**The hepatic BMAL1/AKT/Lipogenesis axis protects against alcoholic liver disease
via promoting PPAR α pathway**

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ABSTRACT

Alcohol liver disease (ALD) is one of the major chronic liver diseases worldwide, ranging from fatty liver, alcoholic hepatitis, cirrhosis, and potentially hepatocellular carcinoma. Epidemiological studies suggest a potential link between ALD and impaired circadian rhythms, but the role of hepatic circadian proteins in the pathogenesis of ALD remains unknown. Here we show that the circadian clock protein BMAL1 in hepatocytes is both necessary and sufficient to protect mice from alcohol liver disease. Ethanol diet-fed mice with liver-specific knockout (*Bmal1*-LKO) or depletion of *Bmal1* develop more severe liver steatosis and injury as well as a simultaneous suppression of both *de novo* lipogenesis and fatty acid oxidation, which can be rescued by the supplementation of synthetic PPAR α ligands. Restoring *de novo* lipogenesis in the liver of *Bmal1*-LKO mice by constitutively active AKT not only elevates hepatic fatty acid oxidation but also alleviates ethanol-induced fatty liver and liver injury. Furthermore, hepatic over-expression of lipogenic transcription factor ChREBP, but not SREBP-1c, in the liver of *Bmal1*-LKO mice also increases fatty acid oxidation and partially reduces ethanol-induced fatty liver and liver injury. *Conclusion:* we identified a novel protective role of BMAL1 in hepatocytes against ALD. The protective action of BMAL1 during alcohol consumption depends on its ability to couple ChREBP-induced *de novo* lipogenesis with PPAR α -mediated fatty oxidation.

INTRODUCTION

Alcohol over-consumption is the primary cause of liver-related mortality in Western countries. In the United States, more than 50% of population consume alcohol (1, 2). As the primary site of alcohol metabolism, the liver is a major target organ of alcohol-induced injury. 3 in 10 adults are heavy alcohol drinkers with a high risk for alcoholic liver disease (ALD) (3). ALD covers a spectrum of disease states that range from simple liver steatosis (fatty liver), steatohepatitis (combined with inflammation), to fibrosis and/or cirrhosis (3). Both genetics and environmental factors contribute to the progression of ALD. Particularly, mice with circadian disruption have been reported to have a higher permeability of intestinal epithelial barrier, a risk factor of alcoholic tissue damage (4). Human studies also indicated that night workers with circadian misalignment are susceptible to alcohol-induced intestinal hyperpermeability with social drinking (5). Although these observations point to a possible role of impairment of circadian rhythms in the pathogenesis of ALD, what and how circadian proteins are involved in ALD remain largely unknown.

The circadian clock functions in the majority of cell types in the human body. In hepatocytes, BMAL1 is one of the essential components of the circadian clock that drives the cyclic expression of a variety of metabolic genes involved in lipid, glucose, and cholesterol metabolism (6). We previously discovered that BMAL1 is also essential for maintaining insulin responsiveness in the liver. *Bmal1* deficiency severely blunts insulin-stimulated AKT phosphorylation and reduces insulin-stimulated *de novo* lipogenesis (7). Upon chronic HFD feeding, hepatic *Bmal1* deficiency results in severe insulin resistance and liver steatosis (8),

indicating that impairment of BMAL1 expression or function may contribute to chronic liver metabolic diseases.

Alcohol exposure has been shown to impair lipid homeostasis in hepatocytes and cause liver injury during the development of ALD (3). It has been reported that ethanol feeding increases SREBP-1c-driven *de novo* lipogenesis while inhibiting the PPAR α activity in the liver (9-11). Administration of a synthetic PPAR α agonist reverses alcoholic fatty liver and improves liver function in mice (9). The inhibitory effect of ethanol on the PPAR α transcriptional activity was observed in cultured hepatocytes, suggesting that ethanol and its metabolites could impact the DNA-binding activity of PPAR α in the liver (10). Currently, it remains unclear how ethanol feeding alters the hepatic PPAR α activity.

Given the pivotal role of PPAR α in liver lipid homeostasis, extensive research has been done on how the PPAR α activity is modulated in response to hormonal and nutritional signals. Unexpectedly, the PPAR α activity is severely down-regulated in hepatocytes lacking FASN, indicating an intricate link between *de novo* lipogenesis and the biogenesis of endogenous PPAR α ligands and the subsequent PPAR α activation in the liver (12). Follow-up studies show *de novo* lipogenesis is a major source of phosphatidyl-choline in hepatocytes that serves as endogenous ligands for PPAR α (13). However, how the connection between *de novo* lipogenesis and PPAR α activation is regulated during ethanol feeding has not been tested. In this study, we explored the role of a key circadian protein BMAL1 in alcoholic liver disease using both hepatocyte-specific gain and loss of function models. Our findings suggest that in hepatocytes, BMAL1 is critical for reducing liver steatosis and hepatocyte apoptosis during chronic alcohol feeding. Moreover, BMAL1 is required for maintaining the PPAR α activity in hepatocytes partially via its ability to promote ChREBP-induced *de novo* lipogenesis.

MATERIALS AND METHODS

Animal Experiments

All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee at the University of Michigan. All animal care and use were in accordance with guidelines of the University of Michigan Institutional Animal Care and Use Committee. *C57BL/6* mice were maintained on 12 hr/12 hr light/dark cycles with ad lib access to food and water. *Bmal1^{Flox/Flox}* mice were generously provided by Dr. Jiandie Lin at the University of Michigan. *Bmal1* liver specific knockout (*Bmal1-LKO*) mice were generated by crossing *Bmal1^{Flox/Flox}* mice with Albumin-Cre mice. Ethanol diet feeding and binge was performed with the protocol described previously (14). For fenofibrate treatment, the mice were daily gavaged in the last 8 days of ethanol diet feeding (20mg/kg body weight). For liver specific knockdown or overexpression, AdshLacZ vs. Ad-sh*Bmal1*, Ad-GFP vs. Ad-Flag-*Bmal1*, Ad-Akt2-CA or Ad-Flag-*Chrebp* adenoviruses were delivered via tail vein injection at a dose of 1×10^{12} plaque-forming units on the first day of ethanol diet feeding.

