CHAPTER 4

LRH-1 plays a central role in hepatic triglyceride metabolism

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ABSTRACT

The transcription factor liver receptor homolog-1 (LRH-1/NR5A2) has been shown to be of vital importance to sustain life in mice. In the liver, LRH-1 is important in the regulation of glucose and bile acid metabolism. Here we show that expression of the LRH-1 gene in liver biopsies of obese human subjects correlates negatively with the extent of NAFLD and NASH, indicating that LRH-1 also may play a crucial role in hepatic triglyceride metabolism. The causality of this effect was investigated in conditional whole-body *Lrh1* knockdown mice. We show here that *Lrh1* knockdown impairs PPARα signaling and decreases fatty acid β-oxidation and ketogenesis, whereas *in vitro Lrh1* overexpression induces *Ppara* expression. Conditional *Lrh1* knockdown mice develop a fatty liver phenotype, characterized by low circulating ketone bodies, high levels of plasma non-esterified fatty acids and hepatic steatosis in concordance with the human data. Conclusion: we show that LRH-1 plays a pivotal role in the control of hepatic triglyceride levels.

INTRODUCTION

Nutrient sensing transcription factors play a critical role in the maintenance of metabolic homeostasis in most species. The concerted action of the transcriptional network fine-tunes the interaction of an organism with its environment. The liver receptor homolog-1 (LRH-1/NR5A2) has a vital role in this network. Systemic disruption of the *Lrh1* gene in mice was shown to be embryonically lethal (1), but the vital importance of LRH-1 is not restricted to the embryonic phase. Recent data show that conditional *Lrh1* knock-out mice die 9-14 days after tamoxifen-induced disruption of the gene (2).

LRH-1 belongs to the NR5A family of nuclear receptors. It binds DNA as a monomer and is closely related to the orphan receptor SF-1. LRH-1 is expressed predominantly in liver and intestine, but also in pre-adipocytes, ovaria, pancreas and various other tissues (3, 4). LRH-1 exerts diverse functions depending on its site of expression. In the liver, LRH-1 is involved in the regulation of bile acid and glucose metabolism (5-9).

Recently, it has been shown that LRH-1 is involved in the control of lipid metabolism as well (10). Analysis of hepatic LRH-1 DNA binding sites by ChIP-seq followed by Gene ontology analysis revealed that LRH-1 binding occurs in proximity of genes related to lipid metabolism. Moreover, the presence of LRH-1 appeared to be required for the anti-steatotic effects of 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC; C12:0/C12:0)(11), a natural LRH-1 agonist (12). These data suggest involvement of LRH-1 in the regulation of hepatic triglyceride metabolism. Abnormal accumulation of triglycerides in the liver, *i.e.*, hepatic steatosis, represents one of the hallmarks of the metabolic syndrome (13). Hepatic steatosis or non-alcoholic fatty liver disease (NAFLD) can remain a benign, non-inflammatory condition without adverse effects, but may also progress to non-alcoholic steatohepatitis (NASH) and cirrhosis, conditions that severely compromise normal liver function and ultimately may result in end-stage liver disease and hepatic carcinoma (14).

To investigate the role of LRH-1 in the pathophysiology of human hepatosteatosis, we determined LRH-1 expression in liver tissue of a cohort of 125 extensively phenotyped obese patients with varying degrees of NAFLD and NASH. These data showed a negative correlation between LRH-1 expression and steatosis in human liver lipid metabolism. To further evaluate the role of LRH-1 in the control of hepatic lipid metabolism we used a conditional whole-body *Lrh1* knockdown mouse model (7). The

advantage of this model is that a hypomorph mouse of the *Lrh1* gene is generated that does survive for over a year. Moreover, due to the residual activity of LRH-1, regulatory network structure is kept intact as much as possible and less compensatory regulation of adjacent network genes can be expected. Our study shows that *Lrh1* knockdown (LRH-1-KD) disturbs hepatic triglyceride homeostasis primarily via downregulation of PPARα, resulting in decreased fatty acid oxidation and ketogenesis. LRH-1-KD mice develop hepatic steatosis specifically in periportal areas of the liver. Circulating ketone bodies are decreased, whereas plasma NEFA levels and medium- and long-chain acylcarnitines are increased. Thus, our data demonstrate that LRH-1 is an important regulator of hepatic lipid metabolism.

EXPERIMENTAL PROCEDURES

Liver biopsies were obtained from patients visiting the obesity clinic of the Antwerp University Hospital, as recently described by Francque *et al.* (15) and analyzed by two experienced pathologist for histological features of NASH. LRH-1 knockdown (LRH-1-KD) mice were obtained commercially from Taconic Artemis and described before (7). All experiments were approved by the Ethical Committee for Animal Experiments of the University of Groningen.

ChiP-Seq data analysis was performed on the dataset kindly provided by Dr. Timothy F Osborne, University of California, USA, and visualized onto UCSC genome browser (16). For gene array, hybridization of cRNA and washing of Affymetrix GeneChip Mouse Genome 430 2.0 arrays was performed according to standard Affymetrix protocols.

AML12 cells (ATCC CRL-2254) were maintained in Dulbecco's Modified Eagle's Medium and HEK293AD cells were grown in DMEM for transient transfection. Adenoviral transductions were performed as described previously (7).

All values are presented as Tukey's Box-and-Whiskers plot using median with 25^{th} to 75^{th} percentile intervals (P_{25}-P_{75}) or bar charts with median +/- range. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, USA).

