Published in final edited form as:
Hepatology. 2010 September ; 52(3): 1134–1142. doi:10.1002/hep.23812.

Pnpla3/adiponutrin deficiency in mice is not associated with fatty liver disease

Weiqin Chen[1], **Benny Chang**[1], **Lan Li**[1], and **Lawrence Chan**[1,2,*]

[1] Diabetes and Endocrinology Research Center (DERC), Section of Diabetes and Endocrinology, Departments of Medicine, Molecular & Cellular Biology and Biochemistry & Molecular Biology, Baylor College of Medicine, Houston, Texas, USA

[2] St. Luke's Episcopal Hospital, Houston, Texas, USA

Abstract

PNPLA3 (Adiponutrin), a novel patatin-like phospholipase domain-containing enzyme, is expressed at high level in fat, but also in other tissues including liver. Polymorphisms in *PNPLA3* have been linked to obesity and insulin sensitivity. Notably, a nonsynonymous variant rs738409(G) allele of the *PNPLA3* gene was found to be strongly associated with both non-alcoholic and alcoholic fatty liver disease. We have generated *Pnpla3*$^{-/-}$ mice by gene targeting. Loss of Pnpla3 has no effect on body weight or composition, adipose mass or development, whether the mice were fed regular chow or high-fat diet or bred into *Lep*$^{ob/ob}$ background. Plasma and liver triglyceride content and plasma aspartate aminotransferase and alanine aminotransferase levels were not different between *Pnpla3*$^{+/+}$ and *Pnpla3*$^{-/-}$ mice while they were on regular chow, fed three different fatty liver-inducing diets, or after they were bred into *Lep*$^{ob/ob}$ background. Hepatic Pnpla5 mRNA levels were similar in wild-type and *Pnpla3*$^{-/-}$ mice, though *adipose* Pnpla5 mRNA level was increased in *Pnpla3*$^{-/-}$ mice. A high sucrose lipogenic diet stimulated hepatic Pnpla3 and Pnpla5 mRNA levels to a similar degree, but it did not affect adipose or liver triglyceride lipase (ATGL, aka Pnpla2) mRNA in *Pnpla3*$^{+/+}$ and *Pnpla3*$^{-/-}$ mice. Finally, *Pnpla3*$^{+/+}$ and *Pnpla3*$^{-/-}$ mice displayed similar glucose tolerance and insulin tolerance tests while on regular chow or three different fatty liver-inducing diets. *Conclusion:* Loss of Pnpla3 does not cause fatty liver, liver enzyme elevation, or insulin resistance in mice.

Keywords

Pnpla3 deficiency; fatty liver disease; insulin resistance

INTRODUCTORY STATEMENT

Fatty liver disease (FLD), especially nonalcoholic fatty liver disease (NAFLD), is characterized by excessive triacylglycerol (TG) accumulation in the liver, and is the most common form of liver disease in western society (1,2). While alcohol abuse, obesity, insulin resistance and diabetes are known to be associated with FLD, genetic factors causing predisposition to hepatic steatosis are not well understood. Recently, several genome-wide association studies (GWAS) have identified a common genetic variant (rs738409 G allele) in the *PNPLA3* gene to be strongly associated with increased liver fat content and susceptibility to nonalcoholic fatty liver disease (NAFLD) (3–5). A subsequent study extends the association of this single nucleotide polymorphism (SNP) also to alcoholic liver

Contact Information: Lawrence Chan, Diabetes and Endocrinology Research Center R614, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. Tel: 713-798-4478, Fax: 713-798-8764, lchan@bcm.edu.

disease (6). *PNPLA3* was recently identified as one of the genetic loci influencing plasma levels of liver enzymes (7), and the rs738409 G allele was also associated with elevated serum levels of aspartate aminotransferase (ALT) and alanine aminotransferase (AST) (3,5). A recent biochemical study showed that this nonsynonymous SNP is associated with an inactive PNPLA3, suggesting a possible direct relation between loss of function of PNPLA3 and an increase in hepatic TG content and inflammation (8).

PNPLA3, also called adiponutrin, belongs to a novel class of patatin-like phospholipase (PNPLA) domain family proteins that also contain a conserved lipase catalytic dyad (Gly-X-Ser-X-Gly and Asp-X-Gly/Ala) (9). Like other PNPLA family proteins such as PNPLA2 (also called adipose triglyceride lipase (ATGL)) and PNPLA5, PNPLA3 possesses both lipase and transacylase activities *in vitro* (10,11). Its expression is highest in fat, though it also occurs in other tissues, including liver, in mice, and is markedly upregulated during adipogenesis (12). In both rodents and humans adipose *PNPLA3* expression is highly upregulated by glucose and insulin but free fatty acid (FFA) and high fat diet produce no effect (13–16). The regulation of PNPLA3 by insulin and glucose is reminiscent of the control of lipogenic enzymes, such as fatty acid synthase and acetyl-CoA reductase (17), and is very different from that of lipolytic enzymes like ATGL (18). Genetic linkage analyses and clinical studies demonstrated that high adipose *PNPLA3* expression occurs with obesity though not all reports are consistent (13–15,19). Indeed, Pnpla3 mRNA levels are increased in *fa*/*fa* obese Zucker rats that lack leptin receptor (12).

