Lipin 1 is an inducible amplifier of the hepatic PGC-1α/PPARα regulatory pathway

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Summary

Perturbations in hepatic lipid homeostasis are linked to the development of obesity-related steatohepatitis. Mutations in the gene encoding lipin 1 cause hepatic steatosis in *fld* mice, a genetic model of lipodystrophy. However, the molecular function of lipin 1 is unclear. Herein, we demonstrate that the expression of lipin 1 is induced by peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α), a transcriptional coactivator controlling several key hepatic metabolic pathways. Gain-of-function and loss-of-function strategies demonstrated that lipin selectively activates a subset of PGC-1α target pathways, including fatty acid oxidation and mitochondrial oxidative phosphorylation, while suppressing the lipogenic program and lowering circulating lipid levels. Lipin activates mitochondrial fatty acid oxidative metabolism by inducing expression of the nuclear receptor PPARα, a known PGC-1α target, and via direct physical interactions with PPARα and PGC-1α. These results identify lipin 1 as a selective physiological amplifier of the PGC-1α/PPARα-mediated control of hepatic lipid metabolism.

Introduction

Overwhelming evidence links obesity-related perturbations in hepatic lipid homeostasis to nonalcoholic fatty liver disease (NAFLD). The incidence of NAFLD has risen dramatically, coincident with the recent epidemic of obesity in the U.S., becoming the most common cause of liver disease (Angulo, 2002; Clark et al., 2002). It is now estimated that 25% or more of the general population and up to 75% of clinically obese patients have developed NAFLD (Browning and Horton, 2004; Youssef and McCullough, 2002; Zafrani, 2004). The pathogenic mechanisms linking obesity to NAFLD are likely related to an oversupply of fatty acid delivery that exceeds the capacity for hepatic fatty acid oxidation, leading to triglyceride accumulation within hepatocytes. Moreover, obesity-related hepatic insulin resistance further exacerbates fatty acid oversupply by inappropriately de-repressing hepatic lipogenesis (Shimomura et al., 2000).

The capacity for mitochondrial fatty acid β-oxidation is a critical determinant of hepatic lipid balance in the context of high levels of circulating fatty acids and triglyceride-rich lipoproteins. The expression of genes encoding enzymes involved in hepatic β-oxidation is under dynamic transcriptional control by the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) (Desvergne and Wahli, 1999). The activity of PPARα is regulated at multiple levels including fatty acid ligand availability and interaction with the highly-inducible transcriptional coactivator, PPARγ coactivator-1α (PGC-1α) (Vega et al., 2000). In addition to PPARα, PGC-1α coactivates a variety of transcription factors controlling cellular energy metabolism through direct protein-protein interactions (Lin et al., 2005a; Spiegelman and Heinrich, 2004). Although PGC-1α is expressed at relatively low levels in adult liver under normal conditions, its expression is markedly activated during fasting and diabetes (Herzig et al., 2001; Yoon et al., 2001) consistent with its role as an activator of genes involved in gluconeogenesis and fatty acid oxidation. Recent work has demonstrated that mice deficient for either PPARα (Kersten et al., 1999; Leone et al., 1999) or PGC-1α (Leone et al., 2005) exhibit fasting-induced hepatic steatosis. Fasting-induced lipid accumulation in these models is likely due to diminished capacity for fatty acid catabolism in the face of increased hepatic delivery of free fatty acids, illustrating the critical role of the hepatic PPARα/PGC-1α system in matching fatty acid oxidative capacity to substrate availability.

The protein encoded by the *Lpin1* gene (lipin 1) has been implicated in the regulation of cellular lipid metabolism in a variety of tissues, including liver. *Lpin1* was discovered using a positional cloning approach to identify the genetic mutation responsible for the fatty liver dystrophic (*fld*) mouse phenotype (Peterfy et al., 2001). *Fld* mice exhibit life-long deficiency in adipocyte differentiation (Reue and Peterfy, 2000; Reue et al., 2000), peripheral neuropathy (Langner et al., 1991), circulating hyperlipidemia (Langner et al., 1989), and neonatal hepatic steatosis associated with diminished rates of hepatic fatty acid oxidation (Rehnmark et al., 1998). Conversely, transgenic lipin 1 overexpression in skeletal muscle or white adipose tissue exacerbates high-fat diet-induced obesity (Phan and Reue, 2005). In higher organisms, genes encoding three lipin family members (lipin 1, lipin 2, and lipin 3) have been identified (Peterfy et al., 2001). In addition, a splice variant *lpin1* transcript encodes an alternative form of this protein containing an insertion of 33 amino acids

(lipin 1β) (Huffman et al., 2002; Peterfy et al., 2005) that may influence its sub-cellular localization and function (Peterfy et al., 2005).

Recently, the yeast homolog of lipin (SMP2 or Pah1) was revealed to have enzymatic activity as a phosphatidic acid phosphohydrolase (PAP) (Han et al., 2006). PAP proteins catalyze the formation of diacylglycerol from phosphatidic acid - the penultimate step in triglyceride synthesis. Defects in other steps in the triglyceride (TAG) synthesis pathway are known to result in lipodystrophy or block adipocyte differentiation (Gale et al., 2006; Vergnes et al., 2006) and this aspect of lipin biology could explain defects in adipose tissue development in the *fld* mouse. However, the relevance of this activity in vivo is still unclear.

Several lines of evidence also suggest a nuclear function for lipin 1. Lipin proteins contain a putative nuclear localization signal and lipin 1 is nuclear-localized in HEK293 and 3T3-L1 cells (Peterfy et al., 2005, 2001). Chromatin immunoprecipitation (ChIP) analyses have recently shown that a yeast homolog of lipin (SMP2) is associated with the promoter regions of genes involved in phospholipid biosynthesis (Santos-Rosa et al., 2005), but lacks nucleic acid-interacting domains required for direct DNA interactions. The specific nuclear functions of lipin 1, including potential mechanisms of transcriptional control, also remain to be delineated.

Using an unbiased gene expression profiling approach, we found that lipin 1 is a hepatic target of the transcriptional coactivator, PGC-1α. Hepatic lipin 1 gene expression is induced by fasting, glucocorticoids, and diabetes in a PGC-1α-dependent manner. Lipin 1 is shown to activate expression of many genes involved in mitochondrial fatty acid oxidative metabolism via transcriptional activation of the gene encoding PPARα and through direct cooperative interactions with PPARα and PGC-1α. These studies place lipin 1 in a key position within the PPARα/PGC-1α regulatory pathway and implicate this factor in the physiologic control of hepatic fatty acid metabolism and oxidative phosphorylation.

Results

Lipin 1 is a fasting-induced PGC-1α target gene in liver

Recently, we found that mice deficient for the transcriptional coactivator PGC-1α (PGC-1α$^{-/-}$ mice) develop fasting-induced hepatic steatosis related to diminished capacity for mitochondrial fatty acid β-oxidation (Leone et al., 2005). To identify PGC-1α target genes relevant to this hepatic phenotype, gene expression profiling (microarray) studies were performed using RNA isolated from livers of fed and fasted wild-type (WT) and PGC-1α$^{-/-}$ mice. These analyses revealed that the gene encoding lipin 1 was robustly induced by fasting in WT mice, but only minimally activated in PGC-1α$^{-/-}$ mice (data not shown). Northern and Western blotting studies confirmed that PGC-1α was required for the full fasting-induced activation of lipin 1 expression (Figure 1A). Lipin expression was also strongly induced in a PGC-1α-dependent manner 5 hr after an acute intraperitoneal injection of the synthetic glucocorticoid dexamethasone (Figure 1B), which stimulates PGC-1α expression (Bernal-Mizrachi et al., 2003). Furthermore, hepatic lipin 1 expression was increased in mouse models of type 1 and type 2 diabetes mellitus (Figure 1C), conditions in which hepatic PGC-1α expression and activity is known to be elevated (Herzig et al., 2001; Rhee et al., 2003; Yoon et al., 2001). Finally, adenoviral-mediated overexpression of PGC-1α markedly induced lipin 1 protein levels in primary mouse hepatocytes and C2C12 skeletal myotubes (Figure 1D). Collectively, these results indicate that PGC-1α is sufficient and required for the fasting-induced activation of lipin 1 gene expression.

Lipin 1 activates the PPARα gene regulatory pathway in liver

To identify relevant pathways downstream of lipin 1, the long form of lipin 1 protein (lipin 1β), which is the predominant lipin 1 isoform in liver (Huffman et al., 2002; Peterfy et al., 2005), was overexpressed in liver of mice using an adenoviral vector (Ad-lipin). Gene expression array studies revealed that lipin 1β induced the expression of 1751 genes and repressed 2235 genes (data not shown). Amongst the genes activated by lipin 1 were the nuclear receptor PPARα (*Ppara*) and many of its target genes involved in fatty acid uptake and utilization (Table S1 in the Supplemental Data available with this article online). In addition, lipin 1 activated the broad program of genes encoding TCA cycle enzymes and proteins involved in mitochondrial oxidative phosphorylation - known targets of PGC-1α (Koves et al., 2005; Lin et al., 2005a). Forced expression of lipin 1 also led to diminished expression of several genes involved in fatty acid and TAG synthesis (Table S1).

