

Alterations in Hepatic Metabolism in *fld* Mice Reveal a Role for Lipin 1 in Regulating VLDL-Triacylglyceride Secretion

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Objective—Lipin 1 controls fatty acid metabolism in the nucleus as a transcriptional regulator and in the cytosol as an enzyme catalyzing the penultimate step in phosphoglycerol triacylglyceride (TAG) synthesis. We sought to evaluate the effects of lipin 1 on hepatic TAG synthesis and secretion by gain-of-function and loss-of-function approaches.

Methods and Results—Rates of TAG synthesis were not impaired in hepatocytes isolated from adult lipin 1-deficient (*fld*) mice and were actually increased in 14-day-old *fld* mice. Additionally, compared to littermate controls, VLDL-TAG secretion rates were markedly increased in *fld* mice of both ages. Lipin 1 overexpression did not alter TAG synthesis rates but significantly suppressed VLDL-TAG secretion. The lipin 1-mediated suppression of VLDL-TAG secretion was linked to the peptide motif mediating its transcriptional-regulatory effects. However, the expression of candidate genes required for VLDL assembly and secretion was unaltered by lipin 1 activation or deficiency. Finally, the hepatic expression of lipin 1 was diminished in obese insulin-resistant mice, whereas adenoviral-mediated overexpression of lipin 1 in liver of these mice inhibits VLDL-TAG secretion and improves hepatic insulin signaling.

Conclusions—Collectively, these studies reveal new and unexpected effects of lipin 1 on hepatic TAG metabolism and obesity-related hepatic insulin resistance. (*Arterioscler Thromb Vasc Biol.* 2008;28:1738-1744)

Key Words: lipin 1 ■ liver ■ triglyceride ■ VLDL secretion ■ dyslipidemia

Obesity, insulin resistance, and diabetes are linked to increased rates of triacylglyceride (TAG)-rich very low-density lipoprotein (VLDL) secretion.¹ Dyslipidemia is a primary risk factor for cardiovascular disease and is linked to a poor prognosis after an ischemic event.^{2,3} With the increased prevalence of obesity and related metabolic diseases, dyslipidemias are emerging as a major public health concern. However, the regulatory mechanisms governing VLDL synthesis, secretion, and degradation are incompletely understood, and factors that play important roles in these processes continue to emerge.

Lipin 1 is an intracellular protein that has emerged as a critical regulator of lipid metabolism.⁴ Spontaneous loss-of-function mutations in the gene encoding lipin 1 in *fatty liver dystrophic (fld)* mice cause a spectrum of metabolic abnormalities, including hepatic steatosis, hyperlipidemia, lipodystrophy, and insulin resistance.⁵⁻⁷ Interestingly, dyslipidemia and hepatic steatosis in *fld* mice spontaneously resolves just before weaning.⁵ However, adult *fld* mice remain lipodystrophic and exhibit insulin resistance and increased susceptibility to atherosclerotic lesion formation.⁶

Recent work has determined that lipin 1 regulates metabolism via activity in both the cytoplasmic and nuclear compartments. Lipin 1 exhibits activity as a Mg²⁺-dependent phosphatidic acid phosphohydrolase (PAP-1) enzyme,⁸⁻¹⁰

which catalyzes the dephosphorylation of phosphatidic acid (PA) to generate diacylglycerol (DAG; Figure 1). This constitutes the penultimate step in the phosphoglycerol TAG synthesis pathway. However, whether lipin 1-associated PAP-1 activity is necessary and sufficient to drive TAG synthesis in liver cells has not been determined. Unlike other enzymes in this TAG synthetic pathway that are integral membrane proteins, lipin 1 is soluble and transiently associates with membranes.¹¹ Moreover, lipin 1 rapidly translocates within the cell in response to phosphorylation by the insulin signaling pathway.⁸ Interestingly, lipin 1 also contains a putative nuclear localization signal and can be nuclear-localized.^{7,12} Lipin 1 has been shown to act as a transcriptional regulator by associating with DNA-bound transcription factors to modulate their activity.^{13,14} For example, lipin 1 interacts with and coactivates the peroxisome proliferator-activated receptor α (PPAR- α) and PPAR- γ coactivator 1 α (PGC-1 α) and enhances the expression of genes involved in fatty acid oxidation.¹³ Collectively, these studies suggest that lipin 1 could play important roles in regulating TAG metabolism through its activity as a PAP-1 enzyme and its transcriptional effects on fatty acid metabolic gene programs.

Availability of neutral lipids, especially TAG, is a driving force for VLDL biogenesis. By promoting TAG synthesis through its PAP-1 activity, lipin 1 might be expected to

