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ORIGINAL ARTICLE

Liver PPAR α is crucial for whole-body fatty acid homeostasis and is protective against NAFLD

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ABSTRACT

Objective Peroxisome proliferator-activated receptor α (PPAR α) is a nuclear receptor expressed in tissues with high oxidative activity that plays a central role in metabolism. In this work, we investigated the effect of hepatocyte PPAR α on non-alcoholic fatty liver disease (NAFLD).

Design We constructed a novel hepatocyte-specific PPAR α knockout (*Ppara*^{hep-/-}) mouse model. Using this novel model, we performed transcriptomic analysis following fenofibrate treatment. Next, we investigated which physiological challenges impact on PPAR α . Moreover, we measured the contribution of hepatocytic PPAR α activity to whole-body metabolism and fibroblast growth factor 21 production during fasting. Finally, we determined the influence of hepatocyte-specific PPAR α deficiency in different models of steatosis and during ageing.

Results Hepatocyte PPAR α deletion impaired fatty acid catabolism, resulting in hepatic lipid accumulation during fasting and in two preclinical models of steatosis. Fasting mice showed acute PPAR α -dependent hepatocyte activity during early night, with correspondingly increased circulating free fatty acids, which could be further stimulated by adipocyte lipolysis. Fasting led to mild hypoglycaemia and hypothermia in *Ppara*^{hep-/-} mice when compared with *Ppara*^{-/-} mice implying a role of PPAR α activity in non-hepatic tissues. In agreement with this observation, *Ppara*^{-/-} mice became overweight during ageing while *Ppara*^{hep-/-} remained lean. However, like *Ppara*^{-/-} mice, *Ppara*^{hep-/-} fed a standard diet developed hepatic steatosis in ageing.

Conclusions Altogether, these findings underscore the potential of hepatocyte PPAR α as a drug target for NAFLD.

INTRODUCTION

Precise control of fatty acid metabolism is essential. Defective fatty acid homeostasis regulation may induce lipotoxic tissue damage, including hepatic steatosis.¹ Peroxisome proliferator-activated receptors (PPARs) are transcription factors that serve as fatty acid receptors and help regulate gene expression in response to fatty acid-derived stimuli.² PPARs act as ligand-activated receptors, controlling

Significance of this study**What is already known on this subject?**

- Peroxisome proliferator-activated receptor α (PPAR α) is a nuclear receptor expressed in many tissues and is responsible for several important metabolic controls, especially during fasting.
- PPAR α is a target for the hypolipidemic drugs of the fibrate family.
- PPAR α is less expressed in the liver of patients with non-alcoholic fatty liver diseases (NAFLD).
- Several PPAR-targeting molecules, including dual agonists, are currently under investigation for NAFLD treatment.

What are the new findings?

- Hepatocyte-restricted PPAR α deletion impairs liver and whole-body fatty acid homeostasis.
- Hepatic PPAR α responds to acute and chronic adipose tissue lipolysis.
- Hepatic PPAR α regulates circadian fibroblast growth factor 21 (FGF21) and fasting-induced FGF21, and is partially responsible for the FGF21 increase in steatohepatitis.
- Hepatocyte-restricted PPAR α deletion is sufficient to promote NAFLD and hypercholesterolaemia during ageing, but does not lead mice to become overweight.

How might it impact on clinical practice in the foreseeable future?

- This work emphasises the relevance and potential of hepatic PPAR α as a drug target for NAFLD.

target gene transcription. The three PPAR isotypes, PPAR α , PPAR β/δ and PPAR γ , display specific tissue expression patterns and control different biological functions,³ but all bind lipids and control lipid homeostasis in different tissues, including the liver.²

A healthy liver does not accumulate lipids, but it plays central roles in fatty acid anabolism and export to peripheral organs, including white

adipose tissue for energy storage.⁴ During dietary restriction, hepatic fatty acid catabolism is also critical for using free fatty acids (FFAs) released from white adipose tissues. PPAR α is the most abundant isotype in hepatocytes and is involved in many aspects of lipid metabolism,^{5–6} including fatty acid degradation, synthesis, transport, storage, lipoprotein metabolism and ketogenesis during fasting.^{7–9} In addition, PPAR α controls glycerol use for gluconeogenesis⁹ as well as autophagy¹⁰ in response to fasting. Moreover, PPAR α regulates the expression of the fibroblast growth factor 21 (FGF21) during starvation.^{11–12} In turn, FGF21 acts as an endocrine hormone targeting various functions including metabolic control.¹³ Finally, PPAR α helps repress the acute-phase response and inflammation in the liver.¹⁴

Obesity can lead to organ and vascular complications.¹⁵ Non-alcoholic fatty liver disease (NAFLD), which are considered the hepatic manifestation of metabolic syndrome, range from benign steatosis to severe non-alcoholic steatohepatitis (NASH), potentially further damaging organs.¹⁶ Sustained elevation of neutral lipid accumulation (mostly triglycerides in hepatocyte lipid droplets) initiates early pathological stages. Different fatty acid sources contribute to fatty liver development, including dietary lipid intake, de novo lipogenesis and adipose tissue lipolysis.⁴ In NAFLD, 60% of fatty acids accumulated in steatotic liver are adipose-derived.¹⁷

Preclinical^{18–21} and clinical²² studies highlight that PPAR α influences NAFLD and NASH. Mice lacking PPAR α develop steatosis during fasting,^{7–8} suggesting the importance of PPAR α activity for using FFA released from adipocytes. However, PPAR α is expressed and active in many tissues, including skeletal muscles,²³ adipose tissues,^{24–25} intestines,²⁶ kidneys²⁷ and heart,²⁸ which all contribute to fatty acid homeostasis. Therefore, it remains unknown whether the increased steatosis susceptibility in mice lacking PPAR α depends on PPAR α activity only in hepatocytes or also in other organs.

Here we investigated consequences of hepatocyte-specific *Ppara* deletion, focusing on effects on fatty acid metabolism in NAFLD, ranging from steatosis to steatohepatitis. We report the first evidence that adipocyte lipolysis correlates with and stimulates NAFLD when hepatocytes are lacking PPAR α . Our data establish that hepatocyte-restricted *Ppara* deletion is sufficient to promote steatosis, emphasising this receptor's relevance as a drug target in NAFLD.

MATERIALS AND METHODS

Animals

Generation of *floxed-Ppara* mice and of *Ppara* hepatocyte-specific knockout (*Ppara*^{hep-/-}) animals is described in online supplementary file 1.

In vivo experiments

In vivo studies followed the European Union guidelines for laboratory animal use and care, and were approved by an independent ethics committee.

Detailed experimental protocols are provided in online supplementary file 1.

Plasma analysis

Plasma FGF21 and insulin, respectively, were assayed using the rat/mouse FGF21 ELISA kit (EMD Millipore) and the ultrasensitive mouse insulin ELISA kit (Crystal Chem) following the manufacturer's instructions. Aspartate transaminase, alanine transaminase (ALT), total cholesterol, LDL cholesterol and HDL

cholesterol were determined using a COBAS-MIRA+ biochemical analyser (Anexplo facility).

Circulating glucose and ketone bodies

Blood glucose was measured using an Accu-Chek Go glucometer (Roche Diagnostics). β -Hydroxybutyrate content was measured using Optium β -ketone test strips with Optium Xceed sensors (Abbott Diabetes Care).

Histology

Paraformaldehyde-fixed, paraffin-embedded liver tissue was sliced into 5 μ m sections and H&E stained. Visualisation was performed using a Leica DFC300 camera.

Liver lipids analysis

Detailed experimental protocols are provided in online supplementary file 1.

Gene expression studies

Total RNA was extracted with TRIzol reagent (Invitrogen). Transcriptomic profiles were obtained using Agilent Whole Mouse Genome microarrays (4 \times 44k). Microarray data and experimental details are available in the Gene Expression Omnibus (GEO) database (accession number GSE73298 and GSE73299). For real-time quantitative PCR (qPCR), 2 μ g RNA samples were reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Online supplementary file 2 presents the SYBR Green assay primers. Amplifications were performed using an ABI Prism 7300 Real-Time PCR System (Applied Biosystems). qPCR data were normalised to TATA-box-binding protein mRNA levels, and analysed with LinRegPCR.v2015.3.

Transcriptomic data analysis

Data were analysed using R (<http://www.r-project.org>). Microarray data were processed using Bioconductor packages (<http://www.bioconductor.org>, v 2.12)²⁹ as described in GEO entry GSE26728. Further details are provided in online supplementary file 1.

