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Delayed liver regeneration after partial hepatectomy in adipose differentiation related protein-null mice

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Abstract

Background & Aims—Adult hepatocytes undergo cell cycle progression and proliferation in response to partial hepatectomy (PH). Transient lipid accumulation within hepatocytes preceding the peak proliferative phase is a characteristic feature of regenerating livers. However, the molecular mediators and mechanisms responsible for lipid accumulation in regenerating livers are not well understood. Adipose differentiation related protein (ADRP; *Plin2*) regulates hepatic triglyceride storage and *Plin2*-deficient (*Plin2*^{-/-}) mice have significantly reduced triglyceride (TG) content in the liver. We sought to determine the functional significance of PLIN2 in liver regeneration in response to PH and toxic liver injury and examined whether absence of *Plin2* expression modulates hepatocyte proliferation and liver regeneration.

Methods—We subjected wild-type (WT) and *Plin2*^{-/-} mice to 70% PH or acute carbon tetrachloride (CCL₄) treatment and examined the hepatic lipid content, the expression profile of lipid metabolism-related genes, the rate of cellular proliferation and the dynamics of liver regeneration in the treated animals.

Results—In response to PH, *Plin2*^{-/-} mice showed decreased hepatic triglyceride accumulation and delayed cell cycle progression, which was associated with impaired liver regeneration. Fatty acid (FA) synthesis and lipid transfer gene expression profile were comparable between *Plin2*^{-/-} and wild-type mice, while VLDL secretion rate was higher in the *Plin2*^{-/-} mice. Downregulated β -oxidation and reduced cytosolic FA level in *Plin2*^{-/-} mice may have contributed to the

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Supplementary data

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attenuation of the liver regeneration capacity in these animals. In parallel experiments, we also observed attenuated hepatic lipid accumulation and proliferation in response to CCl₄-mediated acute toxic liver injury in *Plin2*^{-/-} mice.

Conclusions—We conclude that PLIN2-mediated lipid accumulation and utilization by the liver is important for efficient liver regeneration in response to PH and toxic liver injury.

Keywords

ADRP; *ADFP*; PLIN2; Partial hepatectomy; Lipid oxidation; Triglyceride; Fatty acids

Introduction

The liver is a central metabolic organ that is involved in the synthesis, storage, metabolism, and repartitioning of the macronutrients carbohydrate, protein, and fat. It has a unique capacity to maintain its size for proper function, a property that is quickly activated upon loss of liver mass, as it happens after partial hepatectomy (PH) [1], a maneuver that has been used to study liver regeneration in rodents [2,3]. In this model, the left and medial lobes of the liver are removed, resulting in loss of 70% of the liver mass. As an acute response to PH, most remaining hepatocytes rapidly undergo proliferation, as they also start accumulating large amounts of lipids, including triglycerides (TG), fatty acids (FA), and cholesteryl esters, which are normally stored inside intracellular lipid droplets (LDs). The proliferative response is most marked within the first 3 days, and the original liver mass is restored within a week in rodents [4]. The hepatic LDs are thought to be critical for the process, as lipids stored in these droplets may be used as the source of new cell membrane formation as well as the energy source required for hepatocyte proliferation. However, the exact role of lipid accumulation and utilization in post-PH liver regeneration is poorly understood [5].

Obese patients with fatty livers tend to have poor outcome after liver resection or transplantation [6,7]. In rodents, pre-existing steatosis in high fat diet or genetic models, as well as in other models of augmented hepatic steatosis, are associated with impairment of liver regeneration after PH [8–11]. On the other hand, models such as liver-specific glucocorticoid receptor-inactivated [12] and caveolin 1 deficient mice [13], also display reduced hepatic TG and defective liver regeneration after PH. So, both insufficient and excessive liver fat may negatively impact the normal process of liver regeneration.

Adipose differentiation related protein (*ADRP*; *Plin2*) is a major LD protein readily detected in fatty liver. Interestingly, *Plin2*^{-/-} mice display markedly reduced hepatic TG content [14,15]. In addition, they also exhibit evidence of abnormal lipid homeostasis in other tissues, e.g., reduced cholesteryl ester storage and increased cholesterol efflux in macrophages [16], modestly lowered milk fat [17], and compromised retinyl ester transport and storage in the retina [18]. The reduction of hepatic TG content in *Plin2*^{-/-} mice can be accounted for, wholly or partly, by an increased output of very low density lipoprotein (VLDL) from the liver. Furthermore, in comparison with wildtype mice, the liver of *Plin2*^{-/-} mice displays altered TG compartmentalization; the TG is markedly depleted in most subcellular compartments except the microsomes where the TG concentration is actually increased. To understand the role of *Plin2* and hepatic TG handling in regenerating livers,

we studied the lipid dynamics and the regenerative response to 70% partial hepatectomy and carbon tetrachloride (CCl₄)-mediated toxic liver injury in *Plin2*^{+/+} and *Plin2*^{-/-} mice.

Materials and methods

Animals

We generated and maintained *Plin2*^{-/-} mice in C57BL/6J background as described previously [14]; C57BL/6J mice purchased from Jackson Lab (Bar Harbor, Maine) served as wild-type (WT) controls. All experimental procedures were carried out under a protocol approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine and were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Mice were kept on 12 h dark-light cycle and maintained on standard chow and water before and after the surgery. We performed PH on 8–12 week old male mice by separately ligating and resecting the right upper, left upper (equivalent to median lobe), and left lower lobes (also known as left lateral lobe), which together make up about 70% of the liver [3]. The mortality rate is less than 5% for both the wild-type and *Plin2*^{-/-} mice.

In order to evaluate liver regeneration in response to a different acute liver injury, we treated 8–12 weeks old *Plin2*^{-/-} and wild-type male mice with a single intraperitoneal injection of carbon tetrachloride (CCl₄; 750 µl/kg BW in corn oil) and harvested the liver tissue after 48 and 72 h. BrdU (50 mg/kg BW) was administered intraperitoneally 2 h prior to tissue harvest, tissues were snap frozen in liquid nitrogen and stored in -80 °C for further analysis. For histological analysis, we fixed tissues in 10% formaldehyde and embedded in paraffin or used frozen tissues for further analysis.

Serum chemistry assays

We measured serum triglycerides (Infinity assay by Thermo Electron, Melbourne, Australia), non-esterified free-fatty acids (Wako Chemicals USA, Hercules, CA), Glycerol (Sigma, St. Louis, MO), β-hydroxybutyrate (Cayman Chemical, Ann Arbor, MI) by colorimetric assay. Serum IL-1β, IL-6, and TNF-α (eBioscience, San Diego, CA) were measured using an enzyme immunoassay kit according to the manufacturer's instructions.

