

Retinoid acid-related orphan receptor γ , ROR γ , participates in diurnal transcriptional regulation of lipid metabolic genes

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ABSTRACT

The hepatic circadian clock plays a pivotal role in regulating major aspects of energy homeostasis and lipid metabolism. In this study, we show that ROR γ robustly regulates the rhythmic expression of several lipid metabolic genes, including the insulin-induced gene 2a, *Insig2a*, elongation of very long chain fatty acids-like 3, *Elovl3* and sterol 12 α -hydroxylase, *Cyp8b1*, by enhancing their expression at ZT20–4. The time-dependent increase in their expression correlates with the rhythmic expression pattern of ROR γ . The enhanced recruitment of ROR γ to ROREs in their promoter region, increased histone acetylation, and reporter and mutation analysis support the concept that ROR γ regulates the transcription of several lipid metabolic genes directly by binding ROREs in their promoter regulatory region. Consistent with the disrupted expression of a number of lipid metabolic genes, loss of ROR γ reduced the level of several lipids in liver and blood in a ZT-preferred manner. Particularly the whole-body bile acid pool size was considerably reduced in ROR γ ^{-/-} mice in part through its regulation of several *Cyp* genes. Similar observations were made in liver-specific ROR γ -deficient mice. Altogether, our study indicates that ROR γ functions as an important link between the circadian clock and the transcriptional regulation of several metabolic genes.

INTRODUCTION

The retinoic acid-related orphan receptor γ (ROR γ , NR1F3), a member of the ROR subfamily of nuclear receptors, has been implicated in the control of a variety of physiological processes (1–3). By alternative promoter usage, the ROR γ gene generates two isoforms, ROR γ 1 and ROR γ 2 (ROR γ t). ROR γ t is restricted to several distinct immune cell types and has a critical role in a number of immune processes (1–3). However, the physiological functions of ROR γ 1, which is expressed in various peripheral tissues, including liver, adipose tissue, and kidney, but not brain (4,5), are still poorly understood.

The diurnal oscillations in behavioral activity and physiology are strictly controlled by the circadian clock (6–10). Disruption of the circadian rhythm has been linked to an increased risk for metabolic diseases, including obesity, diabetes, liver steatosis and atherosclerosis (11,12). In several peripheral tissues, including the liver, ROR γ 1 exhibits a robust oscillatory pattern of expression with a peak at ZT16–20 that is controlled by Clock/Bmal1 heterodimers and Rev-Erb nuclear receptors (13–18). Loss of ROR γ reduced peak expression of *Npas2*, *Cry1* and *Rev-Erb α* , but had little effect on the hepatic expression of *Bmal1* and *Clock* (7,14,16,19). Although several studies indicated a connection between ROR γ and the regulation of certain clock and metabolic genes, the precise role of ROR γ is not yet clearly understood. The robust oscillatory regulation of ROR γ 1 expression by the clock machinery raised the possibility that ROR γ might regulate the transcription of certain target genes in a ZT-dependent manner and as such mediates the diurnal regulation of metabolic genes by the clock machinery (16,19–22). However, little is known about physiological

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functions controlled by hepatic ROR γ particularly in lipid metabolism.

To study this hypothesis further, we examined the effect of the loss of ROR γ on the rhythmic expression of a number of genes involved in several lipid metabolic pathways. Our study provides evidence indicating that ROR γ regulates the expression of a number of lipid metabolic genes by multiple mechanisms that involve ZT-(in)dependent as well as (in)direct regulation by ROR γ . We demonstrate that the loss of *ROR γ* reduced peak expression of several lipid metabolic genes linked to fatty acid and cholesterol metabolism. In addition, the loss of ROR γ induced changes in cholesterol, bile acid, triglyceride (TG) and fatty acid metabolism. ROR γ cistrome and promoter analysis demonstrated that the transcription of several of these metabolic genes was regulated directly by ROR γ . These data indicated that ROR γ regulates the diurnal expression of several lipid metabolic genes in liver by a mechanism that involves ZT-dependent recruitment of ROR γ to ROREs in the regulatory regions of these genes. Together, these data support our hypothesis that ROR γ plays an integral role in mediating the transcriptional regulation of certain hepatic metabolic genes downstream of the circadian clock and thereby functions as a link between the circadian clock and its regulation of hepatic metabolism.

MATERIALS AND METHODS

Experimental Animals

Heterozygous C57BL/6 staggerer (*ROR α ^{+/-}*) were obtained from the Jackson Laboratory (Bar Harbor, ME). *ROR γ ^{-/-}* and *ROR α ^{sg/sg}ROR γ ^{-/-}* double knockout (DKO) mice were described previously (16,23). Liver-specific ROR γ knockout mice, referred to as *ROR γ ^{fx/fx}Alb-Cre⁺*, were described previously (22). Mice were supplied *ad libitum* with NIH-A31 formula (normal diet, ND) and water, and maintained at 23°C on a constant 12 h light:12 h dark cycle. Two month-old male mice were fed with a high fat diet (40% kcal fat) (HFD: D12079B Research Diets Inc., New Brunswick, NJ) for 6 weeks. Littermate wild-type (WT) mice were used as controls. All animal protocols followed the guidelines outlined by the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the NIEHS.

RNA isolation and QRT-PCR

To investigate the circadian patterns of gene expression, liver tissues were collected from WT, *ROR γ ^{-/-}*, *ROR α ^{sg/sg}* and *ROR α ^{sg/sg}ROR γ ^{-/-}* DKO mice on a ND every 4 or 6 h over a period of 24 h, and processed overnight in RNeasy[®] solution (Ambion, Austin, TX) at 4°C, and stored at -80°C until use. Total RNA was then extracted using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Liver tissues were also collected from *ROR γ ^{-/-}* mice at ZT8 after retro-orbital injection with empty adenovirus (control) or ROR γ -expressing adenovirus ($n = 6$), from *ROR γ ^{fx/fx}Alb-Cre⁻* and *ROR γ ^{fx/fx}Alb-Cre⁺* mice at ZT8 and ZT20, and from WT and *ROR γ ^{-/-}* mice ($n = 5$) fed with a HFD for 6 weeks

at the ZT indicated. QRT-PCR analysis was performed using SYBR Green I (Applied Biosystems, Foster City, CA, USA) as described previously (16). All the results were normalized by the amount of *Gapdh* mRNA. All primer sequences for QPCR are listed in Supplementary Table S1.

Other molecular, biochemical and histological methods are described in Supplementary Data.

RESULTS

ROR γ regulates the hepatic expression of several lipid metabolic genes

In this study, we demonstrate that the loss of ROR γ significantly impacts the diurnal expression pattern of a number of genes linked to several different lipid metabolic pathways. Loss of ROR γ significantly affected the circadian expression of insulin induced gene 2a (*Insig2a*), ELOVL fatty acid elongase 3 (*Elovl3*) and the sterol 12 α -hydroxylase (*Cyp8b1*). In WT liver, these genes exhibited a robust rhythmic pattern of expression with peak expression at ZT20–4, a few hours after the optimum expression of *ROR γ* at ZT20 (Figure 1A) (16), while peak expression was dramatically repressed in *ROR γ ^{-/-}* mice, blunting their circadian oscillation. In contrast, little difference in the diurnal expression of these genes was observed between WT and *ROR α ^{sg/sg}* mice. Although a recent study provided evidence for direct transcriptional regulation of *Cyp8b1* by ROR α in HepG2 cells (24), our study shows that loss of ROR α had no effect on the diurnal expression of *Cyp8b1*. *Insig2a* and *Elovl3* were repressed to a somewhat greater extent in *ROR α ^{sg/sg}ROR γ ^{-/-}* DKO mice than *ROR γ* single KO mice (Figure 1A and C). These data suggest that these genes are regulated in a highly ROR γ -selective manner. In contrast to the loss of ROR γ , exogenous expression of ROR γ in *ROR γ ^{-/-}* liver tissue by adenovirus significantly increased the expression of *Insig2a*, *Elovl3* and *Cyp8b1* (Figure 1D and Supplementary Figure S1A and B). Reduced hepatic expression of *Insig2a* and *Elovl3* was also observed in *ROR γ ^{-/-}* mice after 16-h fasting (Supplementary Figure S1C). Together, these results indicate that ROR γ plays a critical role in the diurnal regulation of *Insig2a*, *Elovl3* and *Cyp8b1* expression in liver.

