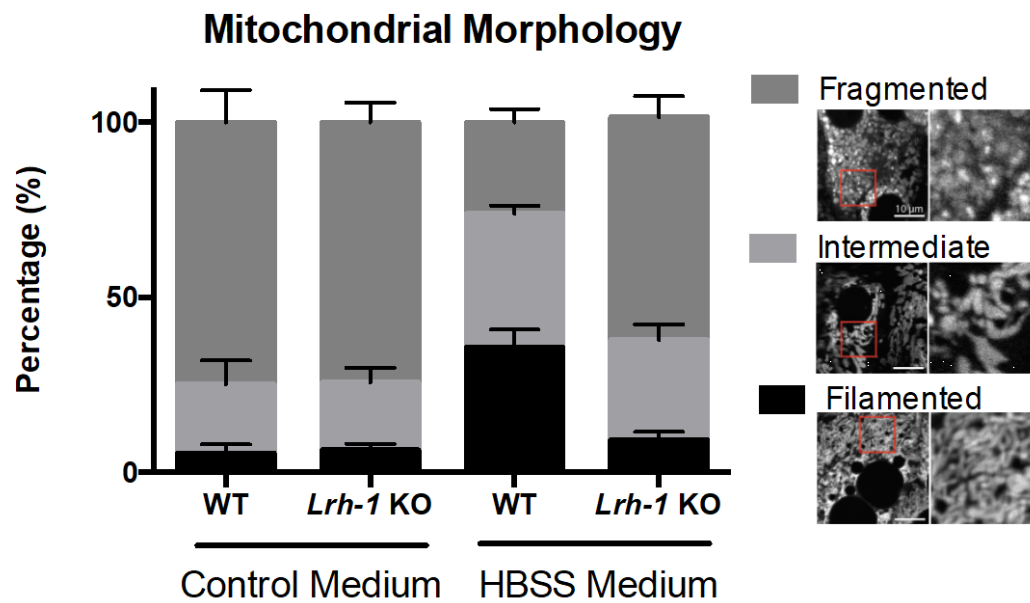
HEP-18-1152
Methyl-sensing nuclear receptor Liver Receptor Homolog-1 regulates mitochondrial biogenesis and beta-oxidation in mouse hepatocytes

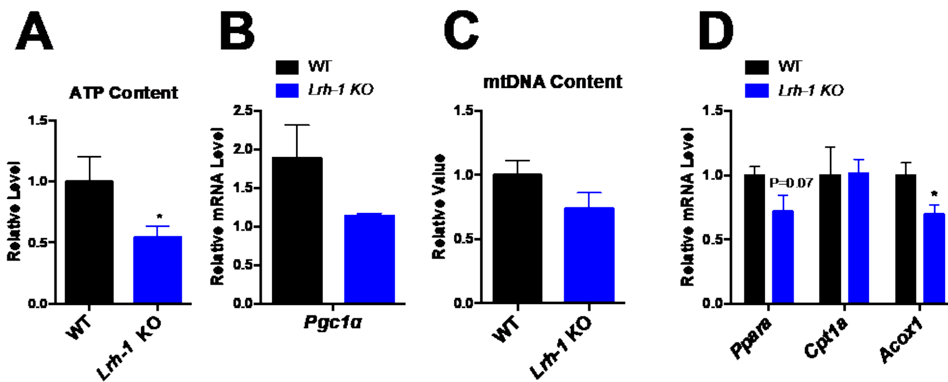
Supporting Information

Supplementary Figures:

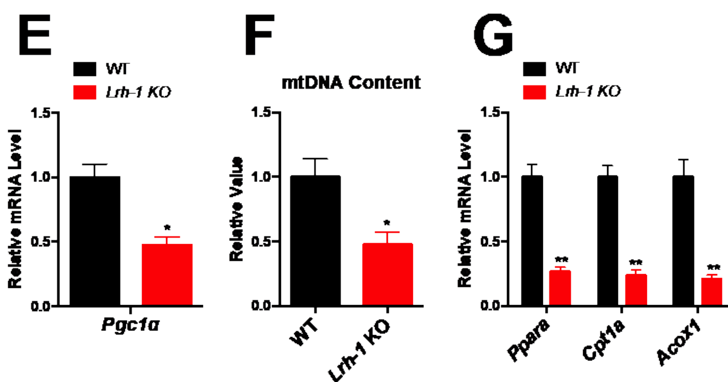


Supplementary Figure 1. *Lrh-1* is required for starvation induced mitochondrial filamentation. A. Mitochondrial morphology of WT and *Lrh-1* KO primary hepatocytes in control medium (William's E) and HBSS medium quantified as percentage of total cells.

