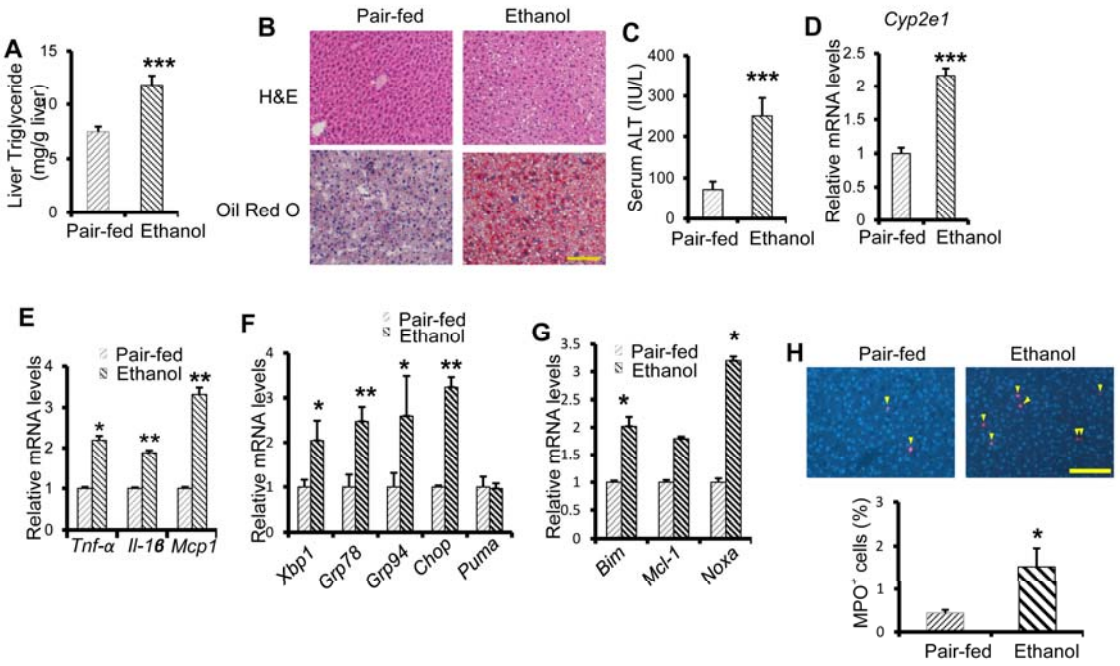
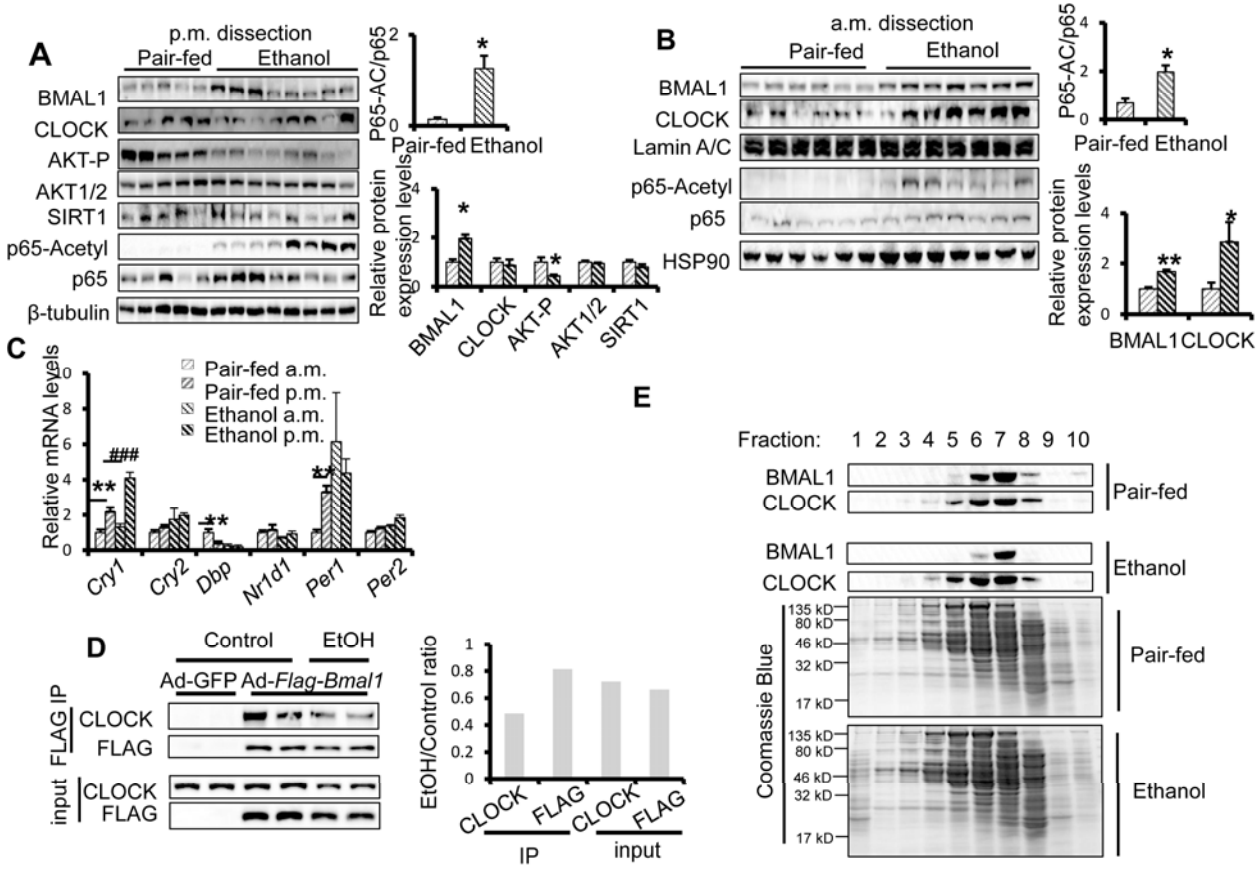
Supplementary information



Supporting Figure 1. Chronic-plus-binge ethanol feeding induces liver injury in WT mice.

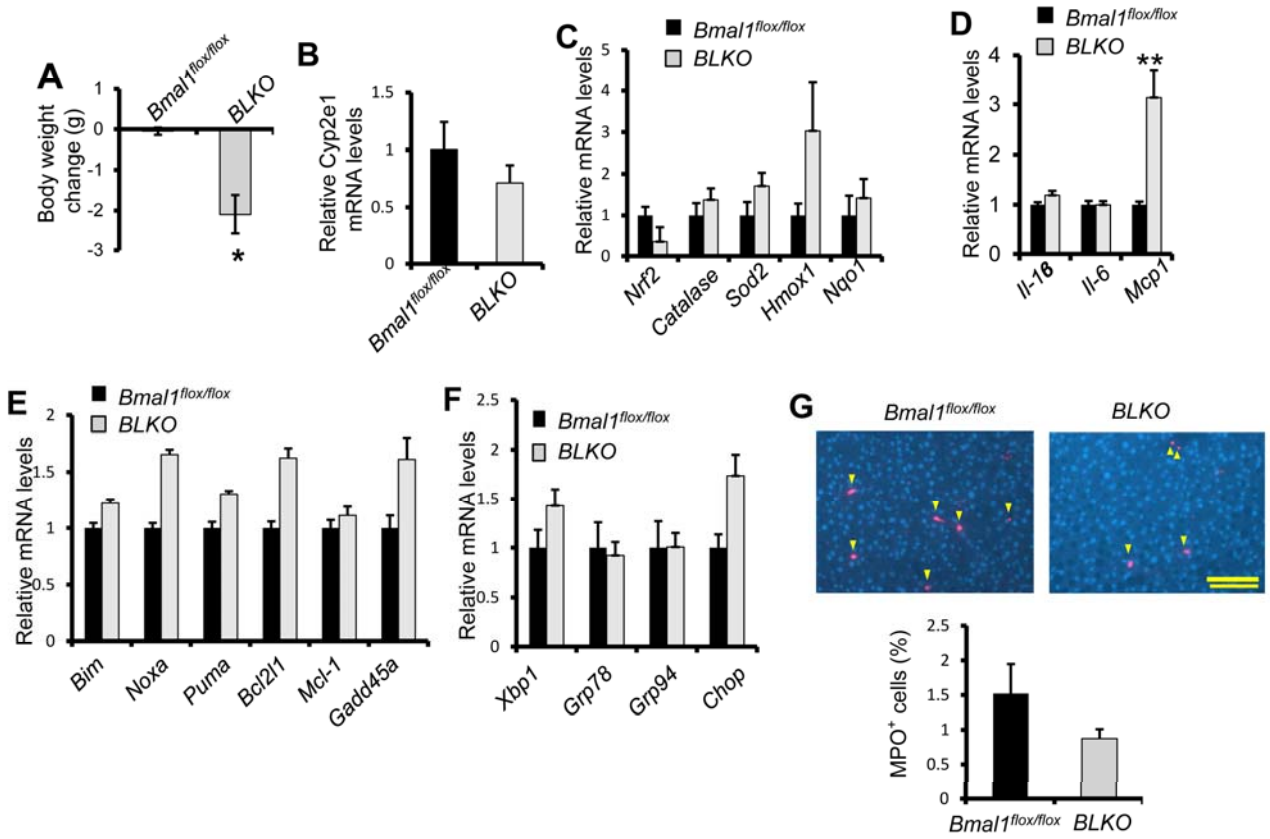
8-wk WT mice were fed either 5% ethanol diet or iso-caloric control diet for 10 days before binged with either ethanol (5g/kg body weight) or maltose dextrin (9 g/kg body weight) at ZT 3, and dissected 9 hr later (n = 5 for control diet and n = 8 for ethanol diet, both males and females). (**A-B**) Ethanol feeding induces liver steatosis. After dissection, liver triglyceride was measured (**A**), and hepatic lipid accumulation was determined by H&E staining and Oil Red O staining (**B**). (**C-H**) Ethanol feeding induces liver injury, assessed by ALT assay (**C**) and RT-qPCR assessing *Cyp2e1* (**D**), inflammation genes (**E**), ER stress markers (**F**) and pro-apoptotic genes (**G**). Immunohistochemistry against Myeloperoxidase (Red, indicated with arrowheads) was utilized to visualize Neutrophils (**H**). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-tailed Student's *t* test. Scale bar = 100 μM.