Electron microscopy

Mouse liver was excised, minced into small pieces and fixed in a solution of 2% paraformaldehyde & 2% glutaraldehyde in PBS for two hours at room temperature. After washing in PBS the tissue samples were post fixed in osmium tetroxide for 45 min at room temperature. Dehydration of the samples was accomplished by transferring the samples through a series of graded ethanol and then 100% propylene oxide. The tissue was then infiltrated by transferring the samples into increasing concentrations of Epon to propylene oxide solutions at 1:3, 1:1, 3:1 then 100% Epon and finally embedded. Sections were made with a Leica EM UC7 ultramicrotome (Leica), stained for 15 min with 7% (saturated) aqueous uranyl acetate, washed, stained with lead citrate, and examined with a JEOL JEM 1400 plus transmission electron microscope (JEOL USA).

Statistics

Statistical analysis was performed using Prism version 6.0 (GraphPad Software, San Diego, CA). Statistical significance was determined either by unpaired two-tailed Student's t-test for comparison between two groups or by one-way ANOVA with Tukey's or Dunnett's post-hoc test for multiple group comparison. All results were given as the mean $\pm$ SEM. Results were considered statistically significant with p value < 0.05 .

Other detailed methods including adenoviral production, serum ALT, liver histology, Nile Red staining, MPO staining, sucrose gradient sedimentation, TUNEL staining, liver triglycerides assay, primary mouse hepatocytes isolation and culture, cDNA synthesis and RT-qPCR are available in the Supplemental Experimental Procedures.

RESULTS

Hepatocyte-specific deletion of BMAL1 sensitizes mice to ethanol feeding-induced liver steatosis and liver injury. To study the role of hepatic molecular circadian clock in the pathogenesis of ALD, we adopted the chronic-plus-binge feeding described by the Gao group to create a moderate ALD in mice (14). Wildtype mice were fed 5% ethanol diet for 10 days before binged with 5g ethanol per kg body weight at Zeitgeber time 3, and dissected 9 hrs later. Ethanol feeding significantly increased liver triglycerides accumulation (**SF1. A-B**), ALT levels and *Cyp2e1* expression level (**SF1. C-D**) along with enhanced expression of pro-inflammatory cytokines, ER stress and pro-apoptotic markers (**SF1. E-G**), as well as increased neutrophil recruitment (**SF1. H**), indicating that this chronic-plus-binge ethanol regimen does induce mild to moderate liver steatosis and injury in WT mice.

We also observed enhanced acetylation of p65, a direct target of SIRT1 (15), in the liver of ethanol-fed mouse livers, indicative of potent suppression of the SIRT1 activity by ethanol feeding. However, ethanol feeding showed no effect on the abundance of SIRT1 (**SF2. A-B**). Given the important role of SIRT1 in regulating hepatic circadian clock (16-19), we hypothesize that ethanol feeding could impact the circadian clock in the liver through SIRT1 inhibition. We found the diurnal rhythms of *Dbp* and *Per1*, two classical clock genes, were lost in the ethanol-fed liver (**SF2. C**). At the protein level, ethanol feeding led to an increase in both BMAL1 and CLOCK protein but a reduction of AKT-P^{S473} in the liver (**SF2. A-B**). We previously showed that SIRT1 protects the BMAL1-CLOCK protein complex formation in the presence of palmitate in hepatocytes (19). To test whether ethanol could impact the BMAL1-CLOCK interaction, we performed anti-FLAG immunoprecipitation in ethanol-treated primary mouse hepatocytes transduced with Ad-Flag-*Bmal1* vs. Ad-GFP. We detected a strong interaction between FLAG-tagged BMAL1 and the endogenous CLOCK in vehicle-treated cells. However, the BMAL1-CLOCK interaction was weakened by ethanol treatment (**SF2. D**). To test whether ethanol feeding impairs the BMAL1-CLOCK complex in mouse liver, we performed sucrose gradient sedimentation using nuclear extracts isolated from both control diet-fed and ethanol diet-fed liver tissues. Consistent with in vitro findings, ethanol feeding reduced the number of fractions in which BMAL1 and CLOCK protein co-migrated (**SF2. E**). In summary, ethanol diet induces selective impairment of the hepatic circadian clock activity in addition to liver steatosis and liver injury.

To evaluate the role of an intact molecular clock in hepatocytes during ALD, we subjected *Bmal1*^{flox/flox} and *Bmal1* liver-specific knockout (*Bmal1*-LKO) to the ethanol diet/binge model. Compared with *Bmal1*^{flox/flox} control, *Bmal1*-LKO mice quickly lost weight on ethanol diet (**SF3. A**), accumulated more lipids in the liver (**Fig. 1 A-B**), developed more pronounced liver injury (**Fig. 1C**), and hepatocyte apoptosis by TUNEL staining and keratin 18 cleavage (**Fig. 1D-E**).

However, increased liver injury in *Bmal1*-LKO liver was independent of gene expression of *Cyp2e1* and markers of oxidative stress, ER stress, inflammation, and apoptosis (**SF3. B-F**). Neutrophil infiltration was comparable between *Bmal1*^{flox/flox} and *Bmal1*-LKO mice (**SF3. G**).

