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The Liver Clock Controls Cholesterol Homeostasis through Trib1 Protein-mediated Regulation of PCSK9/Low Density Lipoprotein Receptor (LDLR) Axis*

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Disruption of the body clock has been recognized as a risk factor for cardiovascular disease. How the circadian pacemaker interacts with the genetic factors associated with plasma lipid traits remains poorly understood. Recent genome-wide association studies have identified an expanding list of genetic variants that influence plasma cholesterol and triglyceride levels. Here we analyzed circadian regulation of lipid-associated candidate genes in the liver and identified two distinct groups exhibiting rhythmic and non-rhythmic patterns of expression during light-dark cycles. Liver-specific inactivation of Bmal1 led to elevated plasma LDL/VLDL cholesterol levels as a consequence of the disruption of the PCSK9/LDL receptor regulatory axis. Ablation of the liver clock perturbed diurnal regulation of lipid-associated genes in the liver and markedly reduced the expression of the non-rhythmically expressed gene Trib1. Adenovirus-mediated rescue of Trib1 expression lowered plasma PCSK9 levels, increased LDL receptor protein expression, and restored plasma cholesterol homeostasis in mice lacking a functional liver clock. These results illustrate an unexpected mechanism through which the biological clock regulates cholesterol homeostasis through its regulation of non-rhythmic genes in the liver.

Organisms evolved diverse strategies to adapt their nutrient and energy metabolism to the light-dark cycles. In mammals, diurnal rhythms of metabolic physiology are coordinated by the biological clock residing in the brain and peripheral tissues (1–3). Global analyses of chromatin occupancy and transcriptional profiling have revealed pervasive rhythmic gene transcription in diverse tissues (4–9). Rhythmic recruitment of the core clock proteins to their genomic targets is accompanied by cyclic changes in the local chromatin landscape and gene expression (4, 8). Furthermore, the concentrations of circulating metabolites exhibit robust diurnal patterns in mice and humans (10–12), likely a consequence of the combined effects of feeding cycles and rhythmic activation of metabolic flux. As such, the temporal organization of metabolic activities is emerging as a fundamental aspect of nutrient and energy homeostasis. Disruption of the body clock has been linked to sleep disorder, cardiovascular disease, and metabolic syndrome (13–15). Mice with impaired clock function developed obesity and displayed key features of metabolic syndrome (16). In humans, circadian misalignment has been causally linked to metabolic disorders such as insulin resistance, hyperphasia, and hyperlipidemia (17, 18).

Plasma lipid levels are influenced by genetic and environmental factors and constitute a major risk factor for cardiovascular disease. Elevated low density lipoprotein (LDL) cholesterol and reduced high density lipoprotein (HDL) cholesterol are associated with an increased risk for atherosclerosis and coronary heart disease, whereas hypertriglyceridemia represents an independent risk factor (19, 20). Recent genome-wide association studies (GWAS)[3] have uncovered a growing number of common genetic variants that influence plasma LDL cholesterol, HDL cholesterol, and triglyceride levels, many of which are located in proximity to the genes that regulate different aspects of lipid metabolism (21–26). Surprisingly, most of the genetic variants associated with plasma lipid levels fall within intronic sequences and putative 5′ and 3′ regulatory regions instead of the coding sequences. These observations raise the possibility that common genetic variants may influence plasma lipid traits through modulating the expression of nearby candidate genes as illustrated by the regulation of Sortilin 1 (Sort1) transcription by a noncoding polymorphism (27).

LDL receptor (LDLR) is required for the uptake and clearance of LDL cholesterol from circulation; its mutations cause

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[3] The abbreviations used are: GWAS, genome-wide association studies; PCSK9, proprotein convertase subtilisin/kexin type 9; LDLR, LDL receptor; Sort1, Sortilin 1; LKO, liver-specific knock-out; ZT, Zeitgeber time; JTK, Jonckheere-Terpstra-Kendall; CETP, cholesterylester transfer protein; LIPC, hepatic lipase; LIPG, endothelial lipase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LPL, lipoprotein lipase; MLXIPL, MLX interacting protein-like; PLTP, phospholipid transfer protein; LCAT, lecithin-cholesterol acyltransferase; HPR, haptoglobin-related protein; GCKR, glucokinase regulator.

