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Liver Receptor Homolog-1 Is a Critical Determinant of Methyl-Pool Metabolism

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Balance of labile methyl groups (choline, methionine, betaine, and folate) is important for normal liver function. Quantitatively, a significant use of labile methyl groups is in the production of phosphatidylcholines (PCs), which are ligands for the nuclear liver receptor homolog-1 (LRH-1). We studied the role of LRH-1 in methyl-pool homeostasis and determined its metabolic effects using the methionine and choline–deficient (MCD) diet, which depletes methyl groups and results in a deleterious decrease in the PC-to-phosphatidylethanolamine ratio. We found that MCD diet–fed, liver-specific LRH-1 knockout mice (*Lrh-1*$^{-/-}$) do not show the expected decreased methyl-pool and PC/phosphatidylethanolamine ratio and are resistant to the hepatitis and fibrosis normally induced by the diet. Adaptive responses observed in wild-type mice on the MCD diet were also observed in *Lrh-1*$^{-/-}$ mice on a normal diet. This includes reduced expression of the highly active glycine-n-methyltransferase and the biliary phospholipid floppase multidrug-resistance protein 2 (Mdr2/Abcb4), resulting in reduced consumption of methyl groups and biliary PC secretion. *In vitro* studies confirm that *Gnmt* and *Mdr2* are primary LRH-1 target genes. Additional similarities between hepatic gene expression profiles in MCD diet–fed wild-type and untreated *Lrh-1*$^{-/-}$ mice suggest that methyl-pool deficiency decreases LRH-1 activity, and this was confirmed by *in vitro* functional results in cells maintained in MCD medium. *Conclusion*: LRH-1 is a novel transcriptional regulator of methyl-pool balance; when the methyl-pool is depleted, decreased LRH-1 transactivation suppresses expression of key genes to minimize loss of labile methyl groups. (HEPATOLOGY 2015; 00:000-000)

Balance of labile methyl groups (choline, betaine, methionine, and folate) and a constant supply of methyl donors (*S*-adenosylmethionine [SAM]) are important for liver function.[1,2] Patients with chronic liver disease such as liver cirrhosis, alcoholic and nonalcoholic fatty liver disease, or hepatocellular carcinoma often have reduced levels of methyl donors.[3-5] In addition, diets deficient in labile methyl groups or genetic manipulation to decrease generation of methyl donors results in liver injury in both humans and animal models, ranging from fatty liver to steatohepatitis and hepatocellular carcinoma.[2,6,7] Conversely, dietary supplementation with methyl groups and donors improves metabolic liver disease and function, which has been the basis for several clinical trials investigating the clinical effects of methyl donors in humans with chronic liver disease.[1] However,

Abbreviations: ChIP, chromatin immunoprecipitation; CYP2E1, cytochrome P450 2E1; Gamt, guanidinoacetate-n-methyltransferase; Gnmt, glycine-n-methyltransferase; LRH-1/NR5A2, liver receptor homolog-1; MCD, methionine/choline-deficient; Mdr2/Abcb4, multidrug-resistance protein 2; mRNA, messenger RNA; NASH, nonalcoholic steatohepatitis; PC, phosphatidylcholine; PCR, polymerase chain reaction; PE, phosphatidylethanolamine; Pemt, phosphoethanolamine-n-methyltransferase; PL, phospholipid; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; si, small interfering; WT, wild type.

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excess of methyl donors can also lead to liver injury.[1,2] Thus, hepatic methyl donor levels need to be maintained within a certain range, and either deficiency or excess can lead to abnormal liver function.

SAM is the principal methyl donor for more than 200 methylation reactions. SAM is generated from methionine through the enzyme methionine-adenosyltransferase, which exists as multiple isoforms.[1,2] Methyltransferases transfer methyl groups from SAM to several different substrates including DNA, RNA, proteins, phosphatidylethanolamine (PE), glycine, and guanidinoacetate. For regeneration of SAM, input of labile methyl groups from either the choline-betaine pathway or the folate cycle is required. The three most abundant methyltransferases are glycine-n-methyltransferase (Gnmt), guanidinoacetate-n-methyltransferase (Gamt), and phosphatidylethanolamine-n-methyltransferase (Pemt), which generate sarcosine, creatine, and distinct phosphatidylcholines (PCs),[8] respectively.[1,2] Gnmt is particularly important as it acts as a cellular buffer for maintaining constant cellular SAM levels.[1,2] Besides its best known role in transmethylation, SAM is linked to cysteine and glutathione biosynthesis through the transulfuration pathway and critically involved in polyamine biosynthesis and radical chemical reactions.[1] The regulation of SAM homeostasis is thought to be primarily based on enzyme activation and inhibition by intermediates of SAM metabolism.[2] Little is known about the potential impact of transcriptional regulation in SAM homeostasis.

Quantitatively, a significant use of labile methyl groups in the liver is generation of PCs in transmethylation reactions using the Pemt pathway.[9] Like the beneficial effects of supplementation of labile methyl groups, PC supplementation shows beneficial "lipotropic" effects on metabolic liver disease, as first described by Best and Huntsman in the 1930s.[10] These observations suggest that at least some of the lipotropic effects of labile methyl groups may be exerted by the action of PCs. Seventy-five years later, PCs were identified as ligands for the nuclear liver receptor homolog-1 (LRH-1/NR5A2),[11,12] and the lipotropic effects of certain PCs have been shown to clearly depend on the presence of LRH-1.[12] However, it is not known whether changes in the methyl-pool affect LRH-1 signaling and if LRH-1 contributes to methyl-pool homeostasis.

To test the potential role of LRH-1 in methyl pool and PC metabolism, we employed the methionine/choline-deficient diet (MCD) model, which markedly alters SAM homeostasis and PC pools and results in metabolic liver injury resembling nonalcoholic steatohepatitis (NASH).[7,13,14] We found that LRH-1 is a direct transcriptional regulator of methyl-donor homeostasis and biliary PC secretion as well as a regulatory target to maintain appropriate methyl-pool levels.

Materials and Methods

For a more detailed description of the methods used, please see the Supporting Information.

Animal Studies and Diets. Generation of Lrh-1 liver-specific knockout ($Lrh\text{-}1^{-/-}$) mice has been previously described.[15] Age-matched floxed Lrh-$1^{f/f}$ littermates served as wild-type (WT) controls. The MCD diet (TD.90262) and the corresponding amino acid control diet (chow, TD.94149) containing 350 g/kg choline dihydrogen citrate and 8.2 g/kg methionine were custom made by Harlan Laboratories (Madison, WI). Male WT and $Lrh\text{-}1^{-/-}$ mice, 8-12 weeks old, were fed either the chow diet or the MCD diet for 2 weeks (acute effects on injury, SAM, and PC metabolism) or 8 weeks (prolonged effects on fibrosis). Methods were approved by Baylor College of Medicine's Institutional Animal Care and Use Committee.

