NASH and fibrosis. However, current research shows that hepatic steatosis results in greater ketogenesis (29,30), suggesting that FA oxidation may contribute to the progression of NAFLD.

In addition to nutritional and metabolic risk factors for NAFLD, hepatitis C, hepatotoxins, inflammatory bowel diseases, and drug toxicity can lead to progressive forms of liver disease (31). Once NAFLD progresses toward fibrosis and cirrhosis liver disease is irreversible. Current treatments include prevention of further damage through dietary intervention and exercise, while severe cases require liver transplantation (32). Due to the increasing prevalence and severity of progressive NAFLD, understanding the development and metabolic complications of simple steatosis is imperative.

The western lifestyle is linked with the development of obesity and metabolic syndrome. It is predicted that as many as 80-90% of obese individuals

have NAFLD (33). To better understand the development of diet induced NAFLD, researchers have attempted to create animal models that mimic the disease. Classically, a methionine and choline deficient diet has been used. This diet exhibits signs of NAFLD including steatosis, inflammation, and fibrosis, but does not coincide with obesity and insulin resistance often seen in human subjects who develop NAFLD (34). On the other hand, use of a high fat diet, 45% fat, results in obesity and insulin resistance, but only causes hepatic steatosis. To achieve hepatic inflammation and fibrosis, mice would have to remain on a high fat diet for 15 months (34). More recently, a diet high in lard, cholesterol, and fructose resulted in 80% of mice developing NASH after 16 weeks (35). These mice also exhibited the metabolic complications that accompany obesity, creating a model very similar to human NAFLD. Another research group designed a rat model that mimicked human NAFLD by feeding a 75% fat diet for only 3 weeks (36). These models more closely mimics the disease progression seen in humans and can better predict what causes the transition from benign steatosis to more severe disease states, however few studies have yet to be performed utilizing these diets.

Regulation and Signaling of Hepatic Free Fatty Acids and Acyl-CoAs

The source of hepatic intracellular FAs includes uptake of circulating NEFA, synthesis from DNL, or products of TG lipolysis at the lipid droplet surface. To be susceptible for further metabolism, intracellular free FAs must be linked to coenzyme A (CoA) via a thioester bond forming an acyl-CoA. Acyl-CoAs serve as substrate for several lipid pathways, including oxidation, TG synthesis, and synthesis of more complex lipid species. ACSLs catalyze the formation of acyl-CoAs, a reaction utilizing ATP, and prevents the efflux of FAs to maintain a concentration gradient for FA uptake (2,7). ACSL isoforms have varying intracellular locations, substrate preferences, and kinetics resulting in selective partitioning of FAs to specific pathways. Hepatic ACSL3 and ACSL5 are important for the conversion of *de novo* synthesized FAs to acyl-CoAs destined to be incorporated into TG (37). ACSL1 is the primary isoform in hepatic tissue and is present on the outer membrane of the mitochondria. ACSL1 is the important isoform for activation of FAs destined for β-oxidation in numerous tissues (38–40) yet hepatic knockout of ACSL1 has minimal effects on fatty acid oxidation (41). Thus, the major ACSL isoform involved in the shuttling of FAs to mitochondrial β-oxidation in the liver has yet to be identified.

Free FAs and acyl-CoAs are considered lipotoxic compounds (2). Therefore, the conversion of FAs to acyl-CoAs and their subsequent metabolism is a rapid process. To maintain low levels of lipotoxic lipids, liver-specific fatty

acid binding protein (LFABP) can bind and transport free FAs as well as acyl-CoAs (42). LFABP is important for the transportation of substrate to metabolic fates. Liver-specific knockout of LFABP results in a reduction in mitochondrial β-oxidation, highlighting the importance of LFABP in trafficking FAs toward oxidation (43). LFABP has a high affinity for FAs reducing cellular concentrations of free FAs. Total liver non-esterified FAs is estimated to range around 100 nM to 500 nM, with free FAs in the low nM range (44). Acyl-CoA binding protein binds acyl-CoAs (45,46) and promotes FA uptake and ACSL activity by removing feedback inhibition of free acyl-CoAs on ACSLs (47). Free cellular acyl-CoAs are kept at a concentration lower than 5 nM primarily due to rapid metabolism (45,46). The concentration of free FAs and acyl-CoAs fluctuates during circadian cues and fed and fasted states, being low during the feeding and reaching the upper limits during fasting (48). The activity of these enzymes and binding proteins regulates the levels and locations of acyl-CoAs and FAs, which likely impacts their effects in cells as discussed below.

Hepatic FAs and acyl-CoAs are prominent signaling molecules important for the activation of transcription factors involved in lipid metabolism. A major class of transcription factors involved in lipid metabolism are peroxisomal proliferator-activated receptors. The primary isoform involved in the transcriptional regulation of proteins involved in hepatic FA disposal is PPARα (44). Activated PPARα forms a heterodimer with retinoic x receptor and can induce transcription by binding to PPAR response elements in the promoter

region of target genes (49). A wide variety of lipid species serve as ligands for PPARα activation. Saturated and monounsaturated FAs ranging from 14-20 carbons (44,50), polyunsaturated fatty acids, eicosanoids (51), as well as phosphatidylcholine species (52) activate PPARα. As such, different physiological conditions that result in high concentrations of these lipid species may be predictive of PPARα activation. NEFA liberated from adipose tissue are taken up by the liver and serve as endogenous ligands for activation of PPARα during fasting (44). LFABP has been suggested to traffic FAs to the nucleus for signaling, yet *in vivo* and *in vitro data* showed mixed results of LFABP on PPARα activation (43,53). As such, the mechanism by which FAs translocate to the nucleus and serve as ligands to PPARα is not well characterized. Both long chain FAs as well as long chain acyl-CoAs are present in the nucleus (54,55). Palmitoyl-CoA antagonizes PPARα activity by inhibiting its binding to co-factors and DNA (55,56), suggesting that controlling the inter-conversion of FAs and acyl-CoAs, particularly in the nucleus, may have a large influence on PPARα activity.

Another transcription factor that exhibits ligand binding to long chain FAs is hepatic nuclear factor 4α (HNF4α). HNF4α is a nuclear transcription factor present in hepatocytes that is necessary for cells to maintain the hepatic phenotype (57). HNF4α is important for the transcription of gluconeogenic genes through interaction with peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) (58,59), as well as genes involved in lipoprotein

synthesis and secretion (60). Binding of FAs to HNF4α is strong, yet has no known affect on activity of the transcription factor (60). Conversely, HNF4α also exhibits ligand binding for acyl-CoAs, various acyl-CoA species can either be inhibitors or activators. Importantly, palmitoyl-CoA is a known activator of HNF4α (61). Thus, FAs and acyl-CoAs serve as important signaling molecules that influence hepatic energy metabolism.

Patients with NAFLD have elevated serum NEFA and enhanced liver uptake of NEFA (62). It is suggested that an accumulation of intracellular FAs causes lipotoxicity, contributing to the progression of NAFLD (18,63). Interestingly, monounsaturated FAs such as oleic and palmitoleic acid do not have the hepatotoxic effects as their saturated counterparts, stearic and palmitic acid. This is primarily due preferred esterification of monounsaturated fatty acids into TG, sequestering them in non-toxic lipid storage (64). In cell culture, saturated FAs are capable of increasing reactive oxygen species (ROS) and subsequently activating the c-Jun NH2-terminal kinase apoptotic pathway in hepatocytes (65,66). Use of etomoxir, a CPT1α inhibitor, rescues palmitate-induced c-Jun NH2-terminal kinase activation, indicating oxidation of palmitic acid is the source of ROS and apoptosis (66). Yet a physiological mix of both monounsaturated and saturated fatty acids prevents the apoptotic pathway caused by saturated fatty acids. Thus, maintaining a delicate balance of intracellular free FAs provides necessary ligands for PPARα activation while simultaneously minimizing lipotoxic effects of free FAs.

Oxidation of Hepatic Fatty Acids

The liver is an oxidative organ, which carries out several pathways involved in FA oxidation. A large majority of liver FA disposal is achieved through mitochondrial β-oxidation. Cytosolic short and medium chain acyl-CoAs can diffuse through the mitochondrial membrane, while longer chain (12-20 carbons) acyl-CoAs require transport through CPT-1α present on the outer membrane of the mitochondria (67). Once in the mitochondrial matrix, acyl-CoAs are metabolized to acetyl-CoA molecules by means of β-oxidation, producing $FADH_2$ and NADH, which donate electrons to the electron transport chain (ETC) (2). Acetyl-CoAs produced from fatty acid oxidation can enter the tricarboxylic acid cycle for complete oxidation to CO_2 or alternatively, excess acetyl-CoAs can be metabolized to ketone bodies in the mitochondrial matrix (2). 3-hydroxy-3-methylglutaryl-CoA synthase 2 catalyzes the initial rate-limiting step in ketone body synthesis, converting acetyl-CoAs to 3-hydroxy-3 methylglutaryl-CoA (68). 3-hydroxy-3-methylglutaryl-CoA is then converted to acetoacetate and finally β-hydroxybutyrate (69). Ketone bodies are released from hepatocytes and re-enter circulation, serving as energy sources for other tissues. Given that the liver is a sole source of ketone bodies (70), the concentration of β-hydroxybutyrate, the primary circulating ketone body, serves as a marker for hepatic mitochondrial β-oxidation and ketogenesis.

As discussed previously, PPARα is important for the activation of genes involved in FA oxidation, including mitochondrial β-oxidation genes CPT1α and 3-hydroxy-3-methylglutaryl-CoA synthase 2, rate-limiting steps for mitochondrial

FA uptake and ketogenesis, respectively (67,68). Another important nuclear transcriptional cofactor is PGC1α, important for FA oxidation and mitochondrial biogenesis (71). It has been demonstrated that PGC1α serves as a co-activator of PPARα enhancing transcription of genes involved in FA oxidation (72). Additionally, PGC1α acts as a co-activator to estrogen-related receptor α, promoting mitochondrial biogenesis (59,73). Therefore, simultaneous activation of both PPARα and PGC1α are essential for maximal mitochondrial oxidative capacity during fasting.

Mitochondrial ROS Generation

Normal mitochondrial oxidation produces a mild stress on the cell resulting in the production of ROS, predominantly superoxide (74). However, ROS production is balanced by mitochondrial antioxidant activity that protects the mitochondria from oxidative stress. Superoxide dismutase catalyzes the conversion of superoxide to hydrogen peroxide, which can then safely be degraded to water and oxygen by antioxidant enzymes, glutathione peroxidase and peroxiredoxin (75). During times of increased FA oxidation, ROS production can exceed antioxidant capacity. A high ratio of ATP/ADP and $NADH/NAD^+$ reduces ATPase activity, and causes an increase in the proton gradient (76). Increased membrane potential can cause feedback inhibition of the proton pumps in the ETC (77). Intermediates in the ETC, primarily at Complex I and III, can then transfer electrons to oxygen forming superoxide (74). ROS production leads to

mitochondrial DNA and protein damage as well as membrane permeabilization and release of apopototic proteins (78), resulting in mitochondrial dysfunction. Superoxide is a highly reactive molecule and can cause the formation of lipid peroxides. Downstream metabolites of lipid peroxidation, such as malondialdehyde and 4-hydroxynonenal, can activate stellate cells that produce collagen and α smooth muscle actin (79). Malondialdehyde and 4-hydroxynonenal also activates inflammatory pathways such as the transcription factor, nuclear factor- κB (28,80). Conversely, inflammatory cytokine tumor necrosis factor alpha (TNFα) can be produced in hepatocytes and by Kupffer cells in the liver during an immune response. TNFα can cause mitochondrial swelling and result in membrane bursting. When mitochondrial membranes burst there is a disconnect between cytochromes in the electron transport chain leading to superoxide production and release of apoptotic proteins (81–83).

During times of oxidative stress, many cell types exhibit expression of uncoupling protein 2 (UCP2) present on the inner membrane of the mitochondria (84). UCP2 serves as a proton leakage source therefore uncoupling the ETC from ATP synthesis. UCP2 has the potential for ameliorating some of the oxidative pressure on mitochondrial respiration (85). UCP2 expression has been shown to protect neurons from apoptosis (86). Similarly, overexpression of UCP2 in cardiomyocytes protects cells from cardiomyopathy by improving FA oxidative capacity (85). In normal physiological conditions, hepatocytes do not express UCP2(84), yet under acute and chronic stress expression is enhanced

(87). UCP2 overexpression lowers the oxidative burden on hepatic mitochondria thereby protecting cells from oxidative damage (88). NASH in humans and rats increases expression of UCP2, which serves as a marker for oxidative stress.

An increase in cellular ROS and lipid peroxides as well mitochondrial dysfunction is a hallmark of NAFLD (26,89,90). Mitochondrial dysfunction, ROS production, and inflammation is a classic “chicken and egg” scenario where each can cause the other, yet all three usually occur simultaneously making it difficult to distinguish what came first (79,91). An increase in FA oxidation has been described as a compensatory mechanism to handle excess FA accumulation (92,93). However, the pathological effect of increased mitochondrial FA oxidation has yet to be studied as a potential cause for ROS production in the progression of NAFLD.

Acyl-CoA Thioesterase Family

As previously stated, FAs must be activated to acyl-CoAs for subsequent metabolism. However, a family of acyl-CoA thioesterases (ACOTs) catalyze the reverse of this reaction, hydrolyzing the thioester bond resulting in the production of coenzyme A and a FA. As such, it is suggested that ACOTs create a futile cycling with ACSLs, due to the use of ATP to form acyl-CoAs (94). ACOTs are ubiquitously expressed in many tissues and varying organelles. Additionally, ACOTs have varying substrate specificity, from acetyl-CoA to short, medium, and long chain acyl-CoAs as well as branch chain acyl-CoAs and bile acids (95).

ACOT activity was first discovered in the cytosol of rat liver, in which palmitoyl-CoA hydrolysis was measured and the enzyme responsible was termed palmitoyl-CoA hydrolase (96). This led to the identification of two cytosolic and two mitochondrial isoforms that differ in molecular weight. The light chain thioesterases (~40kD) were termed cytoplasmic thioesterase I and mitochondrial thioesterase I, while the heavier chains (~110kD) were named cytoplasmic thioesterase II and mitochondrial thioesterase II (97). This was the first distinction between what is now known as Type I and Type II thioesterases. Type I thioesterases are smaller and exhibit 40% homology to N-acyl-transferases, yet do not possess acyl-transferase activity (98). They possess a β-sandwich domain and an α/β hydrolase fold (98). Type I thioesterases consist of ACOTs 1-6, which vary in tissue expression and cellular localization (99). Type II thioesterases (ACOT7-15) are distinguishable from Type I thioesterases because they are larger and possess a hotdog fold domain and a steroidogenic acute regulatory transfer domain (99,100).

ACOTs are present in organelles involved in lipid shuttling and oxidation including cytosol, mitochondria, and peroxisomes. Peroxisomal ACOTs include both Type I and Type II isoforms, ACOT3, ACOT4, ACOT5, ACOT6, ACOT8 and ACOT12 (98). PPARα regulates the expression of peroxisomal ACOTs (98). Peroxisomal ACOTs, primarily those responsible for the hydrolysis of long and medium chain acyl-CoAs. ACOT3, ACOT5, and ACOT8 are speculated to be involved in FA exportation from the peroxisome for further metabolism in the

mitochondria (98). ACOT8 hydrolyzes a wide spectrum of acyl-CoA molecules varying in chain length, saturation, and branching. ACOT8 activity is inhibited by high levels of peroxisomal coenzyme A and is the only ACOT isoform that is inhibited by CoA (101). Acetyl-CoA products from peroxisome β-oxidation are substrates for ACOT12, which is speculated to play a vital role in shuttling acetyl-CoAs out of the peroxisome to be used for cytosolic DNL (102). ACOT2, ACOT13, and ACOT15 are the primary mitochondrial isoforms. ACOT2 is present in the mitochondrial matrix and promotes mitochondrial β-oxidation (103). ACOT13 is located on the outer mitochondrial membrane and is speculated to be important for the cleavage of acyl-CoAs, generating a pool of FAs that serve as substrate for shuttling into the mitochondria through the ACSL1-CPT1 complex (104). ACOT15 is present in the mitochondrial matrix and is essential for cardiolipin synthesis and mitochondrial structure (105).

In humans ACOT1 and ACOT7 are cytosolic isoforms, yet ACOT1 is the only cytosolic Type I acyl-CoA thioesterase in mice (106), while ACOT7 is the primary cytosolic Type II thioesterase. ACOT7 is present in the brain and is important for maintaining adequate neuronal FA oxidation (107). ACOT1 is primarily expressed in the liver, kidney, and heart and shares 93.7% homology to mitochondrial ACOT2 (99). ACOT2 is slightly larger than ACOT1 due to its 42 amino acid N-terminal mitochondrial target signal (99). However, when compared on a Western blot, ACOT2 runs below ACOT1 (108), indicating some potential post translational modification. It is believed that ACOT1 and ACOT2 share

similar activity but are distinct based on their cellular localization.

Acyl-CoA Thioesterase 1

Acyl-CoA Thioesterase 1 was first identified as palmitoyl-CoA hydrolase in rat livers by *Yamada et al.* in 1994 (109). It was then classified as the low molecular weight cytosolic thioesterase I (CTE-1) (97), which today correlates to its Type I thioesterase classification. Recombinant ACOT1 has demonstrated substrate preference toward long chain saturated FAs ranging from 12 to 20 carbons long (110). Activity toward monounsaturated fatty acids is half that of the saturated form and very little activity is detected towards polyunsaturated fatty acids (110). ACOT1 has a Km of 2.6 μM, suggesting a high affinity to acyl-CoAs. High concentrations of long chain acyl-CoAs, greater than 5 μM, inhibits ACOT1 activity (110). ACOT1 has a basic α/β hydrolase characteristic of Type I thioesterases, in which a beta sheet meets an alpha helix creating a sharp γ turn pocket (111). The catalytic triad consists of Serine232, Asp324, and His358. The serine motif consists of GxSxG, which is characteristic of all Type I acyl-CoA thioesterases (110). Mutation of any three of these residues result in complete loss of ACOT1 thioesterase activity (110).

Transcriptional Regulation of ACOT1

Acot1 is a target gene of PPARα (112). Indeed, *Acot1* is the most highly induced gene in response to peroxisome proliferators, such as clofibrate (113). As such, *Acot1* expression is induced during times of enhanced FA oxidation (97,106,113). *Acot1* is transcriptionally regulated by both PPARα and HNF4α through a distal response element (DRE-1) in the promoter region of the gene (112). HNF4α negatively regulates *Acot1* expression; liver knockout of HNF4α resulted in a 15-fold increase in *Acot1* expression (112). During fasting PPARα displaces HNF4α on these regulatory regions of the gene and promotes transcription of *Acot1*. This competitive binding to the *Acot1* DRE-1 is dependent on ligand binding to PPARα and enhanced affinity to the DRE-1 over HNF4α (114). *Ppar*α-null mice express ACOT1 protein during fasting but not with clofibrate treatment (115). In addition, liver-specific *Pparα* knockout mice also express *Acot1* during fasting (116). Together these studies suggest that *Acot1* expression is partially independent of PPARα during fasting.

In addition to PPARα and HNF4α, circadian regulatory proteins PAR domain basic leucine zipper proteins exhibit transcriptional regulation over ACOTs 1-4(117). As such, whole-body knockout of PAR domain basic leucine zipper proteins results in a decrease in circadian expression of these *Acots*. Interestingly, PPARα and target transcripts were also reduced (118). These results highlight a potential feed forward loop in which ACOTs may respond to circadian cues to produce ligands that promote PPARα activation and

subsequent transcription of *Acots*. Others have speculated that ACOTs are responsible for providing FA ligands for PPARα activation (101,106), however only a few studies have evidence to support this theory. Muscle mitochondrial ACOT2 was suggested to produce FAs that transport through UCP3 and serve as signaling molecules during excessive oxidation (119). Similarly, over-expression of ACOT2 in mouse liver increased mitochondrial β-oxidation as well as expression of genes involved in FA oxidation (103). The exact mechanism for how ACOTs provide ligands that activate PPARα has not been elucidated. To date, the involvement of cytosolic ACOT1 in providing FAs ligands has not yet been characterized. Yet ACOT1 activity toward long chain saturated acyl-CoAs (110) suggests potential production of long chain saturated fatty acid ligands known to activate PPARα (50).

ACOT1 Response to Diet and Disease

Although the physiological relevance of ACOT1 has not yet been studied in the liver, ACOT1 has shown physiological relevance in response to diet and disease in other tissues. Fat-free diets reduce the expression of *Acot1* in rodent models, independent of PPARα activity (115). Rats placed on a high fat diet had a 3 fold higher concentration of ACOT1 in heart and Type II muscle during fasting compared to those on a low fat diet (120). Another study indicated that 20 weeks on a high fat diet increased ACOT1 protein 5-fold compared to low-fat diet. These effects were seen independent of metabolic complications due to a high

fat diet (120). Another study feeding a 20% partially hydrogenated fat diet to rats for 29 days demonstrated a 2-fold increase in cytosolic thioesterase activity in the liver (121). Similarly, alloxen diabetic mice have demonstrated an increased expression of cytosolic thioesterase activity in the liver (122). A recent study by Ellis *et al.* suggests that in the liver ACOT1 protein concentration increases during high fat and ketogenic diets (94). These studies demonstrate the importance of dietary lipid in the expression and activity of ACOT1 and that disease states can impact *Acot1* expression.

