Pnpla3/Adiponutrin deficiency in mice does not contribute to fatty liver disease or metabolic syndrome

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Abbreviations:

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAC, bacterial artificial chromosome; BAT, brown adipose tissue; DPSs, digitonin-precipitable sterols; ES cell, embryonic stem cell; ESIMS, electrospray ionization mass spectrometry; GTT, glucose tolerance test; HF, high-fat; HFHS, high-fat high-sucrose diet; ITT, insulin tolerance test; KO, knockout; LFHS, low-fat high-sucrose diet; LFLS, low-fat low-sucrose diet; LXR, liver X receptor; MAT, mesenteric adipose tissue, NAFLD, non-alcoholic fatty liver disease; Pgat, perigonadal adipose tissue; Pnpla3, patatin-like phospholipase domain containing 3 (PNPLA3 for protein, PNPLA3 for human gene or mRNA, Pnpla3 for murine gene or mRNA); SCAT, subcutaneous adipose tissue; MAT, mesenteric adipose tissue; TAG, triacylglycerol; WAT, white adipose tissue; WT, wild-type
ABSTRACT

PNPLA3 (adiponutrin, iPLA₂ε) is an adipose-enriched, nutritionally-regulated gene that belongs to the patatin-like phospholipase domain-containing (PNPLA) family of lipid metabolizing proteins. Genetic variation in the human PNPLA3 gene (i.e. the rs738409 I148M allele) has been strongly and repeatedly associated with fatty liver disease. Although human PNPLA3 has triacylglycerol (TAG) hydrolase and transacylase activities in vitro, its in vivo function and physiological relevance remain controversial. The objective of this study was to determine the metabolic consequences of global targeted deletion of Pnpla3 in mice. We found that Pnpla3 mRNA expression is altered in adipose tissue and liver in response to acute and chronic nutritional challenges. However, global targeted deletion of Pnpla3 in mice did not affect TAG hydrolysis, nor did it influence energy/glucose/lipid homoeostasis or hepatic steatosis/injury. Experimental interventions designed to increase Pnpla3 expression (re-feeding, high-sucrose diet, diet-induced obesity, liver X receptor agonism), likewise failed to reveal differences in the above metabolic phenotypes. Expression of the Pnpla3 paralog, Pnpla5, was increased in adipose tissue but not liver of Pnpla3-deficient mice, but compensatory regulation of genes involved in TAG metabolism was not identified. Together these data argue against a role for Pnpla3 loss-of-function in fatty liver disease or metabolic syndrome in mice.

SUPPLEMENTARY KEY WORDS

Adiponutrin, patatin-like phospholipase domain-containing 3 (Pnpla3), calcium-independent phospholipase A2 epsilon (iPLA₂ε), insulin resistance, metabolic syndrome, fatty liver disease.
INTRODUCTION

Obesity and the metabolic syndrome are major contributors to morbidity and mortality from a variety of diseases affecting virtually all organ systems (1). Obesity is essentially a disorder of lipid accumulation, primarily in the form of triacylglycerols (TAGs) in adipose tissue. TAGs serve as a critical reservoir for lipid metabolites involved not only in energy homeostasis but also other essential cellular processes including membrane synthesis and cell signaling. In the context of chronic energy excess and/or impaired lipid metabolism, TAGs accumulate in metabolically-relevant non-adipose tissues such as liver where they are associated with cellular and systemic metabolic dysfunction (2, 3). TAG accumulation in liver (hepatic steatosis) is the earliest hallmark of non-alcoholic fatty liver disease (NAFLD) and contributes to hepatocyte dysfunction, inflammation (steatohepatitis), fibrosis, cirrhosis, liver failure, and even hepatocellular carcinoma (4). NAFLD frequently occurs with other features of the metabolic syndrome (both in association with and independent of obesity) (4-6) and is the leading cause of liver disease in the Western world (4, 7, 8). However, the mechanisms underlying hepatocellular TAG accumulation and its relationship to lipid-induced toxic metabolic effects (lipotoxicity) remain unclear.

PNPLA3 [alternatively known as adiponutrin and calcium-independent phospholipase A2 epsilon (iPLA2ε)] belongs to a novel family of lipid metabolizing enzymes known as the patatin-like phospholipase domain-containing (PNPLA) family (9). Recently, genetic variation in the human PNPLA3 gene (i.e. the rs738409 I148M allele in particular) has been strongly and repeatedly associated with hepatic TAG content, hepatocellular injury, and progression of NAFLD in humans (10-24). PNPLA3 variants have also been associated with obesity and features of the metabolic syndrome in humans in several studies (16, 25, 26), though subsequent studies have not confirmed this association (10, 12, 18, 22). The mechanisms by which genetic variation in PNPLA3 either predisposes to or protects against NAFLD and/or
metabolic disease are not known, but are obviously of considerable biomedical importance for the prevention and/or treatment of NAFLD and related metabolic disorders.

PNPLA3 was initially identified in 2001 as a membrane-associated, adipose-enriched, protein that is induced during adipogenesis (27). PNPLA3 shares the greatest homology with PNPLA2 [alternatively known as adipose triglyceride lipase (ATGL) and calcium-independent phospholipase A2 zeta (iPLA₂ζ)] which plays an extremely critical role in metabolism by mediating the rate-limiting step in TAG hydrolysis. Like Pnpla2/ATGL, Pnpla3 expression is altered in obesity-related dysmetabolic states (12, 25, 27-29). Also like Pnpla2/ATGL, Pnpla3 is strongly regulated by a variety of important nutritional (27-38), hormonal (25, 27, 35, 39-41), and pharmacological factors (36, 38, 42) – though Pnpla2 and Pnpla3 are usually regulated in the opposite direction from each other. The regulation and subcellular localization of PNPLA3 are most consistent with a role in anabolic lipid metabolism (i.e. lipogenesis). Indeed, lipogenic stimuli dramatically increase Pnpla3 expression not only in adipose tissue (35) but also in liver (38). Interestingly, however, human PNPLA3 has TAG hydrolase (28, 43, 44) and acyl-CoA-independent transacylase activities (43) in cell-free systems in vitro. Furthermore, ectopic overexpression of wild-type (WT) human PNPLA3 in cultured cells (28, 44) or in murine livers (44) does not alter cellular TAG accumulation. Likewise, siRNA-mediated knockdown of endogenous murine PNPLA3 in 3T3-L1 adipocytes does not affect measures of TAG hydrolysis (35). Thus, despite strong evidence implicating PNPLA3 in normal metabolism and disease, the in vivo function and physiological relevance of PNPLA3 remain controversial.