To determine the role of PNPLA3 in fatty liver disease and obesity, we have generated Pnpla3-knockout mice by gene targeting. Loss of Pnpla3 did not affect the liver TG content or serum AST or ALT levels whether the mice were fed normal chow or three different fatty liver-inducing diets, or after they were bred into a genetic obese $Lep^{ob/ob}$ background. Furthermore, $Pnpla3^{-/-}$ mice displayed normal body fat and composition and maintained normal glucose homeostasis and insulin sensitivity. We observed, however, an upregulation of Pnpla5 in the fat depot but not liver of $Pnpla3^{-/-}$ mice. These data indicate that inactivation of Pnpla3 does not lead to hepatic TG accumulation or susceptibility to diet-induced hepatic steatosis, nor does it perturb glucose homeostasis, insulin sensitivity, or adipose development in mice.

EXPERIMENTAL PROCEDURES

Animals

Mice were maintained in a temperature-controlled facility with fixed 12-h-light and 12-h-dark cycles and free access to regular chow and water. Some experiments were done on animals fed with a high-fat diet (HFD; 42% of kcal from fat, Harlan Teklad TD88137), high sucrose very low fat diet (HSD, Harlan Teklad TD03045) and Methionine/choline deficient diet (MCD, MP Biomedicals 0296043910) for various periods of time as indicated. Animals of 8 to 22 weeks old were used throughout this study unless otherwise indicated. All animal experiments were done using protocols approved by the IACUC at Baylor College of Medicine.

Plasma biochemistry

Plasma nonesterified fatty acid (NEFA) (Wako), glycerol (Sigma), glucose (Therma Scientific), total cholesterol, total triacylglycerol levels (Therma DMA), ALT and AST (Teco Diagnostics) were measured by enzymatic assay kits for determination of their concentrations. Serum insulin was measured by enzyme-linked immunosorbent assay (Mercodia).

Tissue lipid analysis

Tissues were homogenized in standard PBS buffer. Lipids were extracted according to Bligh and Dyer (20), and dissolved in 5% triton X-100 in PBS. We measured TG concentration with an Infinity triacylglycerol assay kit (Thermo DMA) and normalized to sample weight.

Glucose tolerance and insulin tolerance tests

For glucose tolerance test (GTT), mice were fasted 6 h or overnight and then injected intraperitoneally (i.p.) a bolus of 1.5 g glucose per kg of body weight. Blood was collected before and at 15, 30, 60 and 120 min after injection. We measured glucose levels using a glucometer (FasTake, LifeScan) and serum insulin level by an enzyme-linked immunosorbent assay (Mercodia). For insulin tolerance test (ITT), mice were fasted for 6 h and injected i.p. with humulin at a final concentration of 0.75 U/kg body weight. Blood was collected for glucose measurements before injection and at 15, 30, 60 and 120 min after injection.

Quantitative RT-PCR

Total RNA was isolated from tissues or cultured cells with TRIzol (Invitrogen) and reverse-transcribed using Superscript II reverse transcriptase using random primers (Invitrogen). We performed real-time quantitative RT-PCR on the Strategene MX3000 real time detection system usingiQ™ SYBR Green PCR reagent kit (Biorad). Primers used were shown in supplemental Table 2.

Statistical analysis

We applied the Student t test for statistical analysis. Differences were considered significant when p values were <0.05. Results were expressed as means ± standard deviation (SD) or standard error (SE) as specified.

RESULTS

Absence of Pnpla3 in mice has no effect on body fat composition and adipose tissue development

The Pnpla3 gene was inactivated by replacing the first 7 exons, including the translation initiation codon and the lipase consensus sequence motif (GXSXG, where G is Glycine, S is Serine, and X is any amino acid), with a neomycin selection cassette (Fig. 1A). Genomic PCR genotyping of tail DNA extracted from wild-type, heterozygous and homozygous littermates are shown in Fig. 1B. Reverse transcription followed by PCR analyses confirmed that there was no Pnpla3 mRNA detectable in the white adipose tissue (WAT) of *Pnpla3*$^{-/-}$ mice (Fig. 1C).

Pnpla3$^{-/-}$ mice were born live with the expected Mendelian mode of inheritance and displayed no overtly abnormal phenotype. They were fertile and nursed their pups normally. The body weights of *Pnpla3*$^{-/-}$ and *Pnpla3*$^{+/+}$ mice showed no difference either while they were fed a normal chow diet (CHD) (Fig. 2A) or a high sucrose very low fat diet (HSD), or high fat diet (HFD) (Fig. 2B), indicating that Pnpla3 deficiency has no effect on body weight. There was also no difference in their 4 h-fasted blood glucose, plasma free fatty acids (NEFA), triacylglycerol (TG), total cholesterol or free glycerol, or overnight fasted plasma insulin level, between *Pnpla3*$^{-/-}$ mice and wild-type littermates while they were fed regular chow or fed HSD or HFD for 10 weeks (Supplementary Table 1). The adiposity index of mice under the regular chow diet determined by Echo Magnetic resonance imaging (MRI) revealed similar body fat content with the fat making up ~7.62% body weight in male wild-type compared with ~8.18% of body weight in male *Pnpla3*$^{-/-}$ mice (Fig. 2C). Their

