Cell Metabolism
Article

Hepatic Deficiency in Transcriptional Cofactor TBL1 Promotes Liver Steatosis and Hypertriglyceridemia

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DOI 10.1016/j.cmet.2011.02.011

SUMMARY

The aberrant accumulation of lipids in the liver ("fatty liver") is tightly associated with several components of the metabolic syndrome, including type 2 diabetes, coronary heart disease, and atherosclerosis. Here we show that the impaired hepatic expression of transcriptional cofactor transducin beta-like (TBL) 1 represents a common feature of mono- and multigenic fatty liver mouse models. Indeed, the liver-specific ablation of TBL1 gene expression in healthy mice promoted hypertriglyceridemia and hepatic steatosis under both normal and high-fat dietary conditions. TBL1 deficiency resulted in inhibition of fatty acid oxidation due to impaired functional cooperation with its heterodimerization partner TBL-related (TBLR) 1 and the nuclear receptor peroxisome proliferator-activated receptor (PPAR) α. As TBL1 expression levels were found to also inversely correlate with liver fat content in human patients, the lack of hepatic TBL1/TBLR1 cofactor activity may represent a molecular rationale for hepatic steatosis in subjects with obesity and the metabolic syndrome.

INTRODUCTION

Triglycerides (TGs) represent the most efficient form of energy storage in mammals. While storage of TGs in adipose tissue is essential for the maintenance of normal energy homeostasis, aberrant lipid accumulation in peripheral tissues, such as liver, pancreas, and skeletal muscle, is considered to be a key feature of severe metabolic disorders (Saltiel, 2001).

In humans, the aberrant accumulation of lipids in the liver (nonalcoholic fatty liver disease, NAFLD) has been reported in 15%–80% of normal and obese individuals, respectively (Ruhl and Everhart, 2004). As a hallmark of the metabolic syndrome, fatty liver conditions are tightly associated with insulin resistance and type 2 diabetes (Seppala-Lindroos et al., 2002) and can trigger a progressive cascade of lipid disorders, ranging from hepatic steatosis to nonalcoholic steatohepatitis (NASH), chronic liver disease, and eventually hepatocellular carcinoma (Park et al., 2010). Furthermore, fatty liver disease has recently been characterized as an independent risk factor for coronary heart disease and atherosclerosis in Western societies (Fabbrini et al., 2009; Gastaldelli et al., 2009).

However, while fatty liver has been connected with numerous impairments of energy homeostasis, the molecular determinants of fatty liver development remain largely unknown.

By wiring distinct transcriptional networks through gene-specific interactions with DNA-binding transcription factors, transcriptional cofactor complexes have been identified as critical checkpoints in the coordination of metabolic programs (Spiegelman and Heinrich, 2004), exemplified by nuclear receptor cofactors peroxisome proliferator-activated receptor coactivator (PGC) 1, receptor-interacting protein (RIP) 140, or the steroid hormone receptor coactivators (SRCs) and their roles in the development of hyperglycemia, hepatic steatosis, and hypertriglyceridemia (Berriel Diaz et al., 2008; Feige and Auwerx, 2007; Herzig et al., 2001; Yoon et al., 2001). In this respect,

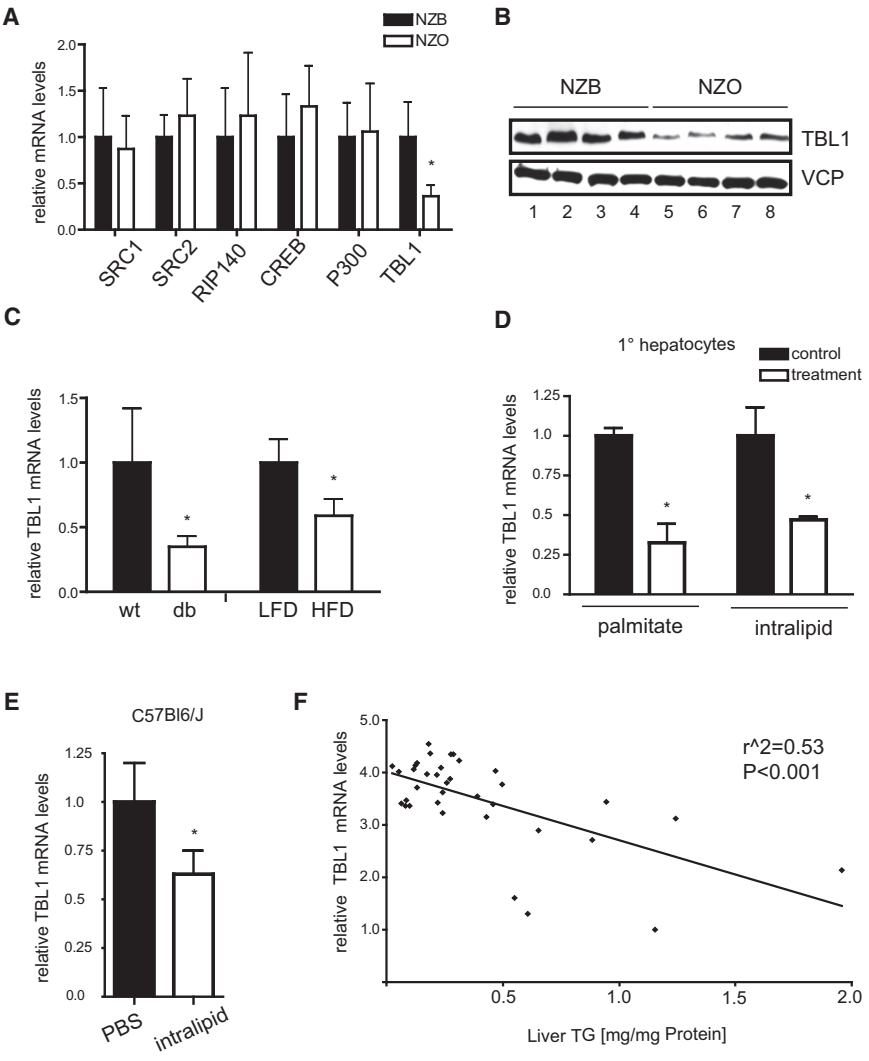

Figure 1. Hepatic Expression of TBL1 Is Reduced in Fatty Livers

(A) mRNA levels of indicated transcriptional cofactors in livers of obese NZO and wild-type NZB mice under random fed conditions ($n \geq 4$).

(B) Western blot of liver extracts from four representative NZO and NZB mice using TBL1 or VCP antibodies.

(C) TBL1 mRNA levels in livers of wild-type or obese db/db mice and wild-type mice fed a high-fat (HFD) or control diet (LFD, 60% and 10% calories from fat, respectively) for 16 weeks ($n \geq 6$).

(D) TBL1 mRNA levels in primary mouse hepatocytes treated with 100 μM palmitate or 5% intralipid for 24 hr ($n \geq 6$).

(E) TBL1 mRNA levels in livers of wild-type mice injected with 100 μl intralipid emulsion ($n = 6$) (means ± SEM). * indicates significance.

(F) Pearson correlation coefficient and P value shown for normalized TBL1 mRNA levels versus TG content in liver samples from human donors ($n = 37$). See also Figures S1 and S7.

particularly the differential gene expression of specific transcriptional regulators between healthy and metabolic disease conditions has been found to often reflect a causal, functional role of these factors in the pathogenesis of type 2 diabetes, cancer cachexia, and obesity, respectively (Berriel Diaz et al., 2008; Herzig et al., 2001; Yoon et al., 2001).

Consequently, we hypothesized that the dysregulation of distinct transcriptional cofactor gene expression under fatty liver conditions directly mirrors alterations of hepatic lipid homeostasis and may causally contribute to the fatty liver phenotype.

In this study, we identify the inhibition of transcriptional cofactor TBL1 gene expression as a conserved and functionally important checkpoint in the manifestation of hepatic steatosis. Originally cloned in relationship to an X-linked human disorder, ocular albinism with late-onset sensorineural deafness (OASD) (Bassi et al., 1999), TBL1 was subsequently identified as a component of a silencing mediator of retinoid and thyroid receptors (SMRT)/nuclear receptor corepressor (NCoR)/histone deacetylase (HDAC) 3 containing cofactor complex (Guenther et al., 2000; Yoon et al., 2005), involved in both gene repression and activation via distinct subsets of nuclear receptors (Perissi et al., 2004). The biological, and in particular, tissue-specific functions of TBL1 in the adult organism, however, remained unknown.

