Nuclear receptor HNF4A transrepresses CLOCK: BMAL1 and modulates tissue-specific circadian networks

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Either expression level or transcriptional activity of various nuclear receptors (NRs) have been demonstrated to be under circadian control. With a few exceptions, little is known about the roles of NRs as direct regulators of the circadian circuitry. Here we show that the nuclear receptor HNF4A strongly transrepresses the transcriptional activity of the CLOCK:BMAL1 heterodimer. We define a central role for HNF4A in maintaining cell-autonomous circadian oscillations in a tissue-specific manner in liver and colon cells. Not only transcript level but also genome-wide chromosome binding of HNF4A is rhythmically regulated in the mouse liver. ChIP-seq analyses revealed cooccupancy of HNF4A and CLOCK:BMAL1 at a wide array of metabolic genes involved in lipid, glucose, and amino acid homeostasis. Taken together, we establish that HNF4A defines a feedback loop in tissue-specific mammalian oscillators and demonstrate its recruitment in the circadian regulation of metabolic pathways.

Significance

Interlocked feedback loops promote robustness and stability in a system and are a feature of circadian clocks in both animal and plants. The mammalian circadian clock is known to consist of two transcriptional feedback loops, relying on the transcriptional activity of the master complex CLOCK:BMAL1 and the feedback regulation by its target genes. Our research extends this knowledge by proposing a feedback loop in peripheral circadian oscillators and highlights the underlying mechanisms mediated by the unappreciated CLOCK:BMAL1 transrepression activity of the circadian nuclear receptor HNF4A. Further, our data suggest that the hepatic roles of HNF4A are circadian.

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Conflict of interest statement: S.A.K., J.B.H., and C.B.G. are coauthors on a 2017 guideline about 24 h in behavior, metabolism, and physiology found in almost all living organisms. This internal clock allows organisms to synchronize their physiology and behavior with the predictable cycle of day and night. Disruption of normal circadian rhythms leads to clinically relevant disorders, including neurodegeneration, diabetes, obesity, and cardiovascular disease (1–4). In mammals, the core circadian circuit composed of transcriptional activators and repressors that form an autoregulatory transcriptional feedback loop is necessary for the generation and regulation of circadian rhythms with an endogenous period close to 24 h. The bHLH-PAS transcription factors (TFs) CLOCK and BMAL1 are heterodimeric transcriptional activators that drive the expression of Period genes (Per1, Per2, and Per3) and Cryptochrome genes (Cry1 and Cry2), by binding E-box cis-regulatory elements at promoters (5–9). Subsequently, the PER–CRY protein complex inhibits transcription of its own genes by directly inhibiting CLOCK:BMAL1 activity (8, 10–13). This feedback loop has been known as the critical hub of the mammalian oscillator.

While many mammalian peripheral tissues have circadian clocks, genes showing circadian expression are markedly diverse within individual tissues of mouse or human, indicating tissue-specific regulation of circadian output relevant to the function of the particular organ (14–18). The core clock may be regulated in a tissue-specific manner as well, supported by observations that explants of diverse mouse tissues express differences in circadian period and phase (16), and that output networks must be driven by the core clock components in a cell type-specific manner. However, such tissue-specific factors responsible for robust tissue-specific circadian rhythmicity remain to be identified.

The nuclear receptor (NR) superfamily is composed of 48 members in human. NRs coordinate processes as diverse as development, reproduction, and many aspects of metabolism. As sensors for lipophilic vitamins, hormones, and dietary lipids, NRs canonically function as ligand-activated transcription factors that regulate the expression of their target genes to affect physiological pathways (19). The importance of NRs in maintaining optimal physiological homeostasis is illustrated in their identification as potential targets for therapeutic drug development to combat a diverse array of diseases, including reproductive disorders, inflammation, cancer, diabetes, cardiovascular disease, and obesity (20). Various NRs have been implicated as targets of the circadian clock, which may contribute to the circadian regulation of nutrient and energy metabolism. Over half of the NR family members are expressed in a rhythmic manner (21). Further, PER2 and CRYs were found to broadly interact with NRs and potentially modulate their transcriptional activity (22–25).

Apart from the circadian control of NR expression and transcriptional activity, other direct links between NR activity and circadian clock function have been identified via NRs’ role as circadian clock inputs. In addition to functions in development, metabolism, and immunity, the nuclear receptors REV-ERBs and retinoic acid receptor-related orphan receptors (RORs) constitute the secondary feedback loop in the circadian cycle, by directly repressing and activating Bmal1 expression, respectively, and in turn being transcriptionally regulated by CLOCK:BMAL1.
Disregulation of REV-ERBs and RORs have dramatic effects on the robustness of circadian oscillators (30–34). However, little is known as to whether other NRs are involved in the regulation of core clock parameters.

