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Methyl-Sensing Nuclear Receptor Liver Receptor Homolog-1 Regulates Mitochondrial Function in Mouse Hepatocytes

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BACKGROUND AND AIMS: Liver receptor homolog-1 (LRH-1; NR5A2) is a nuclear receptor that regulates metabolic homeostasis in the liver. Previous studies identified phosphatidylcholines as potential endogenous agonist ligands for LRH-1. In the liver, distinct subsets of phosphatidylcholine species are generated by two different pathways: choline addition to phosphatidic acid through the Kennedy pathway and trimethylation of phosphatidylethanolamine through phosphatidylethanolamine *N*-methyl transferase (PEMT).

APPROACH AND RESULTS: Here, we report that a PEMT–LRH-1 pathway specifically couples methyl metabolism and mitochondrial activities in hepatocytes. We show that the loss of *Lrh-1* reduces mitochondrial number, basal respiration, beta-oxidation, and adenosine triphosphate production in hepatocytes and decreases expression of mitochondrial biogenesis and beta-oxidation genes. In contrast, activation of LRH-1 by its phosphatidylcholine agonists exerts opposite effects. While disruption of the Kennedy pathway does not affect the LRH-1-mediated regulation of mitochondrial activities, genetic or pharmaceutical inhibition of the PEMT pathway recapitulates the effects of *Lrh-1* knockdown on mitochondria. Furthermore, we show that *S*-adenosyl methionine, a cofactor required for PEMT, is sufficient to induce *Lrh-1* transactivation and consequently mitochondrial biogenesis.

CONCLUSIONS: A PEMT–LRH-1 axis regulates mitochondrial biogenesis and beta-oxidation in hepatocytes. (Hepatology 2020;71:1055-1069).

Liver receptor homolog-1 (*Lrh-1*) is a nuclear receptor that binds as a monomer to a specific response element (5′-TCAAGGTCA-3′) within the promoter and regulatory regions of its target genes.(1) Initial studies of the X-ray crystal structure of the human LRH-1 ligand binding domain expressed in *Escherichia coli* showed that it is occupied by bacterial phospholipids.(2) Further studies suggested that mammalian phospholipids, including both phosphatidylcholines (PCs)(3,4) and phosphatidyl inositols,(5,6) could function as endogenous LRH-1 agonists.

One of the primary targets of LRH-1 in the liver is the nuclear receptor small heterodimer partner (SHP), which functions as a corepressor for LRH-1 and other

Abbreviations: Acox1, acyl-coenzyme A oxidase 1; ATP, adenosine triphosphate; Chka, choline kinase alpha; Cpt1a, carnitine palmitoyltransferase 1-alpha; Cyp8b1, cytochrome P450 8b1; DLPC, dilauroyl-phosphatidylcholine; DZA, 3-deazaadenosine; Esrra/Esrrg, estrogen-related receptor alpha/gamma; FBS, fetal bovine serum; FCCP, 2-[2-[4-(trifluoromethoxy)phenyl] hydrazinylidene]-propanedinitrile; GNMT, glycine N-methyltransferase; KD, knockdown; KO, knockout; LRH-1, liver receptor homolog-1; Mat1a, methionine adenosyltransferase 1a; MDR2, multidrug resistance protein 2; mtDNA, mitochondrial DNA; mTORC1, mammalian target of rapamycin complex 1; NAFLD, nonalcoholic fatty liver disease; NHR-25, nuclear hormone receptor 25; Nrf1, nuclear erythroid 2 p45-related factor 1; OCR, oxygen consumption rate; PC, phosphatidylcholine; Pcyt1a, phosphate cytidylyltransferase 1alpha; Pdk4, pyruvate dehydrogenase kinase 4; PE, phosphatidylethanolamine; Pemt, phosphatidylethanolamine-N-methyltransferase; Pgc-1α, peroxisomal proliferator gamma coactivator-1-alpha; Ppara, peroxisome proliferator–activated receptor alpha; SAH, S-adenosyl homocysteine; SAM, S-adenosylmethionine; SHP, small heterodimer partner; siRNA, small interfering RNA; Tfam, transcription factor A mitochondrial; VLDL, very low-density lipoprotein; WT, wild type.

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nuclear receptors.[7,8] LRH-1 also positively regulates genes encoding bile acid production enzymes, particularly cytochrome P450 8b1 (*Cyp8b1*). *Shp* gene expression is also induced by the bile acid receptor farnesoid X receptor, resulting in a negative feedback loop in which elevated hepatic bile acid levels suppress bile acid production through inhibition of LRH-1 transactivation.[8] Particularly in the agonist bound state, LRH-1 transactivation can be positively regulated by coactivators, with recent structural evidence indicating an important role for peroxisomal proliferator gamma coactivator-1-alpha (PGC-1α; PPARGC1A).[9]

In addition to controlling bile acid homeostasis, several reports suggest that LRH-1 targets different metabolic pathways. We identified dilauroylphosphatidylcholine (DLPC), an unusual PC species with two saturated 12-carbon fatty acid acyl chains, as an exogenous LRH-1 agonist and showed that DLPC treatment could attenuate fatty liver and improve insulin sensitivity by repressing lipogenesis.[4] In the opposite direction, LRH-1 mRNA expression is strongly decreased in livers of human subjects with steatosis or nonalcoholic steatohepatitis,[10] and a recent report confirms that acute knockout (KO) of LRH-1 in mouse liver disrupts lipid metabolism and induces fat accumulation.[11] Another report identifying glucokinase as a primary LRH-1 target links LRH-1 to glucose use and glycogen synthesis.[12]

The nucleus contains a surprisingly large and dynamic endonuclear PC pool that is distinct from the nuclear membrane,[13] suggesting that PCs could act as endogenous agonists. There are two endogenous *de novo* PC synthesis pathways in mammals. The ubiquitous pathway for choline transfer to phosphatidic acid depends on both choline kinase alpha (*Chka*) and phosphate cytidylyltransferase 1alpha (*Pcyt1a*) and is called the "Kennedy pathway." The liver has another pathway based on *S*-adenosylmethionine (SAM)–dependent triple-methylation of phosphatidylethanolamine (PE) through phosphatidylethanolamine *N*-methyl transferase (PEMT). If one of these two pathways is dysfunctional, the other increases its activity to compensate for and maintain total net amount of PC in hepatocytes.[14-18] However, these two pathways produce distinct subsets of PC species that differ in fatty acid side chains; and thus, the compensatory responses can alter overall composition of the PC pool.[14]

The PEMT pathway is tightly linked with SAM metabolism. The ratio of SAM to its demethylated

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product *S*-adenosyl homocysteine (SAH) provides a key index of the status of the endogenous methyl pool. Choline is oxidized to betaine, which provides a methyl group to homocysteine for making methionine. Methionine is then adenylated by methionine adenosyltransferase-1a (*Mat1a*) to generate SAM, the cofactor for diverse methyltransferase enzymes. Depletion of the endogenous methyl pool through either dietary deficiency in methionine and choline or genetic defects in SAM production results in hepatic steatosis.[19] For example, *Mat1a* KO mice are deficient in liver SAM and have impaired fatty acid beta-oxidation capacity and increased fatty acid uptake in the liver.[20,21] Interestingly, these KO mice develop hepatic steatosis with age, which is associated with decreased very low-density lipoprotein (VLDL) secretion linked to decreased PEMT activity.[20] PEMT generates PC species with long acyl chains, such as arachidonic acid or docosahexaenoic acid, that are important for forming VLDL particles.[22] Consistently, *Pemt* KO rat hepatocytes also have decreased VLDL secretion,[22] and *Pemt* polymorphisms in humans confer susceptibility to nonalcoholic fatty liver disease (NAFLD).[23]

