Published online 29 June 2012 *Nucleic Acids Research, 2012, Vol. 40, No. 17* ***8519–8535***
doi:10.1093/nar/gks630

RORγ directly regulates the circadian expression of clock genes and downstream targets *in vivo*

Yukimasa Takeda[1], Raja Jothi[2], Veronique Birault[3] and Anton M. Jetten[1,*]

[1]Cell Biology Section, [2]Systems Biology Group, Biostatistics Branch, Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA and [3]Medicinal Chemistry, GlaxoSmithKline Ltd., Medicines Research Centre, Stevenage, UK

Received May 2, 2012; Revised May 31, 2012; Accepted June 2, 2012

ABSTRACT

In this study, we demonstrate that the lack of retinoic acid-related orphan receptor (ROR) γ or α expression in mice significantly reduced the peak expression level of *Cry1, Bmal1*, *E4bp4*, *Rev-Erbα* and *Per2* in an ROR isotype- and tissue-selective manner without affecting the phase of their rhythmic expression. Analysis of RORγ/RORα double knockout mice indicated that in certain tissues RORγ and RORα exhibited a certain degree of redundancy in regulating clock gene expression. Reporter gene analysis showed that RORγ was able to induce reporter gene activity through the RORE-containing regulatory regions of *Cry1*, *Bmal1, Rev-Erbα* and *E4bp4.* Co-expression of Rev-Erbα or addition of a novel ROR antagonist repressed this activation. ChIP-Seq and ChIP–Quantitative real-time polymerase chain reaction (QPCR) analysis demonstrated that *in vivo* RORγ regulate these genes directly and in a Zeitgeber time (ZT)-dependent manner through these ROREs. This transcriptional activation by RORs was associated with changes in histone acetylation and chromatin accessibility. The rhythmic expression of *RORγ1* by clock proteins may lead to the rhythmic expression of RORγ1 target genes. The presence of RORγ binding sites and its down-regulation in *RORγ*$^{-/-}$ liver suggest that the rhythmic expression of *Avpr1a* depends on RORγ consistent with the concept that RORγ1 provides a link between the clock machinery and its regulation of metabolic genes.

INTRODUCTION

The retinoic acid-related orphan receptors α-γ (RORα-γ or NR1F1-3) constitute a subfamily of the nuclear receptor superfamily (1). Through alternative splicing and promoter usage, each ROR gene produces several isoforms that are expressed in a highly tissue-specific manner. RORs have been implicated in the regulation of embryonic development and in several metabolic and immunological processes (1–12).

RORs are among a number of nuclear receptors involved in the regulation of circadian behavior and clock gene expression (1,8,13–18). In mammals, the suprachiasmatic nucleus (SCN) functions as the central circadian pacemaker that integrates light–dark cycle input and synchronizes the autonomous oscillators in peripheral tissues (19–21). At the molecular level the clockwork consists of an integral network of several interlocking positive and negative transcriptional and translational feedback loops. The heterodimeric complex consisting of brain and muscle ARNT-like (Bmal1) and circadian locomotor output cycles kaput (Clock) or its paralog neuronal PAS domain protein 2 (Npas2), are involved in the positive control of the oscillator, while two cryptochrome (Cry) and three period proteins (Per) are part of the negative control mechanism. Several accessory pathways, in one of which the Rev-Erb nuclear receptors play a role (19–21), further regulate the core loop. Although several studies have provided evidence for a regulatory function of RORs, particularly roles for RORα and RORβ in the SCN, in the regulation of circadian rhythm and clock gene expression, their precise function and regulation are not yet fully understood (13,15,16,18,22–27). Less is known about the role of RORγ in the regulation of circadian rhythm. Recently, we reported that *Npas2* is directly regulated by RORγ suggesting that it may be an important modulator of the circadian clock in peripheral tissues (25).

To obtain greater insights into the roles of RORs in the regulation of circadian rhythm, we examined the effects of the loss of RORα and/or RORγ on clock gene expression in several peripheral tissues of ROR knockout mice. Our data showed that particularly the loss of RORγ reduced the peak expression level of several clock genes in a tissue-selective manner without significantly affecting the phase of their rhythmic expression pattern. The effect of

*To whom correspondence should be addressed. Tel: +1 919 541 2768; Fax: +1 919 541 4133; Email: jetten@niehs.nih.gov

RORα deficiency on the expression of these clock genes was limited largely to the kidney. Loss of both RORα and RORγ in double knockout (DKO) mice had a greater impact on the peak expression levels of these clock genes than single knockouts suggesting a certain degree of redundancy between RORα and RORγ. We demonstrated that RORs regulate the transcription of *Cry1, Bmal1*, *Rev-Erbα* and *E4bp4* directly as indicated by reporter gene and mutation analysis. This transcriptional activation was inhibited by ROR antagonists. Chromatin immunoprecipitation sequencing (ChIP-Seq) and ChIP-QPCR analyses indicated that RORs were associated with these ROR response elements (ROREs) *in vivo* supporting the conclusion that these clock genes are directly regulated by RORs. We further showed that this transcriptional regulation was ZT-dependent and associated with changes in histone acetylation and chromatin structure. Recent studies demonstrated that clock proteins and Rev-Erbs are important regulators of energy homeostasis and lipid/glucose metabolism indicating a connection between the controls of circadian clock and various metabolic pathways (14,20,28,29). Although RORs regulate the expression of several metabolic genes, whether and what role they play in this interrelationship has yet to be determined (4,14,30,31). The rhythmic expression of *RORγ1* by Bmal1/Clock and Rev-Erb might lead to the rhythmic expression of RORγ1 target genes. The in-phase expression pattern of *RORγ1* and the arginine vasopressin receptor 1a gene (*Avpr1a*), the dramatic decrease in *Avpr1a* expression in $ROR\gamma^{-/-}$ liver, and the ChIP-Seq data are consistent with the hypothesis that RORs function as intermediate regulators, providing a link between clock proteins and their regulation of metabolic genes.

MATERIALS AND METHODS

Experimental animals

C57BL/6 staggerer ($ROR\alpha^{sg/sg}$) mice, a natural mutant strain containing a 6.5 kb deletion in *RORα* that prevents translation of the LBD, were purchased from Jackson Laboratories (Bar Harbor, ME, USA) (32). These mice exhibit a very similar phenotype as mice with a targeted disruption of *RORα* (1,33). C57BL/6 $ROR\gamma^{-/-}$ and $ROR\alpha^{sg/sg}ROR\gamma^{-/-}$ DKO mice were described previously (4,10). Mice were supplied *ad libitum* with NIH-A31 formula and water and maintained at 25°C on a constant 12 h light:12 h dark cycle. 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 were approved by the Institutional Animal Care and Use Committee at the NIEHS.

RNA isolation

To study gene expression during circadian time, tissues were excised from WT, $ROR\gamma^{-/-}$ and $ROR\alpha^{sg/sg}$ mice every 4 h over a period of 24 h, processed overnight in RNA*later*® solution (Ambion, Austin, TX, USA) at 4°C, and then stored at –80°C until use. Tissues from DKO and littermate WT mice were collected every 6 h over a period of 24 h. Tissues were homogenized in RNeasy lysis buffer (RLT) (Qiagen, Valencia, CA, USA) with a Polytron PT-3000 (Brinkmann Instruments, Westbury, NY, USA). RNA was then extracted using a QIAshredder column and RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Hepa1-6 stable cell lines and brown adipocytes were directly lysed in RLT buffer and extracted as described for tissues. Cells were treated for 24 h with and without 10 μM of the inverse ROR agonist T0901317 (Sigma-Aldrich, St Louis, MO, USA) (34) or with 1 μM the RORγ-selective antagonist 'A' (R)-N-(1-((4-methoxy-phenyl)sulfonyl)-4-methyl-1,2,3,4-tetrahydroquinolin-7-yl)-2,4,6-trimethylbenzene-sulfonamide provided by GlaxoSmithKline (V. Birault, in preparation) before RNA extraction.

QRT–PCR analysis was performed by SYBR Green I or the TaqMan system (Applied Biosystems, Foster City, CA, USA) to quantify gene expression. The RNA was reverse-transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems). The reactions were carried out in triplicate in a 7300 Real Time PCR system (Applied Biosystems) using 20 ng of cDNA and the following conditions: 2 min at 45°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. All the results were normalized by the amount of 18 S rRNA or *Gapdh* mRNA. Product specificity was confirmed by melting curve analysis. All QRT–PCR primer and probe sequences are listed in Supplementary Table S1.

Hepa1-6, HepG2 and brown preadipocyte stable cell lines

Brown adipocytes were isolated from postnatal day 2 WT and $ROR\gamma^{-/-}$ mice by collagenase digestion as described previously (25). Preadipocytes were immortalized by infection with pBabe retrovirus encoding SV40-Large T antigen as described previously (25). Brown preadipocyte cell lines BAT(RORγ1), BAT(RORα4), BAT(RORγ1E502Q) and BAT(RORα4ΔAF2) stably expressing Flag-RORγ1, Flag-RORα4, mutant Flag-RORγ1(E502Q) or Flag-RORα4ΔAF2 (35) and Hepa1-6(RORα) and Hepa1-6(RORγ) stably expressing Flag-RORα, Flag-RORγ, were generated by infection with the respective pLXIN retrovirus (Clontech, Palo Alto, CA, USA) and subsequent selection in G418 (25). The stable cell lines BAT(E) and Hep1-6(E) containing the pLXIN empty vector were used as controls. The expression and the nuclear localization of ROR proteins were confirmed by western blot analysis and confocal microscopy, respectively. Cell lines established from three to five individual clones were examined in QPCR and ChIP analysis.

ChIP assay

The ChIP assays were performed using a ChIP assay kit from Millipore (Billerica, MA, USA) according to the manufacturer's protocol with minor modifications. In short, livers isolated from WT, $ROR\alpha^{sg/sg}$ and $ROR\gamma^{-/-}$ mice at the circadian time indicated, were homogenized with a polytron PT 3000 (Brinkmann Instruments) and

cross-linked by 1% formaldehyde for 20 min at room temperature. After a wash in phosphate buffered saline (PBS), an aliquot of the cross-linked chromatin was sonicated and incubated overnight with anti-RORα, anti-RORγ or anti-Clock antibody (sc-6062, sc-28559 or sc-6927, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-acetylated histone H3K9Ac antibody (07-352; Millipore). After incubation with protein G agarose beads for 2 h, and several washes, DNA–protein complexes were eluted. The cross-links were reversed by overnight incubation at 65°C in the presence of 25 mM NaCl and subsequent digestion with RNase A and proteinase K, followed by purification of the ChIPed-DNA. The ChIPed-DNA was then amplified by QPCR; all reactions were carried out in triplicate. The procedure for ChIP analysis using Hepa1-6 cells was similar as described for tissues except that 2×10^6 cells were cross-linked with 4% formaldehyde for 10 min and the immunoprecipitation was performed with anti-Flag M2 affinity gel (Sigma-Aldrich). The sequences of the primers used for ChIP-QPCR are listed in Supplementary Table S2.

ChIP-Seq analysis

ChIPed-DNA was prepared as described (36) using RORγ- and RORα-specific antibodies generated against amino acids 129–231 and 121–213 in mouse RORγ1 and RORα4, respectively. ChIP-Seq analysis was performed by the NIH Intramural Sequencing Center and data were analyzed as reported previously (36).

Formaldehyde-assisted isolation of regulatory elements

Formaldehyde-assisted isolation of regulatory elements (FAIRE) was performed as previously described (37). Briefly, mouse livers were cross-linked and sonicated as described for the ChIP assay. Samples were centrifuged and the DNA in the supernatants was isolated by three consecutive extractions with phenol–chloroform–isoamyl alcohol (25:24:1). After the final extraction, the FAIRE samples were reverse cross-linked as described for the ChIP assay. The enrichment of fragmented genomic DNA in the FAIRE samples relative to each input DNA was measured in triplicate by QPCR.

