Abrogation of Hepatic ATP-Citrate Lyase Protects Against Fatty Liver and Ameliorates Hyperglycemia in Leptin Receptor-Deficient Mice

Qiong Wang,[1]* Lei Jiang,[1]* Jue Wang,[1] Shoufeng Li,[1] Yue Yu,[1] Jia You,[1] Rong Zeng,[2] Xiang Gao,[3] Liangyou Rui,[4] Wenjun Li,[1] and Yong Liu[1]

Hepatic steatosis is a hallmark of nonalcoholic fatty liver disease (NAFLD) and a key component of obesity-associated metabolic dysfunctions featuring dyslipidemia, insulin resistance, and loss of glycemic control. It has yet to be completely understood how much dysregulated *de novo* lipogenesis contributes to the pathogenic development of hepatic steatosis and insulin resistance. ATP-citrate lyase (ACL) is a lipogenic enzyme that catalyzes the critical reaction linking cellular glucose catabolism and lipogenesis, converting cytosolic citrate to acetyl-coenzyme A (CoA). Acetyl-CoA is further converted to malonyl-CoA, the essential precursor for fatty acid biosynthesis. We investigated whether dysregulation of hepatic ACL is metabolically connected to hepatic steatosis, insulin resistance, and hyperglycemia. We found that in leptin receptor–deficient *db/db* mice, the expression of ACL was selectively elevated in the liver but not in the white adipose tissue. Liver-specific ACL abrogation via adenovirus-mediated RNA interference prominently reduced the hepatic contents of both acetyl-CoA and malonyl-CoA, markedly inhibited hepatic *de novo* lipogenesis, and protected against hepatic steatosis in *db/db* mice. Surprisingly, liver-specific ACL abrogation markedly inhibited the expression of peroxisome proliferator-activated receptor-gamma and the entire lipogenic program in the liver. Moreover, hepatic ACL deficiency resulted in significantly down-regulated expression of gluconeogenic genes in the liver as well as enhanced insulin sensitivity in the muscle, leading to substantially improved systemic glucose metabolism. *Conclusion:* These findings establish a crucial role of hepatic ACL in lipid and glucose metabolism; therefore, hepatic ACL may serve as a potential target to treat NAFLD and type 2 diabetes. (HEPATOLOGY 2009;49:1166-1175.)

Hepatic steatosis, the excessive accumulation of triglycerides (TGs) in the liver, is an early hallmark of nonalcoholic fatty liver disease (NAFLD).[1,2] NAFLD can progress into more severe liver disorders, including steatohepatitis and hepatic cirrhosis/fibrosis.[3-5] Increasingly recognized as a key component of obesity and the metabolic syndrome, both of which are reaching epidemic proportions worldwide,[6,7] hepatic steatosis/NAFLD is pathogenically linked to dyslipidemia, insulin resistance, and hyperglycemia.[8-10] Currently, effective therapeutic strategies are lacking for the prevention of disease progression in patients with NAFLD.[2]

The liver is a major lipogenic organ that is of central importance in energy storage and conversion. Arising

from obesity-induced impairments of the coordinate regulation of glucose and lipid homeostasis in the liver, hepatic steatosis occurs as a result of abnormally enhanced *de novo* lipid synthesis and fat delivery from excessive adipose tissue, as well as decreased fatty acid oxidation and lipid export.[11] Fatty acid biosynthesis occurs in the cytosol through a series of regulated reactions catalyzed by lipogenic enzymes.[12] ATP-citrate lyase (ACL)[13] is a crucial lipogenic enzyme that regulates the flow of glucose carbons to cytosolic acetyl-coenzyme A (CoA) by catalyzing an ATP-consuming reaction to generate acetyl-CoA from citrate, thereby linking cellular glucose catabolism and *de novo* lipid synthesis. Acetyl-CoA is further converted to malonyl-CoA by acetyl-CoA carboxylase (ACC), the rate-limiting step in *de novo* fatty acid synthesis. Fatty acids are subsequently synthesized from malonyl-CoA by fatty acid synthase (FAS), long-chain elongase (ELOVL6), and stearoyl-CoA desaturase 1 (SCD1); and formation of triglycerides is catalyzed by another series of enzyme, such as glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT). *De novo* lipogenesis is coordinately controlled in response to nutritional, hormonal, and metabolic stimuli, and is mediated by key transcriptional regulators, including the nuclear hormone receptors peroxisome proliferator-activated receptor (PPAR) γ and liver X receptor (LXR) α,[14-16] sterol regulatory element-binding protein (SREBP)-1c,[17] as well as carbohydrate-responsive element-binding protein.[18,19] Although multiple lines of evidence have indicated that dysregulated lipogenesis considerably contributes to NAFLD,[3,20,21] the precise metabolic role of each individual lipogenic enzyme in the progression of hepatic steatosis and insulin resistance has not been fully appreciated until recently.[22-26] Although ACL is absolutely essential in embryonic development and heterozygous knockout mice with ACL haplo-deficiency in a mixed genetic background exhibit normal lipid metabolism,[27] it remains largely unclear whether ACL plays a critical role in obesity-related derangement of glucose and lipid metabolism, or to what degree dysregulation of hepatic ACL activity is pathogenically connected to NAFLD and loss of glycemic control.

Using adenovirus-mediated RNA interference strategy, we investigated the metabolic effects of targeted suppression of hepatic ACL upon liver steatosis and glucose metabolism in leptin receptor-deficient *db/db* mice that spontaneously develop fatty livers. We show that ACL, selectively dysregulated in the livers of *db/db* mice, plays a crucial role in glucose and lipid homeostasis and may serve as a promising therapeutic target for the treatment of NAFLD and diabetes.

Materials and Methods

Animal studies, including adenovirus administration and metabolic measurements, were conducted with all experimental procedures approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Detailed materials and methods are described in the Supplementary Material. For statistical analysis, data are presented as the mean $\pm$ standard error of the mean (SEM) and differences were analyzed by unpaired two-tailed t test between two groups. For glucose tolerance test, comparison was done with two-way analysis of variance.