Liver histology and immunofluorescence

We assessed liver morphology based on 5 µm hematoxylin and eosin-stained paraffin sections. The frequency of nuclear PCNA staining (anti-PCNA antibody; Cell Signaling Technology, Danvers, MA) was determined by examination of at least three random 200× fields and at least 300 cells and nuclei in each tissue section. We stained liver sections for BrdU positive nuclei with the BrdU labeling and detection kit (Roche, Indianapolis, IN) according to manufacturer's instructions. Sections of liver frozen in OCT compound (Sakura Finetek, Torrance, CA) were stained with Oil Red O (Sigma, St. Louis, MO) for analysis of hepatic fat accumulation.

Liver lipid analysis

We homogenized 200 mg liver tissues in 2 ml of PBS, extracted lipids from these homogenates according to Bligh and Dyer [19] and fractioned different lipid species by one dimensional thin layer chromatography (silica Gel-60, Analtech, Newark, DE), using petroleum ether/ether/acetic-acid (85:25:1). Lipids were visualized by incubating the TLC plate in a saturated iodine chamber and quantified by comparing the optical density of samples with lipid standards using Image J software [14,15].

Determination of rate of very low density lipoprotein (VLDL) secretion in vivo

At 24 h after partial hepatectomy, we quantified the rate of VLDL secretion *in vivo* by injecting intraperitoneally Pluronic F-127 (2 mg/g BW in PBS, BASF Corporation, Florham Park, NJ), a lipoprotein lipase inhibitor, and monitored the serum triglyceride before, and 1, 2, 3, and 4 h after injection [14].

Quantitative reverse transcription PCR

We extracted total RNA from the liver by using the Absolutely RNA™ Miniprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. RNA was treated with DNase I, reverse transcribed using the Superscript III First Strand Synthesis System and used for quantitative PCR (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using the iQ™ SYBR Green Supermix (BIO-RAD, Hercules, CA) and an Mx3000P quantitative PCR machine (Stratagene, La Jolla, CA). *Gapdh*, *Hmbs*, and *Eef1g* were used as the housekeeping control genes.

Western blotting

We analyzed total liver protein extracts by 10% SDS-PAGE, and transferred the protein to nitrocellulose membrane (Bio-Rad) at 250 mA for 90 min. Membranes were blocked with 5% non-fat dry milk dissolved in TBST (Tris-buffered saline Tween) for 1 h at RT prior to incubation with antibodies specific for PCNA, cyclins D1 and A (Santa Cruz, CA), PLINs and MTTP (the latter two antibodies were developed in our laboratory [14,15]). To ensure equal loading, we stripped the membranes and reprobated them with anti-β-actin or anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analysis

We expressed all values as means ± S.D. with the exception of quantitative PCR analyses, which were expressed as means ± S.E. Difference between means was analyzed by using Student's *t*-test. A difference was considered significant when $p < 0.05$.

Results

PH induces PLIN2 expression

Partial hepatectomy (PH) is a well-established procedure for studying liver regeneration, and a large body of studies have established the chronological changes of hepatic lipids after PH [4,20,21]. However, in order to identify critical time points for comparative studies with the *Plin2*^{-/-} mice, we first determined the chronology of biochemical and molecular parameters

at various time points after PH in wild-type mice. Our initial characterization of plasma and hepatic lipid profiles and gene expression patterns after PH in the wild-type mice recapitulated previous studies (Supplementary Figs. 1 and 2).

Plin2 (*Adrp*) and *Plin3* (*Tip47*) are major LD proteins that are highly expressed in the liver. We examined the expression pattern of these LD proteins in response to PH and found that *Tip47* mRNA expression is not influenced by PH, whereas *Plin2* mRNA level doubled at 6 h and went up ~7-fold 12 h after PH, returning to baseline by 24 h post-PH (Fig. 1A). The PLIN2 protein expression increased rapidly starting at 3 h and peaked 24 h after PH (Fig. 1B). It remained high at 36 h, but returned to baseline 48 h after PH (Fig. 1B).

***Plin2*^{-/-} mice have attenuated lipid droplet formation and delayed liver regeneration in response to PH**

We have previously shown that *Plin2*^{-/-} mice have marked TG deficiency in the liver under basal conditions [18]. Because *Plin2* was highly upregulated both at transcriptional and posttranscriptional levels after PH (Fig. 1A and B), we decided to use *Plin2*^{-/-} mice as a model to dissect the role of *Plin2* in hepatic TG dynamics during the liver regeneration in response to PH.

Light microscopic examination of remnant liver sections revealed that LD sizes in *Plin2*^{-/-} mice appeared more heterogeneous 24 h after PH (Fig. 1C) with reduced LD formation 36 h and 48 h after PH (Fig. 1C), as compared to wild-type mice. Oil-red-O (ORO) staining of liver sections revealed similar morphological features (Supplementary Fig. 3), though the morphology was not as well defined compared to H & E staining in distinguishing smaller droplets due to solvent (isopropanol) effects [22] (Fig. 1C). We previously noted a similar change in hepatic LD size in *Plin2*^{-/-} hepatocytes in conditions other than PH that are normally associated with fatty liver [14,15]. As compared to wild-type, *Plin2*^{-/-} remnant livers had reduced TG content as analyzed by thin layer chromatography of livers harvested at 24, 36, and 48 h after PH (Fig. 1D and E). Sham operation did not alter the hepatic TG in the wild-type and *Plin2*^{-/-} mice 36 h post-operation (Supplementary Fig. 4A and B).

We next studied the PH-induced liver regeneration in wild-type and *Plin2*^{-/-} mice. The liver regeneration index (the weight of remnant liver divided by initial body weight) rose sharply between 36 to 48 h, and plateaued after at 96 h after PH. In contrast, the liver regeneration in *Plin2*^{-/-} mice was attenuated as compared to wild-type mice (Fig. 1F). In a separate experiment, we calculated the liver regeneration index based on the remnant liver weight and body weight at 48 h post-PH, and confirmed that liver regeneration is significantly impaired in *Plin2*^{-/-} mice (Supplementary Fig. 5A). Notably, body weights of mice recovering from PH were comparable between the wild-type and *Plin2*^{-/-} mice (Supplementary Fig. 5B).