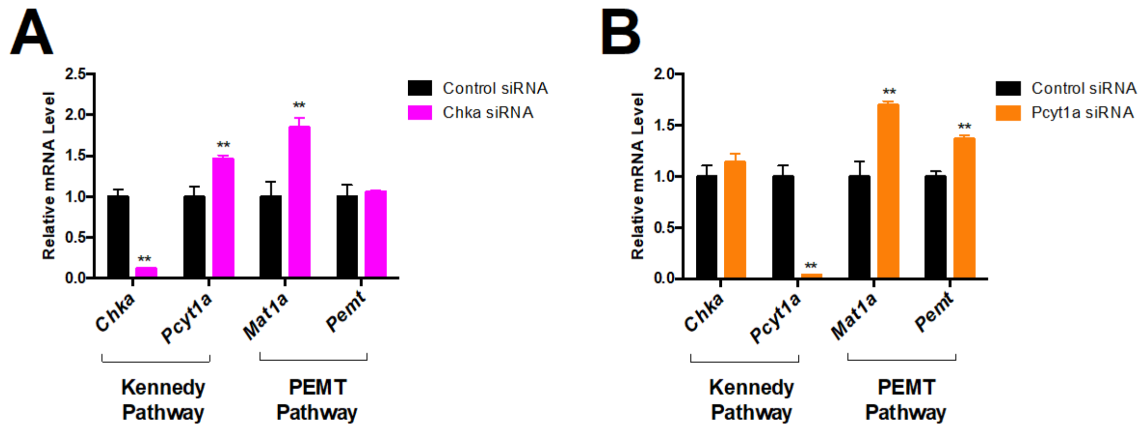
Liver



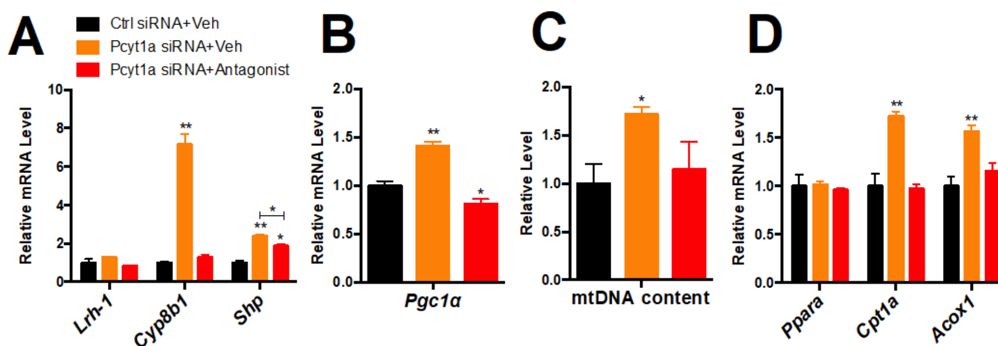
Fresh Hepatocytes



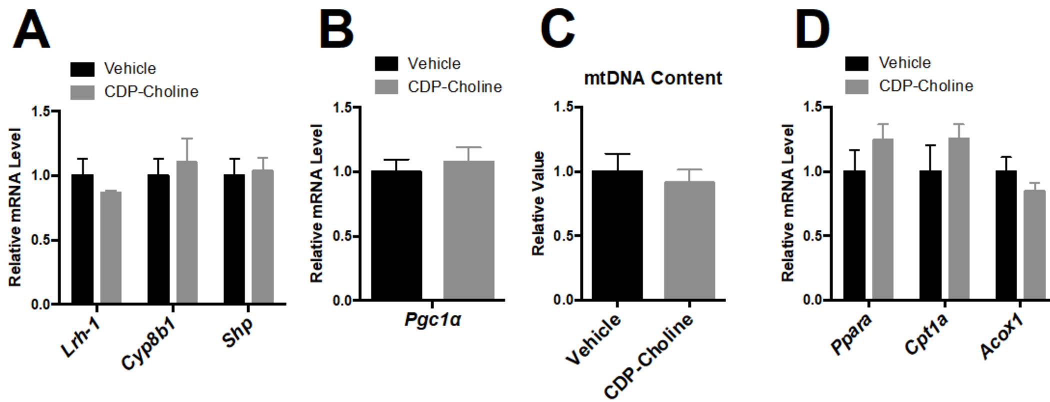
Supplementary Figure 2. Hepatic *Lrh-1* regulates mitochondrial functions. A. ATP content was measured in WT and *Lrh-1* KO liver, or **Figure 1C.** freshly isolated primary hepatocytes prior to plating. **B.** PGC1α mRNA expression WT and in *Lrh-1* KO liver or **E.** freshly isolated primary hepatocytes prior to plating. **C.** Mitochondrial DNA content in WT and *Lrh-1* KO liver, or **F.** fresh primary hepatocytes **D.** mRNA expression of LRH-1 responsive beta-oxidation genes in WT and *Lrh-1* KO liver, or **G.** fresh primary hepatocytes.



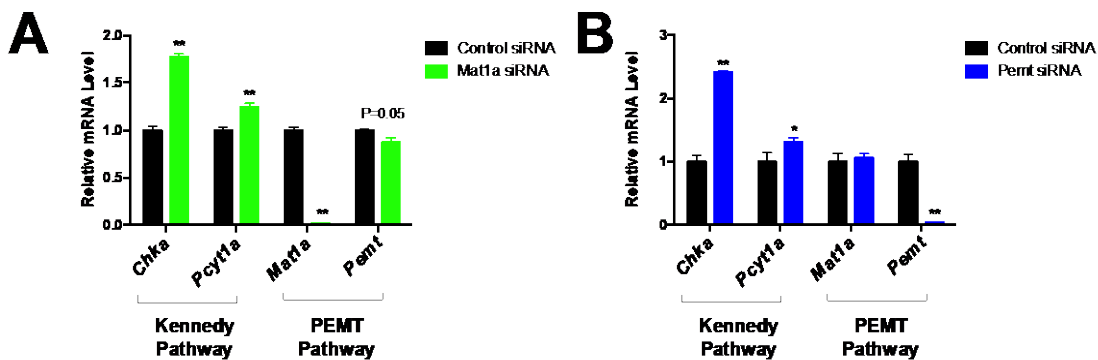