Results

ACL Is Selectively Dysregulated in the Livers of db/db Mice

Leptin is an adipocyte-derived hormone that exerts a broad spectrum of physiological functions in lipid and glucose metabolism, and genetic deletion of its long form receptor results in morbid obesity, fatty liver, and type 2 diabetes.[28] To investigate whether ACL is connected to the derangement of lipid metabolism in the absence of leptin actions, we first assessed the expression levels of ACL in both the white adipose tissue and livers of C57BL/6J *db/db* mice fed a regular chow diet. In comparison with wild-type (WT) littermates, quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) and western immunoblotting analyses showed nearly five-fold and 2.7-fold elevations, respectively, in the messenger RNA (mRNA) and protein expression levels of ACL in the livers of *db/db* mice (Fig. 1A,B). In contrast, comparable ACL expression levels were detected in the white adipose tissue. Consistently, the ACL enzyme activity was also approximately three-fold higher in the livers of *db/db* mice than in WT control mice (Fig. 1C). In parallel, as compared with WT mice, more than three-
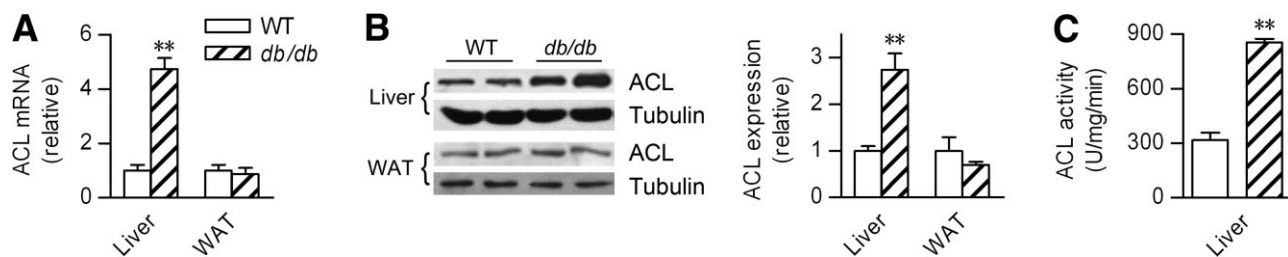
Fig. 1. ACL is selectively dysregulated in the livers of leptin receptor–deficient *db/db* mice. (A) Assessment by quantitative real-time RT-PCR of the mRNA levels of ACL in the liver and white adipose tissue (WAT) in male C57BL/6J WT littermates versus *db/db* mice at 8 weeks of age. (B) ACL protein expression levels were analyzed by immunoblotting in the liver and white adipose tissue of WT littermates versus *db/db* mice. Shown are representative immunoblots for the indicated tissues from two animals of each genotype. The bar graph indicates the relative ACL abundance as quantified by densitometry from the immunoblots after normalization to tubulin. (C) ACL enzyme activities were measured from the liver extracts of WT littermates versus *db/db* mice. For all panels, data are expressed as the mean ± SEM (n = 3-5/genotype). **$P < 0.01$ versus WT.

fold and two-fold increases were observed, respectively, in the mRNA and protein expression levels of the lipogenic regulator PPARγ in the livers but not in the white adipose tissue of *db/db* mice (Supplementary Fig. 1). Thus, ACL expression is dysregulated in a liver-selective manner, which is closely accompanied by the abnormally elevated hepatic expression of PPARγ in *db/db* mice. These results suggest that loss of lipogenic control occurs in the liver as a result of defective leptin actions, presumably contributing to the development of fatty liver.

ACL Is Crucial for the Hepatic Production of Acetyl-CoA and Malonyl-CoA as Well as De Novo Lipogenesis in db/db Mice

The observation that the expression of ACL was prominently elevated in the livers of *db/db* mice prompted us to hypothesize that hepatic ACL mediates leptin's crucial metabolic actions in lipid and glucose homeostasis. To investigate the metabolic effects of targeted suppression of hepatic ACL, we took an RNA interference approach using the recombinant adenovirus to deliver the small hairpin (sh) RNA directed against the coding region of ACL (Ad-shACL). C57BL/6J *db/db* mice were infected with Ad-shACL or Ad-shLacZ adenoviruses via tail vein injection, and sacrificed 15 days after infection. Ad-shACL but not Ad-shLacZ adenoviruses specifically and almost completely abolished ACL expression in the liver (Fig. 2A). In parallel, ACL enzyme activities were also reduced by approximately 95% in the livers of Ad-shACL–infected mice (Fig. 2B). Importantly, evaluation of alanine aminotransferase or aspartate aminotransferase levels in the serum indicated that Ad-shACL did not cause apparent functional damage or inflammation to the livers of *db/db* mice as compared with Ad-shLacZ (Table 1).

Because ACL catalyzes the conversion of cytosolic citrate to acetyl-CoA that is further converted by ACC to malonyl-CoA, the hepatic contents of both acetyl-CoA and malonyl-CoA were measured via LC-tandem mass

spectrometric analysis (LC-MS-MS). Hepatic ACL abrogation resulted in a >50% reduction in the hepatic level of acetyl-CoA (Fig. 2C) and, notably, a >70% decrease in that of malonyl-CoA (Fig. 2D) in *db/db* mice. To examine the effect of ACL knockdown on lipogenesis, we determined the rate of hepatic fatty acid synthesis by measuring the incorporation of ^{3}H-water into hepatic fatty acids. Ad-shACL–mediated knockdown of hepatic ACL decreased hepatic lipogenesis by approximately 78% (Fig. 2E). Hence, these results demonstrate that hepatic ACL is crucial for the production of both acetyl-CoA and malonyl-CoA as well as *de novo* lipogenesis in the liver. Moreover, given the regulatory role of malonyl-CoA as the metabolic intermediate between lipogenesis and fatty acid β-oxidation,[12,29] the reduction in hepatic malonyl-CoA resulting from ACL deficiency may directly elicit significant metabolic alterations in hepatic lipid metabolism.

Ablation of Hepatic ACL Prevents Hepatic Steatosis and Reduces Circulating Levels of Free Fatty Acids in db/db Mice

