RNA-binding protein ZFP36L1 maintains posttranscriptional regulation of bile acid metabolism

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Bile acids function not only as detergents that facilitate lipid absorption but also as signaling molecules that activate the nuclear receptor farnesoid X receptor (FXR). FXR agonists are currently being evaluated as therapeutic agents for a number of hepatic diseases due to their lipid-lowering and antiinflammatory properties. FXR is also essential for maintaining bile acid homeostasis and prevents the accumulation of bile acids. Elevated bile acids activate FXR, which in turn switches off bile acid synthesis by reducing the mRNA levels of bile acid synthesis genes, including cholesterol 7α-hydroxylase (Cyp7a1). Here, we show that FXR activation triggers a rapid posttranscriptional mechanism to degrade Cyp7a1 mRNA. We identified the RNA-binding protein Zfp36l1 as an FXR target gene and determined that gain and loss of function of ZFP36L1 reciprocally regulate Cyp7a1 mRNA and bile acid levels in vivo. Moreover, we found that mice lacking hepatic ZFP36L1 were protected from diet-induced obesity and steatosis. The reduced adiposity and antisteatotic effects observed in ZFP36L1-deficient mice were accompanied by impaired lipid absorption that was consistent with altered bile acid metabolism. Thus, the ZFP36L1-dependent regulation of bile acid metabolism is an important metabolic contributor to obesity and hepatosteatosis.

Introduction

Primary bile acids are synthesized in the liver and stored in the gall bladder and, following a meal, are secreted into the intestine, where they facilitate lipid absorption (1, 2). Virtually all aspects of the enterohepatic circulation involving bile acid synthesis, secretion, conjugation, and resorption in the intestine are dependent on the nuclear receptor farnesoid X receptor (FXR) (encoded by Nr1h4), which is highly expressed in enterocytes and hepatocytes (1). Endogenous bile acids such as chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and cholic acid (CA) (3, 4) are known to activate FXR, while muricholic acid (MCA) is thought to function as an FXR antagonist (5). A number of FXR agonists, including bile acid analogs, are currently being tested in clinical trials as potential treatments for steatosis and cholestasis (6).

Bile acid levels are tightly regulated through a negative feedback loop and end-product feedback inhibition that was first described almost half a century ago (2). Such feedback is critical, as elevated levels of bile acids can be toxic and result in severe metabolic complications including cholestasis, diarrhea, lipid malabsorption, inflammation, and cancer (1). The mechanism for feedback inhibition of bile acid synthesis is highly complex but largely dependent on FXR. In response to elevated bile acid levels, FXR is activated, leading to signaling cascades in both the liver and intestine that result in decreased hepatic mRNA levels of cholesterol 7α-hydroxylase (Cyp7a1), the rate-limiting enzyme of bile acid synthesis (2), and Cyp8b1, which is required for the synthesis of CA (7).

At present, the known mechanisms that lead to decreased Cyp7a1 expression following FXR activation are dependent on transcriptional repression. In one pathway, FXR activates the gene encoding the nuclear receptor SHP (Nr0b2), a classic FXR target gene and transcriptional repressor (8). In a second pathway, FXR activation in intestinal enterocytes induces the expression and subsequent secretion of FGF15 (FGF19 in humans) into the circulation (9). FGF15/19 then binds to a heterodimeric receptor on hepatocytes to initiate a signaling cascade leading to transcriptional repression of Cyp7a1 (10). FGF15/19 signaling was also shown to require SHP (11). Defects in a number of genes in bile acid metabolism pathways are associated with diverse metabolic disturbances, consistent with the premise that maintaining proper bile acid homeostasis is critical for preventing metabolic diseases.

In the present study, we identify and characterize an FXR-regulated posttranscriptional pathway that controls Cyp7a1 mRNA and bile acid levels. We identified ZFP36L1, also known as ZF36L1b, also known as TIS11b (12), BRF1, or RNF162B, as a direct FXR target gene. ZFP36L1 is an RNA-binding protein (RBP) that has been shown to bind to adenylate-uridylate–rich (AU-rich) elements in the 3′-UTRs of specific cytokine mRNAs in immune cells to promote mRNA degradation (13). Here, we used synthetic and endogenous FXR agonists to demonstrate that activation of hepatic FXR leads to a rapid induction of hepatic Zfp36l1 mRNA and protein in vivo and in vitro. Our in vitro studies demonstrate that ZFP36L1 can target both human or mouse Cyp7a1 3′-UTRs. In addition, in vivo ZFP36L1 gain- and loss-of-function studies showed that ZFP36L1 regulates Cyp7a1 mRNA levels and alters bile acid levels. Finally, we demonstrate that mice lacking hepatic
Zfp36l1 (Zfp36L1<sup>%<sup>-/-</sup></sup>) are partially resistant to diet-induced adiposity and steatosis, due, at least in part, to impaired lipid absorption that results from altered bile acid metabolism.

**Results**

Pharmacologic FXR activation with the synthetic FXR agonists GSK2324 or GW4064 results in a greater than 95% reduction of Cyp7a1 and Cyp8b1 mRNA levels within 4 hours (8, 14). To better determine the kinetics of decay of Cyp7a1 and Cyp8b1 mRNA levels following FXR activation, we treated WT and Fxr<sup>−/−</sup> mice with a single dose of vehicle or GSK2324 (30 mg/kg body weight [mpk]) and analyzed gene expression 30 minutes, 1 hour, and 2 hours later. Cyp7a1 mRNA levels declined by more than 50% within 30 minutes of GSK2324 treatment (Figure 1A) suggested that the half-life of murine cell lines that express CYP7A1 or CYP8B1 to be 4.6 hours (15). Thus, our observation that Cyp7a1 mRNA levels decreased by approximately 50% within 30 minutes of GSK2324 treatment (Figure 1A) suggested that a posttranscriptional mechanism might enhance the degradation of preexisting Cyp7a1 mRNA following FXR activation in mice. To determine the half-life of Cyp7a1 mRNA in vivo is challenging, therefore we first considered using isolated primary mouse hepatocytes. However, we observed a near-complete loss of FXR, Cyp7a1, and Cyp8b1 mRNAs within 16 hours of isolation and culturing of primary mouse hepatocytes (Supplemental Figure 1L), suggesting that these cells are not a suitable system by which to study the regulation of bile acid synthesis genes.

