HNF4A defines tissue-specific circadian rhythms by beaconing BMAL1::CLOCK chromatin binding and shaping the rhythmic chromatin landscape

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Transcription modulated by the circadian clock is diverse across cell types, underlying circadian control of peripheral metabolism and its observed perturbation in human diseases. We report that knockout of the lineage-specifying Hnf4a gene in mouse liver causes associated reductions in the genome-wide distribution of core clock component BMAL1 and accessible chromatin marks (H3K4me1 and H3K27ac). Ectopically expressing HNF4A remodels chromatin landscape and nucleates distinct tissue-specific BMAL1 chromatin binding events, predominantly in enhancer regions. Circadian rhythms are disturbed in Hnf4a knockout liver and HNF4A-MODY diabetic model cells. Additionally, the epigenetic state and accessibility of the liver genome dynamically change throughout the day, synchronized with chromatin occupancy of HNF4A and clustered expression of circadian outputs. Lastly, Bmal1 knockout attenuates HNF4A genome-wide binding in the liver, likely due to downregulated Hnf4a transcription. Our results may provide a general mechanism for establishing circadian rhythm heterogeneity during development and disease progression, governed by chromatin structure.

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The circadian clock is a molecular oscillator that aligns behavior and physiology with daily light–dark cycles. The core of the mammalian circadian clock, composed of two interlocked transcriptional feedback loops, relies on chromatin occupancy of the master transcription factor heterodimer BMAL1::CLOCK at the E-box DNA element. BMAL1::CLOCK positively regulates expression of the Period (Per1, Per2, Per3), Cryptochrome (Cry1, Cry2), and Rev-erb (Nr1d1, Nr1d2) genes at the beginning of the feedback cycles. Protein dimer formed by PER and CRY suppresses the transcriptional activity of BMAL1::CLOCK, closing the first feedback loop. The second feedback mechanism is achieved by the nuclear receptor REV-ERBs to repress the transcription of the Arntl (Bmal1) gene (and to a lesser extent on Clock gene)1.

While many peripheral organs have circadian clocks, the identities of rhythmic outputs are considerably divergent across tissues2–6, contributing to organ-specific physiology and disorders associated with circadian misalignment7. However, the molecular mechanisms underlying heterogeneous circadian rhythms remain unclear. Tissue-specific transcription factors have been reported25–27 to our knowledge, how the tissue-specific factors may affect BMAL1::CLOCK recruitment has not been studied. To investigate the influence of HNF4A on BMAL1::CLOCK chromatin occupancy and circadian rhythms, we crossed Hnf4a floxed mice22 with Albumin-Cre mice and Per2-luciferase mice in the same C57BL/6J background to generate liver-specific Hnf4a knockout (Hnf4afl/fl; Alb-Cre/+; Per2-luc−/+; HKO) and control (Hnf4afl/fl; Alb-Cre−/−; Per2-luc−/+; Ctrl) mice (see the “Methods” section). In the HKO liver, RT-qPCR confirmed a ~75% decrease in Hnf4a transcript level accompanied by downregulation of the classic HNF4A target genes ApoC3, Fabp1, Ppara, and Hnf1α (Supplementary Fig. 1a). The liver-to-body-weight ratio was significantly increased for the HKO mice (Supplementary Fig. 1b). Histopathological analyses revealed extensive vacuolization in the HKO hepatocytes and marked lipid accumulation throughout the liver tissue (Supplementary Fig. 1c). Remarkably, in contrast with the premature lethality of HKO mice constructed with Albumin-Cre mice in the FVB genetic background22, the HKO mice we constructed here live to at least the age of 9 months. The Hnf4a knockout liver exhibited more severe pathological lesions and greater changes in gene expression in male mice than the female22,28, although the HCC development rate was sex-independent29. To eliminate sex as a confounder, we used male mice throughout the study. We mapped genome-wide BMAL1 binding profiles in liver samples collected from three HKO mice and three control mice at T76 when BMAL1 binding reaches maximum intensity30. Principal component analyses (PCA) of the three ChIP-seq replicates revealed clustering of samples from the same genotype (Supplementary Fig. 2a). Surprisingly, about 79% (5273 out of 6660) of the total BMAL1 peaks were prominently attenuated in the HKO-reduced BMAL1 binding genes at their target DNA sequences at enhancers. The pioneer TFs recruit histone methyltransferases MLL3/4 to deposit histone mark H3K4me1, whereby the condensed DNA wrapped around histones is loosened12. Completely activated enhancers feature bimodal distribution of histone modifications H3K4me1 and H3K27ac, nucleosome depletion, and recruitment of other transcription factors and coactivators13. Instead of being simply correlated with chromatin accessibility, H3K4me1 has an active regulatory role by serving as docking sites for chromatin remodelers14. Due to the activity of ATP-dependent chromatin remodelers15 and three-dimensional chromatin folding16, chromatin remodeling commonly creates extended accessibility beyond the central nucleosome pioneer TFs bind.

With most (~16%) transcripts exhibiting circadian expression, the liver is the primary organ controlled by the circadian clock4. The hepatic circadian transcripts are highly organ-specific and involved in most principal functions of the liver, including glucose homeostasis, lipogenesis, bile acid synthesis, mitochondrial biogenesis, oxidative metabolism, amino acid turnover, and xenobiotic detoxification. Indeed, environmental or genetic disruption of the circadian clock exacerbates the development of liver diseases such as non-alcoholic fatty liver disease (NAFLD), hepatitis, cirrhosis, and hepatocellular carcinoma (HCC)17. The hepatocyte nuclear factor 4A (HNF4A) is a nuclear receptor specifically expressed in the liver, kidney, pancreas, and intestinal tracts18. Mutation or dysregulation of the Hnf4a gene is associated with human diseases such as maturity-onset diabetes of the young (MODY) and HCC19,20. Whole-body Hnf4a knockout resulted in embryonic lethality, and liver-specific knockout mice displayed severe hepatocyte differentiation defects and premature death by 8 weeks of age21–23. We previously demonstrated that HNF4A modulates peripheral circadian clocks in cell cultures24. Here, we further interrogate the interface between HNF4A and the circadian clock in the liver tissue where they both play critical roles. We find that HNF4A supervises BMAL1 chromatin binding seemingly by remodeling chromatin accessibility. Synchronized with HNF4A recruitment24, mouse liver displayed increased genome-wide chromatin accessibility during the night. Furthermore, the circadian clock contributes to chromatin remodeling likely through regulating HNF4A. Our results reveal a collaborative effort between HNF4A and the clock machinery in shaping tissue-specific chromatin landscape and circadian rhythms that are vital for liver biology.