We next tested whether ACL is connected to obesity-induced development of hepatic steatosis in association with defective leptin actions. In C57BL/6J *db/db* mice that have dramatically enlarged livers, Ad-shACL–mediated hepatic ACL ablation markedly alleviated this hepatomegaly, as shown by a significant decrease in the liver weight (by ≈25%) as compared with Ad-shLacZ–infected control mice (Fig. 3A). Histological examination of liver sections revealed that hepatic ACL abrogation markedly reduced liver steatosis in *db/db* mice, because greatly diminished amounts of lipid droplets were observed in the livers of mice infected by Ad-shACL (Fig. 3B) compared with control mice. Consistently, hepatic ACL ablation dramatically decreased (by ≈50%) the TG contents (Fig. 3C) while slightly increasing the cholesterol levels in the liver. Moreover, abrogation of hepatic ACL resulted in a dramatic reduction (by ≈50%) in the serum levels of free
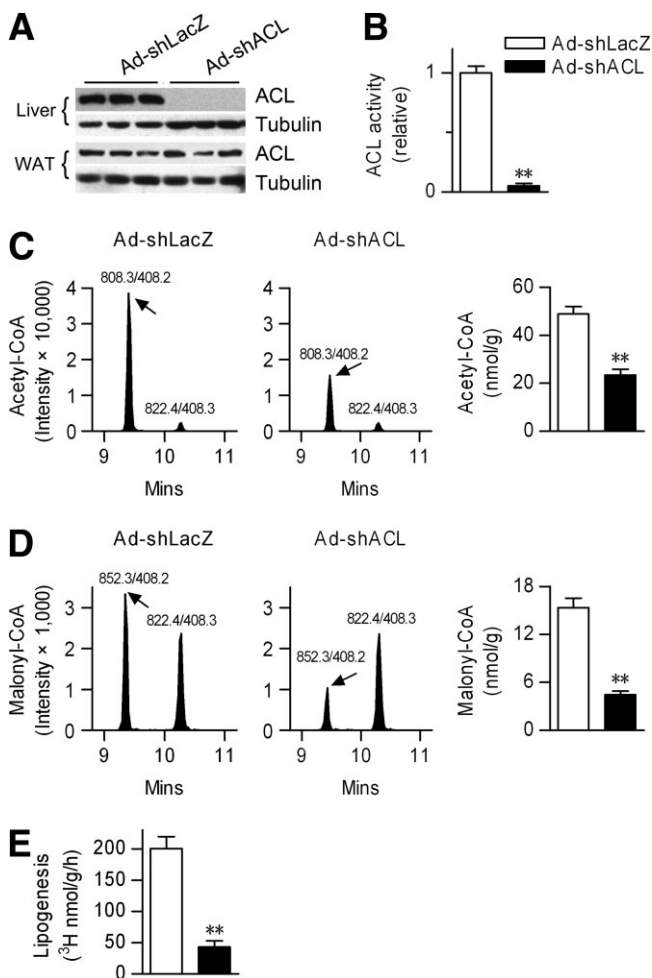
Fig. 2. Hepatic ACL knockdown reduces the hepatic levels of acetyl-CoA and malonyl-CoA as well as hepatic lipogenesis in *db/db* mice. (A-D) Male C57BL/6J *db/db* mice at 8 weeks of age were infected with control adenovirus Ad-shLacZ or targeting adenovirus Ad-shACL via tail-vein injection. At 15 days postinfection, animals were sacrificed in the fed state. (A) Western immunoblot analysis of ACL protein levels from livers and white adipose tissue. Tubulin expression levels were used as the loading control, and each lane represents pooled samples from 2 to 3 mice. (B) The bar graph shows the relative ACL enzyme activities measured from the liver extracts (n = 7-8/group). (C, D) Levels of acetyl-CoA and malonyl-CoA in the liver extracts were determined by LC-MS-MS using the ABI4000 Q-Trap system. Shown are representative tandem mass spectrometry analyses of (C) acetyl-CoA (ion pair at 808.3/408.2) and (D) malonyl-CoA (ion pair at 852.3/408.2) from each individual mouse treated with Ad-shLacZ versus Ad-shACL, with n-propionyl-CoA (ion pair at 822.4/408.3) used as an internal reference. The bar graphs indicate the average levels calculated from standard curves (n = 7-8/group). (E) Male C57BLKS/J *db/db* mice (n = 4/group) at 8 weeks of age were infected with Ad-shLacZ versus Ad-shACL for 12 days. Hepatic lipogenesis rate was determined by measuring the incorporation of ^3H_2O into the fatty acid fractions from the liver of mice injected with ^3H_2O for 1 hour. The bar graphs indicate average levels of the incorporated ^3H-radioactivity into fatty acids. Data in all panels are expressed as the mean ± SEM. **$P < 0.01$ versus Ad-shLacZ-infected control mice.

fatty acids (FFA) but did not alter those of TGs and cholesterol (Fig. 3D). These data thus reveal a crucial link between dysregulated ACL and hepatic steatosis in *db/db* mice, suggesting that targeted suppression of hepatic ACL

can effectively prevent the occurrence of obesity-associated fatty liver.

Deficiency in ACL Results in Suppression of the Entire Lipogenic Program in the Livers of db/db Mice

To investigate the mechanisms whereby hepatic ACL abrogation led to protection against hepatic steatosis, we examined the impact of ACL deficiency upon the gene expression programs of hepatic lipid metabolism, namely the expression of key lipogenic regulators and metabolic enzymes for the biosynthesis of fatty acids and triglycerides, as well as those involved in fatty acid oxidation.

In C57BL/6J *db/db* mice infected with Ad-shACL for 15 days and sacrificed under *ad libitum* feeding conditions, quantitative RT-PCR analysis revealed an approximately 56% reduction in the mRNA expression level of PPARγ but little changes in that of SREBP1c or LXRα (Fig. 4A). Interestingly, hepatic ACL abrogation resulted in considerable increases in the expression of liver X receptor β (LXRβ) (by ≈86%) and modest elevations in the expression of SREBP2 (by ≈26%) (Fig. 4A). This may explain the marginal elevation in hepatic cholesterol levels observed (Fig. 3C), given the role of LXRβ and SREBP2 in cholesterol homeostasis.[30,31] In the fasted state, however, as analyzed in C57BLKS/J *db/db* mice infected by Ad-shACL for 23 days during the subsequent study of glucose homeostasis, ACL deficiency led to similarly repressed levels of PPARγ but significant reductions in both SREBP1c (by ≈56%) and LXRα (by ≈70%) (Supplementary Fig. 2A). On the other hand, no significant changes were detected in the expression of two other lipogenic regulators, PPARγ coactivator 1β (PGC-1β)[32] and carbohydrate-responsive element-binding protein, either under fed or fasted conditions (Fig. 4A; Supplementary Fig. 2A). In parallel with ACL deficiency–induced PPARγ suppression both in the fed and fasted states, the mRNA expression levels were prominently reduced for FAS (by ≈67% and ≈77%, respectively), ELOVL6 (by ≈70% and ≈60%, respectively), GPAT (by ≈62% and ≈42%, respectively) and DGAT2 (by ≈35% and ≈51%, respectively), suggesting a predominant role for PPARγ in regulation of this subset of lipogenic enzymes (Fig. 4B and Supplementary Fig. 2B). Notably, corresponding to the decreased expression of LXRα and SREBP1c in the fasted state, more dramatic suppression was observed for SCD1 (by ≈57%) and DGAT1 (by ≈50%) (Supplementary Fig. 2B), indicating the involvement of the LXRα-SREBP1c pathway[33] in control of their expression.