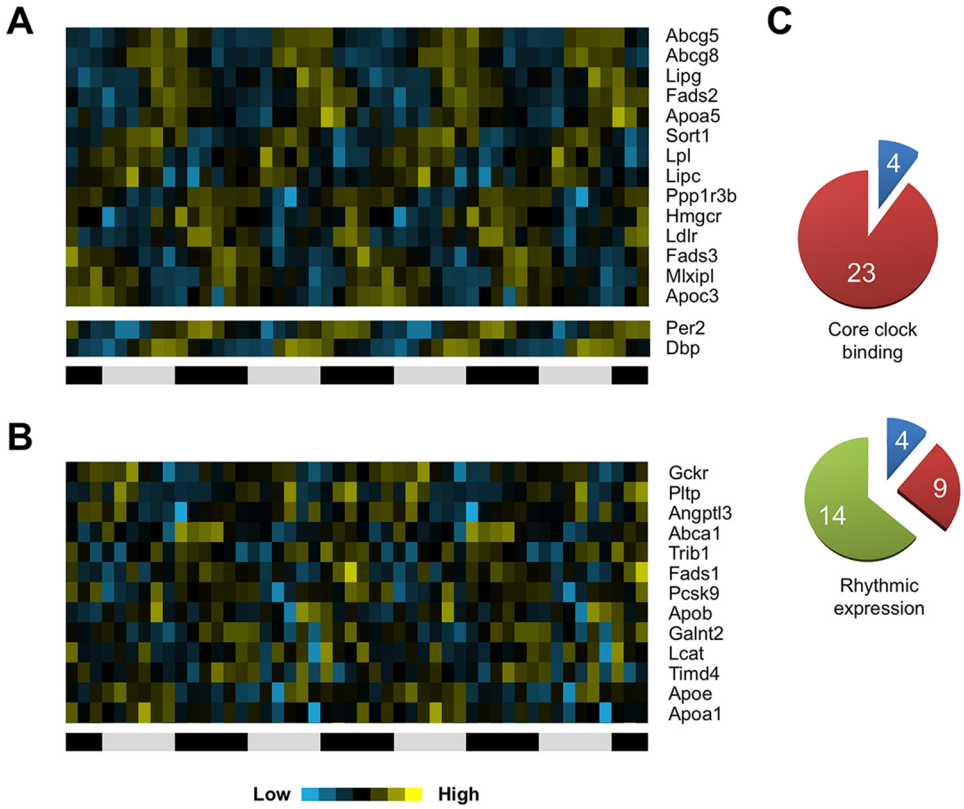

FIGURE 1. **Temporal regulation of GWAS candidate genes associated with blood lipid traits.** TreeView visualization of the rhythmic (*A*) and non-rhythmic (*B*) clusters of the genes associated with plasma lipid levels is shown. Analyses were performed using microarray expression data from GSE54650. Microarray data from 24 time points were plotted in duplicates. Light and dark circadian phases are indicated below. *C*, pie graphs depicting core clock protein occupancy (*top*) and rhythmic mRNA expression (*bottom*). Note that 23 of 27 genes exhibit core clock protein binding, whereas mRNA levels for 14 of these 23 genes are rhythmic.

familial hypercholesterolemia (20). LDLR expression is regulated at the transcriptional and posttranslational levels. The abundance of LDLR protein on plasma membrane is modulated by proprotein convertase subtilisin/kexin type 9 (PCSK9), which promotes endocytosis and subsequent degradation of LDLR (34, 35). Blocking PCSK9 via therapeutic antibodies has been successful in lowering plasma LDL cholesterol levels (28).

In this study, we investigated circadian regulation of lipid-associated GWAS candidate genes in mouse liver. Our analyses identified two groups of genes exhibiting rhythmic and non-rhythmic expression patterns. We demonstrated that conditional ablation of the liver clock resulted in elevated plasma LDL/VLDL cholesterol due to a disruption of the PCSK9/LDLR pathway. At the mechanistic level, we revealed an unexpected mechanism through which the biological clock modulates cholesterol metabolism by targeting Trib1, a non-rhythmically expressed gene in the liver.

Experimental Procedures

Analysis of GWAS Candidate Gene Expression—A list of GWAS loci associated with plasma lipid traits was obtained from the Teslovich *et al.* (24) study using a p value cutoff of 1×10^{-20}. A total of 27 candidate genes were included in our analysis with the exception of CETP, which is not present in the mouse genome. The rhythmicity of these genes was analyzed using the microarray data set GSE54650 with the JTK_CYCLE algorithm (9, 29). A gene with JTK p value of less than 0.005 is considered rhythmic. Clustering analysis of the rhythmic and non-rhythmic genes was performed using Cluster 3.0 and visualized using TreeView software.

Animal Studies—All animal experiments were performed according to procedures approved by the University Committee on Use and Care of Animals. Mice were fed *ad lib* and maintained in 12-h/12-h light-dark cycles. Bmal1 flox/flox mice were purchased from The Jackson Laboratory (stock number 007668) and crossed with albumin-Cre transgenic mice (stock number 003574) to obtain the liver-specific Bmal1 knock-out mice. For *in vivo* adenoviral transduction, Bmal1 flox/flox and Bmal1 liver-specific knock-out (LKO) mice (three to five per group) were transduced with purified adenoviruses through tail vein injection (0.2 OD per mouse) as described previously (30, 31). The titers of all adenoviruses were determined based on the expression of GFP and adenoviral gene AdE4 before use to ensure that similar doses were administered.

Plasma Lipid Analyses—Total plasma cholesterol and triglyceride concentrations were measured using Cholesterol LiquidColor test kits (StanBio Laboratory) and a triglyceride assay kit (Sigma-Aldrich), respectively. Lipoprotein profile analysis was performed using pooled plasma samples from two to three mice by the FPLC method. Plasma HDL and VLDL/LDL cholesterol concentrations were measured using an assay kit (Biovision). For the VLDL secretion assay, plasma samples were

collected at different time points following intravenous injection of tyloxapol (500 mg/kg) and analyzed for total triglycerides. For the measurements of cholesterol biosynthesis rate, mice were injected with D_2O containing 0.9% NaCl at a dose of 4.5% of estimated body water content for a period of 6 h starting at Zeitgeber time (ZT) 0 (6 a.m.) or ZT 12 (6 p.m.). Newly synthesized cholesterol was estimated by mass isotopomer distribution analysis. Plasma PCSK9 concentrations were measured using a Quantikine ELISA kit from R&D Systems.

Gene Expression Analyses—Total RNA was isolated from mouse livers and analyzed by quantitative PCR using the SYBR Green method as described previously (30, 32). Data were normalized to the internal control 36B4. LDLR antibody was purchased from Abcam.

Transient Transfection—HEK293 cells were transiently transfected with vector or FLAG-PCSK9 plasmid with or without Trib1. Total cell lysates and conditioned media were collected for immunoblotting and ELISA analyses, respectively.

Statistics—Data were analyzed using the unpaired two-tailed Student's *t* test for independent groups. A p value of less than 0.05 was considered statistically significant.

