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The Hepatic BMAL1/AKT/Lipogenesis Axis Protects Against Alcoholic Liver Disease in Mice via Promoting PPARα 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 ALD. 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 PPARα 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 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 PPARα-mediated fatty oxidation.

Alcohol over-consumption is the primary cause of liver-related mortality in western countries. In the United States, more than 50% of the population consumes alcohol.[1,2] As the primary site of alcohol metabolism, the liver is a major target organ of alcohol-induced injury. Three 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

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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. A *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 PPARα activity in the liver.[9–11] Administration of a synthetic PPARα agonist reverses alcoholic fatty liver and improves liver function in mice.[9] The inhibitory effect of ethanol on the PPARα transcriptional activity was observed in cultured hepatocytes, suggesting that ethanol and its metabolites could impact the DNA-binding activity of PPARα in the liver.[10] Currently, it remains unclear how ethanol feeding alters the hepatic PPARα activity.

Given the pivotal role of PPARα in liver lipid homeostasis, extensive research has been done on how the PPARα activity is modulated in response to hormonal and nutritional signals. Unexpectedly, the PPARα activity is severely down-regulated in hepatocytes lacking FASN, indicating an intricate link between *de novo* lipogenesis and the biogenesis of endogenous PPARα ligands and the subsequent PPARα 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 PPARα.[13] However, how the connection between *de novo* lipogenesis and PPARα 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 PPARα 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 hour/12 hour light/dark cycles with *ad libitum* access to food and water. *Bmal1*$^{Flox/Flox}$ mice were generously provided by Dr.

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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 and 2% glutaraldehyde in PBS for 2 hours at room temperature. After washing in PBS, the tissue samples were post fixed in osmium tetroxide for 45 minutes 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, and then 100% Epon and finally embedded. Sections were made with a Leica EM UC7 ultra-microtome (Leica), stained for 15 minutes 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 ± 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 Supporting Information.

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 (WT) mice were fed a 5% ethanol diet for 10 days before binged with 5g ethanol per kg body weight at Zeitgeber time 3, and dissected 9 hours later. Ethanol feeding significantly increased liver triglycerides accumulation (Supporting Fig. S1A,B), ALT levels and *Cyp2e1* expression level (Supporting Fig. S1C,D) along with enhanced expression of pro-inflammatory cytokines, ER stress and pro-apoptotic markers (Supporting Fig. S1E–G), as well as increased neutrophil recruitment (Supporting Fig. S1H), indicating

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that this chronic-plus-binge ethanol regimen induces 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 (Supporting Fig. S2A,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 (Supporting Fig. S2C). 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 (Supporting Fig. S2A,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 (Supporting Fig. S2D). 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 (Supporting Fig. S2E). 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*$^{flox/fox}$ and *Bmal1* liver-specific knockout (*Bmal1-LKO*) to the ethanol diet/binge model. Compared with *Bmal1*$^{flox/fox}$ control, *Bmal1-LKO* mice quickly lost weight on ethanol diet (Supporting Fig. SF3A), accumulated more lipids in the liver (Fig. 1A,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 (Supporting Fig. S3B–F). Neutrophil infiltration was comparable between *Bmal1*$^{flox/fox}$ and *Bmal1-LKO* mice (Supporting Fig. S3G).

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*$^{flox/fox}$ vs. *Bmal1-LKO* mice with electron microscopy after ethanol feeding. In the *Bmal1*$^{flox/fox}$ 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.

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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 Adsh*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 (Supporting Fig. S4A) 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-shBmal1 (Fig. 2C), which was further supported by a significant increase in hepatocyte apoptosis detected by TUNEL staining, caspase 3 staining, and immuno-blotting 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 (Supporting Fig. S4B–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 (Supporting Fig. S5A). 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 (Supporting Fig. S5B–D). We also detected comparable levels of serum ALT between *Bmal1$^{flox/flox}$* and *Bmal1-MKO* mice (Supporting Fig. S5E). EM showed intact mitochondrial morphology and mitochondrial fission in the liver of both *Bmal1$^{flox/flox}$* and *Bmal1-MKO* mice (Supporting Fig. S5F). 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 ALDs. Female WT 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* (Supporting Fig. S6A). However, Ad-*Bmal1* injected mice showed approximately 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 AKTP^{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 Supporting Fig. S6, 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 (Supporting Fig. S7). 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 PPARα-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 PPARα 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-shBmal1-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 *Ppara* knockout mice are more sensitive to ethanol-induced liver injury.[26] We suspected that suppression of the PPARα pathway contributes to the severity of ALD in *Bmal1-LKO* and hepatic *Bmal1*-depleted mice. Although we detected about 40% and 20% reduction of *Ppara* mRNA in the *Bmal1-LKO* and *Bmal1*-depleted liver respectively, the PPARα protein abundance in the liver was comparable between *Bmal1*$^{flox/flox}$ and *Bmal1-LKO* mice or between AdshLacZ and Ad-sh*Bmal1* mice (Fig. 4C,F), suggesting that BMAL1 may regulate the biogenesis of endogenous ligands for PPARα without affecting its protein abundance. Thus, we speculated that supplementation