In the fed state, hepatic ACL deficiency also led to dramatically reduced expression (by ≈68%) of PPARα,

Table 1. Physiologic Data and Serum Measurement of *db/db* Mice Infected by Ad-shLacZ and Ad-shACL

	C57BL/6J		C57BLKS/J	
	Ad-shLacZ	Ad-shACL	Ad-shLacZ	Ad-shACL
Body weight (g)	43.0 ± 0.6	40.2 ± 0.9*	40.6 ± 0.9	40.7 ± 0.9
Food intake (g/day)	7.12 ± 0.32	7.14 ± 0.76	7.08 ± 0.53	6.98 ± 0.38
Fat mass (g)	25.35 ± 0.54	23.29 ± 0.50*	22.92 ± 0.76	22.47 ± 0.63
EF weight (g)	1.404 ± 0.034	1.181 ± 0.035*	ND	ND
Fasted glucose (mg/dL)	214.7 ± 33.6	97.3 ± 8.5†	204.8 ± 18.6	100.0 ± 14.9†
β-HB (mM) (fed)	0.28 ± 0.03	0.28 ± 0.02	ND	ND
ALT (U/L) (fasted)	38.99 ± 2.90	34.77 ± 1.88	66.24 ± 6.58	64.94 ± 3.35
AST (U/L) (fasted)	45.69 ± 5.37	59.04 ± 4.62	105.63 ± 10.47	118.87 ± 4.77

All data are from 8-week-old male C57BL/6J (n = 7 per group) or C57BLKS/J *db/db* mice (n=6-7 per group) infected for 15 or 23 days by Ad-shACL versus Ad-shLacZ, except that the levels of alanine transaminase and aspartate transaminase were determined at 8 (for C57BL/6J mice) or 10 (for C57BLKS/J mice) days postinfection. Data are expressed as the mean ± SEM.

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; EF, epididymal fat pad; β-HB, β-hydroxybutyrate; ND, not determined.

*$P < 0.05$, †$P < 0.01$ versus control mice infected by Ad-shLacZ.

the key regulator of β-oxidation,[34] in the liver of *db/db* mice (Fig. 4C). In parallel, the PPARγ target genes, long-chain acyl-CoA dehydrogenase and medium-chain acyl-CoA dehydrogenase, which catalyze the initial steps in mitochondrial fatty acid oxidation, displayed significantly decreased expression levels (by ≈31% and ≈36%, respectively). Notably, acyl-CoA oxidase (ACOX) 1, another PPARα target gene for peroxisomal β-oxidation of fatty acids, was also markedly suppressed (by ≈72%) as a result of hepatic ACL deficiency (Fig. 4C). On the other hand, few changes were detected in the expression of genes related to fatty acid transport, fatty acid transport protein (FATP) 1, CD36, and carnitine palmitoyltransferase (CPT) 1a. Similar hepatic ACL deficiency-induced alterations were also observed in the expression patterns of these genes in the fasted state (Supplementary Fig. 2C).

To confirm that the lipogenic program was blocked as a result of hepatic ACL abrogation, we analyzed the expression levels by western immunoblotting of PPARγ, FAS, and ACC. In fed C57BL/6J *db/db* mice (Fig. 5A), hepatic ACL abrogation markedly diminished (by

≈90%) the protein expression levels of PPARγ and significantly reduced those of FAS (by ≈69%) and ACC1/2 (by ≈68%) (Fig. 5B). Of note, no alterations in the phosphorylation status of ACC1/2 were detected. Conceivably, the decreased ACC1/2 expression, rather than its phosphorylation inactivation, was likely responsible for the more pronounced reduction in the level of malonyl-CoA than acetyl-CoA resulting from ACL abrogation (Fig. 2C,D).

Taken together, these results demonstrate that hepatic ACL abrogation not only resulted in marked decreases in the production of precursor molecules for lipid synthesis, but also triggered metabolic alterations that blunted the entire lipogenic program in the livers of *db/db* mice, leading to efficient inhibition of hepatic *de novo* lipogenesis. The suppressed expression of hepatic lipogenic genes was likely mediated, at least in part, through normalization of the elevated PPARγ expression levels in obese animals. On the other hand, given the observed decreases in the expression of genes related to fatty acid oxidation, lipid disposal through β-oxidation might play a lesser role in
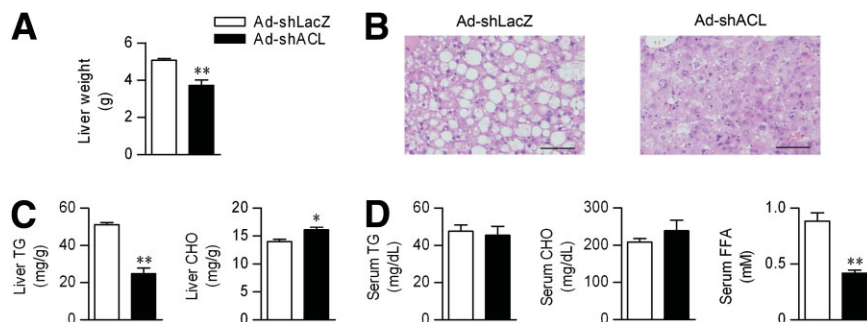

Fig. 3. Hepatic ACL abrogation protects against liver steatosis in *db/db* mice. Male C57BL/6J *db/db* mice at 8 weeks of age were infected with Ad-shLacZ versus Ad-shACL. Measurements were performed at 15 days postinfection. (A) Liver weight. (B) Representative images of hematoxylin-eosin staining of liver sections (n = 5/group) (magnification ×10). The bar indicates 1 mm. (C) TG and cholesterol (CHO) levels in liver extracts. (D) Serum levels of TGs, cholesterol, and FFAs. Data are expressed as the mean ± SEM (n = 7-8/group). *$P < 0.05$ and **$P < 0.01$ versus Ad-shLacZ–infected control mice.
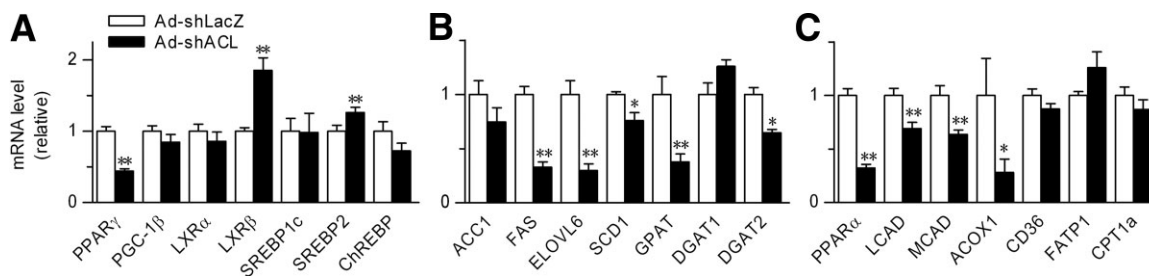
Fig. 4. Effects of hepatic ACL deficiency upon the lipogenic program and the expression of genes related to fatty acid oxidation/transport in the liver of *db/db* mice. Assessment of the mRNA expression levels of the indicated molecules was performed via quantitative RT-PCR from the livers of C57BL/6J *db/db* mice infected by Ad-shLacZ versus Ad-shACL and sacrificed at 15 days postinfection in the fed state (n = 7/group). Shown is the relative expression level of each gene to that of the Ad-shLacZ control group, using glyceraldehyde 3-phosphate dehydrogenase as the internal control. (A) Expression levels of key regulators of lipid metabolism, PPARγ, PGC-1β, LXRα and LXRβ, SREBP1c and SREBP2, and carbohydrate-responsive element-binding protein. (B) Expression levels of enzymes responsible for fatty acid and TG synthesis, ACC1, FAS, ELOVL6, SCD1, GPAT, DGAT1, and DGAT2. (C) Expression levels of genes for fatty acid oxidation and transport, PPARα, long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), acyl-CoA oxidase 1 (ACOX1), CD36, fatty acid transport protein 1 (FATP1) and carnitine palmitoyltransferase 1a (CPT1a). Values are expressed as the mean ± SEM. *$P < 0.05$ and **$P < 0.01$ versus Ad-shLacZ–infected control mice.