The goal of the present study was to better understand the function and physiological relevance of PNPLA3. To do so, we generated mice with global targeted deletion of Pnpla3. We then characterized these Pnpla3 knockout (Pnpla3-KO) mice according to multiple aspects of metabolic phenotype under a variety of metabolic conditions, including those known to induce Pnpla3 expression. In doing so, we found that global targeted deletion of Pnpla3 in mice is not a major determinant of overall energy homoeostasis, glucose homeostasis/insulin action, lipid
homeostasis, or hepatic steatosis/injury. Assuming similar functions of PNPLA3 in mice and humans, these data suggest that PNPLA3 loss-of-function may not be the primary mechanism by which variation in PNPLA3 influences fatty liver disease or metabolic phenotypes.
METHODS

Generation of the \textit{Pnpla3} targeting construct and \textit{Pnpla3}-KO mice.

The \textit{Pnpla3} targeting construct was generated using bacterial artificial chromosome (BAC) recombineering techniques (45, 46). A BAC vector (pBACE3.6) containing \textit{Pnpla3} from the 129SvEV/AB2.2 mouse strain (BAC clone bMQ-381F07) was obtained from the Wellcome Trust Sanger Institute. Nucleotides from -12 to +1927 (1939 bp) of the \textit{Pnpla3} gene were replaced by a neomycin resistance cassette. The resulting targeting construct was electroporated into 129 embryonic stem (ES) cells followed by antibiotic selection under standard conditions. Antibiotic-resistant ES cell clones were screened by Southern blotting to identify clones with homologous recombination. Appropriately targeted ES cells were then injected into C57BL/6 blastocysts and implanted into pseudopregnant females with the assistance of the transgenic core facility at Beth Israel Deaconess Medical Center. Resulting chimeric mice were screened by PCR to identify mice with germline transmission of the targeted allele (WT allele: forward 5' gggccgggtacgtggattagaga, reverse 5' agcctgccccacaaaaag, 296 bp; targeted allele: forward 5' cggccgcttgggtggagag, reverse 5' caggtagccgatcaagcgtatgc, 358 bp). Mice carrying the targeted allele were then backcrossed to C57BL/6 mice for at least 4 generations (N4).

Animals.

For evaluation of adipose- and liver-specific \textit{Pnpla3} mRNA expression in response to high-fat (HF) feeding and leptin-deficiency, tissues were collected from a) overnight-fasted 24 week-old male FVB mice (Taconic) fed chow diet (14 kcal\% fat, Harlan Teklad RD8664) or HF diet (42 kcal\% fat, Harlan Teklad TD88137) since weaning (47), and b) \textit{ad lib}-fed 10 week-old \textit{Lep}^{ob}/\textit{Lep}^{ob} mice (Jackson Laboratories) fed chow since weaning (35), respectively. For evaluation of the effects of global \textit{Pnpla3} deletion on metabolism, experimental mice were derived from breeding 129/C57BL/6(N4-N6) mice heterozygous for the targeted \textit{Pnpla3} allele.
Resulting experimental wild-type (WT, \textit{Pnpla3}^{+/+}) and knockout (KO, \textit{Pnpla3}^{-/-}) mice were fed one of four diets (composition in kcal%): standard chow (Chow; Prolab Isopro RMH 3000; 26% protein / 60% complex carbohydrate in the form of starch / 14% fat), low-fat low-sucrose diet (LFLS, Research Diets D12328N; 16.4% protein / 73% complex carbohydrate in the form of 61% starch and 12% maltodextrin / 10.5% fat), low-fat high-sucrose diet (LFHS; D12329N; 16.4% protein / 73% carbohydrate in the form of sucrose / 10.5% fat), or high-fat high-sucrose diet (HFHS; D12331N; 16.4% protein / 25% carbohydrate in the form of sucrose / 58% fat) from weaning until 19 weeks of age. For evaluation of the metabolic response to pharmacological activation of liver X receptor (LXR), a separate cohort of 12 week-old male mice was fed chow diet containing 0.025% of the potent LXR agonist T0901317 (48, 49) for 6 days (equivalent to \(~50\ \text{mg/kg/d}) as described (38, 50). This dosing regimen was selected based on prior experiments demonstrating induction of hepatic \textit{Pnpla3} mRNA expression under these experimental conditions (38). Mice were housed at Beth Israel Deaconess Medical Center, University of Pittsburgh, or University of Texas-Southwestern Animal Facilities under standard conditions with \textit{ad libitum} access to water and food. All investigations involving animals were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals outlined in the Institute for Laboratory Animal Research (ILAR) Guide for Care and Use of Laboratory Animals. Experimental procedures were approved by the Institutional Animal Care and Use Committees at the institutions noted above.

**Body Composition, energy expenditure, and metabolic measurements.**

Body composition was determined by EchoMRI (Echo Medical Systems). Oxygen consumption (\textit{VO}_2), respiratory exchange ratio (\textit{RER}), and physical activity were determined using a Comprehensive Laboratory Animal Monitoring System (Columbus Instruments). Plasma glucose was measured using a One-touch FastTake glucometer (Lifescan). For glucose tolerance tests (GTTs), mice were injected intraperitoneally with 1.75 g glucose per kg body
weight following a 6h fast. For insulin tolerance tests (ITTs), mice were injected intraperitoneally with regular human insulin (HumulinR; Lilly) at 0.80 units per kg of body weight following a 4h fast. Serum corticosterone was determined by radioimmunoassay as described (47). Other serum parameters were determined using the following kits: insulin (Ultra Sensitive Mouse Insulin ELISA Kit, Crystal Chem), TAG (Infinity Triglycerides Liquid Stable Reagent, Thermo Scientific), NEFA (HR Series NEFA-HR(2) Reagents, Wako Diagnostics), cholesterol (Cholesterol E, Wako Diagnostics), alanine aminotransferase (ALT/SGPT Test, Stanbio Laboratory), and aspartate aminotransferase (AST/SGOT Test, Stanbio Laboratory).

**TAG hydrolase activity.**

TAG hydrolase activity was determined as described (51). Briefly, tissues were homogenized in lysis buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin, pH 7.0). Lysates were then centrifuged for 60 min at 100,000 x g at 4°C to isolate lipid-free infranatants. Triolein substrate containing [9,10-3H]triolien as radioactive tracer was prepared by sonication as described (52). Cytosolic fractions (0.1 ml) with or without 500 ng/assay of purified CGI-58 and/or 25 μM of the HSL-specific inhibitor 76-0079 (NNC 0076-0000-0079, Novo Nordisk) were incubated with 0.1 ml (167 nmol) of substrate at 37°C for 60 min. Reactions were terminated by adding 3.25 ml of methanol/chloroform/heptane (10:9:7) and 1 ml of 0.1 M potassium carbonate, 0.1 M boric acid, at pH 10.5. After centrifugation at 800 x g for 20 min, the radioactivity in 1 ml of the upper phase was determined by liquid scintillation counting.