Reporter gene assay

The RORγ1(–1338/–1) and RORγ1(–1338/–968) promoter regions, and the RORE-containing regulatory regions of *Cry1* (+22976/+23214), *E4bp4* (+5828/+6150), *Bmal1* (–650/+105), *Rev-Erbα* (–657/+38) and *Avpr1a* (–68969/–67651), and *Avpr1a* (–63512/–61359) were inserted into the pGL4.10 or pGL4.27 reporter plasmids. Corresponding mutant pGL4.10/27 plasmids, containing mutations within the E-boxes (CACGTG into CAATTG) or ROREs were generated using a Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The sequences were verified by DNA sequencing. Human hepatoma Huh-7 cells were co-transfected with pCMVβ-Gal, pCMV-Sport6-Bmal1, pCMV-Sport6-Clock, pCMV-Sport6-Cry1 and the pGL4.10 RORγ1(–1338/–1) or pGL4.27-RORγ1(–1338/–968) reporter plasmid as indicated using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h incubation, the luciferase and β-galactosidase were analyzed with a Luciferase Assay Substrate (Promega) and Luminescent β-galactosidase Detection Kit II (Clontech). All transfections were performed in triplicate, and each experiment was repeated at least twice. Except for *Rev-Erbα*, all ROREs were mutated from A/GGGTCA to A/GAA TCA. The RORE1 and RORE2 within the *Rev-Erbα* promoter were mutated from GTGTCACTGGGGCA to ATATCACTGGGGCA and from GTGTCACTGGG GCA to GTGTCACTGAAGCA, respectively.

RESULTS

RORγ1, not *RORα*, exhibited a strong oscillatory pattern of expression in peripheral tissues

As shown in Figure 1, *RORγ1* exhibited a strong oscillatory pattern of expression in mouse liver consistent with previous results with samples from a different set of mice (25). *RORγ1* also exhibited a robust oscillatory pattern of expression in brown adipose tissue (BAT), kidney and small intestines (jejunum) with peak expression around ZT16 (Figure 1), a few hours earlier than in liver. *RORα1* and *RORα4* expression did not show a strong circadian regulation in liver, BAT, WAT or kidney (Figure 1). Because of its negligible level of expression, the circadian regulation of *RORα* was not analyzed in the small intestines. The oscillatory pattern of *RORγ* expression was not changed in BAT or kidney of $RORα^{sg/sg}$ mice. The loss of RORγ did not affect *RORα4* expression in kidney and only slightly reduced its expression in BAT, while it had little effect on the expression of *RORα1* (Figure 1). These data indicated that *RORα* and *RORγ* are regulated largely independently from each other and that *RORγ1* rather than *RORα1* or *RORα4* exhibits a robust oscillatory pattern of expression in these peripheral tissues.

Circadian regulation of clock gene expression in RORα and RORγ null mice

A number of studies have provided evidence for a role of RORs in the transcriptional regulation of several clock genes (13–18,21,23,24). Two recent studies reported on the transcriptional regulation of *Npas2* in $RORγ^{-/-}$ and $RORα^{sg/sg}$ mice (24,25). However, information on the regulation of clock genes by RORs *in vivo* has remained rather limited. We, therefore, compared the circadian expression of several clock genes in more detail in a few peripheral tissues of WT, $RORα^{sg/sg}$ and $RORγ^{-/-}$ mice. In liver, BAT, WAT, kidney and small intestines of WT mice, *Bmal1*, *E4bp4*, *Cry1* and *Rev-Erbα* mRNA reached an optimal level of expression at ZT0 (*Bmal1* and *E4bp4*), ZT16-20 (*Cry1*) and ZT4-ZT8 (*Rev-Erbα*) in agreement with previous studies (Figure 2A and B) (13,18,38–40). In $RORγ^{-/-}$ mice, *Cry1* mRNA expression was consistently suppressed up to 30–40% in all four tissues with the largest decrease seen in the small intestines (Figure 2A). These observations suggested that RORγ plays an important role in the regulation of *Cry1*

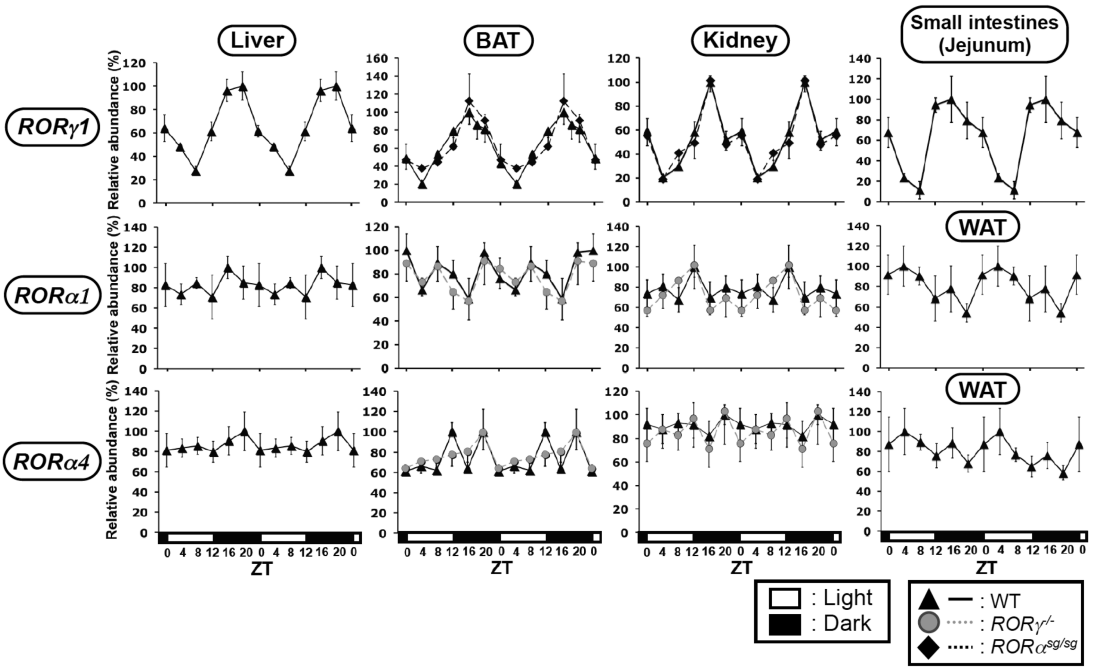

Figure 1. Oscillatory pattern of expression of *RORγ1*, *RORα1* and *RORα4* in mouse BAT, kidney, WAT and small intestines. Liver, BAT, kidney, WAT and small intestines (jejunum) from WT, $RORα^{sg/sg}$ and $RORγ^{-/-}$ mice ($n = 4$) were isolated every 4 h over a period of 24 h. Subsequently, the expression of *RORγ1*, *RORα1* and *RORα4* was analyzed by QRT-PCR. The oscillatory expression patterns in liver were similar to those previously reported (25) using samples from different WT mice. The 24 h expression pattern was double-plotted. The open and solid boxes indicate the 12 h light and dark periods, respectively. Data represent mean ± SD; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by ANOVA.

expression across several peripheral tissues. The oscillatory pattern of expression of *Bmal1* in liver, BAT, kidney and small intestines from $RORγ^{-/-}$ mice was very similar to those in WT mice or slightly reduced at peak levels in some tissues (Figure 2A). A small shift in the circadian phase of *Bmal1* expression was observed in the $RORγ^{-/-}$ small intestines. The peak expression of *Rev-Erbα* was reduced in the liver, BAT and small intestines of $RORγ^{-/-}$ mice, but was not significantly altered in $RORγ^{-/-}$ kidney. *E4bp4* expression was selectively suppressed by about 40–50% at peak levels in $RORγ^{-/-}$ liver and kidney, but changed little in BAT and the small intestines of $RORγ^{-/-}$ mice. Peak expression of *Per2* was somewhat elevated in $RORγ^{-/-}$ liver, slightly diminished in BAT and kidney, and very significantly reduced in the small intestines of $RORγ^{-/-}$ mice. These observations support the hypothesis that RORγ regulates the circadian expression of *Cry1*, *Bmal1*, *Rev-Erbα*, *E4bp4* and *Per2* in a tissue-selective manner.

We next examined the expression of these clock genes in liver, BAT, WAT and kidney of $RORα^{sg/sg}$ mice. As shown for $RORγ^{-/-}$ mice, the phase of the oscillatory pattern of expression of *Bmal1*, *Cry1*, *Rev-Erbα*, *E4bp4* and *Per2* was not significantly altered in any of the $RORα^{sg/sg}$ tissues analyzed (Figure 2B). In contrast, a significant reduction in the peak level of expression of *Bmal1, E4bp4* (ZT18-20) and *Per2* (ZT12) was observed in the kidneys of $RORα^{sg/sg}$ mice. *Rev-Erbα* peak expression at ZT8 was reduced in the kidney and WAT of $RORα^{sg/sg}$ mice, but was unaltered in the liver and BAT. No significant changes in the rhythmic expression pattern of *Cry1* were observed in any of the four tissues of $RORα^{sg/sg}$ mice. These results demonstrated that the loss of RORα affected the expression of certain clock genes in a limited and tissue-selective manner. RORα regulated clock gene expression particularly in the kidney, a tissue in which it is relatively highly expressed (Y. Takeda, data not shown).

RORα and RORγ redundancy

Previous studies have indicated that RORα and RORγ exhibited a certain degree of redundancy with respect to their effects on gene expression (4,7). To examine whether RORα and RORγ exhibited any redundancy in regulating clock gene expression, we examined the circadian expression of clock genes in liver, BAT and kidney collected from $RORα^{sg/sg}RORγ^{-/-}$ DKO mice. As shown in Figure 3 and Supplementary Figure S1, the expression of several clock genes was reduced to a greater extent in certain tissues of DKO mice than those of $RORγ^{-/-}$ or $RORα^{sg/sg}$ single knockout mice. Particularly, *Cry1* and *Bmal1* mRNA expression were down-regulated to a significantly greater extent in the liver and BAT of DKO mice than in single knockout mice. The peak expression of *Npas2* and *Clock* was also notably more reduced in DKO liver compared with their reported expression in single knockout mice (25). Little redundancy between RORα and RORγ was observed in the kidney. These results indicated that in some peripheral tissues RORα and RORγ exhibit a certain degree of redundancy regulating clock gene expression.

RORs regulate clock gene expression through ROREs

RORs bind as monomers to RORE consisting of the consensus sequence AGGTCA preceded by a 6-bp A/T rich region, in the promoter regulatory region of target genes (1). RORE have been identified in several clock genes; *Cry1*, *Bmal1* and *Rev-Erbα* contain two putative RORE, while the *E4bp4* and *Clock* genes contain one site (Supplementary Figure S2A) (13,15,16,18,22,23,38,41,42). However, the significance of these RORE and the role of RORγ1 in clock gene regulation *in vivo* are still far from understood. Both RORα and RORγ were able to induce the *Luc* reporter driven by the RORE-containing regulatory region of *Cry1*, *E4bp4*, *Bmal1*, *Rev-Erbα* and *Clock* in Huh-7 cells. RORα and RORγ induced promoter activity of *Cry1*, respectively, 24- and 8-fold, *Bmal1* 16- and 8-fold, *Rev-Erbα* 7- and 5-fold, 3- and 2-fold (Supplementary Figure S2B) and *Clock* 4- and 2-fold (Supplementary Figure S3A) compared with cells transfected with the empty vector. Except for the RORE2 in *Rev-Erbα*, point mutations in each of these RORE greatly reduced the activation of the *Luc* reporter by either RORα or RORγ. Double mutations in RORE1 and RORE2 in *Cry1* and *Bmal1* almost totally abolished the activation by RORs. These observations suggest that both RORE are important in the regulation of *Cry1* and *Bmal1* and that RORE1 is most critical for the ROR-mediated regulation of *Rev-Erbα, E4bp4* and *Clock* consistent with previous observations (13,15,22). Similar results as for Huh-7 cells were obtained in HEK293 cells (Y. Takeda, data not shown).