The rapid regulation of Cyp7a1 observed following FXR activation (Figure 1A) led us to consider the half-life of Cyp7a1 mRNA. A previous study in which HepG2 cells were used reported the half-life of human CYP7A1 to be 4.6 hours (15). Thus, our observation that Cyp7a1 mRNA levels decreased by approximately 50% within 30 minutes of GSK2324 treatment (Figure 1A) suggested that a posttranscriptional mechanism might enhance the degradation of preexisting Cyp7a1 mRNA following FXR activation in mice. To determine the half-life of Cyp7a1 mRNA in vivo is challenging, therefore we first considered using isolated primary mouse hepatocytes. However, we observed a near-complete loss of FXR, Cyp7a1, and Cyp8b1 mRNAs within 16 hours of isolation and culturing of primary mouse hepatocytes (Supplemental Figure 1L), suggesting that these cells are not a suitable system by which to study the regulation of bile acid synthesis genes.

In the absence of primary hepatocytes or the appropriate murine cell lines that express Cyp7a1, we determined the half-life of CYP7A1 and CYP8B1 mRNA using immortalized human hepatocytes (IHHs) (16). IHHs express many of the bile acid synthesis genes, including CYP7A1 (16). We then treated IHHs with the transcriptional inhibitor actinomycin D for different lengths of time prior to isolation and quantification of mRNA levels to determine mRNA half-lives. The half-lives of human CYP7A1 and CYP8B1 mRNAs were approximately 1 and 4 hours, respectively (Supplemental Figure 1M and N). We then compared the mRNA decay rates observed in IHHs (black lines) with those from GSK2324-treated mice (blue lines) and found that FXR activation with GSK2324 increased the rate of decrease in Cyp7a1, but not in...
Cyp8b1 mRNA levels (Figure 1, E and F). The differences in mRNA half-lives between these conditions are suggestive of the existence of posttranscriptional mechanisms that destabilize Cyp7a1 mRNA.

Two pathways have been identified that are involved in feedback regulation of hepatic Cyp7a1 mRNA levels (1). One involves intestinally derived FGF15 (FGF19 in humans), an FXR target gene that is secreted into the circulation in response to activation of FXR in intestinal enterocytes (9). The second involves induction of SHP, a known transcriptional repressor, following activation of hepatic FXR (8).

To determine whether hepatic FXR is required for the rapid decrease in Cyp7a1 that occurs following FXR activation in vivo, we generated liver-specific FXR-KO mice (FxrL-KO) and littermate control floxed mice (Fxrfl/fl) by crossing Fxrfl/fl mice with albumin-Cre–expressing mice to delete FXR in hepatocytes. We then treated both Fxrfl/fl and FxrL-KO animals with either vehicle or GSK2324 for 30 minutes, 1 hour, 2 hours, and 4 hours. Cyp7a1 mRNA levels in the liver were reduced by 50% within 30 minutes, and the levels were almost completely abolished after 4 hours of GSK2324 treatment of FxrL-KO mice (Figure 2A). In contrast, no inhibition of Cyp7a1 mRNA levels was observed for up to 2 hours after treatment of FxrL-KO mice with GSK2324 (Figure 2A). These data demonstrate that the initial and rapid GSK2324-dependent decrease in Cyp7a1 mRNA requires hepatic FXR. However, 4 hours after GSK2324 treatment of FxrL-KO mice, there was significant inhibition of Cyp7a1 mRNA (Figure 2A), suggesting that this latter inhibition is likely a result of intestinally derived FGF15.

Cyp8b1 mRNA levels were consistently reduced at a slower rate than were Cyp7a1 mRNA levels after GSK2324 treatment, and the initial decline in Cyp8b1 levels that occurred within 2 hours of GSK2324 treatment was dependent on hepatic FXR (Supplemental Figure 2A). The FXR target genes Shp and Bsep were induced in the livers of Fxrfl/fl, but not FxrL-KO, mice treated with GSK2324 (Figure 2B and Supplemental Figure 2B). In contrast, GSK2324 treatment induced Fgf15 and Fabp6 (iBabp) mRNAs in the distal ileum in both FxrL-KO and FxrL-KO mice (Figure 2C and Supplemental Figure 2C), demonstrating that intestinal FXR and the subsequent induction of intestinal FXR target genes were unaffected by loss of hepatic FXR. These results suggest that a specific factor that is controlled by hepatic FXR mediates the rapid decline of Cyp7a1 mRNA levels following FXR activation.
The rapid decrease in Cyp7a1 mRNA levels in response to FXR activation in vivo requires hepatic FXR but is independent of both SHP and induction of intestinal Fgf15. Furthermore, our data suggested that activation of hepatic FXR utilizes a previously unrecognized posttranscriptional mechanism to control Cyp7a1 mRNA levels.

Various posttranscriptional mechanisms have been described that modulate mRNA stability: for example, the binding of microRNAs (miRs) to seed sequences within target mRNA, or the interaction of the mRNA with specific RBPs (18, 19). We previously demonstrated that FXR regulates surprisingly few miRs. Among the direct FXR-regulated miRs we identified are the miR-144/miR-451 cluster (20) and miR-33 (21). Further, miRs are thought to be fine-tuners of gene expression and are therefore unlikely to be rapid regulators of gene expression (19). Thus, miRs seemed to be unlikely candidates for mediating the rapid change in Cyp7a1 mRNA. We next considered RBPs, which represent a large class of genes that are reported to be more abundant in the mammalian genome than are transcription factors (18). RBPs bind RNA targets and either stabilize or destabilize mRNAs, usually in a sequence-specific manner (18).

To determine whether the rapid decrease in Cyp7a1 mRNA observed in response to GSK2324 required the transcriptional repressor SHP, we obtained Shp–/– mice (17) and treated them with vehicle or GSK2324 for 30 minutes, 1 hour, or 2 hours. Notably, Cyp7a1 mRNA levels declined by approximately 50% within 30 minutes of treating Shp–/– mice with GSK2324, indicating that SHP was not required for the rapid decrease in Cyp7a1 mRNA levels (Figure 2D). Treatment of Shp–/– mice with GSK2324 also resulted in a decrease in Cyp8b1 mRNA after 2 hours (Supplemental Figure 2D). The induction of the known FXR target genes Bsep (Figure 2E) and Insig2a (Supplemental Figure 2E) in the liver and induction of Fgf15 in the distal ileum (Figure 2F) were similar in Shp–/– mice, consistent with normal function of hepatic and intestinal FXR signaling in Shp–/– mice.