Supplementary Figure 3. Disrupting the Kennedy pathway increases PEMT pathway related enzyme expression. A. mRNA expression of Kennedy Pathway and PEMT pathway related enzymes was measured in *Chka* KD C3A/HepG2 cells. **B.** Expression of the Kennedy Pathway and PEMT pathway related enzymes was measured in *Pcyt1a* KD C3A/HepG2 cells.



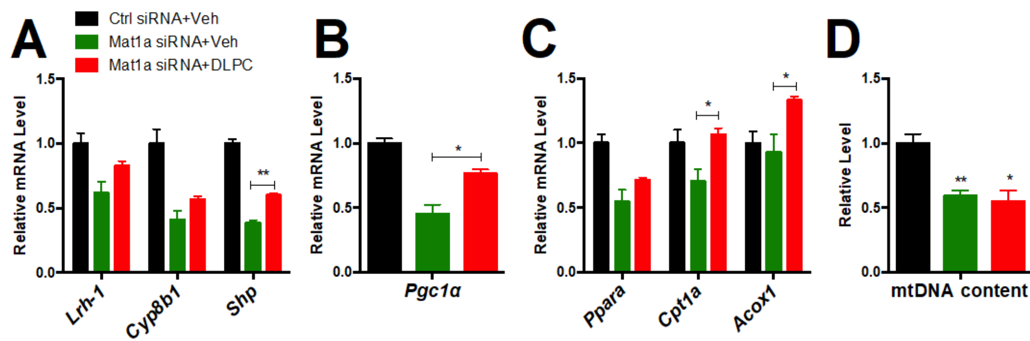
Supplementary Figure 4. LRH-1 antagonist 505601 reduces or abolishes *Pcyt1a* KD induced LRH-1 target, mitochondrial biogenesis and beta-oxidation genes. A. Expression of LRH-1 target genes was measured in *Pcyt1a* KD C3A/HepG2 cells in the presence of LRH-1 antagonist. **B**. Expression of the *Pgc-1α* was measured in *Pcyt1a* KD C3A/HepG2 cells after LRH-1 antagonist treatment. **C.** Mitochondrial DNA content was measured after LRH-1 antagonist treatment to *Pcyt1a* KD C3A/HepG2 cells. **D.** Expression of beta-oxidation genes was measured in *Pcyt1a* KD cells in the presence of LRH-1 antagonist.



