Pnpla3I148M Knockin Mice Accumulate PNPLA3 on Lipid Droplets and Develop Hepatic Steatosis

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A sequence polymorphism (rs738409, I148M) in patatin-like phospholipid domain containing protein 3 (*PNPLA3*) is strongly associated with nonalcoholic fatty liver disease (NAFLD), but the mechanistic basis for this association remains enigmatic. Neither ablation nor overexpression of wild-type *PNPLA3* affects liver fat content in mice, whereas hepatic overexpression of the human 148M transgene causes steatosis. To determine whether the 148M allele causes fat accumulation in the liver when expressed at physiological levels, we introduced a methionine codon at position 148 of the mouse *Pnpla3* gene. Knockin mice had normal levels of hepatic fat on a chow diet, but when challenged with a high-sucrose diet their liver fat levels increased 2 to 3-fold compared to wild-type littermates without any associated changes in glucose homeostasis. The increased liver fat in the knockin mice was accompanied by a 40-fold increase in PNPLA3 on hepatic lipid droplets, with no increase in hepatic PNPLA3 messenger RNA (mRNA). Similar results were obtained when the catalytic dyad of PNPLA3 was inactivated by substituting the catalytic serine with alanine (S47A). *Conclusion:* These data provide the first direct evidence that physiological expression of PNPLA3 148M variant causes NAFLD, and that the accumulation of catalytically inactive PNPLA3 on the surfaces of lipid droplets is associated with the accumulation of TG in the liver. (HEPATOLOGY 2015;61:108-118)

Cirrhosis caused by fatty liver disease is emerging as a major indication for liver transplantation.[1,2] Recent population-based surveys have revealed that one out of every three adults in the United States has hepatic steatosis,[3,4] the first stage of fatty liver disease.[5] The accumulation of hepatic triglyceride (TG) in hepatocytes *per se* is not associated with any apparent adverse clinical sequelae, but a subset of individuals with steatosis develop an inflammatory response and fibrosis; these individuals are at increased risk of developing cirrhosis.[6] This continuum of liver disease is referred to as nonalcoholic fatty liver disease (NAFLD) and individuals with this disorder are at increased risk of hepatocellular carcinomas.[7,8]

Despite the clinical importance of NAFLD, major questions remain unanswered regarding its pathogenesis.[9]

The first molecular handle on the genetic underpinnings of NAFLD was the identification of a missense sequence variation (I148M) in patatin-like phospholipase domain protein family member 3 (PNPLA3), which is associated with increased hepatic TG content and liver enzymes.[10] More than 50 studies have confirmed the association between this variant and the full spectrum of NAFLD, including simple steatosis, nonalcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma.[11]

The mechanism by which the I148M substitution promotes liver fat accumulation remains enigmatic. Recombinant PNPLA3 has TG hydrolase activity that is markedly attenuated by the I148M substitution.[12-14] Based on this observation, it was hypothesized that the accumulation of hepatic TG in the liver was due to the loss of PNPLA3-mediated TG hydrolysis in hepatic lipid droplets. This hypothesis was quickly challenged when two groups reported that *Pnpla3* knockout mice do not

develop fatty liver.[15,16] Moreover, mice expressing high levels of the human mutant protein in the liver (PNPLA3[148MTg] mice) had hepatic steatosis, whereas mice expressing the wild-type protein (PNPLA3[WTTg] mice) had hepatic TG levels that were similar to non-transgenic littermates. Thus, loss of PNPLA3 activity is not sufficient to cause hepatic steatosis and expressing high levels of the enzyme in the liver does not *decrease* hepatic TG content, as would be expected if it functions as a lipase.[17]

Zechner and colleagues showed that PNPLA3 catalyzes the acylation of lysophosphatidic acid to phosphatidic acid, a step in the TG biosynthetic pathway, when expressed in bacteria as a fusion protein with a trigger factor.[18] In this expression system, the 148M variant increased lysophosphatidic acid acyltransferase (LPAAT) activity. LPAAT activity was not detected in recombinant PNPLA3 purified from *Sf9* cells,[13] and was not increased in livers of mice expressing high levels of wild-type PNPLA3 or PNPLA3-148M.[17]

Metabolic studies in the PNPLA3[148MTg] mice provided evidence consistent with PNPLA3-148M conferring both a loss, and a gain, of function. Glycerol release from TG was reduced in primary hepatocytes from PNPLA3[148MTg] mice when compared to the PNPLA3[WTTg] mice. Endogenous fatty acid synthesis was significantly increased in both transgenic lines of mice when compared to non-transgenic animals, with the levels of TG synthesis being higher in PNPLA3[148MTg] animals.

Although the PNPLA3[148MTg] mice developed hepatic steatosis, this model has two important limitations: 1) human PNPLA3 is only 68% identical to the mouse protein and may have nonphysiological effects in mice, and 2) the PNPLA3 transgene is expressed constitutively at very high levels and does not exhibit the ~80-fold changes in response to food intake that characterize the endogenous gene.[19] To address these limitations, we developed mice in which isoleucine at residue 148 of the endogenous *Pnpla3* gene was replaced with methionine. To determine the effect of an obligate loss-of-function mutation in PNPLA3 on hepatic fat content, we generated mice in which the catalytic serine in the patatin domain was replaced by alanine.

Materials and Methods

Animals. The I148M and S47A mutations were introduced into the mouse *Pnpla3* gene by homologous recombination as described in the Supporting Materials. Three founders for each mutation were backcrossed with C57BL/6J females. All mice were maintained on a 12/12-hour light/dark cycle and fed Teklad Mouse/Rat Diet 7001 (Harlan Teklad, Houston, TX) *ad libitum*. For the dietary challenge studies, mice were fed a high-sucrose diet (no. 901683; 74% kCal from sucrose, MP Biomedicals, Santa Ana, CA) or a high-fat diet (D12451; 45% kCal from fat, Research Diets, New Brunswick, NJ) for 4 weeks unless otherwise stated. Prior to each experiment the mice were metabolically synchronized for 3 days by withdrawing food from 8:00 AM till 8:00 PM and providing food from 8:00 PM till 8:00 AM and sacrificed at 8:00 AM at the end of the third refeeding cycle. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at University of Texas Southwestern Medical Center at Dallas.

Body Composition. Total body fat and lean body mass were determined by nuclear magnetic resonance using a Minispec analyzer (Bruker, Billerica, MA).