Hepatocyte cell-cycle entry and proliferation in response to PH is delayed in *Plin2*^{-/-} mice

Adult hepatocytes are highly differentiated quiescent cells which rarely undergo cell division. However, in response to PH almost all hepatocytes underwent proliferation with concerted cell cycle entry and progression. In order to evaluate hepatocyte proliferation in

response to PH, we analyzed liver sections by BrdU incorporation. In the wild-type mice, BrdU-positive nuclei were detectable at 24 h and peaked between 36 to 48 h and near-basal levels were reached by 72 h post-PH. Interestingly, BrdU incorporation was significantly attenuated at 36 h and peaked at 48 h post-PH, suggestive of delayed cell-cycle progression, in *Plin2*^{-/-} mice (Fig. 1G and H). Correspondingly, induction of cyclins D1 and A, key mediators of cell cycle progression at G1 and G1/S phase of cell cycle, as well as PCNA, a marker of proliferating hepatocytes, were attenuated at 36 h post PH in the remnant livers of *Plin2*^{-/-}, as compared to wild-type (Fig. 1I and J). The expression levels of these markers in the remnant livers of *Plin2*^{-/-} mice, however, were comparable to wild-type mice at 48 h post-PH (Fig. 1I and J).

Comparable de novo lipogenesis and lipid uptake in *Plin2*^{-/-} and wild-type mice after PH

To explore the mechanism for the altered lipid homeostasis in *Plin2*^{-/-} mice during liver regeneration after PH, we evaluated mRNA expression of rate-limiting and regulatory genes essential for efficient lipogenesis by quantitative real-time reverse transcription PCR. Basal mRNA expression of *FAS* and *SREBP1c* was comparable between *Plin2*^{-/-} and wild-type mice (Fig. 2A), corroborating with our previous findings [18]. Induction of *FAS* and *SREBP1c* expression in response to PH was also comparable between *Plin2*^{-/-} and wild-type mice at 6 h post PH, time of peak expression post-PH (Supplementary Fig. 2A). We also did not find any difference in *ACCI* and *SCD1* mRNA induction between *Plin2*^{-/-} and wild-type mice (data not shown). Furthermore, the *CD36* mRNA level in *Plin2*^{-/-} mice was not different from that of wild-type mice (Fig. 2B), and no difference was detected in the level of *LDL-R* mRNA (data not shown). Finally, the time course of changes in serum TG, FA, and glycerol for *Plin2*^{-/-} mice after PH was not detectably different from that in wild-type mice (data not shown).

It is known that inflammatory cytokines influence hepatocyte lipogenesis as well as liver regeneration after PH [23–26]. The basal cytokine levels were barely detectable (Fig. 2C). We found that the serum levels of IL-1 β , IL-6, and TNF- α increased after PH and reached their peak at 6 h post PH (data not shown), but the levels of these cytokines were similar in *Plin2*^{-/-} and wild-type mice (Fig. 2D). Furthermore, we also found no difference in the serum level of hepatic growth factor between wild-type and *Plin2*^{-/-} mice under basal conditions and 12 h after PH (Supplementary Fig. 6). These findings suggest that the lower liver regeneration index detected in *Plin2*^{-/-} mice was not accounted for by the serum cytokine levels that stimulate liver regeneration.

Unbridled VLDL secretion in *Plin2*^{-/-} mice after PH

We next examined the rate of VLDL secretion 24 h after PH in wild-type and *Plin2*^{-/-} mice. We measured plasma TG levels at different times after administration of the lipase inhibitor, Pluronic F-127, and found a significantly increased rate of VLDL secretion in *Plin2*^{-/-} mice compared with wild-type mice (Fig. 2E), as we reported previously in the basal state in *Plin2*^{-/-} deficient mice [18,19]. Taken together, these findings suggest that the decreased lipid accumulation in the liver of *Plin2*^{-/-} mice appears not to be related to impaired *de novo* FA synthesis or lipid uptake from the circulation, but the unbridled VLDL secretion in *Plin2*^{-/-} mice seems to offer a partial explanation.

As we have shown before, microsomal triglyceride transfer protein (MTP), the rate limiting enzyme in VLDL secretion, was modestly increased in *Plin2*^{-/-} mice compared to that of the wild-type before PH (Fig. 2F). However, its level was similar at 24 h after PH between the two groups of mice indicating mechanisms other than MTP protein may also be involved.

PH caused an increase of PLIN2 protein in the wild-type mice. As PLIN2 belongs to a family of five related genes (*Plin1–5*) [27], we examined whether loss of PLIN2 caused a compensatory change of other members of the gene family. PLIN1 and 4 were not detectable in the liver of the control or PH mice (data not shown). The PLIN3 protein level was not different between wild-type and *Plin2*^{-/-} mice; however, the protein abundance of PLIN5 was increased in both groups at 24 h after PH and the level was higher in the *Plin2*^{-/-} mice compared to that of the wild-type both before and 24 h after PH (Fig. 2F).

Downregulated β -oxidation in *Plin2*^{-/-} mice after PH

Finally, we examined the expression of genes involved in lipolysis and β -oxidation in the liver of *Plin2*^{-/-} mice, at 36 h after PH, when lipolysis and β -oxidation were at their peak, as observed from the PH of wild-type animals (Supplementary Fig. 2B and C). Interestingly, all of the transcripts that we examined, including those for *PPAR α* and *PPAR γ* (critical regulators of hepatic lipid oxidation), *HSL* (lipolytic enzyme), *CPT1a* (a rate limiting enzyme involved in the transport of long chain FA into mitochondrial matrix for β -oxidation), as well as *LCAD* and *HADH α* (enzymes for β -oxidation), were downregulated in the liver of *Plin2*^{-/-} mice compared to those of wild-type animals (Fig. 3A).

We next analyzed the subcellular distribution of lipids in different cellular compartments by fractionation. We separated liver homogenates isolated from *Plin2*^{-/-} and wild-type mice 36 h after PH into cytosolic and microsomal fractions by differential centrifugation and extracted the lipids from these fractions for TLC analysis. As shown in Fig. 3B and C, TG, and FA content was significantly reduced in the cytosolic fraction of the *Plin2*^{-/-} mice compared to that of the wild-type mice. On the other hand, TG content in the microsomal fraction tended to be higher in the *Plin2*^{-/-} mice as compared with the wild-type, though the difference did not reach statistical significance, unlike what we found previously, in which the microsomal TG was elevated in the *Plin2*^{-/-} mice compared to the wild-type under basal conditions, despite an overall reduction of hepatic TG in *Plin2*^{-/-} mice [14].