Cell Culture of AML-12 and C3HepG2 Cells. Murine AML-12 and human C3A/HepG2 cells were kept in regular Dulbecco's modified Eagle's medium/F-12 medium or MCD medium (see above). All experiments using cell lines were run in triplicate and reproduced at least in two independent experiments.

Protein Isolation and Immunoblotting. The following antibodies and conditions were used: Gnmt (AP1076b; 1:500; Abgent, San Diego, CA) and β-actin (sc-1616 HRP; 1:3000; Santa Cruz Biotechnology, Dallas, TX).

Microarray Analysis. Microarray analysis was performed as described.[15] Pooled total RNA from liver tissue of chow-fed and MCD diet–fed WT and $Lrh\text{-}1^{-/-}$ mice ($n = 3$ per group) was reverse-transcribed and hybridized to the Illumina mouseRefseq-8v2 Expression BeadChip using stand ard protocols (Illumina, San

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Diego, CA). Chips were run in duplicate. Genes up-regulated >0.80log2 (>1.74-fold) and down-regulated >log2-0.80 (<0.57-fold) were regarded as significant and further analyzed.

Determination of LRH-1 Binding Sites. For genome-wide binding of LRH-1, we obtained a BED file from the lab of Osborne[16] and annotated distance to TSS using Galaxy/Cistrome (www.cistrome.org).

Chromatin Immunoprecipitation for Mouse and Human LRH-1 Binding Sites. Livers of WT and LRH-1$^{-/-}$ mice fed with either chow or 2 weeks of the MCD diet (three per group) and pooled tissue from human liver wedge biopsies, which served as normal control samples in a previous study,[17] were processed for chromatin immunoprecipitation (ChIP) experiments using the ChIP-IT High Sensitivity kit (Active Motif, Inc., Carlsbad, CA) and an anti-LRH-1 antibody (R&D Systems, Abingdon, UK; #PP-H2325-00) according to the protocol. Primers for ChIP-polymerase chain reaction (PCR) are provided in Supporting Table S2.

Statistical Analysis. Numbers of mice or replicates for each group used in experiments are indicated in the figure legends. For statistical analysis, analysis of variance with Bonferroni posttesting (experiments with animals and primary hepatocytes) and the Student *t* test (cell line experiments) were used (Sigmastat statistic program; Jandel Scientific, San Rafael, CA). $P < 0.05$ was considered significant. For statistical analysis of microarray experiments, hypergeometric testing relative to the total numbers of transcripts and genes on the Illumina mouseRefseq-8v2 Expression BeadChip (see above) was performed using R software. Error bars represent means ± standard deviation.

Results

Lrh-1$^{-/-}$ *Mice Are Resistant to Hepatitis and Liver Fibrosis Induced by Methyl-Pool Alterations.* LRH-1 regulates diverse aspects of liver metabolism.[12,18,19] Although its natural ligand has not yet been identified, phospholipids (PLs), in particular PCs, are regarded as likely candidates.[11,12,20] About 30% of total hepatic PC production is dependent on methyl-pools through the Pemt pathway.[9] The association between methyl-pool and PC production,[21] along with PCs as potential LRH-1 ligands, suggests that LRH-1 may be involved in regulating methyl-pool metabolism and/or that methyl-pool alterations affect LRH-1 signaling. To explore this, we stressed WT and liver-specific *Lrh-1*$^{-/-}$ mice with a diet completely depleted of methionine and choline, which are essential to feed and maintain methyl-pool cycling (Fig. 1A). The MCD diet typically results in severe liver damage consisting of hepatic lipid droplet accumulation and liver inflammation at the early stage as well as liver fibrosis in later disease stages. Although there are important differences, particularly in insulin sensitivity, the phenotype of MCD diet–fed mice resembles NASH in humans.[7]

Two weeks of the MCD diet induced macrovesicular steatosis in both WT and liver-specific *Lrh-1*$^{-/-}$ mice to comparable amounts. There was no significant difference in macroscopic appearance (not shown), histological evaluation, oil red O staining, or quantitative measurements of hepatic triglyceride, cholesterol, and free fatty acid levels (Supporting Fig. S1A). Decline in body weight ($P = 0.054$) and the liver-to-body weight ratio were also similar between genotypes (Supporting Fig. S1B), and food intake did not differ. However, the expected increase in serum levels of alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase, all biochemical markers of liver injury, observed in WT mice was absent in *Lrh-1*$^{-/-}$ mice (Fig. 1B; Supporting Fig. S1C). Messenger RNA (mRNA) levels of tumor necrosis factor-α and intercellular adhesion molecule, indicators of liver inflammation, were also increased only in MCD WT mice (Fig. 1C). To further test if differences in liver injury at early stages translate into differences in fibrotic response at later injury stages, we treated another set of mice with the MCD diet for 8 weeks. mRNA levels of collagen1a1 as well as direct determination of the fibrotic component hydroxyproline in liver tissue showed increased fibrosis only in MCD diet–fed WT, but not in MCD diet–fed *Lrh-1*$^{-/-}$, mice (Fig. 1D).

Taken together, liver-specific *Lrh-1*$^{-/-}$ mice show markedly diminished liver injury in response to methyl-pool depletion.

Lrh-1$^{-/-}$ *Mice Maintain Normal PL Composition and Methyl-Pool When Methyl-Pool Metabolism Is Altered.* The MCD diet significantly reduces PC pools and decreases the PC/PE ratio, which is critical to maintain membrane integrity.[22,23] We performed a comprehensive hepatic lipidomic analysis to evaluate whether differences in PL content or composition may account for differences in hepatic injury between MCD diet–fed WT and *Lrh-1*$^{-/-}$ mice. Total PC content was significantly decreased after the MCD diet in WT and, to a lesser but not significantly different degree, in *Lrh-1*$^{-/-}$ mice (Fig. 2A). The hepatic PC/PE ratio was markedly decreased in MCD WT mice, and this response was significantly blunted in MCD *Lrh-1*$^{-/-}$ mice (Fig. 2A). The most pronounced differences were observed for PC 18:0/18:2 classes (Supporting Fig. S2A). Lipidomics analysis also revealed that Lyso-PC levels, which are an