Lrh-1 is both a sensor of the state of SAM metabolism in the liver and a critical regulator of the methyl pool. We previously showed that LRH-1 activity is decreased in the livers of mice fed a methionine and choline–deficient diet and that LRH-1 transactivation is decreased in cells maintained in methionine and choline–deficient medium.[24] This is associated with decreased expression of glycine *N*-methyltransferase (*Gnmt)* and multidrug resistance protein 2 (*Mdr2*), two primary targets of LRH-1. GNMT is a major consumer of hepatic SAM, and the phospholipid flippase MDR2 transports phospholipids from hepatocytes to the bile duct. In *Lrh-1* KO liver, the reduction in GNMT preserves the SAM/SAH ratio, and decreased MDR2 activity lowers the loss of labile methyl groups in the form of PC species. Thus, liver-specific *Lrh-1* KO mice are completely resistant to the inflammatory and fibrotic effects of dietary deficiency in methionine and choline.[24]

In striking agreement with this methyl-sensing role, we also showed that in response to environmental deficiency in methionine and its associated methyl metabolites, *Caenorhabditis elegans* accumulates fat in the intestine, the functional homolog of the mammalian liver, and this is mediated by the *C. elegans* LRH-1 relative, the nuclear receptor 5A family member nuclear hormone receptor 25 (NHR-25).[25] Genetic and metabolomics studies revealed that SAM metabolism and its associated PEMT pathway are required for this NHR-25-mediated regulation.[25] Interestingly, *nhr-25* mutant worms also showed more fragmented and less filamentous mitochondria compared to control worms.[25]

Together, these results inspired us to investigate whether and how LRH-1 could regulate fat metabolism by tuning mitochondrial functions in response to changes in the methyl pool. We demonstrate that LRH-1 activation promotes mitochondrial biogenesis and fatty acid beta-oxidation and induces the LRH-1 coactivator PGC-1α. These responses are specifically dependent on the PEMT pathway for PC production but not the Kennedy pathway. We also show that SAM supplementation is sufficient to transactivate LRH-1 to regulate mitochondrial activities. We conclude that LRH-1 functions as a crucial regulator of mitochondrial metabolism and a key sensor of the methyl pool and coordinates their activities.

Materials and Methods

For detailed information on materials and methods, please see the Supporting Information.

ANIMAL STUDIES

12-16 weeks of male mice were used for all the studies. Dr. Steven Kliewer at University of Texas at Southwestern kindly provided with *Lrh-1* fl/fl mouse. Liver-specific *Lrh-1* knockout (LKO) was achieved by *Lrh-1* f/f mice with albumin-cre/+ (Alb-cre) mice obtained from the O'Malley laboratory at the Baylor College of Medicine. All animal studies and procedures were followed by the protocol approved by the Institutional Animal Care and Use Committee of the Baylor College of Medicine. Mice were housed in a temperature controlled room in pathogen-free facilities with a 12-h light, 12-h dark photocycle (07:00 on, 19:00 off) and fed standard chow diet and water *ad libitum*.

CELL CULTURE

Primary hepatocytes were cultured in either William's E medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics or Hank's Balanced Salt Solution (HBSS; 24020117; Invitrogen) containing 10% FBS and 1% penicillin/streptomycin antibiotics. C3A/HepG2 cells and AML12 cells were cultured in Dulbecco's modified Eagle's medium/F-12 (CM017-050; Gendepot) containing 10% FBS and 1% penicillin/streptomycin antibiotics.

PRIMARY HEPATOCYTE ISOLATION

Primary hepatocytes were extracted as reported.[26] Cells were plated in 10-cm plates (1.5×10^7 cells/well), six-well plates (2.5×10^6 cells/well), or XF24 cell culture microplates (12,500 cells/well; 100777-004; Agilent). The cells were cultured in William's E medium containing 10% FBS (12551; Invitrogen).

SMALL INTERFERING RNA TRANSFECTION

C3A/HepG2 cells on six-well plates were transfected with small interfering RNA (siRNA) targeting different genes, using RNAiMAX Lipofectamine (13778150; Invitrogen) for 48 hours. We purchased human *Lrh-1* targeting siRNA (J-003430-07) and nontargeting control siRNA (D-001810-10-05) from Dharmacon. siRNA targeting human *Mat1a* (pooled HSS181024, HSS181023), *Pemt* (pooled HSS145606, HSS170611, HSS145608), *Chka* (pooled HSS 141030, HSS140691), *Pcyt1a* (pooled HSS 1007689, HSS107690), and their nontargeting control siRNA (12935200, 12935300, 12935400) from Invitrogen.

DRUG TREATMENT

See Supporting Information.

REAL-TIME QUANTITATIVE PCR EXPERIMENT

Total RNA was isolated from primary hepatocytes using the Quick-RNA MiniPrep kit (11-328; Zymo Research). Complementary DNA (cDNA) was synthesized by the qScript cDNA synthesis Kit (95047; Quanta Biosciences) with 500 ng of RNA. Gene expression level was determined by real-time PCR using the LightCycler 480 Real-Time PCR System (Roche) with KAPA SYBR FAST Universal qPCR Master Mix (KK 4618; Kapa Biosystems). The relative mRNA level was calculated with the delta delta Ct method and normalized by *36b4*, TATA box binding protein, or cyclophilin expression. Primer information is available upon request.

MITOCHONDRIAL DNA COPY NUMBER MEASUREMENT

See Supporting Information.

LUCIFERASE ASSAY

See Supporting Information.

ADENOSINE TRIPHOSPHATE AND KETONE BODY MEASUREMENT

See Supporting Information.

OXYGEN CONSUMPTION RATE MEASUREMENT

See Supporting Information.

LIPIDOMICS

See Supporting Information.

STATISTICAL ANALYSIS

All experiments were performed at least in biological triplicate. For comparison of multiple groups, analysis of variance was used with Bonferroni's post hoc test. For comparison of two groups, the Student *t* test was used (PRISM program; Graph Pad, La Jolla, CA). $P < 0.05$ was considered significant ($^*P < 0.05$, $^{**}P < 0.005$). Error bars represent means ± standard error of means.

Results

HEPATIC LRH-1 REGULATES MITOCHONDRIAL ACTIVITIES

To test whether LRH-1 regulates mitochondrial functions, we first measured mitochondrial DNA copy number in primary hepatocytes extracted from wild-type (WT) and *Lrh-1* liver-specific KO mice.