Inhibition of ROR-mediated activation of clock gene promoters by Rev-Erbα and ROR antagonists

Previous studies reported that the transcriptional repressor Rev-Erbα can compete with RORs for the binding to RORE (14,15,38,43). Figure 4A shows that exogenous Rev-Erbα expression effectively inhibited the *Cry1*(RORE1/2), *Bmal1*(RORE1/2), *Rev-Erbα*(RORE1)-dependent activation by either RORγ or RORα in a dose-dependent manner, likely by competing with RORα

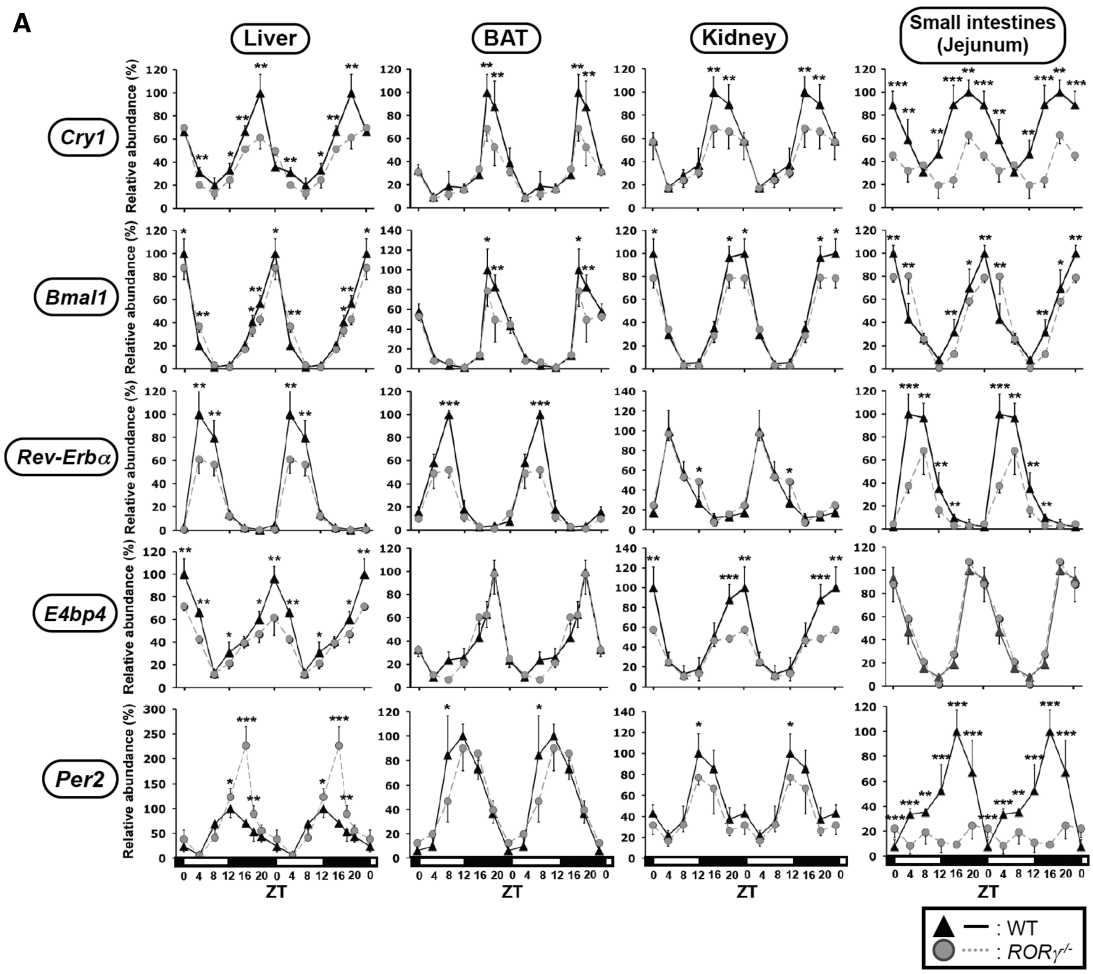

Figure 2. Comparison of the circadian expression pattern of *Cry1*, *Bmal1*, *Rev-Erbα*, *E4bp4* and *Per2* in several peripheral tissues from WT, $ROR\gamma^{-/-}$ and $ROR\alpha^{sg/sg}$ mice. Liver, BAT, kidney and small intestines from WT, $ROR\gamma^{-/-}$ (**A**) and $ROR\alpha^{sg/sg}$ (**B**) mice ($n = 4$) were isolated every 4 h over a period of 24 h and expression of was analyzed by QRT-PCR as indicated. The 24 h expression pattern was double-plotted. Data represent mean ± SD; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by ANOVA.

(continued)

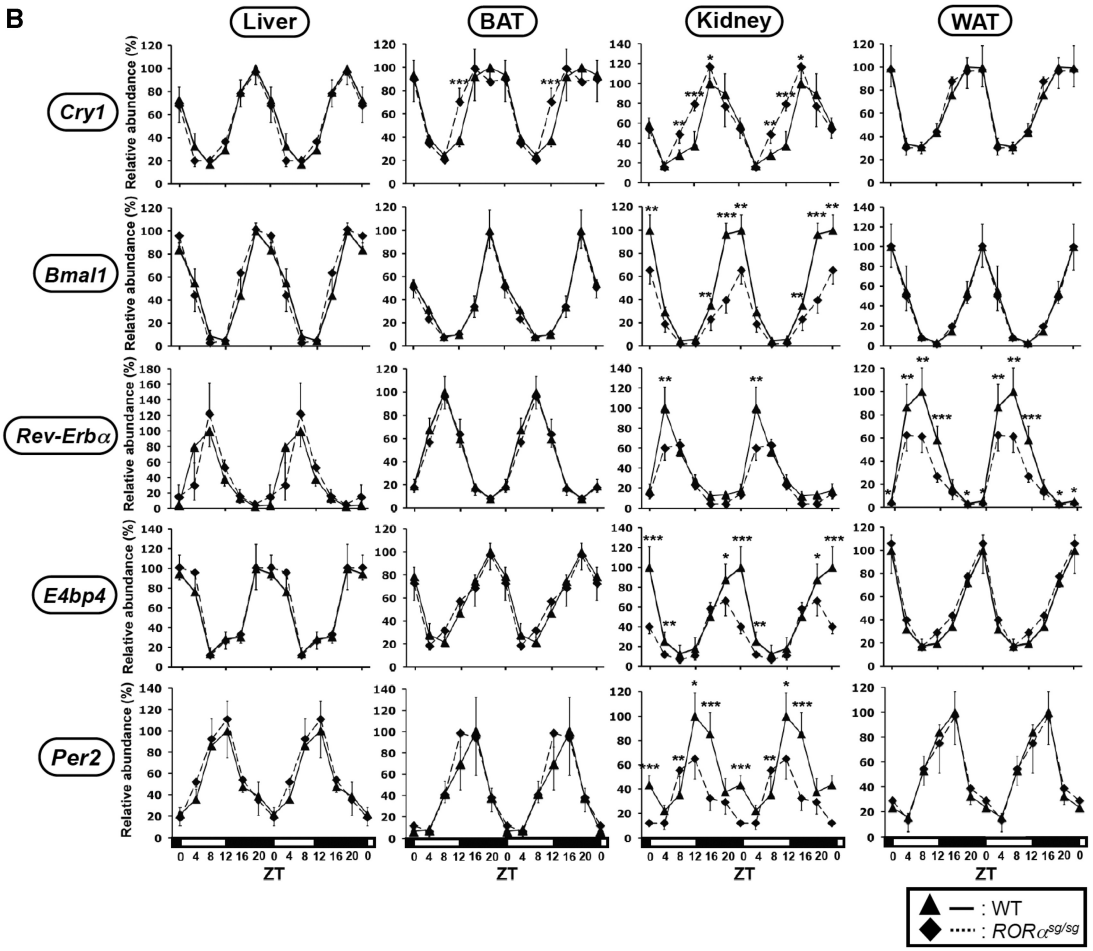

Figure 2. Continued.

and RORγ for the same ROREs. Rev-Erbα had only a small effect on the *E4bp4*(RORE)-dependent activation by RORs (Figure 4A).

Recently several ligands have been identified that function either as ROR agonists or antagonists (34,44–46). To examine the regulation of clock genes by RORs further, we examined the effect of the RORα and RORγ inverse agonist, T0901317, and the RORγ-selective antagonist 'A' on the activation of the RORE-containing regulatory region of several clock genes by RORs. T0901317 significantly inhibited *Cry1*(RORE1/2)-, *Bmal1*(RORE)-, *Rev-Erbα*(RORE)-, *E4bp4*(RORE)- and *Clock*(RORE)-dependent *trans*-activation of the reporter by both RORα and RORγ in Huh-7 cells (Figure 4B and Supplementary Figure S3B). In contrast, the RORγ-selective antagonist 'A' effectively inhibited the RORγ-induced *trans*-activation at concentrations 10× lower than T0901317, but had little effect on the activation mediated by RORα (Figure 4B).

To analyze the role of RORs and the effect of the RORγ-selective antagonist 'A', on the endogenous expression of *Cry1, Bmal1*, *Rev-Erbα* and *E4bp4,* we generated Hepa1-6 cells stably expressing the empty vector, Flag-RORα4 or Flag-RORγ1 (25). Expression of *RORα* and *RORγ* mRNA was greatly elevated in Hepa1-6(RORα4) and Hepa1-6(RORγ1), respectively (Figure 4C). Exogenous expression of *RORα4* did not affect the levels of *RORγ1* mRNA, neither did *RORγ1* affect the expression of *RORα*, consistent with our conclusion that *RORα* and *RORγ1* are regulated independently from each other. Most importantly, RORγ1 and RORα4 significantly increased the endogenous expression of *Cry1, Bmal1*, *Rev-Erbα*, *E4bp4* and *Clock* compared with Hepa1-6(Empty) cells (Figure 4C and Supplementary Figure S3C). The addition of the RORγ-selective antagonist 'A' significantly reduced the expression of clock genes in Hepa1-6(RORγ1) cells, but had little effect in Hepa1-6(RORα4) cells (Figure 4D).

As shown for Hepa1-6 cells, *Baml1*, *Cry1*, *Rev-Erbα* and *E4bp4* expression was also increased in brown preadipocyte cell lines, BAT(RORα4) and BAT(RORγ1), stably expressing Flag-RORα4 and Flag-RORγ1, respectively (Supplementary Figure S4). However, the expression of these clock genes was not changed in BAT(Flag-RORαΔAF2) and BAT(RORγE502Q), expressing Flag-RORαΔAF2 or Flag-RORγE502Q, mutants defective

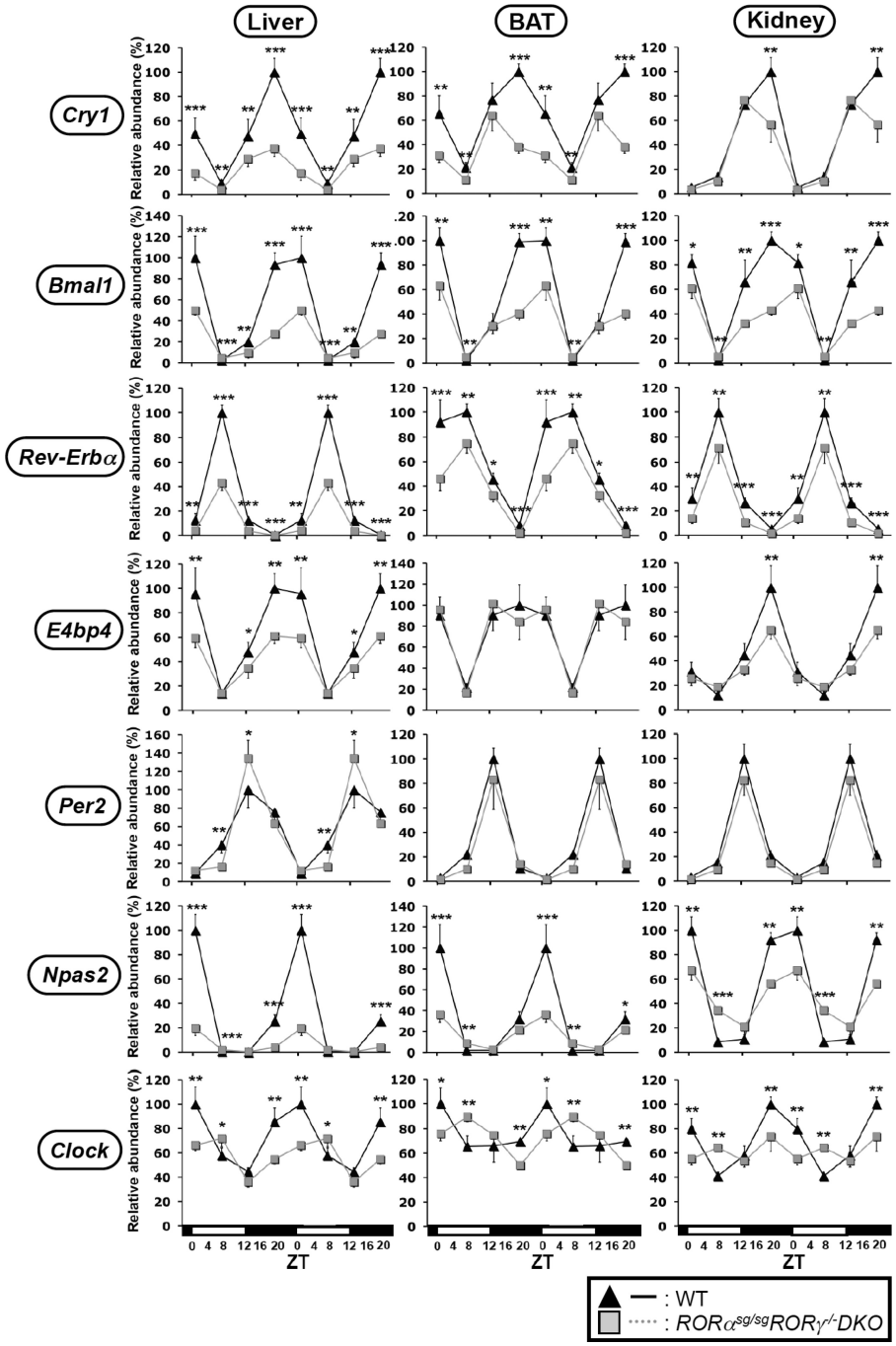

Figure 3. Analysis of the circadian expression pattern of *Cry1, Bmal1, Rev-Erbα*, *E4bp4*, *Per2*, *Npas2* and *Clock* in DKO mice. Liver, BAT and kidney were isolated at ZT2, ZT8, ZT14 and ZT20 from WT and $RORα^{sg/sg}$ $RORγ^{-/-}$ DKO mice ($n = 4$) and the expression of clock genes examined by QRT-PCR. The level of expression was normalized to the expression peak of littermate WT mice controls. Data represent mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA.

in their *trans*-activation function. Treatment of BAT(RORγ1) cells with the inverse agonist T0901317 inhibited the increased expression of *Cry1*, *Bmal1*, *Rev-Erbα* and *E4bp4*. All together, these observations are consistent with the conclusion that the expression of these clock genes is regulated by RORγ and RORα and is dependent on the activation domain of RORs.