To determine whether β -oxidation was altered in *Plin2*^{-/-} mice, we measured serum β -HBA, a ketone body produced after β -oxidation in the liver. The serum β -HBA level of *Plin2*^{-/-} mice at 24 h after PH was significantly lower than that of the wild-type mice (Fig. 3D); a similar trend was found at 12 h after PH, which corroborates with the down-regulation of β -oxidation genes associated with *Plin2* deficiency suggesting a lowering of β -oxidation in the liver after PH in *Plin2*^{-/-} mice.

Liver regeneration after carbon tetrachloride treatment is impaired in *Plin2*^{-/-} mice

To determine if the delayed liver regeneration is unique to PH in the *Plin2*^{-/-} mice or if PLIN2 also plays a role in liver regeneration in response to toxic liver injury, we treated the wild-type and *Plin2*^{-/-} mice with carbon tetrachloride to induce acute liver injury.

As anticipated, the serum markers of liver injury and inflammation, ALT, IL1 β , IL-6, and TNF- α were all elevated at 48 h after CCl₄ treatment (Fig. 4A) in the wild-type mice. The levels of these inflammatory markers subsided at 72 h after treatment (Fig. 4A). *Plin2*^{-/-} mice that received CCl₄ treatment had a similar temporal profile of inflammatory response, but with an attenuated induction of serum markers of inflammation, as compared to wild-type mice. This is quite different from the inflammatory responses after PH (Fig. 2D) in which these two groups of mice had comparable serum cytokine expression.

The hepatic TG levels increased dramatically 48 h after CCl₄ treatment in the wild-type mice with attenuated induction in *Plin2*^{-/-} mice (60% of the wild-type) (Fig. 4B).

Next, we evaluated hepatocyte proliferation by BrdU incorporation at 48 h after CCl₄ treatment. Similar to our findings in response to PH, hepatocyte proliferation in response to CCl₄-mediated injury was impaired in *Plin2*^{-/-} mice. About 30% of the hepatocytes underwent cell proliferation (which were BrdU-positive; Fig. 4C) in the wild-type mice at 48 h post-PH, while only 12% of cells in *Plin2*^{-/-} mice were BrdU-positive (Fig. 4D). Immunostaining for PCNA, a well-established marker for proliferating cells, validated our findings that hepatocyte proliferation in response to CCl₄-mediated injury is impaired in *Plin2*^{-/-} mice (Fig. 4C and D).

In order to understand the molecular changes associated with the differential response of wild-type and *Plin2*^{-/-} mice to CCl₄ hepatotoxicity, we harvested liver tissues at 72 h post-CCl₄ treatment and analyzed these by quantitative RT-PCR. Mirroring our observations in response to PH, mRNA expression of several genes associated with lipolysis (*Hsl*) and β -oxidation (*Ppar- α* , *Cpt1 α* , and *Acadl*) were downregulated in the liver of *Plin2*^{-/-} mice 72 h after CCl₄ treatment (Fig. 4E). The expression of lipogenic genes *Ppar- γ* and *Srebp1c*, and fatty acid transporter, *Cd36* were comparable between wild-type and *Plin2*^{-/-} mice. In addition, the expression of *Stat3* and *Socs3* were downregulated in the *Plin2*^{-/-} mice compared to those of the wild-type at 72 h after CCl₄ treatment (Fig. 4E).

Discussion

It is well known that transient hepatosteatosis occurs at 12 to 48 h after PH in rodents [4,5,26]. Early studies suggested a role for uptake of free fatty acids and subsequent storage as triglycerides in the lipid accumulation in regenerating livers [20,28,29]. Delahunty *et al.* contend that lipid accumulation, early on during liver regeneration, is a consequence of excessive esterification of systemically derived free fatty acids into triglycerides at rates in excess of the liver's ability to secrete as β -lipoproteins and at later times the rate of lipid secretion matches the free fatty acid flux, thus preventing further accumulation [30].

In this study, we first investigated the time course of changes in lipid metabolism in response to PH in wild-type mice. During early liver regeneration, the expression of genes for *de novo* FA synthesis was upregulated at 3 to 6 h, and mRNA for *CD36* was increased from 12 to 24 h after PH. Furthermore, serum FA, and glycerol were increased after PH, and drastically decreased at 24 h after PH. These findings suggest that *de novo* lipogenesis in the liver provides the initial supply of fatty acids for TG synthesis at the early phase (0–6 h) and subsequently uptake of lipolytic products from peripheral tissues serve as a source of hepatic lipids.

The transcript for the major hepatic LD gene *Plin2* is also upregulated at 12 h after PH. Our studies corroborate previous reports that lipid droplet formation and triglyceride accumulation peak at 24 h post-PH (Fig. 1C). We noted that the PLIN2 protein level started to increase at 3 h after PH when the transcription of *Plin2* was unchanged in the wild-type mice (Fig. 1A and B). It appears that both transcriptional as well as post-transcriptional mechanisms influence PLIN2 protein levels in regenerating livers, as PLIN2 protein is known to be regulated by the proteasome degradation pathway, which may be modulated by the availability of intracellular lipids [31,32].

Absence of *Plin2* was previously shown to lead to marked reduction in hepatic TG storage. We therefore took advantage of the *Plin2*^{-/-} mouse model and compared the effect of presence or absence of *Plin2* expression on liver regeneration after PH. *Plin2*^{-/-} mouse liver has a significantly lower TG content than wild-type after PH [14,15]. The *Plin2*^{-/-} hepatocytes recovering from PH maintain the ability to form LDs, however, the size of post-PH hepatic LDs is more heterogeneous in *Plin2*^{-/-} mice, and the number of LDs is substantially lower than that in wild-type mice, an observation similar to what we noted previously in *Plin2*^{-/-} mice [14,15].

Fatty acid synthesis and lipid transfer gene expression profiles were similar between the *Plin2*^{-/-} and wild-type mice prior to and at various times after PH. The serum cytokine levels were also comparable between *Plin2*^{-/-} and wild-type. On the other hand, rate of VLDL secretion in *Plin2*^{-/-} mice was significantly higher than that of wild-type mice. We have reported similar results for these mice at normal state [14], suggesting that *Plin2*^{-/-} mice had unbridled VLDL secretion both before and after PH compared to wild-type mice. Although at the basal condition before PH, the rate limiting enzyme in hepatic VLDL production, MTP, is upregulated in *Plin2*^{-/-} mice, its level at 24 h after PH is not different from that of the wild-type (Fig. 2F). This indicates that other factors may be involved in the elevated VLDL secretion under PH condition in the *Plin2*^{-/-} mice. Therefore, even at a time when the cell needs TG as a source of energy for tissue repair and cellular proliferation, *Plin2*^{-/-} mice show a persistent defect in their ability to retain sufficient amounts of fuel as a source of energy for the tissue growth and repair process.