TABLE 1
Temporal regulation of candidate genes associated with blood lipid traits

Lead trait	Rhythmic	Non-rhythmic
LDL Cholesterol	ABCG5 ABCG8 LDLR SORT1	PCSK9 APOB APOE
HDL Cholesterol	LIPC LIPG PPP1R3B	GALNT2 PLTP ABCA1 LCAT
Total Cholesterol	HMGCR	HPR TIMD4 CILP2
Total Triglycerides	APOC3 APOA5 FADS2 FADS3 LPL MLXIPL	FADS1 APOA1 ANGPTL3 TRIB1 GCKR

Results

Temporal Expression of Genes Associated with Plasma Lipid Traits in the Liver—The biological clock orchestrates major aspects of nutrient and energy metabolism in mammals. Despite this, whether circadian timing cues impinge on the expression of genes associated with blood lipid traits and disease risk has not been explored. To address this, we examined temporal regulation of GWAS candidate genes associated with plasma lipid levels. A total of 27 genes with a p value less than 1×10^{-20} from the Teslovich *et al.* (24) study were selected for expression analysis. We analyzed microarray data set GSE54650 containing liver gene expression at 24 time points sampled every 2 h over a period of 2 days (9). As expected,

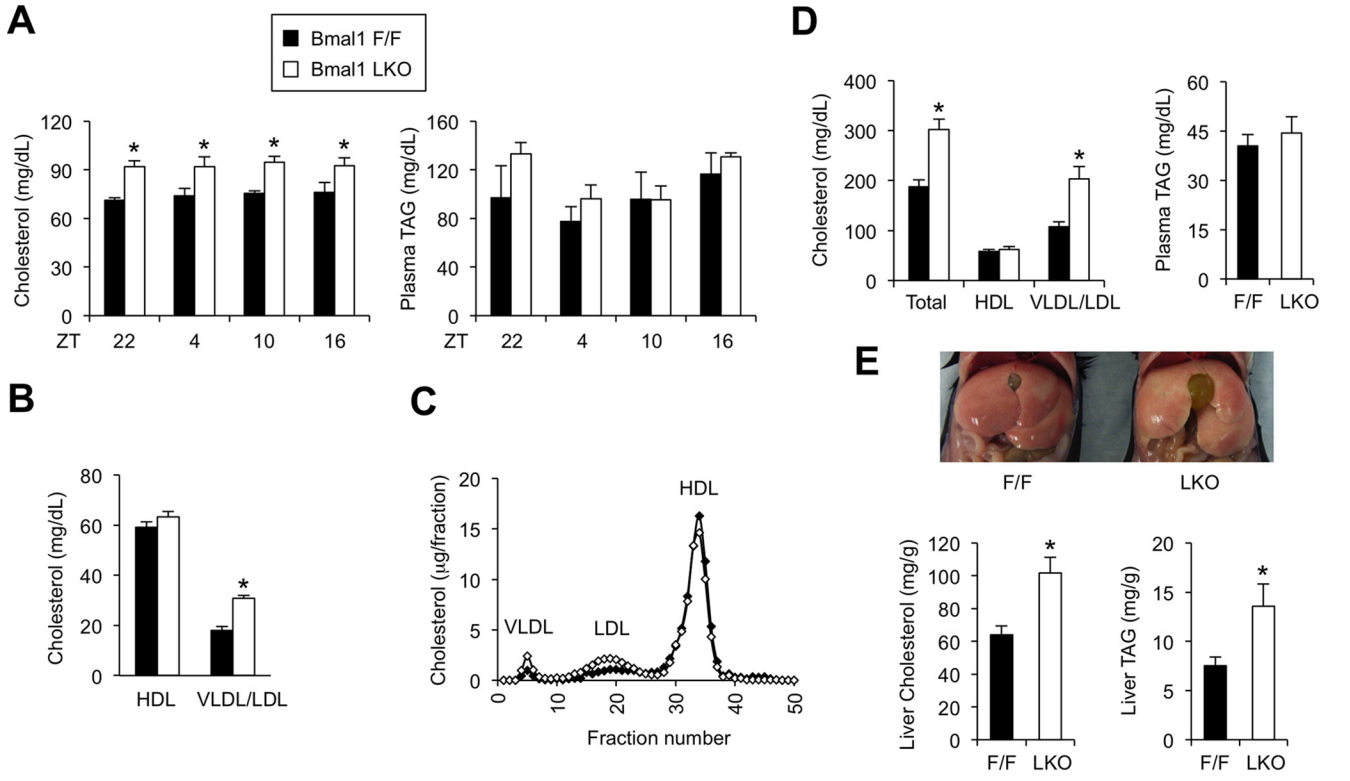

FIGURE 2. **Conditional ablation of the liver clock raises plasma LDL cholesterol.** *A*, plasma cholesterol and triglyceride (*TAG*) concentrations in control (*filled*) and Bmal1 LKO (*open*) mice at different time points ($n = 4–6$). ZT 0 is the onset of the light phase. *B*, plasma HDL and LDL/VLDL cholesterol levels. *C*, lipoprotein profile analysis of pooled plasma. *D*, plasma total, HDL, and LDL/VLDL cholesterol and triglyceride concentrations in mice fed a Western diet. *E*, liver morphology (*top*) and lipid content (*bottom*) in mice fed a high cholesterol diet. *Error bars* represent S.E. *, $p < 0.05$ by two-tailed Student's *t* test. *F/F*, flox/flox.