lean mass was also similar (Fig. 2D). There was also no difference in the adiposity index in HSD or HFD fed *Pnpla3*$^{+/+}$ and *Pnpla3*$^{-/-}$ mice (data not shown). In addition, we detected no difference in the weights of gonadal, subcutaneous, or brown adipose depots in *Pnpla3*$^{+/+}$ and *Pnpla3*$^{-/-}$ mice (data not shown). Experiments on the *in vitro* differentiation of stromal vascular cells (SVC) isolated from *Pnpla3*$^{+/+}$ and *Pnpla3*$^{-/-}$ mice revealed no difference in the efficiency of adipocyte differentiation or TG accumulation between the two genotypes (Supplementary Fig. S1, A & B), which agrees with the fact that the adipose depot mass was similar in these mice. Furthermore, the basal and β-adrenergic agonist-stimulated lipolysis in fully differentiated SVC *in vitro* (Supplementary Fig. S1, C & D) as well as in mice *in vivo* (Supplementary Fig. S1, E & F) was similar between wild-type and *Pnpla3*$^{-/-}$ cells or mice, indicating that, unlike ATGL and hormone sensitive lipase, Pnpla3 does not contribute significantly to basal or β-adrenergic agonist-stimulated lipolysis.

Loss of Pnpla3 does not alter liver TG content or serum ALT and AST levels

The nonsynonymous rs738409 SNP in *PNPLA3* was predicted to cause the loss of PNPLA3 enzymatic activity, a consequence functionally similar to the targeted inactivation of *Pnpla3* in our mouse model (3–6). Microscopic examination of *Pnpla3*$^{-/-}$ mouse liver sections revealed normal histology (data not shown). We analyzed liver TG content in wild-type and *Pnpla3*$^{-/-}$ mice fed regular chow, and after they had been placed on three different fatty liver-inducing diets. As shown in Table 1, mice in C57BL/6 background fed the different fatty liver-inducing diets [including high sucrose very low fat (HSD), high fat (HFD) and the choline/methionine-deficeint (MCD) diets] displayed varying degrees of increased liver TG content compared with mice fed regular chow (CHD). However, there was no significant difference in the degree of hepatic TG accumulation between wild-type and *Pnpla3*$^{-/-}$ mice under each type of diet, indicating that loss of Pnpla3 had no direct impact on liver TG accumulation.

Genetic variations at *PNPLA3* have been reported to be associated with increased serum levels of liver enzymes in human populations (3,5). We found that serum ALT and AST levels varied with the diet conditions (Table 1), being highest in mice fed MCD diet, which may be related to the significant liver damage and inflammation induced by this diet. However, no difference in ALT or AST level was observed between the two genotypes, suggesting that lack of Pnpla3 in mice does not cause an elevated transaminase response in liver either under regular chow or after the mice were fed the different fatty liver-inducing diets.

To further analyze whether loss of Pnpla3 affects fatty liver development associated with a genetic form of obesity, we intercrossed the *Lep*$^{ob/+}$ mice with *Pnpla3*$^{-/-}$ mice to produce *Lep*$^{ob/ob}$/*Pnpla3*$^{+/+}$ and *Lep*$^{ob/ob}$/*Pnpla3*$^{-/-}$ mice. The obesity phenotype was unchanged in these mice. The *Lep*$^{ob/ob}$/*Pnpla3*$^{-/-}$ mice gained similar weight with time as compared to their *Lep*$^{ob/ob}$/*Pnpla3*$^{+/+}$ counterparts (data not shown). Both *Lep*$^{ob/ob}$/*Pnpla3*$^{-/-}$ mice and *Lep*$^{ob/ob}$/*Pnpla3*$^{+/+}$ littermates displayed fatty liver, and there was no difference in the hepatic TG content between the two genotypes (Table 1). Moreover, their serum ALT and AST levels were also not different (Table 1). These data indicate that loss of Pnpla3 in mice has no impact on fatty liver development under basal conditions, after they are on different fatty liver-inducing diets or bred into a genetic model associated with obesity and fatty liver.

Loss of PNPLA3 does not cause glucose intolerance or insulin resistance

Lack of PNPLA3 has been postulated to perturb glucose homeostasis and insulin sensitivity *in vivo* (13,21). As such, we measured the rate of glucose disposal and insulin sensitivity in wild-type and *Pnpla3*$^{-/-}$ mice by glucose tolerance test (GTT) and insulin tolerance test (ITT). After administration of an exogenous glucose load, *Pnpla3*$^{-/-}$ mice and their wild-

type littermates showed similar basal and stimulated blood glucose and insulin levels, indicating a normal glucose disposal rate and insulin secretory response to hyperglycemia in the absence of Pnpla3 (Fig. 3A&B). *Pnpla3*$^{-/-}$ mice and wild-type littermates on a normal chow diet also displayed a similar blood glucose during ITT (Fig. 3C); indicating no significant insulin resistance associated with loss of Pnpla3. We fed these mice a HFD for 15 weeks, and found that the blood glucose levels during the GTT in HFD-fed *Pnpla3*$^{-/-}$ mice was minimally lower than those in wild-type littermates (Fig. 3D). The plasma insulin was, however, similar during the GTT (Fig. 3E); so was the blood glucose response during an ITT (Fig. 3F) in the two HFD-fed groups. Further examination of a cohort fed a HSD for 12 weeks also revealed no difference in either blood glucose or insulin levels during GTT (Supplementary S2, A & B), or blood glucose levels during an ITT (Supplementary Fig. S2, C), between *Pnpla3*$^{-/-}$ mice and *Pnpla3*$^{+/+}$ mice. Finally, we examined the role of Pnpla3 in mice with genetic obese *Lep*$^{ob/ob}$ background and found that *Lep*$^{ob/ob}$*/Pnpla3*$^{-/-}$ and *Lep*$^{ob/ob}$*/Pnpla3*$^{+/+}$ mice displayed similar blood glucose and insulin levels during GTT (Supplementary Fig. S2, D & E), and similar blood glucose levels during ITT (Supplementary Fig. S2, F). Therefore, not only did the absence of Pnpla3 not affect hepatic TG content, it also did not impact the glucose intolerance and insulin resistance that often accompany hepatic steatosis.