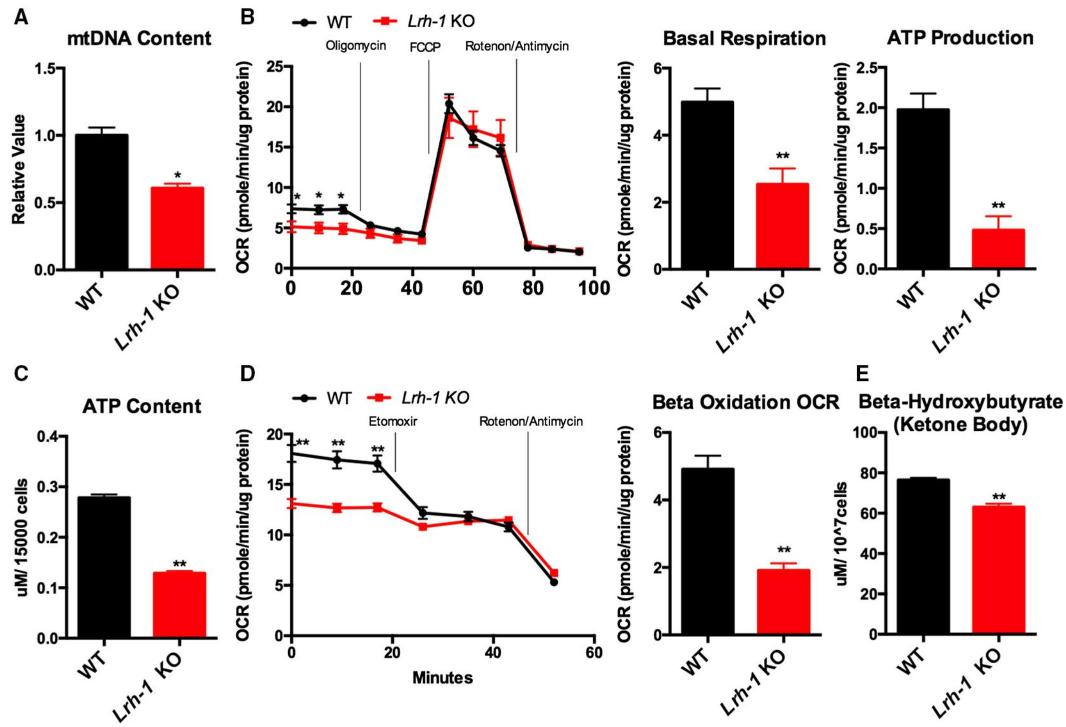

FIG. 1. Hepatic *Lrh-1* regulates mitochondrial functions. (A) Mitochondrial DNA copy number was measured in *Lrh-1* KO and WT primary hepatocytes. (B) OCR in WT and *Lrh-1* KO primary hepatocytes was measured. Basal respiration–linked OCR and ATP production–linked OCR were quantified from *Lrh-1* KO and WT primary hepatocytes. (C) ATP level was measured from *Lrh-1* KO and WT primary hepatocytes. (D) OCR in WT and *Lrh-1* KO primary hepatocytes was measured for endogenous beta-oxidation assay. Beta-oxidation-linked OCR in *Lrh-1* KO and WT primary hepatocytes was quantified. (E) Beta-hydroxybutyrate level was measured from *Lrh-1* KO and WT primary hepatocytes. Abbreviations: FCCP, 2-[2-[4-(trifluoromethoxy)phenyl]hydrazinylidene]-propanedinitrile; mtDNA, mitochondrial DNA.

We found that mitochondrial DNA copy number is reduced in *Lrh-1* KO freshly isolated, plated primary hepatocytes (Fig. 1A). Next, we examined the effect of LRH-1 on mitochondrial metabolic activities using SeaHorse assays. At the basal respiration level, we found that the oxygen consumption rate (OCR) is reduced by 60% in *Lrh-1* KO primary hepatocytes, as assessed by the difference between initial OCR and OCR after rotenone/antimycin treatment (Fig. 1B). Upon inhibition of adenosine triphosphate (ATP) synthase in complex V by oligomycin treatment, OCR is reduced by 80% in *Lrh-1* KO primary hepatocytes (Fig. 1B), which suggests that LRH-1 modulates the ATP-producing capacity in mitochondria. On the other hand, we found that maximal respiration–linked OCR is not affected by LRH-1 because there was no difference between WT and *Lrh-1* KO upon treatment with FCCP (Fig. 1B). We also directly measured ATP content in WT and *Lrh-1* KO primary hepatocytes and found that it was reduced by 66% in *Lrh-1* KO primary hepatocytes (Fig. 1C).

Our previous studies showed that LRH-1 activation decreases fat accumulation in the livers of mice fed a high-fat diet[4] and that NHR-25, the *C. elegans* homolog of LRH-1, regulates mitochondrial dynamics and lipid metabolism in response to different dietary inputs.[25] We thus decided to use SeaHorse to analyze endogenous fatty acid beta-oxidation. We cultured primary hepatocytes in medium with 10% FBS, which contains endogenous fatty acids, and measured OCR in the presence and absence of etomoxir, which inhibits carnitine palmitoyltransferase 1-alpha (CPT1A) to block fatty acid trafficking into mitochondria. We found that the etomoxir treatment reduced OCR to a much smaller degree in *Lrh-1* KO primary hepatocytes (Fig. 1D), suggesting that *Lrh-1* KO reduces mitochondrial beta-oxidation. This was also confirmed by directly measuring beta-hydroxybutyrate, one of the ketone bodies as an indirect beta-oxidation product. As expected, beta-hydroxybutyrate levels were decreased in freshly isolated *Lrh-1* KO primary hepatocytes (Fig. 1E).

Because it has been reported that mitochondrial filamentation boosts beta-oxidation by increasing the efficacy of fatty acids trafficking through the mitochondrial network,[27] we also analyzed mitochondrial morphology using MitoTracker staining. We found no difference in mitochondrial morphology between WT and *Lrh-1* KO primary hepatocytes, despite the reduced beta-oxidation capacity in the *Lrh-1* KO (Fig1D; Supporting Fig. S1). In accord with previous results,[27] when WT cells were cultured in starvation medium (HBSS), mitochondrial filamentation was induced; but this response was absent in *Lrh-1* KO primary hepatocytes (Supporting Fig. S1). Thus, during starvation LRH-1 is required for the induction of mitochondrial filamentation, which is associated with an increased demand of mitochondrial beta-oxidation.[27] However, the reduced beta-oxidation capacity of LRH-1 KO primary hepatocytes in regular medium is independent of changes in mitochondrial morphology.

LRH-1 REGULATES MITOCHONDRIAL BIOGENESIS AND BETA-OXIDATION GENES

To further characterize the molecular mechanisms by which LRH-1 regulates mitochondrial activities, we extracted RNA from primary hepatocytes of WT and *Lrh-1* liver-specific KO mice and examined mRNA expression of genes involved in mitochondrial biogenesis and beta-oxidation. We found that mRNA expression of three transcriptional regulators of mitochondrial biogenesis encoded by the nuclear erythroid 2 p45-related factor 1 (*Nrf1*), *Nrf2*, and *Pgc-1α* genes is significantly decreased >25% in *Lrh-1* KO primary hepatocytes ($P < 0.05$; Fig. 2A). For beta-oxidation, acyl-coenzyme A oxidase 1 (*Acox1*) and *Cpt1a* encode rate-limiting enzymes in the pathway, and peroxisome proliferator–activated receptor alpha (*Ppara*) encodes a key transcriptional activator of the pathway.[28] We found that their expression levels are reduced more than 2 times in *Lrh-1* KO primary hepatocytes (Fig. 2B). On the other hand, other mitochondrial biogenesis and beta-oxidation regulators, transcription factor A mitochondrial (*Tfam*), estrogen-related receptors alpha (*Esrra*) and gamma (*Esrrg*), and pyruvate dehydrogenase kinase 4 (*Pdk4*), were unaffected by *Lrh-1* KO. In accord with the Seahorse studies, these results suggest that LRH-1 is required for the expression of specific genes involved in mitochondrial biogenesis and beta-oxidation in hepatocytes.