Enhanced association of RORα and RORγ with the RORE-containing regulatory regions of other clock genes *in vivo* correlated with increased chromatin accessibility

To determine whether these clock genes were directly regulated by RORα/γ *in vivo*, we analyzed our genome-wide maps of RORα/γ binding sites derived from ChIP-Seq analysis using ChIPed-chromatin from

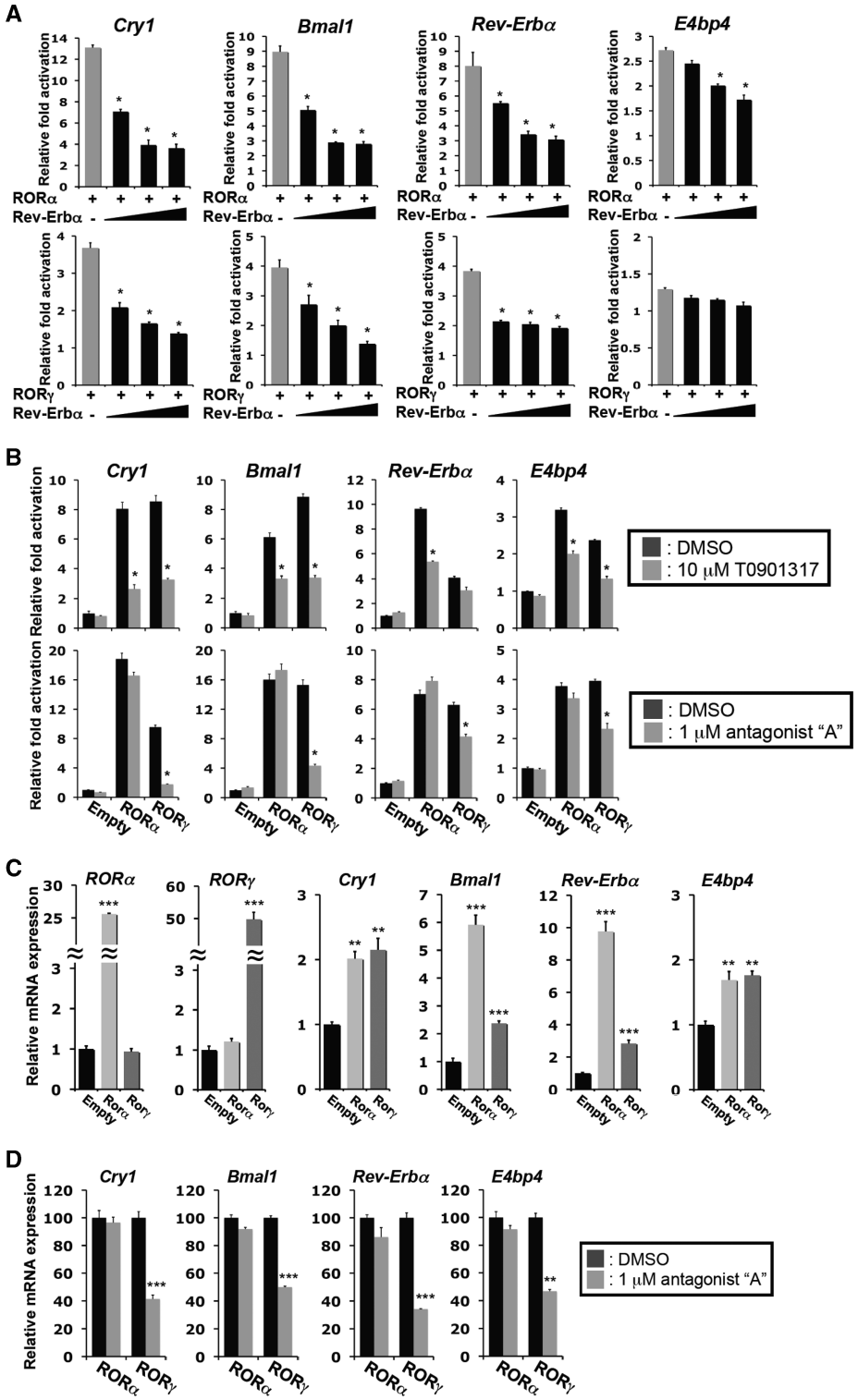

Figure 4. Rev-Erbα and ROR antagonists inhibited the activation of the RORE-containing regulatory regions by RORs. (**A**) Rev-Erbα co-expression inhibited the activation of the *Cry1*, *Bmal1*, *Rev-Erbα* and *E4bp4* regulatory regions by RORs. Huh-7 cells were transfected with p3xFlag-CMV10-RORγ or p3xFlag-CMV10-RORα, pGL4.27 reporter plasmid driven by the RORE-containing regulatory region of *Cry1*, *Bmal1*, *Rev-Erbα* or *E4bp4*, and increasing concentrations (ROR:Rev-Erbα 1:0.3; 1:1; 1:2) of p3xFlag-CMV10-Rev-Erbα. Twenty-four hours later cells were assayed for reporter gene activity. (**B**) Inhibition of transcriptional activation by the ROR antagonists, T0901317 and compound A. Huh-7 cells transfected with p3xFlag-CMV10-RORγ or p3xFlag-CMV10-RORα, and the pGL4.27 reporter plasmid driven by the RORE-containing regulatory region of *Cry1*, *Bmal1*, *Rev-Erbα* or *E4bp4*, were treated with the indicated antagonist. Data represent mean ±SEM; $*P < 0.01$ by ANOVA. (**C**) Exogenous expression of RORγ1 or RORα4 in Hepa1-6 cells increased the expression of *Cry1, Bmal1*, *Rev-Erbα* and *E4bp4*. The expression of clock genes in Hepa1-6 cells ($n = 5$) stably expressing the empty vector, Flag-RORα4 or Flag-RORγ1 was examined by QRT-PCR analysis. The level of clock gene expression in Hepa1-6(Empty) was normalized to 1. (**D**) Treatment of Hepa1-6 cells with the RORγ-selective antagonist compound A (1 μM) repressed the induction of clock gene expression by RORγ, but not that by RORα. Data represent mean ±SEM; $**P < 0.01$, $***P < 0.001$ by ANOVA.

ZT22 mouse liver, for clock gene-associated ROR binding sites. This analysis identified RORα and RORγ binding peaks at RORE-containing regions near or within the *Cry1*, *Npas2, Bmal1* and *E4bp4* genes (Figure 5A). The strongest peaks were associated with *Cry1* and *Npas2*, while a moderate association was found with *Bmal1* and *E4bp4*. Low associations were found with ROREs within *Rev-Erbα* and *Clock* (data not shown). Most importantly, the strong ROR-binding peaks (indicated by arrows) for *Cry1*, *Bmal1* and *E4bp4* corresponded to the RORE-containing regions studied in Supplementary Figure S2. In the case of *Npas2*, the strong ROR-binding peak corresponded to the *Npas2*-RORE described recently (24,25).

To examine the specificity of the recruitment of RORs to the RORE-containing regulatory region of the *Bmal1*, *Cry1*, *Rev-Erbα* and *E4bp4* genes *in vivo*, ChIP-QPCR was carried out using ChIPed liver DNA from WT, $RORα^{sg/sg}$ and $RORγ^{-/-}$ mice (Figure 5B and Supplementary Figure S3D). Both RORα and RORγ were found to be associated with these RORE-containing regulatory regions in WT liver. As expected, ChIP analysis with the RORγ antibody did not show a significant increase in amplification of these regulatory regions in $RORγ^{-/-}$ liver; however, RORγ was still recruited to these RORE regions in $RORα^{sg/sg}$ liver in agreement with the specificity of the RORγ antibody. ChIP analysis with RORα antibody yielded the inverse results. Non-RORE containing regions within *Gapdh* and clock genes, which served as negative controls, showed low amplification.

Because of the strong oscillatory pattern of expression of *RORγ1*, we compared ChIP-QPCR analysis with liver tissues collected at ZT22, a time close to the peak expression of *RORγ1* and *Cry1* and *Bmal1*, with those isolated at ZT10, when these genes are expressed at lower levels. We hypothesized that RORγ1 would be associated with the *Bmal1*(RORE1/2) and *Cry1*(RORE1/2) regions particularly at the time when they are most highly expressed. Figure 5C confirmed that RORγ1 was more efficiently recruited to these regulatory regions in ZT22 liver than in ZT10 liver. No increased amplification was observed with samples from $RORγ^{-/-}$ liver at either ZT. RORγ1 showed a weaker association with the *Rev-Erbα*(RORE1) and *E4bp4*(RORE1) regions (Figure 5C). The recruitment of RORγ1 to the *Rev-Erbα*(RORE1) was higher at ZT22 than ZT10 and correlated inversely with the level of *Rev-Erbα* expression.

The association of RORα and RORγ with the RORE-containing regulatory regions of the *Bmal1*, *Cry1*, *Rev-Erbα* and *E4bp4* was supported by ChIP analysis using an anti-Flag M2 antibody and chromatin isolated from Hepa1-6(Empty), Hepa1-6(RORα4) Hepa1-6(RORγ1) cells. As shown in Figure 5D, RORα and RORγ were strongly associated with ROREs in the *Bmal1*(RORE1/2) and *Cry1*(RORE1/2) regions and to a lesser extend to those of *Rev-Erbα* and *E4bp4*. No significant recruitment of RORs was observed in Hepa1-6(Empty) cells, to distal sites or the *Gapdh* promoter, which served as negative controls. Collectively, the ChIP-Seq and ChIP-QPCR data are in agreement with the concept that both RORα and RORγ regulate several clock genes directly through their interaction with specific ROREs.

Chromatin structure

Changes in gene expression are often associated with alterations in chromatin structure, histone methylation and acetylation. We, therefore, examined whether the association of RORs with the *Bmal1*(RORE1/2) and *Cry1*(RORE1/2) regions correlated with changes in histone acetylation and chromatin accessibility. Levels of histone acetylation, including H3K9Ac have been reported to correlate positively with actively transcribed genes (47). ChIP analysis with DNA samples from livers isolated at ZT10 and ZT22 showed that the level of H3K9Ac associated with the *Cry1*(RORE1/2) and *Bmal1*(RORE1/2) regions was considerably higher at ZT22 than at ZT10 (Figure 6A). This correlated with the high level of association of RORα and RORγ with these regions (Figure 5A and B) and the elevated expression of these clock genes at ZT22. The H3K9Ac signal was significantly reduced in $RORγ^{-/-}$ liver, not affected in $RORα^{sg/sg}$ liver, and further decreased in DKO mice. These observations indicate that the recruitment of particularly RORγ is associated with actively transcribed chromatin and elevated expression of *Bmal1* and *Cry1*.