Results

BMAL1 chromatin binding is attenuated in the Hnf4a knockout liver. Previously we discovered an extensive genome-wide colocalization of HNF4A and BMAL1::CLOCK in the mouse liver24. While physical interactions and genome-wide occupancy between the diurnal regulatory machinery and tissue-specific transcription factors have been reported25–27, to our knowledge, how the tissue-specific factors may affect BMAL1::CLOCK recruitment has not been studied. To investigate the influence of HNF4A on BMAL1::CLOCK chromatin occupancy and circadian rhythms, we crossed Hnf4a floxed mice22 with Albumin-Cre mice and Per2-luciferase mice in the same C57BL/6J background to generate liver-specific Hnf4a knockout (Hnf4afl/fl; Alb-Cre+/−; Per2-luc+/+; HKO) and control (Hnf4afl/fl; Alb-Cre−/−; Per2-luc−/+; Ctrl) mice (see the “Methods” section). In the HKO liver, RT-qPCR confirmed a ~75% decrease in Hnf4a transcript level accompanied by downregulation of the classic HNF4A target genes ApoC3, Fabp1, Ppara, and Hnf1α (Supplementary Fig. 1a). The liver-to-body-weight ratio was significantly increased for the HKO mice (Supplementary Fig. 1b). Histopathological analyses revealed extensive vacuolization in the HKO hepatocytes and marked lipid accumulation throughout the liver tissue (Supplementary Fig. 1c). Remarkably, in contrast with the premature lethality of HKO mice constructed with Albumin-Cre mice in the FVB genetic background22, the HKO mice we constructed here live to at least the age of 9 months. The Hnf4a knockout liver exhibited more severe pathological lesions and greater changes in gene expression in male mice than the female22,28, although the HCC development rate was sex-independent29. To eliminate sex as a confounder, we used male mice throughout the study. We mapped genome-wide BMAL1 binding profiles in liver samples collected from three HKO mice and three control mice at T76 when BMAL1 binding reaches maximum intensity30. Principal component analyses (PCA) of the three ChIP-seq replicates revealed clustering of samples from the same genotype (Supplementary Fig. 2a). Surprisingly, about 79% (5273 out of 6660) of the total BMAL1 peaks were prominently attenuated by Hnf4a removal, including ones located within the E-box-containing core clock genes (Fig. 1a, b). In addition to the clock genes, KEGG and gene ontology (GO) pathway analyses of the HKO-reduced BMAL1 binding genes (Supplementary Data 1) identified enrichment of metabolic pathways, such as glucose and cholesterol metabolism, especially when compared with the unchanged binding sites (Supplementary Fig. 2b, c). Therefore, circadian regulation of these key tissue-specific nodes17 is supervised by HNF4A. The strong impact HNF4A exerted on BMAL1::CLOCK cistrome seemed to occur post-translationally, because BMAL1 transcript and protein levels were not reduced but rather moderately increased in the HKO liver, potentially related to downregulated Nrd1d1 and Nrd1d2 encoding transcriptional repressors of Bmal1 (Fig. 1c, d).

Motif analysis of the HKO-reduced BMAL1 peaks indicated an enrichment of the HNF4A-binding motif, apart from the E-box...
element (Supplementary Fig. 2d). We parsed all BMAL1 peaks into three groups based on signal variation in response to Hnf4a ablation: ones that were reduced (5273 peaks), enhanced (3354 peaks), or not significantly changed (1384 peaks). On average, BMAL1 peaks of higher intensity tended to be more responsive to Hnf4a ablation (Fig. 1e). We also plotted HNF4A ChIP-seq signals when they reach maximum at ZT16 (Supplementary Fig. 2e) at each position of the BMAL1 peaks, finding HNF4A to display higher accumulation at the HKO-reduced BMAL1 peaks, relative to the unchanged peaks (Fig. 1e). In contrast, for transcription factors PPARA, HNF1A, and LXR that were downregulated upon HKO removal (Supplementary Fig. 1a), by analyzing legacy ChIP-seq data, we did not observe their differential accumulation at BMAL1 peaks (Fig. 1e). Consistently, their binding motifs ranked far lower than the HNF4A-binding sequence at the HKO-reduced BMAL1 peaks (Supplementary Fig. 2d). The distance from a BMAL1 peak to the nearest HNF4A peak was significantly smaller in general for the HKO-reduced BMAL1 binding sites than unchanged ones (Supplementary Fig. 2e). Out of the 3517 BMAL1 peaks that colocalize with HNF4A occupancy, 3309 (94%) were greatly reduced by Hnf4a removal (Fig. 1f–h and Supplementary Fig. 2f). These data collectively indicate that
HNF4A directly regulates global BMAL1 chromatin binding in the mouse liver. The underlying mechanisms do not involve gene expression regulation but are likely achieved on chromatin in a spatially restricted manner.