Upregulated Pnpla5 mRNA level in the WAT but not liver of *Pnpla3*$^{-/-}$ mice

Thus far, our data indicate that there was no evident change in hepatic TG content or whole body glucose homeostasis between *Pnpla3*$^{-/-}$ and *Pnpla3*$^{+/+}$ mice under 4 dietary conditions (CHD, HFD, HSD, and MCD) and two genotypes (C57BL/6J *Lep*$^{ob/ob}$ and C57BL/6J *Lep*$^{+/+}$). Since the PNPLA gene family encompasses 3 paralogous gene products in mice, we next examined the dynamics of the three paralogs in the liver under various dietary conditions. We found that the hepatic *Pnpla3* mRNA in wild-type mice was markedly upregulated (~32 fold) by HSD feeding but only moderately by HFD (~5 fold) (Fig. 4A, *left panel*). Interestingly, HSD and HFD both stimulated hepatic *Pnpla5* mRNA expression to the same degree in *Pnpla3*$^{-/-}$ and *Pnpla3*$^{+/+}$ mice (Fig. 4A, *middle panel*). In contrast, as compared with regular chow (CHD), none of the fatty liver-inducing diets (HSD, HFD and MCD) affected the level of *ATGL* mRNA expression (Fig. 4A, *right panel*). It is noteworthy that although MCD diet induced the largest TG accumulation in the liver compared with feeding with other diets (Table 1), it did not have any effect on the mRNA expression of the three different patatin-like family members (Fig. 4A). In any case, there was no evidence of compensatory adjustment in hepatic Pnpla5 or ATGL expression in the absence of Pnpla3 in the liver (Fig. 4A, *middle and right panels*).

We next examined the mRNA expression of PNPLA family genes in perigonadal WAT in wild-type and *Pnpla3*$^{-/-}$ mice. As reported previously (22), and confirmed by us, *Pnpla3* expression in the WAT of wild-type mice was significantly induced by HSD diet (~2.5 fold) and slightly upregulated by HFD diet (~1.5 fold, *not significant*;Figure 4B, *left panel*). Under the same conditions, the expression of *Pnpla5* was not significantly affected (Fig. 4B, open bars, *middle panel*). The mRNA expression of *ATGL* was not altered under the different diets in the wild-type WAT; furthermore, the diets did not affect *ATGL* mRNA in WAT in the two genotypes (Fig. 4B, *right panel*). Interestingly, the mRNA level of *Pnpla5*, normally expressed in WAT at very low level compared with the other two paralogs ((23) and our own data), was upregulated by ~5 fold in *Pnpla3*$^{-/-}$ mice fed regular chow. This upregulation of *Pnpla5* was also observed in the gonadal fat of *Pnpla3*$^{-/-}$ mice fed HSD or HFD, though a little less in the HFD group (Fig. 4B, black bars, *middle panel*). It thus appears that increased mRNA expression of another patatin-like family member, *Pnpla5*, may partly compensate for the loss of Pnpla3 in mice, specifically in WAT, but not in liver.

DISCUSSION

GWAS have identified the Pnpla3/adiponutrin gene to be associated with obesity and insulin sensitivity (13,21,24), and, more recently, with non-alcoholic (3–5), as well as alcoholic, fatty liver disease (6) and elevated AST and ALT (3,5), implicating PNPLA3 in the control of body fat, liver fat and whole-body glucose and lipid homeostasis. However, to our surprise, we found that loss of Pnpla3 in mice does not have any effect on body weight, adiposity or plasma lipid or glucose levels (Fig 1 and Supplemental Table 1), nor does it cause detectable alterations in hepatic TG content or serum ALT and AST levels (Table 1). Furthermore, the whole body glucose homeostasis and insulin sensitivity remained normal. These were evident whether the *Pnpla3*-null mice were fed regular chow, high fat, high sucrose, or a MCD diet or in mice bred into a genetic obesity $Lep^{ob/ob}$ background. We conclude that Pnpla3 appears dispensable for liver TG metabolism and normal adipose development in mice.

Recently, interest in *PNPLA3* gene was kindled by genetic epidemiologic analyses by multiple groups showing a strong association between a nonsynonymous variant (rs738409) in the PNPLA3 gene and hepatic fat content, suggesting the involvement of PNPLA3 in the pathogenesis of hepatosteatosis (3–6,25). This rs738409 variant leads to an I148M substitution which has been shown to inactivate the enzyme by blocking substrate access to the catalytic site (8). If PNPLA3 mediates lipolysis in the liver, one would expect to see increased TG content in the liver of Pnpla3-deficient mice. However, there was no excess hepatic TG in $Pnpla3^{-/-}$ mice (Table 1). We next fed mice with different fatty liver-inducing diets to test whether loss of Pnpla3 engenders increased susceptibility to fatty liver development under dietary stress. However, none of the diets tested produced a detectable difference in hepatic TG content in $Pnpla3^{-/-}$ mice as compared with the wild-type counterparts in any of the cohorts (Table 1). We also did not detect any significant association of serum AST or ALT in $Pnpla3^{-/-}$ mice fed regular chow, or three different fatty liver-inducing diets, or after they were bred into a genetic obesity $Lep^{ob/ob}$ background, though the PNPLA3 locus, and specifically the enzymatically inactive rs738409(G) genetic variant, has been shown to be associated with elevated serum liver enzymes in humans (7,21,24).