For more detailed Experimental Procedures see Supplemental Information.

RESULTS

LRH-1 expression correlates with NASH in human liver

To study LRH-1 expression in human liver, LRH-1 mRNA levels were analyzed in liver biopsies of 125 patients presenting to the Antwerp University Hospital (15). LRH-1 expression was significantly different between patients without or with the presence of NASH according to Brunt *et al.* (17) (p=0.016) with the lowest values in the NASH group. LRH-1 expression was also different according to the steatosis grade (p=0.011), the severity of the necroinflammation as reflected by the NASH Activity Score (NAS) (p=0.004) and fibrosis stage (p=0.014) with highly significant negative correlations in regression analysis (p=0.006, 0.001 and 0.023 for steatosis, NAS and fibrosis (18) respectively). Thus, LRH-1 expression negatively correlates with the severity of NAFLD and NASH in humans.

Phenotypic analysis of conditional LRH-1-KD mice

To further evaluate the role of LRH-1 in the control of hepatic lipid metabolism we used a conditional whole-body *Lrh1* knockdown mouse model. This model circumvents the early embryonic lethality caused by complete *Lrh1* deficiency, by using a conditional short hairpin RNA (shRNA) knockdown strategy (7). *Lrh1* knockdown (LRH-1-KD) mice exhibited less than 5 percent of liver *Lrh1* expression compared to wildtype mice (7). Such so-called hypomorphic alleles are very useful for studying essential genes at the organism level (19).

Chow-fed LRH-1-KD mice displayed a significantly increased liver weight compared to their wildtype littermates and hence an increased liver-to-bodyweight ratio (Table 1). Conversely, gonadal WAT mass was significantly reduced in the LRH-1-KD mice (Table 1).

Hepatic lipid content is increased by *Lrh1* knockdown

Hepatic total fatty acid and triglyceride levels were significantly elevated in LRH-1-KD mice compared to their wildtype littermates (Table 1, Figure 1A/B). No changes were observed in either hepatic protein, cholesterolester or phospholipid levels (Table 1): the latter implying that the increase in fatty acid content is solely due to triglyceride accumulation.

Table 1. Basic characteristics of wildtype and LRH-1-KD mice.

Animal characteristics	Wildtype	LRH-1-KD	Change
Bodyweight (g)	28.7 (25.5 – 29.4)	24.8 (24.0 – 25.8)	
Liverweight (g)	1.0 (1.0 – 1.1)	1.3 (1.2 – 1.3)*	↑
Liver/bodyweight (%)	3.8 (3.7 – 3.9)	5.2 (4.7 – 5.4)*	↑
White adipose tissue weight (g)	0.8 (0.6 – 1.2)	0.3 (0.2 – 0.4)*	↓
White adipose tissue/bodyweight (%)	2.7 (2.5 – 3.5)	1.2 (0.8 – 1.2)*	↓
Plasma parameters	**Wildtype**	**LRH-1-KD**	**Change**
Triglycerides (mM)	0.10 (0.08 – 0.13)	0.12 (0.11 – 0.13)	
Total cholesterol (mM)	1.8 (1.7 – 1.8)	2.1 (2.0 – 2.3)	
NEFA (mM)	0.28 (0.20 – 0.35)	0.38 (0.36 – 0.44)*	↑
β-hydroxybutyrate (mM)	0.42 (0.42 – 0.59)	0.15 (0.14 – 0.27)*	↓
Hepatic metabolic parameters	**Wildtype**	**LRH-1-KD**	**Change**
Triglycerides (µmol/g liver)	19.0 (18.4 – 24.3)	34.0 (32.9 – 34.4)*	↑
Total cholesterol (µmol/g liver)	2.3 (2.1 – 2.6)	3.0 (2.9 – 3.6)*	↑
Free cholesterol (µmol/g liver)	1.9 (1.9 – 1.9)	2.7 (2.4 – 2.9)*	↑
Cholesterolesters (µmol/g liver)	0.5 (0.4 – 0.7)	0.6 (0.2 – 0.7)	
Phospholipids (µmol/g liver)	35.0 (34.0 – 50.3)	36.0 (35.5 – 40.0)	
Proteins (mg/g liver)	182 (146 – 223)	171 (164 – 173)	

*$p < 0.05$

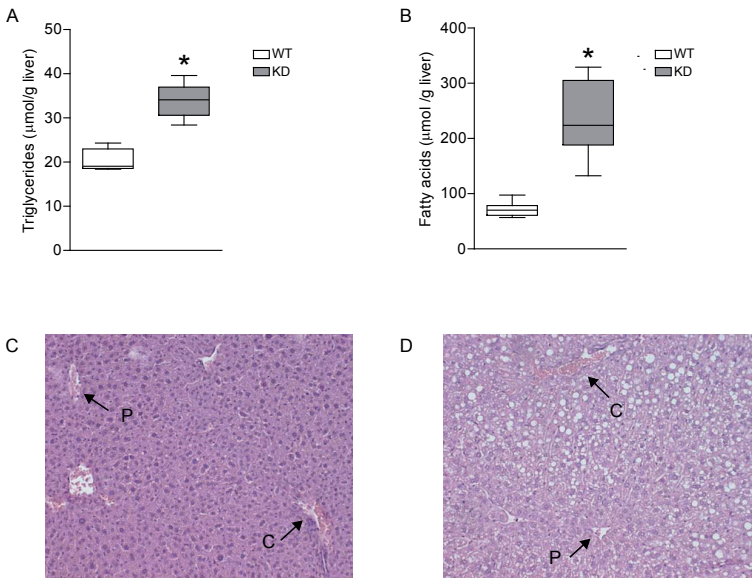

Figure 1. Lrh1 knock-down results in hepatic steatosis. (A) Hepatic triglyceride levels are increased in LRH-1-KD mice. (B) Hepatic fatty acid levels are increased in LRH-1-KD mice. Liver histology upon hematoxylin/eosin staining shows fat accumulation in livers of LRH-1-KD mice (D) but not in wildtype littermates (C), portal and central venes indicated as P and C respectively.

