Title

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Journal

Cancer Cell, 30(6)

ISSN

1535-6108

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Publication Date

2016-12-01

DOI

10.1016/j.ccell.2016.10.007

Peer reviewed

Published in final edited form as:

Cancer Cell. 2016 December 12; 30(6): 909–924. doi:10.1016/j.ccell.2016.10.007.

Circadian Homeostasis of Liver Metabolism Suppresses Hepatocarcinogenesis

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Summary

Chronic jet lag induces spontaneous hepatocellular carcinoma (HCC) in wild-type mice following a mechanism very similar to that observed in obese humans. The process initiates with non-alcoholic fatty liver disease (NAFLD) that progresses to steatohepatitis and fibrosis before HCC detection. This pathophysiological pathway is driven by jet lag induced genome-wide gene deregulation and global liver metabolic dysfunction, with nuclear receptor-controlled cholesterol/ bile acid and xenobiotic metabolism among the top deregulated pathways. Ablation of farnesoid X receptor (FXR) dramatically increases enterohepatic bile acid levels and jet lag-induced HCC, while loss of constitutive androstane receptor (CAR), a well-known liver tumor promoter that mediates toxic bile acid signaling, inhibits NAFLD-induced hepatocarcinogenesis. Circadian disruption activates CAR by promoting cholestasis, peripheral clock disruption, and sympathetic dysfunction.

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ACCESSION NUMBERS

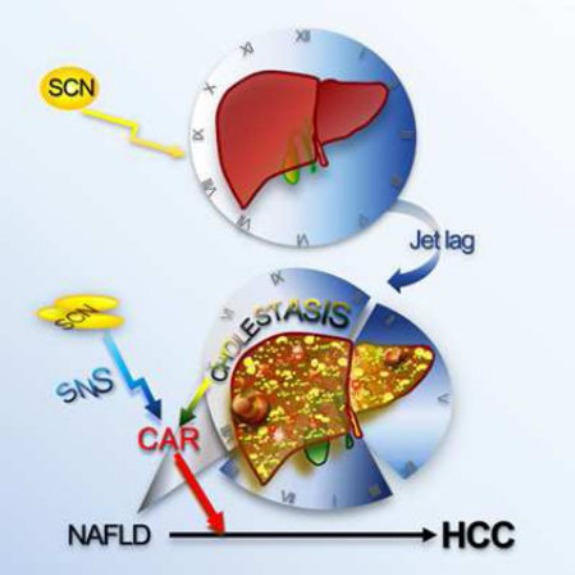
The Gene Expression Omnibus (GEO) accession number for microarray analysis in this study is GSE75475.

Author Contributions

Conceptualization, L.F. and D.D.M.; Methodology, L.F., D.D.M., N.M.K. M.J.F., A.S. and N.P; Investigation, N.M.K., L.F., N.P. and C.A.K.; Statistics, H.V., C.C., N.M.K. and L.F.; Pathology, M.J.F.; Writing-Original Draft, L.F, D.D.M. N.M.K., H.V. and N.P.; Writing–Review & Editing, L.F., D.D.M. and N.M.K.; Resources, C.L. and A.S.; Supervision, L.F.; Funding Acquisition, L.F. and D.D.M.

The authors have no conflicts of interest to declare.

Graphical abstract



Kettner et al. show that experimental chronic jet lag induces persistent deregulation of liver gene expression and metabolism, culminating in the development of hepatocellular carcinoma. The bile acid receptor FXR and xenobiotic receptor CAR play an important role in this process.

Introduction

Hepatocellular carcinoma (HCC), the most common liver malignancy, was previously considered a rare cancer in the U.S., but a nearly 3-fold increase in incidence since the 1980s has made it the fastest rising cause of cancer-related death (El-Serag, 2011). Unlike in developing countries where hepatitis viral infection and aflatoxin contamination are major HCC risk factors, up to 30–50% of HCC diagnosed in the U.S. is resulted from liver metabolic diseases. Among these diseases, non-alcoholic fatty liver disease (NAFLD) is predicted to become the leading cause of HCC in the 21st century as a secondary consequence of the obesity pandemic (Siegel and Zhu, 2009).

NAFLD is found in 30–40% of the general population and up to 95% of those with morbid obesity (Review et al., 2014). Excessive fat accumulation induces liver injury, inflammation and regeneration, which progresses to non-alcoholic steatohepatitis (NASH) in a fraction of patients. This further stimulates the formation of scar tissue (fibrosis) and cirrhosis, both of which predispose to HCC (Michelotti et al., 2013). Although severe NAFLD can lead to hepatocarcinogenesis without NASH and fibrosis (Guzman et al., 2008), NASH and advanced fibrosis increase HCC risk by 15 and 25 folds in humans, respectively (Guzman et al., 2008; Hashimoto et al., 2009). The co-existence of NASH with other metabolic disorders such as leptin resistance and cholestasis significantly accelerates liver fibrosis (Wang et al., 2010).

Like HCC caused by other risk factors, NAFLD-induced HCC is predominantly diagnosed in males, but at older ages and earlier disease stages (Hashimoto et al., 2009). However, no significant difference in overall 5-year survival is found among HCC patients with or without NAFLD-related etiologies (Tokushige et al., 2011). The lack of proper mouse models that develop NAFLD in response to chronic metabolic stress and progress to NAFLD-initiated liver injury, inflammation, fibrosis and HCC as observed in obese human subjects has significantly impaired the study on the mechanism of metabolic syndrome-induced hepatocarcinogenesis (Michelotti et al., 2013). Despite the impending burden of NAFLD-induced HCC, no oncogene addiction loops specific to this disease have been identified (Villanueva et al., 2013).

The prevalence of obesity and NAFLD in our society is coupled with the epidemic of chronic circadian disruption, also termed “social jet lag” (Roenneberg et al., 2012). Circadian dysfunction among night-shift workers and individuals suffering from sleep dyspnea has been identified as a common risk factor of obesity, metabolic disorders, NAFLD and cancer including HCC (Bass and Takahashi, 2010; Fu and Kettner, 2013; Hu et al., 2013; Kao et al., 2012; Kim et al., 2013; Zimberg et al., 2012).

Circadian homeostasis in mammals is maintained by a central clock located in the hypothalamic suprachiasmatic nucleus that constantly synchronizes to external solar light cues and controls subordinate clocks in peripheral tissues via circadian output pathways. Among these pathways, the sympathetic nervous system (SNS) targets peripheral organs via adrenergic receptor-mediated intracellular signaling (Furness, 2006). SNS dysfunction is closely associated with metabolic syndrome, oncogenic activation, neoplastic growth and tumor initiation (Lee et al., 2010; Magnon et al., 2013; Tentolouris et al., 2006). The clock is operated by circadian genes. The current model of the molecular clock is based on the cyclic expression of the bHLH-PAS transcription factors BMAL1 and CLOCK and their downstream targets Cryptochrome (*Cry1* and *Cry2*) and Period (*Per1, Per2* and *Per3*), which encode the repressors of BMAL1/CLOCK heterodimer. The molecular clock also targets clock-controlled genes (CCGs) to couple diverse functions of peripheral organs with daily physical activity (Mohawk et al., 2012). Recent studies have revealed that germline or tissue-specific ablation of core circadian genes promotes genomic instability and accelerates both tumorigenesis and cancer progression in mice (Kettner et al., 2014)

The liver is the focus of circadian regulation of metabolic pathways, especially those controlling the synthesis and metabolism of glucose, lipid, cholesterol and bile acid (Bass and Takahashi, 2010). We have previously reported that mutations in both *Per1* and *Per2* or phase-shifts of the liver clock by restricted feeding both promote uncontrolled intrahepatic bile acid accumulation (cholestasis) and disrupt xenobiotic metabolism in mice (Ma et al., 2009), and that HCC is the second most commonly observed malignancy in circadian gene-mutant mouse models (Lee et al., 2010).

Bile acids are synthesized from cholesterol in the liver and function to promote dietary fat and lipid absorption. As detergents, bile acid intrahepatic levels are tightly controlled by a classic endocrine negative feedback loop in which bile acids act as ligands for the farnesoid X receptor (FXR, NR1H4), which then indirectly suppresses Cyp7A1, the rate limiting

enzyme in bile acid biosynthesis (Goodwin et al., 2000). FXR also plays a broad role in liver function by inhibiting steatosis and inflammatory genes (Lopez-Velazquez et al., 2012). Ablation of *Nr1h4* (hereafter referred to as *Fxr*) not only promotes cholestasis but also NAFLD, NASH and HCC in mice (Kim et al., 2007; Lu et al., 2000; Wang et al., 2008; Watanabe et al., 2004; Yang et al., 2007).

Intrahepatic cholestasis can also activate the constitutive androstane receptor (CAR, NR1I3) (Guo et al., 2003; Huang et al., 2005; Ma et al., 2009; Zhang et al., 2004), a central regulator of xenobiotic metabolism. CAR activation is the basis for phenobarbital promoted non-genotoxic or mutagen-induced hepatocarcinogenesis in mice (Huang et al., 2005; Yamamoto et al., 2004), which is strongly linked to β-catenin activation or mutation (Loeppen et al., 2002), a common molecular lesion in human HCC (Zucman-Rossi et al., 2015). We have recently shown that combined pharmacologic activation of CAR and genetic activation of β-catenin in mice is sufficient to induce liver tumors that share an extensive gene expression signature with a subset of human HCC displaying β-catenin activation (Dong et al., 2015).

Based on the discoveries described above, we hypothesize that chronic circadian disruption is sufficent to induce spontaneous hepatocarcinogenesis by driving persistant liver metabilic dysfunction and oncogenic activation.