The nuclear receptor hepatocyte nuclear factor 4A (HNF4A) binds as a homodimer to the consensus DNA sequence consisting of two direct repeats and controls the expression of downstream genes (35). It is expressed in a tissue-specific manner and plays essential roles in the development and physiology of liver, pancreas, kidney, and intestines, particularly lipid and glucose metabolism and inflammation (36–42). In the current study, we demonstrate that HNF4A potently inhibits the transcriptional activity of CLOCK:BMAL1 heterodimer. Furthermore, our data clearly show that HNF4A is necessary for the regulation of intrinsic circadian oscillations in liver and colon cells, suggesting its role in regulating tissue-specific clock networks.

**Results**

**Identification of HNF4A as a CLOCK:BMAL1 Transrepressor.** The growing body of evidence demonstrating a link between nuclear receptors, metabolic pathways, and intrinsic circadian rhythmicity (21–25) prompted us to test the potential for a broader range of nuclear receptors in mediating this cross-talk. In a physical interaction profiling, we noted a robust binding between core clock proteins and the HNF4A protein, as shown in coimmunoprecipitation experiments using either recombinant or endogenous proteins (Fig. 1A–C). To evaluate the biological significance of the interactions, we hypothesized that either the core clock components regulate the activity of nuclear receptor HNF4A, or HNF4A may transduce metabolic signals as inputs to the circadian system. To test the first hypothesis, we conducted a reporter assay in HEK 293T cells using a luciferase reporter driven by tandem HNF4A consensus binding motifs. Neither CRY1 nor CLOCK:BMAL1 coexpression affected the HNF4A transcriptional activity, indicating that HNF4A activity is unlikely to be directly regulated by these clock proteins (SI Appendix, Fig. S1).

We then tested the second hypothesis, asking whether the core clock feedback loops are regulated by the HNF4A protein. As HNF4A consistently binds CLOCK:BMAL1, we evaluated the effect of HNF4A on CLOCK:BMAL1 activity in endpoint reporter assays using the E-box-dLuc, Per1-Luc, or Per2-dLuc luciferase reporters. Surprisingly, we observed a consistent inhibition of CLOCK:BMAL1 activity by HNF4A coexpression in a dose-dependent pattern (P < 0.05), similar to the canonical clock repressor CRY1 (Fig. 1D–F). Collectively, these findings indicate activity of the nuclear receptor HNF4A in transrepressing another transcription factor CLOCK:BMAL1 and prompted us to evaluate its role in the intact molecular circadian oscillators.

![Fig. 1.](E12306 www.pnas.org/cgi/doi/10.1073/pnas.1816411115 Qu et al.)
HNF4A Is Essential for Tissue-Specific Rhythm Maintenance and Period Regulation. As HNF4A is minimally expressed in bone osteosarcoma U2OS cells, our previous genome-wide RNAi screen (43) was unable to assess its potential as a clock modifier (SI Appendix, Fig. S2). We therefore screened cell lines isolated from various tissue types to identify ones that both express a high level of HNF4A and oscillate upon dexamethasone synchronization. The cell lines we eventually identified are human liver cells Hep3B, mouse liver cells dihXY, and human colon cells SW480, which were genetically modified to stably express Per2-dLuc or Bmal1-Luc reporter gene. To assess the role of HNF4A in regulating circadian oscillations of these cells, we performed gene knockdown by using Hnf4a or Cry1 siRNAs (43) selected for their potency (of ~80%) (SI Appendix, Fig. S3 A–C). Surprisingly, the effect of Hnf4a knockdown in both liver cell lines Hep3B and dihXY was even more disruptive than Cry1 knockdown, leading to arrhythmicity (Fig. 2 A–D). The Hnf4a knockdown has a negative effect on the growth of SW480 colon cells, which is reflected by dampened amplitude of Per2-dLuc (SI Appendix, Fig. S3D). We subtracted the baseline trends, to better visualize the bioluminescence traces, and found that the effect of Hnf4a knockdown on the circadian rhythm was milder and resulted in period shortening by roughly 1 h (Fig. 2 E and F), similar to the Cry1 knockdown (SI Appendix, Fig. S3 E and F). The cell type-specific phenotypes of Hnf4a knockdown are consistent with previous observations for other clock genes (44).