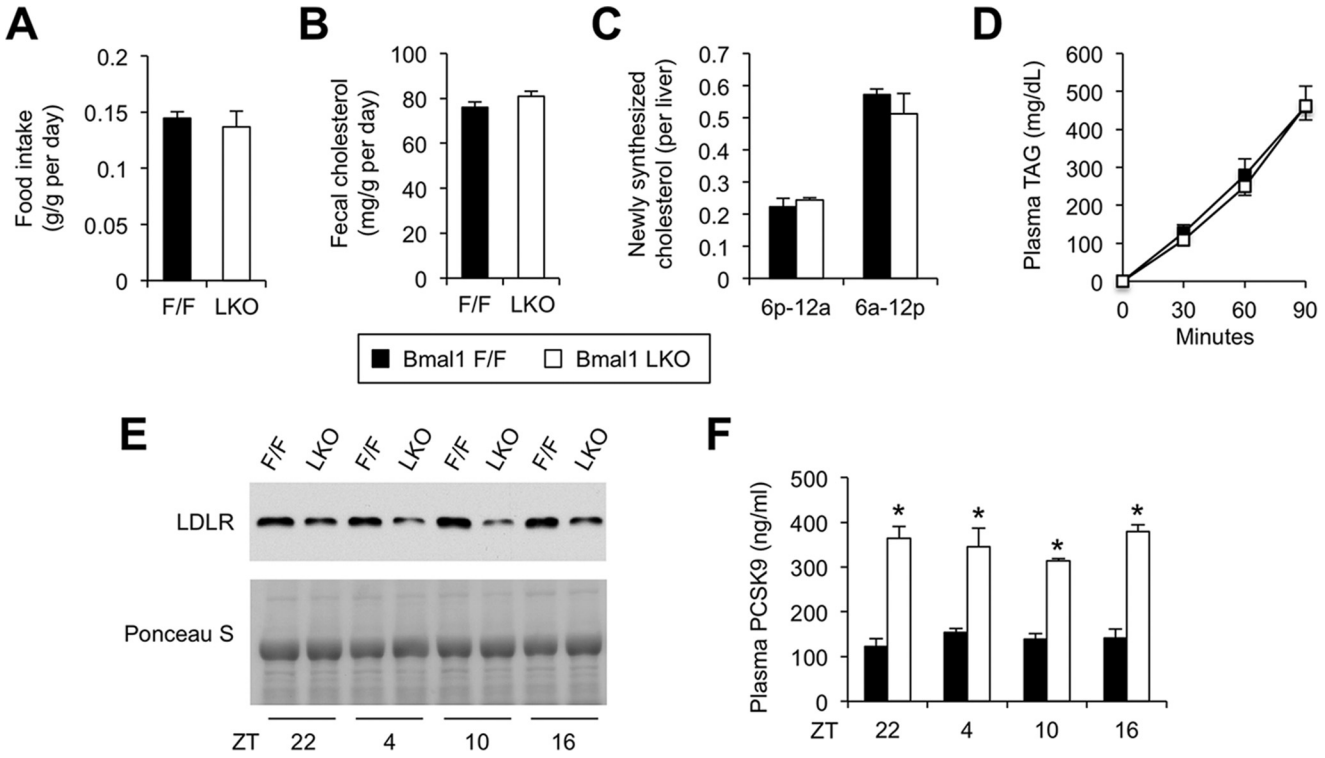

FIGURE 3. **Effects of liver clock ablation on cholesterol metabolism.** *A* and *B*, food intake (*A*) and fecal cholesterol excretion (*B*) in control (Bmal1 flox/flox (*F/F*); *filled*) and Bmal1 LKO (*open*) mice ($n = 4–6$). *C*, cholesterol biosynthesis rate in control and LKO mice during light (6 a.m. (*a*)–12 p.m. (*p*)) and dark (6 p.m.–12 a.m.) phases. *D*, VLDL secretion assay. *E*, LDLR immunoblotting using total liver lysates. A Ponceau S-stained blot is shown as a loading control. *F*, plasma PCSK9 concentrations. *Error bars* represent S.E. *, $p < 0.05$ by two-tailed Student's *t* test. *TAG*, triglyceride.

mRNA expression of core clock genes Per2 and Dbp exhibited robust circadian rhythms (Fig. 1*A*). Using JTK_CYCLE, an algorithm for detecting rhythmic gene expression (9, 29), we identified 14 GWAS candidate genes exhibiting rhythmic expression (Fig. 1*A*). Among the rhythmic genes are those primarily associated with LDL cholesterol (*ABCG5*, *ABCG8*, *LDLR*, and *SORT1*), HDL cholesterol (*LIPC*, *LIPG*, and *PPP1R3B*), total cholesterol (*HMGCR*), and total triglycerides (*APOC3*, *APOA5*, *FADS2*, *FADS3*, *LPL*, and *MLXIPL*) (Table 1). Weak circadian rhythmicity was observed for the remaining 13 genes associated with lipid traits, including *PCSK9*, *APOB*, *APOE*, *GALNT2*, *PLTP*, *ABCA1*, *LCAT*, *HPR*, *TIMD4*, *CILP2*, *FADS1*, *APOA1*, *ANGPTL3*, *TRIB1*, and *GCKR* (Fig. 1*B*). *CETP* was a strong candidate gene associated with plasma lipid levels, but this gene is not present in mice. These observations suggest that circadian control of transcription of the GWAS candidate genes may play an important role in the regulation of plasma lipid levels.

The transcription factors constituting the clock oscillator regulate rhythmic expression of many downstream target genes through direct binding to their chromatin targets. Analysis of a recently published chromatin immunoprecipitation (ChIP)-sequencing data set (4) on core clock proteins revealed that chromatin occupancy by one or more core clock proteins was observed for 23 of 27 GWAS lipid candidate genes in the liver with the exception of *Apoc3*, *Apoa5*, *Timd4*, and *Cilp2* (Fig. 1*C*). Interestingly, nine of 23 genes positive for core clock protein binding exhibited a non-rhythmic expression pattern. For example, chromatin association for Bmal1, Clock, Per1, Per2, Cry1, and Cry2 was observed at multiple genomic sites within the *Trib1* and *Pcsk9* loci, but their mRNA levels exhibited modest circadian rhythmicity. The binding peaks for the core clock proteins appeared to spread across the proximal promoter regions as well as the intronic and coding regions. The observations that the core clock proteins associate with chromatin near the genes that exhibit minimal mRNA rhythmicity suggest that these factors may regulate the transcription of certain target genes in a time-independent manner. Together, these results demonstrate that the biological clock directly regulates the expression of a subset of genes associated with plasma lipid levels.