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of synthetic PPARα ligands could ameliorate ALD in *Bmal1-LKO* mice. As a synthetic PPARα 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 (Supporting Fig. S8), 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 PPARα 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 PPARα signaling by fenofibrate could reverse liver steatosis, liver injury, and mitochondrial impairment in ethanol-fed *Bmal1-LKO* mice.

CONSTITUTIVELY ACTIVE AKT RESTORES PPARα ACTIVITY AND REVERSES LIVER INJURY IN ETHANOL DIET-FED *Bmal1-LKO* MICE

Hepatocyte PPARα signaling plays an essential role in maintaining lipid homeostasis in the liver. PPARα activity is dynamically regulated by nutritional status and various hormonal inputs. It is thought that PPARα activity elevates during fasting and becomes suppressed by feeding. Unexpectedly, hepatocytespecific *Fasn* knockout mice show reduced PPARα activity and liver steatosis, suggesting a positive crosstalk between hepatic *de novo* lipogenesis and PPARα-mediated fatty acid oxidation.[12] It has been proposed that hepatic lipogenesis may be responsible for *de novo* biogenesis of endogenous PPARα 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 PPARα pathways were both repressed in the ethanolfed *Bmal1-LKO* mice, we investigated whether BMAL1-driven *de novo* lipogenesis pathway could impact the PPARα transcription activity. To test this hypothesis, we first determined whether restoring cellular AKT could induce PPARα 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 (Supporting Fig. S9). To test how AKT2-CA overexpression in the liver affects PPARα 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 PPARα targets (Fig. 6B). Notably, over-expression of AKT2-CA showed stronger effects on PPARα 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),

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fewer TUNEL-positive cells and reduced caspase 3 cleavage (Supporting Fig. S10A,B). Interestingly, the protective effects of AKT2-CA overexpression were only observed in *Bmal1-LKO* liver but not in WT mice (Fig. 6F, Supporting Fig. S11). 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 (Supporting Fig. S12A,C). However, only ChREBP was able to potently induce the expression of *β*-oxidation genes (Supporting Fig. S12B,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 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 (Supporting Fig. S13). Interestingly, the protective effect of ChREBP overexpression was only observed in the *Bmal1-LKO* liver but not in that of WT mice (Supporting Fig. S14).

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 Supporting Fig. S15B–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 (Supporting Fig. S15E). In summary, these

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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 (Supporting Fig. S16). 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 PPARα signaling may drive the severity of ALD in the *Bmal1*-deficient condition since PPARα 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 PPARα-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

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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 ethanolfed 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 PPARα-dependent *β*-oxidation pathway. The Semenkovich group used hepatocyte-specific *Fasn* knockout mice as a model to concept that *de novo* lipogenesis in hepatocytes promotes PPARα and *β*-oxidation pathway by generating newly synthesized PPARα-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 PPARα-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 lipodomics 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 ethanolfed 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.

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In summary, we have presented 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* lipogensis in the liver could offer therapeutics to treat human ALD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AKT2-CA	constitutively active AKT2
ALD	Alcohol liver disease
ALT	alanine aminotransferase
EM	electron microscope
HFD	high-fat diet
LKO	liver-specific knockout
MKO	macrophage-specific knockout
TG	triglycerides
WT	wildtype

