Supplementary Materials for

Feedback Regulation of Transcriptional Termination by the Mammalian Circadian Clock PERIOD Complex

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SUPPORTING ONLINE MATERIAL

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Materials and Methods

**FH-Per1 and Per2-FH mice:** The generation of these mice have been described elsewhere (8).

**Tissue Collection:** Mice were entrained to a 12-12-h light-dark cycle for at least two weeks and then transferred to constant darkness. Mice were euthanized under infrared light, and tissues were dissected under light and processed for isolation of nuclei immediately. Studies were performed in accordance with the protocol approved by the Harvard Medical School Standing Committee on Animals.

**Nuclear Lysate Preparation and Co-immunoprecipitation of PER complexes:** Nuclei were isolated from mouse tissues as described (24). For isolation of soluble nucleoplasm, the protocol as described (25) was performed with the following modifications. The nuclear pellet was quickly rinsed with and incubated in Buffer A (10 mM Tris, pH 8, 150 mM NaCl and 1 mM MgCl₂) for 30 min. Lysis was achieved by sonication for 20 min (30 s on, 1 min off cycles) until complete nuclear lysis was observed by microscopy. Tween 20 was added to 1% and in some cases, additional MgCl₂ to 2.5 mM. The lysate was spun over a 30% sucrose cushion in buffer A at 1380 x g at 4°C to pellet insoluble chromatin.

The upper nucleoplasmic layer was removed and incubated with FLAG-M2 agarose beads (Sigma) for 2 h to overnight (4°C) and eluted twice with 250 ug/ml FLAG peptide for 30 min at 4°C. All buffers and solutions were made with distilled RNAse-free water and contained
0.1 mg/ml yeast tRNA and RNase inhibitor (Superase, Ambion; RnaseOUT, Promega).

For co-immunoprecipitation of endogenous PER2 complexes, rabbit PER2-1A antibody (ADI diagnostics) or a control IgG bound to Dynal-Protein G (Invitrogen) or covalently coupled to Dyna-M280 Tosylactivated beads (Invitrogen) were incubated with nucleoplasm for 2 h. Beads were washed 4 times with a total 4 ml of Buffer A containing 0.5% Tween and 250 mM NaCl for a period of 20 min at 4˚C, before being eluted by SDS loading buffer.

Mass Spectrometry: Proteins in immunoaffinity-purified samples were resolved on SDS-PAGE using NUPAGE Novex 4-12% Bis-Tris gel (Invitrogen). A lane for each sample was cut into several gel slices, subjected to trypsin digestion, and analyzed for peptides by LC-MS/MS (Taplin Biological Mass Spectrometry Facility, Harvard Medical School) as described (13).

Antibodies: PER2-1A, PER3-1A were obtained from ADI, DDX5 and DHX9, CDK9, CBP80, Senataxin, XRN2 from Abcam, Pol II (4H8, 8WG16, H14, H5) were all obtained from Covance, Pol II N20 from Santacruz Biotech. Rabbit anti-BMAL1 antibodies were made by Covance as described (26). For western blots, extracts were resolved on NuPAGE 4-12% SDS-PAGE gels (Invitrogen) and blotted onto Nitrocellulose (Amersham) by semi-dry transfer (Bio-RAD). Signals were detected by enhanced chemiluminescence using ECL-Plus (Amersham).

Optiprep Gradient Analysis: FLAG protein complexes eluted by 250 µg/ml peptide were layered on 10-50% Optiprep (Sigma) gradients and spun for 15 h at 30,000 rpm in a SW55 rotor (85,000 x g). 23 fractions were collected and fractions were concentrated by TCA precipitation. TCA precipitates were washed 3 times with 10% TCA followed by 2 washes with 100% Acetone (Sigma), pellets were dried, resuspended in 1X SDS loading dye and analyzed by SDS-PAGE.
**Protein Depletion with shRNAs and siRNAs:** Targeted RNA silencing with shRNAs (DHX9) or siRNAs (DDX5) was performed as described (13). Briefly, BLi cells were grown to 80% confluency, collected after mild trypsin treatment by centrifugation at 100 g for 10 min. Cells and appropriate RNA solutions were resuspended in Nucleofection buffer mix V1 (Lonza) according to manufacturer’s instructions and electroporated using program 7 on the Nucleofector II (Lonza). Following one day of recovery, cells were synchronized and then analyzed for circadian period.