**Ex vivo lipolysis in perigonadal white adipose tissue (WAT) explants.**

*Ex vivo* tissue lipolysis was determined as described (53). Briefly, ~20 mg pieces of perigonadal WAT was incubated in DMEM (Invitrogen) containing 2% fatty acid-free BSA (Sigma) with or without 10 μM isoproterenol (Sigma) at 37°C. NEFA and glycerol release were
determined in aliquots of incubation buffer collected at the times indicated using NEFA-HR(2) reagents (Wako Diagnostics) and Infinity Triglycerides Liquid Stable Reagent (Thermo Scientific), respectively. Results were normalized to tissue protein content as determined by the bicinchoninic acid method (Pierce).

**Biochemical and histological determination of tissue TAG content.**

For biochemical determination of liver TAG content, liver tissue (~20-30 mg) was ground in liquid nitrogen and extracted by the method of Folch (54). Dried lipid extracts were re-dissolved in 60 µL tert-butanol plus 40 µL of a 2:1 mixture of Triton X-114:methanol. TAG content was then determined using Infinity Triglycerides Liquid Stable Reagent (Thermo Scientific). Liver TAG content was confirmed histologically by Oil Red O staining using standard methods (55).

**In vivo cholesterol and fatty acid synthesis.**

*In vivo* rates of cholesterol and fatty acid synthesis were measured in mouse tissues using 3H-labeled water (ICN) as described (56). Mice were fasted for 2 h at the early phase of the light cycle and then injected intraperitoneally with [3H]-H₂O (50 mCi in 0.25 ml isotonic saline). One hour after the injection, mice were sacrificed, and liver, perigonadal WAT, and intrascapular brown adipose tissue (BAT) were collected. Lipids were extracted from tissues and 3H-labeled fatty acids and digitonin-precipitable sterols (DPSs) were determined as described (57, 58). Rates of fatty acid and cholesterol synthesis were calculated as µmol 3H-radioactivity incorporated into fatty acids or DPSs per hour per gram of tissue, respectively.

**Mass spectrometric analysis of lipids.**

The quantitative analysis of TAG molecular species was performed as described (59, 60). Briefly, lipids were extracted from mouse tissues using a modified method of Bligh and Dyer (61) in the presence of internal standards for electrospray ionization mass spectrometric
(ESIMS) analysis. ESIMS analyses were performed using a TSQ Quantum Ultra Plus triple-quadrupole mass spectrometer (ThermoFisher Scientific) equipped with an automated nanospray apparatus (Nanomate HD, Advion Bioscience Ltd.) and Xcalibur system software.

**RNA extraction, reverse transcription, and gene expression analysis.**

Total RNA was extracted from homogenized tissues using RNeasy Lipid Tissue Mini Kit with on-column DNase treatment (Qiagen). Reverse transcription of 1 μg total RNA was performed using random decamers (RETROscript Kit, Ambion, Inc.). Gene expression was determined by qPCR (7300 Real-time PCR System, ABI) using gene-specific primer-probe sets (Taqman Gene Expression Assays, Applied Biosystems) normalized to cyclophilin (forward 5’ ggtggagagcaccaagacaga , reverse 5’ gccggagtcaaatgatg, probe 5’ agccgggacaagccactgaaggat) or 18S ribosomal RNA (Applied Biosystems) reference genes using the standard curve method as described (62). Appropriate analysis was performed to determine that expression of reference genes were unchanged under the experimental conditions described. Accuracy of RNA quantification was optimized by DNase treatment of samples, use of gene-specific primer-probe sets that span intron-exon boundaries, and verification of lack of amplification in no-RT and no-template controls. Experimental parameters conformed to MIQE guidelines (63).

**Statistical analysis.**

Results are expressed as mean ± standard error of the mean (SE). Comparisons were made by unpaired two-tailed Student’s t-test or factorial analysis of variance (ANOVA) followed by determination of simple effects for pairwise comparisons if relevant (SPSS). For repeated measurements, comparisons were made by two-way ANOVA with repeated measures. For all analyses, p values of <0.05 were considered statistically significant.
RESULTS

Pnpla3 mRNA expression in adipose tissue and liver is altered in murine models of fatty liver and metabolic syndrome.

Pnpla3 mRNA expression is highly regulated by acute nutritional and hormonal challenges in both adipose tissue and liver (25, 27-41). To determine the response to chronic nutritional and hormonal challenges that predispose to fatty liver and the metabolic syndrome, we evaluated Pnpla3 mRNA expression in adipose tissue and liver of mice with obesity due to chronic high-fat diet feeding and leptin deficiency (Lep<sup>ob</sup>/Lep<sup>ob</sup> mice) (Fig. 1). In overnight-fasted diet-induced obese mice (Fig. 1A), Pnpla3 expression was increased in all adipose tissue depots. In addition, despite lower Pnpla3 expression in liver relative to adipose tissue, hepatic Pnpla3 expression was also increased (12-fold). Thus, prolonged high-fat feeding [sufficient to induce obesity (47)] increases Pnpla3 expression independent of acute nutritional effects. In ad <i>lib</i>-fed genetically obese leptin-deficient mice (Fig. 1B), Pnpla3 expression was also altered in adipose tissue, but in a depot-specific manner – consistent with prior results from leptin-resistant (Lepr<sup>fa</sup>/Lepr<sup>fa</sup>) rats (29). These data suggest that Pnpla3 may be regulated differently in leptin-deficient/resistant obesity compared to diet-induced obesity. However, similar to diet-induced obesity, Pnpla3 expression was substantially increased in liver (22-fold) in mice with leptin deficiency. Thus, Pnpla3 mRNA expression is altered in adipose tissue and liver of two common models of fatty liver and metabolic syndrome, thereby supporting a potential role for PNPLA3 in the pathogenesis and/or physiological adaptation to these disorders.

Mice with global targeted deletion of Pnpla3 lack Pnpla3 expression in metabolically-relevant tissues.