Acot1 expression in pre-adipocytes is high, but expression diminishes with differentiation, highlighting a potential role in controlling levels of FA oxidation during adipocyte development to increase lipid storage (123,124). ACOT1 activity has been studied more extensively in the heart. Leptin receptor deficient db/db mice, which develop severe obesity-related diseases, have heightened protein levels of ACOT1 in the heart (125). In an insulin resistant state, animals can develop diabetic cardiomyopathy in which the heart becomes enlarged and inflamed due to an enhanced use of FAs as the primary energy source (126). Overexpression of *Acot1* in cardiomyocytes improves diabetic induced cardiomyopathy in mice by reducing FA oxidation and improving ROS levels. These effects were mitigated through enhanced PPARα and PGC1α signaling (125). In addition, *Acot1* overexpression was protective against heart failure during sepsis (127). This highlights the potential involvement of ACOT1 in minimizing oxidative stress and promoting mitochondrial capacity, by removing

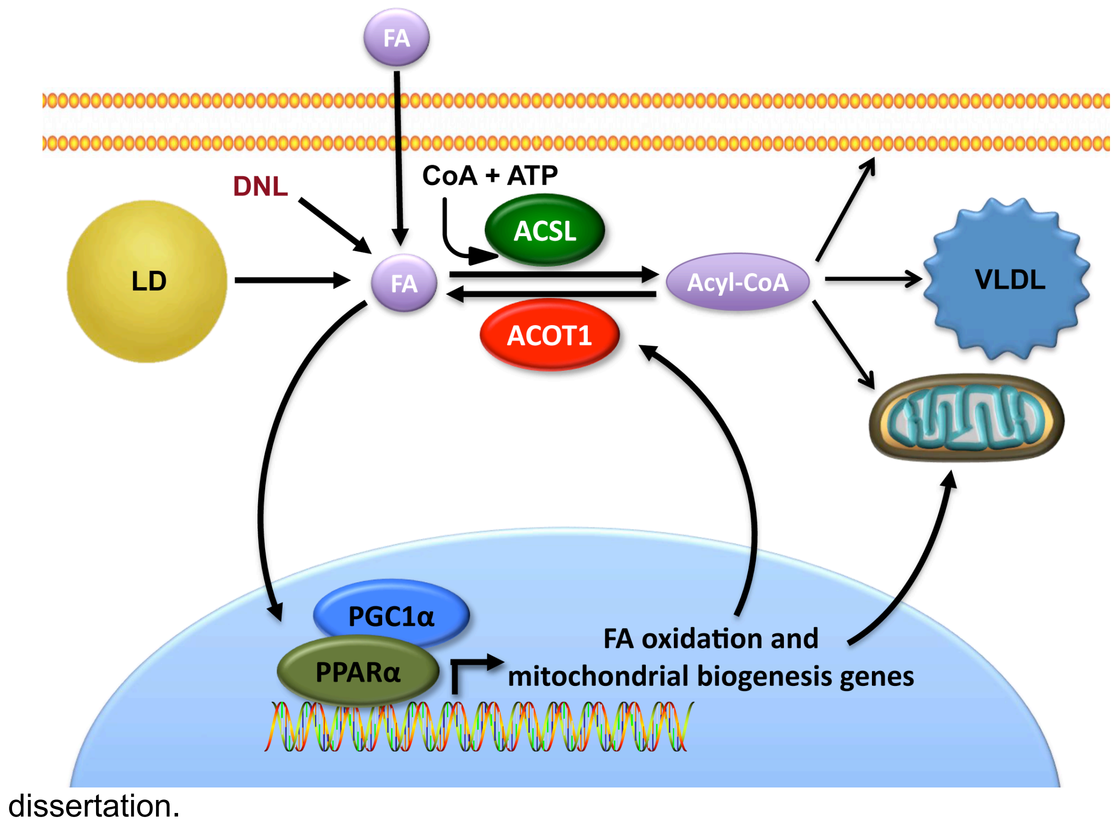
substrate to mitochondrial β-oxidation and increasing signaling toward PPARα and PGC1α. Thus, ACOT1 expression may be elevated in diabetes and cardiomyopathy as a protective mechanism. Investigating the role of ACOT1, the primary hepatic cytosolic ACOT, in hepatic lipid metabolism may provide insight on how oxidative capacity regulated by ACOT1 mitigates progression of liver steatosis to NASH and fibrosis. Despite high expression in the liver, the physiological significance of ACOT1 in the progression of NAFLD has not been investigated.

Current Objectives

The progression of NAFLD from simple steatosis to inflammation, fibrosis, and cirrhosis is described as the two-hit hypothesis. As such, steatosis is the first hit, then the metabolic complications that progress the disease are considered the second hit (28). However, to date, the mechanism of how steatosis leads to oxidative stress, inflammation, and fibrosis is poorly understood. Recent research suggests that steatosis leads to greater FA oxidation in the liver (29,30). However, the contribution of excess FA oxidation to oxidative stress and inflammation is poorly understood, yet *in vitro* studies suggest that oxidation of saturated FAs contributes to oxidative stress (66).

Understanding the intricate regulation of FA and acyl-CoA molecules during fasting is imperative to elucidating the mechanisms by which hepatic steatosis becomes more severe. Acyl-CoA thioesterases are a class of enzymes

that are understudied and may provide insight into how FA metabolic pathways are regulated during feeding and fasting. In order to understand the fundamental importance of hepatic acyl-CoA thioesterase activity, we investigated the physiological relevance of ACOT1 in the forthcoming chapters of this



dissertation.

Figure 1. ACOT1 in hepatic lipid metabolism. Hepatic FAs can be taken up into the cell, synthesized by DNL, or cleaved from TG pools in lipid droplets. Acyl-CoA synthetase family members (ACSL) utilize ATP to form thioester bonds between free FAs and coenzyme A. Acyl-CoAs are then substrate to mitochondrial β oxidation, TG synthesis for VLDL packaging, or the synthesis of other lipid species such as phospholipids. ACOT1 catalyzes the reverse reaction, cleaving acyl-CoAs back to free FAs. Free FAs are known to activate PPARα and

increase expression of genes involved in FA oxidation.

The work in this thesis investigates the physiological importance of ACOT1 in fasting hepatic metabolism. The second chapter investigates ACOT1 via adenovirus-mediated knockdown of ACOT1 for one week during feeding and fasting and under different dietary conditions. Acute *Acot1* knockdown answers the questions: How does ACOT1 regulated lipid metabolism? How does ACOT1 regulate nuclear signaling to PPARα? How does ACOT1 regulate hepatic glucose metabolism? And lastly, how does hepatic lipid metabolism contribute to oxidative stress and inflammation? The third chapter explores the effects of whole-body *Acot1* knockout in male and female mice fed a purified control or a high fat diet. The third chapter answers the questions: How does whole-body *Acot1* ablation impact lipid storage in adipose and liver? How does long-term *Acot1* knockout affect whole-body energy expenditure in relation to lipid and glucose metabolism?

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CHAPTER 2

Acyl-CoA Thioesterase 1 (ACOT1) Regulates PPARα to Couple Fatty Acid Flux With Oxidative Capacity During Fasting

Mallory Franklin wrote this chapter in its entirety

Hepatic acyl-CoA thioesterase 1 (ACOT1) catalyzes the conversion of acyl-CoAs to fatty acids (FAs) and coenzyme A. The physiological importance of hepatic ACOT1 has yet to be determined. Therefore, we sought to determine the role of ACOT1 in hepatic lipid metabolism in C57Bl/6J male mice one week following adenovirus-mediated *Acot1* knockdown. *Acot1* knockdown reduced liver triglyceride (TG) due to enhanced TG hydrolysis and subsequent FA oxidation. *In vitro* experiments demonstrated that *Acot1* knockdown led to greater TG turnover and FA oxidation, suggesting that ACOT1 is important for controlling the rate of FA oxidation. Despite increased FA oxidation, *Acot1* knockdown reduced the expression of peroxisome proliferator-activated receptor α (PPARα) target genes. Similarly, overexpression of *Acot1* increased PPARα reporter activity, an effect that was dependent on ACOT1 catalytic activity, suggesting that ACOT1 regulates PPARα by producing FA ligands. Moreover, ACOT1 exhibited partial nuclear localization during fasting and cAMP/PKA signaling, indicating a potential local regulation of PPARα. As a consequence of increased FA oxidation and a reduction in PPARα activity, *Acot1* knockdown enhanced hepatic oxidative stress and inflammation. The effects of *Acot1* knockdown on PPARα activity, oxidative stress, and inflammation were rescued by supplementation with Wy-14643, a synthetic PPARα ligand. Male mice fed a high fat diet for three months prior to *Acot1* knockdown exhibited greater oxidative stress, inflammation, and markers of fibrosis, a phenotype normally not seen with high fat diet. Finally, *Acot1* knockdown also increased gluconeogenesis and glycogen storage, which was

rescued with Wy-14643, suggesting that PPARα signaling is important for glucose homeostasis. Taken together, we demonstrate that ACOT1 regulates fasting hepatic metabolism by balancing FA oxidative flux and oxidative capacity, which protects from oxidative stress and regulates glucose homeostasis.

Introduction

During times of fasting, FAs are released from adipose tissue and readily taken up by the liver where they can be oxidized in the mitochondria via β-oxidation (1,2). In order to be transported into the mitochondria, FAs must be converted to acyl-CoAs by long chain acyl-CoA synthetases (3). A family of acyl-CoA thioesterases catalyze the reverse reaction – the hydrolysis of the fatty acyl-CoA thioester bond resulting in the production of coenzyme A and a free FA (4). This reaction seemingly removes acyl-CoAs from mitochondrial β-oxidation. In the liver, ACOT1 is the primary cytosolic thioesterase isoform (5). PPARα induces *Acot1* expression through a distal response element in the promoter region of the gene (6,7). However, fasting induces *Acot1* expression in whole-body *Pparα*-null mice as well as liver-specific *Pparα* knockout mice (6,7), suggesting that PPARα is sufficient, but not necessary for *Acot1* expression. Therefore, ACOT1 is speculated to be involved in FA trafficking during periods of increased hepatic FA influx and oxidation (8,9).

PPARα is a transcription factor that governs the expression of genes involved in FA oxidation and is necessary to increase FA oxidative capacity during times of fasting (7). A wide variety of lipid species are speculated to serve as ligands for PPARα activation including free FAs (10–12). In addition to its role in FA oxidation, PPARα promotes the expression of anti-inflammatory genes (13) as well as suppresses expression of inflammatory genes (14). As such, PPARα

ligands are potential therapeutic agents for the prevention or treatment of inflammation (14).

Mitochondrial FA oxidation produces a mild amount of ROS at complex I and III of the electron transport chain (15). This ROS production is balanced by antioxidant activity that protects the mitochondria from oxidative stress (16). However, during times of increased FA oxidation, increased membrane potential can lead to ROS production that can exceed antioxidant capacity leading to oxidative stress (15,17,18). Regulating the rate of FA oxidation in the cell is critical for minimizing ROS and oxidative stress that can occur during times of increased oxidation. Overexpression of *Acot1* in cardiomyocytes reduces FA oxidation and ROS production in mice with diabetic cardiomyopathy (19). These data suggest a potential involvement of ACOT1 in promoting oxidative capacity through substrate regulation and signaling.

During prolonged fasting, the liver is an essential organ for maintaining blood glucose. In the initial stages of fasting, hepatic glycogen is broken down to maintain blood glucose. Once glycogen stores are depleted, hepatic gluconeogenesis continues to provide glucose (20). Gluconeogenesis is an energy-demanding pathway, which is largely driven by ATP produced from hepatic FA oxidation. Therefore alterations in hepatic FA oxidation, could affect glucose production and blood glucose levels during prolonged fasting (21).

Despite high expression in the liver during fasting, the involvement of ACOT1 in lipid metabolism has yet to be characterized. Herein, we identify

ACOT1 as a key enzyme that links FA flux and trafficking to PPARα signaling, ROS, and inflammation, as well as secondary regulation of hepatic glucose production.

Research Design and Methods

Mouse handling. The Institutional Animal Care and Use Committee of the University of Minnesota approved all protocols used in this study. Seven to nine week old male C57BL/6J mice were purchased from Harlan Laboratories (Madison, WI) and housed in a controlled temperature (20°-22°C) and light (12 hrs light/12 hrs dark) vivarium. Mice had free access to water and were fed a purified diet (TD.94045) from Harlan Teklad (Madison, WI). One week before sacrifice, mice received a tail vein injection of an adenovirus harboring either scramble short-hairpin RNA (shRNA) described previously (22) or shRNA targeted to *Acot1* mRNA. *Acot1* shRNA adenovirus was generated from Open Biosystems (Huntsville, AL) based on accession number NM_012006.2 (antisense sequence AAACACTCACTACCCAACTGT). For the studies involving Wy-14643, mice were fed the purified diet supplemented with 0.1% WY-14643 (Selleckchem, Houston, TX), a synthetic PPARα ligand, for 7 days (23) An additional group of mice were fed a 45% high fat diet (TD.06415) for 12 weeks prior to a tail vein injection of the adenovirus treatment. One week post transfection all mice were fasted overnight (16 hrs) and harvested unless noted otherwise.

***In vitro* isotope experiments.** Three days post adenovirus injection, mice were fasted overnight (16 hrs) and primary hepatocytes were isolated via collagenase perfusion and plated on collagen-coated plates. Hepatocytes were cultured in insulin/serum free M199 media to mimic fasting conditions. All experiments were carried out the same day after a 4-hour plating period. Cells were pulsed with trace [^{14}C]oleate along with 500 μM unlabeled oleate bound to BSA (3:1 molar ratio) for 2 hours. A subset of wells were harvested to measure radiolabel incorporation into cellular TG (pulse) and the remaining wells were given radiolabel free media for 6 hours before harvest (chase). Cells and media were harvested to quantify isotope in cellular TG, media TG, and media acid soluble metabolite (ASM) fractions as previously described (24,25).

TG hydrolase activity. TG hydrolase activity was measured from tissue lysates from control and *Acot1* knockdown mice. Lysis buffer consisted of 20 mM Tris-HCl, 150 mM NaCl, and 0.05% Triton X-100, pH 8.0. Lysates were spun at 15,000xg for 15 minutes. Infranatant was collected and mixed with 1,2-Di-O-lauryl-rac glycerol-3-(glutaric acid 6-methylresorufin ester) and incubated at 30°C for 1 hour. Kinetic readings were taken every 2 minutes at 530 nm excitation and 590 nm emission. Readings were compared to a standard curve generated with free resorufin.

Serum β-hydroxybutyrate. Serum was harvested after a 16 hour overnight fast. Serum β-hydroxybutyrate was assessed using β-hydroxybutyrate LiquiColor Kit (Stanbio Laboratory, Boerne, TX).

RNA isolation and analyses. mRNA was isolated from tissue or cells using Trizol Reagent (ThermoFisher, Waltham, MA) according to manufacturer's protocol. cDNA was generated using SuperScript®VILO (Invitrogen, Carlsbad, CA). Quantitative real-time polymerase chain reaction was performed using SYBR Select (ThermoFisher, Waltham, MA).

Protein preparation and Western blotting. Protein was isolated from hepatocytes and liver tissue in lysis buffer and quantified by BCA. ACOT1 protein was determined by Western blotting using 80 µg of protein. Following SDS-PAGE, proteins were transferred to a PDVF membrane and probed for ACOT1 with a custom antibody. The *Mus musculus* ACOT1/ACOT2 antibody was developed in rabbit by injecting the antigen CSVAAVGNTISYKDET-amide (21st Century Biochemicals, Marlboro, MA).

ROS determination. Cellular ROS and reactive nitrogen species (RNS) was determined using the OxiSelect *In Vitro* ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, CA) in which dichlorodihydrofluorescin reacts with ROS or RNS to produce fluorescent 2',7'-dichlorodihydrofluorescein. Readings were measured at

480 nm excitation and 530 nm emission and compared to a standard curve generated with stock 2',7'-dichlorodihydrofluorescein solution.

Plasmids and cloning. DsRed-Express N1 plasmid was purchased from Clonetech (Mountain View, CA). *Acot1* cDNA was amplified from a pCMV6 *Acot1* plasmid purchased from Origene (Rockville, MD). *Acot1* cDNA was cloned into the multiple cloning site of DsRed-Express N1 by restriction enzyme digestion and T4 DNA ligase ligation. S232A point mutation was achieved using the QuikChange site-directed mutagenesis kit from Agilent and primers previously reported (9).

Cell and tissue imaging. Tissue sections were either froze in Tissue-Tek® O.C.T. (VWR, Eagan, MN) or fixed in 10% formalin and subsequently paraffin embedded. Slides were deparaffinized and blocked in 3% BSA, incubated with ACOT1 antibody overnight, stained with DAPI, and mounted. Immunohistochemistry for Cd45 was determined as well as Oil Red O and H&E stains from the University of Minnesota's BioNet Histology and IHC Lab. AML12 cells were cultured in DMEM media containing 10% FBS, 1% pen/strep, and 0.1% insulin-transferrin-selenium solution. Cells were lipid loaded overnight in serum free DMEM with 500 μM oleate, then pre-treated with 30 μM H89, a PKA inhibitor, for one hour before a ten minute treatment with 10 μM of a cell permeable cAMP analogue, 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-

Br-cAMP). Cells were fixed, stained for ACOT1, and DAPI and then mounted. COS7 cells were transfected using Effectene (Qiagen, Germantown, MD) transfection reagent with ACOT1-DsRed-Express. Cells were then treated and fixed as described for the AML12 studies.

PPARα reporter assays. COS7 or L cells were transfected with PPARα reporter construct (pSG5-GAL4-Pparα), firefly luciferase reporter plasmid (DK-MH-UASluc), control Renilla luciferase plasmid (pRLSV40), and empty DsRed-Express N1 plasmid, *Acot1*-DsRed-Express N1, or *Acot1*-S232A-DsRed-Express N1. Cells were treated in serum free media with 500 μM oleate for 16 hours then treated with 8-Br-cAMP or vehicle for 6 hours before harvesting for assessment of reporter activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase activity was normalized to Renilla luciferase activity.

Thioesterase activity assay. Thioesterase activity was determined as previously described (26) with minor modifications. Frozen liver samples were homogenized with a dounce homogenizer for 15 seconds. Homogenates were centrifuged at 100,000xg at 4°C for 1 hour and supernatants were collected. Samples were diluted in assay buffer (50 mM KCl, 10 mM HEPES) and 1mg of protein was loaded per well. DTNB was added at a working concentration of 50μM and read at 405nm at 37°C. Palmitoyl Co-A was added at 20 μM and read

at 405nm at 37°C for 3-5 minutes. CoA concentration was determined against a standard curve. Values were reported as nmoles CoA/min/mg protein.

Liver and serum TG. For Tyloxapol studies, animals received an intraperitoneal injection of tyloxapol (200 mg/kg) after a 16 hour fast and facial vein samples were collected for analysis at 0, 1, 2, and 3 hours. Serum triglyceride (TG) was assessed using the Stanbio LiquiColor® Triglycerides No. 2100 (Stanbio Laboratory, Boerne, TX).

Tissue FAs. Cellular FAs were determined by chloroform methanol extraction method. FA fraction was separated using thin layer chromatography and re-suspended in chloroform. Samples were dried under nitrogen and FAs were converted to fatty acid methyl esters (FAME) by heating them to 90°C for 1 hour in 5% HCl-methanol. FAMEs were re-suspended in hexane and subjected to Gas Chromatography Mass Spectrometry with an internal C17:0 standard for total and species quantification. Nuclear preps were obtained from frozen liver tissue through homogenization in a nuclear lysis buffer containing 10 mM Tris-HCL, 10 mM NaCl, 15 mM $MgCl_2$, 250 mM sucrose, 0.5% NP-40, and 0.1 mM EGTA, pH 7.5. Lysates were separated through a 30% sucrose cushion (10 mM Tris-HCL, 10 mM NaCl, 30% sucrose, 3 mM $MgCl_2$) at 1300xg. Nuclear pellets were washed and a Bradford assay was used to quantify proteins. FAs were collected

via Dole extraction method. Samples were re-suspended in ethanol and quantified using the Wako HR Series NEFA-HR (2) kit.

Pyruvate tolerance test. Eleven-week-old male mice received a tail vein injection of scramble control or *Acot1* shRNA. One-week post transduction mice were fasted overnight and received an intraperitoneal injected of 2 g/kg of sodium pyruvate in sterile saline. Blood glucose was measured via a tail clip at 0, 15, 30, 60, and 90 minutes.

Glycogen. Liver glycogen was quantified by first digesting glycogen to glucose by boiling the sample in 2 M hydrochloric acid for five minutes. Samples were cooled and neutralized with 2 M NaOH. Glucose was then quantified via Wako AutoKit Glucose (Mount View, CA).

Oxygen consumption rate. Seahorse XF analyzer was used to determine total oxygen consumption rate (OCR) in primary hepatocytes insolated from control and *Acot1* knockdown mice after an overnight fast. Cells were seeded in a collagen coated XF cell culture plates at 3 x 10^4 cells/well and incubated at 37° for 4 hours to allow cells to attach. Media was changed to XF Assay media with 5.5 mM glucose, 2 mM glutamax, and 1 mM pyruvate and placed in a CO_2 chamber for one hour. Basal oxygen consumption was measured for 30 minutes, followed by an injection of either BSA control or 500 µM of oleate and OCR was

determined for 30 minutes. Finally antimycin A was injected at 1 μM to determine non-mitochondrial oxygen consumption. Average basal OCR and OCR in response to BSA control or 500μM of oleate was calculated.

Statistical Analysis. Values are expressed as the mean ± SEM. Statistical significance was determined by Student's t-test.

Results

ACOT1 is increased during fasting. To assess the expression pattern and cellular distribution of ACOT1, we harvested mice that were fed or fasted for 16 hours. As expected, fasting resulted in a large induction of *Acot1* mRNA (Fig. 1A), which correlated to an increase in ACOT1 protein expression (Fig. 1B). Importantly, ACOT1 and ACOT2 are 93.7% homologues (27) and therefore the antibody generated for these studies recognizes both ACOT1 and ACOT2. However, ACOT2 is exclusively a mitochondrial protein (26) and previous groups have shown that ACOT2 runs below ACOT1 on Western blots (28). Therefore, to exhibit the specificity of our antibody, we compared our samples to those of mitochondrial preps from mouse liver overexpressing *Acot2* (26). ACOT1 in liver lysates runs above ACOT2 as shown in Supplemental Fig. 1. ACOT2 is barely detectable in whole liver lysates compared to ACOT1. Consistent with the Western blot, ACOT1 exhibited low expression during the fed state but was robustly increased after 16 hours of fasting. As such, our studies primarily focused on mice in the fasted state when ACOT1 is expressed.

ACOT1 has been described as a cytosolic protein, however to assess its cellular localization we stained liver sections for ACOT1. As expected, ACOT1 was not present in the fed state, but was abundant during fasting. Although ACOT1 was largely cytosolic, we observed ACOT1 in the nucleus under fasted conditions (Fig. 1C), which was subsequently confirmed with Western blotting of

nuclear fractions (Fig. 1D), suggesting a potentially undocumented nuclear role of ACOT1.

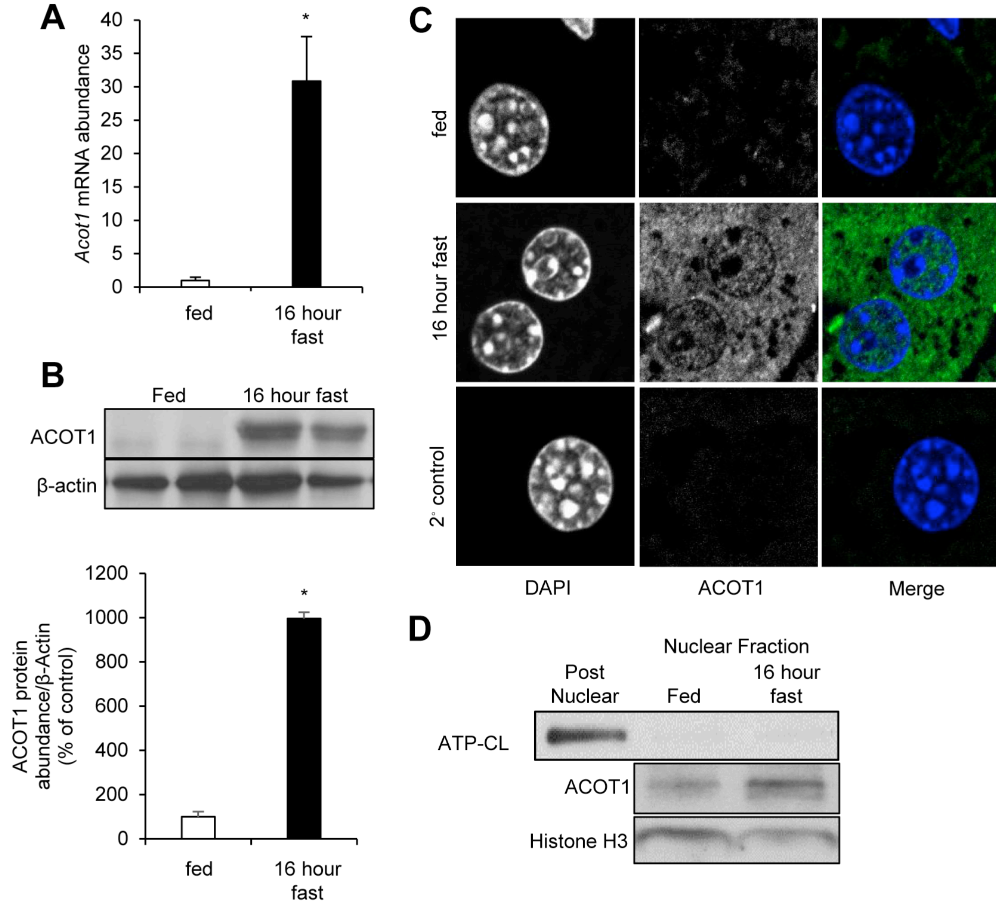
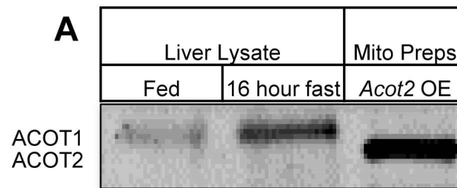


Figure 1. Hepatic ACOT1 is increased in fasting. C57Bl/6J mice were either fed or fasted overnight (16 hrs). (A) *Acot1* expression was increased with fasting as analyzed by RT-PCR (n=5). (B) Western blot and densitometry of ACOT1 in fed and fasted mice. (C) Fixed liver sections from fed or fasted (16 hrs) mice were probed with the ACOT1 primary antibody and Alexa 488 secondary. (D) Western blots of nuclear preps from fed and fasted mice. *P<0.05.