Liver histology upon hematoxylin/eosin staining confirmed fat accumulation in livers of LRH-1-KD mice (Figure 1C/D). Fat appeared to be mainly deposited in periportal regions of the liver lobules, *i.e.*, the predominant site of oxidative energy metabolism (20). Conversely, in perivenous zones, the predominant sites of *de novo* lipid synthesis (20), virtually no fat deposition was observed. As a control, no steatosis was observed in wildtype doxycycline-treated mice (Figure 1) or in mice expressing a control doxycycline-inducible shRNA against DCL1, a protein important for corticogenesis (21) (data not shown), indicating that doxycycline treatment or the expression of a doxycycline-inducible shRNA did not cause the observed disturbance of hepatic triglyceride metabolism.

***Lrh1* knockdown changes metabolic routes involved in lipid metabolism**

In order to get insight in the mechanism underlying hepatic accumulation of triglycerides in LRH-1-KD-mice, a comparative Affymetrix microarray analysis was performed on liver tissue obtained from 4h fasted mice. Gene ontology analysis revealed that *Lrh1* knockdown affects transcriptional networks involved in inflammatory responses and lipid metabolism (Table 2). Several genes found to be upregulated upon *Lrh1* knockdown are mainly involved in inflammatory responses, which is consistent with previous studies showing that *Lrh1* has anti-inflammatory effects and negatively regulates the acute phase response (22-24). Interestingly, downregulated genes appeared to be mainly associated with lipid and steroid metabolism. Careful analysis of this gene set revealed that many of the downregulated genes are actually involved in fatty acid catabolism (Table 2). Quantitative real-time PCR (qPCR) confirmed decreased expression of genes involved in fatty acid oxidation. Expression of *Ppara*, a lipid-sensing nuclear receptor and key regulator of fatty acid β-oxidation, ketogenesis and the adaptive response to fasting (25), was significantly decreased (Figure 2A). Also PPARα target genes involved in beta-oxidation and ketogenesis were downregulated, including carnitine palmitoyltransferase I (*Cpt-1a*), acyl-CoA oxidase (*Acox1*), HMG-CoA synthase (*Hmgcs2*) and fibroblast growth factor 21 (*Fgf21*)(26, 27) (Figure 2A), recently identified as a 'hepatokine' that is controlled by PPARα and stimulates hepatic fatty acid oxidation and ketogenesis (26-30). However, plasma Fgf21 levels were not different between LRH-1-KD mice and wildtype mice (Figure S1).

Table 2. Gene ontology analysis on livers of wildtype and LRH-1-KD mice.

	Function annotation	*p* value	# Molecules	% of gene set
Gene ontology	lipid metabolic process	5.40E-14	63	10.3
	defense response	1.90E-10	43	7.0
	organic acid metabolic process	1.60E-09	43	7.0
	cellular ketone metabolic process	3.10E-09	43	7.0
	sterol metabolic process	1.30E-08	16	2.6
	innate immune response	3.40E-08	18	2.9
	regulation of response to stimulus	1.60E-07	31	5.1
	response to other organism	4.20E-07	25	4.1
	cellular lipid metabolic process	4.40E-07	38	6.2
Upregulated	defense response	5.70E-14	37	9.6
	response to other organism	6.40E-11	24	6.2
	innate immune response	9.50E-11	17	4.4
	regulation of immune system process	2.10E-10	27	7.0
	positive regulation of response to stimulus	1.30E-09	20	5.2
	positive regulation of immune response	3.60E-09	17	4.4
	positive regulation of immune system process	7.30E-09	20	5.2
	immune effector process	9.50E-09	16	4.2
Downregulated	lipid metabolic process	5.70E-20	46	20.2
	sterol metabolic process	9.00E-13	15	6.6
	organic acid metabolic process	3.40E-12	30	13.2
	cellular ketone metabolic process	5.90E-12	30	13.2
	lipid biosynthetic process	7.80E-10	21	9.2
	cellular lipid metabolic process	3.00E-09	26	11.4
	lipid transport	2.40E-05	10	4.4
	lipid localization	4.20E-05	10	4.4

	Gene ID	Name	Function
Lipid metabolic process	19013	peroxisome proliferator activated receptor alpha	nuclear receptor, transcription factor involved in lipid metabolism
	56794	2-hydroxyacyl-CoA lyase 1	hydrolysis of fatty acids in peroxisomes
	433256	acyl-CoA synthetase long-chain family member 5	lipid biosynthesis and fatty acid degradation
	26897	acyl-CoA thioesterase 1	hydrolysis of long-chain acyl-CoAs of C12-C20-CoA in chain length to free fatty acid and CoA
	320024	arylacetamide deacetylase-like 1	hydrolysis of lipids
	12894	carnitine palmitoyltransferase 1a, liver	transport of fatty acids into mitochondria
	76267	fatty acid desaturase 1 and 2	desaturation of fatty acids
	20249	stearoyl-Coenzyme A desaturase 1	desaturation of fatty acids
	22359	very low density lipoprotein receptor	metabolism of apoprotein-E-containing triacylglycerol-rich lipoproteins (such as VLDL)
	56690	malonyl-CoA decarboxylase	malonyl-CoA to acetyl-CoA conversion