the attenuation of hepatic steatosis as a result of hepatic ACL ablation. Consistent with this idea, knockdown of hepatic ACL did not increase serum levels of ketone bodies (Table 1).

Deficiency in Hepatic ACL Improves Systemic Glucose Metabolism and Insulin Sensitivity in db/db Mice

Because hepatic steatosis and circulating FFA elevations are closely associated with obesity, insulin resistance

Fig. 5. Hepatic ACL abrogation markedly diminishes the protein expression levels of PPARγ, FAS, and ACC in the liver of *db/db* mice. The expression of (A) ACL and PPARγ and (B) FAS and ACC1/2 (total and the phosphorylated form) was analyzed via western immunoblotting in the livers of male C57BL/6J *db/db* mice infected for 15 days with Ad-shLacZ versus Ad-shACL. Each lane represents the liver extract analyzed for one animal in the fed state. Relative protein levels were determined by densitometry quantification of the immunoblots after normalization to glyceraldehyde 3-phosphate dehydrogenase, and the phosphorylation level of ACC1/2 was determined from the ratio of pACC1/2 over total ACC1/2. Data are expressed as the mean ± SEM (n = 7/group). **$P < 0.01$ versus Ad-shLacZ–infected control mice.

and hyperglycemia, we next investigated the impact of hepatic ACL ablation upon adiposity, energy balance, and glucose metabolism in *db/db* mice. C57BL/6J *db/db* mice infected with Ad-shACL for 15 days exhibited slightly reduced body weight and fat mass without changes in food intake, but showed dramatically decreased serum glucose levels in the fasted state (Table 1). However, no appreciable changes in metabolic rate, respiratory exchange ratio, or physical activities were detected (Supplementary Fig. 3). Infection of C57BLKS/J *db/db* mice, which manifest more severe insulin resistance and hyperglycemia phenotypes,[35] by Ad-shACL for up to 23 days led to few changes in body weight and fat content (Table 1) despite similar degrees of knock-down of hepatic ACL expression (data not shown). Therefore, hepatic ACL abrogation exerted little action upon energy homeostasis and adiposity, and the observed slight reduction in the body weight of C57BL/6J *db/db* mice might similarly reflect the nonspecific effects of the adenoviral infection as described.[36] As in C57BL/6J *db/db* animals, Ad-shACL–mediated hepatic ACL abrogation in C57BLKS/J *db/db* mice markedly reduced the fasted glucose levels, by ≈38%, ≈58%, and ≈58%, respectively, at 8, 12, and 16 days postinfection compared with control mice infected by Ad-shLacZ (Fig. 6A). Furthermore, glucose tolerance test showed dramatically improved glucose tolerance in Ad-shACL–infected mice at 16 days (Fig. 6B). To exclude the possibility of potential "off-target" effects exerted by Ad-shACL, a second adenovirus (Ad-shACL#) with similar ACL knock-down efficiency was also tested. Consistently, marked amelioration of glucose tolerance and hyperglycemia in *db/db* mice were observed, along with similarly attenuated liver steatosis and repressed expression profiles of lipogenic genes (data not shown). Thus, these results demonstrate that targeted suppression of
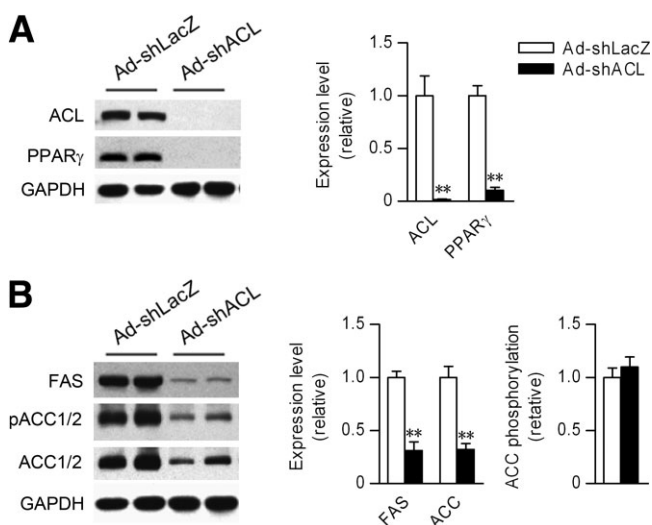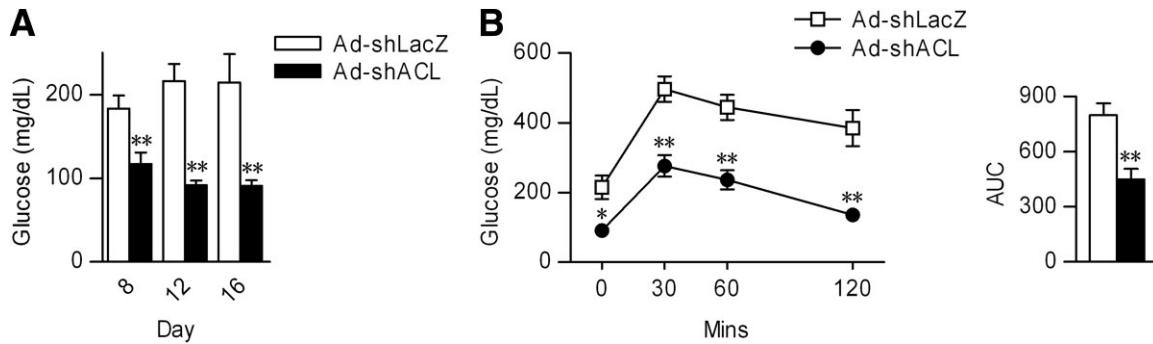
Fig. 6.   Hepatic ACL deficiency ameliorates systemic glycemic control in *db/db* mice. Male C57BLKS/J *db/db* mice at 8 weeks of age were infected with Ad-shLacZ or Ad-shACL. (A) Fasted glucose levels were monitored at 8, 12, and 16 days after adenoviral administration (n = 6-7/group). (B) Glucose tolerance test was performed at 16 days postinfection. The bar graph represents the area under the curve (AUC) for the blood glucose levels from glucose tolerance test measurements (n = 6-7/group). Data are expressed as the mean ± SEM. *$P < 0.05$ and **$P < 0.01$ versus Ad-shLacZ–infected control mice.