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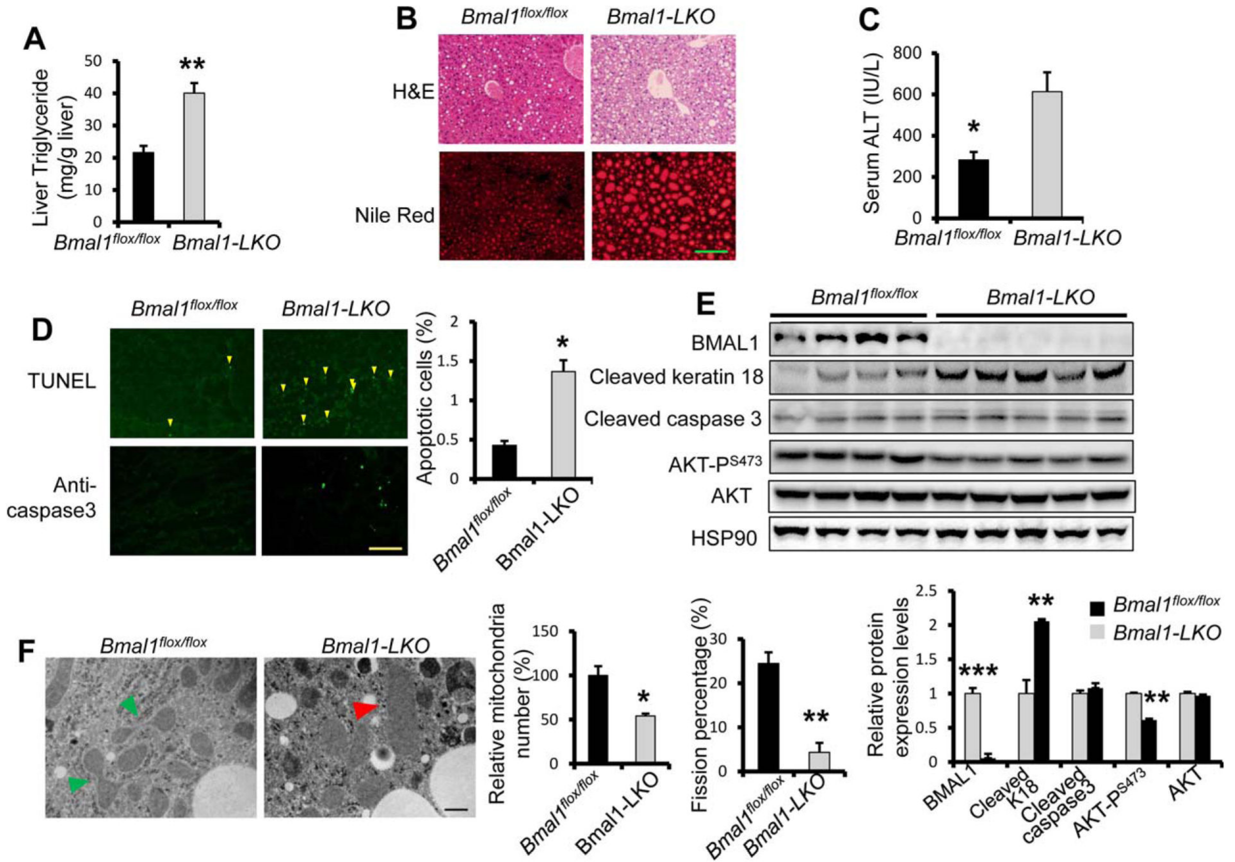

FIG. 1.

Hepatocyte-specific *Bmal1* knockout mice are sensitive to ethanol feeding-induced liver steatosis and liver injury. Eight-week-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 hours later (n 5 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.

