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Post-Translational Regulation of FAS-Mediated PPAR α Activation

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Post-Translational Regulation of FAS-Mediated PPAR α Activation

By

Anne Patricia Louise Jensen-Urstad

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2013

St. Louis, Missouri

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Anne P L Jensen-Urstad

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LIST OF ABBREVIATIONS

| | |
|-------------------|---|
| 16:0/18:1-GPC | 16:0/18:1-glycerophosphocholine |
| ADA | American Diabetes Association |
| ADP | Adenosine diphosphate |
| AHA | American Heart Association |
| AMP | Adenosine monophosphate |
| AMPK | AMP-activated kinase |
| ATP | Adenosine triphosphate |
| CoA | Coenzyme A |
| CEPT1 | Choline/ethanolamine phosphotransferase 1 |
| CETP | Cholesterol ester transfer protein |
| ChRE | Carbohydrate response element |
| ChREBP | Carbohydrate response element binding protein |
| DAG | Diacylglycerol |
| DR | Direct repeat |
| EMA | European Medical Association |
| ER | Endoplasmatic reticulum |
| FAD | Flavin adenine dinucleotide |
| FADH ₂ | Flavin adenine dinucleotide (reduced) |
| FAS | Fatty acid synthase |
| FASKOL | Fatty acid synthase knock-out in liver |
| FBS | Fetal bovine serum |
| FDA | Federal Drug Administration (US) |
| FFA | Free fatty acid |
| GK | Glucokinase |
| HDL | High-density lipoprotein |
| HER2 | Human epidermal growth factor receptor 2 |
| HNF-4 α | Hepatic nuclear factor-4 α |
| IDL | Intermediate-density lipoprotein |
| IRE | Insulin response element |
| LDL | Low-density lipoprotein |
| LXR | Liver X receptor |
| LXRE | Liver X receptor element |
| mTORC1 | Mammalian/Mechanistic target of rapamycin complex 1 |
| NADH | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NCEP | National Cholesterol Education Program (US) |
| PC | Phosphatidylcholine |
| PCTP | Phosphatidylcholine transfer protein |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PPAR α | Peroxisome proliferator-activated receptor α |
| PPAR δ | Peroxisome proliferator-activated receptor δ |
| PPAR γ | Peroxisome proliferator-activated receptor γ |
| PPRE | Peroxisome proliferator response element |
| RXR | Retinoid X receptor |
| Scd1 | Stearoyl-CoA desaturase 1 |
| sdLDL | Small, dense low-density lipoprotein |
| SILAC | Stable isotope labeling of amino acids in culture |
| SRE | Sterol regulatory element |

| | |
|-----------|--|
| SREBP-1c | Sterol regulatory element binding protein 1c |
| TAG | Triacylglycerol (triglyceride) |
| TCA cycle | Tricarboxylic acid cycle |
| USF | Upstream stimulatory factor |
| VLDL | Very low-density lipoprotein |
| WHO | World Health Organization |

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ABSTRACT OF THE DISSERTATION

Post-Translational Regulation of FAS-Mediated PPAR α Activation

by

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Doctor of Philosophy in Molecular Cell Biology

Washington University in St. Louis, 2013

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The liver is a central organ to whole-body metabolism and mediates many of the adaptive responses to changes in nutrient availability, such that the appropriate energy sources are used and blood glucose levels maintained, whether directly after a meal or after a twelve-hour fast. The adaptive responses to fasting in liver are largely mediated by the nuclear receptor peroxisome proliferator-activated receptor α , or PPAR α .

PPAR α can be activated by a de novo synthesized lipid ligand—16:0/18:1-glycerophosphocholine (16:0/18:1-GPC)—the synthesis of which is dependent on fatty acid synthase (FAS), but little is known about the regulation of this pathway. My thesis focused on post-translational mechanisms controlling endogenous activation of PPAR α in the liver and used mouse liver and a hepatocyte cell line as model systems.

In addition to its role in PPAR α activation during fasting, FAS helps store excess calories as fat during feeding. We demonstrated that this paradoxical relationship involves the differential regulation of FAS in at least two distinct subcellular pools: cytoplasmic and membrane-associated FAS, the latter being attached to membranes by a strong peripheral membrane association. To find candidate proteins mediating FAS membrane localization we used a proteomics approach to identify compartment-specific FAS-associated proteins. We identified three proteins—Septin-2, Septin-7, and 40S ribosomal protein S18—that in two different liver model systems associate with FAS exclusively in the membrane fraction.

Because the septins are involved in membrane structuring and scaffolding, these proteins may be involved in FAS membrane localization.

The ratio of cytoplasmic to membrane FAS specific activity was increased with fasting or in the absence of insulin, indicating higher cytoplasmic FAS activity under conditions associated with PPAR α activation. This effect was due to a nutrient-dependent and compartment-selective covalent modification of FAS: cytoplasmic FAS was preferentially phosphorylated during feeding or insulin treatment at Thr-1029 and Thr-1033, which flank a dehydratase domain catalytic residue. Mutating these sites to alanines promoted PPAR α target gene expression. mTORC1, a mediator of the feeding/insulin signal to induce lipogenesis, emerged as a mediator of FAS phosphorylation, inhibiting cytoplasmic FAS activity and reducing PPAR α target gene expression in a FAS-dependent manner.

Next, we investigated the role of ligand transport in FAS-mediated PPAR α activation. 16:0/18:1-GPC is synthesized in the cytoplasm and it is not known how it reaches the nuclear PPAR α . We identified phosphatidylcholine transfer protein (PCTP) as a possible transport protein for this ligand. PCTP knockdown in Hepa1-6 hepatocytes caused dramatic reductions in expression of PPAR α target genes, and PCTP co-immunoprecipitated with PPAR α . Immunofluorescent imaging showed that starvation of cells caused an accumulation of PCTP in the nucleus, consistent with a shuttling function controlled by nutrition. Using mass spectrometry, we demonstrated that PCTP binds 16:0/18:1-GPC. We further showed that the binding of this ligand to PCTP is FAS-dependent: in mice with liver-specific knockout of FAS, the amount of 16:0/18:1-GPC bound to PCTP in the nucleus was significantly reduced.

Together, these findings suggest that multiple modes of post-translational regulation of FAS combined with regulation of lipid delivery by PCTP control fasting-induced PPAR α activation in liver.

EPIGRAPH



"Beauty" (<http://xkcd.com/877>), © 2011 Randall Munroe, used under a Creative Commons Attribution-NonCommercial license: <http://creativecommons.org/licenses/by-nc/2.5/>

Chapter 1:
Introduction and Significance

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HEPATIC LIVER METABOLISM IN HEALTH AND DISEASE

The liver plays a central role in both glucose and lipid metabolism. Through a finely tuned regulation of hepatic glycolysis, gluconeogenesis, glycogen synthesis and glycogenolysis, the liver enables the control of blood glucose levels within certain narrow physiological limits both in the fed and fasted state. This process becomes disturbed in type II diabetes, in which dysregulation of hepatic glucose metabolism combined with reduced glucose uptake by peripheral tissues lead to hyperglycemia. The liver is the hub for lipid and lipoprotein production, secretion, uptake, and breakdown, as well as the primary regulator of plasma cholesterol levels. In healthy individuals, the regulation of lipid metabolism and glucose metabolism in the liver are tightly interlinked, allowing for appropriate substrate utilization both after meals and during periods of fasting (reviewed in [1]). In diabetes, both glucose and lipid metabolism become disturbed, as does this nutrient-responsive adaptation.

Hepatic lipid metabolism: lipoprotein synthesis, secretion, and uptake

Systemic transport of lipids is primarily carried out by lipoproteins, complexes of lipid and protein (apolipoproteins) with a core of neutral lipids (triglycerides, diglycerides, cholesterol esters) and a surface monolayer of amphipathic phospholipids and proteins that promotes interaction of the lipoprotein with the aqueous environment of the blood. As the liver is the key organ for synthesis, secretion, and uptake of lipoproteins, the basics of lipoprotein metabolism will be reviewed below.

Lipoproteins are synthesized and secreted by the liver and, to a smaller extent, by the small intestine. They transport lipid to peripheral tissues (primarily adipose tissue and muscle) and return to the liver for uptake and breakdown or recycling. During their time in

the circulation, lipoproteins are modified by losing lipid to peripheral tissues; by lipid exchange with other lipoproteins, remodeling their relative lipid compositions (such as through the action of cholesterol ester transfer protein (CETP) that exchanges cholesterol esters for triglycerides); and by loss or gain of apolipoproteins. Some lipoproteins are involved in reverse lipid transport, i.e. the transport of lipids from peripheral tissues to the liver (an important example being reverse cholesterol transport by HDL from macrophages to the liver, from which the cholesterol is then excreted as bile).

Lipoproteins can be roughly divided into five classes: chylomicrons and their remnants, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), and high-density lipoprotein (HDL). Generally, the density of a lipoprotein increases as the ratio of protein to lipid increases, hence VLDL is lipid-rich and protein-poor while HDL is protein-rich and lipid-poor. These classes of lipids differ in their lipid composition and their associated apolipoproteins. The apolipoproteins can function as ligands for lipoprotein receptors stimulating their uptake (such as the LDLR binding domain on apoB100) or as activators or inhibitors of various lipid metabolic enzymes.

Important apolipoproteins include apoB100, which is present in VLDL, IDL, and LDL; apoB48 (a truncated version 48% the size of apoB100), which is present in chylomicrons and chylomicron remnants; and apoA1, which is present in HDL. Most apolipoproteins can be synthesized in the liver with the important exception of apoB48, which in humans is only synthesized in the small intestine. ApoB48 lacks the LDL receptor binding capacity and so has a different plasma clearance profile from apoB100. In mice, apoB48-containing lipoproteins are synthesized in the liver as well, an important distinction between mouse and human lipoprotein metabolism (along with the absence of CETP, in mice; hamsters are actually more similar to humans in that they have CETP and cannot synthesize hepatic apoB48, and are so sometimes used as an alternative rodent model for

lipoprotein metabolism [2, 3]).

The liver synthesizes and secretes VLDL (which is hydrolyzed in the circulation to IDL and then LDL) and a precursor form of HDL. VLDL is synthesized in the Golgi and ER of hepatocytes through stepwise lipidation of an apolipoprotein. After entering the circulation, lipoproteins deliver lipids to peripheral tissues by lipolysis and may be remodeled by the gain or loss of apolipoproteins or by lipid exchange via CETP. Finally, LDL and IDL bind to receptors on the surface of hepatocytes (primarily the LDL receptor (LDLR) and LDL-related protein (LRP)). Largely due to the regulation of LDL uptake from circulation via LDLR, the liver is the primary regulator of plasma cholesterol. The receptor-bound lipoproteins are endocytosed into hepatocytes and their lipids are stored or re-packaged into VLDL for secretion. HDL made by liver or intestine takes up cholesterol from peripheral tissues, may be remodeled by lipid exchange by CETP, and is taken up by the liver in part by scavenger receptor-BI (SR-BI), after which its cholesterol is converted to bile acids and excreted.

Chylomicrons are synthesized in the small intestine from dietary fat following a meal and are transported to the vena cava, bypassing the liver to directly deliver lipids (primarily triglycerides) to peripheral tissues. They eventually reach the liver in the form of chylomicron remnants and the cholesterol and remaining triglycerides they carry are taken up by the liver for storage or (re-)secretion.

Hepatic lipid metabolism: extra- and intrahepatic lipid sources

The liver takes up fat from circulation: free fatty acids (FFAs), dietary fats (cholesterol and to a smaller degree triglycerides) from intestinally derived chylomicron remnants, lipids from LDL and IDL, and lipids from HDL (primarily cholesterol). The FFAs are mostly derived from adipose tissue lipolysis that, in a healthy insulin sensitive individual, occurs mainly during fasting. Uptake of FFAs is not regulated, so FFA uptake by the liver is

directly proportional to the FFA concentration in plasma, and should increase during fasting in order to be used for fuel via fatty acid oxidation.

In addition to exogenous lipid uptake, lipids can be synthesized completely *de novo* in the liver by fatty acid synthase (FAS). These lipids can be further incorporated into phospholipids, diglycerides or triglycerides; however, in most cases FAS-derived lipids are a quantitatively minor contributor to stored and secreted lipid, as will be reviewed in detail below. *De novo* synthesis of cholesterol is more quantitatively important, and the bulk of cholesterol synthesis takes place in the liver.

Lipids can be stored in the liver as intrahepatic lipid droplets and released as needed: excessive lipid stored in this manner is referred to as fatty liver. Lipid droplets have a structure resembling lipoproteins: they contain a core of neutral/esterified lipids (such as triglycerides and cholesterol esters) surrounded by an amphipathic phospholipid monolayer and various structural proteins (reviewed in [4] and [5]). A large proportion of the lipid incorporated into VLDL for secretion seems to be derived from intrahepatic lipid droplets [6-8]. The mechanism by which lipids are transferred from lipid droplets to lipoproteins is debated, with evidence both for a hydrolysis-reesterification cycle whereby droplet lipids are hydrolyzed to FFAs that enter the ER and are re-esterified in the ER lumen prior to incorporation into lipoproteins [7, 9], and for direct fusion of lipid droplets (presumably pre-existing in the ER lumen) with pre-VLDL [10].

The fasting response

Under nutrient-replete conditions, the primary fuel of the liver is glucose rather than fat. Dietary fat in the form of chylomicron remnants is taken up by the liver, but fatty acids are not subjected to β -oxidation and instead are incorporated into triglycerides for storage in lipid droplets or secretion in VLDL. *De novo* synthesis of fatty acids by FAS may make a

modest contribution to storing energy as fat when nutrients are present in excess.

“The fasting response” refers to the adaptive changes in metabolism that occur during fasting or starvation in order to maintain blood glucose levels and provide alternative energy in the form of ketones. During fasting, the primary fuel of the liver switches to fat. Plasma insulin levels fall, relieving inhibition of lipases and stimulating lipolysis in peripheral tissues (primarily adipose tissue). This increases the levels of plasma free fatty acids (FFAs), which are taken up by the liver.

In the liver, fatty acids from peripheral tissues and from intrahepatic lipid droplets are catabolized through β -oxidation to form acetyl-CoA, which is either channeled into the TCA cycle or used as substrate to produce ketones that provide energy to other tissues when glucose is scarce. β -oxidation also produces reducing equivalents in the form of FADH₂ and NADH. Energy in the form of ATP from the TCA cycle and electron transport chain (via acetyl-CoA from β -oxidation) and reducing equivalents directly derived from β -oxidation are used to fuel gluconeogenesis. The process of fatty acid catabolism and glucose anabolism during fasting is thus interrelated through the dependency of gluconeogenesis on β -oxidation for energy.

An important protein for the fasting response in the liver is peroxisome proliferator-activated receptor α (PPAR α), which promotes the transcription of genes necessary for the fasting response (such as those encoding enzymes involved in β -oxidation and ketogenesis). PPAR α will be discussed in more detail below.

Hepatic lipid metabolism in the metabolic syndrome and diabetes

The prevalence of type II diabetes is estimated to be 13% among adult Americans [11] and 6% (6.4%) among adults worldwide [12]. The metabolic syndrome is estimated to affect 24% of adult Americans [13] (criteria as defined by the National Cholesterol Education

Program (NCEP)). Both diabetes and the metabolic syndrome are risk factors for cardiovascular disease [14, 15], which is the leading cause of death worldwide [16]. Because the liver is central to controlling systemic glucose and lipid metabolism, it is also central to understanding the pathogenesis of diabetes and for designing treatments. Many of the most important hyperlipidemia and diabetes drugs currently available, including metformin, statins, and fibrates, have the liver as their site of action.

The diagnostic criteria for type II diabetes includes a fasting plasma glucose level over 126 mg/dl or 7.0 mmol/l (according to WHO and ADA recommendations [17, 18]). The hallmark of type II diabetes is insulin resistance, which refers to a state in which the tissues of the body have a decreased sensitivity and responsiveness to insulin. Because of this decreased responsiveness, higher levels of insulin must be produced by the pancreas in order to elicit desired responses, which include stimulating peripheral tissues to take up glucose and suppressing glucose synthesis by the liver. Type II diabetes refers to what happens when, because of resistance to the actions of insulin, the insulin present is no longer enough to maintain appropriate blood glucose levels, leading to hyperglycemia.

The metabolic syndrome refers to risk factors that together increase the risk of diabetes and cardiovascular disease. The criteria for metabolic syndrome differ between institutions (IDF, WHO, NCEP, and AHA), but all include some combination and definition of central obesity, high plasma triglycerides, low HDL cholesterol, high blood pressure, and high fasting blood glucose or insulin resistance. These risk factors are interrelated and tend to cluster together.

In insulin resistance, insulin fails to suppress lipolysis in peripheral tissues even when nutrients are abundant, resulting in high circulating levels of FFAs that are taken up by the liver. Increased FFA uptake and perhaps increased de novo synthesis of fat in the liver overwhelms the capacity for fatty acid oxidation, leading to fat accumulation and eventually

the development of hepatosteatorosis or fatty liver (non-alcoholic fatty liver disease, NAFLD). Fatty liver has been estimated to affect a fifth to a third of adult Americans (21.4% in a nationally representative cohort from 1988 to 1994 [19] and 31% in a cohort from Dallas county from 2000 to 2002 [20]) and is associated with insulin resistance [21] and a high BMI [20, 21]. NAFLD thus constitutes one of the major metabolic disorders along with diabetes and heart disease accompanying the obesity epidemic.

Hepatosteatorosis can progress to nonalcoholic steatohepatitis (NASH), which in addition to fat accumulation is marked by inflammation, hepatocyte injury and/or fibrosis. A significant proportion of patients with NASH develop cirrhosis (10-29% within 10 years [22]), which in turn may progress into hepatocellular carcinoma, a type of cancer with very poor prognosis: the 5-year survival rate is less than 10% [23].

Fatty liver can be diagnosed noninvasively (as was done in the studies estimating NAFLD mentioned above, where ultrasound [19] and proton magnetic resonance spectroscopy were used [20]). Diagnosing the presence of hepatic inflammation or fibrosis, however, requires a liver biopsy. Therapeutic options for NAFLD are very limited; there is currently no medical or surgical treatment for NAFLD approved by the FDA or the EMA. Weight loss or treatment with the insulin-sensitizing thiazolidinedione drugs improve steatorosis and inflammation [24, 25]; the latter, however, cause significant weight gain. There is thus a need for novel non-invasive diagnostic methods and for novel therapies for NAFLD and NASH.

The strong association between insulin resistance and fatty liver has led to speculation of a bidirectional cause-and-effect relationship between the two, in essence a vicious cycle wherein the fatty liver caused by insulin resistance further exacerbates the insulin resistance. However, there are many examples of mouse models that exhibit fatty liver but have normal or improved insulin sensitivity [26, 27], and examples of genetic defects in humans that cause

fatty liver but do not affect insulin sensitivity [26]. These data suggest that fatty liver does not necessarily cause insulin resistance in itself [27].

In addition to fatty liver, changes in hepatic lipoprotein metabolism lead to the dyslipidemia that accompanies the metabolic syndrome and insulin resistance, characterized by the so-called lipid triad: high levels of plasma triglycerides, low levels of HDL cholesterol, and the presence of small, dense LDL (sdLDL) (reviewed in [28]). Additionally, increased postprandial triglyceride and apoB48 levels are a hallmark of diabetic dyslipidemia [29, 30] and is in part due to increased intestinal chylomicron production [31, 32] and decreased clearance of chylomicron remnants by the liver [32, 33]. These lipid abnormalities are atherogenic and promote cardiovascular disease, the leading cause of death among diabetics (52% of type II diabetics in a multinational study by the WHO) [34] and overall (30% worldwide) [16].

The high level of plasma triglycerides is primarily due to an overproduction of triglyceride-rich VLDL by the liver (reviewed in [35]). This, in turn, at least partially originates in the increased amount of triglycerides stored in cytosolic lipid droplets resulting from fatty liver: stored hepatic triglycerides are quantitatively important components of VLDL [6, 7]. In obese patients, the degree of hepatic steatosis correlates positively with VLDL secretion rates [36].

The decrease in HDL cholesterol (both in particle number as reflected by decreased apoA1 levels and in cholesterol content of each particle) is due to increased catabolism of apoA1 by the liver [37, 38] and increased activity of CETP in the plasma in transferring triglycerides from triglyceride-rich lipoproteins such as VLDL to HDL in exchange for cholesterol esters, resulting in a net depletion of HDL-associated cholesterol [39]. The increase in CETP-mediated lipid exchange is, to some degree, stimulated by the high levels of triglyceride-rich VLDL [40]. However, liver insulin receptor knockout (LIRKO) mice, a

model for purely hepatic insulin resistance, do not have fatty liver or hypertriglyceridemia, yet these mice have the low HDL cholesterol (and conversely, cholesterol-enriched VLDL) characteristic of the metabolic syndrome [41]. The studies on LIRKO mice suggest that the features of insulin resistance-associated dyslipidemia can be separated into those due to the hepatic fat accumulation resulting from peripheral insulin resistance (increased plasma triglycerides) and those due to central (hepatic) insulin resistance (decreased HDL cholesterol).

The third component of the lipid triad, the presence of sdLDL, is less directly related to intrahepatic lipid metabolism. CETP in the plasma promotes the transfer of triglycerides from VLDL to LDL, and the resulting triglyceride-rich LDL is a preferred substrate for hepatic lipase on the surface of the liver, which hydrolyses it into sdLDL.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR A

The peroxisome proliferator-activated receptors (PPARs) consist of three known nuclear receptors that have emerged as clinically significant targets for treatment of metabolic syndrome. Each of the three family members, PPAR α , PPAR δ , and PPAR γ , are key metabolic regulators: PPAR α controls fatty acid oxidation and is necessary for the adaptive responses to fasting in the liver, PPAR γ is necessary for adipogenesis and promotes lipid storage in adipose tissue, and PPAR δ is important for muscle and liver lipid metabolism.

Drugs that target PPAR α and PPAR γ are currently on the market for treatment of metabolic diseases: fibrate drugs target PPAR α and are used to treat dyslipidemia (reviewed in [42]) while thiazolidinediones target PPAR γ and are used to treat type II diabetes. Fibrates are carboxylic acids that bind to PPAR α , thereby functioning as agonistic ligands; fenofibrate and gemfibrozil of this drug class are FDA-approved for treatment of hyperlipidemia.

PPAR α activation by fibrates lowers plasma triglycerides and raises HDL cholesterol.

The former is due to increased clearance of plasma triglycerides by the liver [43] and, probably, to the decreased availability of fatty acids for triglyceride synthesis due to increased β -oxidation (in mouse [44] and rat [45] liver following fenofibrate treatment). The increase in HDL cholesterol is dependent on increased production of the HDL apolipoproteins apoAI and apoAII [46, 47].

While PPAR γ agonists have fallen out of favor due to side effects, PPAR α and PPAR δ agonists continue to be of therapeutic interest for treating the metabolic syndrome. At the time of writing, a dual PPAR α /PPAR δ agonist, GFT505, is in phase IIB clinical trials in the US and several EU countries for treatment of non-alcoholic hepatosteatosis, and in numerous pilot studies for treatment of dyslipidemia, obesity, and type II diabetes.

Function of PPAR α in the fasting response

PPAR α is expressed in several metabolically active tissues, including liver, muscle, and heart. In the liver, PPAR α is a key regulator of fatty acid metabolism and necessary for fasting response: PPAR α -null mice are deficient in both ketogenesis and gluconeogenesis and are unable to adapt when challenged with fasting, developing hypoglycemia and hypoketonemia [48]. PPAR α promotes the cellular uptake and catabolism of fatty acids by upregulating genes involved in fatty acid transport, peroxisomal and mitochondrial fatty acid β -oxidation, and ketogenesis, leading to an increased uptake and break-down of fatty acids to generate ketones, reducing equivalents and ATP when PPAR α is activated during fasting [48, 49].

Some important PPAR α target genes/proteins in both mice and humans (reviewed in [50]) that are involved in lipid metabolism include: ACO1 (encoded by *Acox1*), which catalyzes the first step in peroxisomal β -oxidation; carnitine palmitoyl transferase 1 and 2 (CPT1 and CPT2, encoded by *Cpt1a* and *Cpt2*, respectively), which mediate the transport of

long-chain fatty acids across the mitochondrial membrane such that they can be oxidized; FGF21 (encoded by *Fgf21*), a hormone important for ketogenesis; apoAI (encoded by *Apoa1*), the key apolipoprotein in HDL; and ATP-binding cassette transporter 1 (ABCA1, encoded by *ABCA1*), a cholesterol transporter.

PPAR α is also crucial to fasting-induced gluconeogenesis. In the absence of PPAR α , mice become hypoglycemic within just hours of fasting onset (probably reflecting the reduced liver glycogen in these mice, as glucose levels are primarily maintained by glycogenolysis during the first hours of fasting), and the blood glucose continues to drop steeply as fasting progresses, reaching a low of less than half of that of the wild type controls after 24 hours of fasting (45 mg/dl vs. to 100 mg/dl) [48]. “Fasting” in this study and studies on mice and humans cited below refers to complete withdrawal of food but ad lib access to water. Fasting data are relative to ad lib feeding (mice) unless otherwise specified or the latest meal (humans). “Fasting” will be used interchangeably with “starvation.”

The importance of PPAR α for fasting-induced gluconeogenesis may largely be due to the dependency of gluconeogenesis on fatty acid β -oxidation for reducing equivalents in the form of NADH. β -oxidation is a quantitatively important source of NADH: in rat liver, inhibition of β -oxidation with bromooctanoate causes an over 60% reduction in NADH levels [51]. The decreased hepatic glucose production in PPAR α -null mice stems from a dramatically decreased use of lactate as a substrate for gluconeogenesis, despite normal lactate levels [52]. Gluconeogenesis from glycerol, on the other hand, is in fact increased 2.5-fold in PPAR α -null mice [52]. Conversion of one molecule of lactate to pyruvate to eventually glyceraldehyde-3-phosphate (G3P) requires the use of two molecules of NADH whereas conversion of glycerol to G3P requires none, explaining how this compensatory increase in the glycerol-G3P arm of gluconeogenesis in PPAR α -null mice is possible.

To some degree, PPAR α also affects glucose metabolism via expression of

gluconeogenic genes: the expression of pyruvate kinase is decreased 16-fold in fasted PPAR α -null mice [52]. However, the gene encoding phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in gluconeogenesis, is expressed normally in PPAR α -null mice [48, 52].

Hormonal and nutritional regulation of fasting and PPAR α

PPAR α is activated during periods of fasting or starvation. The main physiological effects of PPAR α activation are increased plasma ketone levels and maintenance of euglycemia. Because regulation of glucose levels is complex, ketone levels are a better surrogate for inferring PPAR α activity and will be used as such below. Another surrogate is protein or mRNA expression levels of classical PPAR α target genes in the liver such as those mentioned above. Expression levels of the mRNA for PPAR α itself increases as well during fasting, but follows the same time course as induction of other PPAR α target genes [53], suggesting a positive feedback loop. Ligand activation occurs much sooner and is the primary method of regulation of PPAR α .

Hepatic mRNA levels of the PPAR α target genes ACO1 and CPT1 are unchanged at 4 hours of fasting and increased 2-fold and over 4-fold, respectively, after 8 hours of fasting in mice [53]. Plasma levels of β -hydroxybutyrate, a ketone body formed from fatty acid oxidation/ketogenesis, start increasing after 12 hours of fasting in humans [54] and sometime between 6 and 12 hours of fasting in mice (β -hydroxybutyrate levels are unchanged at 6 hours of fasting [55] but 10-fold increased at 12 hours of fasting [56]), consistent with the time needed for the mRNA to be translated into active proteins promoting ketogenesis. We can conclude that in mice, PPAR α is activated sometime within the first 8 hours of fasting, and its physiological effects become evident within 12 hours of fasting.

The many changes in nutrient and hormone levels that accompany fasting make it

difficult to pinpoint which specific stimulus that causes this activation of PPAR α . During fasting, plasma insulin and leptin levels as well as plasma glucose progressively drop, while the levels of plasma free fatty acids and glucagon rise. In mice, insulin levels are down 70% by six hours of fasting [55]. In humans, insulin levels decrease at a steady rate immediately from the onset of fasting, decreasing by 30% after 6 hours of fasting and 50% after 12 hours of fasting [57]. Leptin levels decrease by 50% after 12 hours of fasting in humans [58]; in mice, leptin levels start decreasing after 4 hours of fasting and are down by 75% after 12 hours of fasting [59]. Plasma free fatty acids increase progressively from the onset of fasting, mirroring the decrease in insulin levels, and have increased by 60-70% at 12 hours of fasting in humans [57] and mice [60]. Glucagon levels are rapidly and dramatically regulated in mice, being increased 5-fold by 6 hours of fasting [55], but much more slowly in humans, where glucagon levels are unchanged after 6 hours of fasting and increased 25% by 12 hours of fasting [57].

In addition, there is species-specific regulation of hormones by fasting. In humans, growth hormone also starts rising after 12 hours of fasting and stimulates lipolysis [57]; in mice, however, growth hormone secretion is actually suppressed by fasting and does not affect lipolysis [59]. Levels of ghrelin, an appetite-stimulating hormone, increase two-fold by 12 hours of fasting in mice [59], but in humans, ghrelin levels actually decrease with fasting (20% by 12 hours and 30% by 24 hours of fasting) [61]. Catecholamines (i.e. epinephrine and norepinephrine) and cortisol levels rise and fall cyclically throughout prolonged fasting in humans with a drop to below fed levels between 15 and 24 hours after fasting onset [57], and are hence probably not involved in the adaptive responses to typical fasting periods.

Among these stimuli, plasma glucose, insulin, glucagon, leptin, and FFA levels are the most likely candidates for physiological regulators of the fasting response via PPAR α , as they are regulated in the same direction in both mice and humans and because of the time

course of their increase or decrease in plasma.

The role of plasma glucose is difficult to study as an independent variable, and little information is available on the effects of glucose deprivation/overload on PPAR α targets in liver cell lines. In cultured primary hepatocytes, mRNA expression of *Acox1*, *Cpt1*, and *Cpt2* were unchanged by incubation with 20 mM glucose for 5 hours compared to 6 mM, but this may have been too short of an incubation time to see gene expression effects [62].

In mice lacking the liver insulin receptor, plasma ketone levels and expression of the PPAR α target gene *Cpt1* are twice as high as in wild type mice during ad lib feeding (during fasting, plasma ketone levels are the same, *Cpt1* expression ~75% higher than in controls) [63], suggesting that hepatic insulin signaling is important for suppression of the fasting response/PPAR α activity during feeding.

Fasting induction of some, but not all, PPAR α target genes is abolished in mice lacking glucagon receptor, and treatment of hepatocytes with glucagon induces expression of PPAR α target genes and fatty acid oxidation, the latter demonstrated to be in a PPAR α -dependent manner [64].

Mice lacking leptin (*ob/ob*) have increased expression of *Cpt1* in the ad lib fed state compared to wild type mice (information is lacking about its fasting induction, however), [65], but interpretation with regards to any direct role of leptin in promoting the fasting response is complicated by the obesity and insulin resistance of the *ob/ob* mice; insulin resistance would blunt insulin suppression of the fasting response in the fed state, and the mice have elevated circulating FFAs. Any role of FFAs in activating the fasting response is difficult to study in vivo due to the lack of any single FFA receptor or transporter that can be genetically modified, but in vitro, various long-chain fatty acids activate PPAR α (demonstrated using a PPRE-containing reporter gene); these experiments were among the first studies on PPAR α [66].

In summary, the interrelationship between circulating nutrients and hormones makes it difficult to study the effects of a single nutrient or hormone on PPAR α . However, thanks to studies on mice lacking liver insulin receptor and on mice lacking (whole-body) glucagon receptor, we can conclude that hepatic insulin signaling is important for suppressing PPAR α activity during feeding, while glucagon signaling is important for induction of PPAR α activity during fasting.

Ligand activation of PPAR α

PPAR α is primarily ligand-activated. Ligand binding causes it to adopt an active conformation and heterodimerize with the nuclear receptor retinoid X receptor-alpha (RXR α), allowing the PPAR α -RXR α complex to bind to peroxisome proliferator response elements (PPREs) on target genes and activate gene transcription [67, 68].

While the fibrate drugs previously described are synthetic agonistic ligands for PPAR α , a physiological ligand for PPAR α was only identified recently [69]: in mouse liver, the phosphatidylcholine (PC) species 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC) acts as an endogenously synthesized PPAR α ligand [69]. The interaction between this particular ligand and PPAR α requires the activity of fatty acid synthase (FAS; described in more detail below) as well as choline-ethanolamine phosphotransferase-1 (CEPT1), the enzyme catalyzing the final step in PC synthesis [69, 70].

It is not known how the 16:0/18:1-GPC ligand reaches PPAR α from CEPT1. CEPT1 is localized to the ER and nuclear membranes (the active site facing the cytoplasm) [71], while PPAR α is localized to the nucleus [72], precluding a direct interaction between CEPT1 and PPAR α . Simple diffusion of the ligand is unlikely to be a physiologically relevant mechanism of transport, as the cytoplasmic movement of amphipathic molecules such as PC is extremely inefficient [73]. A more likely possibility is that the PC ligand is transported to

PPAR α in association with a soluble lipid-binding protein.

FATTY ACID SYNTHASE

Fatty acid synthase (FAS, encoded by *Fasn*) catalyzes the biosynthesis of saturated fatty acids from simple precursors (de novo lipogenesis). The primary product of the FAS reaction is palmitate (C16:0), but stearate (C18:0) and shorter fatty acids may also be produced. FAS substrates are acetyl-CoA, malonyl-CoA, and NADPH. Acetyl-CoA functions as a primer for the reaction, while NADPH provides reducing equivalents. The fatty acid is elongated from the initial acetyl-CoA by repeated condensations with malonyl-CoA, which donates two carbons in each cycle of condensation. Palmitate synthesis thus requires seven cycles of malonyl-CoA addition to an acetyl-CoA primer to yield a saturated, 16-carbon fatty acid.

The FAS protein exists as a homodimer of 273 kDa subunits. Each monomer contains seven protein domains required for fatty acid synthesis: acyl carrier, acyl transferase, β -ketoacyl synthase, β -ketoacyl reductase, β -hydroxylacyl dehydratase, enoyl reductase, and thioesterase (reviewed in [74-76]). However, FAS is only enzymatically active in the dimeric form [77]. The monomers were initially thought to be oriented head-to-tail to form the dimer [78, 79], but recent structural data demonstrate a head-to-head orientation of the monomers that are intertwined at their middle to form an X-shape [80-83]. Mammalian FAS is a type I FAS complex with the domains consolidated in a single peptide; prokaryotes and yeast have a type II FAS with separate proteins catalyzing the individual reactions. Type II FAS complexes capable of synthesizing short-chain (up to 14 carbons) fatty acids are also found in mammalian mitochondria [84].

FAS is a soluble protein and thought to be localized in the cytoplasm, although the specifics of its subcellular localization are largely unexplored. Its tissue distribution is broad

with highest levels in the liver, adipose tissue, and lungs [85, 86]. Whole-body knockout of FAS causes embryonic lethality in mice, suggesting that de novo lipogenesis is necessary early during development [87]. A likely possibility is that FAS is required to provide lipids for cell membranes of the growing embryo. Viable tissue-specific FAS knockout mice have been generated, including a liver-specific knockout (discussed below).

Functions of hepatic FAS: Contribution to stored and secreted lipids

Hepatic FAS synthesizes lipids that are stored as lipid droplets or secreted in VLDL in the fed state. In mice, the contribution of liver FAS to secreted VLDL is minor. *Ob/ob* mice have 10-fold increased hepatic de novo lipogenesis compared to lean mice, but no significant differences in serum triglycerides [88]. In mice with liver-specific knockout of FAS (FASKOL mice), serum triglycerides are normal on a chow diet [69].

The contribution of de novo lipogenesis to secreted triglycerides has been studied in humans in the setting of various diets. On diets low in fat and high in carbohydrate (10% of calories as fat and 75% as carbohydrate), de novo lipogenesis makes a significant contribution to circulating lipids as almost half of VLDL triglyceride is derived from de novo lipogenesis under these conditions [89]. However, a typical Western diet is high in fat as well as carbohydrates. In similar studies using diets higher in fat (30% fat and 55% carbohydrate or 40% fat and 45% carbohydrate), the contribution of de novo lipogenesis to VLDL triglycerides is undetectable or minor, at 0-10% [89, 90]. These diets are more representative of the high fat, high carbohydrate content of a typical Western diet, indicating that under common dietary conditions, de novo lipogenesis is not a significant contributor to VLDL triglycerides. Substituting starch for sugar in a high-carbohydrate diet also decreases the contribution of de novo lipogenesis to 0-1% or 5% [91, 92]. Obese individuals do not appear to have increased FAS-derived VLDL triglycerides compared to lean individuals [90]. Under

the high-fat, high-carbohydrate dietary conditions common in the Western world today, hepatic FAS thus appears to be a minor contributor to VLDL triglycerides.

FAS may contribute to triglycerides stored in hepatic lipid droplets. In rats fed a chow diet, $11 \pm 1\%$ of hepatic triglycerides are derived from de novo lipogenesis [93]. On a high-fat diet, de novo lipogenesis is suppressed and only $1.0 \pm 0.2\%$ of hepatic triglycerides are derived from FAS [93]. FASKOL mice (animals with inactivation of FAS in the liver) on a chow diet have normal, rather than decreased, liver triglyceride content [69]. It thus appears that the contribution of de novo lipogenesis to stored triglycerides is small in healthy liver.

In fatty liver, the contribution of FAS to intrahepatic triglycerides may be greater. *Ob/ob* mice have increased hepatic FAS activity and fatty liver [94], but a mechanistic link between the two has not been established. In humans with non-alcoholic fatty liver disease, one group has reported that $26 \pm 7\%$ of hepatic triglycerides are derived from de novo lipogenesis [95]. It is unknown how this compares to the triglyceride content of healthy human liver. However, even in the setting of hepatic over-accumulation of fat, the contribution of FAS appears to be less than that of fats derived from peripheral tissues or dietary fat.

Functions of hepatic FAS: FAS-dependent ligand activation of PPAR α

When liver-specific fatty acid synthase knockout (FASKOL) mice were generated, they were surprisingly not protected against hepatic lipid accumulation, but instead developed severe hepatic steatosis when on a zero-fat diet or with prolonged fasting [70]. The phenotype of fasted or zero-fat diet-fed FASKOL mice is similar to that of PPAR α -null mice: hypoglycemia, low serum ketone levels, marked hepatic steatosis, and deficient hepatic fatty acid oxidation [49, 70]. Much of this phenotype was corrected by administration of a known PPAR α agonist.

The deficient PPAR α activation in the absence of both FAS and dietary fat led to the hypothesis that “new” fat, derived from de novo lipogenesis or dietary fat, can activate PPAR α , whereas “old” fat, derived from peripheral tissues or stored in the liver, cannot. Hydrolysis of hepatic triglycerides has also been shown to mediate PPAR α activation [96], suggesting that triglycerides of different origins (de novo synthesis vs. free fatty acids entering the liver following lipolysis in peripheral tissues) may occupy separate compartments in the hepatocyte.

In addition to activating PPAR α in liver, FAS has been shown to regulate PPAR α in macrophages [97] and hypothalamus [98] as well; knock-out of FAS in these tissues leads to decreased expression of PPAR α target genes which can be rescued by administration of a PPAR α agonist.

Further study of the FASKOL mouse led to the identification of an endogenous ligand for hepatic PPAR α : the phosphatidylcholine species 16:0/18:1-glycerophosphocholine [69], also described above. The interaction of this species with PPAR α is dependent on the activity of FAS, and inactivation of choline/ethanolamine phosphotransferase 1 (CEPT1), the enzyme catalyzing the final step in the Kennedy pathway for phosphatidylcholine biosynthesis, mimics the FASKOL phenotype [69]. The dependency of PPAR α activity on FAS is likely mediated by its provision of substrate for CEPT1, ultimately producing ligand for PPAR α : long-chain fatty acids generated by FAS may be converted to acyl-CoA and then diacylglycerol, which can combine with phosphocholine to form phosphatidylcholine in the reaction catalyzed by CEPT1.

A summary of the impact of FAS on hepatic triglyceride metabolism is presented in Figure 1.

Modulating hepatic FAS to treat disease

Ob/ob mice have increased hepatic FAS gene expression as well as increased hepatic FAS activity compared to lean mice [94]. Knockdown of the transcription factor carbohydrate response element binding protein (ChREBP), which promotes the expression of FAS as well as other genes, in *ob/ob* liver decreases hepatic lipid accumulation and decreases hepatic lipogenesis, suggesting a link between de novo lipogenesis by FAS and fatty liver [99]. However, in a gene expression profiling study of *ob/ob* animals separated into high glucose and lower glucose groups, mice with lower sugars (and thus likely to be more insulin sensitive) had higher hepatic expression levels of genes encoding lipogenic enzymes, including FAS, as compared to mice with higher sugars [100]. This finding suggests that while activation of lipogenic enzymes in the liver is associated with obesity, this effect is unlikely to be mechanistically linked to insulin resistance.

FAS inhibitors have been tested in mouse models of obesity and diabetes. Treatment of lean or obese mice with the FAS inhibitor C75 causes dramatic weight loss and improvement of hepatic steatosis in obese mice. However, the effect is primarily mediated by reduced food intake through inhibition of hypothalamic FAS (in addition to possible effects of this particular agent that are independent of FAS), obscuring the potential effects of modulating hepatic FAS [101].

The FAS inhibitor platensimycin is concentrated in the liver when administered orally and does not affect food intake [102]. Treatment of high-fructose diet-fed *db/db* mice with platensimycin reduces hepatic FAS activity, hepatic lipid accumulation, and hepatic fatty acid oxidation [102]. These data are consistent with roles for hepatic FAS both as a producer of fat that may accumulate in liver, and as a generator of lipid signals to nuclear receptors such as PPAR α .

These data also highlight a caveat when considering FAS inhibitors as therapy for hepatic steatosis: inhibition of FAS can affect both lipid storage and lipid catabolism, and

under conditions where baseline FAS activity is not particularly high, loss of FAS activity might aggravate rather than ameliorate hepatic steatosis, as seen in the liver-specific FAS knockout mice [70].

Regulation of FAS: Hormonal and nutritional regulation

Hepatic FAS is known to be regulated by insulin, glucagon, cyclic AMP, fructose, glucose, and dietary fat. The long-term effects of hormones and nutrients on FAS expression are clear, but their immediate effects are poorly understood.

Re-feeding mice or rats a high-carbohydrate diet following a prolonged fast causes a robust induction of FAS expression as compared to the fasted or the ad lib-fed state [103-106]. The effect of carbohydrate re-feeding is mediated by both insulin and glucose. Insulin regulates FAS through transcriptional and non-transcriptional mechanisms. Under nutrient-replete conditions, de novo lipogenesis may promote storage of excess energy in the form of hepatic triglycerides. Insulin promotes FAS expression through activation of the transcription factors sterol regulatory element binding protein 1c (SREBP-1c) [107] and upstream stimulatory factors 1 and 2 (USF1 and USF2) [108, 109]. Conversely, glucagon and cyclic AMP inhibit the increase in FAS activity induced by carbohydrate re-feeding in rats [103, 110, 111].

The effect of fasting compared to ad lib feeding on the activity of hepatic FAS is less clear. In mice, a 6 hour fast reduces FAS expression levels by 60% compared to ad lib feeding [105], and in rats, a 24 hour fast reduces FAS expression by over 90% compared to ad lib feeding [106]. However, a 14-hour fast in mice produces no change in FAS activity compared to ad lib-fed mice [112]. One potential explanation for the lack of change in FAS activity in some circumstance could be a relatively long half-life for the FAS protein. It is possible that changes in FAS gene expression might have little effect on FAS enzyme activity

in response to certain physiologically relevant periods of fasting as compared to the ad lib fed condition.