Northern blotting and quantitative real-time RT-PCR analyses were performed to validate the results of the microarray studies. As predicted by the profiling results, infection with Ad-lipin 1 increased hepatic *Ppara* gene expression and the expression of several PPARα target genes involved in fatty acid utilization (*Cpt1a, Acadvl, Acadm, Acox1,* and *Fabp1*)(Figure 2A). Quantitative RT-PCR studies confirmed that expression of genes encoding several TCA cycle enzymes (*Sdha*, *Idh3a*, and *Mdh2*) and additional genes involved in the control of mitochondrial metabolism, including mitochondrial transcription factor A (*Tfam*), a nuclear-encoded transcription factor controlling mitochondrial gene transcription, and downstream genes involved in oxidative phosphorylation (*Cycs*, *Cox2*, and *Atp5b*) were significantly induced by lipin activation (Figure S1). Conversely, lipin 1 activation markedly diminished expression of genes involved in de novo lipogenesis (*Srebf1* and *Fasn*), fatty acid desaturation (*Scd1*), and, to a lesser extent, lipoprotein secretion (*Apoa4* ;Figure 2A). Collectively, these results suggest that lipin activates expression of PPARα/PGC-1α target genes involved in mitochondrial fatty acid catabolism and downstream mitochondrial pathways involved in electron transport and ATP production while suppressing the lipogenic and lipid secretion programs.

To investigate functional correlates of the gene expression results, a series of metabolic studies were performed. As expected, palmitate oxidation rates were significantly increased in hepatocytes isolated from Ad-lipin-infected mice (Figure 2B). The lipin-mediated increase in β-oxidation was blocked by etomoxir, an inhibitor of mitochondrial fatty acid import. Also consistent with an activation of hepatic fatty acid oxidation, levels of plasma β-hydroxybutyrate, a product of ketogenesis, were significantly elevated with lipin 1 overexpression (Figure 2C). Conversely, rates of fatty acid synthesis, as quantified by ^{14}C-acetate incorporation, were diminished in hepatocytes overexpressing lipin 1 (Figure 2D). Moreover, lipin 1 activation led to diminished rates of hepatic TAG secretion in vivo (Figure 2E) and a significant reduction in circulating triglyceride (TAG) and nonesterified fatty acid (NEFA) levels (Figure 2F). Interestingly,

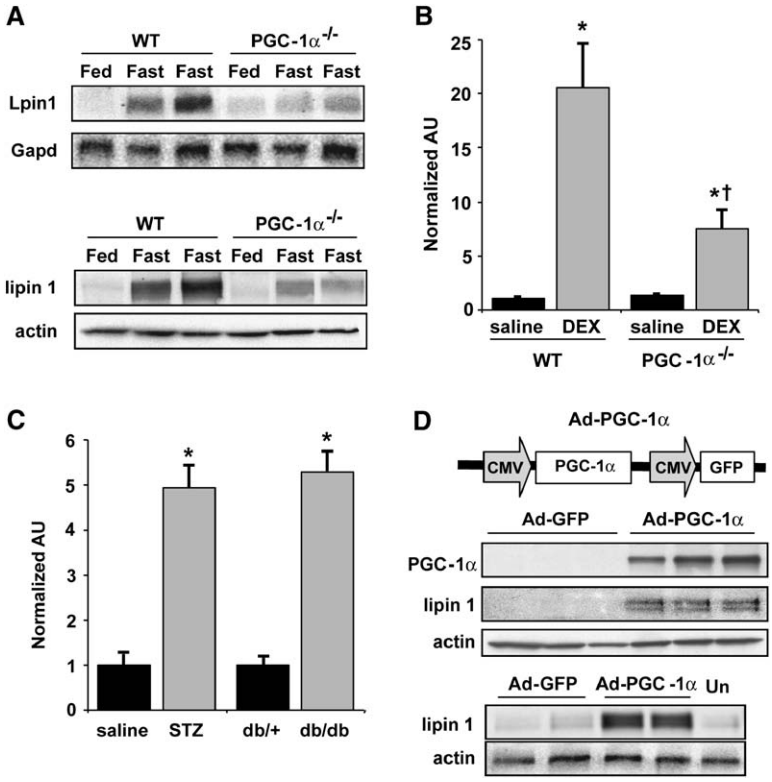

Figure 1. Lipin 1 is a fasting-induced PGC-1α target gene in liver

A) Representative autoradiographs from Northern (top) and Western (bottom) blotting analyses using hepatic RNA or protein from WT and PGC-1α$^{-/-}$ mice given ad libitum access to food or fasted 24 hr.

B) Graph depicts results of RT-PCR analyses to quantify lipin 1 mRNA levels using liver RNA isolated from WT and PGC-1α$^{-/-}$ mice 5 hr after injection with saline or dexamethasone. Values are normalized (= 1.0) to control expression levels. *$p < 0.05$ versus vehicle-injected controls. †$p < 0.05$ versus WT dexamethasone-treated mice.

C) Results of RT-PCR analyses using liver RNA isolated from control and diabetic mice. Type 1 diabetes was induced by streptozotocin injection and *db/db* mice were used as a model of type 2 diabetes. *$p < 0.05$ versus controls.

D) Results of Western blotting analyses using protein isolated from primary mouse hepatocytes (top) or C2C12 skeletal myotubes (bottom) infected with Ad-GFP or Ad-PGC-1α (schematized at the top) and uninfected controls as indicated. Blots were sequentially probed with antibodies shown at left.

steady-state TAG content of liver tissue was actually significantly increased by lipin 1 (Figure 2F), which seems to contradict the effects of lipin 1 on rates of fatty acid catabolism and synthesis. This observation could be explained by hepatic TAG sequestration secondary to diminished TAG secretion, increased fatty acid uptake, or the PAP activity of lipin 1. Collectively, these data indicate that activation of lipin 1 in liver stimulates mitochondrial fatty acid catabolism while suppressing de novo lipogenesis and TAG secretion.

Deactivation of lipin 1 blunts PPARα target gene expression

The role of lipin in the transcriptional regulation of hepatic metabolism was further assessed using loss-of-function approaches. The expression of PPARα/PGC-1α target genes was examined in liver of 7-day-old fld mice, which lack lipin 1 and exhibit hepatic steatosis. The expression of *Ppara* and several genes involved in mitochondrial fatty acid oxidation (*Cpt1a*, *Acadvl, Acadl, Acadm,* and *Fabp1*) was diminished in liver of *fld* mice (Figure 3A), while expression of *Apoa4* was induced, consistent with a previous report (Langner et al., 1989).

To further understand the role of lipin 1 in the control of hepatic fatty acid metabolism, we designed two adenoviral shRNA constructs (sh594 and sh896) to "knockdown" mouse lipin 1 mRNA. Although little effect on lipin 1 expression was observed at baseline, both constructs abolished the induction of lipin 1 protein expression in fasted mice in vivo (Figure 3B). Acute hepatic lipin 1 deficiency in fasted mice significantly diminished the fasting-induced activation of *Ppara* and several PPARα/PGC-1α target genes including *Acadvl*, *Acadm*, and *Fabp1* but only modestly impacted expression of another target, *Cpt1a* (Figure 3B). The increase in plasma β-hydroxybutyrate levels observed with fasting was also significantly blunted by lipin shRNA (Figure 3C). Lipin 1 shRNA administration resulted in a significant increase in hepatic and circulating TAG levels following a 24 hr fast (Figure 3D). Taken together with the observed robust increase in hepatic lipin 1 expression with fasting, these data suggest that lipin 1 plays a critical role in the activation of fatty acid oxidative pathways that occurs with acute fasting.

Lipin 1 activates transcription of the PPARα gene in cooperation with PGC-1α

To determine whether activation of PPARα gene expression by lipin was due to direct transcriptional activating effects, a human PPARα promoter-luciferase reporter was cotransfected into HepG2 hepatocytes with a lipin 1 expression vector in the presence or absence of PGC-1α. We have shown previously that PGC-1α coactivates the PPARα promoter (Huss et al., 2004). Lipin 1 activated the PPARα promoter by approximately 2-fold, an effect that was additive with PGC-1α-mediated coactivation (Figure 4A). The best-characterized regulatory element controlling PPARα gene expression is a nuclear receptor response element originally named the hepatocyte nuclear factor 4 response element (HNF4-RE) that binds and responds to HNF4α, PPARα, and estrogen-related receptor α (ERRα) (Huss et al., 2004; Pineda Torra et al., 2002). Mutation of the PPARα gene promoter HNF4-RE abolished activation by lipin (Figure 4A). Cotransfection studies revealed that lipin 1 also coactivates HNF4α or PPARα, but failed to enhance ERRα-mediated activation of the PPARα promoter (Figure 4B).