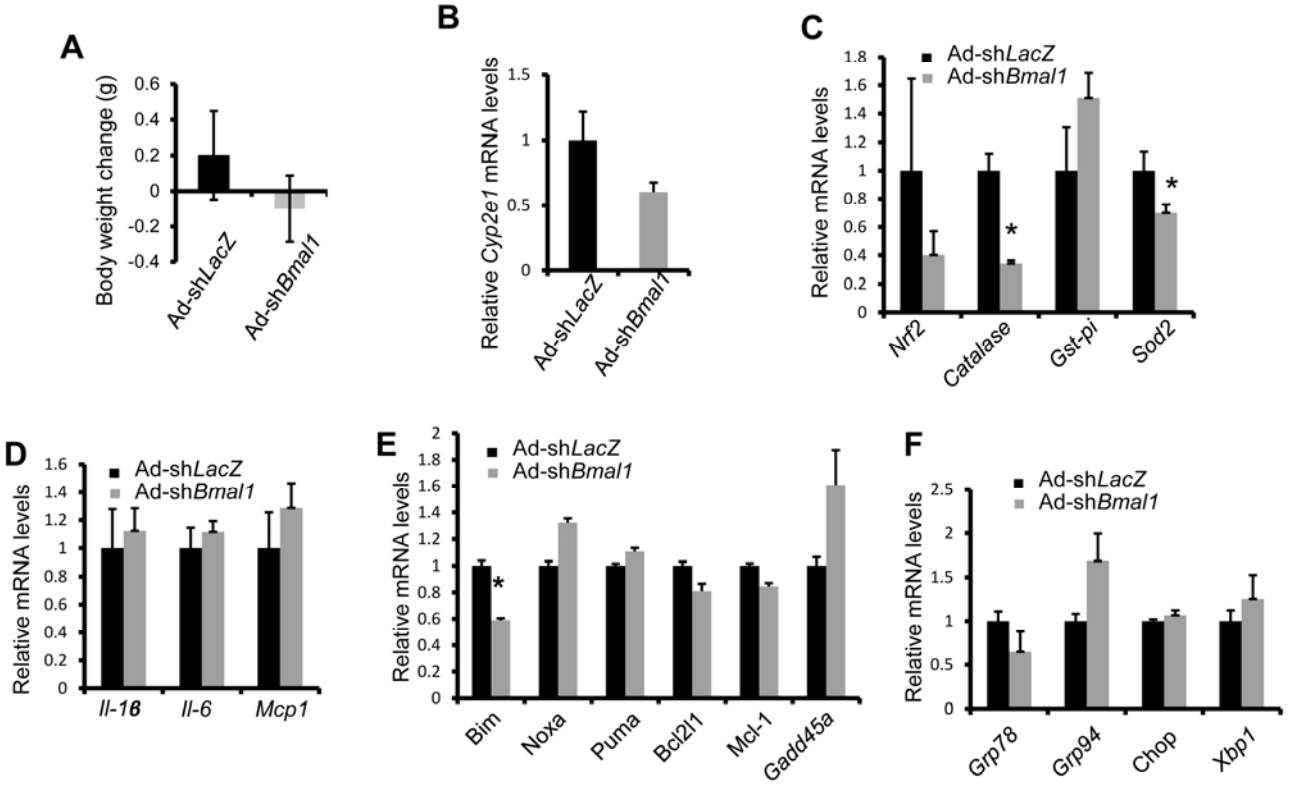
Supporting Figure 2. Ethanol feeding partially impaires the circadian clock in the liver.

(**A-C**) Ethanol consumption disrupted hepatic circadian clock. 8-wk mice were fed either 5% ethanol diet or iso-caloric control diet for 10 days before binged with either 5g ethanol or 9g maltose dextrin per Kg body weight at Zeitgeber time 17 (**B** and **C**, n = 6 for control diet and n = 7 for ethanol diet, both male and females) or Zeitgeber time 3 (**A** and **C**, n = 5 for control diet and n = 8 for ethanol diet, both males and females), and dissected 9h later. BMAL1 and CLOCK protein levels,

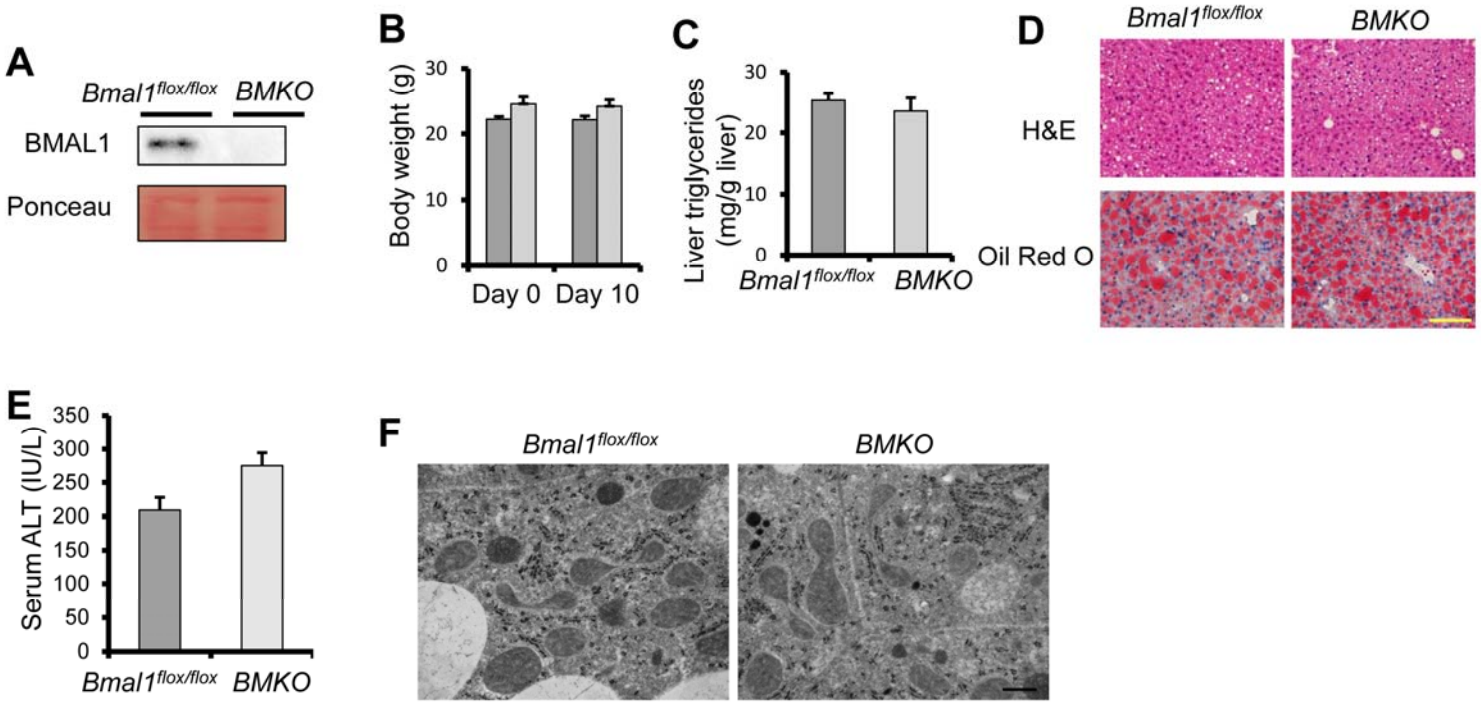
SIRT1 protein level and acetylation level of its target p65, as well as AKT Ser473 phosphorylation level were assessed with western blotting (**A and B**). Circadian clock function was assessed by RT-qPCR checking mRNA level of BMAL1 targets (**C**). (**D**) Primary hepatocytes were isolated from 8-wk male mouse, infected with Ad-GFP or Ad-*Flag-Bmal1*, and then treated with 100mM ethanol for 18 hours. Cell lysate was immunoprecipitated with FLAG antibody conjuncted beads and blocked with BMAL1 or CLOCK antibody. (E) 2 mg nuclei extract of liver of ethanol treated or pair-fed mice was applied to sucrose gradients as described in Methods. 10 fractions (1ml each) were collected (Numbered on top), and separated by SDS – PAGE and immunoblotted with the antibodies against BMAL1 and CLOCK. Coomassie blue staining was used as loading control. *$p < 0.05$, **$p < 0.01$ for **A** and **B** by two-tailed Student's *t* test; **$p < 0.01$, ### $p < 0.001$ by one-way ANOVA with Tukey's test for **C**.