Statistical analysis

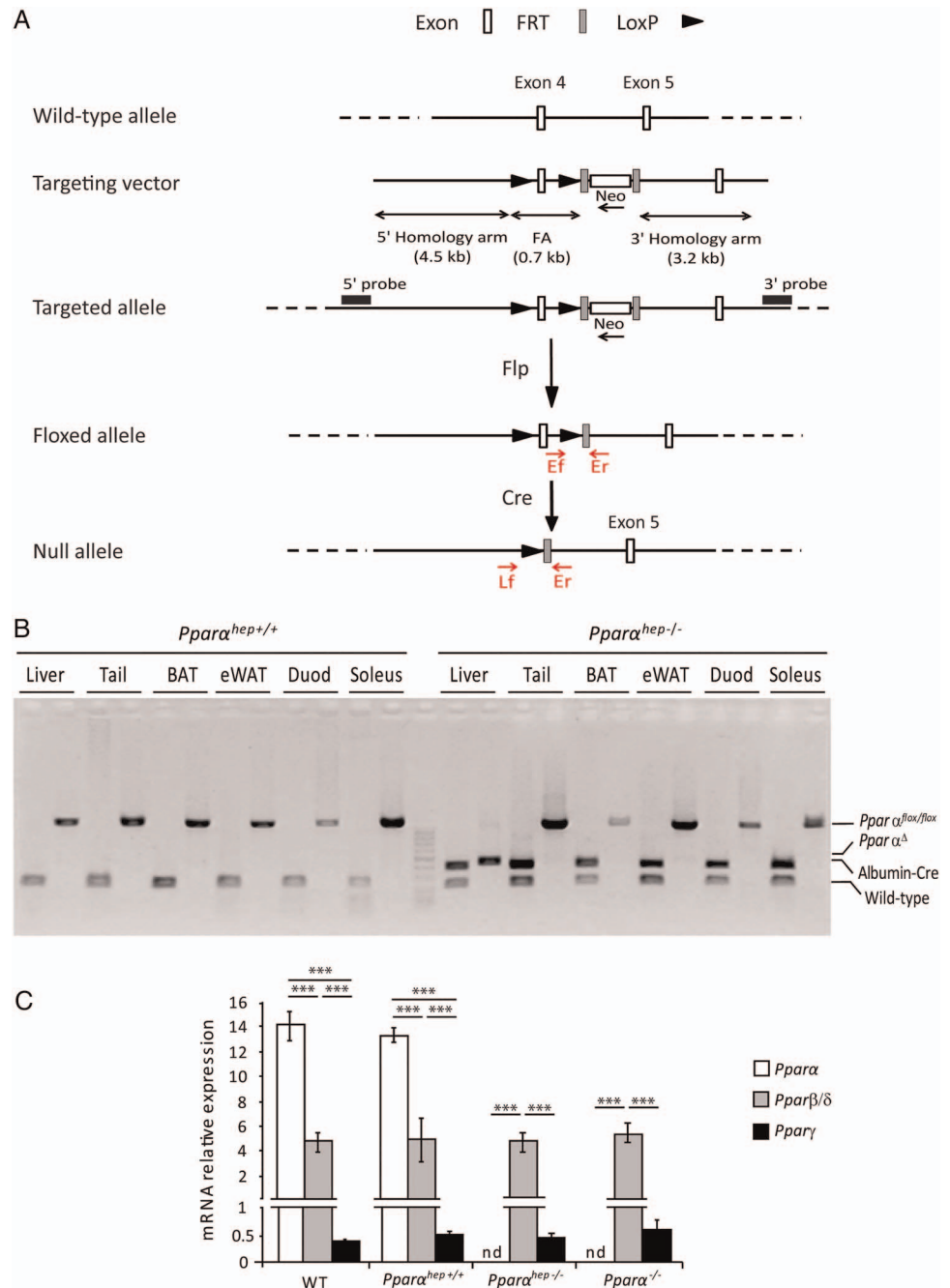
Data were analysed using R (<http://www.r-project.org>). Microarray data were processed using bioconductor packages (<http://www.bioconductor.org>) as described in GEO entry GSE38083. Genes with a *q* value of <0.001 were considered differentially expressed between genotypes. Gene Ontology (GO) Biological Process enrichment was evaluated using conditional hypergeometric tests (GStats package). For non-microarray data, differential effects were analysed by analysis of variance followed by Student's *t*-tests with a pooled variance estimate. A *p* value <0.05 was considered significant.

RESULTS

Generation of hepatocyte-specific PPAR α knockout mice

Progeny carrying the *Ppara*^{flox/flox} alleles (figure 1A), referred to as floxed, were backcrossed in the C57Bl/6J background, and then crossed with *albumin-Cre* mice in the same genetic background, generating a hepatocyte-specific PPAR α knockout (*Ppara*^{flox/flox} *albumin-Cre*^{+/-}) referred to as *Ppara*^{hep-/-} (figure 1B). PPAR α mRNA was not detected in livers from *Ppara*^{hep-/-} mice when compared with floxed and C57Bl/6J mice (figure 1C), suggesting that most hepatic PPAR α expression is from hepatocytes. PPAR α absence in hepatocytes did not alter mRNA expression of other PPAR isotypes (figure 1C).

Figure 1 Characterisation of the hepatocyte-specific peroxisome proliferator-activated receptor α (PPAR α) knockout mouse model. (A) Schematic of the targeting strategy to disrupt hepatic *Ppara* expression. (B) PCR analysis of *Ppara* floxed (*Ppara*^{hep}^{+/+}) and *Albumin-Cre* (*Albumin-Cre*^{+/+}) genes from mice that are liver wild-type (WT), (*Ppara*^{hep}^{+/+}) or liver knockout (*Ppara*^{hep}^{-/-}) for *Ppara* using DNA extracted from different organs. (C) Relative mRNA expression levels of *Ppara*, *Ppar β/δ* and *Ppar γ* from liver samples of WT, liver WT (*Ppara*^{hep}^{+/+}), *Ppara* liver knockout (*Ppara*^{hep}^{-/-}) and *Ppara* knockout (*Ppara*^{-/-}) mice (n=8 mice per group). Data represent mean \pm SEM. ***p \leq 0.005. FA, floxed allele; Flp, flippase; FRT, flippase recognition target; LoxP, locus of X-overP1; nd, not detected; *Ppara* Δ , *Ppara* deletion; WT, the *Albumin-Cre*^{-/-} allele.



Hepatocyte-autonomous effect of fenofibrate on PPAR α activity

To determine whether PPAR α response was hepatocyte-autonomous, we challenged wild-type (WT), floxed *Ppara*^{hep}^{+/+}, *Ppara*^{-/-} and *Ppara*^{hep}^{-/-} mice with the PPAR α agonist fenofibrate. We measured mRNA expressions of PPAR α target genes, including *Cyp4a10* (figure 2A) and *Cyp4a14* (figure 2B). Their expressions were strongly induced by fenofibrate in WT and in floxed *Ppara*^{hep}^{+/+} mice compared with *Ppara*^{-/-} and *Ppara*^{hep}^{-/-} mice. These samples were also used for pangenomic expression profiling through microarray analysis (figure 2C). Differentially expressed gene (DEG) analysis was subjected to hierarchical clustering, highlighting similar expression profiles between WT and floxed *Ppara*^{hep}^{+/+} mice within fenofibrate-treated or vehicle-treated groups. Whole-body *Ppara*^{-/-} and *Ppara*^{hep}^{-/-} mice were unresponsive to fenofibrate, suggesting that fenofibrate-induced hepatic changes were mainly

due to autonomous hepatocyte responses, not secondary to extrahepatic PPAR α activation. GO biological function analysis revealed that fenofibrate upregulated lipid metabolism, and repressed immune and defence response, metabolic responses, and glycosylation and glycoprotein metabolism (figure 2C, groups 1, 2, 6 and 7). However, untreated *Ppara*^{-/-} and *Ppara*^{hep}^{-/-} mice showed marked differences (figure 2C, groups 3, 4, 8 and 9). This implies that the absence of extrahepatic PPAR α has a significant impact on the liver transcriptional profile and underscores the relevance of *Ppara*^{hep}^{-/-} mice to define the hepatocyte autonomous role of the receptor in the control of liver function.

Hepatocyte PPAR α activity is context-specific

The *Ppara*^{hep}^{-/-} model was used to determine whether PPAR α could drive hepatic regulations both in fasting-induced fatty acid catabolism as well as fatty acid anabolism during refeeding. The