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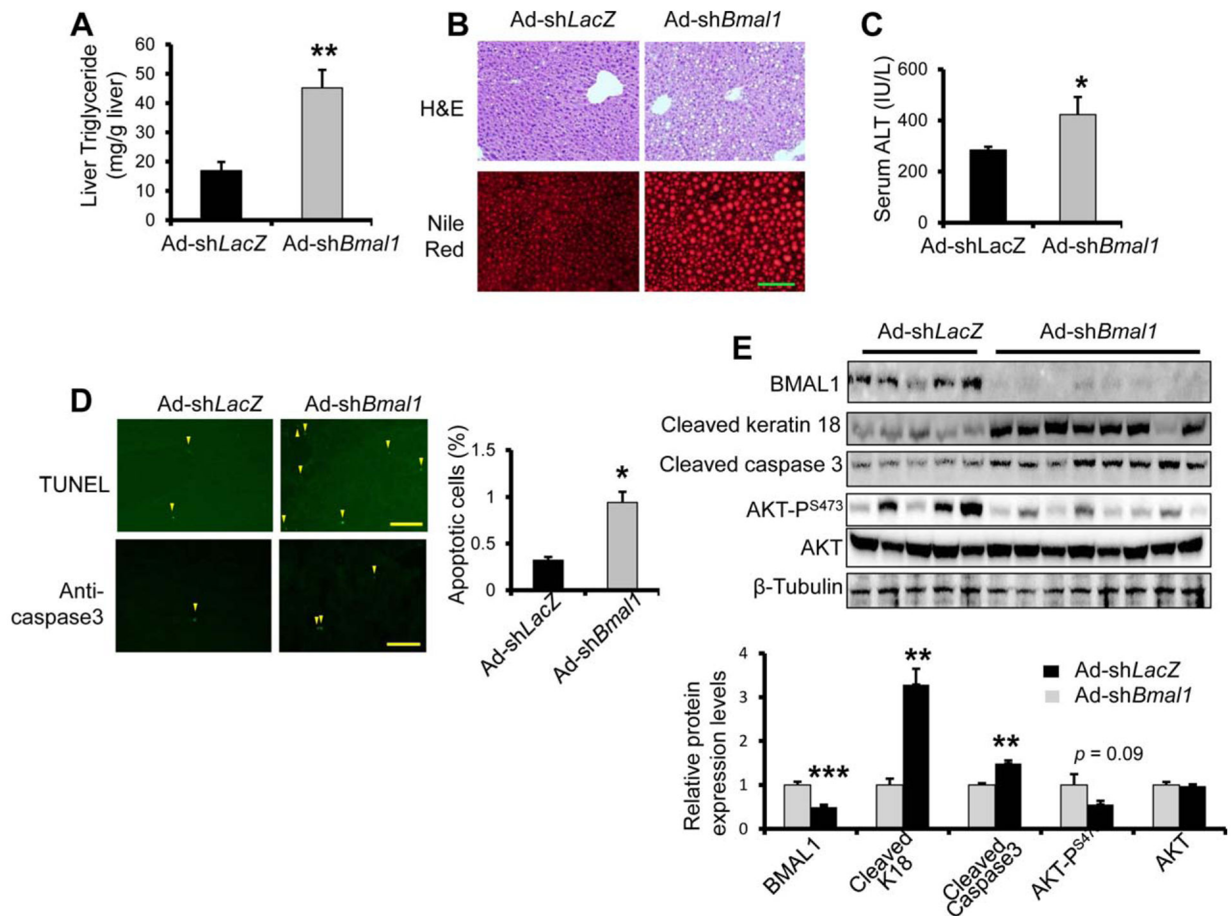

FIG. 2.

Adult-onset hepatocyte *Bmal1* depletion sensitizes mice to ethanol-induced fatty liver and liver injury. Eight-week-old mice were injected with either adenovirus expressing shRNA targeting *Bmal1* (Ad-sh*Bmal1*, n =8) or Ad-shLacZ control (n =5) at MOI 1×10^{12} pfu, fed 5% ethanol diet for 10 days and then binged with ethanol (5 g/kg body weight) at ZT 3 of the eleventh day and were dissected 9 hours 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 uM.