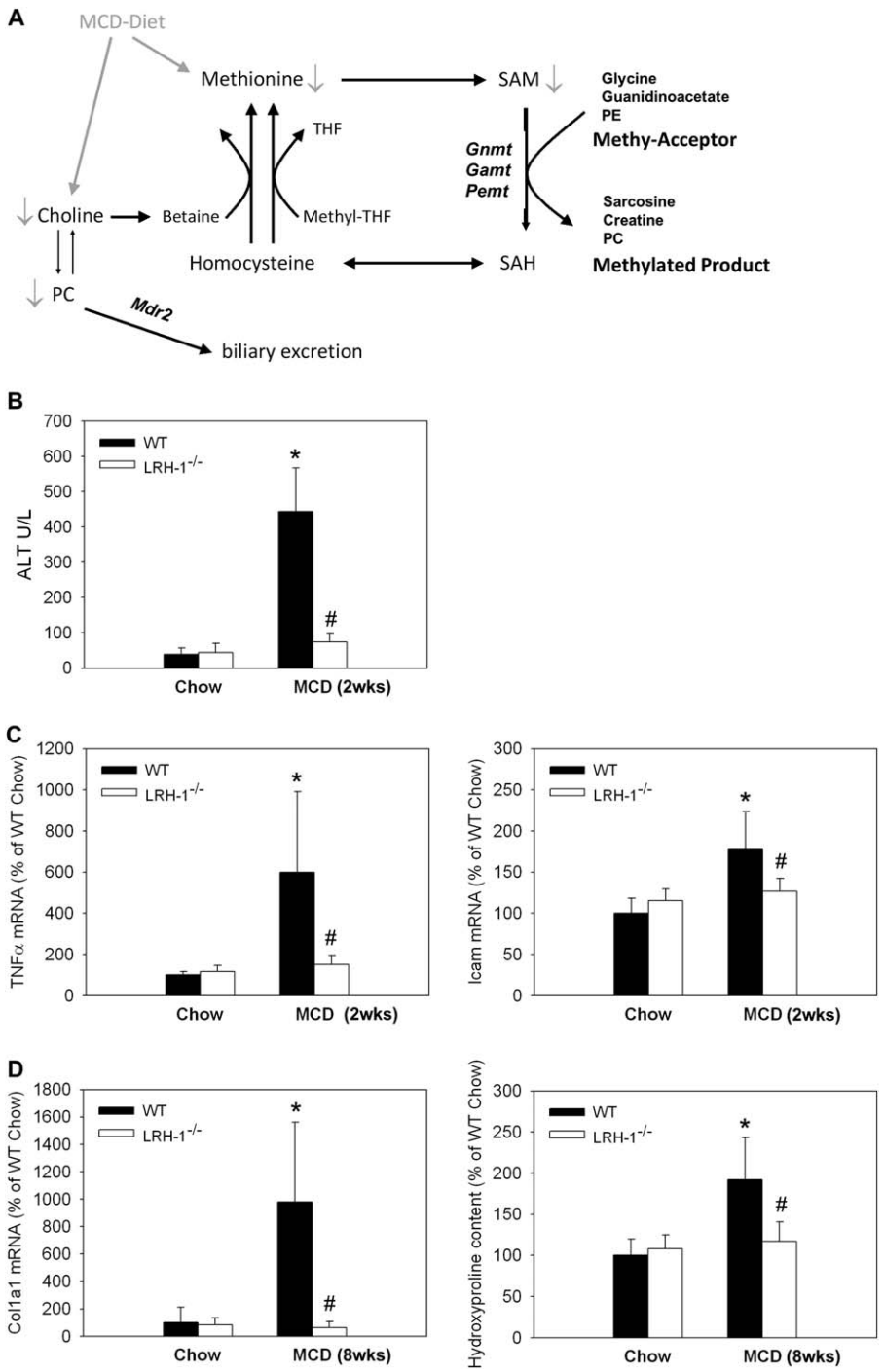

Fig. 1. Lrh-$1^{-/-}$ mice are resistant to hepatitis and liver fibrosis induced by methyl-pool alterations. (A) Schematic of methyl-group cycling. (B,C) WT and liver-specific LRH-1 knockout (*Lrh-*$1^{-/-}$) mice were fed the MCD diet for 2 weeks, and markers of liver injury (alanine aminotransferase serum levels, B) and inflammation (hepatic tumor necrosis factor-α and intercellular adhesion molecule mRNA, C) were measured. n = 7-9 mice per group. (D) WT and liver-specific LRH-1 knockout (*Lrh-*$1^{-/-}$) mice were fed the MCD diet for 8 weeks, and markers of fibrosis (hepatic Col1a1 mRNA and hydroxyproline content) were determined. n = 4 mice per group. $^{*}P < 0.05$, chow versus MCD; $^{\#}P < 0.05$, WT versus *Lrh-*$1^{-/-}$. Error bars represent means ± standard deviation. Abbreviations: ALT, alanine aminotransferase; Icam, intercellular adhesion molecule; THF, tetrahydrofolate; TNFα, tumor necrosis factor-α.

indirect marker of cell stress, inflammation, and injury,[24] were significantly elevated only in MCD diet–fed WT mice, independently confirming differences in injury between genotypes (Supporting Fig. S2B).

A critical determinant of the PC/PE ratio is multidrug-resistance protein 2 (Mdr2/Abcb4), which shuttles PLs from hepatocytes into bile. Various studies have reported that reduction of Mdr2 can stabilize the