Taken together, these data demonstrate that the initial rapid decrease in Cyp7a1 mRNA levels in response to FXR activation in vivo requires hepatic FXR but is independent of both SHP and induction of intestinal Fgf15. Furthermore, our data suggested that activation of hepatic FXR utilizes a previously unrecognized posttranscriptional mechanism to control Cyp7a1 mRNA levels.

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To test this hypothesis and to identify potential FXR-regulated RBPs, we searched the literature for RBPs that degrade mRNA targets and manually checked those for potential FXR response elements (FXREs) using publicly available FXR ChIP-seq (ChIP-seq) data sets (22, 23). This approach
led us to investigate ZFP36L1, since liver FXR ChIP-seq analysis (22) identified several potential FXREs at the mouse Zfp36l1 locus. One FXRE is predicted to lie within the proximal promoter region of Zfp36l1, while a second FXRE is predicted to lie downstream of the gene (Figure 3A). These data suggest that Zfp36l1 may be directly regulated by FXR.

To determine whether the putative FXRE in the Zfp36l1 promoter was functional, we cloned a 2-kb region corresponding to the promoter of Zfp36l1 upstream of a luciferase reporter gene. Cells were then cotransfected with this reporter gene in combination with increasing amounts of an FXR agonist (Figure 3B), suggesting that the promoter of Zfp36l1 was functional, we cloned a 2-kb region corresponding to the promoter of Zfp36l1 upstream of a luciferase reporter gene. As expected, both agonists increased ZFP36L1 mRNA and protein levels and decreased CYP7A1 mRNA levels (Supplemental Figure 3, C and D). Taken together, these results show that FXR activation with either synthetic or endogenous agonists results in the induction of ZFP36L1 in both mouse livers and IHHS, consistent with ZFP36L1 as a direct FXR target gene.

We hypothesized that the regulation of ZFP36L1 by FXR at both mRNA and protein levels would have to be rapid if ZFP36L1 were to be involved in the initial (30 min) decrease in Cyp7a1 mRNA (Figures 1 and 2). To test this hypothesis, we determined how rapidly ZFP36L1 mRNA and/or protein levels were induced in WT, Fxr−/−, FxrL-KO, and Shp−/− mice following short-term treatment with GSK2324. Importantly, we show that the induction of ZFP36L1 mRNA and protein was already observed 30 minutes after GSK2324 treatment of WT (Figure 4, A and B), Fxrfl/fl, Fxr−/− mice (Figure 4, C and D), and Shp−/− mice (Figure 4, E and F, and see the complete unedited blots in the supplemental material). In contrast, Zfp36l1 mRNA was not induced following GSK2324 treatment of Fxr−/− and FxrL-KO mice (Figure 4, A and C). We also determined the half-life of ZFP36L1 mRNA to be approximately 30 minutes in IHHS treated with actinomycin D, suggesting that Zfp36l1 is a short-lived and rapidly regulated mRNA (Supplemental Figure 3E). Finally, we show that ZFP36L1 expression was rapidly induced in IHHS treated with either GW4064 or CDCA (Supplemental Figure 3, F and G). Together, these in vitro and in vivo experiments demonstrate that activation of hepatic FXR results in a rapid (30 min) increase in both ZFP36L1 mRNA and protein levels.

The stability of specific mRNAs has been shown to be dependent on the interaction of the 3′-UTR with various factors, including miRs and RBPs (18). Indeed, ZFP36L1 promotes mRNA degradation after binding to AU-rich sequences in the 3′-UTR of specific cytokines and then recruiting deadenylation complexes (13, 24). Interestingly, the Cyp7a1 3′-UTR is large (2.5 kb in mice, 1.5 kb in human cells), we treated IHHS with either endogenous (CDCA) or synthetic (GW4064) FXR agonists. Both agonists increased ZFP36L1 mRNA and protein levels and
human) and contains multiple conserved AU-rich elements (24). To determine whether ZFP36L1 can regulate Cyp7a1 mRNA stability, we generated a reporter plasmid containing the mouse Cyp7a1 3′-UTR downstream of a constitutively transcribed luciferase reporter. Cotransfection of a plasmid encoding ZFP36L1, together with the mouse Cyp7a1 3′-UTR reporter gene, resulted in a ZFP36L1 dose-dependent decrease in luciferase activity (Figure 5A). The mouse Cyp7a1 3′-UTR has a 200-bp central region that contains several tandem AU-rich elements (Figure 5A). A reporter plasmid containing the mouse Cyp7a1 3′-UTR that lacked this 200-bp region was no longer responsive to coexpression of ZFP36L1 (Figure 5A). The human CYP7A1 3′-UTR also contains a similar 200-bp AU-rich sequence, which, in contrast to the mouse, is found at the 3′ end of the CYP7A1 3′-UTR. Cotransfection of ZFP36L1 with the luciferase reporter gene construct containing the human CYP7A1 3′-UTR also led to a decrease in luciferase activity (Figure 5B). Furthermore, deletion of the 200-bp AU-rich sequence from the 3′-terminus of the UTR resulted in a reporter gene that was unresponsive to cotransfected ZFP36L1 (Figure 5B). We conclude that ZFP36L1 targets both human and mouse CYP7A1 mRNA by binding to AU-rich elements in the 3′-UTR, leading to degradation of the mRNA.

To determine whether ZFP36L1 can also target endogenous Cyp7a1 mRNA in vivo, we generated Zfp36l1-expressing adenovirus particles and infused them into the tail veins of male C57BL/6 mice. Recombinant adenovirus particles resulted in a modest increase in hepatic ZFP36L1 mRNA and protein levels (Figure 5C). Consistent with our in vitro studies involving hybrid reporter genes, enforced ZFP36L1 expression in WT mice significantly
decreased hepatic CYP7A1 mRNA and protein levels (Figure 5D, and see the complete unedited blots in the supplemental material).

We recently reported that expression of most of the 17 genes involved in the synthesis of bile acids from cholesterol is repressed following activation of FXR (14). We therefore examined whether these bile acid synthesis genes were also targeted by Zfp36l1 overexpression. The total amount of bile acids in the gall bladders of adenovirus–treated (Ad–Zfp36l1L-KO) mice was also decreased, but the volume of bile recovered from the gall bladders of adenovirus–treated (Ad–Zfp36l1L-KO) mice was unchanged (Supplemental Figure 4, D and E).