ACL, the lipogenic enzyme that initiates lipid synthesis in the liver, is able to correct hyperglycemia associated with obesity and fatty liver.

To further examine the physiological mechanisms by which deficiency in hepatic ACL improves glucose metabolism in *db/db* mice, we first examined by quantitative RT-PCR the expression levels of key gluconeogenic genes in the liver. Under fasted conditions, C57BLKS/J *db/db* mice at 23 days postinfection by Ad-shACL displayed significant reductions in the mRNA levels of PGC-1α (by ≈50%), the chief gluconeogenic regulator,[37] and its two target genes, phosphoenolpyruvate carboxykinase (by ≈50%) and glucose-6-phosphatase (by ≈75%) (Fig. 7A). Therefore, ACL deficiency–induced repression of the gluconeogenic pathway could contribute to the observed normalization of hyperglycemia in *db/db* mice.

To determine whether hepatic ACL cell-autonomously regulates insulin signaling, insulin-stimulated phosphorylation of Akt and glycogen synthase kinase-3β (GSK-3β) was measured in the liver. When compared with Ad-shLacZ–infected control mice, no apparent increases were observed in the phosphorylation of hepatic Akt or GSK-3β in C57BLKS/J *db/db* mice infected with Ad-shACL for either 17 days (Fig. 7B) or 23 days (data not shown). Consistently, in the primary hepatocytes isolated from C57BL/6J *db/db* mice, ACL knockdown prominently reduced the expression of FAS and ACC but did not significantly alter the insulin-stimulated phosphorylation of Akt and GSK-3β (Fig. 7C). Notably, ACL abrogation both in the liver and in primary hepatocytes led to increased basal levels of GSK-3β phosphorylation (Fig. 7B,C). In contrast, hepatic ACL abrogation led to apparent improvement of insulin resistance in the skeletal muscles. Insulin stimulated phosphorylation of both Akt and GSK-3β in soleus muscles to a greater extent in Ad-shACL–infected than in Ad-shLacZ–infected *db/db* mice

(Fig. 7D; Supplementary Fig. 4). These data demonstrate that amelioration of hepatic steatosis and a reduction of serum FFA levels as a result of hepatic ACL abrogation can improve muscular insulin sensitivity.

Discussion

In the current study, we have established an important role for hepatic ACL, which is dysregulated in an obese and diabetic mouse model with spontaneous progression of liver steatosis, in lipid and glucose metabolism. Our findings illustrate a close pathological link between elevated expression of hepatic ACL and derangement of lipid metabolism in the liver under defective leptin signaling conditions, and further substantiate the contribution of *de novo* lipogenesis to the development of obesity-associated NAFLD and loss of glycemic control.

Our results demonstrate that targeted suppression of hepatic ACL could effectively prevent liver steatosis in *db/db* mice. The restored lipid homeostasis in the liver most likely arises from the suppression of the entire lipogenic program, which is abnormally enhanced in the livers of *db/db* mice. First, deficiency in hepatic ACL significantly reduced the biosynthesis of malonyl-CoA, the essential precursor for fatty acid and TG synthesis. Second, hepatic ACL abrogation prominently normalized the expression level of PPARγ, which was dramatically elevated in *db/db* animals. This in turn is likely responsible for the observed coordinate repression of a broad set of key enzymes related to fatty acid and TG biosynthesis, including ACC1, FAS, ELOVL6, SCD1, GPAT, and DGAT1/2.[38] Consequently, hepatic ACL abrogation markedly inhibited *de novo* fatty acid synthesis in the liver. Consistent with our findings, pharmacological inhibition of ACL results in hypolipidemia in rats.[39]
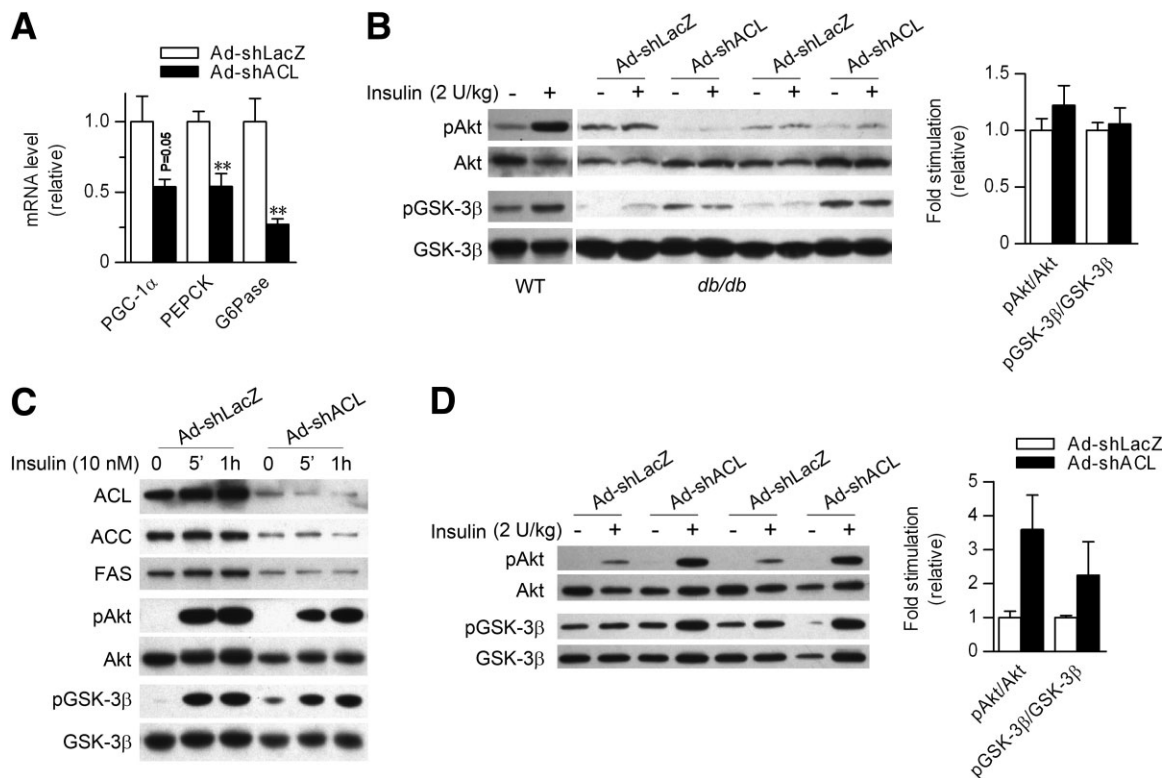
Fig. 7. Knock-down of hepatic ACL suppresses gluconeogenic gene expression and improves muscular insulin sensitivity in *db/db* mice. (A, B) Male C57BLKS/J *db/db* mice at 8 weeks of age were infected with Ad-shLacZ or Ad-shACL for 23 or 17 days. (A) Analysis via quantitative RT-PCR of the relative mRNA levels of PGC-1α, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase) in the livers of mice at 23 days postinfection and sacrificed in the fasted state (n = 6-7/group). (B) Virus-infected *db/db* mice were treated with 2 U/kg insulin at 17 days postinfection, and the phosphorylation of hepatic Akt and GSK-3β was measured by immunoblotting using phospho-Akt or phospho–GSK-3β antibodies. WT mice were used as positive controls. (C) Primary hepatocyte cultures prepared from *db/db* mice were infected with Ad-shACL versus Ad-shLacZ adenoviruses and were stimulated with 10 nM insulin. The expression of ACL, ACC and FAS, and the phosphorylation of Akt and GSK-3β were measured via immunoblotting assays. (D) Analysis via immunoblotting of phosphorylation levels of Akt and GSK-3β, upon stimulation by 2 U/kg insulin versus untreated control, in the soleus muscles of *db/db* mice infected for 17 days. The bar graphs in (B) and (D) indicate the average fold stimulation of Akt and GSK-3β phosphorylation relative to the untreated control group (n = 3/group), with the value from Ad-shLacZ–infected group set as 1. Data are expressed as the mean ± SEM. **$P < 0.01$ versus Ad-shLacZ–infected control mice.