Liver and Plasma Chemistries. Lipids were extracted from livers (~100 mg) using the method of Folch et al.[20] The levels of TG, cholesterol, cholesteryl ester, free cholesterol, and phosphatidylcholine were measured using enzymatic assays (Infinity, Thermo Electron, Madison, WI, and Wako, Richmond, VA) and normalized to sample weight. Lipids were also separated into cholesterol esters, TGs, and phospholipids using thin-layer chromatography as previously described.[21] Lipids in each fraction were hydrolyzed, derivatized with trimethylsilane, and separated by gas-liquid chromatography (GLC) using a Hewlett Packard 6890 Series gas chromatograph (Agilent, Santa Clara, CA). The fatty acids were identified by retention times established using purified fatty acid standards (GLC-744, NU-Chek Prep, Elysian, MN). The percentage of each fatty acid in each fraction was calculated using pentadecanoin (C15:0) as an internal standard.
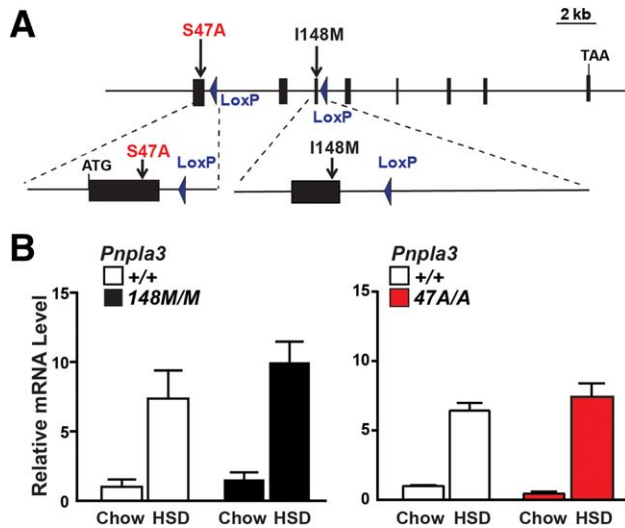
Fig. 1. Generation of *Pnpla3*^{148M} and *Pnpla3*^{47A} KI mice. (A) Diagram of *Pnpla3* targeting strategy. Homologous recombination was used to replace either exon 1 (S47A) or exon 3 (I48M) with the corresponding mutant sequences. The selectable marker (Neo) located between two LoxP sites was then excised with *Cre* recombinase, leaving one LoxP site in the flanking intron. (B) *Pnpla3* RNA levels in livers from female mice (n = 4/group) consuming a chow diet or a high-sucrose diet (HSD) for 4 weeks. Livers were collected at the end of the feeding cycle. Levels of *Pnpla3* mRNA were determined using real-time PCR and normalized to values of chow-fed wild-type mice.

Serum levels of liver enzymes, TG, cholesterol, and glucose were measured using the Vitros 250 system (GMI, Ramsey, MN). Enzymatic assay kits were used to determine serum levels of NEFA (Wako). Serum levels of insulin were measured using an enzyme-linked immunosorbent assay (ELISA) assay (Crystal Chem, Downers Grove, IL).

RNA Expression. Total RNA was isolated from tissues of four mice from each group using RNA STAT-60 (Tel-Test, Friendswood, TX), and real-time polymerase chain reaction (PCR) measurements were performed as previously described.[22] Expression levels of mouse *Pnpla3* hepatic mRNA were measured using oppositely oriented primers (5′-CGAGGCGAGCGG-TACGT-3′ and 5′-TGACACCGTGATGGTGGTTT-3′). Mouse 36B4 mRNA was used as an internal control.

Lipid Droplet Isolation. Hepatic lipid droplets were isolated by centrifugation as described in the Supporting Methods.

Immunoblot Analysis of Lipid Droplet Proteins. Aliquots of resuspended lipid droplet protein (3 μg) were analyzed by quantitative immunoblot analysis as described in the Supporting Methods.

Tissue Morphology. Liver and white adipose tissue were examined by light microscopy as described in Supporting Methods.

Statistics. Data are presented as means ± SEM. Differences between group means were assessed by 2-tailed, unpaired Student *t* tests using GraphPad Prism statistical software (GraphPad Software, San Diego, CA). *P*-values below 0.05 were considered statistically significant.
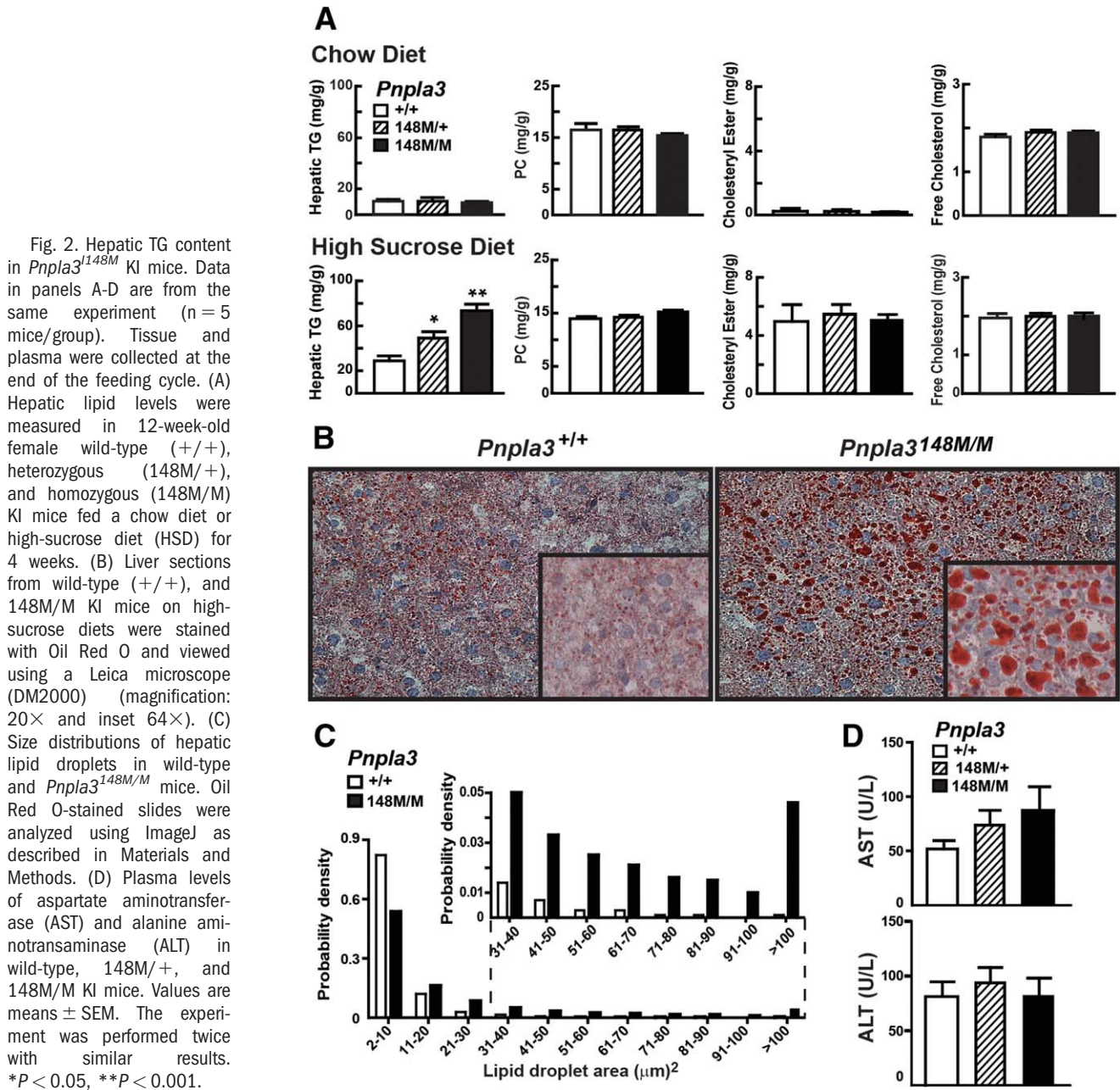
Results