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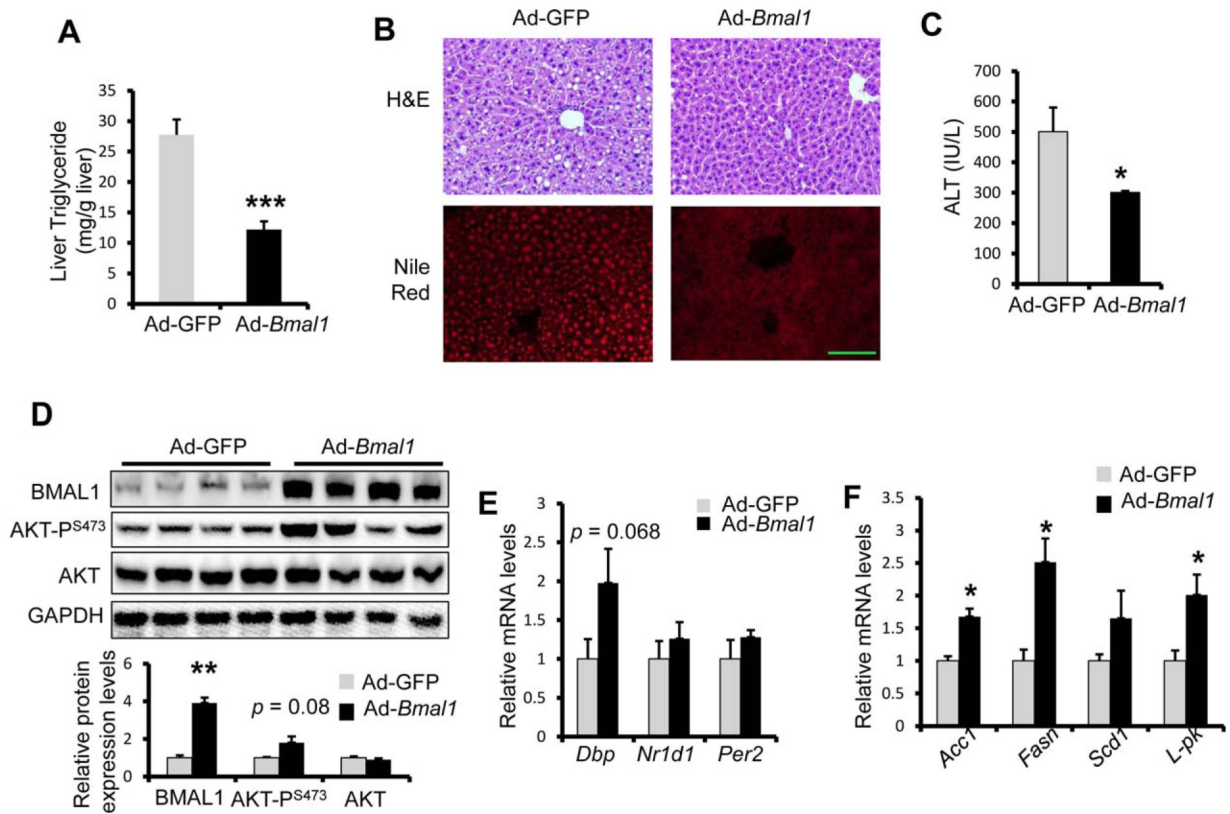

FIG. 3.

Liver-specific *Bmal1* over-expression protects mice from alcohol feeding-induced fatty liver and liver injury. Eight-week-old 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 eleventh day and were dissected 9 hours 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 uM.

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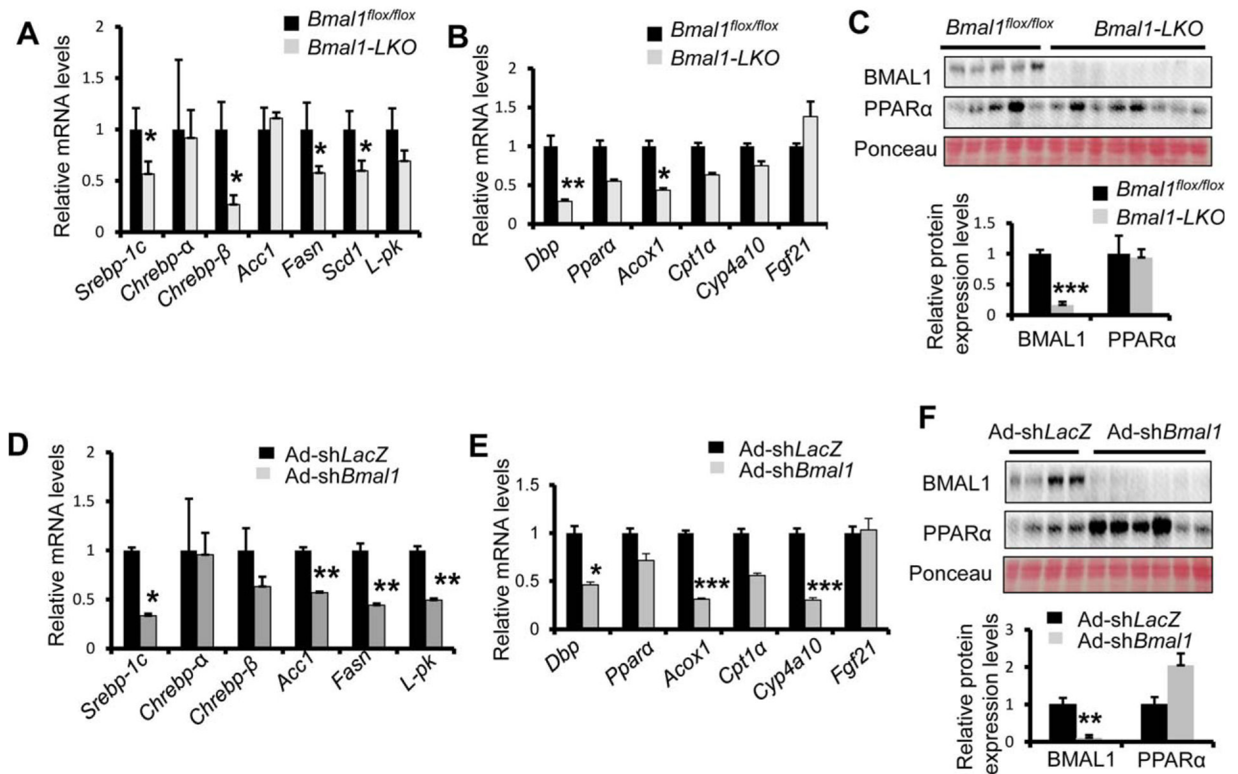