**Hnf4a knockout alters genome-wide epigenetic landscape.** The cooperative loading of transcription factors may involve two mechanisms: (1) a simultaneous loading mediated by protein–protein interactions; (2) a sequential loading that requires a pioneer TF to open up local chromatin for other factors to bind.[13] Notably, we detected physical interactions between BMAL1 and HNF4A in liver cells.[24] To evaluate the possibility of HNF4A recruiting BMAL1 to the genome, we compared enrichments of the E-box element at HKO-unchanged and HKO-reduced BMAL1 binding sites. The “% of targets” and “p-value of enrichment” reported by HOMER analysis indicated that the E-box sequence was present at a similar frequency within the two categories of BMAL1-binding sites (Fig. 1i). Moreover, there were a considerable fraction (1902/5256 = 36%) of HKO-reduced BMAL1-binding sites indeed not displaying exactly overlapping HNF4A occupancy (Fig. 1i). Therefore, it is unlikely for the HNF4A-BMAL1 physical interactions to be generally responsible for the HNF4A-dependent BMAL1 occupancy. We were prompted to ask if HNF4A acts as a pioneer TF and facilitates the accessibility of a broad range of chromatin that is a prerequisite for BMAL1 binding to occur.

The chromatin loading of a pioneer TF initiates increases in accessible/primed enhancers marked by H3K4me1 and subsequent chromatin activation marked by H3K27ac.[13] Therefore, the intensity of H3K4me1 and H3K27ac defines chromatin landscape and is indicative of pioneer TFs’ activity. In agreement with our prediction, we observed a clear reduction in genome-wide H3K4me1 and H3K27ac deposition upon Hnf4a knockout (Fig. 2a, b, and Supplementary Fig. 3a, b), with the HNF4A-binding motif overrepresented at the HKO-reduced sites for both histone marks (Fig. 2c and Supplementary Fig. 3c). To interrogate to what extent HNF4A is involved in the early steps of chromatin remodeling, we looked into H3K4me1 and found it generally reduced at HNF4A-binding sites upon Hnf4a knockout (Supplementary Fig. 3d). In addition, HNF4A tended to accumulate more intensively at the H3K4me1 sites that would be significantly reduced by Hnf4a knockout (about 41.3% of total peaks), relative to the unchanged H3K4me1 sites (Fig. 2d). The distance from an H3K4me1 peak to the nearest HNF4A peak was noticeably smaller for the HKO-reduced H3K4me1 sites (Supplementary Fig. 3e). Similarly, the extent of H3K27ac loss in the HKO liver was positively correlated with the intensity of local HNF4A binding (Supplementary Fig. 3f). Motif analysis of all H3K4me1-marked regions in the control liver revealed maximal enrichment of the HNF4A-binding motif (Fig. 2e), in agreement with a global profiling finding HNF4A occupancy overrepresented in accessible regions of liver chromatin.[33] Taken together, HNF4A potentially serves as a key pioneer TF to open up local chromatin for other factors to bind.

**Fig. 2 Hnf4a knockout alters the genome-wide epigenetic landscape.** a, b Heatmap of H3K4me1 (a) or H3K27ac (b) ChIP-seq signals at ZT6 in control (left) or HKO (right) liver centered at all peaks in control liver. Peaks are ordered vertically by signal strength. c Motif analysis of HKO-deprived H3K4me1 sites. Known consensus motifs are shown with corresponding enrichment significance values. d H3K4me1 peaks in control and HKO livers were partitioned into three categories with DiffBind (the HKO-enriched group has only 23 peaks and could not be plotted), and then the corresponding HNF4A occupancy (at ZT16) at each H3K4me1 site was plotted. Each horizontal line represents a single H3K4me1 site. Peaks were ordered vertically by the strength of the H3K4me1 ChIP signal in control liver. e Motif analysis of all H3K4me1 marked sites in the control liver. Known consensus motifs are shown with corresponding enrichment significance values. f Metaplot showing the average intensity of BMAL1, H3K4me1, and H3K27ac ChIP-seq signals (all at ZT6) in control or HKO livers surrounding HKO-unchanged (upper panel) or HKO-reduced (lower panel) BMAL1 peak centers.
Ectopic HNF4A expression reprograms epigenetic landscape and induces tissue-specific BMAL1 bindings. Next, we sought to assess BMAL1 cistromes before and after HNF4A action in a biological system that has never been exposed to HNF4A protein. We ectopically expressed the adult isoform HNF4A2 in human bone osteosarcoma epithelial U2OS cells where the endogenous Hnf4a expression is negligible. 1742 BMAL1 peaks were moderately reduced by HNF4A2 expression (Fig. 3a and Supplementary Fig. 4a), seemingly resulting from downregulation of the Bmal1 transcription (Fig. 3b). In the meanwhile, we identified 311 BMAL1-binding events that were significantly enhanced or gained de novo in response to HNF4A2 expression, compared...
with the GFP expression group (Fig. 3a and Supplementary Fig. 4a). These HNF4A2-induced BMAL1 peaks were more frequently located at distal or intronic enhancer regions (Fig. 3c) and enriched with the HNF4A2-binding motif ranking second only to the E-box element (Fig. 3d). To interrogate the biological relevance of HNF4A2-induced BMAL1 bindings, we examined whether they occur in cells where HNF4A is naturally expressed. BMAL1 and HNF4A ChIP-seq signals from human liver cancer Hep3B or HepG2 cells were plotted correspondingly at each position of the BMAL1 binding sites we just profiled in U2OS-GFP and U2OS-HNF4A2 cells. Interestingly, BMAL1 ChIP signals in Hep3B cells displayed an analogous pattern to the U2OS-HNF4A2 dataset, i.e. signals at the U2OS-HNF4A2-enriched peak sites were stronger than those at the U2OS-GFP-enriched ones (Fig. 3e), indicating the U2OS-HNF4A2-induced BMAL1 peaks to be specifically expressed in liver cell cultures. Furthermore, endogenously expressed HNF4A in Hep3B or HepG2 cells was found to accumulate more abundantly at the U2OS-HNF4A2-induced BMAL1 peaks than the other sites (Fig. 3e). Therefore, the BMAL1-binding events we have induced in U2OS cells by introducing genome-wide occupancy of HNF4A2 may represent a true aspect of tissue-specific BMAL1 cistromes.