Results

Circadian dysfunction induces NAFLD prior to spontaneous hepatocarcinogenesis

We have reported recently that chronic circadian disruption is sufficient to induce Leptin resistance in mice independent of diet choice and the time or amount of food intake (Kettner et al., 2015). We further studied the role of prolonged circadian disruption in HCC risk by conducting a large scale survival study with C57BL/6J inbred WT and congenic mice lacking *Per1* and *Per*2 (*Per1*$^{-/-}$;*Per2*$^{-/-}$), *Cry1* and *Cry2* (*Cry1*$^{-/-}$;*Cry2*$^{-/-}$), or *Bmal1* in the liver (*Alb*^{cre};*Bmal1*$^{fl/fl}$), under entrained or chronic jet-lagged conditions from 4 to 90 weeks of age. Compared to control littermates maintained in steady 24 hour light/dark (24 hr LD) cycles, jet-lagged mice showed significantly reduced lifespan with disease development leading to early euthanization (Figure 1A), which include neurodegeneration, severe ulcerative dermatitis, aging, cystic renal dysplasia, and cancer (Table 1).

The major cancer types diagnosed in mutant and jet-lagged mice include pancreatic and ovarian tumors, B-lymphoma and HCC (Figures 1B and S1A), with lymphoma and HCC as the leading cause of cancer-related early euthanization. Unlike γ-irradiated mice that are especially susceptible to lymphoma (Fu et al., 2002; Lee et al., 2010), unirradiated jet-lagged WT and mutant mice displayed similar risk of lymphoma and HCC regardless of the differences in the molecular signatures of liver clock disruption in these mice, with HCC incidence specific to each mouse model by 90 weeks of age (Figures 1C, 1D and Table 1). Both sexes of mutant mice displayed increased risk of hepatocarcinogenesis, with males showed greater HCC risk than females. Under entrained condition, *Per*- or *Cry*-mutants developed fewer but larger HCCs first detected at 50 weeks of age, while *Alb*^{cre};*Bmal1*$^{fl/fl}$ mice developed a large number but small HCCs first detected after 70 weeks of age. Chronic jet lag increased both numbers and sizes of tumors in *Per* and *Cry* mutants and also the size

of tumor in HCC bearing $Alb^{cre};Bmal1^{fl/fl}$ mice (Figures 1B, S1A–B and Table 1). Thus, chronic circadian disruption not only increases tumor incidence but also accelerates tumor progression.

Despite the fact that C57BL/6J inbred WT mice are normally resistant to spontaneous and carcinogen-induced HCC (Diwan et al., 1990; Drinkwater and Ginsler, 1986), chronically jetlagged WT mice developed HCC after 78 weeks of age. This was also male dominant and at an age correspondent to the median age of spontaneous HCC diagnosis at 67–72 years in humans (Hashimoto et al., 2009; Kunstyr and Leuenberger, 1975; Yasui et al., 2011) (Figures 1B, S1A–B and Table 1).

Liver pathology in jet-lagged WT and mutant mice, including *Cry*-mutants that are deficient in fat storage in adipose (Kettner et al., 2015), initiated with spontaneous NAFLD onset at a young age. Jet lag also accelerated NAFLD progression in $Alb^{cre};Bmal1^{fl/fl}$ mice (Figures 1E–G and S1C). Together, these findings demonstrates that chronic circadian misalignment is sufficient to disrupt liver clock and induce NAFLD-related HCC in WT mice.
Since ”social jet lag”, but not circadian gene germline or tissue-specific ablation, is the major type of circadian disruption in humans (Roenneberg, 2013), we then focused on studying the mechanism of NAFLD-induced HCC using jet-lagged WT mice, with $Alb^{cre};Bmal1^{fl/fl}$ and $Per1^{-/-};Per2^{-/-}$ mice that display two distinct patterns of liver clock disruption and are easier to breed as controls to confirm the role of the clock in the pathologies observed (Figures 1C and 1D).

Circadian dysfunction induces metabolic syndrome and NAFLD to NASH and fibrosis progression

In addition to developing leptin resistance (Kettner et al., 2015), jet-lagged WT mice also displayed a decrease in serum triglycerides and free fatty acids coupled with an increase in hepatic level of triglycerides and free fatty acids. This was associated with persistent high plasma levels of glucose and insulin, characteristic of insulin resistance (Figure 2A), and a dramatic decrease in hepatic glycogen storage throughout a 24 hr period (Figures 2B and S2).

The development of metabolic syndrome in jet-lagged WT mice is coupled with persistent liver damage as indicated by elevated and deregulated plasma liver parameters, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total bilirubin (Figures 2A and 2C). This is accompanied by hepatomegaly (Figure 2D), increased bile duct proliferation and chronic liver inflammation (Figure 2E), accelerated hepatocyte proliferation and death, and the appearance of fibrosis long before HCC detection (Figure 2F). Thus, chronic circadian disruption not only induces NAFLD but also persistent liver damage and fibrosis long before spontaneous hepatocarcinogenesis. $Per1^{-/-};Per2^{-/-}$ and $Alb^{cre};Bmal1^{fl/fl}$ mice also displayed similar deregulation of serum and hepatic metabolic parameters and liver pathological changes in addition to NAFLD prior to HCC detection (Figure S3).

Circadian disruption induces global metabolic disruption in the liver

To study the mechanism of circadian disruption-induced NAFLD, we conducted a large scale circadian metabolomics study on serum and hepatic carnitines, lipids and prostaglandins, CoA's, and tricarboxylic acid cycle (TCA) metabolites in WT mice at 12 and 30 weeks of age. The majority of metabolites studied displayed robust circadian rhythms in control WT mice, but were deregulated in jet-lagged WT mice. Especially, jet-lagged WT mice displayed increased serum and hepatic levels of lactate and pyruvate, coupled with elevated levels of essential bioprecursors for amino acids, nucleotides, triglyceride and cholesterol synthesis, such as α-ketoglutarate, glycerol 3 phosphate, glutamine, ribose 5 phosphate, acetyl CoA, malate and fumarate, etc. These were associated with deregulation of fatty acid transporting carnitines and accumulation of TCA cycle intermediates, such as isocitrate, succinate, fumarate and malate, an indication of mitochondrial electron transport chain disruption and redox deregulation (Figures 3 and S4–5).

Thus, chronic jet lag induces a global shift in liver metabolism to promote lipid synthesis and storage via accelerating cytoplasmic glycolysis. This is also coupled with elevated intracellular oxidative stress that induces liver damage and increased biosynthetic intermediates that support rapid cell division (Figures 1E–F, 2A–B, S1C, S2 and S4–5), a pattern of metabolic adaptation closely reminiscent of that well-characterized for most types of cancer cells (Warburg, 1956).

Jet lag induces genome-wide gene deregulation with hepatic cholesterol, bile acid and xenobiotic metabolism as top deregulated pathways

To define the mechanism of jet lag induced global disruption of liver metabolism and pathology, we carried out a large scale microarray analysis using total liver RNA prepared from 12 and 30 week old control and jet-lagged WT mice at ZT2, 10 and 18. As expected, chronic jet lag induced persistent and genome-wide gene deregulation in mouse livers (Figure 4A and Table S1–2). This includes overexpression of genes promoting lipid, amino acids and nucleotides biosynthesis and storage, cytoplasmic glycolysis, glycogenolysis, oxidative stress, hepatocyte proliferation and death, cholestasis, and fibrosis, suppression of genes stimulating fatty acid mitochondria transportation and β-oxidation, glycogen synthesis, and tumor suppression, and deregulation of genes controlling cell cycle checkpoints, DNA damage repair, and both innate and adaptive inflammatory responses (Table S3).

Importantly, the jet lag induced gene deregulation signature significantly overlaps with that found in human HCC (Figure 4B), and also includes pathways frequently deregulated in other human cancers, such as pancreatic adenocarcinoma and bladder, small cell lung, prostate, and estrogen-dependent and hereditary breast cancers (Table S4–5) (Hoshida et al., 2008; Huang et al., 2011; Huang et al., 2012; Lee et al., 2006; Woo et al., 2008; Ye et al., 2003; Zucman-Rossi et al., 2015).

The deregulation of key genes, including circadian genes *Bmal1, Clock, Per1, Per2, Cry1* and *Nr1d1* (encoding REV-ERBα), was validated at the mRNA level by RT-PCR (Figure 4C). Together with the results of protein expression studies shown in figure1C and 1D, these

results demonstrated that chronic jet lag is sufficient to disrupt the liver clock independent of circadian gene mutations. Surprisingly, in the livers of *Alb*^{cre}*;Bmal1*$^{fl/fl}$ mice, *Per2* mRNA still displayed a shifted and dampened circadian expression profile, while *Cry1* mRNA was constitutively overexpressed. These findings were consistent with a previous report on *Cry1* mRNA hepatic overexpression in *Alb*^{cre}*;Bmal1*$^{fl/fl}$ mice (Lamia et al., 2008), and with our finding that the negative loop proteins PER2 and CRY1 still display a robust circadian expression in the livers of these mice under entrained condition (Figures 1C–D and 4C).

Dysregulation of homologs of molecular markers of human HCCs, such as *Ctnnb1* (encoding β-catenin), *Myc* and *Trp53* (Zucman-Rossi et al., 2015), was also found at the mRNA and/or protein levels in the livers of jet-lagged WT and circadian gene-mutant mice (Figures 4C–D and S6A–B). However, unlike in mouse thymus where circadian disruption is sufficient to suppress p53 (Lee et al., 2010), chronic jet lag induced a coupled activation of p53 and c-Myc in the livers of WT mice at a young age. Thus, the p53 response to *Myc* oncogenic activation was still intact in the liver at the initial stage of circadian disruption (Figure 4D). This finding agrees with previous reports that the complete loss of p53 function is associated with the progression but not initiation of hepatitis B virus or aflatoxin B1-induced HCC in humans (Hosono et al., 1993; Nose et al., 1993).