Given that a homolog of lipin (SMP2) associates with yeast gene promoters (Santos-Rosa et al., 2005), we next sought to determine whether mammalian lipin could function in an analogous manner on the PPARα gene promoter using chromatin

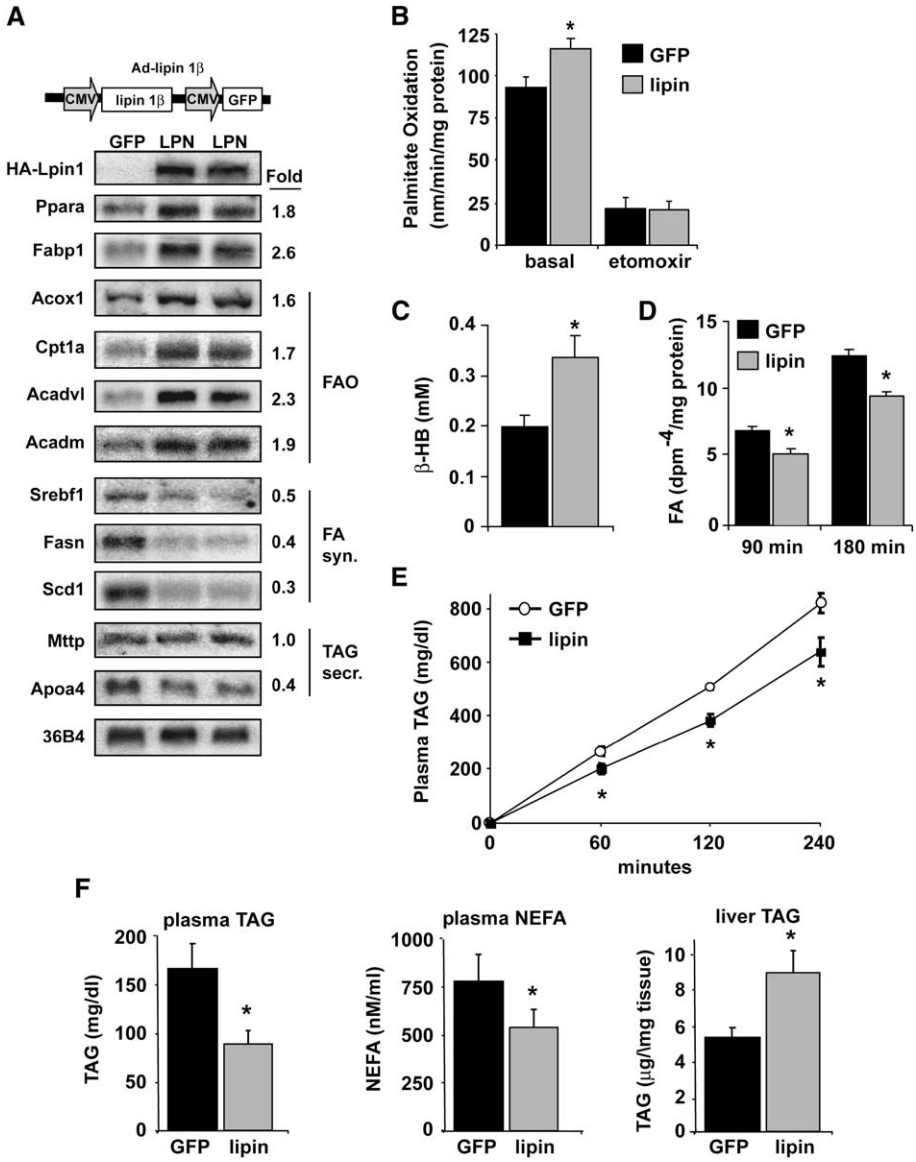

Figure 2. Lipin 1 activates the hepatic PPARα gene regulatory pathway

A) Representative autoradiographs from Northern blotting studies using liver RNA from mice injected intravenously with adenovirus driving expression of lipin 1 (LPN) or green fluorescent protein (GFP). Blots were sequentially hybridized with the cDNA probes listed at left. At right, values represent mean mRNA levels determined by phosphorimaging analyses. The Ad-lipin fold-change value is relative to the normalized (= 1.0) value of GFP signal.

B) The graph depicts mean (±SEM) rates of palmitate oxidation in isolated hepatocytes from mice infected with either Ad-GFP or Ad-lipin under basal conditions and following exposure to the mitochondrial fatty acid import inhibitor, etomoxir. *$p < 0.05$ versus GFP control.

C) Graph depicts plasma β-hydroxybutyrate (HB) levels in mice infected with GFP or lipin 1 adenovirus.

D) Mean rates of de novo fatty acid synthesis from ^{14}C-acetate in isolated hepatocytes infected with lipin and/or GFP are displayed in the graph.

E) The graph depicts plasma triacylglyceride levels in mice infected with Ad-GFP or Ad-lipin and treated with Triton WR-1339 (at time 0) to inhibit lipoprotein lipolysis. *$p < 0.05$ versus GFP control at the same time.

F) Graphs depict mean levels of plasma triacylglycerides (TAG) and Nonesterified fatty acids (NEFA) or liver TAG (right) in mice following infection with Ad-GFP or Ad-lipin after a 4 hr fast.

immunoprecipitation (ChIP) studies. We probed for this interaction using chromatin from hepatocytes isolated from fed or fasted mice and PCR primers flanking the nuclear receptor response element of the *Ppara* gene promoter (Huss et al., 2004). ChIP studies demonstrated that lipin 1 was associated with the *Ppara* promoter and was further enriched when chromatin from fasted mice was employed (Figure 4C). The specificity of this assay was confirmed using chromatin from fasted lipin 1 shRNA-treated mice and mice infected with adenovirus overexpressing lipin 1β. These data indicate that lipin 1 interacts with the *Ppara* promoter in a region known to be transcriptionally-activated by PGC-1α and nuclear receptors (Huss et al., 2004; Pineda Torra et al., 2002). Given that lipin lacks DNA binding domains, we suggest that this interaction is indirect through transcription factor partners such as PPARα as described below.

Lipin enhances PGC-1α-mediated coactivation of PPARα

We sought to determine whether lipin 1 also activated PPARα via posttranslational mechanisms. To this end, a PPAR-responsive reporter (ACO.3X.TKLuc) was transfected into HepG2 cells in the presence or absence of lipin 1, PPARα, and/or PGC-1α expression vectors. Lipin enhanced activation of the reporter in the presence of PPARα, and to a greater extent when both PPARα and PGC-1α were present (Figure 4D). Furthermore, whereas lipin alone had no effect on the activity of a Gal4-PPARα fusion protein, PGC-1α-mediated coactivation of the Gal4-PPARα chimera was significantly enhanced by lipin 1 overexpression; indicating cooperativity among these factors (Figure 4E). In sum, these studies suggest that lipin activates PPARα via transcriptional and posttranslational mechanisms in cooperation with PGC-1α.

Lipin physically interacts with PPARα

Co-immunoprecipitation (Co-IP) studies were performed with adenoviral-driven, epitope-tagged proteins to determine whether lipin 1 interacts directly with PPARα. Immunoprecipitation of lipin co-precipitated PPARα, and to a lesser extent, PPARγ (Figure 5A). Similarly, PPARα or γ co-precipitated lipin 1 (Figure 5A), indicating that lipin 1 interacts with multiple

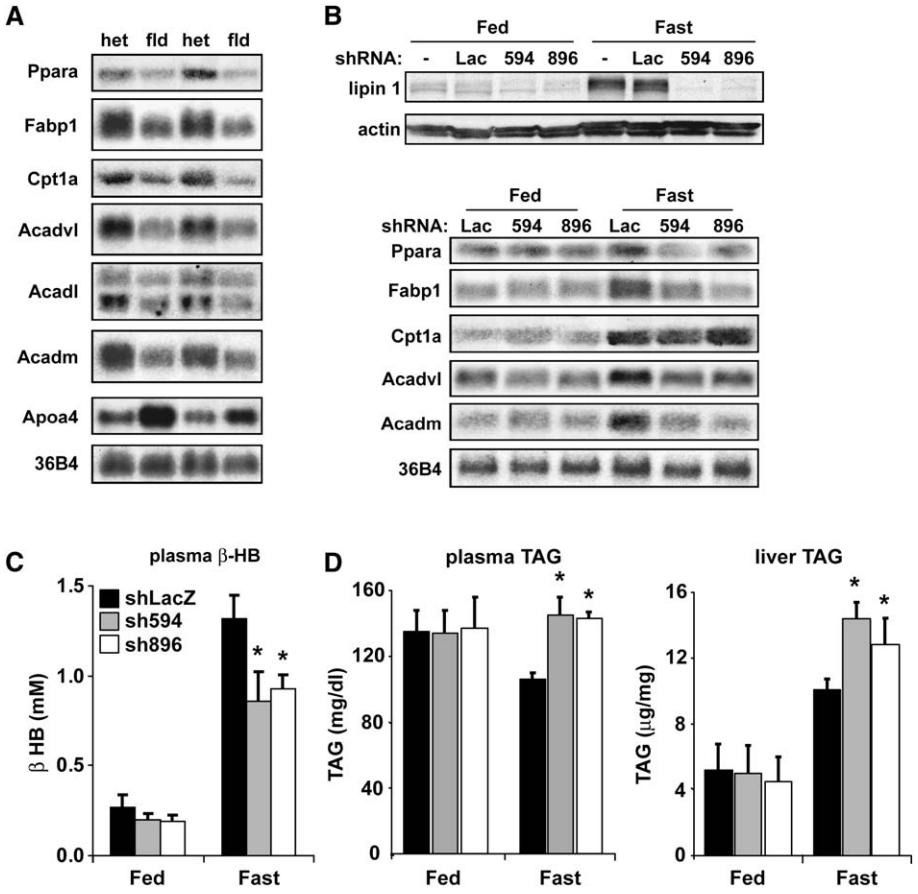

Figure 3. Deactivation of lipin 1 leads to diminished expression of PPARα target genes in liver

A) Representative autoradiographs from Northern blotting analyses using hepatic RNA from fed 7-day-old *fld/fld* or age-matched littermate heterozygote (*fld/+*) control mice. Blots were sequentially hybridized with cDNA probes indicated at left.