Whereas the resultant decrease in the level of malonyl-CoA, a physiological inhibitor of CPT1,[29,40] may lead to increased fatty acid β-oxidation and hepatic lipid disposal, deficiency in hepatic ACL also led to marked suppression of the hepatic expression of PPARα as well as its target genes long-chain acyl-coenzyme A dehydrogenase, medium-chain acyl-coenzyme A dehydrogenase, and acyl-coenzyme A oxidase 1. Therefore, we suspect that ACL deficiency–elicited repression of lipid synthesis, not increased fatty acid oxidation, most likely played a major role in the prevention of fatty liver in the obese mice. This is in line with the fact that no significant changes in the serum levels of β-hydroxybutyrate were detected in the hepatic ACL-deficient *db/db* mice (Table 1).

Hepatic steatosis is known to be closely associated with insulin resistance and hyperglycemia.[11,41] We found that targeted suppression of hepatic ACL led to marked amelioration of hyperglycemia and improvement of insulin resistance. First, the improved glucose metabolism can be

mediated, at least in large part, by the correction of liver steatosis, which may in turn lead to suppression of the gluconeogenic pathway. While the detailed molecular mechanisms responsible for the observed down-regulation of gluconeogenic genes remain to be explored, the decreased expression of PPARα may also contribute to the amelioration of hyperglycemia in hepatic ACL-deficient *db/db* mice, because PPARα is thought to regulate the gluconeogenic program.[42,43] Second, the marked beneficial effects of hepatic ACL abrogation upon glycemic control may arise from the resultant repression of key enzymes in lipid biosynthesis, such as ACC1, SCD1, and ELOVL6. Recently reported studies have revealed that deficiency in these enzymes in the liver can exert profound but distinct impact upon adiposity, hepatic steatosis, gluconeogenesis, and insulin resistance in animal models, presumably via altering the hepatic fatty acid compositions.[23,25,26] Third, hepatic ACL deficiency-elicited improvement of insulin signaling in the muscle reflects the

broad metabolic alterations that may involve cross-talks between different tissues, leading to alleviation of insulin resistance at the whole-body level. We speculate that the observed improvement in systemic glucose metabolism in the hepatic ACL-deficient mice, paralleled by the prominent decreases in the levels of hepatic malonyl-CoA and serum FFAs, is metabolically analogous to that reported for the rodent model through adenoviral overexpression of an active form of malonyl-CoA decarboxylase in the liver.[36] When challenged with a high-fat diet, malonyl-CoA decarboxylase–induced hepatic malonyl-CoA reductions in these diet-induced obese rats lead to improved muscular insulin sensitivity, likely through local changes in the muscular fatty acid metabolite compositions in association with decreased plasma FFAs, which are known to induce insulin resistance in muscles.[36,44] Because *db/db* mice are known to have elevated expression levels of PPARα and PPARγ,[45] it is of particular note that hepatic ACL abrogation could lead to normalization of the expression of both nuclear receptors through mechanisms that are currently not clear. This may in turn serve to amplify the metabolic effects of ACL suppression, bringing about dramatic changes in the lipogenic pathway and, consequently, hepatic fatty acid compositions.

Our results demonstrate that targeted suppression of ACL in the liver, a critical enzyme that regulates the initial step in lipid biosynthesis and is dysregulated in the obese state, not only leads to protection against liver steatosis, but also to marked improvement in whole-body glucose metabolism. Because impaired leptin signaling ("leptin resistance") is closely associated with human obesity, fatty liver, and diabetes,[46] these findings provide direct physiological evidence that hepatic ACL represents an attractive therapeutic target for the treatment of both NAFLD and type 2 diabetes.