Supplemental Figure 1. ACOT1 antibody distinguishes between ACOT1 and ACOT2. (A) The ACOT1 antibody recognizes mouse ACOT1 and ACOT2. Liver homogenates from fed and 16 hour fasted mice were compared to mitochondrial preps from mice overexpressing *Acot2*.

***Acot1* knockdown reduces fasting liver TG by enhancing TG turnover and increases oxidation of endogenous and exogenous FAs.** To determine the contribution of ACOT1 to hepatic energy metabolism, we used adenoviral delivered shRNA to knockdown *Acot1* in the liver. Seven days post transduction, control mice (cont. shRNA) or liver *Acot1* knockdown mice (*Acot1* shRNA) (n=7-9) were fasted for 16 hours prior to sacrifice. We observed a significant reduction (~60%) in both *Acot1* mRNA (Fig. 2A) and protein (Fig. 2B). Immunohistochemistry exhibited reduced cytosolic and nuclear ACOT1 with *Acot1* knockdown (Fig. 2C). This reduction in ACOT1 expression correlated with reduced thioesterase activity (Fig. 2D).

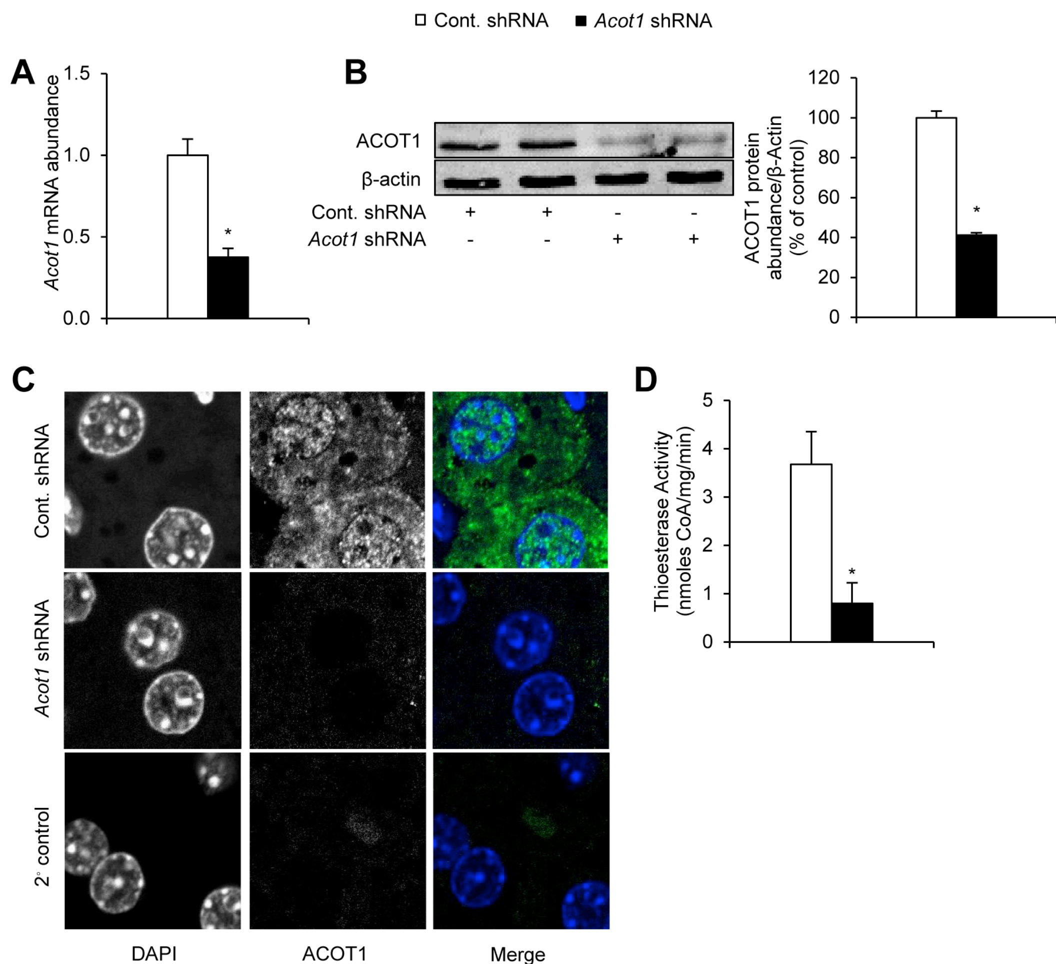


Figure 2. Hepatic *Acot1* knockdown reduces ACOT1 protein and thioesterase activity. *Acot1* expression is reduced in response to *Acot1* knockdown as analyzed by RT-PCR (A) and Western blotting of liver lysates (B) 7 days post transduction (n=7-9). (C) Immunohistochemistry of ACOT1 from fix liver sections indicates reduced ACOT1 protein with *Acot1* knockdown. (D) *Acot1* knockdown reduced thioesterase activity (n=3-4). *P<0.05.

Acot1 knockdown significantly reduced hepatic triglyceride content as determined by enzymatic assays and imaging of lipid droplets in H&E and Oil Red O stained liver sections (Fig. 3A-B). To confirm these effects were specific to the fasted state, we harvested control and *Acot1* knockdown mice in the fed state when *Acot1* is not expressed. As expected, there were no effects of *Acot1* knockdown on liver lipid droplet accumulation in fed mice consistent with its low expression in the fed state (Supplemental Fig. 2A). These results suggest that ACOT1 predominantly regulates hepatic lipid metabolic pathways involved in fasting such as FA oxidation.

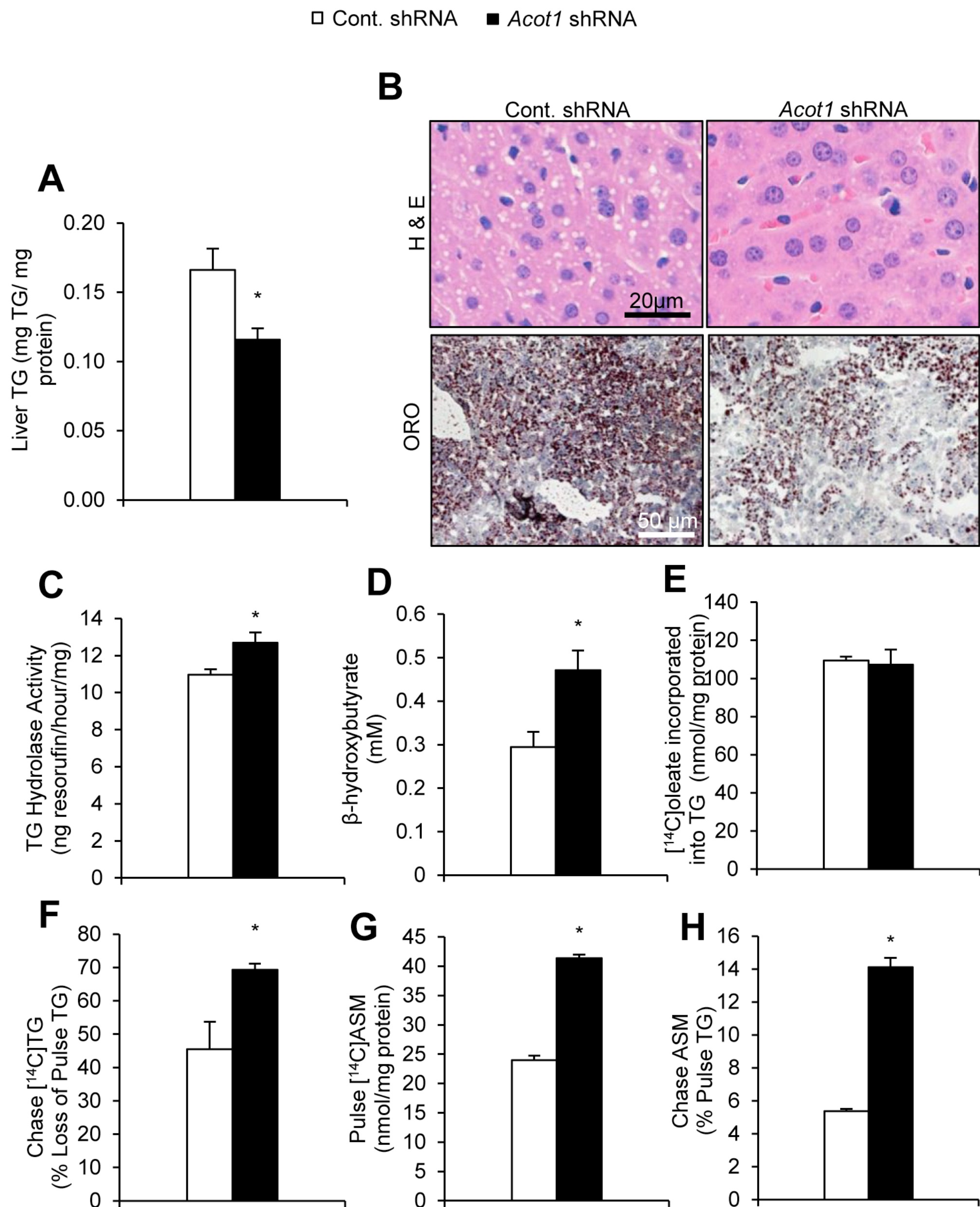
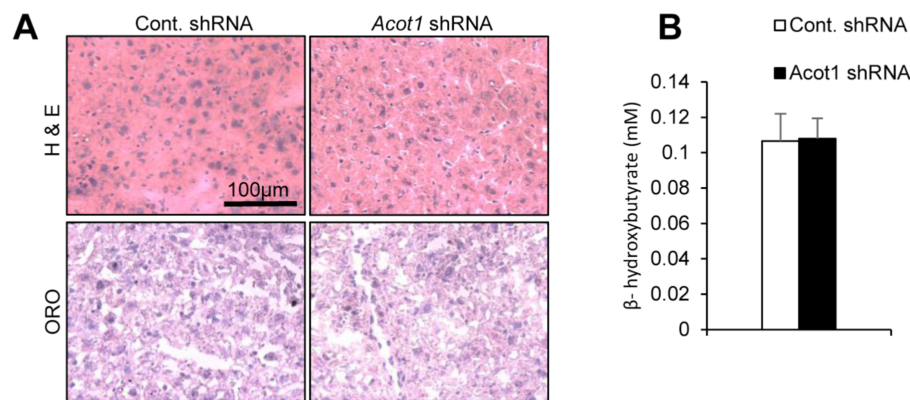


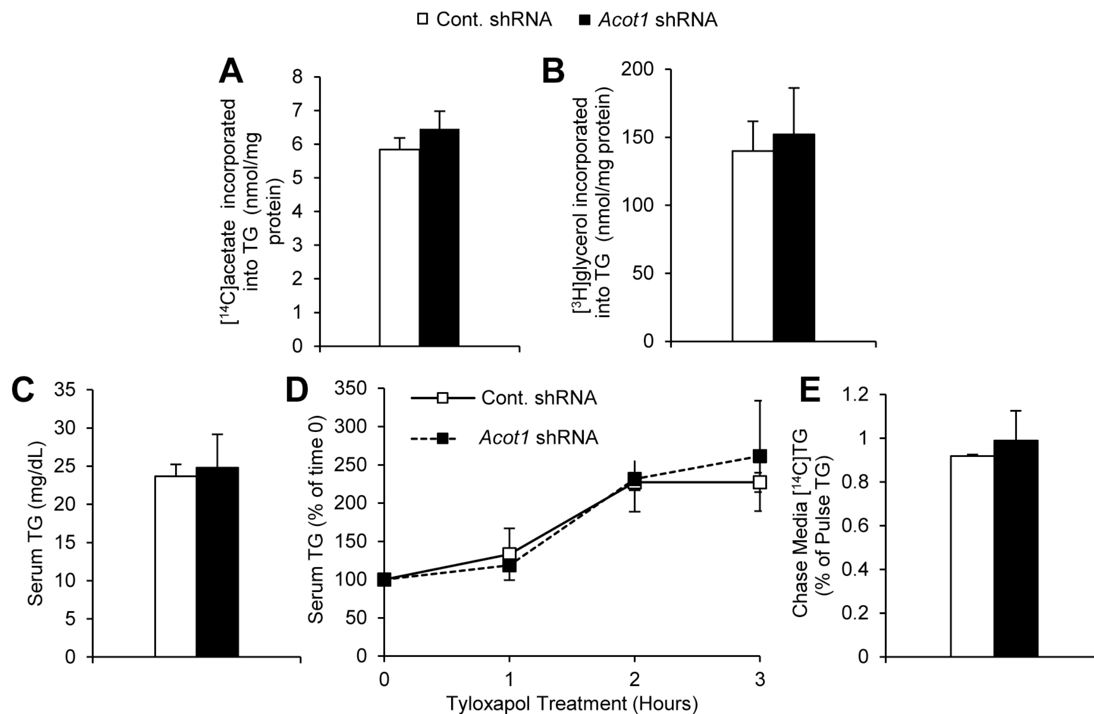
Figure 3. ***Acot1* knockdown increases hepatic FA oxidation.** (A) *Acot1* knockdown reduced hepatic TG. (B) H&E and Oil Red O stains of liver tissue show reduce lipid droplets in the *Acot1* shRNA treatment group (n=7-9). (C) TG hydrolase activity was increased in liver tissue homogenates of mice treated with

Acot1 shRNA (n=4). (D) Serum β-hydroxybutyrate was increased following hepatic *Acot1* knockdown in 16 hour fasted mice. (E) *Acot1* knockdown did not influence [^{14}C]oleate incorporation into cellular triglyceride (TG). (F) TG turnover as measured by [^{14}C]TG loss during the chase period was increased in response to *Acot1* knockdown. *Acot1* knockdown increased the oxidation of exogenous (G) and endogenous (H) FAs as measured by [^{14}C]acid soluble metabolites (ASM) in the media (n=3).



Supplemental Figure 2. *Acot1* knockdown effects on hepatic lipid metabolism in the fed state. Control and *Acot1* knockdown mice were harvested in the fed state. (A) Liver tissue were frozen in Tissue-Tek® O.C.T. for H&E and Oil Red O staining. (B) Serum was assessed for β-hydroxybutyrate.

Although predominantly involved in fasting lipid metabolism, ACOT1 may be reducing hepatic TG through a reduction in *de novo* lipogenesis (DNL) and/or TG synthesis, enhanced TG secretion in the form of very-low density lipoprotein (VLDL), or enhanced TG breakdown and FA oxidation. Thus, we assessed each of these possibilities to confirm exactly which pathway(s) were altered leading to reduced hepatic TG content in response to *Acot1* knockdown during fasting. To assess DNL and TG synthesis, we performed radiolabel experiments in primary hepatocytes isolated from mice transduced with scramble control or *Acot1* shRNA adenoviruses. We incubated cells for 2 hours with [^{14}C]acetate or [^{3}H]glycerol and measured incorporation of the label into the TG fraction. Rates of incorporation of either acetate or glycerol into the TG fraction were similar between treatment groups (Supplemental Fig. 3A-B) suggesting that DNL and TG synthesis are unaffected by *Acot1* knockdown. *Acot1* knockdown had little effect on serum TG (Supplemental Fig. 3C) or hepatic TG secretion based on serum TG from animals treated with tyloxapol (Supplemental Fig. 3D), suggesting no change in VLDL secretion. These results were confirmed with radiolabeling studies in primary hepatocytes, which showed that *Acot1* knockdown had no effect on secreted TG (Supplemental Fig. 3E). Together, these data suggest that changes in DNL, TG synthesis, or VLDL assembly and secretion are unaffected by *Acot1* knockdown.



Supplemental Figure 3. Liver specific knockdown of *Acot1* does not affect DNL, TG synthesis, or VLDL secretion. Pulse-chase experiments were conducted with [^{14}C]glycerol, [^{14}C]acetate, and [^{14}C]oleate. Incorporation of [^{14}C]acetate (A) and [^{14}C]glycerol (B) into the triglyceride (TG) fraction after the 2 hour pulse was similar between treatments. (C-D) *Acot1* knockdown does not alter fasting serum TG (C) or changes in serum TG in response to tyloxapol to measure rates of VLDL secretion (D). Media [^{14}C]TG after the 6 hour chase was similar between groups (E).

We next tested the effects of *Acot1* knockdown on TG hydrolysis. There was a significant increase in TG hydrolase activity in tissue homogenates from livers treated with *Acot1* shRNA compared to control livers (Fig. 3C). Enhanced lipolysis paralleled an increase in serum β-hydroxybutyrate (Fig. 3D) indicative of enhanced FA oxidation. We again confirmed these effects were specific to the fasted since *Acot1* knockdown did not alter β-hydroxybutyrate in fed mice (Supplemental Fig. 2B). These data support a role for ACOT1 in mitigating the flux of acyl-CoAs to mitochondrial β-oxidation in the fasted state. To further assess alterations in FA flux, *in vitro* pulse-chase experiments were performed. Similar to the unchanged TG synthesis from acetate or glycerol (Supplemental Fig. 3A-B), incorporation of [^{14}C]oleate into the cellular TG pool was similar between treatment groups (Fig. 3E). Consistent with increased TG hydrolysis in livers from *Acot1* knockdown mice, turnover of [^{14}C]TG was significantly greater with *Acot1* knockdown in primary hepatocytes (Fig. 3F). Additionally, FA oxidation was significantly increased in both the pulse (Fig. 3G) and chase periods (Fig. 3H) in the *Acot1* knockdown hepatocytes, suggesting there is an increase in oxidation of both endogenously produced and exogenously taken up FAs.

We next assessed oxygen consumption rate (OCR) in primary hepatocytes from control and *Acot1* knockdown mice using the Seahorse XF analyzer. As expected, *Acot1* knockdown hepatocytes exhibited a significant increase in OCR with the addition of FAs compared to BSA control (Fig. 4A-B);

although basal rates were numerically higher they did not reach significance. These results support our findings that *Acot1* knockdown increases oxidation of endogenous and exogenous FAs.

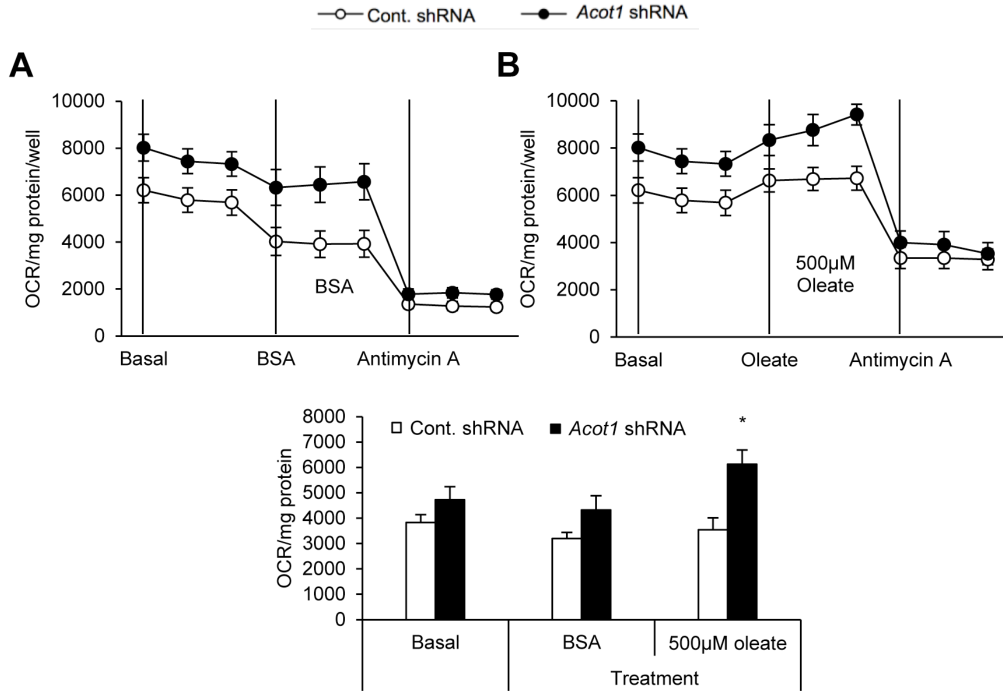
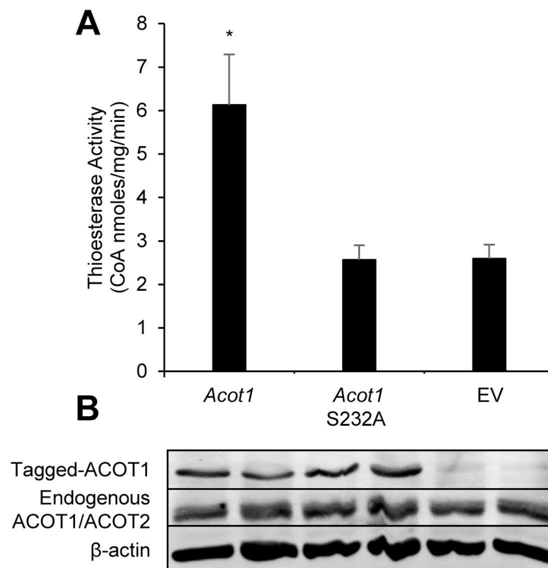
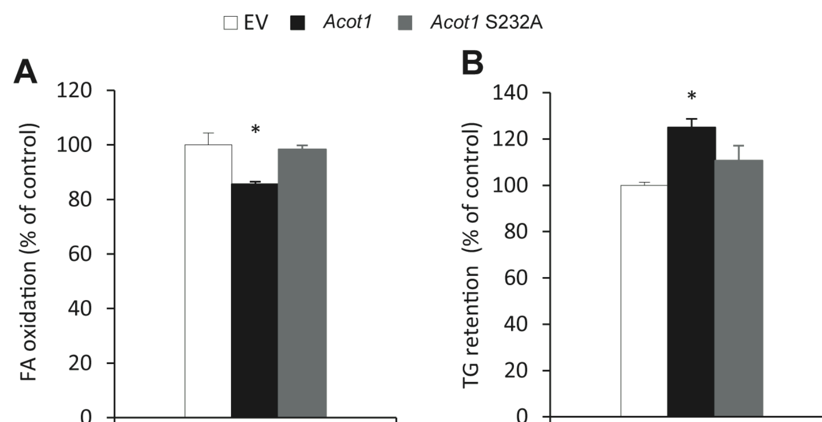


Figure 4. Hepatic *Acot1* knockdown increases oxygen consumption. Primary hepatocytes were isolated from control and *Acot1* knockdown mice. OCR was determined in seahorse analyzer XF in the presence of (A) BSA control or (B) 500μM oleate.

