

SI Materials and Methods

Cell Culture.

HEK 293T, U2OS and HepG2 cells were purchased from the American Type Culture Collection (ATCC). SW480 Per2-dluc reporter cells were a gift from Carrie Partch, Chemistry & Biochemistry Department, UC Santa Cruz. Hep3B and dihXY HCC cell lines with a Bmal1-luc reporter were a gift from Michael Karin, Department of Pathology, UCSD and Jeremy Rich, Department of Medicine, UCSD. HEK 293T, U2OS, SW480, Hep3B and dihXY cells were grown in complete DMEM (Life Technologies cat. #11995065) supplemented with 10% FBS, and 1% penicillin and 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.

Transient Transfection and Endpoint Luciferase Reporter Assays.

Plasmids were transiently transfected in triplicate into HEK293T cells in a 96-well plate using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc. cat. # 11668019) following manufacturer's instructions. The luciferase activity was assayed with Bright-Glo luciferin reagent (Promega) on a microplate reader (Infinite M200, Tecan).

Generation of Lentiviruses and Stable Cell Lines.

Lentiviral vector overexpressing *EGFP*, *Cry1*, *Per2* or *Hnf4a* gene was co-transfected with packaging plasmids psPAX2 and pMD2.G into HEK 293T cells for virus production. Viral supernatants were collected twice, at 48 h and 72 h after transfection, filtered through a 0.45 µm filter and added to U2OS Per2-dluc cells for transduction. The positively infected cells were selected by FACS sorting according to EGFP signal expressed independently by the lentiviral vector.

Cell-based Circadian Assays.

The circadian rhythms of U2OS cells was tested as previously described (3). In brief, U2OS reporter cells harboring Per2-dLuc were plated onto 96-well white solid-bottom plates in normal growth media. After reaching 90-100% confluency, the growth media was changed to explant medium (DMEM (12800-017, Gibco) supplemented with 10% FBS, 10 mM HEPES, 0.38 mg/ml sodium bicarbonate, 0.29 mg/ml L-glutamine, 100 units/ml penicillin, 100 pg/ml streptomycin, 0.1 mg/ml gentamicin, and 1 mM luciferin, pH 7.2). The plate was covered with an optically clear film and set to a microplate reader (Infinite M200, Tecan). The luminescence rhythms of U2OS Per2-dLuc reporter cells were recorded every 60 min for 5 days. The circadian period was calculated using the MultiCycle software (Actimetrics).

The siRNA was transfected into SW480, Hep3B and dihXY cells with Lipofectamine RNAiMAX Reagent (Life Technologies cat. #13778150) according to manufacturer's instructions. siRNA used are human *Cry1* (HSS102308, Invitrogen), mouse *Cry1* (SI02666580, Qiagen), human *Hnf4a* (SI03083773, Qiagen) and mouse *Hnf4a* (SI01068151, Qiagen). After the transfection, SW480, Hep3B and dihXY cells were plated on 35-mm dishes. Cells were synchronized as previously described by a dexamethasone shock (1). In brief, after reaching 90-100% confluency, cell culture media was replaced with HEPES-buffered phenol-free DMEM media containing 100 nM

dexamethasone and 100 μ M D-luciferin. Dishes were covered with 40 mm glass coverslips (Fisher Scientific) and sealed with vacuum grease 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). In all circadian assays, the results shown are representative from at least three independent experiments.

Co-immunoprecipitations and Western Blotting.

HEK 293T cells were transfected with 1 μ g of plasmid expressing BMAL1-FLAG (Bmal1-Flag/p3F), CLOCK-FLAG (Clock-Flag/pcDNA3-intron), PER1-FLAG (Per1-Flag/p3F), PER2-FLAG (Per2-Flag/p3F), CRY1-FLAG (Cry1-Flag/p3F), CRY2-FLAG (Cry2-Flag/p3F), EGFP-FLAG (EGFP-Flag/p3F) or HA-HNF4A2 (HA-HNF4A2/pcDNA3-intron), in 12-well plate format. 40 hours post transfection, cells were washed twice with cold PBS and incubated on ice for 15 min in 100 μ l lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% NP-40) containing 1x EDTA-free protease inhibitor cocktail (Roche) and 1 mM DTT. After centrifuging at 13,000 rpm for 15min, the supernatant was collected and stored at -80°C. Cell lysates overexpressing a Flag-tagged core clock gene and HA-tagged HNF4A2 were mixed and incubated at 30°C for 2 h. Simultaneously, 10 μ l of Dynabeads Protein G (Invitrogen cat. #10004D) was incubated with 2 μ g anti-FLAG monoclonal antibody (F1804, Sigma) at 4°C for 2 h. After three washes, the Dynabeads were incubated with the lysate mix at 4°C for another one hour. IPs were washed three times with wash buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1% NP-40, 2mM EDTA, 10% glycerol) containing 1x EDTA-free protease inhibitor cocktail (Roche) and 1 mM DTT. Dynabeads were boiled in SDS sample buffer containing 5% β -mercaptoethanol. Western blot analyses were performed as previously described (10). Antibodies used in the western blots are: polyclonal anti-FLAG (F7425, Sigma) and anti-HA-Peroxidase (12013819001, Roche).

For endogenous co-immunoprecipitations, HepG2 cells were cultured to reach 80-90% confluency in 10 cm culture dish, rinsed twice with cold PBS and lysed in 1ml lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% NP-40) containing 1x EDTA-free protease inhibitor cocktail (Roche) and 1 mM DTT, by keeping on ice for 30 min. After centrifuging at 13,000 rpm for 15min, the supernatant was collected and stored at -80°C. The immunoprecipitations were performed by incubating 400 μ l HepG2 lysate with mouse IgG (ab18413, abcam), anti-mCRY1 (epitope: residues 506-606) or anti-mBMAL1 (epitope: residues 456-632) generated by Superview Biotechnologies Co. Ltd., Jiangsu, China. overnight at 4°C. 20 μ l Dynabeads Protein G (Invitrogen cat. #10004D) was added to the lysate-antibody mix and incubated for another 2 h. IPs were washed three times with wash buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1% NP-40, 2mM EDTA, 10% glycerol) containing 1x EDTA-free protease inhibitor cocktail (Roche) and 1 mM DTT. Dynabeads were boiled in SDS sample buffer containing 5% β -mercaptoethanol. Western blot analyses were performed as previously described (10). Antibodies used in the western blots are: anti-mCRY1 (epitope: residues 506-606), anti-mBMAL1 (epitope: residues 456-632) and anti-HNF4A (ab181604, abcam).

Quantitative RT-PCR.

Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA)

and group-housed at the Animal Facility of University of Southern California (USC). Mice were housed in a 12-h light/12-h dark (LD) cycle with continuous access to food and water for at least 2 weeks before study. All animal care and experiments were performed under the institutional protocols approved by the Institutional Animal Care and Use Committee (IACUC) at USC.

Liver, pancreas and colon of 8- to 10-week-old male C57BL/6 mice were harvested in 4-h intervals over the course of 24 hours. Total RNA was isolated using TRIzol Reagent according to the manufacturer's instructions (Life Technologies cat. #15596026), and then reverse transcribed to cDNA by using iScript cDNA Synthesis kit (Bio-Rad cat. #1708891). We designed the real-time primers spanning the exon-intron junctions using the IDT primer-designing software PrimerQuest (<https://www.idtdna.com/PrimerQuest>). Primer sequences are:

Rplp0: forward GGCCCTGCACTCTCGCTTTC, reverse TGCCAGGACGCGCTTGT;
Cry1: forward GGGCTGGATCCACCATTAG, reverse TCAAAGACCTTCATCCCTTCTTC;
Cry2: forward GATGGAGGTTCTACTGCAATC, reverse CAGCCTTGGGAACACATCA;
Per2: forward GAGTGTGTGCAGCGCTTAG, reverse GTAGGGTGTGTCATGCGGAAGG;
Hnf4a: forward GTTCTGTCCAGCAGATCAC, reverse GCTCCTTCATAGACTCACACAC;
Bmal1: forward CCCTAGGCCTTCATTGGATTT, reverse GCAAAGGGCCACTGTAGTT.

RT-qPCR analyses were performed as described previously (3) with CFX384 Real-Time PCR Detection System (Bio-Rad).

HNF4A ChIP-seq.

~ 50 mg of blood perfused liver tissue were dissected from C57BL/6J mice, snap frozen in liquid nitrogen and stored at -80°C. Frozen liver tissues were minced in ice-cold PBS using razor blades into 1 mm cubed, and then homogenized by pushing through 1.5-inch 18G needle for 10 times and then 1.5-inch 21G needle for 20 times. Homogenized cells were immediately crosslinked with 1% formaldehyde for 10 min. The crosslink was quenched by adding glycine to a final concentration of 125 µM. After two washes with cold PBS, the nuclei were lysed in 150 µl nuclear lysis buffer (50 mM Tris-HCl pH 8.0, 1% SDS, 10 mM EDTA) containing 1x EDTA-free protease inhibitor cocktail (Roche), by keeping on ice for 5 min. Immediately, to fragment the chromatin, the lysates were sonicated 17 times for 30 sec by using Bioruptor (Diagenode). After centrifuged at 17,000 rpm for 15 minutes at 4°C, the 150 µl fragmented chromatin was diluted 10-fold with IP dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) supplemented with 1x EDTA-free protease inhibitor cocktail, and then incubated with 7 µg anti-HNF4A (abcam cat. ab41898) overnight at 4°C. 100 µl of Dynabeads Protein G (Invitrogen cat. #10004D) was added to the chromatin and incubated for another 3 h. Afterwards, the beads were washed once with low salt wash buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA) and once with high salt wash buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA). 200 µl of IP elution buffer (1% SDS, 100 mM NaHCO₃) was applied to the bead pellet and incubated at 30°C for 15 min. The eluate was added with 5M NaCl to final conc. of 200 mM and reverse-crosslinked at 65°C overnight. To remove RNA and protein, RNase A and proteinase K were subsequently applied by incubating at 45°C for 1 h. Finally, we diluted the DNA with 5 volumes of Qiagen

buffer PB (QIAquick PCR Purification Kit) and purified with QIAquick PCR Purification columns. We eventually resolved our DNA fragments in 30 μ l Qiagen Buffer EB.

ChIP-seq Library Construction and Processing.

We had assistance from the NGS and Microarray Core Facility at The Scripps Research Institute, La Jolla, in sequencing library construction and processing. For library preparation, 2 ng of each sample was prepared using the NEB Ultra DNA Library Prep Kit for Illumina following manufacturer's instructions. Each library was dual size selected with 0.55X and 0.14X Ampure beads followed by PCR amplification with Kappa HiFi 2x PCR mix (15 cycles). PCR product was then quantitated using the Qubit (Thermo Fisher) and BioAnalyzer (Agilent BioAnalyzer 2100) to determine library quantity. Libraries were then gel purified on a 2% agarose gel to remove secondary PCR amplification artifacts, quantitated on the Qubit and loaded onto a NextSeq 500 (Illumina) flow cell for 1 x 76 base single-end sequencing. Approximately 20M reads were generated for each sample.

Bioinformatic Analysis.

ChIP-seq tags of HNF4A were aligned against the reference mouse genome mm9 using Bowtie2 and the peaks were called for each time-point using PePr (6). CLOCK, BMAL1, CRY1 and CRY2 mapped reads were obtained from GSE39977 and the peaks were called using MACS1.4 (7). bedGraph tracks and motif analyses were obtained or performed using HOMER (8). Overlapping peaks were identified using BEDOPS (9) and the Chow-Ruskey diagram was generated using the R package Vennerable (Swinton, 2011). HNF4A ChIP-seq data at ZT4 and ZT16 have been deposited in NCBI GEO under accession number GSE118007.

Quantification and Statistical Analysis.

Data shown are mean \pm SD. The n values represent biological repeats measured independently as specified in each figure legend. Statistical analysis was performed by unpaired Student's t test (two-tailed), with significant differences at $p < 0.05$. The significance of dose dependence in Fig. 1D-F was analyzed using one-way ANOVA. For Fig. 1D GFP titration, $p = 0.205$; CRY1 titration, $p = 1.42 \times 10^{-5}$; HNF4A2 titration, $p = 6.04 \times 10^{-8}$. For Fig. 1E GFP titration, $p = 0.232$; CRY1 titration, $p = 4.79 \times 10^{-5}$; HNF4A2 titration, $p = 0.013$. For Fig. 1F GFP titration, $p = 0.579$; CRY1 titration, $p = 0.00481$; HNF4A2 titration, $p = 9.65 \times 10^{-5}$. The significance of *Hnf4a* transcript variation over the course of 24 hours was analyzed using PROC ANOVA in SAS program.