Development of Pnpla3*^{148M} *and Pnpla3*^{47A} *Knockin (KI) Mice. To develop an animal model that more accurately recapitulates the human phenotype associated with PNPLA3-148M, we altered codon 148 of *Pnpla3* in the mouse genome from AT<u>T</u> to AT<u>G</u>, substituting methionine for isoleucine (*Pnpla3*^{148M}) (Fig. 1A). We also generated a second line of mice in which the catalytic serine at residue 47 was replaced with alanine (*Pnpla3*^{47A} mice). Sanger sequencing confirmed that the mutations were introduced without altering other sequences in the gene (Supporting Fig. 1) and RT-PCR indicated that expression of the mutant and wild-type alleles was similar in mice fed a chow diet and that transcript levels increased by similar amounts when the two lines were fed a high-sucrose diet (74% of kCal from sucrose) (Fig. 1B).

The KI mice were born in the expected Mendelian ratios and both litter sizes and gender distributions were unaltered by introduction of the mutations. No differences in body weight, liver weight, fat mass, or lean mass were detected between the two strains of KI mice and their wild-type littermates (Supporting Fig. 2). Morphological analysis of the adipose tissue revealed no differences in adipocyte size or conformation (Supporting Fig. 3)

Pnpla3*^{148M} *and Pnpla3*^{47A} *KI Mice Develop Hepatic Steatosis on a High-Sucrose Diet. On a chow diet, the levels of TG, phosphatidylcholine (PC), cholesteryl esters, and free cholesterol in the livers of female mice that were heterozygous (*Pnpla3*^{148M/+}) or homozygous (*Pnpla3*^{148M/M}) for the *Pnpla3*^{148M} allele did not differ significantly from those of wild-type littermates (*Pnpla3*^{+/+}) (Fig. 2A, top). After 4 weeks on a high-sucrose diet, hepatic TG content was higher in female *Pnpla3*^{148M/+} and *Pnpla3*^{148M/M} mice than in wild-type animals (Fig. 2A, bottom). Levels of hepatic TG in heterozygous mice were intermediate between those of wild-type and homozygous KI animals. Similar responses were observed in male mice (Supporting Fig. 4A). No differences in circulating lipid levels were seen between the two lines (Supporting Fig. 5A).

Hematoxylin and eosin staining of liver sections from the *Pnpla3*^{148M/M} mice revealed no differences in

Fig. 2. Hepatic TG content in $Pnpla3^{148M}$ KI mice. Data in panels A-D are from the same experiment (n = 5 mice/group). Tissue and plasma were collected at the end of the feeding cycle. (A) Hepatic lipid levels were measured in 12-week-old female wild-type (+/+), heterozygous (148M/+), and homozygous (148M/M) KI mice fed a chow diet or high-sucrose diet (HSD) for 4 weeks. (B) Liver sections from wild-type (+/+), and 148M/M KI mice on high-sucrose diets were stained with Oil Red O and viewed using a Leica microscope (DM2000) (magnification: 20× and inset 64×). (C) Size distributions of hepatic lipid droplets in wild-type and $Pnpla3^{148M/M}$ mice. Oil Red O-stained slides were analyzed using ImageJ as described in Materials and Methods. (D) Plasma levels of aspartate aminotransferase (AST) and alanine aminotransaminase (ALT) in wild-type, 148M/+, and 148M/M KI mice. Values are means ± SEM. The experiment was performed twice with similar results. $*P < 0.05$, $**P < 0.001$.

tissue architecture or cellular morphology (data not shown). Oil Red O staining of neutral lipids was markedly increased in $Pnpla3^{148M/M}$ mice (Fig. 2B) and the size distribution of their lipid droplets was shifted to the right (Fig. 2C), reflecting a striking increase in the number of large droplets (>20 μm^2). In wild-type mice, the median area of the droplets was 4.3 μm^2 and fewer than 5% were greater than 22 μm^2. The median area of lipid droplets in the $Pnpla3^{148M/M}$ mice (8.7 μm^2) was double that of their wild-type littermates, and 28% were greater than 22 μm^2. Despite the increased lipid content of the livers

in the $Pnpla3^{148M/+}$ and $Pnpla3^{148M/M}$ mice, no changes in circulating liver enzymes were observed in these animals (Fig. 2D).