To assess chromatin accessibility, we performed FAIRE, which has been used as a tool to identify actively transcribed genes (37). FAIRE analysis on the *Cry1*(RORE1/2) and *Bmal1*(RORE1/2) regions with chromatin isolated from liver at ZT10 and ZT22 showed that the FAIRE signals were markedly higher at ZT22, a time at which *Cry1* and *Bmal1* are highly expressed, compared with that of ZT10 (Figure 6B). There was no significant change in FAIRE signal at the distal region of the *Cry1* gene. Loss of RORγ or RORα expression led to a reduction in FAIRE signal at ZT22. This observation is consistent with our H3K9Ac data and suggests that the binding of RORs may promote a more open chromatin structure at these regulatory sites.

Transcriptional activation of the *RORγ1* promoter by Clock/Bmal1 heterodimers was repressed by Cry1 and correlated with changes in chromatin accessibility

As shown in Figure 1, *RORγ1* has a strong oscillatory pattern of expression. Previous studies reported that the *RORγ1* promoter contains two successive E-boxes (E1 and E2) and is activated by Clock/Bmal1 heterodimers (18,48). Reporter gene analyses in human hepatoma Huh-7 cells confirmed that Clock/Bmal1 greatly enhanced *RORγ1*(–1338/–1) proximal promoter activity and that mutation of either E1 or E2 significantly reduced the activation, while the double mutation totally abolished the induction by Clock/Bmal1. Co-expression with the transcriptional repressor Cry1 almost completely blocked the activation of the *RORγ1* promoter by Clock/Bmal1 (Figure 7A and B). To determine whether Clock protein was associated with the E-box-containing *RORγ1*(–1179/–1042), we performed ChIP analysis using chromatin isolated from mouse liver and an anti-Clock antibody. Our data showed that Clock protein was

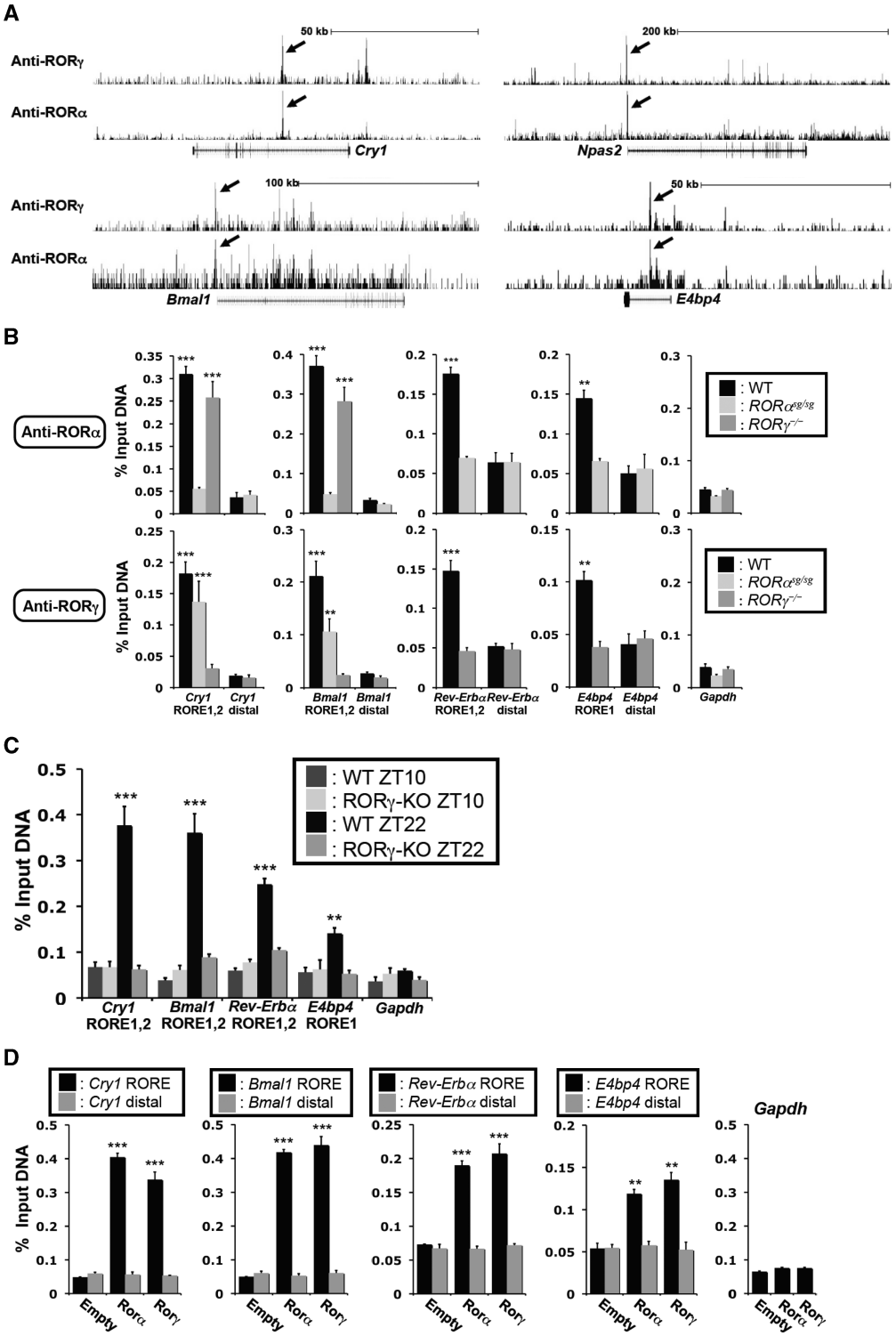

Figure 5. RORα and RORγ were associated with the RORE-containing regulatory regions of *Cry1, Bmal1*, *Rev-Erbα* and *E4bp4 in vivo*. (**A**) Representative view of ChIP-Seq results using either anti-RORγ or -RORα antibody in mouse liver tissue in *Cry1*, *Npas2*, *Bmal1* and *E4bp4* genes. Arrows indicate the peaks corresponding to ROREs studied in this article. Gene tracks were taken from the UCSC Genome Browser using mouse mm9 reference genome. (**B**) Recruitment of RORα and RORγ to the ROREs in *Cry1*, *Bmal1*, *Rev-Erbα* and *E4bp4 in vivo*. ChIP-QPCR analysis was performed with chromatin isolated from livers of WT, $RORα^{sg/sg}$ and $RORγ^{-/-}$ mice ($n = 4$) collected at ZT22 and anti-RORα or -RORγ antibodies. QPCR amplification of distal sites and *Gapdh* were used as negative controls. (**C**) The recruitment of RORγ to the ROREs in *Cry1*, *Bmal1*, *Rev-Erbα* and *E4bp4* was ZT-dependent. ChIP-QPCR was performed using an anti-RORγ antibody and chromatin from WT livers collected at ZT10 (low expression of RORγ) or ZT22 (high expression of RORγ). (**D**) Recruitment of RORα and RORγ to the RORE-containing regulatory regions of *Cry1*, *Bmal1*, *Rev-Erbα* and *E4bp4* in Hepa1-6 cells. ChIP analysis was performed with chromatin isolated from Hepa1-6(Empty), Hepa1-6(RORα4) and Hepa1-6(RORγ1) cells and anti-Flag M2 antibody. QRT-PCR amplification in samples from Hepa1-6(Empty) cells and of the *Gapdh* gene served as negative controls. Data represent mean ±SEM; $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA.

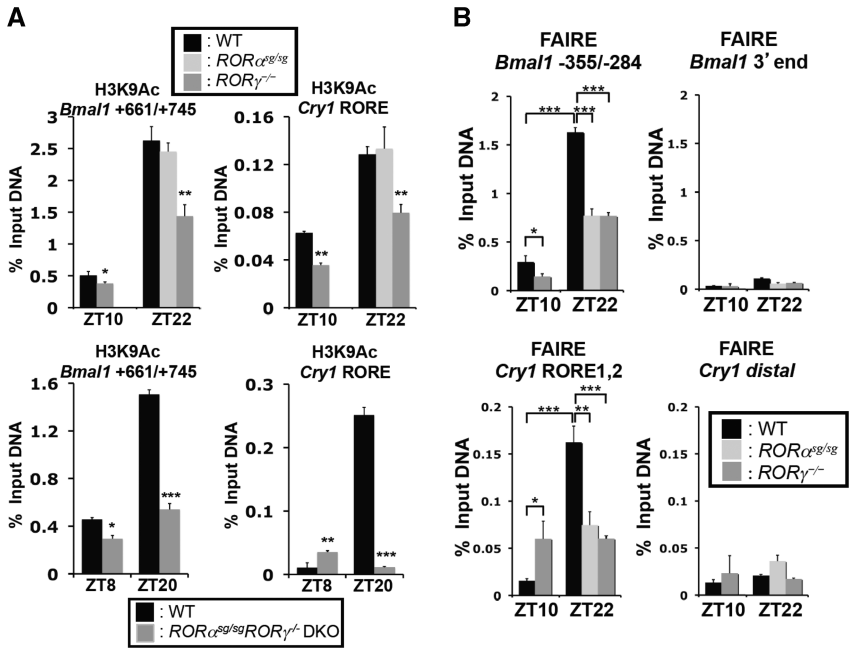

Figure 6. The recruitment of RORα and RORγ to the RORE-containing regulatory regions of *Cry1* and *Bmal1* was associated with chromatin modifications. (**A**) The association of H3K9Ac with the RORE-containing regulatory region of *Cry1* and *Bmal1* was analyzed by ChIP analysis using chromatin samples prepared from liver of WT, $RORα^{sg/sg}$, $RORγ^{-/-}$ and $RORα^{sg/sg}RORγ^{-/-}$ DKO mice ($n = 4$) and an anti-H3K9Ac antibody. (**B**) DNA accessibility at the RORE sites was assessed by FAIRE-QPCR analysis. FAIRE-QPCR at the RORE-containing region indicated and at a downstream distal site was performed using chromatin prepared from WT, $RORα^{sg/sg}$ and $RORγ^{-/-}$ livers collected at ZT10 or ZT22. Data represent mean ±SEM; $*P < 0.05$, $***P < 0.001$ by ANOVA.

recruited to the E-box region of the proximal *RORγ1*(–1179/–1042) promoter region *in vivo* (Figure 7C). The recruitment of Clock protein to the *Cry1* E-box (38) was used as a positive control. Relatively little association of Clock protein was observed with a distant region (at –6.2 kb) of the *RORγ1* promoter, which served as a negative control. The level of Clock recruitment to the *RORγ1*(–1179/–1042) region was not very different between ZT10 and ZT22 similar to what has been reported previously for the recruitment of Clock/Bmal1 to the *Cry1*(E-box) promoter region (38). These data strengthen the previous conclusions (18,48) that Clock/Bmal1 and Cry1 complexes regulate *RORγ1* expression through their interaction with E-boxes in the *RORγ1* promoter.

To assess chromatin accessibility in relation to the level of *RORγ1* expression, we performed FAIRE on the *RORγ1*(–1179/–1042) promoter region with chromatin isolated from liver at ZT10 and ZT22. As shown in Figure 7D, the FAIRE-QPCR signal was markedly higher in samples isolated from liver at ZT22, a time at which *RORγ1* is highly expressed, compared with ZT10 when *RORγ1* expression is low. A low FAIRE-QPCR signal was obtained at a distal site. Our results are consistent with the concept that at ZT22 this region of the *RORγ1* promoter is more accessible and associated with functionally active enhancers. This was supported by ChIP analysis using an H3K9Ac antibody. The level of H3K9Ac associated with the *RORγ1*(–160/–70) region was considerably higher at ZT20 than at ZT8 (Figure 7E). This is consistent with the high level of *RORγ1* mRNA expression at ZT20 and a more open chromatin structure at this site.