Strikingly, nuclear receptor-controlled cholesterol, bile acid and xenobiotic metabolism were among the top deregulated pathways in the livers of jet-lagged WT mice at all ages studied (Table S6). Among these nuclear receptors, the suppression of FXR and induction of CAR, as reported for human HCC (Hoshida et al., 2008; Lee et al., 2006), were found in jet-lagged WT and circadian gene mutant mice, coupled with upregulation of transcription factors stimulating cell proliferation and steatosis such as β-catenin, c-Myc, SREBP1 and PPARγ (encoded by the *Nr1c3*), as well as Cyp2B10, a direct target of CAR activation, and Cyp7A1, the rate limiting enzyme for bile acid synthesis negatively regulated by FXR (Figures 4D–F and S6A–D).

Further analysis revealed that all HCC-prone mouse models in our study displayed significantly elevated serum and hepatic bile acid levels, characteristic of intrahepatic cholestasis, regardless of their molecular mechanisms of liver clock disruption and overall metabolic phenotypes (Figures 1C–D, 4G and S6E). Thus, the coexistence of intrahepatic cholestasis with NAFLD may play a key role in spontaneous hepatocarcinogenesis.

CAR is a CCG that promotes spontaneous HCC by stimulating NAFLD to NASH transition

We and others have previously demonstrated that elevated bile acids, as observed in mice lacking circadian homeostasis, activates CAR (Guo et al., 2003; Huang et al., 2005; Ma et al., 2009; Zhang et al., 2004), which promotes hepatocarcinogenesis independent of exogenous mutagens (Huang et al., 2005; Yamamoto et al., 2004). To define the role of cholestasis and CAR in NAFLD-induced HCC, we studied HCC risk in *Fxr*$^{-/-}$ and *Nr1i3*$^{-/-}$ (here after *Car*$^{-/-}$) C57BL/6J mice. Under entrained conditions, *Fxr*$^{-/-}$ mice displayed the highest levels of intrahepatic bile acids and triglycerides throughout a 24 hr period among all mouse models studied, and also the highest risk of NAFLD and HCC, as expected. Chronic jet lag further accelerated NAFLD development and led to a greater than 2-fold increase in HCC incidence in *Fxr*$^{-/-}$ mice by 90 weeks of age (Figure 5A and Table 1). In contrast,

although *Car*$^{-/-}$ mice were also prone to NAFLD and circadian disruption-induced cholestasis and glycogen storage disease, they showed a dramatically decreased risk of jet lag induced hepatomegaly, inflammation, hepatocyte proliferation and necrosis, fibrosis, and were completely resistant to spontaneous HCC (Figures 5, S7 and Table 1).

Under entrained conditions, *Car*$^{-/-}$ mice had an intact liver clock and maintained a circadian profile of FXR and Cyp7A1 expression (Figures 6A–B). However, they lacked β-catenin nuclear activation and showed dramatically dampened c-Myc and Cyp2B10 protein expression in the liver (Figures 6B–D). CAR mRNA and protein levels also displayed a robust circadian rhythm in 24 hr LD cycles, but were arrhythmic and elevated at most times in the livers of jet-lagged WT mice, coupled with constitutive overexpression of *Cyp2b10* mRNA, a direct consequence of CAR-directed transcriptional activation (Figure 6E). Chronic jet lag disrupted the liver clock in both *Car*$^{-/-}$ and WT mice but failed to fully activate oncogenic, steatotic and inflammatory genes in *Car*$^{-/-}$ mice as compared to jet-lagged WT controls. This, again, was coupled with the lack of direct CAR target *Cyp2b10* mRNA expression in jet-lagged *Car* mutants, which confirmed the ability of jet lag to activate CAR (Figures 6A and 6E). Thus, *Car* is a CCG. Its circadian dysregulation stimulates the pathophysiological progression from NAFLD to HCC.

Sympathetic dysfunction promotes CAR constitutive overexpression

Previous studies have suggested that BMAL1/CLOCK and the PAR-domain basic leucine zipper transcription factor DBP may co-activate *Car* over a 24 hr period in the liver (Gachon et al., 2006; Koike et al., 2012; Ripperger and Schibler, 2006). However, *Bmal1* and *Dbp* were both suppressed by circadian disruption (Figures 1C–D and 4C). Thus, jet lag induced CAR overexpression in WT mice appeared to be controlled by a previously unknown mechanism. We found that apart from multiple D- and E-boxes potentially recognized by DBP and BMAL1/CLOCK, human and mouse *CAR* promoters contain conserved AP1 and CRE motifs potentially activated by SNS signaling (Figures 7A). In vitro transfection assays showed that the *Car* promoter was moderately stimulated by low levels but suppressed by higher levels of AP1 or CREB expression. However, co-expression of AP1 and CREB, even at a moderate level, dramatically stimulated *Car* promoter activity (Figures 7B). We also found that chronic jet lag significantly increased SNS tone in the sleep phase. This was coupled with a constitutive AP1 and Ser133 phospho-CREB (pCREB) nuclear accumulation in mouse livers, which is a well-known consequence of cellular response to SNS-ADRβ-c-AMP-PKA signaling (Figures 7C–E). Ablation of all three β-adrenergic receptors in mice (β-less) completely inhibited AP1 and pCREB nuclear accumulation and also *Car, Fos, Myc* and *Cyp2b10* expression in mouse livers under both entrained and jet lag conditions (Figures 7F–G), which was associated with strong HCC resistance (Figure S8A). Thus, jet lag induced SNS dysfunction may directly deregulate *Car* expression via activation of AP1 and CREB.

To test this hypothesis, we performed in vivo ChIP using anti-BMAL1, c-FOS and pCREB antibodies (Figures S8B–D), primers flanking the AP1, CRE, or E-box 8 sequences in the *Car* promoter, E-box 3, AP1 or CRE motifs in the *Per1* promoter, and liver nuclear extracts from 12 week old WT, *Alb*^{cre}*;Bmal1*$^{fl/fl}$ and β-less mice at ZT2, 6 and 18. In the livers of

WT mice, BMAL1/CLOCK transcriptional activity is low at ZT2 and 18 but peaks at ZT6 (Koike et al., 2012), while nuclear accumulation of AP1 and pCREB is lowest at ZT6, slightly elevated at ZT2 and peaks ZT18 (Figures 7D–E). Thus, ZT2, 6 and 18 are the best times in a circadian cycle to study the interplay between SNS-controlled cell signaling and the liver clock in controlling *Car* expression.

We found that under entrained conditions, pCREB and BMAL1 displayed a coupled and robust rhythmic interaction with both *Car* and *Per1* promoters, which peaked at ZT2 for pCREB and ZT6 for BMAL1. Although c-FOS binding to the *Per1* promoter displayed a strong circadian rhythm and peaked at ZT2, its binding to *Car* promoter was constitutively low. Chronic jet lag abolished BMAL1 binding to both *Car* and *Per1* promoters, significantly dampened c-FOS and pCREB interaction with the *Per1* promoter, but induced a coupled and dramatically increased binding of c-FOS and pCREB to the *Car* promoter at all times studied (Figure 7H), which, as shown by promoter functional analysis, strongly activated *Car* transcription (Figure 7B). As expected, the binding of BMAL1 or c-FOS and pCREB to *Per1* and *Car* promoters was not detected in livers of $Alb^{cre};Bmal1^{fl/fl}$ or β-*less* mice, respectively (Figure 7H). Thus, jet lag induced SNS dysfunction and liver clock disruption are sufficient to promote *Car* dysregulation.

Discussion

Lifestyle changes, but not germline mutations, drive increased cancer risk in modern societies (Anand et al., 2008). One of the major lifestyle changes is chronic social jet lag (Roenneberg, 2013). Human epidemiological studies have revealed a strong link between chronic circadian disruption and cancer risk. However, the diversity of lifestyles, heterogeneity of cancer etiologies and long latency of spontaneous cancer development impose significant difficulties for defining the role of circadian dysfunction in human cancer initiation. Although mouse models with germline or tissue-specific ablation of circadian genes display increased risk of genomic instability, neoplastic growth and cancer (Kettner et al., 2014), the role of circadian dysfunction in spontaneous cancer initiation in humans cannot be closely modeled by these mutant mice as they do not display the same circadian dynamics as normal human individuals in response to changes in environmental cues (Kettner et al., 2015).

We have previously reported that chronic jet lag significantly increases the risk of γ-radiation induced cancer in mice (Lee et al., 2010). In this study, we show that chronic circadian misalignment is sufficient to induce spontaneous hepatocarcinogenesis in mice in the absence of dietary manipulation, exogenous genotoxic stress or germline gene mutations. We found that in addition to leptin resistance, circadian dysfunction also induces hyperinsulinemia, hyperglycemia and dyslipidemia at the organismal level and liver metabolic disorders including NAFLD. Such combined systemic and liver metabolic disorders are also frequently observed among human night-shift workers (Zimberg et al., 2012). We show that jet lag induces a global shift in liver metabolism in mice, which not only promotes fat synthesis and storage via accelerated cytoplasmic glycolysis but also increases oxidative stress and the synthesis of bioprecursors supporting rapid cell division. The majority of jet-lagged WT mice display NAFLD progression to NASH and then fibrosis

at a relatively young age. This is likely due to jet lag induced persistent liver injury and inflammation that triggers a chronic regenerative wound-healing process, a common mechanism of tumor initiation (Michelotti et al., 2013). Overall, this pathophysiological progression closely mimics that described for NAFLD-induced spontaneous hepatocarcinogenesis in humans.

The complex liver pathology in jet-lagged WT mice is driven by global hepatic gene deregulation, which can be detected soon after the initiation of jet lag and displays a pattern overlapping significantly with a human HCC transcriptomic signature, including the induction of key human HCC molecular markers such as *Trp53, Myc* and β-catenin. Thus, jet-lagged WT mice likely also develop spontaneous HCC following a molecular mechanism very similar to that in obese humans.