CYP7A1 is the rate-limiting enzyme involved in the conversion of hepatic cholesterol to primary bile acids (2). Consequently, we next tested the hypothesis that the ZFP36L1-dependent decrease in CYP7A1 mRNA and protein resulted in decreased bile acids. Bile acids turn over very slowly, with only 5% of the bile acid pool being excreted per day, and this loss by excretion is replenished by de novo bile acid synthesis (1). As shown in Figure 5F, biliary bile acid concentration significantly decreased following 5 days of Zfp36l1 overexpression. The residual hepatic ZFP36L1 mRNA detected in the Zfp36l1L-KO mice is likely a result of greater than 80% reduction in total hepatic ZFP36L1 mRNA and protein levels (Figure 6A, and see the complete unedited blots in the supplemental material).

To complement our gain-of-function studies, we next investigated whether loss of Zfp36l1 reciprocally affects bile acid metabolism. Germline deletion of Zfp36l1 results in embryonic lethality (25, 26). Consequently, we obtained floxed Zfp36l1 mice (Zfp36l1fl/fl) (27) and crossed them with albumin-Cre–expressing animals to generate hepatocyte-specific Zfp36l1-KO mice (Zfp36l1fl/fl). Zfp36l1 deletion in hepatocytes resulted in a greater than 80% reduction in total hepatic ZFP36L1 mRNA and protein levels (Figure 6A, and see the complete unedited blots in the supplemental material).

We also determined other metabolic parameters in mice overexpressing Zfp36l1. First, as shown in Figure 5G, plasma total cholesterol levels were significantly elevated following Zfp36l1 overexpression. In contrast, plasma insulin and glucose levels were unaffected in mice following hepatic overexpression of Zfp36l1 (Supplemental Figure 4, F and G). Further, plasma levels of 2 liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were not altered following hepatic Zfp36l1 overexpression (Supplemental Figure 4, H and I). These results emphasize an important role of ZFP36L1 in the regulation of lipid metabolism, given that short-term gain of function of Zfp36l1 in vivo results in reduced Cyp7a1 expression, decreased bile acids, and increased plasma cholesterol levels.
mice were unchanged, although some gene expression levels were increased (Akr1d1, Cyp8b1, Cyp27a1, and Cyp39a1), and Cyp7b1 mRNA expression was decreased (Figure 6C).

Total biliary bile acids were significantly elevated in Zfp36l1-/-KO mice, and the increase was almost exclusive in 1 species of bile acid, namely tauro-β-muricholic acid (T-β-MCA) (Figure 6D). Zfp36l1-/-KO mice also had modestly larger gall bladders, resulting in a larger increase in the total amounts of biliary bile acid (Supplemental Figure 5, A and B). In line with increased bile acid production, Zfp36l1-/-KO mice also had decreased plasma levels of total and HDL cholesterol (Figure 6E). In contrast, the levels of plasma triglycerides (plasma TAG), free fatty acids, ALT, AST, insulin, and glucose were unaffected by loss of hepatic Zfp36l1 (Supplemental Figure 5, C–H). Finally, we detected no gross metabolic abnormalities in Zfp36l1-/-KO mice using indirect calorimetry. Oxygen consumption, carbon dioxide production, and activity were not significantly different in littermate Zfp36l1+/- and Zfp36l1-/-KO mice fed a standard rodent diet (Supplemental Figure 5, I–K).

Together, our combined in vivo gain- and loss-of-function studies strongly support an important role for hepatic ZFP36L1 specifically as a regulator of bile acid homeostasis. To determine whether the FXR/ZFP36L1 pathway is essential for the FXR-dependent bile acid synthesis feedback inhibition, we carried out a time course of GSK2324 treatment of Zfp36l1+/- and Zfp36l1-/-KO mice (Figure 7, A and B). Basal Cyp7a1 mRNA levels were elevated in vehicle-treated Zfp36l1+/- mice (Figure 7B), consistent with the data shown in Figure 6. However, Cyp7a1 mRNA levels, which were rapidly decreased in Zfp36l1+/- mice treated with GSK2324, were still decreased in Zfp36l1-/-KO mice following GSK2324 treatment (Figure 7C). For direct comparison of the rates of Cyp7a1 mRNA degradation, we also show the data for both genotypes (Zfp36l1+/- and Zfp36l1-/-KO) as the fold change relative to the respective vehicle-treated animals (Figure 7D). These data show a modest delay in the loss of Cyp7a1 mRNA levels at 30 minutes and 1 hour following GSK2324 treatment of Zfp36l1+/- as compared with Zfp36l1-/-KO mice (Figure 7D). These latter findings suggest that additional FXR-dependent genes may encode other proteins that also function to degrade Cyp7a1 mRNA. Nonetheless, we conclude that the new pathway whereby FXR activation induces ZFP36L1 mRNA and protein, which subsequently targets Cyp7a1 mRNA for degradation, is not essential for feedback regulation of Cyp7a1. Our results suggest that multiple redundant and complementary pathways target Cyp7a1 via both transcriptional (SHP/FGF15) and posttranscriptional (ZFP36L1) mechanisms to ultimately block bile acid synthesis upon FXR activation (Figure 7E).
Bile acids are both signaling molecules and detergents that facilitate lipid absorption, and mouse models with altered bile acid metabolism have a number of important metabolic phenotypes. For example, transgenic mice that overexpress human CYP7A1 in hepatocytes have increased bile acid synthesis but are also resistant to diet-induced obesity and steatosis (28–30). The precise mechanisms by which elevated CYP7A1 levels lead to changes in obesity or steatosis are not well understood. Given our observations that Zfp36l1l−/− mice have elevated CYP7A1 mRNA and protein as well as increased bile acid levels (Figure 6), we hypothesized that loss of hepatic Zfp36l1 would result in metabolic disturbances similar to those reported in CYP7A1-transgenic mice. We therefore challenged Zfp36l1l−/− and littermate control (Zfp36l1l+/+) mice with a Western-style diet (40 kCal fat, 0.21% cholesterol) for 64 days and measured body weight and fat and lean mass weekly. Zfp36l1l−/− animals fed the Western diet had less body weight gain and reduced fat mass (Figure 8, A and B) as well as reduced lean mass (Supplemental Figure 6A) compared with littermate Zfp36l1l+/+ mice. We obtained similar results when we fed Zfp36l1l−/− and Zfp36l1l−/− animals a diet only enriched in fat (high-fat diet: 45 kCal fat, 0.03% cholesterol), and once again, Zfp36l1l−/− mice were resistant to diet-induced weight gain (Supplemental Figure 6, B–D). To determine whether the reduced body weight and fat mass were due to differences in energy metabolism, we placed littermate Zfp36l1l−/− and Zfp36l1l−/− animals in metabolic chambers and performed comprehensive metabolic analyses after 64 days of Western diet feeding. We observed no significant differences in energy expenditure (Figure 8C), oxygen consumption, carbon dioxide production, or activity (Supplemental Figure 6, E–G), suggesting that loss of hepatic Zfp36l1 did not result in gross energy metabolism abnormalities. However, despite exhibiting decreased body and fat weights, Zfp36l1l−/− mice consumed more food than did their Zfp36l1l+/+ littermates (Figure 8D). Moreover, Zfp36l1l−/− mice were markedly protected from diet-induced hepatosteatosis after 64 days on a Western diet (Figure 8E). Lipid extraction from the livers of control Zfp36l1l+/+ and Zfp36l1l−/− mice confirmed reduced hepatic triglyceride (TAG) and cholesterol levels in Zfp36l1l−/− mice (Figure 8, F and G). Plasma TAG and cholesterol levels were also lower in the Western diet–fed Zfp36l1l−/− mice as compared with levels in control mice (Figure 8, H and I), as were plasma insulin and ALT levels (Figure 8, J and K). Plasma glucose levels were lower, but not significantly, whereas plasma AST levels were not altered (Supplemental Figure 6, H and I). Hepatic expression of the inflammatory cytokines Il1b and Tnfa were also significantly lower in the livers of Western diet–fed Zfp36l1l−/− mice (Supplemental Figure 6J). Together, these changes are consistent with improved hepatic function and improved metabolic handling in Zfp36l1l−/− mice following challenges with fat-enriched diets.