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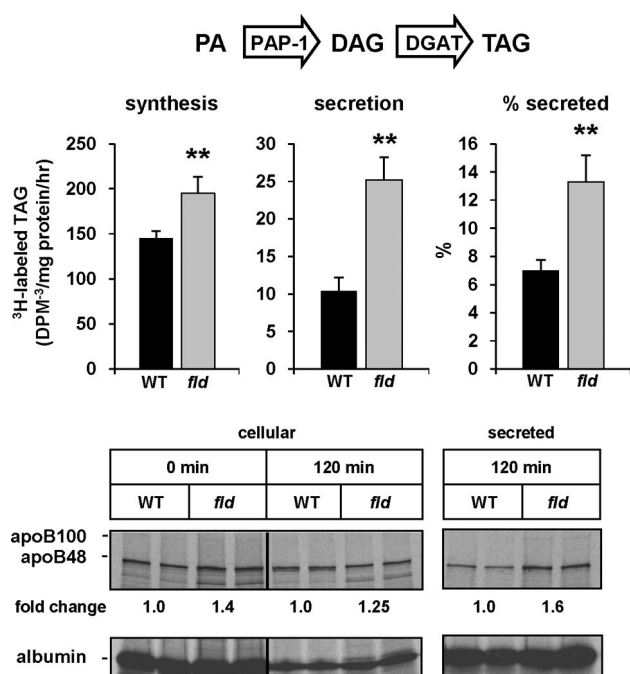


Figure 1. Lipin 1-deficient hepatocytes from 14-day-old mice exhibit high rates of TAG synthesis and secretion. The schematic depicts the terminal steps of the phosphoglycerol TAG synthesis pathway. Lipin proteins catalyze the PAP-1 (Mg^{2+} -dependent) reaction. PA indicates phosphatidic acid; PAP, PA phosphohydrolase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; TAG, triacylglycerol. The graph depicts mean rates of TAG synthesis and secretion by primary hepatocytes isolated from 14-day-old *fld* or wild-type littermate mice. At 6 to 8 hours after isolation, hepatocytes were labeled with $2\text{-}^3\text{H}$ -glycerol for 3 hours, and ^3H -TAG in cell (synthesis) or medium (secretion) were determined. From these values, the secretion efficiency (ie, the percentage of total TAG that was secreted) was also calculated. The mean and SD of 3 independent experiments each done in triplicate are shown. $**P < 0.01$ vs WT hepatocytes. The gel images depict the synthesis and secretion of ^{35}S -labeled apoB48 proteins as described in Methods. Inset values represent mean normalized intensity of the synthesized and secreted ^{35}S -labeled apoB48 proteins.

stimulate hepatic VLDL-TAG secretion. However, overexpression of lipin 1 in liver lowers the circulating TAG concentration in vivo¹³ and neonatal lipin 1-deficient *fld* mice are hyperlipidemic,⁵ suggesting that lipin 1 inhibits rather than promotes hepatic VLDL-TAG secretion. To address this issue, we used both loss-of-function and gain-of-function models to evaluate the effects of lipin 1 on hepatic TAG synthesis and secretion. We also used lipin 1 proteins with site-directed mutations in critical domains to distinguish the role of its PAP-1 and transcriptional coactivator activity. Our data indicate that lipin 1 is not required for robust hepatic TAG synthesis but plays a significant role in regulating hepatic VLDL-TAG secretion.

Experimental Procedures

Mouse Studies

All genetically altered or mutant mice were compared to appropriate strain-matched or littermate controls, and only male mice were studied. In studies using *fld* mice,⁵ homozygous *fld* (*fld/fld*) mice were compared to heterozygous (*fld/+*) or wild-type (*+/+*) littermate control mice (Balb-c strain). With regards to TAG metabolism,

we found no difference between heterozygous and wild-type mice. Hereon, both will be considered “wild-type”. PPAR- α null mice¹⁵ were extensively backcrossed into the C57BL/6 strain. Uncoupling protein 1 diphtheria toxin A (UCP-DTA) mice¹⁶ were compared to nontransgenic littermate controls (FVB strain). All mice, including C57BL/6 controls, were generated from breeding pairs established in the Washington University mouse facility.

Triacylglyceride secretion rates were determined by using Triton WR-1339 injection to inhibit lipoprotein lipolysis, as previously described.¹⁷ Plasma TAG concentrations were determined at 0, 60, and 120 minutes after injection of Triton WR-1339, and rates of TAG secretion were expressed as mg/kg body weight per h.

To examine insulin-stimulated phosphorylation of Akt, mice were fasted overnight and injected i.p. with human insulin (10 mU/g body weight). Mice were killed 5 minutes later and liver was dissected and snap frozen for protein isolation.

All animal experiments were approved by the Animal Studies Committee of Washington University School of Medicine.

Mammalian Cell Culture

Primary cultures of mouse hepatocytes were prepared as described¹⁸ from *fld* or littermate control mice, and metabolic studies were performed 6 to 8 hours later. For studies using adenovirus, hepatocytes were isolated from *fld* or littermate control mice, and after a 2-hour attachment period they were infected with adenovirus to drive overexpression of proteins defined below, then studied after 40 to 48 hours of infection. For studies using lipin 1 mutant constructs, C57BL6J mice were used.

Adenovirus Constructs

The adenoviral constructs driving expression of HA-tagged lipin-1 β or GFP have been previously described.^{8,13} HA-lipin 1 β (D712E) and HA-lipin 1 β (LXXFF) were subcloned into the Ad-track CMV vector and recombined into Ad-EASY to generate adenovirus. For studies using adenovirus in vivo, adenoviral vectors were injected intravenously as previously described.¹³ Mice were used in TAG secretion studies or euthanized for tissue collection 7 days after adenovirus administration.