We found that nuclear receptor-controlled cholesterol, bile acid and xenobiotic metabolism are among top deregulated hepatic pathways in jet-lagged WT mice, and all mouse models studied here displayed cholestasis in addition to NAFLD prior to HCC detection. Especially, HCC prone $Fxr^{-/-}$ mice that display extremely high intrahepatic bile acid levels showed an additional more than 2-fold increase in HCC incidence under jet lag condition. Thus, we suggest that loss of bile acid homeostasis is a direct cause of increased HCC risk in response to persistent circadian disruption.

Two broad mechanisms could link cholestasis to hepatocarcinogenesis. First, as strong hydrophobic detergents, bile acids can induce hepatocyte injury, mitochondrial damage, oxidative stress, DNA damage, and hepatocyte necrosis and apoptosis, all of which drive the progression of NAFLD to NASH, fibrosis and HCC (Audard et al., 2007; Perez and Briz, 2009). Second, and more directly, elevated hepatic bile acid levels, together with an increase in hepatic toxic bile acid species due to microbiota activity associated with circadian disruption (Voigt et al., 2014), activate CAR, which is well known as a potent tumor promoter that drives non-genotoxic hepatocarcinogenesis (Guo et al., 2003; Huang et al., 2005; Yamamoto et al., 2004; Zhang et al., 2004).

The tumor promoting function of CAR is tightly associated with β-catenin activation or mutation (Dong et al., 2015; Loeppen et al., 2002). Jet lag activated CAR, as measured by constitutive overexpression of the CAR target gene *Cyp2b10* over a 24 hr period, was coupled with β-catenin and c-Myc overexpression. Ablation of *Car* completely inhibited nuclear β-catenin expression and suppressed jet lag induced activation of oncogenic, steatotic and inflammatory genes, leading to strong HCC resistance.

In the hierarchical circadian system, peripheral organs rely on signaling from circadian output pathways to maintain synchrony with the central clock. We found that chronic jet lag promotes sympathetic dysfunction and peripheral clock disruption, both of which have been linked to oncogenic activation and increased risk of cancer (Kettner NM, 2016; Lee et al., 2010; Magnon et al., 2013). We demonstrated that jet lag induced sympathetic dysfunction and peripheral clock suppression are sufficient to promote AP1 and CREB oncogenic activation independent of somatic mutations, which drives *Car* overexpression throughout a 24 hr period to mediate persistent toxic bile acid signaling in jet-lagged WT mice.

We propose that chronic circadian disruption induces neuroendocrine dysfunction, leading to a coupled peripheral clock disruption, genome-wide gene deregulation, oncogenic activation and global metabolic dysfunction in the liver. Together, these induce NAFLD and also the progression from NAFLD to HCC. Among top deregulated gene pathways, the nuclear receptor CAR is constitutively activated by a combination of overexpression, in response to SNS dysfunction and peripheral clock disruption, and transactivation, in response to elevated intrahepatic bile acids, to promote spontaneous hepatocarcinogenesis by stimulating the progression from NALFD to NASH and fibrosis (Figure 7I). Inverse agonists to suppress human CAR and agonists for activation of human FXR are both pharmaceutically available (Hirschfield et al., 2015; Huang et al., 2004). Since ablation of CAR prevents spontaneous HCC and loss of FXR increases intrahepatic bile acid levels and stimulates hepatocarcinogenesis, our studies suggest that restoration of bile acid homeostasis and inhibition of CAR activation provide promising complementary strategies for prevention of metabolic syndrome-induced HCC.

Experimental Procedures

Animal Maintenance

All animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (BCM). Detailed experimental procedures for mouse survival, pathological studies and tissue isolation are described in Supplemental Experimental Procedures.

ELISA

Mouse serum, urine and livers were collected and analyzed by ELISA as described in Supplemental Experimental Procedures.

RNA Analysis

Total RNA was isolated from mouse livers and used for RT-PCR. Detailed procedures and RT-PCR primers are described in Supplemental Experimental Procedures.

Plasmid Construction and Reporter Assays

A 3-Kb mouse *Car* promoter was cloned by PCR amplification of C57BL/6J WT mouse genomic DNA, inserted upstream of the luciferase cDNA in the pGL-3 vector, and used for cotransfection assays. Detailed procedures are described in Supplemental Experimental Procedures.

Chromatin immunoprecipitation (ChIP)

Nuclear extracts were prepared from mouse livers at ZT2, 6 and 18. Antibodies against BMAL1, c-FOS or pCREB were used for immunoprecipitation. qPCRs were performed to detect BMAL1, c-FOS and pCREB ChIP signals. Detailed procedures, antibodies and qPCR primers are described in Supplemental Experimental Procedures.

Protein Expression Studies

Western blotting using total liver or nuclear extracts were performed as described in Supplemental Experimental Procedures.

Metabolomics

Targeted measurement of metabolites was carried out by the BCM Dan L Duncan Cancer Center CPRIT Cancer Proteomic and Metabolomic Core using serum and liver samples collected from 12 or 30 week-old WT mice at ZT2, 10 and 18 as described in Supplemental Experimental Procedures.

Microarray

Total liver RNA isolated from 12 and 30 week old WT mice was used for Agilent gene expression microarray at the BCM Genomic and RNA Profiling Core. Detailed procedures are described in Supplemental Experimental Procedures.

Histology and Immunohistochemistry (IHC)

Mouse liver and tumor samples were processed for histological or IHC staining at the Texas Medical Center (TMC) Digestive Disease Center following standard procedures as described in Supplemental Experimental Procedures.

Statistical Analysis

The methods for statistically analyzing data obtained from all experiments performed are described in Supplemental Experimental Procedures. In all analyses, $p < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank D Bolinger, S Khan and P Patel for technical assistance, Drs. LD White for assisting microarray analysis and B Lowell for providing β-*less* mice. This work is funded by grants from NIH/NCI (R01 CA137019-01A) and USDA/ARS (6250-51000-055) to L.F., and CRPIT (MIRA RP150587) to D.D.M and L.F., and partially by NIH/NIDDK (R01 DK46546) to D.D.M., NIH/NIDDK (5T32 DK007696-20) to N.M.K., R01CA137019-05S to C.A.K., the NIH/NCI (P30CA125123) to BCM Genomic and RNA Profiling Core, the Public Health Service Grant DK56338 to TMC Digestive Diseases Center, the CPRIT Core Facility Support Award (RP120092), the NCI Shared Resources funds (2P30CA125123-09) and funds from the BCM Dan L. Duncan Cancer Center to the BCM Metabolomics Core, NSF DMS-1161759 and funds from Alkek Center for Molecular Discovery and the BCM Agilent Technologies Center of Excellence to A.S. and N.P.

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Significance

Non-alcoholic fatty liver disease (NAFLD) is predicted to become the leading cause of hepatocellular carcinoma (HCC) due to the prevalence of obesity and chronic circadian disruption, but the underlying mechanisms are poorly understood. We found that circadian dysfunction promotes NAFLD-induced hepatocarcinogenesis by maintaining persistent liver gene deregulation and metabolic disruption, closely mimicking that observed in obese humans. We demonstrate that the profound circadian dysregulation of nuclear receptor-controlled hepatoprotective pathways, especially those controlled by the bile acid receptor FXR and xenobiotic receptor CAR, plays an essential role in NAFLD-induced HCC. Thus, circadian dysfunction is an independent risk factor of HCC, and restoration of bile acid homeostasis and inhibition of CAR are promising complementary strategies for prevention of metabolic syndrome-induced HCC.

Highlights

- Chronic circadian disruption induces NAFLD and spontaneous hepatocarcinogenesis
- Circadian dysfunction promotes global gene deregulation and metabolic disruption
- The nuclear receptor CAR drives NAFLD to NASH, fibrosis and HCC progression
- Circadian disruption activates CAR via sympathetic dysfunction and Cholestasis