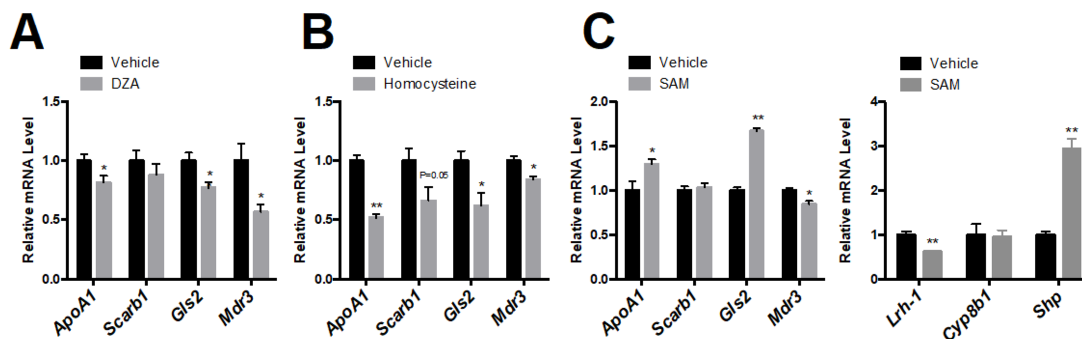
Supplementary Figure 5. CDP-choline treatment does not affect LRH-1 target, mitochondrial biogenesis and beta-oxidation genes. A. Expression of LRH-1 target genes was measured in C3A/HepG2 cells in the presence of CDP-choline for 48hrs. **B**. Expression of Pgc-1α mRNA was measured in C3A/HepG2 cells after CDP-choline treatment. **C.** Mitochondrial DNA content was measured in C3A/HepG2 cells after CDP-choline treatment. **D.** Expression of beta-oxidation genes was measured in C3A/HepG2 cells after CDP-choline treatment.



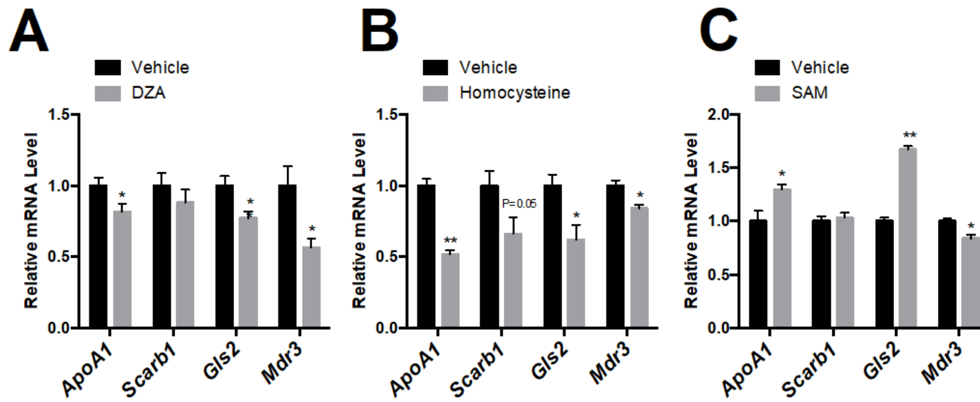
Supplementary Figure 6. Disrupting PEMT pathway increases the Kennedy pathway related enzymes expression. A. mRNA expression of Kennedy Pathway and PEMT pathway related enzymes was measured in *Mat1a* KD C3A/HepG2 cells. **B.** Expression of Kennedy and PEMT pathway related enzymes was measured in *Pemt* KD C3A/HepG2 cells.