RESULTS

Hepatic TBL1 Expression Is Impaired in Fatty Liver Mouse Models and Negatively Correlates with Hepatic TG Content in Humans

In order to identify transcriptional cofactors involved in (dysfunctional) hepatic lipid homeostasis in obesity and/or the metabolic syndrome, we selectively investigated cofactors exemplifying nuclear receptor, inflammatory, and hormonal (cAMP) signaling in liver. In this context, we screened for differential cofactor expression in livers of New Zealand obese (NZO) and lean wild-type New Zealand black (NZB) mice, the former representing a multigenic model of obesity-related fatty liver disease and type 2 diabetes (Leiter and Reifsnyder, 2004). mRNA expression levels of p160 cofactors SRC1 and -2, RIP140, CREB-binding protein (CBP), and P300 remained unchanged between NZO and NZB mice under ad libitum fed conditions (Figure 1A). In contrast, hepatic mRNA, as well as protein expression of transcriptional cofactor TBL1, was significantly repressed in NZO mice (Figures 1A and 1B). To test whether the hepatic inhibition of TBL1 represents a more common feature of obesity-related dyslipidemia, we studied db/db as well as high-fat-diet-fed mice as independent standard models for monogenic and nutrition-induced obesity and type 2 diabetes, respectively (Clee and Attie, 2007; Stienstra et al., 2007). Indeed, TBL1 mRNA and protein levels were found to be reduced in livers of both obesity models as compared with controls (Figure 1C, see Figures S1A and S1B, available online).

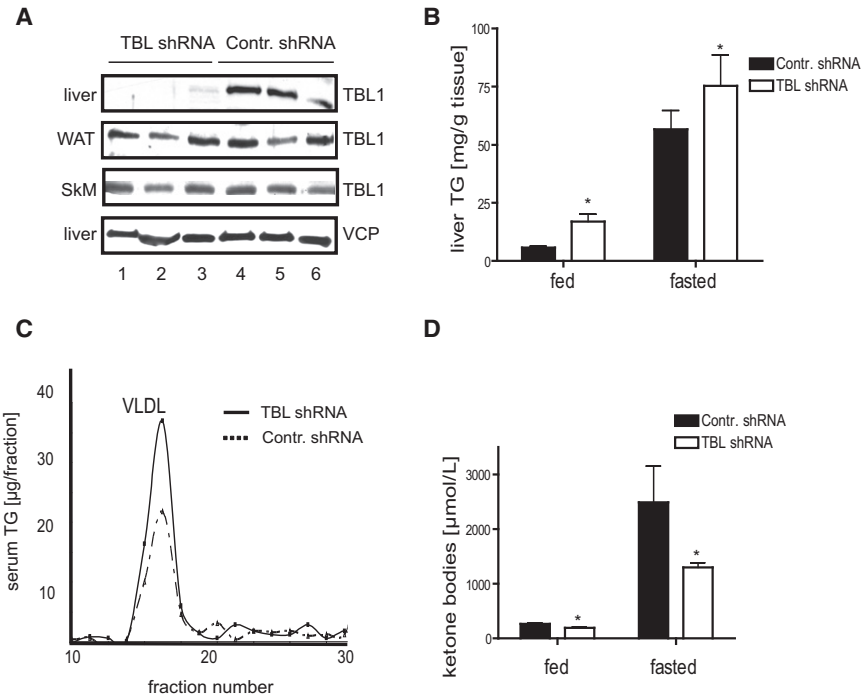

Figure 2. Liver-Specific TBL1 Deficiency Promotes Hepatic Steatosis

(A) Western blot of liver, abdominal WAT, and gastrocnemius muscle (SkM) extracts from fed representative TBL1 (lanes 1–3) or control (lanes 4–6) shRNA adenovirus-injected C57Bl6 mice 7 days after injection using TBL1 and VCP antibodies.

(B–D) Liver (B), lipoprotein-associated serum TG (C), and total serum ketone body (D) levels in the same mice as in (A) ($n \geq 7$) (means ± SEM). * indicates significance. See also Figure S2.

To identify potential triggers of TBL1 gene inhibition under obesity-related conditions, we next monitored TBL1 mRNA levels in response to hormonal stimulation of isolated primary mouse hepatocytes. Neither insulin, cAMP agonist forskolin, nor a glucocorticoid analog affected TBL1 mRNA expression in these cells (data not shown). However, exposure of primary hepatocytes to either palmitate or intralipid emulsion significantly inhibited TBL1 mRNA levels (Figure 1D), showing that fatty acids can impair TBL1 expression in a cell-autonomous manner. Consistent with a fatty acid-sensitive control of TBL1 gene expression, intragastric infusion of a lipid emulsion into wild-type mice also led to a reduction of hepatic TBL1 mRNA and protein expression after 6 hr (Figure 1E, Figure S1C).

Importantly, reduced TBL1 mRNA levels significantly correlated with increased liver TG content in a human patient cohort (Figure 1F, Figures S1D and S7), further substantiating the notion that the impairment of TBL1 expression highlights an unanticipated and conserved feature of steatotic liver metabolism in mice and humans.

Liver-Specific TBL1 Deficiency Promotes Hepatic Steatosis and Hypertriglyceridemia

To address the liver-specific function of TBL1, we disrupted the activity of TBL1 in livers of lean wild-type mice by delivering an adenovirus expressing TBL1-specific or nonspecific control shRNA via tail vein injection. TBL1 shRNA treatment significantly reduced hepatic TBL1 mRNA and protein levels as compared with control shRNA-injected littermates under both fasting and fed conditions (Figure 2A, Figures S2A and S2B), while TBL1 expression in abdominal white adipose tissue (WAT) and skeletal muscle (SkM) remained unaffected (Figure 2A). At day 7 after injection, acute knockdown of TBL1 caused no major differences in serum cholesterol levels (Figure S2C), blood glucose and fed serum insulin levels (Figures S2D and S2E), circulating nonesterified fatty acids (FFAs) (Figure S2F), body weight (Figure S2G), total body fat content (Figure S2H), and markers of liver damage (Figure S2I) compared with controls. In contrast, loss of hepatic TBL1 resulted in a significant increase in fasting liver cholesterol levels (Figure S2C) and particularly in hepatic and very-low-density-lipoprotein (VLDL)-associated serum TG levels in the fed and fasted states (Figures 2B and 2C, Figure S2K), while cholesterol levels in the fed liver and lipoprotein-associated serum cholesterol levels remained unaffected (Figures S2J and S2L), indicating that TBL1 predominantly prevents hepatic TG accumulation in wild-type animals. Further supporting these mouse data, hepatic TBL1 mRNA levels also negatively correlated with serum TG levels in humans (Figure S2M).

Liver TG stores are determined by the relative balance of lipid uptake and release, de novo TG formation (lipogenesis), and fatty acid combustion via β-oxidation (Postic and Girard, 2008). To determine whether changes in the fatty acid oxidation pathway contribute to the observed phenotype, we measured the circulating levels of total ketone bodies in control and TBL1-depleted animals. Consistent with higher hepatic TG content, mice deficient in hepatic TBL1 showed decreased levels of ketone bodies in the serum (Figure 2D), indicating reduced hepatic oxidation of fatty acids. In congruence, genomic expression profiling revealed particularly decreased expression of genes in the mitochondrial and peroxisomal fatty acid oxidation pathways (Figure S2N), indicating that disruption of TBL1 expression promotes steatosis by specifically blunting hepatic fatty acid combustion. Indeed, hepatic VLDL release remained unaffected upon hepatic TBL1 deficiency (Figure S2O).