Adenoviral transductions were carried out by incubating hepatocytes with adenoviruses in a 5% FBS/DMEM media overnight in 24-well plates (0.5 mL/well) or 6-well plates (2 mL/well) at an M.O.I. of 8. Experiments were carried out 40 to 48 hours after adenoviral transduction.

Metabolic Studies on Hepatocytes

To conform to space requirements, Methods for the hepatocyte metabolic studies can be found in the supplemental information.

Gene Expression Analyses

For quantitative polymerase chain reaction (PCR) studies, first-strand cDNA was generated by reverse transcription using total RNA. Real-time RT-PCR was performed using the ABI PRISM 7500 sequence detection system (Applied Biosystems) and the SYBR green kit. Primer sets (available on request) were designed to span exon splice borders. Arbitrary units of target mRNA were corrected by measuring the levels of 36B4 RNA.

Western Blotting Analyses

Western blotting analyses were performed with antibodies directed against the HA epitope tag (Covance), total Akt (Cell Signaling), and phosphoAkt (Ser473 or Thr308; Cell Signaling).

Statistical Analyses

Statistical comparisons were made using analysis of variance (ANOVA) coupled to Scheffe test. All data are presented as means \pm SEM or \pm SD, with a statistically significant difference defined as a probability value < 0.05 .

Results

Increased Rates of TAG Synthesis and Secretion in Hepatocytes From 14-Day-Old Lipin 1-Deficient Mice

We sought to evaluate the effects of lipin 1 deficiency on rates of hepatic TAG synthesis and to probe the possibility that impaired VLDL-TAG secretion leads to hepatic steatosis in mice lacking lipin 1. Rates of TAG synthesis and secretion were actually increased quite significantly in hepatocytes isolated from 14-day-old (P14) *fld* mice compared with littermate controls (Figure 1). Importantly, the percentage of newly-synthesized TAG that was secreted was also increased, suggesting that this effect on secretion is not entirely explained by increased TAG synthesis. Pulse-chase studies in isolated hepatocytes demonstrated that synthesis and secretion of apoB proteins was significantly increased in *fld* hepatocytes (Figure 1). Together, these results demonstrate that a lack of lipin 1 does not impair hepatic TAG synthesis and secretion.

Increased Hepatic VLDL-TAG Secretion by Adult *fld* Mice and Its Reversal With Lipin 1 Overexpression

We also evaluated TAG synthesis and secretion in adult (P42) *fld* mice, which do not exhibit hepatic steatosis and have normal circulating lipid levels.⁵ Rates of TAG synthesis were not altered in adult *fld* mice compared to littermate controls, but rates of TAG secretion and the percentage of newly-synthesized TAG that was secreted were increased in P42 *fld* mice (Figure 2A). Adult *fld* hepatocytes showed no changes in rates of apoB synthesis or secretion (Figure 2B). Moreover, as demonstrated by using density-gradient ultracentrifugation analysis of the secreted ³⁵S-labeled apoB-containing lipoproteins, *fld* hepatocytes secreted more VLDL-sized apoB48-containing particles than hepatocytes from littermate controls (Figure 2C). The hepatocytes of both genotypes secreted very small amounts of apoB100, which distributed only in the VLDL fractions (data not shown). Together, these results indicate that increased particle size of apoB48-containing lipoproteins account for the increased VLDL-TAG secretion by the *fld* hepatocytes.

Because *fld* mice lack lipin 1 in all tissues, the studies above fail to distinguish between the hepatic effects of lipin 1 deficiency and its effects in other peripheral tissues (eg, lack of adipose tissue depots). To evaluate the direct hepatic effects of lipin 1 on VLDL assembly and secretion, lipin 1 β was overexpressed in hepatocytes isolated from *fld* mice. Lipin 1 β overexpression did not affect rates of TAG synthesis, but markedly suppressed TAG secretion, leading to a significant decrease in the efficiency of secretion of newly synthesized TAG (Figure 3A). Synthesis and secretion of apoB was not significantly affected by lipin 1 overexpression (data not shown). Lipin 1 overexpression markedly decreased the VLDL-sized, while increasing the HDL-like particles, in the secreted apoB-containing lipoproteins (data not shown). These results suggest that lipin 1 exerts its effects mainly by modulating the particle size of secreted apoB48-containing lipoproteins.