References

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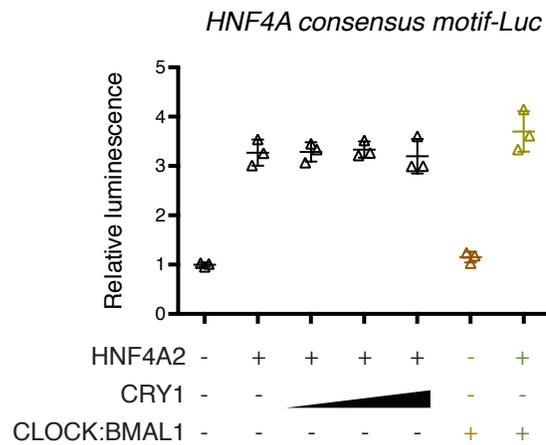


Figure S1. HNF4A activity is not affected by the core clock components. (related to Figure 1) HEK 293T cells were co-transfected with luciferase reporter gene driven by a tandem consensus sequence of HNF4A binding sites (TGGACTTTGAACTaaaTGGACTTTGAACTaaaTGGACTTTGAACTaaaTGGACTTTGAACT), HNF4A, CLOCK:BMAL1, and increasing amounts of CRY1 plasmid (n = 3 for each condition, mean \pm SD).

Green= Pos Control, Blue= Neg Control, Red= Data, Black= Zero line

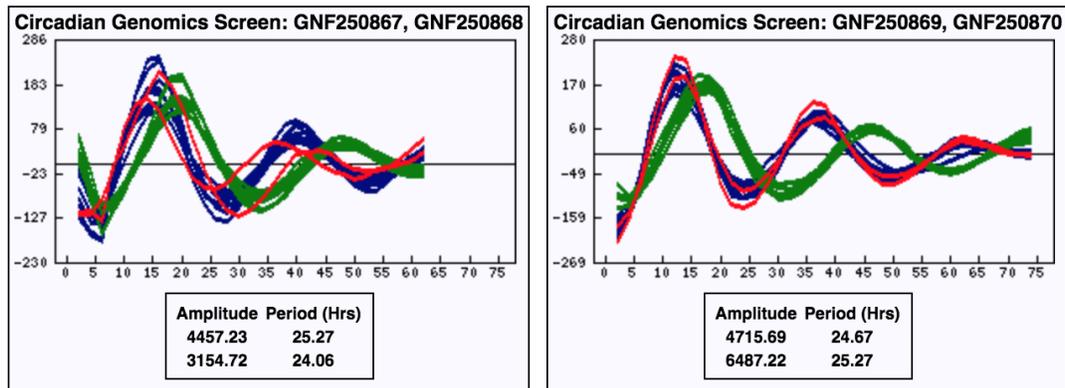


Figure S2. Phenotypes of *Hnf4a* knockdown in genome-wide RNAi screen using U2OS cells.
(related to Figure 2)

Data were retrieved from BioGPS (<http://biogps.org/circadian/>).

Blue: Control siRNA; Green: *Cry2* siRNA; Red: *Hnf4a* siRNAs

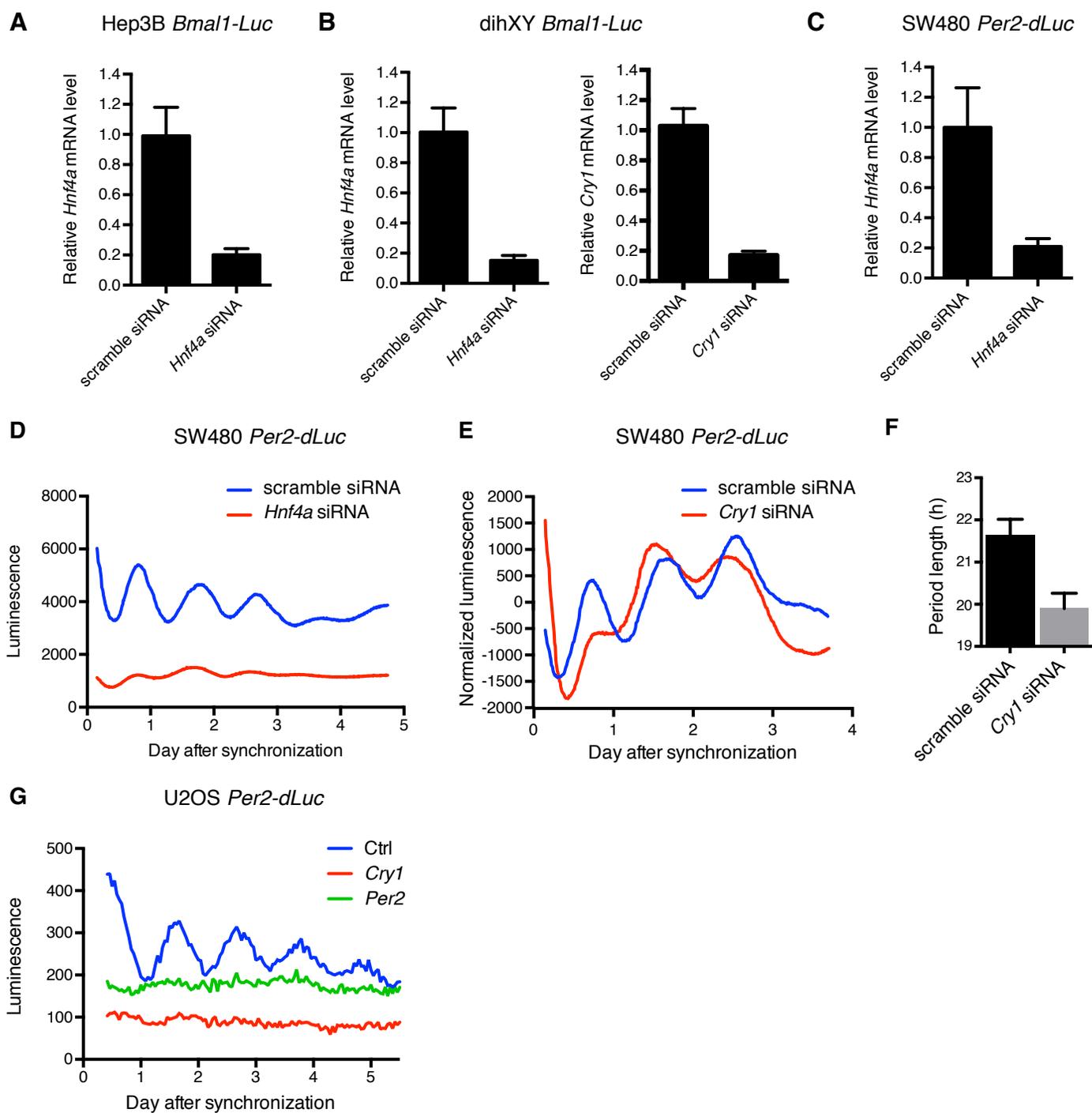


Figure S3. HNF4A is critical for circadian rhythm maintenance and period regulation in liver and colon cells. (related to Figure 2)

(A-C) Efficiency of gene knockdown in human and mouse cell lines. Hep3B (A), diHXY (B), and SW480 (C) cells were transiently transfected with scrambled, *Cry1*- or *Hnf4a*-specific siRNA. *CRY1* and *HNF4A* transcript levels were quantified by RT-qPCR. (D) Effect of control scramble or *Hnf4a* siRNA on *Per2-dLuc* reporter in human colon cells SW480 (n = 3). (E) Effect of control scramble or *Cry1* siRNA on *Per2-dLuc* reporter in human colon cells SW480 (n = 3). (F) Mean period of circadian luminescence rhythms in (E). Data are represented as mean \pm SD (n = 3). Significance was assessed by Student's t test, $p=4.56e-03$ (< 0.05). (G) Effect of expressing *CRY1* or *PER2* on *Per2-dLuc* reporter in U2OS cells.