When we asked whether WT and *Lrh-1* KO livers show similar phenotypes, we found that ATP levels were significantly reduced, while mitochondrial DNA levels and mRNA expression of PGC1α and beta-oxidation genes showed trends in the expected directions that did not reach statistical significance (Supporting Fig. S2). In contrast, analysis of WT and *Lrh-1* KO primary hepatocytes prior to plating showed the same responses as freshly plated primary hepatocytes (Fig. 1C; Supporting Fig. S2). The apparently decreased impact of *Lrh-1* KO in intact livers could be due, at least in part, to the absence of the hepatocyte responses in nonparenchymal cells or the inhibition of the hepatocyte responses by nonparenchymal cells. In addition, we have previously found that LRH-1 is activated by stress responses including endoplasmic reticulum stress[26] and potentially starvation (Supporting Fig. S1). Therefore, it is also possible that the impact of LRH-1 on mitochondrial functions is blunted in unstressed livers but amplified by stresses associated with hepatocyte isolation.

Next, to test whether LRH-1 activation is sufficient to induce mitochondrial biogenesis and beta-oxidation gene expression, we turned to pharmacological gain-of-function studies. DLPC is an LRH-1 agonist,[4] and we confirmed that DLPC treatment induces the expression of the best-characterized LRH-1 target gene, *Shp*, in the mouse hepatocyte cell line AML12 (Fig. 2C). Importantly, DLPC treatrment induced *Pgc-1α* expression, and the induction level is as prominent as that of *Shp* (Fig. 2C). On the other hand, the expression of *Nrf2* or *Esrrg* was not significantly affected by DLPC treatment ($P > 0.05$), and the expression of *Esrra* was significantly ($P < 0.05$) but modestly increased (20%) (Fig. 2C). Thus, we chose to focus on *Pgc-1α* and further investigate its response to DLPC in human C3A/HepG2 cells. We found that *Pgc-1α* is induced by 50% in control cells upon

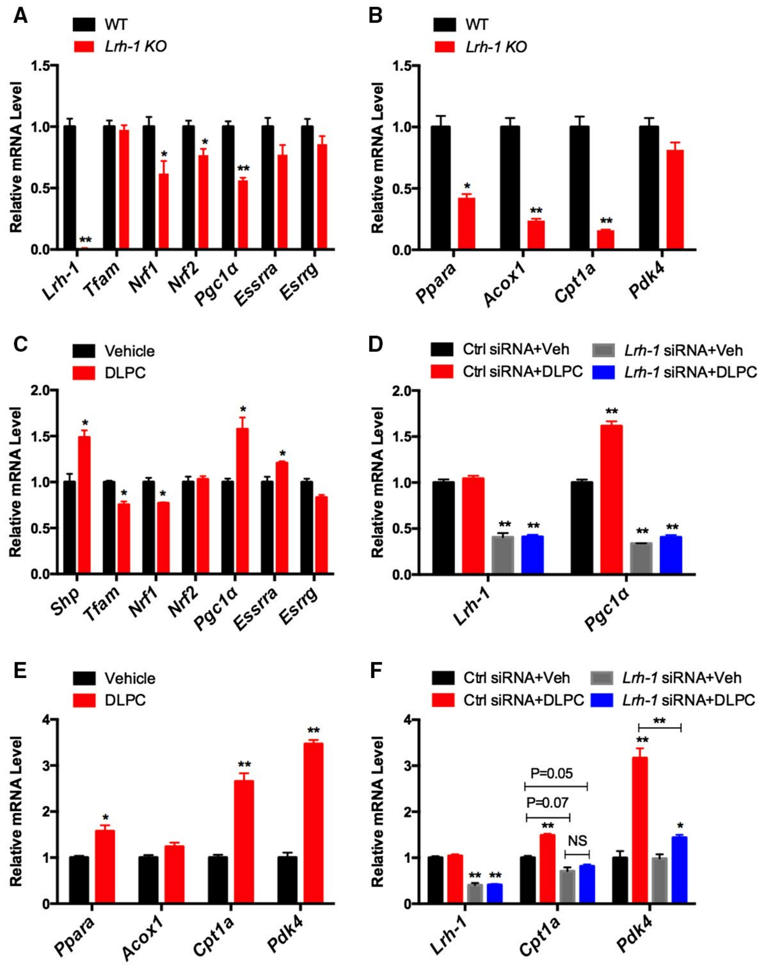

FIG. 2. *Lrh-1* regulates mitochondrial biogenesis and beta-oxidation genes. (A) The expression of mitochondrial biogenesis–related genes was measured from *Lrh-1* KO and WT primary hepatocytes. (B) Expression of beta-oxidation-related genes was measured in *Lrh-1* KO and WT primary hepatocytes. (C) The *Lrh-1* agonist DLPC was administered to the mouse hepatocyte cell line AML12 for 16 hours, and expression of genes important for mitochondria biogenesis was measured. (D) DLPC was administered to control siRNA or *Lrh-1* siRNA transfected C3A/HepG2 cells, and *Pgc1α* expression was measured. (E) DLPC was administered to the mouse hepatocyte cell line AML12 for 16 hours, and expression of genes important for beta-oxidation was measured. (F) DLPC was administered to control siRNA or *Lrh-1* siRNA transfected C3A/HepG2 cells, and *Cpt1a* and *Pdk4* expression was measured.

DLPC treatment, and this induction is fully suppressed in the cells transfected with siRNA targeting *Lrh-1* (Fig. 2D). For beta-oxidation-related genes, we found that *Ppara*, *Cpt1a*, and *Pdk4*, but not *Acox1*, expression is induced upon DLPC treatment (Fig. 2E) and that the induction of *Cpt1a* and *Pdk4* requires *Lrh-1* (Fig. 2F). Together, these studies reveal that LRH-1 regulates mitochondrial activities through controlling specific genes that are crucial for mitochondrial biogenesis and beta-oxidation, and its effects are well conserved in human hepatic cells.

THE KENNEDY PATHWAY DOES NOT CONTRIBUTE TO LRH-1 REGULATION OF MITOCHONDRIAL ACTIVITIES

Because PC species are candidate ligands for LRH-1, managing proper levels of PC species could be crucial to control LRH-1 activity. There are two independent endogenous PC synthesis pathways in mammals.[15] In the Kennedy pathway, the enzymes CHKA and PCYT1A catalyze *de novo* synthesis of PC from choline. In the PEMT pathway, MAT1a