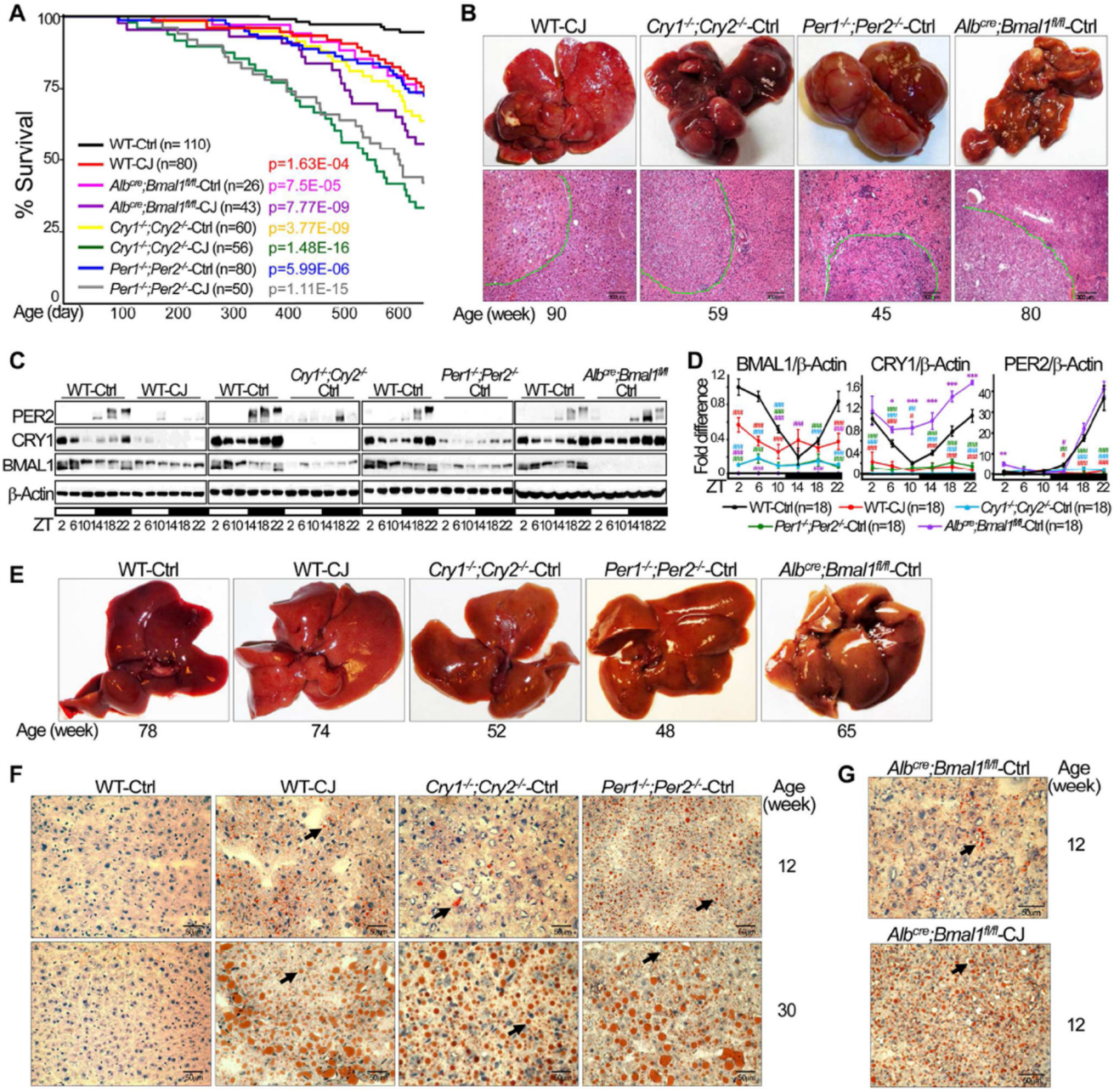

Figure 1. Chronic jet lag induces NAFLD and spontaneous HCC

(A) Kaplan-Meier survival curves show life-spans of WT and circadian gene mutant mice. Ctrl: maintained in steady 24 hr LD cycles; CJ: chronically jet-lagged. p value: Ctrl vs. CJ for each mouse model, Kaplan Meier Statistics. (B) Spontaneous HCC from circadian gene mutant and jet-lagged WT mice. Top: gross image of HCC; bottom: Hematoxylin and eosin (H&E) stained tumor slides show histological diagnosis of HCC, circled by dashed green lines. Scale bars indicate 300 μm. (C) Western blots show BMAL1, CRY1 and PER2 expression in the livers of 12 week old jet-lagged WT and circadian gene mutant mice. ZT: Zeitgeber time, with light on at ZT0 and off at ZT12. (D) Summary of 3 independent Western blotting analyses on hepatic expression of BMAL1, CRY1 and PER2 (higher

protein band only for PER2) with the expression level in control WT mice at ZT2 as the arbitrary unit 1 (student *t* test, ±SEM). *: Increase,$^{\#}$: decrease, comparing to WT controls at the same time, */$^{\#}$p<0.05, **/$^{\#\#}$p<0.01; ***/$^{\#\#\#}$p<0.001. (E, F) Gross liver image (E) and Oil Red O stained slides (F) showing NAFLD development in mutant and jet-lagged WT mice. (G) Representative Oil Red O stained slides show the level of fat accumulation in control (top) and jet-lagged (bottom) *Alb^{cre};Bmal1$^{fl/fl}$* mice at 12 weeks of age. Scale bars in all Oil Red O stained slides indicate 50 μm. Arrows indicate representative fat droplets. See also figure S1.

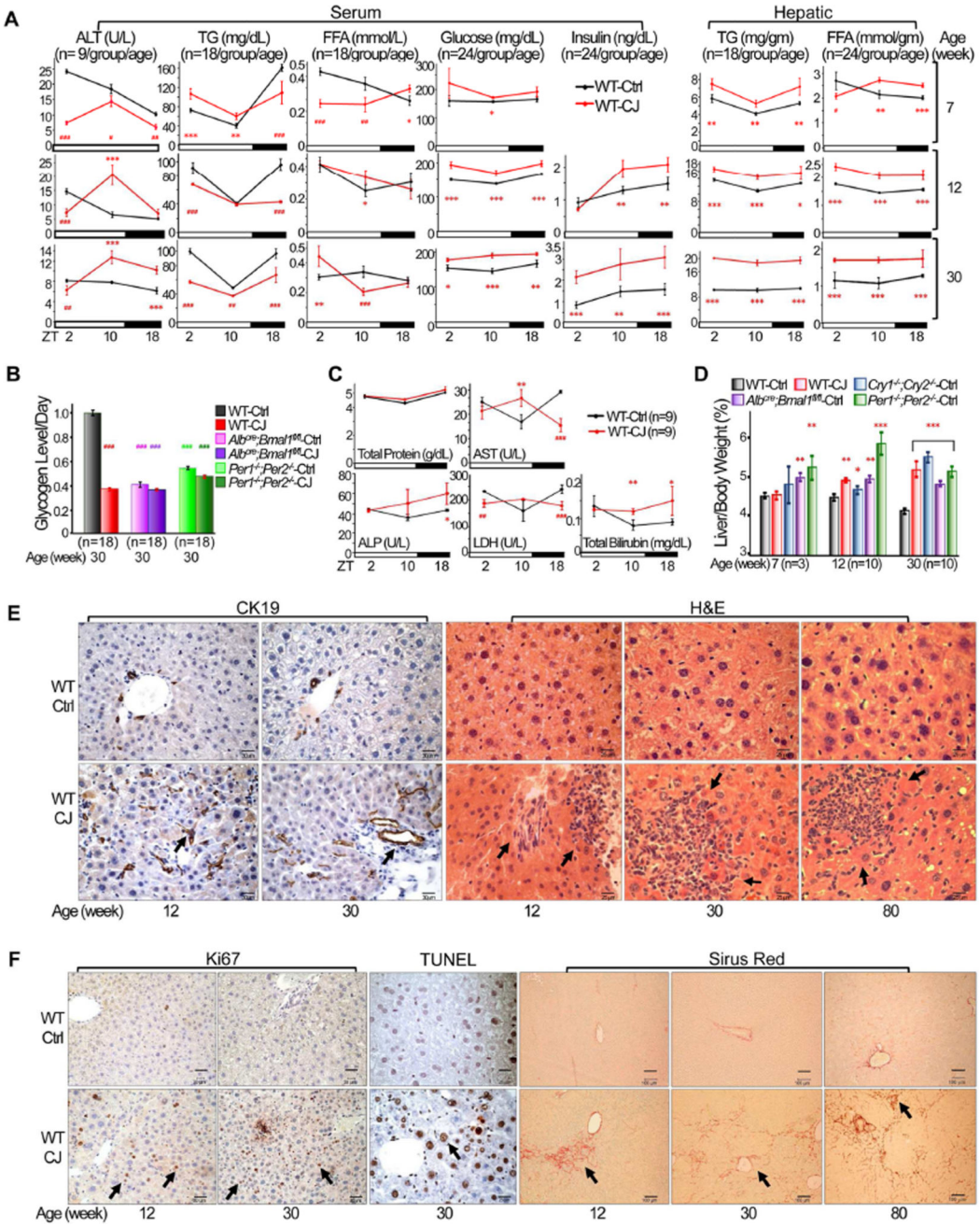

Figure 2. Chronic jet lag induces metabolic syndrome and the progression from NAFLD to NASH and fibrosis

(A) The levels of serum and hepatic biomarkers in control and jet-lagged WT mice. ALT: alanine aminotransferase; TG: triglycerides; FFA: free fatty acids (Student *t* test). (B) Daily hepatic glycogen storage detected by Periodic Acid Schiff (PAS) staining in control and jetlagged WT mice. Values indicate average levels of glycogen storage detected at ZT2, 10 and 18 for each mouse model, with that in control WT mice as the arbitrary unit 1 (Image J/ Color Deconvolution plugin quantification). (C) Serum liver biomarkers in 12 week old jet-lagged WT mice. AST: aspartate transaminase, ALP: alkaline phosphatase, LDH: lactate dehydrogenase (Student *t* test). (D) The ratios of liver vs body weight of circadian gene

mutant and WT mice (Student *t* test). (E) Cytokeratin 19 (CK19) and H&E staining show incidence of intrahepatic bile duct proliferation and inflammation in control and jet-lagged WT mice. Arrows indicate representative proliferating bile ducts (CK19) and areas of hematopoietic cell infiltration (H&E). (F) Ki67, TUNEL and Sirus Red staining show incidence of hepatocyte proliferation and death, and liver fibrosis in control and jet-lagged WT mice. Arrows indicate representative $Ki67^+$ or $TUNEL^+$ hepatocytes (Ki67 or TUNEL), and liver fibrosis (Sirus Red). Scale bars are 60 μm (CK19), 50 μm (HE), 60 μm (Ki67), 50 μm (TUNEL) and 100 μm (Sirus Red) in correspondent slides. *: Increase,$^{\#}$: decrease, comparing to WT controls at the same time or age $*/^{\#}p<0.05$, $**/^{\#\#}p<0.01$; $***/^{\#\#\#}p<0.001$, ±SEM. See also figures S2 and S3.