To confirm the results from acute knockdown experiments (Figures 2A–2D) in an independent setting, wild-type mice were injected with an adeno-associated virus (AAV) expressing a TBL1-specific miRNA or a nonspecific control miRNA under the control of a hepatocyte-specific promoter, allowing the expression of inhibitory miRNAs specifically in liver parenchymal cells but not in other liver cell types for a period of several months (Figure S3A; data not shown). After 12 weeks, TBL1 miRNA delivery led to a more than 50% decrease in hepatic TBL1 mRNA levels (Figure S3B) and almost completely abolished TBL1 protein expression in liver as compared with control

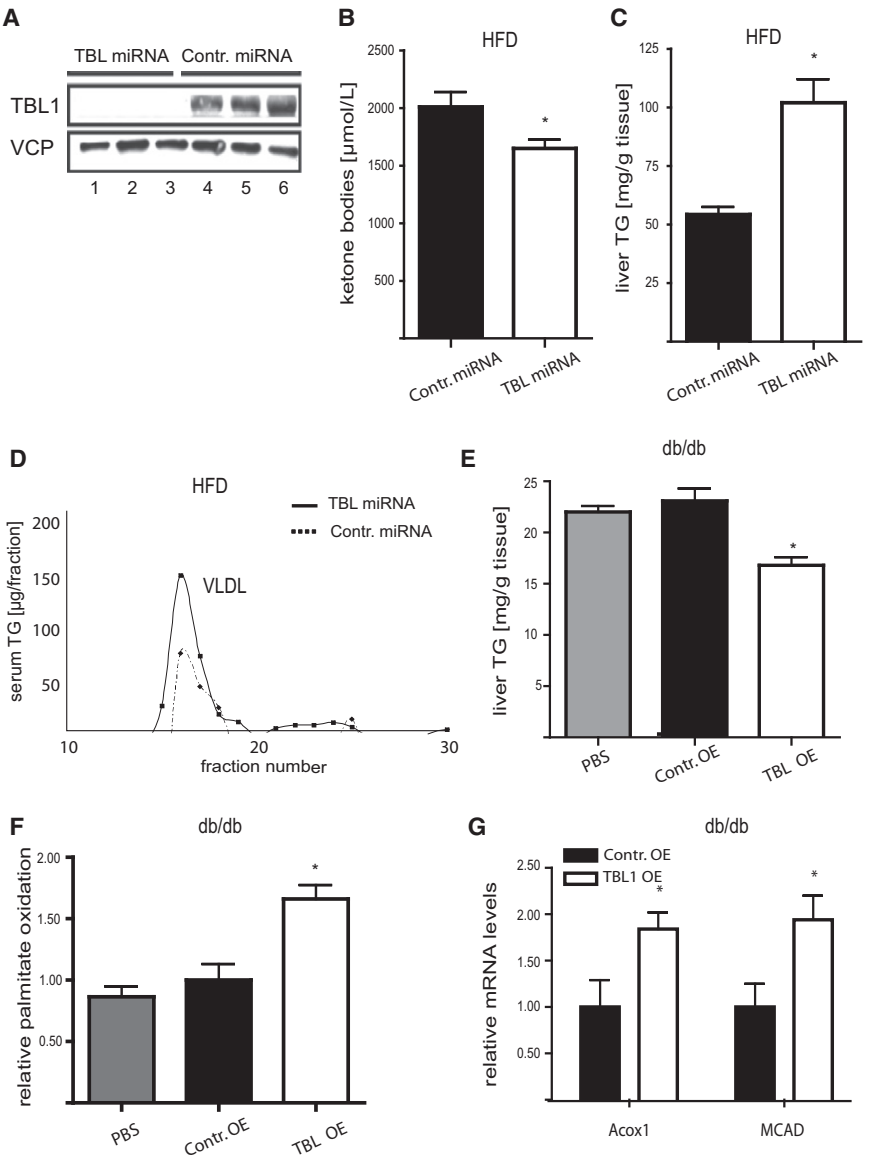

Figure 3. Long-Term Hepatic TBL1 Deficiency Aggravates Liver Lipid Accumulation under High-Fat Diet Conditions

(A) Western blot of liver extracts from representative TBL1 (lanes 1–3) or control (lanes 4–6) miRNA AAV-injected C57Bl6 mice 3 months after injection using TBL1 and VCP antibodies. Mice were kept on a high-fat (HFD) diet (60% calories from fat) for 12 weeks ($n \geq 8$).

(B–D) Total serum ketone body (B), liver TG (C) levels, and TG content of serum fractions by FPLC (D) in the same mice as in (A) ($n \geq 8$). Animals were analyzed in the fasted state.

(E) Liver TG levels after injection of TBL1 overexpression virus in db/db animals ($n = 7$) for 7 days under fed conditions.

(F) Palmitate oxidation determined in liver explants from the same mice as in (E) ($n = 21$).

(G) mRNA levels of fatty acid oxidation genes in livers of the same mice as in (E) ($n = 7$) (means ± SEM). * indicates significance. See also Figure S3.

miRNA- or PBS-injected littermates (Figure S3C and data not shown). Long-term inactivation of TBL1 triggered hepatic TG accumulation (Figure S3D) and an increase in VLDL-associated TG (Figure S3E), while leaving liver (Figure S3F) and serum cholesterol (Figure S3E) as well as blood glucose levels unaffected (Figure S3G). Furthermore, AAV delivery caused no obvious signs of liver damage or hepatic inflammation as determined by unchanged alanine aminotransferase (ALT) serum levels and liver cytokine expression, respectively, when compared with PBS-injected controls (Figure S3P and data not shown).

Chronic excess of caloric intake is the major prerequisite for the worldwide increase in obesity prevalence (Shoelson et al., 2007), prompting us to next explore the effects of hepatic TBL1 deficiency on energy metabolism under long-term high-fat dietary conditions. To this end, AAV miRNA-injected mice were placed on a high-fat (60% calories from fat) diet for 12 weeks. Consistent with results from acute and chronic TBL1 loss-of-function data under chow-fed conditions (Figures 2A–2D, Figures S3B–S3G) and correlating with significantly decreased serum ketone body levels (Figure 3B), hepatic TBL1 deficiency (Figure 3A, Figure S3H) triggered a significant increase in hepatic TG and serum VLDL TG levels under high-fat diet feeding (Figures 3C and 3D), but left other metabolic parameters generally unaffected (Figures S3I–S3L). These data further underlined the notion that hepatic TBL1 is required for the protection against hypertriglyceridemia and hepatic TG overload, and that TBL1 deficiency aggravates fatty liver development and hypertriglyceridemia even under conditions of chronic energy surplus. Indeed, overexpression of TBL1 in livers of steatotic db/db mice (Figure S3M) was sufficient to reduce liver TG levels (Figure 3E, Figure S3N) and to induce hepatic fatty acid oxidation and expression of peroxisomal as well as mitochondrial fatty acid oxidation enzyme genes in these animals (Figures 3F and 3G), while showing no effect on circulating total serum TG levels under these conditions (Figure S3O).

Hepatic TBL1 Acts in Concert with TBLR1 to Regulate Liver Lipid Metabolism

We next sought to explore the mechanistic basis for TBL1 function in lipid homeostasis. Therefore, we acutely disrupted the activity of its highly related heterodimerization partner TBLR1 (Guenther et al., 2000; Yoon et al., 2005) by adenoviral delivery of a TBLR1-specific shRNA into fasted or fed wild-type mice. Noteworthily, in contrast to TBL1, TBLR1 mRNA levels remained unchanged under steatotic conditions in animal models or upon lipid exposure of primary hepatocytes (Figure S1A–S1C).