Supporting Figure 3. Characterization of ethanol-fed *BLKO* and littermate control mice. Body weight **(A)** and RT-qPCR analysis of *Cyp2e1* and genes involved in anti-oxidant, inflammation, pro-survival or pro-apoptosis, and ER stress pathways in the livers of *BLKO* mice and control mice **(B-F).** Immunohistochemistry against Myeloperoxidase (Red, indicated with arrowheads) was utilized to visualize Neutrophils (**G**). *$p < 0.05$, **$p < 0.01$.

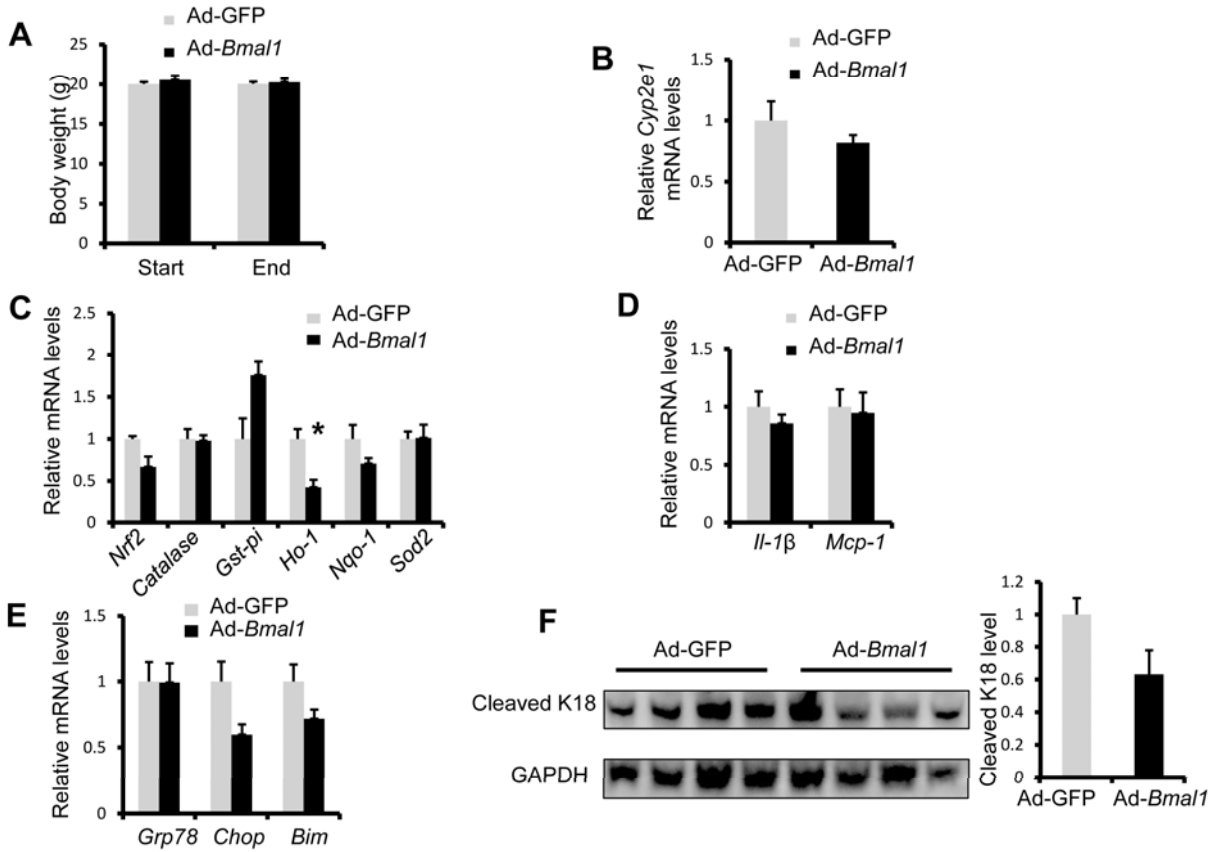


Supporting Figure 4. Characterization of ethanol-fed mice injected with Ad-sh*Bmal1* verse Ad-sh*LacZ*. Body weight **(A)** and RT-qPCR analysis of *Cyp2e1* and genes involved in anti-oxidant, inflammation, pro-survival or pro-apoptotic, and ER stress pathways in the livers of mice injected with either Ad-sh*Bmal1* or Ad-sh*LacZ* **(B-F)**.