Ablation of the Liver Clock Elevates Plasma Cholesterol Levels—To determine the physiological role of the liver clock in plasma lipid homeostasis, we generated mice with liver-specific deletion of Bmal1, a core transcriptional activator of the clock oscillator. Conditional Bmal1 inactivation abolished clock function in the liver without affecting the central clock function (33). Measurements of plasma lipid concentrations indicated that, compared with Bmal1 flox/flox control, total plasma cholesterol was elevated in LKO mice at all time points (ZT 4, 10, 16, and 22) apparently independent of light-dark phases (Fig. 2*A*). In contrast, plasma triglyceride concentrations remained similar between the two groups. Measurements of HDL and non-HDL (LDL/VLDL) cholesterol indicated that liver clock ablation resulted in an ~70% increase in LDL/VLDL cholesterol without affecting HDL cholesterol (Fig. 2*B*). Consistently, lipoprotein profile analysis revealed that the increase of plasma cholesterol in LKO mice was largely due to increased cholesterol levels within the LDL and VLDL fractions (Fig. 2*C*). Cho-

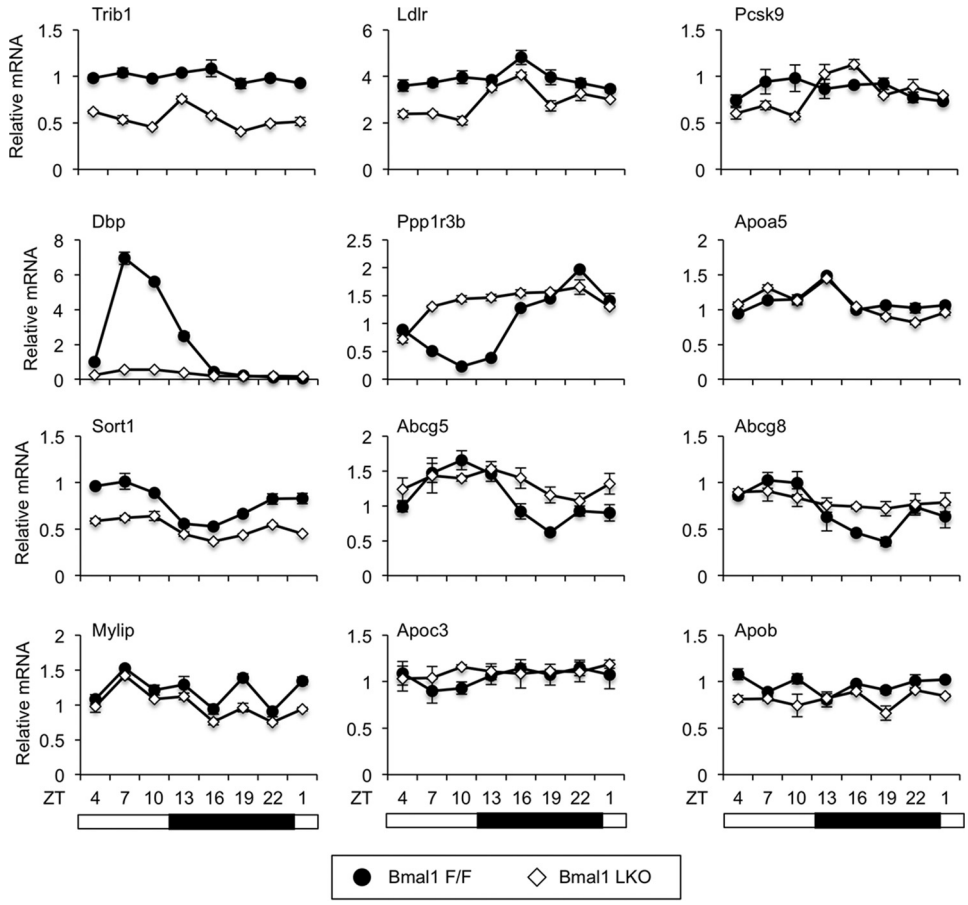

FIGURE 4. **Hepatic gene expression.** Quantitative PCR analysis of liver gene expression at the indicated time points is shown. Pooled liver RNA from three to five control (*filled circles*) and Bmal1 LKO (*open diamonds*) mice was used for each data point with quantitative PCR performed in triplicates. *Error bars* represent S.D. *F/F*, flox/flox.

lesterol in the HDL fractions remained largely unaffected by conditional ablation of the liver clock.

We next examined whether inactivation of the liver clock exacerbates diet-induced hyperlipidemia. We fed control and Bmal1 LKO mice with high fat Western diet supplemented with 2% cholesterol. As expected, Western diet feeding markedly elevated plasma cholesterol in mice compared with standard chow (Fig. 2, *A* and *D*). Total plasma cholesterol levels were ~54% higher in Bmal1 LKO mice than in control (Fig. 2*D*), whereas plasma triglyceride concentrations remained similar between these two groups. Consistent with chow-fed mice, VLDL/LDL, but not HDL, cholesterol was significantly elevated in Bmal1 LKO mice following Western diet feeding. The LKO mouse livers appeared pale in color, a sign of excess lipid accumulation (Fig. 2*E*). Analysis of hepatic lipids revealed that cholesterol and triglyceride content was also significantly higher in Bmal1 LKO mouse livers. Together, these results demonstrate that the liver clock is required for maintaining plasma cholesterol homeostasis.

Absence of the Liver Clock Disrupts the PCSK9/LDLR Axis—To elucidate how clock regulates cholesterol metabolism, we examined different aspects of cholesterol metabolism. Dietary intake and fecal excretion of cholesterol appeared similar between control and Bmal1 LKO mice (Fig. 3, *A* and *B*). Measurements of *de novo* cholesterol biosynthesis indicated that, as expected, the cholesterol synthesis rate exhibited strong diurnal regulation in mice (Fig. 3*C*). Ablation of the liver clock did not appear to perturb the diurnal regulation of cholesterol synthesis. Furthermore, a lipoprotein secretion assay revealed that the hepatic secretion rate of the VLDL particles was largely unaffected by clock deficiency (Fig. 3*D*). Together, these results suggest that elevated LDL/VLDL cholesterol in Bmal1 LKO mice may be due to reduced clearance of these cholesterol-containing lipoprotein particles from circulation. In support of this, we found that LDLR protein levels were markedly reduced in Bmal1 LKO liver lysates at all time points examined (Fig. 3*E*). Surprisingly, LDLR mRNA levels were lower at ZT 4 and 10 but remained similar at ZT 16 and 22 (Fig. 4), suggesting that decreased LDLR mRNA expression could not completely account for reduced LDLR protein expression in the LKO mouse livers.