The loss of ROR γ also significantly reduced the circadian expression of hepatic TG lipase (*Lipc*) and *Elovl2*, which oscillatory patterns are very much in phase with those of *Insig2a*, *Elovl3*, *Cyp8b1* and *ROR γ* (Figure 1E and Supplementary Figure S1D) (16,21,25). The expression of *Elovl5*, which exhibits a weak oscillation, and peak expression of the clock output gene, *Nocturnin* (*Ccrn4l*) at ZT12 were also repressed in *ROR γ ^{-/-}* liver, while the expression of the arrhythmic genes, adiponectin receptor 2 (*AdipoR2*), the fatty acid transporter, *Cd36*, and hydroxy-delta-5-steroid dehydrogenase, *Hsd3b5* was down- or up-regulated at all ZTs (Figure 1E).

In addition to *Cyp8b1*, several other genes involved in bile acid synthesis, including *Cyp7b1* and *Cyp27a1*, were down-regulated in *ROR γ ^{-/-}* liver (Figure 1F). Little or no circadian oscillation was observed for *Cyp7b1* and *Cyp27a1* expression, both of which were repressed in *ROR γ ^{-/-}* liver at all or most ZTs indicating that these genes are regulated in a ZT-independent manner. The latter suggests that

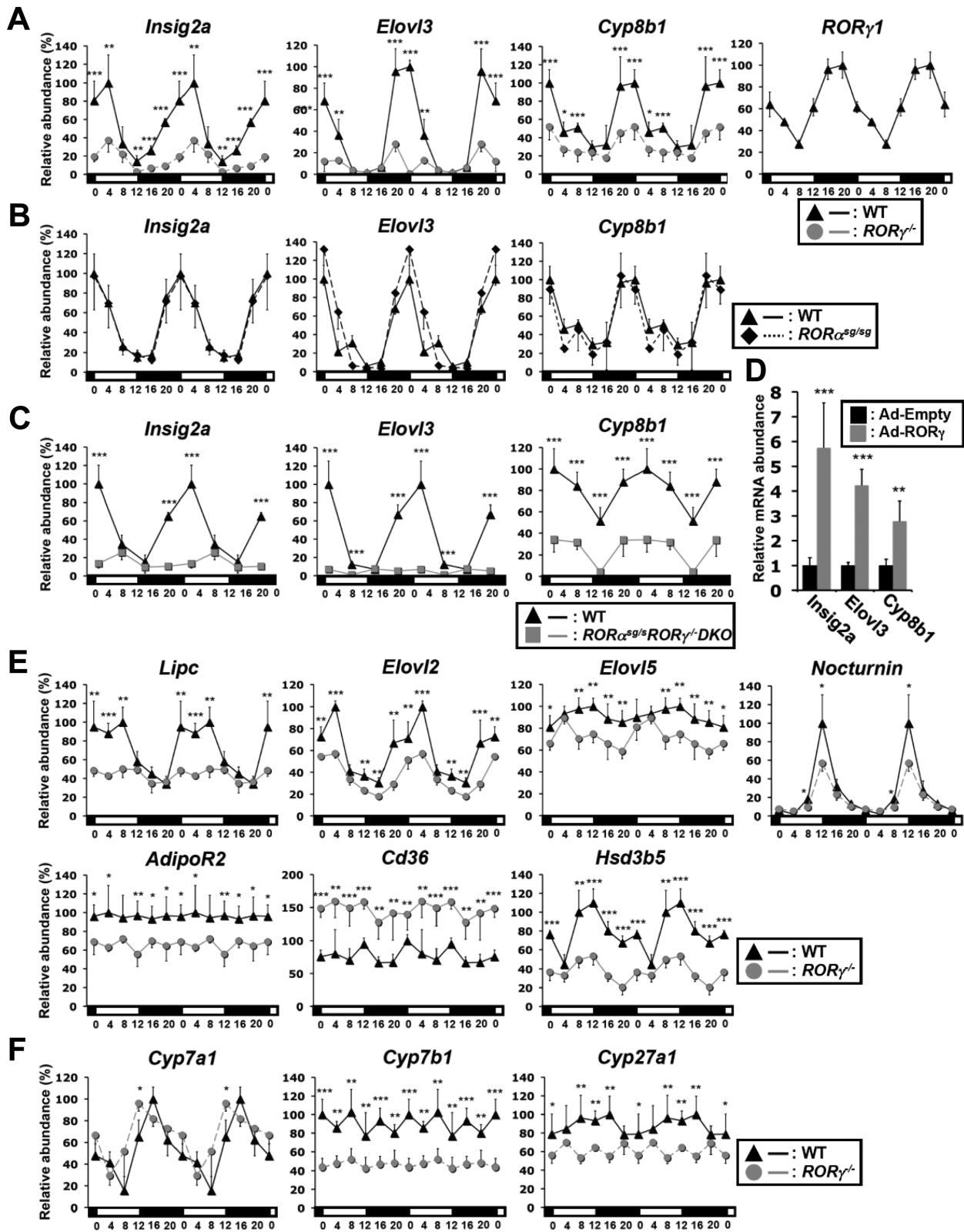


Figure 1. $ROR\gamma$ regulates the circadian expression of *Insig2a*, *Elovl3*, *Cyp8b1* and other lipid metabolic genes. Rhythmic expression patterns of *Insig2a*, *Elovl3* and *Cyp8b1* mRNA in the liver of $ROR\gamma^{-/-}$ (A), $ROR\alpha^{sg/sg}$ (B) and DKO mice (C). The diurnal pattern of $ROR\gamma$ expression in WT liver is shown for comparison (16). Livers ($n = 4$) were collected every 4 or 6 h over a period of 24 h. (D) Adenovirus mediated expression of $ROR\gamma$ in the liver of $ROR\gamma^{-/-}$ mice ($n = 6$) enhanced the *Insig2a*, *Elovl3* and *Cyp8b1* mRNA levels. Circadian expression patterns of several other genes involved in fatty acid/triglycerides metabolism (E) and bile acid synthesis (F) in $ROR\gamma^{-/-}$ mice. The 24 h expression patterns were double-plotted. Data represent mean \pm SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by ANOVA.

their transcription may be regulated indirectly by ROR γ . Although ROR γ is recruited to the *Cyp7a1* promoter, loss of ROR γ had little effect on its level of expression; however, a small shift in circadian phase could be observed (Figure 1F). Exogenous expression of ROR γ by adenovirus enhanced the hepatic expression of *Cyp7b1*, but not that of *Cyp7a1* (Supplementary Figure S1E). Loss of ROR α strongly reduced the hepatic expression of *Cyp7b1* at all ZTs and affected both the level and phase of *Cyp7a1* expression (Supplementary Figure S1F). The repression of *Cyp7b1* in ROR $\alpha^{sg/sg}$ liver is consistent with a previous report showing that *Cyp7b1* is a direct target gene of ROR α (26). Together, these results suggest that although ROR α and ROR γ exhibit a certain degree of redundancy, they largely regulate different sets of *Cyp* genes.