We also measured bile acid parameters in Zfp36l1l−/− mice fed a Western diet. Total biliary bile acid levels were elevated in Western diet–fed Zfp36l1l−/− mice, and the change was largely restricted to elevated T-β-MCA levels (Figure 8L). Moreover, Western diet–fed Zfp36l1l−/− mice had increased hepatic CYP7A1 mRNA and protein levels and altered expression of a number of bile acid synthesis genes (Figure 8, M and N, and see the complete unedited blots in the supplemental material). These changes are consistent with our analysis of mice on a standard rodent diet (Figure 6).

Given the central role of bile acids as detergents, we hypothesized that loss of hepatic Zfp36l1 might affect intestinal lipid absorption, particularly under Western diet-fed conditions, as a consequence of the altered bile acid metabolism. To determine differences in lipid absorption, we first subjected Western diet–fed littermate Zfp36l1l+/+ and Zfp36l1l−/− mice to an intragastric fat challenge and measured circulating triglycerides over a 6-hour period. Zfp36l1l−/− mice had a reduced excursion of plasma TAG levels after the fat challenge, suggesting that loss of hepatic Zfp36l1 resulted in defective lipid absorption (Figure 8O).

Reduced lipid absorption should also result in increased caloric content in the feces, and, indeed, the total energy in the form of calories that was recovered in the feces from Zfp36l1l−/− mice was almost double that in feces from control mice (Figure 8P). Fecal calories were elevated even when expressed per gram, but the increased fecal calorie content was compounded by an increased fecal output by Zfp36l1l−/− mice (Supplemental Figure 6, K and L). Taken together, these data suggest that loss of hepatic Zfp36l1 results in pronounced whole-body metabolic changes that are explained, at least in part, by decreased lipid absorption resulting from altered bile acid metabolism.

Discussion

Here, we identify Zfp36l1 as an FXR target gene that functions to enhance the rate of degradation of Cyp7a1 mRNA. We used gain-and loss-of-function studies to demonstrate that hepatic ZFP36L1 expression is inversely proportional to bile acid levels and, in addition, affects the composition of the bile acid pool. Thus, the current findings identify and characterize a previously unrecognized pathway by which activated FXR rapidly regulates bile acid synthesis. We show that FXR activation in vivo results in an intensely rapid decrease in Cyp7a1 mRNA by a posttranscriptional mechanism that requires hepatic FXR but is independent of the transcriptional represor SHP. To determine the molecular mechanism of the degradation of Cyp7a1 mRNA, we identified the RBP Zfp36l1 (12). The Zfp36l1 gene locus contains multiple putative FXR response elements, consistent with its induction in response to natural (CA) or synthetic (GW4064, GSK2324) FXR agonists in vivo. Further, reporter gene assays indicated the presence of a functional FXRE in the proximal promoter of the Zfp36l1 gene. Importantly, we show that in mice, induction of Zfp36l1 mRNA and protein in response to FXR activation with GSK2324 occurs within 30 minutes. Taken together, these data suggest that rapid FXR-dependent induction of Zfp36l1 leads to a reduction of Cyp7a1 mRNA via a posttranscriptional mechanism, a hypothesis supported by our in vitro reporter gene studies.

We show that luciferase reporter gene linked to either the mouse or human Cyp7a1 3′-UTRs are repressed following overexpression of Zfp36l1. Additional reporter gene assays indicate that ZFP36L1-dependent regulation requires a region containing conserved AU-rich elements in the 3′-UTR of Cyp7a1 mRNA. Indeed, previous studies have shown that Zfp36l1 functions to repress cytokine and cell-cycle mRNAs by binding to AU-rich sequences in their 3′-UTRs (13, 27, 31). However, a role for Zfp36l1 in regulating hepatic mRNAs in vivo, including those involved in lipid and bile acid metabolism, has not been reported. Notably, cytokine mRNAs were unaffected in the liver following treatment of mice with Ad-Zfp36l1 or in Zfp36l1l−/− mice (data not shown).
Our gain- and loss-of-function studies show that there are significant metabolic consequences to modulation of Zfp36l1. Hepatic overexpression of Zfp36l1 in mice resulted in a decrease in both Cyp7a1 and Cyp8b1 mRNA levels. Complementary loss-of-function studies demonstrated that loss of Zfp36l1 in the livers of mice results in an elevated expression of a number of mRNAs, including Cyp7a1 and Cyp8b1, in both normal and Western diet-fed mice. Consistent with these findings, Zfp36l1-KO mice have increased biliary bile acid levels and an altered biliary bile acid composition. These results are consistent with the hypothesis that Zfp36l1 normally functions to repress Cyp7a1 mRNA and reduce bile acid synthesis. The reduction in plasma cholesterol levels that accompanied the increased bile acid levels is also consistent with increased bile acid synthesis in Zfp36l1-KO mice.