A major regulator of hepatic LDLR protein levels is PCSK9, which promotes the endocytosis and subsequent degradation of LDLR (34, 35). In fact, lowering plasma PCSK9 levels has been shown to lower plasma cholesterol through augmenting LDLR-mediated uptake of LDL particles. Measurements of plasma PCSK9 levels indicated that its concentrations more than doubled in LKO mice compared with control (Fig. 3*F*), suggesting that the disruption of the PCSK9/LDLR axis may be a major contributor to the cholesterol phenotype in Bmal1 LKO

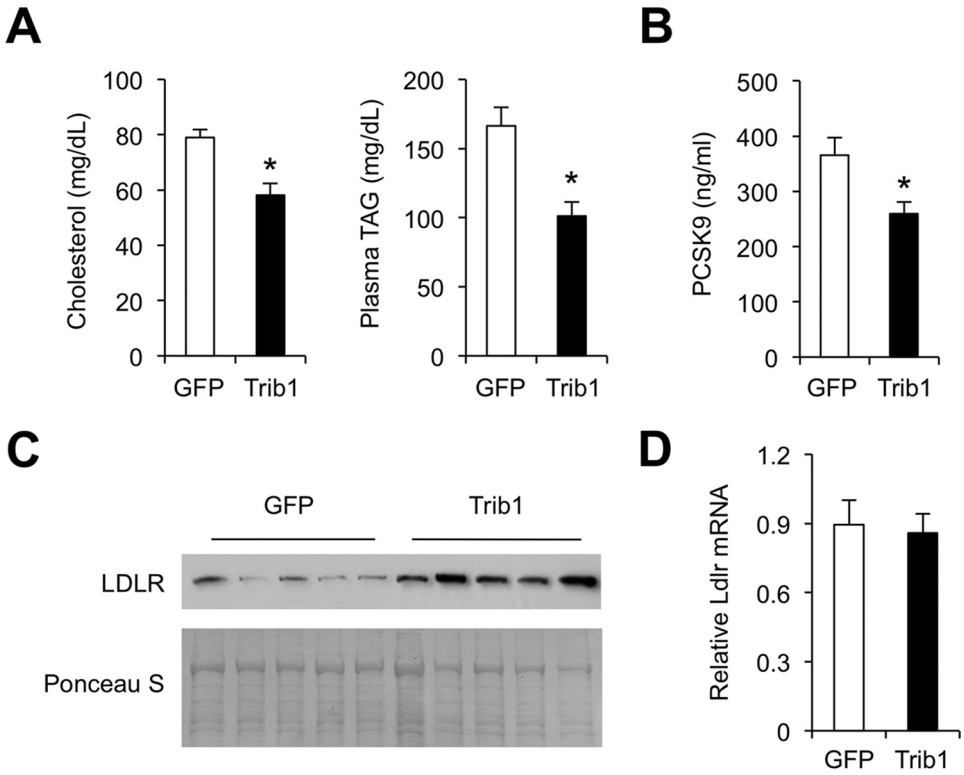

FIGURE 5. **Trib1 acts on the PCSK9/LDLR axis to regulate plasma lipids.** *A*, total plasma cholesterol and triglyceride (*TAG*) concentrations in mice transduced with GFP (*open*; $n = 5$) or Trib1 (*filled*; $n = 5$) adenovirus. *B*, plasma PCSK9 concentrations in transduced mice. *C*, LDLR immunoblotting using total liver lysates. A Ponceau S-stained blot is shown as a loading control. *D*, quantitative PCR analysis of LDLR mRNA expression. *Error bars* represent S.E. *, $p < 0.05$ by two-tailed Student's *t* test.

mice. In contrast, the expression of *Mylip*, a factor that regulates LDLR protein degradation (36), remained largely unaltered by Bmal1 deficiency (Fig. 4). Analyses of hepatic gene expression indicated that rhythmic expression of *Dbp*, a target of the molecular clock, was nearly abolished in Bmal1 LKO mice. Diurnal patterns of mRNA expression for several GWAS candidate genes were also disrupted, including *Ldlr*, *Pcsk9*, *Ppp1r3b*, *Sort1*, *Abcg5*, *Abcg8*, and *Apoc3*, whereas *Apoa5* and *Apob* expression remained similar between the two groups. Interestingly, although *Trib1* expression did not appear to exhibit a strong circadian rhythm, its mRNA levels were significantly decreased at all time points by the ablation of the liver clock.

Trib1 Regulates the PCSK9/LDLR Axis—Genetic variants at the Trib1 gene locus have been identified to associate with plasma triglyceride, LDL cholesterol, and HDL cholesterol levels (24–26). Previous studies have demonstrated that hepatic overexpression of Trib1 lowered plasma lipid levels (37). However, it remains unknown whether Trib1 regulates cholesterol metabolism through the PCSK9/LDLR axis. To determine whether hepatic Trib1 regulates plasma PCSK9 levels, we transduced wild type C57/Bl6J mice with control GFP or Trib1 adenoviruses through tail vein injection. Compared with GFP, adenovirus-mediated Trib1 overexpression in the liver lowered plasma cholesterol and triglyceride concentrations (Fig. 5*A*). Plasma PCSK9 concentration was lowered by ~30% in mice transduced with Trib1 adenovirus (Fig. 5*B*). Consistently, LDLR protein expression in total liver lysates was significantly increased in response to Trib1 overexpression, whereas *Lldr* mRNA levels remained similar between the two groups (Fig. 5, *C* and *D*). These results illustrate that Trib1 may regulate LDLR protein expression in the liver through its modulation of plasma PCSK9 concentrations.

To explore the mechanisms through which Trib1 regulates PCSK9, we transiently transfected HEK293 cells with a PCSK9 expression vector (FLAG-PCSK9) in the absence or presence of Trib1. Immunoblotting analyses indicated that Trib1 significantly reduced PCSK9 protein levels in whole cell lysates from transfected cells (Fig. 6*A*). Importantly, we found that Trib1 significantly reduced PCSK9 secretion into culture media. ELISA of PCSK9 in conditioned medium from transfected HEK293 cells revealed that Trib1 decreased PCSK9 secretion by ~30–40% (Fig. 6*B*). These findings demonstrate that Trib1 functions as a negative regulator of PCSK9 biosynthesis and secretion in a cell-autonomous manner.