While insulin promotes the expression of FAS, insulin also acutely inhibits the enzymatic activity of hepatic FAS, causing a decrease in FAS activity within minutes [112]. This inhibition is dependent on the presence of the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), which is phosphorylated in response to insulin and subsequently associates with FAS [112]. The acute inhibition of FAS by insulin is blunted in hyperinsulinemic *ob/ob* mice [112]. While a clear physiological role for this acute inhibition of FAS activity has not been determined, it is possible that the acute effect on FAS by insulin primarily affects FAS lipid signals that impact PPAR α . Acute inhibition of FAS in response to insulin could then serve to halt the fasting response by PPAR α and decrease fatty acid oxidation when nutrients are abundant. Because the specific activity of FAS is affected, the effect is likely post-translationally mediated. In contrast, the long-term effect of insulin on FAS is transcriptionally mediated and promotes FAS expression, enabling increased storage of energy as fat.

Carbohydrates directly promote the expression of hepatic FAS in the liver in addition to having an indirect effect by stimulating insulin secretion. Feeding mice a high-glucose or high-fructose diet for one week leads to 3-fold and 8-fold, respectively, increases in FAS protein [113]. The effect of glucose on FAS expression is mediated by ChREBP [114-117]. Hepatic metabolism of glucose by glucokinase (GK) is necessary for the glucose-mediated induction of FAS by ChREBP [118]. The insulin-induced activation of SREBP-1c and the glucose-induced activation of ChREBP act synergistically to promote FAS expression [118]. A connection between lipid/carbohydrate sensing and metabolism is suggested by the finding that stearoyl-CoA desaturase (*Scd1*), an enzyme catalyzing the synthesis of oleate, is involved in the carbohydrate-induced induction of FAS and other lipogenic enzymes [113].

Dietary fats inhibit FAS expression to decrease de novo lipogenesis when fats are already abundant. Polyunsaturated fatty acids (PUFAs) may decrease FAS expression through inhibition of SREBP-1c [119] and ChREBP [120] activity. Diets consisting of 10% oil inhibit hepatic FAS activity when fed to rats over the course of 4 weeks, with the greatest reduction in rats fed fish oil [121]. Re-feeding rats a carbohydrate-free, high-fat diet following fasting suppresses FAS gene expression to levels as low as those seen in rats fasted for 24 hours [106].

Regulation of FAS: Transcription and the FAS promoter

Transcriptional regulation of FAS has been well characterized. Much of the work on transcriptional regulation of FAS has been done in rats, but the FAS promoter is highly conserved between species suggesting that studies of the rat FAS promoter are likely to be relevant to mice and humans. Regulatory elements and transcription factor binding sites in the proximal mouse FAS promoter are shown in Figure 2.

As noted above, SREBP-1c is activated by insulin and under appropriate conditions promotes expression of lipogenic genes, including FAS. The FAS promoter contains a sterol regulatory element (SRE) at -150 as well as tandem SREs at positions -72 and -62 that are required for optimal SREBP-1c-mediated activation of FAS expression in rats [122-124].

An inverted CCAAT box at -94 is a binding site for nuclear factor Y (NF-Y) and is necessary for inhibition of FAS expression by cyclic AMP [125, 126]. A binding site for the transcription factor specificity factor 1 (Sp1) is located nearby at -91 [124]. NF-Y and Sp1 proteins interact [127] and mediate sterol-induced FAS expression synergistically with SREBP-1c [124, 128]. Another transcription factor, X-box binding protein 1 (XBP1), increases FAS promoter activity indirectly via SREBP-1c [129].

Also as noted above, ChREBP plays a central role in the glucose-induced

transcriptional regulation of FAS as well as other lipogenic and glycolytic genes in the liver [114-117]. Glucose promotes the nuclear translocation and activation of ChREBP, while polyunsaturated fatty acids and cyclic AMP inhibit ChREBP activity [120, 130]. ChREBP binds to a carbohydrate response element (ChRE) located at -7214 in the distal FAS promoter in rats to activate FAS transcription [131]. ChREBP appears to be the main regulator of glucose-induced FAS expression, as glucose fails to induce an increase in FAS expression in ChREBP-null hepatocytes [114]. Mice fed a high-fructose diet have similar amounts of nuclear ChREBP protein and ChRE-bound ChREBP protein compared to mice fed a high-glucose diet, suggesting that dietary fructose and glucose have comparable effects on ChREBP [132].

In addition to the ChRE, a direct repeat-1 (DR-1) element located between -7110 and -7090 in the distal promoter of rat FAS is necessary for full glucose activation of FAS expression [133]. Hepatic nuclear factor-4 α (HNF-4 α) binds to the DR-1 element and interacts with ChREBP. Ablation of HNF-4 α produces a corresponding decrease in glucose-induced FAS expression [133].

Liver X receptor (LXR), a transcription factor activated by oxysterols, upregulates FAS expression through direct and indirect mechanisms. Indirectly, LXR can promote FAS expression by binding to liver X receptor elements (LXREs) in the promoters of the SREBP [134] and ChREBP [135] genes to promote their transcription. SREBP and ChREBP in turn activate FAS transcription. The LXR-mediated activation of SREBP-1c is the primary mechanism of insulin-induced SREBP activation [134]. The physiological relevance of LXR-mediated transcriptional regulation of ChREBP is debated, as LXR is not necessary for the glucose-induced activation of ChREBP [117]. LXR can also bind directly to LXREs located at positions -686 to -672 of the mouse FAS promoter to activate FAS transcription [136].

An insulin response element (IRE) containing an E-box DNA binding motif is located

at positions -71 to -50 of the FAS promoter, overlapping two tandem SREs. The IRE is necessary for insulin-induced FAS expression [137]. USF1 and USF2 bind to the IRE [109]. Mutation of the E-box prevents USF binding and abolishes insulin-induced FAS expression. However, the importance of USFs in insulin-stimulated FAS expression remains unclear, because mutation of the E-box also prevented SREBP-1c binding [108].

Regulation of FAS: Post-translational regulation

While its transcriptional regulation has been well characterized, little has been known about post-translational regulation of FAS activity.

Transcriptional regulation of FAS may require hours to affect protein levels since both FAS mRNA and protein are fairly stable, buffering sudden changes due to increased transcription and subsequent translation. There are several reports of FAS protein being activated or inhibited in far shorter time frames, as well as reports of changes in FAS activity that do not correlate with changes in FAS protein levels.

Insulin acutely decreases FAS enzyme activity. In hepatoma cells, FAS activity decreases linearly from 2 to 15 minutes after insulin treatment, followed by an increase in FAS activity for 75 minutes [112]. Peroxynitrate inhibits FAS activity in adipocytes within 10 minutes, without any effect on FAS protein levels [138]. Activation and inhibition of FAS without corresponding changes in FAS protein levels have been reported in a variety of cancer cell lines [139-141]. These data suggest the presence of post-translational regulation of FAS.

Phosphorylation has been proposed as a mechanism of FAS regulation in cancer cells, adipocytes, and liver. In livers from pigeons that were fasted and then re-fed, radiolabeled phosphate was incorporated into FAS only in the cytosolic fraction of the liver (the membrane fraction was not studied). The phosphorylation event was associated with low

FAS activity, and dephosphorylation of FAS by incubation with phosphatases caused a 20-fold increase in FAS activity [142]. Another inhibitory phosphorylation was demonstrated in 3T3L1 adipocytes, where FAS threonine phosphorylation was associated with inhibition of FAS activity [138]. This phosphorylation event was shown to require AMP-activated kinase (AMPK), likely through indirect effects since in vitro kinase assays failed to demonstrate any incorporation of labeled phosphate into FAS in the presence of AMPK [138]. These findings suggest the presence of an unidentified intermediate kinase step.

In human and mouse breast cancer cell lines, the finding that large differences in FAS activity between cell lines did not correlate with FAS protein levels prompted an exploration of FAS phosphorylation as an alternative mechanism of FAS regulation [140]. Phosphoserine and phosphothreonine residues were detected in FAS in cell lines from both species, while FAS phosphotyrosine residues were detected in human cells only. Phosphorylation of FAS in these cell lines was associated with greater FAS activity [140]. Recently, tyrosine phosphorylation of FAS was noted in two different human breast cancer cell lines. Both FAS tyrosine phosphorylation and FAS activity were induced by overexpression of human epidermal growth factor receptor 2 (HER2) and decreased by HER2 inhibition, and FAS was phosphorylated when complexed with HER2 [141].

In addition to phosphorylation, FAS was one of a large number of hepatic metabolic enzymes recently found to be lysine acetylated [143]. Acetylation was linked with diverse effects on metabolic enzymes, including protein destabilization, activation, and inhibition, suggesting that acetylation may play a major role in metabolic regulation. Acetylation of FAS could represent a novel mechanism for controlling its activity.

Known examples of post-translational regulation of FAS are summarized in Table 1.

CONCLUSIONS AND DISSERTATION OBJECTIVES

A physiological ligand for hepatic PPAR α was identified in 16:0/18:1-glycerophosphocholine, but is not known how activation of PPAR α by this ligand is regulated—by synthesis, delivery, or breakdown—such that PPAR α is activated within the first few hours of food withdrawal and inactivated as soon as nutrients and insulin become abundant again. The role of FAS in synthesizing this ligand does little to aid our understanding. FAS is thought to be primarily regulated transcriptionally and to be activated after feeding. With the long half-life of the FAS protein, transcriptional regulation of FAS would be irrelevant for the time frame of fasting-induced changes. The postprandial activation of FAS is paradoxical—how can FAS be necessary for endogenous activation of the fasting response, when lipogenesis by FAS supposedly occurs after re-feeding?

Regulation of ligand synthesis aside, how the poorly soluble 16:0/18:1-glycerophosphocholine ligand would reach PPAR α in the nucleus from its site of synthesis on the cytoplasmic side of the ER and nuclear membranes is also unknown. The regulation of ligand transport could be another node of regulation of PPAR α in response to nutrient availability or insulin/glucagon action.

The objective of the research presented in this dissertation is to define the regulation of ligand-based PPAR α activation in the liver. I have focused on two nodes of regulation: that of ligand synthesis by FAS, and that of ligand delivery by a lipid-binding protein. Here, I present research demonstrating subcellular compartmentalization of FAS protein for different physiological functions; post-translational regulation of FAS in the form of compartment-specific inhibitory phosphorylations of a FAS active site in response to insulin or feeding; and nucleo-cytoplasmic transport of endogenously synthesized PPAR α ligand by phosphatidylcholine transfer protein (PCTP).

FIGURES

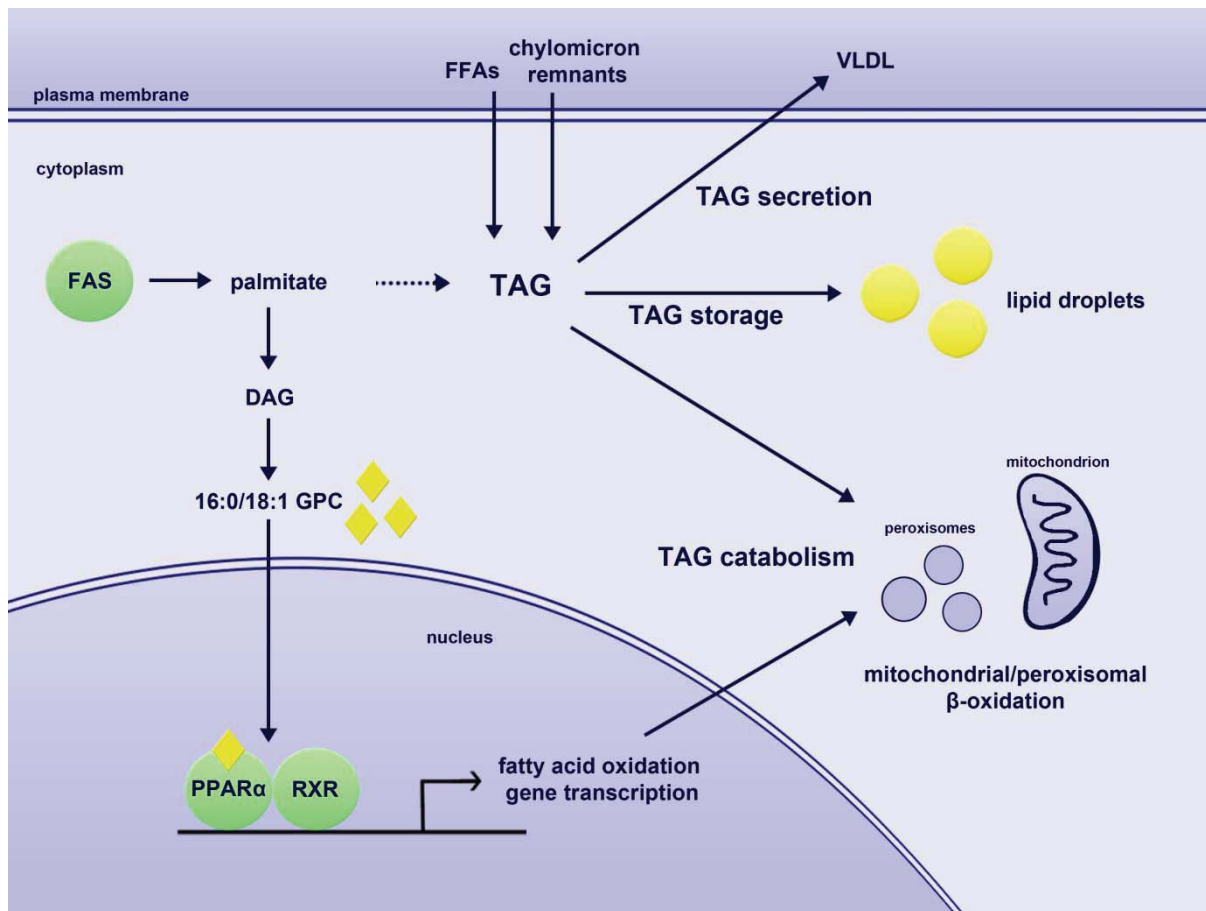


Figure 1. The role of FAS in hepatic lipid metabolism. Fatty acid synthase controls fatty acid catabolism through the synthesis of a ligand for PPAR α , which activates fatty acid oxidation genes. FAS makes a minor contribution of lipids to stored and secreted triglycerides, with the major contributions coming from plasma free fatty acids and dietary fats from chylomicron remnants. Abbreviations: 16:0/18:1 GPC, 16:0/18:1-glycerophosphocholine; DAG, diacylglycerol; FAS, fatty acid synthase; FFA, free fatty acid; PPAR α , peroxisome proliferator-activated receptor alpha; RXR, retinoid X receptor; TAG, triacylglycerol (triglyceride); VLDL, very low-density lipoprotein.

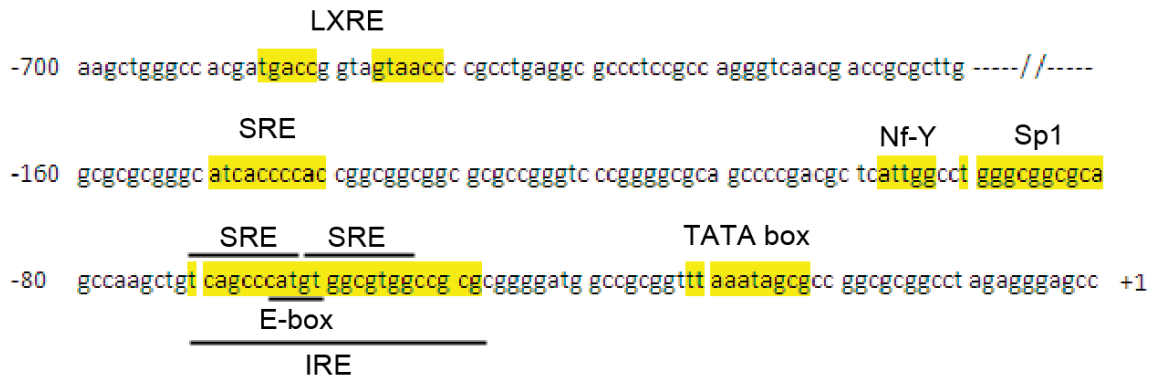


Figure 2. The mouse proximal FAS promoter. Regulatory elements and nuclear factor binding site nucleotides are highlighted in yellow. IRE, insulin response element; LXRE, liver X receptor element; Nf-Y, nuclear factor Y binding site; Sp1, specificity factor 1 binding site; SRE, sterol regulatory element.

TABLES

| Type of post-translational modification | Organism and tissue or cell type | Function |
|--|--|----------------------------------|
| Phosphorylation [142] | Pigeon liver | FAS inhibition |
| Threonine phosphorylation [138] | 3T3-L1 adipocytes (mouse) | FAS inhibition |
| Threonine and serine phosphorylation [140] | NMuMG (mouse mammary epithelial cells), T1 (mouse mammary tumor cells), SKBr3 (human breast carcinoma cells) | Unknown, possibly FAS activation |
| Tyrosine phosphorylation [140, 141] | SKBr3 (human breast carcinoma cells) | FAS activation |
| Acetylation [143] | Human liver | Unknown |

Table 1. Post-translational modifications of FAS.

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Chapter 2:

Differential Subcellular Localization of FAS may be Mediated by Interactions with Septin-2 and Septin-7

ABSTRACT

Fatty acid synthase (FAS) is a lipogenic enzyme functioning both in signaling, by promoting synthesis of lipid ligands and signaling molecules, and in energy balance, by synthesizing fat for storage of calories. In the liver, FAS exists in two distinct subcellular pools: cytoplasmic FAS and membrane FAS. These two pools are differentially regulated and appear to be specialized for different physiological functions.

Membrane FAS is associated with intracellular membranes through a strong peripheral interaction, but the nature of this interaction—whether it is mediated by a lipid anchor, by an interaction with another protein, or otherwise—is unknown. We hypothesized that hepatic FAS associates with membranes through a protein-protein interaction with a membrane-resident protein. To identify candidate proteins for localizing FAS to membranes, we used a proteomics approach to comprehensively identify proteins co-precipitating with FAS in the cytoplasmic and membrane fractions of two different liver model systems: Hepa1-6 immortalized hepatocytes and C57/BL6J mouse liver.

We identified three proteins—Septin-2, Septin-7, and 40S ribosomal protein S18—that in two different liver model systems associate with fatty acid synthase exclusively in the membrane fraction. Because the septins are involved in membrane structuring and scaffolding, these proteins are possible mediators of FAS membrane localization.

INTRODUCTION

The lipogenic enzyme fatty acid synthase (FAS) is highly expressed in liver and involved in several aspects of hepatic metabolism. FAS synthesizes long-chain fatty acids that can be incorporated into hepatic lipid droplets or secreted in lipoproteins. Additionally, FAS is necessary for generating an endogenous ligand for peroxisome proliferator-activated receptor α (PPAR α) in liver [1]. PPAR α is a nuclear receptor and the primary mediator of the fasting response, the adaptive changes in metabolism that occur during fasting or starvation. The involvement of FAS in both synthesis of lipids for energy storage as well as the synthesis of a lipid ligand to activate the fasting response during energy depletion is paradoxical.

FAS is localized to both the cytoplasm as well as to the ER and Golgi membranes [2]. The involvement of FAS in opposing processes (storage of excess energy and the fasting response) is explained by the cytoplasmic FAS and the membrane-associated FAS having distinct physiological functions; cytoplasmic FAS is active during starvation to generate PPAR α ligand, and membrane-associated FAS is active during feeding, probably to generate lipids for incorporation into lipid droplets or VLDL [2].

The association of FAS with the ER and Golgi is mediated by a strong peripheral membrane interaction [2], but the nature of this interaction is unknown. A peripheral membrane association can be mediated by lipid anchoring, by ionic or electrostatic interactions between the protein and membrane lipids, by an interaction between a hydrophobic loop or amphipathic α -helix and the membrane (the α -helix being in-plane with the membrane with the hydrophilic side facing the cytosol or organelle lumen), or by a protein-protein association with another membrane-resident protein.

Mass spectrometric analysis failed to detect any lipid anchors or other post-translational modifications of the FAS protein that could act as anchors [2]. There are currently no known exposed hydrophobic loops or α -helices in the FAS protein. Furthermore,

if FAS were associated with membranes through a direct protein-membrane interaction, then we would expect all FAS molecules to be membrane-associated, or the cytoplasmic FAS molecules to have some sort of modification obscuring the membrane association domain. However, FAS exists in both cytoplasmic and membrane-associated compartments, and no post-translational modifications characteristic of all FAS in a specific compartment could be identified [2].

Instead, we hypothesized that FAS associates with membranes through a protein-protein interaction with a membrane-resident protein, likely an integral membrane protein because of the strength of the interaction between FAS and membranes. If this membrane-resident protein is less abundant than FAS in the cell, then the membrane-resident protein would be saturated with FAS and be the limiting factor determining membrane residence of FAS, thus explaining how the two distinct cytoplasmic and membrane pools of FAS can exist despite the primary structure of the protein being identical between pools.

Here, we used a proteomic approach to identify FAS-associated proteins in the cytoplasmic and membrane fractions of a liver cell line and of mouse liver. We identified three proteins that are FAS-interactors exclusively in the membrane fraction and that were found in both model systems. Out of these, two (Septin-2 and Septin-7) are members of the septin class of proteins, which is known to be involved in membrane structuring, scaffolding, and compartmentalization, marking these FAS-interacting proteins as potential mediators of FAS membrane localization.

MATERIALS AND METHODS

Animals. A six-month old female C57BL/6J mouse was used as a source of liver for the mass spectrometric analysis of proteins.

Cell culture. Hepa1-6 cells were maintained in DMEM + 10% FBS until switching to SILAC (see below) media. The SILAC media contained 10% dialyzed FBS.

SILAC labeling. Hepa1-6 cells were differentially labeled using the stable isotope labeling with amino acids in cell culture (SILAC) technique [3, 4] by growing the cells for 6 passages in SILAC media containing “heavy” $^{13}\text{C}_6$ L-lysine or “light” $^{12}\text{C}_6$ L-lysine (Thermo Scientific), ensuring over 99% incorporation of labeled amino acid into the cellular proteome.

Subcellular fractionation. To fractionate mouse liver, perfused liver from a C57BL/6J mouse was homogenized in 20 mM HEPES buffer (pH 7.4) and centrifuged at 10,000 g for 45 min. The pellet was discarded and the supernatant spun at 179,000 g for 90 min at 4°C. The resulting supernatant (cytoplasm) was transferred to a fresh tube. Because the association of FAS with membranes is resistant to high salt treatment [2], the pellet (crude membrane fraction) was then resuspended in 20 mM HEPES buffer containing 1 M NaCl and incubated for 30 min in order to dissociate irrelevant proteins from the membrane. After spinning the sample again for 90 min, the supernatant was discarded and the pellet (crude membrane fraction) was resuspended in a detergent-containing buffer. All spins were done at 4°C.

To fractionate Hepa1-6 cells, “light” and “heavy” cells were harvested in 20 mM HEPES buffer and centrifuged at 10,000 g for 10 min. The pellets were discarded and the supernatants centrifuged at 179,000 g for 2 h. The supernatants (cytoplasm) were removed and the pellets (crude membrane fraction) were washed and resuspended.

Antibodies and immunoprecipitations. Rabbit polyclonal antibody against FAS (ab22759) from Abcam was used to immunoprecipitated FAS. Non-immune rabbit serum

from Jackson Immunoresearch was used for control immunoprecipitations.

For liver, FAS was immunoprecipitated from 1.2 mg of cytoplasmic or membrane protein by overnight incubation using a polyclonal rabbit anti-FAS antibody. For Hepa1-6 cells, equal amounts of cytoplasmic protein from “light” and “heavy” cells and equal amounts of membrane protein from “light” and “heavy” cells were used for immunoprecipitation of FAS (“light” cells) using a polyclonal rabbit anti-FAS antibody or for a control immunoprecipitation (“light” cells) with non-immune rabbit serum. After overnight incubations at 4°C with antibodies or non-immune serum, IgG agarose beads were added and the samples incubated for another 1 h.

Mass spectrometry. IP beads were washed, boiled in sample buffer, and subjected to SDS-PAGE. The gel was stained with Coomassie, the gel segment corresponding to FAS was excised and further cut into small pieces (1 mm²), destained with 50% CH₃CN containing 25 mM NH₄HCO₃, dehydrated, reduced with 20 mM DTT for 1 h at 55°C, washed and dehydrated, alkylated with 100 mM iodoacetamide for 1 h in the dark at room temperature, then subjected to cycles of washing and dehydration followed by drying in a centrifugal evaporator. In-gel digestion was performed with 0.02 mg/ml trypsin overnight at 37°C. Peptides were extracted from the gel pieces using 5% TFA in 50% CH₃CN and reconstituted in 0.1% FA in 3% CH₃CN.

Samples were then analyzed by LC-MS/MS with a NanoLC-LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) in data dependent mode. Acquired spectra were searched against the Swiss-Prot database through the Mascot server to identify proteins.

RESULTS

To identify candidate proteins localizing FAS to membranes, we comprehensively surveyed FAS-associated proteins in the cytoplasmic and membrane fractions of mouse liver and in a liver cell line by mass spectrometric analysis of proteins pulled down during immunoprecipitation of FAS. To narrow the list of candidates, we looked for proteins that were 1) exclusively found in the membrane fraction, 2) found in the membrane fractions of both model systems, and, preferably, 3) known integral membrane proteins.

FAS-associated proteins in mouse liver cytoplasm and membrane. To identify FAS-associated proteins in mouse liver, perfused liver was fractionated into a crude membrane fraction and a cytoplasmic fraction. FAS was immunoprecipitated from both membrane and cytoplasmic fractions using an anti-FAS antibody and the peptides pulled down were analyzed by mass spectrometry. FAS interactors identified in cytoplasm and membrane of mouse liver are listed in Table 1 and Table 2, respectively, along with the number of peptides identified for each protein and the protein score (or Mascot score) for each protein. A cut-off protein score of 100, reflecting a confidence level over 95% for protein identification, was used to determine likely specific interactors. 49 likely FAS interactors were identified in the cytoplasmic fraction and 50 likely interactors in the membrane fraction.

FAS-associated proteins in Hepa1-6 cell cytoplasm and membrane. To identify FAS-associated proteins in Hepa1-6 cells, a murine immortalized liver cell line, and to exclude as many non-specific interactions as possible, we used a combination of stable isotope labeling with amino acids in cell culture (SILAC) and mass spectrometry. Hepa1-6 cells were labeled as “heavy” or “light” using stable isotope labeling with amino acids in cell culture (SILAC) [3]. FAS was immunoprecipitated from “heavy” cells, while “light” cells were immunoprecipitated using a control antibody. Peptides from both populations were mixed and analyzed by mass spectrometry. The most likely FAS-interactors were found using

the ratio of the intensity of peptides identified in the “heavy” cells versus the control “light” cells, with a minimum ratio of 5 (meaning peak intensity is 5-fold higher for the “heavy” peak than the “light” peak) and a minimum number of 2 peptides detected as the cut-off for likely specific interactors.

FAS interactors in Hepa1-6 cytoplasm and membrane are listed in Table 3 and Table 4, respectively. Excluding FAS, 54 proteins were identified in the cytoplasmic fraction and 23 proteins were identified in the membrane fraction.

FAS-associated proteins exclusively found in the membrane fractions and in both model systems. Three proteins were found that were exclusively present in the membrane fraction and present in both Hepa1-6 cells and mouse liver (Table 5): Septin-2 (encoded by *Sept2*), Septin-7 (encoded by *Sept7*), and 40S ribosomal protein S18 (encoded by *Rps18*).

Integral membrane proteins. None of the three proteins fulfilling the first two criteria (exclusively present in the membrane fraction, and present in both model systems) are integral membrane proteins; Septin-2 and Septin-7 are soluble cytoskeletal proteins while 40S ribosomal protein S18 is a component of ribosomes, which are cytoplasmic or associated with ER membrane (rough ER).

DISCUSSION

While none of the three candidate FAS-interacting proteins that were identified as present exclusively in the membrane fractions in both Hepa1-6 cells and mouse liver were integral membrane protein, the nature of the septin protein class marks Septin-2 and Septin-7 as viable candidates capable of mediating the strong interaction between FAS and intracellular membranes.

The septin proteins are cytoskeletal GTP-binding proteins, forming hexameric and octameric complexes that can assemble into filaments (reviewed in [5]). Functions of the septins include scaffolding and membrane partitioning [5]. In phospholipid-based liposomes, septins tubulate the liposome membrane, creating a membrane “brace” [6]. Septins can regulate protein-protein associations [7] and protein-cytoskeleton interactions [8]. Unfortunately, there are no published studies to date on the function of Septin-2 or Septin-7 in liver or liver cells or in interactions with the ER membrane.

The identification of a ribosomal protein, 40S ribosomal protein S18, in the membrane fraction of both model systems could indicate that FAS binds to the rough ER. The presence of this protein may be a vestige of the abundance of ribosomes associated with the rough ER membrane.

In conclusion, we have identified three proteins—40S ribosomal protein S18, Septin-2, and Septin-7—that in two different liver model systems associate with fatty acid synthase exclusively in the membrane fraction. Rigorous cut-offs for isotope ratio or protein scores ensured a high confidence level for the candidate proteins being correctly identified and specific interactors of FAS. The association of Septin-2 and Septin-7 with membrane-bound FAS and the role of the septins in scaffolding and membrane structuring suggest that these proteins could be important for the membrane localization of FAS. If that is true, these proteins could potentially be modulated to change the proportion of FAS present in

association with membranes and in the cytoplasm, which in turn could affect FAS-mediated PPAR α activation and lipid storage/secretion. Studies on FAS localization and function in cells deficient in Septin-2 or Septin-7 are needed to elucidate the function of the FAS-septin interactions.

TABLES

| Accession ID | Description | Protein score | # of peptides |
|--------------|---|---------------|---------------|
| FAS_MOUSE | Fatty acid synthase | 6979 | 211 |
| ACACA_MOUSE | Acetyl-CoA carboxylase 1 | 3551 | 31 |
| AL1L1_MOUSE | Cytosolic 10-formyltetrahydrofolate dehydrogenase | 1469 | 7 |
| K2C5_MOUSE | Keratin, type II cytoskeletal 5 | 729 | 35 |
| PCCB_MOUSE | Propionyl-CoA carboxylase beta chain, mitochondrial | 706 | 2 |
| K2C6A_MOUSE | Keratin, type II cytoskeletal 6A | 678 | 23 |
| K1C14_MOUSE | Keratin, type I cytoskeletal 14 | 669 | 34 |
| ASSY_MOUSE | Argininosuccinate synthase | 617 | 7 |
| BHMT1_MOUSE | Betaine--homocysteine S-methyltransferase 1 | 614 | 5 |
| F16P1_MOUSE | Fructose-1,6-bisphosphatase 1 | 612 | 3 |
| ALDOB_MOUSE | Fructose-bisphosphate aldolase B | 596 | 2 |
| PYC_MOUSE | Pyruvate carboxylase, mitochondrial | 560 | 9 |
| SAHH_MOUSE | Adenosylhomocysteinase | 520 | 5 |
| K1C17_MOUSE | Keratin, type I cytoskeletal 17 | 515 | 15 |
| SBP1_MOUSE | Selenium-binding protein 1 | 512 | 3 |
| K1C42_MOUSE | Keratin, type I cytoskeletal 42 | 495 | 35 |
| K1C10_MOUSE | Keratin, type I cytoskeletal 10 | 456 | 8 |
| K2C75_MOUSE | Keratin, type II cytoskeletal 75 | 448 | 26 |
| GSTP1_MOUSE | Glutathione S-transferase P 1 | 418 | 3 |
| IGG2B_MOUSE | Ig gamma-2B chain C region | 397 | 2 |
| HSP7C_MOUSE | Heat shock cognate 71 kDa protein | 370 | 3 |
| HPPD_MOUSE | 4-hydroxyphenylpyruvate dioxygenase | 363 | 2 |
| K1C16_MOUSE | Keratin, type I cytoskeletal 16 | 358 | 16 |
| K22E_MOUSE | Keratin, type II cytoskeletal 2 epidermal | 331 | 8 |
| K1C15_MOUSE | Keratin, type I cytoskeletal 15 | 327 | 13 |
| IGKC_MOUSE | Ig kappa chain C region | 326 | 5 |
| K2C1B_MOUSE | Keratin, type II cytoskeletal 1b | 306 | 5 |
| K22O_MOUSE | Keratin, type II cytoskeletal 2 oral | 305 | 11 |
| K1C13_MOUSE | Keratin, type I cytoskeletal 13 | 301 | 14 |
| K2C73_MOUSE | Keratin, type II cytoskeletal 73 | 295 | 4 |
| K2C1_MOUSE | Keratin, type II cytoskeletal 1 | 286 | 10 |
| TBB4B_MOUSE | Tubulin beta-4B chain | 282 | 3 |
| LDHA_MOUSE | L-lactate dehydrogenase A chain | 276 | 3 |
| K2C8_MOUSE | Keratin, type II cytoskeletal 8 | 221 | 10 |
| CAH3_MOUSE | Carbonic anhydrase 3 | 185 | 3 |
| K2C79_MOUSE | Keratin, type II cytoskeletal 79 | 154 | 2 |
| PLAK_MOUSE | Junction plakoglobin | 147 | 2 |
| K2C72_MOUSE | Keratin, type II cytoskeletal 72 | 142 | 2 |
| GSTA3_MOUSE | Glutathione S-transferase A3 | 134 | 2 |
| GPX1_MOUSE | Glutathione peroxidase 1 | 113 | 2 |

Table 1. FAS-associated proteins in mouse liver cytoplasm. A protein score of 100

(representing a confidence level over 95%) and a minimum number of two peptides was used as the cut-off for likely FAS interactors.

| Accession ID | Description | Protein score | # of peptides |
|--------------|--|---------------|---------------|
| FAS_MOUSE | Fatty acid synthase | 6296 | 83 |
| ACACA_MOUSE | Acetyl-CoA carboxylase 1 | 3872 | 11 |
| ATPA_MOUSE | ATP synthase subunit alpha, mitochondrial | 1548 | 29 |
| MVP_MOUSE | Major vault protein | 1339 | 10 |
| GRP78_MOUSE | 78 kDa glucose-regulated protein | 1223 | 3 |
| MCCB_MOUSE | Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial | 857 | 4 |
| K2C5_MOUSE | Keratin, type II cytoskeletal 5 | 807 | 40 |
| MCCA_MOUSE | Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial | 750 | 3 |
| K1C14_MOUSE | Keratin, type I cytoskeletal 14 | 715 | 19 |
| ENPL_MOUSE | Endoplasmic | 710 | 7 |
| PDIA1_MOUSE | Protein disulfide-isomerase | 686 | 7 |
| S27A2_MOUSE | Very long-chain acyl-CoA synthetase | 665 | 6 |
| K2C6A_MOUSE | Keratin, type II cytoskeletal 6A | 663 | 33 |
| RS4X_MOUSE | 40S ribosomal protein S4, X isoform | 596 | 5 |
| K1C42_MOUSE | Keratin, type I cytoskeletal 42 | 585 | 19 |
| TPP2_MOUSE | Tripeptidyl-peptidase 2 | 581 | 4 |
| IGG2B_MOUSE | Ig gamma-2B chain C region | 563 | 5 |
| K1C17_MOUSE | Keratin, type I cytoskeletal 17 | 554 | 9 |
| FMO5_MOUSE | Dimethylaniline monooxygenase [N-oxide-forming] 5 | 548 | 3 |
| CES3_MOUSE | Carboxylesterase 3 | 515 | 3 |
| K2C75_MOUSE | Keratin, type II cytoskeletal 75 | 483 | 31 |
| ATPB_MOUSE | ATP synthase subunit beta, mitochondrial | 469 | 2 |
| RS3_MOUSE | 40S ribosomal protein S3 | 465 | 2 |
| UD11_MOUSE | UDP-glucuronosyltransferase 1-1 =2 | 461 | 3 |
| K1C10_MOUSE | Keratin, type I cytoskeletal 10 | 445 | 15 |
| K1C16_MOUSE | Keratin, type I cytoskeletal 16 | 433 | 5 |
| K22E_MOUSE | Keratin, type II cytoskeletal 2 epidermal | 419 | 12 |
| ACSL5_MOUSE | Long-chain-fatty-acid-CoA ligase 5 | 407 | 3 |
| S27A5_MOUSE | Bile acyl-CoA synthetase | 398 | 4 |
| CP2DA_MOUSE | Cytochrome P450 2D10 | 390 | 2 |
| CP2DQ_MOUSE | Cytochrome P450 2D26 | 365 | 4 |
| SEPT2_MOUSE | Septin-2 | 340 | 4 |
| K1C15_MOUSE | Keratin, type I cytoskeletal 15 | 335 | 9 |
| K22O_MOUSE | Keratin, type II cytoskeletal 2 oral | 322 | 27 |
| K2C1B_MOUSE | Keratin, type II cytoskeletal 1b | 318 | 7 |
| DH11_MOUSE | Corticosteroid 11-beta-dehydrogenase isozyme 1 | 306 | 2 |
| K1C13_MOUSE | Keratin, type I cytoskeletal 13 | 282 | 16 |
| K2C1_MOUSE | Keratin, type II cytoskeletal 1 | 281 | 5 |
| K2C73_MOUSE | Keratin, type II cytoskeletal 73 | 266 | 8 |
| SEPT7_MOUSE | Septin-7 | 250 | 3 |
| CMLO2_MOUSE | Probable N-acetyltransferase CML2 | 240 | 2 |
| K2C8_MOUSE | Keratin, type II cytoskeletal 8 | 232 | 16 |
| PCCB_MOUSE | Propionyl-CoA carboxylase beta chain, mitochondrial | 232 | 4 |
| K2C79_MOUSE | Keratin, type II cytoskeletal 79 | 224 | 8 |

| | | | |
|-------------|-------------------------------------|-----|---|
| K1C19_MOUSE | Keratin, type I cytoskeletal 19 | 219 | 3 |
| KRT85_MOUSE | Keratin, type II cuticular Hb5 | 213 | 2 |
| ALDOB_MOUSE | Fructose-bisphosphate aldolase B | 199 | 2 |
| RS18_MOUSE | 40S ribosomal protein S18 | 189 | 2 |
| K2C7_MOUSE | Keratin, type II cytoskeletal 7 | 133 | 3 |
| HVM51_MOUSE | Ig heavy chain V region AC38 205.12 | 108 | 2 |
| PYGL_MOUSE | Glycogen phosphorylase, liver form | 105 | 2 |

Table 2. FAS-associated proteins in mouse liver membrane. A protein score of 100 (representing a confidence level over 95%) and a minimum number of two peptides was used as the cut-off for likely FAS interactors. Because the association of FAS with membranes is resistant to high salt treatment [2], the pellet containing the crude membrane fraction was resuspended in a buffer containing 1 M NaCl and incubated for 30 min in order to dissociate irrelevant proteins from the membrane, after which the sample was re-centrifuged and the supernatant discarded.

| Accession ID | Description | Heavy/light ratio | # of peptides |
|--------------|--|-------------------|---------------|
| FAS_MOUSE | Fatty acid synthase | 32.87 | 222 |
| ANR40_MOUSE | Ankyrin repeat domain-containing protein 40 | 25.48 | 3 |
| AP2A1_MOUSE | AP-2 complex subunit alpha-1 | 22.59 | 2 |
| DTD1_MOUSE | D-tyrosyl-tRNA(Tyr) deacylase 1 | 12.76 | 2 |
| SNX5_MOUSE | Sorting nexin-5 | 11.36 | 2 |
| GFPT1_MOUSE | Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 1 | 10.7 | 15 |
| TWF1_MOUSE | Twinfilin-1 | 9.94 | 4 |
| ARL1_MOUSE | ADP-ribosylation factor-like protein 1 | 9.83 | 6 |
| ARF1_MOUSE | ADP-ribosylation factor 1 | 9.79 | 18 |
| ARF5_MOUSE | ADP-ribosylation factor 5 | 9.58 | 13 |
| ARF4_MOUSE | ADP-ribosylation factor 4 | 9.17 | 14 |
| UBC12_MOUSE | NEDD8-conjugating enzyme Ubc12 | 8.76 | 4 |
| ATG3_MOUSE | Ubiquitin-like-conjugating enzyme ATG3 | 8.43 | 2 |
| MP2K1_MOUSE | Dual specificity mitogen-activated protein kinase kinase 1 | 8.33 | 3 |
| SAR1A_MOUSE | GTP-binding protein SAR1a | 8.13 | 4 |
| CNN3_MOUSE | Calponin-3 | 8.01 | 27 |
| KTHY_MOUSE | Thymidylate kinase | 7.9 | 2 |
| UBE2Z_MOUSE | Ubiquitin-conjugating enzyme E2 Z | 7.88 | 3 |
| EF2_MOUSE | Elongation factor 2 | 7.72 | 80 |
| CXCR4_MOUSE | C-X-C chemokine receptor type 4 | 7.53 | 3 |
| UBE2N_MOUSE | Ubiquitin-conjugating enzyme E2 N | 7.51 | 6 |
| PYRG1_MOUSE | CTP synthase 1 | 7.44 | 5 |
| SAR1B_MOUSE | GTP-binding protein SAR1b | 7.35 | 7 |
| SAE2_MOUSE | SUMO-activating enzyme subunit 2 | 7.21 | 13 |
| CDK1_MOUSE | Cyclin-dependent kinase 1 | 7.19 | 8 |
| PUR6_MOUSE | Multifunctional protein ADE2 | 6.87 | 26 |
| RAB5A_MOUSE | Ras-related protein Rab-5A | 6.84 | 2 |
| MOL1A_MOUSE | Mps one binder kinase activator-like 1A | 6.83 | 3 |
| RB11A_MOUSE | Ras-related protein Rab-11A | 6.83 | 4 |
| CDK18_MOUSE | Cyclin-dependent kinase 18 | 6.71 | 3 |
| PRS8_MOUSE | 26S protease regulatory subunit 8 | 6.65 | 3 |
| BLK_MOUSE | Tyrosine-protein kinase Blk | 6.45 | 2 |
| ATX10_MOUSE | Ataxin-10 | 6.12 | 10 |
| PDC6I_MOUSE | Programmed cell death 6-interacting protein | 6.05 | 2 |
| TBB2A_MOUSE | Tubulin beta-2A chain | 6.05 | 85 |
| TBB3_MOUSE | Tubulin beta-3 chain | 6.03 | 35 |
| TBB5_MOUSE | Tubulin beta-5 chain | 6 | 104 |
| TBB2C_MOUSE | Tubulin beta-2C chain | 5.99 | 78 |
| TBB6_MOUSE | Tubulin beta-6 chain | 5.97 | 58 |
| TBAL3_MOUSE | Tubulin alpha chain-like 3 | 5.77 | 5 |
| TBA1C_MOUSE | Tubulin alpha-1C chain | 5.73 | 134 |
| TBA1B_MOUSE | Tubulin alpha-1B chain | 5.72 | 134 |
| VILI_MOUSE | Villin-1 | 5.6 | 7 |
| G3PT_MOUSE | Glyceraldehyde-3-phosphate dehydrogenase, testis- | 5.59 | 15 |

| | | | |
|-------------|---|-------|-----|
| | specific | | |
| TBA4A_MOUSE | Tubulin alpha-4A chain | 5.58 | 92 |
| ACTBL_MOUSE | Beta-actin-like protein 2 | 5.54 | 51 |
| TBA1A_MOUSE | Tubulin alpha-1A chain | 5.54 | 134 |
| TLN1_MOUSE | Talin-1 | 5.53 | 3 |
| KPYM_MOUSE | Pyruvate kinase isozymes M1/M2 | 5.48 | 61 |
| CHERP_MOUSE | Calcium homeostasis endoplasmic reticulum protein | 5.445 | 2 |
| IF4A1_MOUSE | Eukaryotic initiation factor 4A-I | 5.37 | 28 |
| EF1A1_MOUSE | Elongation factor 1-alpha 1 | 5.33 | 24 |
| G3P_MOUSE | Glyceraldehyde-3-phosphate dehydrogenase | 5.33 | 77 |
| RAB7A_MOUSE | Ras-related protein Rab-7a | 5.27 | 3 |

Table 3. FAS-associated proteins in Hepa1-6 cytoplasm. A minimum heavy/light ratio of 5

and a minimum of two peptides was used as the cut-off for likely FAS interactors.

| Accession ID | Description | Heavy/light ratio | # of peptides |
|--------------|--|-------------------|---------------|
| FGF22_MOUSE | Fibroblast growth factor 22 | 73.25 | 6 |
| FAS_MOUSE | Fatty acid synthase | 49.87 | 533 |
| RG9D3_MOUSE | RNA (guanine-9-)-methyltransferase domain-containing protein 3 | 48.81 | 3 |
| DTD1_MOUSE | D-tyrosyl-tRNA(Tyr) deacylase 1 | 39.4 | 4 |
| SEPT2_MOUSE | Septin-2 | 29.2 | 6 |
| SEPT9_MOUSE | Septin-9 | 23.41 | 9 |
| G3P_MOUSE | Glyceraldehyde-3-phosphate dehydrogenase | 13.97 | 47 |
| SEPT7_MOUSE | Septin-7 | 8.96 | 2 |
| TCPH_MOUSE | T-complex protein 1 subunit eta | 7.24 | 10 |
| PCBP1_MOUSE | Poly(rC)-binding protein 1 | 6.5 | 10 |
| PCBP2_MOUSE | Poly(rC)-binding protein 2 | 6.48 | 2 |
| SC31A_MOUSE | Protein transport protein Sec31A | 6.41 | 2 |
| TCPD_MOUSE | T-complex protein 1 subunit delta | 6.12 | 22 |
| TCPZ_MOUSE | T-complex protein 1 subunit zeta | 6.1 | 20 |
| HAS1_MOUSE | Hyaluronan synthase 1 | 6.08 | 2 |
| TCPG_MOUSE | T-complex protein 1 subunit gamma | 5.99 | 22 |
| RS18_MOUSE | 40S ribosomal protein S18 | 5.82 | 11 |
| TCPB_MOUSE | T-complex protein 1 subunit beta | 5.7 | 19 |
| EF1A1_MOUSE | Elongation factor 1-alpha 1 | 5.67 | 49 |
| NFH_MOUSE | Neurofilament heavy polypeptide | 5.63 | 5 |
| ABCE1_MOUSE | ATP-binding cassette sub-family E member 1 | 5.47 | 3 |
| SETB2_MOUSE | Histone-lysine N-methyltransferase SETDB2 | 5.32 | 2 |
| UGDH_MOUSE | UDP-glucose 6-dehydrogenase | 5.29 | 2 |

Table 4. FAS-associated proteins in Hepa1-6 membrane. A minimum heavy/light ratio of 5 and a minimum of two peptides was used as the cut-off for likely FAS interactors.

| Description | Protein score | # of peptides | Heavy/light ratio | # of peptides |
|---------------------------|----------------------|----------------------|--------------------------|----------------------|
| Septin-2 | 340 | 4 | 29.2 | 6 |
| Septin-7 | 250 | 3 | 8.96 | 2 |
| 40S ribosomal protein S18 | 189 | 2 | 5.82 | 11 |

Table 5. FAS-associated proteins exclusively present in the membrane fractions and present in both mouse liver and Hepa1-6. Excluding FAS.