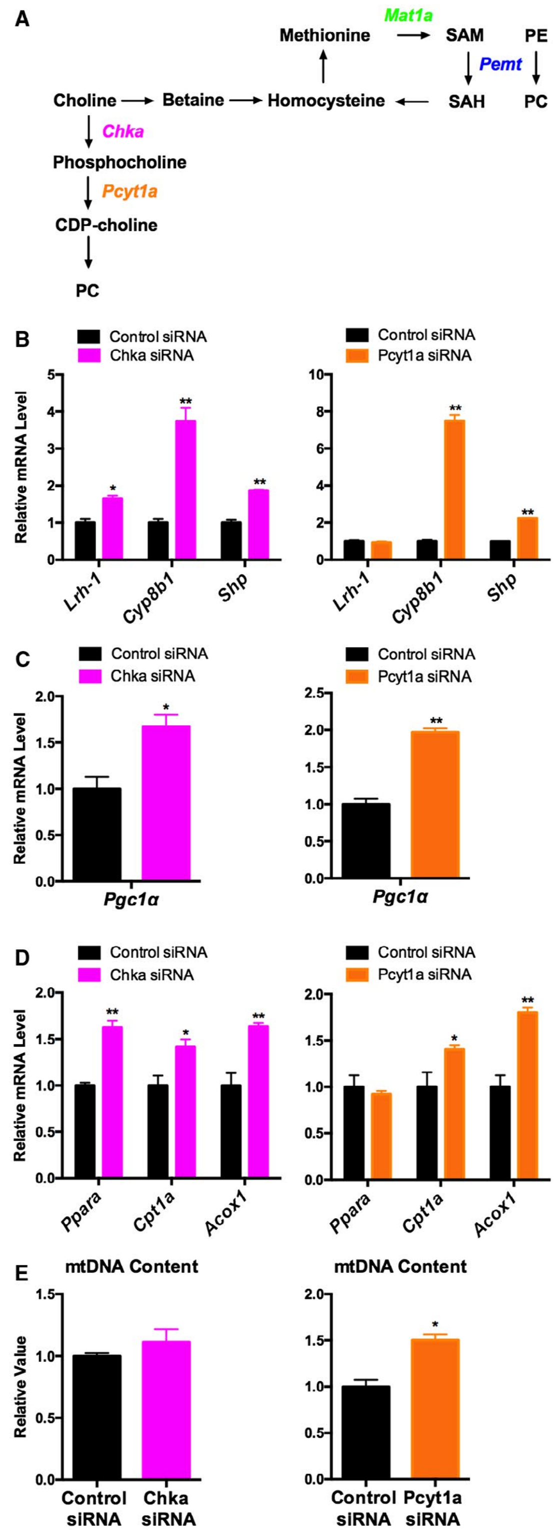

FIG. 3. The Kennedy pathway does not contribute to the *Lrh-1* regulation of mitochondrial activities. (A) Diagram of Kennedy and PEMT pathways. (B) Expression of *Lrh-1* and its target genes was measured in *Chka* KD or *Pcyt1a* KD C3A/HepG2 cells. (C) Expression of *Pgc1α* mRNA was measured in *Chka* KD or *Pcyt1a* KD C3A/HepG2 cells. (D) Expression of beta-oxidation-related genes was measured in *Chka* KD or *Pcyt1a* KD C3A/HepG2 cells. (E) Mitochondrial DNA copy number was measured in *Chka* or *Pcyt1a* KD C3A/HepG2 cells.

generates SAM molecules that are used by PEMT to triply methylate PE to form PC (Fig. 3A). To investigate which pathway contributes to the production of putative PC agonists for LRH-1, we first knocked down either *Pcyt1a* or *Chka* to disrupt the Kennedy pathway. Their siRNA knockdown (KD) in C3A/HepG2 cells resulted in a compensatory increase in the expression of PEMT pathway genes (Supporting Fig. S3). Using lipidomic profiling, we also found that *Pcyt1a* KD did not have a significant impact on total PC levels, but there were approximately 30 specific PC species up-regulated or down-regulated significantly upon *Pcyt1a* KD (Supporting Fig. S9). KD of either *Pcyt1a* or *Chka* also increased, rather than decreased, the expression of *Lrh-1* and its target genes, *Cyp8b1* and *Shp* (Fig. 3B). Although *Lrh-1* expression was increased only 1.5-fold upon *Chka* KD, the *Cyp8b1* induction was up to 4-fold and 8-fold in *Chka* and *Pcyt1a* KD cells, respectively. Together, these results suggest increased LRH-1 transactivation in response to Kennedy pathway KD (Fig. 3B). Supporting this idea, the induction of LRH-1 target genes in *Pcyt1a* KD cells was reduced or abolished by the LRH-1 antagonist 505601[29] (Supporting Fig. S4).

Consistent with the LRH-1 transactivation, mitochondrial biogenesis and beta-oxidation genes were induced by either *Pcyt1a* or *Chka* KD. *Pgc-1α* expression and *Cpt1a* expression are both significantly increased in either *Chka* or *Pcyt1a* KD cells, and *Ppara* is significantly induced by *Chka* KD ($P < 0.05$; Fig. 3C,D). We also found that mitochondrial DNA copy number was increased in *Pcyt1a* KD cells (Fig. 3E). Inhibition of LRH-1 by 505601 suppressed the induction of these mitochondrial genes in *Pcyt1a* KD cells (Supporting Fig. S4). Moreover, cytidine diphosphate (CDP)-choline is the product of PCYT1A and a cofactor required for generating Kennedy pathway–specific PC species.[30] We found that CDP-choline treatment did not affect the expression of mitochondrial biogenesis, beta-oxidation genes, or mitochondrial DNA content (Supporting Fig. S5).

It is apparent that the Kennedy pathway is neither required nor sufficient for the production of PC agonists for LRH-1 to regulate mitochondrial activities. When it is disrupted, LRH-1 is activated instead to promote mitochondrial biogenesis and beta-oxidation. One possible explanation for this response is that induction of the PEMT pathway to compensate for the loss of *de novo* PC synthesis (Supporting Fig. S3) would increase the production of PC agonists for LRH-1.

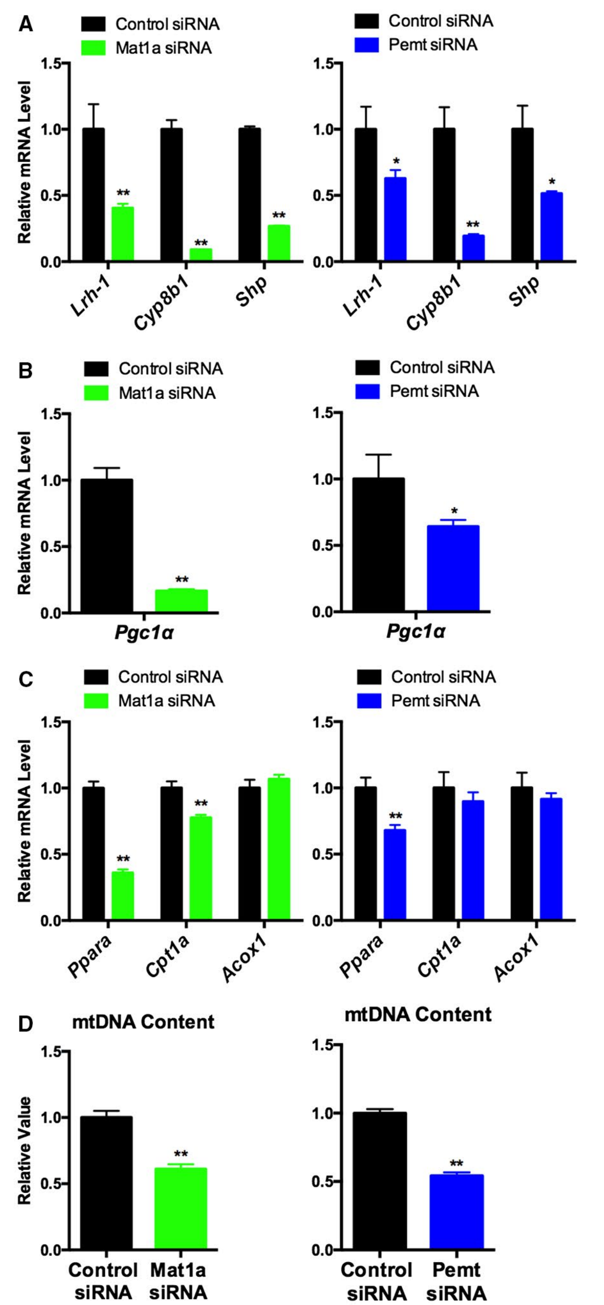

FIG. 4. The PEMT pathway positively regulates the effect of *Lrh-1* on mitochondria. (A) Expression of *Lrh-1* and its target genes was measured in *Mat1a* KD and *Pemt* KD C3A/HepG2 cells. (B) Expression of *Pgc1α* was measured in *Mat1a* KD and *Pemt* KD C3A/HepG2 cells. (C) Expression of beta-oxidation-related genes was measured in *Mat1a* KD and *Pemt* KD C3A/HepG2 cells. (D) Mitochondrial DNA copy number was measured in *Mat1a* KD and *Pemt* KD C3A/HepG2 cells.