To confirm the enzymatic importance of ACOT1 in regulating FA oxidation and TG turnover, we next transfected L cells with an empty DsRed-Express N1 vector (EV), *Acot1* DsRed-Express N1 (*Acot1*), or a catalytically dead mutant *Acot1*-S232A-DsRed-Express N1 (*Acot1* S232A) and performed a pulse-chase with [^{14}C]oleate. Expression and thioesterase activity of these mutants were confirmed in Supplemental Fig. 4. As expected, thioesterase activity was increased with *Acot1* overexpression but was blunted with the *Acot1* S232A mutant, confirming that the mutant was catalytically dead. These results were not due to differences in expression between the two mutants, apparent by the ACOT1 Western blot. Cells transfected with *Acot1* exhibited less FA oxidation and more TG retention (Supplemental Fig. 5) compared the EV and *Acot1* S232A mutant, indicating the catalytic activity of ACOT1 is necessary for its regulation of FA oxidation and TG turnover. Taken together, these data suggest that *Acot1* knockdown reduces hepatic TG levels through increased TG turnover and enhanced oxidation of both exogenous and endogenous FAs.



Supplemental Figure 4. *Acot1* S232A ablates catalytic activity. *Acot1*-DsRed Express N1 (*Acot1*) and *Acot1*-S232A-DsRed Express N1 (*Acot1* S232A) express similarly in COS7 cells (A) compared to the DsRed Express N1 (EV) yet *Acot1*-S232A has no thioesterase activity.



Supplemental Figure 5. ACOT1 overexpression on lipid trafficking. L cells were transfected with *Acot1*-DsRed Express N1 (*Acot1*) and *Acot1*-S232A-

DsRed Express N1 (*Acot1* S232A) and treated with trace [^{14}C]oleate along with 500 μM unlabeled oleate bound to BSA (3:1 molar ratio) overnight, then chased for 6 hours with isotope free media. Compared to the DsRed Express N1 (EV), and catalytically dead mutant, *Acot1*-S232A-DsRed Express N1 (*Acot1* S232A), *Acot1*-DsRed Express N1 (*Acot1*) had less (A) FA oxidation and greater (B) TG retention.

ACOT1 regulates PPARα activity. Since there was an observed increase in FA oxidation, we next measured the expression of target genes of PPARα, the principal transcription factor governing hepatic FA oxidation (7), and peroxisome proliferator-activated receptor gamma co-activator (PGC1α), a co-activator of PPARα (29). Surprisingly, *Acot1* knockdown reduced expression of PPARα and PGC1α target genes (Fig. 5A-B). In addition, catalase protein expression, a marker of peroxisome content, and mitochondrial DNA abundance were reduced with *Acot1* knockdown (Fig. 5C-D). When combined with the aforementioned effects on FA oxidation, these results suggest that ACOT1 uncouples FA oxidation from the upregulation of genes and machinery involved in oxidative metabolism.

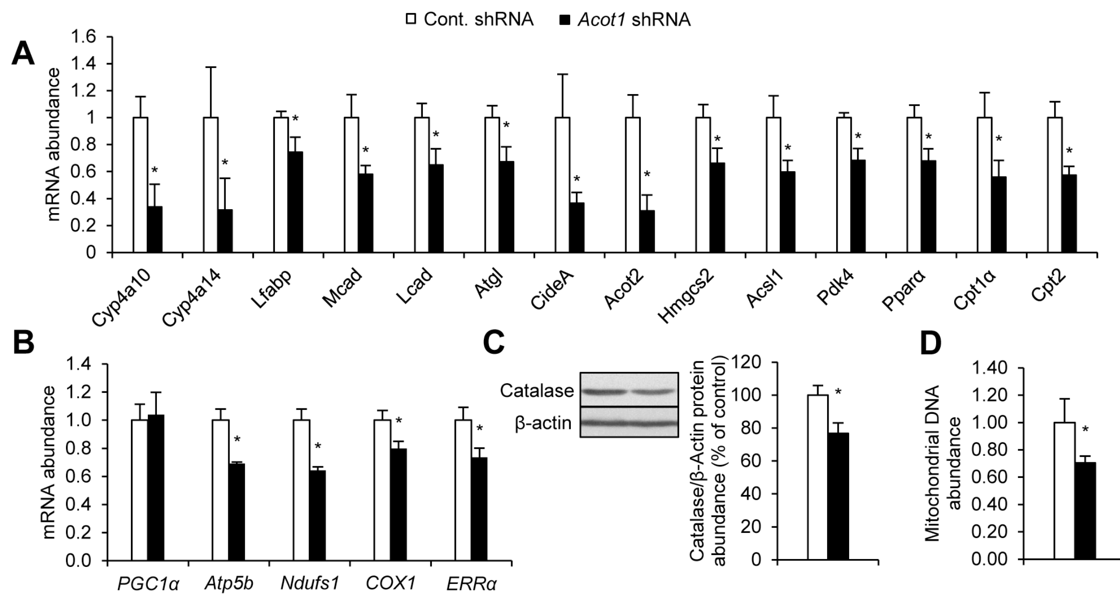


Figure 5. ACOT1 regulates expression of PPARα target genes, PGC1α target genes, and peroxisome and mitochondria abundance. Expression of (A) PPARα target genes and (B) PGC1α target genes were reduced with *Acot1* knockdown (n=7). (C) Catalase protein expression, a marker of peroxisome abundance, was reduced with *Acot1* knockdown; densitometry represents n=5. (D) Mitochondrial DNA abundance was reduced with *Acot1* knockdown.

We next speculated that the overexpression of ACOT1 could drive PPARα activity. To test this, COS7 cells and L cells were transfected with an EV, *Acot1* or *Acot1*-S232A plasmids and treated with vehicle or 8-Br-cAMP. The combination of 8-Br-cAMP and ACOT1 enhanced PPARα reporter activity more than 8-Br-cAMP alone in COS7 cells (Fig. 6A) and L cells (Fig. 6B). Moreover, induction of PPARα reporter activity by ACOT1 was dependent on its catalytic activity, suggesting that ACOT1 regulates PPARα through production of FA ligands.

8-Br-cAMP promotes ACOT1 nuclear translocation. Although characterized as a cytosolic protein, the data in Fig. 1C-D and Fig. 2C, suggest that ACOT1 is also present in the nucleus. Since ACOT1 has no predicted nuclear localization sequence (30), yet has numerous putative PKA phosphorylation sites (31), we suspected that nuclear location of ACOT1 could be driven by elevated cAMP/PKA signaling during fasting. To test the translocation of ACOT1 under β-adrenergic stimulation, AML12 cells were treated with H89, a PKA inhibitor, for 1 hour followed by 8-Br-cAMP for an additional 10 minutes before cells were fixed and stained for ACOT1. Nuclear ACOT1 increased with the addition of 8-Br-cAMP, however this effect was blocked by the PKA inhibitor H89 (Fig. 6C-D). Nuclear localization of ACOT1 was also confirmed in COS7 cells transfected with *Acot1* DsRed-Express N1 treated with vehicle or 8-Br-cAMP (Fig. 6E). ACOT1 signal was detectable in the nucleus in response to 8-Br-cAMP, but not when

treated with a vehicle. Previous groups have identified both FAs and acyl-CoAs in the nucleus (32), in which acyl-CoAs antagonize PPARα and FAs activate PPARα (33). In addition, saturated fatty acids have been shown to be endogenous to PPARα (34), while ACOT1 shows substrate preference toward saturated acyl-CoA species (9). These data suggest a link between ACOT1 substrate and PPARα ligands. Combined with our previous data showing ACOT1 catalytic activity is necessary for PPARα reporter activity (Fig. 6A-B), we speculate that nuclear ACOT1 regulates nuclear pools of saturated acyl-CoAs and free FAs to control PPARα activity during fasting. Surprisingly, we found no difference in cytosolic or nuclear free FAs with *Acot1* knockdown (Supplemental Fig. 6). Free FAs are lipotoxic substances that are tightly regulated inside cells (1). It is possible that the ACOT1-derived FA pool may be too small or transient to see distinct differences in total quantity or that FAs are selectively channeled to influence PPARα activity. Primarily ACOT1 shows preference toward saturated acyl-CoAs (9), therefore activity may not change the entire nuclear pool of FAs, rather selectively increase the presence of free saturated FAs.

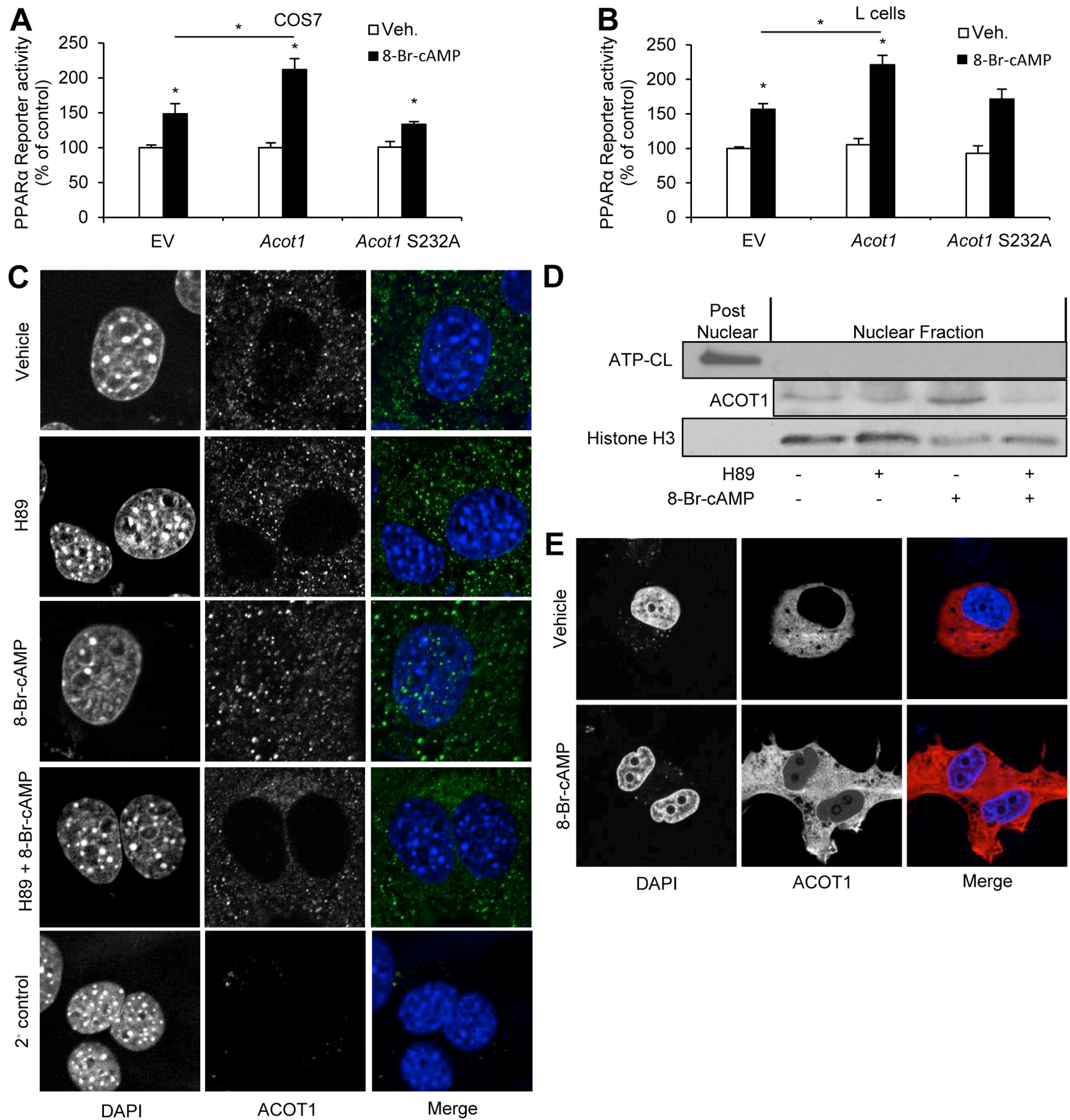
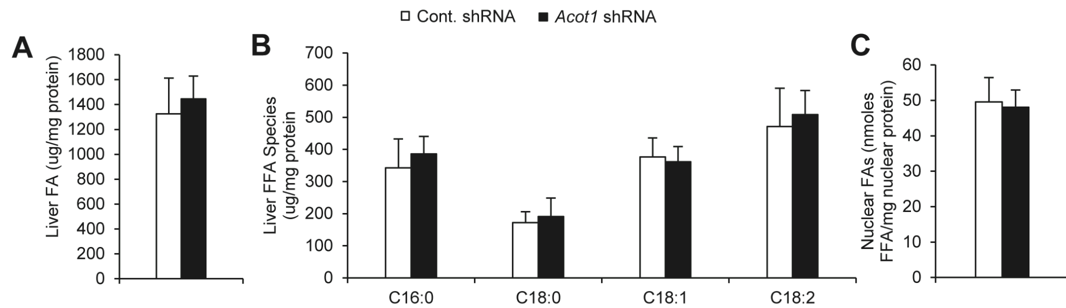


Figure 6. ACOT1 regulates PPARα activity and partially localizes to the nucleus. (A) COS7 and (B) L cells were transfected with an empty DsRed Express N1 (EV), *Acot1*-DsRed Express N1 (*Acot1*), or *Acot1*-S232A-DsRed Express N1, and reporter assay components (n=6-12). Cells were harvested and luciferase activity was assessed. ACOT1 translocated to the nucleus in AML12

cells in response to treatment with 8-Br-cAMP. Pretreatment with H89 blocked the translocation of ACOT1 in response to 8-Br-cAMP in (C) immunofluorescent staining and (D) nuclear fractionation and Western blotting. (E) An ACOT1 fusion construct (DsRed) showed partial nuclear localization in response to 8-Br-cAMP in COS7 cells. *P<0.05.



Supplemental Figure 6. Hepatic *Acot1* knockdown had no effect on hepatic FA content. (A) Total liver FAs were not changed with *Acot1* knockdown as well as no significant change in abundance of (B) specific FA species. (C) Nuclear preps were isolated from liver tissue FAs were extracted via DOLE extraction method, and quantified.

ACOT1 is important for mitigating oxidative stress due to enhanced FA oxidation during fasting. Enhanced FA oxidation can lead to the production of ROS in the electron transport chain leading to oxidative stress (35). In response to *Acot1* knockdown, we observed enhanced FA oxidation (Fig. 3D) yet a reduction in oxidative machinery as indicated by reduced expression of PPARα targets (Fig. 5A-E). Such effects could lead to enhanced ROS generation and oxidative stress. Supportive of this logic, *Acot1* knockdown livers exhibited significantly more ROS than the control livers (Fig.7A). In addition to increased ROS, *Acot1* knockdown increased the expression of oxidative stress markers heme oxygenase 1 (*Ho-1*) and uncoupling protein 2 (*Ucp2*) (Fig. 7B). Increases in oxidative stress promotes inflammation (15) and PPARα is well-documented to be important for anti-inflammatory pathways (13,14). Thus, the reduction in PPARα targets along with increased oxidative stress suggests *Acot1* knockdown could promote inflammation. Indeed, there was a significantly increase in expression of inflammatory markers *Tnfα, Cd11c, Il-1β,* and *F4/80* (Fig. 7C). Consistent with enhanced inflammatory gene expression, Cd45 immunohistochemistry revealed enhanced immune cell infiltration in livers with *Acot1* knockdown (Fig. 7D). Similarly, immune cell infiltration can also be observed in the H&E micrographs (Fig. 3B). These results suggest that ACOT1 mitigates oxidative stress and inflammation during fasting, potentially through enhancing PPARα activity.

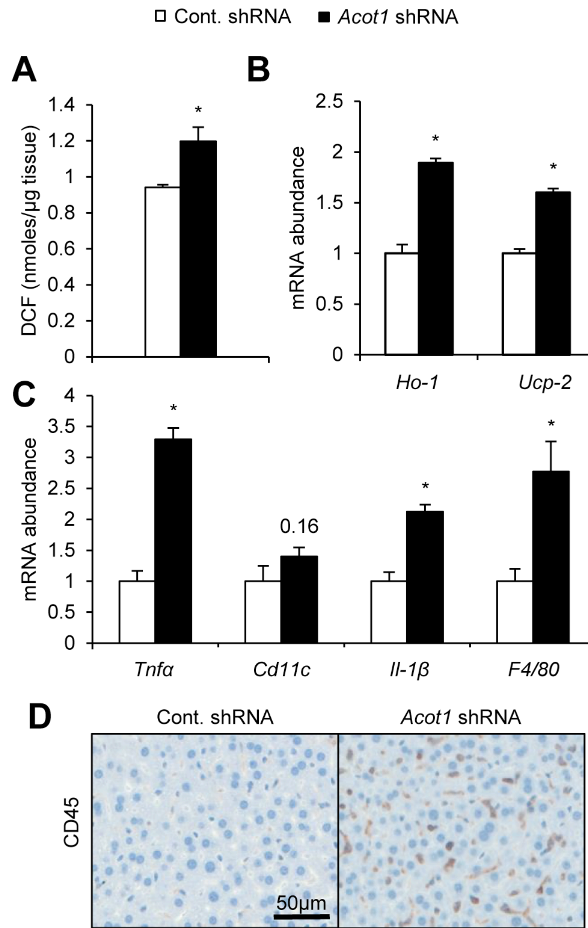
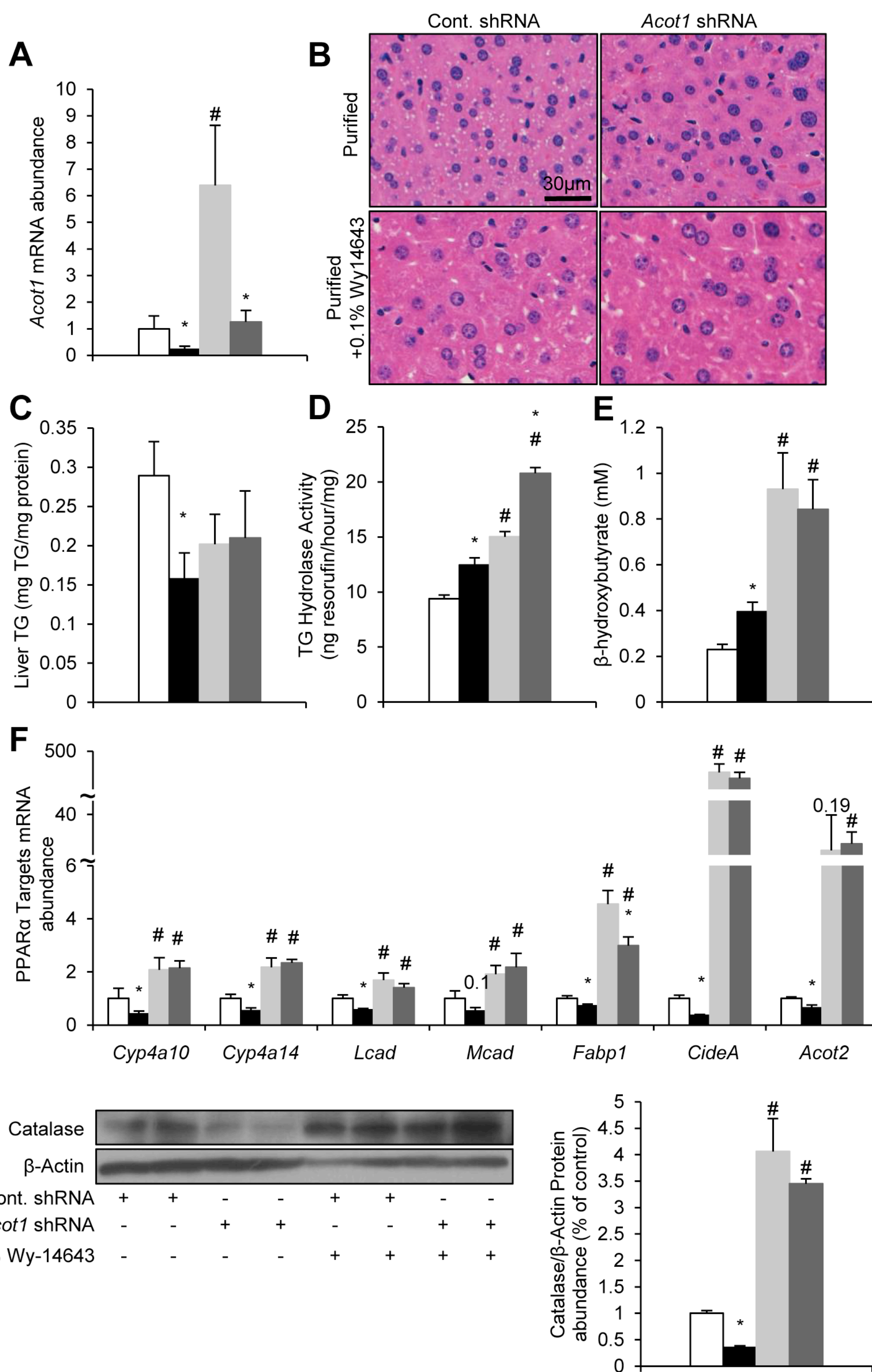


Figure 7. Hepatic *Acot1* knockdown increases inflammation and oxidative stress in mice. (A) *Acot1* knockdown increased intracellular ROS measured by OxiSelect *in vitro* ROS/RNS Kit (n=4). (B) *Acot1* knockdown increased expression of the oxidative stress genes heme oxygenase 1 (*Ho-1*) and uncoupling protein 2 (*Ucp2*). (C) Expression of inflammatory markers are increased in mice treated with *Acot1* shRNA (n=7). (D) CD45 immunohistochemistry stains from liver tissues revealed increased immune cell infiltration following *Acot1* knockdown (n=2). *P<0.05.