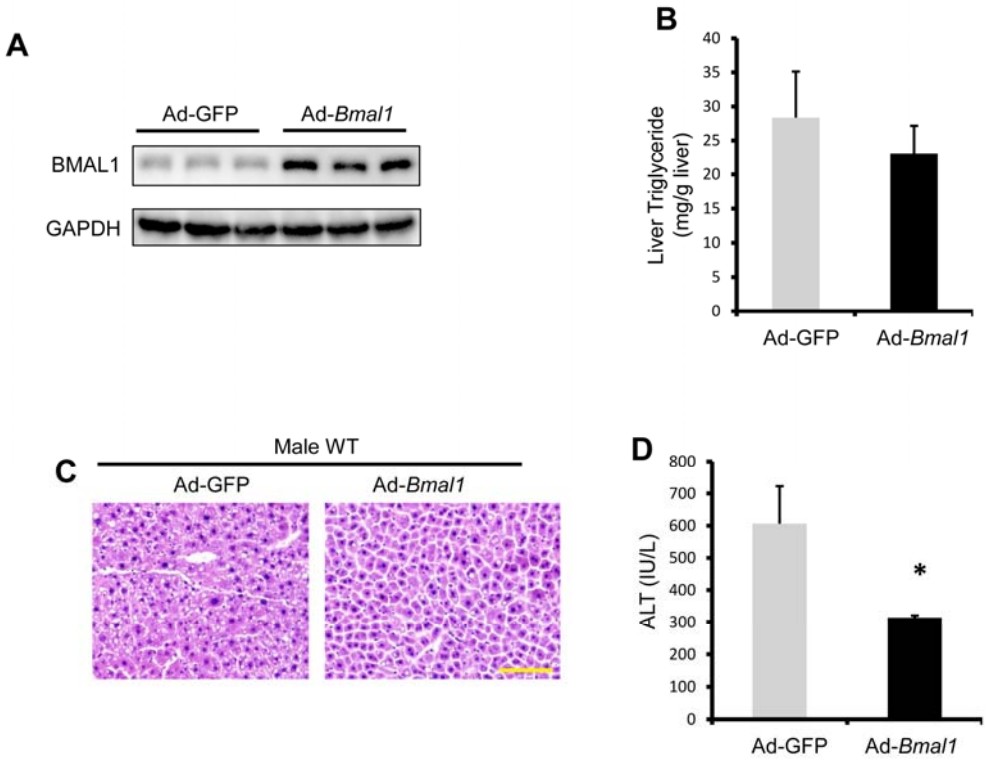


Supporting Figure 5. Macrophage BMAL1 is dispensable in protecting mice against alcohol-induced liver injury. 8-wk *Bmal1*$^{Flox/Flox}$ *Lysm-Cre (+)* mice (*BMKO*, n = 6) and *Bmal1*$^{Flox/Flox}$ littermates (n = 5) were fed as described in Figure 1. (**A**) *Bmal1* deletion in macrophages was confirmed by immuno-blotting with anti-BMAL1 in macrophages isolated from liver . (**B**) Body weight changes during ethanol diet feeding. Lipid accumulation was assessed by liver triglyceride assay (**C**), H&E staining and Oil Red O staining (**D**). Liver injury was assessed by serum ALT assay (**E**). Electron microscopy (magnification at 40,000x) was performed to visualize mitochondria (**F**). Mitochondria undergoing fission are indicated with

green arrowheads and swollen mitochondria with red arrowheads. *$p < 0.05$, **$p < 0.01$ by two-tailed Student's *t* test. Scale bar = 100 μM for **D**; Scale bar = 400 nM for **F**.

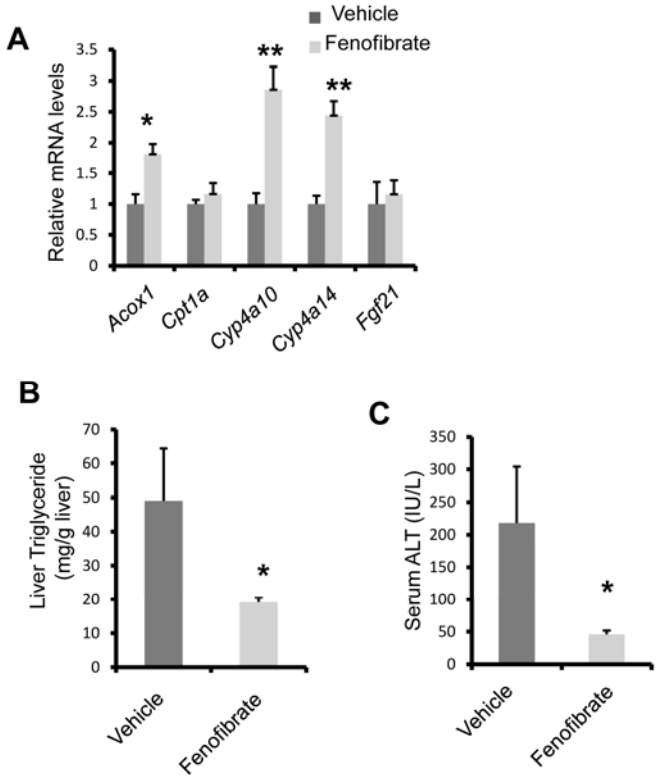


Supporting Figure 6. Effects of hepatic *Bmal1* overexpression in ethanol-induced liver injury. Body weight **(A)** and RT-qPCR analysis of *Cyp2e1* and genes involved in anti-oxidation, inflammation, and ER stress pathways **(B-E)**, and cleaved K18 protein levels **(F)** in the livers of mice injected with Ad-*Bmal1* or Ad-GFP after ethanol feeding.



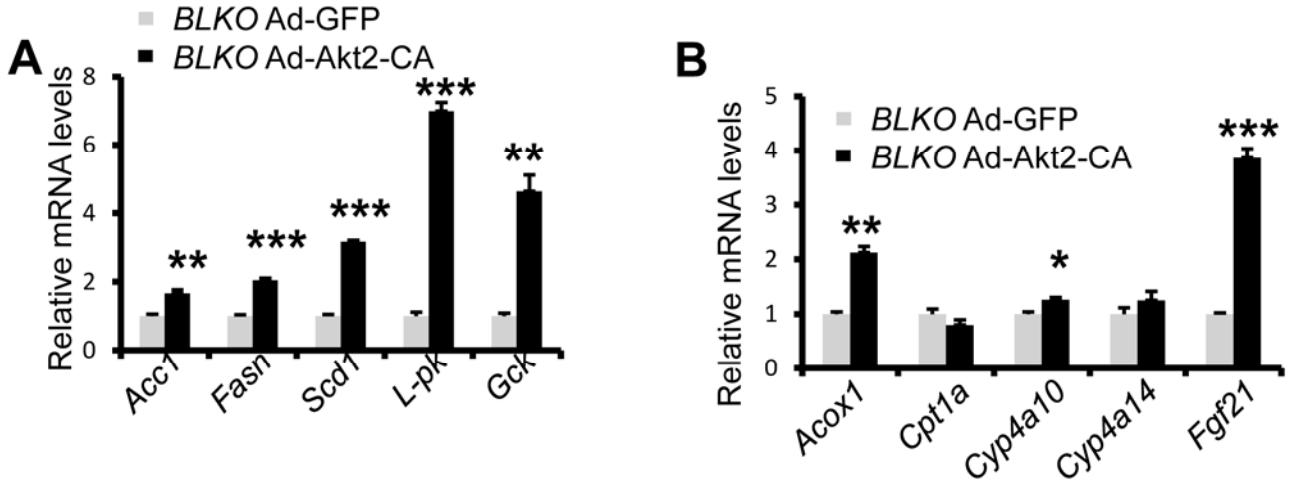
Supporting Figure 7. Liver-specific *Bmal1* over-expression partially protects male mice from alcohol feeding-induced fatty liver and liver injury.

8-wk mice were injected with adenovirus expressing either *Bmal1* (Ad-*Bmal1,* n = 3) or GFP control (Ad-GFP, n = 3), and then fed 5% ethanol diet for 10 days and binged with 5 g ethanol per kg body weight at ZT 3 of the 11th day and were dissected 9 hr later. (**A**) *Bmal1* overexpression was confirmed by western blotting against BMAL1. (**B-D**) *Bmal1* overexpression lowers ethanol-induced liver toxicity. Hepatic triglyceride assay (**B**) and H&E staining (**C**) were utilized to assess hepatic lipid accumulation. Liver injury was assessed with ALT assay (**D**). *$p < 0.05$, ***$p < 0.001$ by two-tailed Student's *t* test. Scale bar = 100 uM.



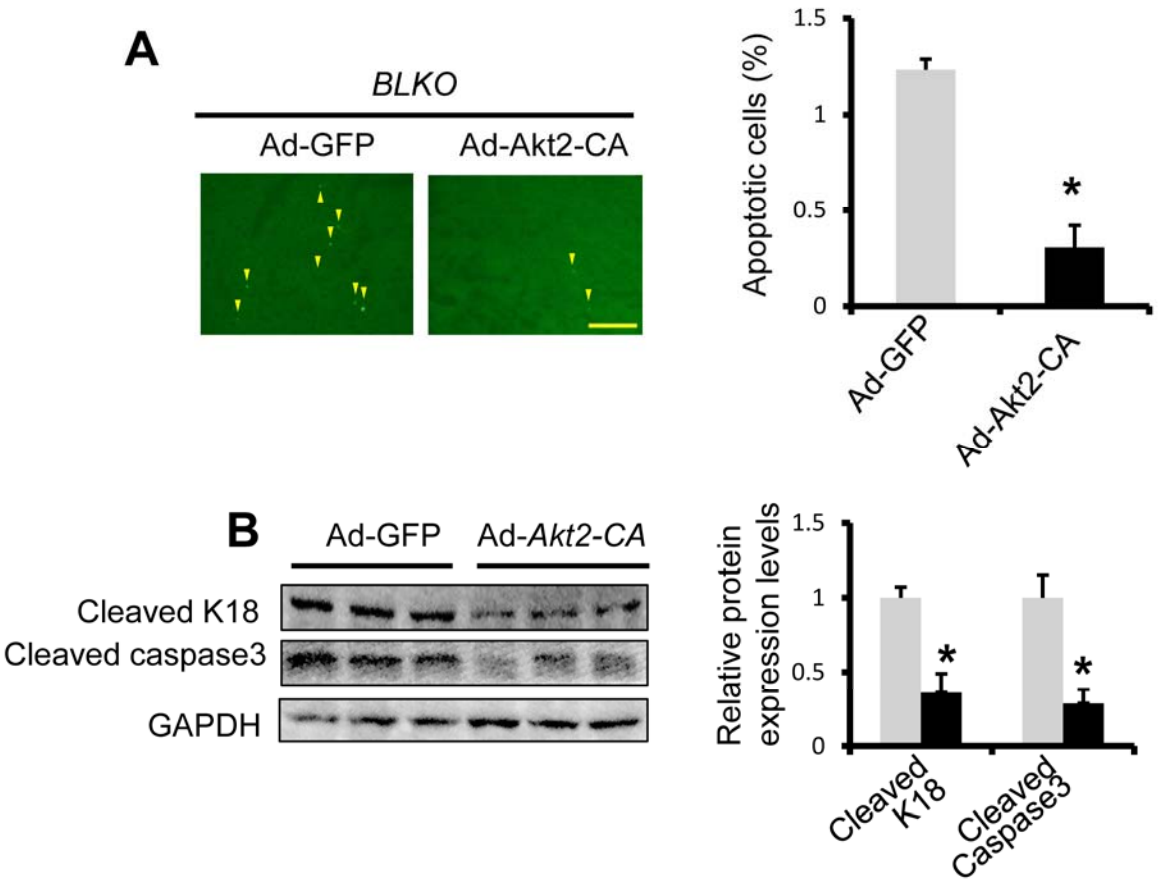
Supporting Figure 8. Fenofibrate administration ameliorates ethanol-induced liver injury in *WT* mice.