β -oxidation was not altered in *Plin2*^{-/-} mice under basal conditions [14]. However, the hepatic mRNA levels of lipolytic enzyme (*HSL*), β -oxidation (*CPT1a*, *LCAD*, *HADH α*), and key transcriptional regulators of lipid metabolism (*PPAR α* , and *PPAR γ*) were uniformly downregulated in *Plin2*^{-/-} mice compared to wild-type mice 36 h after PH (Fig. 3A). The difference in gene expression was accompanied by a distinct delay in liver regeneration and

impaired hepatocyte proliferation response in *Plin2*^{-/-} mice after PH (Fig. 1F). It is likely that the elevated lipolysis and β -oxidation induced by PH, 36–48 h post PH, are necessary for optimal liver regeneration in wild-type mice. Reduced hepatic lipids after PH in *Plin2*^{-/-} mice may hamstring the hepatocytes from regulating the genes involved in lipolysis and β -oxidation that are required for full recovery.

Intracellular partitioning of lipids was different in *Plin2*^{-/-} mouse livers as compared with those of wild-type mice. Not only was TG affected, but FA content in the cytosolic fraction of *Plin2*^{-/-} mice was also reduced compared with wild-type mouse livers after PH. It is possible that reduced cytosolic FA in the hepatocytes of *Plin2*^{-/-} mice could adversely affect the optimal activation of PPAR α , which controls the expression of genes involved in β -oxidation.

In response to partial hepatectomy, caveolins, key components of caveolae, exhibit a dramatic redistribution from the cell surface to the newly formed lipid droplets within hepatocytes. *Cav-1*^{-/-} mice fail to accumulate hepatic TG and show impaired liver regeneration and decreased survival after PH [13]. The distinct defects in hepatic triglyceride accumulation and hepatocyte proliferation in response to PH in *Cav-1*^{-/-} mice primarily stems from its genetic defect in lipid droplet formation [13,33]. It is interesting to note the similarities between *Cav-1*^{-/-} and *Plin2*^{-/-} mice in their response to PH, i.e., inherent defects in efficient lipid droplet formation early on in response to PH have a profound impact on the subsequent hepatocyte proliferative response. Our finding of delayed hepatocyte proliferation in response to PH in *Plin2*^{-/-} mice further underscores the importance of optimal lipid droplet formation and triglyceride accumulation in regenerating livers.

Impaired β -oxidation was also found to hamstring liver regeneration after PH. Adiponectin-deficient mice that show downregulation of genes necessary for efficient β -oxidation also have impaired liver regeneration [33]. PPAR α -null mice in the sv129 genetic background have reduced β -oxidation, increased hepatic TG content, and suppressed liver regeneration capacity [10]. Interestingly, however, Newberry *et al.* [34] found that PPAR α -null mice in the C57BL/6 genetic background have increased hepatic TG content but normal post-PH liver regeneration. The same authors used other mouse models with altered hepatic TG content, and showed that hepatic TG content does not appear to affect liver regeneration and hepatocyte proliferation after PH in the models tested. In view of the different findings in different laboratories, it is reasonable to conclude that absolute levels of hepatic TG content alone may not control the capacity of liver regeneration following PH. It is likely a combination of alterations of hepatic TG content and the underlying genetic and metabolic changes that may impact the capacity of the liver to regenerate optimally after PH. Liver regeneration is a multi-factorial process under the influence of numerous humoral and hemodynamic factors which activate multiple redundant cell signaling cascades and pathways essential for survival and proliferation in order to ensure efficient liver regeneration. Therefore, it is not surprising that despite delays in hepatocyte proliferation in *Plin2*^{-/-}, as evidenced by attenuated induction of markers of proliferation at 36 h post-PH, restoration of liver mass is comparable to wild-type mice at 168 h post-PH.

Finally, we examined the liver regeneration in these mice in a model based on CCl₄ liver toxicity. Again, *Plin2*^{-/-} mice showed a delayed liver regenerative response after CCl₄ treatment compared to wild-type mice (Fig. 4C). However, the mechanism may not be exactly the same as that of PH. Although the gene expression profiles suggested a defect in β-oxidation in *Plin2*^{-/-} mice (Fig. 4E), we noted that the inflammatory cytokine levels such as Tnf-α, IL-1β, and IL-6 were also attenuated in *Plin2*^{-/-} mice (Fig. 4A), whereas these factors were not different between *Plin2*^{-/-} and the wild-type mice in the PH model. We speculate that the CCl₄ may have enticed a more global inflammatory response in the animals resulting in a difference in plasma cytokine profile compared to PH. As these cytokines are known to stimulate liver regeneration [35], it is likely that the delayed liver regeneration in *Plin2*^{-/-} mice responding to CCl₄ toxicity was also due to the attenuated levels of these cytokines – an observation that should be investigated in the future.

We also noticed that the *STAT3* mRNA level was decreased 72 h after CCl₄ treatment in the *Plin2*^{-/-} compared to the wild-type mice (Fig. 4E). Although STAT3 exerts its function through protein phosphorylation, a previous study has shown that liver specific *Stat3* knockout mice had decreased DNA synthesis in response to CCl₄ treatment compared to the wild-type mice [36].