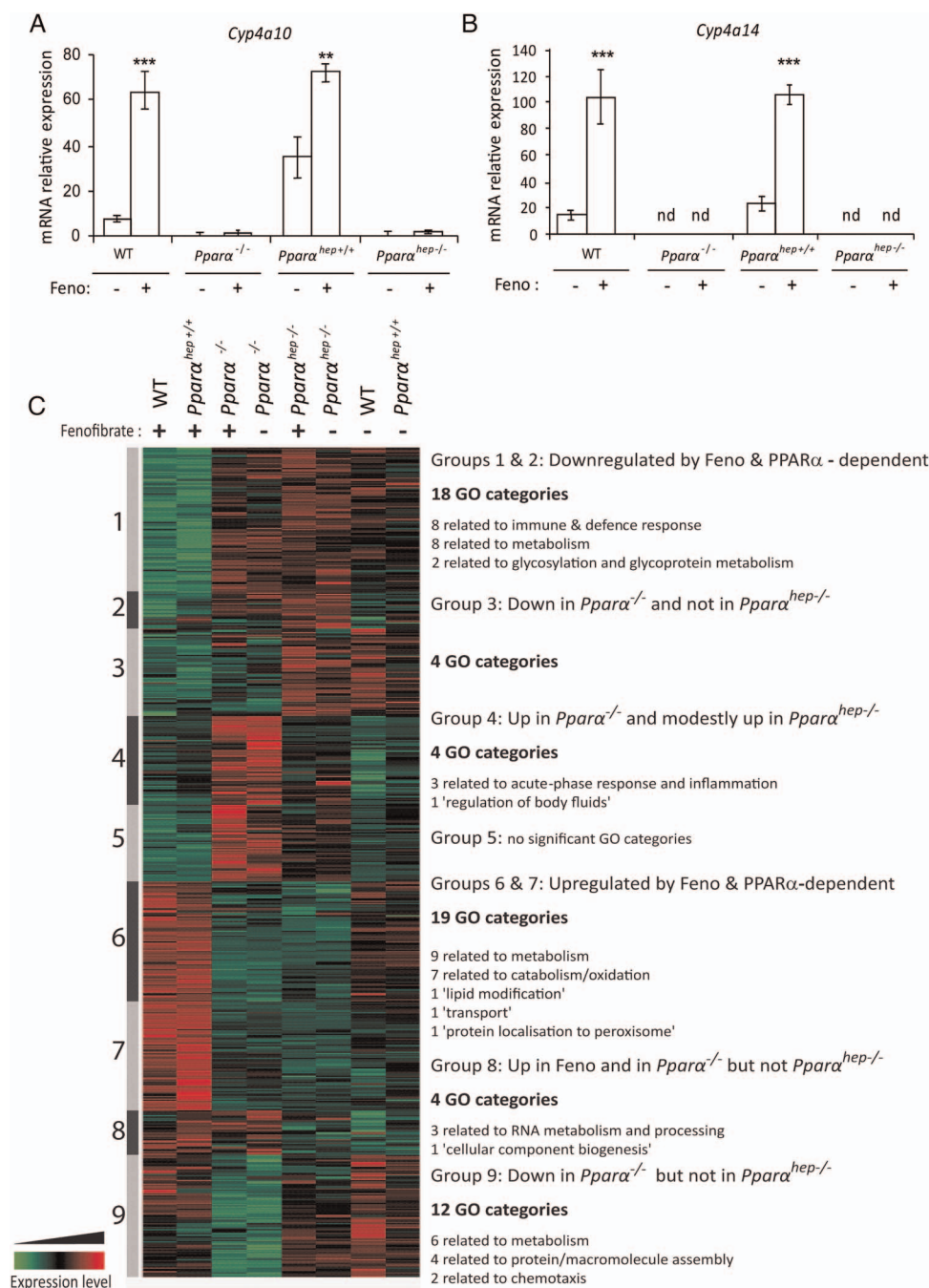


Figure 2 Pharmacological peroxisome proliferator-activated receptor α (PPAR α) activation using fenofibrate reveals hepatocyte-specific PPAR α -dependent biological functions. Liver samples from wild-type (WT), PPAR α knockout (*Ppara*^{-/-}), liver WT (*Ppara*^{hep+/+}) and PPAR α hepatocyte knockout (*Ppara*^{hep-/-}) mice treated with fenofibrate (Feno, +) or vehicle (-) by oral gavage for 14 days were collected. (A and B) The relative gene expression of two specific PPAR α target genes *Cyp4a10* (A) and *Cyp4a14* (B) was measured by qRT-PCR. Data represent mean \pm SEM. ** $p \leq 0.01$, *** $p \leq 0.005$. (C) Heat map representing data from a microarray experiment performed with liver samples. Hierarchical clustering is also shown, which allows the definition of nine gene clusters. Gene Ontology (GO) analysis of each cluster revealed significant biological functions ($p \leq 0.05$). nd, not detected.

fasting-refeeding experimental design was validated by measuring glycaemia (figure 3A) and expression of fatty acid synthase (*Fasn*), which encodes the rate-limiting enzyme in lipogenesis (figure 3B). Both were low during fasting, intermediary in ad libitum-fed animals, and high in refed animals. *Cyp4a14* (a well-known PPAR α target) expression was low or undetectable in *Ppara*^{hep-/-} animals, and strongly upregulated with fasting in WT mice (figure 3C).

Next we evaluated the hepatic transcriptome expression pattern using microarrays. We performed hierarchical clustering (figure 3D). Most PPAR α -dependent changes were observed in fasted mouse livers. Venn diagrams were used to show nutritional status-related PPAR α -dependent changes (figure 3E). Among the significant DEGs, 3048 were related to fasting, 390 to ad libitum-fed animals and 156 to refed mice, suggesting context-specific PPAR α activity. The results further highlighted

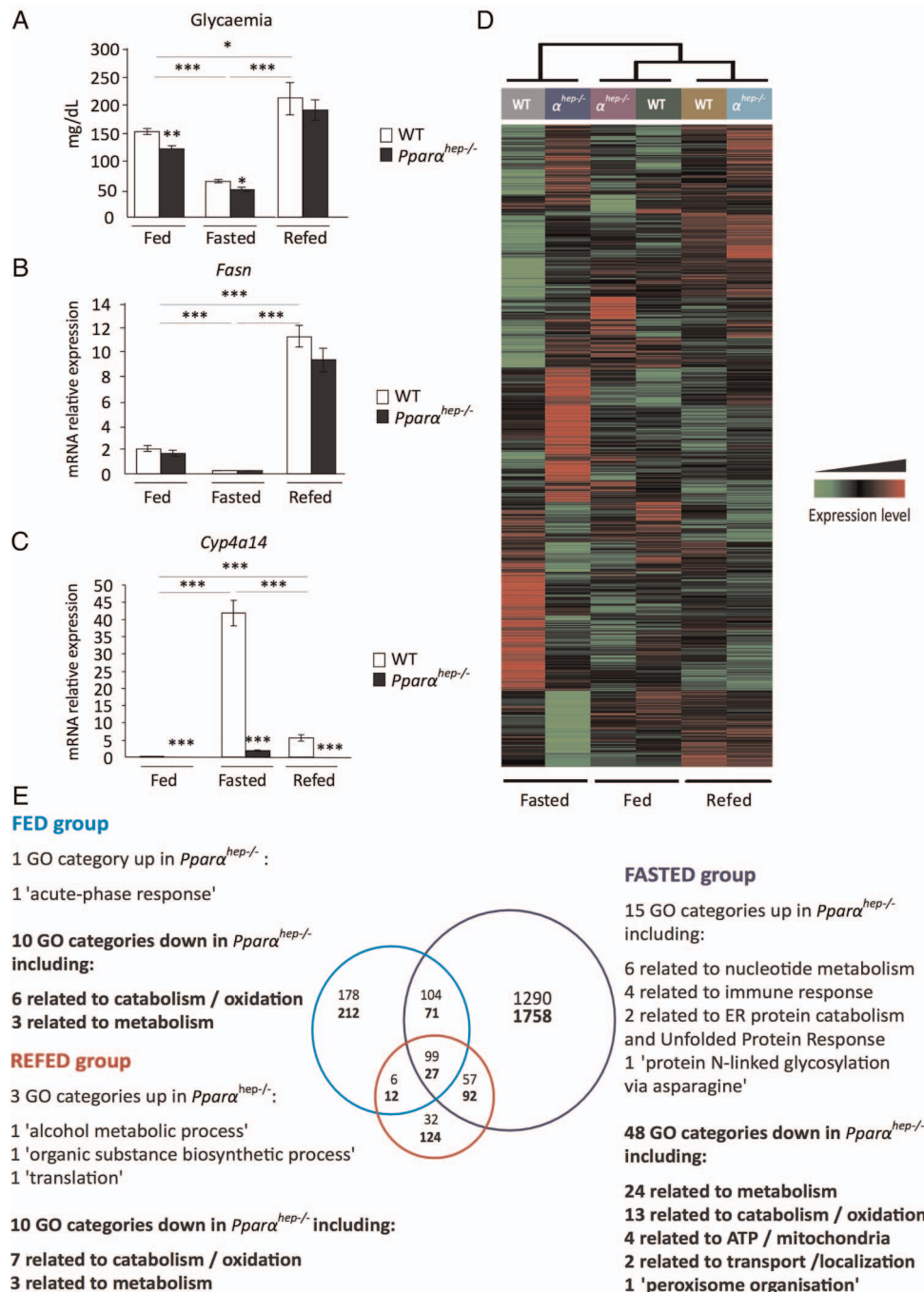


Figure 3 Hepatocyte-specific peroxisome proliferator-activated receptor α (PPAR α) function is dependent on nutritional status. Wild-type (WT) and PPAR α liver knockout (*Ppara*^{hep-/-}) male 8-week-old mice were fed ad libitum, fasted for 24 h, or fasted for 24 h and refed for 24 h. All mice were killed at ZT14, and sera and livers were collected. (A) Quantification of circulating glucose levels. (B, C) Relative mRNA expressions of *Fasn* (B) and *Cyp4a14* (C) in liver samples quantified by qRT-PCR. Data represent mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$. (D) Heat map was performed based on average gene expression levels from WT ($n=12$ (6 WT and 6 *Ppara*^{hep+/+})) and from *Ppara*^{hep-/-} ($n=6$). (E) Venn diagram and associated Gene Ontology (GO) function analysis ($p \leq 0.05$), GO categories corresponding to functions down in the absence of PPAR α are in bold, GO categories corresponding to functions up in the absence of PPAR α are in regular font.

that fasting, rather than feeding or refeeding, triggered the broader PPAR α -dependent hepatocytic response, with most upregulated genes related to metabolism (figure 3E). However, the expression of several genes was identified as PPAR α dependent regardless of the nutritional condition tested (fasting, but also feeding and refeeding). These genes are mostly downregulated in the absence of PPAR α and pathway analysis highlights

their involvement in mitochondrial fatty acid catabolism (see online supplementary file 3).