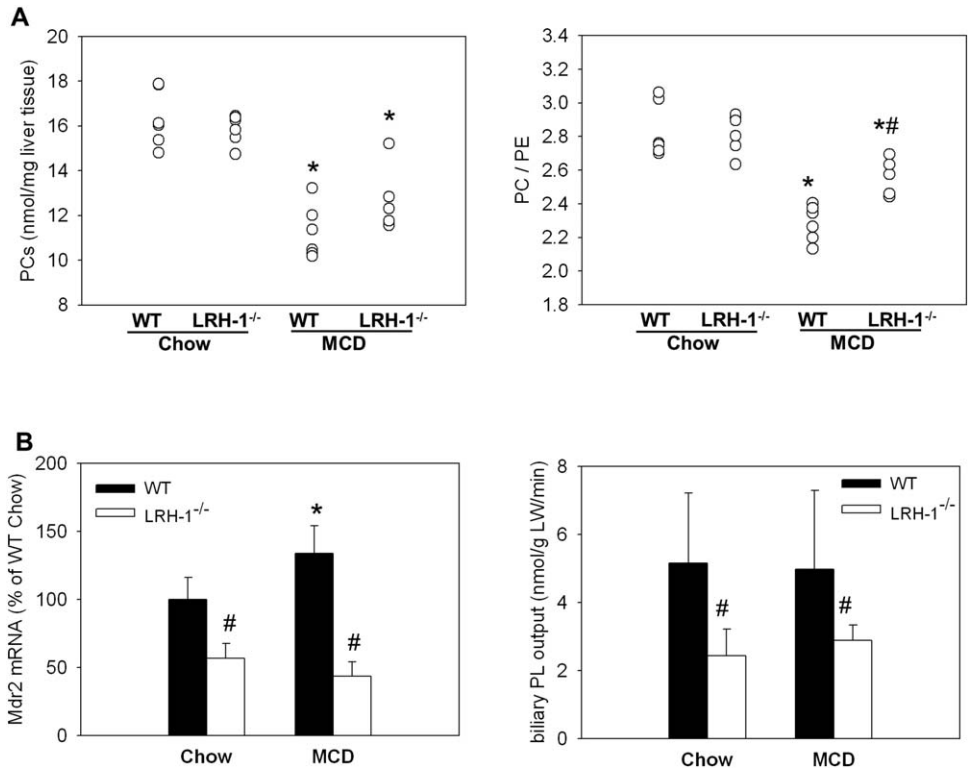

Fig. 2. Lrh-1$^{-/-}$ mice maintain normal PL composition when methyl-pool metabolism is altered. WT and liver-specific LRH-1 knockout (*Lrh-1*$^{-/-}$) mice were fed the MCD diet for 2 weeks. (A) Comprehensive mass spectrometry–based lipidomics analysis of PC and PE species. n = 5-6 mice per group. (B) Mdr2 mRNA levels (n = 7-9 mice per group) and biliary PL output (n = 4-6 mice per group). *$P < 0.05$, chow versus MCD; $^{\#}P < 0.05$, WT versus *Lrh-1*$^{-/-}$. Error bars represent means ± standard deviation.

PC/PE ratio and diminish injury resulting from MCD or depleted methyl-pools.[22,23] Chow-fed and MCD diet–fed *Lrh-1*$^{-/-}$ mice exhibited only 57% and 44% Mdr2 mRNA expression compared to their WT littermates, respectively (Fig. 2B). Functionally, reduced Mdr2 expression resulted in significantly lower biliary PL output in *Lrh-1*$^{-/-}$ mice than in WT animals (Fig. 2B). This suggests that reduced expression and function of Mdr2 in *Lrh-1*$^{-/-}$ mice may contribute to reduced injury in MCD diet–fed *Lrh-1*$^{-/-}$ mice by stabilizing the critical PC/PE ratio.

The MCD diet also significantly reduces methyl donors, particularly SAM. Imbalance in SAM, in particular reduced SAM levels, is a hallmark of several liver diseases including NASH.[1] We performed another comprehensive metabolomic analysis of the methyl-pool to determine if additional differences in methyl-pool metabolites may also contribute to differences in injury (Supporting Fig. S3A). Levels of betaine, a direct product of choline, were robustly decreased to 26% and 32% in WT and *Lrh-1*$^{-/-}$ mice after 2 weeks of the MCD diet, respectively (Supporting Fig. S3B). SAM levels and the SAM-to-*S*-adenosylhomocysteine (SAH) ratio as an indicator of cellular methylation capacity were markedly decreased in MCD diet–fed WT livers after 2 weeks. In contrast, LRH-1$^{-/-}$ mice showed higher SAM and SAM/SAH starting levels (not significant) and maintained significantly higher SAM levels and SAM/SAH ratio after 2 weeks of MCD diet feeding. Of note, SAM levels and the SAM/SAH ratio of MCD diet–fed LRH-1$^{-/-}$ mice were comparable to levels in normal WT mice (Fig. 3A).

LRH-1 Is a Transcriptional Regulator of Key Enzymes of the Methyl-Pool Cycle. A number of genes involved in methyl-pool metabolism were decreased in chow-fed or MCD diet–fed *Lrh-1*$^{-/-}$ mice (Supporting Fig. S4). In particular, Gnmt was significantly underexpressed in *Lrh-1*$^{-/-}$ mice at both the mRNA and protein levels (Fig. 3B). Gnmt is the most abundant hepatic methyltransferase and thereby a critical determinant of SAM usage. Metabolically, reduced Gnmt expression in *Lrh-1*$^{-/-}$ mice resulted in a trend toward reduction of its product sarcosine and accumulation of its starting metabolite glycine (Fig. 3C; Supporting Fig. S3A). Thus, reduced expression of Gnmt may contribute to reduced injury in MCD diet–fed *Lrh-1*$^{-/-}$ mice by maintaining SAM pools and methylation capacity.

We next evaluated whether the observed reduction of key genes of PC and methyl-pool metabolism, in