The effects of *Acot1* knockdown can be rescued by PPARα agonism. To further explore the potential role of ACOT1 in supplying FA ligands for PPARα activation, we attempted to rescue the effects of ACOT1 knockdown by feeding mice a diet supplemented with a synthetic PPARα ligand, Wy-14643. Mice were treated with adenovirus as described above and fed a purified control diet or a diet supplemented with 0.1% Wy-14643 (36) for one week. As expected Wy-14643 significantly increased expression of ACOT1, however, *Acot1* shRNA adenovirus significantly blunted this induction (Fig. 8A). Wy-14643 ablated the effects of *Acot1* knockdown on reducing liver TG (Fig. 8B-C). However, *Acot1* knockdown and Wy-14643 independently and together increased TG hydrolase activity (Fig. 8D). As seen above, *Acot1* knockdown increased serum β-hydroxybutyrate in mice fed the control diet, while Wy-14643 significantly increased serum β-hydroxybutyrate to a similar level between treatment groups (Fig. 8E). Moreover, Wy-14643 rescued the expression of PPARα target genes in mice treated with *Acot1* shRNA (Fig. 8F) as well as catalase protein expression (Fig. 8G). Since Wy-14643 is able to rescue the *Acot1* knockdown phenotype, it is likely that ACOT1 is responsible for providing FA ligands for activation of PPARα.



Cont. shRNA Purified diet
Acot1 shRNA Purified diet
Cont. shRNA Purified diet + 0.1% Wy
Acot1 shRNA Purified diet + 0.1% Wy
A
Acot1 mRNA abundance
B
Cont. shRNA
Acot1 shRNA
Purified
Purified +0.1% Wy14643
30μm
C
Liver TG (mg TG/mg protein)
D
TG Hydrolase Activity (ng resorufin/hour/mg)
E
β-hydroxybutyrate (mM)
F
PPARα Targets mRNA abundance
0.1
0.19
Cyp4a10
Cyp4a14
Lcad
Mcad
Fabp1
CideA
Acot2
G
Catalase
β-Actin
Cont. shRNA
Acot1 shRNA
0.1% Wy-14643
Catalase/β-Actin Protein abundance (% of control)

Figure 8. Wy-14643 rescues effects of *Acot1* knockdown. (A) *Acot1* mRNA following adenovirus treatments and 0.1% Wy-14643 supplementation (n=7-9). (B) H&E staining shows that Wy-14643 reduced and normalized lipid droplet accumulation in the *Acot1* knockdown treatment group. (C) Wy-14643 normalized liver TG levels between control and *Acot1* shRNA treatment groups. (D) TG hydrolase activity and (E) serum β-hydroxybutyrate concentrations are increased and normalized following Wy-14643 in *Acot1* shRNA treatment groups (n=4). (F) Wy-14643 increased PPARα target gene expression and rescued the effects of *Acot1* knockdown. (G) Catalase protein expression is increased by Wy-14643 and normalized in livers of *Acot1* shRNA treated mice (n=2). *$P<0.05$ vs. cont. shRNA group. $^{\#}P<0.05$ vs. control diet.

We next explored the possibility that Wy-14643 could also rescue the oxidative stress and inflammation observed in response to *Acot1* knockdown. Wy-14643 was able to normalize the expression of oxidative stress marker *Ho-1*, but was unable to normalize *Ucp2* expression in *Acot1* knockdown livers (Fig. 9A). Wy-14643 also normalized the expression of inflammatory genes and prevented immune cell infiltration in livers of *Acot1* knockdown mice (Fig. 9B-C). In summary, these data suggest that ACOT1 regulates PPARα as a means to influence hepatic energy metabolism, oxidative stress, and inflammation.

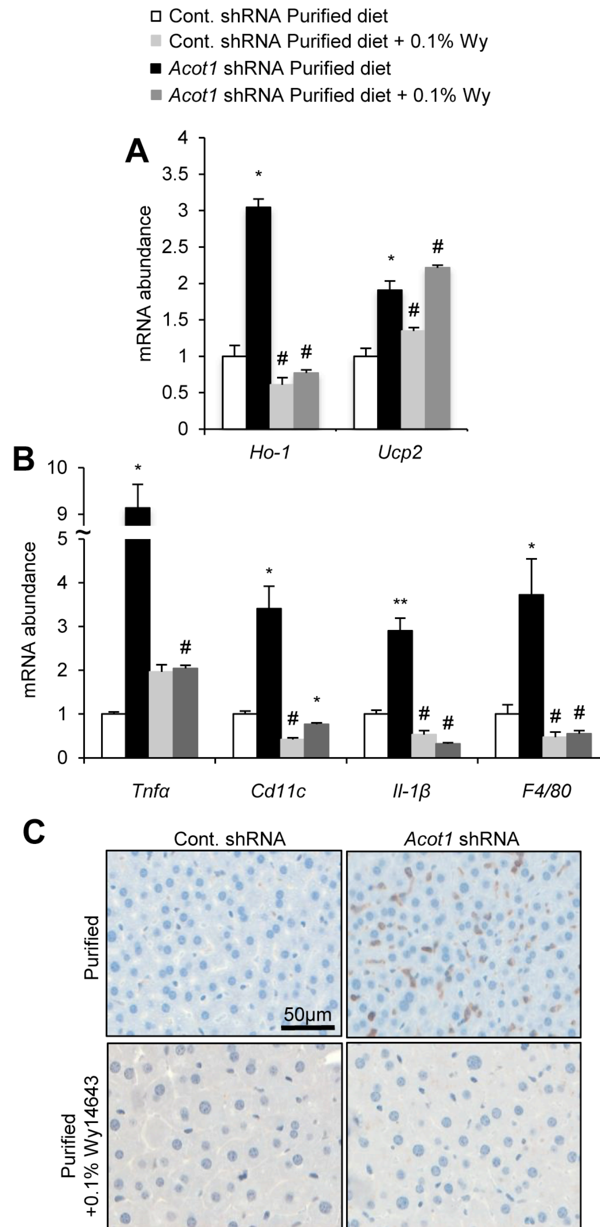


Figure 9. Wy-14643 rescues inflammation and oxidative stress markers. (A-B) Wy-14643 normalized the expression of most oxidative stress markers (A) and reduced the inflammatory markers observed with *Acot1* knockdown (n=7-9) (B). (C) Wy-14643 normalized macrophage infiltration assessed by Cd45

immunohistochemistry staining (n=2). *P<0.05 vs. cont. shRNA group. #P<0.05 vs. control diet.

The effects of *Acot1* knockdown in diet induced steatosis. NAFLD is a disease characterized by hepatic lipid accumulation. However, hepatic steatosis can then progress to inflammation and fibrosis (37). High fat diet feeding is known to cause steatosis in mice, however, it is unable to generate the fibrotic responses seen in NAFLD (38). Previously, we observed increased oxidative stress and inflammation in the absence of steatosis (Fig. 7). As such, we investigated the effects of *Acot1* knockdown in mice fed a high fat diet, which should induce steatosis but not result in fibrosis. Mice were fed a 45% fat diet for 12 weeks prior to an adenovirus delivery of cont. shRNA or *Acot1* shRNA. One week later mice were fasted overnight (16 hrs) and sacrificed. As seen previously, *Acot1* knockdown significantly decreased hepatic *Acot1* expression (Fig. 10A) and ACOT1 protein (Fig. 10B). As expected, abundant lipid droplet accumulation was observed in livers of mice fed the high fat diet, but *Acot1* knockdown had similar hepatic TG content compared to controls (Fig. 10C-D). *Acot1* knockdown increased serum β-hydroxybutyrate (Fig. 10E) despite unchanged expression of PPARα (Fig. 10F) or PGC1α (Fig. 10G) target genes. Despite the lack of reductions in gene expression, mitochondria content was reduced with *Acot1* knockdown (Fig. 10H). Most importantly, *Acot1* knockdown solicited a robust induction of inflammatory and oxidative stress markers (Fig.

10I), including α smooth muscle actin, a marker of fibrosis. *Acot1* knockdown also increased hepatic ROS/RNS species (Fig. 10J) and greater Sirius Red staining, a marker of fibrosis (Fig. 10K). Together these data suggest that under high fat diet induced steatosis, ACOT1 is protective against inflammation and oxidative stress that leads to fibrosis.

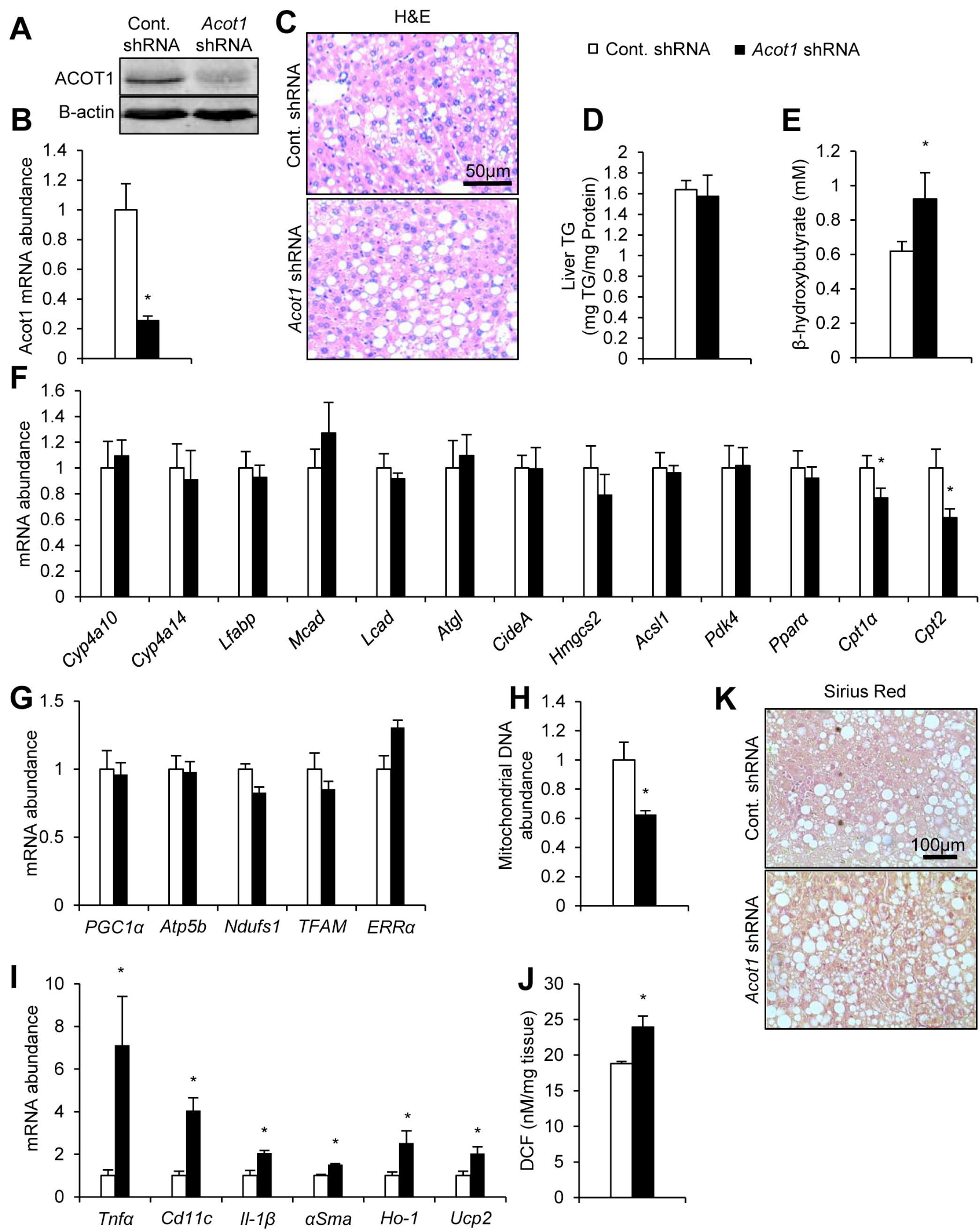


Figure 10. Hepatic *Acot1* knockdown exacerbates complications on a high fat diet. *Acot1* expression is reduced as measured by (A) Western blotting and (B) RT-PCR (n=8-10). *Acot1* knockdown had no effect on hepatic TG (C) H&E

and (D) liver TG quantification. (E) *Acot1* knockdown increased serum β-hydroxybutyrate. *Acot1* knockdown had no effect on (F) PPARα and (G) PGC1α target gene expression, yet significantly reduced total (H) mitochondrial abundance. (I) *Acot1* knockdown increased expression of inflammatory and oxidative stress genes. (J) *Acot1* knockdown increased intracellular ROS measured by OxiSelect *in vitro* ROS/RNS Kit (n=4). (K) Sirius Red staining indicated more fibrosis with *Acot1* knockdown.

ACOT1 regulates hepatic glucose metabolism. Since we observed a significant increase in hepatic FA oxidation and ketone production (Fig. 3), we speculated that *Acot1* knockdown might have an impact on fasting glucose storage and production. FA oxidation is the primary energy provider for hepatic gluconeogenesis (20). As such, previous groups have demonstrated that FA oxidation can drive hepatic gluconeogenesis (21). To test if hepatic FA oxidation could drive gluconeogenesis we assessed fasting serum glucose levels and found that *Acot1* knockdown significantly increased fasting glucose (Fig. 11A) We next performed a pyruvate tolerance test to assess gluconeogenesis in 16 hour fasted control and *Acot1* knockdown mice. There was a significant increase in glucose production in response to *Acot1* knockdown (Fig.11B-C). Together these results suggest that *Acot1* knockdown results in enhanced fasting gluconeogenesis.

Interestingly, hepatic glycogen was also robustly upregulated in *Acot1* knockdown mice (Fig. 11D-E). PPARα has been shown to increase glycogenolysis and rats fed a synthetic PPARα ligand exhibited depleted glycogen stores (39), however, the mechanism by which PPARα regulates hepatic glycogen synthesis or degradation is not well understood. Since Wy-14643 was able to normalize FA oxidation and PPARα target genes in *Acot1* knockdown mice (Fig. 8), we speculated that hepatic glucose metabolism would also be normalized. As expected, Wy-14643 reduced hepatic glycogen stores compared to control, but did not reach significance. Wy-14643 also normalized hepatic glycogen stores between control and *Acot1* knockdown mice (Fig. 12A-B). These results suggest hepatic glycogen stores seen with *Acot1* knockdown were due to reduced PPARα signaling.

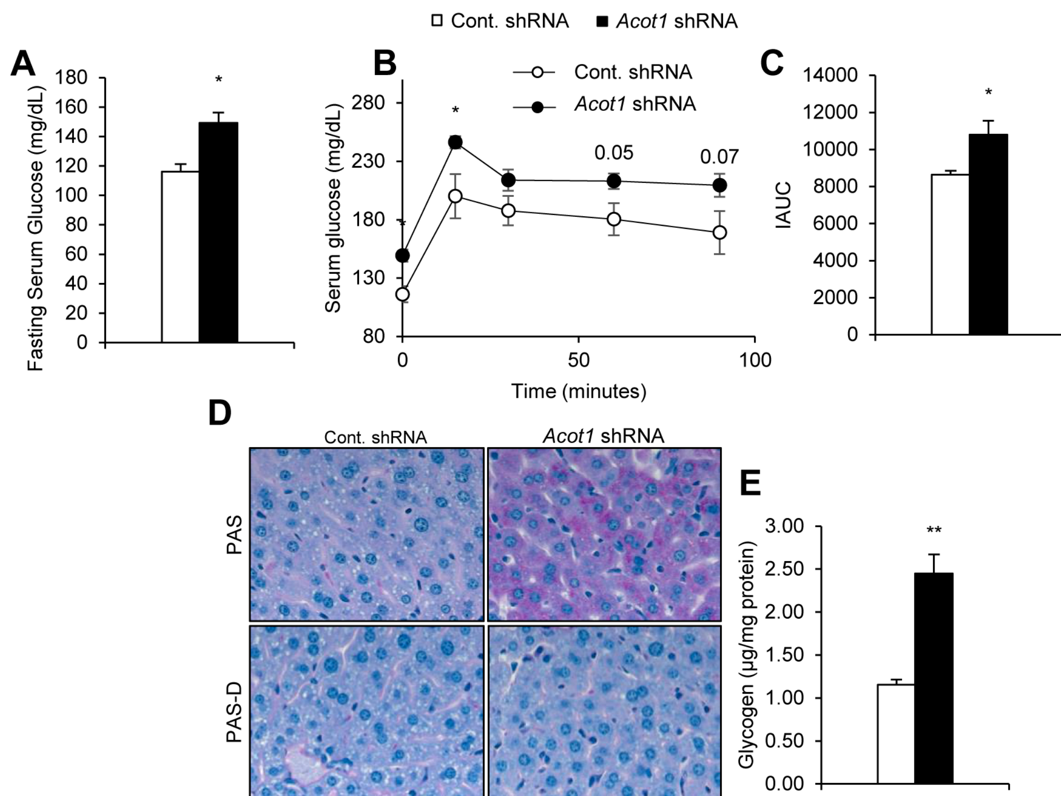


Figure 11. *Acot1* knockdown increases hepatic glycogen storage. (A) Fasting serum glucose was greater in *Acot1* knockdown mice. (B-C) *Acot1* knockdown slightly increased hepatic glucose production during a pyruvate tolerance test. (D) Periodic-Acid Schiff (PAS) and PAS with diastase (PAS-D) stains indicated greater glycogen storage in *Acot1* knockdown mice. (E) Hepatic glycogen was quantified by digesting glycogen and subsequently measuring glucose.

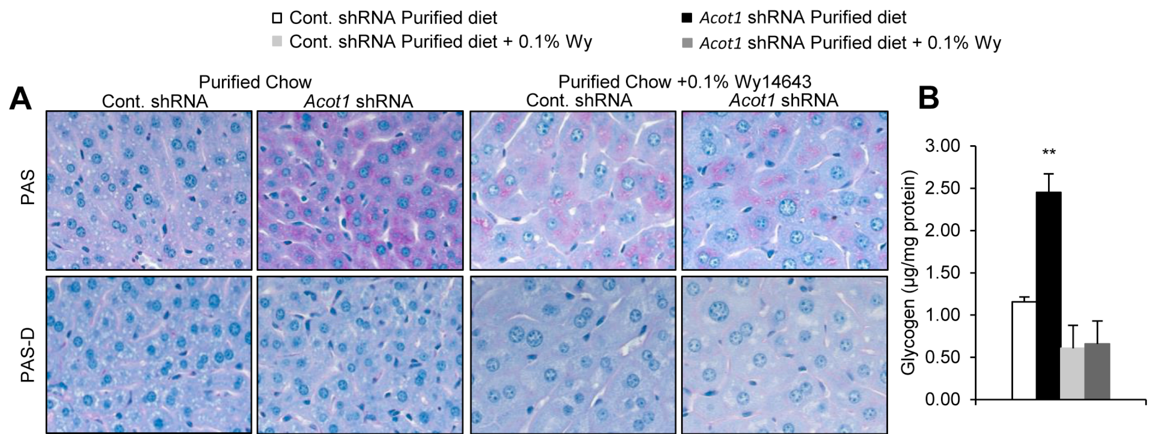


Figure 12. Wy-14643 normalized hepatic glucose metabolism. Wy-14643 normalized hepatic glycogen stores in *Acot1* knockdown measured by (A) PAS/PAS-D stains and (B) glycogen quantification.

Discussion

The liver imports FAs proportional to their concentration in the blood. Thus, increased rates of FA metabolism must match this uptake during times of fasting. Upon entering the liver, hepatic FAs are first converted to acyl-CoA molecules prior to their entry into most metabolic pathways (3). ACOT1 catalyzes the reverse reaction, producing free FAs (5), which should slow the flux of FAs to downstream metabolic pathways. Consistent with this logic, hepatic knockdown of *Acot1* increased the oxidation of FAs *in vivo* and *in vitro* (Fig. 3), while having no observed effect on TG synthesis or VLDL secretion (Supplemental Fig. 3). Taken together, these results suggest that ACOT1 specifically regulates FAs destined to oxidative pathways, potentially by removing acyl-CoAs as substrates. By slowing FA oxidation, ACOT1 may serve to protect the liver from detrimental effects of excessive FA oxidation.

PPARα is a dominant transcription factor in the liver, responsible for the upregulation of genes involved in FA oxidation during fasting (7,40). *Acot1* knockdown led to enhanced FA oxidation, which would be expected to correlate to greater expression of several PPARα target genes. However, PPARα targets were reduced (Fig. 4A) following *Acot1* knockdown despite the greater oxidative rate. Since FAs serve as endogenous ligands to activate PPARα (10), we speculated that ACOT1 provides FA ligands to activate PPARα. Therefore, administration of a synthetic PPARα ligand (e.g.Wy-14643) should rescue the effects of hepatic *Acot1* knockdown on PPARα signaling. Wy-14643 treatment

largely rescued the phenotype resulting from ACOT1 knockdown including normalization of FA oxidation and PPARα target genes (Fig. 8). We also tested if ACOT1 thioesterase activity was necessary for its regulation of PPARα. Expressing ACOT1 in COS7 or L cells increased PPARα reporter activity during 8-Br-cAMP stimulation. However, a catalytically dead ACOT1 mutant (*Acot1* S232A) had no effect on PPARα activity compared to the empty vector control (EV) (Fig. 6A-B), supporting the theory that ACOT1 thioesterase activity provides FA ligands to regulate PPARα. However, we found no difference in cellular FAs (Supplemental Fig. 6). As such, we surmise that ACOT1 specifically produces a local small pool of FAs to activate PPARα. Further studies are needed to determine if ACOT1 and PPARα interact, which would suggest ACOT1 hands FA ligands directly to PPARα.

ACOT1 overexpression alone was not sufficient to increase PPARα reporter activity, only during cAMP/PKA signaling did ACOT1 solicit PPARα activation (Fig. 6A-B). We observed nuclear localization of ACOT1 in response to 8-Br-cAMP (Fig. 6C-E), suggesting nuclear localization may be necessary for ACOT1 to activate PPARα. Similarly, *Acot1* knockdown reduced nuclear ACOT1 (Fig. 2C) and subsequently reduced PPARα transcripts (Fig. 5A). Thus, these data show that ACOT1 produces FA ligands to activate PPARα, increasing FA oxidative capacity. This regulation may be dependent on the nuclear localization of ACOT1 in response to fasting and cAMP/PKA signaling.

Normal mitochondrial oxidation produces a mild stress on cells resulting in the production of ROS (15). However, ROS production is balanced by mitochondrial antioxidant activity that protects the mitochondria from oxidative stress. During times of increased FA oxidation, ROS can exceed antioxidant capacity and lead to oxidative stress (41). In addition, PPARα signaling limits oxidative stress (42,43) and promotes the expression of anti-inflammatory genes (13). There was greater hepatic ROS and expression of oxidative stress markers in hepatic *Acot1* knockdown mice (Fig. 5A-B) that paralleled elevated rates of FA oxidation (Fig. 3) and reduced PPARα target gene expression (Fig. 5A). Additionally, there was greater expression of inflammatory genes and immune cell infiltration in response to *Acot1* knockdown (Fig. 7C-D). Thus, the observed inflammation in response to *Acot1* knockdown could be due to reduced PPARα dependent anti-inflammatory gene expression. Wy-14643 treatment rescued the oxidative stress and inflammation seen in *Acot1* knockdown livers (Fig. 9). As such, ACOT1 appears to protect the liver from oxidative stress and inflammation via promotion of PPARα signaling.