***Insig2a* is directly regulated by ROR γ through RORE**

The regulation of the rhythmic expression of metabolic genes by ROR γ might involve both direct and indirect mechanisms. ChIP-Seq analysis showed that ROR γ was recruited to three sites, referred to as *Insig2a* (A, B, C), that are respectively at (−491/+177), (−3829/−2985) and (−17428/−15521) in the promoter of *Insig2a* (Figure 2A), a gene playing an important regulatory role in lipid metabolism (27,28). Reporter gene analysis revealed that ROR γ increased the transactivation of the reporter via *Insig2a*(A) and *Insig2a*(B) sites, respectively, 14 \times and 5 \times (Figure 2A and B), while no activation was observed through the distal site C (not shown). Deletion analysis of *Insig2a*(A) and *Insig2a*(B) suggested that the activation was mediated through, respectively, the −160/−53 and −3592/−3452 region, which include a RORE-like motif. Mutation of the putative RORE sequence within *Insig2a*(A) and *Insig2a*(B) resulted in a significant reduction in ROR γ -mediated transactivation, suggesting that the transcriptional activation is mediated through direct binding of ROR γ to these ROREs. Addition of an ROR γ -selective antagonist inhibited *Insig2a*(A) and *Insig2a*(B) activation by ROR γ in a dose-responsive manner (Figure 2C). A previous report showed that Rev-Erb α , a transcriptional repressor that binds RORE-like motifs, regulates the expression of *Insig2a* (29). Figure 2D shows that Rev-Erb α repressed the ROR γ -mediated as well as the endogenous activation of *Insig2a*(B) by about 55%, but had no effect on *Insig2a*(A)-mediated activation. These results suggest that the circadian regulation of *Insig2a* by Rev-Erb α may involve competition with ROR γ for the binding to *Insig2a*(B) RORE.

ChIP-QPCR analysis indicated that the association of ROR γ with the *Insig2a*(B) RORE was greater at ZT22 than at ZT10. The increased recruitment at ZT22 correlates with the time at which ROR γ most robustly regulated *Insig2a* (Figure 2E). The high expression of *Insig2a* at ZT22 was also associated with increased H3K9 acetylation (Figure 2F). Both the recruitment of ROR γ and H3K9 acetylation at *Insig2a*(B) were significantly reduced in the liver of ROR $\gamma^{-/-}$ mice. Thus, the increased association of ROR γ and H3K9 acetylation, a marker for transcriptionally active chromatin, at ZT22 correlated with enhanced *Insig2a* transcription.

Because *Insig2a* regulates the proteolytic activation of the sterol regulatory element binding transcription factor 1c (Srebp-1c) and its subsequent translocation to the nucleus (27,28,30), the effect of the down-regulation of *Insig2a* in ROR $\gamma^{-/-}$ liver on Srebp-1c processing was examined. Both the precursor (135 kDa) and proteolytic (55–60 kDa) forms of Srebp-1c were detected in whole liver lysates prepared at ZT4; however, the level of the processed form was consistently increased in ROR $\gamma^{-/-}$ liver (Figure 3A) as well as nuclear Srebp-1 protein (Figure 3B). The increase in nuclear Srebp-1 protein at ZT4 in ROR $\gamma^{-/-}$ mice was associated with a temporal increase in the expression of the Srebp-1 target genes, *Fasn* and *Elovl6*, at ZT4 (Figure 3C). Loss of ROR γ reduced *Srebp-1c* expression at ZT12–16. This was associated with a down-regulation of *Fasn* and *Elovl6* expression. Thus, loss of ROR γ appears to affect Srebp-1c at several levels.

***Elovl3* is directly regulated by ROR γ through RORE**

Elovl3, which circadian expression was also strongly regulated by ROR γ (Figure 1A), contains a putative RORE located at −836 bp in its proximal promoter (Figure 4A). Reporter analysis showed that ROR γ caused a 5-fold increase in the activation of the *Elovl3*(−919/−1) promoter. Mutation (*Elovl3*(ROREm)) and deletion (*Elovl3*(−504/−1) and *Elovl3*(−42/−1)) of the putative RORE significantly reduced the activation by ROR γ . Rev-Erb α was able to inhibit the activation of the *Elovl3*(−919/−1) promoter by competing with ROR γ for RORE binding (Figure 4B). The ROR γ -mediated transactivation was also repressed by the ROR γ -selective antagonist (Figure 4C). ChIP analysis showed that the association of ROR γ with the *Elovl3* proximal promoter was increased particularly at ZT22 along with enhanced H3K9 acetylation (Figure 4D and E). This association was greatly reduced in ROR $\gamma^{-/-}$ liver. Together, these observations suggest that ROR γ regulates the diurnal expression of *Elovl3* directly through RORE interaction.

Analysis of hepatic fatty acids by GC-MS showed that the composition of palmitoleic acid was increased at ZT4 and decreased at ZT20 in ROR $\gamma^{-/-}$ mice (Supplementary Figure S2). The percentage of palmitic acid, oleic acid and linoleic acid at ZT4 was either increased or decreased, while that of docosahexaenoic acid (DHA) was significantly lower in ROR $\gamma^{-/-}$ liver at both ZT4 and ZT20. Fatty acids were also analyzed in ROR $\gamma^{-/-}$ serum collected at ZT4 (Supplementary Table S2). Consistent with the changes in liver, palmitic acid, palmitoleic acid, oleic acid were increased in ROR $\gamma^{-/-}$ mice, while DHA was reduced. These results indicate that loss of ROR γ significantly affects the composition of fatty acids, which may at least in part be related to changes in the regulation of several *Elovl* family genes.

Loss of ROR γ causes changes in triglyceride and bile acid levels

We next examined whether the changes in the hepatic expression of lipid metabolic genes in ROR $\gamma^{-/-}$ mice were associated with alterations in lipid homeostasis. Serum TG levels tended to be lower in ROR $\gamma^{-/-}$ (ND) mice particularly after fasting (Supplementary Figure S3A and B).