Chromatin landscape was confirmed to be remodeled by HNF4A2 expression, according to ChIP-seq profiling of H3K4me1 and H3K27ac (Fig. 3f and Supplementary Fig. 4b–d). In line with that observed at the gained BMAL1 peaks, the HNF4A2-enhanced H3K4me1 and H3K27ac sites were more likely located in distal or intronic enhancer regions (Supplementary Fig. 4e, f), concordant with a general recognition that lineage-specifying transcription factors exert physiologic effects through interactions with tissue-specific enhancers. The U2OS-HNF4A2-enhanced H3K4me1 sites were confirmed to enrich more HNF4A occupation than the other sites in liver cells (Fig. 3g), suggesting that HNF4A2 binding is directly responsible for the induced H3K4me1 deposition. The subset of H3K4me1 sites that were mildly reduced by HNF4A2 expression, considering the minimal on-site HNF4A localization in liver cells (Fig. 3g), likely resulted from indirect effects of HNF4A2 ectopic expression. Lastly, distinct from the other BMAL1 peaks, the HNF4A2-induced BMAL1 peaks were marked by locally enhanced deposition of H3K4me1 and H3K27ac upon HNF4A expression (Fig. 3h and Supplementary Fig. 4g). To exhibit the HNF4A2-reprogrammed BMAL1, H3K4me1, and H3K27ac peaks in higher resolution, we present genome tracks of representative genes (SLC25A42, DOK4, CDHR2, and PLPP3) in Fig. 5 and Supplementary Fig. 5. The fetal HNF4A isoforms lacking the N-terminal activation domain AF-1 relative to the adult isoforms are specifically expressed in the embryonic liver and diseased liver. They occupy much the same set of genome loci as the adult isoforms do yet exhibit a lower transcriptional activity. We found that ectopically expressing the fetal isoform HNF4A8 induced tissue-specific BMAL1 binding likewise (Supplementary Fig. 6), arguing that HNF4A4-regulated BMAL1 recruitment is variable during liver development and disease transition. Taken together, we programmed tissue-specific BMAL1 bindings by remodeling E-box-containing enhancers which are otherwise actively masked by nucleosomes. The chromatin landscape was confirmed to be remodeled by HNF4A2 expression, according to ChIP-seq profiling of H3K4me1 and H3K27ac (Fig. 3f and Supplementary Fig. 4b–d). In line with that observed at the gained BMAL1 peaks, the HNF4A2-enhanced H3K4me1 and H3K27ac sites were more likely located in distal or intronic enhancer regions (Supplementary Fig. 4e, f), concordant with a general recognition that lineage-specifying transcription factors exert physiologic effects through interactions with tissue-specific enhancers. The U2OS-HNF4A2-enhanced H3K4me1 sites were confirmed to enrich more HNF4A occupation than the other sites in liver cells (Fig. 3g), suggesting that HNF4A2 binding is directly responsible for the induced H3K4me1 deposition. 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BMAL1 and HNF4A ChIP-seq signals from human liver cancer Hep3B or HepG2 cells were plotted correspondingly at each position of the BMAL1 binding sites we just profiled in U2OS-GFP and U2OS-HNF4A2 cells. Interestingly, BMAL1 ChIP signals in Hep3B cells displayed an analogous pattern to the U2OS-HNF4A2 dataset, i.e. signals at the U2OS-HNF4A2-enriched peak sites were stronger than those at the U2OS-GFP-enriched ones (Fig. 3e), indicating the U2OS-HNF4A2-induced BMAL1 peaks to be specifically expressed in liver cell cultures. Furthermore, endogenously expressed HNF4A in Hep3B or HepG2 cells was found to accumulate more abundantly at the U2OS-HNF4A2-induced BMAL1 peaks than the other sites (Fig. 3e). Therefore, the BMAL1-binding events we have induced in U2OS cells by introducing genome-wide occupancy of HNF4A2 may represent a true aspect of tissue-specific BMAL1 cistromes.

Circadian rhythms are disturbed by Hnf4a knockout and Hnf4a-MODY mutation. We previously showed that Hnf4a knockout caused varying degrees of circadian rhythm disruption in cell cultures including period shortening and complete arrhythmia. Consistently, tissue explants of HKO liver harbored a shorter period of Per2-Luc oscillation ex vivo (Fig. 4a, b). Control and HKO liver tissues were collected every four hours from mice housed under a 12-h light:12-h dark cycle (LD 12:12). RT-qPCR quantification of the core clock transcripts revealed robust circadian oscillations in the control liver, while a damping was observed after Hnf4a ablation (Fig. 4c). This phenotype was especially clear for Dbp, Nrd1d1, and Nrd1d2 (Fig. 4c), genes that are distinct from the other E-box-containing clock genes and lose expression in the Bmal1 knockout mice. Downregulation of the three Bmal1::CLOCK-dependent genes was confirmed by dimmed local H3K4me1 and H3K27ac signals and associated with dysregulated BMAL1 recruitment (Fig. 4d and Supplementary Fig. 7). Since Hnf4a is minimally expressed outside the liver, kidney, pancreas, and intestinal tracts, we do not expect it to act on the master circadian clock in the SCN or animal behaviors.