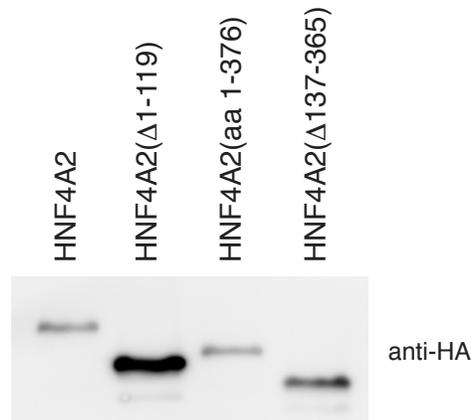


Figure S5. Expression quantification of HNF4A fragments. (related to Figure 4)
HEK 293T was transiently transformed with the same amount of HNF4A fragment plasmids.
And Western blot was performed to quantify their expression level.

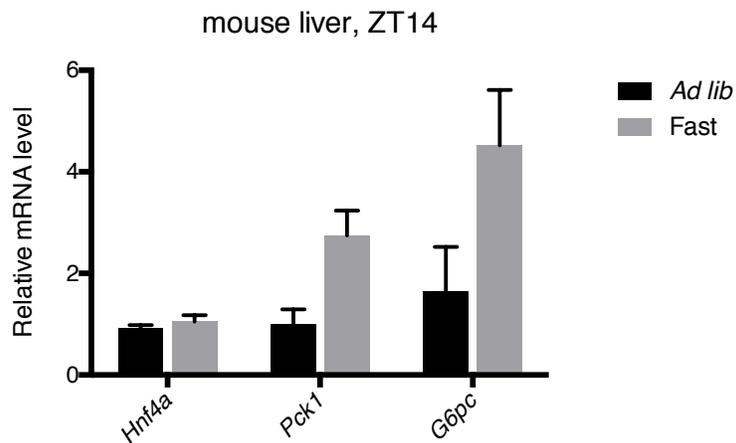


Figure S6. Quantification of *Hnf4a* mRNA levels after fasting. (related to Figure 5)
Mice were fed normally or fasted from ZT4 to ZT14. Liver tissues were collected at ZT14 and the transcripts were quantified by RT-qPCR (n=5). Significance was assessed by Student's t test, $p=0.058(>0.05)$ for *Hnf4a*; $p=0.0001(<0.05)$ for *Pck1*; $p=0.002(<0.05)$ for *G6pc*.

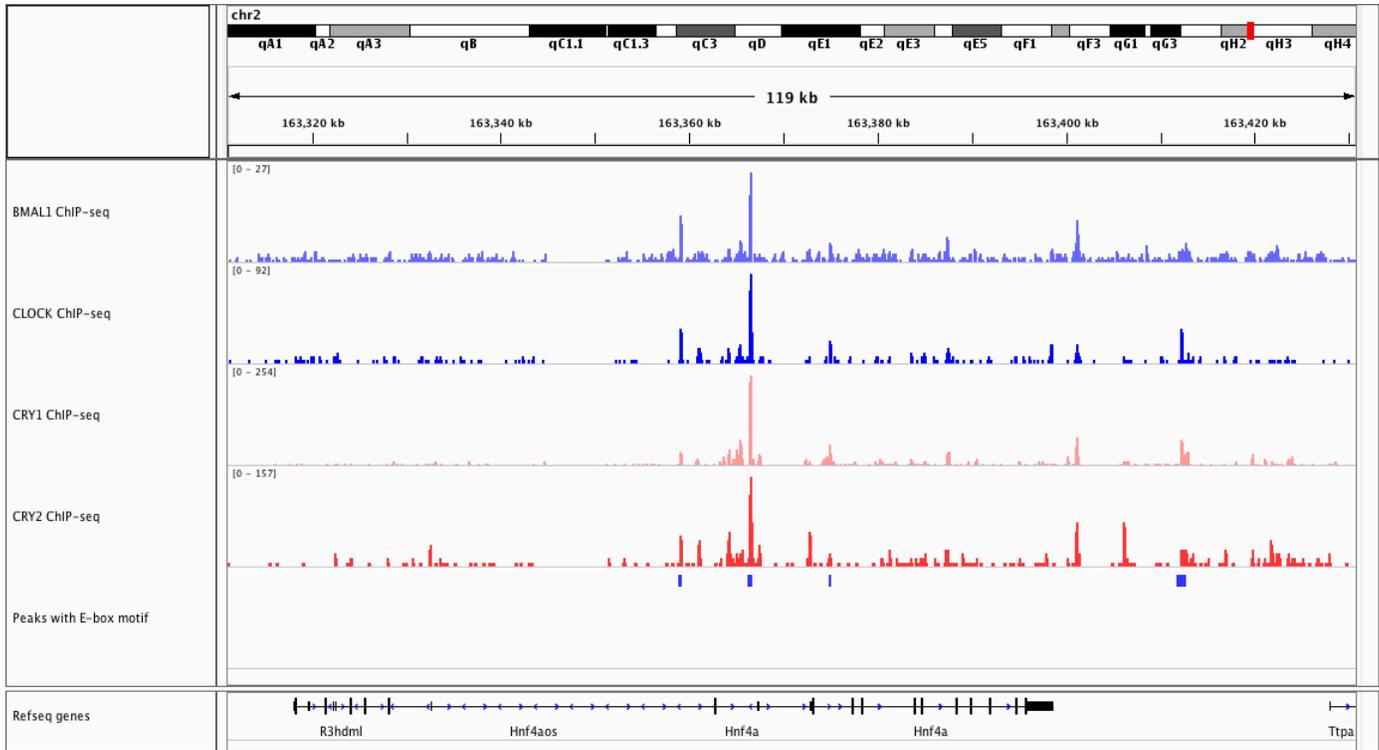


Figure S7. Core clock proteins localize at the *Hnf4a* gene. (related to Figure 5)