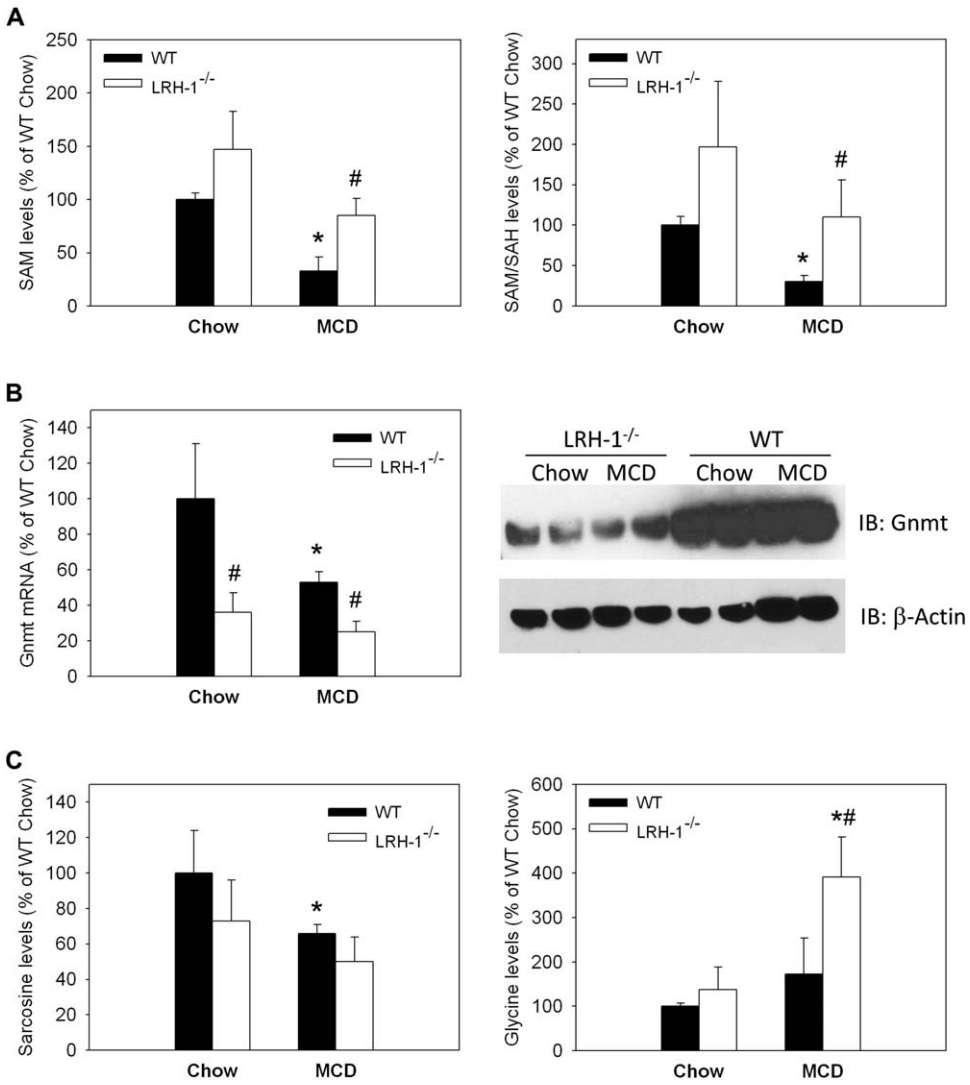

Fig. 3. Lrh-1$^{-/-}$ mice maintain methyl-pools when methyl-pool metabolism is altered. WT and liver-specific LRH-1 knockout (*Lrh-1*$^{-/-}$) mice were fed the MCD diet for 2 weeks. (A) Mass spectrometry–based analysis of SAM and SAM/SAH ratio as readout for transmethylation capacity. n = 3-4 mice per group. (B) mRNA levels (n = 7-9 mice per group) and immunoblot analysis (four mice per group were pooled and blotted in duplicate) of the main hepatic methyltransferase Gnmt. (C) Gnmt catalyzes the transmethylation of glycine into sarcosine. Mass spectrometry–based analysis of sarcosine and glycine. Sarcosine represents both sarcosine and alanine isomeric compounds. n = 3-4 mice per group. *$P < 0.05$, chow versus MCD; $^{\#}P < 0.05$, WT versus *Lrh-1*$^{-/-}$. Error bars represent means ± standard deviation.

particular Mdr2 and Gnmt, is a direct result of loss of LRH-1. Small interfering RNA (siRNA)-mediated knockdown of LRH-1 in the murine hepatocyte-derived AML12 cell line resulted in significant reduction of Mdr2 and Gnmt mRNA (28% and 21% of si-scramble-treated control cells, respectively) (Fig. 4A). Conversely, treatment of AML12 cells with the specific LRH-1 ligand dilauroyl-sn-glycero-3-phosphocholine resulted in a significant increase of Mdr2 and Gnmt mRNA levels (238% and 470% of vehicle-treated control cells, respectively) (Fig. 4B). Direct LRH-1 binding to the Gnmt promoter was significantly enriched 1.5-fold in LRH-1 ChIP-PCR experiments, with significantly less binding in WT MCD and no binding in LRH-1$^{-/-}$ mice (Fig. 4C). In line with the ChIP-PCR results, cotransfection assays using the minimal −658-kb promoter of Gnmt containing the LRH-1 binding site showed dose-dependent LRH-1 transactivation. Specific mutation of the binding site at Gnmt −143 and −68 abolished LRH-1 responsiveness (Fig. 4D). For the potential LRH-1 binding sites within the −1-kb Mdr2 promoter region we could detect only modest binding by ChIP-PCR and only modest LRH-1 transactivation when using the −763 Mdr2 promoter region (not shown). We therefore conclude that the regulation of murine Mdr2 by LRH-1 may be indirect, with additional factors (e.g., bile acids) required, or that binding sites outside the screened minimal promoter are