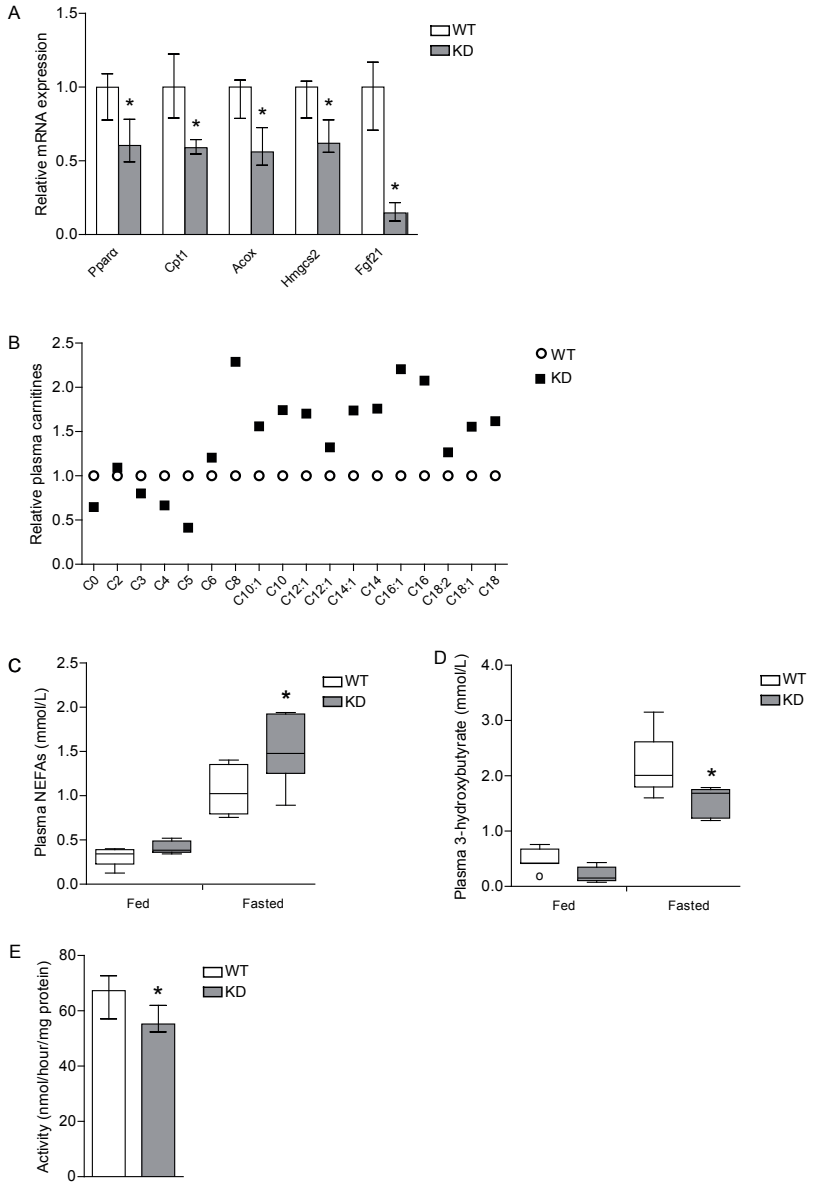

Figure 2. Decreased β-oxidation in LRH-1-KD animals. (A) Decreased gene expression of *Ppara* and its target genes is in LRH-1-KD mice. (B) Relative levels of medium- and long-chain acylcarnitines are increased in plasma of LRH-1-KD mice. Plasma NEFA levels (C) are increased, whereas 3-hydroxybutyrate levels (D) are decreased in fasted LRH-1-KD mice. (E) *Ex vivo* [9,10-^{3}H] myristic acid oxidation is decreased in primary hepatocytes isolated from LRH-1-KD mice compared to wildtype mice.

***Lrh1* knockdown decreases hepatic oxidation of fatty acids**

Thus, knockdown of *Lrh1* appears to affect hepatic lipid metabolism, particularly the breakdown of fatty acids in mitochondria and/or peroxisomes. Hepatocytes oxidize fatty acids to form acetyl-CoA that can subsequently be used for ketone body production. Defects in fatty acid oxidation are therefore routinely detected by acylcarnitine spectrum analysis. Determination of hepatic and plasma acylcarnitine profiles revealed accumulation of medium- and long-chain acylcarnitines (C6-C18) in both compartments of LRH-1-KD mice compared to wildtype mice (Figure 2B), suggestive of incomplete oxidation of fatty acids. An increase in plasma non-esterified fatty acid (NEFA) levels was also observed in LRH-1-KD mice (Table 1, Figure 2C), suggesting that decreased hepatic oxidation results in their accumulation in plasma. Moreover, β-hydroxybutyrate, a marker of fatty acid oxidation and subsequent ketogenesis in liver, was decreased in plasma of LRH-1-KD mice under fed and fasted conditions (Table 1, Figure 2D). To directly assess hepatic fatty acid oxidation, *ex vivo* myristic acid oxidation was measured in primary hepatocytes isolated from wildtype and LRH-1-KD mice by assessing the rate of conversion of [9,10-^{3}H] myristic acid into $^{3}H_2O$. Primary hepatocytes isolated from LRH-1 KD mice indeed showed decreased rates of β-oxidation compared to wildtype hepatocytes (Figure 2E).