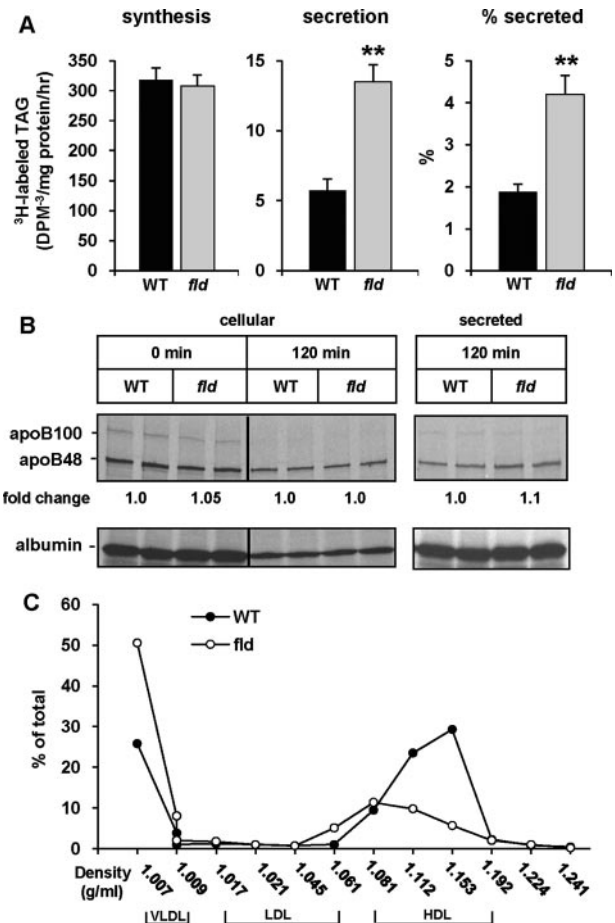


Figure 2. Hepatocytes from adult *fld* mice exhibit normal rates of TAG synthesis and increased VLDL-TAG secretion. A, The graph depicts mean rates of TAG synthesis and secretion by primary hepatocytes isolated from 42-day-old *fld* or wild-type littermate mice as described in Figure 1 and Methods. The mean and SD of 3 independent experiments each done in triplicate are shown. ** $P < 0.01$ vs WT hepatocytes. B, The gel images depict the synthesis and secretion of ³⁵S-labeled apoB proteins as described in Methods. Inset values represent mean normalized intensity of the synthesized and secreted ³⁵S-labeled apoB48 proteins. C, Density distribution of apoB48-containing lipoprotein particles secreted by hepatocytes isolated from adult *fld* mice (*fld*) and littermate control (WT). Hepatocytes were continuously labeled with ³⁵S-methionine for 3 hours in the presence of 0.3 mmol/L oleate, and ³⁵S-labeled apoB-containing lipoproteins in media were fractionated and quantified by density-gradient ultracentrifugation analysis and immunoprecipitation as described in Methods. Each data point represents mean of 2 independent experiments.

To establish the physiological relevance of the above findings in vivo, we examined the effect of liver-specific complementation of lipin 1 in *fld* mice. Lipin 1 β was overexpressed in liver of *fld* mice and their littermate controls by adenovirus injection and VLDL secretion was assessed after injection with Triton-1339, which inhibits VLDL lipolysis. Under basal conditions (infection with Ad-GFP), there was a 2-fold increase in VLDL secretion in the *fld* mice compared to the littermate controls (Figure 3B). Overexpression of lipin 1 in the liver significantly decreased rates of VLDL-TAG secretion in both *fld* and control mice, with a more pronounced effect in *fld* mice. Altogether, these find-

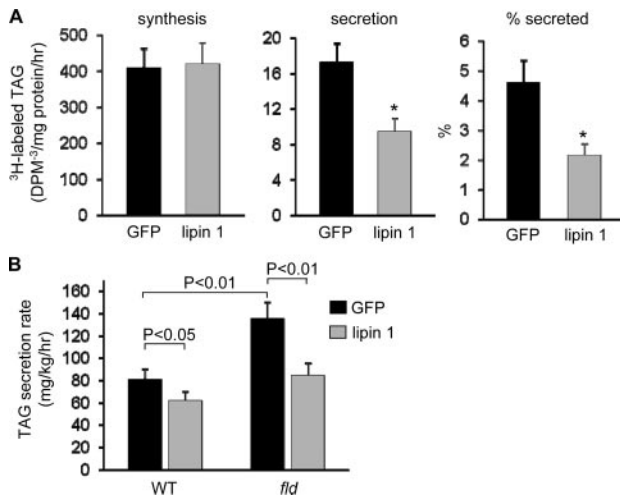


Figure 3. Lipin 1 overexpression suppresses hepatic VLDL-TAG secretion in *fld* mice. A, Hepatocytes isolated from livers of adult *fld* mice were infected with adenoviruses expressing GFP alone (GFP) or lipin 1 plus GFP (lipin 1) and studied 48 hours later. Synthesis and secretion of TAG was determined in the presence of exogenous oleate as described in Figure 1 and Methods at 48 hours after viral transduction. The mean and SD of 3 independent experiments each done in triplicate are shown. ** $P < 0.01$ vs GFP control. B, The graph depicts mean and SD ($n = 4$) of rates of hepatic VLDL-TAG secretion in vivo determined using Triton WR-1339 from 4 adult male WT or *fld* mice infected with Ad-GFP or Ad-lipin 1 β .

ings suggest that lipin 1 functions directly in the liver to suppress VLDL-TAG secretion.

The Lipin 1 LXXIL Motif Is Required for its Effects on TAG Metabolism

The two known molecular functions of lipin 1 relate to its activity as a PAP-1 enzyme and its role as a transcriptional regulator. Two previously described¹³ lipin 1 proteins with site-directed mutations in key domains were used to determine which molecular function was involved in this biological effect. Lipin 1 (D712E) lacks PAP-1 activity but retains the ability to interact with and coactivate PPAR- α , whereas lipin 1 (LXXFF) lacks both the ability to interact with PPAR- α and PAP activity.¹³ We found that the overexpressed D712E mutant retained the ability to suppress TAG secretion in WT mice, indicating that PAP activity is dispensable for these effects (Figure 4A). In contrast, overexpression of the LXXFF allele failed to suppress TAG secretion, implying a critical role of the transcriptional regulatory function of lipin 1 in mediating its inhibitory effects on VLDL-TAG secretion.