Regulation of the rhythmic expression of RORγ1 target genes

We previously proposed that in addition to their modulation of clock gene expression, RORs might function as a link between the clock machinery and the circadian regulation of down-stream genes (1). The loss of RORs has been shown to affect the rhythmic expression of several genes involved in glucose and lipid metabolism, including perilipin 2 (*Plin2* or *Adfp*) and arginine vasopressin receptor 1a (*Avpr1a*) (17,31). Figure 8A shows that as for *Npas2* and *Cry1*, the peak expression of *Avpr1a* at ZT0, a time at which RORγ1 is most highly expressed, was greatly diminished in $RORγ^{-/-}$ liver. The loss of RORα had little effect on *Avpr1a* expression, while the pattern of *Avpr1a* expression in DKO liver was very similar to that in $RORγ^{-/-}$ liver. These observations indicate that *Avpr1a* expression is selectively regulated by RORγ1. Analysis of our genome-wide map of RORγ binding sites showed strong association of RORγ with two regions, A and B, upstream from the *Avpr1a* transcription start site (Figure 8B). RORγ was able to induce region A-dependent *trans*-activation, but not region B-mediated *trans*-activation (Figure 8C). ChIP analysis showed that RORγ was recruited to region A at ZT22 (Figure 8D), but not at ZT10, as observed for clock genes (Figure 5C). Although overexpression of RORα induced

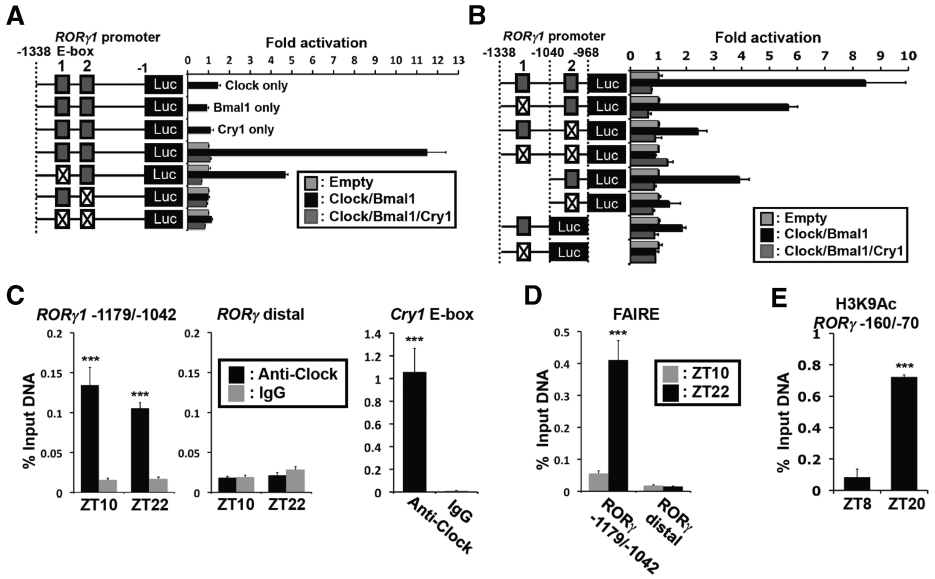

Figure 7. The activation of the *RORγ1* promoter by Bmal1/Clock was repressed by Cry1, and associated with recruitment of Clock and increased accessibility to the *RORγ1*(E-box1, 2) site. (**A, B**) Activation of the *RORγ1* promoter by Bmal1/Clock. Huh-7 cells were co-transfected with pGL4 reporter plasmids containing the wild type or mutant *RORγ1*(–1338/–1) (A) or *RORγ1*(–1338/–968) (B) promoter region, pCMV-β-Gal, empty vector, and pCMV-Sport6 expression plasmids for Clock, Bmal1 and Cry1 as indicated. The E-box 1 and 2 (solid boxes) were mutated from CACGTG to CAATTG (crossed box) as indicated. (**C**) Recruitment of Clock protein to the *RORγ1*(E-box1,2) promoter site *in vivo*. ChIP-QPCR at the *RORγ1*(–1179-/–1042) site was performed using an anti-Clock antibody and chromatin isolated from mouse livers collected at ZT10 or ZT22. ChIP with an anti-IgG and QPCR amplification of a distal site (–6.2 kb) of *RORγ1* were used as negative controls. ChIP analysis targeting the E-box in *Cry1* was used as a positive control. The experiment was repeated twice independently. (**D**) Chromatin accessibility at the *RORγ1(*E-box1, 2) region was assessed by FAIRE-QPCR analysis using chromatin samples prepared from WT livers isolated at ZT10 or ZT22. FAIRE-QPCR at a distal *RORγ1* site was used as a negative control. (**E**) Activation of the *RORγ* promoter correlated with an increase in the association of H3K9Ac. ChIP-QPCR analysis was performed using an anti-H3K9Ac antibody and chromatin isolated from mouse livers collected at ZT8 or ZT20. Data represent mean ±SEM; $***P < 0.001$ by ANOVA.

region A-dependent *trans*-activation (Figure 8C), RORα was only weakly associated with region A and B (Figure 8B and D). Together, these observations suggest that RORγ1, rather than RORα, is involved in the direct regulation of the rhythmic *Avpr1a* expression.

DISCUSSION

In this study, we demonstrate that RORα4 and RORα1 have a weak and that RORγ1 exhibits a robust oscillatory pattern of expression in BAT, WAT, kidney and jejunum as we reported previously for liver (25). We further show that the loss of RORγ did not significantly alter the expression pattern of RORα1 or RORα4, while the loss of RORα had little effect on the oscillatory expression pattern of RORγ1 in either BAT or kidney suggesting that RORα and RORγ are regulated independently from each other under the conditions tested. To obtain further insights into the roles of RORα and RORγ in circadian regulation *in vivo*, we compared the oscillatory expression pattern of *Cry1*, *Baml1*, *E4bp4*, *Rev-Erbα* and *Per2* in several tissues from RORα and RORγ single knockout and RORα/RORγ DKO mice. The loss of either RORα or RORγ, or both had either a small or no effect on the phase of the rhythmic expression of these clock genes (Figures 2 and 3). However, the loss of RORα or RORγ affected the peak expression of several clock genes in a tissue- and ROR isotype-selective manner. Loss of RORγ significantly reduced the peak expression of *Cry1*, *Rev-Erbα*, *E4bp4* and *Per2* in most tissues tested and caused a small reduction in *Bmal1* peak expression. The increased levels of *Per2* in $RORγ^{-/-}$ liver might be related to the observed, reduced expression of *E4bp4,* which has been reported to function as a negative regulator of *Per2* (49). The effect of the loss of RORα on the expression of *Bmal1*, *Rev-Erbα*, *E4bp4* and *Per2* was limited mainly to the kidney, a tissue in which RORα is relatively highly expressed, while *Cry1* expression was little changed in $RORα^{sg/sg}$ kidney. We reported previously that the loss of RORγ, but not the loss of RORα, reduced peak expression of *Npas2* and *Clock* (25). These observations indicated that both RORs, but particularly RORγ, are modulators of clock gene expression in several peripheral tissues. The tissue selectivity of clock gene regulation by RORα and RORγ might be due to differences in their level of expression and/or transcriptional activity.

We previously reported that RORα and RORγ exhibit a certain degree of functional redundancy in liver and Th17 cells. The hepatic expression of several Phase I and Phase II metabolic genes was affected to considerably greater extent in DKO than in single knockout mice (4) and both RORα and RORγ have been shown to promote Th17 differentiation and *Il-17* expression (50). We, therefore, were interested in determining whether RORα and RORγ exhibited any redundancy in regulating clock gene expression in liver, kidney and BAT. Comparison of *Cry1*,

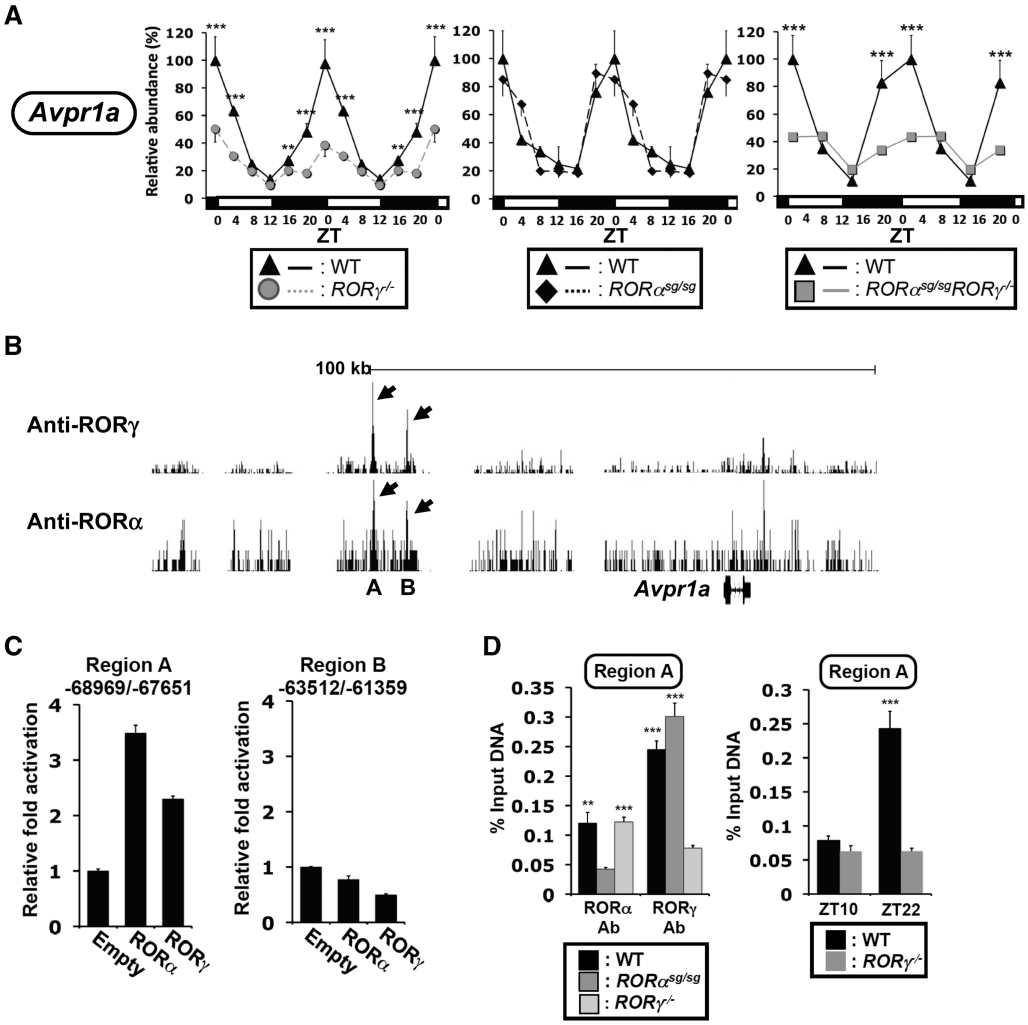

Figure 8. Regulation of the circadian expression of *Avpr1a* by RORγ. Liver from WT, $ROR\gamma^{-/-}$, $ROR\alpha^{sg/sg}$ and $ROR\alpha^{sg/sg}ROR\gamma^{-/-}$ DKO mice ($n = 4$) were isolated every 4 h over a period of 24 h and *Avpr1a* mRNA was quantified by QRT-PCR. The 24 h expression pattern was double-plotted. Data represent mean ± SD; $^{**}P < 0.01$ and $^{***}P < 0.001$ by ANOVA. (**B**) Analysis of our genome-wide map of ROR binding sites showed strong association of RORγ with two regions, A and B, upstream from the *Avpr1a* transcription start and weak association of RORα. Data were derived from ChIP-Seq analysis using ChIPed chromatin from liver and an anti-RORγ or -RORα antibody. (**C**) Exogenous expression of RORα/γ was able to induce region A-dependent *trans*-activation, but not region B-mediated *trans*-activation in Huh-7 cells. (**D**) ChIP-QPCR analysis was performed with chromatin isolated from livers of WT, $ROR\alpha^{sg/sg}$ and $ROR\gamma^{-/-}$ mice ($n = 4$) collected at ZT22 and an anti-RORα or -RORγ antibody (left panel). The recruitment of RORγ to region A was ZT-dependent. ChIP-QPCR was performed using an anti-RORγ antibody and chromatin from WT livers collected at ZT10 (low expression of RORγ) or ZT22 (high expression of RORγ) (right panel). Data represent mean ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA.

Bmal1, *Rev-Erbα*, *E4bp4*, *Per2*, *Npas2* and *Clock* expression between single knockout and DKO mice showed a complex picture. The expression of *Cry1*, *Bmal1*, *Rev-Erbα*, *Npas2* and *Clock* in particular was reduced to a significantly greater degree in liver and BAT of DKO mice than in single knockout mice (Figure 3 and Supplementary Figure S1). A greater reduction in *Cry1*, *Bmal1* and *Rev-Erbα* expression was also observed in DKO kidney; however, little redundancy was observed in the small intestines (jejunum), where RORα is not significantly expressed. Our data suggest that RORα and RORγ exhibit a degree of functional redundancy in their regulation of clock gene expression that explains some of the synergistic effects observed in some tissues of DKO mice. It is interesting to note that Rev-Erbα and Rev-Erbβ, which can compete with RORs for the same bindings sites, also work together in regulating clock gene expression (51,52).