To evaluate whether other changes in lipid metabolism could contribute to the development of hepatic steatosis, the hepatic fatty acid profile was analyzed. In addition to accumulation of non-essential fatty acids, LRH-1-KD mice exhibited increased concentrations of the essential fatty acids linoleic acid (C18:2ω6) and linolenic acid (C18:3ω3) (Figure 3A) which cannot be synthesized *de novo* and are hence derived from the diet. The expression of several genes involved in fatty acid synthesis such as *Lxra*, *Srebp1a*, *Srebp1c*, and *Acc1* remained unchanged (Figure 3B), whereas the expression of S*cd1* and the LRH-1 target gene *Fas* was even decreased. Assessment of *de novo* lipogenesis by MIDA (31), revealed no differences in lipogenesis between wildtype and LRH-1-KD mice (Figure 3C), which is consistent with the observed location of fat deposition, as no fat accumulation was observed in the perivenous zone, where lipogenesis occurs.

Hepatic export of very low-density lipoprotein (VLDL) particles was not different between LRH-1-KD and control mice (Figure 3D). The mRNA expression levels of apoprotein B100 (*apoB*) and microsomal triglyceride transfer protein (*Mttp*), both essential for VLDL synthesis, remained unchanged in livers of LRH-1-KD mice (data not shown).

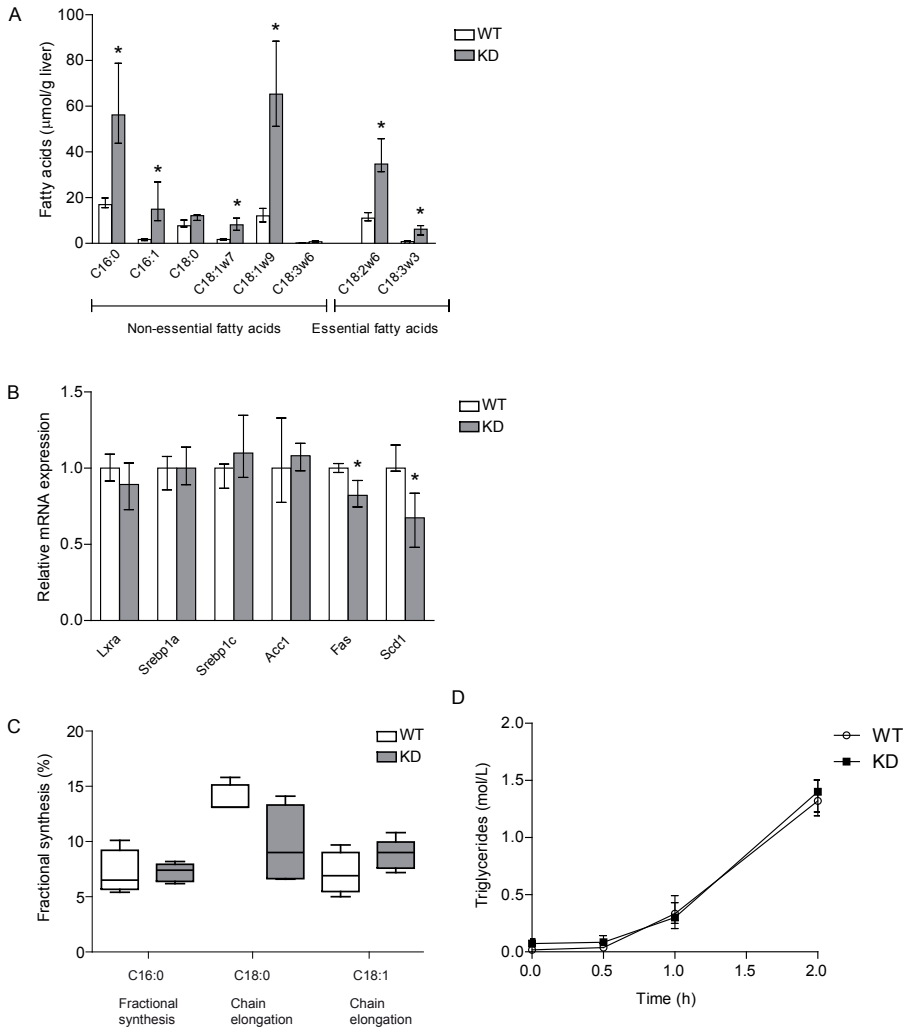

Figure 3. *De novo* lipogenesis and VLDL production are unaltered in LRH-1-KD mice. (A) Accumulation of both essential and non-essential fatty acids in livers of LRH-1-KD mice. (B) Expression of genes involved in *de novo* lipogenesis is unaltered or decreased in LRH-1-KD mice. (C) *De novo* lipogenesis measured by MIDA analysis is unchanged in LRH-1-KD mice. (D) The rate of very low-density lipoprotein (VLDL) production by the liver is similar in LRH-1-KD and wildtype mice.

In addition, total plasma TG concentrations and TG distribution in plasma lipoproteins were found to be unchanged between wildtype and LRH-1-KD animals (Table 1, Figure S2). Finally, total intestinal fatty acid absorption was calculated and found to be not different between wildtype and LRH-1-KD mice (data not shown).