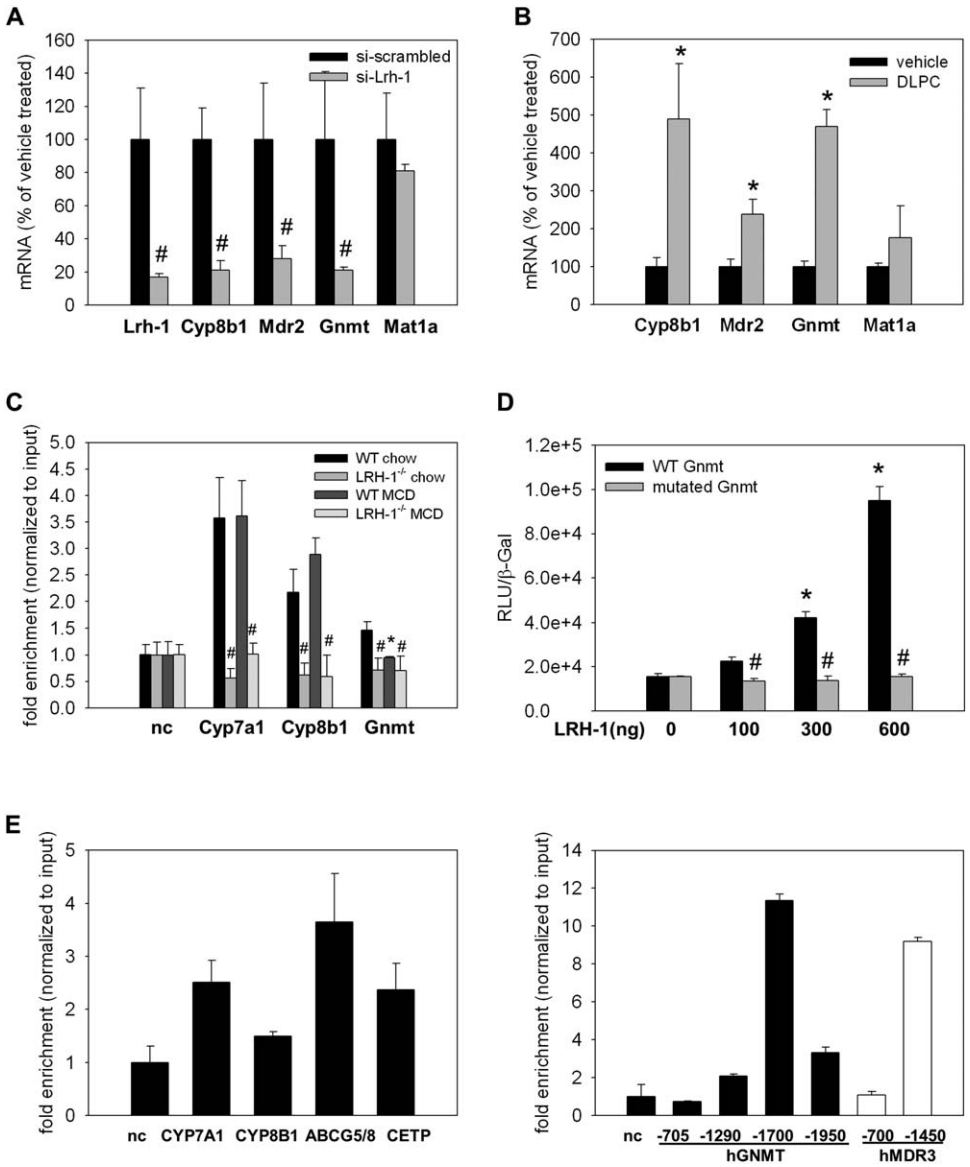

Fig. 4. LRH-1 is a transcriptional regulator of key enzymes of the methyl-pool cycle. (A) AML-12 cells were treated with scrambled siRNA or a pool of three different Lrh-1 siRNAs for 24 hours. The classical transcriptional Lrh-1 target Cyp8b1 is significantly reduced in line with reduction of mRNA levels of Mdr2 and Gnmt. (B) AML-12 cells were treated with vehicle or the LRH-1 ligand dilauroyl-sn-glycero-3-phosphocholine for 24 hours. mRNA levels of the classical Lrh-1 target gene Cyp8b1 are significantly increased in line with an induction of Mdr2 and Gnmt mRNA. n = triplicates. $^{*}P < 0.05$, vehicle versus dilauroyl-sn-glycero-3-phosphocholine; $^{\#}P < 0.05$, si-scrambled versus si-Lrh-1. Error bars represent means ± standard deviation. (C) WT and liver-specific LRH-1 knockout (*Lrh-1*$^{-/-}$) mice were fed the MCD diet for 2 weeks. LRH-1 ChIP-PCR for LRH-1 binding sites in the promoters of Cyp7a1, Cyp8b1, and Gnmt; n = 3 mice per group. (D) Luciferase assay using the minimal promoter of Gnmt (−661 to TSS) unmutated (WT Gnmt) or after mutation of the ChIP binding sites at −143 (mutated Gnmt). (E) LRH-1 ChIP-PCR from pooled liver tissue from normal control liver biopsies for classical LRH-1 target genes (left panel) and for human GNMT binding sites (right panel, black bars) and MDR3 binding sites (right panel, white bars). Abbreviations: CETP, cholesterol ester transfer protein; DLPC, dilauroyl-sn-glycero-3-phosphocholine; β-Gal, β-galactosidase; Mat, methionine-adenosyltransferase; nc, negative control; RLU, relative light units.

involved. Importantly, we did find direct binding of LRH-1 to the human GNMT and MDR3 promoter regions (Fig. 4E), indicating that LRH-1 may also be an important regulator of human methyl-pool metabolism.

Analysis of a published murine LRH-1 ChIP-Seq data set[16] showed LRH-1 binding sites at around −238 in the promoter of the *Mdr2* gene and −79 in the *Gnmt* gene promoter (Table 1). Further *in silico* analysis of promoter regions of the most essential genes involved in methyl-pool metabolism (27 genes as referenced in recent extensive reviews[1,2] as there is no separate gene ontology category for methyl-pool metabolism) revealed LRH-1 binding sites within 10 kb of the transcription starting site for 74.1% of these methyl-pool cycle genes compared to 27.7% LRH-1 binding sites for the entire gene pool ($P = 6.57e\text{-}7$ by hypergeometric testing)

Table 1. Genes Involved in Methyl-Pool/C1 Metabolism With LRH-1 Binding Sites

Gene	TSS2pCenter	Gene	TSS2pCenter
Ada	–	Mthfd1	−6
Adk	–	Mthfd2	+8330
Ahcy	−4191	Mthfr	−28|+2084
Amd2	−1145|+132	Mthfs	–
Bhmt	−138	Mtr	–
Bhmt2	−18	Mtrr	–
Cbs	–	Pemt	−8721|9507
Chdh	−3363|-9427|+13|+2876|+4206|+8940	Sgms1	−721|+1079
Cth	+411	Smpd1	−66
Dhfr	–	Srm	−201
Gamt	−58|-5241|+3822|+9964	Tyms	−124
Gnmt	−79|-4161|+6930		
Mars1	−40	Nr0b2	−209|-4217|-9950
Mars2	−54	Cyp8b1	−59|-3536
Mat1a	−7795	Mdr2	−238|-4727|-8665
Mat2a	+5799		

The LRH-1 ChIP-Seq data set published by Chong et al.[16] was used to screen for LRH-1 binding sites within 10 kb of the transcription start site. Because there is no gene ontology category for C1 metabolism, we used genes regarded as playing key roles in C1 metabolism according to recent comprehensive reviews.[1,2]
Abbreviations: Mat, methionine-adenosyltransferase; TSS2pCenter, transcription start site to center of peak.

(Table 1). Taken together, these results demonstrate that LRH-1 directly regulates multiple genes critically involved in PC and methyl-pool metabolism, suggesting a more general role for LRH-1 in regulating and maintaining methyl-pool metabolism.

Methyl-Pool Depletion Induces an LRH-1 Antagonistic Profile. To critically test the similarity of chow-fed *Lrh-1*$^{-/-}$ mice to MCD diet–fed WT mice, we compared their mRNA microarray signatures. The 117 genes that changed in WT livers upon MCD feeding (ΔMCD; 26.3% of all genes that changed upon MCD-feeding) were also changed in hepatic *Lrh-1*$^{-/-}$ mice (ΔLRH-1; 32.6% of all genes that changed in hepatic *Lrh-1*$^{-/-}$ mice compared to WT mice), yielding a highly significant overlap ($P = 1.17e-103$). Genes that were up-regulated by the MCD diet (ΔMCD up; 68 out of 286, 23.7%) were likely to be up-regulated in *Lrh-1*$^{-/-}$ mice (ΔLRH-1 up; 68 out of 201, 33.8%), and genes that were down-regulated by the MCD diet (ΔMCD down; 39 out of 159, 24.5%) were likely to be down-regulated in *Lrh-1*$^{-/-}$ mice (ΔLRH-1 down; 39 out of 158, 24.7%) (Fig. 5A). Of the genes that were down-regulated in *Lrh-1*$^{-/-}$ mice, 61% have LRH-1 binding sites within 10 kb of the transcription start ($P = 1.31e-18$); and this is shared by 48% of genes down-regulated by the MCD diet ($P = 3.36e-08$) (not shown). Thus, the MCD diet induces a transcriptional profile that is highly comparable to that of *Lrh-1*$^{-/-}$ mice.

To directly assess the impact of methyl-pool depletion on LRH-1 signaling, we carried out transient transfections with an LRH-1 responsive luciferase reporter. MCD medium induced a striking time-dependent and concentration-dependent decline in LRH-1 luciferase activity (Fig. 5B,C). Importantly, MCD medium did not significantly affect viability and did not decrease transactivation by the constitutive androstane receptor as an example of another nuclear receptor (Supporting Fig. S4A,B). Moreover, the MCD diet significantly reduced endogenous mRNA levels of classical LRH-1 target genes (i.e., cytochrome P450 8b1 [CYP8b1], CYP7A1) and major methyl-pool metabolic genes (i.e., GNMT and GAMT) in C3AHepG2 cells (Fig. 5D). Overall, these experiments indicate that methyl-pool depletion reduces LRH-1 signaling and that this in turn reduces transcription of several methyltransferases, the major methyl-pool consuming enzymes.