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Chapter 3:
Nutrient-Dependent Phosphorylation
Channels Lipid Synthesis to Regulate PPAR α

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ABSTRACT

PPAR α is a nuclear receptor that coordinates liver metabolism during fasting. Fatty acid synthase (FAS) is an enzyme that stores excess calories as fat during feeding, but also activates hepatic PPAR α by promoting synthesis of an endogenous ligand. Here we show that the mechanism underlying this paradoxical relationship involves the differential regulation of FAS in at least two distinct subcellular pools: cytoplasmic and membrane-associated. In mouse liver and cultured hepatoma cells, the ratio of cytoplasmic to membrane FAS specific activity was increased with fasting, indicating higher cytoplasmic FAS activity under conditions associated with PPAR α activation. This effect was due to a nutrient-dependent and compartment-selective covalent modification of FAS. Cytoplasmic FAS was preferentially phosphorylated during feeding or insulin treatment at Thr-1029 and Thr-1033, which flank a dehydratase domain catalytic residue. Mutating these sites to alanines promoted PPAR α target gene expression. Rapamycin-induced inhibition of mTORC1, a mediator of the feeding/insulin signal to induce lipogenesis, reduced FAS phosphorylation, increased cytoplasmic FAS enzyme activity, and increased PPAR α target gene expression. Rapamycin-mediated induction of the same gene was abrogated with FAS knockdown. These findings suggest that hepatic FAS channels lipid synthesis through specific subcellular compartments that allow differential gene expression based on nutritional status.

INTRODUCTION

PPAR α (peroxisome proliferator-activated receptor α), one of three known members of a nuclear receptor family targeted to treat lipid disorders, diabetes and obesity, is highly expressed in the liver. Its induction by fasting promotes lipid uptake, fatty acid β -oxidation, ketogenesis, and gluconeogenesis [1, 2]. Ligand binding to PPAR α causes it to heterodimerize with RXR α , allowing activation of gene transcription at PPREs [3, 4]. Synthetic PPAR α ligands such as fibrates, used for human lipid disorders [5], have been known for decades, but potential endogenous ligands were identified only recently [6, 7]. Mice with liver-specific deletion of the lipogenic enzyme fatty acid synthase (FAS) have impaired PPAR α activity [8], and FAS activates PPAR α by producing an endogenous phospholipid ligand [6]. FAS also activates PPAR α in brain and macrophages [9, 10].

Mammalian FAS synthesizes long chain fatty acids, primarily palmitate, through the activities of seven functional domains: acyl carrier, acyl transferase, β -ketoacyl synthase, β -ketoacyl reductase, β -hydroxyacyl dehydratase, enoyl reductase, and thioesterase [11]. Like PPAR α , FAS is highly expressed in liver [12]. In times of nutrient excess, hepatic FAS converts carbohydrate to lipid that is stored in lipid droplets or secreted in the form of VLDL [13]. Nutrient excess is associated with elevated levels of insulin, known to induce FAS expression.

These accepted physiological roles for PPAR α and FAS appear to conflict with the observation that inactivation of FAS impairs PPAR α activation. How might FAS activate a process stimulated by feeding such as insulin-responsive lipogenesis and yet also activate a process stimulated by fasting such as the induction of PPAR α -dependent gene expression?

We hypothesized that distinct subcellular pools of FAS mediate these disparate effects. Compartmentalization would permit regulation of an FAS pool generating lipids for signaling that would be distinct from an FAS pool generating lipids for energy storage. In

support of this hypothesis, we demonstrate that FAS at two separate subcellular locations is differentially regulated by nutrients and insulin, that this regulation involves preferential dehydratase domain phosphorylation for the FAS pool that regulates PPAR α , and that the effects of the kinase mTORC1 on PPAR α activity require FAS.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice at 8 weeks of age were provided ad libitum access to chow diet (Purina #5053) or fasted for 18 h. All mice were kept on Aspen bedding and had free access to water. Protocols were approved by the Washington University Animal Studies Committee.

FAS enzyme activity assay. Using a modification of a previously described assay [14], 20 μ l of sample at 1 μ g protein/ μ l was added to 70 μ l of assay buffer (0.14 M potassium phosphate buffer [pH 7.0], 1.4 mM EDTA [pH 8.0], 1.4 mM DTT, 0.24 mM NADPH, 0.1 mM acetyl-CoA). The rate of NADPH oxidation was monitored at 340 nm at baseline and again after adding 10 μ l of 0.85 mg/ml malonyl-CoA (Sigma). The substrate-dependent rate was determined by subtracting the baseline NADPH oxidation rate from the rate after addition of malonyl-CoA. The rate of NADPH oxidation was normalized to FAS protein levels as determined by Western blotting and densitometry to determine specific activity.

Subcellular fractionation. Perfused liver from C57BL/6J mice was homogenized in 20 mM HEPES buffer (pH 7.4), centrifuged at 100 g for 30 min, and the pellet was discarded. The supernatant was centrifuged at 500 g for 60 min; 1,200 g for 20 min; 10,000 g for 20 min; 20,000 g for 30 min; 40,000 g for 30 min; 70,000 g for 30 min; 100,000 g for 60 min; and 179,000 g for 75 min. After each spin, the pellet was washed and resuspended, while the supernatant was centrifuged again. All spins were done at 4°C. To obtain crude membrane and cytoplasmic fractions from mouse liver, freshly isolated perfused liver was homogenized in HEPES buffer and centrifuged at 10,000 g for 45 min at 4°C. The resulting pellet was discarded and the supernatant centrifuged at 179,000 g for 180 min at 4°C. The supernatant (cytoplasm) and pellet (crude membrane) were collected, and the pellet was washed and resuspended in HEPES buffer. To obtain membrane and cytoplasmic extracts from Hepa1-6 cells, a Subcellular Protein Fractionation Kit for Cultured Cells (78840) from Thermo Fisher

Scientific was used according to the manufacturer's protocol.

Antibodies. Rabbit polyclonal antibodies against FAS (ab22759), PMP70 (ab3421), and phosphothreonine (ab9337) were from Abcam. Mouse monoclonal antibody against α -tubulin (sc-5286) and rabbit polyclonal antibodies against Cav1 (sc-894) and β -tubulin (sc-9104, used to control for loading in Western blotting experiments) were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against PDI (226), GM130 (2296), Na^+/K^+ ATPase (3010), Akt (9272), Phospho-Akt (S473) (9271), S6 ribosomal protein (2217), and phospho-S6 ribosomal protein (Ser235/236) (2F9/4856) and rabbit monoclonal antibodies against p70 S6 Kinase (2708) and CoxIV (4850) were from Cell Signaling Technology.

FAS solubility. Solubility assays were performed as previously described [15] with minor modifications. Membranes were isolated from mouse liver by ultracentrifugation and resuspended in buffer containing 20 mM HEPES buffer (pH 7.4), 1 mM EDTA, and 255 mM sucrose. The membrane fraction was subjected to treatment with various solvents (1 M NaCl, 0.1 M Na_2CO_3 at pH 11.5, 1% SDS or 1% Triton X-100) and then centrifuged once more (4°C, 180,000 g, 30 min). The resulting pellets and supernatants were analyzed by Western blotting.

Cell culture. Hepa1-6 and Hek293T cells were maintained in DMEM + 10% FBS. Prior to insulin treatment for FAS activity assays, Hepa1-6 cells were cultured in DMEM + 0.5% FBS for 6 h. All insulin treatments were performed in DMEM + 10% FBS.

Pulse-chase. Confluent Hepa1-6 cells in 6 cm dishes were incubated in methionine-free media for 30 min. The cells were then pulsed with 500 μCi of ^{35}S -methionine per dish. After 1 h cells for the "0" time point were harvested. For subsequent time points, cells were washed with PBS, chased with non-radioactive complete media, and incubated for an additional 45, 90, or 180 minutes before harvesting. Cells were fractionated into cytoplasm and membrane as described above. FAS was immunoprecipitated from each fraction, samples

were subjected to SDS-PAGE, the gel transferred onto PVDF membrane, and the bands corresponding to labeled FAS visualized by autoradiography. Autoradiograms were then analyzed by densitometry.

RT-PCR. Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed using an iScript™ cDNA synthesis kit (Invitrogen). Semi-quantitative RT-PCR was performed using SYBR® Green reagent (Applied Biosystems) with an ABI Prism 7700 PCR instrument.

Mutagenesis and plasmid construction. A retroviral plasmid, pBABE-Puro, containing human FAS [16] generated by Max Loda (Dana Farber) was utilized to generate FAS phosphosite mutants. A 3.4 kb fragment of FAS/pBABE-Puro including the two putative phosphorylation sites (hFAS S1028 and T1032) and two flanking BsrGI sites was amplified by PCR and subcloned into an intermediate Topo vector. Site-directed mutagenesis of the Topo-FAS plasmid changed the codons corresponding to S1028 and T1032 to alanines, yielding two single mutants. The S1028A/T1032A double mutant was made by sequential mutagenesis, using the S1028A mutant as a template. Mutated FAS fragments were then excised and cloned back into pBabe-Puro using the two BsrGI sites to generate mutant, full length FAS cDNAs. Mutations as well as correct orientation of the re-inserted FAS fragments were verified by DNA sequencing.

GFP-tagged FAS was generated by amplifying the cDNA encoding FAS from pBabe-Puro-FAS by RT-PCR, adding restriction sites for XhoI and EcoRI on the 5' and 3' ends, respectively. The amplified product was cloned into pEGFP-C3 using the XhoI and EcoRI sites, yielding an N-terminally GFP-tagged FAS construct.

Lentiviral shRNA-mediated knockdown and human FAS expression. A plasmid encoding a mouse FAS shRNA (TRCN0000075703) was obtained from Open Biosystems. The packaging vector psPAX2 (12260) and envelope vector pMD2.G (12259)

were obtained from Addgene. Hek293T cells at 70% confluence in a 15 cm dish were transfected using Lipofectamine 2000 with 8 μg psPAX2, 2.25 μg pMD2.G, and 9 μg shRNA. After 48 h, media was collected and filtered through 0.45 μm syringe filters. Polybrene was added and the media used to treat 50-70% confluent Hepa1-6 cells. After 24 h, the media was aspirated and replaced with media containing retroviral particles encoding human FAS (see below). Forty-eight h after addition of the retroviral media, cells were selected with puromycin. After another 48 h, cells were harvested and knockdown of mouse FAS as well as expression of human FAS were assessed.

To generate retroviral particles encoding human FAS, Hek293T cells in 10 cm dishes were transfected using Lipofectamine 2000 with 3 μg FAS plasmid and 3 μg ψA helper plasmid. After 48 h, media were collected, filtered using 0.45 μm syringe filters, polybrene was added, and the media was used to treat 50-70% confluent Hepa1-6 cells. After 48 h, 2 $\mu\text{g}/\text{ml}$ puromycin was added, and after an additional 48 h, cells were harvested.

In experiments assessing PPAR α target gene expression in cells expressing mutant FAS, the endogenous murine FAS of Hepa1-6 cells was knocked down prior to retroviral expression of human FAS as described above.

PPRE-luciferase reporter assay. Media containing lentiviral particles encoding shRNA for murine FAS and media containing retroviral particles encoding wild-type or S1028A/T1032A double mutant human FAS were prepared as described above. 70% confluent Hepa1-6 cells in 10 cm dishes were treated with lentiviral media for 24 h, after which the media was aspirated and replaced with retroviral media for either wild type or S1028A/T1032A FAS. After another 24 h, the media was again aspirated and replaced with fresh media containing puromycin.

After two days of puromycin selection, the media was aspirated, replaced with charcoal-stripped media (which is essentially fatty-acid free, minimizing PPAR activation by

lipids in the growth media), and incubated for one hour. For all following steps, charcoal-stripped media was used. Hepa1-6 cells were transfected with plasmids encoding 3x PPRE-luciferase and *Renilla* luciferase by electroporation. The electroporation for each 10 cm dish of cells was done as follows: 5 µg of PPRE-luciferase plasmid and 5 µg of *Renilla* luciferase plasmid were added to the bottom of a cuvette. Cells were harvested by trypsinization and spun after adding media. The media was aspirated and cells were washed once with PBS. The PBS was aspirated and cells resuspended in 0.5 ml PBS and transferred to the cuvette. The cuvette containing cells and DNA was electroporated at 360 V and 250 µF (time constant of 4.5-5 sec⁻¹). 1 ml of media was immediately added to the cuvette. Cells were transferred to a 15 ml tube and media containing puromycin was added up to 6 ml. Cells were allowed to recover for 10 min, then plated.

One day following transfection, cells were harvested by scraping, washed with room-temperature PBS three times, resuspended in PBS, and plated on a 96-well plate. Luminescence from firefly luciferase and *Renilla* luciferase was then measured using Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. The relative amounts of PPRE-luciferase activity were calculated as the ratio between firefly luciferase to *Renilla* luciferase luminescence.

Mass spectrometry. To identify post-translational modifications in hepatic FAS, perfused C57BL/6J mouse livers were homogenized in lysis buffer containing 1% Triton X-100. The lysate was spun at 10,000 g for 45 min and the pellet discarded. FAS was immunoprecipitated from 10 mg of the lysate by overnight incubation using a polyclonal rabbit anti-FAS antibody. IP beads were washed, boiled in sample buffer, and subjected to SDS-PAGE. The gel was stained with Coomassie, the gel segment corresponding to FAS was excised and further cut into small pieces (1 mm²), destained with 50% CH₃CN containing 25 mM NH₄HCO₃, dehydrated, reduced with 20 mM DTT for 1 h at 55°C, washed and

dehydrated, alkylated with 100 mM iodoacetamide for 1h in the dark at room temperature, then subjected to cycles of washing and dehydration followed by drying in a centrifugal evaporator. In-gel digestion was performed with 0.02 mg/ml trypsin overnight or 0.02 mg/ml chymotrypsin for 6 h at 37°C. Peptides were extracted from the gel pieces using 5% TFA in 50% CH₃CN and reconstituted in 0.1% FA in 3% CH₃CN.

Immobilized metal ion affinity chromatography (IMAC) was used to enrich the sample for phosphopeptides. The sample was incubated with IMAC beads for 1 h at room temperature. Peptides were eluted from the beads in IMAC buffer and the sample diluted with 0.1% FA in 3% CH₃CN. Samples were then analyzed by LC-MS/MS with a NanoLC-LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) in data dependent mode. Acquired spectra were searched against Swiss-Prot database through Mascot server to identify the protein and its post-translational modification. Non-enriched samples were also run to allow a universal search for protein modifications as well as to search for acetyl modifications.

To identify FAS modifications specific to membrane-associated FAS and cytoplasmic FAS, membrane and cytoplasmic fractions were isolated from C57BL/6J mice as described above. FAS was immunoprecipitated from equal amounts of membrane and cytoplasmic lysates (1-10 mg/each) by overnight incubation using a polyclonal rabbit anti-FAS antibody. The samples were then subjected to SDS-PAGE and analyzed as described above.

Statistics. Data are presented as mean \pm standard error of the mean. Comparisons between two groups were performed using an unpaired, two-tailed t-test. ANOVA was used for comparisons involving more than two groups.

RESULTS

Hepatic FAS is present in subcellular compartments. FAS synthesizes palmitate, and FAS deficiency in liver decreases PPAR α target genes. If the effect of FAS deficiency on PPAR α simply reflects palmitate availability, then exogenous palmitate should rescue the effect. It did not. Treatment of Hepa1-6 cells with 50 μ M palmitate failed to rescue expression of the PPAR α target gene ACO following FAS knockdown (Figure 1A). Higher concentrations of palmitate (125-500 μ M) were toxic (data not shown).

Since the FAS knockdown effect was not rescued with exogenous palmitate, it is plausible that not only the product of the FAS reaction but also the location of its synthesis mediates downstream effects. Dogma holds that FAS is a cytoplasmic enzyme. To determine if FAS is also present at other sites, we fractionated mouse liver FAS by ultracentrifugation (Figure 1B). FAS co-fractionated with the cytoplasmic marker S6K, but also with markers for several organelles. Immunofluorescent staining for FAS in murine Hepa1-6 liver cells demonstrated co-localization of FAS with ER and Golgi markers but not peroxisomal or mitochondrial markers (Figure 1C). FAS did not appear in the nucleus (Figure 1C).

Membrane-associated and cytoplasmic FAS are differentially regulated. FAS is induced by insulin and nutrients [12]. Surprisingly, the specific activity of mouse liver cytoplasmic FAS was not increased in the fed state when insulin levels are high (Figure 2A). Membrane-associated FAS specific activity was increased with feeding (Figure 2B). The cytoplasmic/membrane activity ratio in liver was increased with fasting, when PPAR α is activated (Figure 2C). In Hepa1-6 cells, a transformed liver cell line, insulin significantly decreased cytoplasmic FAS activity (Figure 2D), an effect that was not seen in the membrane fraction (Figure 2E). As with mouse liver, the cytoplasmic/membrane activity ratio in Hepa1-6 cells was increased in the absence of added insulin (Figure 2F), a mimic of fasting.

To begin to address the possibility that membrane-associated FAS is an artifact of

preparation, we treated isolated fractions with different solvents. Membrane-associated FAS resisted solubilization by 1 M NaCl, remaining in the pelleted fraction, but was largely solubilized by 0.1 M Na₂CO₃ (Figure 3A). Treatment with detergent (1% SDS or 1% Triton X-100) solubilized most FAS protein (Figure 3A). These results suggest [17-19] that FAS manifests a strong peripheral membrane interaction.

A pulse-chase study showed that radiolabelled FAS decreased over time in the membrane-associated and cytoplasmic compartments (Figure 3B), suggesting that there is no ordered flux of protein from one compartment to another over the time course of this experiment. There was no discernible change in the distribution of FAS between membrane and cytoplasm when cells were treated with insulin (Figure 3C).

Given the presence of a putative open reading frame (with a potential alternative start codon) 5' to the published first exon of both mouse and human FAS, we considered the possibility that compartmentalized FAS represented differential splicing leading to non-identical protein isoforms, only one of which is membrane-targeted. However, mass spectrometric analysis of FAS in membrane and cytoplasm failed to detect the predicted alternative amino acids at the N-terminus, and identified the published FAS protein sequence as being N-terminally acetylated (Figure 3D). This modification, which marks the N-terminus of most eukaryotic proteins [20], was present in membrane and cytoplasmic fractions of FAS, precluding the existence of additional N-terminal sequence. All regions of the FAS protein were similarly represented in each fraction, decreasing the possibility that compartment location is determined by altered protein sequence due to a process such as exon exclusion (data not shown).

Collectively, these results suggest that the enzyme activities of cytoplasmic and membrane-associated FAS are differentially regulated, a phenomenon that does not appear to be due to intracellular trafficking of the protein or differences in its primary structure.

Cytoplasmic FAS is preferentially phosphorylated. To address the possibility that differential regulation of cytoplasmic and membrane-associated FAS is caused by a covalent modification, we immunoprecipitated hepatic FAS from fasting and fed mice then tested different fractions for the presence of phosphothreonine by Western blotting. Cytoplasmic FAS in fed mice was strongly threonine phosphorylated, a modification that was almost undetectable in fasted mice (Figure 4A). Phosphorylation of membrane-associated FAS was low under both conditions (Figure 4A). In Hepa1-6 cells, insulin treatment (a mimic of feeding) stimulated threonine phosphorylation of cytoplasmic but not membrane-associated FAS (Figure 4B).

Analysis of FAS protein from unfractionated mouse liver by mass spectrometry revealed only a single peptide that was threonine phosphorylated. This modification was detected at two residues, Thr-1029 and Thr-1033 (a representative spectrum is shown in Figure 5A). When liver FAS was separated into cytoplasmic and membrane-associated fractions and subjected to the same analysis, the phosphorylated peptide was found predominantly in the cytoplasm (Figure 5B) despite similar total amounts of the peptide in both fractions (data not shown). These results suggest that the phosphorylated FAS species detected in the cytoplasm with feeding or insulin (Figure 4A,B) is modified at Thr-1029 and Thr-1033.

These residues are in the dehydratase domain of FAS. The function of this domain requires two catalytic residues, His-878 and Asp-1032, and a third residue, Gln-1036, that maintains the orientation of the catalytic residues [21]. The phosphorylated residues we identified (denoted by * in Figure 5C) are in close proximity to the catalytic residue D1032 and the structural residue Q1036 (denoted by # in Figure 5C). Sequence alignment of the dehydratase regions from different species revealed that in addition to strict conservation of the active site residues D1032 and Q1036 (denoted by #), the phosphoresidues we identified

are also conserved as either serines or threonines in humans, mice, rats, *D. melanogaster*, and *C. elegans* (boxes, Figure 5D).

Since the evolutionary conservation of these phosphorylation sites suggests involvement in FAS function, we mutated S1028 and T1032 in human FAS (corresponding to the T1029 and T1033 in murine FAS) to alanines, generating two single mutants (S1028A and T1032A) and one double mutant (S1028A/T1032A) (Figure 5E, mutated sites are indicated by boxes and the active site residues by #). Wild type or mutant human FAS was then expressed in Hepa1-6 cells following knockdown of endogenous mouse FAS. Compared to cells expressing wild-type human FAS, cells expressing the S1028A mutation had increased levels of the PPAR α target gene CPT1 (Figure 5F), while cells expressing the T1032A mutation did not show changes in PPAR α target genes (Figure 5G). However, expression of the double mutant S1028A/T1032A was associated with increased levels of both ACO and CPT1 (Figure 5H). To test whether this effect could truly be mediated by PPAR transcriptional activity, we performed a PPRE-luciferase reporter assay on Hepa1-6 cells. After expression of wild type or S1028A/T1032A double mutant FAS and knockdown of endogenous mouse FAS, cells were transfected with plasmid encoding three tandem peroxisome proliferator response elements (PPREs) fused to a firefly luciferase reporter gene. Luciferase activity was five-fold increased in cells expressing S1028A/T1032A double mutant FAS compared to wild type FAS (Figure 5I), indicating that the effect of FAS mutant expression on the PPAR α target genes ACO and CPT1 is indeed likely to be mediated by a change in PPAR transcriptional activity. These data suggest that the inability to phosphorylate FAS at these two sites disinhibits FAS enzyme activity to promote PPAR α target gene expression.

mTORC1 phosphorylates and inactivates FAS and inhibits PPAR α activity. mTORC1 was recently identified as a physiologically important negative regulator of hepatic PPAR α

[22]. mTOR, the kinase component of mTORC1, is a serine/threonine kinase that preferentially phosphorylates sites with hydrophobic residues at the +1 position [23]. Since the phosphorylated residues we identified have the highly hydrophobic phenylalanine (F1030) and methionine (M1034) at the +1 positions, we addressed a role for mTORC1 in FAS phosphorylation. Treating Hepa1-6 cells with the mTORC1 inhibitor rapamycin for 30 min abolished the insulin-induced increase in cytoplasmic FAS threonine phosphorylation (Figure 6A) and was associated with an increase in cytoplasmic FAS specific activity (Figure 6B). Treatment of these cells with Torin 1 at 250 nM also abolished insulin-induced FAS phosphorylation (data not shown). Treating Hepa1-6 cells with rapamycin for 24 h (a sufficient time to reach a new steady state for mRNA levels) decreased expression of the PPAR α target gene CPT1 (Figure 6C). These findings confirm those made in a different system [22] and extend that work by implicating FAS in the mTORC1-PPAR α axis.

To better define the interaction between mTORC1, FAS, and PPAR α , FAS was knocked down in Hepa1-6 cells followed by rapamycin treatment. FAS knockdown, confirmed in the presence of rapamycin (Figure 6D), decreased CPT1 expression (Figure 6E). The induction of CPT1 levels with rapamycin occurring with FAS expression (Figure 6C) was lost with FAS knockdown (Figure 6E, solid bar). These results suggest that in this cell line under these conditions, the induction of the PPAR α target gene CPT1 caused by inhibition of mTORC1 is FAS-dependent.

DISCUSSION

FAS synthesizes lipid for energy storage and participates in the generation of a lipid ligand involved in the activation of fatty acid oxidation. Energy storage occurs with feeding and activation of fatty acid oxidation occurs with fasting. To clarify how the same enzyme mediates both processes, we pursued the possibility that distinct pools of FAS are differentially regulated in the liver.

We found FAS in the cytosol, but also localized FAS to organelles (Figure 1) through a strong peripheral membrane interaction (Figure 3A). FAS specific activity was relatively higher with feeding/insulin in membranes and relatively higher with fasting in the cytosol (Figure 2). This effect did not appear to involve movement of FAS between compartments or primary sequence differences between these pools of FAS. Instead, this activity difference was associated with preferential phosphorylation of cytoplasmic (but not membrane) FAS with feeding (Figure 4) at conserved sites within a catalytic domain (Figure 5). Mutation of these sites increased endogenous PPAR α target gene expression as well as activity of a PPRE-dependent reporter gene (Figure 5) consistent with disinhibition of FAS in the absence of phosphorylation. Inhibition of mTORC1 with rapamycin decreased FAS phosphorylation, increased cytosolic FAS enzyme activity, and increased expression of the PPAR α target gene CPT1, an effect that was FAS-dependent (Figure 6). One interpretation of these findings is that hepatic FAS exists in at least two differentially regulated subcellular pools, cytoplasmic and membrane-associated (Figure 7). Cytoplasmic FAS is phosphorylated with feeding to limit PPAR α activation, and dephosphorylated with fasting to promote PPAR α activation.

Our findings provide molecular definition and physiological context to an observation made nearly four decades ago in birds. Using pigeon liver as a model and exclusively studying FAS in the cytoplasm, Qureshi and colleagues found that feeding induced ^{32}P incorporation into FAS, which was associated with a loss of enzyme activity [24]. In vitro

treatment with phosphatases dephosphorylated FAS and restored enzyme activity. The authors of this study did not identify a physiological role for this covalent modification and it is not known if the phosphosites we found are conserved in pigeon FAS due to the unavailability of sequence data for this species. Regardless, our work suggests that the phosphorylation state of cytoplasmic FAS may channel lipid flow to impact phospholipids inducing gene expression in the nucleus.

Physiological, mass spectrometric, and crystal structure data indicate that phospholipids interact with nuclear receptors [6, 25-29]. FAS appears to be linked to PPAR α through phosphatidylcholine synthesis mediated by the Kennedy pathway [6]. Viewed with previous studies showing that phosphorylation regulates the CDP-choline branch of the Kennedy pathway [30, 31], our identification of functionally relevant FAS phosphorylation sites raises the possibility that phosphorylation at several nodes within a cascade of lipid signaling from the cytoplasm to the nucleus coordinates FAS-mediated PPAR α activation.

Palmitate is the direct product of the FAS reaction. If the mere availability of palmitate were required to activate PPAR α , exogenous palmitate would correct FAS deficiency. However, the addition of palmitate to liver cells with FAS deficiency does not restore defects in PPAR α -dependent genes (Figure 1) and elevated serum palmitate levels that accompany inactivation of liver FAS in mice does not rescue impaired activation of PPAR α -dependent genes [8]. Thus, palmitate produced by FAS appears to be compartmentalized, a notion supported by our finding of preferential phosphorylation depending on cellular location and nutritional state.

There is precedent for compartmentalization in metabolism. Exogenous administration of T3, the active form of thyroid hormone that can be produced locally from its precursor T4, does not rescue gene expression defects in the setting of hypothyroidism. But administration of T4, which is metabolized to generate T3 locally, restores downstream

effects [32]. There is also precedent for compartmentalization in lipid signaling. Phosphatidic acid derived from glycerolipid synthesis has effects on mTORC2 that are opposite from those induced by phosphatidic acid derived from membrane lipolysis [33]. These observations are consistent with our model (Figure 7). In the fed state, cytoplasmic FAS is phosphorylated to limit lipid production resulting in PPAR α activation, while membrane FAS, less susceptible to phosphorylation, likely produces lipids for energy storage or export. Given the rapid demands of lipid synthesis prompted by transition from the fasting to the fed state, the induction of membrane FAS may be predominantly substrate driven through allosteric activation by the glycolytic intermediate fructose-1,6-bisphosphate [34].

mTORC1 may control the reciprocal activity of FAS in different compartments. mTORC1 is activated by insulin and nutrients, prefers substrates like those we identified in the dehydratase domain, and is known to suppress PPAR α in the liver [22]. FAS and mTORC1 appear to interact in the central nervous system where the physiological effects of FAS inhibition are blunted by rapamycin [35], consistent with our model suggesting that mTORC1 inhibition would increase FAS activity.

Our work provides evidence that hepatic FAS is in the cytoplasm as well as peripherally associated with membranes. These two pools are differentially regulated by nutrients and insulin, and differentially susceptible to phosphorylation, thus providing a conceptual framework for understanding how FAS-mediated PPAR α activation is linked to the fasting state. Pharmacologically targeting modulators of FAS phosphorylation or localization could allow the selective regulation of one pool of FAS. While complete inhibition of FAS in liver leads to loss of PPAR α activation and consequently fatty liver, specifically targeting FAS-mediated lipid storage and avoiding the undesirable inhibition of PPAR α in this manner could, in theory, be an effective treatment for fatty liver and other disorders associated with nutrient excess.

FIGURES

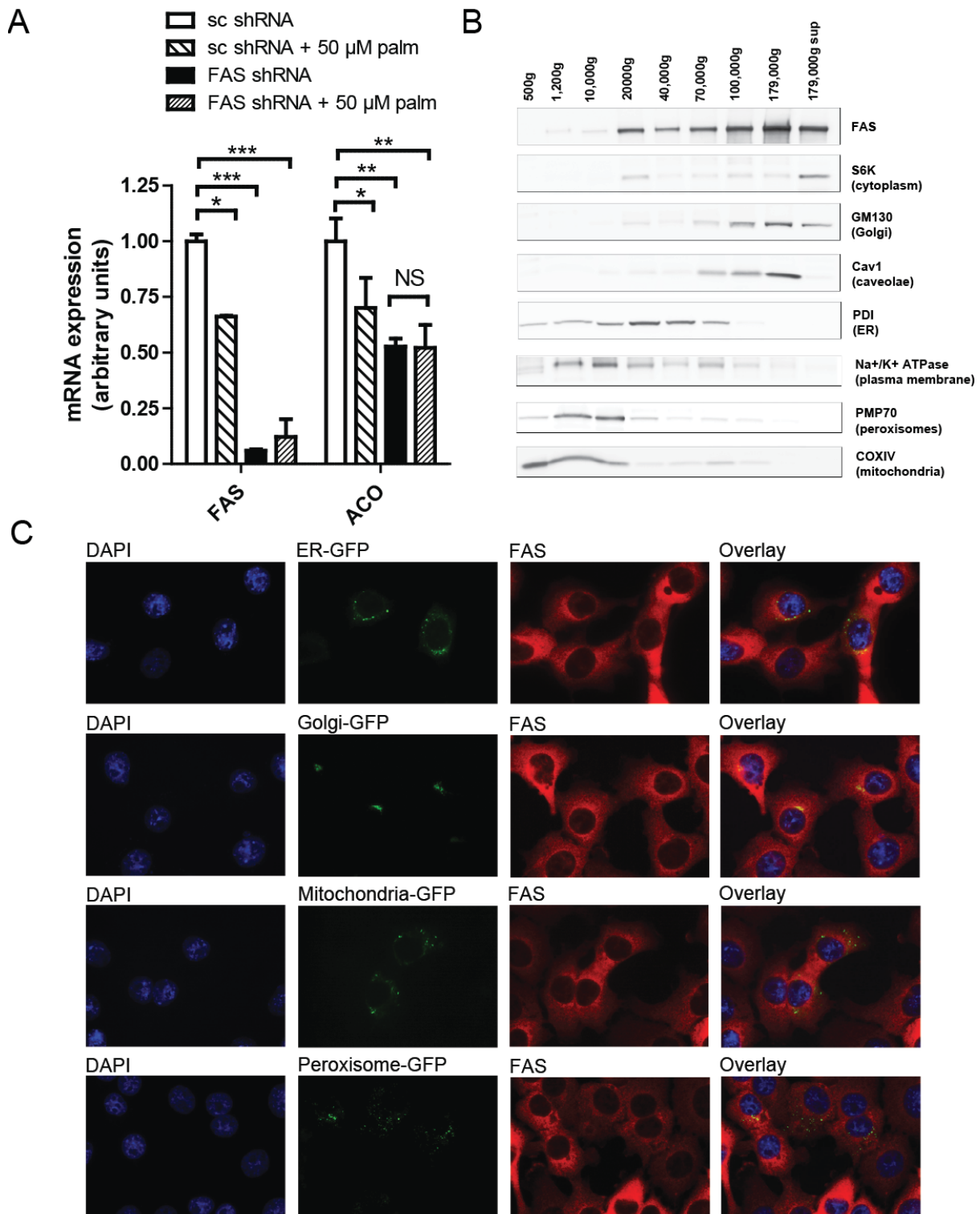


Figure 1. Hepatic FAS is not exclusively cytoplasmic. (A) Expression levels of FAS (left) and the PPAR α -dependent gene ACO (right) in Hepa1-6 cells. Cells were treated with a control (scrambled, sc) shRNA or an FAS shRNA in the presence of exogenous BSA-

conjugated palmitate or vehicle (BSA alone) for 8 h. *indicates $P \leq 0.05$. **indicates $P \leq 0.005$. ***indicates $P \leq 0.0005$. (B) Subcellular distribution of FAS protein in mouse liver by differential centrifugation followed by Western blotting. Organelle markers: S6K = P70/S6 kinase (cytoplasmic marker), GM130 = Golgi Matrix protein 130 (Golgi marker), Cav1 = Caveolin1 (caveolae marker), PDI = protein disulfide isomerase (endoplasmic reticulum marker), Na^+/K^+ ATPase (plasma membrane marker), PMP70 = Peroxisomal Membrane Protein 70 (peroxisomal marker), COXIV = Cytochrome C OXidase IV (mitochondrial marker). (C) Immunofluorescent staining of FAS and expression of GFP-tagged organelle markers in murine Hepa1-6 cells. Nuclei stained with DAPI are presented on the far left, GFP images are presented second from left, FAS images are presented second from right, and merged GFP/FAS images are presented on the far right.

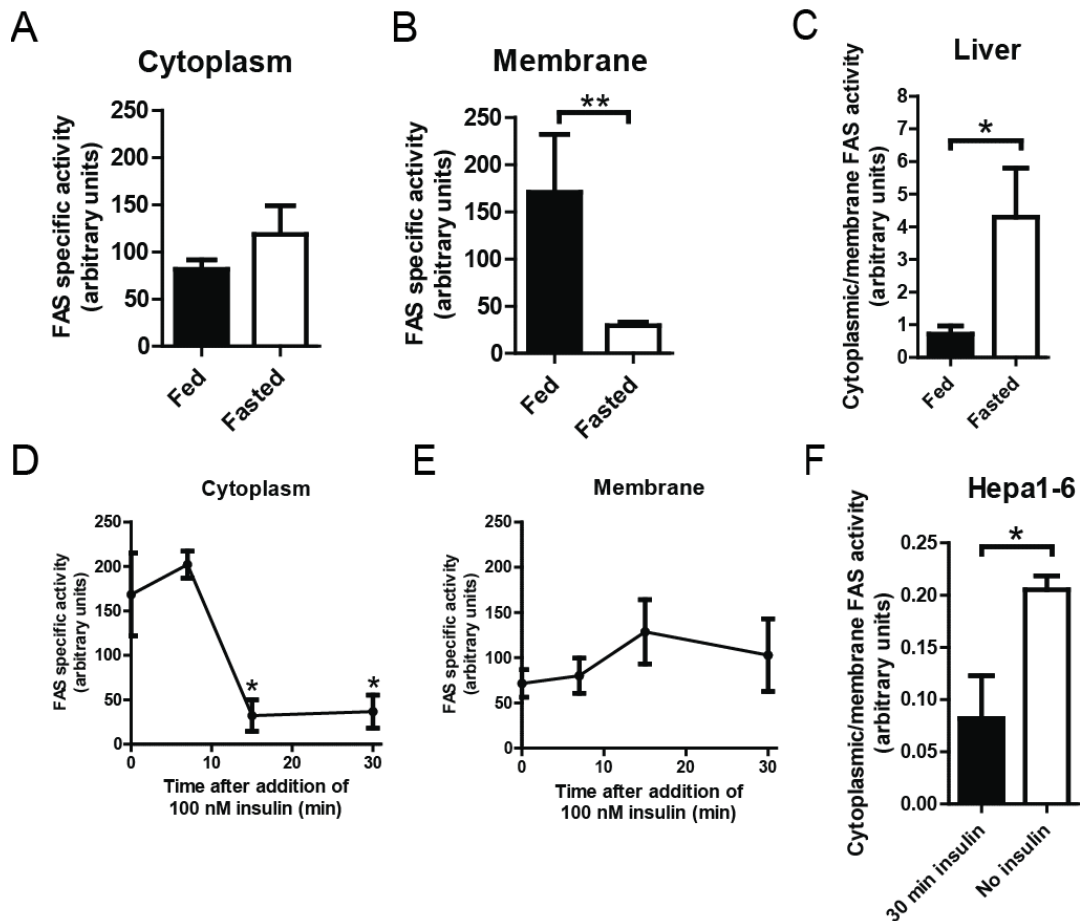


Figure 2. Differential regulation of the activities of membrane-associated FAS and cytoplasmic FAS. (A) Specific activity of FAS in the cytoplasmic fraction of mouse liver. Mice were either fed ad lib (fed) or fasted for 18h (fasted). Activity was normalized to FAS protein levels as measured by Western blotting. N=9/group. (B) Specific activity of FAS in the membrane (Golgi/ER) fraction of mouse liver. Mice were either fed ad lib (fed) or fasted for 18h (fasted). Activity was normalized to FAS protein levels as measured by Western blotting. N=5/group. **indicates $P \leq 0.005$. (C) FAS specific activities shown in A and B expressed as the ratio of FAS specific activity in cytoplasm to FAS specific activity in membrane. *indicates $P \leq 0.05$. (D) Specific activity of FAS in the cytoplasmic fraction of Hepa1-6 cells. Cells were treated with 100 nM insulin for indicated times. Activity was normalized to FAS protein levels as measured by Western blotting. N=3/group. *indicates $P \leq 0.05$. (E) Specific activity of FAS in the membrane (Golgi/ER) fraction of Hepa1-6 cells.

Cells were treated with 100 nM insulin for indicated times. Activity was normalized to FAS protein levels as measured by Western blotting. N=3/group. (F) FAS specific activities shown in D and E expressed as the ratio of FAS specific activity in cytoplasm to FAS specific activity in membrane.