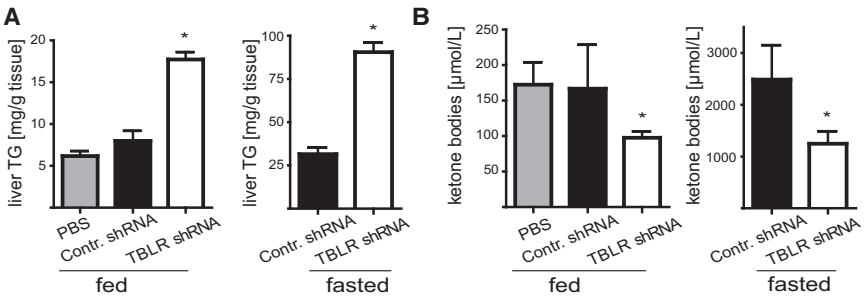

Figure 4. Knockdown of Hepatic TBLR1 Phenocopies Liver TBL1 Deficiency

(A and B) Liver TG (A) and total serum ketone body (B) levels in control or TBLR1 shRNA adenovirus-injected C57Bl6 mice 7 days after injection under fed and fasted conditions ($n \geq 7$) (means ± SEM). * indicates significance. See also Figure S4.

shRNA-mediated loss of hepatic TBLR1 (Figures S4A and S4B) produced a phenocopy of TBL1 deficiency, as it elevated liver TG and serum VLDL TG levels by 2- to 3-fold (Figure 4A, Figures S4C and S4E), reduced circulating ketone body levels (Figure 4B), and had no major impact on the other metabolic parameters tested (Figures S4D–S4I). Also, long-term inactivation of TBLR1 by AAV-mediated miRNA delivery (Figure S4J) resulted in liver and serum TG accumulation (Figure S4K and S4L) and decreased ketone body levels (Figure S4M), leaving liver and serum cholesterol levels as well as blood glucose, serum insulin, and liver damage marker levels unaffected (Figure S4L, data not shown).

These results suggested that TBL1 and TBLR1 fulfill similar roles in hepatic lipid metabolism. Consequently, we performed double knockdown experiments in wild-type mice by coinjection of TBL1 and TBLR1 shRNA adenoviruses (Figure 5A, Figure S5A). In contrast to the 2- to 3-fold elevation of hepatic TG levels upon single TBL1 or TBLR1 knockdown, respectively, simultaneous ablation of both TBL1 and TBLR1 resulted in an up to 9-fold increase in liver TG (Figure 5B, Figures S5B and S5C). Conversely, combined overexpression of TBL1 and TBLR1 in primary hepatocytes from steatotic db/db mice significantly reduced the cellular TG content of these cells (Figure 5C, Figure S5D), verifying the regulatory function of TBL1/TBLR1 for hepatic lipid homeostasis in a cell-autonomous manner. In line with the reduction of ketone body levels upon TBL1/TBLR1 single and double knockdown (Figure 5D), palmitate oxidation rates in livers from TBL1/TBLR1-deficient animals were diminished (Figure 5E). Consistently, gene expression profiling revealed that TBL1/TBLR1 knockdown particularly blunted the expression of genes in the mitochondrial and peroxisomal fatty acid oxidation pathway and concomitantly led to increased expression of lipogenic genes as compared to controls (Figure 5F). Consistent with the reduced expression of hepatic VLDL/ApoB reuptake transporters (Narvekar et al., 2009; Yen et al., 2008) (Figure S5E), clearance of human VLDL from serum (Figure S5F) and lipid clearance after intravenous lipid infusion (Figure S5G) were both impaired upon hepatic TBL1/TBLR1 deficiency. In congruence, hepatic TBL1/TBLR1 deficiency caused an almost 3-fold elevation of serum TG levels when compared to control shRNA- or PBS-injected littermates (Figure S5H). These results demonstrated that the TBL1/TBLR1 cofactor complex synergistically exerts a critical regulatory function in the prevention of hepatic steatosis and hypertriglyceridemia. Interestingly, hepatic steatosis induced by TBL1/TBLR1 deficiency did not impact intrahepatic insulin signaling strength (Figure S5I), but rather mildly improved systemic glucose clearance and insulin sensitivity in an oral glucose and insulin tolerance test, respectively (Figure 5G).

TBL1/TBLR1 Cooperate with Nuclear Receptor PPARα

Thus far, our results suggested that the antisteatotic effect of the hepatic TBL1/TBLR1 cofactor complex predominantly relies on its activating function for genes involved in fatty acid oxidation (Figure 5F). The nuclear receptor PPARα has been identified as a master regulator of fatty acid oxidation in the liver (Kersten et al., 1999), prompting us to test whether the TBL1/TBLR1 complex might serve as a physiological coactivator for PPARα in this context. Inhibition of TBL1 expression in primary mouse hepatocytes reduced the induction of prototypic PPARα target genes upon treatment of the cells with a PPARα-specific ligand (Figure 6A), demonstrating that TBL1 is indeed required for full PPARα transcriptional activity in a cell-autonomous manner. In agreement, overexpression of TBL1/TBLR1 in primary db/db hepatocytes activated the expression of PPARα target genes in the presence and absence of PPARα ligand (Figure 6B), and single TBL1 overexpression caused the induction of the same genes in db/db livers in vivo (Figure 6C).

Next, we employed our TBL1/TBLR1 double knockdown strategy in PPARα knockout mice (Kersten et al., 1999). Consistent with previous reports (Rakhshandehroo et al., 2010), PPARα-deficient animals displayed elevated hepatic and serum TG levels as well as diminished circulating ketone body levels as compared with wild-type littermates (Figures 6D and 6E, Figures S6A and S6B). As shown above, TBL1/TBLR1 double deficiency produced a similar phenotype as PPARα loss of function in wild-type mice (Figures 6D and 6E, Figures S6A–S6E), while exerting no significant effect in PPARα knockout animals in terms of serum and hepatic TG levels (Figures 6D and 6E) as well as circulating ketone bodies (Figure S6B). This substantiated the hypothesis that TBL1/TBLR1 serve as coactivators for the PPARα transcriptional complex in hepatic lipid homeostasis. Additional TBL1/TBLR1 loss-of-function experiments in PPARα/PPARβ/δ double knockout mice (Bedu et al., 2007) produced identical results to the PPARα single knockout model (Figure S6F), underlining the specific importance of the PPARα isoform for TBL1/TBLR1 action in this context.

To verify the functional connection between TBL1/TBLR1 and PPARα independently, we performed chromatin immunoprecipitation assays using liver extracts from wild-type and PPARα knockout mice. Remarkably, prototypic PPARα target gene promoters (cytochrome P450 [CYP] 4A10 and CYP4A14) were