Similar results were obtained in $Pnpla3^{47A}$ KI mice. On chow diets the lipid levels of $Pnpla3^{47A/+}$ and $Pnpla3^{47A/A}$ KI mice were similar to those of their wild-type littermates in liver (Fig. 3A, top; Supporting Fig 4B, top) and in plasma (Supporting Fig. 5B). On the high-sucrose diet, hepatic TG content was significantly higher in both the female (Fig. 3A, bottom) and male (Supporting Fig. 4B, bottom) $Pnpla3^{47A/A}$ mice than in their wild-type littermates. The hepatic
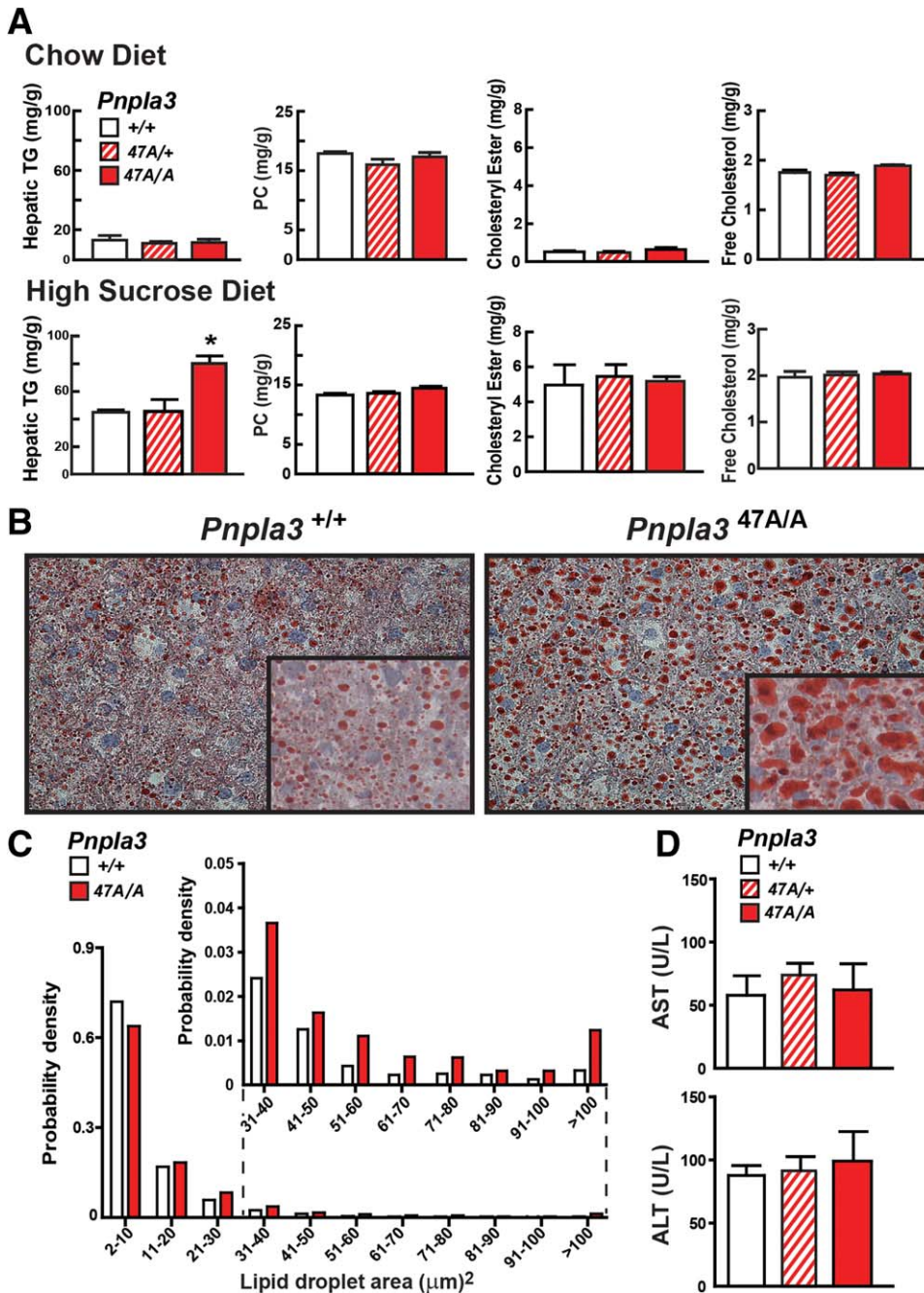
Fig. 3. Hepatic TG content in *Pnpla3*^{S47A} mice. Data in panels A-D are from the same experiment (n = 7 mice/group). Tissue and plasma samples were collected at the end of the feeding cycle. The experiment was repeated with similar results. (A) Hepatic lipid levels were measured in 13-week-old female wild-type (+/+), S47A heterozygous (S47A/+) and homozygous (S47A/A) KI mice fed a chow diet or high-sucrose diet (HSD) for 4 weeks. (B) Liver sections from sucrose-fed wild-type and S47A/A KI mice were stained with Oil Red O and viewed using a Leica microscope (DM2000) (magnification: 20×, inset 64×). (C) Size distributions of hepatic lipid droplets in wild-type and homozygous (S47A/A) KI mice. Oil Red O-stained slides were analyzed using ImageJ as described in the Materials and Methods. (D) Plasma levels of liver enzymes (AST and ALT). Values are means ± SEM. *P = 0.001.

TG content of mice heterozygous for the S47A substitution did not differ significantly from that of wild-type animals. Hepatic levels of PC, cholesteryl esters, and free cholesterol did not differ between the lines on either diet (Fig. 3A) and no differences in circulating liver enzymes (Fig. 3D) or plasma lipids (Supporting Figure 5B) were apparent on the high-sucrose diet. The increase in hepatic TG content was associated with an increase in the number of large, Oil Red O-positive lipid droplets in the livers of the *Pnpla3*$^{S47A/A}$ animals (Fig. 3B,C).

No Change in Hepatic mRNA Profile in PNPLA3 KI Mice. High-level expression of human PNPLA3-148M in livers of transgenic mice was associated with increased mRNA levels of sterol regulatory element binding protein-1c (SREBP-1c), which orchestrates the up-regulation of the fatty acid synthesis pathway,[23] and several of its target genes,[17] suggesting that the elevated TG content associated with the PNPLA3-148M variant is due to accelerated TG synthesis. In mice consuming high-sucrose diets, hepatic mRNA levels of SREBP-1c and its target genes were similar in
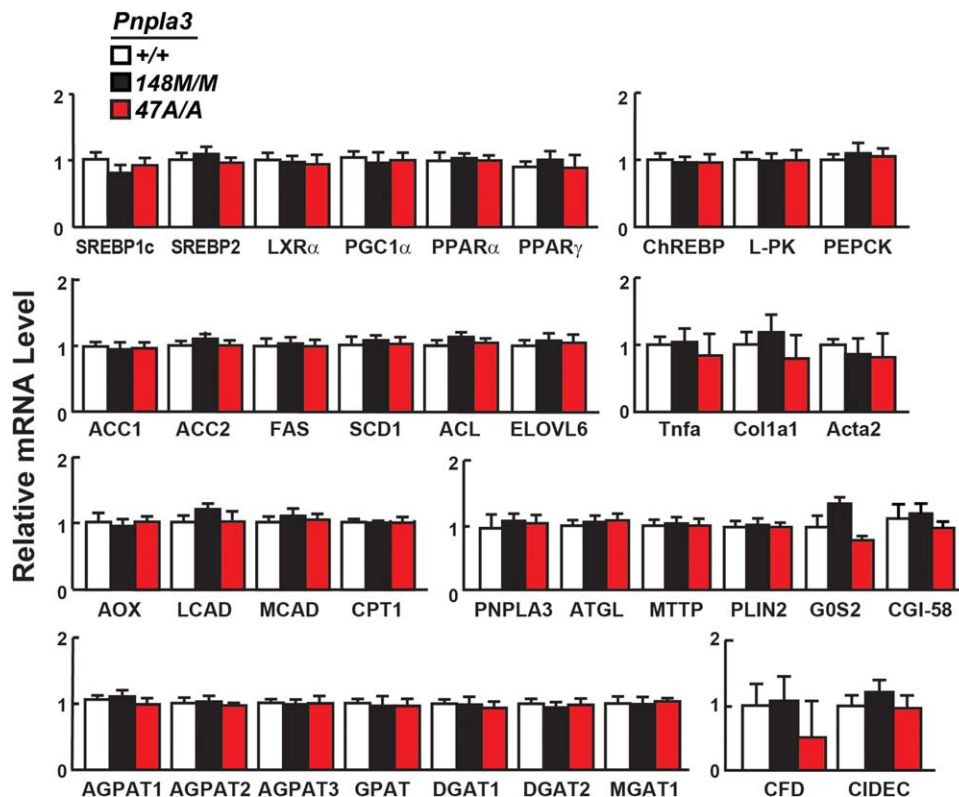
Fig. 4. Relative mRNA levels of selected genes in livers of *Pnpla3*[148M/M] and *Pnpla3*[47A/A] mice with wild-type littermates (n = 4/group). The mice used in this experiment are described in the legends to Figs. 2 and 3. mRNA levels were quantified by real-time PCR, normalized to levels of 36B4, and expressed relative to levels in wild-type mice. Values are means ± SEM. SREBP1c, sterol regulatory element binding protein 1 isoform C; SREBP2, sterol regulatory element binding protein 2; LXRα, liver X receptor alpha; PGC-1α, PPARγ coactivator 1α; PPARα, peroxisome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma; ChREBP, carbohydrate-responsive element-binding protein; L-PK, liver pyruvate kinase; PEPCK, phosphoenoylpyruvate carboxykinase; ACC1, acetyl-CoA carboxylase-1; ACC2, acetyl-CoA carboxylase 2; FAS, fatty acid synthase; SCD1, stearoyl-CoA desaturase-1; ACL, ATP citrate lyase; ELOVL6, ELOVL family member 6; TNFα, tumor necrosis factor alpha; COL1α1, collagen type-1α1; ACTA2, α-smooth muscle actin; AOX, acyl-CoA oxidase-1; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; CPT1, carnitine palmitoyltransferase1; ATGL, adipose triglyceride lipase; MTTP, microsomal TG transfer protein; PLIN2, perilipin 2; G0S2, G0/G1switch 2; CGI-58, comparative gene identification 58; AGPAT1-3, 1-acylglycerol-3-phosphate O-acyltransferase 1-3; GPAT, glycerol-3-phosphate acyltransferase; DGAT1, diglyceride acyltransferase-1; DGAT2, diglyceride acyltransferase-2; MGAT1, monoacylglycerol O-acyltransferase 1; CFD, complement factor D (adipsin); CIDEC, cell death-inducing DFFA-like effector C. The experiment was repeated twice and the results were similar.