Hnf4a mutations were frequently identified in patients with MODY, a rare form of diabetes. In agreement, disrupting Hnf4a expression in mouse islets or insulinoma cells resulted in impaired glucose-stimulated insulin secretion. Interestingly, insulin secretion by pancreatic β cells is rhythmic, and perturbation of the circadian cycles contributes to diabetes. Our results provide an excellent opportunity for investigating whether HNF4A-MODY mutations connect clock dysregulation...
to the development of diabetes. R85W is a mutation within the DNA-binding domain of HNF4A that was repeatedly identified in MODY patients.\(^{42,43}\) To investigate this connection, we generated homozygous R85W mutation using CRISPR-Cas9 and surprisingly found the mutant cells to exhibit fundamentally disrupted circadian rhythms (Fig. 4e and Supplementary Fig. 8a), resembling cells carrying Hnf4a homozygous knockout (Fig. 4f and Supplementary Fig. 8b). Therefore, HNF4A-MODY patients may express deregulated circadian rhythms which potentially contribute to the disease’s pathogenesis and progression.

**HNF4A governs liver-specific circadian transcription.**

The chromatin remodeling activity and rhythmic recruitment\(^{24}\) of HNF4A prompted us to test whether the hepatic chromatin landscape is dynamically shaped throughout the day. We performed ChIP-seq analyses of H3K4me1 and H3K27ac with wild-type mouse livers collected at ZT16, the peak time of HNF4A binding\(^{24}\), or the antiphase ZT6. Interestingly, genome-wide deposition of H3K4me1 or H3K27ac was overall higher at ZT16 and surprisingly found the mutant cells to exhibit fundamentally chromatin remodeling activity and rhythmic recruitment\(^{24}\) of Hnf4a resembling cells carrying WT Hnf4a knockout and HNF4A-MODY mutation. a, b Liver tissue explants were isolated from mice of indicated genotypes and recorded for Per2-Luc bioluminescence. Period lengths of Per2-Luc oscillation were plotted (means ± SD, n = 5 or 6) and statistical significance was determined by two-tailed Student’s t-test (a). Representative bioluminescence records show Per2-Luc circadian profiles in control or HKO liver (b). c Control and HKO mouse livers were harvested at 4-h intervals over the course of 24 h. Transcript level of genes was analyzed by using RT-qPCR. Displayed are the means ± SD (n = 3 or 4) normalized to non-oscillating Rplp0 expression levels. P-values determined by two-tailed Student’s t-test were displayed. d IGV genome tracks showing HNF4A (at ZT16), BMAL1 (at ZT6), H3K4me1 (at ZT6), and H3K27ac (at ZT6) enrichment at the Hnf4a+/- and Hnf4a+/-/ mice livers collected at ZT16, the peak time of HNF4A formation ChIP-seq analyses of H3K4me1 and H3K27ac with wild-type mouse livers collected at ZT16, the peak time of HNF4A prompted us to test whether the hepatic chromatin (Fig. 5a, b and Supplementary Fig. 9a, b). ATAC-seq that assesses deposition of H3K4me1 or H3K27ac was overall higher at ZT16 and surprisedly found the mutant cells to exhibit fundamentally chromatin remodeling activity and rhythmic recruitment\(^{24}\) of Hnf4a resembling cells carrying WT Hnf4a knockout and HNF4A-MODY mutation. a, b Liver tissue explants were isolated from mice of indicated genotypes and recorded for Per2-Luc bioluminescence. Period lengths of Per2-Luc oscillation were plotted (means ± SD, n = 5 or 6) and statistical significance was determined by two-tailed Student’s t-test (a). Representative bioluminescence records show Per2-Luc circadian profiles in control or HKO liver (b). c Control and HKO mouse livers were harvested at 4-h intervals over the course of 24 h. Transcript level of genes was analyzed by using RT-qPCR. Displayed are the means ± SD (n = 3 or 4) normalized to non-oscillating Rplp0 expression levels. P-values determined by two-tailed Student’s t-test were displayed. d IGV genome tracks showing HNF4A (at ZT16), BMAL1 (at ZT6), H3K4me1 (at ZT6), and H3K27ac (at ZT6) enrichment at the Dbp gene in liver tissues, based on normalized ChIP-seq read coverage. Track heights are indicated. e, f Representative effect of Hnf4a(R85W) point mutation (e) or Hnf4a knockout (f) on Bmal1-Luc oscillation in human Hep3B cells (n = 3).
were no longer greater than those at ZT424 (Fig. 6a and Supplementary Fig. 11b). KEGG and GO term analyses of (4576 out of 32,201) of total HNF4A ChIP-seq peaks reduced and controlled mice (Ctrl), identifying about 14% (2000 out of 14,800) of total HNF4A peaks; 10.3% for BKO-unchanged peaks) (Supplementary Fig. 11g, h), therefore, it is unlikely for chromatin recruitment mediated by protein–protein interactions to play a dominant role in the regulation. Instead, we found Hnf4a transcription steadily downregulated by 20–30% upon Bmal1 removal at all sampling times (Fig. 6d). Since Bmal1 directly binds to the Hnf4a gene body (Supplementary Fig. 11i), BMAL1::CLOCK likely modulates HNF4A chromatin binding through transcriptional regulation. Analogous to Per2 transcripts, although dampened, Hnf4a oscillation was not abolished by Bmal1 removal (Fig. 6d). Since night-enhanced Hnf4a expression was not altered by fasting24, mechanisms rather than feeding behavior may be involved in clock-independent Hnf4a oscillation.