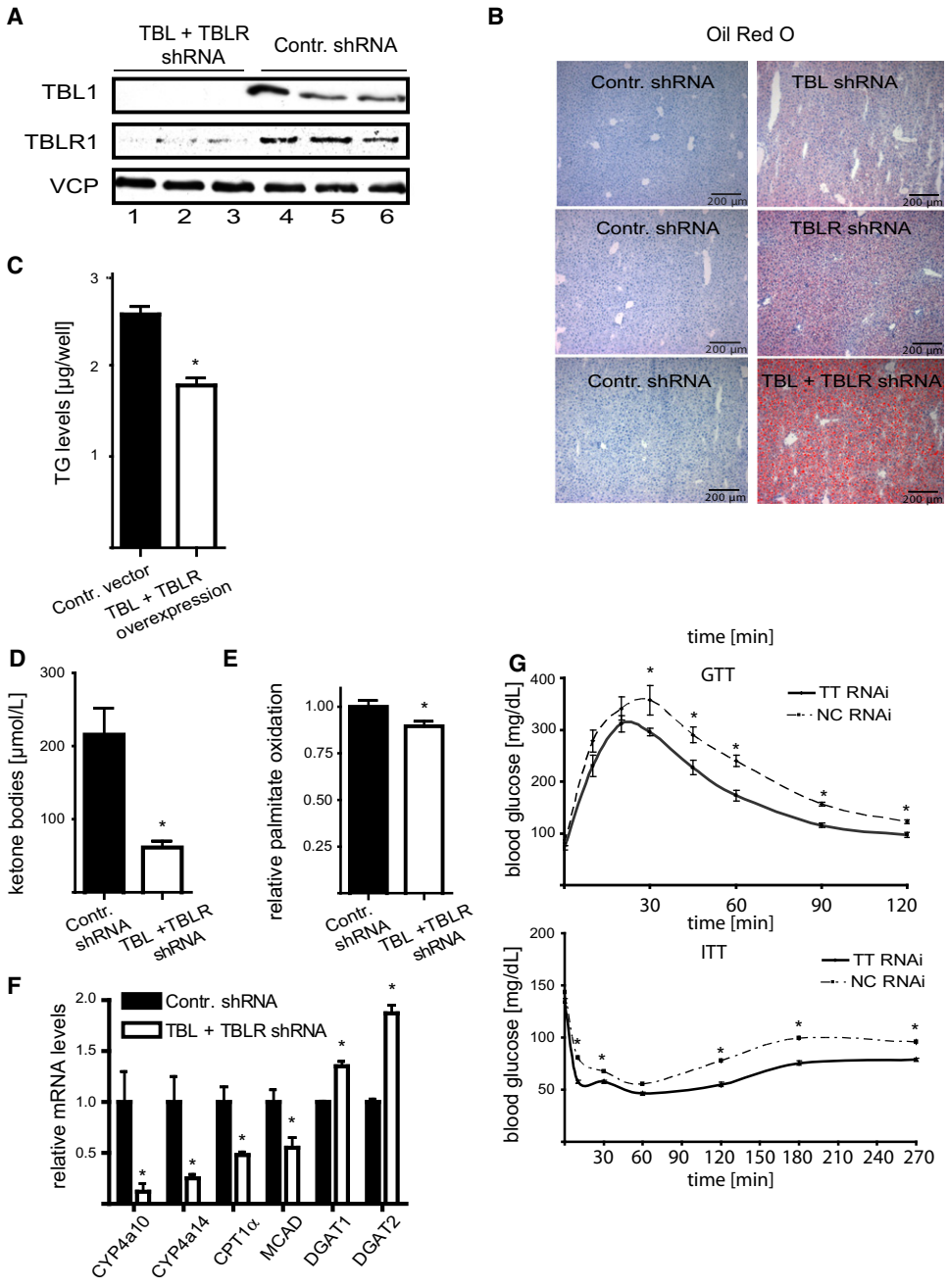

Figure 5. TBL1 Controls Hepatic Steatosis in Synergism with TBLR1

(A) Western blot of liver extracts from representative TBL1/TBLR1 (lanes 1–3) or control (lanes 4–6) shRNA adenovirus-injected C57Bl6 mice 7 days after injection under fed conditions using TBL1, TBLR1, and VCP antibodies.

(B) Liver sections from representative C57Bl6 mice under fed conditions injected with either control, TBL1, TBLR1, or both TBL1/TBLR1 shRNA adenoviruses 7 days after injection stained with oil red O. Lipids stain positive (red color) with oil red O.

(C) TG levels in primary mouse hepatocytes from db/db mice transfected with TBL1 and TBLR1 overexpression plasmids for 48 hr (n = 12).

(D) Total serum ketone body levels in the same mice as in (A) ($n \geq 7$).

(E) Palmitate oxidation in liver slices from TBL1/TBLR1 or control shRNA adenovirus-injected C57Bl6 mice (n = 18).

(F) mRNA levels of fatty acid oxidation and lipogenic genes in livers of the same mice as in (A) ($n \geq 7$).

(G) Glucose tolerance test (GTT) and insulin tolerance test (ITT) in TBL1/TBLR1 (TT) or control shRNA (NC) adenovirus-injected C57Bl6 mice 7 days after injection (means ± SEM). * indicates significance. See also Figure S5.

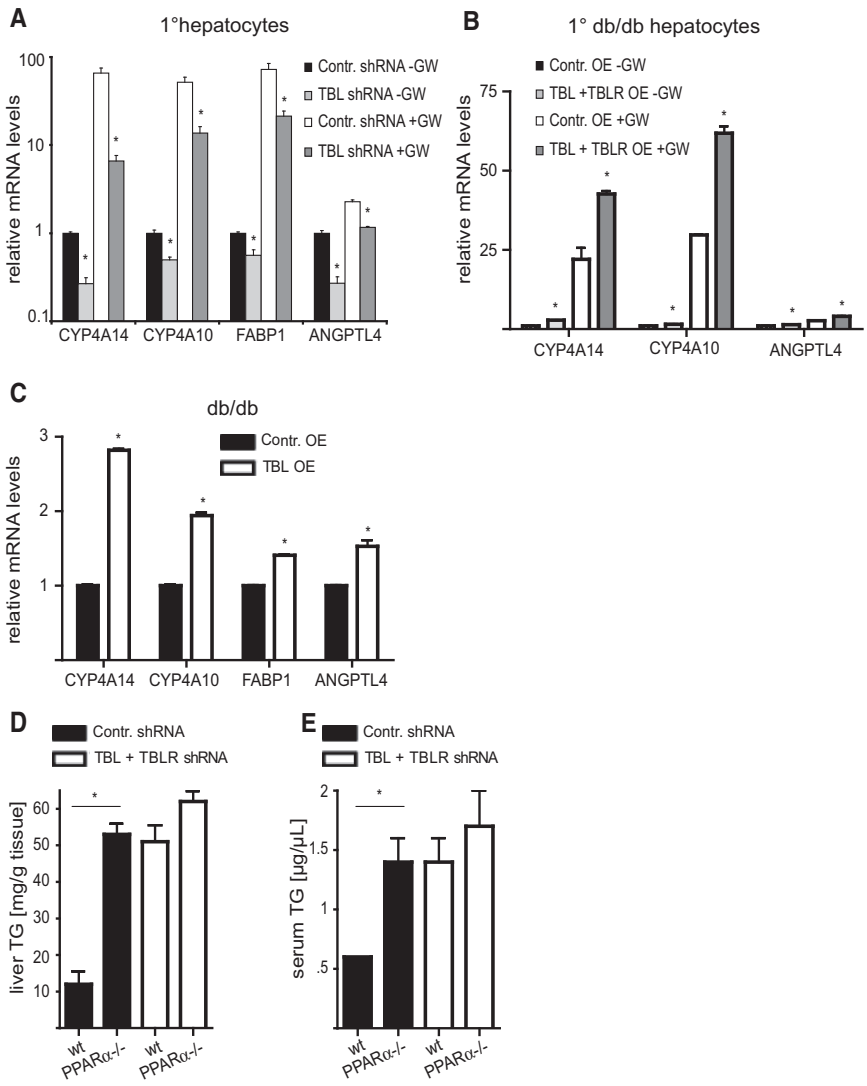

Figure 6. TBL1/TBLR1 Regulate Liver Lipid Metabolism via PPARα

(A) mRNA levels of fatty acid oxidation genes in primary mouse hepatocytes infected with control or TBL1 shRNA adenovirus (moi = 75). Twenty-four hours after infection, cells were treated with the PPARα ligand GW7647 (1 μM) for 24 hr before harvesting ($n \geq 4$).

(B) mRNA levels of fatty acid oxidation genes in primary db/db hepatocytes infected with control or TBL1/TBLR1 overexpression adenovirus (moi = 10). Twenty-four hours after infection, cells were treated with the PPARα ligand GW7647 (1 μM) for 24 hr before harvesting ($n \geq 6$).

(C) mRNA levels of fatty acid oxidation genes in db/db animals injected with control or TBL1 overexpression adenovirus 7 days after injection ($n = 7$).

(D and E) Liver (D) and serum (E) TG levels in wild-type (WT) and *PPARα*$^{-/-}$ mice injected with either control or TBL1/TBLR1 shRNA adenoviruses 7 days after injection under fed conditions ($n \geq 7$). See also Figure S6. Means ± SEM. * indicates significance.

efficiently recovered from liver immunoprecipitates using TBL1 and TBLR1 but not control IgG antibodies in wild-type livers (Figure 7A). TBL1 and TBLR1 promoter binding was largely reduced in PPARα knockout mice (Figure 7A), showing that the TBL1/TBLR1 coactivator complex is directly recruited to metabolic target gene promoters in a PPARα-sensitive manner in vivo. Indeed, TBL1 and TBLR1 were found to physically interact with PPARα in GST pull-down (Figure 7B) as well as in coimmunoprecipitation studies of H4IIE hepatocytes (Figure 7C). Furthermore, abrogation of TBL1 and TBLR1 substantially impaired the activity of a PPARα-driven reporter gene in Hepa1c1 hepatocytes (Figure 7D) and led to an increased recruitment of the NCoR/HDAC3 corepressor complex to PPARα fatty acid oxidation target genes in livers of mice, accompanied by decreased histone H4 acetylation at these promoter sites (Figure 6E). In parallel to the increased recruitment of corepressor complex components (i.e., HDAC3) to PPARα target promoters, TBL1/TBLR1 deficiency triggered the release of known PPARα coactivators (i.e., Sirtuin1, Baf60a) from these promoter sites in cultured Hepa1c1 hepatocytes (Figure 6F), demonstrating a role for TBL1/TBLR1 in the PPARα corepressor/coactivator exchange on fatty acid oxidation genes in a cell-autonomous manner.