8-wk *WT* mice were fed 5% ethanol diet for 10 days. From the 3rd day of ethanol diet feeding, mice were gavaged daily with Fenofibrate (20 mg/ kg body weight) till the last day of ethanol diet feeding. On the 11th day, mice were binged with 5g ethanol/kg body weight at ZT 3, and were dissected 9 hr later (n = 4 for veicle and n = 6 for fenofibrate, both males and females). (**A**) PPARα activation by Fenofibrate was confirmed by the induction of PPAR*α* target genes with RT-qPCR. (**B-C**) Fenofibrate treatment reduces ethanol feeding-induced liver steatosis (**B**) and liver injury (**C**) in *WT* mice. *$p < 0.05$, **$p < 0.01$ by two-tailed Student's *t* test.

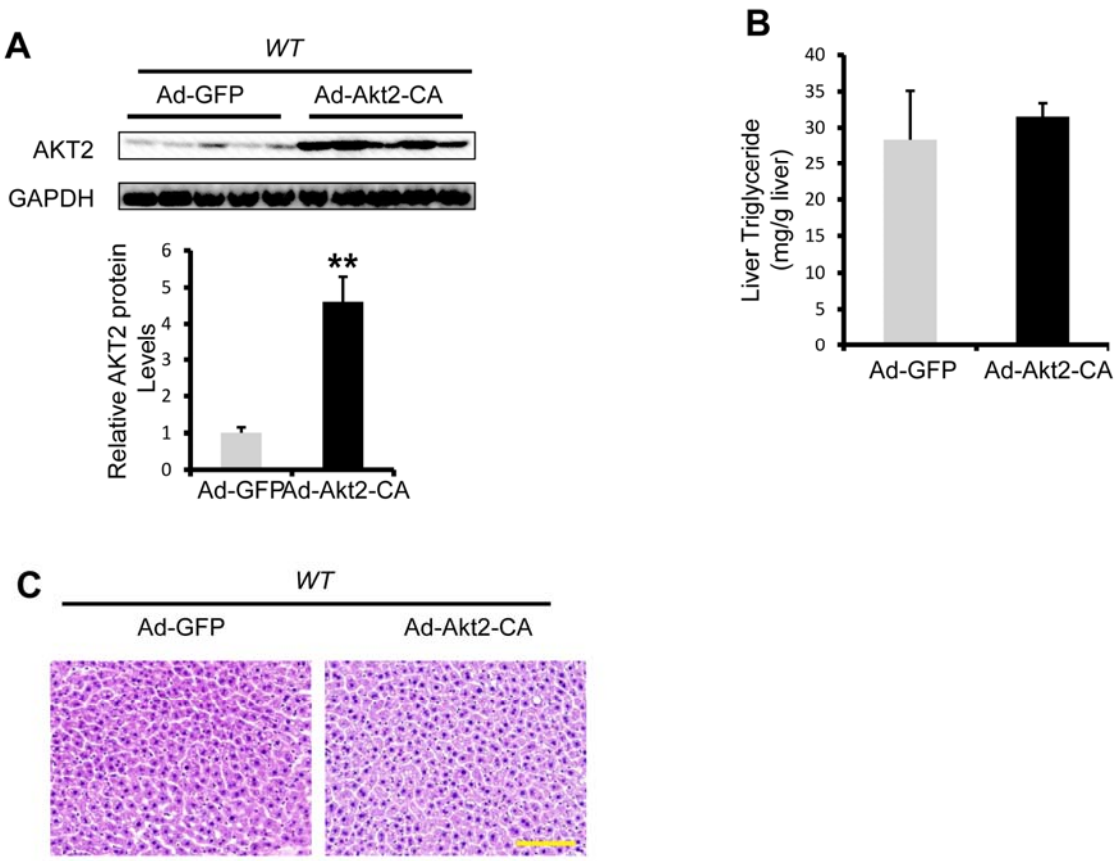


Supporting Figure 9. Constitutively active AKT enhances the expression of β-oxidation genes in *Bmal1*$^{-/-}$ hepatocytes.

Primary hepatocytes isolated from *Bmal1*$^{-/-}$ mouse were transduced with adenovirus expressing a constitutively active *Akt2* (**Ad-*Akt2*-CA**) or Ad-GFP. 24 h later, cells were harvested and the expression levels of lipogenic genes (**A**) and PPARα targets (**B**) were assessed with RT-qPCR. *$p < 0.05$ and **$p < 0.01$ by two-tailed Student's *t* test.

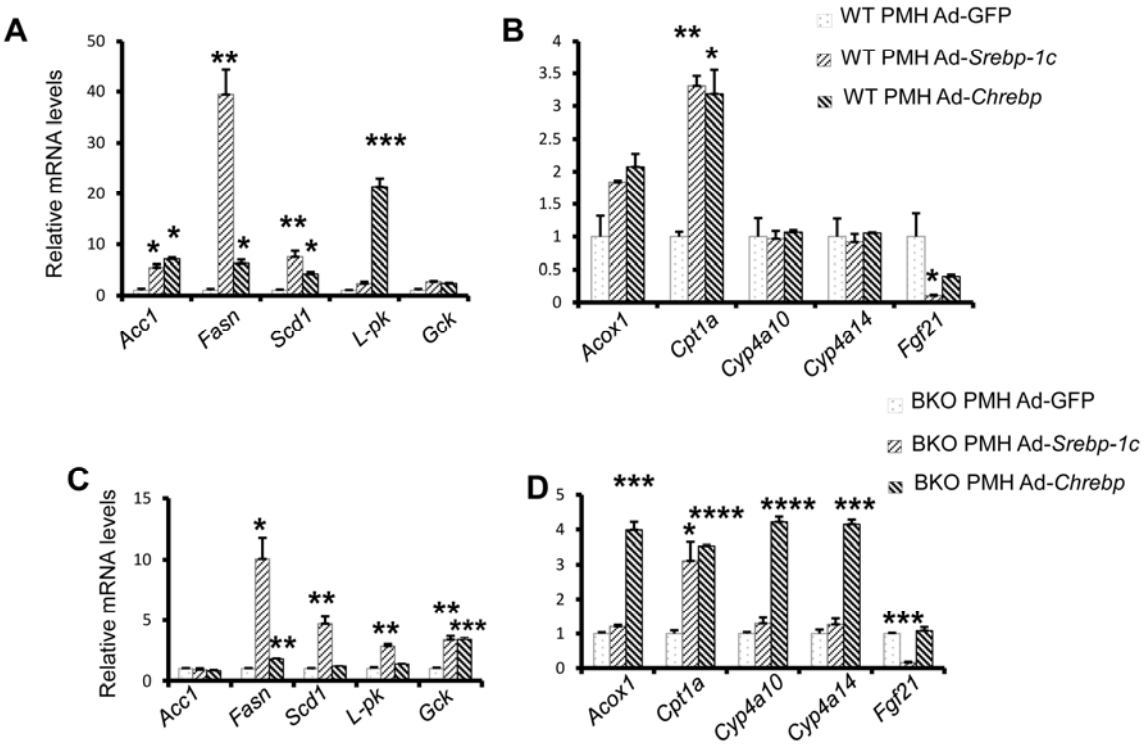


Supporting Figure 10. Constitutively active AKT suppresses hepatocyte apoptosis in ethanol-fed *BLKO* mice. (A) TUNEL staining of frozen liver section from ethanol-fed BLKO mice injected either Ad-GFP or Ad-AKT-CA. (**B**) Immunoblotting of apoptotic markers in the liver lysates isolated from ethanol-fed BLKO mice injected either Ad-GFP or Ad-AKT-CA.