Biological function analyses revealed that both transcriptional activation and repression were PPAR α sensitive (figure 3E). The functions of PPAR α -sensitive repressions (GO categories up in *Ppara*^{hep-/-} mice) varied with context, and included GO categories not directly related to metabolism, including acute-phase

response (fed), translation (refed) and protein glycosylation (fasted).

Hepatocyte PPAR α is required for liver and whole-body fatty acid homeostasis in fasting

We next used *Ppara*^{hep-/-} mice to determine the contribution of hepatocyte PPAR α , and compared it with *Ppara*^{-/-} and WT mice. We measured FFA and β -hydroxybutyrate (ketonaemia) levels in fasted and non-fasted mice (figure 4A). Plasma FFA was elevated in fasting mice of all three genotypes, but was significantly higher in *Ppara*^{hep-/-} and *Ppara*^{-/-} mice compared with controls. Fasting strongly increased ketone body levels in WT mice and to a lesser degree in *Ppara*^{hep-/-} and *Ppara*^{-/-} mice. This suggests that hepatic PPAR α is required for FFA disposal and for β -hydroxybutyrate production. Correspondingly, fasting *Ppara*^{hep-/-} and *Ppara*^{-/-} mice showed elevated hepatic triglycerides and cholesterol esters (figure 4B), and substantial centrilobular steatosis (figure 4C), confirming that hepatic PPAR α expression is required for fasting-induced FFA catabolism. PPAR α absence led to defective expressions of PPAR α target genes (figure 4D), including those involved in fatty acid catabolism and processing in lipid droplets (figure 4E). As a consequence of PPAR α deficiency in hepatocytes, *Ppara*^{hep-/-} mice exhibit a distinct fasting-induced fatty acid profile with a significant increase in oleic acid (C18:1n-9) and linoleic acid (C18:2n-6) when compared with WT mice (see online supplementary file 4).

Hepatocyte-specific *Ppara* deletion impairs constitutive and fasting-induced FGF21 expression

FGF21 is a hepatokine mainly produced by the liver. We examined liver *Fgf21* mRNA expression (figure 5A) and plasma FGF21 levels (figure 5B) in fed and fasted animals. We identified a constitutive expression peak during the day (ZT8) in both groups, and a fasting-triggered night-time peak (ZT16). In *Ppara*^{hep-/-} mice, we examined whether fasting-induced FGF21 expression/production was strictly dependent on PPAR α hepatic activity. *Ppara*^{-/-} and *Ppara*^{hep-/-} mice showed very low plasma FGF21 protein at ZT8 or at ZT16 with fasting (figure 5C).

Since FGF21 has been shown to reduce steatosis and lipotoxic lipids^{13 30} we questioned whether the absence of FGF21 determines fasting-induced steatosis observed in *Ppara*^{hep-/-} and *Ppara*^{-/-} mice. FGF21 expression was rescued by adenoviral delivery both in *Ppara*^{hep-/-} and in *Ppara*^{-/-} mice (figure 5D). Comparable expression of FGF21 (figure 5E) was obtained in liver of WT, *Ppara*^{hep-/-} and in *Ppara*^{-/-} mice. FGF21-sensitive genes such as *G6pd* and *Scd1* showed significantly different expression in response to FGF21 overexpression (figure 5E). However, FGF21 only reduced hepatic triglycerides and cholesterol esters in WT mice, but not in *Ppara*^{hep-/-} and in *Ppara*^{-/-} mice (figure 5F, G). These results indicate that the fasting-induced steatosis occurring in *Ppara*^{hep-/-} and in *Ppara*^{-/-} mice does not depend on the lack of FGF21. This is in line with our observations that FGF21- and PPAR α -sensitive target genes are different (see online supplementary file 5A). Moreover, it is also consistent with the observation that FGF21 overexpression does not rescue the expression of PPAR α target genes and conversely that PPAR α -sensitive regulations occur in *Fgf21*^{-/-} mice (see online supplementary file 5B, C).

In addition to their defective fatty acid catabolism, *Ppara*^{-/-} mice are hypoglycaemic and hypothermic during fasting.⁷ Because FGF21 is important for glucose homeostasis and for thermogenesis,¹³ we investigated the role of hepatocyte PPAR α in controlling fasting glycaemia and body temperature. Both

Ppara^{hep-/-} and *Ppara*^{-/-} mice were hypoglycaemic and hypothermic compared with WT mice during fasting. However, this phenotype was much stronger in fasted *Ppara*^{-/-} mice compared with fasted *Ppara*^{hep-/-} mice (figure 5H-J), indicating that extra-hepatic PPAR α strongly influenced whole-body glucose homeostasis and temperature independent of hepatocytic PPAR α activity and FGF21 production.

Fasting-enhanced hepatocytic PPAR α activity is time-restricted and sensitive to adipocyte lipolysis

We next tested the kinetics of other fasting-induced hepatic PPAR α activity in vivo. We used several measures of PPAR α activity, including *Fgf21* (figure 5A) and *Vanin1*, *Cyp4a10*, *Cyp4a14* and *Fsp27* mRNAs (figure 6A), since these genes were most sensitive to fasting and to fenofibrate, and were strictly PPAR α dependent (see online supplementary files 6–10A). Plasma FFA and glucose levels were also measured during fasting (figure 6B). FFA were markedly increased in the early night (ZT14–ZT16). The FFA pattern was correlated with the PPAR α mRNA expression profile and expressions of *Fgf21*, *Vanin1*, *Cyp4a10*, *Cyp4a14* and *Fsp27* (figures 5A and 6A). This strongly suggested that FFA released from adipocytes during fasting-influenced hepatic PPAR α expression and activity without inflammatory response since hepatic *Tnfa* mRNA expression was not sensitive to fasting. We further determined that acute treatment of fasted mice with the β 3-adrenergic receptor agonist CL316243 enhanced circulating FFA levels in WT and *Ppara*^{hep-/-} mice (figure 6C), and increased expressions of *Fgf21*, *Cyp4a14*, *Vanin1*, *Cyp4a10* and *Fsp27* in WT mice but not *Ppara*^{hep-/-} mice (figure 6D) without inducing *Tnf α* in response to fasting or in response to CL316243 (see online supplementary file 10C and D). These data support a role for acute adipocyte lipolysis as a signal for hepatocyte PPAR α activity during fasting.

Hepatocyte PPAR α is required for protection in steatohepatitis

We next examined whether the hepatocytic PPAR α response to chronic lipolysis occurred during methionine-deficient and choline-deficient diet (MCD)-induced weight loss. In rodents, this diet rapidly promotes lipolysis in adipocytes, resulting in steatohepatitis. On the MCD diet, mice of each genotype showed weight loss (figure 7A), steatosis (figure 7B), and increased hepatic triglycerides, cholesterol esters (figure 7C) and plasma ALT (figure 7D). Compared with WT, *Ppara*^{hep-/-} and *Ppara*^{-/-} mice showed greater steatosis and liver damage, suggesting a more severe MCD diet-induced phenotype without hepatocyte PPAR α . MCD also induced increased expressions of *Cyp4a14* and *Vanin1* in WT mice, but not *Ppara*^{hep-/-} or *Ppara*^{-/-} mice (figure 7E). *Fgf21* mRNA (figure 7E) and circulating FGF21 (figure 7F) were increased through a mechanism that is partly dependent on hepatic PPAR α . Overall, hepatocyte-specific *Ppara* deletion aggravated MCD diet-induced liver damage, correlating with defective PPAR α -dependent pathway upregulation in response to chronic lipolysis.

Additionally, we questioned whether hepatocyte PPAR α may also be required for the protection of the liver during early hits in steatosis such as those occurring in response to short-term exposure to a high-fat diet (HFD). Over 2 weeks of HFD, mouse liver accumulated hepatic triglycerides and cholesterol esters. Importantly, this steatosis was twice higher in *Ppara*^{hep-/-} mice than in WT mice, and was further elevated in *Ppara*^{-/-} mice (see online supplementary file 11). Altogether, these data suggest that hepatic PPAR α is essential in hepatoprotection.