Using U2OS Per2-dLuc cells, we generated stable lines expressing EGFP, CRY1, PER2, or HNF4A. Additional expression of CRY1 and PER2 proteins led to arrhythmicity along with greatly reduced Per2-dLuc reporter signals (SI Appendix, Fig. S3G). In contrast, ectopic expression of the Hnf4a gene significantly dampened the amplitude and lengthened the period (Fig. 2 G and H). Taken together, our data suggest that HNF4A is essential for circadian rhythm of cells where it is endogenously expressed and significantly alters the intrinsic circadian clock when ectopically expressed. We thus identify HNF4A to be a central regulator of tissue-specific circadian rhythms in liver and colon cell lines.

HNF4A Acts Differently from Other CLOCK:BMAL1 Repressors. As HNF4A is able to bind CRY proteins as well (Fig. 1 A and C), we asked whether its CLOCK:BMAL1 repression is attributable to an indirect mechanism bridged by CRYs. We applied the CLOCK:BMAL1 LL/AA mutant which can no longer be sequestered by CRY1 (45, 46). Even though the activation of Per1-Luc reporter by CLOCK:BMAL1 LL/AA mutant is less pronounced than the wild-type complex, our results show that CRY1 repression was substantially abolished (Fig. 3A). In contrast, HNF4A could still prominently suppress the mutant complex, suggesting that HNF4A inhibits CLOCK:BMAL1 by a mechanism independent of CRY1 (Fig. 3B). This conclusion was further supported by our observation that the knockdown of two Cry genes (43) did not affect the extent of HNF4A-dependent repression of CLOCK:BMAL1 activity (SI Appendix, Fig. S4A).

Apart from CRYs, other protein–protein interaction dependent CLOCK:BMAL1 repressors have recently emerged as key regulators of the circadian networks, e.g., CHRONO, DEC1/2, and PASD1 (47–50). Cancer/testis antigen PASD1 was identified to repress the CLOCK:BMAL1 activity depending upon exon...
19 of the Clock gene (47). Deletion of exon 19 was found to attenuate the transactivation potential of the transcription factor (6, 7). We found that HNF4A could still potently repress the residual activity of the CLOCKΔ19:BMAL1 complex (SI Appendix, Fig. S4B). Consequently, there appears to be a different targeting mechanism leveraged by HNF4A in achieving CLOCK:BMAL1 repression activity.

**Multiple HNF4A Domains Are Involved in CLOCK:BMAL1 Repression.**

Like most other nuclear receptors, HNF4A contains six structural domains that are necessary to mediate disparate functions: an N-terminal activation function (AF)-1 domain; a DNA-binding domain (DBD); a putative ligand-binding domain (LBD); a flexible hinge between the DBD and LBD; a C-terminal activation domain (AF-2), and a repressor region (F domain) that recruits corepressors and inhibits access of coactivators to AF-2 (51–56).

We used various genetic tools to characterize the transrepression activity of HNF4A. The predominant HNF4A splice variants, HNF4A1, HNF4A2, and HNF4A8, driven by tissue-specific promoters P1 or P2, are considered to have different physiological roles in development and transcriptional regulation of target genes. P1-driven HNF4A1 and HNF4A2, which differ exclusively by 10 amino acids in the F domain, are specifically expressed in the adult liver and kidney; whereas the P2-driven HNF4A8 is not found in these tissues but in fetal liver and the adult pancreas. Relative to HNF4A1/2, HNF4A8 lacks the N-terminal activation domain AF-1 (57–59). We further tested the effect of HNF4G, which shares nearly identical DBD and LBD domains with HNF4A. Intriguingly, all HNF4A isoforms we tested, together with HNF4G, potently repressed CLOCK:BMAL1 activity (Fig. 4A). It suggests that the HNF4 proteins share a common transrepression activity in various peripheral tissues where they are

**Fig. 3.** HNF4A targets CLOCK:BMAL1 differently from CRY1. (A and B) HNF4A is able to suppress a CLOCK:BMAL1 mutant which interferes with sequestration by CRY1. HEK 293T cells were cotransfected with reporter gene Per1-Luc, wild-type or mutant CLOCK:BMAL1, and increasing amounts of CRY1 (A) or HNF4A (B) plasmid (n = 3 for each condition, mean ± SD). Percentages on the Top indicate the degree of CLOCK:BMAL1 repression activity.