Discussion

Homeostasis of methyl donors is important for liver physiology and is thought to be maintained through enzyme activation and inhibition by methyl-pool metabolites. Here, we describe a new level of transcriptional regulation by the nuclear receptor LRH-1. LRH-1 directly regulates expression of the most abundant methyltransferase, Gnmt, which balances SAM levels within a critical range. LRH-1 also regulates expression of the biliary PL export floppase Mdr2, which channels biliary PC loss. When methyl-pools are low, like in methionine/choline-depleted states, LRH-1 signaling is down-regulated, reducing SAM breakdown by suppression of Gnmt and biliary PC loss by suppression of Mdr2. Under harsh conditions of methionine and choline depletion, mice with genetic loss of LRH-1 are

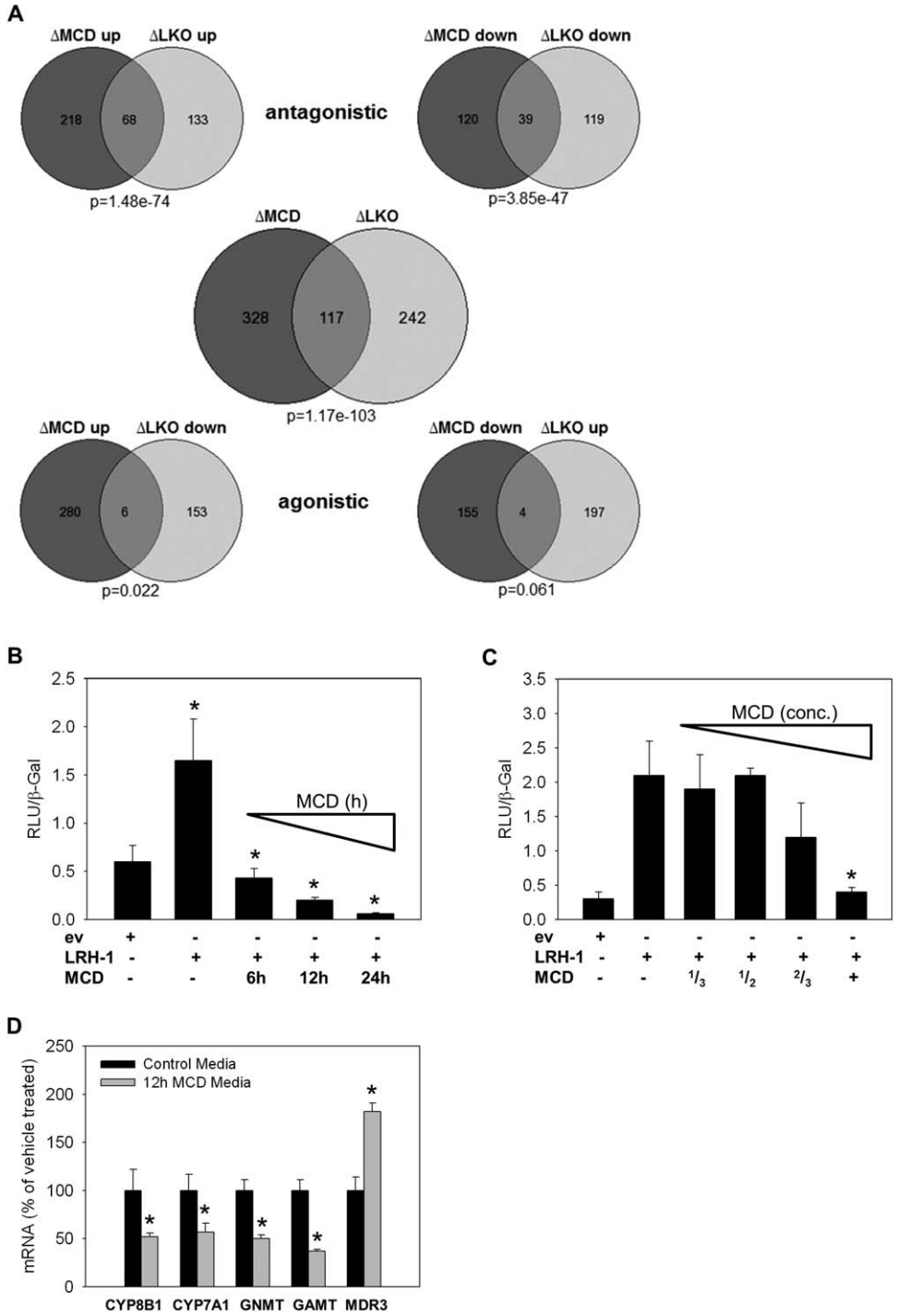

Fig. 5. Methyl-pool depletion induces an LRH-1 antagonistic profile. (A) mRNA microarray analysis for chow-fed WT and *Lrh-1*$^{-/-}$ as well as MCD diet–fed WT and *Lrh-1*$^{-/-}$ mice (three mice per group were pooled and run in duplicate). Genes up-regulated >0.80log2 (>1.74-fold) and down-regulated >log2-0.80 (<0.57-fold) were further analyzed. ΔMCD indicates deregulated genes of WT chow versus WT MCD, and ΔLKO indicates deregulated genes of WT chow versus *Lrh-1*$^{-/-}$ chow. (B,C) C3HepG2 cells were transfected with an LRH-1 luciferase reporter, β-galactosidase, and either LRH-1 construct or a corresponding empty vector and incubated for the indicated time points or concentrations with regular Dulbecco's modified Eagle's medium/F-12 medium or MCD medium. MCD medium resulted in a time-dependent decrease in LRH-1 luciferase reporter activity (B). After 12-hour incubation, LRH-1 reporter activity showed a trend for reduced activity when two-thirds of regular medium was substituted by MCD medium and was significantly reduced when full MCD medium was used (C). n = triplicate. (D) C3HepG2 cells were incubated for 12 hours with regular control or MCD medium. mRNA levels of the classical LRH-1 target genes CYP8B1 and CYP7A1 as well as GNMT and GAMT were significantly down-regulated. n = triplicate. *$P < 0.05$, control medium versus MCD medium. Error bars represent means ± standard deviation. Abbreviations: ev, empty vector; RLU, relative light units.

therefore resistant to detrimental effects of the MCD diet (Supporting Fig. S5).