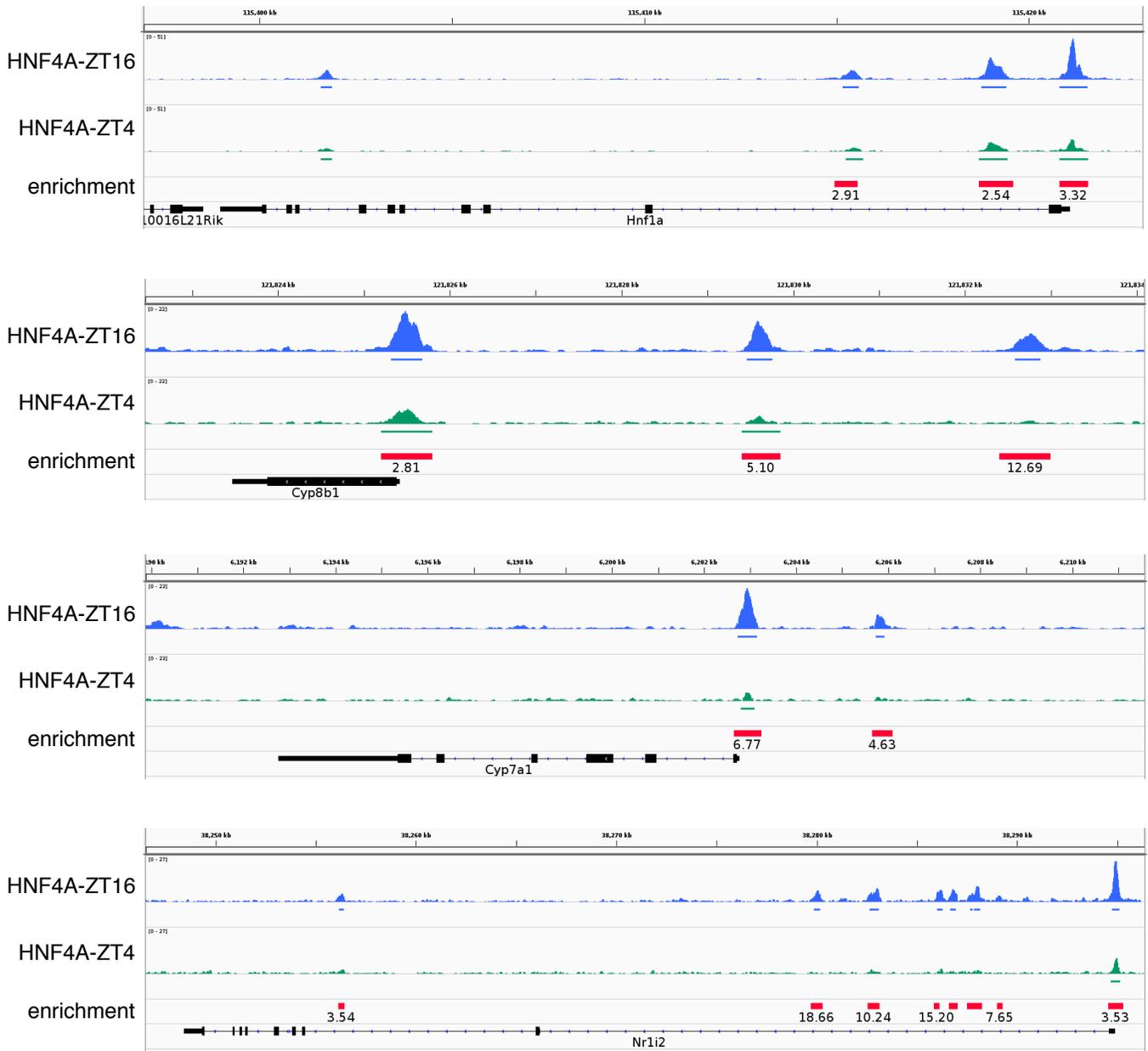
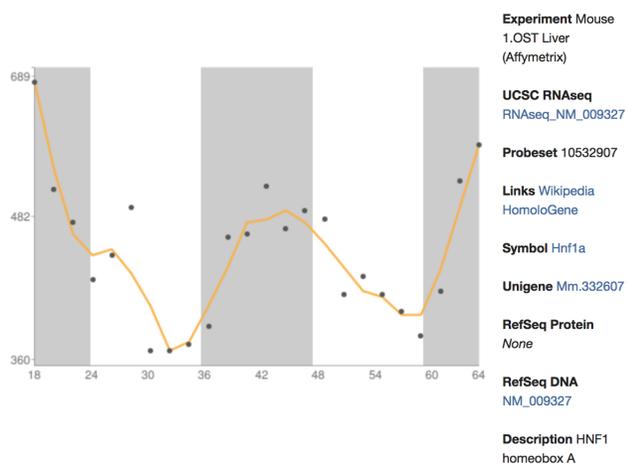


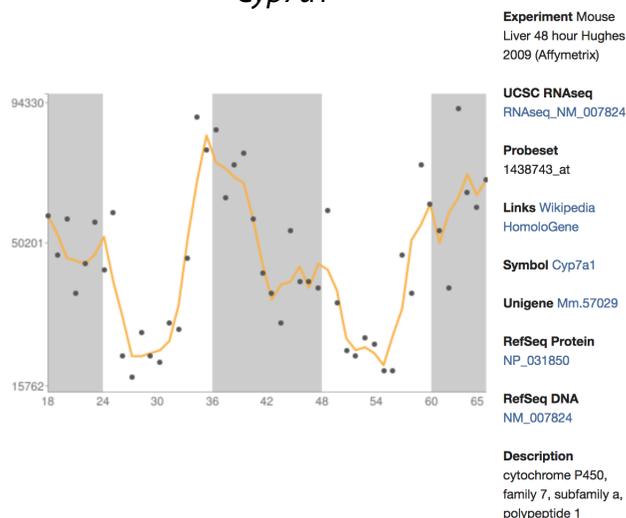
Figure S8. HNF4A binding tracks of selected genes showing an enrichment at ZT16 relative to ZT4.
(related to Figure 6)