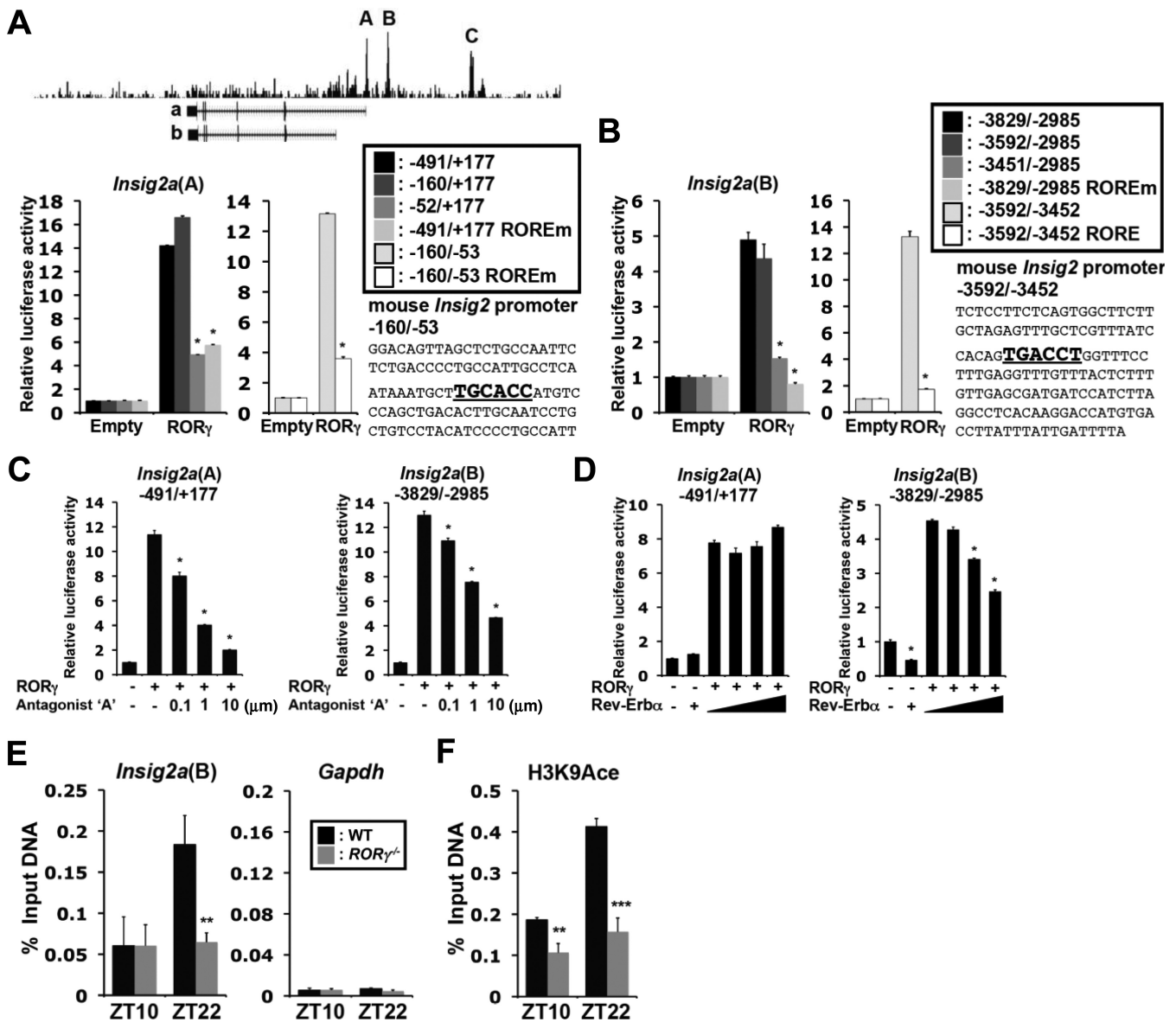


Figure 2. ROR γ directly regulates the transcription of *Insig2a*. (A and B) ChIP-Seq analysis using anti-ROR γ antibody and chromatin prepared from mouse liver tissue identified several ROR γ -binding sites, A–C, in the *Insig2a* gene. Huh-7 cells were co-transfected with p3xFlag-CMV10-ROR γ and a pGL4 reporter plasmid containing the WT or mutated *Insig2a*(A) (–491/+177) or *Insig2a*(B) (–3829/–2985). The ROREs are shown in bold. (C) The ROR γ -selective antagonist ‘A’ inhibited the *Insig2a*(A)- and *Insig2a*(B)-mediated transactivation. (D) Rev-Erb α repressed the *Insig2a*(B)-mediated activation by ROR γ , but not the *Insig2a*(A)-mediated activation. (E) ChIP-QPCR was performed using an anti-ROR γ antibody and chromatin prepared from WT and ROR γ ^{–/–} liver tissues ($n = 4$) collected at ZT10 (low expression of ROR γ) and ZT22 (high expression of ROR γ). Amplification of *Gapdh* gene was used as a negative control. (F) ChIP-QPCR was performed using anti-H3K9Ac.

ROR γ ^{–/–} mice fed a HFD for 6 weeks exhibited considerable changes in TGs without a significant change in their body weights (Supplementary Figure S3C). Serum TG levels were significantly reduced at all ZTs in ROR γ ^{–/–} (HFD) mice (Figure 5A), whereas hepatic TGs were reduced particularly at ZT4 and ZT8 (Figure 5B). The level of circulating free fatty acids, glycerol and total ketone bodies were lower particularly at ZT0 and ZT4 (Figure 5C), whereas that of hepatic free fatty acids was increased in ROR γ ^{–/–} (HFD) mice at ZT4 (Figure 5D), both of which indicates lower fatty acid consumption in ROR γ ^{–/–} (HFD) mice (31). Serum and hepatic TGs were similarly reduced

in ROR γ ^{–/–} (HFD) mice at ZT4 after 16-h fasting and so were levels of free fatty acids and glycerol (Supplementary Figure S3D and E). Adenovirus expression of ROR γ in ROR γ ^{–/–} (HFD) mice did not rescue serum TG levels, suggesting that longer term expression of ROR γ as well as proper circadian pattern of ROR γ expression might be required to impact lipid homeostasis (Supplementary Figure S3F). Similarly to ROR γ ^{–/–} (ND) mice, the hepatic expression of several genes, including *Insig2a*, *Elovl3* and *Lipc* was reduced in ROR γ ^{–/–} (HFD) mice (Supplementary Figure S4A–C).

Figure 6A shows that serum cholesterol and circulating

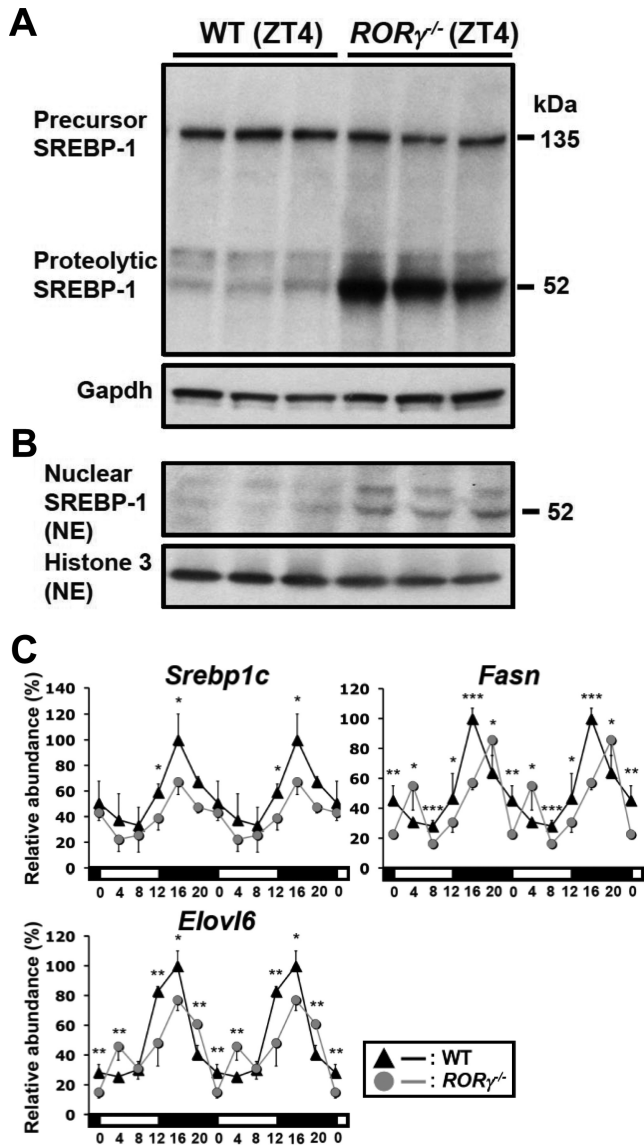


Figure 3. Effects of the loss of ROR γ on Srebp-1. (A and B) Comparison of precursor Srebp-1 (135 kDa) and processed Srebp-1 (52–60 kDa) levels in whole liver lysates and nuclear extracts from WT and ROR γ ^{-/-} mice collected at ZT4. (C) Circadian expression profiles of *Srebp-1c*, and the target genes, *Fasn* and *Elov16*, in the liver of WT and ROR γ ^{-/-} mice. Data represent mean \pm SD, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by ANOVA.