In summary, our results suggest that *Plin2* upregulation at 12 h after PH may be an important component of a concerted homeostatic response to liver injury and loss of liver mass leading to LD accumulation within hepatocytes. The early accumulation of lipids within hepatocytes could serve as a source of β-oxidation during the peak hepatocyte proliferative phase in regenerating livers. *Plin2*^{-/-} mice display decreased hepatic lipid accumulation and impaired liver regeneration. Lipid insufficiency in the liver of *Plin2*^{-/-} mice is not the result of decreased lipogenesis or lipid uptake, but the consequence of an unbridled VLDL secretion. *Plin2*^{-/-} mice also exhibit decreased cytosolic FA levels and attenuated β-oxidation in regenerating livers, as evidenced by decreased expression of β-oxidation-related genes and lower serum β-HBA levels post-PH. Collectively, a combination of these defects in lipid accumulation and utilization during critical periods of liver regeneration may compromise the liver regeneration capacity in *Plin2*^{-/-} mice after PH. Moreover, our findings suggest that optimal subcellular distribution of lipids maintained by *Plin2* is necessary for optimal liver regeneration after PH. These findings highlight the significance of metabolic control of liver regeneration and provide additional new insights on the role of lipid droplet-associated proteins in efficient liver regeneration in response to loss of liver mass and toxic liver injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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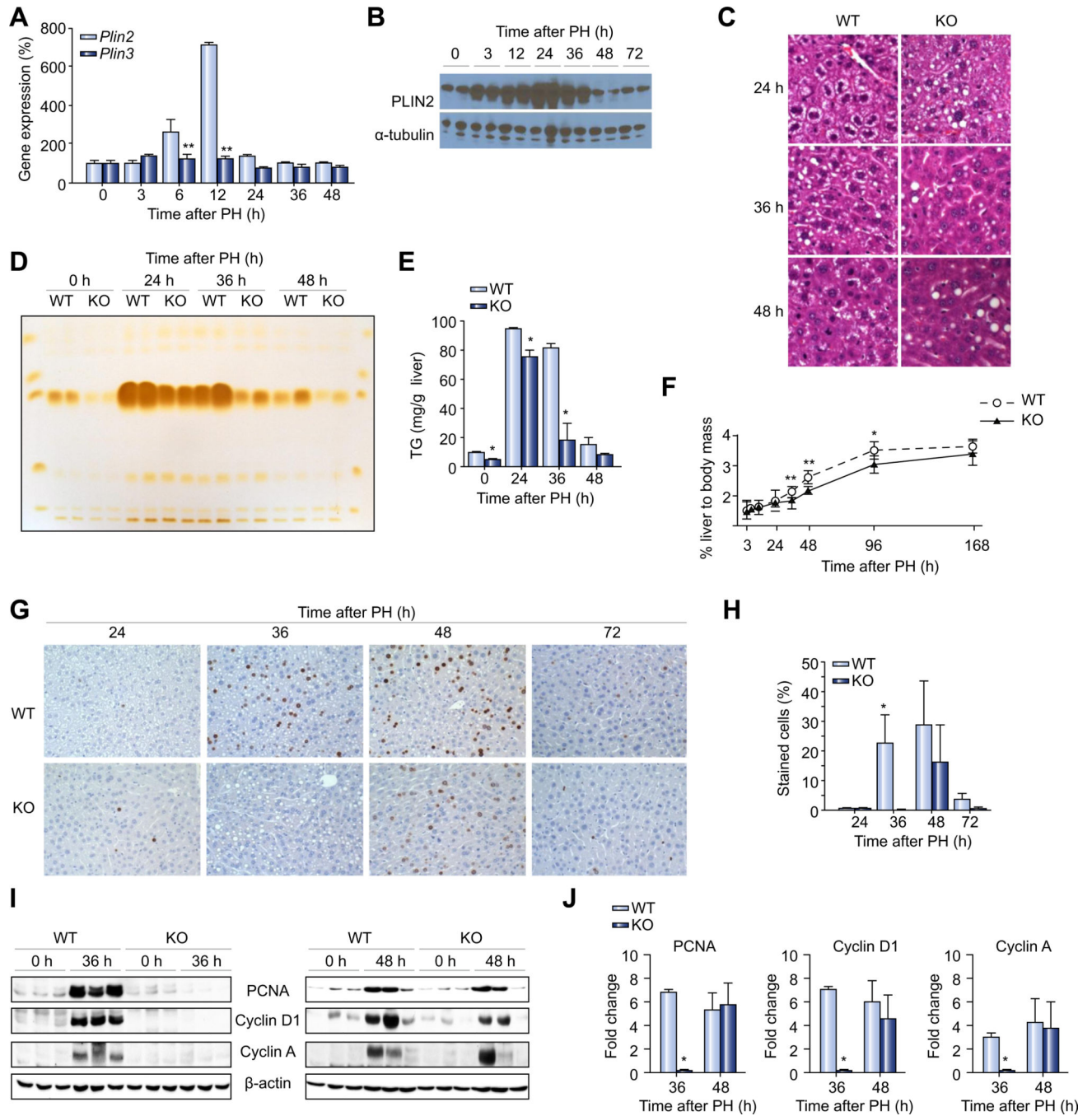


Fig. 1. Hepatic lipids, lipid droplet protein and regeneration after PH

(A) Quantitative RT-PCR analysis of mRNA expression of *Plin2* and *Plin3* lipid droplet protein genes (n = 4 of each group) in wild-type mice before (0 h) or various time points after PH. (B) PLIN2 protein expression at 0, 3, 12, 24, 36, 48, and 72 h after PH. Two representative samples from each time point are shown and α -tubulin was used as an internal control for equal loading. (C) Microscopic examination of H&E stained liver sections from wild-type mice and *Plin2*^{-/-} mice 24, 36, 48 h after PH. (D) Thin-layer chromatography analysis of lipids isolated from livers of wild-type and *Plin2*^{-/-} mice at basal (0 h) and 24, 36, 48 h after PH. (E) Quantitative densitometry of hepatic TG contents in wild-type, and *Plin2*^{-/-} mice (based on TLC results; n = 4). (F) Liver regeneration index in wild-type and *Plin2*^{-/-} mice (n = 5–16 of each group) determined by dividing the weight of liver

at indicated time points with the body weight before PH. (G) Immunohistochemical analysis of BrdU incorporation at 24, 36, 48, and 72 h post-PH of wild-type and *Plin2*^{-/-} mice. (H) Percent of BrdU positive cells in wild-type and *Plin2*^{-/-} mice at indicated time points after PH (n = 3). (I) Western blotting of total liver homogenates of resected lobes (0 h) and remnant livers (36 h and 48 h post-PH) of wild-type and *Plin2*^{-/-} mice. (J) Densitometric analysis of band intensities of Western blotting for PCNA, Cyclin D1 and Cyclin A. In all panels, data are expressed as means ± S.D. (n = 3). **p* < 0.05, ***p* < 0.01 vs. wild-type group. WT, wild-type; KO, *Plin2*^{-/-}. (This figure appears in colour on the web.)