Hnf1a



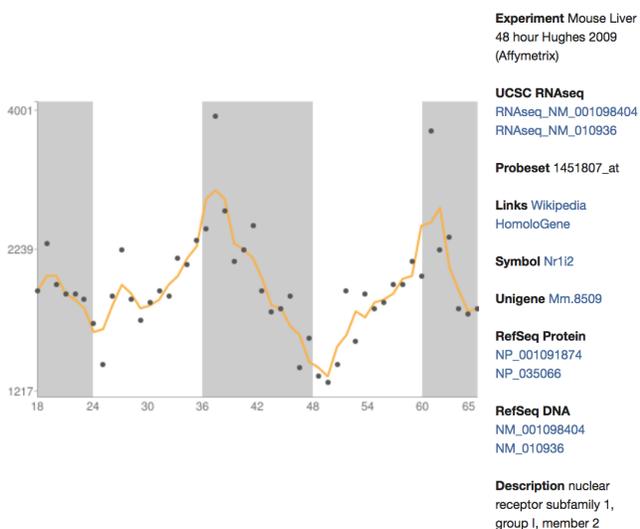
	p-Value	q-Value	period	phase
JTK	0.000322162	0.00594128	24.0	20.0
Lomb Scargle	0.0149	0.264495	23.892	21.62
DeLichtenberg	0.9495	1.0	24.0	NA

Cyp7a1



	p-Value	q-Value	period	phase
JTK	2.07e-05	0.000503311	24.0	15.0
Lomb Scargle	0.000407	0.0108616	23.946	22.91
DeLichtenberg	0.0122	0.568452	24.0	NA

Nr1i2



	p-Value	q-Value	period	phase
JTK	2.11e-07	1.03e-05	23.0	13.5
Lomb Scargle	0.000274	0.00827708	22.345	18.19
DeLichtenberg	0.1058	0.754894	24.0	NA

Figure S9. Circadian expression profiles of *Hnf4a* target genes. (related to Figure 6)
 Data were retrieved from <http://circadb.org>

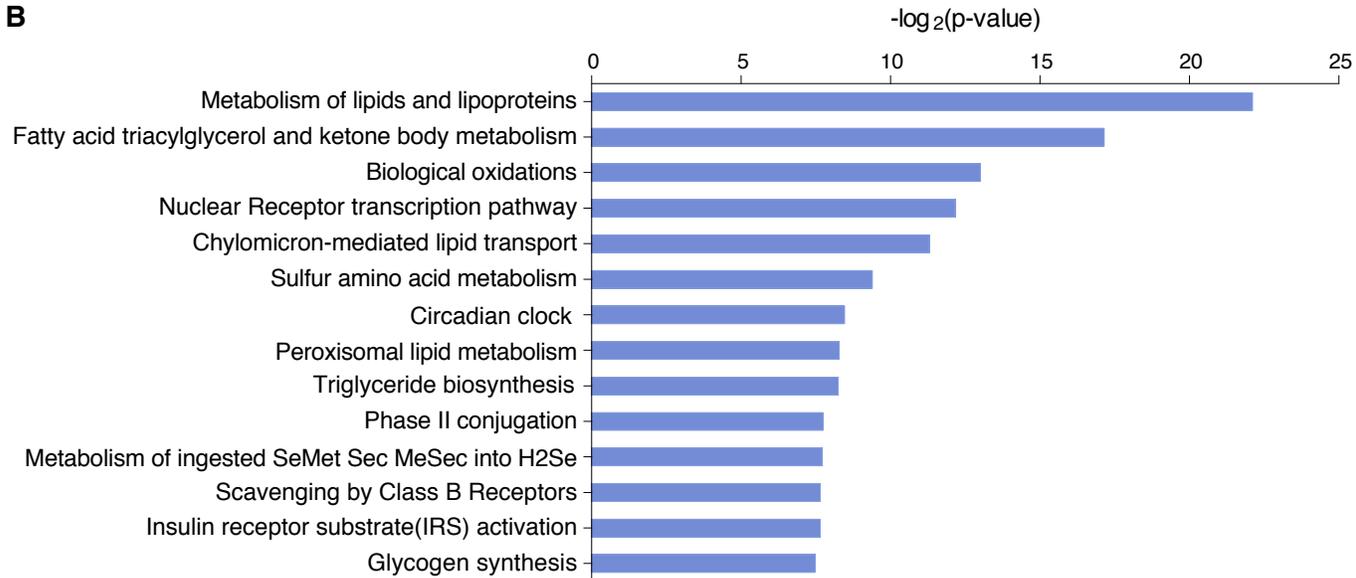
A

Total target sequences = 1740
 Total background sequences = 45802
 * - possible false positive

Rank	Motif	P-value	log P-value	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-387	-8.917e+02	56.78%	13.65%	48.0bp (63.9bp)	HNF4G/MA0484.1/Jaspar(0.959) More Information Similar Motifs Found
2		1e-227	-5.243e+02	54.83%	19.80%	50.6bp (71.2bp)	NPAS(bHLH)/Liver-NPAS-ChIP-Seq(GSE39860)/Homer(0.997) More Information Similar Motifs Found
3		1e-152	-3.511e+02	25.11%	5.71%	49.5bp (67.8bp)	CEBPA/MA0102.3/Jaspar(0.950) More Information Similar Motifs Found
4		1e-93	-2.144e+02	41.95%	20.25%	56.3bp (65.7bp)	VDR/MA0693.2/Jaspar(0.893) More Information Similar Motifs Found
5		1e-62	-1.450e+02	17.64%	6.00%	53.0bp (69.0bp)	FOXM1(Forkhead)/MCF7-FOXM1-ChIP-Seq(GSE72977)/Homer(0.946) More Information Similar Motifs Found
6		1e-36	-8.402e+01	5.29%	0.99%	53.3bp (60.0bp)	HNF1b(Homeobox)/PDAC-HNF1B-ChIP-Seq(GSE64557)/Homer(0.956) More Information Similar Motifs Found
7		1e-33	-7.702e+01	6.84%	1.79%	48.0bp (63.1bp)	CUX1(Homeobox)/K562-CUX1-ChIP-Seq(GSE92882)/Homer(0.910) More Information Similar Motifs Found
8		1e-24	-5.714e+01	18.56%	10.25%	54.0bp (64.8bp)	FXR(NR).JRI/Liver-FXR-ChIP-Seq(Chong_et_al.)/Homer(0.922) More Information Similar Motifs Found
9		1e-22	-5.132e+01	3.51%	0.72%	56.1bp (64.8bp)	RAR:RXR(NR).DR5/ES-RAR-ChIP-Seq(GSE56893)/Homer(0.732) More Information Similar Motifs Found
10		1e-17	-3.922e+01	1.32%	0.11%	50.5bp (59.8bp)	PGR(NR)/EndoStromal-PGR-ChIP-Seq(GSE69539)/Homer(0.777) More Information Similar Motifs Found