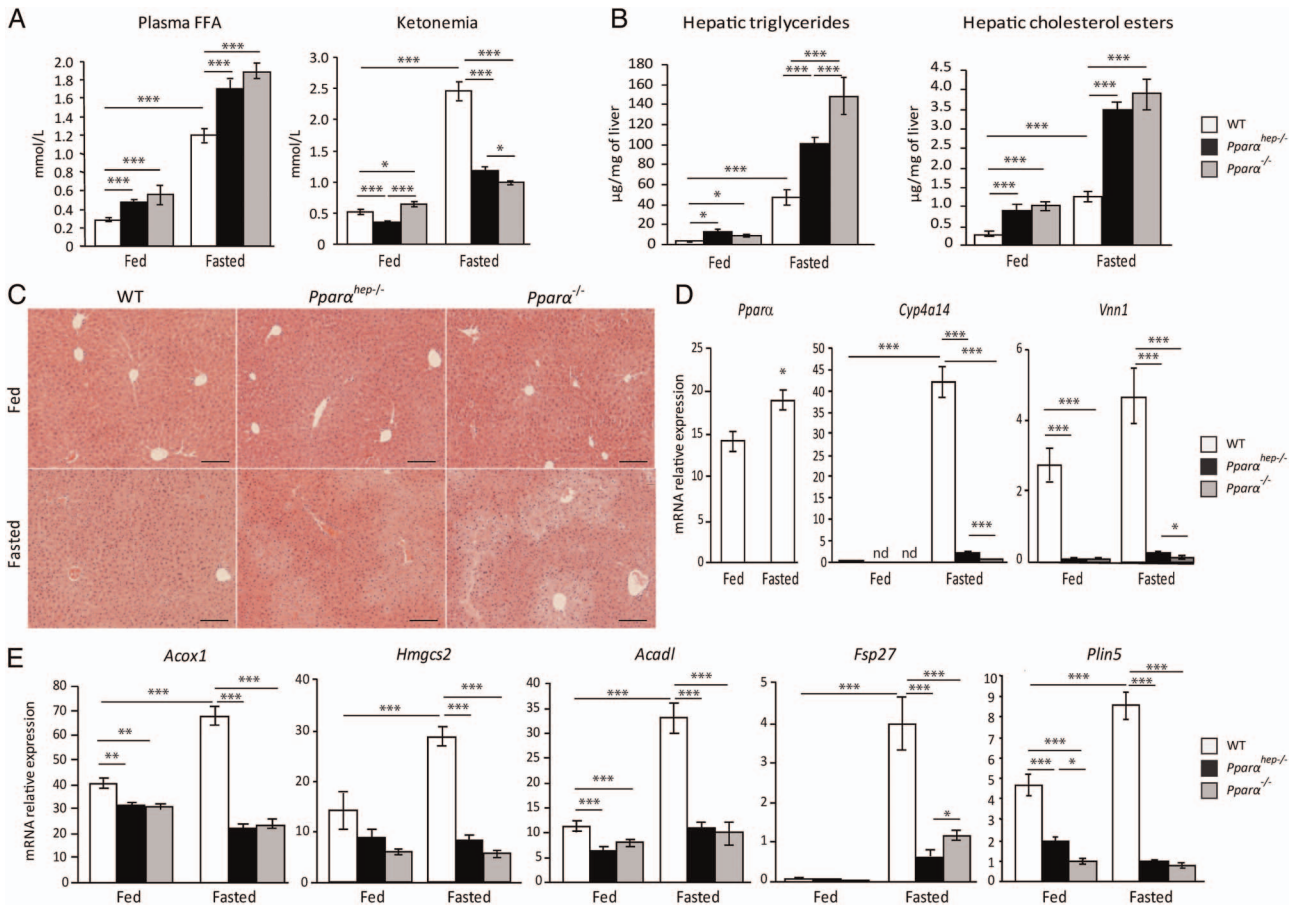


Figure 4 Fasting is the major inducer of hepatic peroxisome proliferator-activated receptor α (PPAR α) activity. Wild-type (WT), hepatocyte-specific PPAR α knockout (*Ppara*^{hep-/-}) and total PPAR α knockout (*Ppara*^{-/-}) mice were fed ad libitum or fasted for 24 h and then killed. (A) Quantification of plasma free fatty acids (FFAs) and ketone bodies (ketonaemia). (B) Hepatic triglycerides and cholesterol esters hepatic levels. (C) Representative pictures of H&E staining of liver sections. Scale bars, 100 μ m. (D) Relative mRNA expression levels of *Ppara*, *Cyp4a14* and *Vnn1* in liver samples determined by qRT-PCR. (E) Quantification of mRNA expression of *Acox1*, *Hmgcs2*, *Acadl*, *Fsp27* and *Plin5* by qRT-PCR. Data shown as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$.

Hepatocyte PPAR α deficiency leads to steatosis and hypercholesterolaemia but not excess weight gain in ageing mice

Lastly, we questioned the long-term consequences of hepatocyte-specific *Ppara* deletion during ageing. More specifically, since PPAR α is broadly expressed in metabolic tissues, we aimed at clarifying whether the steatosis that develops in aged whole-body *Ppara*^{-/-} mice is due to the hepatocytic defect in PPAR α activity. WT, *Ppara*^{hep-/-} and *Ppara*^{-/-} mice were fed a standard diet over 1 year. *Ppara*^{-/-} mice, but not *Ppara*^{hep-/-} mice, grew overweight with ageing (figure 8A–C). Both *Ppara*^{hep-/-} and *Ppara*^{-/-} mice showed spontaneous centrilobular steatosis (figure 8D), elevated hepatic triglycerides and hepatic cholesterol esters (figure 8E), as well as hypercholesterolaemia (see figure 8F online supplementary file 12) without hyperglycaemia (figure 8G). Overall, hepatocyte-specific PPAR α deficiency was sufficient to induce spontaneous steatosis and disrupt whole-body fatty acid as well as cholesterol homeostasis, but did not affect weight gain and diabetes during ageing.

DISCUSSION

NAFLD are a spectrum of diseases presenting a major public health concern that is strongly linked with obesity. Most accumulated hepatic fatty acids in NAFLD come from increased non-esterified FFA in the fasting state.¹⁷ Thus, it is essential to

define the mechanisms by which the liver adapts to this influx. FFA processing largely involves the fatty acid oxidative pathway, coupled to ketogenesis allowing the liver to use lipids,³¹ which is critical during fasting and requires transcriptional regulation of rate-limiting enzymes.³²

Whole-body *Ppara*^{-/-} mice show impaired coping with prolonged fasting, resulting in defective fatty acid oxidation and steatosis, hypoglycaemia and hypothermia. However, PPAR α also contributes to metabolic homeostasis through expression in other tissues. Here we investigated the impact of hepatocyte-specific PPAR α deletion on liver physiology and lipid metabolism in vivo. To our knowledge, this is the first report that selective PPAR α deletion in hepatocytes (*Ppara*^{hep-/-}) was sufficient to promote hepatic steatosis.

PPAR α is targeted by several fibrate drugs,³³ and by pan-agonists for PPAR isotypes²¹ that are currently in clinical trials for NASH treatment. Using *Ppara*^{hep-/-} mice, we demonstrated an autonomous transcriptional response of hepatocytes to fenofibrate, indicating that fibrates' effects on the liver gene expression are largely independent from those in extrahepatic tissues. Moreover, liver gene expression profiles markedly differed between untreated *Ppara*^{-/-} and *Ppara*^{hep-/-} mice, suggesting that extrahepatic PPAR α activity substantially influenced the hepatic transcriptome.

Food restriction induces PPAR α activity, and endogenous PPAR α ligand production requires hepatic lipogenesis, which