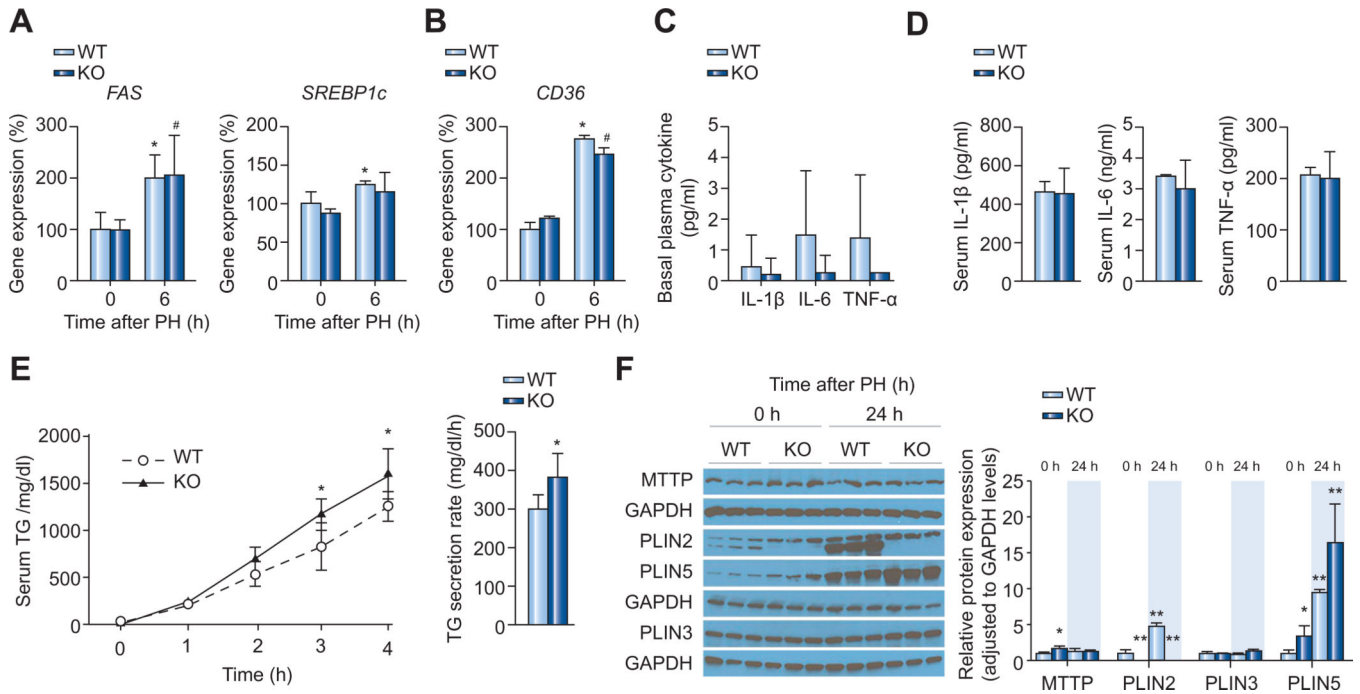


Fig. 2. Mechanisms of hepatic TG reduction in the liver of *Plin2*^{-/-} compared to that of the wild-type (WT) mice after PH
 Quantitative RT-PCR analysis of mRNA expression of (A) lipogenic genes, *FAS* and *SREBP1c*, and (B) the fatty acid transporter, *CD36*, in wild-type and mice at indicated time after PH. (n = 4 of each group). (C) Basal plasma cytokine levels in wild-type and *Plin2*^{-/-} mice. (D) Serum cytokine levels measured at 6 h after PH (n = 4–5 of each group). Plasma IL-1β, IL-6, and TNF-α were measured by using ELISA kits (n = 4). (E) VLDL secretion determined at 24 h after PH (n = 5 of each group). Plasma TG levels were determined at indicated time after i.p. injection of Pluronic F-127, a lipoprotein lipase inhibitor at 2 mg/g BW (left panel). We calculated the rate of VLDL secretion (right panel) using data obtained between 1 and 4 h when the secretion was occurring at near-linear rate. (F) Western blot analyses of MTP and LD proteins (PLIN2, 3, and 5 are shown; PLIN4 was undetectable) before (0 h) and 24 h after PH in the wild-type and *Plin2*^{-/-} mice (left panel). Three independent polyacrylamide gels were analyzed and GAPDH was used as an internal control in each case. Quantitative analyses of proteins (right panel) were done using Image J software. W, wild-type; KO, *Plin2*^{-/-} mice. In all panels, data are expressed as means ± S.D. **p* < 0.05, ***p* < 0.01 vs. wild-type group. (This figure appears in colour on the web.)