high and low density lipoprotein (HDL and LDL, respectively) levels were significantly reduced in ROR γ ^{-/-} (HFD) mice at all ZTs analyzed, but the reduction was more pronounced at ZT0–4. Hepatic cholesterol levels were also reduced at all ZTs (Figure 6B). However, the loss of ROR γ caused an increase rather than a decrease in peak (around ZT16) expression of *HMG-CoA reductase*, which encodes a rate-limiting enzyme in cholesterol synthesis in the liver (Supplementary Figure S4D). Hepatic bile acids were reduced 40–50% in ROR γ ^{-/-} (HFD) mice at all ZTs, whereas levels of serum bile acids, which reached a peak at ZT0, were greatly reduced at ZT0 and ZT4 in ROR γ ^{-/-} (HFD) mice (Figure 6C). Inversely, expression of exogenous ROR γ in ROR γ ^{-/-} liver by adenovirus injection increased hep-

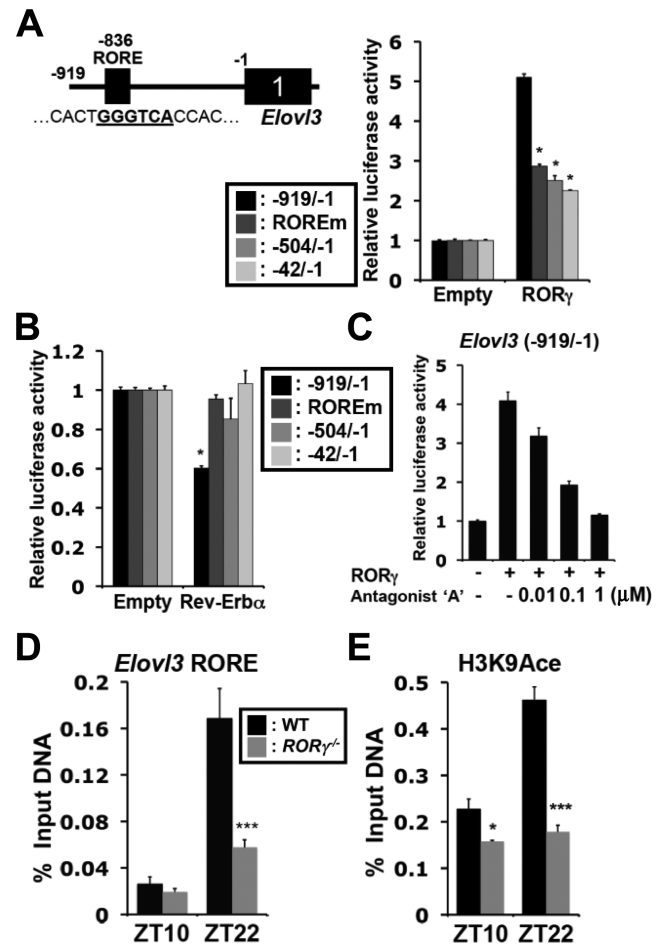


Figure 4. ROR γ directly regulates the transcription of *Elov13*. (A) Huh-7 cells were co-transfected with p3xFlag-CMV10-ROR γ and pGL4 reporter plasmid containing the WT or the RORE-mutated *Elov13*(-1 to -919) promoter region. (B) Rev-Erb α repressed the *Elov13* promoter through the RORE. (C) Inhibition of the ROR γ -induced activation of the *Elov13* promoter by the ROR γ -selective antagonist. (D and E) ChIP analysis. The increased association of ROR γ and elevated H3K9 acetylation at the *Elov13* promoter is ZT-dependent. Data represent mean \pm SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by ANOVA.

atic bile acids (Figure 6D). Levels of total bile acids were also reduced in feces and gallbladder of ROR γ ^{-/-} (HFD) mice as well as the whole-body pool size (the sum of bile acids in liver, gallbladder and small intestines) (Figure 6E). These observations suggest that not increased lipid excretion (Figure 6F), but lower cholesterol levels in the liver combined with a reduction in the expression of genes regulating bile acid synthesis, including *Cyp8b1*, *Cyp27a1* and *Cyp7b1*, might be responsible for reduced bile acid generation in ROR γ -deficient mice. Together these results indicate that ROR γ plays an important role in the diurnal regulation of fatty acid, cholesterol and TG homeostasis. Several other nuclear receptors, particularly FXR and LXR, have been implicated in the regulation of cholesterol and bile acid metabolism (32–34). To examine whether the changes in cholesterol and bile acid metabolism in ROR γ ^{-/-} mice were related to alterations in the expression of other nuclear receptors, we analyzed the expression of *Fxr*, *Lxr*, *Car* and

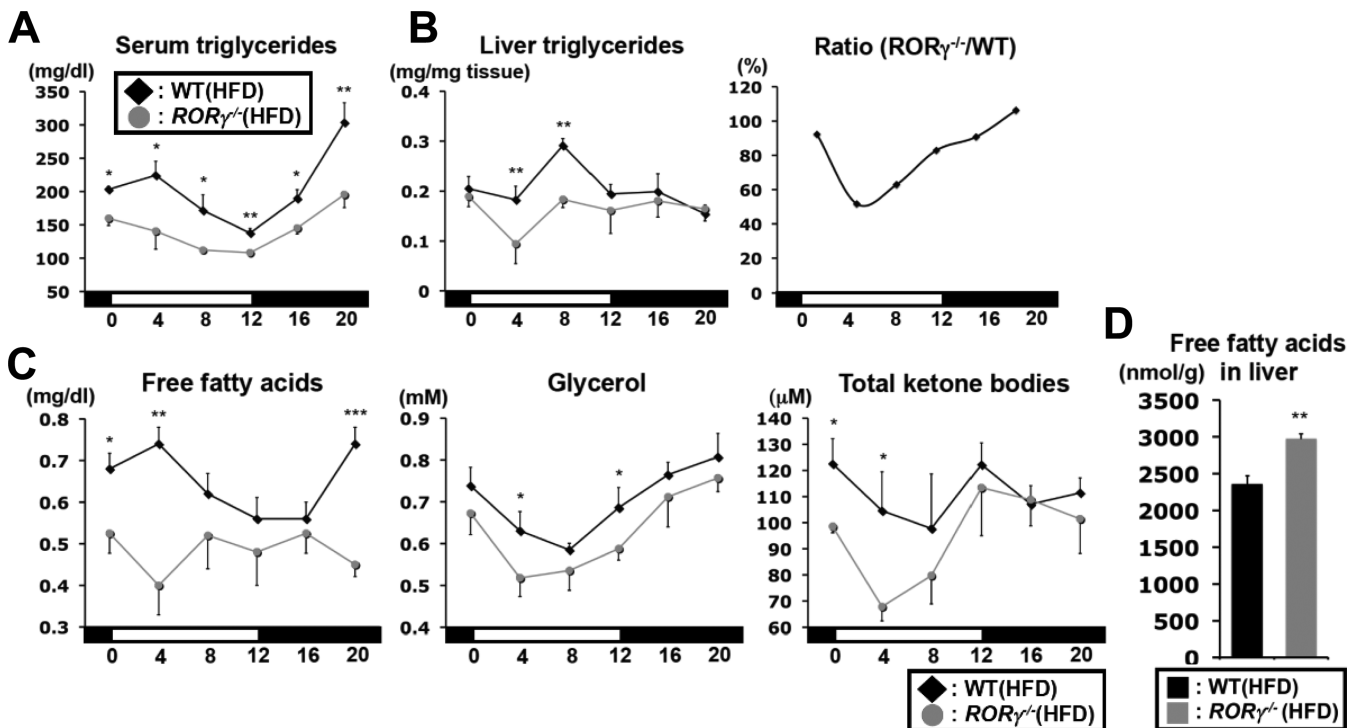


Figure 5. Changes in triglyceride and fatty acid levels in *RORγ*^{-/-}(HFD) mice. Serum (A) and hepatic (B) triglyceride levels were examined WT(HFD) and *RORγ*^{-/-}(HFD) mice ($n = 5$) every 4 h over a period of 24 h. Liver triglycerides at each ZT were plotted as a ratio between *RORγ*^{-/-}(HFD) and WT(HFD) mice. (C) Daily patterns of free fatty acids, glycerol and total ketone bodies were measured in serum from WT(HFD) and *RORγ*^{-/-}(HFD) mice ($n = 5$). (D) Free (unesterified) fatty acids extracted from liver tissue at ZT4 ($n = 8$) were quantified by LC-MS/MS. Data represent mean \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by ANOVA.