Mitochondrial dysfunction has been implicated in the pathogenesis of ALD (20). The liver of mice with hepatocyte deficiency in *Bmal1* showed a defect in mitochondrial fission following a 40-wk high-fat diet (8). To test whether *Bmal1*-LKO mice also exhibited mitochondrial defects, we evaluated the mitochondrial morphology in the liver of *Bmal1*^{flox/flox} vs. *Bmal1*-LKO mice with electron microscopy after ethanol feeding. In the *Bmal1*^{flox/flox} liver, mitochondria displayed normal shape and active fission. However, we observed swollen mitochondria without fission in the liver of *Bmal1*-LKO after ethanol diet (**Fig. 1F**). In summary, *Bmal1* deficiency in hepatocytes results in augmented liver steatosis and liver injury with mitochondrial impairment following ethanol diet independently of ER stress, oxidative stress, and induction of pro-apoptotic genes.

Mice with acute depletion of *Bmal1* in hepatocytes are more susceptible to ethanol-induced fatty liver and liver injury.

To test how acute depletion of hepatic *Bmal1* in adult mice responds to ethanol feeding, we knocked down hepatic *Bmal1* in adult WT mice by administrating Ad-sh*Bmal1* via tail vein prior to ethanol feeding. Compared with Ad-sh*LacZ* control, Ad-sh*Bmal1*-injected mice showed negative gain in body weight (**SF4. A**) but greater liver lipid accumulation (**Fig. 2A-B**) by liver triglycerides assay and Nile Red staining. Liver H&E staining also revealed increased lipid droplets in the liver of Ad-sh*Bmal1* mice (**Fig. 2B**). Serum ALT assay detected greater liver injury in the mice of Ad-sh*Bmal1* (**Fig. 2C**), which was further supported by a significant increase in hepatocyte apoptosis detected by TUNEL staining, caspase 3 staining, and immunoblotting for apoptotic markers including cleaved keratin 18 and caspase 3 (**Fig. 2D-E**).

Interestingly, acute *Bmal1* knockdown did not result in altered expression of *Cyp2e1*, markers of inflammation, ER stress, and apoptosis (**SF4. B-F**). Altogether, our data suggest that acute hepatic clock deficiency by depleting *Bmal1* also sensitizes mice to ethanol-induced fatty liver and liver injury.

Macrophage BMAL1 is dispensable in protecting mice from ALD.

A significant body of work has established the critical role of macrophage activation in the pathogenesis of ALD (21, 22). The circadian clock is also present in macrophages and plays an important role in regulating inflammatory cytokine production (23). We hypothesized that deletion of *Bmal1* in macrophages may also impact the severity of ALD in mice. We therefore generated macrophage-specific deletion of *Bmal1* (*Bmal1*-MKO) with *Lysosome-Cre* and confirmed that the BMAL1 protein was largely absent in liver residential macrophages Kupffer cells isolated from *Bmal1*-MKO livers (**SF5. A**). Following ethanol feeding protocol, both *Bmal1*^{flox/flox} and *Bmal1*-MKO displayed similar body weight, liver TAG and lipid droplet formation by H&E staining and Oil-Red-O staining (**SF5. B-D**). We also detected comparable levels of serum ALT between *Bmal1*^{flox/flox} and *Bmal1*-MKO mice (**SF5. E**). EM showed intact mitochondrial morphology and mitochondrial fission in the liver of both *Bmal1*^{flox/flox} and *Bmal1*-MKO mice (**SF5. F**). Taken together, BMAL1 in macrophages is likely to be dispensable during the pathogenesis of ALD.

Liver-specific *Bmal1* over-expression protects mice from alcohol feeding induced fatty liver and liver injury.

So far we have demonstrated that lack of BMAL1 expression is detrimental to hepatocytes when challenged by ethanol feeding. It remains unclear whether over-expression of BMAL1 is sufficient to protect mice from alcoholic liver diseases. Female wild type mice were injected with either Ad-GFP or Ad-*Bmal1* prior to alcohol feeding/binge treatment. We first verified BMAL1

over-expression in the liver by immunoblotting with anti-FLAG. Over the course of alcohol feeding, mice injected with Ad-GFP showed similar body weight as mice with Ad-*Bmal1* (**SF6. A**). However, Ad-*Bmal1* injected mice showed about 50% reduction in liver TG content, which was further supported by H&E staining and Nile-Red staining (**Fig. 3A-B**). Moreover, Ad-*Bmal1* expressing mice showed reduced serum ALT, suggesting BMAL1 over-expression protects mice against ethanol-induced liver injury (**Fig. 3C**). Moreover, BMAL1 over-expression led to enhanced AKT-P^{S473} phosphorylation in the liver of alcohol-fed female mice (**Fig. 3D**). To determine whether this protection against ethanol-induced liver injury is gender-specific, we also assessed liver steatosis and injury in ethanol-fed male WT mice after injection of Ad-*Bmal1*. As shown in **SF6**, BMAL1 overexpression indeed effectively reduced serum ALT, but to a lesser degree liver steatosis in male WT mice after ethanol feeding. Together these data support that ectopic expression of BMAL1 in the liver is sufficient to protect mice from alcohol feeding-induced fatty liver and liver injury.

What is the underlying mechanism for the protective role of BMAL1? Given the role of BMAL1 as a key regulator of the circadian clock, we first examined the expression of BMAL1 targets in the liver of Ad-*Bmal1*-injected female mice following alcohol feeding. To our surprise, we only detected modest induction of *Dbp*, *Nr1d1*, and *Per2* in the liver (**Fig. 3E**). In contrast, we observed robust induction of lipogenic genes (*Fasn*, *Acc1*, and *Scd1*) and the glycolytic gene *Lpk* (**Fig. 3F**). Moreover, the expression of genes in ER stress, apoptosis, and oxidative stress were comparable between Ad-GFP and Ad-*Bmal1* group (**SF7**). These data suggest that BMAL1 overexpression may protect mice against ALD in part via its ability to regulate hepatic lipid metabolism, particularly *de novo* lipogenic pathway.