THE PEMT PATHWAY POSITIVELY REGULATES THE EFFECT OF *Lrh-1* ON MITOCHONDRIA

To directly assess the predicted role of the PEMT pathway, we knocked down either *Mat1a* or *Pemt* with siRNA in C3A/HepG2 cells. As expected, *Mat1a* or *Pemt* KD increased mRNA expression of two Kennedy pathway–related enzymes, *Chka* and *Pcyt1a* (Supporting Fig. S6). *Pemt* KD did not significantly affect overall PC levels but did decrease levels of a large number of individual PC species, while increasing few others (Supporting Fig. S10). Interestingly, the identities of the PC species affected by *Pcyt1a* KD and *Pemt* KD were quite different (Supporting Fig. S12).

In accord with the prediction that the PEMT pathway is responsible for generating endogenous LRH-1 agonists, *Mat1a* or *Pemt* KD decreased LRH-1 target gene expression, including *Cyp8b1* and *Shp*, as well as that of *Lrh-1* itself (Fig. 4A). Despite the 40% reduction in *Lrh-1* expression upon *Pemt* KD, the decrease in *Cyp8b1* was twice as much, 80% (Fig. 4A). Furthermore, we found that mitochondrial biogenesis and beta-oxidation genes *Pgc-1α* and *Ppara* are reduced by either *Mat1a* or *Pemt* KD and that *Cpt1a* is reduced by *Mat1a* KD (Fig. 4B,C). We also found that mitochondrial DNA content levels are reduced in both *Mat1a* and *Pemt* KD cells (Fig. 4D). Importantly, treatment with the exogenous agonist DLPC restored the expression of LRH-1 target genes, including *Shp*, *Pgc-1α*, *Cpt1a*, and *Acox1*, that were down-regulated by *Mat1a* KD, although reduced mitochondrial DNA content was not rescued (Supporting Fig. S7).

Together, these results indicate that the PEMT pathway specifically contributes to the production of PC agonists for LRH-1 activation and expression of *Lrh-1* itself.

SAM SUPPLEMENTATION INCREASES LRH-1 TRANSACTIVATION IN A *PEMT*-DEPENDENT MANNER

The PEMT pathway requires SAM to synthesize PC from PE. Given the importance of the PEMT pathway in regulating LRH-1 activities, we tested whether and how SAM contributes to LRH-1 transactivation. We first used a luciferase reporter driven by the LRH-1 responsive *Shp* promoter and showed that SAM supplementation increases luciferase activity by 1.5-fold in C3A/HepG2 cells that endogenously express *Pemt* and *Lrh-1*. When the cells were transiently transfected with an LRH-1 expression vector, basal luciferase activity increased, as expected, and SAM supplementation further increased reporter activity by 1.5-fold compared to vehicle-treated, LRH-1-expressing cells (Fig. 5A). In contrast, in *Lrh-1* KD C3A/HepG2 cells SAM supplementation did not induce luciferase reporter activity, confirming the *Lrh-1* dependence of this response (Fig. 5A). Moreover, SAM supplementation also failed to induce luciferase reporter activity in *Pemt* KD cells (Fig. 5B), suggesting that the increased LRH-1 transactivation in response to SAM is also *Pemt*-dependent. In addition, SAM supplementation did not alter total PC levels but did increase and decrease levels of a large number of individual PC species (Supporting Fig. S11). The identities of the PC species affected by SAM supplementation were quite different from those affected by *Pcyt1a* or *Pemt* KD (Supporting Fig. S12).

To further support the link between SAM and PEMT in the induction of LRH-1 target gene expression, we used both 3-deazaadenosine (DZA), a specific inhibitor of PEMT enzymatic activity,[22] and a high-dose acute homocysteine treatment, which disrupts the flow from SAM to SAH.[17] We found that both DZA and acute homocysteine treatments decrease mRNA expression of *Lrh-1* target genes including *Cyp8b1* and *Shp* (Fig. 5C,D). Next, we fused the *Lrh-1* ligand binding domain to the galectin 4 DNA binding domain to drive an appropriate luciferase reporter and examined the effects of DZA and acute homocysteine treatments on LRH-1 transactivation. We found that both treatments reduced luciferase reporter activity (Fig. 5C,D). Expression of several other LRH-1 target genes, including apolipoprotein A1 (*Apoa1*), scavenger receptor class B member 1, glutaminase 2 (*Gls2*), and *Mdr3*, was also reduced upon DZA or homocysteine treatment. In the opposite direction, however, only *Apoa1* and *Gls2* showed induction upon SAM treatment, which might be due to different transcriptional regulation or negative feedback mediated by *Shp* (Supporting Fig. S8). Together, these results support that the activity of LRH-1 is regulated by SAM through the PEMT pathway.

To characterize whether SAM also regulates mitochondrial activities, we measured mitochondrial biogenesis and beta-oxidation genes. Interestingly, we