← HNF4-binding motif
 ← E box

B



C

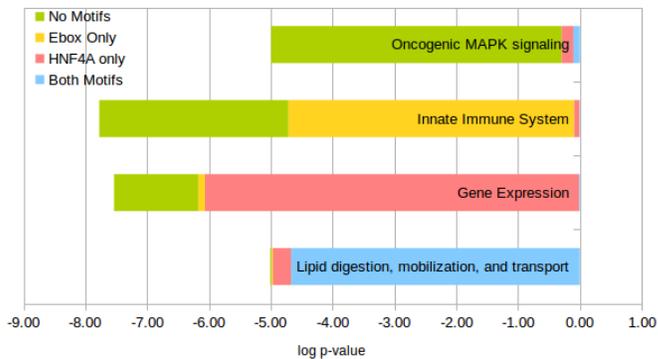
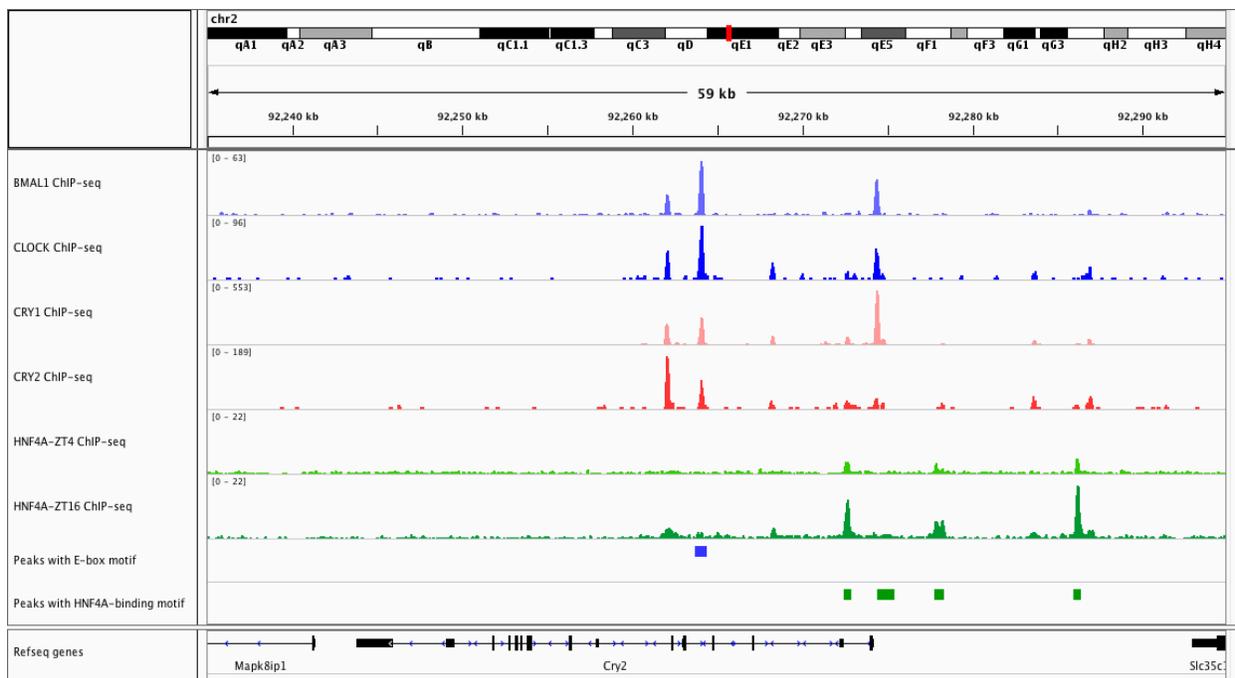
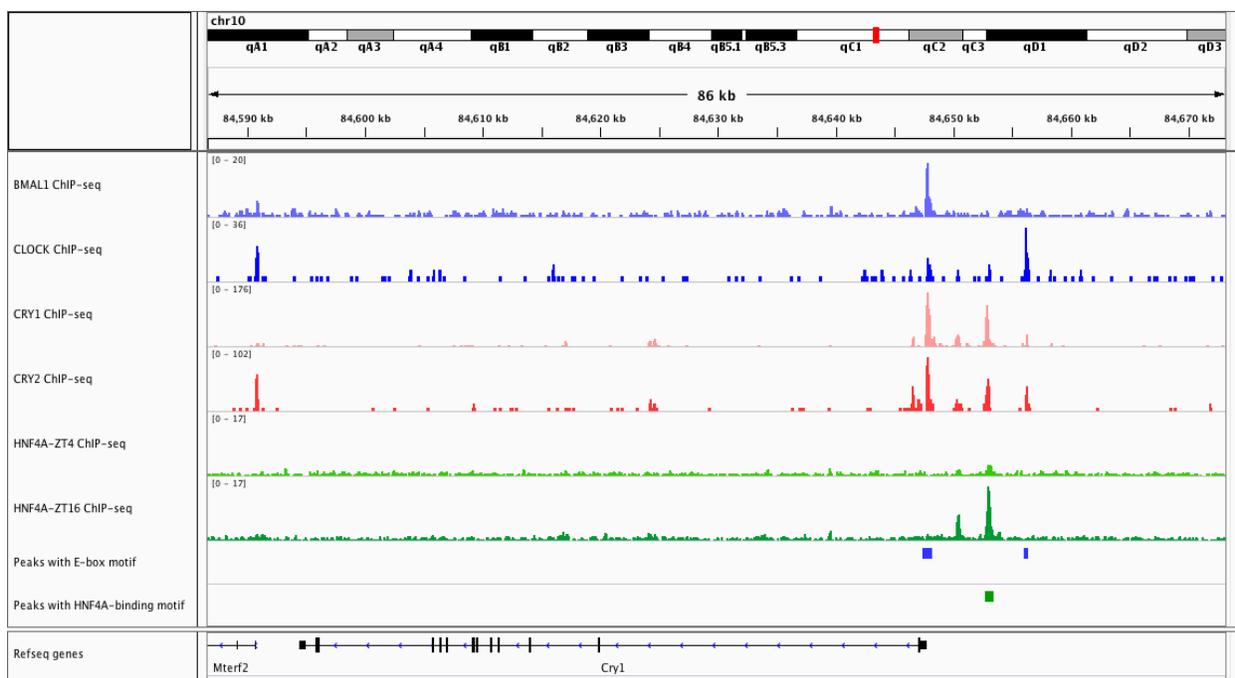
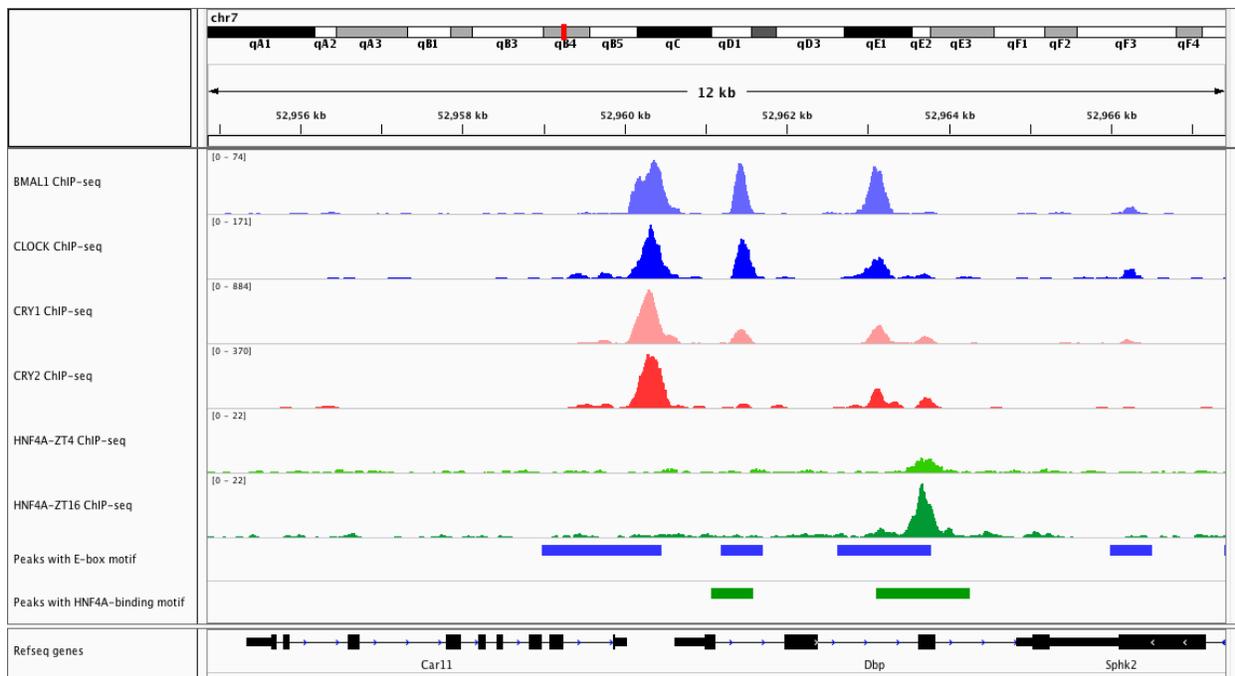