Hepatocyte BMAL1 protects ALD via promoting PPAR α -mediated β -oxidation

We further investigated how BMAL1 could impact hepatic lipid metabolism during the pathogenesis of ALD. Previously, we discovered that hepatocyte BMAL1 is required for post-prandial *de novo* lipogenesis (7), while others reported that BMAL1 regulates the PPAR α expression and fatty acid oxidation in hepatocytes (24, 25). Dysregulated hepatic lipid metabolism is one of major pathological feature of ALD, which is characterized by increased *de novo* lipogenesis and suppressed fatty acid oxidation. We therefore hypothesize that BMAL1 may protect hepatocytes against ethanol toxicity by maintaining lipid homeostasis in the liver.

We first measured the mRNA expression of lipid metabolism in the mouse liver with various levels of *Bmal1* expression following ethanol feeding. Compared with *Bmal1*^{flox/flox} mice, we observed reduced expression of genes in *de novo* lipogenesis and fatty acid oxidation in *Bmal1*-LKO liver (**Fig. 4A-B**). A similar down-regulation of *de novo* lipogenesis and fatty acid oxidation genes in Ad-sh*Bmal1*-injected liver was also observed vs. Ad-sh*LacZ* mice (**Fig. 4D-E**), suggesting that *Bmal1* deficiency leads to a suppression of both *de novo* lipogenesis and fatty acid oxidation pathways.

Previous studies demonstrated that *Ppar* α knockout mice are more sensitive to ethanol-induced liver injury (26). We suspected that suppression of the PPAR α pathway contributes to the severity of ALD in *Bmal1*-LKO and hepatic *Bmal1*-depleted mice. Although we detected about 40% and 20% reduction of *Ppar* α mRNA in the *Bmal1*-LKO and *Bmal1*-depleted liver respectively, the PPAR α protein abundance in the liver was comparable between *Bmal1*^{flox/flox} and *Bmal1*-LKO mice or between Ad-sh*LacZ* and Ad-sh*Bmal1* mice (**Fig. 4C&F**), suggesting that BMAL1 may regulate the biogenesis of endogenous ligands for PPAR α without affecting its protein abundance. Thus, we speculated that supplementation of synthetic PPAR α ligands could ameliorate ALD in *Bmal1*-LKO mice. As a synthetic PPAR α agonist, fenofibrate has been

clinically used to treat hyperlipidemia (27, 28). Of note, we firstly used ethanol-fed WT mice to confirm the efficacy of fenofibrate. At the dose of 20 mg/kg, daily treatment of fenofibrate was sufficient to reduce liver steatosis and serum ALT (**SF8**), consistently with the previous report (29). We therefore tested our hypothesis by treating *Bmal1-LKO* mice daily with either corn oil or fenofibrate during the course of ethanol feeding. As expected, fenofibrate stimulated the expression of PPAR α target genes in the liver of *Bmal1-LKO* mice such as *Acox1*, *Cpt1a*, and *Cyp4a10* (**Fig. 5A**). Fenofibrate treatment reduced lipid accumulation in the liver of *Bmal1-LKO* mice (**Fig. 5B-C**). Additionally, fenofibrate lowered serum ALT and reduced TUNEL-positive hepatocytes (**Fig. 5D-E**). Electron microscopy also showed higher density of mitochondria in active fission in the liver of fenofibrate-treated *Bmal1-LKO* mice (**Fig. 5F**). Taken together, we found that reactivation of PPAR α signaling by fenofibrate could reverse liver steatosis, liver injury, and mitochondrial impairment in ethanol-fed *Bmal1-LKO* mice.

Constitutively active AKT restores PPAR α activity and reverses liver injury in ethanol diet-fed *Bmal1-LKO* mice.

Hepatocyte PPAR α signaling plays an essential role in maintaining lipid homeostasis in the liver. PPAR α activity is dynamically regulated by nutritional status and various hormonal inputs. It is thought that PPAR α activity elevates during fasting and becomes suppressed by feeding. Unexpectedly, hepatocyte-specific *Fasn* knockout mice show reduced PPAR α activity and liver steatosis, suggesting a positive crosstalk between hepatic *de novo* lipogenesis and PPAR α -mediated fatty acid oxidation (12). It has been proposed that hepatic lipogenesis may be responsible for *de novo* biogenesis of endogenous PPAR α ligands (12, 13, 30). We also demonstrated previously that restoring AKT activity by expressing constitutively active AKT2 (**AKT2-CA**) can rescue the defect of *de novo* lipogenesis in the *Bmal1* knockout liver (7). Since *de novo* lipogenesis and PPAR α pathways were both repressed in the ethanol-fed *Bmal1-LKO*

mice, we investigated whether BMAL1-driven *de novo* lipogenesis pathway could impact the PPAR α transcription activity. To test this hypothesis, we first determined whether restoring cellular AKT could induce PPAR α target genes in hepatocytes with *Bmal1* deficiency. In *Bmal1-LKO* hepatocytes, over-expression of AKT2-CA increased the expression of not only lipogenic genes but also a subset of β -oxidation genes (**SF9**). To test how AKT2-CA overexpression in the liver affects PPAR α target gene expression in *Bmal1-LKO*, we injected *Bmal1-LKO* mice with either Ad-GFP or Ad-Akt2-CA prior to ethanol feeding. Overexpression of AKT2-CA was confirmed by immunoblotting for total AKT2 and GSK3 β -P^{S9}, a direct phosphorylation target of AKT (**Fig. 6A**). In ethanol-fed *Bmal1-LKO* mouse livers, over-expression of AKT2-CA induced lipogenic genes, and more importantly increased several PPAR α targets (**Fig. 6B**). Of note, over-expression of AKT2-CA showed stronger effects on PPAR α targets in vivo than in primary hepatocytes, possibly due to a longer duration of expression.