DISCUSSION

TBL1 was originally cloned in relationship to an X-linked human disorder, OASD, in which deletion of TBL1 was suggested to be responsible for the hearing defect (Bassi et al., 1999). Along with the highly homologous TBLR1 protein, TBL1 was subsequently identified as a component of a nuclear receptor SMRT/NCoR/HDAC3 corepressor complex (Guenther et al., 2000; Yoon et al., 2005).

Our data now provide insights into tissue-specific functions of the TBL1/TBLR1 cofactor complex with further implications for the pathogenesis of obesity-related NAFLD. The results of our studies are consistent with a model in which hyperlipidemic conditions are correlated with the downregulation of hepatic TBL1 gene expression, as shown for monogenic (db/db) and multigenic (NZO and high-fat diet) mouse obesity models, as well as for human liver samples. An increased exposure of the liver to lipids/fatty acids might at least in part explain the TBL1 inhibition under these conditions, although other and/or additional stress signals might also contribute to the control of TBL1 gene transcription. In any case, TBL1 inhibition is sufficient to partly block hepatic fatty acid oxidation and to promote liver TG accumulation, thereby establishing a more permanent inhibitory loop for TBL1 gene expression and further aggravating hepatic steatosis under these conditions.

Intriguingly, the steatosis caused by liver-specific inactivation of TBL1/TBLR1 did not impair components of the hepatic insulin

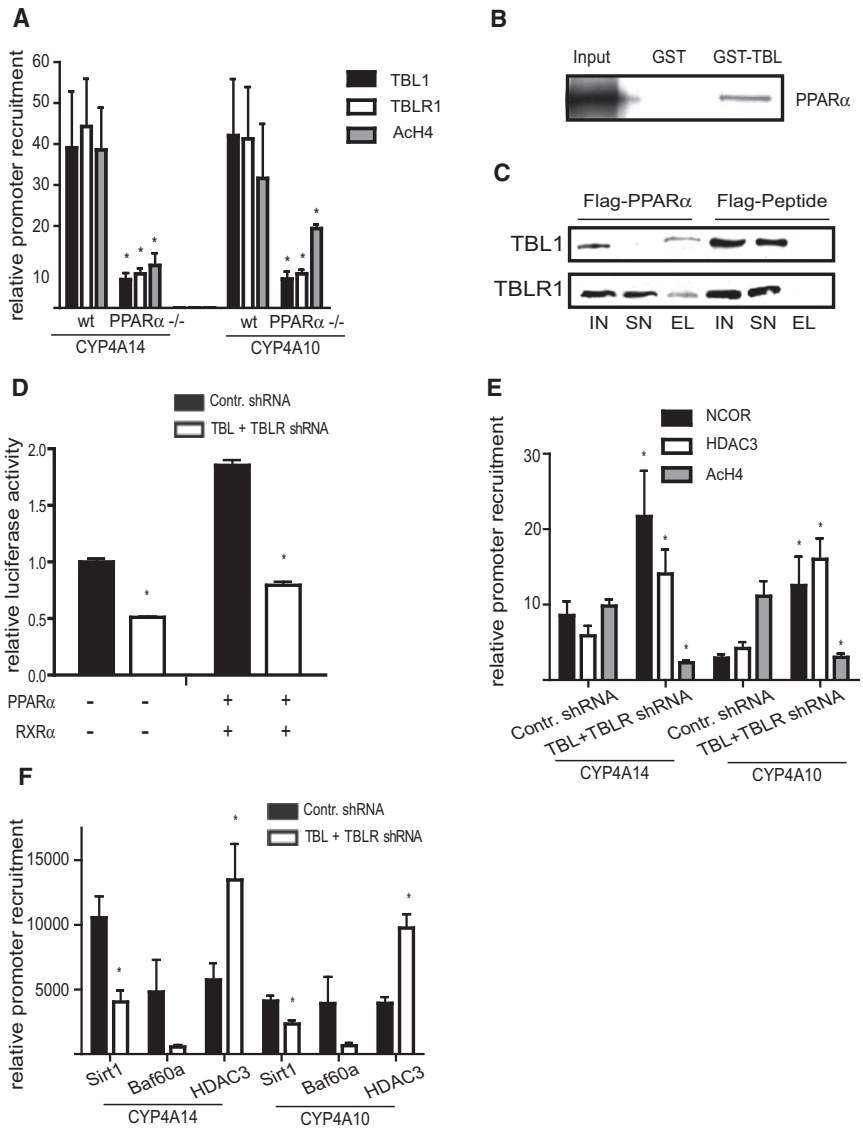

Figure 7. TBL1/TBLR1 Regulate Liver Lipid Metabolism via Direct Interaction with PPARα

(A) Chromatin immunoprecipitation (ChIP) assay of liver extracts from fasted wild-type or *PPARα*$^{-/-}$ mice using TBL1, TBLR1, and acetylated histone H4 (AcH4)-specific antibodies. Precipitated fragments were analyzed by real-time PCR using promoter primers against CYP4A10 and CYP4A14. Data show recruitment relative to IgG and chromatin input ($n \geq 5$).

(B) Pull-down assays performed with full-length TBL1 fused to GST and GST alone as a control. GST fusion proteins were incubated with in vitro translated full-length PPARα. Bound proteins were resolved by SDS-PAGE and visualized by autoradiography. Input lanes represent 20% of the input.

(C) Coimmunoprecipitation of TBL1, TBLR1, and PPARα from H4IIE hepatocytes transfected with Flag-PPARα or an empty vector expressing the Flag peptide alone using an anti-Flag M2 antibody. Bound proteins were resolved by SDS-PAGE and subsequently detected by western blot. IN, input; SN, supernatant; EL, elution.

(D) Transient transfection assay of Hepa1c1 hepatocytes cotransfected with a 3xPPRE-Luc reporter plasmid (containing PPAR response elements) together with plasmids encoding PPARα, retinoid X receptor (RXR)α, and TBL1/TBLR1-specific or nonspecific control shRNA constructs as indicated.

(E) ChIP assay using liver extracts of wild-type mice injected with either control or TBL1/TBLR1 shRNA adenoviruses as in a using antibodies specific for NCOR, HDAC3, AcH4, and nonspecific IgG. Precipitated fragments were analyzed by real-time PCR as in (A). Data show recruitment relative to IgG and chromatin input ($n \geq 5$).

(F) ChIP assay using Hepa1c1 hepatocytes transfected with Flag-tagged cofactors or an empty vector expressing the Flag peptide alone, infected with either control or TBL1/TBLR1 shRNA adenoviruses using Flag agarose. Precipitated fragments were analyzed by real-time PCR as in (A). Data show recruitment relative to Flag peptide and chromatin input (means ± SEM). * indicates significance.

signaling axis but rather led to the improvement of systemic glucose tolerance and insulin sensitivity. While hepatic steatosis is tightly associated with insulin resistance in humans and animals (Angulo and Lindor, 2001; Marchesini et al., 1999), the causal and potentially mutual links between fatty liver and insulin resistance remain to be defined. Whereas insulin resistance and the accompanying hyperinsulinemia have been described to trigger lipogenesis and liver lipid accumulation via the sterol regulatory element-binding (SREBP)-1c transcription factor pathway (Shimomura et al., 1999a, 1999b), studies on the influence of hepatic lipid accumulation on systemic insulin sensitivity resulted in ambiguous conclusions: while hepatic overexpression of lipoprotein lipase or glycerol-3-phosphate acyltransferase triggers hepatic steatosis and systemic insulin resistance (Kim et al., 2001; Nagle et al., 2007), transgenic mice overexpressing lipogenic acyl-CoA diacylglycerol acyltransferase genes specifically in the liver are characterized by hepatic lipid accumulation in the absence of impaired insulin sensitivity or dysfunctional glucose homeostasis (Monetti et al., 2007), indicating that hepatic steatosis under certain conditions can be dissociated from the occurrence of insulin resistance and its adverse effects on glucose metabolism.