**Fig. 11c, d.** HNF4A inhibits Wnt/β-catenin signaling and cell cycle progression, potentially underlying its tumor-suppressive roles52. In comparison, genome-wide binding of the well-characterized hepatic pioneer factor FOXA213 was barely affected by Bmal1 knockout (Supplementary Fig. 11e, f). BMAL1 co-occupancy was only slightly more enriched at the BKO-reduced HNF4A binding sites (19.2% colocalized with BMAL1 binding) than the control sites (14.0% for total HNF4A peaks; 10.3% for BKO-unchanged peaks) (Supplementary Fig. 11g, h), therefore, it is unlikely for chromatin recruitment mediated by protein–protein interactions to play a dominant role in the regulation. Instead, we found Hnf4a transcription steadily downregulated by 20–30% upon Bmal1 removal at all sampling times (Fig. 6d). Since Bmal1 directly binds to the Hnf4a gene body (Supplementary Fig. 11i), BMAL1::CLOCK likely modulates HNF4A chromatin binding through transcriptional regulation. Analogous to Per2 transcripts, although dampened, Hnf4a oscillation was not abolished by Bmal1 removal (Fig. 6d). Since night-enhanced Hnf4a expression was not altered by fasting24, mechanisms rather than feeding behavior may be involved in clock-independent Hnf4a oscillation.

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significantly enhanced histone modifications included Npas2 (Fig. 7c).

Motif analysis of the BKO-enhanced H3K4me1 (n = 264) or H3K27ac (n = 134) peaks identified the ROR response element (RORE), binding motif of transcriptional repressors REV-ERBs (Supplementary Fig. 12a-d). Interestingly, at the BKO-reduced H3K4me1 (n = 987) or H3K27ac (n = 101) peaks, we did not identify the E-box element but instead found an enrichment of nuclear receptor binding sites, with the HNF4A-binding motif top-ranked (Supplementary Fig. 12a-f). About 4.6% of total H3K4me1 peaks display colocalization with BMAL1 binding within a distance of 500 bp. This degree of BMAL1 colocalization remained similar for the BKO-unchanged (4.5%) and BKO-reduced (4.0%) subgroups of H3K4me1 sites (Fig. 7f and Supplementary Fig. 12g), indicating BMAL1 occupancy not enriched at the BKO-reduced H3K4me1 sites. In contrast, H3K4me1 peaks having HNF4A colocalization increased from a background of 19.0 to 27.5% for the BKO-reduced sites, and decreased to 16.6% for the BKO-unchanged sites (Fig. 7f and Supplementary Fig. 12h). We consider HNF4A co-occupancy enriched at the BKO-reduced H3K4me1 sites, given that only 42% of the HKO-reduced H3K4me1 peaks exhibited HNF4A colocalization within the same distance of 500 bp (Supplementary Fig. 12i). We noticed that HNF4A occupancy was selectively reduced at the BKO-reduced H3K4me1 sites compared with the unchanged sites (Supplementary Fig. 12i). Importantly, we plotted H3K4me1 and H3K27ac ChIP-seq signals at each position of the BMAL1 or HNF4A peaks to find both histone marks specifically attenuated by BKO at HNF4A peaks (Fig. 7g) rather than at BMAL1 peaks (Fig. 7h). Therefore, BMAL1::CLOCK occupancy does not directly regulate active epigenetic modifications at ZT16 but through positively modulating HNF4A. Taken together, it is unlikely for BMAL1 to regulate HNF4A cistrome through chromatin remodeling.

**Discussion**

Our findings demonstrate that HNF4A may act as a pioneer TF creating tissue-specific repertoires of accessible *cis*-regulatory elements. Consistently, the HNF4-binding element was top-scoring in accessible chromatin regions in the intestinal duodenal epithelium. HNF4A was essential for maintaining active histone signature H3K27ac in the intestine and liver. While HNF4A was an established fundamental liver development
regulator, it was not as well characterized in the process of chromatin remodeling as another hepatic TF FOXA/HNF313,56,57. Nevertheless, we found the HNF4A-binding motif for chromatin remodeling as another hepatic TF FOXA/HNF3 does or engages ATP-dependent enzymes to expand the “openness” of local chromatin13.

The REV-ERB regulation of metabolic genes was reported to require chromatin recruitment by hepatic transcription factors10,60. The activity of pancreatic cycling gene expression displayed a correlation with the binding of the pancreas-specific transcription factor PDX115. Despite these insights into the role of the lineage-specifying TFs, there has been a gap in understanding the molecular basis of tissue-specific rhythmicity whose misalignment is closely associated with organ-specific disorders7. By using loss-of-function and gain-of-function genetic models, we demonstrate that the lineage-specifying HNF4A is critical and in some cases sufficient for establishing liver-specific BMAL1 cistrome, seemingly independent of direct recruitment but by means of providing permissive chromatin structure. Our results may provide a molecular basis for tissue-specific BMAL1::CLOCK cistromes depending on the chromatin structures likely arising in the future to clarify whether HNF4A can independently displace histones like FOXA/HNF3 does or engages ATP-dependent enzymes to expand the “openness” of local chromatin13.

Fig. 7 Bmal1 knockout alters the epigenetic landscape in the liver, seemingly due to attenuated HNF4A activity. a, b Heatmap of H3K4me1 (a) or H3K27ac (b) ChIP-seq signals at ZT16 in control (left) or BKO (right) liver and centered at all peaks in control liver. Peaks are ordered vertically by signal strength. c, d MA plot showing differential H3K4me1 (c) or H3K27ac (d) peaks in control and BKO livers, using a threshold of FDR < 0.05. The x-axis represents the mean number of reads (log scaled) within the peaks across all samples. The y-axis represents the log fold change between the two samples. e IGV genome tracks showing BMAL1 (at ZT6), HNF4A (at ZT16), H3K4me1 (at ZT16), and H3K27ac (at ZT16) enrichment at Nrf1d2 or Npas2 genes in control or BKO liver, based on normalized ChIP-seq read coverage. Track heights are indicated. f Percentages of three groups of H3K4me1 sites colocalizing with BMAL1 or HNF4A peaks. Peak numbers for percentage calculation are in Supplementary Fig. 12. g, h Metaplot showing average intensity of HNF4A, H3K4me1, and H3K27ac ChIP-seq signals (all at ZT16) in control or BKO livers surrounding HNF4A peak centers in control liver. h Metaplot showing the average intensity of BMAL1 (at ZT6), H3K4me1 (at ZT16), and H3K27ac (at ZT16) ChIP-seq signals in control or BKO livers surrounding BMAL1 peak centers in control liver.