The regulation of *Cry1*, *E4bp4*, *Baml1* and *Rev-Erbα* by RORα4 and RORγ1 was supported by data showing that exogenous expression of RORα4 or RORγ1 in Hepa1-6 and BAT cells increased the expression of all four clock genes (Figure 4C and Supplementary Figure S4). Mutant ROR receptors lacking *trans*-activating activity were unable to enhance expression of these clock genes indicating that the induction of clock genes by RORs is dependent on the activation function of RORs. This was supported by data showing that ROR antagonists significantly reduced the induction of *Bmal1*, *Cry1*, *E4bp4* and *Rev-Erbα* mRNA by RORα and RORγ.

RORs regulate gene transcription directly by binding ROREs in the regulatory region of target genes (1). Previous studies have identified putative ROREs in a number of clock genes (13,15,18,22–25,41). Reporter gene analysis showed that both RORα and RORγ were able to activate the *Luc* reporter under the control of the RORE-containing regions of *Cry1*, *Bmal1*, *Rev-Erbα*, *E4bp4* or *Clock* (Supplementary Figures S2 and S3). Mutation of either the RORE1 or RORE2 in *Bmal1* or *Cry1* partially reduced this activation, whereas mutation of both ROREs totally abolished the activation of the *Luc* reporter suggesting that both RORE1 and RORE2 are required for the optimal activation of these genes by RORα and RORγ. In the case of *E4bp4*, *Rev-Erbα* and *Clock* the activation by RORα and RORγ was mediated through RORE1. The activation of these regulatory regions was inhibited by co-expression with Rev-Erbα, a transcriptional repressor that can compete with RORs for RORE binding (14,15,41,43). Moreover, treatment with the RORα and RORγ inverse agonist T0901317 significantly inhibited the activation by either RORα or RORγ, while the RORγ-selective antagonist 'A' inhibited the induction by RORγ, but not that by RORα (Figure 4 and Supplementary Figures S3 and S4). These observations are consistent with the concept that RORα and RORγ enhance the expression of clock genes directly by binding ROREs in their promoter regulatory region and further confirm that this induction requires the activation function of RORs. The mouse and human *Cry1*(RORE1/2) are distal from the transcription start site (TSS) and located within introns at +23086/23141 and +66014/66068, respectively. ROR activator complexes bound to distal ROREs might exert coordinated control of *Cry1* expression via a physical interaction with protein complexes at the proximal promoter or TSS (53).

Our genome-wide maps of RORα/γ binding sites in liver identified major RORα and RORγ binding peaks near or within the *Cry1*, *Npas2, Bmal1* and *E4bp4* genes (Figure 5A). These sites corresponded to the ROREs studied in this article or reported previously (18,24,25). The recruitment of RORs to these regions was further supported by ChIP-QPCR analysis using ChIPed DNA from liver tissue and Hepa1-6(RORγ1) or Hepa1-6(RORα4) cells. The specificity of ROR binding was indicated by data showing that no significant recruitment of RORα or RORγ was observed in, respectively, $RORα^{sg/sg}$ and $RORγ^{-/-}$ liver or to non-RORE containing distal regions of the respective clock gene or *Gapdh*. Because *RORγ1* exhibits a robust circadian pattern of expression, we determined whether the recruitment of RORγ1 to the regulatory regions was ZT-dependent. This was supported by ChIP-QPCR analysis showing higher levels of association of RORγ1 with the RORE-containing regulatory regions of *Cry1*, *Bmal1* and *E4bp4* at ZT22, when *RORγ* as well as these clock genes are relatively highly expressed, than at ZT10, when these genes are expressed at low levels. Moreover, increased competition for RORE binding by Rev-Erbα/β, which are highly expressed at ZT10, is likely a major factor in the reduced interaction of RORs with ROREs at this ZT. Unexpectedly, the recruitment of RORγ1 to the *Rev-Erbα*(RORE) was also higher at ZT22 than ZT10 despite the low expression of *Rev-Erbα* at ZT22 indicating that binding of RORγ is not necessarily sufficient to enhance transcription of *Rev-Erbα*. ChIP-Seq analysis suggests that this association is relatively weak suggesting that RORs may play a lesser role in regulating *Rev-Erb* expression. Collectively, our promoter analyses, and ChIP-Seq data support the concept that the up-regulation of *Cry1*, *Npas2*, *Bmal1* and *E4bp4* transcription by RORs *in vivo* occurs by a direct mechanism and involves recruitment of RORγ1 and RORα to ROREs within the regulatory regions of these genes. It is interesting to note that the Rev-Erb binding sites associated with *Bmal1* and *E4bp4* identified by ChIP-Seq analysis are the same as the ROR binding sites identified by our ChIP-Seq analysis (52) consistent with competition and regulation through the same binding sites. However, for *Cry1* the major ROR binding sites were different from those described for Rev-Erb suggesting that RORs and Rev-Erbs may not always regulate clock gene expression through competition for the same response elements.

In many instances activation of transcription is accompanied by changes in chromatin structure. Actively transcribed regions are associated with an open chromatin structure and accompanied by changes in histone acetylation and methylation. ChIP analysis with an antibody against H3K9Ac, a marker for actively transcribed chromatin, showed that the level of H3K9Ac association with the *Bmal1* and *Cry1* regulatory regions in WT liver was higher at ZT22 than at ZT10 (Figure 6A). This association was diminished in $RORγ^{-/-}$ liver, but not altered in $RORα^{sg/sg}$ liver. FAIRE, which has been used as a tool to map open chromatin (37), revealed that the FAIRE signal was significantly increased at the *Cry1*(RORE1/2) and *Bmal1*(RORE1/2) regulatory region in liver at ZT22 compared with ZT10 consistent with increased chromatin accessibility at these sites. This correlates with the increased association of H3K9Ac and the relatively high level of *Cry1* and *Bmal1* expression at ZT22 (Figure 6B). The loss of RORγ caused a significant reduction in FAIRE signal at the *Cry1*(RORE1/2) and *Bmal1*(RORE1/2) region consistent with a positive relationship between RORγ binding, chromatin remodeling and transcriptional activation of these genes.

In addition to the circadian regulation of clock genes by RORs, RORγ itself exhibits a robust rhythmic expression in several peripheral tissues that is controlled by clock proteins (Figure 1) (13,15,18,25). The positive regulation of *RORγ1* is partially mediated through the binding of Bmal1-Clock heterodimers to two E-boxes in the proximal promoter of *RORγ1* consistent with a previous study (48). Moreover, one of the E-boxes (E2) within *RORγ* has been reported to be able to drive the oscillation of a reporter in cultured cells, indicating the importance of this E-box in the circadian regulation of *RORγ* by Clock/Bmal1 dimers (18). In this study, we show that the repressor Cry1 effectively blocked this activation (Figure 7). Cry1 likely mediates this repression through inhibition of the transcriptional activity of Bmal1/Clock heterodimers (54). ChIP analysis using an anti-Clock antibody showed that Clock protein was recruited to the (E1/2-box)

sites in the *RORγ1* promoter in liver consistent with the hypothesis that *RORγ1* transcription is regulated directly by Bmal1-Clock heterodimers *in vivo*. Unexpectedly little difference was observed in Clock recruitment between ZT10 and ZT22, times of low and high RORγ expression, respectively. Similarly, a previous study demonstrated that the degree of Bmal1-Clock binding did not correlate with the level of activation of *Cry1* transcription (38). These observations suggested that binding of Bmal1-Clock is not necessarily sufficient to activate transcription of *RORγ1*. The latter may be due to a repressor function of the Bmal1-Clock complex and the type of co-repressors or co-activators recruited to the heterodimer, or crosstalk with other transcriptional factors (55). In contrast to Clock binding, the association of H3K9Ac and the FAIRE signal was significantly increased at the *RORγ1*(E-box) promoter region in liver at ZT22 compared with ZT10 indicating that the transcriptional activation of *RORγ1* correlated with increased chromatin accessibility consistent with an association between open chromatin structure and functionally active enhancers. In addition to its regulation by Bmal1/Clock, Rev-Erbs are recruited to the regulatory region of *RORγ* (52), consistent with the concept that they are also involved in the regulation of the rhythmic expression of RORγ.

In summary, study of RORα, RORγ and DKO mice showed that ROR receptors modulate the expression of several clock genes in an ROR isoform- and tissue-dependent manner. Particularly RORγ1 appears to affect peak expression of clock genes in several peripheral tissues. Study of DKO mice, ChIP-Seq and ChIP-QPCR analysis indicated a certain degree of redundancy between RORα and RORγ and demonstrated that *in vivo* RORα/γ regulate these genes directly and in a ZT-dependent manner through their interaction with ROREs. We provided evidence that this transcriptional regulation is associated with changes in chromatin structure. Our results are consistent with the concept of a reciprocal relationship between the regulation of clock genes and *RORγ1* (Supplementary Figure S5). Recent studies demonstrated that clock proteins are important regulators of energy homeostasis and lipid/glucose metabolism suggesting a connection between the regulation of the circadian clock and various metabolic pathways (4,14,20,30,31). We previously proposed that RORs might function as a link between clock proteins and their regulation of metabolic genes (1). One might predict that the Bmal1/Clock/Rev-Erb-mediated rhythmic expression of *RORγ1* leads to the rhythmic expression of RORγ1 target genes, including metabolic genes, such as *Avpr1a*. This concept is supported by observations showing that the oscillatory expression of *RORγ1* and *Avpr1a* are in phase, that the loss of RORγ greatly reduces *Avpr1a* peak expression, and the presence of RORγ binding sites (Figure 7) (17). In this scenario, the rhythmic expression of downstream target genes, such as *Avpr1a*, depends on the rhythmic expression of *RORγ1* and to a certain degree on Rev-Erb through its regulation of *RORγ1* and/or its competition with RORγ1 for *Avpr1a*(RORE) binding (Supplementary Figure S5). Analysis of additional RORγ1 target genes is required to obtain further support for this hypothesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2 and Supplementary Figures 1–5.

ACKNOWLEDGEMENTS

The authors thank Drs Kristin Lichti-Kaiser and Gary ZeRuth for their comments on the manuscript and Laura Miller (NIEHS) for her great assistance with the mice.

FUNDING

Intramural Research Program of the National Institute of Environmental Health Sciences, National Institutes of Health [Z01-ES-101586]. Funding for open access charge: National Institute of Environmental Health Sciences, National Institutes of Health.

Conflict of interest statement. None declared.

REFERENCES

1. Jetten,A.M. (2009) Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. *Nucleic Recept. Signal.*, **7**, e003.
2. Gold,D.A., Baek,S.H., Schork,N.J., Rose,D.W., Larsen,D.D., Sachs,B.D., Rosenfeld,M.G. and Hamilton,B.A. (2003) RORalpha coordinates reciprocal signaling in cerebellar development through sonic hedgehog and calcium-dependent pathways. *Neuron*, **40**, 1119–1131.
3. Steinmayr,M., Andre,E., Conquet,F., Rondi-Reig,L., Delhaye-Bouchaud,N., Auclair,N., Daniel,H., Crepel,F., Mariani,J., Sotelo,C. *et al.* (1998) Staggerer phenotype in retinoid-related orphan receptor alpha-deficient mice. *Proc. Natl Acad. Sci. USA*, **95**, 3960–3965.
4. Kang,H.S., Angers,M., Beak,J.Y., Wu,X., Gimble,J.M., Wada,T., Xie,W., Collins,J.B., Grissom,S.F. and Jetten,A.M. (2007) Gene expression profiling reveals a regulatory role for ROR alpha and ROR gamma in phase I and phase II metabolism. *Physiol. Genomics*, **31**, 281–294.
5. Lau,P., Fitzsimmons,R.L., Raichur,S., Wang,S.C., Lechtken,A. and Muscat,G.E. (2008) The orphan nuclear receptor, RORalpha, regulates gene expression that controls lipid metabolism: staggerer (SG/SG) mice are resistant to diet-induced obesity. *J. Biol. Chem.*, **283**, 18411–18421.
6. Jaradat,M., Stapleton,C., Tilley,S.L., Dixon,D., Erikson,C.J., McCaskill,J.G., Kang,H.S., Angers,M., Liao,G., Collins,J. *et al.* (2006) Modulatory role for retinoid-related orphan receptor alpha in allergen-induced lung inflammation. *Am. J. Respir. Crit. Care Med.*, **174**, 1299–1309.
7. Yang,X.O., Nurieva,R., Martinez,G.J., Kang,H.S., Chung,Y., Pappu,B.P., Shah,B., Chang,S.H., Schluns,K.S., Watowich,S.S. *et al.* (2008) Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*, **29**, 44–56.
8. Schaeren-Wiemers,N., Andre,E., Kapfhammer,J.P. and Becker-Andre,M. (1997) The expression pattern of the orphan nuclear receptor RORbeta in the developing and adult rat nervous system suggests a role in the processing of sensory information and in circadian rhythm. *Eur. J. Neurosci.*, **9**, 2687–2701.
9. Jia,L., Oh,E.C., Ng,L., Srinivas,M., Brooks,M., Swaroop,A. and Forrest,D. (2009) Retinoid-related orphan nuclear receptor