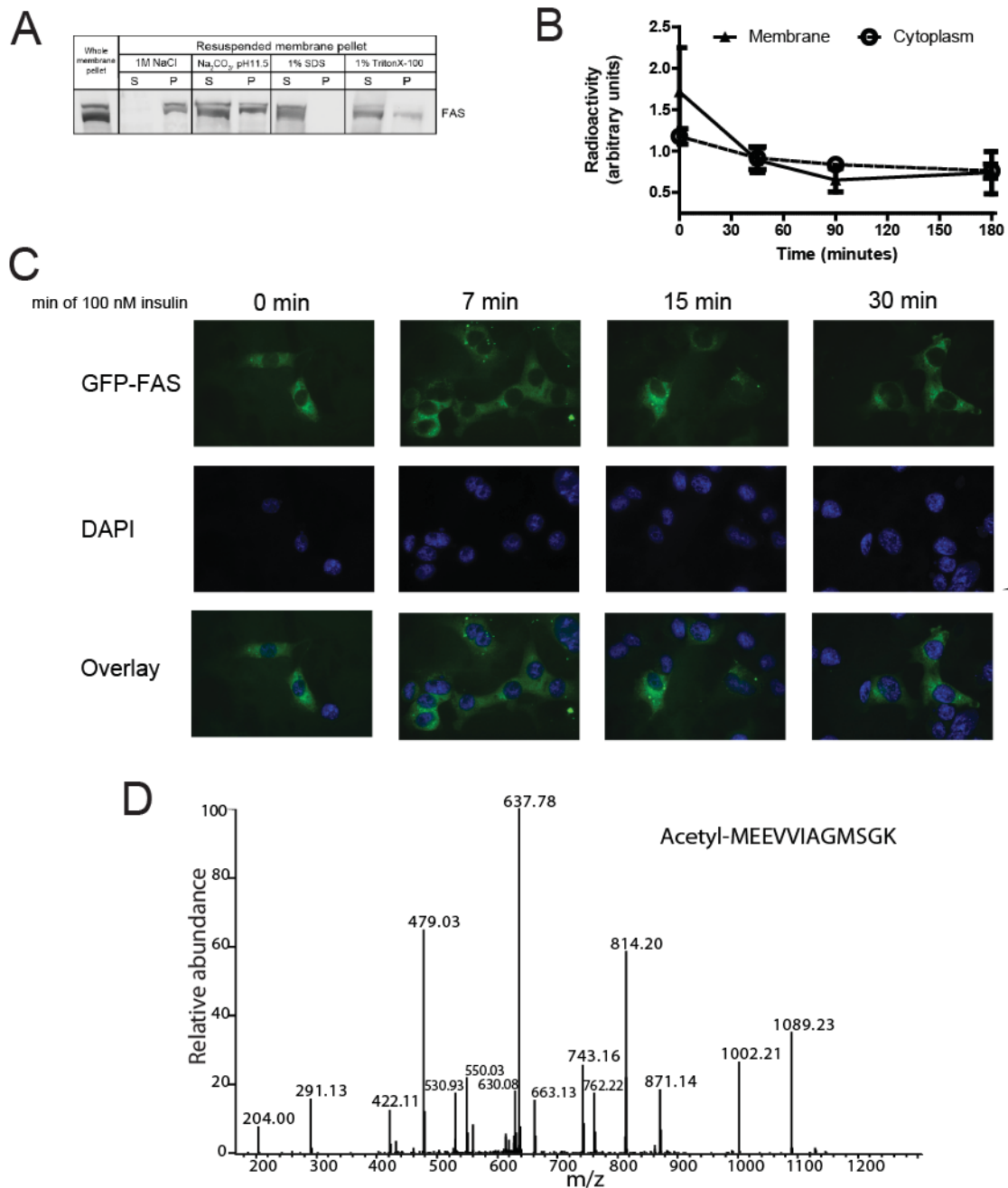


Figure 3. Distinct characteristics of membrane and cytoplasmic FAS. (A) Detection of FAS protein by Western blotting in pellets and supernatants of membrane fractions following high-salt, carbonate, and detergent treatments. Mouse liver homogenate was fractionated by differential centrifugation into cytoplasm (not shown) and membrane pellet (lane 1). The pellet was resuspended, exposed to solvents as indicated, and again centrifuged to separate pellet (P) from the new supernatant (S). (B) Pulse-chase analysis of FAS protein in

membrane and cytoplasm of Hepa1-6 cells. Cells were pulsed with ^{35}S -labeled methionine for 1 h, then chased with media containing non-labeled methionine for the indicated times. (C) Expression of GFP-tagged human FAS in Hepa1-6 cells treated with insulin for the indicated times. Images demonstrate no detectable shifts of FAS between cytoplasmic and membrane sites with insulin treatment. (D) Representative spectrum of N-terminally acetylated peptide of FAS. N-terminal acetylation effectively marks the initial amino acid of the protein, precluding the existence of additional expressed N-terminal exons that might constitute distinct FAS isoforms.

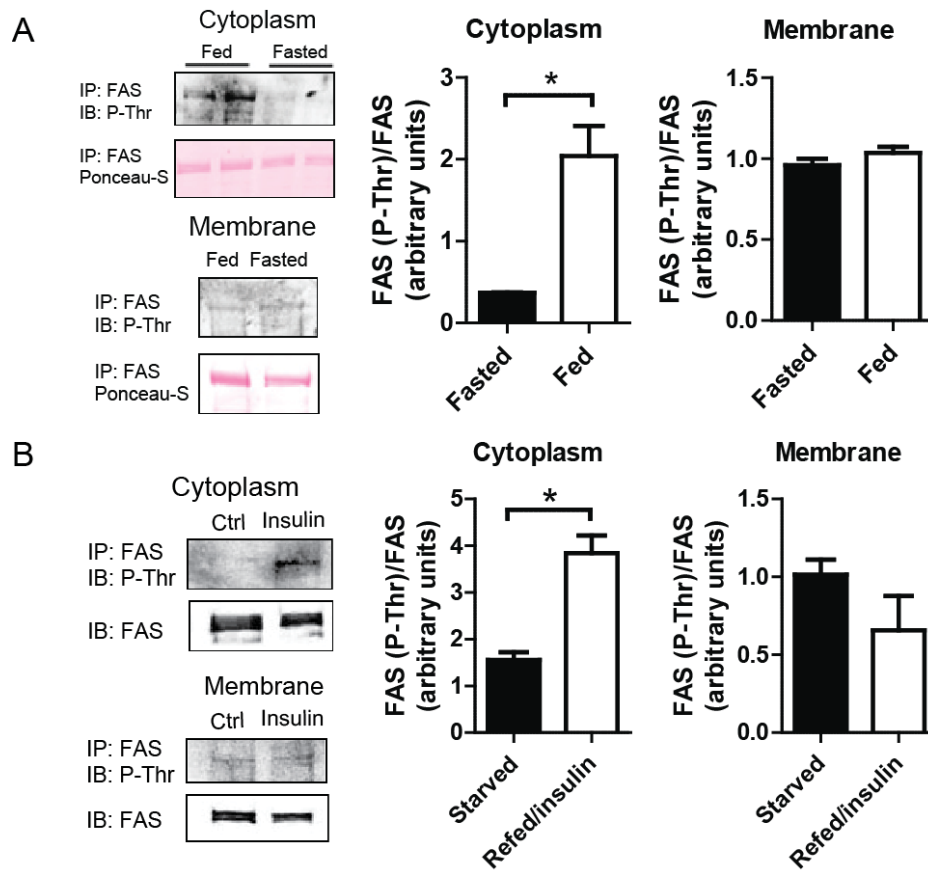


Figure 4. Cytoplasmic FAS is threonine phosphorylated with feeding or insulin

treatment. (A) FAS threonine phosphorylation in response to feeding in mouse liver. FAS was immunoprecipitated from cytoplasmic and membrane fractions and analyzed for phosphothreonine by Western blotting. Mice were either fed ad lib (fed) or fasted for 18 h (fasted). Representative blots are shown. Data are averages of two independent experiments. *indicates $P \leq 0.05$.

(B) FAS threonine phosphorylation in response to insulin in Hepa1-6 cells. FAS was immunoprecipitated from Hepa1-6 cytoplasmic and membrane fractions and analyzed for phosphothreonine by Western blotting. Cells were cultured in 0.5% FBS media for 4 h prior to harvest (starved), or in 0.5% FBS media for 4 hours, then treated with 1 nM insulin in 10% FBS media for 15 min (refed/insulin). Representative blots are shown. Data are averages of two independent experiments. *indicates $P \leq 0.05$.

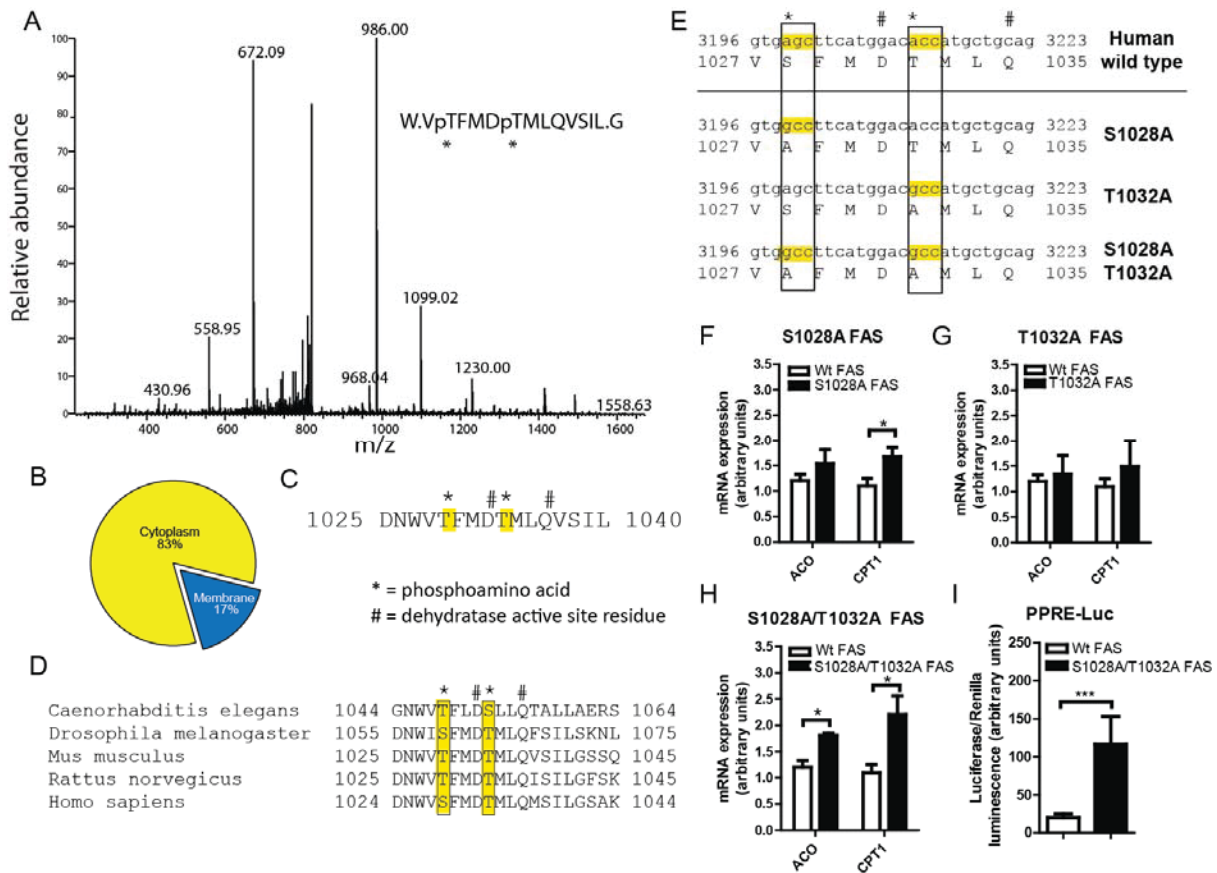


Figure 5. Phosphorylation of cytoplasmic FAS at the dehydratase domain catalytic site controls downstream PPAR α target gene expression. (A) Representative spectrum of the FAS P-T1029/P-T1033 phosphopeptide from wild type mouse liver. (B) Distribution of P-T1029/P-T1033 phosphopeptides identified by mass spectrometry in cytoplasm and membrane fractions of mouse liver. While the proportion of phosphorylation differed based on fraction, peptide abundances (phosphorylated + non-phosphorylated) were similar for the membrane and cytoplasm fractions (not shown). (C) Position of P-T1029 and P-T1033 amino acid residues in relation to the FAS dehydratase domain active site residues. D1032 is one of two dehydratase domain catalytic residues in FAS. (D) Sequence alignment of the FAS putative phospho-amino acids and dehydratase domain active sites in several species. (E) Diagram of the FAS phosphosite mutants in human FAS. (F) RT-PCR analyses of PPAR α target gene expression in Hepa1-6 cells expressing wild type or S1028A mutant FAS. Endogenous FAS was knocked down using lentiviral shRNA for murine FAS. Wild type or

mutant human FAS was expressed using retroviruses. Data are averages of three independent experiments. *indicates $P \leq 0.05$. (G) RT-PCR analyses of PPAR α target gene expression in Hepa1-6 cells expressing wild type or T1032A mutant FAS. Assay performed as in (F). Data are averages of three independent experiments. (H) RT-PCR analyses of PPAR α target gene expression in Hepa1-6 cells expressing wild type or S1028A/T1032A mutant FAS. Assay performed as in (F). Data are averages of three independent experiments. *indicates $P \leq 0.05$. (I) PPRE-luciferase activity in Hepa1-6 cells expressing wild type or S1028A/T1032A mutant FAS. Wild type or mutant human FAS was expressed using retroviruses. Endogenous FAS was knocked down using lentiviral shRNA for murine FAS. Cells were co-transfected with plasmids encoding 3xPPRE-firefly luciferase and *Renilla* luciferase. PPRE-luciferase activity is reported as the ratio of firefly/*Renilla* luciferase luminescence. N=3-6/group. ***indicates $P \leq 0.0005$.

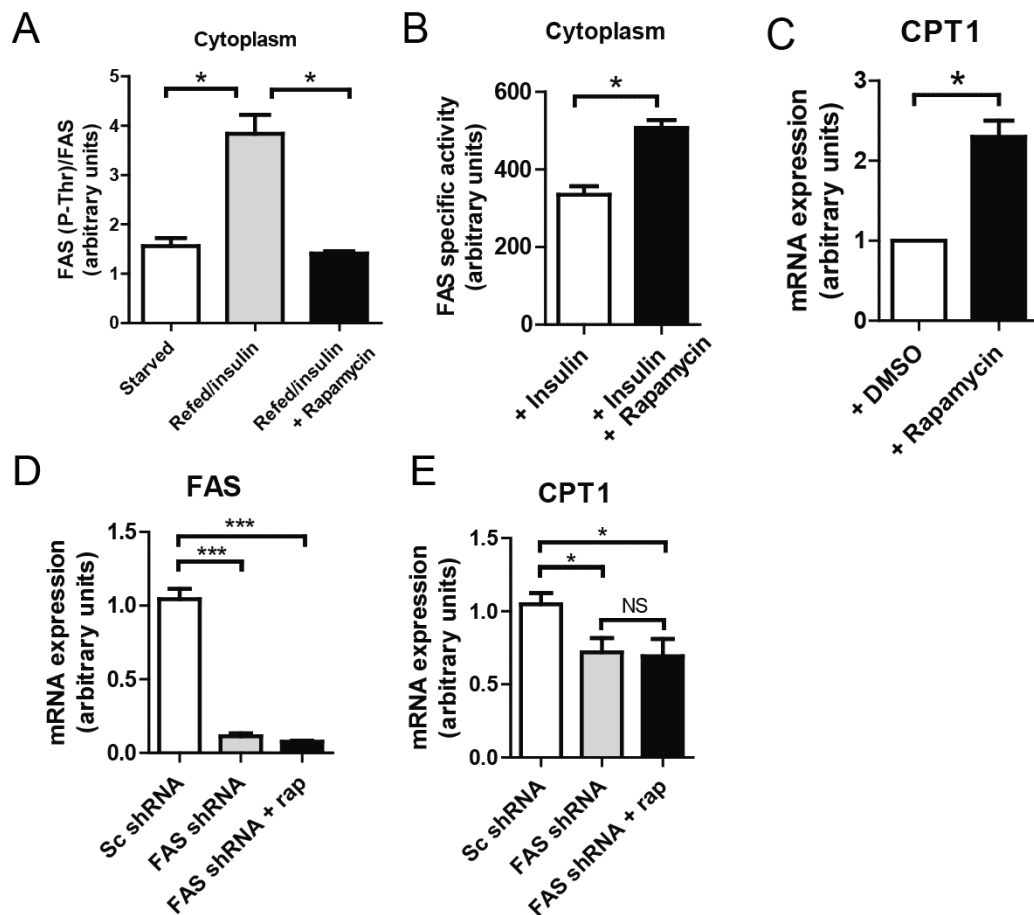


Figure 6. FAS phosphorylation is inhibited by rapamycin and impacts CPT1 expression.

(A) Cytoplasmic FAS phosphorylation in response to rapamycin in Hepa1-6 cells. Hepa1-6 cells were treated with vehicle, 100 nM insulin, or 100 nM insulin + 100 nM rapamycin for 30 min. The cytoplasmic fractions were isolated, then FAS was immunoprecipitated and analyzed for phosphothreonine by Western blotting. *indicates $P \leq 0.05$. (B) Cytoplasmic FAS activity in response to rapamycin in Hepa1-6 cells. Cells were treated with 100 nM insulin and vehicle (DMSO) or 100 nM insulin + 100 nM rapamycin for 30 min and FAS enzyme activity was assayed. Activity was normalized to FAS protein levels as measured by Western blotting. Data are averages of two independent experiments. *indicates $P \leq 0.05$. (C) CPT1 expression levels in response to rapamycin in Hepa1-6 cells. Cells were treated with vehicle (DMSO) or 100 nM rapamycin for 24 h. Data are averages of two independent experiments. *indicates $P \leq 0.05$. (D) FAS expression levels following FAS knockdown in Hepa1-6 cells.

N=3-5/group. ***indicates $P \leq 0.0005$. (E) CPT1 expression levels in response to rapamycin following FAS knockdown in Hepa1-6 cells. N=3-5/group. *indicates $P \leq 0.05$. NS indicates not significant.

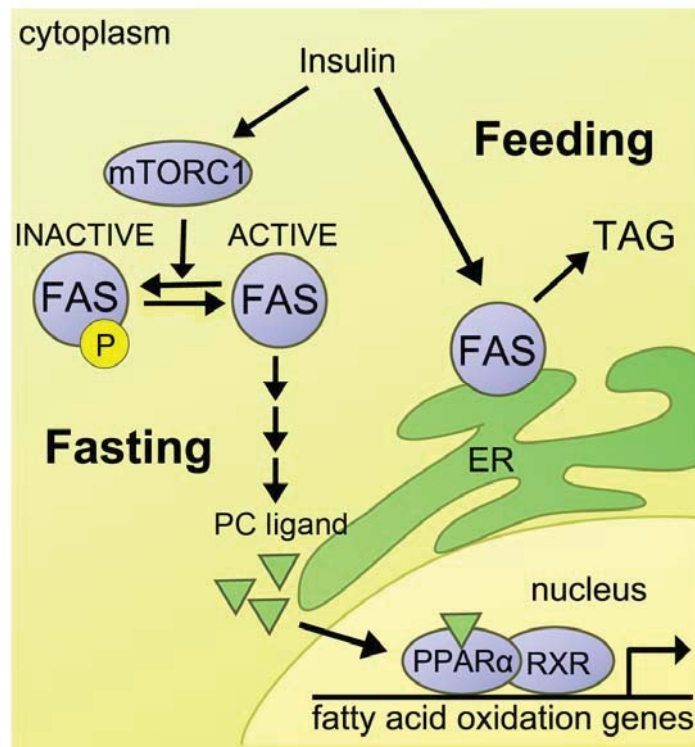


Figure 7. Schematic depiction of insulin/feeding-regulated FAS phosphorylation and FAS-mediated PPAR α activation. In the fed state, mTORC1 promotes phosphorylation of FAS, thus limiting downstream generation of a phosphatidylcholine ligand that activates PPAR α -dependent gene expression. In the fasting state, dephosphorylated FAS in the cytoplasm is permissive for the generation of the ligand activating PPAR α -dependent gene expression. Abbreviations: PC = phosphatidylcholine, RXR = retinoid X receptor, TAG = triacylglycerol.

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Chapter 4:

**Phosphatidylcholine Transfer Protein Activates PPAR α
in the Liver by Nucleo-Cytoplasmic Ligand Shuttling**

ABSTRACT

The nuclear receptor PPAR α is a key regulator of lipid metabolism in the liver and the target of the fibrate drugs, used to treat dyslipidemia. Hepatic PPAR α is activated by an endogenous phosphatidylcholine (PC) ligand, the production of which is dependent on fatty acid synthase (FAS) and choline-ethanolamine phosphotransferase-1 (CEPT1), the latter catalyzing the final step in PC synthesis. It is not known how this lipid reaches the nuclear PPAR α from the extranuclear CEPT1. Here, we provide evidence that phosphatidylcholine transfer protein (PCTP) shuttles PC ligand to PPAR α in liver.

PCTP knockdown in Hepa1-6 hepatocytes caused dramatic reductions in expression of PPAR α target genes, and PCTP co-immunoprecipitated with PPAR α , suggesting this effect may be due to a direct interaction. Immunofluorescent imaging showed that PCTP is found in both cytoplasm and nucleus, and starvation of cells caused an accumulation of PCTP in the nucleus, consistent with a shuttling function controlled by nutrition.

Using mass spectrometry, we demonstrated that PCTP binds 16:0/18:1-GPC. We further showed that the binding of this ligand to PCTP is FAS-dependent: in mice with liver-specific knockout of FAS, the amount of 16:0/18:1-GPC bound to PCTP in the nucleus was significantly reduced. In the cytoplasm, there was no significant difference in binding. In mice with whole-body knockout of *Pctp*, the amount of 16:0/18:1-GPC bound to PPAR α in the nucleus may be reduced compared to wild type mice.

Taken together, these data suggest that PCTP activates PPAR α in the liver by promoting delivery of endogenously synthesized lipid ligand to PPAR α in the nucleus.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease in the Western world and a common comorbidity of the metabolic syndrome [1]. In the United States, 10–35% of Americans are believed to have fatty liver [2]. While fatty liver in itself can be benign, it often progresses into nonalcoholic steatohepatitis (NASH), which in turn can lead to liver cirrhosis and hepatocarcinoma. As of 2013, there are no therapies for NAFLD that are approved by the FDA in the United States or the EMA in the European Union. A pharmacological treatment for fatty liver could thus benefit a large fraction of the population.

One of the major pathways for modulating fat metabolism and transport in the liver is through the nuclear receptor peroxisome proliferator-activated receptor α (PPAR α). In the liver, PPAR α is essential for the fasting response and regulates gluconeogenesis, fatty acid oxidation, and lipoprotein metabolism in response to changes in nutrient availability [3, 4]. Mice lacking PPAR α are fasting-intolerant and develop hepatosteatosis [3, 4].

PPAR α is ligand-activated, and in the liver, the phosphatidylcholine (PC) species 16:0/18:1-glycerophosphocholine (16:0/18:1-GPC) functions as an endogenous ligand activating PPAR α during periods of fasting or starvation [5]. Synthesis of this ligand and activation of PPAR α is dependent on the presence of fatty acid synthase (FAS) and of choline/ethanolamine phosphotransferase-1 (CEPT1), which catalyzes the final step in phosphatidylcholine synthesis by the Kennedy pathway [5, 6].

How, then, does ligand synthesized by CEPT1 reach PPAR α ? CEPT1 is located at the ER and the nuclear membranes [7] with the active site facing the cytoplasm [8], precluding a direct interaction with the nuclear PPAR α . Because the cytoplasmic movement of amphipathic molecules such as phosphatidylcholine is extremely inefficient [9], simple diffusion of the ligand is an unlikely mechanism of transport. A more likely possibility is that

phosphatidylcholine ligand is transported to PPAR α in association with a protein.

Phosphatidylcholine transfer protein (PCTP) is a small, soluble lipid-binding protein of unclear physiological function. It is also known as StARd2 as it contains a StART (StAR-related lipid-transfer) domain. PCTP binds phosphatidylcholine exclusively, at a 1:1 ratio [10]. It is highly expressed in the liver [11] and found both in cytoplasm and in the nucleus [11]. Mice with whole-body knock-out of PCTP have decreased mRNA expression of PPAR α target genes in the liver [12]. These characteristics make PCTP an ideal candidate for a ligand-delivering chaperone for hepatic PPAR α .

Here, we present evidence that PCTP activates PPAR α in the liver through the endogenous FAS-CEPT1 pathway by promoting ligand delivery to PPAR α in the nucleus. Any of the nodes of the FAS-PCTP-CEPT1 pathway of PPAR α activation could potentially represent novel targets for treatment of fatty liver.

RESULTS

Loss of PCTP decreases hepatic PPAR α target gene expression. Gene-chip analysis of hepatic mRNA in full-body PCTP knock-out mice (*Pctp*^{-/-} mice) indicates that the mice have decreased PPAR α target gene expression compared to controls [12]. We tested the effect of PCTP ablation in Hepa1-6 cells, a murine hepatoma cell line. Following knockdown of *Pctp* with shRNA, expression of the PPAR α targets ACO and CEPT1 was dramatically reduced, suggesting a similar role for PCTP with respect to PPAR α in this in vitro system (Figure 1A). If the effect of PCTP on PPAR α is due to PCTP delivering ligand to PPAR α , it would be expected that the two proteins physically interact. To test this, we expressed Myc-tagged PCTP in Hek293T cells and did a pull-down using an anti-Myc antibody. PPAR α co-immunoprecipitated with Myc (Figure 1B), suggesting a physical interaction between these proteins.

PCTP shuttles between cytoplasm and PPAR α in the nucleus in a nutrient-dependent manner. As PPAR α is activated under conditions of low nutrients, we hypothesized that the movement of PCTP would be regulated by nutritional stimuli such that there are greater amounts of PCTP available to PPAR α in the nucleus during starvation. To test this, we imaged Myc-tagged PCTP in Hepa1-6 cells under control or serum-starved conditions using an antibody against the Myc epitope. Under control conditions, PCTP was evenly distributed throughout the cell (Figure 2A, left). Under serum-starved conditions, the majority of cells remained unchanged; however, we found that in a significant proportion of cells, Myc-PCTP distinctly accumulated in the nucleus (Figure 2A, right). The reason for the heterogeneous PCTP distribution between cells is unclear.

To confirm the nuclear localization of PPAR α in this cell line and under these conditions, we imaged FLAG-tagged PPAR α in Hepa1-6 cells under control or serum-starved conditions. FLAG-PPAR α was exclusively nuclear under either condition (Figure 2B).

Nuclear PCTP binds 16:0/18:1-GPC in vivo in liver in a FAS-dependent manner. To determine which phosphatidylcholine (PC) species bind to hepatic PCTP in vivo and their dependence on FAS, we administrated adenovirus encoding FLAG-tagged PCTP (Ad-PCTP-FLAG) or, as a control, untagged GFP to wild type or FASKOL mice. Four days after adenovirus injection and after fasting the mice for 18 h, the livers were harvested and fractionated into cytoplasm and nucleus under conditions unlikely to disturb the ligand/protein interaction. We immunoprecipitated FLAG-tagged PCTP using an antibody directed at the FLAG epitope. Immunoprecipitate eluates showed a dominant band the size of PCTP during immunoblotting with an anti-FLAG antibody (representative blot shown in Figure S1).

Lipids from the eluates were analyzed by mass spectrometry. There was no detectable PC signal for eluates from mice injected with GFP adenovirus (data not shown). Several peaks corresponding to PC species were detected in wild type Ad-PCTP-FLAG mice (representative spectra shown in Figure 3A and 3D), the most abundant being the peaks with mass to charge ratio (m/z) of 758.6 and 760.6. M/z 758.6 corresponds to 16:1/18:1-GPC or 16:0/18:2-GPC, while m/z 760.6 corresponds to the PPAR α ligand 16:0/18:1-GPC. These peaks were detected in both the cytoplasmic and the nuclear eluates. As a control, the samples were also analyzed for phosphatidylethanolamine (PE). There was no detectable PE signal (data not shown), consistent with the highly selective PC-binding by PCTP.

Since activation of PPAR α by 16:0/18:1-GPC is dependent on the lipogenic pathway including FAS and CEPT1 [5], we tested whether the presence of 16:0/18:1-GPC in the FLAG-PCTP eluates is dependent on the presence of FAS using mice with liver-specific ablation of FAS (FASKOL mice). Representative spectra for PC are shown in Figure 3B and 3E. In the cytoplasm, there was no difference in the amount of 16:0/18:1-GPC bound to FLAG-PCTP (normalized to FLAG protein levels) between wild type and FASKOL mice

(Figure 3F). In the nucleus, however, the amount of 16:0/18:1-GPC ligand in the FLAG eluate was significantly reduced in mice lacking FAS (Fig. 3E). M/z 758.6 (corresponding to 16:1/18:1-GPC or 16:0/18:2-GPC) was likewise reduced in the nucleus of mice lacking FAS (see representative spectra in Figure 3A-B; not quantified).

One interpretation of these data is that FAS participates in the biosynthesis of a lipid ligand that is delivered to the nucleus by PCTP. The lack of change in cytoplasmic 16:0/18:1-GPC levels in the FLAG eluates could reflect participation of cytoplasmic PCTP in additional functions that involve its binding to dietary or membrane phosphatidylcholine as well, obscuring any difference solely from loss of FAS.

PPAR α may bind 16:0/18:1-GPC in vivo in liver in a PCTP-dependent manner. To test whether the binding of the 16:0/18:1-GPC ligand to PPAR α is dependent on PCTP, we administered adenovirus encoding FLAG-tagged PPAR α (Ad-FLAG-PPAR α) to *Pctp*^{-/-} mice or wild type littermates, then analyzed lipids in the FLAG immunoprecipitate eluate as above. Immunoblotting with anti-FLAG antibody showed a band the size of PPAR α in the nuclear fraction (representative blot shown in Figure S2). There was no detectable FLAG-tagged protein in the cytoplasmic fraction, as expected from the nuclear localization of PPAR α (data not shown).

We analyzed FLAG-PPAR α eluates for phospholipids by mass spectrometry. No phosphatidylethanolamine or phosphatidylserine was detected (data not shown). Similar to PCTP, the dominant peaks for PC were m/z 758.6 and 760.6, corresponding to 16:1/18:1-GPC/16:0/18:2-GPC and 16:0/18:1-GPC, respectively (Figure 4A). The relative amounts of this lipid were over 60% lower in *Pctp*^{-/-} mice than wild types (representative spectra are shown in Figure 4A and 4B); however, this difference was not statistically significant (quantification in Figure 4C; p = 0.20).

DISCUSSION

The final enzyme in the biosynthesis of an endogenous ligand for PPAR α , CEPT1, is exclusively extranuclear, while hepatic PPAR α is exclusively nuclear. We pursued the hypothesis that the phosphatidylcholine-binding protein PCTP functions as a transport protein for the 16:0/18:1-GPC PPAR α ligand in the liver, shuttling between the cytoplasm and PPAR α in the nucleus to deliver ligand and thereby activate PPAR α during periods of starvation.

We found that PCTP moved between cytoplasm and nucleus in a nutrient-dependent manner, tending to accumulate in the nucleus under periods of starvation (Figure 2A). PCTP co-precipitated with PPAR α (Figure 1B), suggesting a physical association between the two proteins that could serve to transfer lipid. PCTP bound 16:0/18:1-GPC, and in the nucleus, it did so in a manner dependent on FAS (Figure 3), indicating that the ligand delivered to the nucleus by PCTP is indeed derived from the FAS-catalyzed de novo lipogenic pathway previously described [5]. Binding of the 16:0/18:1-GPC ligand to PPAR α may in turn be dependent on the presence of PCTP (Figure 4), supporting a role for PCTP in delivering this ligand to hepatic PPAR α .

Because of the capacious ligand-binding domain of PPAR α , there are likely several endogenous ligands with varying transactivation capabilities and tissue distribution. It follows, then, that there may be several ligand-binding chaperones with varying specificities and distributions for transport of lipid ligands with poor solubility, both for PPAR α and for the other PPARs. It will be of interest to characterize the transport mechanisms for endogenous PPAR ligands in other tissues as well, as this could potentially provide new ways of selectively or concomitantly modulating desired sets of PPARs to achieve a therapeutic effect.

MATERIALS AND METHODS

Animals. *Pctp*^{-/-} mice (gift from David Cohen, Brigham and Women's Hospital, generated as described [13]) and littermate control mice were on an FVB/NJ genetic background. *Pctp*^{-/-} mice were genotyped using Klentaq and the following primer sets: 5'-CCCTTCTTGCCGTCCTC-3' and 5'-TACGTCTACACCCGCCAG-3' resulting in a 162 bp PCTP fragment and 5'-TGTC AAGACCGACCTGTCCG-3' and 5'-TATTCGGCAAGC-AGGCATCG-3' resulting in a 447 bp fragment of the Neomycin resistance gene replacing the *Pctp* gene in knock-out alleles. Liver-specific FAS knock-out mice (FASKOL mice) and littermate controls were on a C57BL/6J background and were genotyped using previously described primer sets [6].

Mice were provided ad libitum access to chow diet (Purina #5053) or fasted for 18 h. All mice were kept on Aspen bedding during fasting/feeding experiments and had free access to water. Protocols were approved by the Washington University Animal Studies Committee.

Antibodies. Rabbit monoclonal antibody against p70 S6 Kinase (2708) was from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibody against Myc (sc789) and rabbit polyclonal antibody against PPAR α (sc9000) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against FLAG (F1804) was from Sigma (St. Louis, MO).

Cell culture. Hepa1-6 cells, Hek293, and Hek293T cells were maintained in DMEM + 10% FBS.

Lentiviral shRNA-mediated knockdown. Plasmid encoding shRNA for mouse PCTP (TRCN0000105217) was obtained from Open Biosystems (Huntsville, AL). Packaging vector psPAX2 (12260) and envelope vector pMD2.G (12259) were obtained from Addgene (Cambridge, MA). 70% confluent Hek293T cells in a 15 cm dish were transfected using Lipofectamine 2000 with 8 μ g psPAX2, 2.25 μ g pMD2.G, and 3 μ g mouse PCTP shRNA.

After 48 h, media was collected and filtered through 0.45 µm syringe filters. Polybrene was added and the media was used to treat 50-70% confluent Hepa1-6 cells. After 24 h, the media was aspirated and replaced with fresh media. 48 h after addition of retroviral media, cells were selected with puromycin. After another 48 h, cells were harvested and knockdown of PCTP was assessed.

RT-PCR. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using iScript™ cDNA synthesis kit (Invitrogen). Quantitative RT-PCR was performed using SYBR® Green reagent (Applied Biosystems, Carlsbad, CA) with an ABI Prism 7700 PCR instrument.

Plasmid constructs. Plasmid encoding Myc-tagged PCTP was a gift from David Cohen (Brigham and Women's Hospital).

Transfection. Hepa1-6 cells were transfected by electroporation. 25 µg plasmid DNA was used per confluent 10 cm dish of Hepa1-6 cells (or $\sim 1 \times 10^7$ cells). Plasmid DNA was added to the bottom of a cuvette. Cells were trypsinized and spun down after adding regular media. Media was aspirated and the cells washed in PBS and spun again. PBS was aspirated and cells were resuspended in 0.5 ml PBS and transferred to the cuvette. The cuvette with cells and DNA was electroporated at 360 V and 250 µF (time constant of 4.5-5 sec⁻¹) and 1 ml of media was immediately added to the cuvette. Cells were transferred to a 15 ml tube and media was added up to 5-10 ml. Cells were allowed to recover for 10 min after which they were plated. For immunofluorescent imaging, cells were plated directly onto fibronectin-covered glass coverslips (BD Biosciences) in 6-well dishes and imaged 2 days following transfection.

Hek293T cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 10 µg of plasmid was used per 10 cm dish of $\sim 90\%$ confluent Hek293T cells. Cells were harvested for immunoprecipitation the following day.

Myc immunoprecipitation. 0.5 mg protein lysate from Hek293T cells in 1 ml of buffer containing 0.5% NP-40 was incubated with 20 μ l anti-Myc antibody on a rocker at 4°C overnight. 50 μ l agarose beads were then added and the sample incubated for another 1 h on a rocker at 4°C. The beads were spun down, the supernatant aspirated, and the beads were washed with HNTG buffer 3 times before boiling the beads in 2X loading buffer for Western blotting and loading the entire volume onto a protein gel.

Adenoviruses. Adenovirus encoding FLAG-tagged wild type mouse PPAR α and adenovirus encoding a GFP marker only were gifts from T. C. Leone and D. P. Kelly. To make adenovirus encoding FLAG-tagged wild type mouse PCTP, murine Hepa1-6 cell cDNA was used as template to amplify full-length *Pctp* by PCR with primers adding a 5' BamHI site followed by a FLAG tag on the 5' side and an XhoI site on the 3' side: 5'-ACAACAGGATCCACCATGGATTACAAGGATGACG- 3' and 5'-ACAACACTC-GAGTTAGGTTTTCTTGTGGTAGTTC-3'. The amplified *Pctp* was cut using BamHI and XhoI, ligated with pAdTrack-CMV vector (encoding a GFP marker) that had been cut with BglIII and XhoI, and recombined with Ad-Easy1 vector.

Adenoviruses were packaged in Hek293 cells and purified with cesium chloride ultracentrifugation. Optimal dosing of the adenoviruses was determined by assaying for adenoviral protein expression by Western blotting and the survival of mice injected with varying doses of adenovirus. Adenovirus was injected in 100 μ l total volume. Livers were harvested four days following injection for nuclear extraction and FLAG immunoprecipitation for mass spectrometry.

Nuclear extraction. Perfused liver from *Pctp*^{-/-} mice, FASKOL mice, or appropriate control mice was harvested and ~100 mg liver was added directly into a glass homogenizing tube on ice containing 1 ml of cold non-detergent-containing hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, protease and phosphatase

inhibitors). After 5 min incubation on ice, the liver was manually homogenized using a glass pestle. After an additional 10 min incubation, the homogenate was centrifuged at 8000 g at 4°C for 20 min. The supernatant (cytoplasmic fraction) was removed to a fresh tube. The pellet was resuspended in nuclear extraction buffer (10 mM HEPES, 0.42 M NaCl, 25% glycerol, 1.5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, protease and phosphatase inhibitors), placed on a rotating shaker at 4°C for 1 h, then centrifuged at 18,000 g for 10 min. The supernatant (nuclear fraction) was removed to a fresh tube.

FLAG immunoprecipitation. Cytoplasmic or nuclear extract (~3 mg of cytoplasmic protein or ~600 µg nuclear protein in a total volume of 750 µl) was incubated with 50 µl anti-FLAG M2-Agarose affinity gel beads (A2220, Sigma) overnight at 4°C on a rotating shaker. Five washes (wash buffer: 50 mM Tris HCl [pH 7.4], 100 mM NaCl, protease and phosphatase inhibitor cocktail) were followed by elution by competition with excess 3x FLAG peptide (F4799, Sigma; 150 ng/ml). A 25 µl aliquot of the eluted sample was processed for immunoblotting. The remainder was transferred to glass tubes along with 1 ml of chloroform per sample and processed for mass spectrometry.

Mass spectrometry. Lipids were extracted from the samples, mixed with 14:0/14:0-GPC as an internal standard for PC, and analyzed as [M+H]⁺ ions by positive ion ESI/MS.

Statistics. Data are shown as mean ± SEM. Comparisons between two groups were performed using an unpaired, two-tailed t-test. Comparisons between more than two groups were performed using ANOVA.

FIGURES

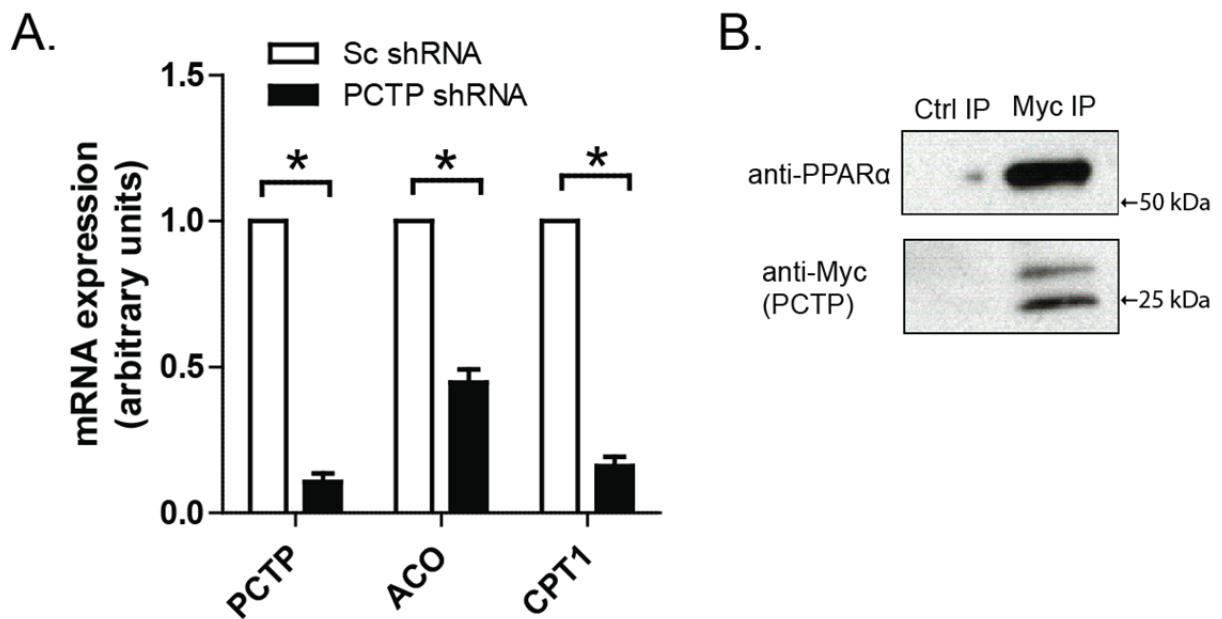


Figure 1. PCTP promotes hepatic PPAR α target gene expression and physically associates with PPAR α . (A) mRNA expression of *Pctp*, *Aco*, and *Cpt1* as measured by real-time RT-PCR in Hepa1-6 cells with or without knock-down of *Pctp* using shRNA. Expression levels are normalized to the ribosomal gene *L32*. *indicates $P \leq 0.05$. (B) Co-immunoprecipitation of PPAR α and Myc-tagged PCTP. Myc-PCTP was overexpressed in Hek293T cells and immunoprecipitated using anti-Myc antibody. The immunoprecipitate eluates were analyzed by Western blotting as shown for PPAR α and Myc (PCTP).

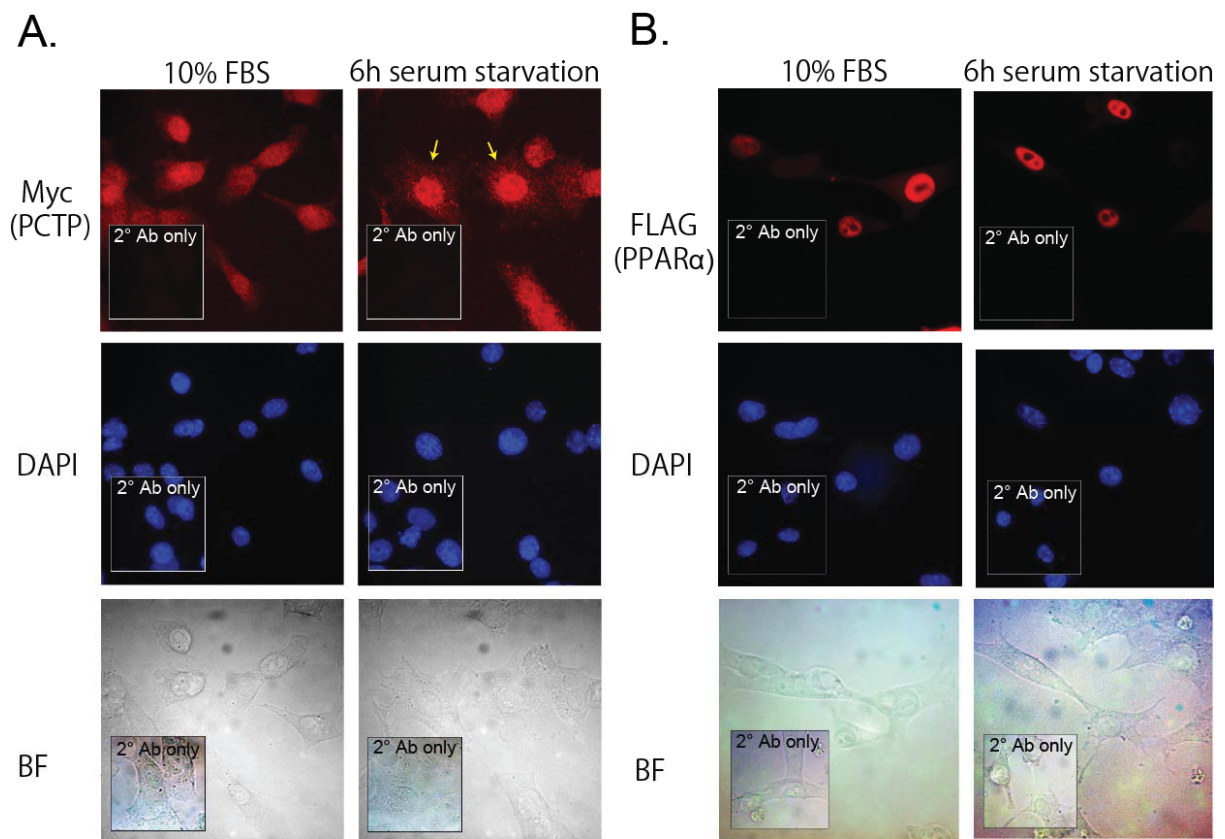


Figure 2. PCTP shuttles between cytoplasm and the nucleus in a nutrient-dependent manner. (A) Representative images of immunofluorescent staining of Myc-tagged PCTP in Hepa1-6 cells during control or serum-starved conditions. Hepa1-6 cells were transfected with Myc-PCTP by electroporation two days prior to experiment. Cells pictured on the left were kept in media containing 10% FBS (fresh at 6 h prior to fixation). Cells pictured on the right were starved in media containing 0.5% FBS for 6 h prior to fixation. BF denotes bright-field. Insets show cells stained with secondary antibody only. (B) Representative images of immunofluorescent staining of FLAG-tagged PPAR α in Hepa1-6 cells under control or serum-starved conditions (as described for panel A). Cells were treated with FLAG- PPAR α adenovirus two days prior to harvest. There were no cells under either condition where extra-nuclear PPAR α staining was visible.