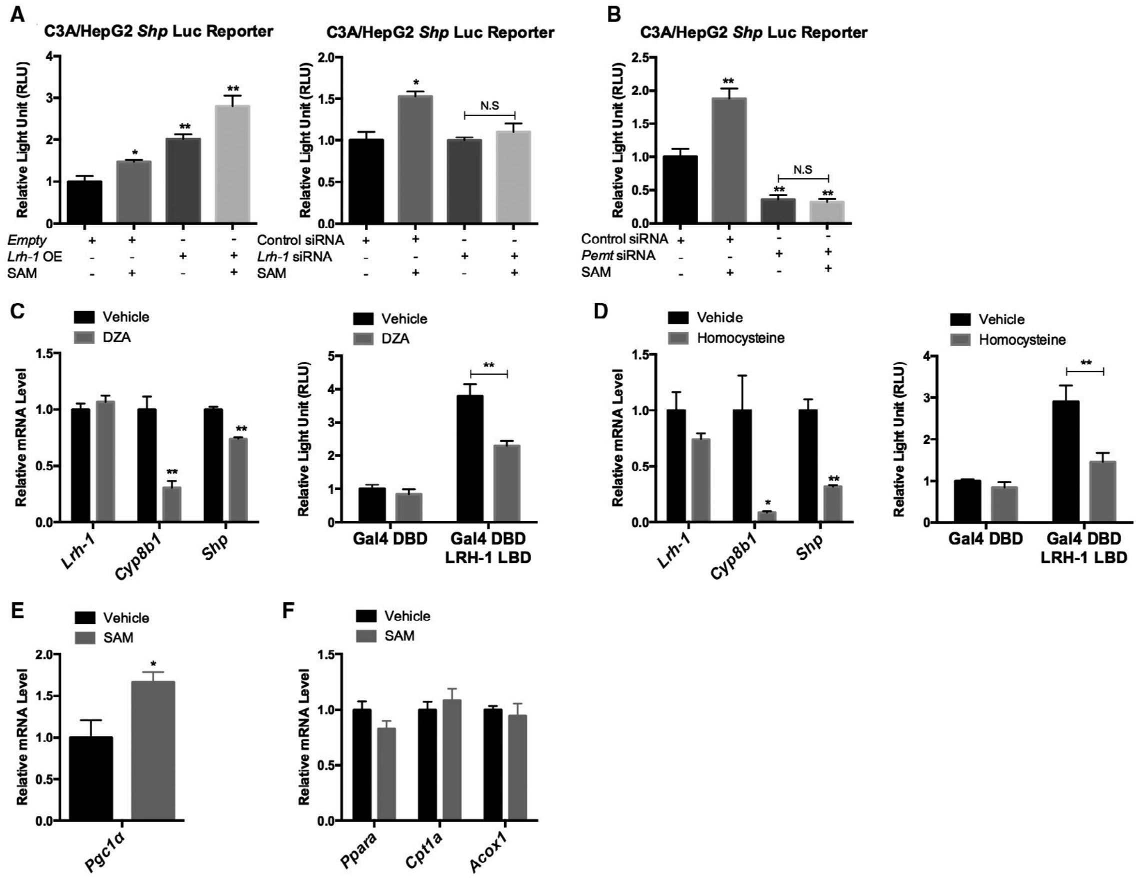

FIG. 5. SAM mediates the transactivation of LRH-1 in a PEMT-dependent manner. (A) *Shp* luciferase reporter activity was measured upon 10 hours of SAM supplementation (100 μM) to *Lrh-1* or empty vector transfected C3A/HepG2 cells. *Shp* luc reporter activity was measured upon 10 hours of SAM supplementation (100 μM) to *Lrh-1 KD* C3A/HepG2 cells. (B) *Shp* luciferase reporter activity was measured upon 10 hours of SAM supplementation (100 μM) to *Pemt* KD C3A/HepG2 cells. (C) Expression of *Lrh-1* and *Lrh-1* target gene was measured upon DZA (30 μM) treatment for 6 hours. LRH-1 ligand binding domain fused Gal4 DBD reporter activity was measured upon DZA (10 μM) treatment for 6 hours after C3A/HepG2 cells were transfected with luciferase reporter vectors for 12 hours. (D) Expression of *Lrh-1* and *Lrh-1* target gene was measured upon homocysteine (5 mM) treatment for 8 hours. LRH-1 ligand binding domain fused Gal4 DBD reporter activity was measured upon homocysteine (2 mM) treatment for 6 hours after C3A/HepG2 cells were transfected with luciferase reporter vectors for 12 hours. (E) Expression of *Pgc-1α* was measured in SAM-treated (200 μM) or vehicle-treated C3A/HepG2 cells for 48 hours. (F) Expression of beta-oxidation-related genes was measured in SAM-treated (200 μM) or vehicle-treated C3A/HepG2 cells for 48 hours. Abbreviations: DBD, DNA binding domain; OE, overexpression.

found that SAM administration to C3A/HepG2 cells induces *Pgc-1α* expression levels by 1.5-fold (Fig. 5E). On the other hand, beta-oxidation-related gene expression was not significantly changed (Fig. 5F), which might be due to other effects of SAM on lipid metabolism and/or transcriptional regulation. In accord with the induction of *Pgc-1α* expression (Fig. 5E), SAM administration to C3A/HepG2 cells increased mitochondrial DNA content by 1.5-fold (Fig. 6A). As a result of increased mitochondrial biogenesis, SAM administration increased ATP levels by 2-fold in C3A/HepG2 cells (Fig. 6B) and increased basal respiration and maximal respiration (Fig. 6C,D).

Together, our studies support a model in which the one-carbon metabolic cycle regulates SAM synthesis through *Mat1a*, and SAM is then fed into PC synthesis through the PEMT pathway. Specific PC species generated by PEMT act as agonists to transactivate LRH-1 and consequently regulate mitochondrial biogenesis and beta-oxidation (Fig. 7).

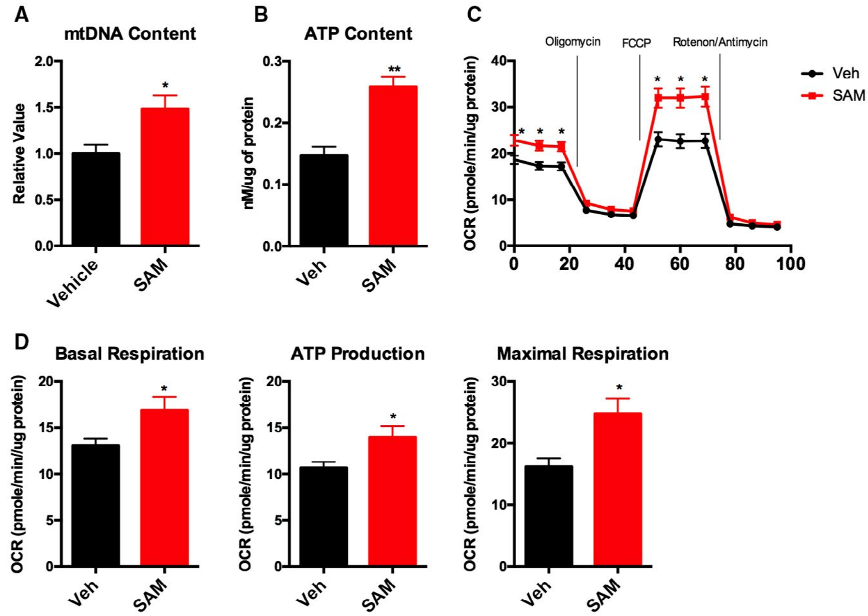

FIG. 6. SAM supplementation promotes mitochondrial biogenesis and mitochondrial functions. (A) Mitochondrial DNA copy number was measured from SAM-treated (200 μM) or vehicle-treated C3A/HepG2 cells for 48 hours. (B) ATP content was measured after SAM (200 μM) treatment for 24 hours. (C) OCR was measured in C3A/HepG2 cells after SAM (200 μM) treatment for 24 hours. Basal respiration, ATP production, and maximal respiration were quantified.