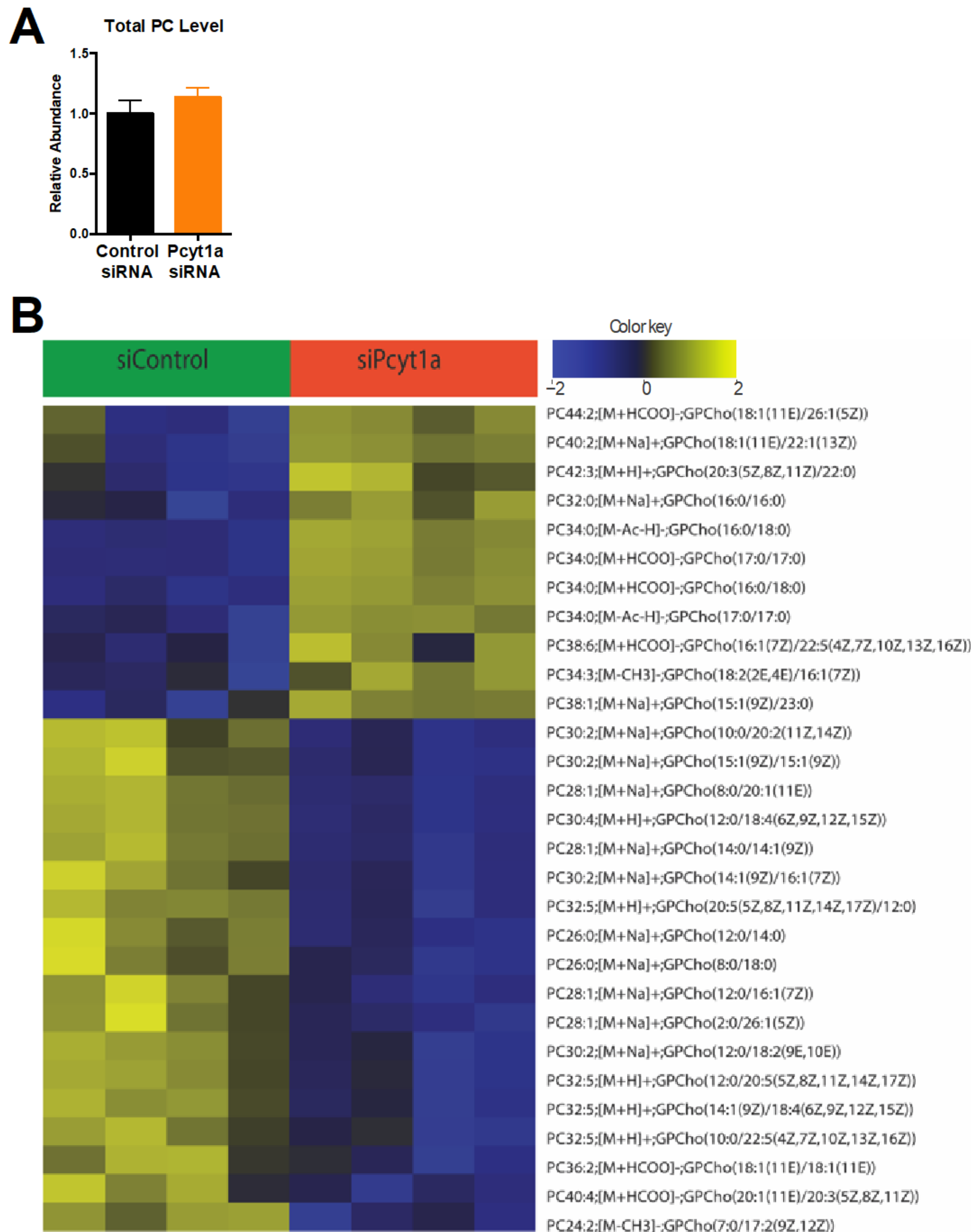
Supplementary Figure 7. DLPC treatment partially rescues *Mat1a* KD reduced LRH-1 target, mitochondrial biogenesis and beta-oxidation genes. A. Expression of LRH-1 target genes was measured in *Mat1a* KD C3A/HepG2 cells in the presence or absence of DLPC. **B.** Expression of the *Pgc-1α* was measured in *Mat1a* KD C3A/HepG2 cells in the presence or absence of DLPC. **C.** Mitochondrial DNA copy number was measured after DLPC treatment to *Mat1a* KD C3A/HepG2 cells. **D.** Expression of beta-oxidation genes was measured in *Mat1a* KD cells in the presence or absence of DLPC**.**