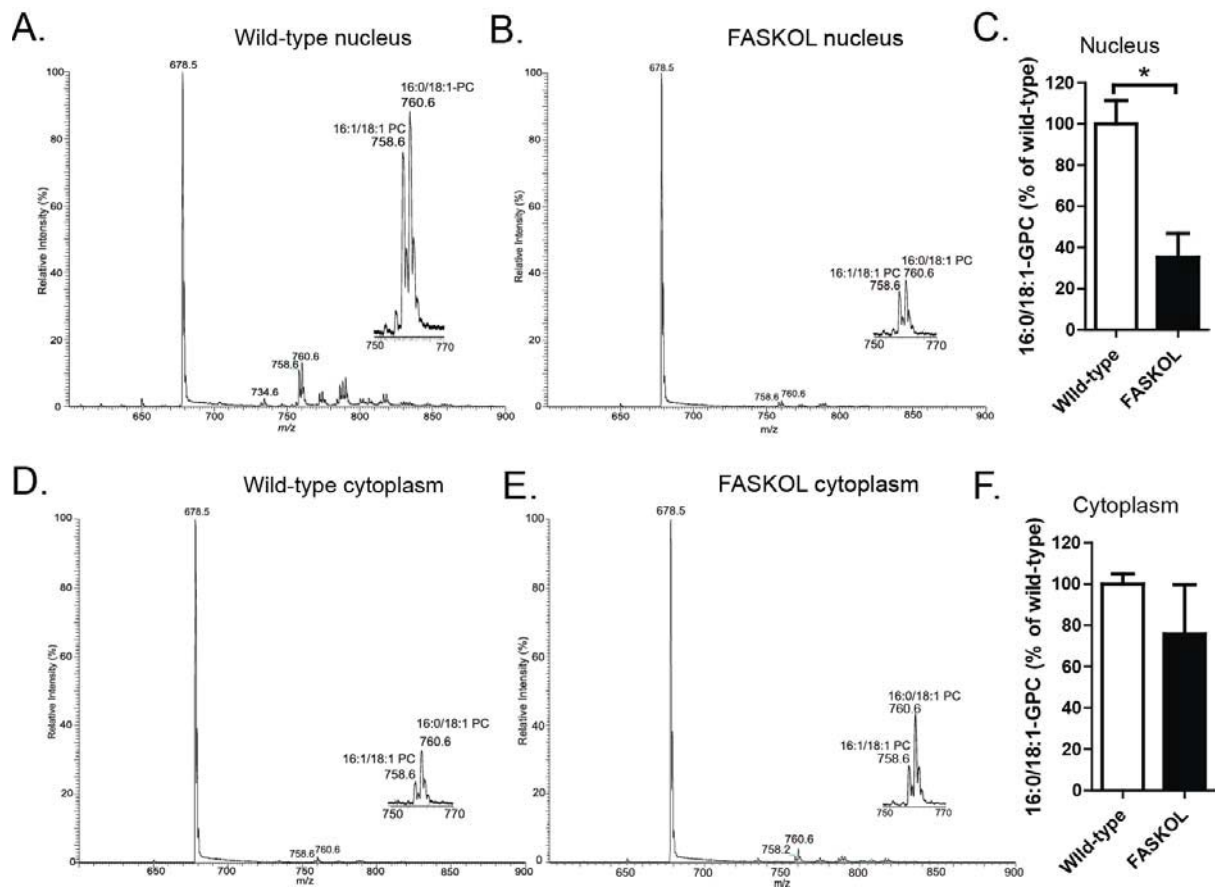


Figure 3. Nuclear PCTP binds 16:0/18:1-GPC in vivo in liver in a FAS-dependent manner. (A-B) and (D-E) Representative mass spectra of phosphatidylcholine bound to FLAG-PCTP immunoprecipitated from nuclear and cytoplasmic fractions of liver from FASKOL mice or wild type littermates. The insets show magnification of peaks including that corresponding to 16:0/18:1-GPC. (C) Quantification of 16:0/18:1-GPC abundance in nuclear PCTP expressed as percentage of wild type, as measured by mass spectrometry. *indicates $P \leq 0.05$. (F) Quantification of 16:0/18:1-GPC abundance in cytoplasmic PCTP expressed as percentage of wild type, as measured by mass spectrometry.

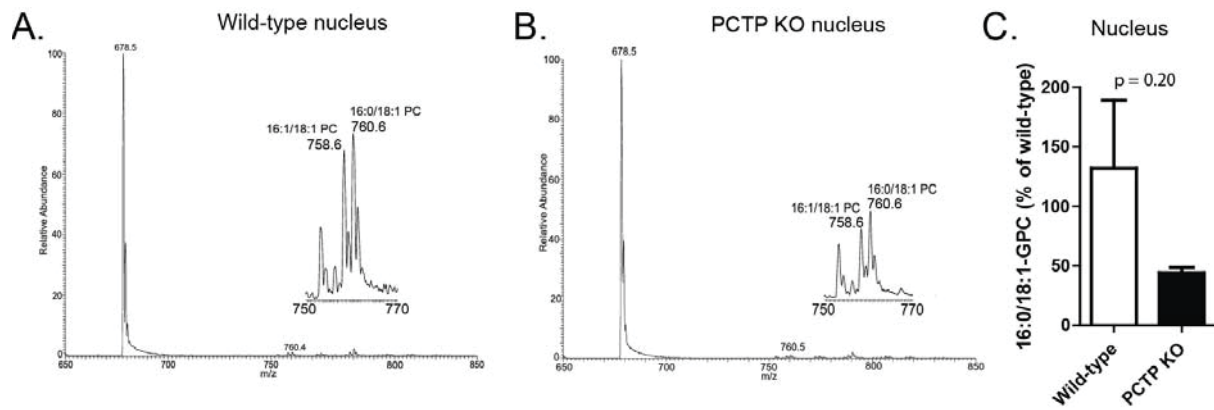


Figure 4. PPAR α binds 16:0/18:1-GPC in vivo in liver in a PCTP-dependent manner.

(A-B) Representative mass spectra of phosphatidylcholine bound to FLAG-PPAR α immunoprecipitated from nuclear fractions of liver from *Pctp*^{-/-} mice and wild type littermates. The insets show magnification of peaks including that corresponding to 16:0/18:1-GPC. (C) Quantification of 16:0/18:1-GPC abundance in nuclear PPAR α expressed as percentage of wild type, as measured by mass spectrometry.

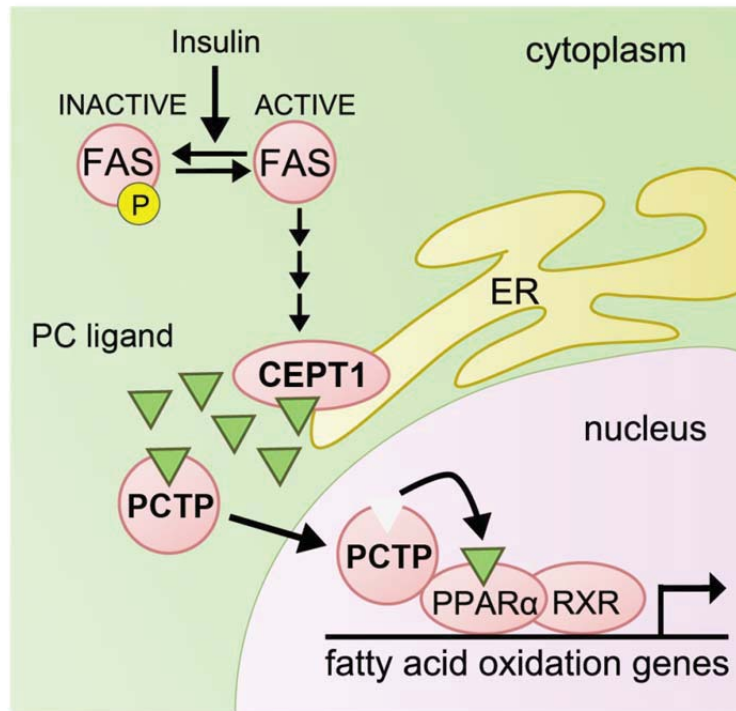


Figure 5. Proposed model of PCTP-mediated ligand delivery to PPAR α . In the fasting state, FAS is active and promotes synthesis of an endogenous PC ligand for PCTP, which can then enter the nucleus and deliver the ligand to PPAR α . PPAR α then promotes transcription of genes involved in fatty acid oxidation and ketogenesis. Abbreviations: CEPT1, choline/ethanolamine phosphotransferase 1; FAS, fatty acid synthase; PC, phosphatidylcholine; PCTP, phosphatidylcholine transfer protein, PPAR α , peroxisome proliferator-activated receptor alpha; RXR = retinoid X receptor.

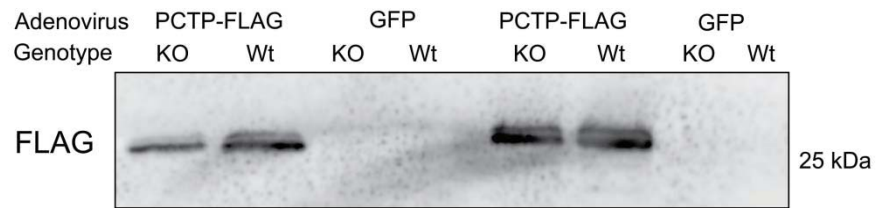


Figure S1. FLAG protein levels in liver from mice injected with PCTP-FLAG or GFP adenovirus. “KO” refers to FASKOL mice. “Wt” refers to wild type, FAS $lox^{+/+}$ mice not expressing Cre.

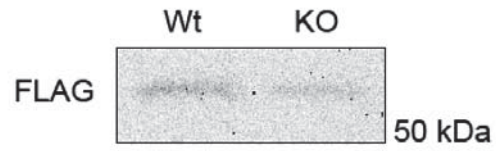


Figure S2. FLAG protein levels in liver from mice injected with PPAR α -FLAG adenovirus. “KO” refers to a *Pctp*^{-/-} mouse. “Wt” refers to a wild type littermate.

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Chapter 5:
Conclusions

The studies presented in this dissertation demonstrate how the post-translational regulation of a signaling lipid—16:0/18:1-glycerophosphocholine—ensures appropriate metabolic responses to changes in nutrient and hormone levels.

This lipid activates the hepatic fasting response via PPAR α , and we have shown that its activity is regulated both at the level of its synthesis and by its subcellular location. In Chapter 2 and 3, we demonstrated that PPAR α activation by 16:0/18:1-GPC is controlled by multiple modes of post-translational regulation of its biosynthesis by fatty acid synthase (FAS). In Chapter 4, we demonstrated that the nucleo-cytoplasmic transport of 16:0/18:1-GPC by phosphatidylcholine transfer protein (PCTP) regulates PPAR α activation.

Here, I will briefly discuss these findings in the context of four things I learned from my graduate research.

1. The cytoplasm is a big place

In Chapter 3, I presented evidence that FAS protein in the liver is compartmentalized into two separate subcellular locations, each with its own distinct pattern of regulation and physiological function. In Chapter 2, a possible mechanism for the membrane localization of FAS was provided by identifying two cytoskeletal proteins, Septin-2 and Septin-7, that associate with FAS exclusively in the membrane fraction.

The dual location of FAS was surprising, as FAS is a cytoplasmic protein according to textbooks (the term “cytosolic FAS” is used to distinguish it from mitochondrial type II FAS in some sources [1]). The discovery that liver FAS concentrates at the ER and Golgi was a chance observation. But the textbooks are correct: FAS is not a membrane-spanning protein or a secreted protein and does not exist within any intracellular organelles. “Cytoplasmic” is simply too broad a term to be useful in describing location. As exemplified by FAS, a protein freely floating in the cytoplasm can be very different in function and regulation from the

same protein peripherally associated with the cytoplasmic side of a membrane—even though they are both, technically, cytoplasmic proteins.

2. Some things are not new, just forgotten

Even more surprising was the discovery that cytoplasmic FAS is active during fasting, while membrane FAS is active during feeding; our hypothesis for months had been that the unexpected membrane-bound FAS would be responsible for the correspondingly unexpected role of FAS in PPAR α activation. Actually measuring FAS activity in mouse liver after ad lib feeding or fasting, however, disrupted this hypothesis (Chapter 3, Figure 2).

I found it worrisome that the “classical,” cytoplasmic pool of FAS was regulated in the opposite direction of how it was supposed to be regulated—let alone that the changes in specific activity by necessity involved post-translational regulation, another feature that FAS was not thought to possess. However, a careful literature search revealed that we were not the first to observe feeding-induced inhibition of FAS activity nor post-translational regulation of FAS activity. In fact, some of the earliest studies on FAS demonstrated changes in FAS specific activity, allosteric regulation, and post-translational modifications of FAS. In 1968, Salih Wakil’s group demonstrated allosteric regulation of FAS specific activity by 1,6-fructose-bisphosphate, a glycolytic intermediate [2], and in 1975, John Porter’s group published an account of inhibitory phosphorylation of FAS during feeding in pigeon liver [3].

These were rigorously performed studies on the biochemistry of the FAS protein, something of a rarity today when FAS mRNA levels are far easier to measure and so a common proxy for FAS activation. They also provided completely novel and, in the case of the Porter study, unexpected information about the regulation of FAS. In spite of this, neither study had ever been cited in a review article; rather, reviews on FAS tend to focus on the studies showing induction of FAS mRNA by re-feeding following starvation and to define FAS regulation as transcriptional and feeding-induced [4-6]. The lack of impact of these

studies can in part be attributed to their age; they used the term “fatty acid synthetase” which has become less common, and may so be missed during literature searches for “fatty acid synthase.” But it also emphasizes the importance of reading primary literature, even (or especially) when it is relatively old. As a side note, I was delighted to find the 1975 article on inhibition of FAS by phosphorylation; it set my mind at ease that our results were real, yet the study was old enough that our work would still be considered novel. Sometimes old is new again.

3. Housekeeping gene \neq unregulated gene

In Chapter 3, we demonstrated rapid, post-translational regulation of FAS, a housekeeping gene. FAS enzymatic activity was controlled through two phosphorylations on threonines 1029 and 1033 of murine FAS, flanking a crucial catalytic residue. These phosphorylations were found exclusively in cytoplasmic FAS, where insulin or feeding induced phosphorylation of FAS by mTORC1 leading to an inhibition of FAS-mediated PPAR α activation.

In the textbook Molecular Biology of the Cell, a housekeeping gene is defined as a “[g]ene serving a function required in all the cell types of an organism, regardless of their specialized role” [7]. These include genes involved in transcription and translation (such as ribosomal proteins and heat shock proteins), metabolism (such as genes involved in lipid and glucose metabolism), and cell structure (such as actin and myosin). The term is useful and accurate to indicate genes and proteins that are ubiquitous and necessary for life, but becomes problematic when it is assumed that “constitutive” expression is equivalent to “unregulated” expression. If a protein is necessary for life, would that diminish the likelihood of its regulation by physiological or pathophysiological stimuli, or, by extension, of its involvement in signaling cascades requiring some manner of regulation? There is no reason to believe that to be the case.

An example of this misconception is the use of certain housekeeping genes as internal standards or loading controls for protein or mRNA levels, such as the glycolytic enzyme GAPDH, when the levels of many housekeeping genes and proteins are in fact regulated in response to various drugs, experimental conditions, cell cycle stages, age etc. (reviewed and studied in [8]). But more importantly, this misconception puts a damper on interest in research on housekeeping proteins. A gene assumed not to be regulated is simply not as interesting for studying dynamic signaling processes.

The “housekeeping” attribute of a protein or gene does make it difficult to study: unless compensatory mechanisms are at play, a mouse with full-body knock-out of a housekeeping gene by definition cannot be generated, as the housekeeping protein fulfills a function required for life. The embryonic lethality resulting from knock-out of many housekeeping genes obscures more subtle functions and any physiological or pathophysiological regulation that would occur in adult mice. In the case of FAS, whole-body knock-out causes early embryonic lethality (probably due to an inability to synthesize cell membranes), but tissue-specific knock-outs have revealed a number of signaling functions of FAS specific for different tissues that are sometimes finely regulated [9-14]. How many other important signaling roles and levels of regulation of housekeeping proteins are unexplored simply because they are assumed not to exist, or too difficult to study?

4. Not all lipids are the same

Just like not all proteins are the same, there is a staggering variety of lipids in nature and not all lipids are functionally equivalent. Rather, different lipids (or the same lipid in different places) play a variety of roles in cell signaling, structure, and energy storage, such as the function of 16:0/18:1-GPC in activating PPAR α (and not just 16:0/18:1-GPC, but FAS-derived 16:0/18:1-GPC specifically). While the variety of functions of such a diverse class of

biomolecules shouldn't come as a big surprise, the importance of lipids in signaling has only gained attention in the past fifteen years. As an illustration of this, the phrases "lipid signaling" and "signaling lipid" appeared 584 times in articles in the PubMed database over the past fifteen years (as of 6/20/2013), and prior to that only 44 times since the first usage of "lipid signaling" in the literature in 1988.

The increasing interest in signaling lipids partly results from improvements in mass spectrometry, allowing for rapid identification of low-abundance lipid molecules. It also represents a recognition that the abundance of distinct lipid species may reflect a corresponding variety in functions. To me, this recognition ties in with the third point in emphasizing just how much we still do not know about cell biology, even in research fields that have been studied for decades, such as lipids and housekeeping genes. Thanks to the enormous increase in our knowledge of biology over the past century, a textbook in molecular biology may make it seem as if most cell biology has already been worked out. The challenge lies in seeing the gaps in our knowledge.

The most important classes of larger biomolecules in living organisms are proteins, nucleic acids, lipids and polysaccharides. It would be easy to dismiss any of the latter classes as simply "fats" and "sugars" meant for storing and transporting energy, yet the sheer number of unique lipid species and polysaccharide modifications suggest that there is much we do not yet know. Developments in mass spectrometry have improved our ability to fill these gaps in knowledge.

Establishing lipid-protein relationships for signaling lipids will be crucial, as the existence of protein targets facilitate pharmacological and genetic manipulation for research or therapeutic purposes. For all the lipid species of yet unknown function, there will be known and novel proteins involved in their synthesis, transport, and catabolism. Identification of signaling lipids may provide novel pathways that can be targeted to treat human disease,

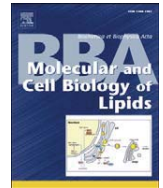
and knowledge of these proteins would provide feasible pharmacological targets to treat metabolic disease.

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Appendix A

Jensen-Urstad APL, Semenkovich CF. “Fatty acid synthase and liver triglyceride metabolism: housekeeper or messenger?” *Biochimica et Biophysica Acta*. **2012**. 1821(5):747-53.



Review

Fatty acid synthase and liver triglyceride metabolism: Housekeeper or messenger? ☆

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ABSTRACT

Fatty acid synthase (FAS) catalyzes the de novo synthesis of fatty acids. In the liver, FAS has long been categorized as a housekeeping protein, producing fat for storage of energy when nutrients are present in excess. Most previous studies of FAS regulation have focused on the control of gene expression. However, recent findings suggest that hepatic FAS may also be involved in signaling processes that include activation of peroxisome proliferator-activated receptor α (PPAR α). Moreover, reports of rapid alterations in FAS activity as well as findings of post-translational modifications of the FAS protein support the notion that dynamic events in addition to transcription impact FAS regulation. These results indicate that FAS enzyme activity can impact liver physiology through signaling as well as energy storage and that its regulation may be complex. This article is part of a Special Issue entitled Triglyceride Metabolism and Disease.

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1. Introduction

The liver is involved in the uptake, synthesis, storage, secretion, and catabolism of fatty acids and triglycerides. Fatty acid synthase (FAS), the enzyme catalyzing de novo synthesis of fatty acids, is traditionally thought of as a housekeeping protein, producing fatty acids that can be used for energy storage, membrane assembly and repair, and secretion in the form of lipoprotein triglycerides. However, the contribution by FAS to secreted triglycerides appears to be negligible compared to other sources of fat under common dietary conditions. An unexpected role for FAS as a signaling enzyme emerged with the finding that FAS can affect fatty acid oxidation through PPAR α , the main mediator of the fasting response in the liver.

The possibility that FAS may be involved in promoting fat catabolism in addition to its known function of synthesizing fat raises new questions regarding the regulation of FAS. Are there multiple pools of FAS with distinct functions, allowing separate control of FAS-mediated signaling and FAS-mediated energy storage? How is FAS-PPAR α signaling regulated in response to nutritional and hormonal stimuli, and how is it possible for FAS to be activated or inhibited rapidly? FAS has been thought to be regulated mostly at the transcriptional level, which might preclude an immediate response by FAS to changes in nutritional or hormonal stimuli since FAS mRNA is fairly stable. A role for FAS in signaling suggests the presence of rapid, post-translational mechanisms of FAS regulation. This review will address

physiological functions of hepatic FAS, its regulation by nutrients and hormones, and mechanisms of regulation.

FAS may be a therapeutic target for treating fatty liver and dyslipidemia [1]. Both are common features of the metabolic syndrome [2,3], which affects ~1 in 4 Americans [4]. Both are also independent risk factors for coronary artery disease [5–7], the most common cause of death worldwide. Identification of regulatory proteins and pathways distinguishing housekeeping FAS from signaling FAS could potentially lead to novel therapeutics that selectively target FAS function.

1.1. Hepatic triglyceride metabolism

Under nutrient-replete conditions, the primary fuel of the liver is glucose rather than fat. Fatty acids are not subjected to β -oxidation and instead are incorporated into triglycerides for storage in lipid droplets or secretion in very low-density lipoproteins (VLDL). Dietary fat in the form of chylomicron remnants is taken up by the liver; de novo synthesis of fatty acids by FAS may make a modest contribution to storing energy as fat when nutrients are present in excess.

During fasting, lipolysis in peripheral tissues (primarily adipose tissue) increases the levels of plasma free fatty acids (FFAs), which are taken up by the liver. Activation of the transcription factor peroxisome proliferator-activated receptor α (PPAR α) mediates the adaptive response to fasting by promoting the transcription of genes involved in the uptake and catabolism of fatty acids [8–11]. Fatty acids derived from peripheral tissues or intrahepatic lipid droplets are catabolized through β -oxidation to produce ketone bodies, which are used as fuel when glucose is scarce.

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In insulin resistance, insulin fails to suppress lipolysis in peripheral tissues even when nutrients are abundant, resulting in high circulating levels of FFAs that are taken up by the liver. Increased FFA uptake and perhaps increased de novo synthesis of fat in the liver overwhelms the capacity for fatty acid oxidation, leading to fat accumulation and eventually the development of hepatosteatosis or fatty liver.

There are thus three main sources of FFAs that contribute to liver triglyceride: plasma, de novo synthesis, and dietary fat delivered by chylomicron remnants. Triglycerides are secreted in VLDL, stored in lipid droplets, or catabolized through the action of lipases and β -oxidation. Fatty acid synthase appears to participate in liver triglyceride metabolism both by contributing de novo synthesized lipids for storage and secretion under nutrient-replete conditions and by promoting β -oxidation of fatty acids through activation of PPAR α under nutrient-deficient conditions.

1.2. Fatty acid synthase

Fatty acid synthase (FAS, encoded by *Fasn*) catalyzes the biosynthesis of saturated fatty acids from simple precursors (de novo lipogenesis). The primary product of the FAS reaction is palmitate (C16:0), but stearate (C18:0) and shorter fatty acids may also be produced. FAS substrates are acetyl-CoA, malonyl-CoA, and NADPH. Acetyl-CoA functions as a primer for the reaction, while NADPH provides reducing equivalents. The fatty acid is elongated from the initial acetyl-CoA by repeated condensations with malonyl-CoA, which donates two carbons in each cycle of condensation. Palmitate synthesis thus requires seven cycles of malonyl-CoA addition to an acetyl-CoA primer to yield a saturated, 16-carbon fatty acid.

The FAS protein exists as a homodimer of 273 kDa subunits. Each monomer contains seven protein domains required for fatty acid synthesis: acyl carrier, acyl transferase, β -ketoacyl synthase, β -ketoacyl reductase, β -hydroxylacyl dehydratase, enoyl reductase, and thioesterase [12] (reviewed in Refs. [13–15]). However, FAS is only enzymatically active in the dimeric form [12]. The monomers were initially thought to be oriented head-to-tail to form the dimer [16,17], but recent structural data demonstrate a head-to-head orientation of the monomers that are intertwined at their middle to form an X-shape [18–21]. Mammalian FAS is a type I FAS complex with the domains consolidated in a single peptide; prokaryotes and yeast have a type II FAS with separate proteins catalyzing the individual reactions. Type II FAS complexes capable of synthesizing short-chain (up to 14 carbons) fatty acids are also found in mammalian mitochondria [22].

FAS is a soluble protein and thought to be localized in the cytoplasm, although the specifics of its subcellular localization are largely unexplored. Its tissue distribution is broad with highest levels in the liver, adipose tissue, and lungs [23,24]. Whole-body knockout of FAS causes embryonic lethality in mice, suggesting that de novo lipogenesis is necessary early during development [25]. A likely possibility is that FAS is required to provide lipids for cell membranes of the growing embryo. Viable tissue-specific FAS knockout mice have been generated, including a liver-specific knockout (discussed below).

2. Function of FAS in hepatic lipid metabolism

2.1. Contribution of de novo synthesized lipids to stored and secreted hepatic triglycerides

Hepatic FAS synthesizes lipids that are stored as lipid droplets or secreted in VLDL in the fed state. In mice, the contribution of liver FAS to secreted VLDL is minor. *Ob/ob* mice have 10-fold increased hepatic de novo lipogenesis compared to lean mice, but no significant differences in serum triglycerides [26]. In mice with liver-specific knockout of FAS (FASKOL mice), serum triglycerides are normal on a chow diet [27].

The contribution of de novo lipogenesis to secreted triglycerides has been studied in humans in the setting of various diets. On diets low in fat and high in carbohydrate (10% of calories as fat and 75% as carbohydrate), de novo lipogenesis makes a significant contribution to circulating lipids as almost half of VLDL triglyceride is derived from de novo lipogenesis under these conditions [28]. However, a typical Western diet is high in fat as well as carbohydrates. In similar studies using diets higher in fat (30% fat, 55% carbohydrate or 40% fat, 45% carbohydrate), the contribution of de novo lipogenesis to VLDL triglycerides is undetectable or minor, at 0–10% [28,29]. These diets are more representative of the high fat, high carbohydrate content of a typical Western diet, indicating that under common dietary conditions, de novo lipogenesis is not a significant contributor to VLDL triglycerides. Substituting starch for sugar in a high-carbohydrate diet also decreases the contribution of de novo lipogenesis to 0–1% or 5% [30,31]. Obese individuals do not appear to have increased FAS-derived VLDL triglycerides compared to lean individuals [29]. Under the high-fat, high-carbohydrate dietary conditions common in the Western world today, hepatic FAS thus appears to be a minor contributor to VLDL triglycerides.

FAS may contribute to triglycerides stored in hepatic lipid droplets. In rats fed a chow diet, $11 \pm 1\%$ of hepatic triglycerides are derived from de novo lipogenesis [32]. On a high-fat diet, de novo lipogenesis is suppressed and only $1.0 \pm 0.2\%$ of hepatic triglycerides are derived from FAS [32]. FASKOL mice on a chow diet have normal, rather than decreased, liver triglyceride content [27]. It thus appears that the contribution of de novo lipogenesis to stored triglycerides is small in healthy liver.

In fatty liver, the contribution of FAS to intrahepatic triglycerides may be greater. *Ob/ob* mice have increased hepatic FAS activity and fatty liver [33], but a mechanistic link between the two has not been established. In humans with non-alcoholic fatty liver disease, one group has reported that $26 \pm 7\%$ of hepatic triglycerides are derived from de novo lipogenesis [34]. It is unknown how this compares to the triglyceride content of healthy human liver. However, even in the setting of hepatic over-accumulation of fat, the contribution of FAS appears to be less than that of fats derived from peripheral tissues or dietary fat.

2.2. Regulation of triglyceride metabolism through signaling lipids: ligand activation of PPAR α

PPAR α is a member of a family of ligand-activated nuclear receptors important for modulating metabolism and inflammation. During fasting, PPAR α promotes lipid uptake and catabolism of fatty acids through β -oxidation to produce ketone bodies [9–11].

When liver-specific fatty acid synthase knockout (FASKOL) mice were generated, they were surprisingly not protected against hepatic lipid accumulation, but instead developed severe hepatic steatosis when on a zero-fat diet or with prolonged fasting [35]. The phenotype of fasted or zero-fat diet-fed FASKOL mice is similar to that of PPAR α null mice: hypoglycemia, low serum ketone levels, marked hepatic steatosis, and deficient hepatic fatty acid oxidation [10,35]. Much of this phenotype was corrected by administration of a known PPAR α ligand. The deficient PPAR α activation in the absence of both FAS and dietary fat led to the hypothesis that “new” fat, derived from de novo lipogenesis or dietary fat, can activate PPAR α , whereas “old” fat, derived from peripheral tissues or stored in the liver, cannot. Hydrolysis of hepatic triglycerides has also been shown to mediate PPAR α activation [36], suggesting that triglycerides of different origins (de novo synthesis vs. free fatty acids entering the liver following lipolysis in peripheral tissues) may occupy separate compartments in the hepatocyte. In addition to activating PPAR α in liver, FAS has been shown to regulate PPAR α in macrophages [37] and hypothalamus [38] as well.

Further study of the FASKOL mouse led to the identification of an endogenous ligand for hepatic PPAR α : the phosphatidylcholine species 16:0/18:1-glycerophosphocholine [27]. The interaction of this species with PPAR α is dependent on the activity of FAS, and inactivation of choline/ethanolamine phosphotransferase 1 (CEPT1), an enzyme catalyzing the final step in phosphatidylcholine biosynthesis, mimics the FASKOL phenotype [27]. FAS thus appears to contribute to PPAR α activity by promoting the synthesis of one of its ligands.

A summary of the impact of FAS on hepatic triglyceride metabolism is presented in Fig. 1.

2.3. Modulating hepatic FAS to treat disease

Ob/ob mice have increased hepatic FAS gene expression as well as increased hepatic FAS activity compared to lean mice [33]. Knockdown of the transcription factor carbohydrate response element binding protein (ChREBP), which promotes the expression of FAS as well as other genes, in *ob/ob* liver decreases hepatic lipid accumulation and decreases hepatic lipogenesis, suggesting a link between de novo lipogenesis by FAS and fatty liver [39]. However, in a gene expression profiling study of *ob/ob* animals separated into high glucose and lower glucose groups, mice with lower sugars (and thus likely to be more insulin sensitive) had higher hepatic expression levels of genes encoding lipogenic enzymes, including FAS, as compared to mice with higher sugars [40]. This finding suggests that while activation of lipogenic enzymes in the liver is associated with obesity, this effect is unlikely to be mechanistically linked to insulin resistance.

FAS inhibitors have been tested in mouse models of obesity and diabetes. Treatment of lean or obese mice with the FAS inhibitor C75 causes dramatic weight loss and improvement of hepatic steatosis in obese mice. However, the effect is primarily mediated by reduced food intake through inhibition of hypothalamic FAS (in addition to possible effects of this particular agent that are independent of FAS), obscuring the potential effects of inhibiting hepatic FAS [41].

The FAS inhibitor platensimycin is concentrated in the liver when administered orally and does not affect food intake [1]. Treatment of high-fructose diet-fed *db/db* mice with platensimycin reduces hepatic FAS activity, hepatic lipid accumulation, and hepatic fatty acid oxidation [1]. These data are consistent with roles for hepatic FAS both as a producer of fat that may accumulate in liver, and as a generator of lipid signals to nuclear receptors such as PPAR α .

These data also highlight a caveat when considering FAS inhibitors as therapy for hepatic steatosis: inhibition of FAS can affect both lipid storage and lipid catabolism, and under conditions where baseline FAS activity is not particularly high, loss of FAS activity might aggravate rather than ameliorate hepatic steatosis, as seen in the liver-specific FAS knockout mice [35].

3. Regulation of FAS activity

Transcriptional regulation of FAS has been well characterized, but little is known about the post-translational regulation of FAS activity. Similarly, long-term effects of hormones and nutrients on FAS expression are clear but their immediate effects are poorly understood.

3.1. Hormonal and nutritional regulation of FAS

Hepatic FAS is known to be regulated by insulin, glucagon, cyclic AMP, fructose, glucose, and dietary fat.

Re-feeding mice or rats a high-carbohydrate diet following a prolonged fast causes a robust induction of FAS expression as compared to the fasted or the ad lib-fed state [42–45]. The effect of carbohydrate re-feeding is mediated by both insulin and glucose. Insulin regulates FAS through transcriptional and non-transcriptional mechanisms. Under nutrient-replete conditions, de novo lipogenesis may promote storage of excess energy in the form of hepatic triglycerides. Insulin promotes FAS expression through activation of the transcription factors sterol regulatory element binding protein 1c (SREBP-1c) [46] and upstream stimulatory factors 1 and 2 (USF1 and USF2) [47,48].

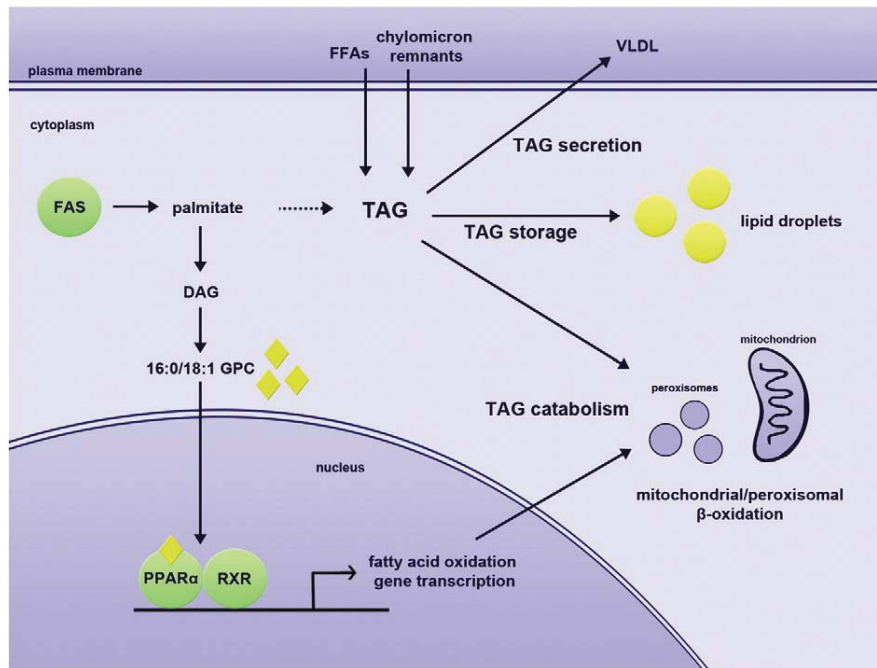


Fig. 1. The role of FAS in hepatic triglyceride metabolism. Fatty acid synthase controls fatty acid catabolism through the synthesis of a ligand for PPAR α , which activates fatty acid oxidation genes. FAS makes a minor contribution of lipids to stored and secreted triglycerides, with the major contributions coming from plasma free fatty acids and dietary fats from chylomicron remnants. 16:0/18:1 GPC, 16:0/18:1-glycerophosphocholine; DAG, diacylglycerol; FAS, fatty acid synthase; FFA, free fatty acid; PPAR α , peroxisome proliferator-activated receptor alpha; RXR, retinoid X receptor; TAG, triacylglycerol (triglyceride); VLDL, very low-density lipoprotein.

Conversely, glucagon and cyclic AMP inhibit the increase in FAS activity induced by carbohydrate re-feeding in rats [42,49,50].

The effect of fasting compared to ad lib feeding on the activity of hepatic FAS is less clear. In mice, a 6 hour fast reduces FAS expression levels by 60% compared to ad lib feeding [44], and in rats, a 24 hour fast reduces FAS expression by over 90% compared to ad lib feeding [45]. However, a 14 hour fast in mice produces no change in FAS activity compared to ad lib-fed mice [51]. One potential explanation for the lack of change in FAS activity in some circumstances could be a relatively long half-life for the FAS protein. It is possible that changes in FAS gene expression might have little effect on FAS enzyme activity in response to certain physiologically relevant periods of fasting as compared to the ad lib fed condition.

While insulin promotes the expression of FAS, insulin also acutely inhibits the enzymatic activity of hepatic FAS, causing a decrease in FAS activity within minutes [51]. This inhibition is dependent on the presence of the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), which is phosphorylated in response to insulin and subsequently associates with FAS [51]. This acute inhibition of FAS by insulin is blunted in hyperinsulinemic *ob/ob* mice [51]. While a clear physiological role for this acute inhibition of FAS activity has not been determined, it is possible that the acute effect on FAS by insulin primarily affects FAS lipid signals that impact PPAR α . Acute inhibition of FAS in response to insulin could then serve to halt the fasting response by PPAR α and decrease fatty acid oxidation when nutrients are abundant. Because this acute change alters the specific activity of FAS, the effect is likely post-translationally mediated. In contrast, the long-term effect of insulin on FAS is transcriptionally mediated and promotes FAS expression, enabling increased storage of energy as fat.

Carbohydrates directly promote the expression of hepatic FAS in the liver in addition to having an indirect effect by stimulating insulin secretion. Feeding mice a high-glucose or high-fructose diet for 1 week leads to 3-fold and 8-fold, respectively, increases in FAS protein [52]. The effect of glucose on FAS expression is mediated by ChREBP [53–56]. Hepatic metabolism of glucose by glucokinase (GK) is necessary for the glucose-mediated induction of FAS by ChREBP [57]. The insulin-induced activation of SREBP-1c and the glucose-induced activation of ChREBP act synergistically to promote FAS expression [57]. A connection between lipid/carbohydrate sensing and metabolism is suggested by the finding that stearoyl-CoA desaturase (SCD1), an enzyme catalyzing the synthesis of oleate, is involved in the carbohydrate-induced induction of FAS and other lipogenic enzymes [52].

Dietary fats inhibit FAS expression to decrease de novo lipogenesis when fats are already abundant. Polyunsaturated fatty acids (PUFAs) may decrease FAS expression through inhibition of SREBP-1c [58] and ChREBP [59] activity. Diets consisting of 10% oil inhibit hepatic FAS activity when fed to rats of over the course of 4 weeks, with the greatest reduction in rats fed fish oil [60]. Re-feeding rats a carbohydrate-free, high-fat diet following fasting suppresses FAS gene expression to levels as low as those seen in rats fasted for 24 h [45].

3.2. Transcription and the FAS promoter

Much of the work on transcriptional regulation of FAS has been done in rats, but the FAS promoter is highly conserved between species suggesting that studies of the rat FAS promoter are likely to be relevant to mice and humans. Regulatory elements and transcription factor binding sites in the proximal mouse FAS promoter are shown in Fig. 2.

As noted above, SREBP-1c is activated by insulin and under appropriate conditions promotes expression of lipogenic genes, including FAS. The FAS promoter contains a sterol regulatory element (SRE) at –150 as well as tandem SREs at positions –72 and –62 that are required for optimal SREBP-1c-mediated activation of FAS expression in rats [61–63].

An inverted CCAAT box at –94 is a binding site for nuclear factor Y (NF-Y) and is necessary for inhibition of FAS expression by cyclic AMP [64,65]. A binding site for the transcription factor specificity factor 1 (Sp1) is located nearby at –91 [63]. NF-Y and Sp1 proteins interact [66] and mediate sterol-induced FAS expression synergistically with SREBP-1c [63,67]. Another transcription factor, X-box binding protein 1 (XBP1), increases FAS promoter activity indirectly via SREBP-1c [68].

Also as noted above, ChREBP plays a central role in the glucose-induced transcriptional regulation of FAS as well as other lipogenic and glycolytic genes in the liver [53–56]. Glucose promotes the nuclear translocation and activation of ChREBP, while polyunsaturated fatty acids and cyclic AMP inhibit ChREBP activity [59,69]. ChREBP binds to a carbohydrate response element (ChRE) located at –7214 in the distal FAS promoter in rats to activate FAS transcription [70]. ChREBP appears to be the main regulator of glucose-induced FAS expression, as glucose fails to induce an increase in FAS expression in ChREBP-null hepatocytes [53]. Mice fed a high-fructose diet have similar amounts of nuclear ChREBP protein and ChRE-bound ChREBP protein compared to mice fed a high-glucose diet, suggesting that dietary fructose and glucose have comparable effects on ChREBP [71].

In addition to the ChRE, a direct repeat-1 (DR-1) element located between –7110 and –7090 in the distal promoter of rat FAS is necessary for full glucose activation of FAS expression [72]. Hepatic nuclear factor-4 α (HNF-4 α) binds to the DR-1 element and interacts with ChREBP. Ablation of HNF-4 α produces a corresponding decrease in glucose-induced FAS expression [72].

Liver X receptor (LXR), a transcription factor activated by oxysterols, upregulates FAS expression through direct and indirect mechanisms. Indirectly, LXR can promote FAS expression by binding to liver X receptor elements (LXREs) in the promoters of the SREBP [73] and ChREBP [74] genes to promote their transcription. SREBP and ChREBP in turn activate FAS transcription. The LXR-mediated activation of SREBP-1c is the primary mechanism of insulin-induced SREBP activation [73]. The physiological relevance of LXR-mediated transcriptional regulation of ChREBP is debated, as LXR is not necessary for the glucose-induced activation of ChREBP [56]. LXR can also bind directly to LXREs located at positions –686 to –672 of the mouse FAS promoter to activate FAS transcription [75].

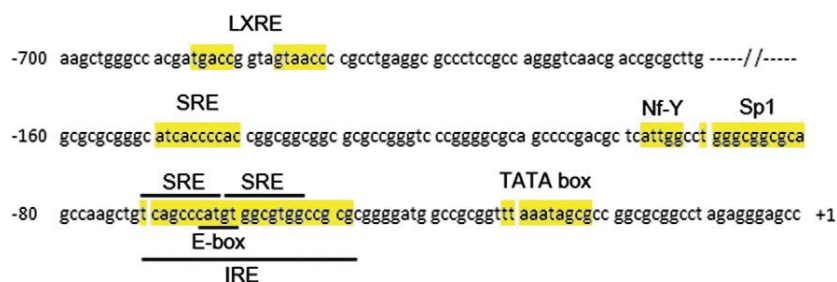


Fig. 2. The mouse proximal FAS promoter. Regulatory elements and nuclear factor binding site nucleotides are highlighted in yellow. IRE, insulin response element; LXRE, liver X receptor element; NF-Y, nuclear factor Y binding site; Sp1, specificity factor 1 binding site; SRE, sterol regulatory element.

An insulin response element (IRE) containing an E-box DNA binding motif is located at positions –71 to –50 of the FAS promoter, overlapping two tandem SREs. The IRE is necessary for insulin-induced FAS expression [76]. USF1 and USF2 bind to the IRE [48]. Mutation of the E-box prevents USF binding and abolishes insulin-induced FAS expression. However, the importance of USFs in insulin-stimulated FAS expression remains unclear, because mutation of the E-box also prevented SREBP-1c binding [47].

3.3. Post-translational regulation of FAS

Transcriptional regulation of FAS may require hours to affect protein levels since both FAS mRNA and protein are fairly stable, buffering sudden changes due to increased transcription and subsequent translation.