It is unclear whether the lack of phenotype in lipid accumulation in the liver of $Pnpla3^{-/-}$ mice can be explained by an indirect effect resulting from an adipose-specific upregulation of Pnpla5, a paralogous PNPLA family protein, which expression remains very low and unchanged in the liver of $Pnpla3^{-/-}$ mice. PNPLA5 is highly conserved among different species and is located immediately upstream of the *PNPLA3* gene in the human, rat and mouse genome. PNPLA5 exhibits both lipase and transacylase activities *in vitro* and its mRNA in WAT is regulated by changes in energy balance that is not too dissimilar from that seen with Pnpla3 (23). It should be noted that Pnpla3 and Pnpla5 mRNA levels in liver are much lower than those in WAT in mice. Pnpla5 mRNA especially is barely detectable in mouse liver ((23) and our data). On the other hand, we showed that a lipogenic high sucrose diet treatment leads to a marked stimulation of the transcripts of both Pnpla3 and Pnpla5 in the liver (Fig. 4A). A HFD also upregulated these mRNAs but MCD failed to do so, a finding that may be related to the different mechanism whereby MCD induces hepatic TG accumulation (26). Although the regulatory pattern of Pnpla3 by lipogenic diets could suggest an involvement in an anabolic process, whether Pnpla3 normally plays a direct role in hepatic lipogenesis is unclear. It is interesting that its ablation in mice does not affect hepatic TG accumulation under multiple dietary conditions.

He et al. recently reported that adenovirus-mediated overexpression of human PNPLA3 I148M mutant in liver increased hepatic TG content while overexpression of the normal

human PNPLA3 failed to lower TG (8). The seemingly paradoxical observation suggests that Pnpla3 may not be a primary liver TG metabolizing enzyme and it is possible that the I148M mutant has a dominant negative action on other liver TG hydrolases. In our study, the upregulation of *Pnpla5* mRNA only happens in the WAT but not in the liver of *Pnpla3*$^{-/-}$ mice. Such adipose tissue-specific upregulation of *Pnpla5* mRNA was consistently seen among the different cohorts receiving the different dietary manipulations (Fig. 4B), implicating a dynamic interaction between Pnpla3 and Pnpla5 in WAT. On the other hand, we cannot rule out inter-species differences of PNPLA3 or PNPLA5 action or expression between humans and mice. For example, one very recent study suggested that PNPLA3 expression is higher in the liver than in the WAT of humans (27), in contrast to mice where its expression is significantly higher in adipose tissue than in liver (our data and (23)).

In conclusion, our study constitutes the initial study demonstrating that loss of Pnpla3 in mice has no effect on hepatic TG accumulation. The observation of the upregulation of Pnpla5 specifically in fat but not liver in *Pnpla3*$^{-/-}$ mice is intriguing. It is tempting to speculate that upregulated adipose Pnpla5 expression may be a confounding factor that underlies, or possibly modulates, the association between the rs738409(G) allele and the presence or absence of fatty liver at the individual level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are indebted to our coworkers in the Chan laboratory, especially Dr. Vijay Yechoor, Dr. Minako Imamura and Dr. Yisheng Yang, for suggestions and discussions. We are also grateful to Dr. Saul J Karpen for a critical review of the manuscript.

Financial Support: This research was supported by grants from the US National Institutes of Health HL-51586 (to L.C.) and P30DK079638 for a Diabetes and Endocrinology Research Center. W. Chen was supported by a postdoctoral fellowship from American Heart Association South Central Affiliate No.0825134F.

List of Abbreviations

PNPLA	patatin-like phospholipase
FLD	fatty liver disease
NAFLD	nonalcoholic fatty liver disease
ALT	aspartate aminotransferase
AST	alanine aminotransferase
CHD	regular chow diet
HSD	high sucrose very low fat diet
HFD	high fat diet
MCD	methionine-choline deficient diet
GTT	glucose tolerance test
ITT	insulin tolerance test