Supplementary Figure 8. Homocysteine or DZA treatment reduces and SAM treatment induces LRH-1 target genes. A. Expression of LRH-1 target genes was measured in C3A/HepG2 cells after DZA treatment for 8hrs. **B**. Expression of LRH-1 target genes was measured in C3A/HepG2 cells after homocysteine treatment for 8rhs **C.** Expression of LRH-1 target genes was measured in C3A/HepG2 cells in the presence of SAM for 48hrs.

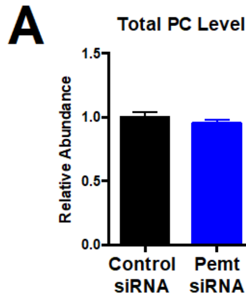


Supplementary Figure 9. Homocysteine or DZA treatment reduces and SAM treatment induces LRH-1 target genes. A. Expression of LRH-1 target genes was measured in C3A/HepG2 cells after DZA treatment for 8hrs. **B**. Expression of LRH-1 target genes was measured in C3A/HepG2 cells after homocysteine treatment for 8rhs **C.** Expression of LRH-1 target genes was measured in C3A/HepG2 cells in the presence of SAM for 48hrs.



Supplementary Figure 10. PC species with different fatty acyl chains are regulated by *Pcyt1a*. A. Total level of PC was measured from *Pcyt1a* KD C3A/HepG2 cells. **B.** Comprehensive lipidomics analysis was done with C3A/HepG2 cells transfected with siRNA targeting *Pcty1a*.

A



B

siControl
siPemt
Color key
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0
2

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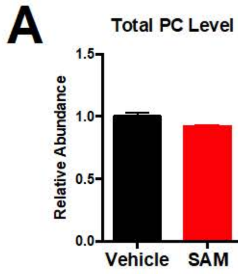
Supplementary Figure 11. PC species with different fatty acyl chains are regulated by *Pemt*. A. Total level of PC was measured from *Pemt* KD C3A/HepG2 cells. **B.** Comprehensive lipidomics analysis was done with C3A/HepG2 cells transfected with siRNA targeting *Pemt*.