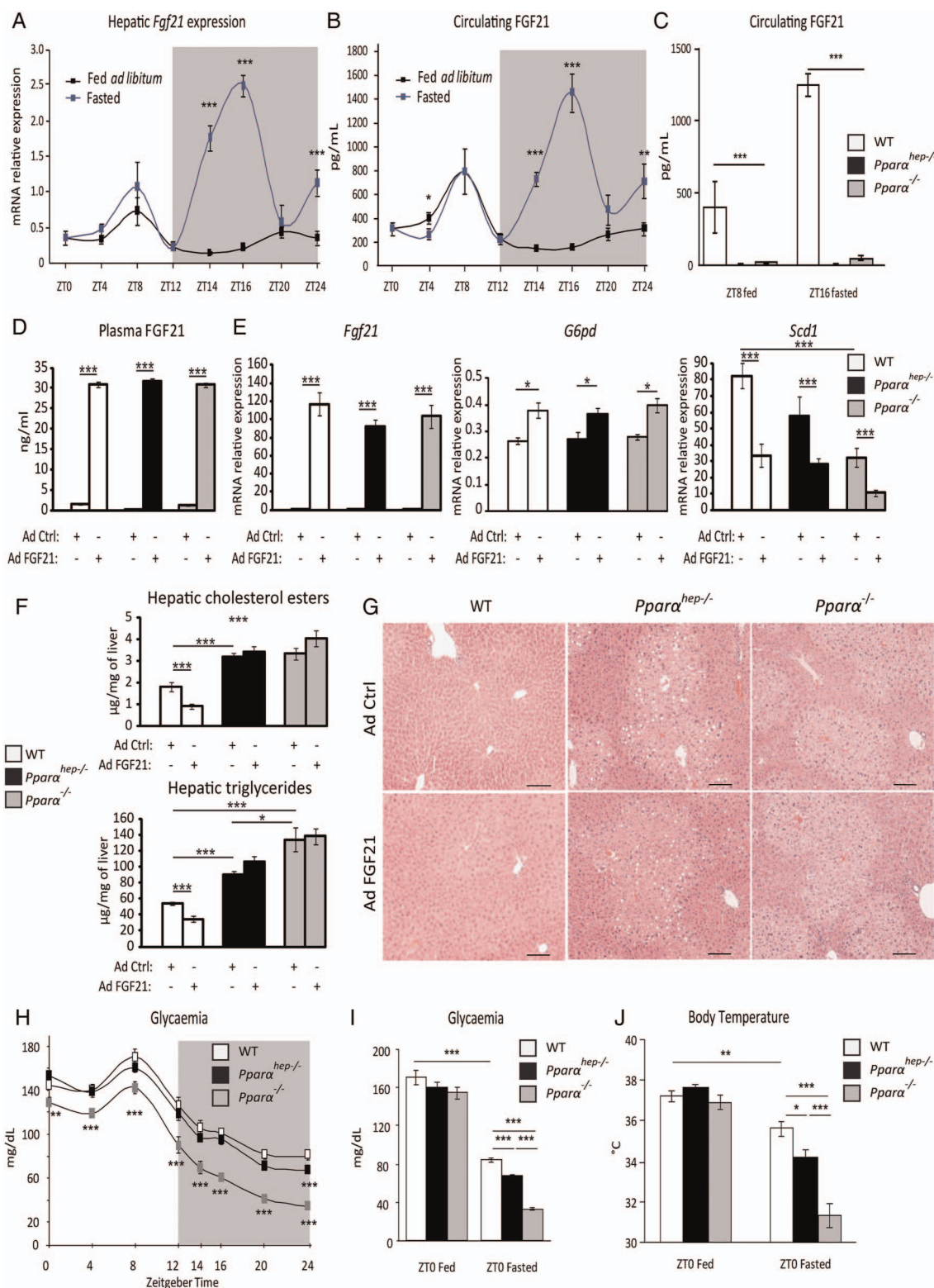


Figure 5 Hepatocyte and extrahepatocyte peroxisome proliferator-activated receptor α (PPAR α) regulate fibroblast growth factor 21 (FGF21), glycaemia and body temperature during fasting. (A and B) Eleven-week-old male mice of the C57Bl/6J background were fed ad libitum or fasted for 24 h, and were killed around the clock from ZT0 to ZT24. (A) *Fgf21* mRNA was quantified by qRT-PCR. (B) Quantification of circulating FGF21 levels by ELISA. (C) Twelve-week-old wild-type (WT), PPAR α -hepatocyte knockout (*Ppara^{hep-/-}*) and PPAR α knockout (*Ppara^{-/-}*) male mice were fed ad libitum or fasted for 16 h and blood was collected at ZT8 (ZT8 fed) or at ZT16 (ZT16 fasted). FGF21 plasma level was determined by ELISA. (D–G) Male mice of WT, *Ppara^{hep-/-}* and *Ppara^{-/-}* genotypes were infected with an adenoviral construct containing cDNA of *Fgf21* or an empty vector. Mice were sacrificed after a 24 h fasting period at ZT14. (D) Quantification of circulating FGF21 levels by ELISA. (E) *Fgf21*, *G6pd* and *Scd1* mRNAs were quantified by qRT-PCR. (F) Quantification of hepatic cholesterol esters and triglycerides. (G) Representative pictures of H&E staining of liver sections. Scale bars, 100 μ m. (H) Plasma glucose level was monitored over a 24 h fasting period from ZT0 to ZT24 in WT, *Ppara^{hep-/-}* and *Ppara^{-/-}* mice. (I, J) Plasma glucose (I) and body temperature (J) were determined at ZT0 in fed mice or at ZT0 in mice fasted for 24 h. Data are shown as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$.

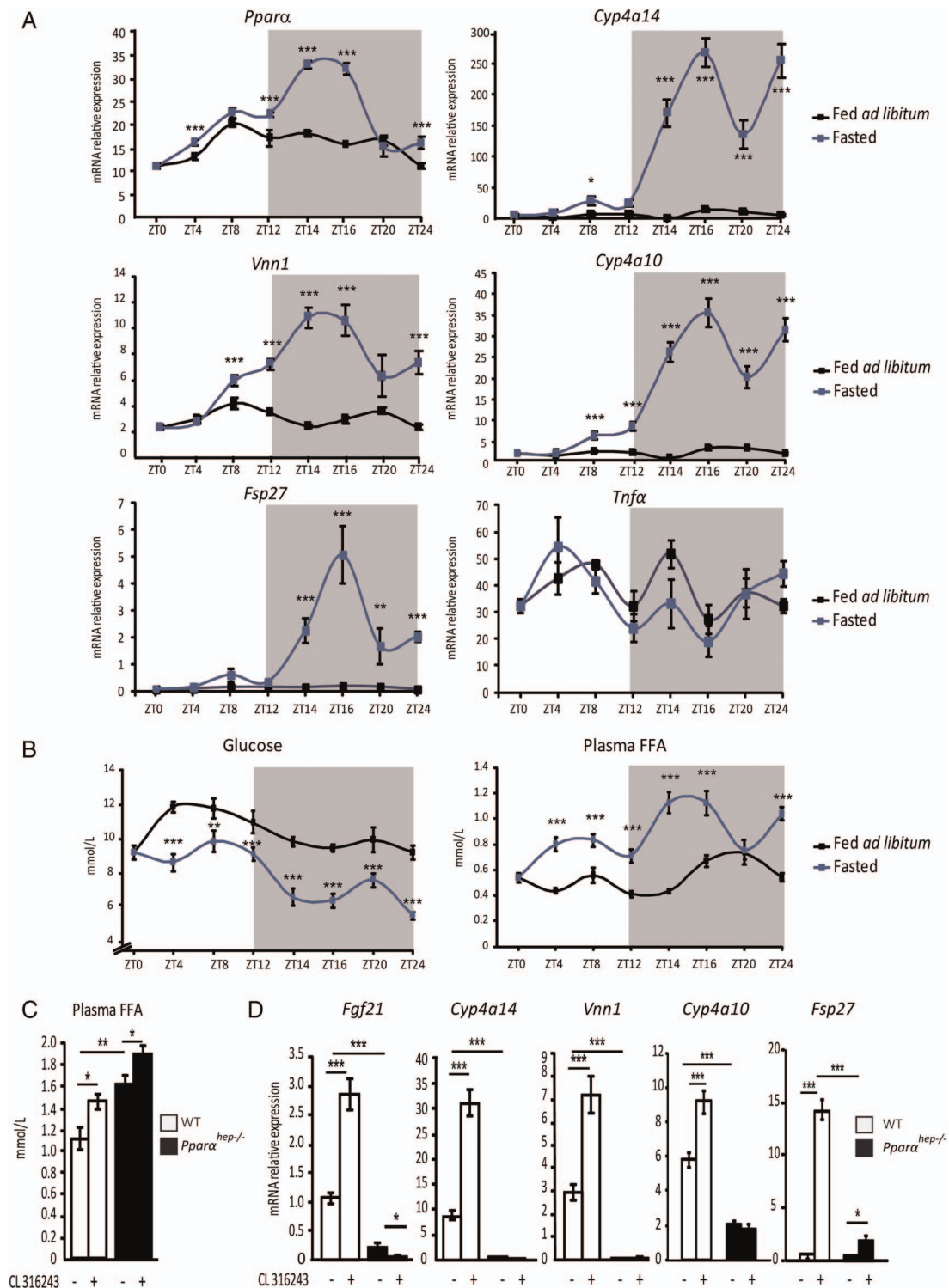


Figure 6 Hepatocyte peroxisome proliferator-activated receptor α (PPAR α) activity is induced by adipose tissue lipolysis. (A and B) Liver samples were collected from male wild-type (WT) C57Bl/6J mice that were fed ad libitum (black curve) or fasted (blue curve) over 24 h. (A) Hepatic mRNA expression levels of *Ppara*, *Cyp4a14*, *Vnn1*, *Cyp4a10*, *Fsp27* and *Tnfa* were quantified by qRT-PCR. (B) Plasma glucose and free fatty acids (FFA) were measured. (C and D) WT and PPAR α hepatocyte-specific knockout (*Ppara*^{hep-/-}) mice were treated with the β_3 -adrenergic receptor agonist CL316243 at ZT6 and then killed at ZT14. (C) Quantification of plasma FFA. (D) Relative mRNA expression levels of *Fgf21*, *Cyp4a14*, *Vnn1*, *Cyp4a10* and *Fsp27* were measured by qRT-PCR. Data are shown as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$.

increases upon feeding.^{34, 35} Thus, PPAR α may be important during fasting-induced lipid catabolism and in the response to anabolic fatty acid-derived signals. Our data revealed the context dependency of PPAR α hepatocytic activity defined by DEGs. This activity was clearly the highest during fasting.