Given the large body of work indicating that PPAR- α ligands suppress hepatic TAG secretion,¹⁹ we also tested whether lipin 1 was exerting its effects via activation of PPAR- α . However, lipin 1 β retained the ability to suppress TAG secretion in hepatocytes from PPAR- α null mice (Figure 4B), indicating that these effects are independent of PPAR- α .

Lipin 1 Suppresses *Scd1* Expression but Does Not Regulate Expression of *Apob* and *Mtp*

The maintenance of normal rates of hepatic TAG synthesis in *fld* mice perhaps suggested that other components of the TAG

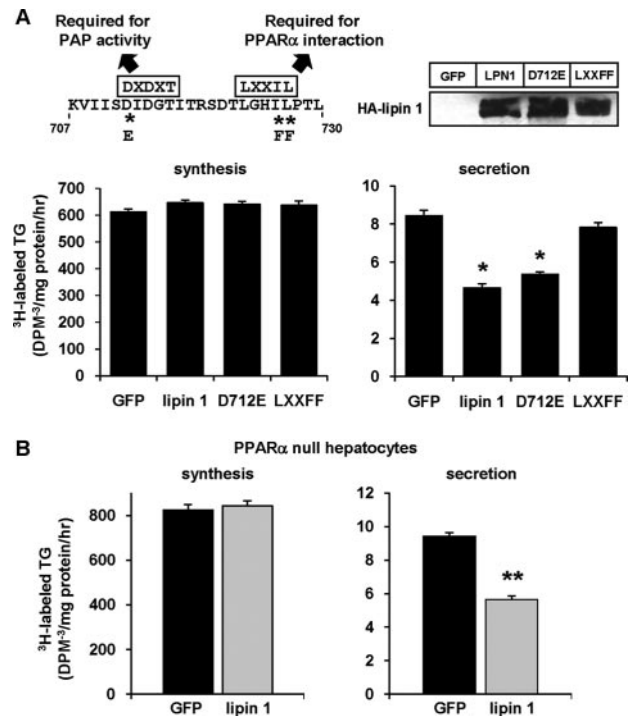


Figure 4. The LXXIL motif of lipin 1 is required to suppress VLDL-TAG secretion in mouse hepatocytes. A, The amino acid sequence of C-terminal lipin 1 motifs required for PAP-1 activity (DDXDT) and nuclear receptor interaction (LXXIL) are shown. Asterisks denote amino acid residues mutated in D712E and LXXFF mutant lipin 1 alleles. The autoradiograph of representative Western blotting analyses for the HA tag of lipin 1 demonstrates that wild-type, D712E, and LXXFF lipin 1 proteins are robustly expressed at a similar level in hepatocytes by their respective adenoviral vectors. Primary hepatocytes were isolated from 6-week-old C57BL/6 mice and infected with adenovirus driving expression of wild-type, D712E, or LXXFF lipin 1 β or GFP (control). The graphs depict mean rates of TAG synthesis and secretion. The mean and SD of 3 independent experiments each done in triplicate are shown. * $P < 0.05$ vs GFP control and lipin 1 (LXXFF). B, The graphs depict mean rates of TAG synthesis and secretion by primary hepatocytes isolated from PPAR- α null mice. The mean and SD of 3 independent experiments done in triplicate are shown. ** $P < 0.01$ vs GFP control.

synthesis pathway, including other lipin family members, might be upregulated. However, quantitative RT-PCR analyses demonstrated that the expression of *Lpin2* and *Lpin3* was unchanged in *fld* mice or with lipin 1 overexpression (Table). We also observed no change in the expression of other enzymes in this pathway (*Dgat1*, *Dgat2*, and *Gpam*; data not shown). The expression of steroyl-coenzyme A (CoA) desaturase (*Scd1*), which may promote TAG synthesis and secretion, was markedly increased in neonatal and adult *fld* mice (Table) yet suppressed by lipin 1 overexpression.

We next evaluated the expression of genes involved in VLDL metabolism. Consistent with increased rates of apoB synthesis by neonatal *fld* hepatocytes, the expression of apoB (*Apob*) was strongly induced in 10-day-old *fld* mice compared to controls (Table), though the relevance of transcriptional regulation of *Apob* is unclear given the high level of posttranslational regulation of apoB secretion. The expression of the gene encoding apoA4 (*Apoa4*) was also markedly increased in *fld* mice. In contrast, the expression of the genes

Table. Expression of Genes Involved in TAG Metabolism With Deactivation or Overexpression of Lipin 1