One of the major functions of bile acids is to facilitate lipid absorption, and it is clear that not all bile acids act in the same manner (1, 2). For example, Cyp8b1−/− mice have increased T-β-MCA levels and no detectable tauro-CA. These same Cyp8b1−/− mice have increased CYP7A1 enzymatic activity and an increased biliary acid pool, yet decreased cholesterol absorption (7). Interestingly, Cyp8b1−/− mice were recently shown to be resistant to steatosis and weight gain (32). The improved steatosis and decreased adiposity are similar to our findings in Zfp36l1-KO mice, which also had increased biliary acid levels and, in particular, increased levels of T-β-MCA. Thus, the different bile acid composition of Zfp36l1-KO mice may account, at least in part, for the decrease in lipid absorption and the increase in fecal calorie content as well as the resistance to diet-induced adiposity and the decrease in steatosis. While the changes in plasma triglycerides following an oral lipid challenge were modest, the changes in body weight and adiposity are likely reflective of cumulatively defective lipid absorption over the 64-day period that the animals were on the diet.

Previous studies have defined two different FXR-dependent pathways, one requiring hepatic SHP and the other being dependent on intestinally derived FGF15/19, that are involved in the repression of Cyp7a1 transcription (8, 9). Both these pathways have also been shown to work together, as recombinant FGF19 was unable to reduce Cyp7a1 mRNA levels in Shp−/− mice (11). We recently described an FXR-dependent pathway that functions through another transcriptional repressor, MAFG, which does not directly repress Cyp7a1. MAFG functions to repress numerous genes in the bile acid synthesis pathway, including Cyp8b1, the regulatory gene of CA synthesis (14). The relative contribution of each of these pathways to feedback regulation of bile acid synthesis has not been comprehensively established. Previous studies using Shp−/− mice showed that treatment with GW4064 failed to repress Cyp7a1 mRNA levels (33, 34), supporting the idea that SHP has a role in FXR-dependent repression of this gene. In contrast, we and others have reported that Cyp7a1 mRNA levels decline following treatment of Shp−/− mice with either GW4064 (35), CA (33, 34), or GSK2324, as we have shown here. At present, it is unclear whether these divergent results are due to the different genetic backgrounds of the animal models or to differences in the modes of administration, tissue uptake, or pharmacokinetics of the FXR agonists. Consequently, the exact role and importance of SHP in the repression of Cyp7a1 remains to be clearly elucidated, although our data suggest that SHP is not essential for feedback regulation of bile acid synthesis.

The finding that treatment of Zfp36l1−/− mice with GSK2324 results in reduced Cyp7a1 expression, albeit at a slower rate than in their WT littersmates, suggests that Zfp36l1 is also not essential for feedback regulation of bile acid synthesis. Nonetheless, the findings that hepatic loss of ZFP36L1 results in elevated levels of Cyp7a1 and bile acids, reduced adiposity, and steatosis indicate that this protein functions in controlling Cyp7a1 mRNA. Importantly, here we show that GSK2324 treatment of WT, Shp−/−, and Zfp36l1−/− mice reduces Cyp7a1 mRNA levels. In contrast, Cyp7a1 mRNA levels were unaltered in Fxr−/− mice treated with GSK2324. Taken together, we conclude that FXR utilizes multiple redundant pathways to mediate feedback inhibition of bile acid synthesis. Redundancy in feedback inhibition of bile acid synthesis was also proposed in the original reports characterizing Shp−/− mice (33, 34).

Our identification of ZFP36L1 as a regulator of Cyp7a1 mRNA and bile acid composition provides a pathway that complements the known transcriptional repression pathways that decrease bile acid synthesis. ZFP36L1 may function to rapidly decrease preexisting Cyp7a1 mRNA levels, and subsequent transcriptional repression mechanisms in response to FGF15 and/or SHP then maintain reduced levels of Cyp7a1 mRNA under conditions of elevated bile acids and/or FXR activation. In addition to ZFP36L1, we have identified at least 1 other RBP that appears to be FXR regulated and can target and degrade Cyp7a1 mRNA (data not shown). However, we have not yet characterized this pathway in detail. It remains to be determined whether the different RBPs that are responsive to FXR activation target Cyp7a1 mRNA function in concert.

Studies conducted over a decade ago by Davis and colleagues had proposed that Cyp7a1 might be regulated by posttranscriptional mechanisms (24). However, no molecular mechanism was identified at that time, although the AU-rich elements present in the Cyp7a1 UTR were found to be required for the proposed posttranscriptional regulation (24). Since ZFP36L1 requires these AU-rich elements to repress Cyp7a1 mRNA levels, we believe we have identified a factor involved in the posttranscriptional regulation of Cyp7a1.
ZFP36L1 can bind RNA directly but does not have the capability of degrading mRNAs by itself. Rather, ZFP36L1 binds mRNA targets in a sequence-specific fashion and then recruits additional proteins, including deadenylases, that then mediate mRNA degradation (13). Thus, ZFP36L1 acts as a guide for deadenylase complexes by recognizing specific motifs on mRNAs that are to be targeted for degradation. ZFP36L1 is known to bind AU-rich elements, which are common in 3′-UTRs of many genes, including Cyp7a1. However, it is clear that not all genes that contain AU-rich elements are regulated in the same way. The mRNA targets of ZFP36L1 are probably cell specific and depend not only on the presence of other specific proteins but also on the abundance of target mRNAs. Thus, the specificity of ZFP36L1 for particular targets is likely a combination of mRNA sequences and secondary structure, mRNA abundance, and protein-protein interactions. Additional studies will be required to identify and characterize the protein components in the liver that are involved in regulating Cyp7a1 mRNA stability and degradation.

ZFP36L1 has also been implicated in human disease, as GWAS identified variants in the ZFP36L1 locus associated with Crohn’s disease and inflammatory bowel disease (IBD) (36). Since altered bile acid metabolism has also been implicated in diseases of the gut, our studies may provide an additional mechanism to explain, at least in part, how variants in the ZFP36L1 locus are associated with IBD and Crohn’s disease.