During fasting, hepatocyte-specific PPAR α deletion resulted in steatosis, increased plasma FFA and impaired ketone bodies. This supports the concept that FFA released from adipose stores during fasting may activate PPAR α for hepatic use. Accordingly, we found that *Ppara*^{hep-/-} mice accumulate high oleic and

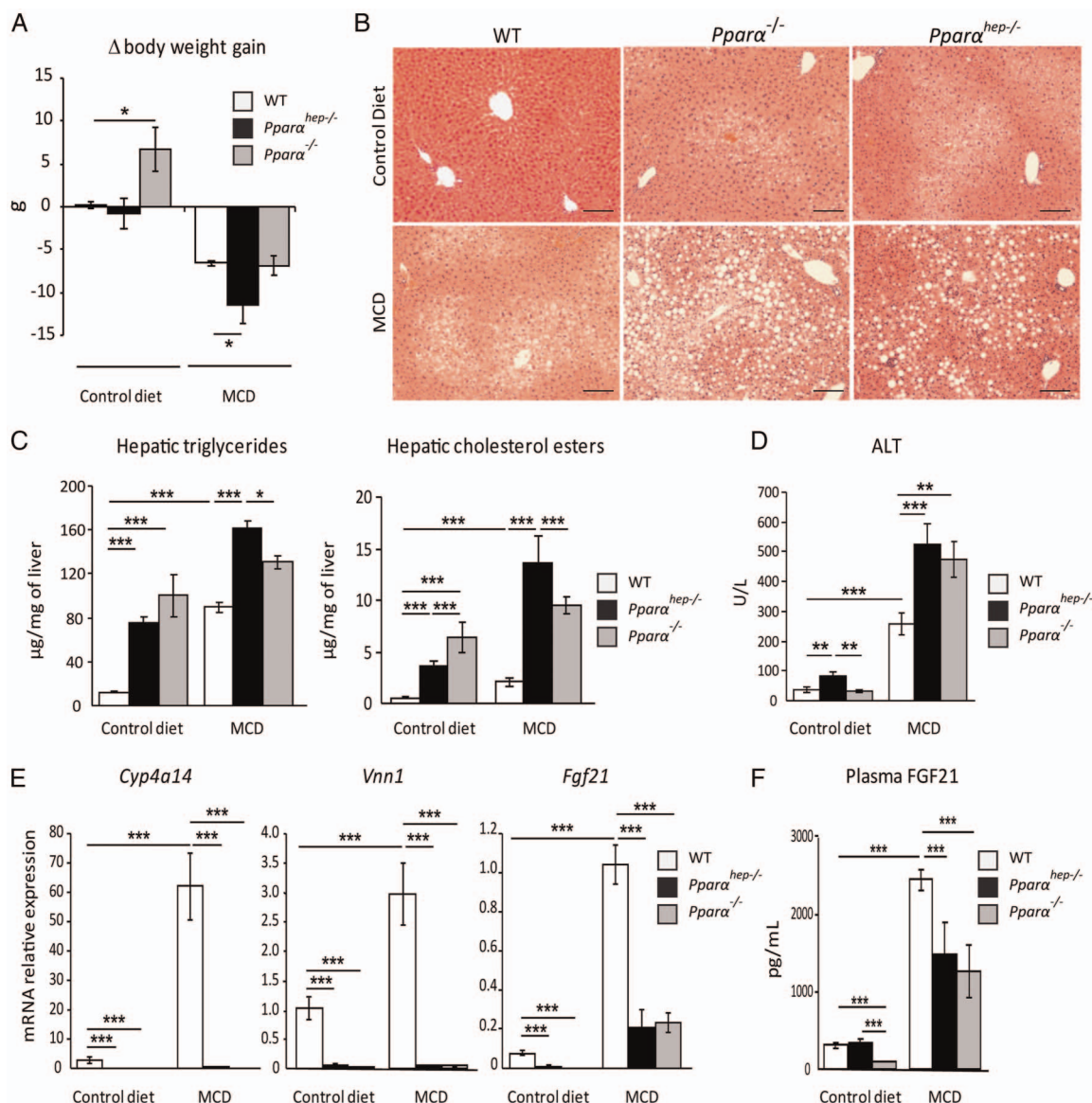


Figure 7 Liver peroxisome proliferator-activated receptor α (PPAR α) deficiency aggravates non-alcoholic steatohepatitis in response to a methionine-deficient and choline-deficient diet (MCD). Wild-type (WT), PPAR α hepatocyte knockout (*Ppara*^{hep-/-}) and PPAR α knockout (*Ppara*^{-/-}) mice were fed a MCD or a control diet for 2 weeks and were killed at ZT8. (A) Body weight gain was measured over 2 weeks. (B) Representative pictures of H&E staining on liver sections. Scale bar, 100 μ m. (C) Quantification of hepatic triglycerides and cholesterol esters. (D) Alanine transaminase activity level in plasma. (E) Hepatic mRNA expression levels of *Cyp4a14*, *Vnn1* and *Fgf21*. (F) Plasma levels of fibroblast growth factor 21 (FGF21). Data are shown as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$.

linolenic acids in the liver during fasting (see online supplementary file 4), which is in agreement with the fact that both of them are the main fatty acids stored in the white adipose tissues of mice fed a chow diet.³⁶ Importantly, we found a high correlation between the kinetics of circulating FFA increase and expression of PPAR α and several of its target genes. Moreover, treatment with a β 3-adrenergic receptor agonist further enhanced this response in vivo through PPAR α but did not induce detrimental FFA-sensitive response driven by toll-like receptor 4 (TLR4). This is likely due to the mixture of FFA released from the adipose stores. Indeed, fatty acids that accumulated in the liver of *Ppara*^{hep-/-} mice during fasting were mostly oleic (C18:1n-9) and linoleic acids (C18:2n-6), and not only saturated fatty acids such as palmitic acid (C16:0). Interestingly, it has been shown that palmitic acid cannot activate TLR4 in the presence of unsaturated FFA.³⁷

Overall, our data highlight hepatic PPAR α activity regulation by fatty acids released from adipocytes. This contrasts with the previous evidence that PPAR β/δ rather than PPAR α may act as a FFA sensor.³⁸ However, our data support the possibility that this adipose-derived signal is time-restricted and specifically efficient in early night. Moreover, other pathways likely influence PPAR α activity by providing ligands.^{34 35 39 40} Several insulin-sensitive signalling mechanisms influence hepatic PPAR α , and adipocyte lipolysis is insulin sensitive.⁴¹ Thus, insulin may coordinate hepatic PPAR α , both through cell-autonomous mechanisms and adipocyte lipolysis inducing interorgan communication mediated by FFA release. Our findings also correspond with the recent evidence that adipocyte lipolysis may regulate hepatic *Fgf21*.⁴² Circulating FGF21 was strictly dependent on hepatocytic PPAR α activation during fasting. Most circulating FGF21 is liver-derived⁴³ and *Ppara*^{-/-} mice

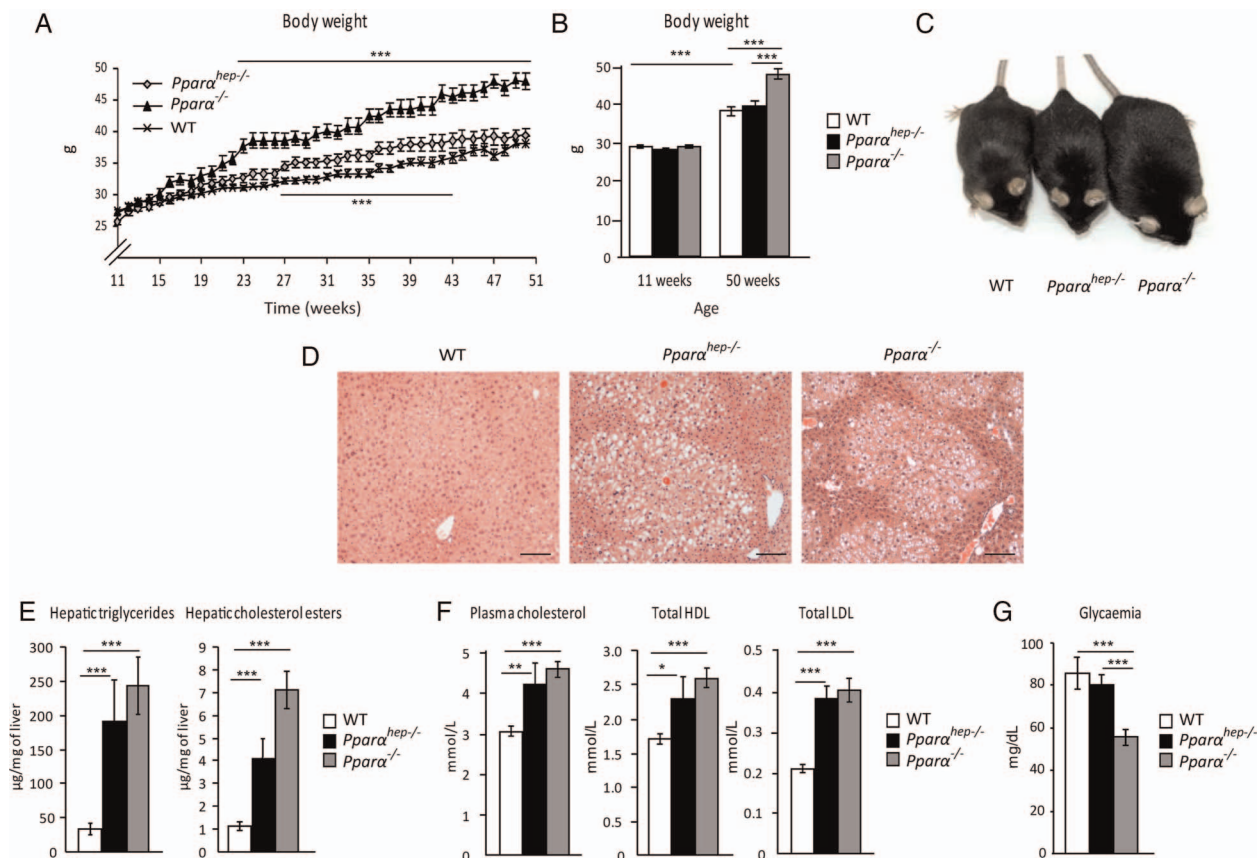


Figure 8 Mice deficient in hepatic peroxisome proliferator-activated receptor α (PPAR α) develop spontaneous hepatic steatosis during ageing. Wild-type (WT), PPAR α hepatocyte knockout (*Ppara*^{hep-/-}) and PPAR α knockout (*Ppara*^{-/-}) mice were fed a chow diet for 51 weeks. All mice were killed at ZT16 in a non-fasted state. (A) Body weight gain was followed over time. (B) Comparison of body weight between weeks 11 and 50. (C) Representative pictures of 52-week-old mice of the three genotypes. (D) Representative images of H&E staining of liver sections. Scale bar, 100 μ m. (E) Quantification of hepatic triglycerides and cholesterol esters. (F) Measurement of plasma total cholesterol, HDL cholesterol and LDL cholesterol. (G) Fasting glycaemia. Data are shown as mean \pm SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.005$.