	WT Hepatocytes		<i>fld</i> Hepatocytes			
	P8 <i>fld</i>	P42 <i>fld</i>	Ad-GFP	Ad-Lipin 1 β	Ad-GFP	Ad-Lipin 1 β
<i>Lpin2</i>	1.1 \pm 0.38	1.3 \pm 0.15	1.0 \pm 0.10	1.1 \pm 0.18	1.1 \pm 0.27	1.1 \pm 0.20
<i>Lpin3</i>	1.0 \pm 0.29	1.3 \pm 0.24	1.0 \pm 0.25	1.3 \pm 0.09	1.4 \pm 0.24	1.1 \pm 0.28
<i>Scd1</i>	8.4 \pm 3.04*	6.7 \pm 2.54*	1.0 \pm 0.18	0.2 \pm 0.31**	4.2 \pm 1.29*	1.6 \pm 1.17**
<i>Apoa4</i>	25.1 \pm 4.42*	3.4 \pm 0.21*	1.0 \pm 0.18	0.5 \pm 0.16**	2.1 \pm 0.16*	0.9 \pm 0.15**
<i>Apob</i>	1.9 \pm 0.36*	1.1 \pm 0.31	1.0 \pm 0.25	0.9 \pm 0.11	1.2 \pm 0.35	0.9 \pm 0.23
<i>Apoc3</i>	0.9 \pm 0.37	0.8 \pm 0.42	1.0 \pm 0.07	0.6 \pm 0.14**	1.4 \pm 0.16	0.7 \pm 0.14**
<i>ApoE</i>	0.9 \pm 0.35	0.9 \pm 0.17	1.0 \pm 0.02	0.9 \pm 0.05	0.9 \pm 0.11	0.7 \pm 0.10
<i>Mttp</i>	1.4 \pm 0.33	1.1 \pm 0.26	1.0 \pm 0.10	1.0 \pm 0.09	0.9 \pm 0.09	0.8 \pm 0.11

Left, Values represent mean (\pm SEM) hepatic expression of the indicated genes expressed as arbitrary units (AU) normalized (=1.0) to the mean expression in WT control mice (n=5 for P8 mice and n=6 for P42 mice). * P <0.05 compared to age-matched WT littermate mice.

Right, Hepatocytes were isolated from P42 WT or *fld* mice, pooled within genotypes, and then infected with adenovirus overexpressing lipin 1 β or GFP at time of plating. RNA was isolated 48 h later. Values represent mean (\pm SEM) expression of the indicated genes normalized (=1.0) to the mean expression in WT GFP hepatocytes. Results represent 6 independent wells of hepatocytes pooled from 3 mice of each genotype. * P <0.05 compared to WT hepatocytes. ** P <0.05 vs Ad-GFP controls of the same genotype.

encoding apoE (*ApoE*), apoC3 (*Apoc3*), and microsomal TAG transfer protein (*Mttp*) were not changed. Among other genes evaluated in adult *fld* mice (P42), only the expression of *Apoa4* was altered (Table).

We next quantified the expression of genes involved in VLDL metabolism in response to lipin 1 overexpression in hepatocytes from wild-type and *fld* mice. Lipin 1 overexpression did not affect the expression of *Apob*, *ApoE*, or *Mttp*. However, the expression of *Apoa4* and *Apoc3* was markedly suppressed by lipin 1 β overexpression in hepatocytes of both *fld* and littermate control mice. Consistent with our metabolic studies, the transcriptional effects of lipin 1 were also observed with overexpression of lipin 1(D712E) but not the LXXXFF allele (supplemental Figure I). Collectively, these data indicate that the effects of lipin 1 are not associated with altered expression of *Apob* and *Mttp*; the two obligatory components of the VLDL-TAG exporting machinery.

Lipin 1 Suppresses VLDL-TAG Secretion and Increases Hepatic Insulin Sensitivity in UCP-DTA Mice

To explore a potential link between lipin 1 expression and increased VLDL-TAG production associated with metabolic syndrome, we used UCP-DTA mice,¹⁶ which are obese, insulin-resistant, and hyperlipidemic because of transgenic ablation of most of the brown adipose tissue. As previously noted,¹⁶ UCP-DTA mice were hypertriglyceridemic (Figure 5A). We also found that rates of TAG secretion were dramatically increased in insulin-resistant UCP-DTA mice versus NTG littermates (Figure 5A). Interestingly, hepatic expression of lipin 1 was significantly diminished in UCP-DTA mice (Figure 5A).

To determine whether restoration of lipin 1 expression corrected the metabolic derangements in VLDL metabolism in UCP-DTA mice, we overexpressed lipin 1 in the liver of UCP-DTA mice using an adenovirus. Liver-specific lipin 1

overexpression lowered the fasting plasma TAG concentration (Figure 5B) and reversed TAG hyper-secretion in UCP-DTA mice (Figure 5B). Lipin 1 overexpression also markedly increased insulin-mediated phosphorylation of Akt at serine 473 and threonine 308 in the liver of UCP-DTA mice (Figure 5C) despite having no effect on hepatic TAG levels (data not shown). Collectively, these data indicate that overexpression of lipin 1 in liver of UCP-DTA mice is sufficient to improve hepatic insulin signaling and suppress VLDL-TAG secretion in this obese mouse model.

Discussion

Lipin 1 is emerging as an important regulator of lipid homeostasis via its function as a PAP-1 enzyme in the phosphoglycerol TAG synthesis pathway and in the nucleus as a transcriptional regulator. Herein, we found that lipin 1 was neither required for nor sufficient to induce hepatic TAG synthesis, but instead, lipin 1 markedly suppressed hepatic VLDL-TAG secretion. The repressive effects of lipin 1 were independent of its PAP activity but required the domain that mediates its interaction with nuclear receptor transcription factors. Finally, we found that hepatic *Lpin1* expression was markedly diminished in obese, insulin-resistant UCP-DTA mice and that hepatic overexpression of lipin 1 in these mice suppressed VLDL-TAG secretion and increased insulin-stimulated phosphorylation of Akt.