Pnpla3[148M/M], *Pnpla3* [47A/A] and wild-type animals (Fig. 4). We also found no strain-specific changes in genes involved in TG lipolysis (hormone sensitive lipase [HSL], adipocyte TG lipase [ATGL]), fatty acid oxidation, hepatic inflammation, or fibrosis.

No Major Alterations in Fatty Acid Composition of Major Hepatic Lipids. Previously, we found a significant reduction in the proportion of long-chain polyunsaturated fatty acids in liver TG from mice expressing high levels of human PNPLA3-148M.[17] The fatty acid profiles of hepatic TG, cholesteryl esters, and phospholipids were remarkably similar in the KI mice and their wild-type littermates (Fig. 5). Stearate (C18:0) was modestly decreased in the liver TG fraction of *Pnpla3*[148M/M] mice, while palmitoleate and linoleate were slightly higher in liver TGs of *Pnpla3*[S47A/A] animals, but no consistent differences

were observed in the two KI strains. Thus, the increased hepatic TG content in the two KI strains was not associated with any obvious alteration in the spectrum of fatty acids of hepatic glycerolipids.

Insulin Resistance Is Comparable in Wild-Type and PNPLA3*[148M] *Mice Fed a High-Fat Diet. To determine if the KI mice develop more severe insulin resistance than do wild-type animals, we challenged them with a high-fat diet (45% of kCal) for 12 weeks. High-fat feeding led to comparable increases in body weight and plasma levels of insulin in wild-type and KI mice (Supporting Fig. 6A). Fat feeding increased hepatic fat content 3 to 4-fold in the *Pnpla3*[148M/M] KI (41 ± 9 mg/g) and wild-type (51 ± 18 mg/g) mice compared to chow-fed animals (13 ± 5 mg/g). Fasting glucose (163 ± 25 mg/dL versus 154 ± 14 mg/dL) and insulin (1.6 ± 0.6 versus 2.1 ± 1.0) levels did not
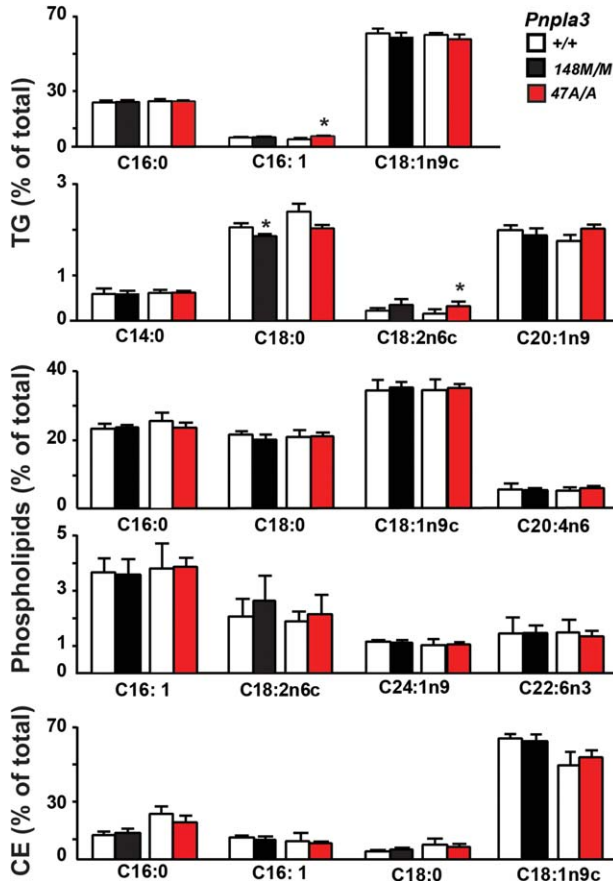
Fig. 5. Fatty acid composition of hepatic lipids in wild-type, Pnpla3$^{I148M/M}$, and Pnpla3$^{S47A/A}$ KI mice. Lipids were extracted from livers of 12-week-old female mice (n = 6/group) fed a high-sucrose diet for 4 weeks as described in the Materials and Methods. The TG, phospholipid, and cholesterol ester fractions were separated by TLC, hydrolyzed, and derivatized with trimethylsilane. The fatty acid methylesters were quantified by gas chromatography. Each value represents the mean ± SEM. *$P < 0.05$. The experiment was repeated and the results were similar.

differ between the groups, and glucose tolerance testing (performed after 10 weeks of high-fat feeding) did not elicit any significant differences between the two groups of mice (Supporting Fig. 6B). A GTT was also performed in mice fed a chow or a high sucrose diet for 4 weeks and the results were similar (Supporting Fig. 6C). Thus, expression of PNPLA3-148M did not affect the development of insulin resistance in mice, which is congruent with the lack of association with either homeostasis model of assessment, insulin resistance (HOMA-IR) or diabetes in humans.[24]

Lipid Droplets From the Sucrose-Fed But Not Fat-Fed Pnpla3$^{148M/M}$ *and* Pnpla3$^{47A/A}$ *Mice Accumulate PNPLA3 and CGI-58 (ABHD5).* On a high-sucrose diet, levels of PNPLA3 mRNA were similar in the livers of wild-type and KI mice (Fig. 6A). Liver TG content was 2-fold higher in KI mice (Fig.