show very little FGF21.^{11 12} Other transcription factors can also regulate hepatic *Fgf21* expression⁴⁴⁻⁴⁸ and PPAR α is also expressed in extrahepatic tissues.¹³ Our findings in *Ppara*^{hep-/-} mice showed very little FGF21 without hepatic PPAR α in both fed and fasted states. *Ppara*^{-/-} mice are hypoglycaemic and hypothermic during fasting⁷ and FGF21 is known for its endocrine effect on glucose homeostasis and thermogenesis.¹³ However, compared with fasted *Ppara*^{-/-} mice, fasted *Ppara*^{hep-/-} mice showed reduced hypoglycaemia and hypothermia while FGF21 was equally absent in both models. This indicates that extrahepatic PPAR α strongly influenced whole-body glucose homeostasis and temperature independently of hepatocyte PPAR α and FGF21 production during fasting. In addition, while FGF21 prevents steatosis in different mouse models^{13 30} and FGF21 reduces hepatic lipids in WT mice, its overexpression is not sufficient to protect from lipid accumulation in *Ppara*^{hep-/-} and in *Ppara*^{-/-} mice. Therefore, the absence of FGF21 is not the primary cause for the steatosis observed in *Ppara*^{hep-/-} mice.

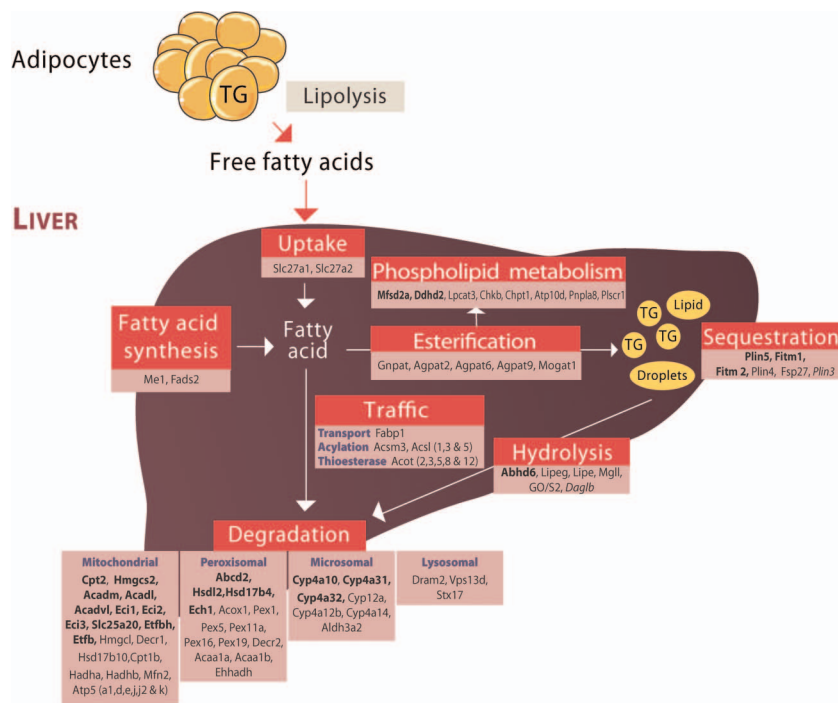
Lack of hepatic PPAR α impairs the liver's ability to use FFA from acute lipolysis, resulting in steatosis. MCD diet-induced weight loss^{49 50} also correlated with hepatic PPAR α activity, suggesting that chronic lipolysis elevates hepatocytic PPAR α activity in non-fasted mice. In agreement with the findings in whole-body PPAR α -deficient mice,²⁰ our data demonstrated that the absence of hepatocytic PPAR α was sufficient to increase MCD diet-induced liver damage. FGF21 expression/circulating levels

increased in steatohepatitis, supporting the possibility that elevated FGF21 may reflect liver stress without fasting. This MCD diet-induced FGF21 increase was not strictly PPAR α -dependent, consistent with the findings that amino acid deprivation induces hepatic FGF21 expression through ATF4.⁴⁴ PPAR α presence led to greater FGF21 increase, and may contribute to hepatoprotection from lipotoxic lipid accumulation.³⁰

MCD diet is widely used for preclinical NASH studies. However, it has many limitations, including the important weight loss that occurs in mice fed such diet. Therefore, we also tested the role of hepatocyte PPAR α in lipid homeostasis in response to a short-term HFD feeding, which is sufficient to initiate early neutral lipid accumulation that may promote NAFLD. *Ppara*^{hep-/-} mice showed marked increase in hepatic steatosis in response to 2 weeks of HFD feeding (see online supplementary file 11) suggesting that hepatocyte PPAR α plays a dual role in exogenous (dietary) as well as in endogenous (released from adipocyte lipolysis) fatty acid homeostasis.

Previous studies have shown that *Ppara*^{-/-} mice show a significant alteration of systemic lipid metabolism that leads to hepatic steatosis in ageing mice. Since PPAR α is active in skeletal muscles,²³ adipose tissues,^{24 25} intestines,²⁶ kidneys²⁷ and heart,²⁸ which all contribute to fatty acid homeostasis, it is impossible to determine whether the spontaneous steatosis that occurs in ageing *Ppara*^{-/-} mice originates from a defect in the hepatocytic PPAR α activity. This led us to investigate ageing-related differences between *Ppara*^{-/-} and *Ppara*^{hep-/-}

Figure 9 Overview of hepatocyte-specific peroxisome proliferator-activated receptor α (PPAR α)-regulated genes involved in fatty acid metabolism. This figure was designed based on transcriptome analysis of PPAR α -dependent gene expression in hepatocytes. Genes listed in regular font are induced by fenofibrate and by fasting in wild-type (WT) but not in *Ppara*^{hep-/-} mice. Genes in italics are repressed by fenofibrate and by fasting in WT but not in *Ppara*^{hep-/-} mice. Genes referenced in bold are downregulated in *Ppara*^{hep-/-} compared with WT mice, whatever the conditions.



mice. During ageing, *Ppara*^{-/-} mice became overweight and developed steatosis, while *Ppara*^{hep-/-} mice only suffered steatosis. Therefore, neither obesity nor hyperglycaemia, which are both known to promote NAFLD,^{15 16} is responsible for the steatosis observed in mice with hepatocyte-specific PPAR α deletion.

Furthermore, both *Ppara*^{-/-} and *Ppara*^{hep-/-} ageing mice were hypercholesterolaemic. This is likely due to the dysregulation of apolipoproteins gene expression as well as cholesterol transport (Abcg8) as revealed in microarray analysis (see online supplementary file 12A). It is also possible that the cholesterol biosynthesis pathway driven by SREBP-2 may be dysregulated in the absence of PPAR α since some of the SREBP-2 genes are elevated in *Ppara*^{-/-} and/or in *Ppara*^{hep-/-} mice (see online supplementary file 12B). Therefore, this suggests that drugs that activate hepatocytic PPAR α will likely influence whole-body fatty acid and cholesterol homeostasis.

Altogether, our extensive analysis performed in *Ppara*^{hep-/-} mice has allowed us to extend the evidence for the central role of PPAR α in hepatocyte fatty acid homeostasis (figure 9). PPAR α is strikingly essential to many aspects of fatty acid homeostasis including degradation through oxidative pathways. Our work provides the first demonstration that hepatocyte-specific PPAR α deletion impairs whole-body fatty acid homeostasis during fasting, MCD and HFD feeding as well as in ageing. These findings underscore the central role of PPAR α in the clearance of dietary fatty acids and of FFA released from adipocytes, the major source of lipid accumulation in NAFLD. These data highlight the relevance of PPAR α as a drug target for NAFLD treatment.

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Contributors AM initiated the project, designed experiments, performed experiments, analysed the data and wrote the paper. AP, EF, SD, YL, FL, MR, CL, FB and AI contributed to design experiments, perform experiments and to analyse the data. VB designed and performed a critical experiment. JB-M, TAS, PC and LL provided critical analysis and technical support. SL contributed to analyse the data. GM, FR and TP provided critical materials and contributed to design the project. NL, CP and DL critically contributed to design the project and supervised experiments. WW provided critical reagents, designed the project, analysed the data and wrote the paper. HG designed the project, performed experiments, analysed the data and wrote the paper.

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