FIG. 4.

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 PPARα activity in BLKO mice liver after ethanol treatment. Mice were treated as described in Fig. 1. Relative mRNA levels of lipogenic genes (A) and PPARα target genes (B), and protein levels of PPARα (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 PPARα activity after ethanol treatment. Relative mRNA levels of lipogenic genes (D) and PPARα target genes (E), and protein levels of PPARα (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.

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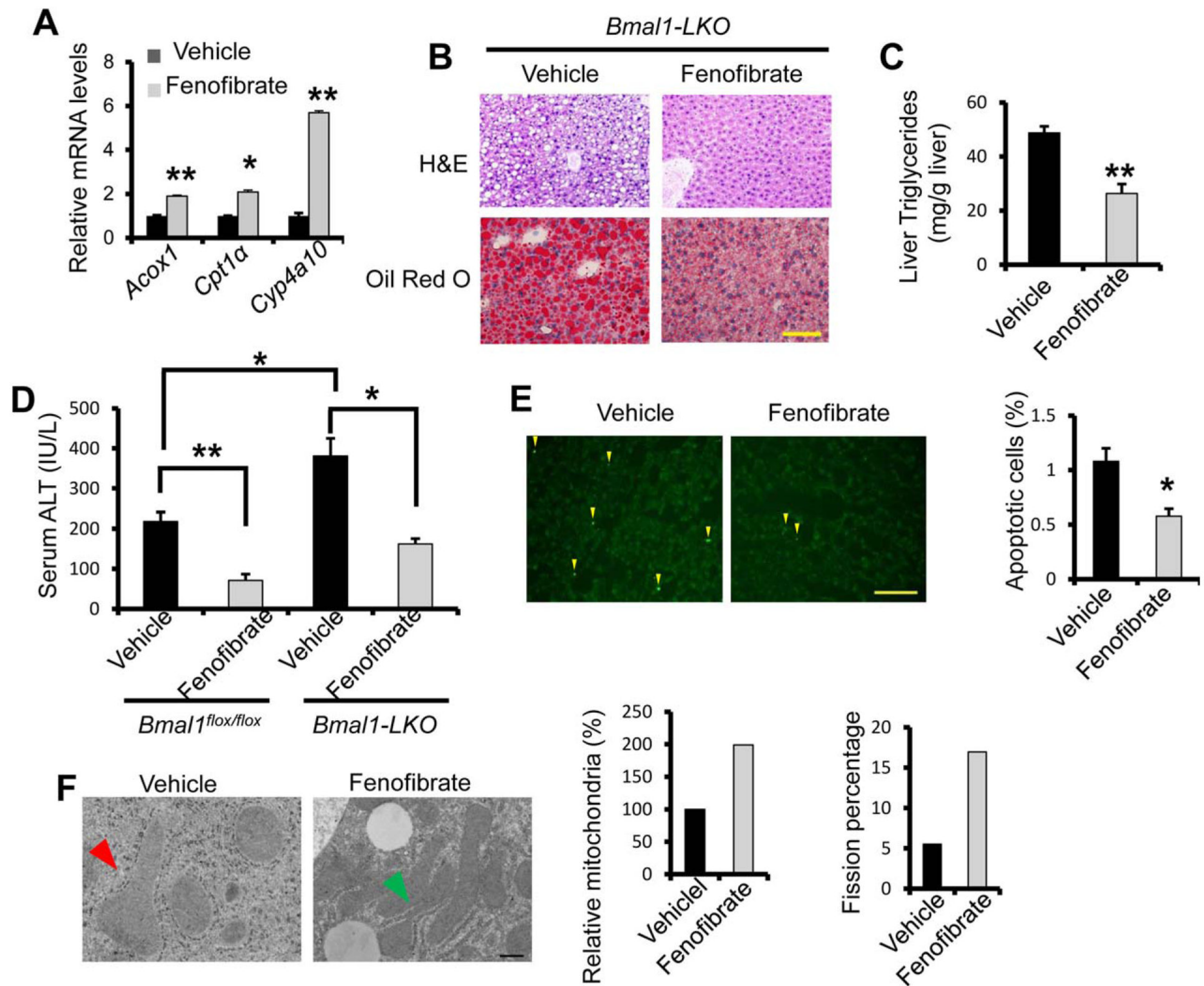