6B). Thus, the differences in hepatic TG associated with sucrose feeding were not due to differences arising from altered PNPLA3 mRNA levels. Quantitative immunoblotting revealed a dramatic (40-fold) increase in PNPLA3 levels in lipid droplets from Pnpla3$^{148M/M}$ and Pnpla3$^{47A/A}$ mice compared to their wild-type littermates (Fig. 6C,D). No differences in levels of ATGL or HSL, the two major TG lipases in lipid droplets, were seen among the four groups. In contrast to these findings, the levels of CGI-58, a cofactor of ATGL that was barely detectable in the wild-type animals, were increased ∼5-fold in lipid droplets from both Pnpla3$^{148M/M}$ and Pnpla3$^{47A/A}$ mice. The major differences in levels of both PNPLA3 and CGI-58 were not accompanied by changes in levels of the three perilipins that are most abundant in hepatic lipid droplets: PLIN2, PLIN3, and PLIN5. The selective accumulation of CGI-58 in the lipid droplets suggested that PNPLA3, which resembles ATGL, may sequester CGI-58 on the lipid droplet, resulting in a reduction in TG hydrolase activity by ATGL.

To determine if PNPLA3-148M accumulates secondary to the development of fatty liver, we measured PNPLA3 mRNA and protein in livers from mice fed a high-fat diet for 12 weeks (Supporting Fig. 6A). Hepatic levels of PNPLA3 mRNA were similar in wild-type and KI animals (Fig. 7A), but in both lines the levels were much lower on the high-fat diet ($C_t = 24.5$) than on the high-sucrose diet ($C_t = 22$). Despite the 3 to 4-fold increase in hepatic TG levels in fat-fed mice (Fig. 7B), PNPLA3 protein was barely detectable in the lipid droplets of wild-type or the KI animals (Fig. 7C). Quantitative immunoblotting of lipid droplets from the fat-fed mice indicated that PNPLA3 levels were 6-fold higher in the Pnpla3$^{148M/M}$ mice than in their wild-type littermates (Fig. 7C,D). Nonetheless, PNPLA3 levels in both strains were far lower in fat-fed than in sucrose-fed animals. Levels of CGI-58 were increased 2.5-fold in the KI mice.

PNPLA3, But Not CGI-58 Accumulates on Lipid Droplets of Liver-Specific PNPLA3^{148MTg} *Mice.* To determine if the accumulation of CGI-58 on the lipid droplets of the KI was causally related to the accumulation of TG, we examined the levels of CGI-58 on the lipid droplets of mice expressing mutant human PNPLA3. If lipid accumulation in the mutant KI mice is due to sequestration of CGI-58 by PNPLA3, a similar increase in CGI-58 should be seen in the PNPLA3^{148MTg} mice. In mice fed a high-sucrose diet for 4 weeks, levels of mouse PNPLA3 and CGI-58 mRNA were similar in livers from the transgenic and wild-type animals (Fig. 8A). The level of human PNPLA3 mRNA was ∼30% higher in the
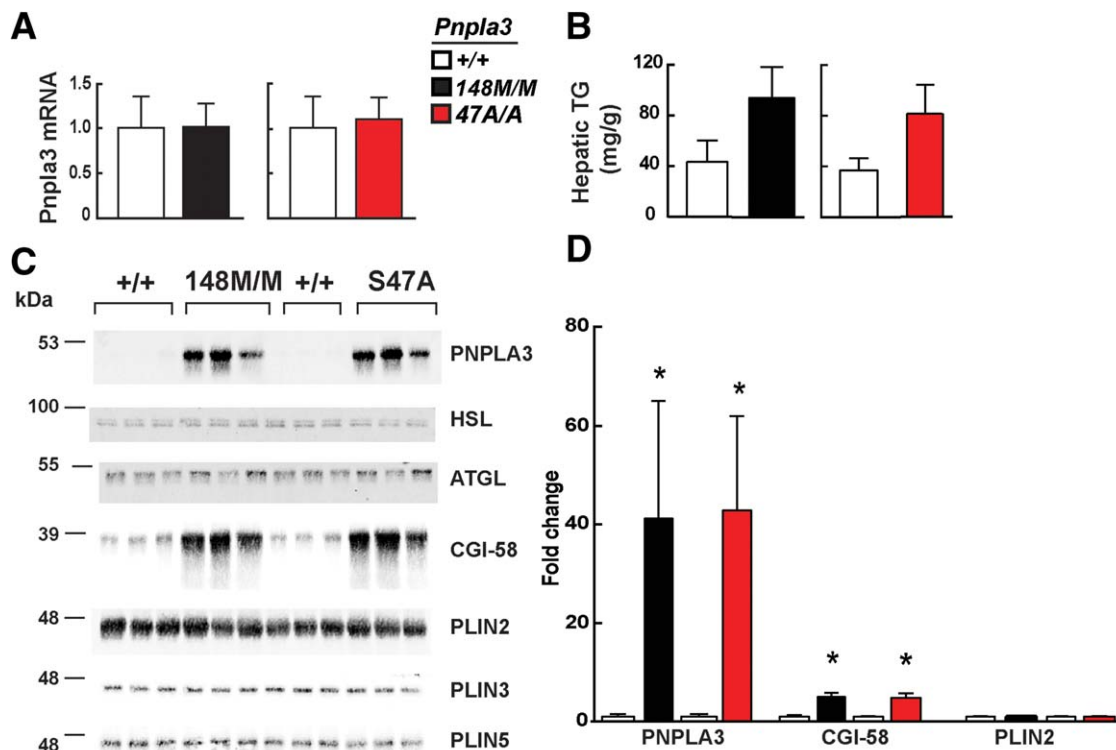
Fig. 6. Accumulation of PNPLA3 and CGI-58 protein on hepatic lipid droplets of $Pnpla3^{148M/M}$ and $Pnpla3^{47A/A}$ KI mice. Livers were harvested from 11-week-old female wild-type (+/+), $Pnpla3^{148M/M}$, and $Pnpla3^{47A/A}$ mice (n = 5/group) fed a high-sucrose diet for 4 weeks. (A) Hepatic PNPLA3 mRNA levels were determined using real-time PCR, as described in the Materials and Methods. (B) Hepatic TG levels were measured by enzymatic assay as described in the Materials and Methods. (C) Quantitative immunoblot analysis of lipid droplets (LD) isolated from 3 mice per group. A total of 3 μg of LD protein was size-fractionated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and immunoblot analysis was performed as described in the Materials and Methods. (D) Immunoblots were quantitated using infrared fluorescent imaging as described in the Materials and Methods. Values are mean $\pm$ SEM. *$P < 0.05$. The experiment was repeated twice and the results were similar.