The physiological and cellular mechanisms that cause hepatic steatosis in neonatal *fld* mice is incompletely understood. Hepatic steatosis is common in lipodystrophic mouse models^{20,21} and in humans with genetic or acquired forms of lipodystrophy.^{22,23} This may relate to insufficient capacity to store fatty acids in adipose depots leading to hepatic TAG accretion, which would be an overt problem in *fld* mice exposed to TAG-rich maternal milk. In addition, neonatal *fld* mice exhibit a significant defect in fatty acid oxidation,²⁴ consistent with hepatic steatosis in other models of fatty acid oxidation deficiencies.²⁵ The present study excludes defects

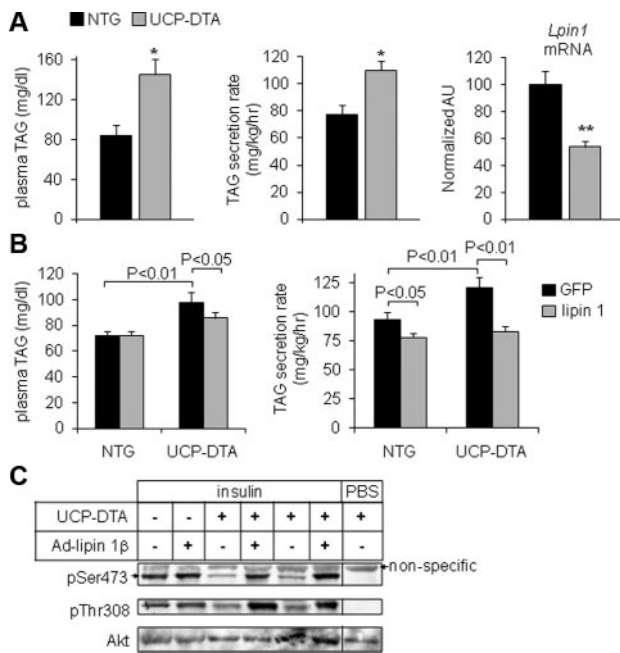


Figure 5. Lipin 1 overexpression suppresses hepatic VLDL-TAG secretion and increases insulin signaling in UCP-DTA mice. **A**, Left, The graph depicts plasma TAG levels in nontransgenic (NTG) or uncoupling protein 1 diphtheria toxin A (UCP-DTA) mice after a 4-hour fast. * $P < 0.05$ vs NTG mice ($n = 5$). Middle, The graph depicts mean rates of hepatic VLDL-TAG secretion in NTG or UCP-DTA mice determined using Triton WR-1339 as described in Methods. * $P < 0.01$ vs NTG ($n = 4$). Right, The graph depicts the expression of lipin 1 β in liver of NTG and UCP-DTA mice as quantified by quantitative RT-PCR. ** $P < 0.01$ vs NTG control ($n = 6$). **B**, Left, The graph depicts mean and SD ($n = 4$) of plasma TAG levels in NTG or UCP-DTA mice infected with Ad-GFP or Ad-lipin 1 β after a 4-hour fast. Right, The graph depicts mean and SD ($n = 5$) of in vivo rates of hepatic TAG secretion in NTG or UCP-DTA mice infected with Ad-GFP or Ad-lipin 1 β . **C**, The autoradiographs depict representative results of Western blotting analyses using hepatic protein lysates from NTG and UCP-DTA mice injected with insulin or saline. Duplicate blots were probed with antibodies against Akt phosphorylated at serine 473 (ser473), threonine 308 (thr308), or total Akt protein.

in VLDL-TAG secretion, which can also cause fatty liver,^{17,18} as causing to the steatotic phenotype of *fld* mice. Although *fld* mice almost completely lack PAP-1 activity in most extrahepatic tissues, previous work has shown that at least residual PAP-1 activity remains in liver,^{8,10} probably because of high hepatic expression of another lipin family protein, lipin 2.¹⁰ However, we also show that overexpression of lipin 1 β is not sufficient to drive increased TAG synthesis. Together, these data likely suggest that the PAP-1 reaction is not rate-limiting for phosphoglycerol TAG synthesis.

Although lipin 1 activity does not influence rates of TAG synthesis in hepatocytes, our gain-of-function and loss-of-function studies uniformly revealed that lipin 1 suppresses VLDL-TAG secretion. This is consistent with the original report demonstrating that neonatal *fld* mice are hypertriglyceridemic and lipoproteinemic.⁵ We found that VLDL-TAG secretion was also increased in hepatocytes isolated from adult *fld* mice, which exhibit normal lipemia.^{5,6} Increased catabolism of VLDL-TAG by peripheral tissues may explain the normal plasma lipid levels in adult *fld* mice.^{26,27} It is also

tempting to speculate that an increased capacity for VLDL-TAG secretion is one compensatory mechanism leading to the temporal resolution of the neonatal fatty liver observed in *fld* mice.⁵ Although increased VLDL-TAG secretion by *fld* hepatocytes could be an adaptive response to lack of adipose tissue, our overexpression data indicate that lipin 1 also plays a direct role in regulating hepatic VLDL-TAG secretion.