Pxr in *RORγ*^{-/-} liver. As shown in Supplemental Figure S4E, loss of ROR γ had little effect on the diurnal pattern of expression of these receptors.

In addition to the reduced hepatic TGs and increased free fatty acid levels (Figure 5) in *RORγ*^{-/-}(HFD) mice at daytime, histological examination of liver sections showed that WT(HFD) mice exhibited more of a microvesicular steatosis phenotype, while *RORγ*^{-/-}(HFD) mice exhibited a more macrovesicular form of steatosis (Supplementary Figure S5A and Table S3). *RORγ*^{-/-} mice also exhibited perivascular inflammation. This was supported by increased expression of various inflammatory genes (Supplementary Figure S5B and Table S4). Blood urea nitrogen was not changed, suggesting that kidney function was not impaired (Supplementary Figure S4F).

Changes in lipid metabolism in liver-specific *RORγ* knockout mice

To determine whether the effects on gene expression were based on the hepatocyte-specific loss of ROR γ rather than the loss of ROR γ in other tissues, we analyzed the expression of several lipid metabolic genes in liver-specific ROR γ -deficient mice (*RORγ*^{flx/flx} *Alb-Cre*⁺). Consistent with our observations in ubiquitous *RORγ* knockout mice, the expression of *Insig2a*, *Elovl3*, *Lipic* and *Hsd3b5* was reduced particularly at ZT20, while the expression of *Cyp8b1*, *Cyp7b1* and *Cyp27a1* was decreased both at ZT8 and ZT20 (Figure 7A). TG, cholesterol and bile acid levels in serum

and liver were significantly reduced at ZT0 or ZT4 in *RORγ*^{flx/flx} *Alb-Cre*⁺ mice fed a HFD (Figure 7B), although the reductions were smaller than in ubiquitous *RORγ*^{-/-} mice. These observations indicate that the changes in gene expression and lipid metabolism were directly related to the loss of hepatic ROR γ expression.

DISCUSSION

The regulation of many metabolic functions in the liver are linked to the daily timing of feeding and under the control of the circadian clock machinery (8,9,35). This includes transcriptional regulation of many metabolic genes by clock proteins, including Bmal1 and Clock (6,10,36,37), as well as by other transcription factors and, as we show in this study, ROR γ 1 (7,14,16,17). Our data indicate that the regulation of lipid metabolic genes by ROR γ is complex and mediated by multiple mechanisms that involve ZT-(in)dependent as well as (in)direct regulation by ROR γ (Figure 7C). The loss of ROR γ results in significantly reduced peak expression of several hepatic genes, including *Insig2a*, *Elovl3*, *Elovl2*, *Cyp8b1* and *Lipic*, without affecting their circadian phase (Figure 1) suggesting that their regulation is mediated by a ZT-dependent mechanism. ChIP-Seq analysis showing an association of ROR γ with the regulatory region of several of these genes, including *Insig2a* and *Elovl3*, suggested that these genes are regulated directly by ROR γ . Studies showing that the diurnal expression of ROR γ is directly regulated by the circadian clock machinery (15,16,18), together