$PNPLA3^{148MTg}$ mice than in the mice expressing the wild-type human PNPLA3 transgene, although this difference was not statistically significant. The levels of human PNPLA3 were increased dramatically in liver lysates (Supporting Fig. 7) and in hepatic lipid droplets isolated from mice expressing the PNPLA3-148M transgene when compared to the mice expressing wild-type PNPLA3 (Fig. 8B). The levels of CGI-58 on hepatic lipid droplets did not differ significantly between the PNPLA3-148M and PNPLA3-WT transgenic mice. Thus, the accumulation of CGI-58 is not a prerequisite for the development of hepatic steatosis in association with expression of mutant PNPLA3.

Discussion

The major finding of this study is that $Pnpla3^{148M/M}$ KI mice develop diet-dependent hepatic steatosis. The I148M substitution did not lead to increased hepatic TG levels in chow-fed mice, but elicited a 2 to 3-fold elevation in liver fat content in sucrose-fed animals (Fig. 2). This finding is congruent with the observa-

tion in humans that the PNPLA3-148M variant is associated with appreciably greater liver fat accumulation in obese than in lean individuals.[24] Moreover, the increase in liver TG of KI mice was comparable in magnitude to that observed in humans homozygous for the PNPLA3-148M variant,[10] and was not associated with any changes in circulating levels of lipids (Supporting Fig. 5) or in glucose homeostasis. Similarly, the variant has little effect on plasma lipid levels or glucose metabolism in humans,[10] except in the extremely obese, where it is associated with lower plasma TG levels.[25] Taken together, these data provide the first direct evidence that physiological expression of the PNPLA3-148M variant causes hepatic steatosis.

The phenotypic differences among PNPLA3 knockout, transgenic, and KI mice reiterate the limitations of genetically modified animals as models of human pathophysiology. Nonetheless, comparisons among these lines provide new insights into the biochemical sequelae of the I148M substitution and allow significant refinement of current models of how the 148M variant promotes steatosis. The accumulation of
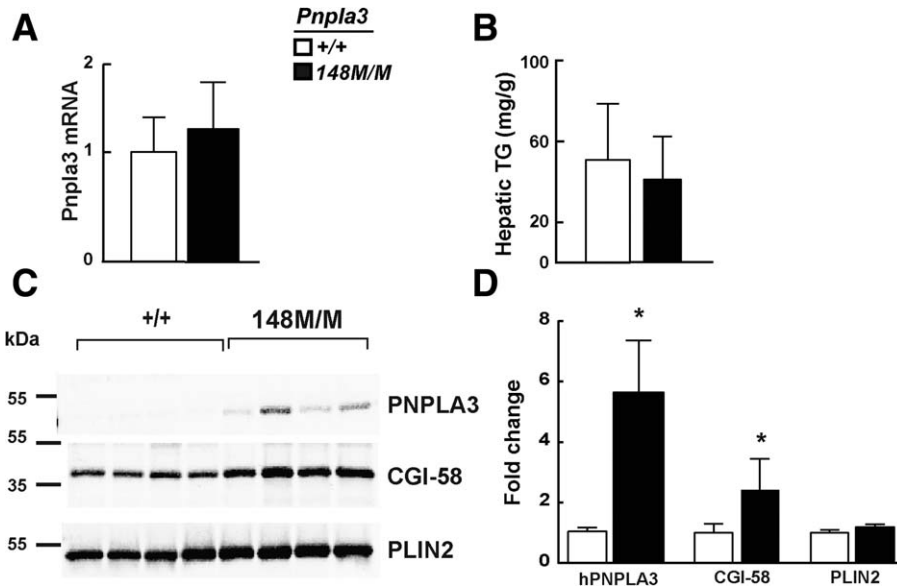
Fig. 7. PNPLA3 on lipid droplets from fat-fed mice. Livers were harvested from 17-week-old female wild-type (+/+) and Pnpla3[148M/M] mice (n = 5/group) fed a high-fat diet for 12 weeks. (A) Hepatic PNPLA3 mRNA levels were determined using real-time PCR, as described in the Materials and Methods. (B) Hepatic TG levels were measured by enzymatic assay as described in the Materials and Methods. (C) Immunoblot analysis of lipid droplets isolated from 4 mice per group was performed as described in the legend to Fig. 6, and (D) quantified as described in the Materials and Methods. Values are means ± SEM. *$P < 0.05$.

hepatic TG in Pnpla3[148M] mice indicates that the steatosis observed previously in PNPLA3[148M] transgenic mice[17] is not an artifact of high-level expression of the human protein. Furthermore, the similar phenotypes of the Pnpla3[47A/A] and PNPLA3[148M] KI indicate that the I148M substitution is equivalent to a loss of catalytic function, and that the association with steatosis is not a unique property of the 148M isoform. However, catalytic inactivation of PNPLA3 is not equivalent to loss of protein expression (as illustrated by the lack of steatosis in Pnpla3 knockout mice). Rather, PNPLA3-associated hepatic steatosis in mice requires the presence of the catalytically inactive protein, not simply the absence PNPLA3 activity.

Does the 148M variant also confer a gain-of-function? In the gain-of-function model proposed by Zechner and colleagues, the I148M substitution promotes TG synthesis by increasing the LPAAT activity of the enzyme.[18] A limitation of this model is that it implies an enzymatic activity that is independent of the catalytic serine, since Pnpla3[S47A] mice also express the fatty liver phenotype. Moreover, transgenic mice overexpressing wild-type human PNPLA3 did not have increased hepatic fat content, as might have been expected if the protein has LPAAT activity. Furthermore, rates of phosphatidic acid turnover and TG synthesis in transgenic mice expressing the 148M isoform were similar to those of mice expressing wild-type PNPLA3.[17]

An alternative gain-of-function hypothesis is that PNPLA3-148M promotes formation of a signaling molecule that stimulates TG synthesis. In mice overexpressing human PNPLA3-148M protein in the liver, mRNA levels of several SREBP-1c target genes involved in fatty acid TG synthesis were significantly increased.[17] How-

ever, no such changes were seen in mRNA expression profiles of the 148M KI mice. Thus, the steatosis in the KI mice appears not be due to the production of a signaling molecule that activates a transcriptional program promoting fatty acid and TG synthesis. We also found significant differences in the composition of hepatic TG-fatty acids in transgenic mice that could potentially alter SREBP-1c activity.[17] These changes were not observed in KI mice and are therefore unlikely to be causally related to the steatosis associated with the 148M variant.