References

1. Lewis JR, Mohanty SR. Nonalcoholic fatty liver disease: a review and update. Dig Dis Sci. 2010; 55:560–578. [PubMed: 20101463]
2. 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. [PubMed: 15864352]
3. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet. 2008; 40:1461–1465. [PubMed: 18820647]
4. Kotronen A, Johansson LE, Johansson LM, Roos C, Westerbacka J, Hamsten A, Bergholm R, et al. A common variant in PNPLA3, which encodes adiponutrin, is associated with liver fat content in humans. Diabetologia. 2009; 52:1056–1060. [PubMed: 19224197]
5. Sookoian S, Castano GO, Burgueno AL, Gianotti TF, Rosselli MS, Pirola CJ. A nonsynonymous gene variant in the adiponutrin gene is associated with nonalcoholic fatty liver disease severity. J Lipid Res. 2009; 50:2111–2116. [PubMed: 19738004]
6. Tian C, Stokowski RP, Kershenobich D, Ballinger DG, Hinds DA. Variant in PNPLA3 is associated with alcoholic liver disease. Nat Genet. 2010; 42:21–23. [PubMed: 19946271]
7. Yuan X, Waterworth D, Perry JR, Lim N, Song K, Chambers JC, Zhang W, et al. Population-based genome-wide association studies reveal six loci influencing plasma levels of liver enzymes. Am J Hum Genet. 2008; 83:520–528. [PubMed: 18940312]
8. He S, McPhaul C, Li JZ, Garuti R, Kinch L, Grishin NV, Cohen JC, et al. A sequence variation (I148M) in PNPLA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. J Biol Chem. 2010; 285:6706–6715. [PubMed: 20034933]
9. Rydel TJ, Williams JM, Krieger E, Moshiri F, Stallings WC, Brown SM, Pershing JC, et al. The crystal structure, mutagenesis, and activity studies reveal that patatin is a lipid acyl hydrolase with a Ser-Asp catalytic dyad. Biochemistry. 2003; 42:6696–6708. [PubMed: 12779324]
10. Jenkins CM, Mancuso DJ, Yan W, Sims HF, Gibson B, Gross RW. Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. J Biol Chem. 2004; 279:48968–48975. [PubMed: 15364929]
11. Coleman RA, Lee DP. Enzymes of triacylglycerol synthesis and their regulation. Prog Lipid Res. 2004; 43:134–176. [PubMed: 14654091]
12. Baulande S, Lasnier F, Lucas M, Pairault J. Adiponutrin, a transmembrane protein corresponding to a novel dietary- and obesity-linked mRNA specifically expressed in the adipose lineage. J Biol Chem. 2001; 276:33336–33344. [PubMed: 11431482]
13. Johansson LE, Hoffstedt J, Parikh H, Carlsson E, Wabitsch M, Bondeson AG, Hedenbro J, et al. Variation in the adiponutrin gene influences its expression and associates with obesity. Diabetes. 2006; 55:826–833. [PubMed: 16505250]
14. Liu YM, Moldes M, Bastard JP, Bruckert E, Viguerie N, Hainque B, Basdevant A, et al. Adiponutrin: A new gene regulated by energy balance in human adipose tissue. J Clin Endocrinol Metab. 2004; 89:2684–2689. [PubMed: 15181042]
15. Moldes M, Beauregard G, Faraj M, Peretti N, Ducluzeau PH, Laville M, Rabasa-Lhoret R, et al. Adiponutrin gene is regulated by insulin and glucose in human adipose tissue. Eur J Endocrinol. 2006; 155:461–468. [PubMed: 16914601]
16. Faraj M, Beauregard G, Loizon E, Moldes M, Clement K, Tahiri Y, Cianflone K, et al. Insulin regulation of gene expression and concentrations of white adipose tissue-derived proteins in vivo in healthy men: relation to adiponutrin. J Endocrinol. 2006; 191:427–435. [PubMed: 17088412]
17. Foufelle F, Girard J, Ferre P. Regulation of lipogenic enzyme expression by glucose in liver and adipose tissue: a review of the potential cellular and molecular mechanisms. Adv Enzyme Regul. 1996; 36:199–226. [PubMed: 8869748]
18. Haemmerle G, Lass A, Zimmermann R, Gorkiewicz G, Meyer C, Rozman J, Heldmaier G, et al. Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. Science. 2006; 312:734–737. [PubMed: 16675698]

19. Gauguier D, Froguel P, Parent V, Bernard C, Bihoreau MT, Portha B, James MR, et al. Chromosomal mapping of genetic loci associated with non-insulin dependent diabetes in the GK rat. Nat Genet. 1996; 12:38–43. [PubMed: 8528248]

20. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959; 37:911–917. [PubMed: 13671378]

21. Johansson LE, Lindblad U, Larsson CA, Rastam L, Ridderstrale M. Polymorphisms in the adiponutrin gene are associated with increased insulin secretion and obesity. Eur J Endocrinol. 2008; 159:577–583. [PubMed: 18728122]

22. Polson DA, Thompson MP. Adiponutrin mRNA expression in white adipose tissue is rapidly induced by meal-feeding a high-sucrose diet. Biochem Biophys Res Commun. 2003; 301:261–266. [PubMed: 12565853]

23. Lake AC, Sun Y, Li JL, Kim JE, Johnson JW, Li D, Revett T, et al. Expression, regulation, and triglyceride hydrolase activity of Adiponutrin family members. J Lipid Res. 2005; 46:2477–2487. [PubMed: 16150821]

24. Johansson LE, Johansson LM, Danielsson P, Norgren S, Johansson S, Marcus C, Ridderstrale M. Genetic variance in the adiponutrin gene family and childhood obesity. PLoS One. 2009; 4:e5327. [PubMed: 19390624]

25. Kantartzis K, Peter A, Machicao F, Machann J, Wagner S, Konigsrainer I, Konigsrainer A, et al. Dissociation between fatty liver and insulin resistance in humans carrying a variant of the patatin-like phospholipase 3 gene. Diabetes. 2009; 58:2616–2623. [PubMed: 19651814]