B) Results of Western blotting analyses using protein isolated from liver of mice infected with adenoviral vectors driving expression of shRNA designed to "knock-down" LacZ (control) or lipin 1 (sh594 and sh896) and given ad libitum access to food or fasted for 24 hr. Blots were probed with antibodies shown at left. (below) Representative autoradiographs from Northern blotting analyses using hepatic RNA from mice injected intravenously with adenovirus driving expression shRNA to "knock-down" LacZ or lipin 1. Blots were sequentially hybridized with cDNA probes indicated at left.

C and D) Graphs depict mean (± SEM) levels of (**C**) plasma β-HB, (**D**) plasma TAG (left), or liver TAG (right) in mice infected with adenoviral shRNA vectors given ad libitum access to food or fasted for 24 hr. *p < 0.05 versus LacZ shRNA mice with the same dietary status.

PPAR family members. GST pulldown assays further confirmed these findings and demonstrated that the degree of the interaction was not altered by the presence of exogenous ligand (data not shown). The ligand-independent nature of the interaction is consistent with transfection studies showing minimal ligand influence on lipin-mediated coactivation of PPARα (data not shown).

To map the lipin domains mediating the interaction between lipin and PPARα, GST pull-down studies were performed utilizing truncated forms of lipin protein. GST-lipin fusion constructs containing either amino acids 1-641 (ΔC) or 640-924 (ΔN) of lipin 1 protein were employed. Whereas GST-lipinΔN interacted strongly with PPARα (Figure 5B), GST-lipinΔC interacted only weakly, suggesting that, although multiple interacting domains exist, the primary lipin-PPARα interaction occurs between amino acids 640–924 of the lipin protein. The intensity of the interaction between GST-lipinΔN and PPARα was roughly equivalent to the previously-characterized (Vega et al., 2000) interaction between GST-PGC-1α and PPARα (Figure 5B).

The region of lipin 1 mediating the interaction between lipin 1 and PPARα (residues 640-924) contains the previously described C-terminal lipin domain (CLIP) that is homologous across the lipin family (Figure 5C). We found that lipin 1α and β as well as lipin 3 interacted with GST-PPARα in GST pull-down assays (Figure 5D), suggesting that this is a general function of lipin proteins. In addition, both lipin 1α and β coactivated PPARα in transfection studies (Figure 5E). Inspection of the amino acid sequence of lipin 1 between residues 640 and 924 failed to reveal a signature α-helical leucine-rich (LXXLL) motif, through which numerous nuclear receptor coregulators interact with nuclear receptors (Savkur and Burris, 2004). However, two conserved hydrophobic domains, an inverted IXXII (IISDI; residues 709–713) and an LXXIL (LGHIL; residues 722–726), were identified within this region. Interestingly, the IIXXI domain overlaps with the DXDXT consensus sequence of the PAP domain (Figure 5C). When lipin 1 proteins containing site-directed mutations of the LXXIL or IIXXI motifs were employed in pull-down assays, we found that mutation of the IIXXI motif (I mut) did not affect the ability of lipin 1 to interact with or coactivate PPARα, whereas conversion of the LXXIL motif to LXXFF (L mut) significantly diminished the interaction and coactivation (Figures 5D and 5E). Site-directed mutation of the key aspartate within the PAP catalytic domain of lipin 1 (D712E), did not impact the interaction with, or coactivation of, PPARα (Figures 5D and 5E). The D712E, LXXFF, and FFXXI lipin 1 mutants all lack PAP activity (Figure 5E). The observation that the FFXXI and D712E mutants retain the ability to interact with and coactivate PPARα indicate that the PAP activity of lipin 1 is distinct from its coactivator function.

Hydrophobic residue-rich α-helical domains are known to mediate interactions with the with the coregulator recruitment domain (AF2) of nuclear receptors. GST pull-down assays utilizing a mutated PPARα protein in which the AF2 domain was deleted (PPARα 450) demonstrated a diminution in the intensity of the interaction with GST-lipinΔN (Figure 5F). However, a significant interaction was still detected. The lipin-PPARα interaction was nearly abolished when an additional 162 amino acids (PPARα 288) comprising most of the ligand binding domain of

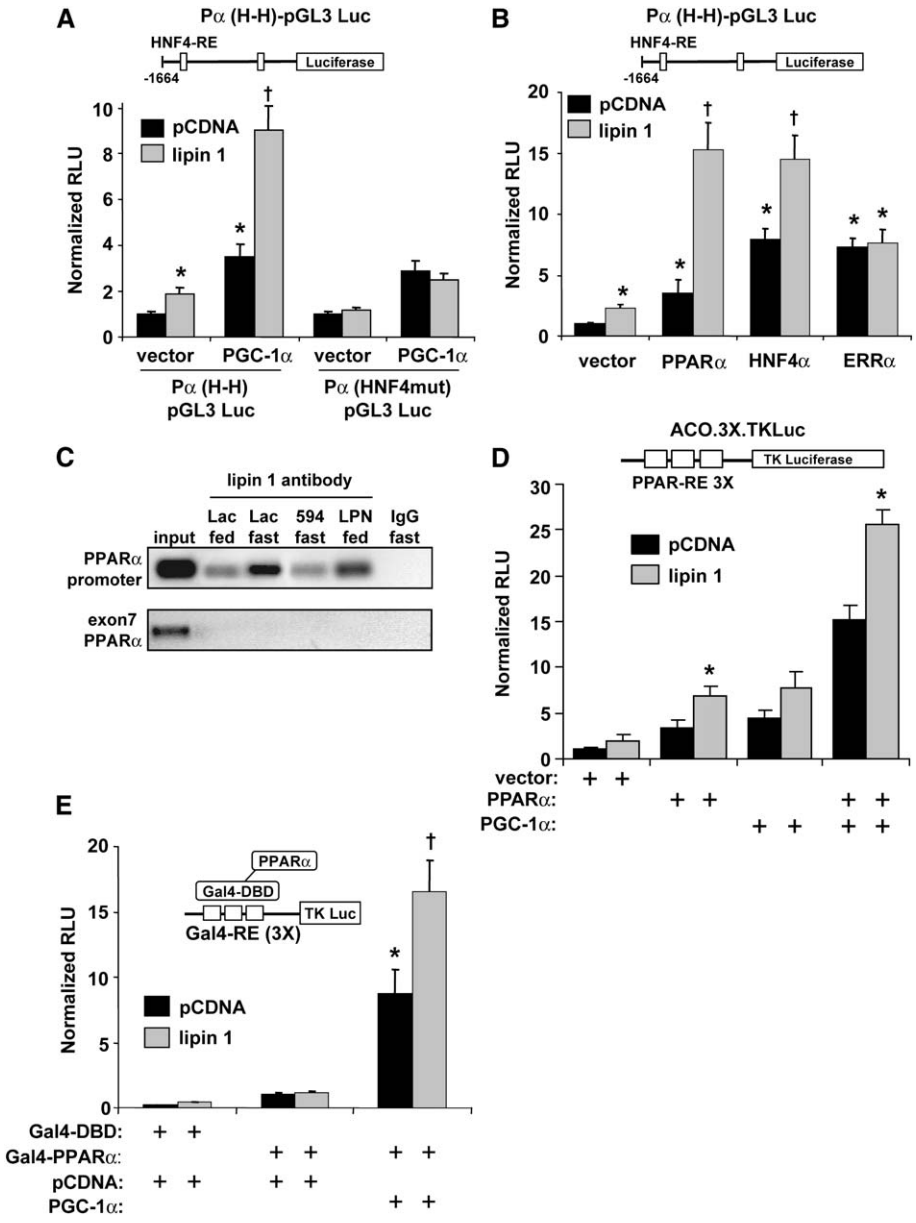

Figure 4. Lipin activates transcription of the PPARα gene

Bars in all graphs represent mean (± SEM) luciferase activity in relative luciferase units (RLU) corrected for renilla luciferase activity and normalized (= 1.0) to the value of empty expression vector-transfected cells.

A) A schematic representation of the PPARα promoter construct (Pα(H-H)-pGL3) is shown at top. Pα(H-H)-pGL3 or a PPARα promoter-reporter harboring mutations in the HNF4-RE (Pα(HNF4mut)-pGL3) was cotransfected into HepG2 cells in the presence or absence of lipin 1 and/or PGC-1α expression vectors. *p < 0.05 versus the value of pcDNA control (vector). †p < 0.05 versus lipin 1 alone or PGC-1α overexpression alone.

B) Results of studies in which Pα(H-H)-pGL3 was cotransfected into HepG2 cells with lipin 1 and/or PPARα, HNF4α, or ERRα expression vectors. *p < 0.05 versus pcDNA controls (vector). †p < 0.05 versus corresponding transcription factor overexpression alone.

C) The image depicts the results of ChIP assays using chromatin from hepatocytes of fed or fasted mice infected with adenovirus driving expression LacZ shRNA (Lac), lipin 1 shRNA (594), or lipin 1 overexpression (LPN). Chromatin bound proteins were immunoprecipitated using antibody directed against lipin 1 or IgG control. "Input" represents 0.2% of the total chromatin used in the IP reactions. For PCR reactions for samples wherein lipin was overexpressed, the DNA input was reduced by 75%. PCR primers were designed to amplify a region of the *Ppara* gene promoter or exon 7 (negative control).