To better understand the physiological relevance of PNPLA3, including its contribution to fatty liver and metabolic syndrome, we generated a murine model with global targeted deletion
of *Pnpla3* (*Pnpla3*-KO mice) (Fig. 2A-B). As expected, *Pnpla3* mRNA expression was decreased in a gene-dose dependent manner in mice carrying the targeted allele and was completely absent in metabolically-relevant tissues of *Pnpla3*-KO mice where *Pnpla3* is normally expressed (*i.e.* adipose tissue, adrenal gland, and liver) (Fig. 2C) (35). Similar mRNA expression results were obtained for primer-probe sets spanning exon 1-2 (proximal) (Fig. 2C), 4-5 (mid) and 7-8 (distal) of the *Pnpla3* gene (data not shown). Gene expression analysis further confirmed that endogenous *Pnpla3* expression was 100 to 1000-fold higher in adipose tissue than in liver. Despite differences in adrenal *Pnpla3* expression, no differences in adrenal histopathology (data not shown) or am serum corticosterone levels (WT 25.3±15.2 vs KO - 27.1±13.8 ng/mL) were identified between genotypes. Finally, *Pnpla3*-KO mice were viable, fertile, and produced in the expected Mendelian ratios.

**Global targeted deletion of *Pnpla3* does not affect adipose tissue TAG hydrolysis.**

Because human PNPLA3 has TAG hydrolase activity in cell-free systems *in vitro* (43, 44), we next evaluated TAG hydrolysis in adipose tissue of *Pnpla3*-KO mice (Fig. 3). *Pnpla3* is most highly expressed in perigonadal WAT and BAT (35). However, *in vitro* TAG hydrolase activity in lysates of perigonadal WAT (Fig. 3A) and BAT (data not shown) did not differ between genotypes, either when assayed alone or in the presence of the PNPLA2/ATGL co-activator CGI58 and/or the hormone sensitive lipase (HSL) inhibitor 76-0079. Likewise, basal and isoproterenol-stimulated glycerol (Fig. 3B) and NEFA (data not shown) release from perigonadal WAT explants did not differ between genotypes. Finally, there was no compensatory up-regulation of mRNA for *Pnpla2/ATGL* or *HSL*, the primary enzymes mediating TAG hydrolysis, in WAT (Fig. 3C and 3D, respectively) or BAT (data not shown). These data suggest that PNPLA3 does not contribute significantly to TAG hydrolysis in murine tissues.

**Global targeted deletion of *Pnpla3* does not affect energy homeostasis.**
Because PNPLA3 could have activities other than TAG hydrolysis that could influence metabolism, we performed comprehensive metabolic phenotyping of Pnpla3-KO and WT mice in response to chronic nutritional challenges known to induce Pnpla3 expression (i.e. high-sucrose feeding and diet-induced obesity). Specifically, mice were fed a standard chow diet as well as three diets with identical protein content but differing in sucrose and fat content - a “control” low-fat low-sucrose diet (LFLS), a low-fat high-sucrose diet (LFHS), and a high-fat high-sucrose diet (HFHS) from weaning until 19 weeks of age. Importantly, gene expression analysis of terminal liver and perigonadal WAT confirmed substantially higher Pnpla3 mRNA expression in tissues from WT compared with Pnpla3-KO mice for all diet groups (see Fig. 7 & Supplemental Table 1). Nevertheless, no differences were observed between genotypes for body weight (Fig. 4A-D), % fat mass (Fig. 4E-H), % lean body mass (Chow - WT 70.28±2.17 vs KO 69.11±1.80; LFLS – WT 64.84±1.64 vs KO 65.55±1.09; LFHS – WT 62.45±1.70 vs KO 62.06±2.01; HFHS – WT 57.04±2.39 vs KO 50.15±4.81%), cumulative food intake (Chow - WT 1438±55 vs KO 1444±55; LFLS – WT 1515±24 vs KO 1466±36; LFHS – WT 1472±48 vs KO 1495±77; HFHS – WT 1738±89 vs KO 1764±41 kcal/mouse/19w), or fat pad/tissue weights (data not shown). Likewise no differences were identified between genotypes for oxygen consumption, respiratory exchange ratios (RER), or physical activity in the ad lib-fed, fasted, or re-fed states (8 week-old males, n=4-6/group; data not shown). Thus, Pnpla3 deficiency does not alter overall energy homeostasis in response to chronic nutritional challenges.

Global deletion of Pnpla3 does not affect systemic glucose tolerance, insulin sensitivity, or serum lipid homeostasis.

To determine the effect of Pnpla3 deficiency on systemic lipid/glucose homeostasis and insulin action, we next evaluated body weight and serum parameters in response to acute nutritional challenges (ad lib feeding, fasting, re-feeding) in mice fed LFLS, LFHS, and HFHS diets from weaning until 12 weeks of age (Table 1). As expected, both diet and feeding status
had significant main effects on body weight and serum parameters, consistent with the appropriate metabolic responses. Further analysis revealed significant interactions between diet and genotype for NEFA (NEFA were lower in KO than WT in LFHS-fed mice but trended higher in KO than WT in HFHS-fed mice) and between feeding status and genotype for cholesterol (cholesterol was higher in KO than WT in re-fed mice). However, no main effects were identified for genotype on body weight, glucose, insulin, TAGs, NEFAs, or cholesterol. Furthermore, no consistent or reproducible differences between genotypes were identified for these parameters in terminal blood from ad lib-fed 19 week-old mice from the above diet groups, in terminal blood from 11 week-old chow-fed mice fasted for intervals ranging from 4-16 hours, or in terminal blood from 8-10 week-old chow-fed mice fasted for 12h followed by 12h of re-feeding LFHS diet (data not shown). Finally, both genotypes from the above diet groups had similar serum glucose responses to intraperitoneal glucose (Fig. 5A-D) and insulin (Fig. 5E-H) challenges, indicating no differences in systemic glucose tolerance or insulin sensitivity, respectively. These data argue against a significant effect of genotype on these parameters, but suggest that Pnpla3 may influence NEFA and/or cholesterol homeostasis under specific nutritional/metabolic conditions.