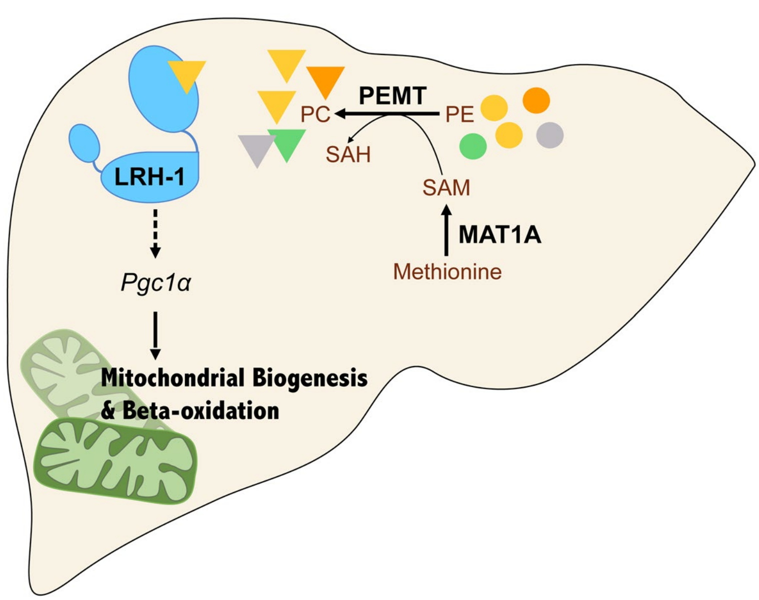

FIG. 7. PEMT-mediated PC synthesis transactivates LRH-1 and promotes mitochondrial biogenesis and beta-oxidation. Working model. SAM generated by MAT1a is used by PEMT to synthesize specific PC species, which act as agonist ligands to transactivate LRH-1 to induce mitochondrial biogenesis and beta-oxidation in hepatocytes.

Discussion

Our previous results linked LRH-1 and its *C. elegans* homolog NHR-25 to fat metabolism and mitochondrial dynamics.[25] Here, we confirm this association and extend it to mitochondrial biogenesis and beta-oxidation. We also define an SAM–PEMT–LRH-1 pathway in which SAM metabolism couples with the PEMT pathway to tune the LRH-1-mediated regulation of mitochondrial activities. This mechanism provides a molecular basis for our previous finding that LRH-1 activity is decreased in the livers of mice fed a methionine and choline–deficient diet, suggesting that LRH-1 functions as both a sensor and a regulator of methyl pool homeostasis.[24] Consistently, activity of the *C. elegans* LRH-1 relative NHR-25 is also sensitive to the environmental methyl pool that determines endogenous SAM levels and PC synthesis through the PEMT pathway.[25] Beyond the link between NHR-25 and mitochondrial morphological dynamics, our results uncover a role for LRH-1 in regulating mitochondrial biogenesis and beta-oxidation, which are directly linked with fat metabolism.

A previous report described altered mitochondrial function in global *Pemt* KO livers.[31] In accord with our studies, the mitochondrial area was decreased. Gluconeogenesis was significantly compromised, but in hepatocytes maintained in the absence of fatty acids, glycolysis was increased. This increase was correlated with the expected decrease in the mitochondrial PC/PE ratio. Expression of mitochondrial biogenesis and fatty acid oxidation genes was not altered, perhaps due to compensatory adaptations in mitochondrial functions that are not directly linked to *Pemt* deficiency. In our studies, it is likely that acute disruption of PEMT by KD reveals direct effects of PEMT deficiency on mitochondrial functions. Importantly, our results from *Mat1a* KD and SAM supplementation are consistent with those from *Pemt* KD, providing strong support for the role of the SAM–PEMT–LRH-1 pathway in regulating mitochondrial functions.

Many studies have linked increased SAM levels to reduced fat accumulation in the liver and decreased SAM levels to the opposite. Decreasing the SAM/SAH ratio or depleting the methyl pool causes fat accumulation in hepatocytes. This has been attributed to a reduction in VLDL-mediated lipid export related to decreased PEMT activity.[20] Based on our findings, we propose an additional mechanism, in which decreased methyl-pool or SAM levels decrease levels of endogenous LRH-1 agonist(s) and therefore decrease mitochondrial biogenesis and beta-oxidation, leading to fat accumulation. A high level of homocysteine, homocysteinemia, is often observed in the serum of NAFLD patients.[32] High homocysteine levels inhibit SAM to SAH flow.[33] Based on our result that interrupting SAM to SAH flow by *Mat1a* KD or acute homocysteine treatments decreases *Lrh-1* signaling (Figs. 4A and 5D), it is possible that NAFLD patients with homocysteinemia might have defective *Lrh-1* signaling and mitochondrial malfunction.

A recent report identified mammalian target of rapamycin complex 1 (mTORC1) as another downstream target of SAM.[34] SAM is directly sensed by an inhibitory mTORC1 binding partner called SAMTOR. SAM binding dissociates SAMTOR from mTORC1 and activates mTORC1 signaling. In the opposite direction, methionine starvation depletes SAM and inactivates mTORC1 signaling. Activation of mitochondrial energy metabolism in coordination with ESRRA[35] is one of the many pathways regulated by mTORC1. Our results suggest that the SAM–PEMT–LRH-1 pathway could reinforce this mTORC1 function by augmenting mitochondrial number and function.

Although the Kennedy pathway is thought to account for approximately 70% of PC production in hepatocytes, it is clearly dispensable for generation of the putative but still unknown endogenous PC agonists of LRH-1. Instead, LRH-1 transactivation is increased upon disruption of the Kennedy pathway. On the other hand, inhibition of the PEMT pathway by *Mat1a* or *Pemt* KD decreased LRH-1, strongly implicating the SAM–PEMT pathway in LRH-1 transactivation and providing a direct mechanism for LRH-1 response to the labile methyl pool. It is interesting that this pathway preferentially produces PC species with longer fatty acid side chains,[36] suggesting that such species may be preferential LRH-1 agonists. However, our biochemical studies agree with previous results[37] that many different PC species are able to bind the LRH-1 ligand binding domain *in vitro* (data not shown). Furthermore, lipidomics profiling failed to identify any specific candidate PC species that fit the predicted LRH-1 agonist profile: increased by *Pcyt1a* KD and SAM supplementation but decreased by *Pemt* KD (Supporting Fig. S12).

Therefore, more functional studies are required to address the important question regarding the nature of endogenous LRH-1 agonists. Particularly in light of previous results with phosphoinositols,[5,6] it is likely that LRH-1 has multiple agonists, potentially with distinct regulatory effects.

When regulating mitochondrial biogenesis, LRH-1 activation induces an increase in mitochondrial DNA content that is associated with an increase in the expression of PGC-1α. PGC-1α is not only a well-known inducer of mitochondrial biogenesis[38] but also an LRH-1 coactivator.[9] This suggests the existence of a self-supporting feed-forward loop in which LRH-1 activation reinforces its own transactivation through increased activity of its key coregulator. The identification of *Pemt*[39,40] for PC production, and potentially several genes for the generation of specific longer-chain unsaturated PC species[11] as LRH-1 target genes, suggests an additional loop in which LRH-1 induces expression of its own agonist(s). Upon fasting, PGC-1α levels are induced in response to protein kinase A activation of cAMP response element-binding protein,[38] and PGC-1α activity can also be increased through phosphorylation by adenosine monophosphate–activated protein kinase and deacetylation by Sirtuin 1.[41] Thus, increased PGC-1α activity in response to nutrient deprivation could provide an additional nutritional input to regulate LRH-1 transactivation. It is intriguing that the potential PGC-1α agonist feed-forward loops are strongly opposed by the previously described role of LRH-1 as an inducer of its potent negative regulator, SHP.[8] The existence of both positive and negative feedback loops substantially increases the complexity of LRH-1 regulatory circuits, with difficult-to-predict implications for the magnitudes of responses and their kinetics over time.

In summary, we conclude that *Lrh-1* together with PEMT-mediated PC synthesis is required for mitochondrial biogenesis and beta-oxidation. This highlights *Lrh-1* as a potential target to manipulate mitochondrial activities, with beneficial impact on type 2 diabetes and other diseases associated with elevated liver fat.

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