Bile acids are both signaling molecules and detergents, thus, we also determined whether loss of Zfp36l1 in the liver results in broader metabolic dysfunction. Compared with their WT littermates, Zfp36l1−/− mice had markedly reduced obesity, without changes in energy expenditure compared with their littermate WT controls. Zfp36l1−/− mice were also protected from diet-induced hepatosteatosis. We propose that these metabolically beneficial effects are due to defects in lipid absorption, since Zfp36l1−/− mice had a reduced response following an intragastric fat challenge and increased fecal calorie output. Interestingly, CYP7A1-transgenic mice exhibit many of the same phenotypes we have described here in Zfp36l1−/− mice, including resistance to obesity and protection from steatosis (28–30). However, the mechanisms leading to the changes in obesity, steatosis, and lipid absorption in CYP7A1-transgenic mice remain unknown (28–30). Nonetheless, our studies suggest that many of the phenotypic changes we observed in Zfp36l1−/− mice were a direct result of increased CYP7A1 activity, increased bile acid synthesis, and an altered bile acid pool size and composition that then affected lipid absorption in the gut. The prevalence of nonalcoholic fatty liver disease (NAFLD) and obesity has increased worldwide, and the pathogenesis of NAFLD is poorly understood (37). Our studies suggest that dysregulated bile acid metabolism is probably important in NAFLD progression.

Methods

Animals and treatments. All animals were bred and housed in a pathogen-free animal facility. C57BL/6 liver– and intestine–specific Fxr−/− mice and their respective (littermate) Fxr+/+ WT controls as well as whole-body Fxr−/− mice were generated as previously described (20). C57BL/6 littermate Zfp36l1−/− (Zfp36l1lox/lox) and Zfp36l1−/− mice were generated by crossing Zfp36l1lox/lox mice (31) with albumin-Cre–expressing animals (The Jackson Laboratory). Whole-body Shp−/− mice (17) were obtained and backcrossed at UCLA for 6 further generations on a C57BL/6 background. All mice were maintained on a 12-hour light/12-hour dark cycle. In general, animals were used at 60 days of age, fasted at 9 am for 4 to 5 hours, and then sacrificed between 1 pm and 2 pm for treatments with FXR agonists, GSK2324 was dissolved in water and administered once to mice via i.p. injection at 30 mpk for 30 minutes, 1 hour, 2 hours, or 4 hours before sacrifice. Vehicle-treated mice were injected with water for randomly assigned time points (between 30 min and 4 h). All mice were fasted for 4 hours and sacrificed at the same time of day (1–3 pm) to ensure a consistent circadian time point. Studies with GW4064 and GSK2324 at 60 mpk for 3 days were described previously (14).

Adenovirus production. All adenoviruses were prepared in biosafety level 2–category (BSL-2–category) facilities. Briefly, DNAs for mouse Zfp36l1 were cloned from whole-liver cDNA into a pAdTrack CMV plasmid and prepared as described previously (14). For animal experiments, 1 × 10⁶ PFU were infused into either male or female C57BL/6 mice for 5 to 7 days before tissue collection.

Bile acid analysis. Gall bladders were removed after a 4- to 6-hour fast, bile was removed, and the contents were stored at –80°C. Bile acid species were measured using an HPLC system as described previously (14). Conjugated bile acids were analyzed by HPLC (Shimadzu) using a Kinetex 5m C-18 100A 250 × 4.6 mm column (Phenomenex) with isocratic elution at 0.75 ml/min. The eluting solution was composed of a mixture of methanol and 0.01 M KH₂PO₄ (67.4% v/v), adjusted to an apparent pH of 5.25 with H₂PO₄. Bile acids were quantified by measuring their absorbance at 205 nm and identified by matching their relative retention times with those of known standards.

Total bile acids were quantified with a calorimetric assay (Diazyme) using standards prepared with Na-tauro-CA. For biliary bile acids, samples were diluted at 1:1,000, and 10 μl was assayed. Bile acid amounts were calculated by multiplying the concentrations per microliter with the volume of bile in each gall bladder.

RNA isolation and quantitative real-time PCR. Liver samples (approximately 100 mg) were removed from mice and immediately flash frozen in liquid nitrogen and then stored at –80°C. Frozen tissue was homogenized in QIAzol (QIAGEN) and extracted according to the manufacturer’s instructions. RNA was then treated with DNase (DNaseI; Ambion, Life Technologies, Thermo Fisher Scientific) for 1 hour at 37°C and re-extracted with phenol/chloroform/isoamyl alcohol (25:24:1, pH 6.6; Life Technologies, Thermo Fisher Scientific). cDNA was synthesized from 500 ng RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences, Thermo Fisher Scientific) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) standards were prepared from an aliquot from each cDNA reaction, which was then pooled. Standards were then diluted over a 3-log range (dilution range of 1:5, 1:10, 1:50, 1:100, 1:500, and 1:1,000). qRT-PCR was performed with primers designed to cross exon-exon boundaries using the Roche UPL primer design website (https://lifescience.roche.com/en_us/brands/universal-probe-library.html). qPCR was performed in triplicate for each sample in a 384-well format using Kapa LC480 SYBR Green Mix (Kapa Biosystems). qPCR was performed using a Lightcycler 480 (Roche), and concentrations were determined from the standard curve using the efficiency-corrected method (second derivative maximum, Roche). Relative quantification was determined by normalizing the expression of each gene to a housekeeping gene. Two housekeeping genes (36B4 and Tbp) were used to normalize gene expression data. The primer sequences were previously described (14), except for Zfp36l1 (forward: TTTCCACACACCCAGATCCT; reverse: TGAGCAACCTTGTACCCCTTGCC).
FXR agonist treatments and mRNA half-life determination in cultured cells. IHHs were seeded in 6-well plates and treated with 5 mg/ml actinomycin D (Sigma-Aldrich) dissolved in DMSO for various time intervals. All cells treated with actinomycin D for various time periods were harvested at the same time. CDCA (Sigma-Aldrich) or GW4064 (Sigma-Aldrich) was dissolved in vehicle (DMSO) and added to cells plated at 70% to 80% confluency. IHHs were treated at the concentrations and for the durations specified in the figure legends. Cells were then washed in PBS and harvested in QIAzol, and RNA was isolated using a miRNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Gene expression was determined as described above. The primer sequences were previously described (14), except for huZFP36L1 (forward: GATGACCACCAACCTCGT; reverse: TCTGGGAGCCTATATGCTGACATC).