Since Pnpla3 is positively regulated by the lipogenic nuclear transcription factor LXR (38), we additionally measured phenotypic parameters in a separate cohort of 12 week-old, 4h-fasted, chow-fed male mice that were treated with the potent LXR agonist T0901317 for 6 days. This treatment regimen has previously been shown to induce hepatic Pnpla3 mRNA expression by 6 to 16-fold over the duration of the treatment (38), and would thereby be expected to enhance phenotypic differences between genotype groups. As expected, T0901317 treatment increased serum cholesterol and hepatic TAG content to levels comparable to its previously reported effects under similar experimental conditions (50), thereby supporting the effectiveness of the treatment. However again, no differences were observed between genotypes for body weight (WT 27.27±1.21 vs KO 28.50±0.47 g), individual (data not shown) or total fat pad weight (WT 3.80±0.43 vs KO 3.27±0.56 % of body weight), liver weight (WT 7.70±0.49 vs KO
7.51±0.32 % of body weight), hepatic TAG content (WT 85.07±22.39 vs KO 89.94±19.61 mg/g tissue), hepatic cholesterol content (WT 1.86±0.17 vs KO 1.85±0.33 mg/g tissue), blood glucose (WT 367±13 vs KO 380±10 mg/dL), serum TAG (WT 75.33±15.33 vs KO 72.00±7.23 mg/dL), serum NEFA (WT 0.81±0.04 vs KO 0.82±0.06 mEq/L), or serum cholesterol (WT 188.79±20.79 vs KO 186.0±15.89 mg/dL). Thus, Pnpla3 deficiency does not alter systemic glucose or lipid homeostasis in response to pharmacological induction by an LXR agonist.

**Global deletion of Pnpla3 does not contribute to hepatic steatosis or injury.**

Recently, genetic variation in the human PNPLA3 gene has been strongly and repeatedly associated with fatty liver disease in humans. We, therefore, assessed the effect of Pnpla3 deficiency on hepatic lipid content and injury (Fig. 6). Although the expected increases in hepatic TAG content were observed for LFHS and HFHS diet groups, no differences between genotypes were identified for liver weights (data not shown) or liver TAG content between genotypes for any of the diet groups, whether measured biochemically (Fig. 6A) or histologically by Oil Red O staining (data not shown). Since transacylase activity of PNPLA3 could result in physiologically significant changes in TAG molecular species without a net overall change in TAG mass, we further characterized TAG molecular species using electrospray ionization tandem mass spectrometry (59, 60). However, individual TAG molecular species also did not differ between genotypes for liver (Fig. 6B) or WAT (data not shown). Serum AST (Fig. 6C) and ALT (Fig. 6D), markers of hepatic injury, also did not differ between genotypes. Given the above interactive effects on serum NEFA and cholesterol, we additionally evaluated *in vivo* cholesterol and fatty acid metabolism in a separate cohort of 8-9 week old chow-fed mice using tritiated water. However, no differences in hepatic incorporation of [3H]-H₂O into digitonin precipitable sterols (i.e. cholesterol) (Fig. 6E) or NEFAs (Fig. 6F) were identified. Similar results were obtained for NEFA biosynthesis in WAT and BAT (Fig. 6F). These data suggest that Pnpla3 deficiency does not contribute to hepatic lipid metabolism, steatosis, or injury.
Global deletion of *Pnpla3* promotes compensatory regulation of *Pnpla5* mRNA but not other genes/proteins involved in TAG metabolism.

To identify genes and/or pathways that might be compensating for *Pnpla3* deficiency, we evaluated mRNA expression of genes involved in TAG metabolism in liver and perigonadal WAT of *ad lib*-fed WT and *Pnpla3*-KO mice fed chow, LFLS, LFHS, or HFHS diet from weaning until 19 weeks of age (Fig. 7 and Supplemental Table 1). As expected, both diet and genotype had significant main effects on *Pnpla3* mRNA expression in both WAT (Fig. 7A) and liver (Fig. 7B). The diet-induced effect was particularly dramatic in liver where *Pnpla3* mRNA was induced over 100-fold in LFHS-fed mice. It is worth noting that the LFLS diet (61% starch, 12% maltodextrin) also induced hepatic *Pnpla3* mRNA expression by 29-fold compared to chow (60% starch) and 2-fold compared to HFHS-diet (25% sucrose), suggesting a positive regulatory effect of the more palatable/digestible maltodextrin carbohydrate component in the former and/or a suppressive regulatory effect of dietary fat in the latter. As expected, significant main effects of diet on mRNA expression of numerous genes involved in TAG metabolism were identified in both liver and WAT (Supplemental Table 1). However, no main effects of genotype on mRNA expression of these genes were identified for either tissue. In contrast, *Pnpla5*, a *Pnpla3* paralog with unclear function, was significantly increased in WAT of KO compared with WT mice (Fig. 7C). *Pnpla5* mRNA expression in liver was extremely low and could not be detected in liver of WT or KO mice, even after 60 cycles using undiluted cDNA prepared from 1 μg of RNA. Thus, *Pnpla3* deficiency promotes compensatory up-regulation of *Pnpla5* (in WAT only) but not other genes involved in TAG metabolism.
DISCUSSION

Substantial data supports an important role for PNPLA3 in normal metabolism and disease including the following: i) PNPLA3 shares significant homology with proteins known to play critical roles in metabolism (9, 64, 65), ii) PNPLA3 is highly regulated by important nutritional/metabolic factors (25, 27-42), iii) PNPLA3 expression is altered in obese/dysmetabolic states (25, 27-29), iv) PNPLA3 has lipid hydrolase and transacylase activities in vitro (28, 43, 44), and v) genetic variation in PNPLA3 is associated with NAFLD in humans (10-26). Despite this strong evidence, the in vivo function and physiological relevance of PNPLA3 remain unclear. In this study, we determine the metabolic consequences of global targeted deletion of Pnpla3 in mice. We confirm that Pnpla3 mRNA expression is indeed altered in adipose tissue and liver in response to acute and chronic nutritional challenges. Importantly, however, global targeted deletion of Pnpla3 does not affect TAG hydrolysis or influence metabolic phenotypes including energy homoeostasis, glucose homeostasis/insulin action, lipid homeostasis, or hepatic steatosis/injury. Furthermore, experimental interventions designed to increase Pnpla3 expression and enhance differences between genotypes (i.e. re-feeding, high sucrose diet, diet-induced obesity, and pharmacological activation by LXR), likewise fail to reveal any differences in the above metabolic phenotypes. It is worth noting that, while this manuscript was in preparation, another group published similar data in an independently-generated model of global Pnpla3 deletion and additionally failed to identify any metabolic consequences of Pnpla3 deficiency in mice challenged with a methacholine-deficient diet or with leptin deficiency (66). Together these data argue against a role for Pnpla3 loss-of-function in NAFLD and the metabolic syndrome, at least in mice.