There are several reports of FAS protein being activated or inhibited in far shorter time frames, as well as reports of changes in FAS activity that do not correlate with changes in FAS protein levels. Insulin acutely decreases FAS enzyme activity. In hepatoma cells, FAS activity decreases linearly from 2 to 15 min after insulin treatment, followed by an increase in FAS activity for 75 min [51]. Peroxynitrate inhibits FAS activity in adipocytes within 10 min, without any effect on FAS protein levels [77]. Activation and inhibition of FAS without corresponding changes in FAS protein levels have been reported in a variety of cancer cell lines [78–80]. These data suggest the presence of post-translational regulation of FAS.

Phosphorylation has been proposed as a mechanism of FAS regulation in cancer cells, adipocytes, and liver. In livers from pigeons that were fasted and then re-fed, radiolabeled phosphate was incorporated into FAS in the cytosolic fraction. The phosphorylation event was associated with low FAS activity, and dephosphorylation of FAS by incubation with phosphatases caused a 20-fold increase in FAS activity [81]. Another inhibitory phosphorylation was demonstrated in 3T3-L1 adipocytes, where FAS threonine phosphorylation was associated with inhibition of FAS activity [77]. This phosphorylation event was shown to require AMP-activated kinase (AMPK), likely through indirect effects since *in vitro* kinase assays failed to demonstrate any incorporation of labeled phosphate into FAS in the presence of AMPK [77]. These findings suggest the presence of an unidentified intermediate kinase step.

In human and mouse breast cancer cell lines, the finding that large differences in FAS activity between cell lines did not correlate with FAS protein levels prompted an exploration of FAS phosphorylation as an alternative mechanism of FAS regulation [79]. Phosphoserine and phosphothreonine residues were detected in FAS in cell lines from both species, while FAS phosphotyrosine residues were detected in human cells only. Phosphorylation of FAS in these cell lines was associated with greater FAS activity [79]. Recently, tyrosine phosphorylation of FAS was noted in two different human breast cancer cell lines. Both FAS tyrosine phosphorylation and FAS activity were induced by overexpression of human epidermal growth factor receptor 2 (HER2) and decreased by HER2 inhibition, and FAS was phosphorylated when complexed with HER2 [80].

In addition to phosphorylation, FAS was one of a large number of hepatic metabolic enzymes recently found to be lysine acetylated [82]. Acetylation was linked with diverse effects on metabolic enzymes, including protein destabilization, activation, and inhibition, suggesting that acetylation may play a major role in metabolic regulation. Acetylation of FAS could represent a novel mechanism for controlling its activity.

Known examples of post-translational regulation of FAS are summarized in Table 1.

4. Conclusions and future directions

Hepatic FAS is generally thought to be a housekeeping protein, synthesizing fatty acids for the partitioning and storage of excess

Table 1
Post-translational modifications of FAS.

| Type of post-translational modification | Organism and tissue or cell type | Function |
|---|--|----------------------------------|
| Phosphorylation [81] | Pigeon liver | FAS inhibition |
| Threonine phosphorylation [77] | 3T3-L1 adipocytes (mouse) | FAS inhibition |
| Threonine and serine phosphorylation [79] | NMuMG (mouse mammary epithelial cells), T1 (mouse mammary tumor cells), SKBr3 (human breast carcinoma cells) | Unknown, possibly FAS activation |
| Tyrosine phosphorylation [79,80] | SKBr3 (human breast carcinoma cells) | FAS activation |
| Acetylation [82] | Human liver | Unknown |

energy. However, the contribution of FAS to stored and secreted triglycerides is minor under most physiological conditions. Studies of mice deficient in hepatic FAS have demonstrated that FAS also serves as a signaling protein, controlling the activation of PPAR α under nutrient-deficient conditions to promote the adaptive response to fasting.

FAS is regulated in part through effects on gene expression. However, rapid changes in enzyme activity associated with alterations in nutritional status suggest that post-translational mechanisms underlie enzymatic responses to external stimuli. An approach to understanding these dynamic effects might include identifying post-translational modifications of FAS, characterizing FAS subcellular localization, searching for FAS-interacting proteins, and pursuing other mechanisms that enable immediate control of FAS activity.

The existence of separate physiological functions for FAS implies that it might be possible to develop function-specific therapies. Exclusively modulating the cellular FAS pool that promotes fatty acid oxidation or exclusively modulating the pool that promotes synthesis of lipids for storage could provide new treatment options for fatty liver and other serious obesity-related conditions.

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Appendix B

Lodhi IJ, Yin L, **Jensen-Urstad AP**, Funai K, Coleman T, Baird JH, El Ramahi MK, Razani B, Song H, Fu-Hsu F, Turk J, Semenkovich CF. “Inhibiting adipose tissue lipogenesis reprograms thermogenesis and PPAR γ activation to decrease diet-induced obesity.” *Cell Metabolism*. **2012**. 16(2):189-201.

Inhibiting Adipose Tissue Lipogenesis Reprograms Thermogenesis and PPAR γ Activation to Decrease Diet-Induced Obesity

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SUMMARY

De novo lipogenesis in adipocytes, especially with high fat feeding, is poorly understood. We demonstrate that an adipocyte lipogenic pathway encompassing fatty acid synthase (FAS) and PexRAP (peroxisomal reductase activating PPAR γ) modulates endogenous PPAR γ activation and adiposity. Mice lacking FAS in adult adipose tissue manifested increased energy expenditure, increased brown fat-like adipocytes in subcutaneous adipose tissue, and resistance to diet-induced obesity. FAS knockdown in embryonic fibroblasts decreased PPAR γ transcriptional activity and adipogenesis. FAS-dependent alkyl ether phosphatidylcholine species were associated with PPAR γ and treatment of 3T3-L1 cells with one such ether lipid increased PPAR γ transcriptional activity. PexRAP, a protein required for alkyl ether lipid synthesis, was associated with peroxisomes and induced during adipogenesis. PexRAP knockdown in cells decreased PPAR γ transcriptional activity and adipogenesis. PexRAP knockdown in mice decreased expression of PPAR γ -dependent genes and reduced diet-induced adiposity. These findings suggest that inhibiting PexRAP or related lipogenic enzymes could treat obesity and diabetes.

INTRODUCTION

A relentless increase in mean global body weight since 1980 has resulted in an estimated 1.5 billion overweight people worldwide, of which a half billion are obese (Finucane et al., 2011). Obesity leads to diabetes, which is associated with premature death from many causes (Seshasai et al., 2011). Obesity is caused by positive energy balance leading to expansion of adipocyte mass. However, adipocytes possess functional pathways that might be targeted to complement therapies altering energy balance. De novo lipogenesis, an adipocyte function

that requires the multifunctional enzyme fatty acid synthase (FAS) (Semenkovich, 1997), is one such potential target since adipose tissue FAS has been implicated in obesity and insulin resistance in humans (Moreno-Navarrete et al., 2009; Roberts et al., 2009; Schleinitz et al., 2010).

Fatty acid synthase catalyzes the first committed step in de novo lipogenesis. The magnitude of de novo lipogenesis is different in rodents and people. Lipogenesis is thought to be a relatively minor contributor to whole body lipid stores in a present-day human consuming a typical high fat diet (Aarsland et al., 1996; Letexier et al., 2003; McDevitt et al., 2001). However, pharmacologic or genetic manipulation of enzymes in the lipogenic pathway can have profound metabolic consequences (Postic and Girard, 2008), suggesting that de novo lipogenesis might serve a signaling function independent of the generation of lipid stores (Lodhi et al., 2011). Consistent with this concept, FAS in liver is part of a lipogenic pathway involved in the generation of a ligand for peroxisome proliferator-activated receptor α (PPAR α) (Chakravarthy et al., 2009), a key transcriptional regulator of fatty acid oxidation.

PPARs, consisting of PPAR α , PPAR δ and PPAR γ , are ligand activated transcription factors that form obligate heterodimers with the retinoid X receptor (RXR) and regulate metabolism (Wang, 2010). Ligand binding results in a conformational change in the receptor, promoting dissociation of repressors, recruitment of coactivators, and subsequent activation of target gene expression. This nuclear receptor family was identified and named based on activation by chemicals that promote proliferation of peroxisomes (Dreyer et al., 1992; Issemann and Green, 1990).

Peroxisomes participate in the oxidation of certain fatty acids as well as the synthesis of bile acids and ether lipids (Wanders and Waterham, 2006). These single membrane-enclosed organelles are present in virtually all eukaryotic cells. In adipocytes they tend to be small and were referred to as microperoxisomes by Novikoff and colleagues, who documented a large increase in peroxisome number during the differentiation of 3T3-L1 adipocytes (Novikoff and Novikoff, 1982; Novikoff et al., 1980).

We sought to evaluate the role of de novo lipogenesis in adipocyte function and metabolism. Here we show that a lipogenic pathway encompassing FAS and PexRAP (peroxisomal reductase activating PPAR γ), an enzyme localized to peroxisomes

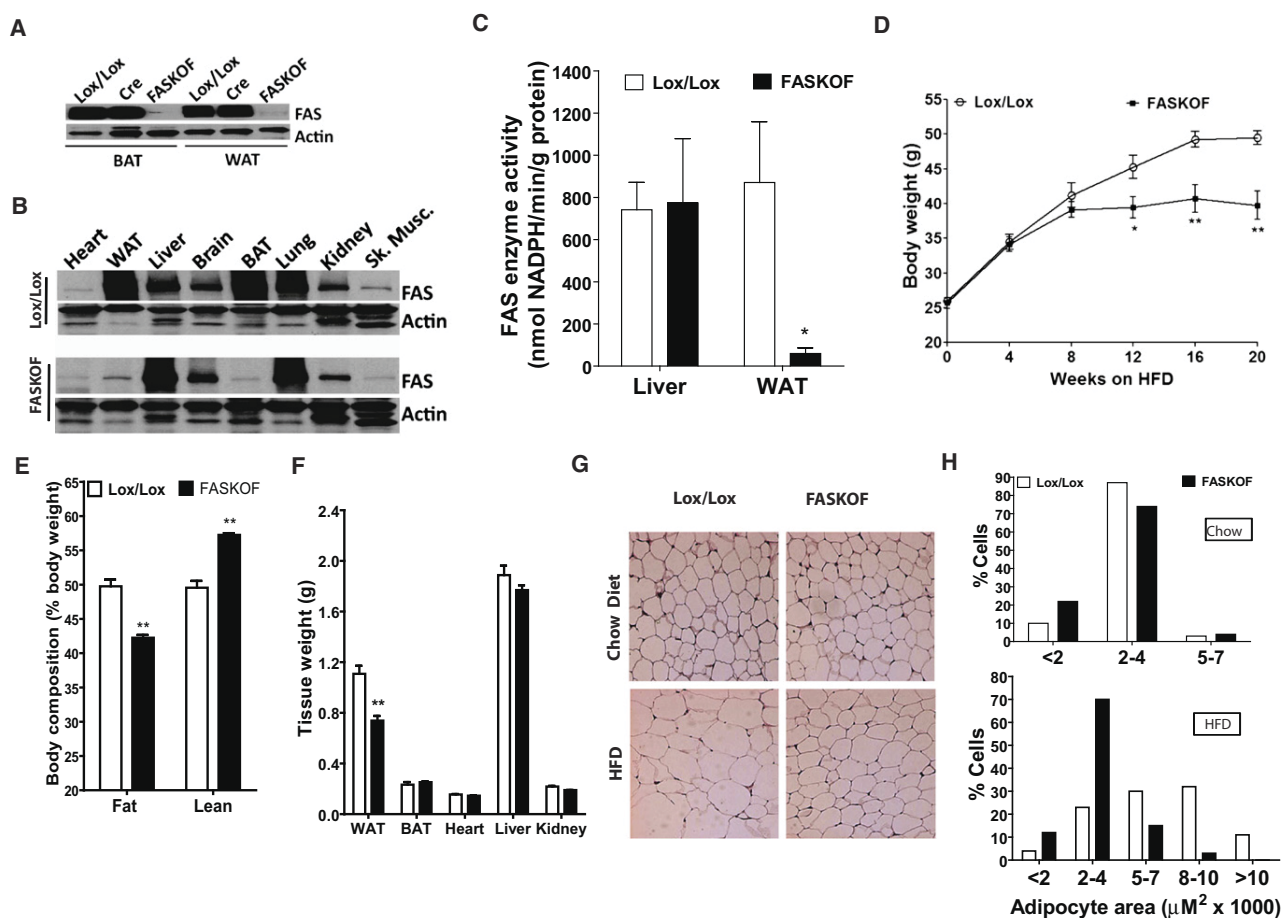


Figure 1. Targeted Deletion of Adipose Tissue FAS Decreases Adiposity

(A) FAS protein by western blot in brown (BAT) and white (WAT) adipose tissue of *Lox/Lox* control (without Cre), adiponectin-Cre control (without floxed alleles), and FASKOF mice.
 (B) Tissue distribution of FAS protein by western blot. An apparent increased expression of hepatic FAS protein in FASKOF mice was not consistently observed.
 (C) FAS enzyme activity assay. * $p = 0.031$. $N = 4$ /genotype.
 (D) Body weight of HFD-fed control and FASKOF male mice. Similar results were also obtained in two additional feeding experiments with different cohorts of male mice. * $p = 0.03$. ** $p = 0.0068$ at 16 weeks, 0.0028 at 20 weeks. $N = 6$ -8/genotype. Additional data including females are provided in Table S1.
 (E) MRI analysis of body composition in HFD-fed mice. ** $p < 0.0001$. $N = 6$ /genotype.
 (F) Tissue weights of HFD-fed control and FASKOF mice. ** $p = 0.005$. $N = 6$ /genotype.
 (G) Histologic appearance of WAT harvested from chow-fed or HFD-fed mice.
 (H) Adipocyte size distribution determined with the NIH Image J program. Error bars in (C)–(F) represent SEM.

and encoded by a previously unidentified mammalian gene, contributes to the endogenous activation of PPAR γ and modulates adiposity with high fat feeding.

RESULTS

Targeted Deletion of Adipose Tissue FAS

We generated FAS knocked out in fat (FASKOF) mice by crossing FAS^{lox/lox} mice (Chakravarthy et al., 2005) with adiponectin-Cre transgenic mice (Eguchi et al., 2011). FASKOF mice, born at the expected Mendelian frequency, were overtly normal. FAS protein was decreased in white and brown adipose tissue of FASKOF relative to Cre only (without lox sites) and *lox/lox* (without Cre) control mice (Figures 1A and 1B). FAS protein content was not decreased in whole brain extracts of FASKOF

mice (Figure 1B). FAS mRNA assayed by quantitative RT-PCR was the same in the hypothalamus of FASKOF and *lox/lox* mice (not shown), suggesting that phenotypes are not likely to be due to CNS effects (Lu et al., 2011; Ryan et al., 2011). FAS enzyme activity was decreased in fat but not liver of FASKOF mice (Figure 1C). Hepatic FAS enzyme activity was not significantly increased in the setting of decreased adipose tissue FAS activity (Figure 1C).

Chow-fed FASKOF and control mice weighed the same. However, feeding a high fat diet (HFD) elicited a phenotypic difference. HFD-fed FASKOF mice weighed less (Figure 1D) and had less adiposity as well as more lean tissue compared to controls (Figure 1E). The adiposity effect was seen in both sexes and also in the setting of high carbohydrate/zero fat diet feeding (Table S1). Epididymal fat pads (white adipose tissue,

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WAT), but not other tissues, from HFD-fed FASKOF mice weighed less than those from control mice (Figure 1F). White adipocytes isolated from FASKOF mice were smaller than adipocytes from control mice with HFD feeding (Figures 1G and 1H), but there was no effect with chow feeding. Genotype and diet had no effect on adipocyte cell number (not shown), perhaps reflecting induction of adiponectin-Cre expression following adipose tissue development.

The weight of brown adipose tissue (BAT) was not different in mice fed HFD. However, when adult mice were fed a high carbohydrate/zero fat diet, which maximizes effects due to FAS deficiency, the BAT depot in FASKOF mice weighed significantly less than that of control mice (Figure S1A). The histologic appearance of BAT was not different between genotypes in mice fed HFD, but lipid stores were depleted (Figure S1B) and the PPAR γ target genes CD36, HSL, and ATGL were decreased (Figure S1C) in the BAT depot from FASKOF mice fed a high carbohydrate/zero fat diet.

Hepatic histologic appearance (Figure S1D) and lipid content (Figure S1E) were not different between control and FASKOF animals.

Altered Thermogenesis in FASKOF Mice

Food intake was not different between FASKOF and control mice on any diet (Table S1). When studied on a HFD prior to development of statistically significant differences in body weight, FASKOF mice had increased energy expenditure compared to controls (Figure 2A). Systemic glucose tolerance and insulin sensitivity were enhanced in HFD-fed FASKOF mice (notable for less adiposity) compared to controls (Figures 2B and 2C), but with chow feeding (a condition associated with similar degrees of adiposity in each genotype) there was no difference in glucose tolerance between FASKOF and control mice (Figure S1F). Consistent with the observation that decreased adiposity improves insulin sensitivity in numerous animal models (Elchebly et al., 1999; Masuzaki et al., 2001; Yang et al., 2005; Yuan et al., 2001), levels of phospho-Akt relative to total Akt were increased in skeletal muscle of HFD-fed FASKOF mice (data not shown). Serum leptin was lower (perhaps reflecting decreased adiposity) but adiponectin was unaffected in HFD-fed FASKOF mice (Table S1). Given effects of FAS deletion on other PPAR γ genes (see below), it is possible that not all targets of PPAR γ , including adiponectin, are affected by FAS deletion. Monitored physical activity was not increased in FASKOF animals (Figure S1G).

Body temperature was not different between control and FASKOF mice at room temperature, there were no apparent brown fat-like adipocytes in the epididymal fat of FASKOF mice, and FASKOF epididymal fat did not have increased expression of the brown fat gene UCP1 (not shown). However, UCP1 expression was strikingly increased in inguinal fat from HFD-fed FASKOF mice as compared to controls (Figure 2D). Expression of PRDM16, a transcriptional coregulator involved in the development of classic BAT as well as brown fat-like adipocytes in subcutaneous white adipose tissue (WAT) (Seale et al., 2011), was also increased as were levels of the brown fat genes Cidea and PGC1 α (Figure 2D). PPAR α is known to induce UCP1 expression (Barbera et al., 2001), and mRNA levels for PPAR α as well as the PPAR α -dependent genes CPT1 and

ACO were increased in inguinal fat (Figure 2D). Since PPAR α promotes fatty acid oxidation, we assayed this process in homogenates of WAT as the release of CO $_2$ from radiolabelled palmitate. In HFD-fed mice, fatty acid oxidation was increased in FASKOF as compared to control mice in inguinal but not epididymal WAT (Figure 2E). To maximize effects due to FAS deficiency, we fed mice a high carbohydrate/zero fat diet and analyzed inguinal fat. Under these conditions, inguinal fat mRNA levels for UCP1, Cidea, and PGC1 α were increased (Figure S1H). UCP1 protein was increased in inguinal fat from FASKOF as compared to control mice by both western blotting (Figure 2F) and immunocytochemistry (Figure 2G). With cold exposure, FASKOF mice maintained their body temperature at a significantly higher level than control mice (Figure 2H), suggesting that increased brown fat-like cells in subcutaneous WAT of FASKOF are physiologically relevant.

FAS Promotes PPAR γ Activation and Adipogenesis

PPAR γ is necessary and sufficient for adipogenesis (Tontonoz and Spiegelman, 2008) but also mediates HFD-induced hypertrophy of adipocytes (Hosooka et al., 2008; Kubota et al., 1999). Moreover, PPAR γ is thought to promote fat development at the expense of myogenesis (Hu et al., 1995; Seale et al., 2008). Previous studies suggested that lipogenic pathways may be required for activating PPAR γ by generating its endogenous ligand (Kim and Spiegelman, 1996; Kim et al., 1998) and influencing adipogenesis (Schmid et al., 2005).

Since HFD-fed FASKOF mice have decreased adiposity and reduced adipocyte hypertrophy (Figure 1), we explored the possibility that FAS is involved in PPAR γ activation and adipogenesis using mouse embryonic fibroblasts (MEFs) from FAS^{lox/lox} animals. Expression of Cre using an adenovirus (Ad-Cre) in these cells decreased FAS protein and impaired adipogenesis (Figures 3A and 3B). Defective adipogenesis induced by FAS deficiency was rescued by treatment with the PPAR γ activator rosiglitazone (Figure 3B, bottom panels), likely due to induction of processes (involving CD36, LPL, and other proteins) that facilitate uptake of lipids from the culture media.

We next transfected HEK293 cells with cDNAs for PPAR γ and a PPAR-dependent luciferase reporter in the presence or absence of FAS knockdown. FAS deficiency decreased luciferase reporter activity, an effect that was rescued with rosiglitazone, suggesting that FAS regulates PPAR γ transcriptional activity (Figure 3C). To address possible contributions of ligand-independent effects of FAS knockdown on PPAR γ transactivation, we performed luciferase reporter assays using cells transfected with constitutively active PPAR γ (VP16-PPAR γ) or wild-type PPAR γ . FAS knockdown reduced luciferase reporter activity in cells transfected with WT PPAR γ , and the effect was significantly greater than in cells transfected with VP16-PPAR γ (Figure 3D). Knockdown of FAS in primary MEFs decreased expression of the PPAR γ target genes aP2 and CD36 but increased expression of the early myogenesis markers MyoD and myogenin, effects that were reversed with rosiglitazone (Figure 3E). Consistent with induction of myogenic markers, FAS inactivation was associated with myotube formation under promyogenic culture conditions (Figure 3F). FAS knockdown decreased levels of proteins regulated by PPAR γ in 3T3-L1 adipocytes (Figure 3G). PPAR γ target gene expression was

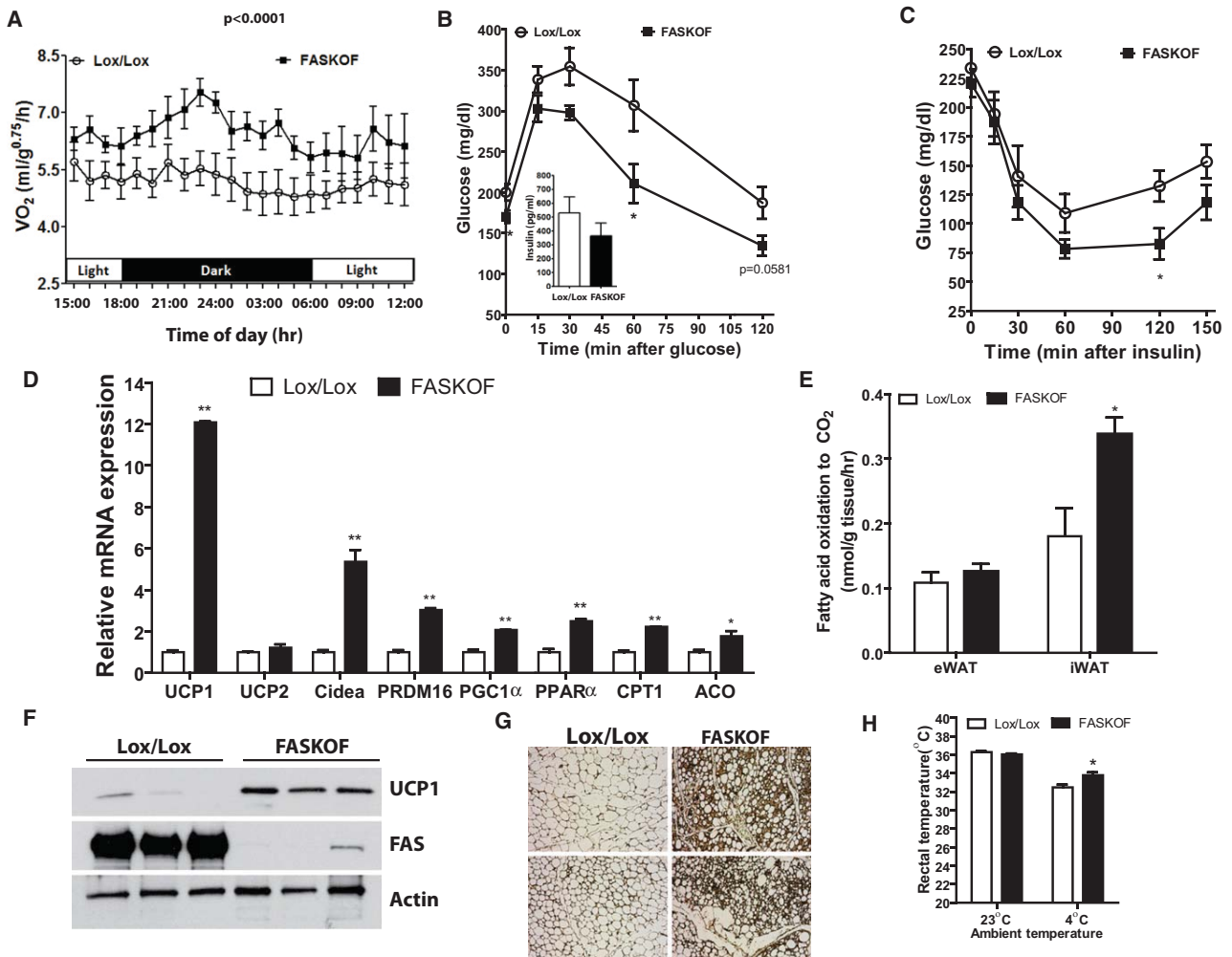


Figure 2. Altered Metabolism in Mice with Adipose-Specific Knockout of FAS

(A) Oxygen consumption (VO₂) by indirect calorimetry in HFD-fed mice. Indicated p value by ANOVA. N = 8–10/genotype.
 (B) Glucose tolerance testing in HFD-fed mice. $p = 0.0477$ at 0 min, 0.0415 at 60 min. N = 6–8/genotype. Serum insulin values at 30 min point shown in the inset.
 (C) Insulin tolerance testing in the mice of (B). * $p = 0.039$.
 (D) RT-PCR analysis of gene expression in inguinal WAT of HFD-fed control and FASKOF male mice. Gene expression analysis in inguinal WAT of ZFD-fed mice is presented in Figure S1H. ** $p < 0.0001$ for UCP1, 0.0017 for Cidea, 0.0001 for PRDM16, 0.0008 for PGC1 α , 0.0012 for PPAR α , and 0.0001 for CPT1; * $p = 0.042$ for ACO.
 (E) Measurement of fatty acid oxidation in epididymal (eWAT) and inguinal (iWAT) fat of control and FASKOF mice fed HFD. * $p = 0.0355$ for HFD iWAT. N = 3 animals/genotype for each diet.
 (F) Western blot analysis in inguinal WAT of ZFD-fed control and FASKOF male mice. Each lane represents a separate mouse.
 (G) Immunocytochemical analysis of UCP1 expression in inguinal WAT of ZFD-fed control and FASKOF mice. Images are from two separate mice per genotype.
 (H) Rectal temperature of ZFD-fed control and FASKOF mice at room temperature (23°C) and after 1 hr exposure to 4°C. N = 6–8 animals/genotype. * $p = 0.011$. Error bars in (A)–(E) and (H) represent SEM.

restored in these murine cells with FAS knockdown by expressing human FAS (Figure 3H). PPAR γ target genes were also decreased in the adipose tissue of FASKOF mice (Figure 3I). To determine if FAS deficiency is affecting PPAR γ expression as opposed to its transcriptional activity, we fed mice a high carbohydrate/zero fat diet to maximize effects due to FAS deficiency and subjected gonadal WAT to western blotting. There was no effect on PPAR γ protein mass while protein levels of the PPAR γ target aP2 were decreased in FASKOF as compared to control mice (Figure 3J). One plausible interpretation of these

results is that FAS is part of a lipogenic pathway that regulates adipogenesis at the expense of myogenesis by generating endogenous ligands for PPAR γ that promote its transcriptional activity.

Identification of FAS-Dependent Diacyl and Alkyl Ether Lipid Species Bound to PPAR γ

There are probably numerous PPAR γ endogenous ligands that may be generated under conditions requiring alterations in adipocyte function, but ligands are initially produced early during

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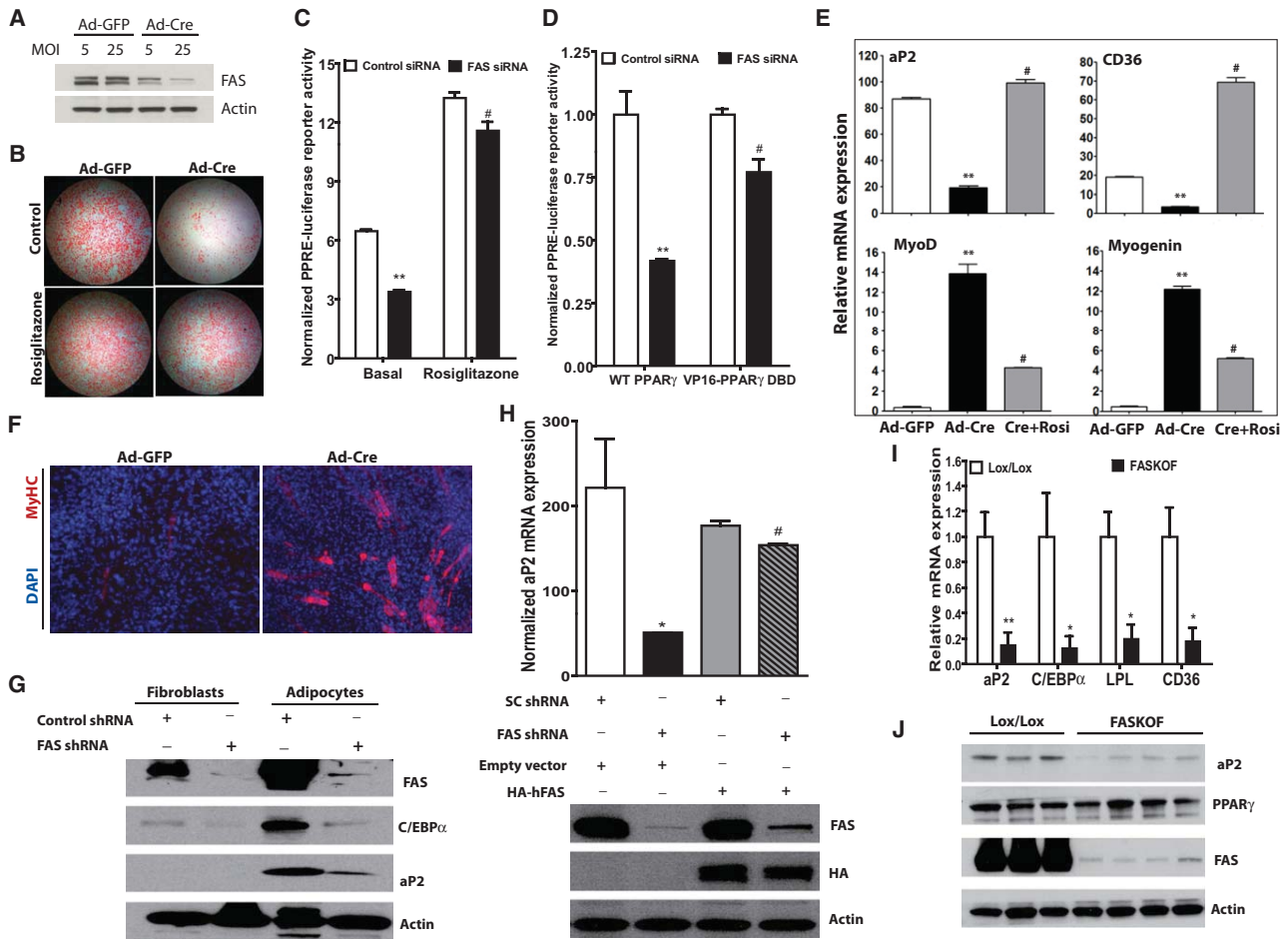


Figure 3. FAS Is Required for Adipogenesis and PPAR γ Activation

(A) Western blot analysis of FAS knockdown in primary MEFs from FAS^{lox/lox} mice treated with an adenovirus expressing GFP or Cre at the indicated multiplicity of infection (moi).
 (B) Oil red O staining of FAS^{lox/lox} MEFs treated with Ad-GFP or Ad-Cre and differentiated to adipocytes in the presence or absence of rosiglitazone.
 (C) HEK293 cells treated with control or FAS siRNA were transfected with plasmids encoding PPRE-luciferase, *Renilla* luciferase and wild-type PPAR γ in the presence or absence of rosiglitazone. **p < 0.0001 versus control, #p < 0.0001 versus FAS siRNA basal. N = 3/condition.
 (D) HEK293 cells treated with control or FAS siRNA were transfected with plasmids encoding PPRE-luciferase, *Renilla* luciferase and wild-type PPAR γ or VP16-PPAR γ DBD (an N-terminal fragment of PPAR γ encompassing the DNA binding domain fused to the VP16 transactivation domain). **p < 0.0001 versus control, #p < 0.0001 versus FAS siRNA/WT PPAR γ . N = 3/condition.
 (E) RT-PCR analysis of gene expression in FAS-deficient (Ad-Cre-treated) or control (Ad-GFP-treated) MEFs subjected to the adipogenesis protocol. **versus Ad-GFP, p = 0.0060 for aP2, 0.0010 for CD36, 0.0051 for MyoD, 0.0007 for Myogenin. #versus Ad-Cre, p = 0.0015 for aP2, 0.0013 for CD36, 0.0099 for MyoD, 0.0019 for Myogenin.
 (F) FAS-deficient (Ad-Cre-treated) or control (Ad-GFP-treated) MEFs cultured to promote myogenesis and stained with a skeletal muscle myosin heavy chain antibody.
 (G) Detection of proteins induced by PPAR γ in 3T3-L1 fibroblasts and adipocytes treated with control or FAS shRNA.
 (H) Restoration of PPAR γ target gene expression with human FAS using 3T3-L1 adipocytes with endogenous knockdown of FAS. 3T3-L1 cells stably expressing retrovirally encoded human FAS were infected with a lentivirus expressing scrambled control (SC) or mouse FAS shRNA. The cells were induced to differentiate into adipocytes. The upper panel shows real-time PCR analysis of aP2 expression and the bottom panel shows a western blot with antibodies against FAS, HA, and actin. *p = 0.0224 (versus SC shRNA, empty vector). #p < 0.0001 (versus FAS shRNA, empty vector).
 (I) RT-PCR analysis of gene expression in control and FASKOF gonadal WAT. **p = 0.007. *p = 0.0493 for C/EBP α , 0.010 for LPL, 0.039 for CD36. N = 4/genotype.
 (J) Western blot analysis in gonadal WAT of ZFD-fed control and FASKOF female mice. Each lane represents a separate mouse. Error bars in (C)–(E), (H), and (I) represent SEM.

adipocyte differentiation (Tzamelis et al., 2004). To isolate such putative FAS-dependent ligands, we used mass spectrometry after infecting MEFs with an adenovirus encoding FLAG-tagged PPAR γ and inducing differentiation (Figure 4A). PPAR γ was iso-

lated by affinity from cells in the presence or absence of FAS knockdown (Figure 4B) and associated lipids were analyzed by mass spectrometry (Figure 4C). We identified several phosphatidylcholine species with diacyl (ester bond-linked) or 1-O-alkyl

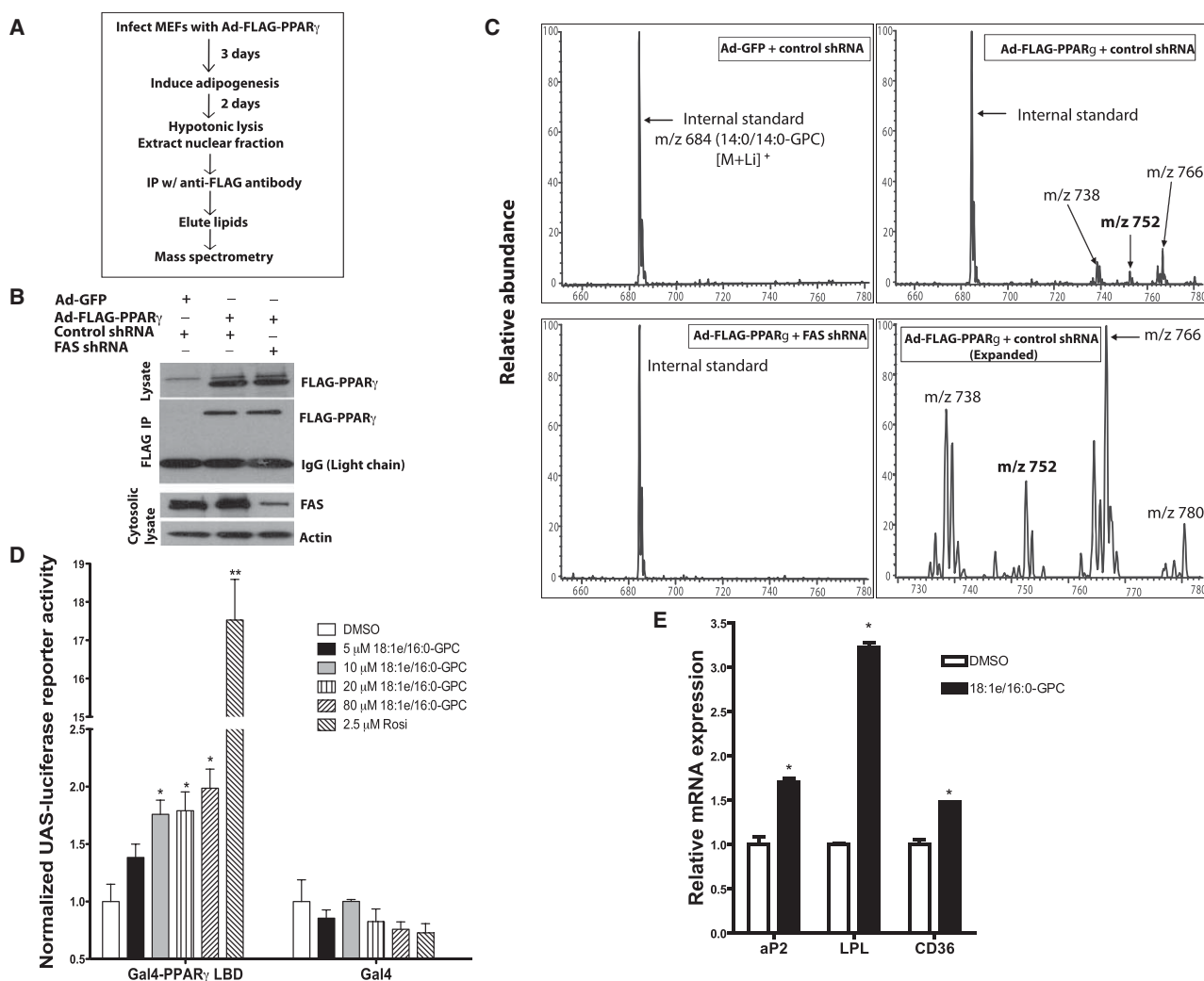


Figure 4. Isolation of FAS-Dependent Diacyl and 1-O-alkyl Ether Phosphatidylcholine Species Associated with PPAR γ

(A) Strategy for detection of PPAR γ -associated lipids.

(B) Detection of FLAG-PPAR γ protein immunoprecipitated from adipocytes treated with control or FAS shRNA.

(C) Mass spectrometric analyses of [M+Li]⁺ ions of glycerophosphocholine (GPC) lipids bound to FLAG-PPAR γ or control protein (GFP) immunoprecipitated from control and FAS knockdown adipocytes. Ions of *m/z* 752 and 780 represent 1-O-alkyl GPC species.

(D) CV-1 cells were transfected with plasmids encoding UAS-luciferase, *Renilla* luciferase and Gal4-PPAR γ LBD (a C-terminal fragment of PPAR γ encompassing the ligand binding domain fused to the Gal4 DNA binding domain) or Gal4 alone. The cells were treated with 18:1e/16:0-GPC (corresponding to *m/z* 752 in C), rosiglitazone, or DMSO. After 48 hr, UAS-luciferase reporter activity was measured and normalized to *Renilla* luciferase reporter activity. ***p* = 0.0001. **p* = 0.018 (10 μ M), 0.024 (20 μ M), 0.019 (80 μ M).

(E) 3T3-L1 cells were induced to differentiate in DMEM+10% FBS with supplemental dexamethasone, insulin and IBMX in the presence of 20 μ M 18:1e/16:0-GPC or DMSO. After 3 days, the cells were retreated with the GPC in media containing supplemental insulin alone. The next day, the cells were harvested for RNA extraction and real-time PCR analysis. The data are representative of 3 separate experiments. **p* = 0.0147 (aP2), 0.0006 (LPL), 0.0102 (CD36). Error bars in (D) and (E) represent SEM.

(ether bond-linked) side chains associated with PPAR γ that were competitively displaced by rosiglitazone (not shown). Alkyl ether lipids were particularly enriched in PPAR γ samples compared to controls (Table S2). The species at *m/z* 752 [M+Li]⁺ was most frequently associated with PPAR γ and tandem mass spectrometry identified it as 1-O-octadecenyl-2-palmitoyl-3-glycerophosphocholine (18:1e/16:0-GPC) (Figure S2). We synthesized this alkyl ether lipid and used it to treat cultured cells. 18:1e/16:0-GPC increased PPAR γ -dependent luciferase reporter activity

in a dose-dependent fashion (Figure 4D) but was less potent than rosiglitazone. We found that 20 μ M 18:1e/16:0-GPC significantly increased the expression of PPAR γ target genes in differentiating 3T3-L1 adipocytes (Figure 4E).

In order to provide insight into the interaction between 18:1e/16:0-GPC and the PPAR γ ligand binding domain (LBD), we developed a GST-pulldown assay of PPAR γ ligand binding based on the ligand-dependent interaction between an N-terminal region of CBP1 and the PPAR γ LBD (Gelman et al.,

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1999). 18:1e/16:0-GPC increased the interaction between the GST-tagged PPAR γ LBD and the myc-tagged CBP1 N terminus in a dose-dependent manner (Figure S3A). However, 18:1e/16:0-GPC did not increase the interaction between the GST-tagged LBD of a different nuclear receptor, PPAR α , and the myc-tagged CBP1 N terminus (Figure S3B). To provide additional evidence that this ether lipid enhances PPAR γ transcription due to agonism, we added 18:1e/16:0-GPC to terminally differentiated 3T3-L1 adipocytes. Both 18:1e/16:0-GPC and rosiglitazone increased LPL gene expression in differentiated adipocytes that were treated with a control shRNA prior to induction of differentiation (Figure S3C). In cells prevented from differentiating into adipocytes by FAS knockdown, treatment with either 18:1e/16:0-GPC or rosiglitazone after completion of the differentiation protocol (with dexamethasone, IBMX, and insulin followed by additional insulin treatment) did not restore full LPL expression (Figure S3C). FAS deficiency decreased expression of PPAR γ -dependent genes (Figure 3I) while increasing expression of PPAR α -dependent genes (Figure 2D). When FAS expression was knocked down in 3T3-L1 cells that were subsequently induced to differentiate into adipocytes, the FAS-deficiency-associated increase in ACO gene expression was significantly decreased when cells were differentiated in the presence of the selective PPAR α antagonist GW6471 (Figure S3D). These results suggest that an FAS-dependent ether lipid interacts with PPAR γ but not PPAR α and that FAS deficiency is associated with decreased activation of PPAR γ and increased activation of PPAR α .

Cloning and Characterization of PexRAP

Ether lipid synthesis in mammals occurs through the peroxisomal acyl dihydroxyacetone phosphate (DHAP) pathway, allowing synthesis of lysophosphatidic acid as an alternative to direct acylation of glycerol 3-phosphate. This pathway is obligatory for synthesis of ether lipids including platelet activating factors and plasmalogens (Hajra and Das, 1996; Hajra et al., 2000; McIntyre et al., 2008) (Figure 5A). The terminal enzyme activity in this pathway, acyl/alkyl DHAP reductase, was purified and characterized from guinea pig liver (LaBelle and Hajra, 1974), but the gene encoding this protein has not been identified in mammals (McIntyre et al., 2008). Since a yeast enzyme (Ayr1p) (Athenstaedt and Daum, 2000) that catalyzes this reaction has been cloned and characterized, we used this sequence to identify DHRS7b, a protein of unknown function, as a mammalian ortholog (Figure 5B). We renamed this protein PexRAP. Gradient fractionation of 3T3-L1 adipocytes showed that PexRAP is enriched in fractions containing peroxisomal markers, such as PMP70 and catalase (Figure 5C). Myc-tagged PexRAP coimmunoprecipitated with Pex19 (peroxisomal biogenesis factor 19, an import receptor for peroxisomal membrane proteins) (Figure 5D), and this interaction was confirmed in pulldown experiments using GST-PexRAP (Figure 5E).