RORbeta is an early-acting factor in rod photoreceptor development. *Proc. Natl Acad. Sci. USA*, **106**, 17534–17539.
10. Kurebayashi,S., Ueda,E., Sakaue,M., Patel,D.D., Medvedev,A., Zhang,F. and Jetten,A.M. (2000) Retinoid-related orphan receptor gamma (RORgamma) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. *Proc. Natl Acad. Sci. USA*, **97**, 10132–10137.
11. Sun,Z., Unutmaz,D., Zou,Y.R., Sunshine,M.J., Pierani,A., Brenner-Morton,S., Mebius,R.E. and Littman,D.R. (2000) Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science*, **288**, 2369–2373.
12. Ivanov,I.I., McKenzie,B.S., Zhou,L., Tadokoro,C.E., Lepelley,A., Lafaille,J.J., Cua,D.J. and Littman,D.R. (2006) The Orphan Nuclear Receptor RORgammat Directs the Differentiation Program of Proinflammatory IL-17(+) T Helper Cells. *Cell*, **126**, 1121–1133.
13. Akashi,M. and Takumi,T. (2005) The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. *Nat. Struct. Mol. Biol.*, **12**, 441–448.
14. Duez,H. and Staels,B. (2010) Nuclear receptors linking circadian rhythms and cardiometabolic control. *Arterioscler. Thromb. Vasc. Biol.*, **30**, 1529–1534.
15. Guillaumond,F., Dardente,H., Giguere,V. and Cermakian,N. (2005) Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J. Biol. Rhythms*, **20**, 391–403.
16. Sato,T.K., Panda,S., Miraglia,L.J., Reyes,T.M., Rudic,R.D., McNamara,P., Naik,K.A., FitzGerald,G.A., Kay,S.A. and Hogenesch,J.B. (2004) A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron*, **43**, 527–537.
17. Liu,A.C., Tran,H.G., Zhang,E.E., Priest,A.A., Welsh,D.K. and Kay,S.A. (2008) Redundant function of REV-ERBalpha and beta and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. *PLoS Genet.*, **4**, e1000023.
18. Ueda,H.R., Hayashi,S., Chen,W., Sano,M., Machida,M., Shigeyoshi,Y., Iino,M. and Hashimoto,S. (2005) System-level identification of transcriptional circuits underlying mammalian clocks. *Nat. Genet.*, **37**, 187–192.
19. Asher,G. and Schibler,U. (2011) Crosstalk between components of circadian and metabolic cycles in mammals. *Cell Metab.*, **13**, 125–137.
20. Bass,J. and Takahashi,J.S. (2010) Circadian integration of metabolism and energetics. *Science*, **330**, 1349–1354.
21. Zhang,E.E. and Kay,S.A. (2010) Clocks not winding down: unravelling circadian networks. *Nat. Rev. Mol. Cell Biol.*, **11**, 764–776.
22. Raspe,E., Mautino,G., Duval,C., Fontaine,C., Duez,H., Barbier,O., Monte,D., Fruchart,J., Fruchart,J.C. and Staels,B. (2002) Transcriptional regulation of human Rev-erbalpha gene expression by the orphan nuclear receptor RORalpha. *J. Biol. Chem.*, **277**, 49275–49281.
23. Delerive,P., Chin,W.W. and Suen,C.S. (2002) Identification of Reverb(alpha) as a novel ROR(alpha) target gene. *J. Biol. Chem.*, **277**, 35013–35018.
24. Crumbley,C., Wang,Y., Kojetin,D.J. and Burris,T.P. (2010) Characterization of the core mammalian clock component, NPAS2, as a REV-ERBα/RORα target gene. *J. Biol. Chem.*, **285**, 35386–35392.
25. Takeda,Y., Kang,H.S., Angers,M. and Jetten,A.M. (2011) Retinoic acid-related orphan receptor γ (RORγ) directly regulates neuronal PAS domain protein 2 (Npas2) transcription in vivo. *Nucleic Acids Res.*, **39**, 4769–4782.
26. VanDunk,C., Hunter,L.A. and Gray,P.A. (2011) Development, maturation, and necessity of transcription factors in the mouse suprachiasmatic nucleus. *J. Neurosci.*, **31**, 6457–6467.
27. Masana,M.I., Sumaya,I.C., Becker-Andre,M. and Dubocovich,M.L. (2007) Behavioral characterization and modulation of circadian rhythms by light and melatonin in C3H/HeN mice homozygous for the RORbeta knockout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **292**, R2357–2367.
28. Yin,L., Wu,N. and Lazar,M.A. (2010) Nuclear receptor Rev-erbα: a heme receptor that coordinates circadian rhythm and metabolism. *Nucl. Recept. Signal.*, **8**, e001.
29. Schibler,U. and Naef,F. (2005) Cellular oscillators: rhythmic gene expression and metabolism. *Curr. Opin. Cell Biol.*, **17**, 223–229.
30. Lau,P., Fitzsimmons,R.L., Pearen,M.A., Watt,M.J. and Muscat,G.E. (2011) Homozygous staggerer (sg/sg) mice display improved insulin sensitivity and enhanced glucose uptake in skeletal muscle. *Diabetologia*, **54**, 1169–1180.
31. Kang,H.S., Okamoto,K., Takeda,Y., Beak,J.Y., Gerrish,K., Bortner,C.D., DeGraff,L.M., Wada,T., Xie,W. and Jetten,A.M. (2011) Transcriptional profiling reveals a role for RORalpha in regulating gene expression in obesity-associated inflammation and hepatic steatosis. *Physiol. Genomics*, **43**, 818–828.
32. Hamilton,B.A., Frankel,W.N., Kerrebrock,A.W., Hawkins,T.L., FitzHugh,W., Kusumi,K., Russell,L.B., Mueller,K.L., van Berkel,V., Birren,B.W. *et al.* (1996) Disruption of the nuclear hormone receptor RORalpha in staggerer mice. *Nature*, **379**, 736–739.
33. Dussault,I., Fawcett,D., Matthyssen,A., Bader,J.A. and Giguere,V. (1998) Orphan nuclear receptor RORalpha-deficient mice display the cerebellar defects of staggerer. *Mech. Dev.*, **70**, 147–153.
34. Kumar,N., Solt,L.A., Conkright,J.J., Wang,Y., Istrate,M.A., Busby,S.A., Garcia-Ordonez,R.D., Burris,T.P. and Griffin,P.R. (2010) The benzenesulfoamide T0901317 [N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluorometh yl)ethyl]phenyl]-benzenesulfonamide] is a novel retinoic acid receptor-related orphan receptor-alpha/gamma inverse agonist. *Mol. Pharmacol.*, **77**, 228–236.
35. Kurebayashi,S., Nakajima,T., Kim,S.C., Chang,C.Y., McDonnell,D.P., Renaud,J.P. and Jetten,A.M. (2004) Selective LXXLL peptides antagonize transcriptional activation by the retinoid-related orphan receptor RORgamma. *Biochem. Biophys. Res. Commun.*, **315**, 919–927.
36. Narlikar,L. and Jothi,R. (2012) ChIP-Seq data analysis: identification of protein-DNA binding sites with SISSRs peak-finder. *Methods Mol. Biol.*, **802**, 305–322.
37. Giresi,P.G., Kim,J., McDaniell,R.M., Iyer,V.R. and Lieb,J.D. (2007) FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Res.*, **17**, 877–885.
38. Etchegaray,J.P., Lee,C., Wade,P.A. and Reppert,S.M. (2003) Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature*, **421**, 177–182.
39. Ptitsyn,A.A., Zvonic,S., Conrad,S.A., Scott,L.K., Mynatt,R.L. and Gimble,J.M. (2006) Circadian clocks are resounding in peripheral tissues. *PLoS Comput. Biol.*, **2**, e16.
40. Zvonic,S., Ptitsyn,A.A., Conrad,S.A., Scott,L.K., Floyd,Z.E., Kilroy,G., Wu,X., Goh,B.C., Mynatt,R.L. and Gimble,J.M. (2006) Characterization of peripheral circadian clocks in adipose tissues. *Diabetes*, **55**, 962–970.
41. Preitner,N., Damiola,F., Lopez-Molina,L., Zakany,J., Duboule,D., Albrecht,U. and Schibler,U. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell*, **110**, 251–260.
42. Ukai-Tadenuma,M., Yamada,R.G., Xu,H., Ripperger,J.A., Liu,A.C. and Ueda,H.R. (2011) Delay in feedback repression by cryptochrome 1 is required for circadian clock function. *Cell*, **144**, 268–281.
43. Austin,S., Medvedev,A., Yan,Z.H., Adachi,H., Hirose,T. and Jetten,A.M. (1998) Induction of the nuclear orphan receptor RORgamma during adipocyte differentiation of D1 and 3T3-L1 cells. *Cell Growth Differ.*, **9**, 267–276.
44. Kallen,J.A., Schlaeppi,J., Bitsch,F., Geisse,S., Geiser,M., Delhon,I. and Fournier,B. (2002) X-ray structure of the RORα LBD at 1.63A: structural and functional data that cholesterol or a cholesterol derivative is the natural ligand of RORα. *Structure*, **10**, 1697–1707.
45. Jin,L., Martynowski,D., Zheng,S., Wada,T., Xie,W. and Li,Y. (2010) Structural basis for hydroxycholesterols as natural ligands of orphan nuclear receptor RORgamma. *Mol. Endocrinol.*, **24**, 923–929.

46. Huh,J.R., Leung,M.W., Huang,P., Ryan,D.A., Krout,M.R., Malapaka,R.R., Chow,J., Manel,N., Ciofani,M., Kim,S.V. *et al.* (2011) Digoxin and its derivatives suppress TH17 cell differentiation by antagonizing RORgammat activity. *Nature*, **472**, 486–490.
47. MacDonald,V.E. and Howe,L.J. (2009) Histone acetylation: where to go and how to get there. *Epigenetics*, **4**, 139–143.
48. Mongrain,V., Ruan,X., Dardente,H., Fortier,E.E. and Cermakian,N. (2008) Clock-dependent and independent transcriptional control of the two isoforms from the mouse Rorgamma gene. *Genes Cells*, **13**, 1197–1210.
49. Ohno,T., Onishi,Y. and Ishida,N. (2007) A novel E4BP4 element drives circadian expression of mPeriod2. *Nucleic Acids Res.*, **35**, 648–655.
50. Yang,X.O., Pappu,B.P., Nurieva,R., Akimzhanov,A., Kang,H.S., Chung,Y., Ma,L., Shah,B., Panopoulos,A.D., Schluns,K.S. *et al.* (2008) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity*, **28**, 29–39.
51. Bugge,A., Feng,D., Everett,L.J., Briggs,E.R., Mullican,S.E., Wang,F., Jager,J. and Lazar,M.A. (2012) Rev-erbalpha and Rev-erbbeta coordinately protect the circadian clock and normal metabolic function. *Genes Dev.*, **26**, 657–667.
52. Cho,H., Zhao,X., Hatori,M., Yu,R.T., Barish,G.D., Lam,M.T., Chong,L.W., DiTacchio,L., Atkins,A.R., Glass,C.K. *et al.* (2012) Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. *Nature*, **485**, 123–127.
53. Miele,A., Bystricky,K. and Dekker,J. (2009) Yeast silent mating type loci form heterochromatic clusters through silencer protein-dependent long-range interactions. *PLoS Genet.*, **5**, e1000478.
54. Sahar,S. and Sassone-Corsi,P. (2009) Metabolism and cancer: the circadian clock connection. *Nat. Rev. Cancer*, **9**, 886–896.
55. Grimaldi,B., Nakahata,Y., Sahar,S., Kaluzova,M., Gauthier,D., Pham,K., Patel,N., Hirayama,J. and Sassone-Corsi,P. (2007) Chromatin remodeling and circadian control: master regulator CLOCK is an enzyme. *Cold Spring Harb. Symp. Quant. Biol.*, **72**, 105–112.