26. Koteish A, Diehl AM. Animal models of steatosis. Semin Liver Dis. 2001; 21:89–104. [PubMed: 11296700]

27. Huang Y, He S, Li JZ, Seo YK, Osborne TF, Cohen JC, Hobbs HH. A feed-forward loop amplifies nutritional regulation of PNPLA3. Proc Natl Acad Sci U S A. 107:7892–7897. [PubMed: 20385813]

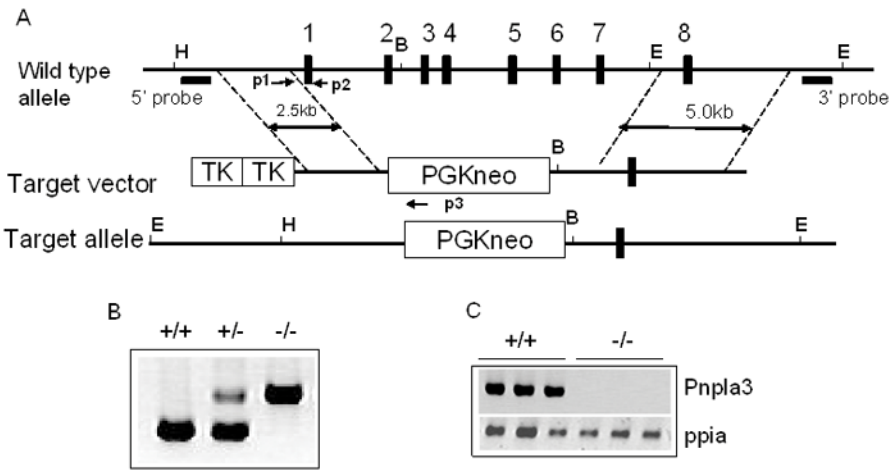

Fig. 1. Generation of *Pnpla3*$^{-/-}$ mice

A, We used a replacement vector that replaces the sequence encompassing exon 1 through exon 7 and part of intron 7 of the murine *Pnpla3* gene with the *neo* gene driven by a PGK promoter. Two thymidine kinase cassettes (TK) were ligated in tandem to the 5′ end of the targeting vector. H, *Hin*dIII; B, *Bam*HI; E, *Eco*RI. **B,** Genomic PCR genotyping produced bands of the predicted 522bp (p1&p3) and 344bp (p1&p2) for mutant and wild-type alleles, respectively. **C,** RNA from WAT of *Pnpla3*$^{+/+}$ and *Pnpla3*$^{-/-}$ littlemates was extracted, reverse transcribed followed by PCR analysis. No transcripts of Pnpla3 were detected in the RNA isolated from the WAT of *Pnpla3*$^{-/-}$ mice.

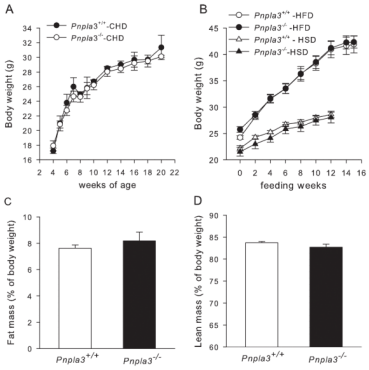

Fig. 2. *Pnpla3* deficiency has no effect on body weight or body fat

Growth curves of wild-type and *Pnpla3*$^{-/-}$ mice fed a regular chow (CHD) (**A**), and high fat (HFD) starting at 6 weeks, or high sucrose very low fat diet (HSD) starting at 4 weeks of age (**B**) for indicated no. of weeks. **C** & **D,** Echo-MRI analysis of 13 week old male wild-type and *Pnpla3*$^{-/-}$ mice fed chow diet. Total fat (**C**) and lean mass (**D**) were normalized to body weight. Each group contains 10 mice. No significant differences were found.

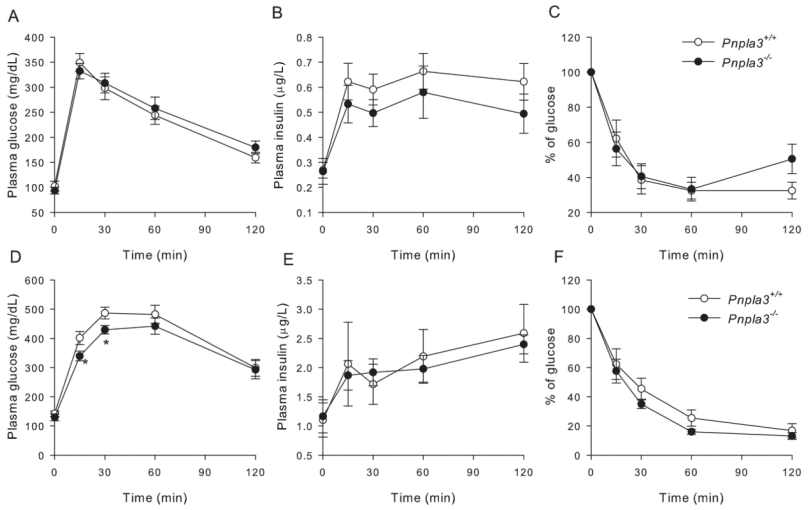

Fig. 3. Glucose tolerance test (GTT) and insulin tolerance test (ITT) in *Pnpla3*$^{-/-}$ and *Pnpla3*$^{+/+}$ mice fed regular chow or high fat diet