rhythmic whose mis-expression changes during development. To gain mechanistic insights, direct nucleosome binding studies will be needed in future to clarify whether HNF4A can independently displace histones like FOXA/HNF3 does or engages ATP-dependent enzymes to expand the “openness” of local chromatin13. Of local chromatin13.
from early events in tissue development. Systematic profiling of 20 diverse human cell types identified ~25% of genes displaying cell-type-specific expression that is explained by alterations in chromatin structures. Our discoveries suggest that tissue-specific chromatin landscape profoundly shapes the circadian rhythms, providing a unifying mechanism for circadian rhythm heterogeneity across tissue types. We recently reported that the genome-wide BMAL1:CLOCK occupancy in glioblastoma stem cells was more expanded as compared with normal neural stem cells. Given that chromatin structure alterations are prevalent in tumor tissues, our findings may provide additional insights into reprogrammed circadian clocks now found in cancer and many other disease states.

Liver-specific Hnf4a removal undermined BMAL1 occupancy at most of its target genes including the E-box-containing core clock genes (Fig. 1b), downregulated transcription of the BMAL1:CLOCK-dependent core clock genes Dbp, Nr1d1, and Nr1d2 (Fig. 4c), and shortened the period of Per2-Luc oscillation (Fig. 4a, b). Potentially resulting from altered clock genes (Fig. 1b), downregulated transcription of the at most of its target genes including the E-box-containing core repression activity we have characterized can serve as a second mechanism for HNF4A to transcriptionally activity and a simultaneous increase in BMAL1::CLOCK recruitment during the day and secure the circadian rhythms through two mechanisms: (1) to facilitate BMAL1::CLOCK recruitment during the day and secure the operation of the core clock; (2) to open chromatin maximally during the night and promote predawn-clustered expression of tissue-specific circadian outputs.

Finally, the rhythmic HNF4A genome binding was disrupted by chronic jet lag (Fig. 6a). BMAL1 promoted efficient genome binding of HNF4A, likely independent of protein–protein interactions or chromatin remodeling but through activating Hnf4a transcription (Figs. 6 and 7). Bmal1 knockout only slightly altered H3K4me1 and H3K27ac at ZT16 (Fig. 7a–d). The RORE element was enriched at BKO-enhanced modification sites; the BKO-reduced modification sites did not enrich E-box element or BMAL1 colocalization but were associated with HNF4A binding (Fig. 7f–h). Therefore, BMAL1:CLOCK modulates hepatic epigenetic landscape potentially by activating target genes, namely Rev-erbs and Hnf4a. These results incidentally support our main finding that HNF4A shapes the hepatic chromatin landscape. The circadian clock regulates HNF4A transcription and rhythmic DNA binding whereby it contributes to the hepatic epigenetic landscape.

**Methods**

**Animal experiments.** All animal care and experiments were performed under the institutional protocols approved by the Institutional Animal Care and Use Committee (IACUC, #20826) at the University of Southern California. Hnf4a floxed mice (The Jackson Laboratory #004665) were crossed with Albumin-Cre mice (The Jackson Laboratory #003574) and Per2-luciferase reporter mice (The Jackson Laboratory #006852) to obtain Hnf4a+/−;Alb-Cre+/−;Per2-luc+/− and Hnf4a+/−;Alb-Cre+/−;Per2-luc+/− mice, which were then mated to obtain Hnf4a−/−;Alb-Cre−/−;Per2-luc−/− (HKO) and Hnf4a−/−;Alb-Cre−/−;Per2-luc−/− (Control) littersmates. Bmal1 floxed mice (The Jackson Laboratory #007688) were crossed with mice expressing Albumin-Cre (The Jackson Laboratory #003574) to obtain Arntl+/−;Alb-Cre+/− and Arntl−/−;Alb-Cre−/− mice which were then mated to obtain Arntl−/−;Alb-Cre−/− (BKO) and Arntl−/−;Alb-Cre−/− (Control) littersmates. In all experiments, male mice between 10 and 12 weeks of age were used. In all experiments except the jet lag treatment, mice were housed in a room with controlled temperature of 21–23°C and humidity of 35–40% under a 12-h light/12-h dark (LD) cycle with free access to food and water. The chronic jet lag treatment was performed by housing experimental mice in the light-tight cabinet and switching lighting conditions between two light onset schedules which are apart by 8 h every 3 days from 7 to 11 week of age.

**Plasmid constructs, lentivirus production, and generation of stable cell lines.** EGFp, Hnf4a2 (OriGene, #RC217863), and Hnf4a3 (OriGene, #RC238302) genes were subcloned into the lentiviral vector pLV-EF1a-IRES-Ametrine. The constructs were co-transfected with the envelope and packaging plasmids into HEK 293T cells for lentivirus production. Viral supernatants were collected twice, at 48 and 72 h after transfection, pooled and filtered through 0.45 μm filters, and then added to U2OS cells for transduction. The positively transduced U2OS cells were selected by FACS sorting according to the Ametrine signals.

**Cell culture and circadian assays.** Hnf4a knockout and MODY Hnf4a(R85W) mutation in Hep3B cells expressing Bmal1-Luc reporter were generated using CRISPR-Cas9. HEK 293T, U2OS, and Hep3B cells were grown in complete DMEM (Life Technologies cat. #11995065) supplemented with 10% FBS and 1% penicillin/streptomycin. HepG2 cells were grown in Ham’s F12 (Corning Cellgro 10-080-CV) supplemented with 10% FBS, and 1% penicillin and streptomycin. All cells were grown in a 37 °C incubator maintained at 5% CO2. The Hep3B cell line was a gift from Dr. Michael Karin at UCSD, originally purchased from ATCC (HB-8064). HEK 293T, U2OS, and HepG2 cell lines were directly purchased from ATCC (CRL-3216, HTB-96, HB-8065).