FIG. 5.

Fenofibrate administration rescues ethanol-induced liver injury in *BLKO* mice. Eight-week-old *BLKO* mice were fed 5% ethanol diet for 10 days. From the third 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 eleventh day, mice were binged with 5g ethanol/kg body weight at ZT3, and were dissected 9 hours later (n = 5 for control and n = 7 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 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 uM for B and E; Scale bar $_5$ 400 nM for F.

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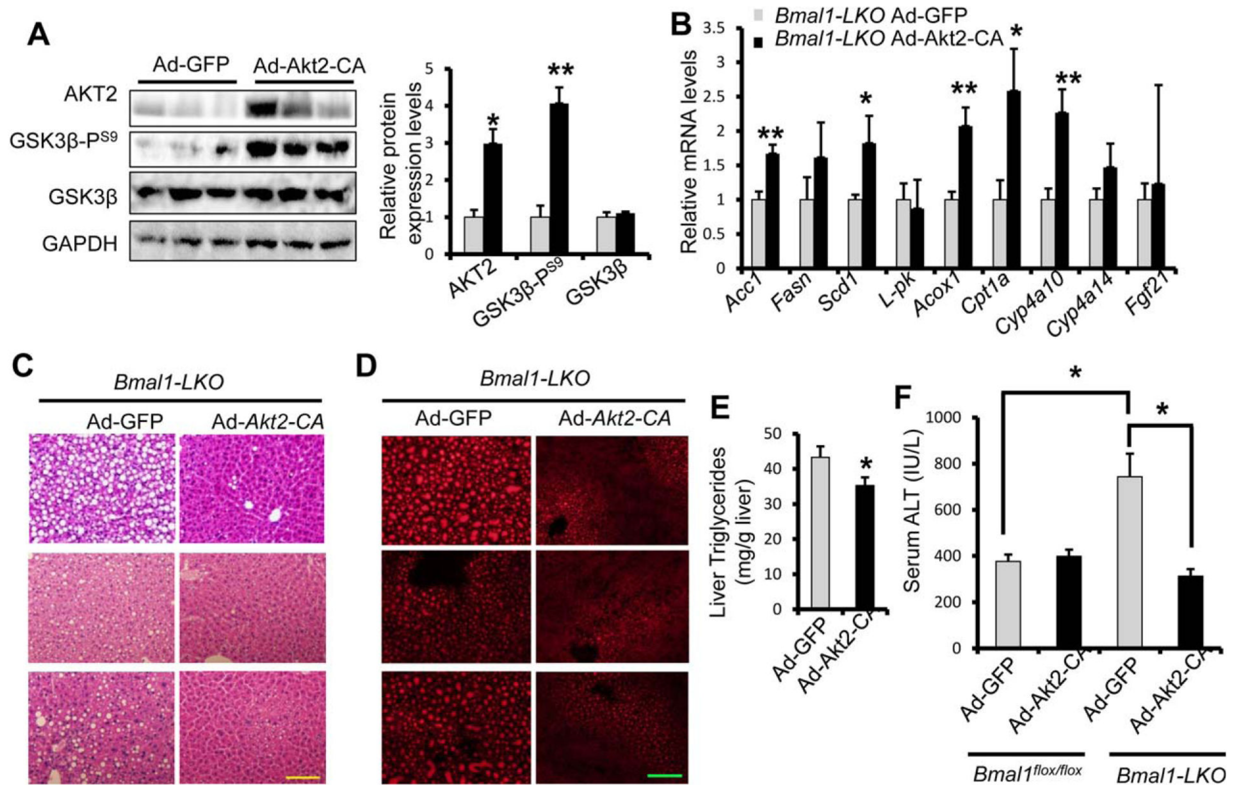

FIG. 6.