The MCD diet results in significant liver injury resembling NASH.[7,13] The resulting fat accumulation, which comprises a relatively benign component of injury, has been linked to the lack of choline.[14,25] The severe inflammatory/hepatitis aspect of injury, which leads to fibrosis and eventually cirrhosis, has been attributed rather to the lack of methionine and subsequent pronounced reduction of SAM and glutathione levels.[14,25] It is well known that the deleterious phenotype of the MCD diet can completely be rescued by supplementing MCD diet–fed animals with SAM, highlighting its central role.[14] The underlying hepatoprotective mechanisms of SAM include improved membrane fluidity, decreased tumor necrosis factor-α expression, suppression of collagen synthesis by hepatic stellate cells, rise in mitochondrial glutathione levels, change in DNA methylation, inactivation of CYP2E1, and protection against apoptosis.[1,26] We found that MCD diet–fed *Lrh-1*$^{-/-}$ mice have indistinguishable macrosteatosis compared to WT counterparts yet are completely protected from hepatitis. This suggests that the higher SAM levels observed in *Lrh-1*$^{-/-}$ mice are an important part of the protective effect.

The three most abundant hepatic methyltransferases are Gnmt, Gamt, and Pemt. We observed that both Gnmt and Gamt are significantly reduced in *Lrh-1*$^{-/-}$ mice under baseline chow-fed conditions. The contribution of Gamt to overall SAM homeostasis is low,[27] but Gnmt, comprising 1%-3% of hepatic cytosolic protein, is critical for maintaining constant SAM levels.[1] Gnmt knockout mice show markedly increased SAM levels, SAM/SAH levels, and hypermethylation capacity.[28,29] Gnmt knockout mice also exhibit fatty liver that is attributed to rerouting SAM into triglyceride synthesis through a novel pathway based on Pemt and PC breakdown, which may contribute to steatosis in MCD diet–fed *Lrh-1*$^{-/-}$ mice.[21] When Gnmt knockout mice are fed a diet deficient in methionine, SAM levels decrease/normalize and they are protected from steatotic liver injury.[21] Our results suggest that LRH-1-dependent reduced expression of Gnmt may constitute a mechanism to maintain SAM levels and hepatic integrity in conditions when methyl donors are scarce.

In response to the MCD diet, WT mice counteract further methyl-donor usage by down-regulation of Gnmt and, at least transcriptionally, Gamt. This adaptive response of WT mice is similar to the basal state of *Lrh-1*$^{-/-}$ mice. More broadly, the global gene expression profile of MCD diet–fed WT animals indicates striking overlap with that of chow-fed *Lrh-1*$^{-/-}$ mice. A simple interpretation of this is that methionine/choline depletion may either deplete an endogenous LRH-1 agonist or actively increase an endogenous LRH-1 antagonist. In either case, LRH-1 would be acting as both an active sensor and a modulator of the methyl-pool. Alternatively, LRH-1 activity may also be modulated posttranslationally[30] independent of ligand by methyl-pool responsive pathways.

The importance of Mdr2 for PC-pool homeostasis is evident from the observation that feeding *Pemt* knockout mice a choline-depleted diet causes hyperacute liver failure within 3 days.[22] These mice entirely lack the ability to generate PC from either the choline-dependent classical PC biosynthesis pathway or the alternative SAM-dependent Pemt pathway.[9] However, the lethal phenotype is rescued by knockout of Mdr2, the "phospholipid floppase" transporter required for PC secretion into bile.[22] These striking results are explained by the fact that the amount of PC in a mouse's normal daily biliary secretion is equivalent to the total pool of PC in its liver.[31] In contrast to the very efficient recycling of bile acids, less than half of the biliary PC is returned to the liver,[31] resulting in a significant net loss of methyl groups as well as fatty acids in the acyl side chains. Homozygous Mdr2 knockout mice almost completely lack biliary PC output, and heterozygous Mdr2 knockout mice show a 50% decrease.[32,33] When heterozygous Mdr2 knockout mice were challenged with the MCD diet, they also showed significantly less liver injury.[23] The inability to generate PC decreases the PC/PE ratio, which is important to maintain membrane fluidity and integrity. Choline-starved Pemt knockout mice experience a dramatic decrease in their PC/PE ratio, which is reversed when *Mdr2* is knocked out.[22] We found that Mdr2 transcripts are down-regulated by 50% in *Lrh-1*$^{-/-}$ mice and that this was associated with a significant decrease in biliary PL output. We also observed a significantly higher PC/PE ratio in MCD diet–fed *Lrh-1*$^{-/-}$ mice. This suggests that LRH-1-dependent reduction of Mdr2 is an additional component of the observed hepatoprotective response to methyl-pool deprived states.

PC synthesis by the Pemt pathway preferentially generates PC species rich in polyunsaturated fatty acids such as PC with (20:4) and PC with (22:6).[8,34] However, the most pronounced differences in our lipidomic approach were in PC (18:0/18:2) classes, which are mainly synthesized by the cytidine 5′-diphosphocholine pathway. The specific ligand of LRH-1, dilauroyl-sn-glycero-3-phosphocholine, PC (12:0/12:0), is also not synthesized by the PEMT pathway. This argues against the Pemt pathway having an important role.

In contrast to the adaptive down-regulation of the methyltransferases Gnmt and Gamt in MCD diet–fed WT mice, we observed a modest increase in Mdr2 expression. The underlying molecular mechanism is not clear, but we speculate that increased bile acid levels upon MCD feeding[35] may increase Mdr2 expression through the well-established effects of the bile acid receptor farnesoid X receptor on Mdr2 expression.[36] Consistent with this, serum bile acid levels were modestly but significantly higher in MCD diet–fed WT mice (Supporting Fig. S6A), which was paralleled by induction of adaptive hepatobiliary transporters (i.e., predominantly Mrp4 and Ostβ) (Supporting Fig. S6B). Elevated serum bile acid levels may be secondary to steatohepatitis in MCD diet–fed WT mice[35] and therefore would be expected to remain normal in MCD diet–fed *Lrh-1*$^{-/-}$ mice.

Although our results show a protective role of LRH-1 loss in the MCD model of NASH, the effects are specific to this model and cannot be transferred to other models of NASH or NASH-induced fibrosis. These results also do not imply that LRH-1 ligands would aggravate NASH or NASH fibrosis because it has clearly been shown that LRH-1 agonism improves steatosis and insulin resistance in models of steatotic liver injury[12] and inhibits acute-phase and inflammatory responses in the liver.[37]

Thus, we report a novel role for LRH-1 in transcriptional control of key genes of PC and methyl-pool metabolism. Methyl-pool depletion results in an LRH-1 antagonistic response which promotes maintenance of methyl-pools. Lastly, LRH-1 antagonists may allow adaptation to methyl-pool depleted states and may represent a potential therapeutic direction for human liver diseases.

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