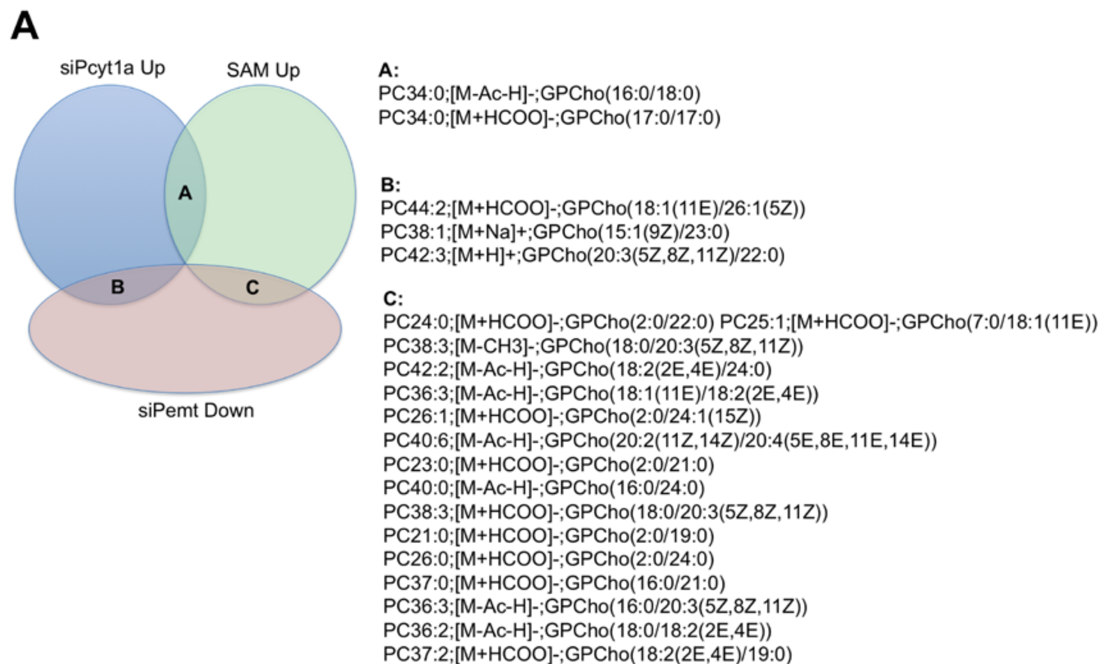
B

Vehicle SAM

Color key

-2 0 2

Supplementary Figure 12. PC species with different fatty acyl chains are regulated by SAM treatment. A. Total level of PC was measured from vehicle control or SAM treated C3A/HepG2 cells. **B.** Comprehensive lipidomics analysis was done with C3A/HepG2 cells treated with SAM for 16hrs.



Supplementary Figure 13. Distinct PC species are affected by SAM supplementation, *Pcyt1a* KD, or *Pemt* KD was. A. Overlaps of comprehensive lipidomics analysis of C3A/HepG2 cells after SAM treatment or *Pemt* silencing or *Pcyt1a* silencing are indicated.

Supplementary Experimental Procedures:

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 0ff) and fed standard chow diet and water *ad libitum*.

Luciferase Assay. When C3A/HepG2 cells reached to 80% confluence, cells were re-plated onto 12-well plates as 1:6 ratio. On the next day, cells were transfected with Shp-luc (200ng/well), pCMX hLRH1 vector (200ng/well), or empty vector (200ng/well), using lipofectamin 2000(11668019; Invitrogen) for 24hrs. Then, SAM (A7007; Simga) were administered to the cells for 10hrs at 100μM under charcoal stripped FBS. To study the *Pemt* or *Lrh-1* dependency, C3A/HepG2 cells on 24 well plates were transfected with siRNA targeting *Pemt* (10pmol/well) (Invitrogen) and Shp-luc (200ng/well) together for 48hrs, using lipofectamin 2000(11668019; Invitrogen). Then the cells were treated with SAM at 100μM for additional 10hrs under charcoal stripped FBS. For LRH-1 ligand binding domain fused Gal-4 DBD reporter assay, C3A/HepG2 cells were transfected with G5-tk-luc (200ng/well), pCMX Gal4 DBD only (100ng/well), pCMX Gal4 DBD LRH-1 LBD (100ng/well), and pSV40 beta-galactosidase vector (50ng/well) for 12hrs using lipofectamine 2000(11668019; Invitrogen). Then the cells were treated with homocysteine at 2mM for 6hrs under regular FBS or DZA at 10μM for 6hrs under charcoal stripped FBS. For pCMX Gal4 DBD - LRH-1 LBD vector, LRH-1 ligand binding domain (amino acids residue 185-541) was cloned into the pCMX-Gal4 DBD empty vector (PMID16439367).

Mitochondrial DNA Copy Number Measurement. Total DNA was isolated from primary hepatocytes using Qiagen DNA/RNA Miniprep kit (80204; Qiagen). Then its DNA was used as a template to run qPCR with the primers specific for mitochondrial DNA. *Nd1* and *Nd4* primers were used for mitochondrial DNA detection and their quantitative CT values were normalized by the CT values from nuclear DNA encoded primer, targeting *b2m*. Primer information is upon request.

Primary Hepatocytes Isolation. Mice were anesthetized with Isofluorane, then 25G needle was inserted into the inferior vena cava. Cutting the portal vein, liver was perfused with Earle's balanced salt solution (EBSS) containing 5mM EGTA. Following EBSS perfusion, Hank's balanced salt solution containing collagenase and trypsin inhibitor was used to perfuse the liver. The liver was removed and then massaged to obtain dissociated cells in hepatocytes wash medium (17704-024; Invitrogen). This suspension was layered onto percoll (P4927; Sigma) and only viable hepatocytes were washed with hepatocyte wash medium. Then the cells were plated onto the 10cm plate, 6 well plate or XF24 cell culture microplate (100777-004;Agilent). The cells were cultured in William's E medium (12551; Invitrogen)