Although lipin mutation studies indicate that lipin 1 acts via a transcriptional mechanism, lipin 1 did not suppress the expression of *Apob* and *Mttp* genes, the two obligatory factors for VLDL assembly and secretion. Although genetic variations in the *Apoc3/Apoa4* gene cluster are linked to altered lipemia and incidence of cardiovascular disease,^{28,29} based on the data from overexpression and knockout models,^{30–32} it is unlikely that regulation of *Apoc3/Apoa4* expression per se can explain the observed effects of lipin 1 on hepatic VLDL-TAG secretion. *Scd1* was also found to be increased in *fld* mice of both ages and was repressed by lipin 1 overexpression. *Scd1* null mice exhibit diminished hepatic TAG stores and VLDL secretion,³³ potentially suggesting that it could play a role in the observed biological effects.

Finally, we recently reported that hepatic lipin 1 gene expression is inversely related to body mass index and plasma insulin concentration in extremely obese human volunteers.³⁴ Lipin 1 gene expression was also reactivated by marked weight loss after gastric bypass surgery. The increase in lipin 1 expression in the liver was coincident with a significant decline in hepatic TAG content and rates of hepatic VLDL-TAG secretion after gastric bypass surgery in these subjects.³⁵ Consistent with this, we find that lipin 1 gene expression is diminished in obese insulin-resistant UCP-DTA mice. More importantly, we show that lipin 1 overexpression reduces hepatic TAG secretion rates in these mice and improves insulin-mediated phosphorylation of Akt. Given the data presented herein, future strategies for modulating lipin 1 activity in liver might prove useful in the treatment of insulin resistance and dyslipidemia. Obviously, a better understanding of the full gamut of lipin 1 effects on lipid metabolism is required before it can be seriously considered as a therapeutic target.

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Disclosures

None.

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Alterations in Hepatic Metabolism in *fld* Mice Reveal a Role for Lipin 1 in Regulating VLDL-Triacylglyceride Secretion

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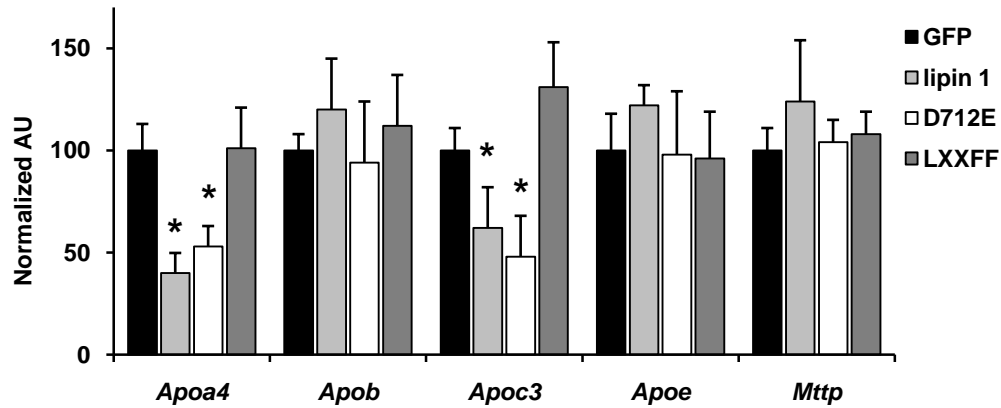
Metabolic Studies on Hepatocytes. To measure of rates of synthesis and secretion of TAG, hepatocytes were incubated with a 2-³H-glycerol-containing DMEM (10 µCi/ml) for the specified time periods without exogenous fatty acids (for experiments involving hepatocytes from *fld* mice and their littermates) or with 0.3 mM oleate complexed to BSA (for experiments involving adenovirus-infection). ³H-labeled TAG was isolated from cells and medium and ³H-radioactivity was determined as described.^{1,2} Data were expressed as DPM/mg protein/h.

To determine apolipoprotein B (apoB) synthesis and secretion, hepatocytes were pulsed with ³⁵S-methionine for 25 min and chased for 0 or 120 min with media containing 100 X excess amounts of unlabeled methionine as described.^{1,2} The ³⁵S-labeled apoB and albumin in cells and media were immunoprecipitated and separated on a gradient SDS-gel (3-15%) and quantified as described.^{1,2}

To determine the density distribution of the apoB-containing lipoproteins secreted by the liver, hepatocytes isolated from *fld* and the littermate control were continuously labeled with ³⁵S-Met for 3 h in 60-mm dishes in the presence of 0.3 mM oleate as described above. The media were then subjected to density-gradient ultracentrifugation in a $d = 1.006-1.25$ g/ml KBr density gradient as described.² Fractions (12 fractions) were aspirated and dialyzed, thereafter, they were subjected to immunoprecipitation and ³⁵S-labeled apoB in each fraction were separated by SDS-PAGE and quantified as described above.

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Supplemental Figure 1



Supplemental Figure 1. Primary hepatocytes were isolated from 6 week old C57BL/6 mice and infected with adenovirus driving expression of wild-type, D712E, or LXXFF lipin 1b and/or GFP (control). The graphs depict mean of the genes denoted at bottom. The mean and SEM is shown (n=8). *p<0.05 versus GFP control and lipin 1(LXXFF).