For circadian assays, Hep3B cells were plated on 35-mm dishes and synchronized as previously described by a dexamethasone shock. In brief, cell culture media was replaced with HEPES-buffered phenol-free DMEM medium containing 100 nM dexamethasone and 100 μM D-luciferin. Dishes were covered with 40 mm glass coverslips (Fisher Scientific) and sealed with vacuum grease to prevent evaporation. Luminescence signals were monitored every 10 min using the LumiCycle luminometer (Actimetrics) at 37 °C without supplementary CO2. Results shown are representative of at least three independent experiments.

**Liver explant circadian assays.** Mice were anesthetized with isoflurane and euthanized by cervical dislocation, and then livers were rapidly removed and kept at −80°C before the experiments.
on ice. Liver sections (2–3 mm² – 3 mm) were cultured in explant medium (DMEM supplemented with 10% FBS, 400 μM NaOH, 1% PSC, and 1 mM l-lysine, pH 7.2). Dishes were covered with 40 μm glass coverslips (Fisher Scientific) and sealed with vacuum glue to prevent evaporation. The luminescence signals of reporter cells were monitored every 10 min using a LumiCycle luminometer (Actimetrics) at 37 °C without added CO₂. The circadian period was calculated using the LumiCycle software (Actimetrics). Results shown are representative of at least three independent experiments.

Western blotting. Frozen mouse liver tissue was homogenized in RIPA buffer containing 1× EDTA-free protease inhibitor cocktail (Roche) by using Omni Tissue Homogenizer (Omni International). The concentration of total protein was determined by Bio-Rad Protein Assay and then equalized to 15 μg/μl. 25 μg of total protein was used for western blot assay which was performed as previously described. Antibodies used in the western blots are anti-BMAL1 (Cell signaling, #14020), anti-H3K4me1 (Abcam, ab8895), anti-H3K27ac (Abcam, ab4729), and anti-FOXA2 (Abcam, ab256493).

ChIP-seq analysis. Single-end ChIP-seq reads were trimmed using Trimmomatic (v0.36) and then aligned to hg38 or mm10 genome with Bowtie2 (v2.3.4.1). BAM files were processed using SAMtools (v1.10) and PCR duplicates were removed with PicardTools (v2.18.3). Peaks were called in MACS2 (v2.1.2) using default settings and IgG mock ChIP files for normalization. BAM files of replicate samples were merged using SAMtools. BIGWIG track coverage files were generated from merged BAM files using the DeepTools (v3.0.0) bamCoverage command with RPGC normalization. Heatmaps and metaphots were generated by the computeMatrix, plotHeatmap, and plotProfiles functions of DeepTools (v3.0.0) using BIGWIG files (replicates merged) and scaled regions. DiffBind (v3.2.7) was used to make PCA plots. Statistically significantly differential peaks were called and MA plots were generated by using the DESeq2 method within DiffBind, which selected differential regions based on ChIP signals in each replicate and FDR-corrected p-value of 0.05.

HOMER (v4.11.1) mergePeaks program was used to identify overlapping binding loci of two transcription factors. In order to define the sites as “overlapping”, peak centers of the two binding sites must be at a distance ≤500 bp. Note that the peak numbers may not add up exactly since the function automatically resolves redundant peaks by dropping one peak from each fragment during analysis. Motif enrichment analysis was performed using HOMER findMotifsGenome.pl command and scanned ±200 bp from the peak center for binding sites of transcription factors, and ±750 bp for histone modifications. HOMER annotatePeaks.pl command was used to make annotations of genomic features. The functional analyses of GO term (“Biological Process” sub-ontology) and KEGG pathway were performed using the clusterProfiler package in R or DAVID (https://david.ncifcrf.gov).

ATAC-seq experiments. The ATAC-seq procedure was based on a published method. Briefly, liver tissues were pulverized with mortar and pestle in liquid nitrogen and followed by nuclei permeabilization, tagmentation, library preparation, and Illumina HiSeq paired-end sequencing.

ATAC-seq analysis. Paired-end ATAC-seq reads were trimmed using Trimmomatic (v0.36) and then mapped to mm10 mouse genome using Bowtie2 (v2.3.4.1). SAMtools (v1.10) was used to generate BAM files, remove PCR duplicates, and remove mitochondrial DNA. MACS2 version 2.1.2 was used for peak calling with the following parameters: --nomodel --broad --shift 0 --extsize 200 --keep-dup all.

Quantification and statistical analysis. The significance of differences between peak distance, period length, and gene expression was evaluated by unpaired Student’s t-test (two-tailed), with significance levels adjusted using the False Discovery Rate method. For GO term and KEGG pathway analyses, clusterProfiler calculated p-values using a hypergeometric distribution which was then adjusted for multiple comparison.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support this study are available from the corresponding authors upon reasonable request. Raw data (fastq files) and final processed data (bigWig and peak files) for NGS experiments are available on GEO under accession code GSE157452, GSE35262 and E-MTAB-941 were used to analyze PPARA, HNF1A, and LXRβ binding at BMAL1 binding sites. GSE39860 and SRA025656 were used for reanalysis of H3K4me1 circadian rhythms. CircaDB [http://circa.db.hogeneschlab.org/] was used for identification of circadian transcripts. Source data are provided with this paper.

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Author contributions
M.Q. designed the study, performed data collection and bioinformatic analyses, and wrote the manuscript. H.Q. and Z.I. performed the majority of bioinformatic and statistical analyses. S.A.K. advised on study design, supported manuscript preparation, and supervised the project.

Competing interests
The authors declare no competing interests.

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