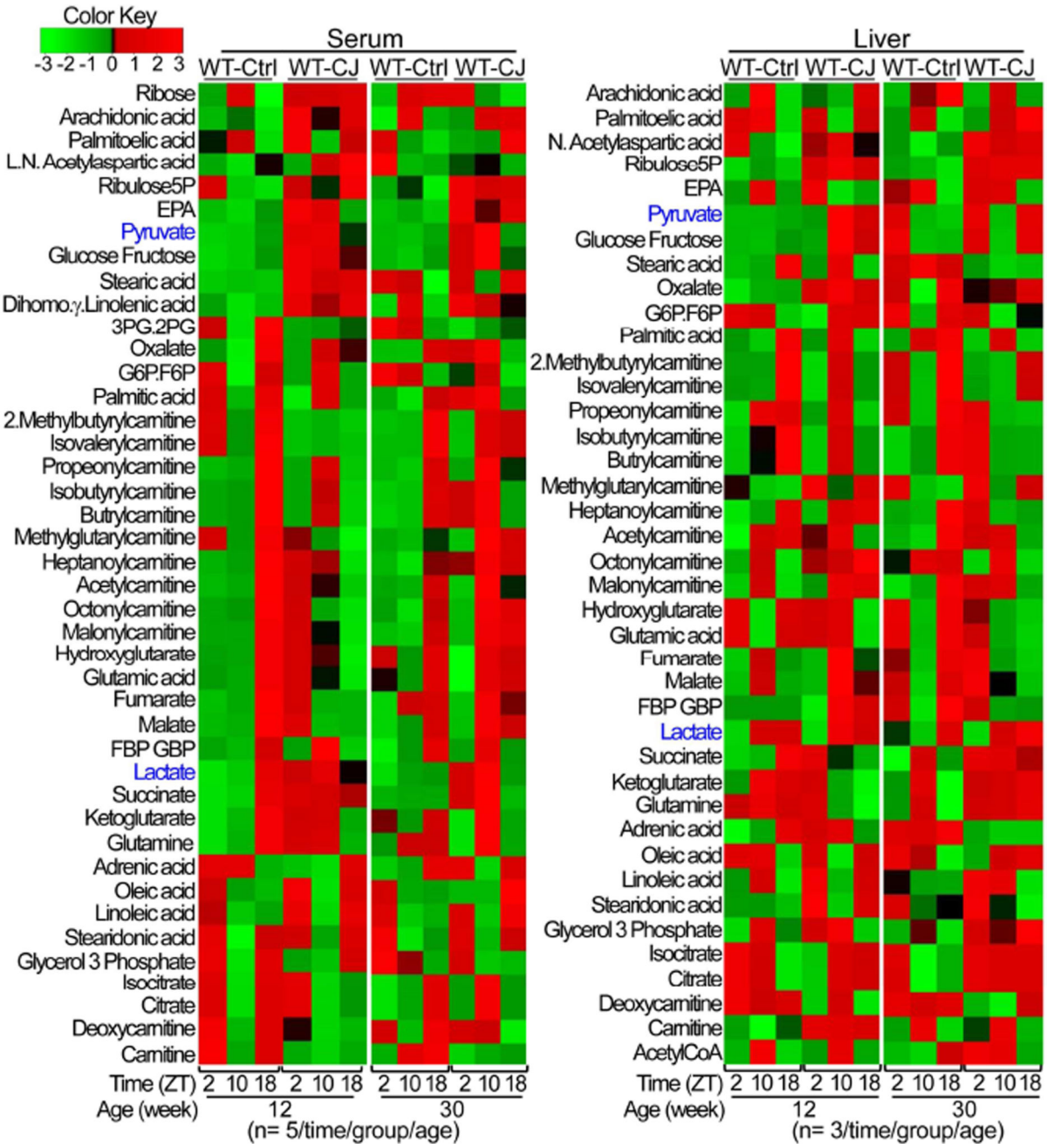

Figure 3. Global disruption of liver metabolism in jet-lagged WT mice

Hierarchical clustering heat maps show serum (left panel) and hepatic (right panel) carnitines, lipids and prostaglandins, CoA's and TCA metabolites in control WT mice and the persistent deregulation of these metabolites in jet-lagged WT mice. See also figures S4 and S5.

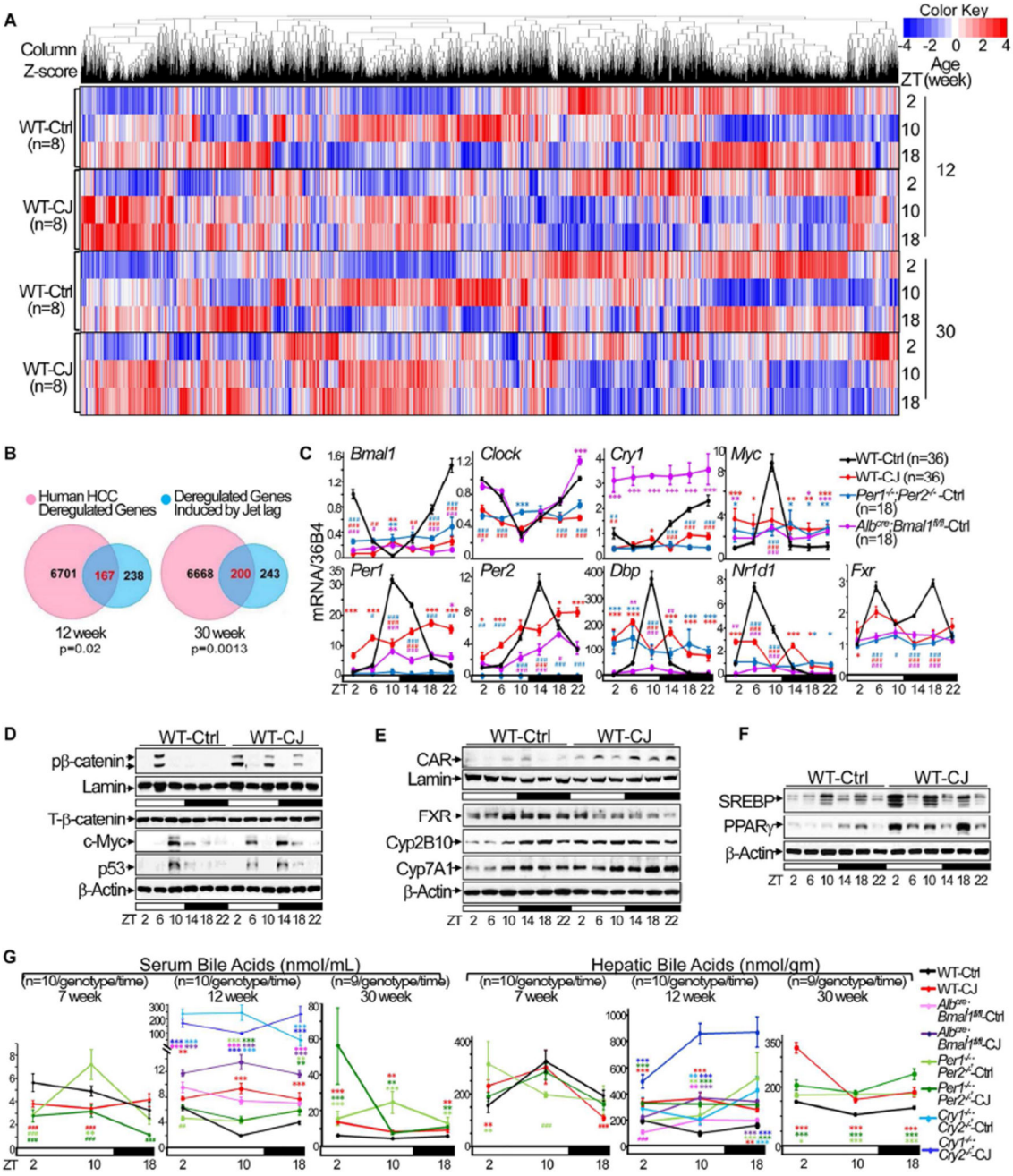

Figure 4. Chronic jet lag induces genome-wide gene deregulation in mouse livers

(A) Hierarchical clustering heat map shows expression of hepatic genes in the livers of control and jet-lagged WT mice. (B) Venn diagrams showing persistent overlap of deregulated liver transcriptomic signatures in jet-lagged WT mice with that of human HCC from 12 to 30 weeks of age (hypergeometric test). (C) The summary of 3 independent RT-PCR studies on the expression of core circadian genes, *Myc* and *Fxr* in the livers of 12 week-old circadian gene mutant and WT mice, with levels detected in control WT mice at ZT2 as the arbitrary unit 1. (D–F) Western blots show the expression of Ser552 phospho-β-catenin (pβ-catenin), total β-catenin (T-β-catenin), c-Myc and p53 in (D), FXR, CAR,

Cyp2B10 and Cyp7A1 in (E), and SREBP1 and PPARγ in (F) in the livers of 12 week old WT mice. (G) Serum and hepatic bile acid levels over a 24 hr period in circadian gene-mutant and WT mice at 7, 12 and 30 weeks of age. *: Increase,$^{\#}$: decrease, comparing to WT controls at the same time, */$^{\#}$p<0.05, **/$^{\#\#}$p<0.01; ***/$^{\#\#\#}$p<0.001, student *t* test, ±SEM. See also figure S6 and Table S1–6.