MitoTracker staining. The lyophilized MitoTracker Green FM (M7514; Invitrogen) was dissolved in high-quality, anhydrous dimethylsulfoxide (DMSO) to make 1mM stock solution, kept frozen at ≤ –20°C, and protected from light. On the day of experiment, making serial dilution from the stock solution to the final concentration of 100nM in the William's E medium or HBSS medium, and keeping warm at 37°C. Primary hepatocytes on glass bottom cell culture dishes (89125-438; VWR, 250,000 cells/plate) were washed with pre-warmed PBS, and then cultured with pre-warmed William's E medium or HBSS medium for 5~6 hours. After that, we replaced medium with 1ml of the freshly-made pre-warmed staining solution, which is 100nM MitoTracker Green in the corresponding medium. Cells were incubated at 37°C for 30 minutes. After staining, cells were washed three times with the corresponding medium, and incubated for another 1 hour in the corresponding medium (2ml). Mitochondrial morphology was examined by using con-focal fluorescence microscope with a water condenser, 60X objective, and digitally zoomed at 3X. The excitation wavelength is 490nm, and the emission wavelength is 516nm. Based on the mitochondrial morphology, each cell was categorized into three groups: fragmented, intermediate, or filamented.

ATP and Ketone Body Measurement

ATP content was measured from 15000 primary hepatocytes and 10000 C3A/HepG2 cells using ATP level measurement kit (ab113849; Abcam). Ketone Body measurement kit (700190; Cayman) was used to quantify Beta-hydroxybutyrate per 18*10^6 primary hepatocytes.

Drug Treatment. AML12 cells on 6 well plates were treated with DLPC for 16hrs at 100μM in DMEM/F-12 containing 10% charcoal stripped FBS. 48hrs after C3A/HepG2 cells were transfected with siRNA (Dharmacon), cells were treated with beta-cyclodextrin conjugated di-

lauroyl phosphatidylcholine (1:1 ratio) (DLPC) (850335; Avanti) at 100μM for 12hrs in DMEM/F-12 containing 10% charcoal stripped FBS.

After 6hrs of siMat1a transfection to C3A/HepG2 cells, the cells were treated with DLPC at 50uM for 24hrs in DMEM/F-12 containing 10% charcoal stripped FBS.

After 8hrs of siPcyt1a transfection to C3A/HepG2 cells, LRH-1 antagonist (505601; Calbiochem) was administered to the cells for 40hrs at 20uM.

For DZA (9000785; Cayman) or Homocysteine (H4628; Sigma) treatment, C3A/HepG2 cells on 6 well plates were treated with DZA for 6-8hrs at 30μM or with homocysteine for 8hrs at 5mM.

For SAM treatment, C3A/HepG2 cells were treated with SAM(A1007; Sigma) for either 24hrs or 48hrs in DMEM/F-12 containing 10% charcoal stripped FBS at 200uM. Fro CDP-choline treatment, CDP-choline (C0256; Sigma) was administered to C3A/HepG2 cells for 48hrs in DMEM/F-12 containing 10% charcoal stripped FBS at 200uM.

Oxygen Consumption Rate Measurement. 12500 primary hepatocytes were plated on XF24 cell culture microplate (100777-004;Agilent) and cultured in William's E medium overnight. The rest of process was performed according to manufacturer's protocol. For endogenous beta-oxidation SeaHorse assay, etomoxir at 20μM (11969; Cayman) and rotenone/antimycin at 0.5μM (103015-100;Agilent) were used to treat the cells in William's E medium containing 10% FBS. For SeaHorse assay, oligomycin at 2μM, FCCP at 1μM, rotenone/antimycin at 0.5μM (103015-100; Agilent) were administered to the cells in SeaHorse XF assay medium (102365-100; Agilent) supplemented with glucose and pyruvate. For SeaHorse mitostress assay with C3A/HepG2 cells, 12500 cells were plated on XF96 cell culture microplate (101085-004;Agilent) and cultured in DMEM/F12 medium containing 10% FBS overnight. Then SAM at 200uM was administered to the cells in DMEM/F12 medium containing 10% charcoal stripped FBS for 24hrs. After medium was replaced with SeaHorse XF assay medium supplemented with glucose and pyruvate, the plate were stayed in CO2 free incubator for an hour before running the assay. Oligomycin at 2uM, FCCP at 1uM, and rotenone/antimycin at 0.5uM were used for SeaHorse mitostress assay.