Next, we examined the impact of AKT2-CA over-expression on the severity of ALD in *BLKO* mice. H&E staining and Nile-Red staining showed reduced lipid accumulation in the liver of AKT2-CA injected mice (**Fig. 6C-D**) in addition to reduced liver TG (**Fig. 6E**). Restoring of AKT activity also reduced liver injury detected by lower ALT level (**Fig. 6F**), fewer TUNEL-positive cells and reduced caspase 3 cleavage (**SF10. A-B**). Interestingly, the protective effects of AKT2-CA overexpression were only observed in *Bmal1-LKO* liver but not in WT mice (**Fig. 6F, SF11**). Taken together, restoring hepatocyte AKT is sufficient to reverse ALD in *Bmal1-LKO* mice. Furthermore, increasing AKT activity in *Bmal1-LKO* mice not only promotes *de novo* lipogenesis but also enhances the expression of β -oxidation genes. To our knowledge this is the first evidence showing that active AKT signaling pathway enhances both *de novo* lipogenesis and β -oxidation while protecting against ALD.

Overexpression of ChREBP in the liver partially rescues liver injury in ethanol-fed *Bmal1*-LKO mice

Both ChREBP and SREBP-1c are two major lipogenic transcription factors in hepatic lipid biosynthesis (31). SREBP-1c has been implicated as a direct downstream effector of AKT-mTORC1 signaling in insulin-induced *de novo* lipogenesis (32), whereas ChREBP has been more associated with high carbohydrate feeding, in particular, fructose feeding (33, 34). A recent study showed loss of *Chrebp* may sensitize mice from binge-drinking-induced liver injury (35). We observed that *L-pk*, the hallmark target of ChREBP, was reduced in the liver of both *Bmal1*-LKO and mice with liver specific depletion of *Bmal1* but induced in Ad-*Bmal1* liver (**Fig. 3F, Fig. 4A&D**), indicating a functional link between BMAL1 and ChREBP during the process of *de novo* lipogenesis. Furthermore, we evaluated the effects of ChREBP over-expression in primary hepatocyte isolated from *Bmal1*^{flox/flox} and *Bmal1*-LKO mice on the expression of enzymes in lipogenesis and fatty acid oxidation. We also included SREBP-1c as a comparison. Consistent with their roles in driving *de novo* lipogenesis, both SREBP-1c and ChREBP overexpression significantly induced key genes of lipogenic program (**SF12 A&C**). However, only ChREBP was able to potently induce the expression of β -oxidation genes (**SF12 B&D**). Of note, compared with *Bmal1*^{flox/flox} hepatocytes, *Bmal1*-LKO hepatocytes showed a more robust induction of β -oxidation genes in response to ChREBP overexpression. These data for the first time unveiled a diverging effect of ChREBP and SREBP-1c on β -oxidation in the liver.

These observations prompted us to ask whether over-expression of ChREBP in liver could affect the degree of liver injury in ethanol-fed *Bmal1*-LKO mice. Over-expression of FLAG-ChREBP in the liver after Ad-*Flag-Chrebp* adenovirus injection was confirmed by immunoblotting with anti-FLAG (**Fig. 7A**). Compared with Ad-GFP injection, Ad-*Flag-Chrebp* injection induced hepatic expression of β -oxidation genes (**Fig. 7B**), reduced fat accumulation in

the liver of *Bmal1*-LKO mice (**Fig. 7C-E**). We also detected significantly reduced apoptosis in the liver in addition to modestly decreased serum ALT (**SF13**). Interestingly, the protective effect of ChREBP overexpression was only observed in the *Bmal1*-LKO liver but not in that of WT mice (**SF14**).

Meanwhile, we also evaluated whether over-expression of SREBP-1c in the liver could impact the degree of liver injury in ethanol-fed *Bmal1*-LKO mice. As shown in **SF15 B-D**, ectopic expression of SREBP-1c failed to reduce liver steatosis and liver injury after ethanol diet. Moreover, SREBP-1c overexpression reduced the expression of β -oxidation genes in ethanol-fed *Bmal1*-LKO mice (**SF15 E**). In summary, these data suggest that ChREBP overexpression in the *Bmal1*-LKO liver efficiently reverses alcoholic fatty liver and reduces liver injury. Our study also demonstrated a clear distinction between ChREBP and SREBP-1c in hepatic regulation of *de novo* lipogenesis and fatty acid oxidation during ethanol feeding.

To investigate the clinical relevance of the BMAL1-AKT-ChREBP axis in human ALD, we examined the abundance of BMAL1, AKT-P^{S473}, AKT, and ChREBP in the liver samples from patients with alcoholic hepatitis vs. normal controls. Indeed, BMAL1 protein levels were severely decreased in the livers of patients with ALD compared with normal controls (**SF16**). In the same liver samples, the levels of AKT-P^{S473} were greatly reduced. Moreover, ChREBP protein levels were reduced in the liver of ALD patients. Collectively, our data suggest that the impairment of the BMAL1-AKT-ChREBP axis could be involved in the development of human ALD.

DISCUSSION

In the current study, we presented evidence supporting that the intact circadian clock in the liver is required to protect mice from ethanol feeding-induced liver steatosis and injury. Either chronic or acute loss of *Bmal1* aggravates liver steatosis and injury after chronic ethanol-binge feeding.

Impaired PPAR α signaling may drive the severity of ALD in the *Bmal1*-deficient condition since PPAR α agonist fenofibrate rescues liver injury in *Bmal1*-LKO mice. Further investigations reveal an intimate relationship between the BMAL1-AKT-ChREBP axis-mediated de novo lipogenesis and PPAR α -induced fatty acid oxidation. In summary, our work demonstrated a pivotal role of the circadian clock protein BMAL1 in the liver in protecting against ALD via maintaining hepatic lipid homeostasis.