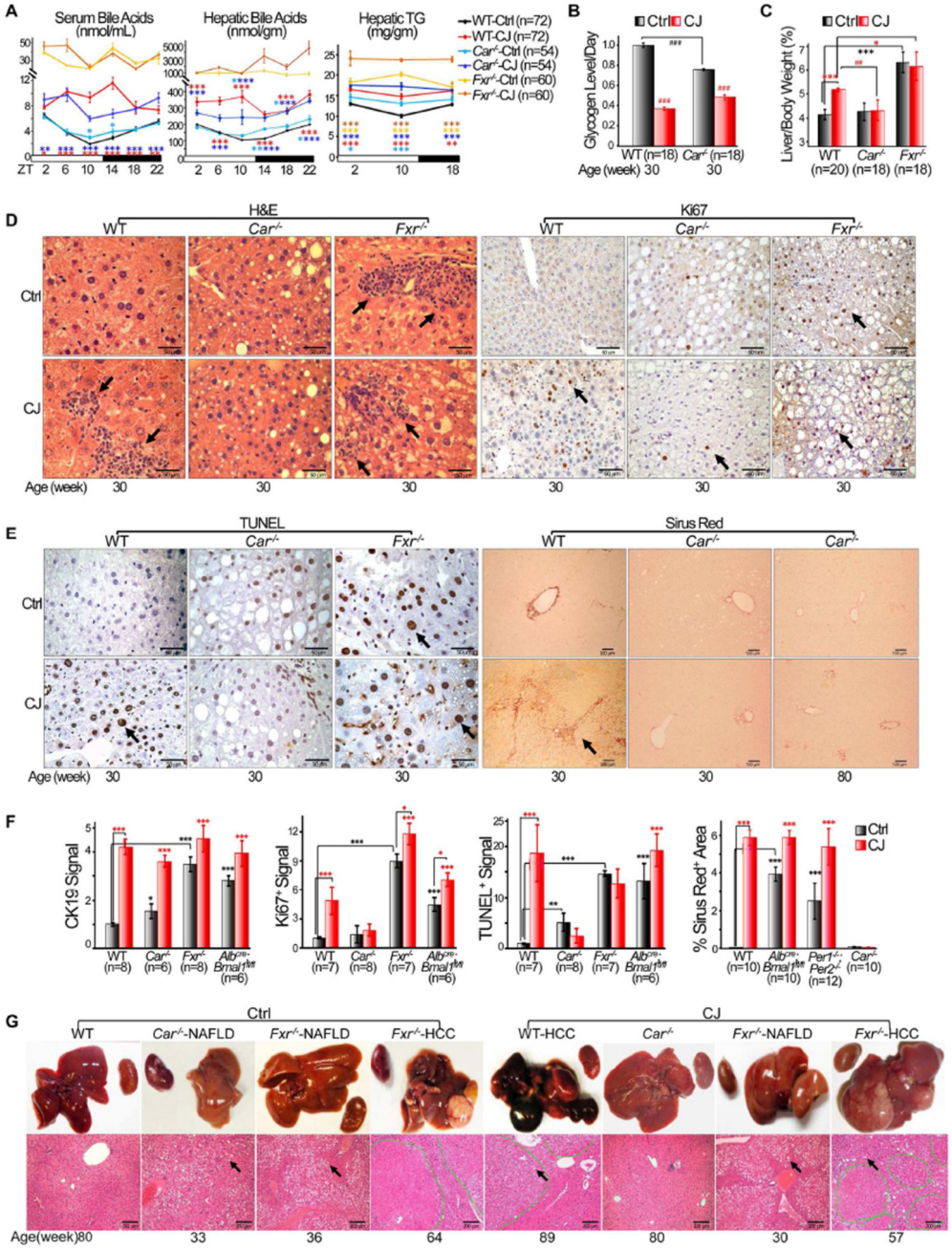

Figure 5. CAR stimulates NAFLD to NASH and fibrosis progression

(A) Circadian profiles of serum and hepatic bile acids and triglyceride (TG) in 12 week old control and jet-lagged WT, *Car*$^{-/-}$ and *Fxr*$^{-/-}$ mice (Student *t* test). (B) Daily hepatic glycogen storage in control and jet-lagged WT and *Car*$^{-/-}$ mice detected by PAS staining. Values indicate average levels of hepatic glycogen storage detected at ZT2, 10 and 18 for each mouse model, with that in control WT mice as the arbitrary unit 1 (Image J/Color Deconvolution plugin quantification). (C) The ratios of liver vs. body weight of WT, *Car*$^{-/-}$ and *Fxr*$^{-/-}$ mice at 30 weeks of age (Student *t* test). (D) H&E and Ki67 staining detect liver inflammation and hepatocyte proliferation in control and jet-lagged WT, *Car*$^{-/-}$, *Fxr*$^{-/-}$

mice. Arrows indicate representative areas of hematopoietic cell infiltration in H&E and Ki67^{+} hepatocytes in Ki67 slides. Scale bars indicate 50 and 60 μm in the H&E and Ki67 slides, respectively. (E) TUNEL and Sirus Red staining show incidence of hepatocyte death (TUNEL) and fibrosis (Sirus Red) in the livers of WT, *Car*$^{-/-}$, *Fxr*$^{-/-}$ mice. Arrows indicate representative TUNEL^{+} hepatocytes (TUNEL) and areas of liver fibrosis (Sirus Red). Scale bars indicate 50 and 100 μm in the TUNEL and Sirus Red slides, respectively. (F) The level of bile duct and hepatocyte proliferation (Student *t* test), hepatocyte necrosis (Student *t* test), and liver fibrosis (Image J) in 30 week old control and jetlagged WT and mutant mice. All analyzed at 10× magnification with CK19, Ki67 and TUNEL signals detected in the livers of control WT mice as the arbitrary unit 1. (G) Gross image (top) and histological diagnosis of HCC by H&E staining (bottom) show NAFLD in jet-lagged WT, *Car*$^{-/-}$ and *Fxr*$^{-/-}$ mice and HCCs found in jet-lagged WT and *Fxr*$^{-/-}$ mice. Kidneys that do not show significant changes in size among different mouse models are included in gross images. Tumors in H&E slides are circled by dashed green lines. Arrows indicate representative fat droplets in H&E slides. Scale bars indicate 200 μm. *: Increase,$^{\#}$: decrease, comparing to WT control samples at the same age or time, $^{*/\#}p<0.05$, $^{**/\#\#}p<0.01$; $^{***/\#\#}p<0.001$, ±SEM. See also figure S7.

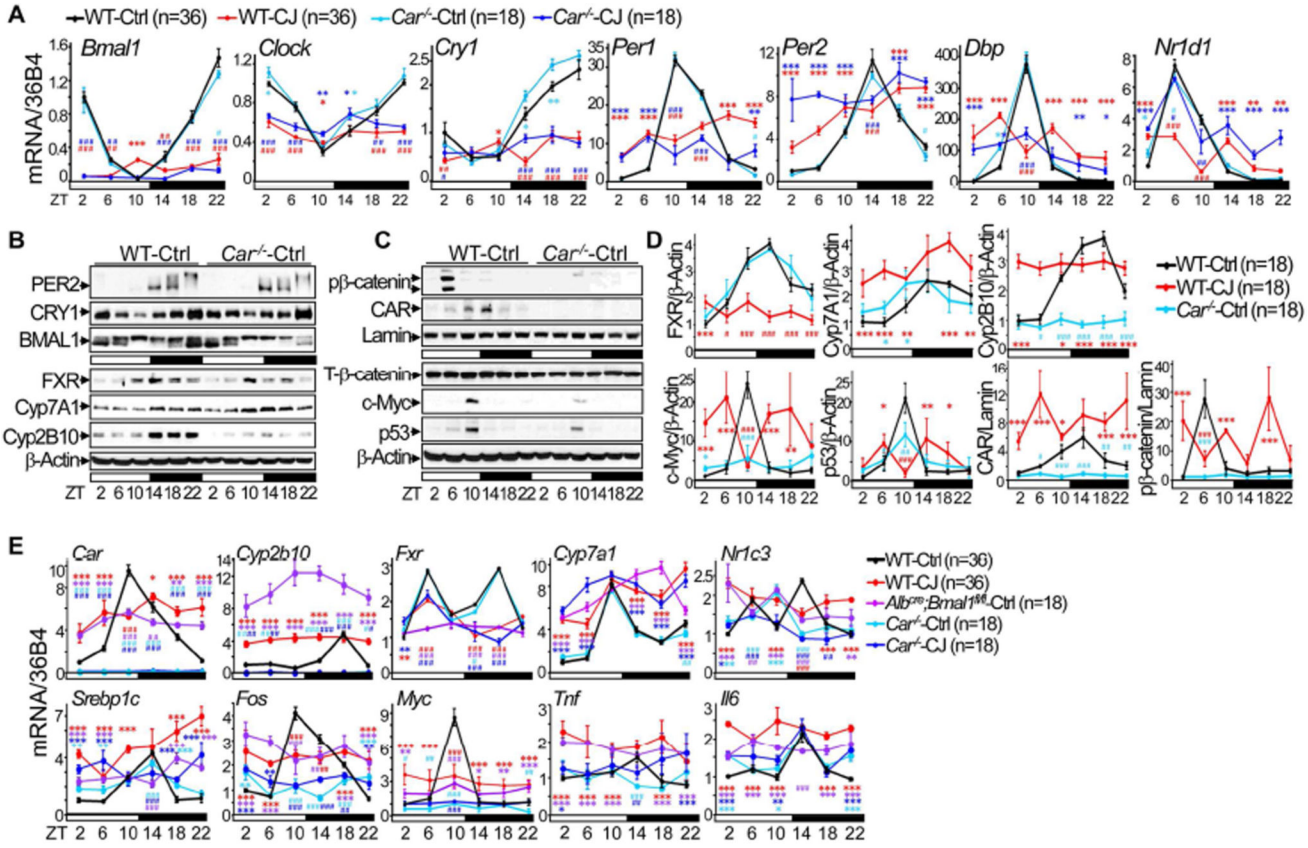

Figure 6. *Car* is a clock-controlled gene

(A) The summary of 3–6 independent RT-PCR studies on core circadian gene expression in WT and *Car*$^{-/-}$ mouse livers at 12 weeks of age, with the expression level in control WT mice at ZT2 as the arbitrary unit 1. (B, C) Western blots show the expression of BMAL1, CRY1, PER2, FXR, Cyp7A1 and Cyp2B10 (B), and CAR, p53, c-Myc, phospho-β-catenin (pβ-catenin) and total β-catenin (T-β-catenin) (C) in WT and *Car*$^{-/-}$ mouse livers at 12 weeks of age. (D) The summary of 3 independent Western blotting on hepatic expression of FXR, CAR, c-Myc, pβ-catenin, p53, Cyp7A1 and Cyp2B10 in 12 week old WT and *Car*$^{-/-}$ mice, with the expression level in control WT mice at ZT2 as the arbitrary unit 1. (E) The summary of 3–6 independent RT-PCR studies on the expression of *Car, Cyp2b10, Fxr, Cyp7a1, Myc, Fos, Pparγ*, *Srebp1c, Tnf* and *Il6* in the livers of 12 week-old WT, *Car*$^{-/-}$ and *Alb*^{cre}*;Bmal1*$^{fl/fl}$ mice, with the expression level in control WT mice at ZT2 as the arbitrary unit 1. *: Increase,$^{\#}$: decrease, comparing to WT control samples at the same time, */$^{\#}$p<0.05, **/$^{\#\#}$p<0.01; ***/$^{\#\#\#}$p<0.001, student *t* test, ±SEM.