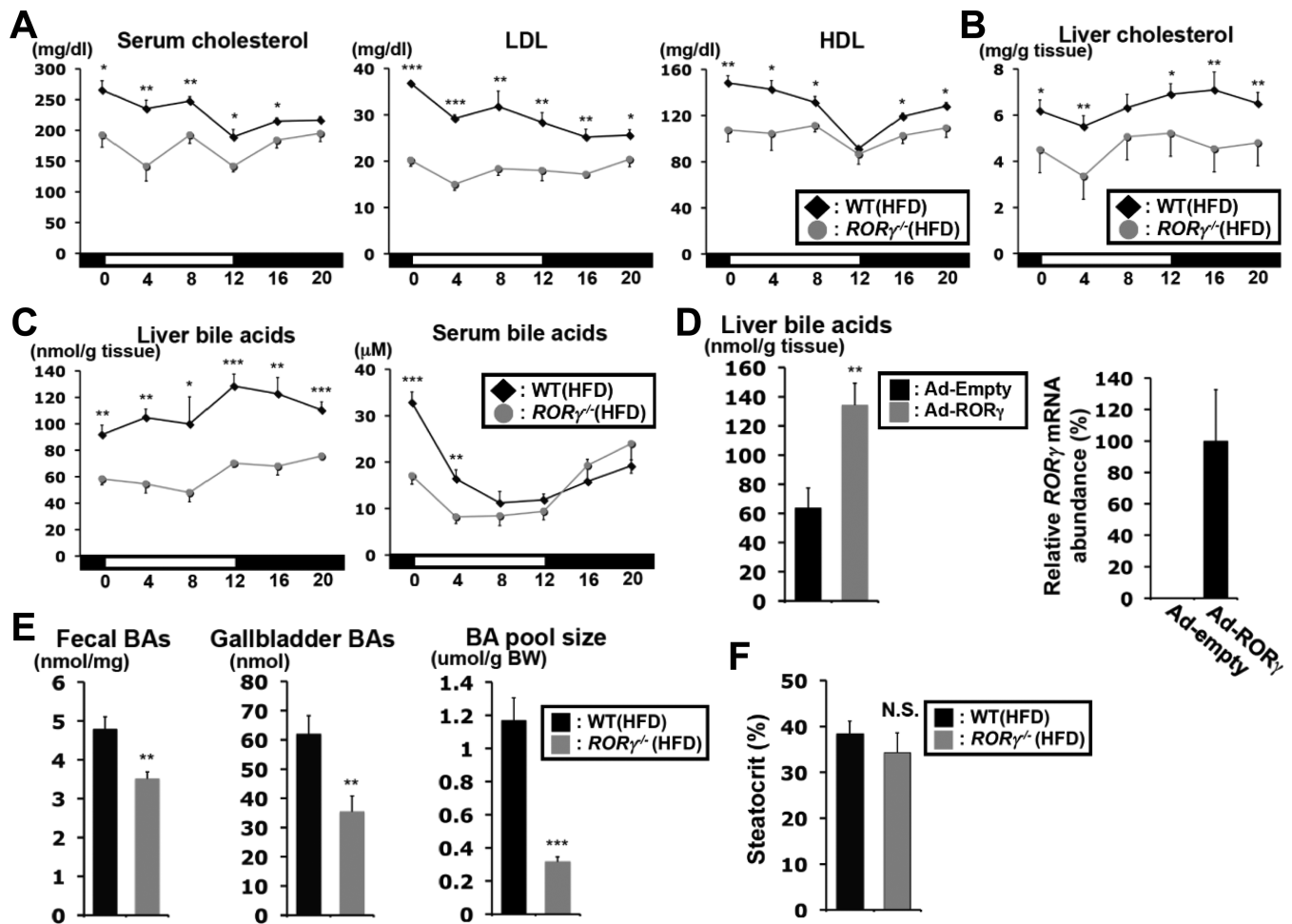


Figure 6. Loss of ROR γ affects the hepatic and circulating levels of cholesterol and bile acids. (A) Comparison of the cholesterol, LDL and HDL levels in serum collected from WT(HFD) and ROR $\gamma^{-/-}$ (HFD) mice ($n = 5$) every 4 h over a period of 24 h. (B) Comparison of the hepatic cholesterol levels ($n = 5$). (C) Total bile acids in the liver and serum from WT(HFD) and ROR $\gamma^{-/-}$ (HFD) mice ($n = 5$). (D) Total bile acids were increased in the liver of ROR $\gamma^{-/-}$ (HFD) mice ($n = 6$) injected with adenovirus expressing ROR γ . Exogenous expression of ROR γ was detected by QRT-PCR. Data represent mean \pm SD. (E) Total bile acids in feces, gallbladder and the whole-body (whole liver, gallbladder and small intestines) were measured in WT(HFD) and ROR $\gamma^{-/-}$ (HFD) mice ($n = 7-9$). (F) Comparison of steatocrit in feces ($n = 8$). Data represent mean \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by ANOVA.

with our findings that the phase of the diurnal pattern of expression of several of these genes closely follows that of ROR γ , suggest a connection between the diurnal regulation of these metabolic genes, the rhythmic expression of ROR γ , and the circadian clock. These observations are further consistent with our hypothesis that ROR γ functions as a transcriptional mediator that regulates the rhythmic expression of certain metabolic genes downstream of the clock machinery (Figure 7C). Although the expression of certain genes, including *Cyp7b1*, was reduced in both ROR $\gamma^{-/-}$ and ROR $\alpha^{sg/sg}$ mice suggesting a certain degree of redundancy, loss of ROR α had little effect on the expression of *Insig2a* and *Elovl3*, suggesting that these genes are regulated in a highly ROR γ -selective manner. This, together with our recent ChIP-Seq analysis (22), indicates that ROR α and ROR γ largely regulate different sets of metabolic genes and therefore control different metabolic pathways.

Analysis of the ROR γ cisome suggested that ROR γ directly regulates the transcription of a number of lipid

metabolic genes (Figure 2 and Supplementary Figure S1D). This was supported by our promoter analyses showing that ROR γ was able to activate the *Insig2a* and *Elovl3* promoters through ROREs in their respective upstream promoter region. The enhanced recruitment of ROR γ to ROREs within *Insig2a* and *Elovl3* promoters at ZT22 and the higher level of histone H3K9 acetylation are consistent with the increased activation of these promoters at ZT20-4 (Figures 2 and 4). Together, these data are consistent with the concept that ROR γ positively regulates the transcription of these genes by binding ROREs in their promoter region in a ZT-dependent manner and around the time when ROR γ is most highly expressed. These results support our hypothesis that ROR γ functions as an intermediary regulator between the circadian clock machinery and the transcriptional regulation of the rhythmic expression of certain lipid metabolic genes (Figure 7C). However, some of these genes might be directly regulated Bmal1/Clock in conjunction with direct regulation by ROR γ .

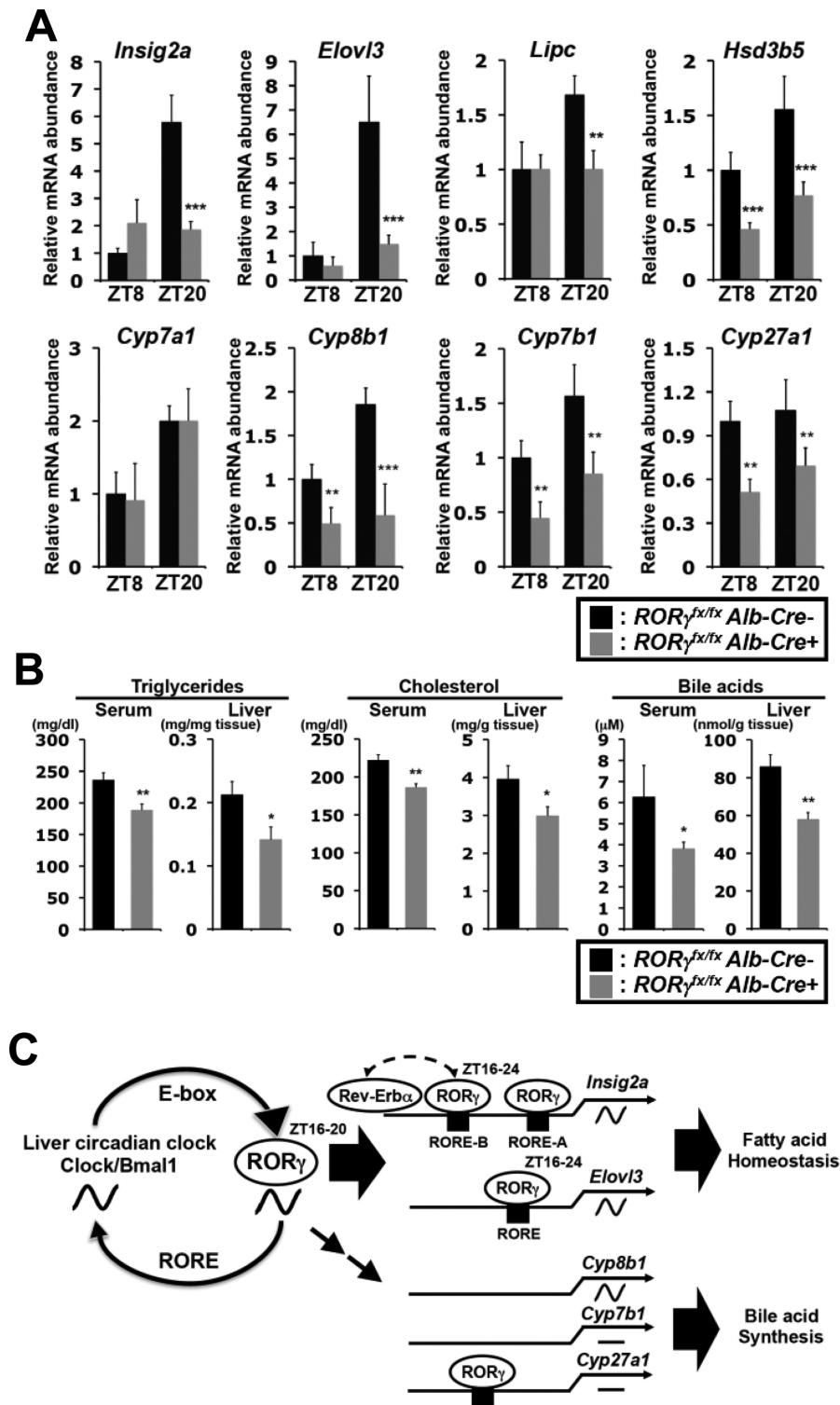


Figure 7. Analysis of liver-specific *RORγ*-deficient mice. (A) Comparison of the expression of several lipid metabolic genes in liver from *RORγ^{flox/flox} Alb-Cre* negative and positive mice ($n = 4-5$) at ZT8 and ZT20. Data represent mean \pm SD. (B) Triglycerides, Cholesterol and bile acids levels in serum and liver collected from *RORγ^{flox/flox} Alb-Cre* negative and positive mice on HFD ($n = 8$) at ZT0 or ZT4. Data represent mean \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by ANOVA. (C) Regulation of lipid metabolic genes by *RORγ* in liver involves multiple mechanisms. *RORγ*, which circadian expression is under direct control of the clock machinery, strongly regulates the peak expression of *Insig2a*, *Elovl3* and *Cyp8b1* in phase with the optimal expression of *RORγ*. ChIP-Seq and promoter analyses indicated that the diurnal expression of some metabolic genes, such as *Insig2a* and *Elovl3*, are regulated directly by *RORγ*. This is consistent with the concept that *RORγ* functions as a link between the circadian clock and its regulation of several metabolic genes and downstream metabolic outputs. However, certain metabolic genes, including *Cyp7b1* and *Cyp27a1*, are regulated by *RORγ* in a ZT-independent manner likely involving indirect mechanisms. Association of *RORγ* with *Cyp27a1* may suggest regulation by both direct and indirect mechanisms. Although, *RORγ* regulates *Cyp8b1* in a ZT-dependent manner, no evidence was obtained to support regulation through a direct mechanism.