D) Schematic of the heterologous ACO.3X.TKLuc reporter, which contains a multimerized (3×) PPARα-responsive element derived from the promoter of the mouse acyl-CoA oxidase gene linked to a viral thymidine kinase (TK) minimal promoter with a luciferase reporter is shown at top. HepG2 cells were cotransfected with ACO.3X.TKLuc and lipin 1, PPARα, and/or PGC-1α expression vectors. *p < 0.05 versus pcDNA vector control.

E) A schematic depicting the heterologous UAS. TKLuc reporter, which contains a multimerized (5×) yeast-derived Gal4-responsive element upstream of TKLuc. UAS.TKLuc was cotransfected into CV1 cells with expression vector driving expression of a Gal4-PPARα fusion protein (or Gal4 DNA binding domain (DBD) control) in the presence or absence of lipin 1 and/or PGC-1α expression vectors. *p < 0.05 versus pcDNA vector control. †p < 0.05 versus PGC-1α overexpression alone.

PPARα were deleted (Figure 5F). Collectively, these data suggest that lipin physically interacts with PPARα via residues spanning the AF2 and ligand binding domains.

To determine whether lipin might also interact with other PGC-1α partner proteins known to control expression of genes involved in fatty acid oxidation and mitochondrial metabolism, we examined the interaction of lipin 1 with PPARβ, HNF4α, ERRα and the glucocorticoid receptor (GR). GST-lipinΔN interacted strongly with PPARβ, HNF4α, and GR, but only weakly with ERRα (Figure 5F), a finding that coincides with the observation that lipin coactivates PPARα and HNF4α, but not ERRα, on the PPARα promoter (Figure 4B). Thus, lipin 1 interacts selectively with several PGC-1α partner proteins involved in the control of fatty acid oxidation.

Lipin interacts with PGC-1α

Co-IP studies were performed to determine whether lipin 1 interacts directly with PGC-1α. An interaction between endogenous lipin 1 and PGC-1α proteins was demonstrated in Co-IP studies using liver protein isolated from fed and fasted mice (Figure 6A). The quantity of PGC-1α and lipin 1 protein that immunoprecipitated with a lipin 1 antibody was significantly increased under fasting conditions, but only in WT mice as neither protein was immunoprecipitated in PGC-1α-deficient mice (Figure 6A). GST-lipinΔN, but not the GST-lipinΔC, protein interacted strongly with full-length PGC-1α protein in pull-down assays (Figure 6A). GST-lipinΔN also interacted with a truncated PGC-1α protein containing only the first 338 amino acids (Figure 6B). However, the lipin/PGC-1α interaction diminished in a stepwise manner when PGC-1α protein was further truncated by deletions from amino acids 338 to 120, a region that contains 3 LXXLL motifs (L1, L2, and L3) known to be required for interactions with many PGC-1α partner proteins, including nuclear receptors (Figure 6B). Combined site-directed mutations in L1, 2, and 3 (mL1/2/3) markedly diminished the lipin-PGC-1α interaction (Figure 6B).

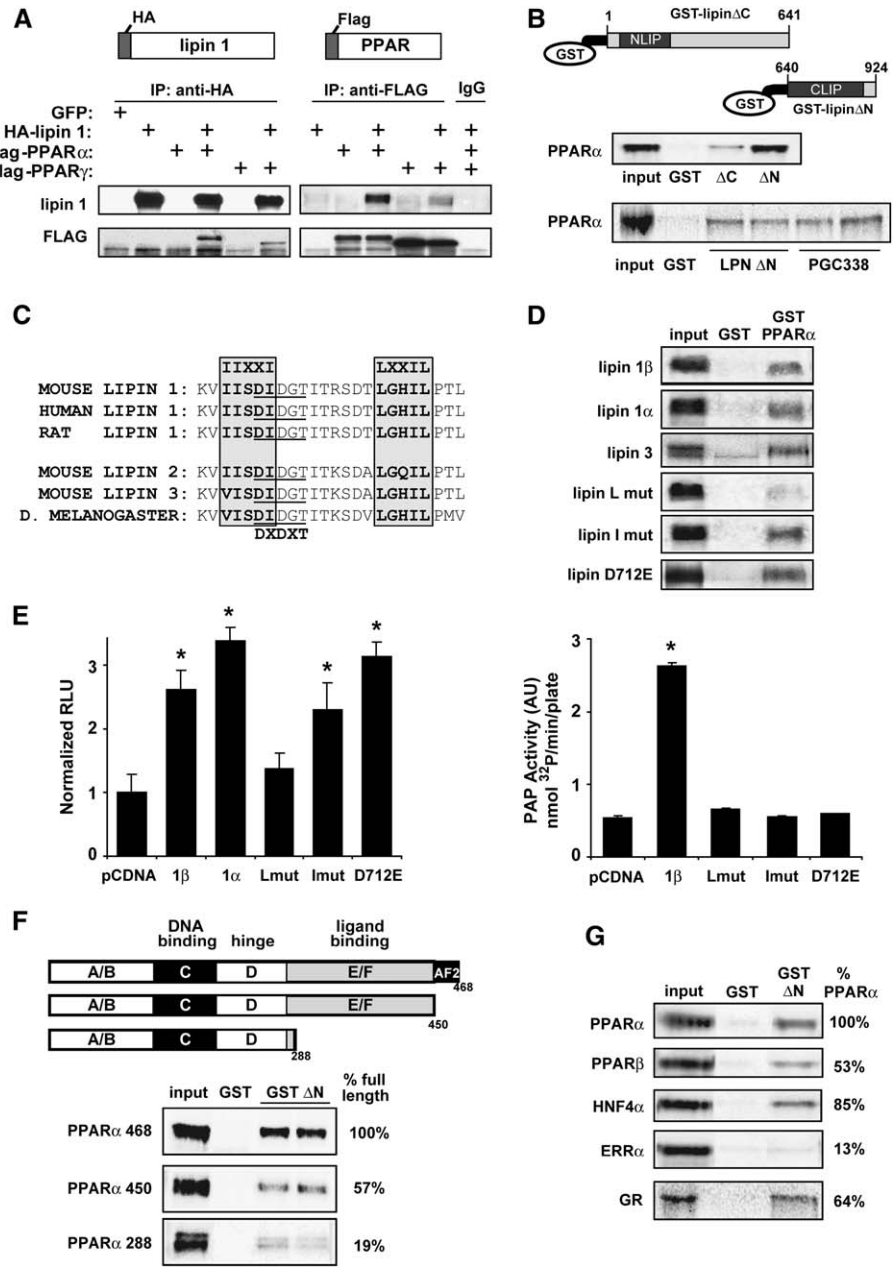

Figure 5. An LXXIL motif in lipin 1 mediates the interaction between lipin 1 and the C terminus of PPARα

A) Results of Western blotting analyses using protein isolated from HepG2 hepatocytes infected with Ad-GFP, Ad-lipin 1, Ad-PPARα, or Ad-PPARγ as indicated at top. Proteins were immunoprecipitated using antibodies directed against fusion tags (HA-lipin 1, Flag-PPARα, or Flag-PPARγ), followed by Western blotting with antibodies raised against lipin 1 or Flag proteins. Irrelevant IgG control IP is shown at far right.

B) At top, schematics depicting GST-lipin fusion proteins lacking the C terminus (lipinΔC) or the N terminus (lipinΔN) are shown. The shaded areas denote previously described conserved regions (NLIP and CLIP). The upper autoradiograph depicts representative results of GST pull-down assays performed with GST control or GST-lipin proteins and ^{35}S-labeled PPARα. The lower autoradiograph displays results of GST pull-down assays comparing the interaction between ^{35}S-labeled PPARα and GST-lipinΔN or GST-PGC-1α(1-338). 10% of the input radiolabeled protein was run in parallel lanes for comparison.

C) Amino acid sequence alignments of a portion of the CLIP domain of lipin family proteins with IIXXI and LXXIL motifs outlined by boxes. The consensus (DXDXT) sequence for the PAP domain is underscored.

D) The autoradiograph depicts representative results of GST pull-down assays performed with GST control or GST-PPARα fusion protein and recombinant lipin proteins (wild-type and site-directed mutations).

E) (left) The graph depicts results of transfection studies using HepG2 cells cotransfected with ACO, 3×.TKLuc and expression vectors driving expression of various lipin 1 proteins (wild-type and site-directed mutations). (right) The graph depicts mean PAP activity in lysates of 293 transfected with vector control (pCDNA-GFP) and various lipin 1 proteins (wild-type and site-directed mutations). *$p < 0.05$ versus pcDNA vector control.

F) A representative autoradiograph from GST pull-down assays performed with C-terminal fragment of lipin fused to GST (GST-lipinΔN) with ^{35}S-labeled PPARα containing 468 (full-length), 450, or 288 amino acids (see schematic at top) is shown. All truncation mutants begin with aa 1 and mutant designations indicate C-terminal ends.

G) The autoradiograph depicts representative results of GST pull-down assays performed with PPARα, PPARβ, ERRα, GR, and GST-lipin 1ΔN protein. The quantity of input protein pulled down by lipin 1 corrected to corresponding input lanes and normalized (= 100%) to PPARα values, is shown at right.