Restoring AKT activity rescues both *de novo* lipogenesis and PPARα pathway and reverses liver injury in *Bmal1-LKO* mice. Eight-week-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 eleventh day, and were dissected 9 hours later. (A) AKT2 activity was confirmed by western blotting with anti-AKT2 and anti-GSK3β 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 μM.

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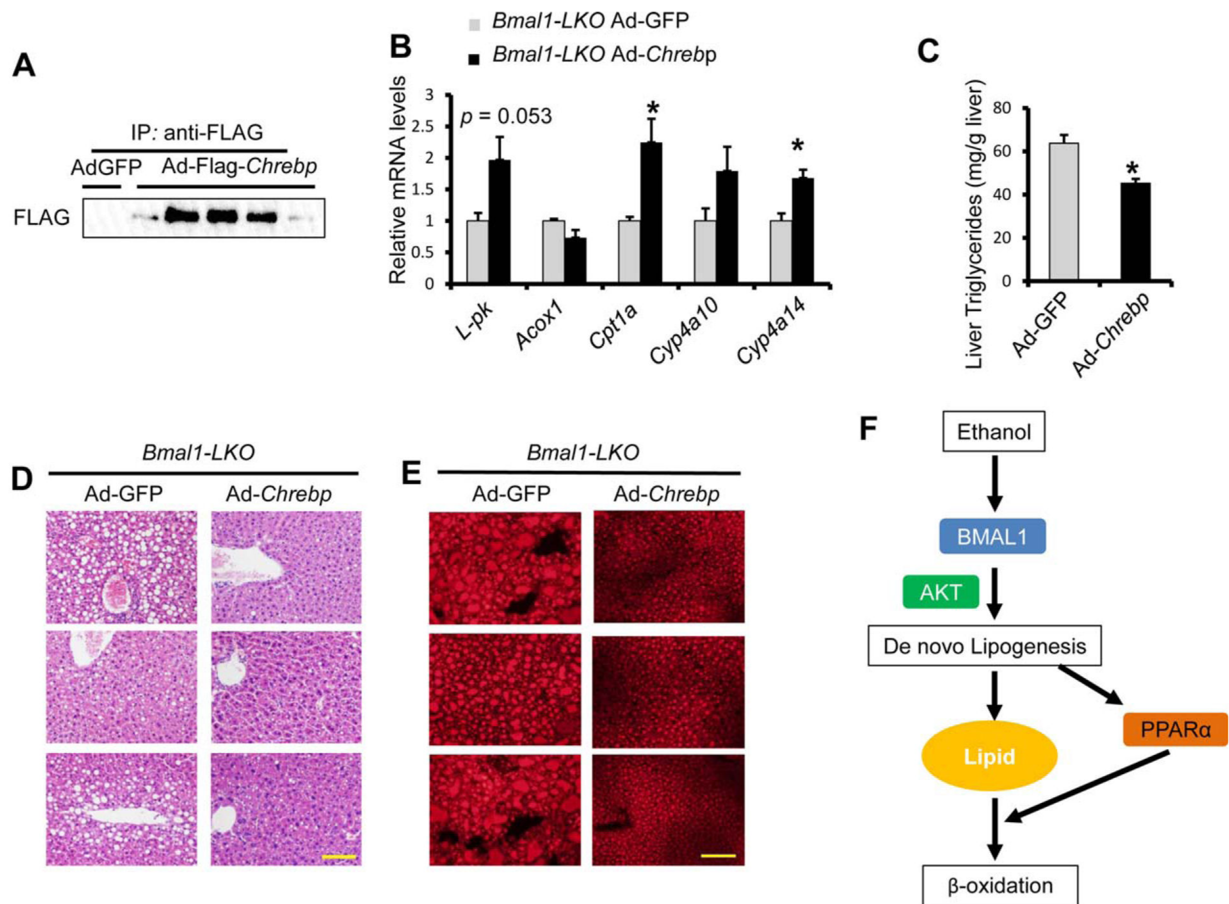

FIG. 7.

Hepatic overexpression of ChREBP restores the PPARα activity and partially ameliorates liver injury in ethanol-fed *Bmal1-LKO* mice. Eight-week-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 hours 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 PPARα 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 PPARα. *$p < 0.05$, by two-tailed Student's t test. Scale bar = 100 μM