Western blot analysis. Liver samples (approximately 100 mg) were homogenized in 1 ml RIPA buffer supplemented with protease inhibitor complex (Cell Signaling Technology). Protein was quantified using the BCA assay (Thermo Fisher Scientific), and 50 μg protein was used for Western blot analysis. PVDF membranes (EMD Millipore) were probed with antibodies overnight, and HRP detection was performed using ECL Reagent (Sigma-Aldrich) or ECL Prime (GE Healthcare) according to the manufacturer’s instructions. Anti-ZFP36L1 antibody (BRF1/2, catalog 2119; Cell Signaling Technology; 1:1,000); anti-CYP7A1 antibody (catalog MABD42; Sigma-Aldrich; 1:1,000); protein disulfide isomerase (PDI) (catalog 3501; Cell Signalling Technology; 1:1,000); and secondary anti-rabbit HRP-conjugated antibody (GE Healthcare; 1:10,000) were used. HRP signal detection was determined electronically using an Amersham Imager 600UV system (GE Healthcare), and exposure was determined using GE software strictly set below the saturation point.

Promoter and UTR reporter analysis. A mouse Zfp36l1 promoter (2 kb) luciferase reporter construct was generated by amplifying these regions from mouse genomic DNA using KAPA HiFi polymerase (Kapa Biosystems) and cloning them into a pGL4.10[luc2] plasmid (Promega). Mouse or human Cyp7a1 UTR sequences were cloned into pcDNA3.1 (+) containing luciferase (luc2 gene; Promega) inserted at the beginning of the multiple cloning site. Luciferase reporter constructs were transfected using Fugene HD (Promega), according to the manufacturer’s instructions, into human IHHs plated onto 48-well dishes (n = 6 wells/condition). After 24 hours, medium was replaced, and for promoter reporter experiments, cells were treated with either vehicle (DMSO) or GW4064 (1 μM in DMSO) in medium containing 10% charcoal-stripped serum (Omega Scientific) for a further 24 hours.

Diet feeding studies and metabolic analysis. The Western-style diets (high-fat, high-cholesterol; catalog D12451) were purchased from Research Diets. CA-containing diets (0.5%) and global control diets were purchased from Envigo (Custom Diets). Body composition was determined weekly using NMR (Bruker Minispec) by measuring body fat mass and lean mass. For metabolic assessment, the comprehensive laboratory animal monitoring system (CLAMS); Columbus Instruments). Data for VO₂, VCO₂, and RER were used to calculate energy expenditure, as described previously (38).

Food consumption and calorimetric analysis. Littermate Zfp36l1±/± and Zfp36l1−/− mice were fed a Western diet for 64 days and then individually housed for 2 days in cages containing metal grills to allow for acclimation. After 48 hours, mice were moved to a new clean cage with a metal grill, and food consumption was monitored every 24 hours for 48 hours. After 48 hours, fecal content was collected, dried, and weighed. Bomb calorimetric analysis of dried fecal samples was performed at the UT Southwestern Metabolic Phenotyping Core. Calorimeters were expressed per gram of feces and per total mass excreted for each animal.

Plasma and liver lipid analysis. Plasma lipids were analyzed as previously described (20). For hepatic lipid analysis, liver tissue was snap frozen in liquid nitrogen, and total lipids were extracted by a modified Folch extraction method (39). Briefly, 50–100 mg liver tissue was homogenized in methanol. Lipids were extracted overnight in chloroform/methanol (2:1, v/v) and filtered. The extraction tube was rinsed twice with chloroform/methanol (2:1, v/v) and combined with the filtrate. To induce phase separation, 0.043% magnesium chloride was added, and the upper aqueous phase and interface (containing salted out proteins) were removed. The lower organic phase was dried under a nitrogen stream. The dried lipid was reconstituted in 1% Triton X-100 in chloroform (3 ml per 0.1 g tissue) and dried down once more under a nitrogen stream. The dried lipid was reconstituted in 1.8% Triton X-100 in water for lipid quantification using a calorimetric triglyceride or cholesterol assay kit (Thermo Fisher Scientific). Liver sections were prepared from paraformaldehyde tissue fixed overnight at the UCLA Translational Pathology Core Laboratory (TPCL) and stained with H&E.

Plasma metabolites and enzymes. EDTA plasma samples from animals were stored at −80°C after collection. Plasma ALT and AST (Teco Diagnostics) and plasma glucose levels were determined according to the manufacturer’s instructions (Stanbio Laboratory). Plasma insulin levels were determined by ELISA (Alpco).

Statistics. All error bars represent the mean ± SEM. Significance was calculated using a 2-tailed, paired Student’s t test for comparison of 2 groups (Microsoft Excel) or a 1- or 2-way ANOVA (GraphPad Prism, GraphPad Software) for comparison of more than 2 groups. For dietary studies over time, we used a 2-way repeated-measures ANOVA (GraphPad Prism Software). A repeated-measures 2-way ANCOVA (SPSS Inc.) was performed for analysis of metabolic parameters, with body weight, fat mass, and lean mass as defined covariates. Where Mauchly’s test for sphericity failed, significance was calculated using the Greenhouse-Geisser correction. A P value of less than 0.05 was considered statistically significant.

Study approval. All animal experiments were performed according to NIH guidelines and were approved by the Office of Animal Research Oversight (OARO) at UCLA.

Author contributions

Study concept and design: TDAV; data acquisition: EJT, BLC, JC, AC, EL, PM, and TDAV; data analysis and interpretation: EJT, BLC, and TDAV; statistical analysis: BLC and TDAV; CLAMS: BLC and TS; mouse model: MT; technical support: JC, AC, EL, and PM; writing of the manuscript: EJT and TDAV; revision of the manuscript: EJT, TS, MT, and TDAV; study supervision: TDAV.

Acknowledgments

We thank Peter Edwards for his guidance and input on this manuscript. We also thank Peter Tontonoz and Steve Bensinger and the members of their laboratories (UCLA) for feedback and suggestions. We thank Tim Willson and David Deaton (GSK, RESEARCH ARTICLE

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Research Triangle Park, North Carolina, USA) for GSK2324; Bryan Goodwin and Helen Hartman (Pfizer, Collegeville, Pennsylvania, USA) for the Shp−/− mice; Richard Davis (UCLA) for help with gavage experiments; and Laurent Vergnes (UCLA) for the IHHs. EJT is supported by NIH grants HL118161 and HL136543; TS is supported by NIH grant HL128822; MT is supported by Bio-technology and Biological Sciences Research Council grants BB/J004472/1 and BB/J00152X/1; and TQDAV is supported by NIH grants HL122677 and DK112119 and in part by HL028481 and DK102559. TQDAV was also supported by the UCLA Clinical and Translational Science Institute (UL1TR000124); the UCLA/UCSD Diabetes Research Center (DK063491); and the American Heart Association (SDG18440015).

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