Therefore, it is tempting to speculate that the inhibition of TBL1 gene expression in fatty livers and the concomitant impact on intrahepatic lipid accumulation may represent a protective rather than a harmful mechanism for overall systemic energy homeostasis under certain metabolic conditions that await further in-depth analysis in future studies.

The differential response of TBL1 and TBLR1 gene expression to fatty acid/lipid exposure and hyperlipidemic conditions suggests that TBL1 represents the main regulatory site in the TBL1/TBLR1-dependent lipid gene network. This hypothesis is indeed supported by both mouse and human data, as hepatic TBLR1 mRNA levels did not respond to acute and chronic lipid

exposure in animals or cells, nor did they correlate with hepatic and serum TG levels in human samples. Also, TBL1 gene knockdown did not impair TBLR1 gene expression directly (data not shown). However, due to the functional synergism between TBL1 and TBLR1 in terms of hepatic lipid homeostasis, reduced TBL1 levels may push the stoichiometry of the TBL1/TBLR1 transcriptional complex on lipid metabolizing target genes toward a functionally less favorable state, thereby determining the overall regulatory impact on liver lipid homeostasis and explaining the functional synergism of the TBL1/TBLR1 complex in this setting. Indeed, previous studies have shown that TBL1 and TBLR1 are mutually dependent on each other for their respective recruitment to DNA in specific gene promoter contexts (Li and Wang, 2008). As single TBL1 or TBLR1 gene knockdown also produced metabolic phenotypes that were not (fully) compensated by the other binding partner, both TBL1 and TBLR1 may additionally not only fulfill common but also specific roles in hepatic energy homeostasis. Future analyses of distinct and overlapping target gene networks are expected to provide insights into a potential functional specification within the TBL1/TBLR1 cofactor complex with respect to liver lipid handling and systemic energy homeostasis.

Although transcriptional cofactors exert their biological function by definition through the interaction with various DNA-binding partners within a given cellular context (Rosenfeld and Glass, 2001), a large portion of TBL1/TBLR1 effects in hepatic lipid handling seems to depend on PPARα, suggesting that this nuclear receptor represents a main mechanistic checkpoint for TBL1/TBLR1 action in this setting. Indeed, hepatic TBL1/TBLR1 deficiency produced a phenocopy of the PPARα gene knockout with respect to liver TG levels, and loss of PPARα was sufficient to significantly blunt the effects of TBL1/TBLR1 gene knockdown which was not further influenced by the additional absence of PPARβ/δ, thereby pointing toward a certain degree of TBL1/TBLR1 interaction partner preference within the PPAR transcription factor family. Though part of a corepressor complex, TBL1 and TBLR1 seem to also be necessary for the activation of distinct subsets of nuclear receptors and other transcription factor families through the recruitment of the ubiquitin conjugating/19S proteasome complex and the subsequent degradation of corepressors and attraction of coactivators (Perissi et al., 2004). In agreement with this model, absence of TBL1/TBLR1 indeed promoted an enrichment of NCoR and HDAC3 on PPARα target gene promoters involved in fatty acid oxidation, while triggering the release of known PPARα coactivators from these sites.

Together, our studies identify the TBL1/TBLR1 transcriptional coactivator complex as a critical regulatory checkpoint in the prevention of fatty liver development. Consequently, the loss of TBL1 expression in fatty livers as documented in both mouse models as well as human patient samples may represent an important determinant of susceptibility to NAFLD and obesity-related dyslipidemia.

EXPERIMENTAL PROCEDURES

Recombinant Viruses

Adenoviruses expressing TBL1- or TBLR1-specific or nonspecific shRNA under control of the U6 promoter or the TBL1 cDNA under control of the CMV promoter were cloned as described previously (Herzig et al., 2001, 2003). Viruses were purified by the cesium chloride method and dialyzed against phosphate-buffered-saline buffer containing 10% glycerol prior to animal injection.

For long-term inactivation of TBL1 and TBLR1, AAVs encoding TBL1 and TBLR1-specific miRNA under the control of a hepatocyte-specific promoter were established. For details, please see the Supplemental Information.

Animal Experiments

Male 8- to 12-week-old C57Bl6, db/db, NZO/NZB, and PPARα (−/−) mice were obtained from Charles River Laboratories (Brussels, BEL) and maintained on a 12 hr light-dark cycle with regular unrestricted diet. For starvation experiments, animals were fasted for 24 hr or random fed with free access to water. For adenovirus injections, 1×10^9 plaque-forming units (pfu) per recombinant virus were administered via tail vein injection. In each experiment, seven to eight animals received identical treatments and were analyzed under fasted or fed conditions as indicated. In high-fat diet experiments, C57Bl6 mice were injected with 5×10^{11} AAV particles per mouse and fed a high-fat diet (60% energy from fat, Research Diets D12492) for a period of 12 weeks. In additional experiments, PPARα (−/−) and PPARα/β/δ (−/−) double knockout mice (Bedu et al., 2007) were injected with recombinant adenoviruses as above.

For intralipid infusions, mice were fasted for 16 hr, and 100 μl of a 20% (v/v) intra-lipid emulsion in saline (Sigma, Munich, GER) was administered intravenously. Tissue was collected 6 hr after infusion and analyzed by quantitative PCR as described below. Insulin and glucose tolerance tests were performed as described previously (Michael et al., 2000).

Organs including liver, epididymal fat pads, and gastrocnemius muscles were collected after the corresponding time periods, weighed, snap-frozen, and used for further analysis. Total body fat content was determined by an Echo MRI body composition analyzer (Echo Medical Systems, Houston, TX). Animal handling and experimentation was done in accordance with National Institutes of Health (NIH) guidelines and approved by local authorities.

Blood Metabolites

Serum levels of glucose, insulin, TGs, cholesterol, total ketone bodies, and nonesterified fatty acids (NEFAs) were determined using an automatic glucose monitor (One Touch, Lifescan, Neckargemünd, DEU) or commercial kits (MP Biomedicals, Orangeburg, NY; Mercodia, Uppsala, SE; Sigma, Munich, DEU; RANDOX, Crumlin, NIR; WAKO, Neuss, DEU).

Fast Protein Liquid Chromatography

Serum from seven mice per experimental group was pooled and subjected to fast protein liquid chromatography as previously described (Lichtenstein et al., 2007). Cholesterol and TG were measured in the eluted fractions using commercial kits as above.

Hepatic VLDL Release

VLDL production was determined after tyloxapol (Sigma, Munich, DEU) injection as described (Mandard et al., 2006).

VLDL Clearance

Human VLDL was isolated from fasted serum samples by ultracentrifugation as described (Redgrave et al., 1975). Briefly, 3.5 ml serum was put in a polyallomer tube SW40Ti and mixed with 1.39 g KBr, overlayered with 3 × 2.8 ml of a NaCl/KBr solution (D = 1.063, 1.019, and 1.006 g/ml) and run for 18 hr at 40,000 rpm. Human VLDL (20 μg) was injected into each animal, and serum samples were taken at 2, 10, 30, 60, and 120 min. Serum human ApoB-100 levels were measured using a human-specific ApoB ELISA. For the ELISA, we used a primary coating antibody generated against human apoB-100 (mAb47, kindly supplied by J. Witztum, University of San Diego, USA), in a concentration of 2 μg/well IgG protein in TBS/EDTA/BHT and a secondary biotinylated polyclonal antibody raised in goat against human ApoB in a concentration of 4 ug/well in 1.5% BSA/TBS/0.1% Tween. To prevent nonspecific binding, plates were blocked with 1.5% BSA/TBS/0.1% Tween. Samples were diluted 1:25. Absorbance was read 30 min after addition of TMB and termination of the reaction with 2 M H2SO4 at 450 nm (Groot et al., 1991).

Tissue Lipid Extraction

Hepatic lipids were extracted as described previously (Herzig et al., 2003), and TG and total cholesterol content was determined using commercial kits as above. Values were calculated as milligrams (TG and cholesterol) per gram wet tissue.