Taken together, these data indicate that hepatic fat accumulation in LRH-1-KD mice is not due to alterations in either *de novo* synthesis of fatty acids, triglyceride export in VLDL particles or increased intestinal lipid absorption, but rather to suppressed β-oxidation capacity.

LRH-1 binds the LRH-1 site in the PPARα promoter and regulates its transcriptional activity

Whereas whole-body knockdown of *Lrh1* was shown to impair the expression of *Ppara* and its target genes, adenoviral-mediated overexpression of *Lrh1* in HepA1.6 cells consistently induced the mRNA expression of *Ppara* (Figure 4A). The effects of LRH-1 on fatty acid β-oxidation and ketogenesis *in vivo* and on PPARα and its target genes suggest that LRH-1 might regulate PPARα expression. Recently, Chong *et al.* (10) suggested a role for LRH-1 in lipid metabolism based on a non-biased genome-wide ChIP-seq approach on mouse liver. Using this ChIP-seq dataset, LRH-1 binding to the PPARα promoter was visualized. Several LRH-1 binding peaks were identified in the PPARα proximal promoter (-1500 bp upstream of the transcription start site)(Figure 4B). In contrast, no peaks were detected on the FGF21 promoter (Figure 4C).

Alignment of the mouse PPARα promoter sequence with the known LRH-1 consensus binding site identified several motifs with partial (-670, -861) or total (-571) homology with the consensus at -861, -670 and -571 base pairs upstream of the PPARα transcription start site (Figure 5A). To determine whether LRH-1 directly binds to those sites electrophoretic mobility shift assays were performed. Only the -670 and -571 sites specifically bound LRH-1 under these conditions with the -571 site displaying the strongest affinity for LRH-1 (Figure 5B). Interestingly, these two sites match with the strongest peak within the *Ppara* gene as determined by the LRH-1 ChIP-seq data analysis (Figure 4B). These strong protein-DNA complexes were efficiently competed by preincubation with increasing amounts of unlabeled wildtype probes, but not by the mutated oligonucleotides (Figure 5C). These results demonstrate that LRH-1 binds to the PPARα promoter *in vitro* and *in vivo*. Furthermore, a dose-dependent increase

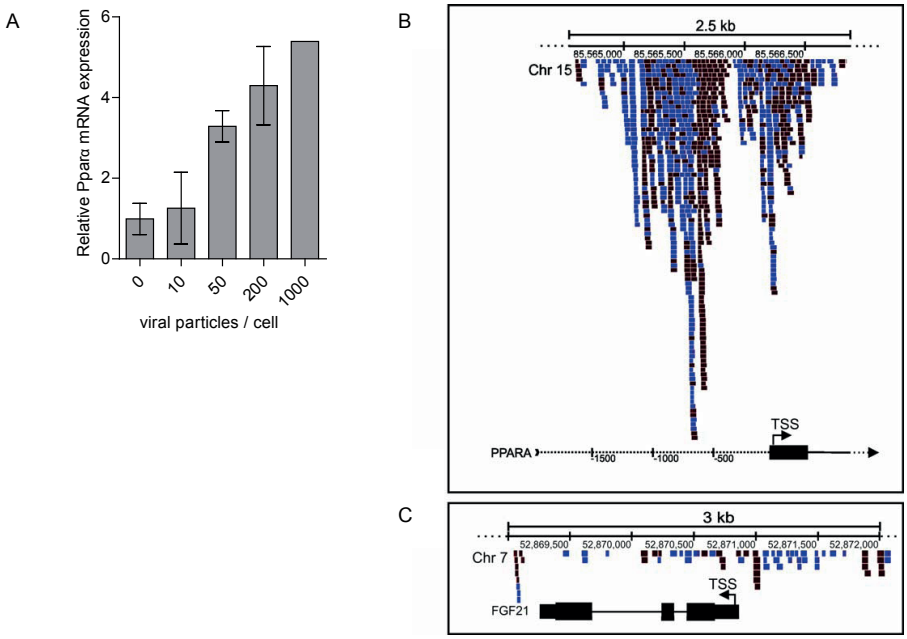

Figure 4. LRH-1 is recruited to the mouse PPARα promoter. (A) mRNA expression of *Ppara* is dose-dependently increased by adeno-LRH-1 transduction in HepA1.6 cells. Promoter regions of PPARα and FGF21 were inspected for the presence of LRH-1 ChIP-seq peaks visualized onto UCSC genome browser. (B) Representative view of ChIP-seq peaks on the mouse PPARα promoter. Promoter region and transcription start site (TSS) are indicated as described (50). (C) Representative view of ChIP-seq peaks on mouse FGF21 gene and promoter region. TSS is indicated as described (25). Shown are chromosomal locations according to the July 2007 Mouse Genome Assembly (mm9). Blue and red tags represent sequence reads from opposite DNA strands. The ability of LRH-1 to bind putative response elements in the PPARα gene was examined by EMSA as outlined in Materials and Methods.

in transcriptional activity was observed when a luciferase reporter gene driven by the -926 to +131 sequence of the mouse PPARα promoter was co-transfected with increasing amounts of LRH-1 (Figure 5D). Thus, PPARα regulation by LRH-1 occurs at the transcriptional level.