Although genotype had no main effects on phenotype overall, two noteworthy interactions were identified in our study: 1) serum NEFAs were lower in LFHS-fed Pnpla3-KO mice (but tended to be higher in HFHS-fed mice); and 2) serum cholesterol was higher in re-fed Pnpla3-
KO mice. These data suggest that Pnpla3 deficiency may influence NEFA and/or cholesterol homeostasis under certain metabolic conditions (i.e. chronic high carbohydrate feeding and/or the postprandial state), perhaps in combination with other as yet unknown factors (i.e. stress/glucocorticoids, concurrent genetic/environmental influences, etc.). These findings may be particularly relevant to humans who consume increasing quantities of simple carbohydrates (particularly fructose) and who spend a significant amount of time in the postprandial state (67). Interestingly, Kolleritis, et al., recently identified an association between the rs738409 (I148M) PNPLA3 variant and serum apoB-containing lipoprotein concentrations in humans (16). However, numerous other studies have failed to identify an association between PNPLA3 variants and cholesterol/lipoprotein concentrations (10, 22, 23). Furthermore, in the present study, we were not able to identify differences between genotypes for NEFA or cholesterol metabolism using *in vivo* tritiated water methods. The lack of consistency of these findings across outcomes stresses the need for further exploration of these results. Finally, it is also worth noting that hepatic TG content, though not statistically different, was consistently slightly higher in Pnpla3-KO mice in our studies (Fig. 6A, 6B, and unpublished data). Thus, it is possible that alternative metabolic conditions, enhanced statistical power, and/or a more homogeneous background strain could unmask previously unrecognized phenotypes in Pnpla3-KO mice.

Thus, the question remains – what is the function of PNPLA3? The regulation and subcellular localization of PNPLA3 are most consistent with a role for PNPLA3 in anabolic lipid metabolism (i.e. lipogenesis), and yet *in vitro* functional data support a role in TAG hydrolysis (and/or transacylation) (43, 44). The latter could account for a metabolically neutral phenotype, but the physiological significance of such an activity is unclear. Thus far, overexpression and/or knockdown of PNPLA3 in cells (28, 35, 44) and now also global targeted deletion in mice (66) have failed to identify significant effects of PNPLA3 on metabolic phenotypes including TAG metabolism. Furthermore, no clear compensatory regulation of key enzymes involved in TAG synthesis or hydrolysis were identified in Pnpla3-KO mice. Interestingly, however, Pnpla5
mRNA expression was increased in WAT of Pnpla3-KO mice, thereby corroborating findings by Chen et al. (66). Pnpla5 (also known as GS2-like) is a paralog of Pnpla3. Like PNPLA3, PNPLA5 has been shown to have TAG hydrolase activity, and ectopic expression reduces incorporation of radiolabeled FFA’s into TAG in HEK293 cells (28). However, another study failed to identify any TAG hydrolase activity for PNPLA5 (68). Furthermore, it is not clear that PNPLA5 is expressed at physiologically-significant levels, at least in liver, of humans (65) or mice (28). Our data support these findings since, unlike Chen et al., we did not detect significant expression of Pnpla5 mRNA in liver. Thus, it is possible that PNPLA5 may compensate for loss of PNPLA3 in WAT but not liver in this model. Understanding the function of PNPLA5 may provide clues to the function of PNPLA3 and vice versa.

Nevertheless, numerous studies have demonstrated a strong association between genetic variation in PNPLA3 and hepatic steatosis, hepatic injury, and progression of NAFLD (10-24). The human rs738409 variant is of particular interest because it contains a C→G nucleotide change that encodes for a methionine in place of isoleucine at position 148 (I148M mutation). Computer modeling suggests that this mutation might alter substrate access to the putative catalytic serine residue in the conserved GXSXG motif (44). Indeed, both the human I148M mutant as well as a mutant in which the catalytic serine has been replaced by an alanine (S47A) lack in vitro TAG hydrolase activity (28, 44), suggesting that the I148M allele contributes to disease via loss of TAG hydrolytic function. Interestingly, adenoviral overexpression of these mutants (I148M and S47A) in murine liver enhances hepatic TAG accumulation, even in the presence of normal endogenous expression of the WT murine allele, which is not consistent with simple loss-of-function. Thus, the I148M and S47A mutants may also affect TAG metabolism by mechanisms distinct from loss of TAG hydrolytic function. For example, PNPLA3 may have additional substrates and/or functions that directly or indirectly promote TAG synthesis and that are enhanced by the above mutations. Certainly, a predominant role for PNPLA3 in lipid synthesis would be more consistent with its regulation. It remains possible that
species-specific differences exist in function, expression, and/or regulation of PNPLA3. Indeed, human and rodent PNPLA3 differ considerably at their C-termini and exhibit different expression patterns (i.e. human PNPLA3 mRNA is more highly expressed in liver) (38). However, the impact of these differences on function and physiology remains to be determined.

In summary, our data provide strong evidence against a critical role for PNPLA3 in murine TAG hydrolysis and also provide further important corroborative evidence against a role for Pnpla3 deficiency/loss-of-function in the pathogenesis of NAFLD and the metabolic syndrome in mice. It is important to note, however, that our data do not rule out a potential contribution of PNPLA3 gain-of-function or of specific genetic PNPLA3 variants (i.e. the rs738409 I148M mutant) to lipid metabolism or metabolic disease in humans. The data connecting genetic variation in PNPLA3 to human disease are extremely compelling and clearly warrant continued efforts to understand the precise function and physiological relevance of this gene – which unfortunately remain unclear nearly a decade after its initial identification. Purification and systematic analysis of its enzymatic activities and substrate specificity in vitro are necessary to more clearly characterize its function, but are challenging given the numerous potential substrates/activities and inherent differences between in vitro and in vivo systems. Thus, these studies should, therefore, also be complemented by further in vivo metabolic characterization of mice with targeted genetic “knock-in” of the I148M allele as well as overexpression of WT and/or mutant PNPLA3 alleles in metabolically relevant tissues. Such studies will be crucial for determining whether therapeutic manipulation of Pnpla3 might be useful in the prevention and/or treatment of NAFLD and related metabolic disorders.
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FIGURE LEGENDS

Fig 1. Pnpla3 mRNA expression in animal models of fatty liver and metabolic syndrome. (A) Pnpla3 mRNA expression in brown (BAT), perigonadal (PGAT), subcutaneous (SCAT), and mesenteric (MAT) adipose tissue, and liver of overnight-fasted male FVB mice fed chow (14 kcal% fat) or high-fat diet (HFD, 42 kcal% fat) from weaning until 24 weeks of age (n=10 per group, male, 24 week-old, overnight-fasted). (B) Pnpla3 mRNA expression in the above adipose tissue depots and liver of wild-type (WT) or leptin-deficient (Lep\textsuperscript{ob}/Lep\textsuperscript{ob}) mice (n=6 per group, male, 10 week-old, ad lib-fed, chow diet). Pnpla3 expression is normalized to 18S ribosomal RNA and expressed relative to Pnpla3 expression in BAT of the chow-fed (A) or wild-type (B) control groups, respectively. * p<0.05 by Student's t-test.