References

1. Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. HEPATOLOGY 2003;37:1202-1219.
2. Adams LA, Lindor KD. Nonalcoholic fatty liver disease. Ann Epidemiol 2007;17:863-869.
3. Browning JD, Horton JD. Molecular mediators of hepatic steatosis and liver injury. J Clin Invest 2004;114:147-152.
4. Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. HEPATOLOGY 2006;43(Suppl 1):S99-S112.
5. Brunt EM. Pathology of fatty liver disease. Mod Pathol 2007;(Suppl 1):S40-S48.
6. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. HEPATOLOGY 2003;37:917-923.
7. Wieckowska A, McCullough AJ, Feldstein AE. Noninvasive diagnosis and monitoring of nonalcoholic steatohepatitis: present and future. HEPATOLOGY 2007;46:582-589.
8. Samuel VT, Liu ZX, Wang A, Beddow SA, Geisler JG, Kahn M, et al. Inhibition of protein kinase Cepsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease. J Clin Invest 2007;117:739-745.
9. Watanabe S, Yaginuma R, Ikejima K, Miyazaki A. Liver diseases and metabolic syndrome. J Gastroenterol 2008;43:509-518.
10. Rector RS, Thyfault JP, Wei Y, Ibdah JA. Non-alcoholic fatty liver disease and the metabolic syndrome: an update. World J Gastroenterol 2008;14:185-192.
11. Postic C, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. J Clin Invest 2008;118:829-838.
12. Wakil SJ, Stoops JK, Joshi VC. Fatty acid synthesis and its regulation. Ann Rev Biochem 1983;52:537-579.
13. Srere PA. The Citrate Cleavage Enzyme. I. Distribution and purification. J Biol Chem 1959;234:2544-2547.
14. Yu S, Matsusue K, Kashireddy P, Cao WQ, Yeldandi V, Yeldandi AV, et al. Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma 1 (PPAR-gamma 1) overexpression. J Biol Chem 2003;278:498-505.
15. Herzig S, Hedrick S, Morantte I, Koo SH, Galimi F, Montminy M. CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-γ. Nature 2003;426:190-193.
16. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, et al. Role of LXRs in control of lipogenesis. Genes Dev 2000;14:2831-2838.
17. Shimano H, Yahagi N, Amemiya-Kudo M, Hasty AH, Osuga Ji, Tamura Y, et al. Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. J Biol Chem 1999;274:35832-35839.
18. Uyeda K, Repa JJ. Carbohydrate response element binding protein, ChREBP, a transcription factor coupling hepatic glucose utilization and lipid synthesis. Cell Metab 2006;4:107-110.
19. Denechaud PD, Bossard P, Lobaccaro JM, Millatt L, Staels B, Girard J, et al. ChREBP, but not LXRs, is required for the induction of glucose-regulated genes in mouse liver. J Clin Invest 2008;118:956-964.
20. Lewis GF, Carpentier A, Adeli K, Giacca A. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. Endocr Rev 2002;23:201-229.
21. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest 2005;115:1343-1351.
22. Mao J, DeMayo FJ, Li H, bu-Elheiga L, Gu Z, Shaikenov TE, et al. Liver-specific deletion of acetyl-CoA carboxylase 1 reduces hepatic triglyceride accumulation without affecting glucose homeostasis. Proc Natl Acad Sci U S A 2006;103:8552-8557.
23. Savage DB, Choi CS, Samuel VT, Liu ZX, Zhang D, Wang A, et al. Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. J Clin Invest 2006;116:817-824.
24. Chakravarthy MV, Pan Z, Zhu Y, Tordjman K, Schneider JG, Coleman T, et al. "New" hepatic fat activates PPARalpha to maintain glucose, lipid, and cholesterol homeostasis. Cell Metab 2005;1:309-322.
25. Miyazaki M, Flowers MT, Sampath H, Chu K, Otzelberger C, Liu X, et al. Hepatic stearoyl-CoA desaturase-1 deficiency protects mice from carbohydrate-induced adiposity and hepatic steatosis. Cell Metab 2007;6:484-496.
26. Matsuzaka T, Shimano H, Yahagi N, Kato T, Atsumi A, Yamamoto T, et al. Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. Nat Med 2007;13:1193-1202.
27. Beigneux AP, Kosinski C, Gavino B, Horton JD, Skarnes WC, Young SG. ATP-citrate lyase deficiency in the mouse. J Biol Chem 2004;279:9557-9564.
28. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. Nature 1998;395:763-770.

29. McGarry JD, Mannaerts GP, Foster DW. A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. J Clin Invest 1977;60:265-270.

30. Zelcer N, Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. J Clin Invest 2006;116:607-614.

31. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 2002;109:1125-1131.

32. Lin J, Yang R, Tarr PT, Wu PH, Handschin C, Li S, et al. Hyperlipidemic effects of dietary saturated fats mediated through PGC-1β coactivation of SREBP. Cell 2005;120:261-273.

33. Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, et al. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev 2000;14:2819-2830.

34. Lefebvre P, Chinetti G, Fruchart JC, Staels B. Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis. J Clin Invest 2006;116:571-580.

35. Mu JL, Naggert JK, Svenson KL, Collin GB, Kim JH, McFarland C, et al. Quantitative trait loci analysis for the differences in susceptibility to atherosclerosis and diabetes between inbred mouse strains C57BL/6J and C57BLKS/J. J Lipid Res 1999;40:1328-1335.

36. An J, Muoio DM, Shiota M, Fujimoto Y, Cline GW, Shulman GI, et al. Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance. Nat Med 2004;10:268-274.

37. Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, et al. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 2001;413:131-138.

38. Matsusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, et al. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. J Clin Invest 2003; 111:737-747.

39. Pearce NJ, Yates JW, Berkhout TA, Jackson B, Tew D, Boyd H, et al. The role of ATP citrate-lyase in the metabolic regulation of plasma lipids. Hypolipidaemic effects of SB-204990, a lactone prodrug of the potent ATP citrate-lyase inhibitor SB-201076. Biochem J 1998;334:113-119.

40. McGarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 1997; 244:1-14.

41. Dentin R, Benhamed F, Hainault I, Fauveau V, Foufelle F, Dyck JRB, et al. Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice. Diabetes 2006;55:2159-2170.

42. Leone TC, Weinheimer CJ, Kelly DP. A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders. Proc Natl Acad Sci U S A 1999;96:7473-7478.

43. Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. J Clin Invest 1999;103:1489-1498.

44. Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, et al. Mechanism of free fatty acid-induced insulin resistance in humans. J Clin Invest 1996;97:2859-2865.

45. Memon RA, Tecott LH, Nonogaki K, Beigneux A, Moser AH, Grunfeld C, et al. Up-regulation of peroxisome proliferator-activated receptors (PPAR-alpha) and PPAR-gamma messenger ribonucleic acid expression in the liver in murine obesity: troglitazone induces expression of PPAR-gamma-responsive adipose tissue-specific genes in the liver of obese diabetic mice. Endocrinology 2000;141:4021-4031.

46. Myers MG, Cowley MA, Munzberg H. Mechanisms of leptin action and leptin resistance. Annu Rev Physiol 2008;70:537-556.