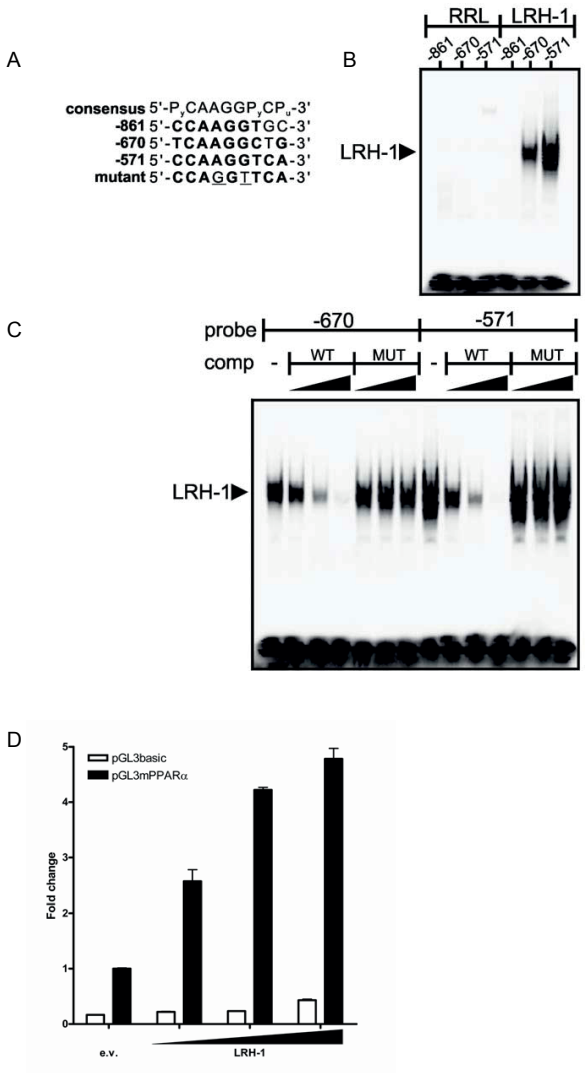

Figure 5. LRH-1 binds to two adjacent regions of the mouse PPARα promoter and increases its transcriptional activity. (A) Partial sequences of the oligonucleotides probes (antisense strand only) corresponding to the putative LRH-1 responses elements from the PPARα gene and the mutated derivative of this site (mutant). The sequences -861, -670 and -571 starting respectively 861, 670 and 571 base pairs downstream of the PPARα TSS are aligned with the consensus LRH-1 response element (matching bases are indicated by bold-typed letters and mutated bases are underlined). (B) EMSA was performed using *in vitro* synthesized LRH-1 or unprogrammed rabbit reticulocyte lysate (RRL) and ^{32}P-labeled probes as indicated. (C) Competition EMSA was performed using *in vitro* synthesized LRH-1 and increasing concentrations (2X, 5X, 25X) of unlabeled competitor probes or mutated, unlabeled probe as indicated. (D) AML12 cells were transfected with a luciferase reporter construct driven by the mouse PPARα promoter or with pGL3basic as a control and increasing amounts of LRH-1 expression vector (50, 100, 200 ng) or of the empty expression vector (e.v.). Luciferase activity was assayed 48h after transfection. RLU values were normalized to pGL3mPPARα basal activity.

***Lrh1* knockdown mice show a decrease in white adipose tissue mass**

Compared to wildtype mice, LRH-1-KD mice show decreased gonadal white adipose tissue mass (Table 1). Furthermore, total body fat content measured by DEXA scanning was decreased in these mice (Figure S3A). In line with these results, mRNA expression of hormone-sensitive lipase (*Hsl*) and adipose triglyceride lipase (*Atgl*) was increased in WAT isolated from LRH-1-KD mice (Figure S3B). However, HSL and ATGL protein levels were not changed in the knockdown animals (Figure S3C).

DISCUSSION

Nutrient-sensing transcription factors play key roles in the maintenance of organismal energy homeostasis and are active in complex networks. In this study, we show that the expression of the pivotal transcription factor LRH-1 in livers of a large cohort of patients with NAFLD, correlated negatively with the extent of steatosis as well as with the severity of the necroinflammatory changes and fibrosis in NASH. Using an inducible knockdown mouse model, we subsequently investigated the molecular mechanism by which LRH-1 may control hepatic triglyceride metabolism. We identified PPARα to be an important bona fide downstream target of LRH-1 in the liver. LRH-1 is able to directly bind and activate PPARα, which is known as a major regulator of hepatic fatty acid metabolism, particularly during fasting: its actions promote uptake, utilization and catabolism of fatty acids and ketogenesis. FGF21 is a downstream target of PPARα that also stimulates hepatic fatty acid oxidation, ketogenesis and energy metabolism (26-28, 30, 32). In LRH-1 KD mice the expression of *Fgf21* was decreased, however plasma levels were unchanged. Since no promoter binding was detected from ChIP-seq data (10), decreased expression is more likely to represent an indirect effect via PPARα. The reduction in PPARα signaling in LRH-1-KD mice resulted in decreased fatty acid oxidation and suppressed ketogenesis, leading to the development of hepatic steatosis because excess free fatty acids are stored as triglycerides. These results are in line with recent data showing that activation of LRH-1 by the phospholipid 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC; C12:0/C12:0) has anti-steatotic effects (11). Upregulation of inflammatory responses in LRH-1-KD mice, as evident from microarray data analysis, might contribute to progression of hepatic steatosis to NASH, which is in line with the human data.