The hepatic expression of several arrhythmic genes, including *Cyp7b1*, *Elovl5*, *AdipoR2*, *Cd36* and *Hsd3b5*, was reduced in *ROR γ ^{-/-}* liver at all ZTs suggesting that they are regulated by ROR γ in a ZT-independent manner. Moreover, our ChIP-Seq analysis showed no evidence for the recruitment of ROR γ to some of these genes such as *Cyp7b1*, *Cd36* and *Hsd3b5*, suggesting that they may be regulated by ROR γ through an indirect mechanism (Figure 7C). Although peak expression of *Cyp8b1* is reduced in *ROR γ ^{-/-}* mice, ChIP-Seq (Supplementary Figure S1D), ChIP and reporter analyses (Takeda, Y., unpublished data) did not support direct regulation of *Cyp8b1* by ROR γ suggesting that it may also be regulated by an indirect mechanism.

The regulation of lipid metabolic genes is complex and other nuclear receptors, including LXRs and FXR, PXR and CAR (32,33), have been implicated in the regulation of lipid metabolism. Several studies have provided evidence for crosstalk between different nuclear receptor signaling pathways at multiple levels that include competition for the same DNA binding site, synergistic activation through adjacent binding sites, as well as regulation of the expression of one receptor by another (22). Thus, genes indirectly regulated by ROR γ might be regulated through changes in the expression of other nuclear receptors or transcription factors. However, the loss of ROR γ expression did not significantly change the diurnal pattern of expression of *Lxr*, *Pxr*, *Fxr* and *Car* (Supplementary Figure S4E) suggesting that changes in lipid metabolism did not relate to alterations in the level of expression of these receptors. Since the activity of these nuclear receptors is regulated by their interaction with (ant)agonists, including various oxysterols, bile acids and xenobiotics, an interesting alternative possibility is that metabolic changes in *ROR γ ^{-/-}* liver might alter the level of certain nuclear receptor (ant)agonists thereby changing the activation of these receptors and subsequently the transactivation of their target genes. Crosstalk between receptor pathways is further complicated by the observations showing that certain oxysterol function as ligands for both LXR and ROR receptors (2,32,38).

Crosstalk between RORs and Rev-Erb receptors has been well established (1,7). Rev-Erbs, which function as transcriptional repressors, can bind similar ROREs as RORs and compete with RORs for binding thereby antagonizing their action. A recent study reported that the loss of Rev-Erb α eliminated the repression of *Insig2a* expression at ZT8–12, the time of optimal Rev-Erb α expression, thereby almost totally abolishing the rhythmic expression of *Insig2a* (29). Our data showing that Rev-Erb α was able to repress *Insig2a*(B)- and *Elovl3*(RORE)-dependent activation by ROR γ is consistent with this. Together these observations suggest that the rhythmic expression of *Insig2a* and *Elovl3* involves repression by Rev-Erb α at ZT8–12, whereas the optimal expression of *Insig2a* and *Elovl3* at ZT20–4 is mediated in part by ROR γ . These observations support the conclusion that ROR γ plays a critical role in the positive transcriptional regulation of the *Insig2a* and *Elovl3* expression at ZT16–24 (Figure 7C).

Insig2a regulates hepatic fatty acid synthesis by inhibiting proteolytic activation of Srebp-1c (27,28,39). The downregulation of *Insig2a* in *ROR γ ^{-/-}* liver resulted in increased processing and a higher level of nuclear Srebp-1c and a

small temporal increase in Srebp-1c target gene expression at ZT4 (Figure 3A–C). However, the relative increase in processed Srebp-1c had only a limited effect on the expression of its target genes suggesting that additional mechanisms of regulation might be involved, such as regulation by Lipin-1 (40). Srebp-1c expression and activation is controlled at multiple levels and further study is needed to determine the precise role of Srebp-1c in the regulation of lipid homeostasis by ROR γ .

The altered expression of lipid metabolic genes in *ROR γ ^{-/-}* mice was associated with changes in serum and hepatic lipid composition and hepatic histology. These changes were more pronounced and distinct in *ROR γ ^{-/-}* (HFD) mice than in mice fed an ND. The latter might be related to different mechanisms of regulation of lipid metabolism between ND and HFD mice and the higher lipid levels in serum and liver in mice fed an HFD. In addition, differences in the type and amount of lipids between WT(HFD) and *ROR γ ^{-/-}* (HFD) livers may be linked to the increase in macrovesicular steatosis observed in *ROR γ ^{-/-}* mice.

The most distinctive change seen in *ROR γ ^{-/-}* mice was a reduction in bile acid levels in liver and serum as well as the whole body pool size. This reduction might at least in part be due to the repression of the hepatic expression of several genes involved in bile acid synthesis pathways, including *Cyp8b1*, *Cyp7b1* and *Cyp27a1*. These findings suggest that ROR γ positively regulates hepatic bile acid synthesis. This is supported by data showing that exogenous expression of ROR γ in liver enhanced the level of hepatic bile acids and that liver-specific ROR γ -deficient mice also showed a reduction in bile acid levels (Figures 6D and 7B). Recent studies showed that ROR α and ROR γ regulate the expression of several sulfotransferases, including *Sult2A1*, which has been implicated in bile acid synthesis (41), through their binding to RORE in their promoter (42,43). Thus, the combined regulation of several sterol metabolic genes by ROR γ is likely responsible for the changes in cholesterol and bile acid homeostasis observed in *ROR γ ^{-/-}* mice.

We further show that ROR γ deficiency causes a change in fatty acid composition in liver and serum (Supplementary Figure S2 and Table S2), which might be in part related to the observed alterations in the expression of several *Elovl* genes (Figure 1A and E) and likely involves changes in the expression of other lipid metabolic genes. Although TGs in serum and liver were reduced in *ROR γ ^{-/-}* mice (Figure 5A and B), it is not likely due to increased fatty acid consumption, but rather due to reduced rate of fatty acid/TG synthesis because the reduction of serum free fatty acids, glycerol and total ketone bodies indicates lower fatty acid consumption (Figure 5C). In addition, the reduced blood levels of TGs and free fatty acids in *ROR γ ^{-/-}* (HFD) mice might be a contributory factor in the improved insulin sensitivity observed in these mice (22,44,45).

In summary, our study indicates that the transcriptional regulation of lipid metabolic genes by ROR γ is complex and involves multiple mechanisms. The regulation by ROR γ can be ZT-dependent and -independent and occur through a direct or indirect mechanism as shown for *Insig2a* and *Cyp7b1*, respectively. The circadian regulation of ROR γ expression by the clock machinery together with our

observations that ROR γ directly regulates the transcription of a number of lipid metabolic genes, support our hypothesis that ROR γ acts downstream of clock proteins and functions as an important link between the circadian clock machinery and its regulation of certain metabolic genes. Our study indicates that ROR γ is an important participant in the diurnal regulation of lipid metabolism in the liver.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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