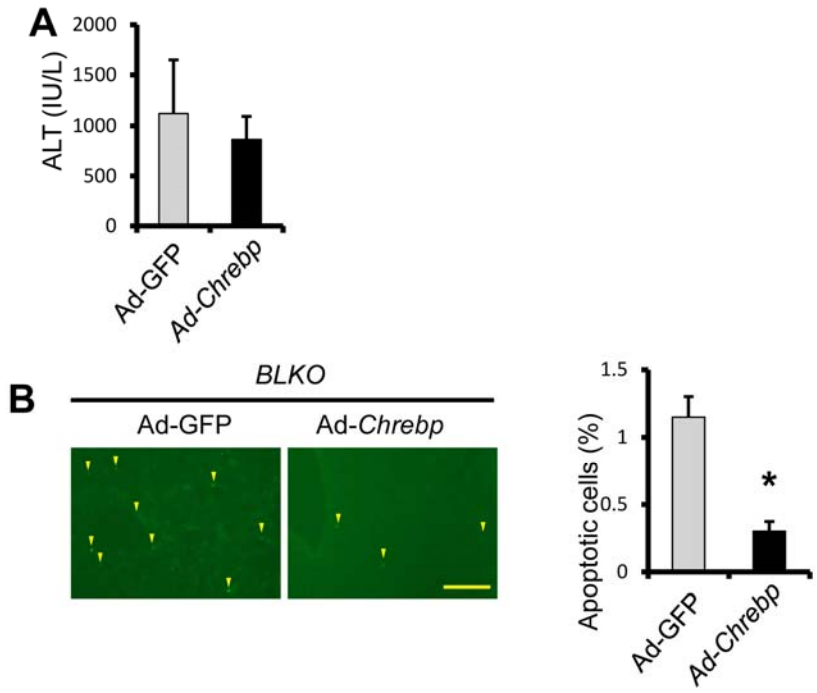


Supporting Figure 11. Over-expressing constitutively active AKT in WT mice liver fails to reverse liver injury induced by ethanol feeding.

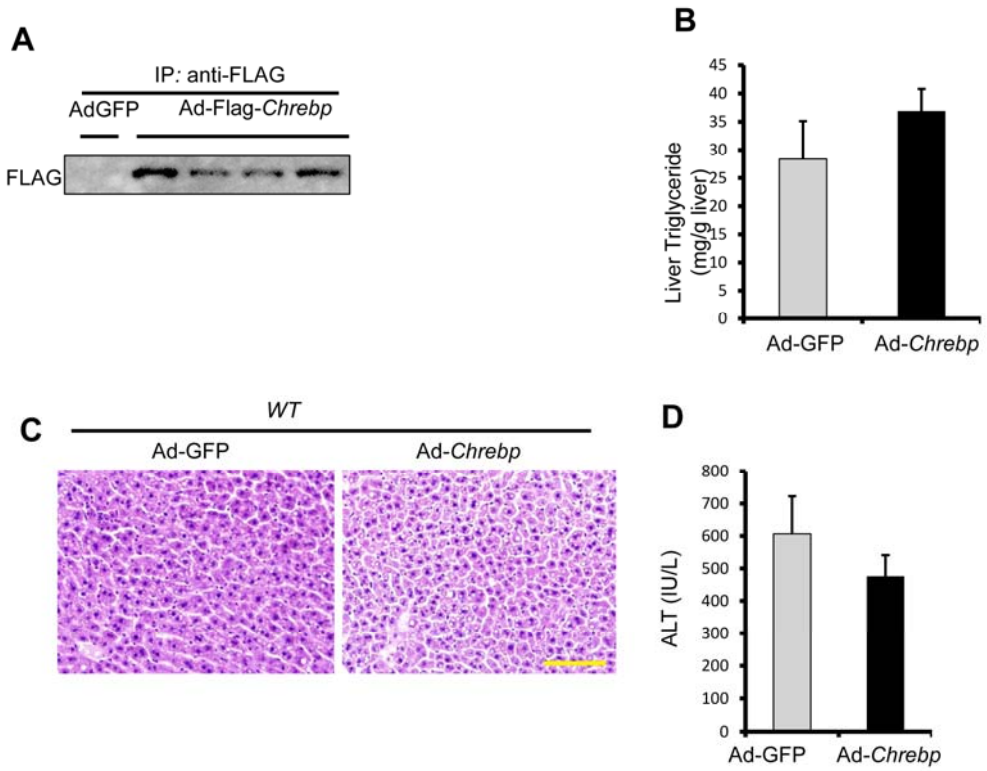
8-wk old *WT* mice were injected with Ad-*Akt2*-CA or Ad-GFP (n = 5), and then fed 5% ethanol diet for 10 days, binged with 5g ethanol/kg body weight at ZT 3 on the 11th day, and were dissected 9 hr later. (**A**) AKT2 overexpression was confirmed by western blotting with anti-AKT2. (**B**) AKT activation fails to ameliorate ethanol-induced liver steatosis and injury in *WT* mice, assessed with liver triglyceride assay (**B)** and H&E staining (**C**). ** $p < 0.01$ by two-tailed Student's *t* test. Scale bar = 100 uM.



Supporting Figure 12. Over-expression of ChREBP but not SREBP1c induces the expression of β-oxidation genes in *Bmal1*$^{-/-}$ hepatocytes. Primary hepatocytes isolated from WT (**A** & **B**) and *Bmal1*$^{-/-}$ (**C** & **D**) mouse were transduced with adenovirus expressing Ad-GFP, or Ad-*Srebp1c* (nuclear form) or Ad-*Chrebp*. 24 hr later, cells were harvested and the expression levels of lipogenic genes (**A** & **C**) and PPARα targets (**B** & **D**) were assessed with RT-qPCR. *$p < 0.05$ and **$p < 0.01$ by two-tailed Student's *t* test.

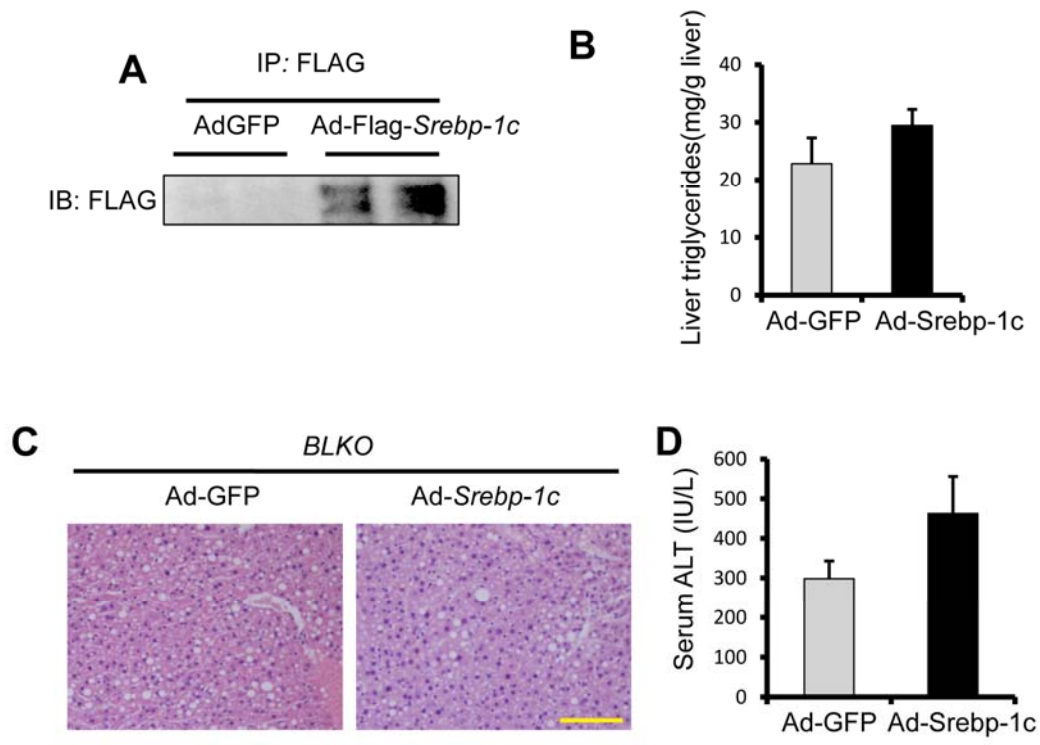


Supporting Figure 13. Hepatic ChREBP overexpression partially ameliorates ethanol-induced liver injury in *BLKO* mice. **(A)** Serum ALT assay and **(B)** TUNEL staining of liver sections.