GTT was performed on 12-week-old male wild-type and *Pnpla3*$^{-/-}$ mice after 15 h fasting using 1.5 g/kg BW glucose by i.p. injection. Plasma glucose levels (**A**) and Insulin levels (**B**) during the GTT test were presented as means ± SE. ITT was performed on 6 h fasted 17–18 week-old male wild-type and *Pnpla3*$^{-/-}$ mice with i.p. injection of humulin at 0.75 unit/kg BW. Plasma glucose levels are presented as % of change from gluocse level at time 0 (**C**). GTT was performed on wild-type and *Pnpla3*$^{-/-}$ mice fed HFD for 15 weeks as above. Plasma glucose levels (**D**) and Insulin levels (**E**) during the GTT test are presented as means ± SE. F, ITT analyses on 6 h fasted mice that had been fed HFD for 20 weeks after i.p. injection of humulin at 2.0 unit/kg BW. Plasma glucose levels are presented as % of glucose change by means ± SE.

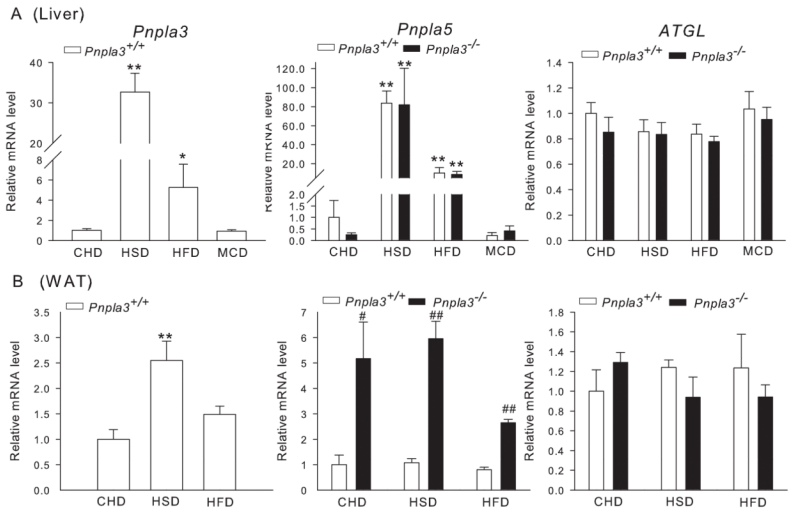

Fig. 4. Quantitative analysis of mRNA expression of PNPLA family members in the liver and WAT of *Pnpla3*$^{-/-}$ and *Pnpla3*$^{+/+}$ mice fed different diets

Total RNA was extracted and reverse transcribed from liver (**A**) and WAT (**B**) of wild-type and *Pnpla3*$^{-/-}$ mice fed CHD (18 weeks), HSD (15 weeks), HFD (20 weeks) or MCD (2 weeks, only for liver analysis) diets. We performed real-time PCR to analyze the mRNA level of Pnpla family proteins, including Pnpla3, Pnpla5 and ATGL (Pnpla2). Data were normalized to cyclophilin A and compared with CHD-fed wild-type mice. Data were presented as means ± SE. Each group contains 5–7 mice. *: $p< 0.05$; **: $p< 0.005$ vs CHD fed wild-type mice. #: $p< 0.05$; ##: $p< 0.005$ vs wild-type mice fed the same diet.

Table 1

Liver triacylglycerol content, serum ALT and AST levels in wild-type and *Pnpla3*$^{-/-}$ mice fed regular chow or different fatty liver-inducing diets or after they were bred into genetic obese *Lep*$^{ob/ob}$ background.

Diets	Liver TGs (mg/g tissue)		ALT (U/L)		AST (U/L)	
	Pnpla3$^{+/+}$	Pnpla3$^{-/-}$	Pnpla3$^{+/+}$	Pnpla3$^{-/-}$	Pnpla3$^{+/+}$	Pnpla3$^{-/-}$
Regular chow diets/2 genetic backgrounds						
C57BL/6J	0.087±0.03	0.071±0.02	19.4±4	21.6±4.3	54.3±1.2	55.5±12.1
***Lep*$^{ob/ob}$**	0.42±0.05	0.41±0.07	162±6	142±12	159±15	154±24
Fatty liver inducing diets/C57BL/6J background						
HSD	0.127±0.03	0.127±0.02	19.3±4.2	21.3±12.5	34.1±3.4	36.3±13.3
HFD	0.27±0.03	0.25±0.05	79±39	71±23	53±19	46±22
MCD	0.26±0.05	0.28±0.03	183±34	169±20	151±31	162±45

All mice were sacrificed after 4 h fasting. Mice under regular chow diets were sacrificed at 18 week old (C57BL/6J) and 24 week old (*Lep*$^{ob/ob}$ background). Wild-type and *Pnpla3*$^{-/-}$ mice were fed from 6 weeks old with HFD for 20 weeks and HSD for 15 weeks, respectively, before sacrifice; 18 week old mice were fed with MCD diet for 2 weeks before sacrifice. Each group contains at least 6–8 mice.

Each value represents the mean ± SE. No significant differences were observed in the liver TGs, ALT and AST levels between the wild-type and *Pnpla3*$^{-/-}$ mice. HSD, high sucrose very low fat diet; HFD, high fat diet; MCD, Methionine- choline deficient diet; TG, triacylglycerol.