Recent studies suggest a potential crosstalk between alcohol metabolism and circadian rhythms could impact the progression and severity of ALD (4, 36). We observed severe liver steatosis and liver injury following ethanol feeding in two different mouse models with circadian clock deficiency in the liver, pointing to the protective role of an intact hepatic circadian clock against ALD. Interestingly, our study revealed that it is the circadian clock in hepatocytes rather than macrophages that protects the liver in response to ethanol feeding, highlighting the cell type-specific function of the circadian clock. Our data suggest that hepatic circadian deficiency or impairment could be a risk factor for the development of advanced ALD. It has been reported that high-fat diet greatly dampens the hepatic circadian clock even before the development of obesity (37). Social factors such as jet-lag can also disturb the hepatic circadian clock (16). Therefore, restoring or maintaining a normal circadian clock could be key to the prevention or treatment of ALD.

Our study also suggests that BMAL1 overexpression is also sufficient in protecting mice from alcoholic liver injury. Liver-specific expression of BMAL1 using adenovirus provides almost full protection against ALD in both female and male mice. This exciting finding suggests that elevating the BMAL1 abundance in hepatocytes might be an effective approach to treat ALD. In our study, we also found that BMAL1 protein is modestly increased in the liver of ethanol-fed

WT mice although the clock activity is suppressed. It is well known that the *Bmal1* mRNA oscillates in hepatocytes during a 24-hr cycle and multiple transcription factors are implicated in its mRNA oscillation. In contrast, the BMAL1 protein levels remain steady with modest changes throughout the circadian cycle (38). It has been suggested that BMAL1 protein abundance and activity are subject to multiple post-translational modifications including O-linked glycosylation, acetylation, and phosphorylation (38-41). We suspect that elevated BMAL1 protein in the ethanol-fed WT mouse liver is a net result of altered multiple upstream signaling pathways. Given that BMAL1 overexpression is sufficient to protect mice from ALD, the modest induction of BMAL1 in the liver of ethanol-fed WT mice might be a compensatory mechanism to preserve the clock activity in hepatocytes.

Based on our findings, one of the major downstream pathways could be AKT-ChREBP-lipogenesis. During ethanol feeding, BMAL1 over-expression up-regulates liver AKT activity and ChREBP target gene expression, whereas *Bmal1* deficiency reduces liver AKT activation and ChREBP target gene expression. Restoring AKT or ChREBP in *Bmal1*-LKO mice attenuates fatty liver and more importantly activates the β -oxidation pathway. How BMAL1 regulates the ChREBP transcriptional activity via AKT remains to be determined in future study. A recent study suggests AKT can activate ChREBP in brown adipose tissue upon cold exposure (42). It is intriguing to speculate that a similar pathway is conserved in hepatocytes in the context of ethanol feeding.

Our findings have shed light on an under-appreciated function of hepatocyte *de novo* lipogenesis in promoting PPAR α -dependent β -oxidation pathway. The Semenkovich group used hepatocyte-specific *Fasn* knockout mice as a model to establish a novel concept that *de novo* lipogenesis in hepatocytes promotes PPAR α and β -oxidation pathway by generating newly

synthesized PPAR α -specific ligands (12, 13). For alcoholic fatty liver disease, a canonical model is that lipogenesis over-activation leads to liver steatosis and lipotoxicity. However, a recent report showed that, although ethanol feeding induced liver steatosis in mice, lipogenic gene expression was repressed (43). Our findings support this concept in *Bmal1-LKO* mice by demonstrating hepatic BMAL1 acts as a central regulator that coordinates *de novo* lipogenesis and PPAR α -dependent β -oxidation in hepatocytes when challenged with ethanol feeding. Given the fact that BMAL1, ChREBP, and SREBP-1c all can stimulate the transcriptional program of *de novo* lipogenesis, it will be interesting to use unbiased lipidomics to differentiate lipid end-products specific for each lipogenic factor after over-expression.

The Bailey group showed that chronic ethanol consumption disrupts the core circadian clock and diurnal rhythms of metabolic genes in the liver (44). The Duffield group also showed the circadian clock impairment in the mouse liver with alcohol-induced steatosis (45). In agreement with these two studies, our study showed disrupted diurnal rhythms of core clock genes and a potent suppression of SIRT1 activity in the ethanol-fed mouse liver. Compared with the two studies, our study used a different chronic feeding/binge protocol. Therefore, there are noticeable differences in the type and pattern of clock genes oscillations affected by the duration of feeding, degree of liver steatosis, and severity of injury. It is likely that ethanol feeding impacts the expression of circadian genes in both clock-dependent and clock-independent manners.

In summary, we have presented novel evidence that BMAL1, a key circadian protein, is both necessary and sufficient to protect mice against ALD. Our findings identified the BMAL1-AKT-ChREBP-lipogenesis axis as a major signaling route in the pathogenesis of ALD. Our study

suggests that activation of BMAL1-dependent *de novo* lipogenesis in the liver could offer novel therapeutics to treat human ALD.

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Figure legends

Figure 1. Hepatocyte-specific *Bmal1* knockout mice are sensitive to ethanol feeding-induced liver steatosis and liver injury

8-wk old *Bmal1*^{Flox/Flox} littermates and *Bmal1*^{Flox/Flox} *Alb-Cre*(+) (*Bmal1*-LKO) mice were fed 5% ethanol diet for 10 days before binge feeding with ethanol (5 g/kg body weight) at Zeitgeber Time (ZT)3 and dissected 9 hr later (n = 4 for *Bmal1*^{Flox/Flox} and n = 5 for *Bmal1*-LKO, both male and females). (A) Hepatic triglyceride levels. (B) H&E staining and Nile red staining. (C) ALT assay to assess liver injury. (D) TUNEL staining and immune-fluorescence against cleaved caspase3 to assess apoptosis. (E) Western blotting analysis of hepatic apoptotic markers. (F) Transmission electron microscopy (magnification 40,000X). Mitochondria undergoing fission are indicated with green arrowheads and swollen mitochondrial with red arrowheads. **p* < 0.05, ***p* < 0.01 by two-tailed Student's *t* test. Scale bar = 100 μM for B and D; Scale bar = 400 nM for F.