NAFLD is an inclusive title for a spectrum of liver disease states that includes steatosis, nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis (37). NAFLD is initially characterized by lipid droplet accumulation, followed by enhanced oxidative stress and inflammation that progresses the disease to NASH. Since *Acot1* knockdown had a significant impact on hepatic oxidative stress and inflammation in mice on a control diet potentially due to greater

oxidative metabolism, we speculated that *Acot1* knockdown could progress high fat diet induced hepatic steatosis to a fibrotic state. Unlike under basal conditions, weeklong *Acot1* knockdown did not significantly reduce hepatic TG, yet increased serum β-hydroxybutyrate. *Acot1* knockdown did not solicit the same effects on PPARα and PGC1α as seen in mice fed the control diet, but increased oxidative stress and inflammation ultimately leading to fibrosis. Current evidence suggests that high fat diets increases FA oxidation (44) which correlates with the increase in serum β-hydroxybutyrate we see in mice fed a high fat diet (Fig. 10E) compared to basal mice (Fig. 3D). This increase in FA oxidation is associated with the oxidative stress and inflammation that occurs as the disease progresses (45). Our findings support the importance of FA oxidation and its complications in the progression of NAFLD from steatosis to fibrosis.

Lastly, we investigated the role of FA oxidation on glucose homeostasis during prolonged fasting with *Acot1* knockdown. Hepatic FAs can inhibit glycogenolysis and FA oxidation can increase gluconeogenesis (21,37,46). As expected, *Acot1* knockdown resulted in greater glucose production and glycogen storage after a 16 hour fast (Fig. 11). Normally after a 16 hour fast glycogen is depleted by ~80% (47). Therefore these results suggest that *Acot1* knockdown causes FA oxidation that promotes gluconeogenesis and conserves hepatic glycogen stores.

To our knowledge, these are the first studies to characterize the physiological importance of ACOT1 in hepatic fasting lipid metabolism. This

study highlights a significant role for ACOT1 in regulating FA oxidation, PPARα activity, oxidative stress, and inflammation during fasting. In support of a protective role of ACOT1, humans with fewer copy numbers of the 14q24.3 locus where ACOT1 resides are more likely to develop NASH, a disease in which oxidative stress and inflammation are the defining characteristics (48). Similarly, overexpression of ACOT1 improves diabetic cardiomyopathy by reducing FA oxidation and ROS production (19). Thus, when combined, the few studies evaluating ACOT1 all highlight a potential beneficial role of ACOT1 in mitigating the negative effects of aberrant lipid metabolism.

Based on our current findings, ACOT1 plays a pivotal role in protecting livers by regulating FA oxidation and the ensuing oxidative stress and inflammation while simultaneously promoting PPARα activity (Fig. 13). In addition, ACOT1 locates to the nucleus during fasting, suggesting a potential local regulation of PPARα via the production of FA ligands. Thus, this work highlights the importance of ACOT1 as a branch point in regulating FA flux to oxidative pathways and controlling the transcriptional regulation of FA oxidation.

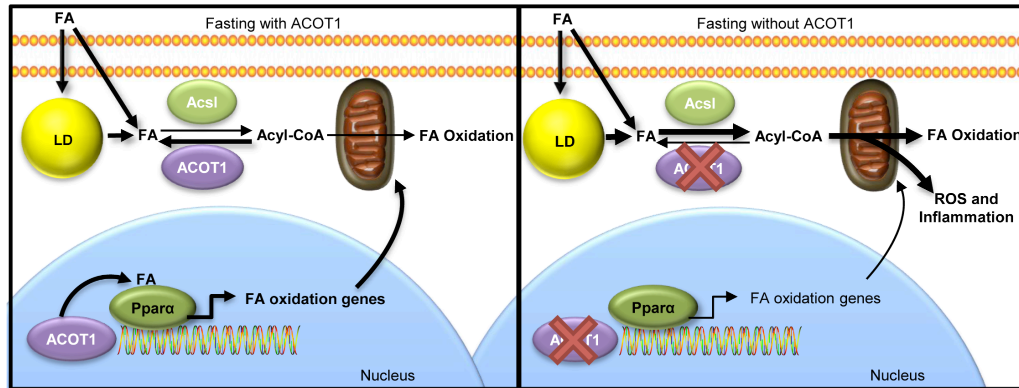


Figure 13. Proposed mechanism of ACOT1 in fasting liver lipid metabolism. ACOT1 expression during fasting mitigates FA flux toward oxidation in the cytosol. Under fasting and elevated cAMP/PKA stimulation, nuclear ACOT1 is important in the activation of PPARα. Fasting with reduced ACOT1 enhances FA oxidation with reduced PPARα activity that leads to oxidative stress and inflammation. Therefore, ACOT1 is an important enzyme for balancing oxidative capacity, oxidative stress, and inflammation with PPARα activity during fasting.

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Chapter 3

Whole-Body *Acot1* Knockout Protects From Adiposity and Hepatic Steatosis

Mallory Franklin wrote this chapter in its entirety

Acyl-CoA thioesterase 1 (ACOT1) catalyzes the hydrolysis of long chain acyl-CoAs to free fatty acids (FAs) and coenzyme A. ACOT1 is expressed predominantly in oxidative tissues such as liver, heart, and kidney during times of enhanced oxidative needs, such as fasting or exercise. The physiological relevance of ACOT1 in these tissues is poorly understood. We previously demonstrated that acute hepatic *Acot1* knockdown significantly reduced hepatic triglyceride (TG), enhanced gluconeogenesis, and resulted in oxidative stress and inflammation. Therefore, this study sought to determine the effects of whole-body *Acot1* knockout in mice fed a purified control diet (control) or a 45% fat diet (HFD) for 12 weeks. *Acot1* knockout protected from diet induced obesity in male and female mice. *Acot1* knockout reduced adipocyte size, but increased the number of adipocytes, suggesting greater hyperplasia and less hypertrophy. However, discrepancies in weight gain were not accounted for by changes in energy expenditure, locomotion, or food intake. *Acot1* knockout also reduced hepatic lipid accumulation during fasting and protected mice from HFD induced steatosis. Reduced hepatic TG correlated to a reduction in hepatic reactive oxygen species (ROS). However, an oral glucose tolerance test (OGTT) revealed impaired glucose tolerance in *Acot1* knockout mice. In conclusion, these studies suggest that *Acot1* is physiologically relevant in adiposity and ectopic lipid accumulation in basal conditions and in response to high fat feeding.

Introduction

Dietary fat, *de novo* lipogenesis (DNL), and reduced FA oxidation contribute to increased lipid storage, a defining feature of obesity and the metabolic syndrome (1). Increased adiposity leads to ectopic lipid accumulation in tissues such as liver, heart, and muscle. (2). Hepatic TG accumulation is the initial characteristic of non-alcoholic fatty liver disease (NAFLD). Progressive states of NAFLD are defined by increased oxidative stress, inflammation, and impaired mitochondrial function (3). In addition, the development of NAFLD is strongly correlated to the risk of insulin resistance (4). Although TG accumulation itself is not considered harmful, numerous lipid intermediates act as signaling molecules that contribute to the pathophysiology of ectopic lipid accumulation (5). As such, understanding the regulation of lipid channeling and signaling may explain the mechanism by which ectopic lipid leads to inflammation and insulin resistance.

A family of acyl-CoA synthetases generate acyl-CoA molecules from free FAs and coenzyme A (6). Acyl-CoAs are then substrates for many metabolic pathways including FA oxidation or synthesis of complex lipid species including TG (7). A family of acyl-CoA thioesterases are responsible for cleaving acyl-CoA molecules back to free FAs (8). These acyl-CoA thioesterases are believed to serve as regulator enzymes, maintaining the level of acyl-CoAs and free FAs (8–11). In particular, ACOT1 is the primary cytosolic thioesterase found in oxidative tissue, including the heart, liver, kidney, and muscle. ACOT1 responds to circadian cues including feeding and fasting, exercise, and light and dark cycles

(12–14). ACOT1 predominately cleaves long chain acyl-CoA molecules (15,16), rendering it an important enzyme for regulating the concentration and metabolic fate of both fatty acids and long chain acyl-CoAs.

We previously described the effects of acute *Acot1* knockdown in the liver. *Acot1* knockdown resulted in enhanced FA oxidation and reduced peroxisome proliferator-activated receptor α (PPARα) target gene expression. This combination led to increased oxidative stress and inflammation, a phenotype typical in advanced stages of NAFLD (17,18). These results suggest that ACOT1 may play a beneficial role in attenuating oxidative stress and inflammation. Therefore, we sought to determine the metabolic effects of chronic global whole-body *Acot1* ablation.

Research Design and Methods

Mouse handling. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. ACOT1 heterozygous mice were purchased from the NIH knockout mouse project. Heterozygous mice were bred and offspring were genotyped at 3 weeks of age to identify wild type (WT) and knockout (KO) littermates. DNA was isolated from tail clips using DNeasy blood and tissue kit from Qiagen (Germantown, MD) and genotyped based on PCR protocols provided by the NIH knockout mouse project. Primers to identify the gene and cassette were used to genotype litters. Mice were housed in a controlled temperature (20°-22°C) and light (14 hrs light/10 hrs dark) vivarium with free access to water. At 8 weeks of age, WT and KO male and female mice were fed a purified diet (TD.94045; control) or a 45% fat diet (TD.06415; HFD) from Harlan Teklad (Madison, WI) for 12 weeks. Mice were fasted overnight (16 hrs) prior to sacrifice.

Metabolic chambers. Male mice fed a control or HFD for ten weeks were transferred to the metabolic core facility one week prior to study initiation to adapt to the environment. Oxygen consumption (VO_2), and carbon dioxide production (VCO_2) were measured via Oxymax Comprehensive Lab Animal Monitoring System by (Columbus Instruments, Columbus, OH) for 24 hours in the fed ad libitum state and 24 hours in the fasted state. Respiratory exchange ratio (RER) and energy expenditure (kcals/h) were generated from formulas provided by the

manufacturer. VO_2, VCO_2, and energy expenditure are presented as estimated marginal means after an ANCOVA analysis with body weight as the covariate (19). Statistical analysis was determined with post-hoc Tukey test.

TG and serum analyses. Hepatic TG was determined via chloroform:methanol extraction method. Hepatic TG and serum TG were quantified using the the Stanbio LiquiColor® Triglycerides No. 2100 assay (Stanbio Laboratory, Boerne, TX). Serum NEFAs were determined with a Wako HR Series NEFA-HR (2) kit. Serum β-hydroxybutyrate was assessed using a β-hydroxybutyrate LiquiColor Kit (Stanbio Laboratory, Boerne, TX).

RNA isolation and analyses. mRNA abundance was determined via RNA isolation using the Trizol Reagent (ThermoFisher, Waltham, MA). cDNA was made using SuperScript®VILO (Invitrogen, Carlsbad, CA) and SYBER Select (ThermoFisher, Waltham, MA) was used to quantitative mRNA abundance via RT-PCR.

Protein preparation and Western blotting. Protein was isolated from tissues via homogenization and sonication in lysis buffer. Protein concentration was quantified by BCA. ACOT1 abundance and knockout was determined by Western blot with a custom antibody described in chapter two.

ROS determination. Cellular hepatic ROS was determined using the OxiSelect *In Vitro* ROS/RNS Assay Kit (Cell Biolabs, Inc., San Diego, CA). Samples were measured at 480 nm excitation and 530 nm emission and compared to a standard curve.

Oral glucose tolerance test. Glucose tolerance was assessed at ten weeks on diet, after an overnight fast (16 hrs). Blood glucose was taken at baseline from a tail clip, and then take 15, 30, 60, and 90 minutes after an oral gavage of 20% dextrose solution (2g/kg body weight).

Tissue imaging. Tissue sections were fixed in 10% formalin and subsequently paraffin embedded or cryosections were made by submerging samples in Tissue Tek ® O.C.T. Compound from Sakura Finetek (Torrance, CA) and freezing in isopentane. Slides were deparaffinized and blocked in 3% BSA, incubated with ACOT1 antibody overnight, stained with DAPI, and mounted. Oil red O and H&E stains were generated from the University of Minnesota's BioNet Histology and IHC Lab.

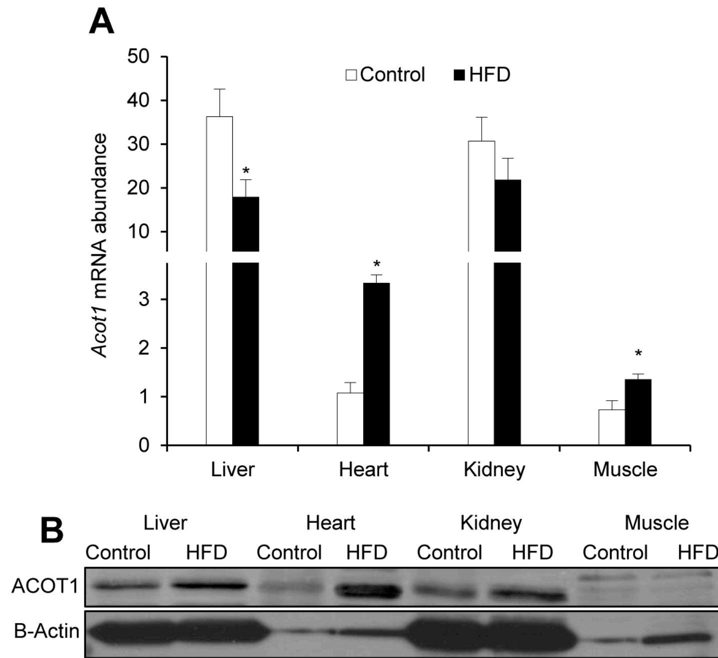
Thioesterase activity assay. Thioesterase activity was determined in male mice fed a purified diet. Frozen liver samples were homogenized and centrifuged 100,000g at 4°C for 1 hour and supernatants were collected. One mg of protein was added per well in assay buffer (50 mM KCl, 10 mM HEPES). DTNB was

added at a working concentration of 50 μM and read at 405nm at 37°C. Palmitoyl Co-A was added at 20 μM and read at 405nm at 37°C for 3-5 minutes. CoA concentration was determined against a standard curve. Values are reported as nmoles CoA/min/mg protein.

Statistical analysis. Values are expressed as the mean ± SEM. Statistical significance was determined by student t-tests or two-way ANOVA tests with Post-hoc student's t-test where appropriate. Statistical significance for the metabolic cage data was determined as described above.

Results

A high fat diet differentially regulates *Acot1* expression in tissues. Previous groups have demonstrated that *Acot1* mRNA is increased after 12 weeks on a HFD in liver, heart, kidney, and muscle, and ACOT1 protein is increased in liver and heart (10). We assessed the expression of *Acot1* as well as ACOT1 protein in liver, heart, kidney, and muscle of our WT mice fed a control or HFD for 12 weeks (Supplemental Fig. 1A-B). *Acot1* mRNA abundance was increased in heart and muscle, however there was no change in the kidney and a reduction in *Acot1* expression in liver in response to the HFD. ACOT1 protein was upregulated in mice fed a HFD in liver, heart, and kidney, but was undetectable in muscle. The discrepancy between *Acot1* expression and ACOT1 protein in liver and kidney suggest that the protein increase may be driven by changes in ACOT1 degradation or posttranscriptional mechanisms rather than transcription.



Supplemental Figure 1. ACOT1 in control and HFD. (A) *Acot1* expression and (B) ACOT1 protein in oxidative tissues from male mice fed a control or HFD for 12 weeks.

Generation of *Acot1* knockout mice. To investigate the physiological role of ACOT1, *Acot1* knockout mice (VG12838) were purchased from the NIH knockout mouse project. The mice were generated via a deletion in Chromosome 12 where *Acot1* resides, deleting 7,874 base pairs that were replaced with a 6,084 base pair ZEN-Ub1 cassette (Fig. 1A). Littermates from *Acot1* heterozygous breeding pairs were genotyped using primer sets to identify *Acot1* DNA as well as the presence of the cassette (Fig. 1B). ACOT2 is a mitochondrial thioesterase and shares 93.7% homology to ACOT1 (20), and runs slightly below ACOT1 in

SDS-PAGE gels (21). We confirmed ACOT1 knockout and assessed ACOT2 compensation by Western blotting (Fig. 1C) as well as RT-PCR (Fig. 1D). *Acot1* knockout was confirmed in oxidative tissues, liver, heart, and kidney in male mice fed a control diet for three months prior to being fasted overnight (Fig. 1C). Liver and kidney had more ACOT1 protein compared to the heart. As such, the heart seems to express more ACOT2 than ACOT1 especially with *Acot1* knockout (Fig. 1C-D). Lastly, we measured acyl-CoA thioesterase activity in WT and KO livers of male mice. As expected, KO mice had less thioesterase activity than their WT littermates (Fig. 1E). However total thioesterase activity wasn't ablated, likely due to the presence of other cytosolic thioesterases such as ACOT7 (22).

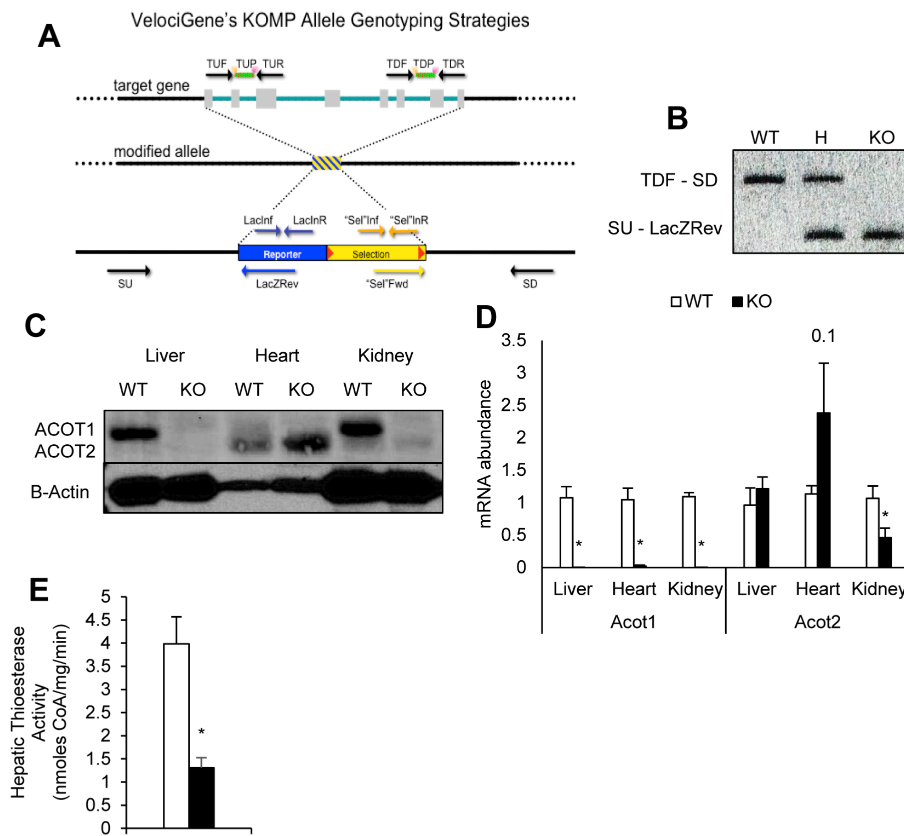


Figure 1. *Acot1* knockout. C57Bl/6J mice were genotyped at 3 weeks of age following exposure to purified diet for 3 months and an overnight (16 hrs) fast. (A) Genotyping schematic from the NIH KOMP. (B) Example gel electrophoresis results from genotyped samples. (C) ACOT1/ACOT2 protein (D) and mRNA abundance of *Acot1* and *Acot2* were assessed in oxidative tissue of WT and KO male mice fed a control diet. (E) Hepatic thioesterase activity was assessed between WT and KO male mice. $^{*}P<0.05$ vs. WT.

***Acot1* knockout protects from diet-induced adiposity.** Male and female WT and KO mice on either a control or HFD were weighed weekly for 12 weeks. Male KO mice exhibited significantly lower weights at the beginning of the study and continued to be lighter than WT mice throughout the study (Figure 2A,C) regardless of diet. Unlike the males, female mice started at similar weights, yet female KO mice on a HFD gained significantly less weight compared to the WT controls (Figure 2B,C). We next assessed adipose depot weights. Male KO mice fed a HFD had similar epididymal adipose depots (Fig. 2D), but reduced inguinal adipose compared to WT controls (Fig. 2E). Female KO mice fed a HFD had significant reductions in both epididymal (Fig. 2D) and inguinal (Fig. 2E) adipose tissue. These results are supported by studies that suggest males are prone to inguinal adiposity while females gain fat in both inguinal and epididymal depots (23). Together, these data suggest that *Acot1* knockout protects male and female mice from diet-induced adiposity.

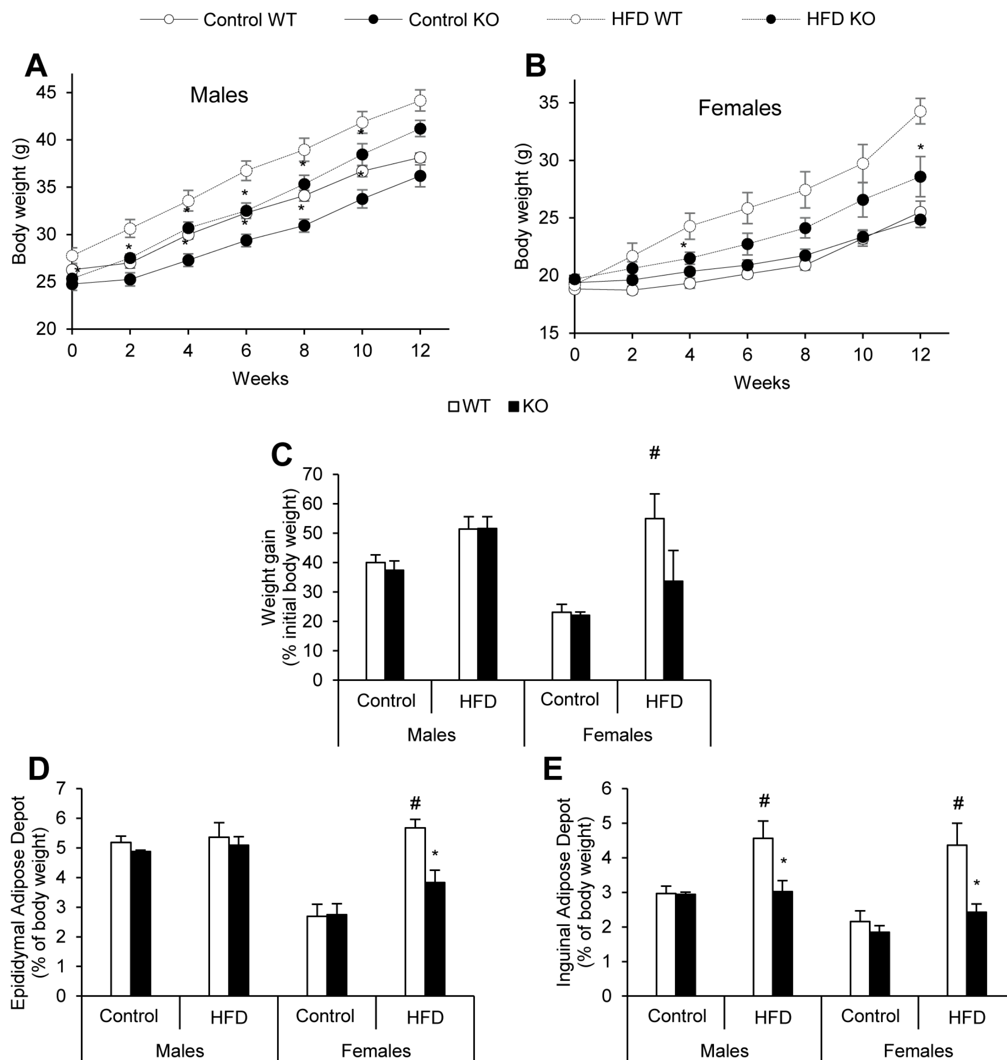


Figure 2. *Acot1* knockout effected body weight and adiposity. (A) Male and (B) female WT and *Acot1* KO mice were weighed every week for the 12 week study and average (C) percent weight gain was calculated from week 0 to week 12. (D) Epididymal and (E) inguinal tissue weights in male and female WT and KO mice fed a control or HFD and fasted overnight. *P<0.05 vs. WT. $^{\#}$P<0.05 vs. control diet.