Lipin 1 augments the transcriptional activity of PGC-1α

As an initial step to investigate the mechanism whereby lipin 1 enhances the activity of PGC-1α, its effect on a Gal4-PGC-1α chimeric protein was assessed. We found that lipin 1 coactivated full-length Gal4-PGC-1α, as well as a Gal4-PGC-1α fusion protein containing the first 120 amino acids of PGC-1α, in CV1 cells (Figure 6C). These results suggested that the direct interaction of lipin 1 with PGC-1α increases its transcriptional activating function. Like PGC-1α, lipin 1 does not possess histone acetylase (HAT) activity. We therefore examined whether lipin 1 served to "dock" other coactivators possessing HAT activity. We found that overexpressed lipin 1 co-immunoprecipitated with endogenous p300 protein in IP using mouse liver extracts (Figure 6D).

To determine whether the observed PGC-1α/lipin 1/p300 interaction increased HAT activity on a PPARα target promoter, ChIP studies were performed on the *Acadm* gene promoter using antibodies against lipin, PGC-1α, or acetylated histones and chromatin isolated from fasted mouse liver. Hepatic *Acadm* expression is markedly induced by fasting (Leone et al., 1999) and as expected, the association of lipin 1 and PGC-1α with the *Acadm* promoter was increased in fasted mouse liver coincident with an increase in histone acetylation (Figure 6E). Interestingly, lipin 1 shRNA abolished the fasting-induced increase in PGC-1α occupation and histone acetylation of the *Acadm* promoter. Taken together, these findings suggest that lipin increases the recruitment of PGC-1α and other coactivators with HAT activity, such as p300 to target promoters, resulting in an augmentation of transcription.

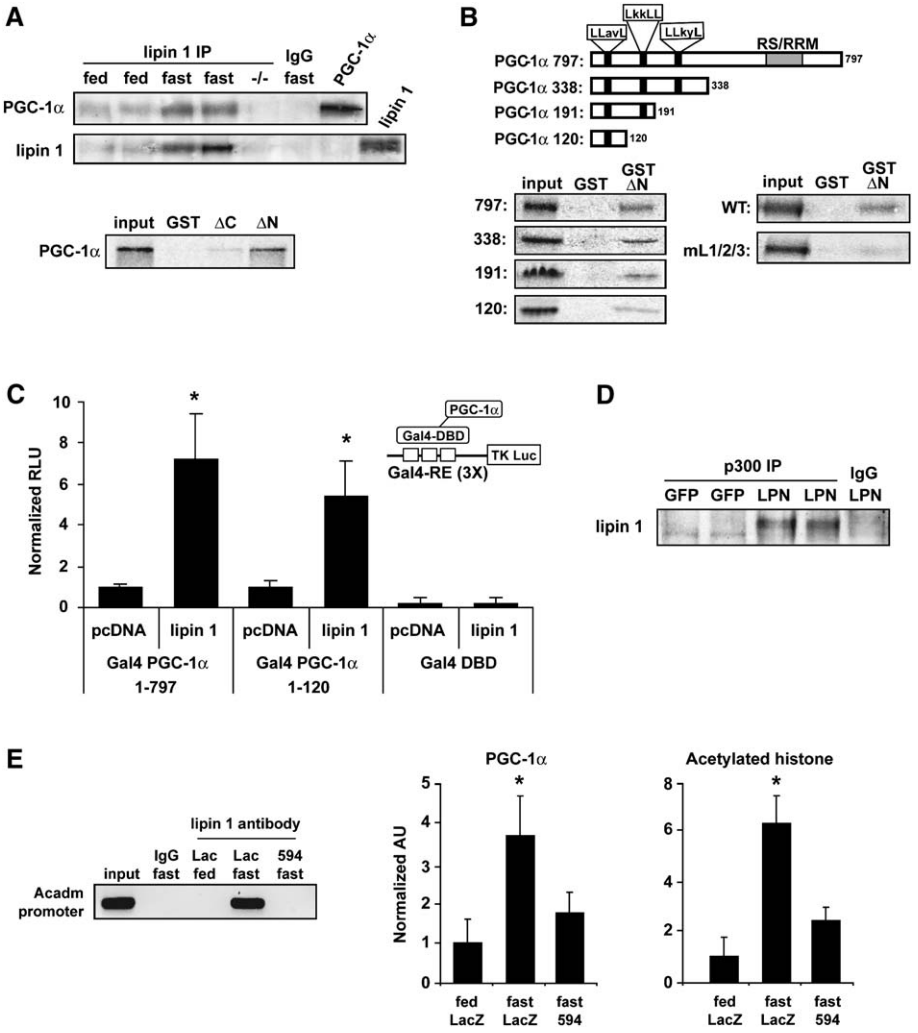

Figure 6. Lipin 1 interacts directly with PGC-1α

A) Results of Co-IP-Western blotting analyses using protein isolated from liver of fed or fasted WT mice. Proteins were immunoprecipitated using lipin 1 antibody followed by Western blotting analyses using lipin 1 or PGC-1α antibodies. Lysates from fasted PGC-1$\alpha^{-/-}$ mice (−/−) or WT lysates using nonspecific IgG antibodies are shown as negative controls and whole-cell lysates from lipin or PGC-1α overexpressing cells included as positive controls. (bottom) Representative autoradiographs depict results of GST pull-down assays performed with GST-lipin fusion proteins and recombinant PGC-1α.

B) Schematic depicts full-length PGC-1α protein (top) including the relative location of 3 LXXLL motifs required for interaction with nuclear receptors. C-terminal PGC-1α truncation mutants are also shown. Autoradiographs depict results of GST pull-down assays performed with GST control or GST-lipinΔN with ^{35}S-labeled PGC-1α (full-length [797], 338, 191, or 120 amino acids [see schematic at top]) or full-length PGC-1α harboring mutations in L1/2/3 (mL1/2/3) is shown. All truncation mutants begin with aa 1 and mutant designations indicate their C-terminal ends.

C) A schematic depicting the heterologous UAS.TKLuc reporter is shown inset. UAS.TKLuc was cotransfected into CV1 cells with expression vector driving expression of a Gal4-PGC-1α fusion protein containing amino acids 1–797 or 1–120 of PGC-1α protein (or Gal4 DNA binding domain [DBD] control) in the presence or absence of lipin 1. *$p < 0.05$ versus pCDNA vector control.

D) Results of Co-IP-Western blotting analyses using protein isolated from liver of mice infected with adenovirus driving expression of lipin 1 and/or GFP. Proteins were immunoprecipitated using p300 antibody followed by Western blotting analyses using lipin 1 antibody. Nonspecific IgG antibody was utilized with lipin overexpressing lysates as negative controls.

E) The image depicts the results of ChIP assays using chromatin from hepatocytes of fed or fasted mice infected with adenovirus driving expression LacZ shRNA (Lac), or lipin 1 shRNA (594). Chromatin bound proteins were immunoprecipitated using antibody directed against lipin 1 or IgG control. "Input" represents 0.2% of the total chromatin used in the IP reactions. Graphs depict the results of SYBR GREEN PCR analyses to quantify the results of ChIP experiments using antibodies directed against PGC-1α or acetylated histone H3 and chromatin from fed or fasted mouse liver infected with adenovirus driving expression LacZ shRNA (LacZ) or lipin 1 shRNA (594). PCR primers were designed to amplify a region of the *Acadm* gene promoter known to respond to PGC-1α.

Lipin-mediated activation of hepatic fatty acid oxidation requires PGC-1α

To determine the requirement of PPARα or PGC-1α for lipin-mediated increases in target genes and hepatic fatty acid oxidation, WT, PPARα$^{-/-}$, or PGC-1α$^{-/-}$ mice were injected with adenovirus overexpressing lipin-1β or GFP. The effects of lipin were only partly altered in PPARα null mice. Specifically, the lipin-mediated induction of a subset of target genes (*Fabp1* and *Acadvl*) was blunted in the PPARα$^{-/-}$ mice whereas other targets (*Cpt1a* and *Acadm*) did not require the presence of PPARα (Figure S2). It is likely that other transcription factors such as PPARβ/δ and/or HNF4α, both of which are known to regulate *Acadm* and other PPAR targets, support lipin-mediated activation in the absence of PPARα.

In contrast to the results obtained with PPARα$^{-/-}$ mice, when PGC-1α$^{-/-}$ mice were employed, Northern blotting analyses revealed that activation of *Ppara*, *Cpt1a*, *Acadvl*, and *Acadm* expression in response to lipin 1 was completely abolished in PGC-1α$^{-/-}$ mice (Figure 7A). Moreover, plasma β-hydroxybutyrate levels were not increased following lipin 1 overexpression in PGC-1α$^{-/-}$ mice as was observed in WT controls (Figure 7B). Interestingly, plasma TAG levels and *Apoa4* expression were not suppressed by lipin activation in PGC-1α$^{-/-}$ mice (Figure 7C), which seems at odds with the known stimulatory effects of PGC-1α on *Apoa4* expression. Liver TAG content was increased by lipin 1 in PGC-1α$^{-/-}$ mice (Figure 7C), potentially due in part to the PAP activity of lipin or the reduced mitochondrial oxidative capacity of PGC-1α-deficient mice (Burgess et al., 2006; Leone et al., 2005). These findings indicate that PGC-1α is required for the lipin 1-mediated activation of hepatic fatty acid oxidation.