Trib1 Rescues the Cholesterol Phenotype in LKO Mice—Because Trib1 expression was significantly reduced in Bmal1 LKO mouse livers, we next examined whether adenovirus-mediated expression of Trib1 may rescue the hypercholesterolemia phenotype in Bmal1 LKO mice. We transduced Western diet-fed control and LKO mice with GFP or Trib1 adenoviruses and monitored their plasma lipid levels. Total plasma cholesterol was elevated in Bmal1 LKO mice (Fig. 7*A*). Compared with GFP, Trib1 significantly lowered cholesterol levels in LKO mice to the levels observed in control mice. Lipoprotein analyses indicated that LDL/VLDL cholesterol was reduced in response to adenovirus-mediated Trib1 expression in the liver (Fig. 7*B*). Importantly, Trib1 also rescued aberrantly high plasma PCSK9

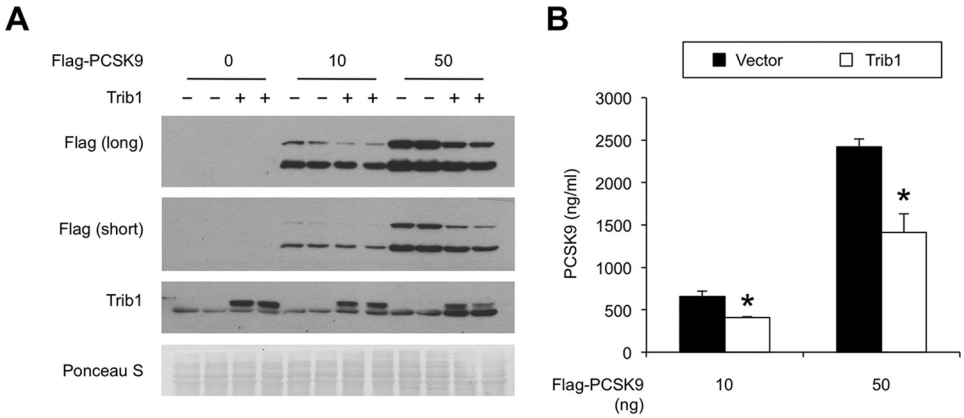

FIGURE 6. **Trib1 regulates PCSK9 protein expression and secretion.** *A*, immunoblots of total cell lysates from HEK293 cells transiently transfected with 0, 10, or 50 ng of FLAG-PCSK9 plasmid with vector (−) or Trib1 (+) plasmid. *B*, PCSK9 concentrations in culture media from cells transfected with FLAG-PCSK9 plasmid in the presence of vector (*filled*) or Trib1 (*open*) plasmid. *Error bars* represent S.D. *, $p < 0.05$ by two-tailed Student's *t* test.

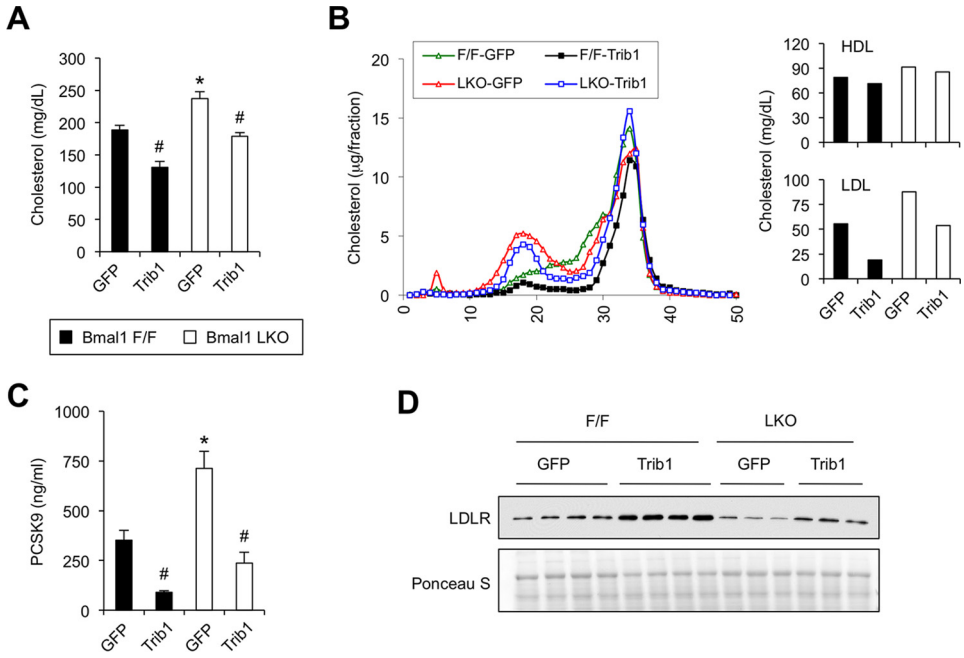

FIGURE 7. **Adenovirus-mediated expression of Trib1 rescues PCSK9/LDLR expression and plasma cholesterol levels.** *A*, total plasma cholesterol levels in Western diet-fed control (flox/flox (*F/F*); *filled*) and Bmal1 LKO (*open*) mice transduced with GFP or Trib1 adenoviruses ($n = 4–6$). *B*, lipoprotein profile analysis of pooled plasma from transduced mice. Areas under the curve for LDL and HDL fractions are shown on the *right*. *C*, plasma PCSK9 concentrations in transduced mice. *D*, LDLR immunoblotting using total liver lysates. A Ponceau S-stained blot is shown as a loading control. *Error bars* represent S.E. *, $p < 0.05$ for LKO *versus* Bmal1 flox/flox; #, $p < 0.05$ for Trib1 *versus* GFP by two-tailed Student's *t* test.

levels in Bmal1 LKO mice to those of control mice (Fig. 7*C*) and increased LDLR protein expression in transduced livers (Fig. 7*D*). Together, these results demonstrate that hepatic Trib1 expression is sufficient to rescue the dysregulation of the PCSK9/LDLR axis in mice lacking a functional liver clock. As Trib1 expression itself is largely non-rhythmic, our studies revealed an unexpected role for Trib1 as a non-canonical target of circadian signaling in the liver that links the biological clock to cholesterol homeostasis.