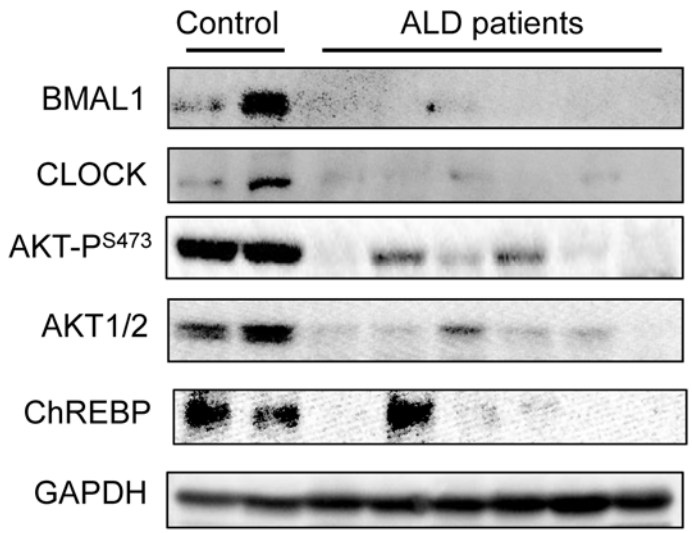
Supporting Figure 14. Hepatic ChREBP overexpression does not rescue ethanol-induced liver injury in *WT* mice

8-wk old *WT* mice were injected with Ad-*Flag-Chrebp* or Ad-GFP (n = 4 for each group, females), and fed with ethanol diet to induce liver injury. (**A**) Hepatic FLAG-ChREBP overexpression was confirmed by immunoprecipitation with anti-FLAG beads and western blotting with anti-FLAG. (**B-D**) ChREBP overexpression fails to ameliorate ethanol-induced liver steatosis and injury in *WT* mice, assessed with liver triglyceride assay (**B)** and H&E staining (**C**), and serum ALT assay (**D**).



Supporting Figure 15. Restoring SREBP-1c does not rescue liver injury in ethanol-fed *BLKO* mice

8-wk old *BLKO* mice were injected with Ad-*Flag-Srebp-1c* or Ad-GFP (n = 3 for each group, females), and fed with ethanol diet to induce liver injury. (**A**) Hepatic SREBP-1c overexpression was confirmed by western blotting with anti-FLAG after immunoprecipitation anti-FLAG beads. (**B-D**) Effects of SREBP-1c overexpression on ethanol-induced liver steatosis in *BLKO* mice with liver triglyceride assay (**B**), H&E staining (**C**), and liver injury with serum ALT assay (**D**).



Supporting Figure 16. Decreased protein expression of BMAL-AKT-lipogenesis axis in the liver samples of human ALD patients. Immunoblotting of BMAL1, AKT-P^{S473}, AKT and ChREBP protein levels in the liver of normal control verse and patients with alcoholic hepatitis.

Additional materials and methods

Adenoviral Generation

The *Bmal1* shRNA shuttle construct was made by ligating the targeting oligo nucleotide sequence into the pEntry/U6 vector (Invitrogen). The targeting sequence for mouse *Bmal1* is 5'-CATCGATATGATAGATAACG-3'. Ad-sh*Bmal1* plasmid was generated through Gateway LR recombination between pEntry/U6-sh*Bmal1* and pAdBlock-iT vector (Invitrogen). The adenoviral *Bmal1* and *Chrebp* shuttle vector was made by cloning the full-length *Bmal1* or *Chrebp* cDNA into the pShuttle-IRES-GFP (Agilent). pAdEasy Bmal1-FLAG-IRES-GFP viral DNA was made by in vitro recombination between pShuttle-Bmal1-FLAG-IRES-GFP and the pAdEasy-1 plasmid in BJ5183-AD-1 competent cells (Agilent) after electroporation. All the adenoviruses were produced in 293AD packaging cells (Agilent) after Lipofectamine-mediated transfection and concentrated after ultracentrifuge in cesium chloride gradient solutions. The constitutively active AKT2 adenovirus was kindly provided by Dr. Morris Birnbaum at the University of Pennsylvania.

Primary Mouse Hepatocyte Isolation and Culture

Primary mouse hepatocytes were isolated from C57BL/6 male mice (9-10 weeks) using the protocol described before (1). The liver was perfused with EBSS (Invitrogen) with 0.5 mM EGTA for 5 min, followed by perfusion with 100 U/ml type I collagenase (*Worthington*) via the inferior vena cava for 5 min. After dissection, hepatocytes were released by scattering, passed through a 100-μm cell strainer, and then spun at 50 × *g* for 1 min. The pellet was re-suspended in DMEM and then spun at 50 × *g* for 10 min in a Percoll gradient to remove dead hepatocytes. Viable cells were washed with DMEM at 50 × *g* for 10 min and checked by trypan blue staining. PMHs in DMEM with 5% FBS were seeded at a density of 2×10^5 cells/ well of 12-well-plate. Adenovirus was transduced 6 hours after seeding. The cells were harvested 24h after seeding for protein or RNA preparation.

Mouse Kupffer cell isolation

A Kupffer cell–enriched fraction was obtained from frozen liver of C57BL/6 male mice (9–10 weeks) using the protocol described before, with minor modifications (2). Briefly, the livers frozen in -80°C were kept in -20°C for 30 min, incubated in 10% formalin in 4°C for 2 hrs, and then washed with PBS for three times. After mincing, the liver was incubated in Type I Collagenase (1mg/ml in 10mM $CaCl_2$, 10mM HEPES and 4 mM NaOH in HBSS) in 37 °C for 2hrs with shaking. After being filtered with a 100-μm cell strainer, hepatocytes were pelleted at 50 xg for 10 min, and the supernatant containing non-parenchymal cells was further centrifuged at 400 xg, re-suspended in DMEM medium with 5% fetal bovine serum (FBS), and separated by centrifugation on a 25%-40% Percoll gradient. The Kupffer-cell fraction located at the interface between the 25% and the 40% Percoll layers was lysed with RIPA buffer and subjected to western blotting analysis.

cDNA Synthesis and qPCR

Total cellular RNA extraction was performed with TRIzol reagent (Invitrogen) and chloroform. cDNA was synthesized with the Verso cDNA kit (*ThermoFisher Scientific*, Surrey, UK) and subjected to qPCR using Radiant™ Green 2X qPCR Mix (*Alkali Scientific*) on an ABI 7900 HT thermal cycler (Applied Biosys-tems, Foster City, CA). The value of each cDNA was calculated using the ΔΔCt method and normalized to the value of the house-keeping gene control, 18S ribosomal RNA. The data were plotted as fold of change. The qPCR primer sequences are listed below.