Figure 2. Adult-onset hepatocyte *Bmal1* depletion sensitizes mice to ethanol-induced fatty liver and liver injury.

8-wk mice were injected with either adenovirus expressing shRNA targeting *Bmal1* (Ad-sh*Bmal1*, n=8) or Ad-shLacZ control (n=5) at MOI 1x10¹² pfu, fed 5% ethanol diet for 10 days and then binged with ethanol (5 g/kg body weight) at ZT 3 of the 11th day and were dissected 9 hr later. Liver samples were subjected to triglyceride assay (A), H&E staining and Nile Red staining (B) to assess hepatic lipid accumulation, ALT assay to assess liver injury (C), TUNEL staining and caspase3 immuno-staining to assess apoptosis (D), and Western analysis of apoptotic markers (E). **p* < 0.05, ***p* < 0.01 by two-tailed Student's *t* test. Scale bar = 100 μM.

Figure 3. Liver-specific *Bmal1* over-expression protects mice from alcohol feeding-induced fatty liver and liver injury.

8-wk mice were injected with adenovirus expressing either *Bmal1* (Ad-*Bmal1*, n = 8) or GFP control (Ad-GFP, n = 5), and then fed 5% ethanol diet for 10 days and binged with 5 g ethanol per kg body weight at ZT3 of the 11th day and were dissected 9 hr later. (A-B) *Bmal1* overexpression lowers ethanol-induced liver toxicity. Hepatic triglycerides assay (A), H&E staining and Nile red staining (B) were utilized to assess hepatic lipid accumulation. Liver injury was assessed with ALT assay (C). (D-F) *Bmal1* overexpression activates hepatic lipogenesis. AKT phosphorylation was assessed with western blotting (D). Circadian genes (E) and lipogenic genes (F) were assessed with RT-qPCR. * $p < 0.05$, *** $p < 0.001$ by two-tailed Student's *t* test. Scale bar = 100 μ M.

Figure 4. Hepatic *Bmal1* deficiency results in decreased gene expression of both *de novo* lipogenesis and β -oxidation in the liver of ethanol-fed mice.

(A-C) Hepatocyte-specific deficiency of *Bmal1* results in decreased PPAR α activity in *BLKO* mice liver after ethanol treatment. Mice were treated as described in Figure1. Relative mRNA levels of lipogenic genes (A) and PPAR α target genes (B), and protein levels of PPAR α (C) in the livers of *Bmal1*-LKO mice (n=5), were compared to those of *Bmal1*^{Flox/Flox} mice (n=4). (D-F) Adult-onset *Bmal1* depletion in the liver decreases the PPAR α activity after ethanol treatment. Relative mRNA levels of lipogenic genes (D) and PPAR α target genes (E), and protein levels of PPAR α (F) in the livers of Ad-sh*Bmal1*-injected mice (n=8), were compared with those of Ad-sh*LacZ* control (n=5). * $p < 0.05$, *** $p < 0.001$ by two-tailed Student's *t* test.

Figure 5. Fenofibrate administration rescues ethanol-induced liver injury in *BLKO* mice.

8-wk *BLKO* 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 ZT3, and were dissected 9 hrs later (n = 5 for control and n = 7 for fenofibrate, both males and females). (A)

PPAR α 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 in *Bmal1-LKO* mice, assessed by H&E staining and Oil-Red-O staining **(B)** and liver triglycerides assay **(C)**. **(D-F)** Fenofibrate treatment ameliorates ethanol-induced liver injury in *Bmal1-LKO* mice. Liver injury was assessed with ALT assay **(D)**, TUNEL staining **(E)** and electron microscopy (magnification 40,000X) **(F)**. Mitochondria undergoing fission were 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 μ M for **B** and **E**; Scale bar = 400 nM for **F**.

Figure 6. Restoring AKT activity rescues both de novo lipogenesis and PPAR α pathway and reverses liver injury in *Bmal1-LKO* mice.

8-wk old *Bmal1-LKO* mice were injected with Ad-Akt2-CA ($n = 6$) or Ad-GFP ($n = 5$), and then fed 5% ethanol diet for 10 days, binged with 5g ethanol/kg body weight at ZT3 on the 11th day, and were dissected 9 hr later. **(A)** AKT2 activity was confirmed by western blotting with anti-AKT2 and anti-GSK3 β phosphorylation. **(B)** Restoring AKT activity induces both de novo lipogenic genes and β -oxidation genes with RT-qPCR. **(C-F)** AKT activation reduces ethanol-induced liver steatosis and injury in *Bmal1-LKO* mice with H&E staining **(C)** and Nile Red staining **(D)**, liver triglycerides assay **(E)** and serum ALT assay **(F)**. * $p < 0.05$, ** $p < 0.01$ by two-tailed Student's t test. Scale bar = 100 μ M.

Figure 7. Hepatic overexpression of ChREBP restores the PPAR α activity and partially ameliorates liver injury in ethanol-fed *Bmal1-LKO* mice.