In both humans and mice, the PNPLA3-148M isoform promotes liver TG accumulation under dietary conditions that expose the liver to high levels of insulin: obesity and insulin resistance in humans, and sucrose-feeding in mice. Moreover, the effect of the 148M variant in humans is amplified by diets rich in carbohydrates.[26] Sucrose feeding may promote hepatic steatosis by increasing fatty acid and TG synthesis in the liver and also by stimulating PNPLA3 expression.[19] Increasing the availability of fatty acids to the liver is not sufficient to explain the effects of the 148M variant on liver TG content: Pnpla3[148M] KI mice fed a high-fat diet, which increases fatty acid flux to the liver but up-regulates PNPLA3 expression only modestly, did not develop hepatic steatosis. Conversely, high-level expression of PNPLA3-148M increased liver TG content even in chow-fed animals.[12,17] Thus, the diet-dependence of PNPLA3-148M-associated steatosis may reflect the need for diet-induced stimulation of PNPLA3 expression. Additional studies will be required to fully define the relationship between PNPLA3 genotype, diet composition, and liver fat content in both mice and humans.

Kumashiro et al.[27] recently reported that antisense oligonucleotide-mediated knockdown of PNPLA3
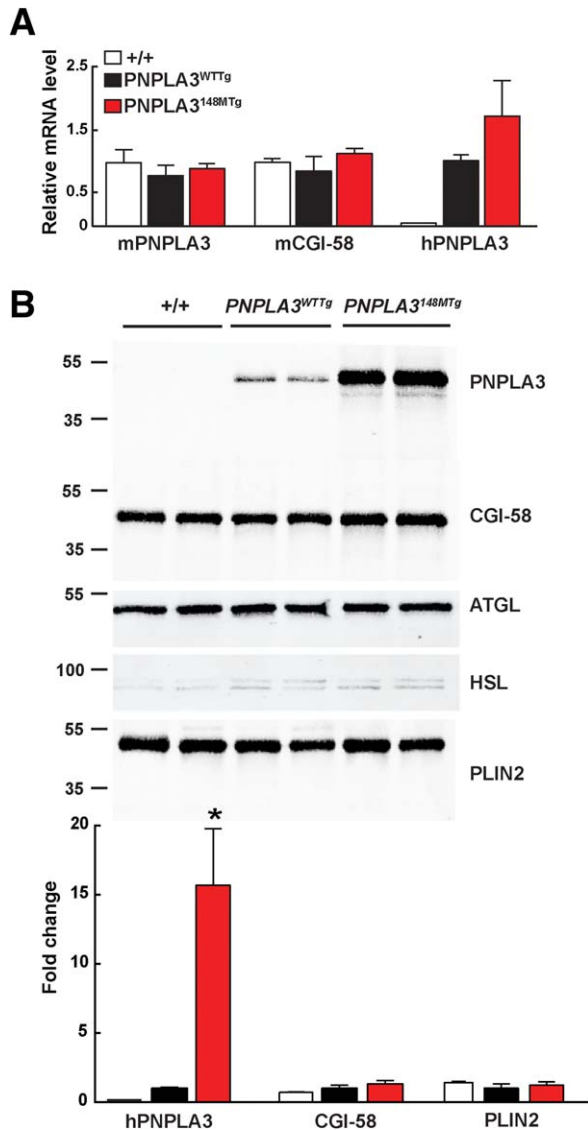
Fig. 8. Accumulation of PNPLA3 and CGI-58 protein on hepatic lipid droplets of *PNPLA3^WTTg* and *PNPLA3^148MTg* mice. Lipid droplets and mRNA were prepared from livers of 11-week-old male mice after 4 weeks on a high-sucrose diet. (A) Relative levels of endogenous and human PNPLA3 and CGI-58 mRNA in wild-type (+/+), *PNPLA3^WTTg*, and *PNPLA3^148MTg* transgenic mice. (B) Immunoblot analysis of lipid droplet proteins from wild-type (+/+), *PNPLA3^WTTg*, and *PNPLA3^148MTg* transgenic mice. *$P < 0.01$. The experiment was repeated twice and the results were similar.

prevents hepatic steatosis, improves glucose tolerance, and increases hepatic insulin sensitivity in fat-fed rats. However, chronic inactivation (or overexpression) of PNPLA3 in mice was not associated with changes in glucose homeostasis.[15-17] Nor did we find any association between the PNPLA3-148M isoform and insulin resistance in this study. Our results are similar to those of multiple studies in humans, including three population-based studies,[10,28,29] which found no association between the I148M variant and either diabetes or HOMA-IR. Moreover, euglycemic clamp studies

failed to identify defects in glucose homeostasis in individuals with the I148M variant.[30-32]

A striking and unanticipated consequence of sucrose feeding in the KI mice was a marked accumulation of mutant PNPLA3 on lipid droplets. Despite similar levels of hepatic PNPLA3 mRNA, lipid droplets from livers of both 148M and 47A KI animals had ~40-fold more PNPLA3 protein than wild-type animals. The accumulation of inactive PNPLA3 on lipid droplets may cause hepatic TG accumulation associated with the I148M substitution. The inactive protein may limit TG hydrolysis, possibly by restricting droplet access to, or by sequestering, a factor required for lipolysis. Immunoblot analysis indicated that the accumulation of mutant PNPLA3 was not associated with a reduction in ATGL or hormone-sensitive lipase on lipid droplets, but CGI-58 (ABHD5), an essential cofactor of ATGL,[33] was ~5-fold more abundant on droplets from livers of 148M KI mice than on those of their wild-type littermates. Thus, we speculated that PNPLA3-148M promotes hepatic steatosis by sequestering CGI-58, thereby limiting hydrolysis of lipid droplet TG by ATGL. The marginal increase in CGI-58 accumulation on lipid droplets of *PNPLA3^148MTg* mice despite a 2-fold increase in liver fat content and massive accumulation of the mutant PNPLA3 protein (Fig. 7) argues against this hypothesis.

The I148M substitution also resulted in a marked rightward shift in the size distribution of hepatic lipid droplets, such that very large droplets ($>100 \mu m^2$) comprised less than 0.1% of the total in wild-type mice, but almost 5% in the *Pnpla3^148M* KI animals. Since the volume of these very large droplets (~750 μm^3) is ~40 times larger than that of the median-sized droplet (19 μm^3), the major portion of hepatic TG in *Pnpla3^148M* mice is contained in a small fraction of the droplets. This finding raises the possibility that the 148M isoform alters the partitioning of proteins among different-sized lipid droplets, rather than between the lipid droplets and cytosol. Partitioning of proteins among diverse lipid droplets would not be detected by the methods used in this study. Experiments are under way to further characterize the protein and lipid composition of the lipid droplets in which PNPLA3 resides.

We cannot exclude the possibility that the accumulation of mutant PNPLA3 on lipid droplets is a consequence, rather than a cause, of hepatic steatosis. Steatosis was not associated with high levels of the 148M isoform in fat-fed mice, but PNPLA3 mRNA was markedly reduced in livers of these animals. In cultured cells, addition of free fatty acids to the medium induces lipid droplet formation and stabilizes PNPLA3, leading to increased expression of the protein.[19] In

mice, expression of the 148M isoform is associated with an increase in large lipid droplets, which may stabilize the mutant allele. Further studies will be required to elucidate the relationships between PNPLA3 accumulation, lipid droplet size, and hepatic steatosis.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.27242/suppinfo.