Discussion

Mounting evidence implicates lipin 1 in the regulation of lipid and energy metabolism. Herein, we demonstrate that the expression of lipin 1 is rapidly induced in liver by fasting, glucocorticoids, and insulin-deficiency in a PGC-1α-dependent manner. Gain-of-function and loss-of-function strategies revealed that lipin

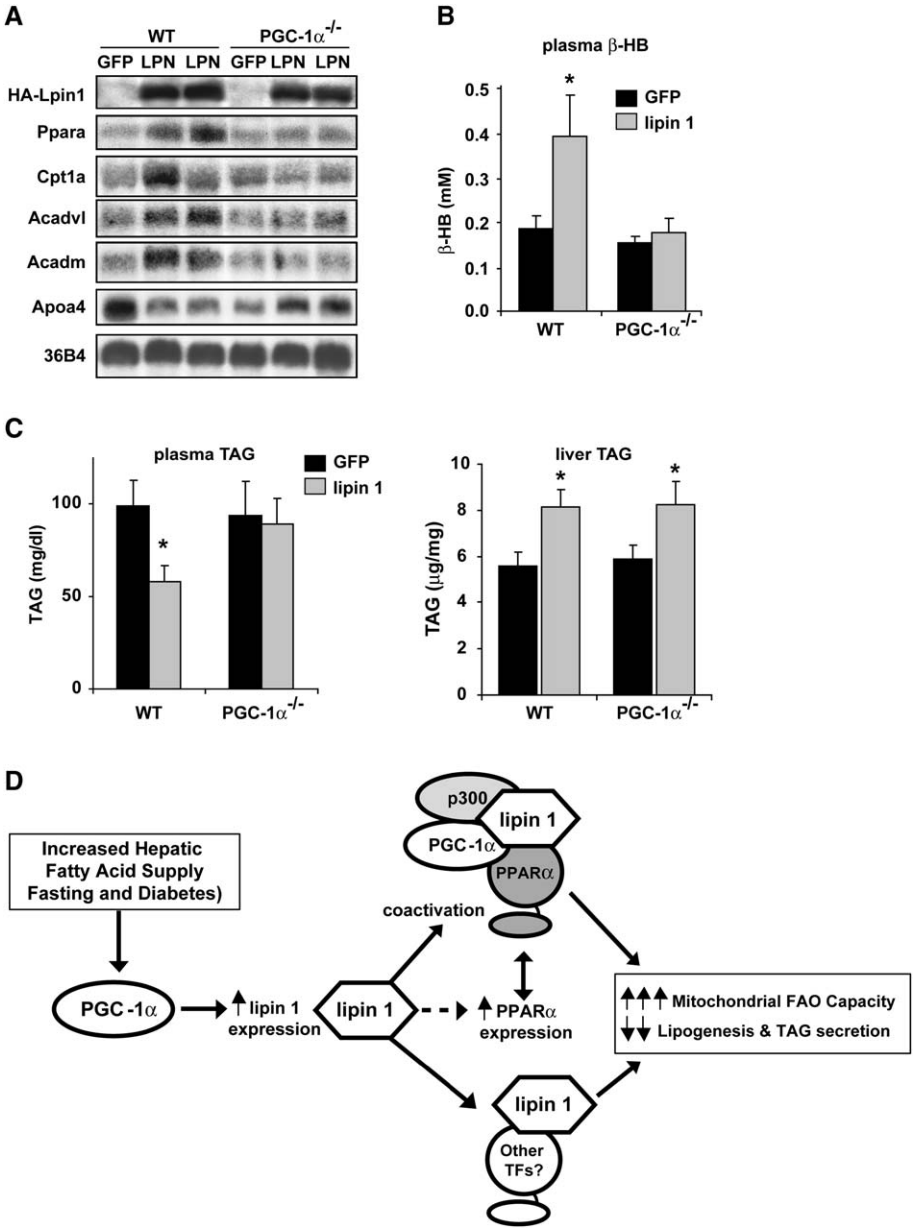

Figure 7. Lipin 1 requires PGC-1α for target gene activation in liver

A) Representative autoradiographs from Northern blotting analyses using hepatic RNA from WT and PGC-1α$^{-/-}$ mice injected intravenously with adenovirus driving expression of lipin 1 (LPN) and/or green fluorescent protein (GFP). Blots were sequentially hybridized with the cDNA probes listed at left.

B) Graph depicts plasma β-hydroxybutyrate (HB) levels in WT and PGC-1α$^{-/-}$ mice infected with GFP or lipin 1 adenovirus. *$p < 0.05$ versus GFP controls.

C) Graph depicts plasma (left) or liver (right) triglyceride levels in WT and PGC-1α$^{-/-}$ mice infected with GFP or lipin 1 adenovirus. *$p < 0.05$ versus GFP controls.

D) A schematic representation of the involvement of lipin 1 in the hepatic response to fasting. Physiologic stimuli that dictate an increased cellular capacity mitochondrial fatty acid oxidation, like fasting and diabetes, induce PGC-1α gene expression which, in turn, activates expression of lipin 1. Thus, lipin 1 increases PPARα activity by two mechanisms: transcriptional activation of the PPARα gene and direct coactivation of PPARα in cooperation with PGC-1α and p300. Lipin is an inducible "booster" that serves to amplify select pathways downstream of PPARα/PGC-1α to meet the demands of increase delivery of fatty acids to the liver.

serves as an inducible coactivator of the PGC-1α-PPARα circuit to increase hepatic capacity for mitochondrial fatty acid oxidation. In addition, lipin 1 suppresses hepatic lipogenesis and triglyceride secretion, while increasing triglyceride stores. These results implicate lipin as an amplifier of the PGC-1α-PPARα regulatory limb in liver (Figure 7D) to maintain hepatic lipid balance in the context of physiologic and pathophysiologic conditions (fasting and diabetes) that increase lipid delivery to the liver.

PGC-1α serves as a transcriptional coactivator for multiple metabolic pathways in liver including fatty acid oxidation, gluconeogenesis, and mitochondrial respiration (Lin et al., 2005a; Spiegelman and Heinrich, 2004). The PGC-1α-PPARα circuit functions to increase hepatic capacity for mitochondrial fatty acid oxidation and ketogenesis in response to short-term starvation, when fatty acids serve as the chief energy substrate (Kersten et al., 1999; Leone et al., 2005, 1999). We present evidence that places lipin 1 within the PGC-1 regulatory pathway. Our results indicate that fasting triggers the induction of lipin 1 expression resulting in the activation of select limbs of the PGC-1α cascade to increase expression of genes involved in fatty acid β-oxidation, the TCA cycle, and the mitochondrial respiratory chain (Figure 7D). Lipin activates the PPARα/PGC-1α regulatory pathway via multiple mechanisms including cooperation with PPARα and PGC-1α as well as transcriptional activation of the PPARα gene. Our results also demonstrate that lipin 1 is capable of interacting with other hepatic transcription factors including HNF-4α, PPARβ, and PPARγ. However, lipin 1 appears to confer selectivity within the PGC-1α cascade given that it does not significantly coactivate or interact with ERRα, which is highly dependent on PGC-1α for its activity (Huss et al., 2002). In addition, lipin overexpression leads to diminished lipogenesis and TAG secretion – both pathways recently defined as targets of the PGC-1s (Lin et al., 2005b; Rhee et al., 2006; Wolfrum and Stoffel, 2006). This last result is puzzling given the mounting evidence

that PGC-1α stimulates lipoprotein secretion. The mechanisms whereby lipin can inhibit hepatic TAG secretion are an area of active investigation and will hopefully be unveiled by additional studies.

Our finding that lipin 1 serves nuclear functions is consistent with reports that the yeast homolog, SMP2, regulates transcription (Santos-Rosa et al., 2005). Interestingly, SMP2 (also known as *Pah1*) was also recently identified as a phosphatidic acid phosphohydrolase (PAP) enzyme that catalyzes the penultimate step in the triglyceride synthesis pathway (Han et al., 2006). Mammalian lipin 1 proteins also possess PAP activity, suggesting that this function is preserved in higher organisms (Han et al., 2006) and (Figure 5E). We suggest that lipin is a multi-functional protein that regulates lipid metabolism at several levels. It is tempting to speculate that in response to increased hepatic free fatty acid delivery during fasting, the PAP activity of lipin serves to rapidly increase esterification of fatty acids into TAG whereas the gene regulatory effects are aimed at bolstering the capacity for β-oxidation and suppressing fatty acid synthesis.

The transcriptional regulatory effects of lipin 1 on hepatic lipid metabolism are consistent with the hepatic phenotype of *fld* mice. *Fld* mice are hyperlipidemic (Langner et al., 1989) and isolated hepatocytes from neonatal *fld* mice exhibit reduced FAO rates (Rehnmark et al., 1998). Our results indicate that hepatic lipin 1 activation lowers circulating lipid levels by reducing triglyceride secretion and stimulating hepatic β-oxidation. We also found that lipin 1 activates *Fabp1* and represses *Apoa4* expression, which are down-regulated (*Fabp1*) and activated (*Apoa4*), respectively in *fld* mouse liver (Langner et al., 1989). Interestingly, consistent with the finding that lipin 1 increases PPARα expression, studies of pre-adipocytes from *fld* mice demonstrate that lipin 1 is required for the induction of PPARγ expression which drives adipocyte differentiation (Peterfy et al., 2005; Phan et al., 2004). Given that we detected a protein-protein interaction between lipin 1 and PPARγ, it is possible that lipin 1 also serves as a coactivator of PPARγ during adipogenesis.