8-wk old *Bmal1-LKO* mice were injected with Ad-Flag-Chrebp ($n = 5$) or Ad-GFP ($n = 3$), and then fed 5% ethanol diet for 10 days and binged with ethanol 5g/kg body weight at ZT3, and were dissected 9 hr later. **(A)** Hepatic ChREBP overexpression by adeovirus was confirmed by

immunoprecipitation with anti-FLAG beads and western blotting with anti-FLAG. **(B)** Hepatic ChREBP overexpression activates the expression of *L-pk* and PPAR α target genes measured by RT-qPCR. **(C-E)** Hepatic ChREBP overexpression reduces ethanol feeding-induced liver steatosis in *Bmal1-LKO* mice with liver triglycerides assay **(C)** H&E staining **(D)** and Nile Red staining **(E)**. **(E-F)** Hepatic ChREBP overexpression ameliorates ethanol-induced liver injury in *Bmal1-LKO* mice with serum ALT assay **(E)**. **(F)** Model depicting that BMAL1 limits ethanol-induced lipotoxicity via increased β -oxidation by activating de novo lipogenesis to provide ligands for PPAR α . * $p < 0.05$, by two-tailed Student's t test. Scale bar = 100 μ M

Figure 1.

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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 impairs 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-shBmal1* verse *Ad-shLacZ*. 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-shBmal1* or *Ad-shLacZ* (**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 μ M 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 μ M.

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 vehicle and n = 6 for fenofibrate, both males and females). (A) PPARα activation by Fenofibrate was confirmed by the induction of PPARα 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 μ M.

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 PPAR α 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- μ m cell strainer, and then spun at 50 $\times$ g for 1 min. The pellet was re-suspended in DMEM and then spun at 50 $\times$ g for 10 min in a Percoll gradient to remove dead hepatocytes. Viable cells were washed with DMEM at 50 $\times$ g for 10 min and checked by trypan blue staining. PMHs in DMEM with 5% FBS were seeded at a density of 2 $\times$ 10⁵ 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 CaCl₂, 10mM HEPES and 4 mM NaOH in HBSS) in 37 °C for 2hrs with shaking. After being filtered with a 100- μ m 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 Biosystems, Foster City, CA). The value of each cDNA was calculated using the $\Delta\Delta C_t$ 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
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<i>18s RNA</i>	5'-TTGACGGAAGGGCACCACCAG-3'	5'-GCACCACCACCCACGGAATCG-3'
<i>Acc1</i>	5'-GAAGCCACAGTGAATCTCG-3'	5'-GATGGTTTGGCCTTTACAT-3'
<i>Acox1</i>	5'-TGCTGCAGACGGCCAGGTTTC-3'	5'-GGCCAGACTGCCACCTGCTG-3'
<i>Bcl2-l1</i>	5'-GCTGCATTGTTCCCGTAGAG-3'	5'-GTTGGATGGCCACCTATCTG-3'
<i>Bim</i>	5'-CGGTCCTCCAGTGGGTATTT-3'	5'-TATGGAAGCCATTGCACTGAGA-3'
<i>Cd36</i>	5'-CCAAGCTATTGCGACATGATT-3'	5'-CCGAACACAGCGTAGATAGACC-3'
<i>Chop</i>	5'-CTGCCTTTCACCTTGAGAC-3'	5'-CGTTTCCTGGGGATGAGATA-3'
<i>Chrebpa</i>	5'-CGACACTCACCCACCTCTTC-3'	5'-TTGTTTCAGCCGGATCTTGTC-3'
<i>Chrebpb</i>	5'-TCTGCAGATCGCGTGGAG-3'	5'-CTTGTCCCGGCATAGCAAC-3'
<i>Cpt1a</i>	5'-TCTGCATGTTTGACCCAAAA-3'	5'-TTGCTGGAGATGTGGAAGAA-3'
<i>Cyp4a10</i>	5'-GGAGCTCCAATGTCTGAGAAGAGT	5'-TCTCTGGAGTATTCTTCTGAAAAAGGT
<i>Cyp4a14</i>	5'-TCTCTGGCTTTTCTGTACTTTGCTT	5'-CAGAAAGATGAGATGACAGGACACA
<i>Fasn</i>	5'-TTGGCCCAGAACTCCTGTAG-3'	5'-CTCGCTTGTCGTCTGCCT-3'
<i>Gadd45a</i>	5'-TGGTGACGAACCCACATTCA-3'	5'-CGGGAGATTAATCACGGGCA-3'
<i>Gck</i>	5'-CCCTGAGTGGCTTACAGTTC-3'	5'-ACGGATGTGGAGTGTGAAGC-3'
<i>Grp78</i>	5'-GGTGCAGCAGGACATCAAGTT-3'	5'-CCCACCTCCAATATCAACTTGA-3'
<i>L-PK</i>	5'-CTGGAACACCTCTGCCTTCTG-3'	5'-CACAATTTCCACCTCCGACTC-3'
<i>Mcl-1</i>	5'-TTCTTTCGGTGCCTTTGTGGC-3'	5'-AACCCATCCCAGCCTCTTTGTT-3'
<i>Mttp1</i>	5'-CTCCACAGTGCAGTTCTCACA-3'	5'-AGAGACATATCCCCTGCCTGT-3'
<i>Ppara</i>	5'-CCTTCTACGCTCCCGACCCA-3'	5'-CCATGTCCATAAATCGGCACCA-3'
<i>Puma</i>	5'-TACGAGCGGCGGAGACAAG-3'	5'-GTGTAGGCACCTAGTTGGGC-3'
<i>Scd1</i>	5'-GCCGAGCCTTGTAAAGTTCTG-3'	5'-CCTCCTGCAAGCTCTACACC-3'
<i>Srebp-1c</i>	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 (7 μ L acetic acid/mg liver tissue). After quick spinning, 200 μ L of supernatant was transferred to a 1.5 mL tube containing 800 μ L of chloroform/methanol (2:1, v/v). After centrifuge for 10 min at 10000xg at room temperature, 450 μ L 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 μ L ethanol and incubated at 55°C for 20 min. 3 μ L 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 magnification 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 μ l/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 Alphamager (Cell Biosciences).

Primary antibodies against AKT α 1/ α 2 (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 μ L of sample was loaded for western blotting against BMAL1 and CLOCK, and Coomassie blue staining to examine protein loading.

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