***Acot1* knockout reduces adiposity.** Recent studies suggest that ACOT1 is important in lipid accumulation during differentiation of pre-adipocytes to adipocytes (24). As such reduced adiposity may be driven by impaired maturation of adipocytes, reducing their storage capacity. DEXA scans confirm that male KO mice indeed had reduced body fat when fed a control or HFD (Fig. 3A). To investigate adiposity further, we fixed tissue from both epididymal and inguinal adipose depots for H&E staining (Fig. 3B). Surprisingly, KO mice exhibited smaller adipocyte size (Fig. 3C) and more adipocytes (Fig. 3D). These results suggest that KO mice have reduced hypertrophy, supporting the theory that ACOT1 promotes lipid storage. Next, we measured inflammatory gene expression in inguinal adipose tissue (Fig. 3E). There was a reduction in inflammatory markers in KO mice fed a HFD, but there was no difference in macrophage recruitment as measured by *F4/80* and *Cd11c* expression. Together these data suggest that *Acot1* knockout reduces adipocyte size and increases adipocyte number, and protects adipocytes from inflammation.

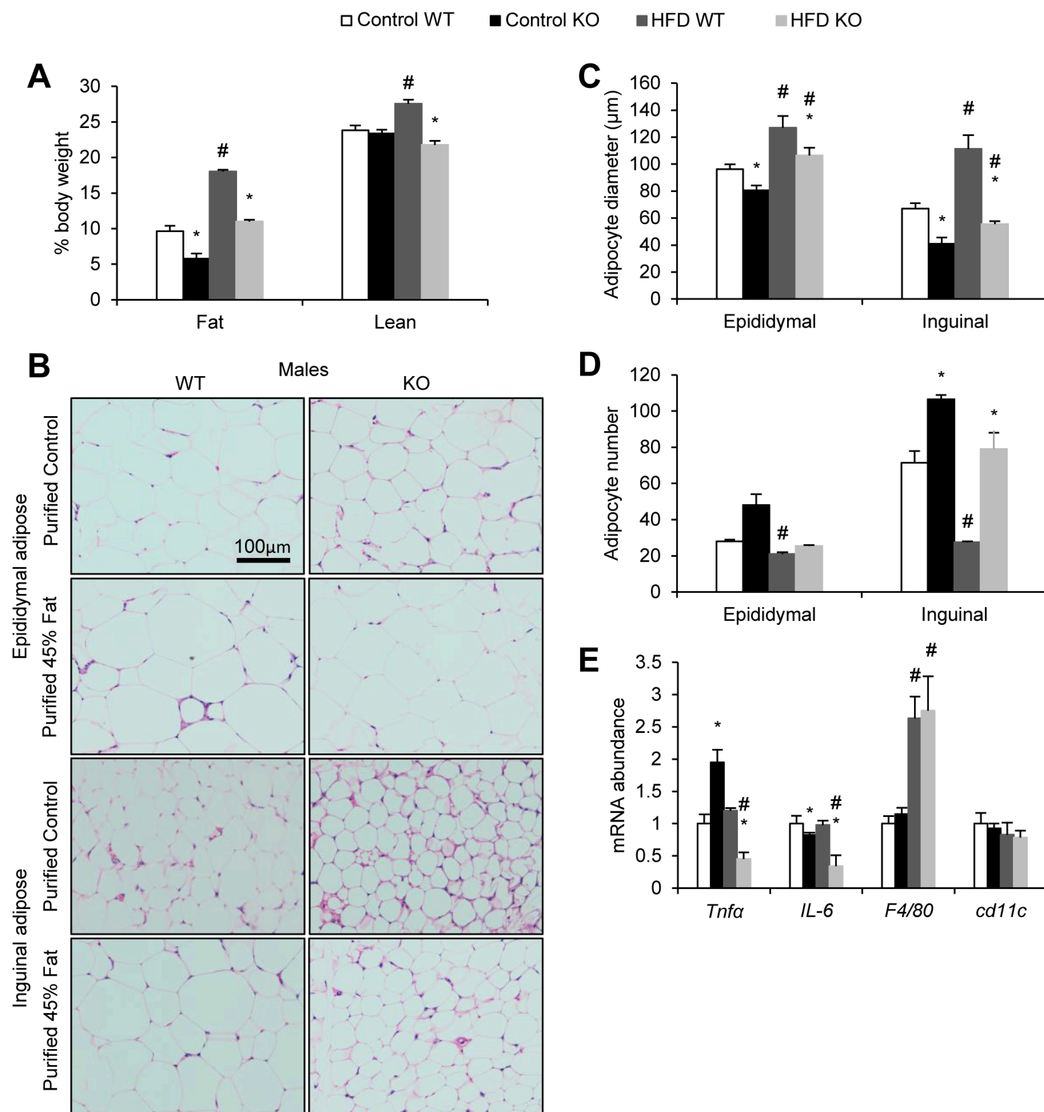


Figure 3. *Acot1* knockout reduced adiposity. (A) DEXA scans of male WT and KO mice. (B) H&E stains from male WT and KO mice. *Acot1* knockout results in (C) smaller adipocytes in both control and HFD dietary conditions as well as (D) more adipocytes in epididymal and inguinal adipose depots. (E) Inflammatory markers in inguinal adipose tissue.

***Acot1* knockout has no effect on whole-body energy metabolism.**

To determine if global *Acot1* ablation affects whole-body energetics we subjected male WT and KO mice fed a control or HFD for 10 weeks to metabolic chambers. Since *Acot1* expression is regulated by nutritional state (13), mice were examined for 24 hours in the fed state and 24 hours in the fasted state. There was no significant difference in VO_2 (Fig. 4A-C) and VCO_2 (Fig. 4D-F) for WT and KO male mice on a control or HFD. We next assessed respiratory exchange ratios (RER) to determine substrate oxidation. As expected HFD significantly lowered RER in the light and dark phases in the fed state, and in the light phase during fasting, suggesting greater lipid utilization (Fig. 5A-C). However, there was no significant difference between WT and KO mice. There was no significant difference in energy expenditure, however, KO mice fed a HFD were trending toward greater energy expenditure in the dark cycle (Fig. 5D-F). Consistent with unaltered energy expenditure, there were also no differences in locomotion between WT and KO mice on either diet (Supplemental Fig. 2). In addition, food intake could not account for the discrepancy in weight and adiposity in WT and KO mice (Supplemental Fig. 3). These results do not identify a clear mechanism by which *Acot1* knockout reduces adiposity. We observed a significant discrepancy in weight of male mice at the start of the study, suggesting that ACOT1 may be important in pre- and post-natal development leading to impaired lipid utilization and storage at least in males (24).

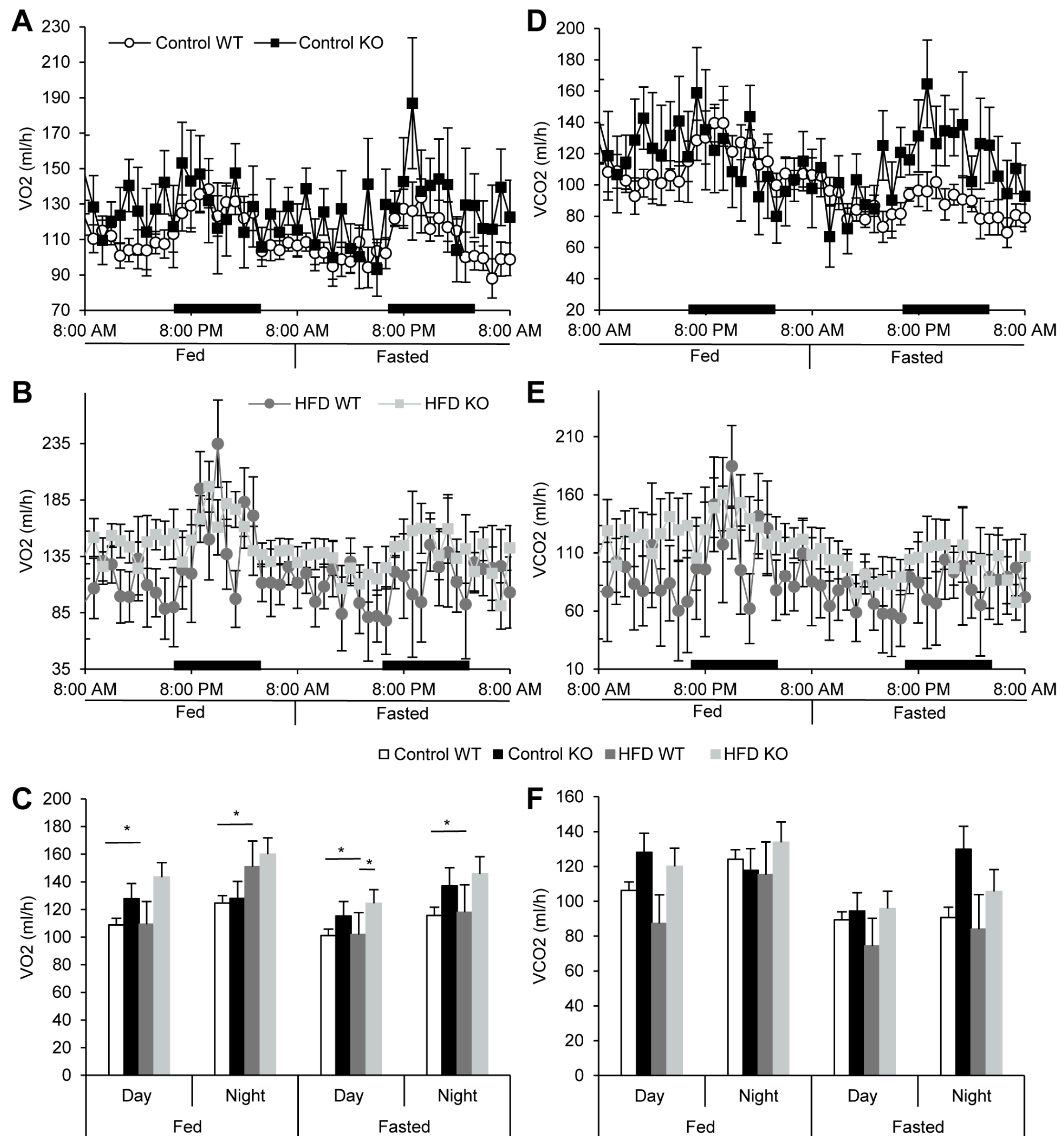


Figure 4. Indirect calorimetry of male WT and KO mice. (A-C) Oxygen consumption rate (VO_2) and (D-F) carbon dioxide production (VCO_2) of WT and KO males fed a control (A,D) or HFD (B,E) for 10 weeks (n=3-4). Average (C) VO_2 and (F) VCO_2 during the night (10 hrs dark cycle) and day (14 hrs light cycle) in fed (24 hrs) and fasted (24 hrs) conditions. All values are represented

as estimated marginal means generated from an ANCOVA analysis with body weight as the covariate. Statistics were determined via Tukey Post-hoc test.

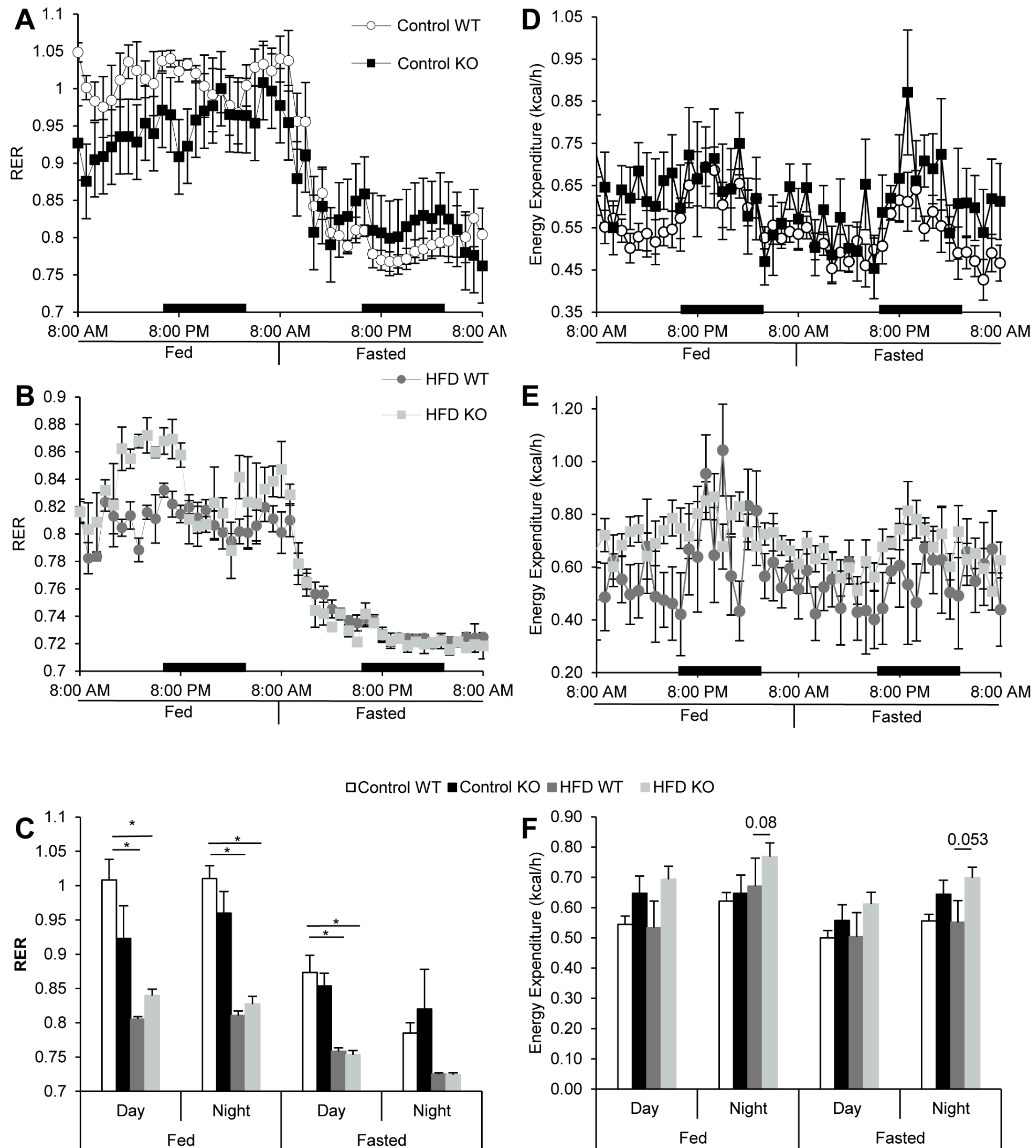
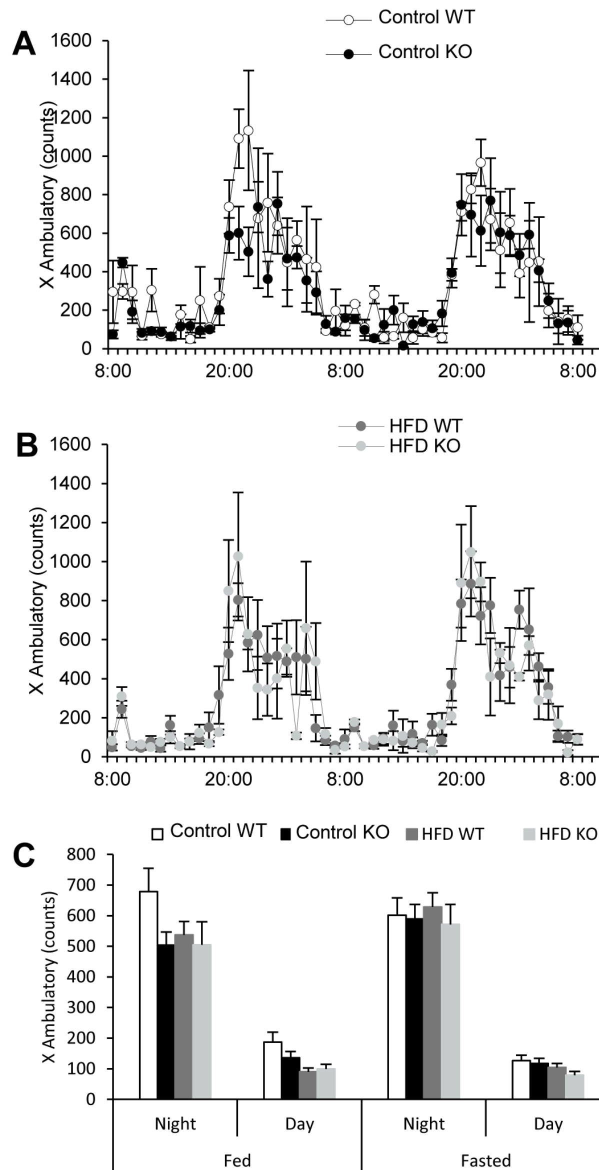
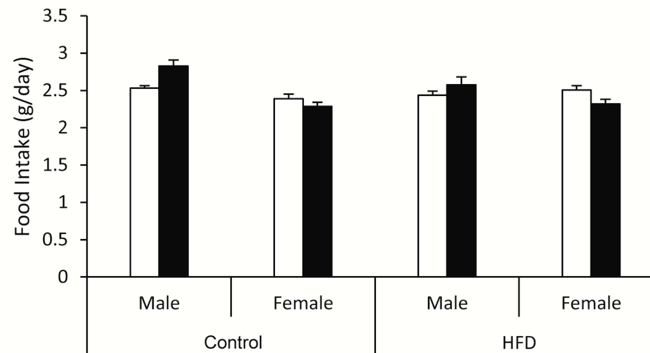


Figure 5. Respiratory exchange ratio and energy expenditure in male WT and KO mice. (A-C) Respiratory energy ratio (RER) and (D-F) energy expenditure for WT and KO mice fed a control (A,D) or a HFD (B,E) for 10

weeks. (n=3-4). Average RER (C) and energy expenditure (F) during the night (10 hrs dark cycle) and day (14 hrs light cycle) in fed (24 hrs) and fasted (24 hrs) conditions. Energy expenditure is represented as estimated marginal means generated from an ANCOVA analysis with body weight as the covariate. Statistics were determined via Tukey Post-hoc test.



Supplemental Figure 2. Locomotion of WT and KO male mice on control and HFD. (A-C) Ambulatory movement was recorded for 48 hours during which mice were either in fed state or were fasted.



Supplemental Figure 3. Food intake in male and female mice. Average food intake was determined under control or HFD conditions.

***Acot1* knockout does not alter fasting serum glucose but impairs glucose tolerance.** Previously we reported that hepatic *Acot1* knockdown enhances gluconeogenesis and greater glycogen stores under fasting conditions. To assess if chronic *Acot1* knockout would elicit a similar effect, we measured fasting glucose in male and female mice. As expected, HFD increase fasting glucose in both male and female mice (Fig. 6A). However, *Acot1* knockout did not further increase fasting glucose but rather reduced fasting glucose in female KO mice on a HFD. We next assessed glycogen stores via Periodic acid-Schiff stain with and without diastase. There was no significant difference in fasting glycogen stores between WT and KO mice (Fig. 6B). These results suggest that *Acot1* knockout had no effect on fasting hepatic glucose homeostasis.

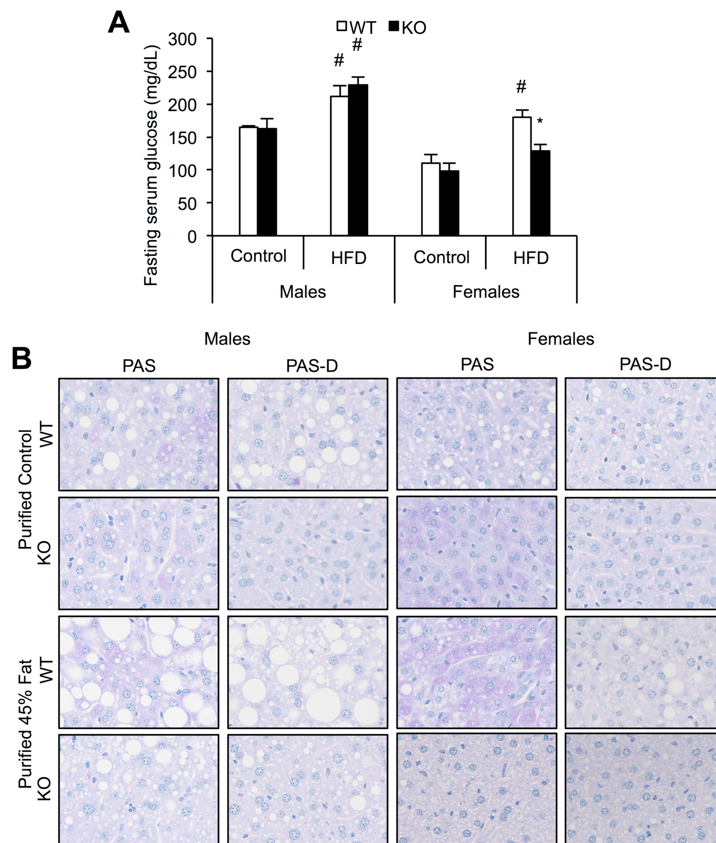


Figure 6. *Acot1* knockout mice exhibit normal glucose metabolism during fasting. (A) Fasting serum glucose was measured in male and female mice. (B) Periodic-Acid Schiff Staining (PAS) and Periodic-Acid Schiff staining with diastase (PAS-D) were performed on fixed liver sections to assess hepatic glycogen stores.

Since we saw significant differences in body weight and adiposity (Fig. 2) between WT and KO mice, we next investigated glucose tolerance via an oral glucose tolerance test. Ten weeks after dietary interventions, mice were fasted for 16 hours then received an oral gavage of glucose (2 g/kg). Male KO mice had

impaired glucose clearance compared to WT in response to both control and HFD (Fig. 7A,C), whereas female KO mice had impaired glucose clearance only under HFD conditions (Fig. 7B,C). HFD resulted in higher serum insulin, yet there was no significant difference between WT and KO mice (Fig. 7D). Together these results suggest that *Acot1* knockout has no effect on fasting glucose production but impairs glucose tolerance during feeding.