**Fig. 4.** Multiple HNF4A functional domains are required for CLOCK:BMAL1 transrepression. (A) HNF4A isoforms and HNF4G repress CLOCK:BMAL1 activity similarly. HEK 293T cells were cotransfected with reporter gene Per1-Luc, CLOCK:BMAL1, and increasing amounts of HNF4 plasmid (n = 3 for each condition, mean ± SD). (B) Domain analysis of HNF4A2. HEK 293T cells were cotransfected with reporter gene Per1-Luc, CLOCK:BMAL1, and increasing amounts of HNF4A2 fragment plasmid (n = 3 for each condition, mean ± SD).
specifically expressed, and the unshared structural domains or protein sequences are dispensable for the activity. To explore contribution of some major functional domains, we generated constructs expressing HNF4A2 fragments. We found that deletion of the AF-1 and DBD domains (Δ 1–119), LBD (Δ 137–365), or the F domain (amino acids 1–376) did not decrease the protein expression (SI Appendix, Fig. S5) but substantially abolished an efficient repression of the CLOCK:BMAL1 activity (Fig. 4B). To potently transrepress the CLOCK:BMAL1 transcriptional activity, our data reflect requirements of the DBD, LBD, and F domains within the intact HNF4 protein.

HNF4A mRNA Level and Chromatin Binding Are Rhythmic in Mouse. Our combined data suggest that HNF4A shares many of the canonical properties of a core clock protein (60). We further examined Hnf4a mRNA expression in liver, pancreas, and colon of C57BL/6J mice over a light/dark (LD) cycle, and found it to be rhythmic on a 24-h timescale, with a cyclic pattern (peak at night) and modest but detectable amplitude comparable to the Cry2 gene (SAS PROC ANOVA, P < 0.001, Fig. 5). HNF4A has been known to play central roles in dietary lipid absorbance, bile acid synthesis, conjugation, and transport (38, 61–63). However, the accumulation of HNF4A transcripts in the early evening is unlikely to be induced by diet, as mice that are normally fed and fasted have similar levels of Hnf4a transcript at zeitgeber time 14 (ZT14), in contrast to the remarkable differences in gluconeogenic genes Pck1 and G6pc (SI Appendix, Fig. S6). By analyzing ChiP-seq data (64), we observed binding of CLOCK, BMAL1, and CRYs at the Hnf4a gene (SI Appendix, Fig. S7), suggesting that Hnf4a transcription is directly modulated by the core clock proteins.

To address whether the HNF4A functions are also rhythmically regulated, we used chromatin immunoprecipitation to evaluate its genome-wide binding at ZT4 and ZT16 in the mouse liver, which correspond to the trough and peak of Hnf4a transcript abundance. Surprisingly, out of 20,230 sites identified at ZT16, 17,839 are differentially occupied by HNF4A at ZT4, when only 6,163 peak sites were identified using the same cutoff (Fig. 6A). Consistently, genome-wide HNF4A occupancy is clearly more extensive at ZT16 compared with ZT4 (Fig. 6B). In particular, we observed strong rhythmicity of HNF4A binding at genes involved in well-characterized HNF4A-regulated processes (SI Appendix, Fig. S8). HNF4A induces the expression of Hnf1a, another tissue-specific transcription factor that plays essential roles in key developmental processes (65). HNF4A is also required for bile acid synthesis by directly targeting Cyp7a1 (63, 66) and is required for the expression of the xenobiotic nuclear receptor PXR (NR1I2) (67). Indeed, all of the three target genes show oscillating expression in the mouse liver with phases similar to Hnf4a, by exhibiting higher levels during the night (SI Appendix, Fig. S9, data derived from the CircarDB database) (68). In aggregate, our data strongly suggest that the oscillation of tissue-specific nuclear receptor HNF4A directly determines the rhythmicity of an array of its target genes, such that temporal regulation of various aspects of physiology can be achieved.

Notably, some phosphorylation events in HNF4A protein were also rhythmic, peaking around ZT15 (69). It will be of interest to investigate whether the rhythmic chromatin binding also involves a post-translational mechanism, apart from the rhythmicity in mRNA level.

HNF4A and CLOCK:BMAL1 Cooccupy Clock and Metabolic Genes. To assess the genome-wide effects of HNF4A-mediated CLOCK:BMAL1 transrepression, we compared cistromes of HNF4A (ZT16), BMAL1 (ZT8), and CLOCK (ZT8) (64) and calculated their overlaps. At the center of the Chow–Ruskey plot, 1,911 loci in the genome are bound by all three factors (Fig. 6C). Motif analysis of the common binding sites shows a significant enrichment for the canonical CLOCK:BMAL1 E-box motif (CACCGTG) and HNF4A-binding motif (SI Appendix, Fig. S10A). Interestingly,

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**Fig. 5.** Gene Hnf4a is rhythmically expressed in vivo. Mouse liver (A), pancreas (B), and colon (C) were harvested at 4-h intervals over the course of 24 h. Transcript levels were analyzed by using RT-qPCR. Displayed are the means ± SD (n = 5 mice) normalized to nonoscillating Rplp0 expression levels.
Appendix

### Discussion

Our findings in this study highlight the fundamental roles played by the nuclear receptor HNF4s (including HNF4A and HNF4G) in maintaining the tissue-specific circadian oscillations in the intestinal and liver cells. Similarly, the Bmal1 activator RORγ, also a nuclear receptor, controls the amplitude of clock genes in peripheral tissues without affecting rhythms in the SCN, where it is not expressed (29, 33). The identification of tissue-specific clock regulators reveals one mechanism generating tissue-specific circadian rhythms and allows precise modulation of clock networks in specific organs.