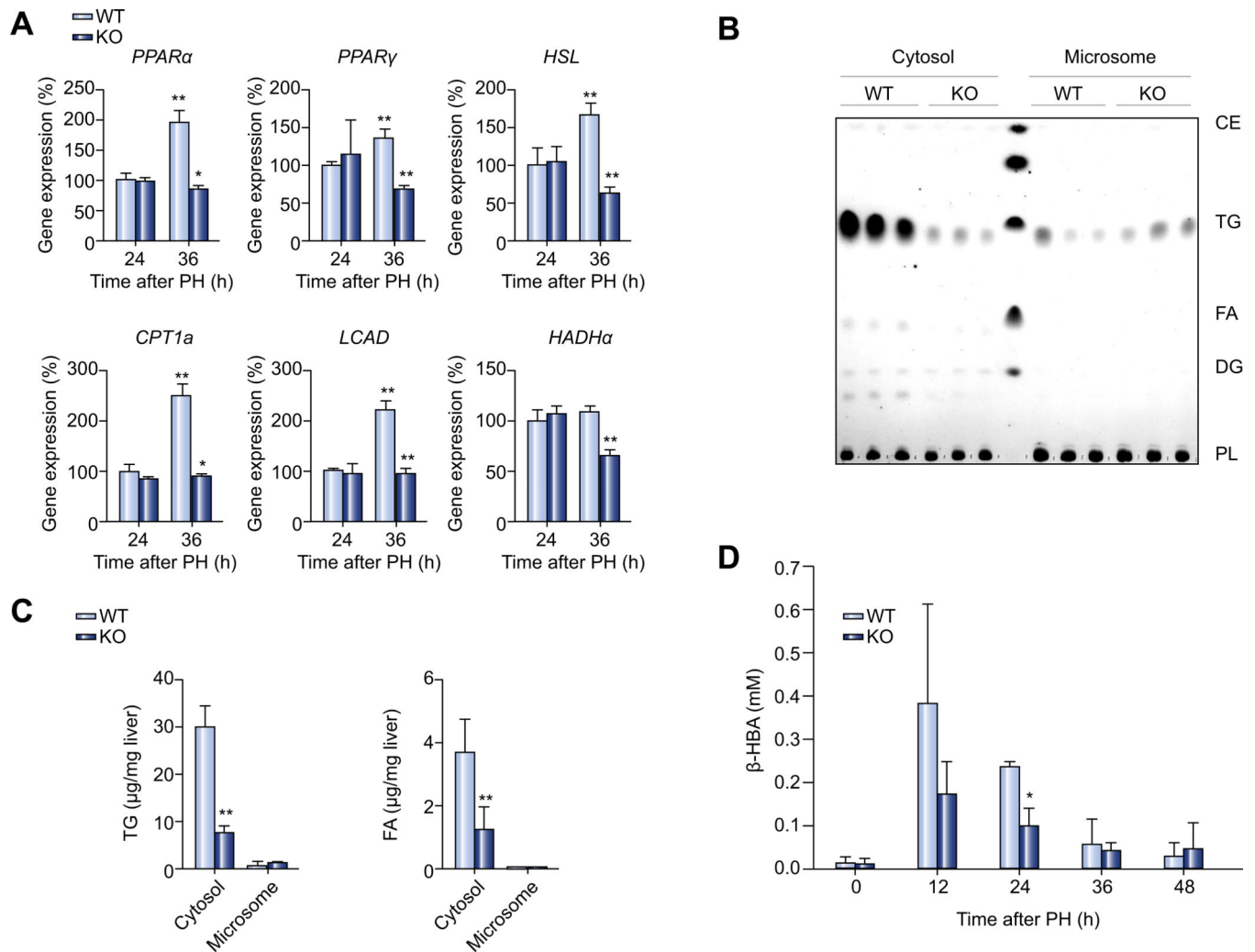


Fig. 3. β -oxidation and lipid partitioning in the liver of wild-type and *Plin2*^{-/-} mice after PH

(A) Quantitative RT-PCR analysis of mRNA expression of genes related to lipid metabolism at 24 h (24) and 36 h (36) after PH (n = 4). (B) Thin-layer chromatography analysis of lipids in cytosolic and microsomal compartments isolated from wild-type and *Plin2*^{-/-} livers 36 h after PH. (C) Quantification of cytosolic and microsomal triglyceride and fatty acids content in livers of wild-type, and *Plin2*^{-/-} mice 36 h after PH (n = 5). (D) Serum β -HBA from wild-type and *Plin2*^{-/-} mice (n = 5) at basal and different time points after PH. In all panels, data are expressed as means \pm S.D. * p < 0.05 and ** p < 0.01 vs. wild-type group. WT, wild-type; KO, *Plin2*^{-/-}; TG, triglyceride; FA, fatty acids.

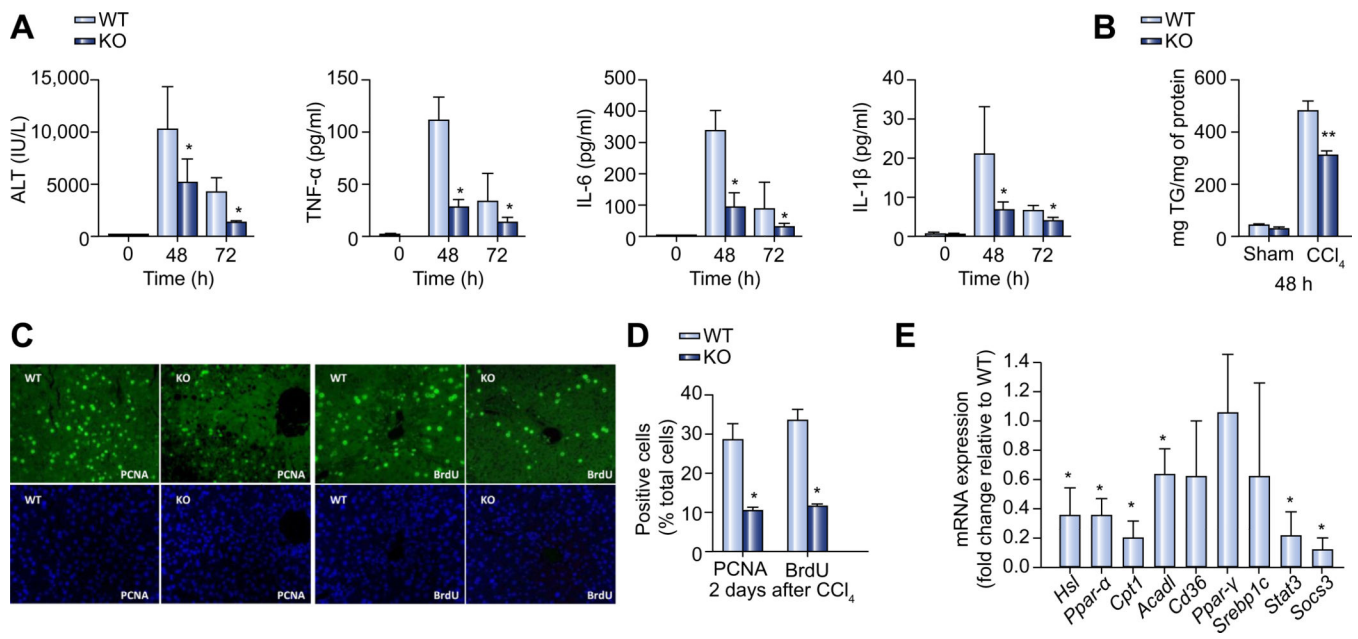


Fig. 4. Impaired liver regeneration after CCl₄ treatment in *Plin2*^{-/-} mice

(A) Plasma liver enzyme (ALT) and inflammatory cytokine (TNF-α, IL-6, and IL-1β) levels before (0 h) and 48 and 72 h after CCl₄ treatment in wild-type and *Plin2*^{-/-} mice (n = 6). (B) Hepatic TG content in wild-type and *Plin2*^{-/-} mice 48 h after CCl₄ or sham treatments. (C) PCNA and BrdU staining (upper panel) of liver sections from 48 h after CCl₄ treatment in wild-type and *Plin2*^{-/-} mice. DAPI (lower panel) staining was used to identify nuclei and for cell counting. (D) Percent of PCNA and BrdU positive cells 2 days (48 h) after CCl₄ treatment in the wild-type and *Plin2*^{-/-} mice. (E) Quantitative analysis of mRNA expression 72 h after CCl₄ treatment. Relative mRNA expression in *Plin2*^{-/-} mice was shown by using house-keeping genes as controls and expressed as ratios to the levels of the wild-type. **p* < 0.05 and ***p* < 0.01 between wild-type and *Plin2*^{-/-} mice (n = 6). WT, wild-type; KO, *Plin2*^{-/-}. (This figure appears in colour on the web.)