To demonstrate that PexRAP mediates its predicted enzyme activity, we knocked down PexRAP expression in MEFs (Figure 5F) and found decreased levels of 1-O-alkyl ether phospholipids as well as certain diacyl phospholipids (Figure 5G), some of which also arise from the DHAP pathway. 18:1e/16:0-GPC was detected in these experiments as *m/z* 746 [M+H]⁺ since these experiments were performed with protonated species; 18:1e/

16:0-GPC was detected as *m/z* 752 using lithiated species in Figure 4C. PexRAP protein was detected in multiple tissues, but levels were low in skeletal muscle (Figure 5H). The overall expression of PexRAP in BAT was relatively low and BAT primarily expressed a shorter isoform (which lacks 9 amino acid residues at the N terminus), suggesting that PexRAP may have a different role in BAT compared to WAT. Both PexRAP and FAS proteins markedly increase early during differentiation of 3T3-L1 adipocytes, prior to similar increases in proteins such as C/EBP α and aP2 known to be induced by PPAR γ activation (Figure 5I). Thus, PexRAP is peroxisomal, its inactivation decreases lipids associated with PPAR γ , and its temporal relationship during differentiation with other adipocyte proteins suggests that it could be involved in adipogenesis.

PexRAP Is Required for Adipogenesis

To address the role of PexRAP in adipogenesis, we knocked down its expression in 3T3-L1 cells. Adipogenesis (assessed by both Nile red staining and triglyceride content) was abrogated with PexRAP knockdown and rescued with rosiglitazone (Figures 6A and 6B), suggesting that PexRAP, like FAS (Figure 3B), affects PPAR γ activation. Knockdown of PexRAP or DHAP acyltransferase (DHAPAT, the enzyme immediately upstream of PexRAP, Figure 5A) in 3T3-L1 adipocytes decreased expression of PPAR γ target genes (Figure 6C). Rosiglitazone treatment rescued the effect of PexRAP or DHAPAT knockdown on PPAR γ target genes (Figure 6D).

PexRAP Knockdown in Mice Alters Body Composition and Metabolism

We translated these observations to HFD-fed C57BL/6J mice, characterized by increased adiposity and insulin resistance. A series of PexRAP antisense oligonucleotides (ASOs) were screened for effectiveness (not shown) and results of PexRAP knockdown for two of the most promising are shown in Figure 7A using Hepa1-6 cells. ASO2 was selected for use in mice. Intraperitoneal administration of ASO2 at up to 20 mg/kg twice a week for three weeks resulted in a dose-dependent decrease in PexRAP protein in WAT and liver (but not in brain or skeletal muscle, Figures 7B and S4A). Mice were fed a HFD for four weeks to increase adiposity and then animals were injected twice a week with 20 mg/kg of ASO2 or the control ASO for 24 days while HFD was continued. ASO treatment had no effect on liver function tests or liver histology (not shown). Liver fat content was nearly significantly lower ($p = 0.072$) with ASO treatment. Food intake was unaffected (Table S3). However, this intervention decreased expression of PexRAP as well as PPAR γ target genes (including PPAR γ itself) in WAT (Figure 7C). PexRAP knockdown in HFD-fed mice also decreased adiposity, increased leanness, and decreased fasting glucose (Figure 7D and Table S3). Glucose tolerance was improved and insulin levels were lower in HFD-fed mice treated with the PexRAP ASO (Figures 7E and 7F).

DISCUSSION

These studies suggest that depletion of FAS in adipose tissue suppresses HFD-induced obesity. FAS is a minor contributor to cellular lipid stores with high fat feeding (Aarsland et al.,

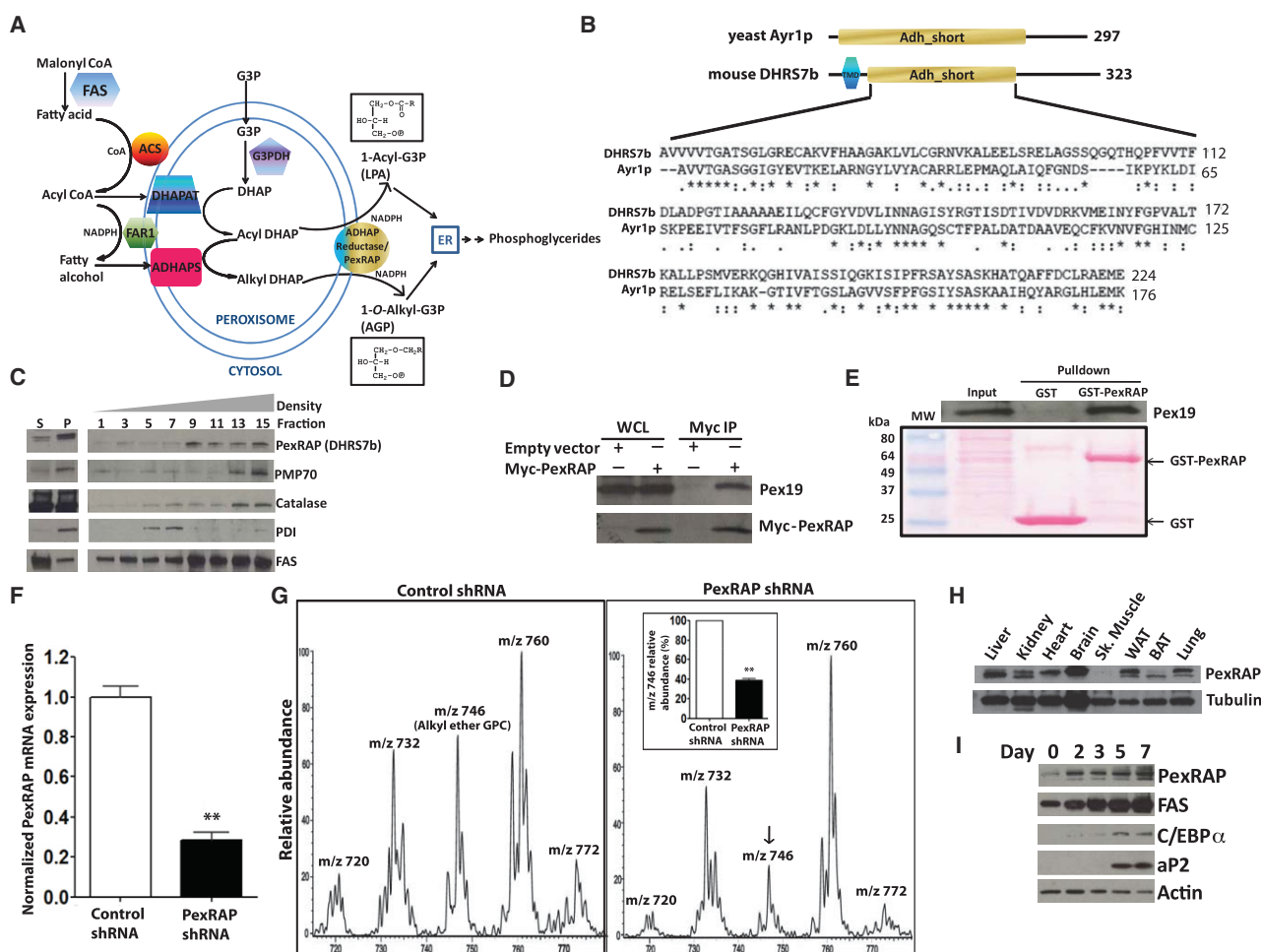


Figure 5. Cloning and Characterization of the Terminal Component in the Mammalian Peroxisomal Ether Lipid Synthetic Pathway

(A) The peroxisomal acyl-DHAP pathway of lipid synthesis. FAS, fatty acid synthase; ACS, acyl CoA synthase; G3PDH, glycerol 3-phosphate dehydrogenase; DHAP, dihydroxyacetone phosphate; DHAPAT, DHAP acyltransferase; FAR1, fatty acyl CoA reductase 1; ADHAPS, alkyl DHAP synthase; ADHAP Reductase, acyl/alkyl DHAP reductase activity; LPA, lysophosphatidic acid; AGP, 1-O-alkyl glycerol 3-phosphate.
 (B) Mouse Dhrs7b is homologous to yeast acyl DHAP reductase, Ayr1p. TMD, transmembrane domain; Adh_short, short chain dehydrogenase/reductase domain.
 (C) PexRAP (peroxisomal reductase activating PPAR γ , detected using anti-Dhrs7b antibody) is enriched in peroxisomal fractions isolated from 3T3-L1 adipocytes. S, supernatant; P, pellet after sedimentation.
 (D) Pex19 coimmunoprecipitates with Myc-tagged PexRAP. WCL, whole cell lysates.
 (E) Pex19 interacts with PexRAP in GST pull-down experiments using 3T3-L1 adipocytes.
 (F) RT-PCR analysis of PexRAP expression with PexRAP knockdown in MEFs. **p = 0.0084.
 (G) Mass spectrometric analyses of [M+H]⁺ ions of GPC lipids in MEFs after PexRAP knockdown. Quantification of the 1-O-alkyl ether GPC lipid peak at m/z 746 [M+H]⁺ (identical to the lithium adduct at m/z 752 in Figure 4C) is shown in the inset. **p = 0.0009.
 (H) Mouse tissue distribution of PexRAP protein by western blotting.
 (I) Protein abundances of PexRAP and FAS increase prior to increases in C/EBP α and aP2 during differentiation of 3T3-L1 adipocytes. Error bars in (F) and (G) (inset) represent SEM.

1996; Letexier et al., 2003; McDevitt et al., 2001); HFD feeding decreases FAS expression (Coupe et al., 1990; Kersten, 2001). Thus, it is unlikely that the reduced adiposity observed in the HFD-fed FASKOF mice was due to the inability to synthesize fatty acids per se. Rather, our results suggest that inhibiting a lipogenic pathway initiated by FAS increases thermogenesis and reduces activation of PPAR γ . Increased energy expenditure comes not from effects on classic BAT but instead by inducing

the formation of brown fat-like (“brite”) cells in subcutaneous adipose tissue (Seale et al., 2011).

In addition to inducing brown fat-like cells in subcutaneous fat, FAS deletion decreased PPAR γ transcriptional activity. It is possible that these transcriptional effects are unrelated or only partially related to the phenotype of resistance to diet-induced obesity caused by increased brite cells. PPAR γ agonism can induce UCP1 gene expression and produce small adipocytes in

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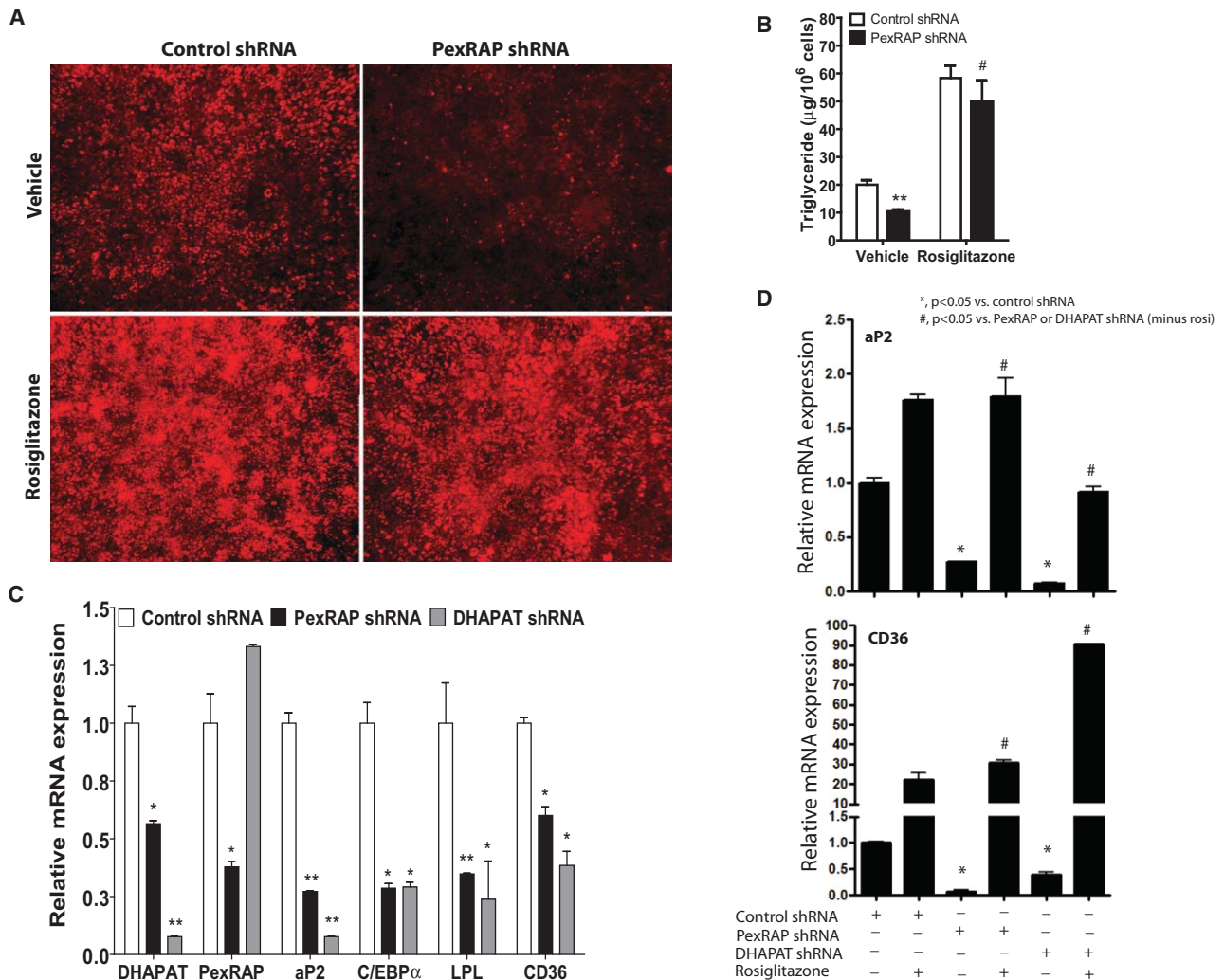


Figure 6. PexRAP Is Required for Adipogenesis and PPAR γ Activation

(A) Nile red staining of 3T3-L1 adipocytes treated with control or PexRAP shRNA in the presence or absence of rosiglitazone. (B) Triglyceride content for the cells of (A). **p = 0.0066 versus control, #p = 0.0071 versus PexRAP shRNA vehicle. N = 3/condition. (C) RT-PCR analysis of gene expression following PexRAP or DHAPAT knockdown. P versus control: DHAPAT, *0.0278, **0.007; PexRAP, *0.040; aP2, **0.0060 for PexRAP shRNA and 0.0058 for DHAPAT shRNA; C/EBP α , *0.0160 for PexRAP shRNA and 0.0165 for DHAPAT shRNA; LPL, **0.0014, *0.0450; CD36, *0.0113 for PexRAP shRNA and 0.0132 for DHAPAT shRNA. N = 3–5/condition. (D) Rosiglitazone treatment rescues the effect of PexRAP or DHAPAT knockdown on PPAR γ target gene expression. 3T3-L1 cells infected with lentivirus expressing control, PexRAP, or DHAPAT shRNA were induced to differentiate into adipocytes and then treated with 2.5 μM rosiglitazone. Expression of PPAR γ target genes was analyzed by quantitative RT-PCR. For aP2, exact p values from left to right = 0.0038, 0.0119, 0.0024, 0.0032. For CD36, p values = 0.0022, 0.0015, 0.0110, < 0.0001. Error bars in (B)–(D) represent SEM.

WAT (de Souza et al., 2001; Fukui et al., 2000; Tiraby et al., 2003), similar to the FASKOF mouse phenotype, and yet FASKOF mice have decreased PPAR γ activation. However, pharmacologic agonism of PPAR γ promotes adiposity, while FASKOF mice have less adiposity. Effects on PPAR α with FAS deletion could provide a plausible explanation. PPAR α and its targets are induced in FASKOF adipose tissue (Figure 2D). PPAR α inhibition decreases induction of the PPAR α target gene ACO with FAS knockdown in 3T3-L1 cells (Figure S3D). PPAR α agonism can also induce UCP1 gene expression (Barbera et al., 2001) as

well as decrease adipocyte size (Tsuchida et al., 2005), and the acute effects of PPAR α activation on UCP1 gene expression may exceed those of PPAR γ (Pedraza et al., 2001). Decreased PPAR γ transcriptional activity could reflect the lack of a FAS-associated lipid ligand, allowing increased PPAR α activity and induction of UCP1. In support of this notion, induction of UCP1 and the development of brown fat-like cells by FNDC5 (cleaved to form irisin) occurs in part through PPAR α (Boström et al., 2012).

Our data point to a pathway (Figure 7G, left) in which lipids synthesized by FAS serve as substrate for PexRAP, which

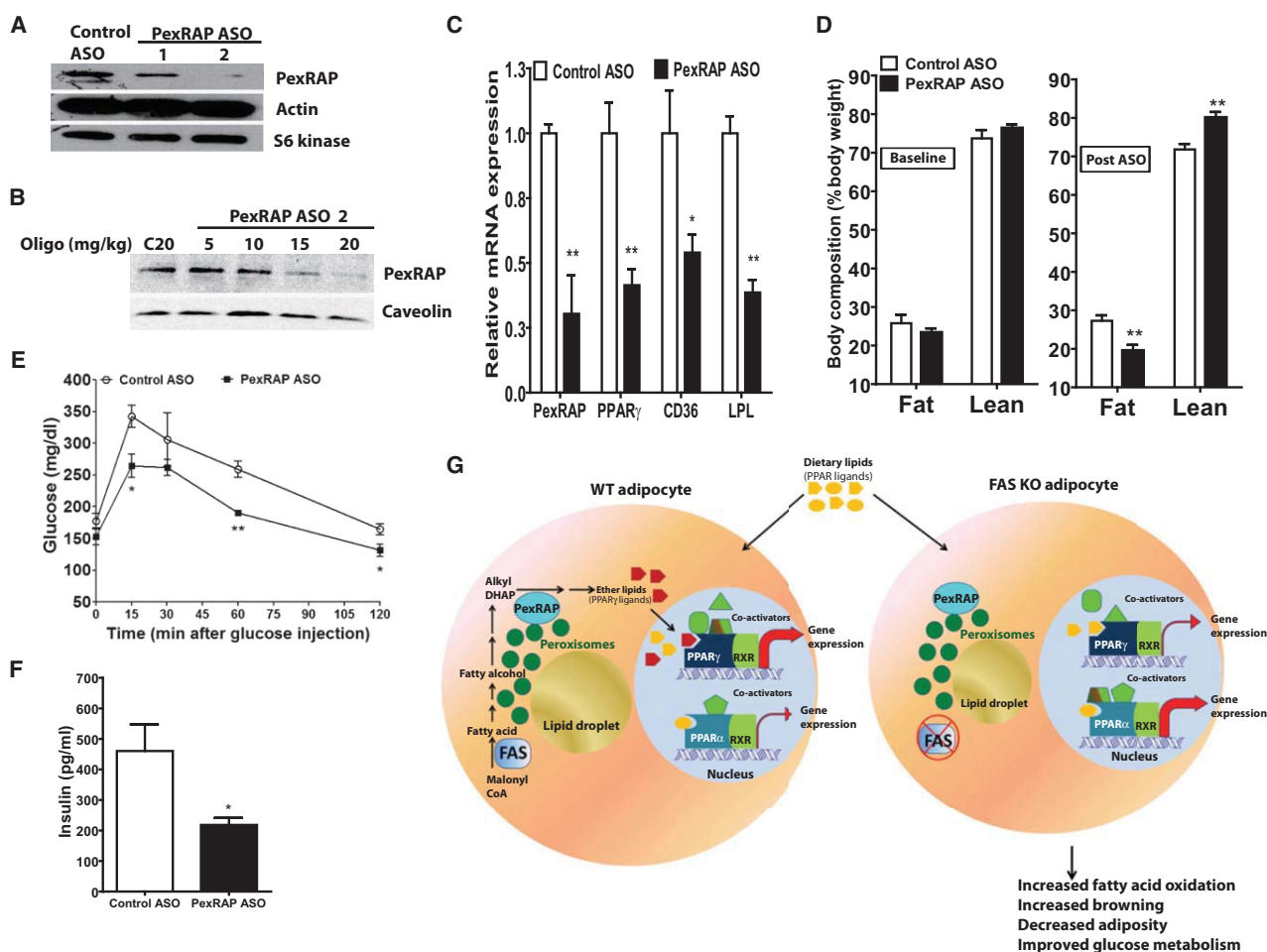


Figure 7. Knockdown of PexRAP in Mice Alters Body Composition and Metabolism

(A) PexRAP knockdown using antisense oligonucleotides (ASOs) in Hepa1-6 cells.
 (B) Western blot analysis using epididymal WAT of C57BL/6J mice treated intraperitoneally with the indicated doses of control or PexRAP ASO twice a week for 3 weeks.
 (C) RT-PCR analysis of epididymal WAT expression following ASO treatment. P versus control: PexRAP **0.0078; PPAR γ **0.0051; CD36 *0.0420; LPL **0.0030. N = 4/condition.
 (D) Body composition by MRI following 4 weeks of HFD feeding (baseline) and after 3.5 weeks of ASO treatment while still eating a HFD. **p = 0.0098 for fat, 0.0071 for lean. N = 4/condition.
 (E) Glucose tolerance testing in HFD-fed mice following ASO treatment. *p = 0.0220 at 15 min and 0.0434 at 120 min. **p = 0.0019.
 (F) Insulin levels at the 30 min point from (E). *p = 0.0363.
 (G) Models of PPAR γ and PPAR α gene expression in WT and FAS-deficient adipocytes. Error bars in (C)–(F) represent SEM.

generates alkyl ether lipids that are potential endogenous PPAR γ ligands. Disruption of FAS (Figure 7G, right) decreases these ether lipids, altering the coactivator milieu to favor PPAR α -dependent gene expression.

Several lines of evidence support the concept that a lipogenic pathway localized to peroxisomes is important for endogenous activation of PPAR γ . The PPAR family was named because of the ability to be activated by agents that increase the number of peroxisomes (Dreyer et al., 1992; Issemann and Green, 1990). The number of peroxisomes is dramatically increased during differentiation of 3T3-L1 adipocytes (Novikoff and Novikoff, 1982; Novikoff et al., 1980), a PPAR γ -dependent process. Consistent with our observation that PexRAP expression is

induced during adipogenesis, previous studies suggest that the activities of various enzymes in the peroxisomal ether lipid synthetic pathway increase during differentiation of 3T3-L1 adipocytes (Hajra et al., 2000).

There is precedent for PPAR γ activation by alkyl ether lipids. Azelaoyl PAF (1-O-hexadecyl-2-O-(9-carboxyoctanoyl)-sn-glycerol-3-phosphocholine), reported to be equipotent to rosiglitazone (Davies et al., 2001), and 1-O-alkyl glycerol 3-phosphate (McIntyre et al., 2003; Tsukahara et al., 2006), synthesized directly by PexRAP (see Figure 5A), are thought to be PPAR γ ligands. Because PPAR γ has a capacious ligand binding pocket (Schupp and Lazar, 2010), it is possible that instead of a single authentic endogenous ligand, multiple lipids are recruited to

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the receptor depending on the physiological context, with variable effects on transcriptional activity (Lodhi et al., 2011; Schupp and Lazar, 2010).

ASO-mediated inhibition of PexRAP decreased adiposity and improved glucose metabolism, probably by activating thermogenesis in subcutaneous WAT. In studies to be reported elsewhere, we have observed that PexRAP deficiency in adipose tissue achieved by crossing adiponectin-Cre mice with floxed PexRAP animals robustly induces UCP1 expression in subcutaneous but not epididymal WAT.

Adipose-specific knockout of PPAR γ in mice has yielded conflicting results: one group reported lipodystrophy and insulin resistance (He et al., 2003), while another found enhanced insulin sensitivity (Jones et al., 2005). Certain human PPAR γ mutations cause lipodystrophy and insulin resistance, likely through a dominant-negative effect to disrupt interaction with coactivators (Agostini et al., 2006). In our studies, neither ASO-mediated PexRAP knockdown in mice nor Cre-mediated adipose-specific FAS knockout in mice produced lipodystrophy. Both decreased adiposity and improved glucose metabolism. PPAR γ haploinsufficiency in mice (Kubota et al., 1999; Miles et al., 2000) also decreases adiposity and increases insulin sensitivity, but this genetic effect is not limited to adipose tissue. A Pro12Ala PPAR γ mutation in mice (Heikkinen et al., 2009) and humans (Huguenin and Rosa, 2010) decreases (but does not abolish) PPAR γ transcriptional activity and results in decreased adiposity and increased insulin sensitivity, although this mutation is not adipose-specific.

Inhibiting FAS or the peroxisomal enzyme PexRAP in adipose tissue alters body composition and improves glucose metabolism in the setting of a high fat diet. Both represent attractive targets for novel diabetes and obesity therapies.

EXPERIMENTAL PROCEDURES

Animals

Mice with a floxed FAS locus (FAS^{lox/lox}) (Chakravarthy et al., 2005) were crossed with transgenic mice (a gift from Evan Rosen, BI Deaconess) expressing Cre recombinase under the control of the adiponectin promoter (Eguchi et al., 2011) to obtain FASKOF mice that were studied after backcrossing ≥ 7 times with pure C57BL/6J mice. Genotyping was performed using previously described primer sets and diets included Purina 4043 control chow, Harlan Teklad TD 88137 high fat diet, and Harlan Teklad TD03314 high carbohydrate/zero fat diet. Unless indicated otherwise, male FASKOF mice and control littermates at 8–12 weeks of age were used for experiments. For antisense oligonucleotide studies, 8-week-old C57BL/6J mice were used. All protocols were approved by the Washington University Animal Studies Committee.

Cell Culture

Primary mouse embryonic fibroblasts (MEFs) were isolated at 13.5 days post conception from FAS^{lox/lox} embryos as previously described (Razani et al., 2001) and maintained in DMEM+10% FBS. MEFs were differentiated to adipocytes by treatment with 1 μ M dexamethasone, 5 μ g/ml insulin and 0.5 mM IBMX for 14 days, followed by supplemental 5 μ g/ml insulin alone for an additional 4 days. 3T3-L1 cells were maintained in DMEM+10% NCS and differentiated to adipocytes as previously described (Lodhi et al., 2007). CV-1, HEK293 and HEK293T cells were maintained in DMEM+10% FBS.

Lentiviral shRNA-Mediated Knockdown

Plasmids encoding shRNA for mouse FAS (TRCN0000075703), PexRAP (TRCN0000181732 and 0000198546), and DHAPAT (TRCN0000193539) were obtained from Open Biosystems (Huntsville, AL). Packaging vector

psPAX2 (12260), envelope vector pMD2.G (12259), and scrambled shRNA plasmid (1864) were obtained from Addgene. 293T cells in 10 cm dishes were transfected using Fugene 6 with 2.66 μ g psPAX2, 0.75 μ g pMD2.G, and 3 μ g shRNA plasmid. After 48 hr, media were collected, filtered using 0.45 μ m syringe filters, and used to treat cells. After 36 hr, cells were selected with puromycin and knockdown was assessed after an additional 48 hr.

Identification of Alkyl Ether GPC Lipids Associated with PPAR γ

The strategy for detecting endogenous lipids associated with PPAR γ involved adenovirus-mediated expression of FLAG-tagged PPAR γ or GFP (as control) in cells induced to differentiate into adipocytes. Nuclear fractions, prepared from cell lysates and subjected to hypotonic lysis as described (Chakravarthy et al., 2009), were incubated with an antibody recognizing the FLAG epitope to capture the PPAR γ construct and any associated lipids under conditions (no detergent or high salt elution buffers) unlikely to disrupt potential ligand/nuclear factor interaction.

Affinity matrix eluates (with equal protein content) of nuclear fractions from cells treated with Ad-GFP (as a control) or Ad-PPAR γ were subjected to lipid extraction. These extracts were mixed with an internal standard [(14:0/14:0)-GPC] and analyzed as [M+Li]⁺, [M+H]⁺, or [M+Na]⁺ ions by positive ion ESI/MS or as [M+CH₃CO₂]⁻ ions by negative ion ESI/MS (Hsu and Turk, 2007; Hsu et al., 2003). To determine the identity of the lithiated lipid species of m/z 752, we performed multigenerational tandem MS on a linear ion trap instrument. Collisionally activated dissociation (CAD) was employed to deduce structures of R₁ and R₂ substituents. Additional details are provided in Figure S3.

GST-PexRAP Pull-Down Assays

3T3-L1 adipocytes were lysed using a buffer containing 50 mM HEPES (pH 7.4), 4 mM EDTA, 2 mM EGTA, 2 mM sodium pyrophosphate, 1% Triton X-100, 10 mM NaF, and protease inhibitors. Lysates were mixed with an equal volume of the same buffer lacking Triton X-100, then 5 μ g of GST or GST-PexRAP was added and samples were rocked at 4°C. After 2 hr, samples were centrifuged at 2500 \times g for 1 min, beads were washed 5 times with 1 ml of the lysis buffer containing Triton X-100, then 2X SDS-PAGE sample buffer was added and samples were subjected to SDS-PAGE.

Antisense Oligonucleotides

ASOs were synthesized by TriLink Biotechnologies (San Diego, CA). The first 5 and last 5 nucleotides were substituted with 2' O-methyl RNA bases; all of the bases had phosphorothioate linkages. The PexRAP ASO (RNA bases underlined) is: 5' GGUUGGTGTGTCTGTCCUCG 3'. The control oligonucleotide is: 5' CCUUCCTGAAGGTTCCUCC 3'. Both were purified by anion exchange HPLC, lyophilized, reconstituted with 0.9% normal saline, and then injected intraperitoneally.

Statistical Analysis

Data are expressed as mean \pm SEM. Comparisons between two groups were performed using an unpaired, two-tailed t test. ANOVA was used for more than two groups and post testing was performed using Tukey's post test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at <http://dx.doi.org/10.1016/j.cmet.2012.06.013>.

ACKNOWLEDGMENTS

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Appendix C

Jensen-Urstad APL, Song H, Lodhi IJ, Funai K, Yin L, Coleman T, Semenkovich CF.

“Nutrient-dependent phosphorylation channels lipid synthesis to regulate PPAR α .” *Journal of Lipid Research*. 2013. 54(7):1848-59.

Nutrient-dependent phosphorylation channels lipid synthesis to regulate PPAR α

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Abstract Peroxisome proliferator-activated receptor (PPAR) α is a nuclear receptor that coordinates liver metabolism during fasting. Fatty acid synthase (FAS) is an enzyme that stores excess calories as fat during feeding, but it also activates hepatic PPAR α by promoting synthesis of an endogenous ligand. Here we show that the mechanism underlying this paradoxical relationship involves the differential regulation of FAS in at least two distinct subcellular pools: cytoplasmic and membrane-associated. In mouse liver and cultured hepatoma cells, the ratio of cytoplasmic to membrane FAS-specific activity was increased with fasting, indicating higher cytoplasmic FAS activity under conditions associated with PPAR α activation. This effect was due to a nutrient-dependent and compartment-selective covalent modification of FAS. Cytoplasmic FAS was preferentially phosphorylated during feeding or insulin treatment at Thr-1029 and Thr-1033, which flank a dehydratase domain catalytic residue. Mutating these sites to alanines promoted PPAR α target gene expression. Rapamycin-induced inhibition of mammalian/mechanistic target of rapamycin complex 1 (mTORC1), a mediator of the feeding/insulin signal to induce lipogenesis, reduced FAS phosphorylation, increased cytoplasmic FAS enzyme activity, and increased PPAR α target gene expression. Rapamycin-mediated induction of the same gene was abrogated with FAS knockdown. These findings suggest that hepatic FAS channels lipid synthesis through specific subcellular compartments that allow differential gene expression based on nutritional status.—Jensen-Urstad, A. P. L., H. Song, I. J. Lodhi, K. Funai, L. Yin, T. Coleman, and C. F. Semenkovich. **Nutrient-dependent phosphorylation channels lipid synthesis to regulate PPAR α .** *J. Lipid Res.* 2013. 54: 1848–1859.

Supplementary key words de novo lipogenesis • starvation • peroxisome proliferator-activated receptor α

Peroxisome proliferator-activated receptor (PPAR) α , one of three known members of a nuclear receptor family

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targeted to treat lipid disorders, diabetes, and obesity, is highly expressed in the liver. Its induction by fasting promotes lipid uptake, fatty acid β -oxidation, ketogenesis, and gluconeogenesis (1, 2). Ligand binding to PPAR α causes it to heterodimerize with retinoid X receptor (RXR) α , allowing activation of gene transcription at peroxisome proliferator response elements (PPRE) (3, 4). Synthetic PPAR α ligands, such as fibrates, used for human lipid disorders (5) have been known for decades, but potential endogenous ligands were identified only recently (6, 7). Mice with liver-specific deletion of the lipogenic enzyme fatty acid synthase (FAS) have impaired PPAR α activity (8), and FAS activates PPAR α by producing an endogenous phospholipid ligand (6). FAS also activates PPAR α in brain and macrophages (9, 10).

Mammalian FAS synthesizes long-chain fatty acids, primarily palmitate, through the activities of seven functional domains: acyl carrier, acyl transferase, β -ketoacyl synthase, β -ketoacyl reductase, β -hydroxyacyl dehydratase, enoyl reductase, and thioesterase (11). Like PPAR α , FAS is highly expressed in liver (12). In times of nutrient excess, hepatic FAS converts carbohydrate to lipid that is stored in lipid droplets or secreted in the form of VLDL (13). Nutrient excess is associated with elevated levels of insulin, known to induce FAS expression.

These accepted physiological roles for PPAR α and FAS appear to conflict with the observation that inactivation of FAS impairs PPAR α activation. How might FAS activate a process stimulated by feeding such as insulin-responsive lipogenesis and also activate a process stimulated by fasting such as the induction of PPAR α -dependent gene expression?

We hypothesized that distinct subcellular pools of FAS mediate these disparate effects. Compartmentalization would permit regulation of an FAS pool generating lipids for signaling that would be distinct from an FAS pool generating lipids for energy storage. In support of this hypothesis,

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; mTORC1, mammalian/mechanistic target of rapamycin complex 1; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor.

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we demonstrate that FAS at two separate subcellular locations is differentially regulated by nutrients and insulin, that this regulation involves preferential dehydratase domain phosphorylation for the FAS pool that regulates PPAR α , and that the effects of the kinase mammalian/mechanistic target of rapamycin complex 1 (mTORC1) on PPAR α activity require FAS.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice at eight weeks of age were provided ad libitum access to chow diet (Purina #5053) or fasted for 18 h. All mice were kept on Aspen bedding and had free access to water. Protocols were approved by the Washington University Animal Studies Committee.

FAS enzyme activity assay

Using a modification of a previously described assay (14), 20 μ l of sample at 1 μ g protein/ μ l was added to 70 μ l of assay buffer [0.14 M potassium phosphate buffer (pH 7.0), 1.4 mM EDTA (pH 8.0), 1.4 mM DTT, 0.24 mM NADPH, 0.1 mM acetyl-CoA]. The rate of NADPH oxidation was monitored at 340 nm at baseline and again after adding 10 μ l of 0.85 mg/ml malonyl-CoA (Sigma). The substrate-dependent rate was determined by subtracting the baseline NADPH oxidation rate from the rate after addition of malonyl-CoA. The rate of NADPH oxidation was normalized to FAS protein levels as determined by western blotting and densitometry to determine specific activity.

Subcellular fractionation

Perfused liver from C57BL/6J mice was homogenized in 20 mM HEPES buffer (pH 7.4) and centrifuged at 100 *g* for 30 min, and then the pellet was discarded. The supernatant was centrifuged at 500 *g* for 60 min; 1,200 *g* for 20 min; 10,000 *g* for 20 min; 20,000 *g* for 30 min; 40,000 *g* for 30 min; 70,000 *g* for 30 min; 100,000 *g* for 60 min; and 179,000 *g* for 75 min. After each spin, the pellet was washed and resuspended, while the supernatant was centrifuged again. All spins were done at 4°C. To obtain crude membrane and cytoplasmic fractions from mouse liver, freshly isolated perfused liver was homogenized in HEPES buffer and centrifuged at 10,000 *g* for 45 min at 4°C. The resulting pellet was discarded, and the supernatant centrifuged at 179,000 *g* for 180 min at 4°C. The supernatant (cytoplasm) and pellet (crude membrane) were collected, and the pellet was washed and resuspended in HEPES buffer. To obtain membrane and cytoplasmic extracts from Hepa1-6 cells, a Subcellular Protein Fractionation Kit for Cultured Cells (78840) from Thermo Fisher Scientific was used according to the manufacturer's protocol.

Antibodies

Rabbit polyclonal antibodies against FAS (ab22759), PMP70 (ab3421), and phosphothreonine (ab9337) were from Abcam. Mouse monoclonal antibody against α -tubulin (sc-5286) and rabbit polyclonal antibodies against Cav1 (sc-894) and β -tubulin (sc-9104, used to control for loading in western blotting experiments) were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against PDI (226), GM130 (2296), Na⁺/K⁺ ATPase (3010), Akt (9272), phospho-Akt (S473) (9271), S6 ribosomal protein (2217), and phospho-S6 ribosomal protein (Ser235/236) (2F9/4856), and rabbit monoclonal antibodies against p70 S6 kinase (2708) and CoxIV (4850) were from Cell Signaling Technology.

FAS solubility

Solubility assays were performed as previously described (15) with minor modifications. Membranes were isolated from mouse liver by ultracentrifugation and resuspended in buffer containing 20 mM HEPES buffer (pH 7.4), 1 mM EDTA, and 255 mM sucrose. The membrane fraction was subjected to treatment with various solvents (1 M NaCl, 0.1 M Na₂CO₃ at pH 11.5, 1% SDS or 1% Triton X-100) and then centrifuged once more (4°C, 180,000 *g*, 30 min). The resulting pellets and supernatants were analyzed by western blotting.

Cell culture

Hepa1-6 and Hek293T cells were maintained in DMEM + 10% FBS. Prior to insulin treatment for FAS activity assays, Hepa1-6 cells were cultured in DMEM + 0.5% FBS for 6 h. All insulin treatments were performed in DMEM + 10% FBS.

Pulse-chase study

Confluent Hepa1-6 cells in 6 cm dishes were incubated in methionine-free media for 30 min. The cells were then pulsed with 500 μ Ci of ³⁵S-methionine per dish. After 1 h, cells for the "0" time point were harvested. For subsequent time points, cells were washed with PBS, chased with nonradioactive complete media, and incubated for an additional 45, 90, or 180 min before harvesting. Cells were fractionated into cytoplasm and membrane as described above. FAS was immunoprecipitated from each fraction, samples were subjected to SDS-PAGE, the gel was transferred onto PVDF membrane, and the bands corresponding to labeled FAS were visualized by autoradiography. Autoradiograms were then analyzed by densitometry.

RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) and reverse transcribed using an iScriptTM cDNA synthesis kit (Invitrogen). Semiquantitative RT-PCR was performed using SYBR[®] Green reagent (Applied Biosystems) with an ABI Prism 7700 PCR instrument.

Mutagenesis and plasmid construction

A retroviral plasmid, pBabe-Puro, containing human FAS (16) generated by Max Loda (Dana Farber) was utilized to generate FAS phosphosite mutants. A 3.4 kb fragment of FAS/pBabe-Puro, including the two putative phosphorylation sites (hFAS S1028 and T1032) and two flanking BsrGI sites, was amplified by PCR and subcloned into an intermediate Topo vector. Site-directed mutagenesis of the Topo-FAS plasmid changed the codons corresponding to S1028 and T1032 to alanines, yielding two single mutants. The S1028A/T1032A double mutant was made by sequential mutagenesis, using the S1028A mutant as a template. Mutated FAS fragments were then excised and cloned back into pBabe-Puro using the two BsrGI sites to generate mutant, full-length FAS cDNAs. Mutations as well as correct orientation of the reinserted FAS fragments were verified by DNA sequencing.

Green fluorescent protein (GFP)-tagged FAS was generated by amplifying the cDNA encoding FAS from pBabe-Puro-FAS by RT-PCR, adding restriction sites for XhoI and EcoRI on the 5' and 3' ends, respectively. The amplified product was cloned into pEGFP-C3 using the XhoI and EcoRI sites, yielding an N-terminal GFP-tagged FAS construct.

Lentiviral shRNA-mediated knockdown and human FAS expression

A plasmid encoding a mouse FAS shRNA (TRCN0000075703) was obtained from Open Biosystems. The packaging vector psPAX2 (12260) and envelope vector pMD2.G (12259) were obtained from Addgene. Hek293T cells at 70% confluence in a 15 cm dish were transfected using Lipofectamine 2000 with 8 μ g

psPAX2, 2.25 μ g pMD2.G, and 9 μ g shRNA. After 48 h, media was collected and filtered through 0.45 μ m syringe filters. Polybrene was added and the media used to treat 50–70% confluent Hepa1-6 cells. After 24 h, the media was aspirated and replaced with media containing retroviral particles encoding human FAS (see below). Forty-eight hours after addition of the retroviral media, cells were selected with puromycin. After another 48 h, cells were harvested and knockdown of mouse FAS as well as expression of human FAS were assessed.

To generate retroviral particles encoding human FAS, Hek293T cells in 10 cm dishes were transfected using Lipofectamine 2000 with 3 μ g FAS plasmid and 3 μ g ψ A helper plasmid. After 48 h, media were collected, filtered using 0.45 μ m syringe filters, then polybrene was added, and the media was used to treat 50–70% confluent Hepa1-6 cells. After 48 h, 2 μ g/ml puromycin was added, and after an additional 48 h, cells were harvested.

In experiments assessing PPAR α target gene expression in cells expressing mutant FAS, the endogenous murine FAS of Hepa1-6 cells was knocked down prior to retroviral expression of human FAS as described above.

PPRE-luciferase reporter assay

Media containing lentiviral particles encoding shRNA for murine FAS and media containing retroviral particles encoding wild-type or S1028A/T1032A double-mutant human FAS were prepared as described above. Seventy percent confluent Hepa1-6 cells in 10 cm dishes were treated with retroviral media for either wild-type or S1028A/T1032A FAS for 24 h, after which the media was aspirated and replaced with lentiviral media. After another 24 h, the media was again aspirated and replaced with fresh media containing puromycin.

After two days of puromycin selection, the media was aspirated, replaced with charcoal-stripped media, and incubated for 1 h. Charcoal-stripped media was also used for subsequent steps. Hepa1-6 cells were transfected with plasmids encoding 3 \times PPRE-luciferase and *Renilla* luciferase by electroporation. The electroporation for each 10 cm dish of cells was done as follows: 5 μ g of PPRE-luciferase plasmid and 5 μ g of *Renilla* luciferase plasmid were added to the bottom of a cuvette. Cells were harvested by trypsinization and spun after adding media. The media was aspirated, and cells were washed once with PBS. The PBS was aspirated, and cells were resuspended in 0.5 ml PBS and transferred to the cuvette followed by electroporation at 360 V and 250 μ F (time constant of 4.5–5 s⁻¹). One milliliter of media was added to the cuvette, cells were transferred to a 15 ml tube, and media containing puromycin was added up to 6 ml. Cells were allowed to recover for 10 min, then plated.