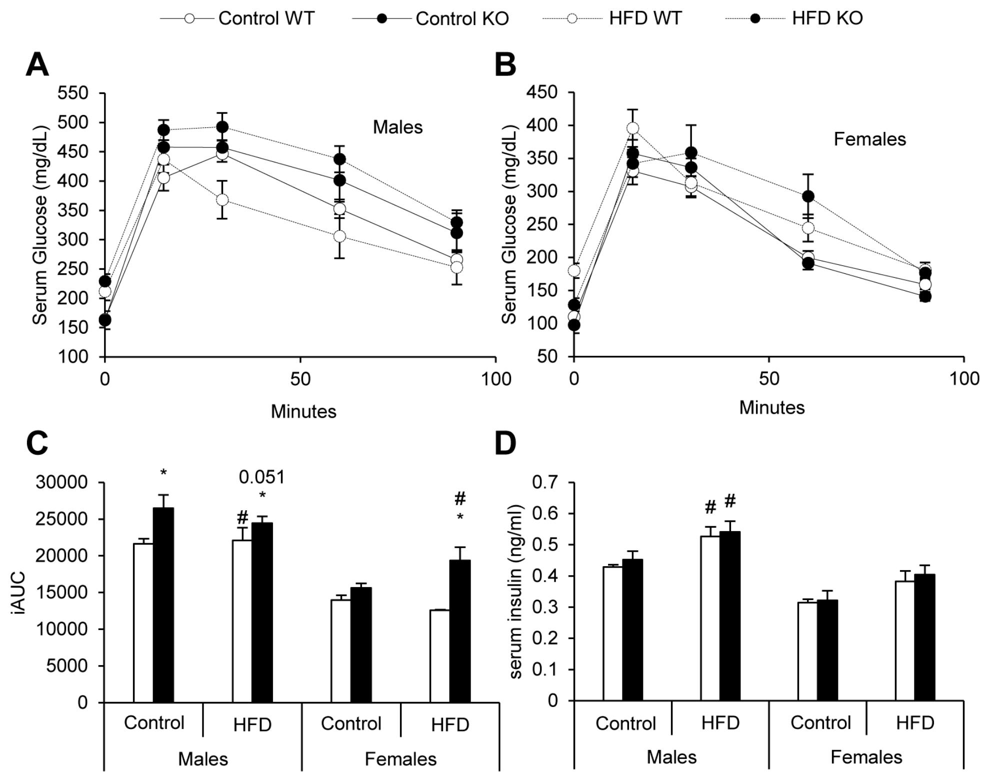
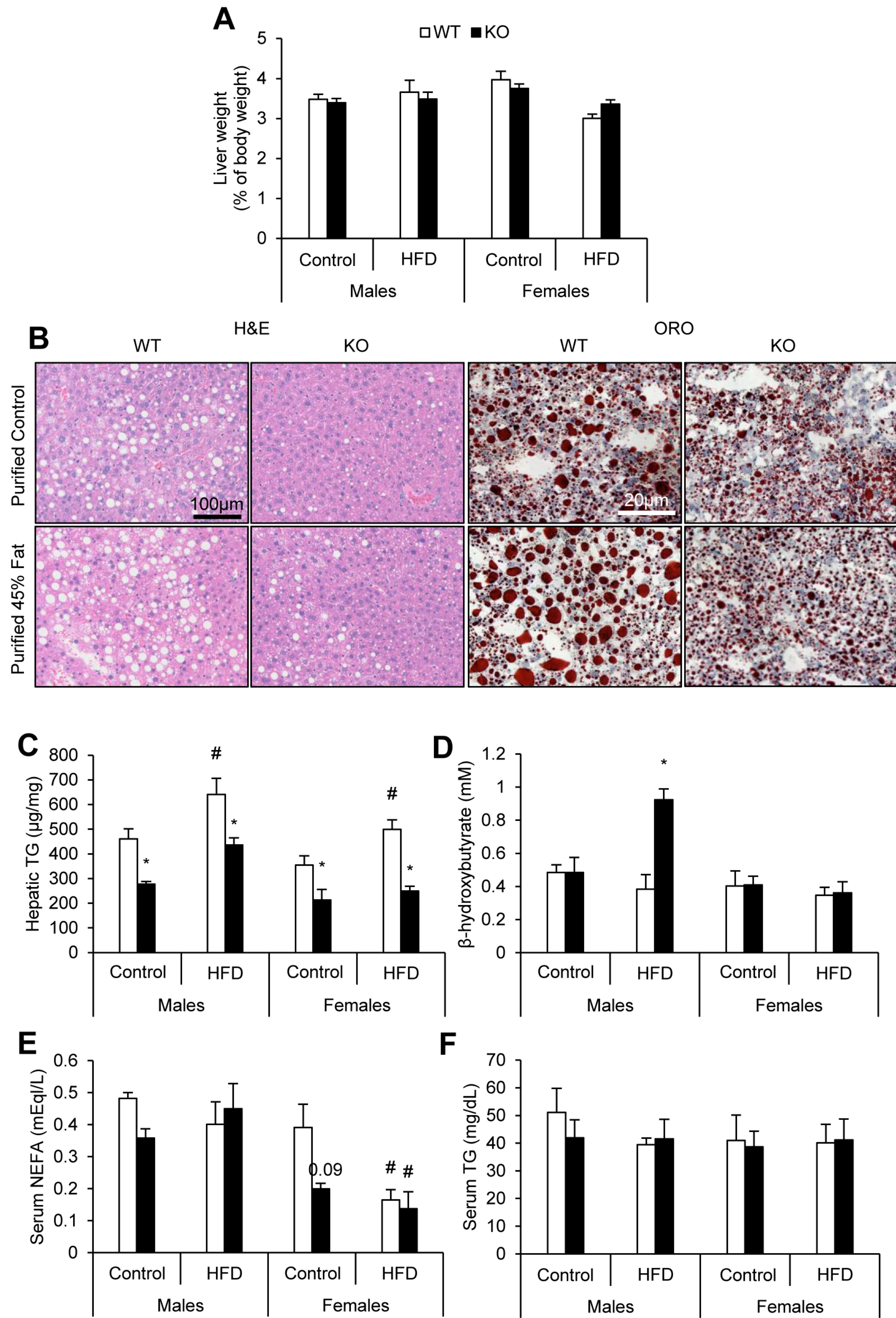


Figure 7. *Acot1* knockout disrupts glucose tolerance. OGTTs were performed on (A) male and (B) female mice on a control and HFD for 10 weeks prior to an overnight fast (16 hrs) (n=4-7). (C) *Acot1* knockout reduced glucose clearance after an OGTT as evident by iAUC. (D) Fasting serum insulin was in male and female mice. *P<0.05 vs. WT. #P<0.05 vs. control diet.

***Acot1* knockout reduces fasting liver TG and protects from HFD induced steatosis.** Because *Acot1* is highly expressed in the fasted liver, we assessed the effects of whole-body *Acot1* knockout on hepatic lipid homeostasis after a 16 hour fast. There was no significant difference in liver weight between any of the groups (Fig. 8A). However, KO mice exhibited significant reduction in hepatic TG seen in H&E and Oil Red O staining (Fig. 8B) as well as chemical analysis (Fig. 8C). Hepatic TG content during fasting is influenced by many factors including rate of oxidation, flux of non-esterified fatty acids (NEFAs) coming from adipose tissue, and secretion of very low-density lipoproteins (VLDL). There was a significant increase in serum β-hydroxybutyrate, indicative of increased FA oxidation in KO males on a HFD compared to WT, an effect that was absent in females (Fig. 8D). Serum NEFAs were not significantly different in male mice but trended lower in female KO mice fed a control diet (Fig. 8E). Finally, there were no changes in serum NEFA and serum TG (Fig. 8F), consistent with our previous work showing hepatic ACOT1 does not affect VLDL secretion.



A
WT
KO
Liver weight (% of body weight)
Control
HFD
Males
Females
B
H&E
ORO
Purified Control
Purified 45% Fat
100µm
20µm
C
Hepatic TG (µg/mg)
D
β-hydroxybutyrate (mM)
E
Serum NEFA (mEql/L)
0.09
F
Serum TG (mg/dL)

Figure 8. *Acot1* knockout reduced hepatic TG. (A) Percent liver weight in male and female WT and KO mice fed a control or HFD and fasted overnight. (B) H&E and Oil Red O staining of hepatic tissue from male WT and KO mice. (C) ACOT1 KO in male and female mice reduced hepatic TG. (D) ACOT1 KO males on a HFD show significant increased serum β-hydroxybutyrate. (E) Serum NEFA is reduced with ACOT1 KO in male and female mice fed a control diet. (F) There was no significant difference in serum TG. *$P<0.05$ vs. WT. $^{\#}P<0.05$ vs. control diet.

We next assessed expression of genes involved in hepatic lipid metabolism, including FA oxidation and FA synthesis. Male KO mice on a control diet had reduced expression of PPARα target genes, while the HFD significantly reduced expression of PPARα target genes in both WT and KO mice (Fig. 9A). Additionally, KO mice on a control diet had reduced PGC1α target gene expression. While HFD reduced PGC1α target genes in WT mice, *Acot1* knockout seemed to rescue this effect (Fig. 9B). These results suggest that ACOT1 is important for PPARα activity. *Acot1* knockout also reduced the expression of genes involved in FA synthesis in mice fed a control diet, but not a HFD (Fig. 9C). These results suggest reduced hepatic TG could be in part driven by diet-specific mechanisms in *Acot1* knockout mice. Male KO mice on a control diet have reduced hepatic TG due to reduced DNL, while males KO on a HFD have enhanced FA oxidation.

HFD is known to induce hepatic steatosis, but is not sufficient to increase progression of NAFLD including inflammation and fibrosis (25). However, acute *Acot1* knockdown in the liver increases inflammation and fibrosis in the absence of steatosis. Since our current study also resulted in decreased hepatic steatosis, we next investigated hepatic ROS in our model. As expected, HFD increased hepatic ROS production, however *Acot1* knockout reduced ROS in both diets (Fig. 9D). Similar to a reduction in oxidative stress markers, KO mice also had reduced inflammatory markers when fed a control diet. Surprisingly, we did not see an increase in inflammatory markers on a HFD as expected and KO mice exhibited a slight increase in these markers (Fig. 9E). Together these results suggest that whole-body *Acot1* knockout reduces hepatic oxidative stress and inflammation in mice fed a control diet.

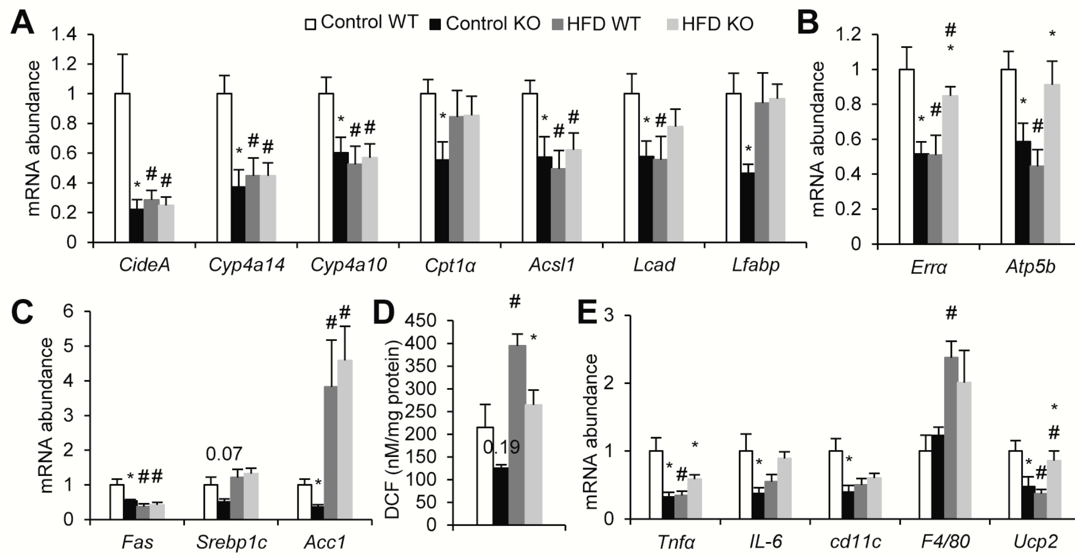


Figure 9. *Acot1* knockout altered expression of genes involved in hepatic lipid homeostasis. (A) Hepatic PPARα target genes, (B) PGC1α target genes, and (C) FA synthesis genes in male WT and KO mice fed a control and HFD. (D) Hepatic ROS as well as (E) inflammatory gene expression. *$P<0.05$ vs. WT. $^{\#}P<0.05$ vs. control diet.

Discussion

Ectopic lipid stores are a defining characteristic of obesity-related diseases including NAFLD and insulin resistance (2,5). Ectopic TG storage is not considered pathogenic, rather lipid intermediates contribute to the development of inflammation and insulin resistance (5,26). ACOT1 is responsible for the cleavage of long chain acyl-CoAs back to free FAs. As such, ACOT1 is believed to regulate the flux of lipid intermediates through metabolic pathways. However, the relevance of ACOT1 in adiposity, ectopic lipid accumulation, and subsequent development of NAFLD and insulin resistance has not been explored. Therefore, we investigated the effects of whole-body *Acot1* knockout on adiposity and ectopic lipid metabolism.

Male *Acot1* knockout mice exhibited lower body weights throughout the study regardless of diet. Conversely, female mice exhibit similar weight gain on a control diet, however KO female mice were resistant to weight gain on a HFD (Fig. 2). These results suggest considerably different phenotypes between male and female KO mice. Metabolic cage studies were unable to elucidate a difference in energy expenditure in male mice fed a control diet, however, there was a trend toward increased energy expenditure in male KO mice fed a HFD (Fig. 4-5). There was also no observed difference in locomotion or food intake. The reduction in body weight was accounted for by reduced adiposity (Fig. 2E,F). *Acot1* knockout correlated to smaller adipocytes in both epididymal and inguinal adipose depots (Fig. 3C-D). Previous groups have suggested that ACOT1 is

important for lipid accumulation during adipocyte differentiation (24), while others suggest upregulation of ACOT1 increases lipid storage (27). These results suggest that weight difference may be due to impaired lipid storage capacity with *Acot1* knockout.

Adiposity, ectopic lipid, and inflammation influence glucose homeostasis and insulin sensitivity. We observed slight glucose intolerance in KO mice, evident by OGTT (Fig. 6), yet KO mice had reduced adiposity (Fig. 3A), less hepatic lipid (Fig. 8B,C) and less inflammation (Fig. 9D,E). Therefore, impaired glucose tolerance seen in *Acot1* knockout mice may be a result of impaired lipid intermediate metabolism. These results suggest that ectopic lipid is not essential to develop glucose intolerance, which is supported by other studies that uncouple TG accumulation from glucose intolerance and insulin resistance (28).

Previously we demonstrated that hepatic *Acot1* knockdown resulted in reduced hepatic TG in the fasted state due to enhanced FA oxidation. As expected, both male and female *Acot1* knockout mice had reduced hepatic TG when fed a control diet and were protected from hepatic steatosis when fed a HFD (Fig. 8B,C). Hepatic lipid stores are influenced by uptake of NEFA, DNL, oxidation of FAs, or secretion of TG in VLDL. However, the observed decrease in hepatic TG was not fully explained by consistent changes in serum β-hydroxybutyrate, serum NEFA, or serum TG across every group (Fig. 8D-F). As such, male mice fed a HFD have enhanced FA oxidation that may contribute to the observed reduced hepatic TG (Fig. 8D), an effect not observed in male mice

fed the control diet or female mice. These results were based on a snapshot in time and do not describe the flux of these metabolites through these pathways. Therefore we assessed RER in male mice to determine if KO mice had greater lipid metabolism, however there was no significant difference in RER (Fig. 5A-C). To further investigate lipid metabolism pathways, we measured gene expression involved in FA synthesis and FA oxidation in male mice. *Acot1* knockdown reduced expression of FA synthesis genes and PPARα and PGC1α target genes on a control diet, but not a HFD. As such, male mice fed a control diet have reduced hepatic lipid likely due to reduced DNL (Fig. 9C). These results suggest that diet influences the mechanism by which *Acot1* knockout is able to protect from hepatic lipid accumulation.

Based on our current study, ACOT1 is involved in the development and pathogenesis of obesity and ectopic lipid storage. In the absence of ACOT1, mice were protected from adipocyte hypertrophy and hepatic lipid droplet accumulation. This phenotype correlated to reduced ROS production and expression of inflammatory genes, however exhibited slight glucose intolerance, suggesting that insulin sensitivity is affected by lipid species intermediates and not TG accumulation. Together, these results suggest that ACOT1 increases lipid storage potential in male and female mice fed a purified control diet or a 45% high fat diet.

Acknowledgements

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Chapter 4

Conclusions and Perspectives

Mallory Franklin wrote this chapter in its entirety

The prevalence of obesity and obesity related diseases continue to rise in the United States. Currently over one third of all Americans suffer from obesity (1), contributing an extra $1700 to annual healthcare costs per person (2). Of the many comorbidities of obesity, NAFLD represents one of the most prominent as 80% of all obese individuals have the disease (3). As such, it is speculated that as the rates of obesity climb in the United States so do the rates of NAFLD. Unfortunately, NAFLD is often undiagnosed and many patients go untreated for years leading to disease progression and greater liver damage (4). Outside of diet and exercise interventions that promote weight loss, there is currently no treatment for NAFLD. If the disease progresses to cirrhosis, damage can be permanent, requiring liver transplantation (5). As such, there is great need in understanding the metabolic pathways that propagate the progression of NAFLD.

Thioesterases are highly conserved enzymes, exhibiting similar gene clusters in humans, mice, and rats (6). ACOT1 was first discovered in 1994 as the primary cytosolic thioesterase found in oxidative tissues (7). Since its initial discovery, the physiological importance of ACOT1 has been investigated in pre-adipocytes (8), cardiomyocytes (9,10), and mesenteric lymph nodes (11). Our group is the first to investigate the importance of ACOT1 in hepatic fasting metabolism and whole-body lipid homeostasis.

Literature suggests that hepatic FA oxidation is governed by PPARα activity and expression of target genes (12). However, we observed increased FA oxidation in the absence of increased PPARα target gene expression during

acute *Acot1* knockdown. Therefore, we suggest that ACOT1 creates the balance between FA oxidation and PPARα activity. Other groups have demonstrated a link between FA oxidation and ACOT1 driven expression of PPARα target genes. Liver-specific *Cpt2* knockout mice have impaired FA oxidation resulting in an increase of cellular long chain acyl-CoAs. *Acot1* expression is enhanced in this model to remove long chain acyl-CoAs by producing free FAs. Subsequently, the increased production of free FAs was suggested to increase PPARα target gene expression seen in liver-specific *Cpt2* knockout mice (13). These results support the findings in our study that ACOT1 maintains the balance between lipid oxidation and PPARα signaling through mitigating the pool of acyl-CoAs to free FAs.

NAFLD is defined by hepatic steatosis, which then progresses to inflammation and fibrosis. However there are few disease states in which inflammation and fibrosis manifest in the absence of hepatic steatosis, including type III glycogen storage disease (14) and viral hepatitis (15). In our current studies, *Acot1* knockdown reduced hepatic steatosis but increased oxidative stress, inflammation, and fibrosis. We demonstrate that *Acot1* knockdown enhanced FA oxidation and subsequently increased gluconeogenesis (16). These results suggest that the cause of NALFD progression is not lipid accumulation in the liver, but the subsequent oxidation of excess lipid. Investigating the correlation between advanced NAFLD and these metabolic

pathways (17,18) provides insights into disease progression and identifies potential points of intervention to treat or prevent NAFLD.

Ectopic lipid storage is also correlated to the development of insulin resistance (19). Previous work from our laboratory and others have suggested a disconnect between hepatic TG and insulin resistance. Adipose triglyceride lipase knockdown prevents hepatic TG hydrolysis and sequesters TG in lipid droplets leading to massive accumulation of hepatic TG but protection from insulin resistance and glucose intolerance (20). Conversely, *Acot1* knockout reduced ectopic lipid accumulation yet resulted in a mild glucose intolerance. These results were likely driven by dysregulation in lipid intermediates due to *Acot1* knockout. These results support the current paradigm that inert TG accumulation in lipid droplets is not pathogenic, rather other lipid intermediates lead to insulin resistance (21).

Unlike our results with acute *Acot1* knockdown, chronic *Acot1* knockout solicited a very different phenotype. Whole-body *Acot1* knockout had reduced adiposity and hepatic TG, likely due to implications of adipocyte maturation and lipid utilization. Little research has investigated the role of ACOT1 in adipocyte differentiation. However, in brown adipocyte differentiation, *Acot1* is expressed in the initial stages of differentiation where it is speculated to remove long chain acyl-CoAs from oxidation and promote lipid accumulation. Once differentiated, *Acot1* expression reduces and *Acot2* expression is enhanced (8).

Understanding the contribution of ACOT1 in adipocyte maturation may explain the discrepancy in body weight and observed adiposity.

ACOT1 has previously been identified as a cytosolic protein (22–24). However, our research has shown ACOT1 to be a nuclear protein during prolonged fasting and under β-adrenergic stimulation. ACOT1 does not possess a known nuclear localization sequence. However, our studies suggest that ACOT1 translocation is dependent on PKA mediated post-translational modification. Nuclear Western blotting for ACOT1 identified a slight shift in ACOT1 protein size also suggesting nuclear localization may be driven by protein modification. In preliminary data, we explored potential phosphorylation sights of ACOT1, generating point mutations to mimic de-phosphorylation and phosphorylation, to determine their role in nuclear localization (data not shown). We were unable to confirm a specific phosphorylation sight that would explain the nuclear localization. Further studies are needed to determine the mechanism by which ACOT1 becomes nuclear.

In addition to ACOT1, there are other thioesterases that have had profound implications on metabolic health. ACOT13, a thioesterase found on the outer membrane of the mitochondria also regulates hepatic FA oxidation (25). ACOT13 produces free FAs on the mitochondrial surface rendering substrate for the ACSL1/CPT1α pathway for oxidation (26). Hepatic ACOT2 resides in the mitochondrial matrix where it also promotes the oxidation of FAs. Overexpression of *Acot2* results in greater FA oxidation by mitigating the buildup of mitochondrial

long chain acyl-CoAs (27). Lastly, ACOT7, a type II thioesterase found in the cytosol appears to play a minimal role in regulating FA oxidation in the liver however plays a critical role in adipose and neuronal lipid metabolism (28,29). Thioesterases are an understudied group of enzymes that play key roles in lipid trafficking and signaling. Future studies may provide great insights in the flux of lipid intermediates through metabolic pathways.

In summary, ACOT1 is a major regulator of hepatic lipid oxidation, removing acyl-CoAs from oxidation and providing nuclear FAs to activate PPARα. Together these roles regulate lipid intermediates, hepatic gluconeogenesis, and protects from oxidative stress, inflammation, and insulin resistance. Further studies will be needed to determine the exact mechanism by which ACOT1 translocates to the nucleus and its role in lipid storage capacity. Furthering our understanding of ACOT1 may shed light on the involvement of oxidative metabolism in the progression of NAFLD from simple steatosis to fibrosis and inflammation.

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