Our observation that lipin 1 coactivates PPARα/PGC-1α in liver appears to contradict recent work demonstrating diminished expression of PPAR target genes in skeletal muscle of lipin-overexpressing transgenic mice and activation of fatty acid oxidation pathways in skeletal muscle of *fld* mice (Phan and Reue, 2005). However, transgenic lipin 1 overexpression was shown to exert distinct tissue-specific effects on PPAR target gene expression in muscle versus adipose tissue (Peterfy et al., 2005; Phan and Reue, 2005). It is possible, therefore, that the regulatory effects of lipin 1 on PPAR or other nuclear receptors is influenced by cell-specific factors or signaling pathways. Lipin 1 has been shown to be phosphorylated in response to insulin (Huffman et al., 2002), a hormone with profound regulatory effects upon intermediary metabolism and gene expression. Cellular signaling events including, but not limited to, insulin signaling could alter cellular localization or interactions with coactivator or corepressor proteins in a cell-specific manner.

The fasting inducibility of lipin and its role as an activator of FAO and mitochondrial function strongly suggest that this factor serves to match oxidative capacity with fatty acid delivery to maintain hepatic lipid balance. Consistent with this notion, lipin 1 suppresses hepatic lipogenesis and TAG secretion, while increasing TAG storage. Increased delivery of fatty acids and excessive hepatic lipogenesis are known to contribute to the development of obesity-related NAFLD (Browning and Horton, 2004). It is believed that in this disease state, the endogenous and exogenous supply of fatty acids exceeds the capacity of the hepatocyte to oxidize this substrate. Similarly, chronic alcohol exposure also leads to lipid accumulation, potentially by reducing the oxidative capacity of the hepatocyte (You and Crabb, 2004). Given the effects of lipin 1 on hepatic oxidative capacity and circulating TAG and free fatty acid levels, this factor could prove to be a therapeutic target for obesity-related dyslipidemia, NAFLD, and alcoholic liver disease. However, given that our observations that lipin increases hepatic TAG accumulation, it is difficult at this point to predict the relative benefits versus detrimental effects of chronic lipin 1 activation on hepatic lipid balance.

In conclusion, we have found that lipin 1 is a hepatic PGC-1α target gene that is robustly induced in response to conditions that dictate increased fatty acid uptake and oxidative flux such as fasting, glucocorticoids, and diabetes. Lipin 1 is also a PGC-1α-interacting protein that coactivates selective targets of the PGC-1α coactivation circuitry, including the entire PPAR family. These results provide a possible mechanism for the selective activation of distinct pathways within the PGC-1α regulatory cascade in response to physiologic stimuli, in this case, serving to increase hepatic oxidative capacity and reduce lipogenic flux. Accordingly, we postulate that lipin 1 serves as an inducible link between PGC-1α and a subset of its target transcription factors to augment the capacity for hepatic mitochondrial fatty acid catabolism and oxidative phosphorylation.

Experimental procedures

Animal studies

PGC-1α$^{-/-}$ (Leone et al., 2005) and PPARα$^{-/-}$ mice (Lee et al., 1995) have been described. Diabetic *db*/*db* mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were sacrificed at 10 weeks of age for tissue collection. Breeding pairs of *fld* mice (*fld*/*fld* × *fld*/+) were obtained from Jackson Laboratories and 7-day-old *fld*/*fld* and *fld*/+ (heterozygote) littermate control mice were sacrificed for tissue collection.

Short-term fasting studies were performed with individually-housed mice which were either food deprived for 24 hr (beginning at 0900) or given ad libitum access to standard rodent chow. Insulin-deficient diabetes was induced by intraperitoneal injection of the pancreatic β cell toxin, streptozotocin (180 mg/kg body weight). Diabetic and saline-injected control mice were sacrificed 7 days after injection. A single bolus intraperitoneal injection of dexamethasone 21-phosphate (500 μg/kg) was administered 5 hr prior to sacrifice for tissue collection. Hepatic lipin 1 overexpression or shRNA-mediated knockdown was achieved by intravenous administration of adenoviral vectors as previously described (Bernal-Mizrachi et al., 2003). Mice were sacrificed 5 days after adenovirus administration.

Mammalian cell culture and transient transfections

CV1 and HepG2 cells were maintained in DMEM-10% fetal calf serum. Transient transfections were performed by calcium-phosphate coprecipitation.

The wild-type and HNF4-RE mutant human PPARα promoter (gift of B. Staels), $(ACO)_3$-TK-Luc, Gal4-responsive $(UAS)_3$-TK-Luc (gift of D. Moore), and Gal4-PPARα and Gal4-PGC-1α fusion protein expression vector constructs have been described (Barger et al., 2000; Huss et al., 2004; Pineda Torra et al., 2002; Vega et al., 2000). PGC-1α and PPARα were overexpressed using pCDNA3.1-Myc/His.PGC-1α and pEFBOS-PPARα expression constructs, respectively.

Northern and Western blotting analyses

Northern blotting analyses were performed as described (Kelly et al., 1989) using radiolabeled cDNA probes. Western blotting studies were performed with whole-cell lysates using rabbit-derived antibodies directed against lipin 1 (Huffman et al., 2002), Flag epitope, (Sigma Chemical, St. Louis, MO), PGC-1α (Santa Cruz Biotechnology), or actin (Sigma Chemical Co.).

In Co-IP experiments, cells were lysed and incubations performed in NP40-containing lysis buffer (20 mM Tris HCl, 100 mM NaCl, 0.5% NP40, 0.5 mM EDTA, 0.5 mM PMSF, and protease inhibitor cocktail). Proteins were IPed using protein A-conjugated agarose beads and antibodies directed against lipin 1 (Huffman et al., 2002), p300 (Upstate Biotechnology), or the epitope tags of HA-lipin 1 (Covance Research Products), Flag-PPARα or Flag-PPARγ (Sigma Chemical Co), or PGC-1α-myc (Santa Cruz Biotechnology). Precipitated proteins were electrophoresed on acrylamide gels followed by Western blotting analyses.

Adenoviral constructs

The adenoviral constructs driving expression of myc-tagged PGC-1α and/or green fluorescent protein (GFP) has been previously described (Lehman et al., 2000). Similarly, full-length HA-tagged lipin 1β and GFP was overexpressed using the Ad-EASY system. Lipin 1 shRNA constructs were designed to target nucleotides 594–613 (sh594) or 896–915 (sh896) of the lipin 1 transcript and were cloned into the Invitrogen pENTR vector and then recombined with the BLOCK-iT adenoviral RNAi expression system (Invitrogen). Adenoviral-driven shRNA constructs targeted to LacZ were utilized as vector controls.

GST pull-down

GST pull-down studies were performed as described (Huss et al., 2002). GST fusion protein constructs containing amino acids 1-641 or 640-924 of lipin 1 protein were generated using EcoRI digest of expression vector constructs containing the truncated cDNAs and subsequent ligation into pCMX vector backbone. The GST-PPARα and GST-PGC-1α 338 (amino acids 1–338) expression constructs have been described (Vega et al., 2000). The 891 (lipin 1α) and 924 (lipin 1β) amino acid forms of lipin 1 and full-length lipin 3 cDNAs were fused to N-terminal HA tags and overexpressed using pCDNA3.1. The lipin IIXXI and LXXIL motifs (amino acid 709–713 and 722–726) of lipin 1β protein were changed to FFXXI and LXXFF by site directed mutagenesis (Quickchange; Strategene Inc.) using the pcDNA3.1-HA.lipin 1β vector as a template. The aspartate 712 residue was changed to glutamate (D712E) by the same technique. Full-length recombinant ^{35}S-labeled protein was synthesized using the TNT quickcoupled in vitro transcription/translation system (Promega) and the respective pcDNA3 expression vectors. To produce PPARα protein lacking the AF2 domain or the ligand binding domain, stop codons were introduced at codon 450 or 288 respectively. Truncated PGC-1α proteins were generated by introducing stop codons at codon 338, 191, or 120. PGC-1α proteins containing mutations in the L1/L2/L3 motifs have been described (Huss et al., 2002).

Statistical analyses

Statistical comparisons were made using analysis of variance (ANOVA) coupled to Scheffe's test. All data are presented as the mean ± SEM, with a statistically significant difference defined as a p value < 0.05.

Supplemental data

Supplemental data include Supplemental Experimental Procedures, Supplemental References, two supplemental tables, and two supplemental figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/3/199/DC1/.

Acknowledgments

The authors wish to thank Mary Wingate for her excellent assistance in manuscript preparation and Dr. Janice M. Huss for technical guidance with microarray and ChIP studies as well as critical manuscript reading. B.N.F. was supported by an NIDDK K01 award (KO1 DK062903). This work was also supported by National Institutes of Health grants R01 DK28312 (to J.C.L.), RO1 DK45416, RO1 HL58427, P30 DK52574, P30 DK56341, and P60 DK20579.

Received: February 24, 2006
Revised: June 26, 2006
Accepted: August 14, 2006
Published: September 5, 2006

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Accession numbers

The microarray data from lipin overexpressing mice have been deposited in the NCBI Gene Expression Omnibus (GEO: http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE5538.