We report here a noncanonical transrepression activity of HNF4A reminiscent of the antiinflammatory activities of glucocorticoid receptor (GR) that are caused predominantly by regulating the key proinflammatory transcription factors nuclear factor-κB (NF-κB) and activator protein 1 (AP-1). Consistent with our observations here, nuclear receptor GR physically interacts with NF-κB or AP-1 heterodimers and represses their transcriptional activity (70, 71). The exact mechanism by which GR inhibits the proinflammatory factors is still unclear, although several models have been proposed. The chromatin recruitment of GR to the vicinity of the proinflammatory factors could be achieved by “tethering,” through the protein–protein interactions. Apart from this, at some promoters, it could also be mediated by a “composite binding” in which GR binding to both the target transcription factor and DNA is required. Afterward, the first mode of GR-mediated transrepression is attributable to a direct repression of the DNA binding and/or transactivation functions of the proinflammatory factors, dependent on the specific physical interactions. In addition, there are indirect modes that may involve competition for limiting amounts of coactivator, recruitment of corepressor, and interference with the basal transcriptional machinery (BTM) (72–74).

Our deletion analysis suggests involvement of the DBD, LBD, and F domains of HNF4A in the transrepression of CLOCK:BMAL1. Similarly, GR DBD and LBD domains are also demonstrated to be required for its transrepression function (75–77). In HNF4A, the C-terminal F domain is known as a repressor region that is responsible for recruitment of corepressor SMRT/NCOR2. The requirement of the F domain may imply a role for corepressor recruitment in the process of CLOCK:BMAL1 inhibition. Taken together, we propose an activity of HNF4A in
transrepressing CLOCK:BMAL1, the core positive arm of the molecular clock, and consequently a central role in the maintenance of circadian oscillations. Supporting this model, we observed cooccupancy of HNF4A and CLOCK:BMAL1 at clock genes Dloop, Cry1, Cry2, Per2, and Per3 (SI Appendix, Fig. S11). An HNF4-binding motif was identified at most of these cooccupied loci, suggesting that throughout the HNF4A-mediated CLOCK:BMAL1 transrepression at these clock genes, the composite binding mode may be at play. Hence, similar to GR, HNF4A may bind to the regulatory regions of the clock genes, transrepressing the cooccupying CLOCK:BMAL1 via physical interactions.

Extensive chromosome colocalization of clock components and tissue-specific TFs have been implied to contribute to the circadian control of metabolic pathways (78, 79). In particular, the liver-lineage determining TF hepatocyte nuclear factor 6 (HNF6) tethers the circadian nuclear receptor Rev-erbα, leading to the repression of target gene and circadian regulation of hepatic lipid metabolism (80, 81). Our findings also suggest HNF4A to be a central regulator in the circadian control of metabolisms, which could be accomplished through activities at two distinct levels, depending on the chromatin environment. First, the extensive cooccupancy of HNF4A and CLOCK:BMAL1 strongly suggest that, apart from the core clock, HNF4A negatively modulates the transcriptional activity of CLOCK:BMAL1 in diverse metabolic pathways (82–84). Further, the circadian expression and genome binding of the nuclear receptor HNF4A itself, particularly at loci not cooccupied by CLOCK:BMAL1, is implied to directly result in the circadian expression of downstream metabolic genes responsible for bile acid synthesis and xenobiotic metabolism. HNF1α, another liver-specific TF that shows robust oscillations at the transcription level most likely due to targeting by HNF4A, potentially serves as another link between circadian regulation and metabolic pathways. Taken together, HNF4A provides an interesting link between the molecular regulation of core clock proteins and the metabolic pathways, a subject for future studies.

Materials and Methods

For details on luciferase assays, circadian assays, co-IP, qPCR, HNF4A ChIP-seq, and data analysis, please see SI Appendix, SI Materials and Methods. All animal care and experiments were performed under the institutional protocols approved by the Institutional Animal Care and Use Committee at the University of Southern California.

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