Ex Vivo Palmitate Oxidation

Mice were dissected, and three 10–20 mg liver pieces were incubated in a 48-well plate containing Krebs Ringer buffer with 1% fatty acid free BSA, 5 mM glucose, and 1 mM palmitic acid [^{3}H palmitate, 5μCi/μL] (Perkin Elmer, Waltham, USA) for 2 hr at 37°C and 5% CO_2. The reaction was stopped by adding chloroform/methanol (2:1). Subsequently the aqueous phase was separated from the organic phase. The aqueous phase was analyzed in a β-counter. All chemicals if not mentioned otherwise were purchased from Sigma (Munich, DEU). All measurements were made in triplicates.

Quantitative Taqman RT-PCR

Total RNA was extracted from homogenized mouse liver or cell lysates using Qiazol reagent (QIAGEN, Hilden, DEU). cDNA was prepared by reverse transcription using the M-MuLV enzyme and Oligo dT primer (Fermentas, St. Leon-Rot, DEU). cDNAs were amplified using assay-on-demand kits and an ABIPRISM 7700 Sequence detector (Applied Biosystems, Darmstadt, DEU). RNA expression data were normalized to levels of TATA-box binding protein (TBP) RNA.

Protein Analysis

Protein was extracted from frozen organ samples or cultured hepatocytes in cell lysis buffer (Rose et al., 2007), and 20 μg of protein was loaded onto a 8% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane. Western blot assays were performed as described (Herzig et al., 2001) using antibodies specific for TBL1 (Abcam, Cambridge, UK), TBLR1 (Novus Biologicals, Littleton, USA), phospho Ser473 Akt, total Akt (Cell Signaling, Boston, USA), phospho Ser9 glycogen synthase kinase (GSK) 3, total GSK3 (Upstate, Lake Placid, NY), or valosin-containing protein (VCP) (Abcam, Cambridge, UK).

Histochemistry and Immunohistochemistry

For hematoxylin and eosin (H&E) and oil red O lipid staining, 5 μm cryosections of liver embedded in Tissue Tek OCT compound (Sakura, NL) were fixed in Baker's formol. Nuclei were counterstained with hematoxylin.

Chromatin Immunoprecipitation Assay

Liver tissue powder samples from fasted (24 hr) wild-type or PPARα (−/−) mice; from wild-type animals injected with control or TBL1/TBLR1 adenovirus; or from cellular lysates of Hepa1c1 cells transfected with Flag-tagged Sirtuin1, Baf60a, or HDAC3 were fixed with formaldehyde in PBS, and ChIP assays were performed as described (Canettieri et al., 2003) using antibodies specific for acetylated histone H4 (Upstate, Lake Placid, NY), TBL1, TBLR1, NCOR, HDAC3, unspecific IgG (Santa Cruz, Heidelberg, DEU), or Flag agarose (Sigma, Munich, DEU). Precipitated DNA fragments were analyzed by PCR amplification using primers directed against the murine cytochrome P450 (CYP), 4A10, and CYP4A14 gene promoters (SABiosciences, MD, USA).

Coimmunoprecipitation

H4IIE hepatocytes were cotransfected with a Flag-PPARα or an empty Flag vector. Subsequently, cells were lysed, centrifuged, and the supernatant incubated with anti-Flag M2 Agarose (Sigma, Munich, DEU) for 2 hr. The immunoprecipitates were subsequently analyzed by western blot as described.

GST Pull-Down

GST fusion proteins (pGEX5.1, pGEX5.1-GST_TBL1) were produced in BL21 cells and affinity purified using glutathione Sepharose (Amersham Biosciences, Darmstadt, DEU). In vitro transcription/translation was performed using the TNT T7/T3 quick coupled transcription/translation system (Promega, Mannheim, DEU) according to the manufacturer's instructions. GST and in vitro translated proteins were incubated at 4°C overnight. After extensive washing, GST-precipitated proteins were separated by SDS-PAGE and detected by autoradiography as described (Zschiedrich et al., 2008).

Plasmids and RNA Interference

Oligonucleotides targeting mouse TBL1 (5′-GCGAGGATATGGAACCTTAAT-3′) or TBLR1 (5′-GCATAAAGGTCCTATATTTGC-3′) were annealed and cloned into the pENTRY RNAi vector (Invitrogen, Karlsruhe, DEU). Nonspecific oligonucleotides (5′-GATCTGATCGACACTGTAATG-3′) with no significant homology to any mammalian gene sequence were used as nonsilencing controls in all experiments. The PPRE-Luc reporter plasmid and expression vectors for PPARα and retinoid X receptor (RXR) were kindly provided by M. Downes, La Jolla, CA. Expression vectors for Flag-tagged TBL1 and TBLR1 were generated by standard PCR-based methods and cloned into the pcDNA3.1 expression vector (Promega, Mannheim, DEU).

Cell Culture and Transient Transfection Assays

Murine Hepa1c1 hepatocytes were transfected using polyethylenimine (PEI) reagent as described (Reed et al., 2006). Cell extracts were prepared 48 hr after transfection, and luciferase assays were performed as described (Herzig et al., 2001), normalizing to the activity from a cotransfected β-galactosidase expression plasmid. Primary mouse hepatocytes were isolated and cultured as described (Klingmuller et al., 2006). For further details, please see the Supplemental Information.

Human Liver Samples

Subjects

Thirty-seven participants were enrolled into a human cross-sectional study between February 2009 and March 2010. For eligibility, subjects had to be ≥18 years old. All patients underwent liver surgery for different medical reasons and gave their written informed consent at least 24 hr prior to the study. The experimental protocol was approved by the local ethics committee. Subjects who underwent chemotherapy prior to surgical intervention, subjects with a history of liver cirrhosis, who received organ transplantation, or who suffered from acute or chronic inflammatory diseases were excluded. Long-term therapy with glucocorticoids or antirheumatic drugs, drug abuse, excessive alcohol consumption, or mental dysfunction was also considered to be exclusion criteria.

Experimental Design

Upon arrival at the hospital, all volunteers underwent intensive evaluation of medical history and physical examination. Anthropometric measurements and arterial blood pressure according to Riva Rocci were obtained. Liver samples were collected intrasurgically from healthy tissue and were confirmed to be histologically normal. After resection, liver samples were snapfrozen in liquid nitrogen within seconds and stored at −80°C until further analysis.

Frozen liver tissue was ground in liquid nitrogen to a homogenous powder; 50 mg of tissue was homogenized in 5 ml of 10 mM sodium phosphate buffer containing 1 mM EDTA and 1% polyoxyethylene 10 tridecylethan, using an Ultra-Turrax (IKA Werke, Germany). Samples were centrifuged (10 min, 20,000 × g), and the supernatant was incubated at 70°C for 5 min. TG (SIGMA, USA) and protein (DC protein assay, Bio-Rad, USA) levels were analyzed in triplicates.

Statistical Analysis

Statistical analyses were performed using a two-way analysis of variance (ANOVA) with Bonferroni-adjusted post tests, or t test in one-factorial designs, respectively. The significance level was $p = 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at doi:10.1016/j.cmet.2011.02.011.

ACKNOWLEDGMENTS

P.K., A.J., F.M., A.J.R., A.R., D.S., S.K., C.R., K.M.-D., C.S., C.v.L., S.S., G.M.D.-T., and M.B.D performed experiments. J.K., W.W., N.G., A.P., and P.P.N. designed and generated viral systems, knockout mouse models, statistical analyses, and human studies, respectively. M.S. collected human material. S.H. designed and directed research and wrote the manuscript. We thank Michael Downes, Lisa Johnsen, Florian Rösch, Hans-Georg Joost,

Dagmar Kindler, Annette Kopp-Schneider, Stefan Scherneck, Matthew Summers, Jiandie Lin, and Alexander Tups for reagents, helpful discussions, and experimental support. This work was supported by grants from the Deutsche Forschungsgemeinschaft He3260/4-1 and He3260/2-1, the European Foundation for the Study of Diabetes, and the Network Aging Research Heidelberg/Mannheim to S.H.

Received: August 18, 2010
Revised: November 3, 2010
Accepted: January 20, 2011
Published: April 5, 2011

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