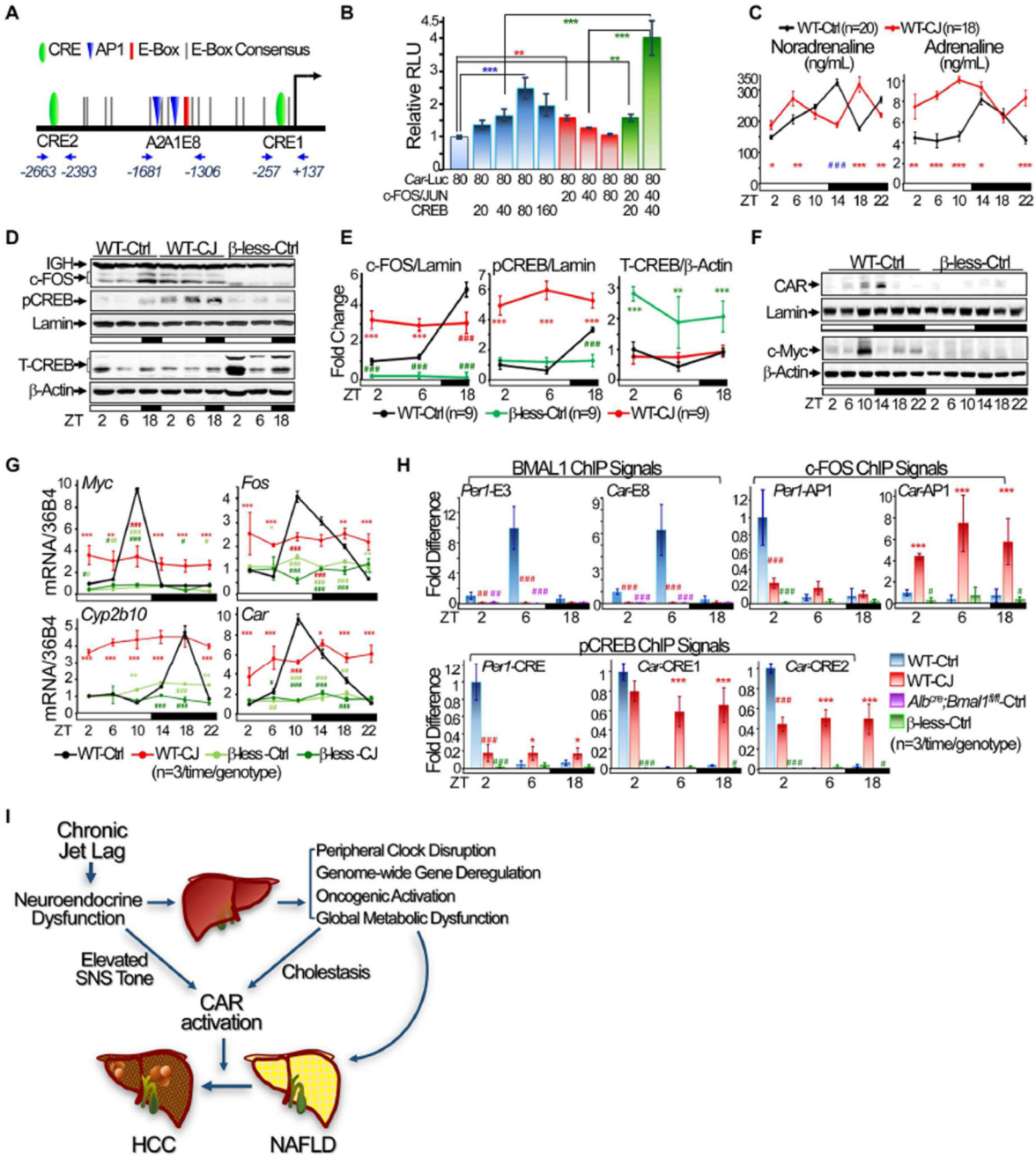

Figure 7. SNS dysfunction induces *Car* activation

(A) The schematic illustration of conserved E-boxes, AP1 (A1 and A2) and CRE (C1 and C2) binding motifs and ChIP qPCR primers in the *Car* promoter. (B) The summary of 4–5 independent co-transfection assays studying the role of AP1 and CREB in *Car* promoter activation. (C) Urine catecholamine assays show sympathetic tone over a 24 hr period in control and jet-lagged WT mice at 12 weeks of age. (D) Western blots show nuclear expression of c-FOS and S133 phopho-CREB (pCREB) (top panel), and total CREB (T-CREB) (bottom panel) levels in liver nuclear extracts of WT and β-less mice at 12 weeks of age. (E) The summary of 3 independent Western blotting studying the hepatic expression of

c-FOS, pCREB and T-CREB in WT and β-less mice at 12 weeks of age. F. Western blots show CAR and c-Myc expression in the livers of 12 week old β-less mice. G. The summary of 3 independent RT-PCR studies on hepatic expression of *Fos, Myc, Car* and *Cyp2b10* mRNAs in control and jet-lagged WT and β-less mice at 12 weeks of age. (H) BMAL1, c-FOS and pCREB ChIP signals on *Car* and *Per1* promoters in the livers of WT, β-less and $Alb^{Cre};Bmal1^{fl/fl}$ mice at 12 week of age, with ChIP signals for each transcription factor detected in control WT mice at ZT2 as the arbitrary unit 1. Negative control IgGs and qPCR primers are explained in Supplemental Experimental Procedures. *: Increase,$^{\#}$: decrease, comparing to WT control mice at the same time, */$^{\#}$p<0.05, **/$^{\#\#}$p<0.01; ***/$^{\#\#\#}$p<0.001, student *t* test, ±SEM. (I) A model for the role of chronic circadian disruption in NAFLD-induced hepatocarcinogenesis. See also figure S8.

Table 1

Incidence of HCC, Hepatomegaly and NAFLD in Jet-lagged WT and Mutant Mice by 90 Weeks of Age

C57BL/6J Inbred Mouse Models	% HCC	Age HCC found (week)	p1*	% Hepatomegaly at 16 week	% NAFLD at 90 week	p2**
WT Ctrl (n=110)	0	N/A		15.5	20	
WT CJ (n=80)	8.75[a]	78–90	0.00694	75	96.3	2.2E-16
Cry1$^{-/-}$;*Cry2*$^{-/-}$Ctrl (n=60)	11.7[b]	51–90	7.87E-05	68.3	93.3	2.27E-16
Cry1$^{-/-}$;*Cry2*$^{-/-}$CJ (n=56)	17.9[c]	43–90	1.26E-05	69.7	96.4	2.2E-16
Per1$^{-/-}$;*Per2*$^{-/-}$Ctrl (n=80)	12.5[d]	50–90	1.34E-04	86.25	100	1.7E-16
Per1$^{-/-}$;*Per2*$^{-/-}$CJ (n=50)	26[e]	42–90	1.14E-08	86	100	1.7E-16
Alb^{cre};*Bmal1*$^{fl/fl}$ Ctrl (n=26)	11.53[f]	74–90	2.3E-03	73.1	92.3	1.28E-09
Alb^{cre};*Bmal1*$^{fl/fl}$CJ (n=43)	11.63[g]	65–90	1.71E-03	95.35	100	7.2E-16
Car$^{-/-}$Ctrl (n=24)	0	N/A	N/A	25	87.5	4.2E-08
Car$^{-/-}$CJ (n=25)	0	N/A	N/A	0	68	0.0005
Fxr$^{-/-}$Ctrl (n=25)	29.2	57–90	1.67E-09	100	100	2.87E-11
Fxr$^{-/-}$CJ (n=31)	61.3	57–90	4.00E-13	100	100	6.46E-13

Non-Cancer related early euthanization: 8.8%

[a], 18.3%

[b], 17.5%

[d]or 30.2%

[g]early euthanization from 15–82 weeks of age due to overgrowth of teeth, severe ulcerative dermatitis, or neurological disorders.

[c]32.1% early euthanization from 12–77 weeks of age due to overgrowth of teeth, severe ulcerative dermatitis, cystic renal dysplasia, or neurological disorders.

[e]30% early euthanization from 35–79 weeks due to overgrowth of teeth, cystic renal dysplasia, severe ulcerative dermatitis, or neurological disorders.

[f]15.4% early euthanization from 58–81 weeks of age due to ulcerative dermatitis.

*p1: Comparing HCC incidence with WT control mice, Kaplan-Meier statistics.

**p2: Comparing NAFLD incidence with WT control mice, two sample Wilcoxon test.