Discussion

Rhythmic regulation of metabolic gene programs synchronizes body metabolism to the feeding and light-dark cycles. Although disruption of the body clock has been linked to the development of metabolic disorders, the extent to which circadian timing interacts with the disease-associated genetic network remained poorly understood. Recent GWAS have identified a large number of common genetic variants associated with blood lipid levels and cardiovascular disease risk. In this study, we analyzed circadian regulation of lipid-associated candidate genes in the liver and identified two distinct groups exhibiting rhythmic and non-rhythmic patterns of expression during light-dark cycles. To our surprise, the liver clock is required for diurnal regulation of genes in both groups and plays a critical role in maintaining plasma cholesterol homeostasis. At the mechanistic level, we identified Trib1 as a non-rhythmically expressed clock-regulated gene that targets the PCSK9/LDLR axis to control hepatic LDLR protein expression and plasma LDL cholesterol levels (Fig. 8). These results reveal an unexpected mechanism through which the biological clock influ-

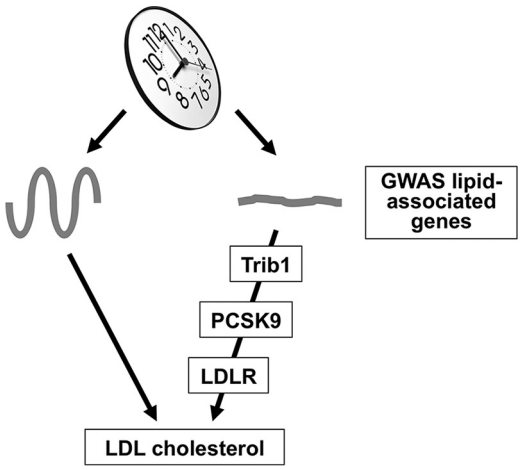

FIGURE 8. **A working model for clock regulation of plasma cholesterol homeostasis.** The liver clock regulates the expression of rhythmic and non-rhythmic genes associated with plasma lipid traits. The non-rhythmic gene Trib1 is a target of the liver clock that acts on the PCSK9/LDLR axis to regulate plasma LDL cholesterol.

ences lipid metabolism through its regulation of non-rhythmically expressed genes.

The core clock transcription factors and coactivators regulate the diurnal rhythms of metabolic gene expression and metabolic homeostasis. Perturbations of the liver clock resulted in impaired hepatic gluconeogenesis, autophagy, hepatic lipid metabolism, and mitochondrial dynamics and oxidative metabolism (38–45). Using liver-specific Bmal1 knock-out mice, we demonstrated that the liver clock was required for maintaining plasma cholesterol homeostasis. Bmal1 LKO mice had elevated plasma LDL/VLDL cholesterol when fed standard chow or Western diet while having normal HDL cholesterol. Interestingly, whole body Bmal1 knock-out mice also had elevated LDL/VLDL cholesterol levels (46), suggesting that this lipid phenotype may be largely due to Bmal1 deficiency in the liver. Analyses of different aspects of cholesterol metabolism revealed that dietary intake, fecal excretion, and *de novo* synthesis of cholesterol were similar between control and Bmal1 LKO mice. Furthermore, the secretion of VLDL into the circulation was also largely unaffected by the ablation of clock in the liver. On the contrary, we found that LDLR protein levels were significantly reduced in LKO mouse livers accompanied by elevated plasma PCSK9 levels. Given the central role of PCSK9 and LDLR in the regulation of LDL cholesterol metabolism, our findings strongly suggest that the dysregulation of the PCSK9/LDLR axis is likely responsible for hyperlipidemia caused by clock deficiency. Interestingly, Rev-erbα deficiency also resulted in elevated plasma cholesterol levels (47). In this case, both LDL and HDL cholesterol appeared to be increased in Rev-erbα knock-out mice, likely as a result of imbalance in cholesterol biosynthesis and catabolism. Whether the PCSK9/LDLR pathway is altered in Rev-erbα deficiency remains unknown. It is possible that different clock proteins exert distinct effects on pathways relevant for lipid metabolism and homeostasis.

A somewhat surprising finding here is that Bmal1 deficiency affected the expression of both rhythmic and non-rhythmic groups of the candidate genes associated with plasma lipid levels. Notably, mRNA expression of Trib1, a gene recently found to regulate plasma triglyceride and cholesterol metabolism (37), was severely depressed across all circadian time points in Bmal1 LKO mouse livers. The physiological significance of this down-regulation in plasma lipid regulation was supported by adenovirus-mediated rescue studies. Tail vein injection of Trib1 adenovirus lowered plasma LDL cholesterol in LKO mice to levels observed in control mice. Importantly, hepatic expression of Trib1 reduced plasma PCSK9 concentrations, leading to increased LDLR protein levels in the liver of transduced mice. As such, impaired Trib1 expression appeared to serve an important role in mediating the hyperlipidemic effects of Bmal1 deficiency on plasma cholesterol homeostasis. Although Trib1 mRNA levels exhibited weak rhythmicity in the liver, we cannot rule out the possibility that its activity may be cyclically regulated. Chromatin occupancy by clock proteins has been detected within the Trib1 gene locus. How the core clock transcription factors regulate Trib1 gene expression in a time-independent manner remain unknown. Given the widespread chromatin occupancy of clock proteins on non-rhythmic genes (4, 8), it is possible that the biological clock exerts influences on the transcription of both rhythmic and non-rhythmic genes. Together, our studies identified an unusual mechanism linking the biological clock to metabolic physiology where a non-rhythmic clock-regulated gene exerts profound effects on cholesterol homeostasis.

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