Forward	Reverse

18s RNA	5'-TTGACGGAAGGGCACCACCAG-3'	5'-GCACCACCACCCACGGAATCG-3'
Acc1	5'-GAAGCCACAGTGAAATCTCG-3'	5'-GATGGTTTGGCCTTTCACAT-3'
Acox1	5'-TGCTGCAGACGGCCAGGTTC-3'	5'-GGCCAGACTGCCACCTGCTG-3'
Bcl2-l1	5'-GCTGCATTGTTCCCGTAGAG-3'	5'-GTTGGATGGCCACCTATCTG-3'
Bim	5'-CGGTCCTCCAGTGGGTATTT-3'	5'-TATGGAAGCCATTGCACTGAGA-3'
Cd36	5'-CCAAGCTATTGCGACATGATT-3'	5'-CCGAACACAGCGTAGATAGACC-3'
Chop	5'-CTGCCTTTCACCTTGGAGAC-3'	5'-CGTTTCCTGGGGATGAGATA-3'
Chrebpα	5'-CGACACTCACCCACCTCTTC-3'	5'-TTGTTCAGCCGGATCTTGTC-3'
Chrebpβ	5'-TCTGCAGATCGCGTGGAG-3'	5'-CTTGTCCCGGCATAGCAAC-3'
Cpt1a	5'-TCTGCATGTTTGACCCAAAA-3'	5'-TTGCTGGAGATGTGGAAGAA-3'
Cyp4a10	5'GGAGCTCCAATGTCTGAGAAGAGT	5'-TCTCTGGAGTATTCTTCTGAAAAAGGT
Cyp4a14	5'-TCTCTGGCTTTTCTGTACTTTGCTT	5'-CAGAAAGATGAGATGACAGGACACA
Fasn	5'-TTGGCCCAGAACTCCTGTAG-3'	5'-CTCGCTTGTCGTCTGCCT-3'
Gadd45a	5'-TGGTGACGAACCCACATTCA-3'	5'-CGGGAGATTAATCACGGGCA-3'
Gck	5'-CCCTGAGTGGCTTACAGTTC-3'	5'-ACGGATGTGGAGTGTTGAAGC-3'
Grp78	5'-GGTGCAGCAGGACATCAAGTT-3'	5'-CCCACCTCCAATATCAACTTGA-3'
L-PK	5'-CTGGAACACCTCTGCCTTCTG-3'	5'-CACAATTTCCACCTCCGACTC-3'
Mcl-1	5'-TTCTTTCGGTGCCTTTGTGGC-3'	5'-AACCCATCCCAGCCTCTTTGTT-3'
Mttp1	5'-CTCCACAGTGCAGTTCTCACA-3'	5'-AGAGACATATCCCCTGCCTGT-3'
Pparα	5'-CCTTCTACGCTCCCGACCCA-3'	5'-CCATGTCCATAAATCGGCACCA-3'
Puma	5'-TACGAGCGGCGGAGACAAG-3'	5'-GTGTAGGCACCTAGTTGGGC-3'
Scd1	5'-GCCGAGCCTTGTAAGTTCTG-3'	5'-CCTCCTGCAAGCTCTACACC-3'
Srebp-1c	5'-AACGTCACTTCCAGCTAGAC-3'	5'-CCACTAAGGTGCCTACAGAGC-3'

Liver Triglyceride Measurements

Hepatic total lipids were extracted according to Bligh and Dyer (2). The liver tissues were weighed and homogenized in 1% acetic acid (7uL acetic acid/mg liver tissue). After quick spinning, 200 µL of supernatant was transferred to a 1.5 mL tube containing 800 µL of chloroform/methanol (2:1, v/v). After centrifuge for 10 min at 10000xg at room temperature, 450 µL of organic phase was transferred to a new tube from the bottom layer, and the tubes were left in a fume hood overnight. Total lipids were dissolved in 200 µL ethanol and incubated at 55°C for 20 min. 3 µL were used for measurement with the Triglycerides Liquid Reagent (*POINTE*, T7532-500).

Liver Histology analysis

For Oil Red O (ORO) staining, frozen section slides were fixed in pre-chilled 10% formalin for 10 minutes, air dried for 10min and incubated in absolute propylene glycol for 5 min to avoid carrying water into ORO. Then the slides were stained in pre-warmed 0.5% ORO solution for 10 min in 60°C water bath, differentiated in 85% propylene glycol solution for 2-5 minutes, counter stained with hematoxylin and eosin for 5 seconds, and visualized under microscope.

TUNEL staining was performed with in situ cell death detection kit from Roche. Briefly, the frozen sections of liver were fixed with 4% paraformaldehyde, permeabilized with a buffer containing 0.5% Triton X-100 and 0.05% SDS, and then incubated with reaction buffer. The slides were then counter stained with DAPI and visualized under microscope. TUNEL positive cells were quantified in 200x magnitude fields of 3 mouse livers from each group, and normalized to DAPI staining numbers counted with Image J.

Immunoblotting

For whole cell lysate preparation, primary mouse hepatocytes were washed once in 1 x PBS buffer and lysed in RIPA buffer supplemented with 1 x protease inhibitor (Roche Applied Science). Liver tissues were weighed and homogenized in RIPA buffer (8 ul/mg tissue weight). After whole cell lysates were pre-cleared at maximal speed at 4 °C in a microfuge, the protein concentration of each supernatant was measured using Bio-Rad reagent. Equal amounts of protein samples were separated in 9% SDS-poly-acrylamide gels and transferred to PVDF membrane (*Millipore*). The membranes were incubated in primary antibodies at 4°C overnight. HRP-conjugated secondary antibodies against mouse, rabbit, or goat IgG and Western Lightning ECL substrate were used for detecting chemi-luminescence on an HD2 AlphaImager (*Cell Biosciences*).

Primary antibodies against AKTα1/α2 (SC-1619), BMAL1 (SC-8550) and ChREBP (SC-33764) were all from *Santa Cruz*. Primary antibodies against phosphorylated AKT at Ser-473 (4060s) were from *Cell Signaling*. HRP-conjugated FLAG antibody was from *Sigma* (A8592). HRP-conjugated secondary anti-rabbit (A6154), anti-mouse (A4416), and anti-goat (A5420) were from *Sigma*. Anti-caspase3 (160745) was from *Cayman*. Anti-β-actin was from the Developmental Studies Hybridoma Bank in the Department of Biology, University of Iowa. Anit-keratin18 antibody was a generous gift from Dr. Bishr Omary from the Department of Molecular & Integrative Physiology at the University of Michigan Medical School.

Immunohistochemistry

The frozen sections of liver were fixed with 4% paraformaldehyde, permeabilized with a buffer containing 0.5% Triton X-100 and 0.05% SDS, and then incubated in PBS with 10% goat serum for 20 minutes. After washing with PBS with 0.1% Tween-20, the slides were incubated with cleaved Caspase3 antibody (*Cell Signaling*) at 4°C overnight. Next day, the slides were washed three times with PBS with 0.1% Tween-20, and incubated with secondary antibody Alexa Fluor Plus 488 (A32723, *Life Technologies*) for 30 min, washed three times, mounted with ProLong™ antifade mountants (P36931, *Life Technologies*), and visualized under microscope. For myeloperoxidase immuno-staining, formalin fixed and paraffin embedded liver slides were hydrated by sequentially incubating in xylene (5 min, three time), 100% ethanol (2 min, twice), 95% ethanol (2 min, twice), 70% ethanol (2 min, once) and water (2 min, once). Then the slides were boiled in 10 mM sodium citrate buffer for 12 minutes to unmask antigen. After cooling down at room temperature for 2 hr, the slides were blocked in 10% horse serum for 1 hr, and then incubated with anti-myeloperoxidase antibody (Abcam, ab208670, 1:1000 dilution) at 4°C overnight. Next day, the slides were washed three times with PBS with 0.1% Tween-20, and incubated with secondary antibody Alexa Fluor Plus 647 (A21245, *Life Technologies*) for 30 min, washed three times, mounted and visualized under microscope.

Sucrose-gradient centrifugation

Sucrose-gradient centrifugation was performed as previously described (3). Briefly, 2 mg nuclei extract of liver was layered on top of a discontinuous 8%, 12%, 16%, 20% and 25% sucrose gradient (in RIPA buffer). After ultracentrifugation at 36,000 r.p.m. for 18 hr in a SW40 rotor, the bottom of the tube was punched with a needle to collect fractions (1 ml/tube). After mixing with 5x SDS loading buffer, 40 µL of sample was loaded for western blotting against BMAL1 and CLOCK, and Coomassie blue staining to examine protein loading.

References

1. Zhang D, Tong X, Arthurs B, Guha A, Rui L, Kamath A, Inoki K, et al. Liver clock protein BMAL1 promotes de novo lipogenesis through insulin-mTORC2-AKT signaling. Journal of Biological Chemistry 2014;289:25925-25935.

2. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology 1959;37:911-917.
3. Chou C-F, Riopel CL, Rott LS, Omary MB. A significant soluble keratin fraction in ‘simple’epithelial cells. Lack of an apparent phosphorylation and glycosylation role in keratin solubility. Journal of Cell Science 1993;105:433-444.