Figure S10. Analysis of HNF4A and CLOCK:BMAL1 co-occupancy. (related to Figure 6)

(A) De novo discovery of over-represented motifs at BMAL1-CLOCK-HNF4A overlapped loci. The top ten motif matrices predicted are shown. (B) Reactome pathway analysis of BMAL1-CLOCK-HNF4A co-occupied genes. (C) Reactome pathway analysis for four motif categories of BMAL1-CLOCK-HNF4A co-occupied genes.



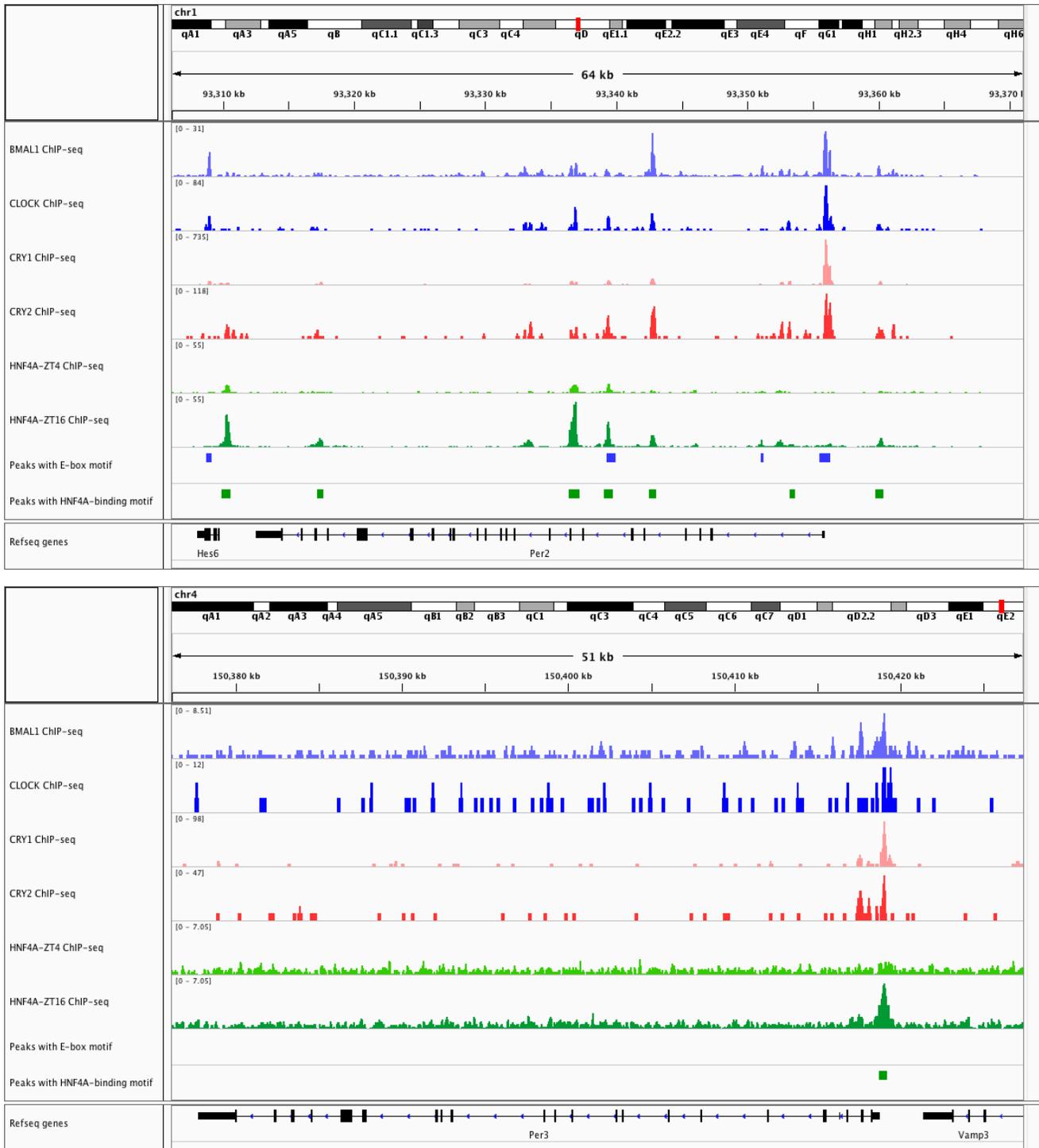


Figure S11. CLOCK:BMAL1 and HNF4A co-occupy binding sites at core clock genes.
 (related to Figure 6)
 Peaks that have E-box or HNF4A motifs are indicated.