One day following transfection, cells were harvested by scraping, washed with room-temperature PBS three times, resuspended in PBS, and plated on a 96-well plate. Luminescence from firefly luciferase and *Renilla* luciferase was then measured using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. PPRE-luciferase activity was calculated as the ratio of firefly luciferase to *Renilla* luciferase luminescence.

Mass spectrometry

To identify posttranslational modifications in hepatic FAS, perfused C57BL/6J mouse livers were homogenized in lysis buffer containing 1% Triton X-100. The lysate was spun at 10,000 g for 45 min, and the pellet was discarded. FAS was immunoprecipitated from 10 mg of the lysate by overnight incubation using a polyclonal rabbit anti-FAS antibody. IP beads were washed, boiled in sample buffer, and subjected to SDS-PAGE. The gel was stained with Coomassie, the gel segment corresponding to FAS

was excised and further cut into small pieces (1 mm²), destained with 50% CH₃CN containing 25 mM NH₄HCO₃, dehydrated, reduced with 20 mM DTT for 1 h at 55°C, washed and dehydrated, alkylated with 100 mM iodoacetamide for 1 h in the dark at room temperature, then subjected to cycles of washing and dehydration followed by drying in a centrifugal evaporator. In-gel digestion was performed with 0.02 mg/ml trypsin overnight or 0.02 mg/ml chymotrypsin for 6 h at 37°C. Peptides were extracted from the gel pieces using 5% TFA in 50% CH₃CN and reconstituted in 0.1% FA in 3% CH₃CN.

Immobilized metal ion affinity chromatography (IMAC) was used to enrich the sample for phosphopeptides. The sample was incubated with IMAC beads for 1 h at room temperature. Peptides were eluted from the beads in IMAC buffer, and the sample was diluted with 0.1% FA in 3% CH₃CN. Samples were then analyzed by LC-MS/MS with a NanoLC-LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) in data-dependent mode. Acquired spectra were searched against Swiss-Prot database through Mascot server to identify the protein and its posttranslational modifications. Nonenriched samples were also run to allow a universal search for protein modifications as well as to search for acetyl modifications.

To identify FAS modifications specific to membrane-associated FAS and cytoplasmic FAS, membrane and cytoplasmic fractions were isolated from C57BL/6J mice as described above. FAS was immunoprecipitated from equal amounts of membrane and cytoplasmic lysates (1–10 mg/each) by overnight incubation using a polyclonal rabbit anti-FAS antibody. The samples were then subjected to SDS-PAGE and analyzed as described above.

Statistics

Data are presented as mean \pm standard error of the mean. Comparisons between two groups were performed using an unpaired, two-tailed *t*-test. ANOVA was used for comparisons involving more than two groups.

RESULTS

Hepatic FAS is present in subcellular compartments

FAS synthesizes palmitate, and FAS deficiency in liver decreases PPAR α target genes. If the effect of FAS deficiency on PPAR α simply reflects palmitate availability, then exogenous palmitate should rescue the effect. It did not. Treatment of Hepa1-6 cells with 50 μ M palmitate failed to rescue expression of the PPAR α target gene ACO following FAS knockdown (Fig. 1A). Higher concentrations of palmitate (125–500 μ M) were toxic (data not shown).

Since the FAS knockdown effect was not rescued with exogenous palmitate, it is plausible that not only the product of the FAS reaction but also the location of its synthesis mediates downstream effects. Dogma holds that FAS is a cytoplasmic enzyme. To determine whether FAS is also present at other sites, we fractionated mouse liver FAS by ultracentrifugation (Fig. 1B). FAS cofractionated with the cytoplasmic marker S6K but also with markers for several organelles. Immunofluorescent staining for FAS in murine Hepa1-6 liver cells demonstrated colocalization of FAS with endoplasmic reticulum (ER) and Golgi markers

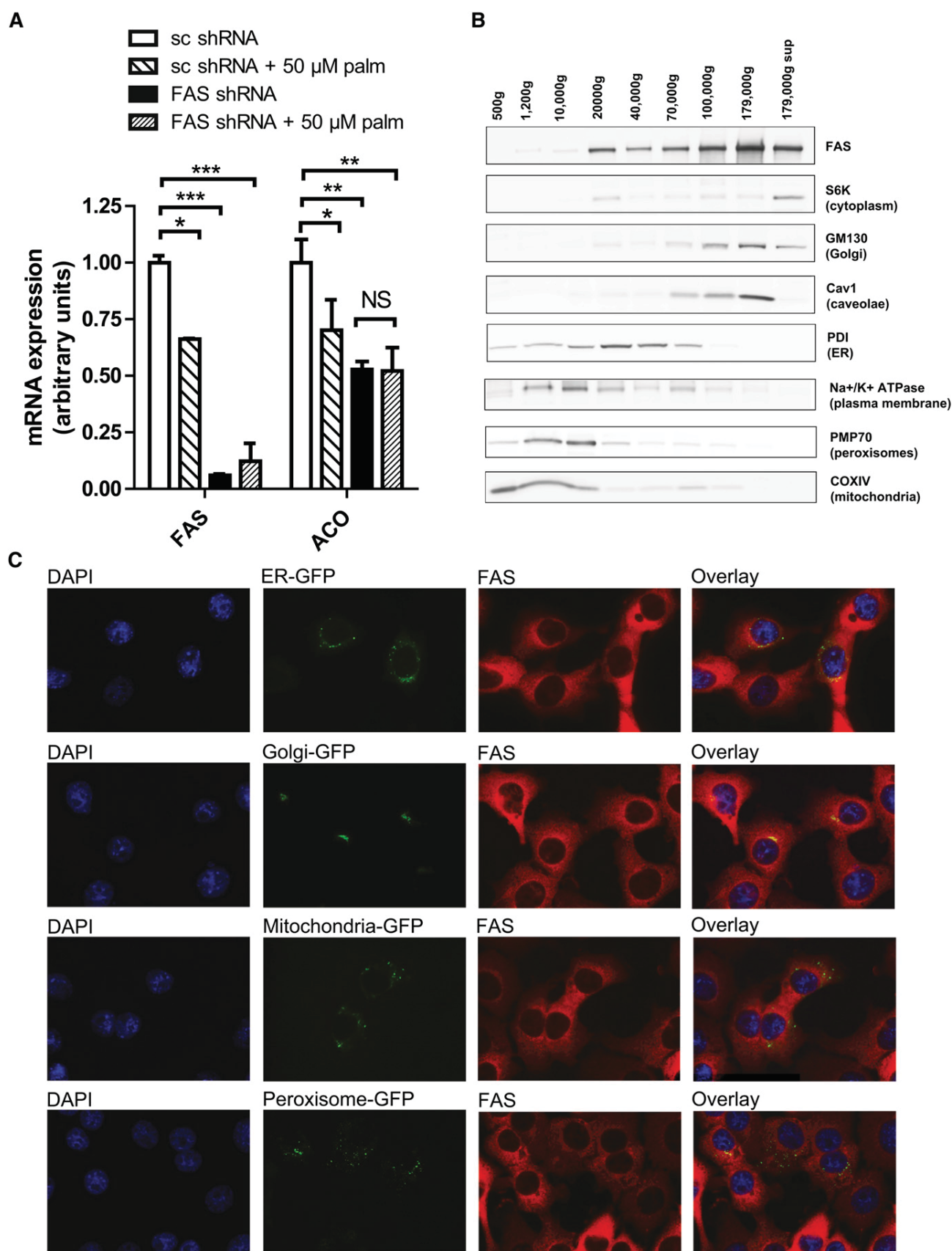


Fig. 1. Hepatic FAS is not exclusively cytoplasmic. (A) Expression levels of FAS (left) and the PPAR α -dependent gene ACO (right) in Hepal-6 cells. Cells were treated with a control (scrambled, sc) shRNA or an FAS shRNA in the presence of exogenous BSA-conjugated palmitate or vehicle (BSA alone) for 8 h. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.0005$. NS, not significant. (B) Subcellular distribution of FAS

but not peroxisomal or mitochondrial markers (Fig. 1C). FAS did not appear in the nucleus (Fig. 1C).

Membrane-associated and cytoplasmic FAS are differentially regulated

FAS is induced by insulin and nutrients (12). Surprisingly, the specific activity of mouse liver cytoplasmic FAS was not increased in the fed state when insulin levels are high (Fig. 2A). Membrane-associated, FAS-specific activity was increased with feeding (Fig. 2B). The cytoplasmic/membrane activity ratio in liver was increased with fasting, when PPAR α is activated (Fig. 2C). In Hepa1-6 cells, a transformed liver cell line, insulin significantly decreased cytoplasmic FAS activity (Fig. 2D), an effect that was not seen in the membrane fraction (Fig. 2E). As with mouse liver, the cytoplasmic/membrane activity ratio in Hepa1-6 cells was increased in the absence of added insulin (Fig. 2F), a mimic of fasting.

To begin to address the possibility that membrane-associated FAS is an artifact of preparation, we treated isolated fractions with different solvents. Membrane-associated FAS resisted solubilization by 1 M NaCl, remaining in the pelleted fraction, but it was largely solubilized by 0.1 M Na₂CO₃ (Fig. 3A). Treatment with detergent (1% SDS or 1% Triton X-100) solubilized most FAS protein (Fig. 3A). These results suggest (17–19) that FAS manifests a strong peripheral membrane interaction.

A pulse-chase study showed that radiolabeled FAS decreased over time in the membrane-associated and cytoplasmic compartments (Fig. 3B), suggesting that there was no ordered flux of protein from one compartment to another over the time course of this experiment. There was no discernible change in the distribution of FAS between membrane and cytoplasm when cells were treated with insulin (Fig. 3C).

Given the presence of a putative open reading frame (with a potential alternative start codon) 5' to the published first exon of both mouse and human FAS, we considered the possibility that compartmentalized FAS represented differential splicing leading to nonidentical protein isoforms, only one of which is membrane-targeted. However, mass spectrometric analysis of FAS in membrane and cytoplasm failed to detect the predicted alternative amino acids at the N-terminus, and it identified the published FAS protein sequence as being N-terminally acetylated (Fig. 3D). This modification, which marks the N-terminus of most eukaryotic proteins (20), was present in membrane and cytoplasmic fractions of FAS, precluding the existence of an additional N-terminal sequence. All regions of the FAS protein were similarly represented in each fraction, decreasing the possibility that compartment location was determined by altered protein

sequence due to a process such as exon exclusion (data not shown).

Collectively, these results suggest that the enzyme activities of cytoplasmic and membrane-associated FAS are differentially regulated, a phenomenon that does not appear to be due to intracellular trafficking of the protein or differences in its primary structure.

Cytoplasmic FAS is preferentially phosphorylated

To address the possibility that differential regulation of cytoplasmic and membrane-associated FAS is caused by a covalent modification, we immunoprecipitated hepatic FAS from fasting and fed mice, and then tested different fractions for the presence of phosphothreonine by western blotting. Cytoplasmic FAS in fed mice was strongly threonine phosphorylated, a modification that was almost undetectable in fasted mice (Fig. 4A). Phosphorylation of membrane-associated FAS was low under both conditions (Fig. 4A). In Hepa1-6 cells, insulin treatment (a mimic of feeding) stimulated threonine phosphorylation of cytoplasmic but not membrane-associated FAS (Fig. 4B).

Analysis of FAS protein from unfractionated mouse liver by mass spectrometry revealed only a single peptide that was threonine phosphorylated. This modification was detected at two residues, Thr-1029 and Thr-1033 (a representative spectrum is shown in Fig. 5A). When liver FAS was separated into cytoplasmic and membrane-associated fractions and subjected to the same analysis, the phosphorylated peptide was found predominantly in the cytoplasm (Fig. 5B) despite similar total amounts of the peptide in both fractions (data not shown). These results suggest that the phosphorylated FAS species detected in the cytoplasm with feeding or insulin (Fig. 4A, B) is modified at Thr-1029 and Thr-1033.

These residues are in the dehydratase domain of FAS. The function of this domain requires two catalytic residues, His-878 and Asp-1032, and a third residue, Gln-1036, that maintains the orientation of the catalytic residues (21). The phosphorylated residues we identified (denoted by * in Fig. 5C) are in close proximity to the catalytic residue D1032 and the structural residue Q1036 (denoted by # in Fig. 5C). Sequence alignment of the dehydratase regions from different species revealed that in addition to strict conservation of the active site residues D1032 and Q1036 (denoted by #), the phosphoresidues we identified are also conserved as either serines or threonines in humans, mice, rats, *D. melanogaster*, and *C. elegans* (boxes in Fig. 5D).

Since the evolutionary conservation of these phosphorylation sites suggests involvement in FAS function, we mutated S1028 and T1032 in human FAS (corresponding to the T1029 and T1033 in murine FAS) to alanines, generating two single mutants (S1028A and T1032A) and one double mutant (S1028A/T1032A) (Fig. 5E, mutated sites

protein in mouse liver by differential centrifugation followed by western blotting. Organelle markers: S6K = P70/S6 kinase (cytoplasmic marker), GM130 = Golgi Matrix protein 130 (Golgi marker), Cav1 = Caveolin1 (caveolae marker), PDI = protein disulfide isomerase (endoplasmic reticulum marker), Na⁺/K⁺ ATPase (plasma membrane marker), PMP70 = peroxisomal membrane protein 70 (peroxisomal marker), and COXIV = cytochrome C oxidase IV (mitochondrial marker). (C) Immunofluorescent staining of FAS and expression of GFP-tagged organelle markers in murine Hepa1-6 cells. Nuclei stained with DAPI are presented on the far left, GFP images are presented second from left, FAS images are presented second from right, and merged GFP/FAS images are presented on the far right.

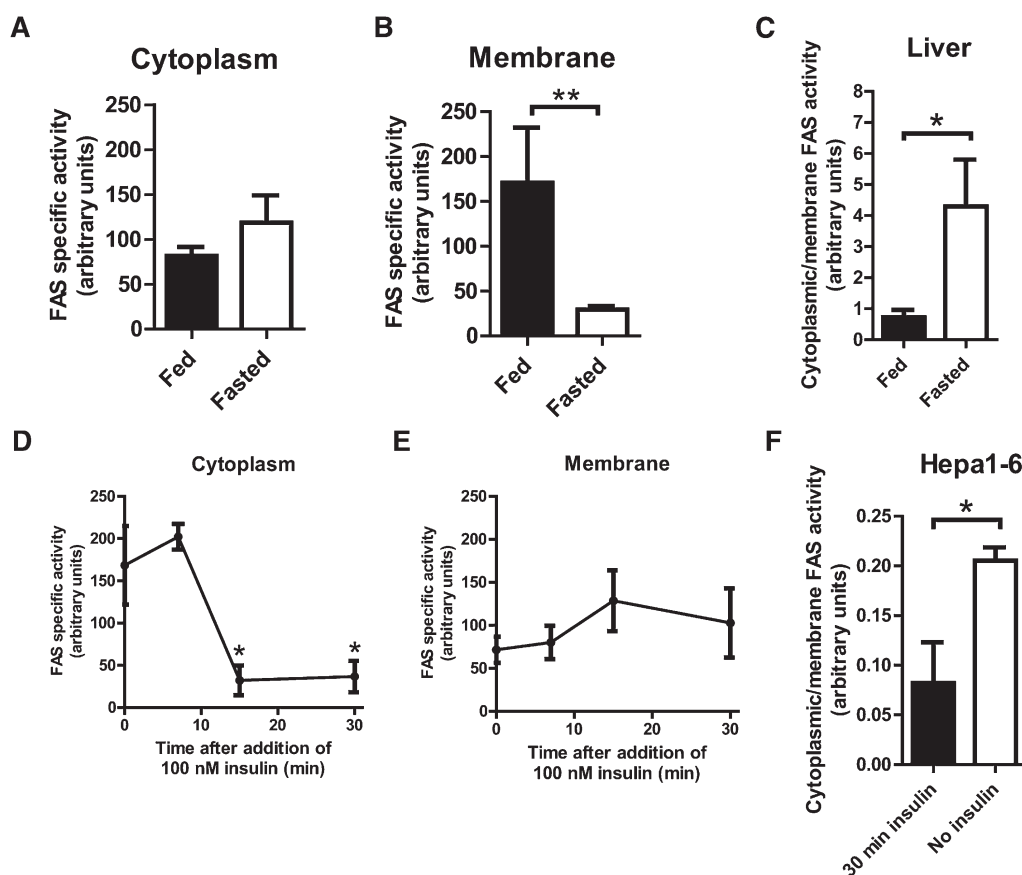


Fig. 2. Differential regulation of the activities of membrane-associated FAS and cytoplasmic FAS. (A) Specific activity of FAS in the cytoplasmic fraction of mouse liver. Mice were fed ad lib (fed) or fasted for 18 h (fasted). Activity was normalized to FAS protein levels as measured by western blotting. $N = 9$ /group. (B) Specific activity of FAS in the membrane (Golgi/ER) fraction of mouse liver. Mice were fed ad lib (fed) or fasted for 18 h (fasted). Activity was normalized to FAS protein levels as measured by western blotting. $N = 5$ /group. $**P \leq 0.005$. (C) FAS-specific activities in (A) and (B) expressed as the ratio of FAS-specific activity in cytoplasm to FAS-specific activity in membrane. $*P \leq 0.05$. (D) Specific activity of FAS in the cytoplasmic fraction of Hepa1-6 cells. Cells were treated with 100 nM insulin for indicated times. Activity was normalized to FAS protein levels as measured by western blotting. $N = 3$ /group. $*P \leq 0.05$. (E) Specific activity of FAS in the membrane (Golgi/ER) fraction of Hepa1-6 cells. Cells were treated with 100 nM insulin for indicated times. Activity was normalized to FAS protein levels as measured by western blotting. $N = 3$ /group. (F) FAS-specific activities in (D) and (E) expressed as the ratio of FAS-specific activity in cytoplasm to FAS-specific activity in membrane. $*P \leq 0.05$.

are indicated by boxes and the active site residues by #). Wild-type or mutant human FAS was then expressed in Hepa1-6 cells following knockdown of endogenous mouse FAS. Compared with cells expressing wild-type human FAS, cells expressing the S1028A mutation had increased levels of the PPAR α target gene CPT1 (Fig. 5F), whereas cells expressing the T1032A mutation did not show changes in PPAR α target genes (Fig. 5G). However, expression of the double-mutant S1028A/T1032A was associated with increased levels of both ACO and CPT1 (Fig. 5H). To implicate PPAR transcriptional activity in this effect, we performed a PPRE-luciferase reporter assay. After expression of wild-type or S1028A/T1032A double-mutant FAS and knockdown of endogenous mouse FAS, cells were transfected with a plasmid encoding three tandem PPREs fused to a firefly luciferase reporter gene. Luciferase activity was increased in cells expressing the S1028A/T1032A double-mutant FAS

compared with wild-type FAS (Fig. 5I), suggesting that effects of the FAS mutant on PPAR α target genes are mediated by PPAR α transcriptional activity. One interpretation of these data is that the inability to phosphorylate FAS disinhibits FAS enzyme activity to promote PPAR α transcription.

mTORC1 phosphorylates and inactivates FAS and inhibits PPAR α activity

mTORC1 was recently identified as a physiologically important negative regulator of hepatic PPAR α (22). mTOR, the kinase component of mTORC1, is a serine/threonine kinase that preferentially phosphorylates sites with hydrophobic residues at the +1 position (23). Since the phosphorylated residues we identified have the highly hydrophobic phenylalanine (F1030) and methionine (M1034) at the +1 positions, we addressed a role for

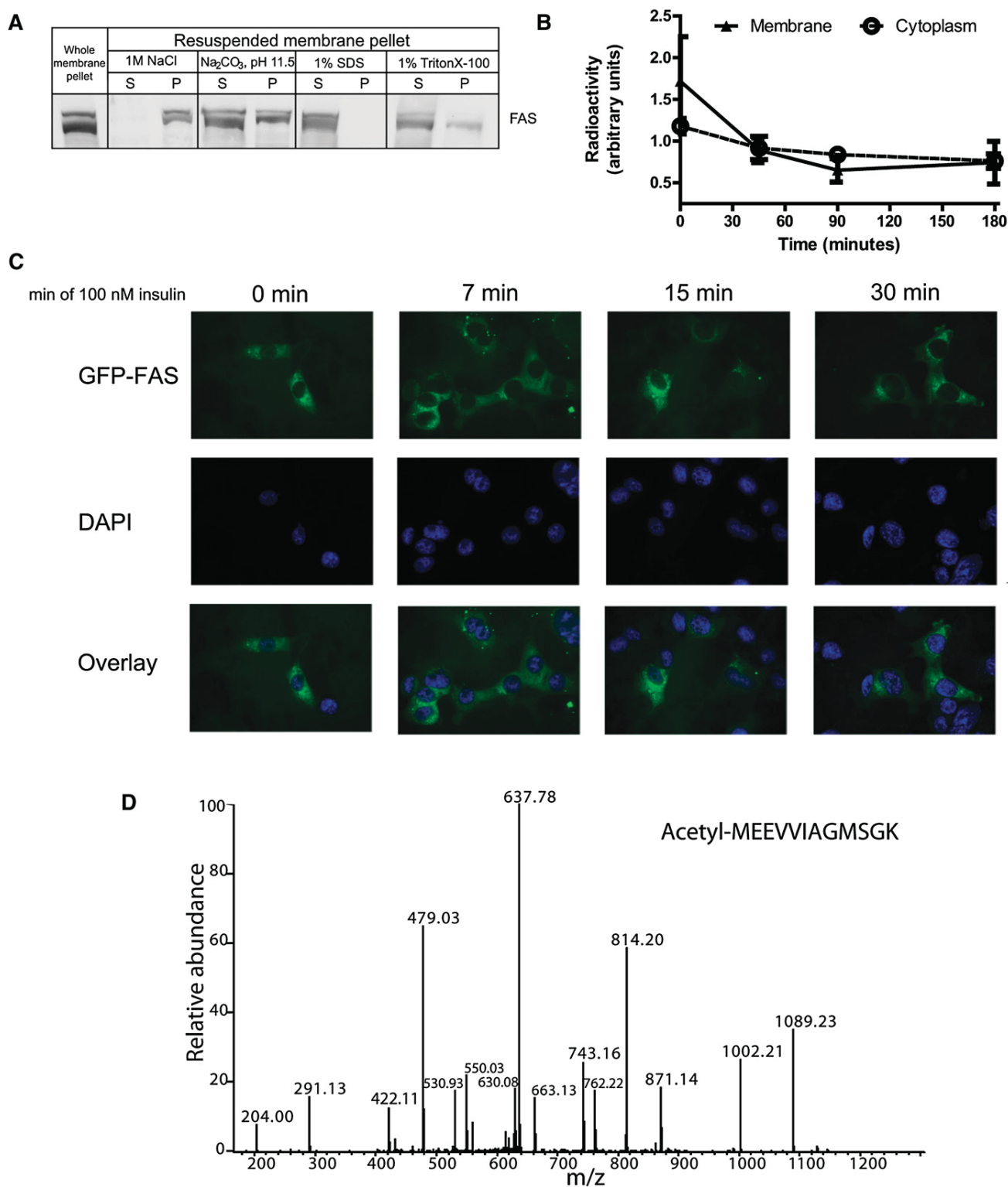


Fig. 3. Distinct characteristics of membrane and cytoplasmic FAS. (A) Detection of FAS protein by western blotting in pellets and supernatants of membrane fractions following high-salt, carbonate, and detergent treatments. Mouse liver homogenate was fractionated by differential centrifugation into cytoplasm (not shown) and membrane pellet (lane 1). The pellet was resuspended, exposed to solvents as indicated, and again centrifuged to separate pellet (P) from the new supernatant (S). (B) Pulse-chase analysis of FAS protein in membrane and cytoplasm of Hepa1-6 cells. Cells were pulsed with ³⁵S-labeled methionine for 1 h, then chased with media containing nonlabeled methionine for the indicated times. (C) Expression of GFP-tagged human FAS in Hepa1-6 cells treated with insulin for the indicated times. Images demonstrate no detectable shifts of FAS between cytoplasmic and membrane sites with insulin treatment. (D) Representative spectrum of N-terminally acetylated peptide of FAS. N-terminal acetylation effectively marks the initial amino acid of the protein, precluding the existence of additional expressed N-terminal exons that might constitute distinct FAS isoforms.

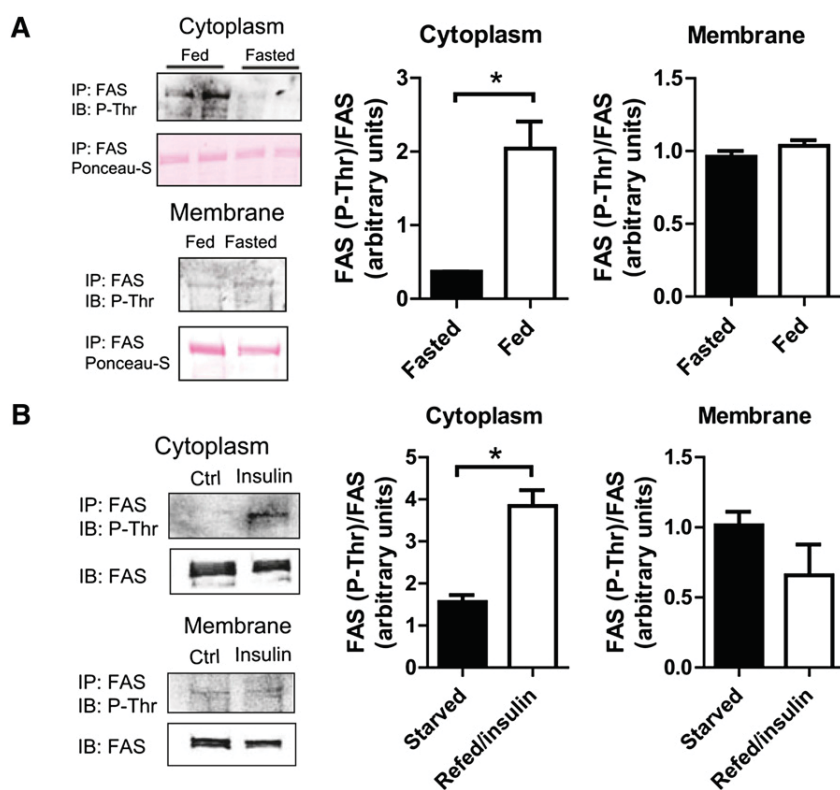


Fig. 4. Cytoplasmic FAS is threonine phosphorylated with feeding or insulin treatment. (A) FAS threonine phosphorylation in response to feeding in mouse liver. FAS was immunoprecipitated from cytoplasmic and membrane fractions and analyzed for phosphothreonine by western blotting. Mice were fed ad lib (fed) or fasted for 18 h (fasted). Representative blots are shown. Data are averages of two independent experiments. $*P \leq 0.05$. (B) FAS threonine phosphorylation in response to insulin in Hepa1-6 cells. FAS was immunoprecipitated from Hepa1-6 cytoplasmic and membrane fractions and analyzed for phosphothreonine by western blotting. Cells were cultured in 0.5% FBS media for 4 h prior to harvest (starved) or in 0.5% FBS media for 4 h, then treated with 1 nM insulin in 10% FBS media for 15 min (refed/insulin). Representative blots are shown. Data are averages of two independent experiments. $*P \leq 0.05$.

mTORC1 in FAS phosphorylation. Treating Hepa1-6 cells with the mTORC1 inhibitor rapamycin for 30 min abolished the insulin-induced increase in cytoplasmic FAS threonine phosphorylation (Fig. 6A) and was associated with an increase in cytoplasmic FAS-specific activity (Fig. 6B). Treatment of these cells with Torin 1 at 250 nM also abolished insulin-induced FAS phosphorylation (data not shown). Treating Hepa1-6 cells with rapamycin for 24 h (a sufficient time to reach a new steady state for mRNA levels) increased expression of the PPAR α target gene CPT1 (Fig. 6C). These findings confirm those made in a different system (22) and extend that work by implicating FAS in the mTORC1-PPAR α axis.

To better define the interaction between mTORC1, FAS, and PPAR α , FAS was knocked down in Hepa1-6 cells followed by rapamycin treatment. FAS knockdown, confirmed in the presence of rapamycin (Fig. 6D), decreased CPT1 expression (Fig. 6E). The induction of CPT1 levels with rapamycin occurring with FAS expression (Fig. 6C) was lost with FAS knockdown (Fig. 6E, solid bar). These results suggest that in this cell line under these conditions, the induction of the PPAR α target gene CPT1 caused by inhibition of mTORC1 is FAS-dependent.

DISCUSSION

FAS synthesizes lipid for energy storage and participates in the generation of a lipid ligand involved in the activation of fatty acid oxidation. Energy storage occurs with feeding, and activation of fatty acid oxidation occurs with fasting. To clarify how the same enzyme mediates both processes, we pursued the possibility that distinct pools of FAS are differentially regulated in the liver.

We found FAS in the cytosol, but we also localized FAS to organelles (Fig. 1) through a strong peripheral membrane interaction (Fig. 3A). FAS-specific activity was relatively higher with feeding/insulin in membranes and relatively higher with fasting in the cytosol (Fig. 2). This effect did not appear to involve movement of FAS between compartments or primary sequence differences between these pools of FAS. Instead, this activity difference was associated with preferential phosphorylation of cytoplasmic (but not membrane) FAS with feeding (Fig. 4) at conserved sites within a catalytic domain (Fig. 5). Mutation of these sites increased endogenous PPAR α target gene expression as well as activity of a PPRE-dependent reporter gene (Fig. 5), consistent with disinhibition of FAS in the

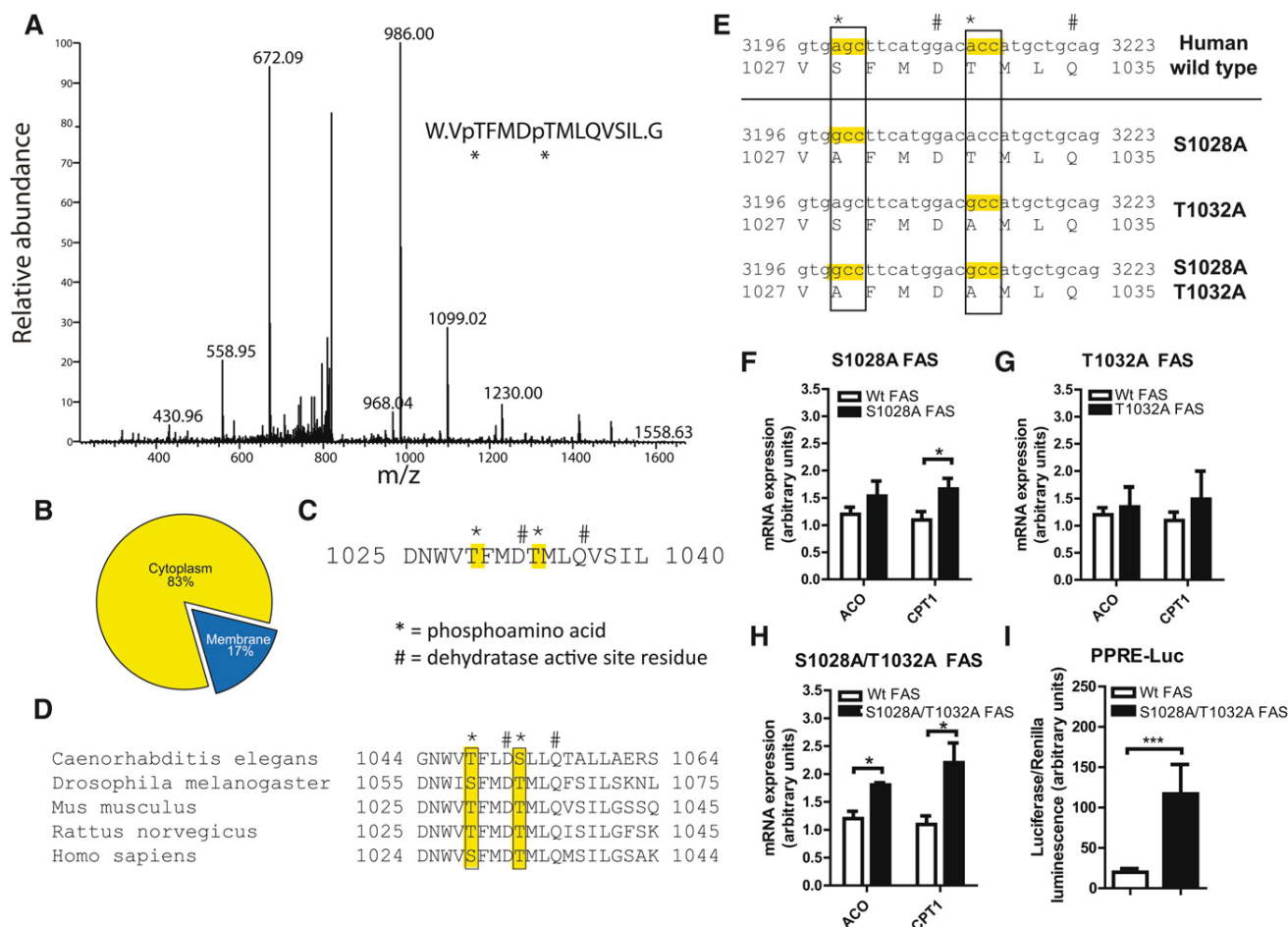


Fig. 5. Phosphorylation of cytoplasmic FAS at the dehydratase domain catalytic site controls downstream PPAR α target gene expression. (A) Representative spectrum of the FAS P-T1029/P-T1033 phosphopeptide from wild-type mouse liver. (B) Distribution of P-T1029/P-T1033 phosphopeptides identified by mass spectrometry in cytoplasm and membrane fractions of mouse liver. Although the proportion of phosphorylation differed based on fraction, peptide abundances (phosphorylated + nonphosphorylated) were similar for the membrane and cytoplasm fractions (not shown). (C) Position of P-T1029 and P-T1033 amino acid residues in relation to the FAS dehydratase domain active site residues. D1032 is one of two dehydratase domain catalytic residues in FAS. (D) Sequence alignment of the FAS putative phosphoamino acids and dehydratase domain active sites in several species. (E) FAS phosphosite mutants in human FAS. (F) RT-PCR analyses of PPAR α target gene expression in Hepa1-6 cells expressing wild-type or S1028A mutant FAS. Endogenous FAS was knocked down using lentiviral shRNA for murine FAS. Wild-type or mutant human FAS was expressed using retroviruses. Data are averages of three independent experiments. * $P \leq 0.05$. (G) RT-PCR analyses of PPAR α target gene expression in Hepa1-6 cells expressing wild-type or T1032A mutant FAS. Assay performed as in (F). Data are averages of three independent experiments. (H) RT-PCR analyses of PPAR α target gene expression in Hepa1-6 cells expressing wild-type or S1028A/T1032A mutant FAS. Assay performed as in (F). Data are averages of three independent experiments. * $P \leq 0.05$. (I) PPRE-luciferase activity in Hepa1-6 cells expressing wild-type or S1028A/T1032A mutant FAS. Wild-type or mutant human FAS was expressed using retroviruses. Endogenous FAS was knocked down using lentiviral shRNA for murine FAS. Cells were cotransfected with plasmids encoding 3 \times PPRE-firefly luciferase and *Renilla* luciferase. PPRE-luciferase activity is reported as the ratio of firefly/*Renilla* luciferase luminescence. $N = 3$ –6/group. *** $P \leq 0.0005$.

absence of phosphorylation. Inhibition of mTORC1 with rapamycin decreased FAS phosphorylation, increased cytosolic FAS enzyme activity, and increased expression of the PPAR α target gene CPT1, an effect that was FAS-dependent (Fig. 6). One interpretation of these findings is that hepatic FAS exists in at least two differentially regulated subcellular pools, cytoplasmic and membrane-associated (Fig. 7). Cytoplasmic FAS is phosphorylated with feeding to limit PPAR α activation, and it is dephosphorylated with fasting to promote PPAR α activation.

Our findings provide molecular definition and physiological context to an observation made nearly four decades ago in

birds. Using pigeon liver as a model and exclusively studying FAS in the cytoplasm, Qureshi and colleagues found that feeding induced ^{32}P incorporation into FAS, which was associated with a loss of enzyme activity (24). In vitro treatment with phosphatases dephosphorylated FAS and restored enzyme activity. The authors of this study did not identify a physiological role for this covalent modification, and it is not known whether the phosphosites we found are conserved in pigeon FAS due to the unavailability of sequence data for this species. Regardless, our work suggests that the phosphorylation state of cytoplasmic FAS may channel lipid flow to impact phospholipids inducing gene expression in the nucleus.

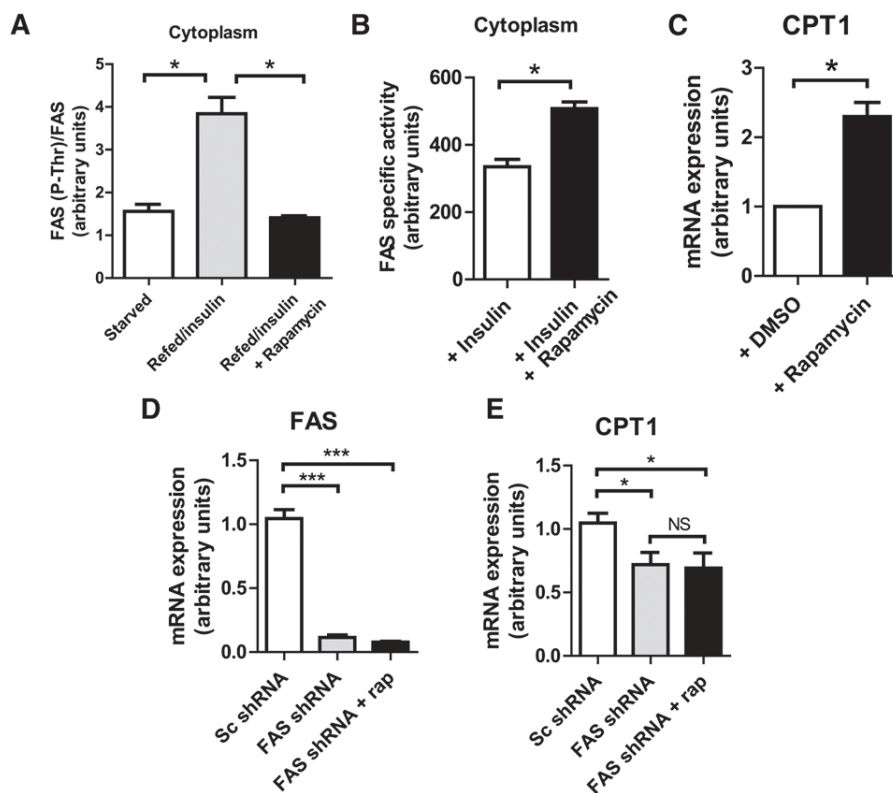


Fig. 6. FAS phosphorylation is inhibited by rapamycin and impacts CPT1 expression. (A) Cytoplasmic FAS phosphorylation in response to rapamycin in Hepa1-6 cells. Hepa1-6 cells were treated with vehicle, 100 nM insulin, or 100 nM insulin + 100 nM rapamycin for 30 min. The cytoplasmic fractions were isolated, and then FAS was immunoprecipitated and analyzed for phosphothreonine by western blotting. $*P \leq 0.05$. (B) Cytoplasmic FAS activity in response to rapamycin in Hepa1-6 cells. Cells were treated with 100 nM insulin and vehicle (DMSO) or 100 nM insulin + 100 nM rapamycin for 30 min, and then FAS enzyme activity was assayed. Activity was normalized to FAS protein levels as measured by western blotting. Data are averages of two independent experiments. $*P \leq 0.05$. (C) CPT1 expression levels in response to rapamycin in Hepa1-6 cells. Cells were treated with vehicle (DMSO) or 100 nM rapamycin for 24 h. Data are averages of two independent experiments. $*P \leq 0.05$. (D) FAS expression levels following FAS knockdown in Hepa1-6 cells. $N = 3-5$ /group. $***P \leq 0.0005$. (E) CPT1 expression levels in response to rapamycin following FAS knockdown in Hepa1-6 cells. $N = 3-5$ /group. $*P \leq 0.05$. NS, not significant.

Physiological, mass spectrometric, and crystal structure data indicate that phospholipids interact with nuclear receptors (6, 25–29). FAS appears to be linked to PPAR α through phosphatidylcholine synthesis mediated by the Kennedy pathway (6). Viewed with previous studies showing that phosphorylation regulates the CDP-choline branch of the Kennedy pathway (30, 31), our identification of functionally relevant FAS phosphorylation sites raises the possibility that phosphorylation at several nodes within a cascade of lipid signaling from the cytoplasm to the nucleus coordinates FAS-mediated PPAR α activation.

Palmitate is the direct product of the FAS reaction. If the mere availability of palmitate were required to activate PPAR α , exogenous palmitate would correct FAS deficiency. However, the addition of palmitate to liver cells with FAS deficiency does not restore defects in PPAR α -dependent genes (Fig. 1), and elevated serum palmitate levels that accompany inactivation of liver FAS in mice does not rescue impaired activation of PPAR α -dependent genes (8). Thus, palmitate produced by FAS appears to be compartmentalized, a notion supported by our finding of

preferential phosphorylation depending on cellular location and nutritional state.

There is precedent for compartmentalization in metabolism. Exogenous administration of T3, the active form of thyroid hormone that can be produced locally from its precursor T4, does not rescue gene expression defects in the setting of hypothyroidism. But administration of T4, which is metabolized to generate T3 locally, restores downstream effects (32). There is also precedent for compartmentalization in lipid signaling. Phosphatidic acid derived from glycerolipid synthesis has effects on mTORC2 that are opposite from those induced by phosphatidic acid derived from membrane lipolysis (33). These observations are consistent with our model (Fig. 7). In the fed state, cytoplasmic FAS is phosphorylated to limit lipid production resulting in PPAR α activation, while membrane FAS, less susceptible to phosphorylation, likely produces lipids for energy storage or export. Given the rapid demands of lipid synthesis prompted by transition from the fasting to the fed state, the induction of membrane FAS may be predominantly substrate-driven through allosteric

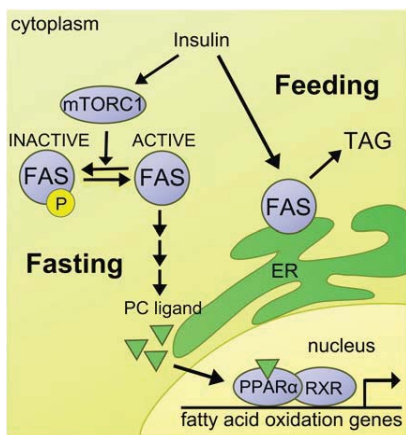


Fig. 7. Schematic depiction of insulin/feeding-regulated FAS phosphorylation and FAS-mediated PPAR α activation. In the fed state, mTORC1 promotes phosphorylation of FAS, thus limiting downstream generation of a phosphatidylcholine ligand that activates PPAR α -dependent gene expression. In the fasting state, dephosphorylated FAS in the cytoplasm is permissive for the generation of the ligand activating PPAR α -dependent gene expression. PC, phosphatidylcholine; TAG, triacylglycerol.

activation by the glycolytic intermediate fructose-1,6-bisphosphate (34).

mTORC1 may control the reciprocal activity of FAS in different compartments. mTORC1 is activated by insulin and nutrients, prefers substrates like those we identified in the dehydratase domain, and is known to suppress PPAR α in the liver (22). FAS and mTORC1 appear to interact in the central nervous system where the physiological effects of FAS inhibition are blunted by rapamycin (35), consistent with our model suggesting that mTORC1 inhibition would increase FAS activity.

Our work provides evidence that hepatic FAS is in the cytoplasm as well as peripherally associated with membranes. These two pools are differentially regulated by nutrients and insulin, and they are differentially susceptible to phosphorylation, thus providing a conceptual framework for understanding how FAS-mediated PPAR α activation is linked to the fasting state. These observations could have clinical implications. Selective pharmacological targeting of FAS to achieve inhibition of lipid storage without impairing PPAR α activation could treat fatty liver and other disorders associated with nutrient excess. **||**

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