Since we used a conditional whole-body *Lrh1* knockdown mouse model, the contribution of decreased *Lrh1* expression in other tissues to the overall phenotype cannot be ruled out. LRH-1-KD mice show decreased white adipose tissue mass and increased plasma NEFA levels. However, in WAT *Lrh1* expression is very low compared to expression in the liver or the intestinal tract. The expression of *Hsl* and *Atgl* in WAT was increased, but protein levels were not different (Figure S3). This suggests that - at least at the time of sacrifice - enhanced lipolysis was not present. We cannot rule out that this occurred at an earlier time point leading to decreased levels of WAT, thereby increasing the lipid load on the liver and exacerbating hepatic fat accumulation. Previously we have shown that LRH-1-KD mice exhibit decreased expression of intestinal *Fgf15* (7), which may contribute to the development of a fatty liver. It has been shown that the human ortholog FGF19 influences hepatic beta-oxidation (33, 34). However, also plasma TG-raising effects of FGF19 have been shown (35), possibly through different FGF receptors and target tissues. The exact role of FGF15/19 in lipid metabolism therefore remains to be determined.

Previously, two hepatic *Lrh1* knock-out models have been studied and showed no major differences in hepatic triglyceride content (6, 8). Apart from differences in background strain which may influence the phenotype, we speculate that complete knock-out of a gene, even when organ specific, induces a much more pronounced compensatory effect of adjacent network genes which may partly rescue the phenotype. Indeed, surprising differences were seen between embryonic and inducible *Lrh1* knock-out mouse models. For example, *Lrh1* deficiency in livers of albumin-Cre mice did not significantly alter *Cyp7a1* mRNA levels, whereas acute knock-out of hepatic *Lrh1* in *Lrh-1fl/fl* mice did decrease basal *Cyp7a1* mRNA levels (36) and conditional *Lrh1* knockdown mice could not upregulate *Cyp7a1*(7). Moreover, *Lrh1*$^{+/-}$ mice show a remarkable distinct phenotype (37, 38), whereas (conditional) systemic disruption of *Lrh1* is lethal (1). Thus, we speculate that due to its vital importance, chronic LRH-1 depletion leads to important compensatory regulation of adjacent network genes.

Given our results that LRH-1 knockdown leads to accumulation of hepatic triglycerides, we hypothesized that liver-specific *Lrh1* overexpression might protect against high fat diet-induced steatosis. Wildtype mice fed a high fat diet were injected with PBS or a self-complementary adeno-associated virus containing either the murine *Lrh1* gene, or GFP as a control. Unfortunately, only a 3-fold induction of hepatic *Lrh1* mRNA

expression could be achieved without substantial changes in the expression of well-known target genes, whereas GFP was observed (using fluorescence microscopy) in 80% of hepatocytes transfected with scAAV-GFP (data not shown). Apparently, liver *Lrh1* expression is tightly regulated by feedback control mechanisms, which complicates overexpression experiments *in vivo*.

Hepatic LRH-1 is essential for the expression of CYP8B1, a key enzyme in the synthesis of the primary bile acid species cholic acid (CA). Hence, depletion of liver *Lrh1* decreases the contribution of CA-derived bile acids to the bile acid pool (6-8). It has recently been reported that secondary bile acids, in particular deoxycholic acid (DCA) that is derived from CA, can have impact on hepatic triglyceride metabolism by potently inhibiting the NEFA importer protein FATP5(39) in the liver. Furthermore, 12-hydroxylated bile acids such as CA and DCA have been linked to dyslipidemia (40), providing another site of interaction between LRH-1 and triglyceride homeostasis. In addition, Chong *et al.* suggested that LRH-1 recruits the bile acid activated nuclear receptor FXR to lipid metabolic genes, thereby regulating genes of lipid metabolism in concert with FXR (10). Alterations in FXR activity may therefore also contribute to the observed phenotype.

To ensure energy supply during alternating periods of fasting and feeding, hepatic lipid and carbohydrate metabolism are tightly synchronized (41, 42). Several nuclear receptors, such as PPARα, show a strong circadian expression pattern (43, 44) and may link nutrient sensing (a.o. by fatty acids) to circadian control of metabolism. Co-ordinated regulation of both lipid and bile acid metabolism, couples intake of lipid substances to synthesis and secretion of bile acids to ensure proper lipid solubilization in the intestine. Because LRH-1 interacts with different nuclear receptors, transcription factors, including PGC-1α, SHP and SIRT1 (3, 45, 46, 46-49) and target genes involved in lipid and bile acid metabolism, it may be a key component of the coordinated response necessary to relay circadian signals into metabolic responses.

It should be noted that although there is a striking concordance between LRH-1 expression and hepatic steatosis in mice and man, there are also differences. The mice show a decrease in WAT, humans obviously not. Another interesting difference is that the steatosis in mice is periportal whereas steatosis in humans is mostly pericentral. Importantly, however, the LRH-1-KD mice show upregulation of inflammatory pathways which is in line with the correlation between LRH-1 expression and the severity of NASH in the human cohort.

In conclusion, LRH-1 is a key player in the metabolic network controlling hepatic lipid homeostasis. In human liver LRH-1 is negatively correlated with NASH severity. Targeted activation of LRH-1 may therefore be beneficial and additional to fibrates in combatting hepatic steatosis and its complications. On the other hand, when inhibiting LRH-1 as a potential target for contraception (50), lipid metabolic side-effects should be monitored.

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