Fig 2. Generation of mice with global targeted deletion of Pnpla3 (Pnpla3-KO mice). (A) The native (wild-type, WT) Pnpla3 allele and targeting (knockout, KO) construct (not drawn to scale). Bacterial artificial chromosome (BAC) recombineering was used to replace 1939 bp of the native Pnpla3 gene from position -12 to +1927 including the ATG start signal and the entire exon 1 (which contains the GXSXG motif with the putative critical catalytic serine residue of the patatin domain) by a neomycin resistance cassette. Embryonic stem (ES) cell electroporation, selection, and screening were then performed using standard techniques. (B) Southern blots of ES cell clones confirming homologous recombination of the target construct at upstream (BamH1) and downstream (HindIII) ends. BACs containing the WT and targeted (KO) allele are shown as controls. Lanes 1 and 2 demonstrate positive ES cell clones. Lane 3 demonstrates a negative ES cell clone. All gels were run under identical conditions. Images shown were run on separate gels. (C) Pnpla3 mRNA expression in perigonadal white adipose tissue (WAT), brown adipose tissue (BAT), adrenal gland, and liver using a primer-probe set spanning exons 1-2 (Pnpla3\textsubscript{Exon1-2}) (n=7-10/group, mixed gender, 7-8 week-old, ad lib-fed, chow diet). Similar results
were obtained for primer-probe sets spanning exons 4-5 and 7-8 (data not shown). Pnpla3 expression is normalized to 18S ribosomal RNA and expressed relative to Pnpla3 expression in WAT of the control group.

**Fig 3. TAG hydrolysis in Pnpla3-KO mice.** (A) TAG hydrolase activity was determined in cytosolic fractions of perigonadal WAT lysates of WT and KO mice (n=6/group, male, 16-17 week-old, *ad lib*-fed, chow diet) in the presence or absence of the Pnpla2 coactivator CGI58 and/or the HSL-specific inhibitor 76-0079. Similar results were obtained for BAT (data not shown). (B) Glycerol release over time from perigonadal WAT explants of WT and KO mice (n=3/group, male, 8-10 week-old, *ad lib*-fed, chow diet) in the absence (basal) or presence of 10 μM isoproterenol (stimulated, stim). Comparable results were obtained for NEFA release (data not shown). (C-D) mRNA expression of Pnpla2 (C) and HSL (D) in periogonadal WAT of WT and KO mice (n=7-10/group, mixed gender, 7-8 week-old, *ad lib*-fed, chow diet). Expression is normalized to 18S ribosomal RNA and expressed relative to gene expression in the WT control group. No significant differences were identified.

**Fig 4. Energy homeostasis in Pnpla3-KO mice.** (A-D) Body weight in grams (g) of WT and KO mice fed chow (A), low-fat low-sucrose (LFLS) (B), low-fat high-sucrose (LFHS) (C), or high-fat high-sucrose (HFHS) (D) diets from weaning until 19 weeks of age. (E-H) Fat mass as a percent of total body mass (%) of WT and KO mice fed chow (E), LFLS (F), LFHS (G), or HFHS (H) diets from weaning until 19 weeks of age (n=6-11/group, male). No significant differences were identified.

**Fig 5. Glucose tolerance and insulin sensitivity in Pnpla3-KO mice.** (A-D) Glucose tolerance tests for WT and KO mice fed chow (A), low-fat low-sucrose (LFLS) (B), low-fat high-sucrose (LFHS) (C), or high-fat high-sucrose (HFHS) (D) diets (n=6-11/group, male, 14 week-
Mice were injected intraperitoneally with 1.75 g glucose per kg body weight following a 6h fast, and plasma glucose was determined at the times indicated. (E-H) Insulin tolerance tests for WT and KO mice fed chow (E), LFLS (F), LFHS (G), or HFHS (H) diets (n=6-11/group, male, 16 week-old). Mice were injected intraperitoneally with regular human insulin at 0.80 units per kg of body weight following a 4h fast, and plasma glucose was determined at the times indicated. No significant differences were identified.

**Fig 6. Lipid homeostasis in Pnpla3-KO mice.** (A) Liver TAG content normalized to tissue weight in WT and KO mice fed low-fat low-sucrose (LFLS), low-fat high-sucrose (LFHS), or high-fat high-sucrose (HFHS) diets (n=6-11/group, male, 19 week-old, ad lib-fed). (B) TAG molecular species normalized to protein content in liver of WT and KO mice fed LFHS diet (n=7-10/group, male, 19 week-old, ad lib-fed). TAG molecular species in liver lipid extracts were determined by electrospray ionization mass spectrometric analysis using C51:3 as an internal standard (tri 17:1). TAG species listed on the x-axis are identified by the total number of carbons in the fatty acid moieties and the total number of double bonds in those fatty acids moieties (i.e. C51:3 represents a glycerol backbone esterified to three fatty acids with 17 carbons and 1 double bond each). (C-D) Serum aspartate aminotransferase (AST) (C) and alanine aminotransferase (ALT) (D) in WT and KO mice fed LFLS, LFHS, or HFHS diets (n=6-11/group, male, 19 week-old, ad lib-fed). (E-F) In vivo cholesterol (E) and fatty acid (F) biosynthesis in liver, BAT, and perigonadal WAT of WT and KO mice (n=5/group, male, 8-9 week-old, chow diet). Fatty acid and cholesterol biosynthesis rates were calculated as micromoles of 3H-radioactivity from [3H]-H2O incorporated into fatty acids or digitonin-precipitable sterols (DPSs), respectively, per gram of tissue per hour as described in methods. * p<0.05 by Student’s t-test (for effect of diet, only comparisons in the WT group are shown).
Fig 7. Gene expression in WAT and liver of Pnpla3-KO mice. Pnpla3 mRNA expression in (A) perigonadal white adipose tissue (WAT) and (B) liver, and (C) Pnpla5 mRNA expression in WAT of ad lib-fed 19 week-old male wild-type (WT) and Pnpla3 knockout (KO) mice fed chow, low-fat low-sucrose (LFLS), low-fat high sucrose (LFHS), or high-fat high sucrose (HFHS) diet since weaning (n=6-11/group). Genes of interest are normalized to cyclophilin as a reference gene and expressed relative to gene expression in the WT chow-fed control for each gene/tissue. Significant main effects (p<0.05) of diet (d) and genotype (g) as well as interactions between diet and genotype (d x g) are indicated in upper right corner. Simple effects for pairwise comparisons are indicated over the appropriate bars (g for comparison of KO vs WT; d for comparison of special diet vs chow control). Expression data for additional genes are found in Supplemental Table 1.
Table 1. Body weight and serum parameters in fed, fasted, and re-fed WT and Pnpla3-KO mice fed LFLS, LFHS, and HFHS diets.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype</th>
<th>LFLS</th>
<th>LFHS</th>
<th>HFHS</th>
<th>p&lt;0.05</th>
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<tbody>
<tr>
<td>Weight (g)</td>
<td>WT</td>
<td>26.81±0.63</td>
<td>24.09±0.67</td>
<td>27.51±0.42</td>
<td>27.14±1.18</td>
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<td></td>
<td>KO</td>
<td>25.89±0.94</td>
<td>23.19±0.89</td>
<td>26.05±0.89</td>
<td>28.86±1.43</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>WT</td>
<td>137±14</td>
<td>96±9</td>
<td>143±9</td>
<td>116±9</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>129±6</td>
<td>109±8</td>
<td>125±10</td>
<td>139±9</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>WT</td>
<td>0.29±0.08</td>
<td>0.12±0.04</td>
<td>7.01±3.09</td>
<td>0.64±0.12</td>
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<tr>
<td></td>
<td>KO</td>
<td>0.24±0.10</td>
<td>0.16±0.06</td>
<td>7.61±3.01</td>
<td>0.94±0.09</td>
</tr>
<tr>
<td>TAG (mg/dL)</td>
<td>WT</td>
<td>75.70±5.16</td>
<td>64.24±2.94</td>
<td>134.14±12.87</td>
<td>134.14±12.87</td>
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<tr>
<td></td>
<td>KO</td>
<td>71.18±11.54</td>
<td>53.42±2.53</td>
<td>145.37±14.45</td>
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<tr>
<td>NEFA (mEq/L)</td>
<td>WT</td>
<td>0.55±0.09</td>
<td>0.99±0.09</td>
<td>0.57±0.04</td>
<td>0.55±0.08</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>0.56±0.04</td>
<td>1.15±0.03</td>
<td>0.53±0.06</td>
<td>0.34±0.03</td>
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<tr>
<td>Cholesterol (mg/dL)</td>
<td>WT</td>
<td>98.97±7.72</td>
<td>120.55±11.40</td>
<td>122.12±11.75</td>
<td>102.98±4.86</td>
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<tr>
<td></td>
<td>KO</td>
<td>92.69±5.18</td>
<td>102.68±10.50</td>
<td>155.11±12.98</td>
<td>98.21±3.18</td>
</tr>
</tbody>
</table>

12 week-old male wild-type (WT) and Pnpla3 knockout (KO) mice fed low-fat low-sucrose (LFLS), low-fat high sucrose (LFHS), or high-fat high sucrose (HFHS) diet since weaning were weighed, and blood was collected in the morning following overnight ad lib feeding (Fed), following an overnight 12h fast (Fasted), or following a 24h fast plus 12h re-feeding (Re-fed) (n=6-11/group). Significant main effects (p<0.05) of diet (d), feeding status (f), and genotype (g) as well as interactions between these factors are indicated in the right-hand column. No main effects were identified for genotype for any of the above parameters. Since main effects of diet and
feeding status are consistent with the appropriate metabolic responses, results of post-hoc analysis for simple effects for these factors are omitted for clarity. However, results of post-hoc analysis for interactions between these factors and genotype are as follows:\textsuperscript{d x g} Significant interactions between diet and genotype were observed for NEFA, with NEFA tending to be higher in KO compared to WT in the HFHS-fed group (p=0.061) but lower in the LFHS-fed group. \textsuperscript{fx g} Significant interactions between feeding status and genotype were observed for cholesterol with KO having higher cholesterol than WT in the re-fed group. p<0.05 using factorial ANOVA followed by simple effects for pairwise comparisons.
Figure 1

A. Chow vs. HFD

- BAT
- PGAT
- SCAT
- MAT

Relative expression (Pnpla3/18S)

B. WT vs. Lep<sup>ob</sup>/Lep<sup>ob</sup>

- BAT
- PGAT
- SCAT
- MAT

Relative expression (Pnpla3/18S)

Liver

* indicates significant difference.
Figure 2

A. Wild-type allele

Targeting construct

BamH1 HindIII

5’ Probe

-12 bp

HindIII

3888 bp homology arm

BamH1 HindIII

+1927 bp homology arm

1

B. Upstream Screen (BamH1)

Downstream Screen (HindIII)

WT KO 1 2 3

Wild-type allele (11725 bp)

Targeted allele (7916 bp)

WT KO 1 2 3

Wild-type allele (10763 bp)

Targeted allele (5627 bp)

C. Relative Expression

(Pnpla3 Exon1-2/18S)

WT Het KO WAT BAT Adrenal

Liver

0.0000 0.0005 0.0010 0.0015 0.0020

0.0000 0.0001 0.0002 0.0003 0.0004 0.0005 0.0006 0.0007 0.0008 0.0009 0.0010

0.0 0.2 0.4 0.6 0.8 1.0 1.2

0.0 0.2 0.4 0.6 0.8 1.0
Figure 5

A. Chow-WT vs. Chow-KO
B. LFLS-WT vs. LFLS-KO
C. LFHS-WT vs. LFHS-KO
D. HFHS-WT vs. HFHS-KO

E. Chow-WT vs. Chow-KO (% initial)
F. LFLS-WT vs. LFLS-KO (% initial)
G. LFHS-WT vs. LFHS-KO (% initial)
H. HFHS-WT vs. HFHS-KO (% initial)
Figure 6

A. Liver TAG content (mg/g tissue)

B. Liver TAG content (pmol/mg protein)

C. Serum AST (U/L)

D. Serum ALT (U/L)

E. [3H]water incorporation into NEFAs (mmol/g/h)

F. [3H]water incorporation into DPS (mmol/g/h)
Figure 7

A. WAT

Relative expression (Pnpla3/Cyclophilin)

B. Liver

Relative expression (Pnpla3/Cyclophilin)

C.

Relative expression (Pnpla5/Cyclophilin)