**Real-Time Monitoring of Circadian Oscillations:** BLi cells (13) at 90% confluence were synchronized by addition of DMEM containing 10 µM forskolin. After 2 h, the medium was changed to DMEM, 10% FBS, 100 U/ml penicillin, 100 mg /ml streptomycin, 250 mM d-Luciferin. Bioluminescence was continuously recorded (LumiCycle, Actimetrics) and analyzed as described (13).

**Quantitative RT-PCR:** Quantitative RT-PCR was performed as described (27).

**Chromatin Immunoprecipitation and Deep Sequencing:** RNA Polymerase II-ChIP was performed overnight with N20 antibody against RNA Polymerase II as described (28), on chromatin prepared by sonication on a Covaris platform from pooled liver nuclei obtained from 2 mice, sacrificed every 4 h from CT2 to CT22. After 3 sequential washes, crosslinks were reversed at 65°C for 6 h, DNA was eluted, size selected for fragments between 250 and 400 bp using Pippin Prep followed by barcoded library generation on the SPRIworks system, amplified for 16 cycles and single-end sequenced on the Illumina Hi-Seq 2000 platform (Center for Cancer Computational Biology, Dana Farber Cancer Institute). Total reads varied from 24x10^7 to ~70x10^7 reads, and values were normalized after removal of duplicates (which ranged from 75% to 92% across samples). For the input sample, purified chromatin from all 6 time-points were
pooled, barcoded, amplified, and sequenced in parallel. This file was used as a control for subtraction of background for MACS analysis (29). Output BED or WIG files were uploaded to the UCSC database, the Integrated Genome Browser (IGB) browser or Galaxy browser (http://main.g2.bx.psu.edu/) for visualization and analysis.

**Chromatin Immunoprecipitation-qPCR:** For DHX9 and PER2 ChIP, nuclei were purified as described (24) after sequentially crosslinking liver extracts in PBS containing 2 mM Disuccinimidyl Glutarate (DSG, Pierce) for 13 min at room temperature followed by 1% formaldehyde for another additional 7 min. ChIP was performed as above, followed by 4 sequential washes in RIPA buffer for 20 min total and 2 washes in TE. Complexes were eluted in buffer containing 100 mM NaHCO3 and 1% SDS for 30 min at room temperature by vortexing. Following elution, NaCl was added to final concentration of 0.45 M followed by de-crosslinking by boiling for 15 min. Eluted DNA was purified by Qiagen columns and analyzed by qPCR. For ChIP from cultured cells, cells were washed in warm PBS and then incubated in PBS-1% formaldehyde for 10 min at 37°C. The cells were washed with cold PBS-5% BSA and then a second wash with cold PBS before being scraped and spun in cold PBS at 2000 rpm for 2 min. The cell pellet was then resuspended in lysis buffer, sonicated, and processed as described above.

**Chromatin RNA Immunoprecipitation:** Chromatin was prepared as above. For isolation of pre-mRNA associated with PER2, following ChIP with PER2 (ADI) or control rabbit IgG antibody (Sigma), the beads were washed with RIPA buffer 3 times for 15 min and RIPA-1000 mM NaCl once prior to reversing crosslinks at 65°C for 4 h as described (30) with suggested modifications (31). Following reversal of crosslinks, the samples were treated with RNase-free DNAse (Promega) for 30 min at room temperature, followed by DNAse inactivation
with EDTA. RNA was further extracted by Trizol, and RNA from 4 such experiments were pooled prior to cDNA synthesis using random primers.

**qPCR primers:**

*Per1 pre-mRNA (3 sets)*

5’ set
Forward: 5’-ccagggcctcagagtccca-3’
Reverse: 5’-ggccatctctacacccctt-3’

3’ set
Forward: 5’-gccaataaggcagagcgtgt-3’
Reverse: 5’-ggaagagctctggctgagga-3’

for ChRIP:
Forward: 5’-gagtctacgtgcctcggaa-3’
Reverse: 5’-aggcattgttctgcagct-3’

For DDX5, PER2 and Pol II ChIP 3’ primers:
Forward: 5’-ccacctgttaaggccagtg-3’
Reverse: 5’-agagtcgatgctgccagt-3’

*PABPc1 pre-mRNA*

Forward: 5’-ccatcagcctcatctgagc-3’
Reverse: 5’-tccttaaggcgctcatc-3’

*Per2 pre-mRNA*
Forward: 5’-ccaagtgaagggccgagca-3’
Reverse: 5’-ccgagccggcggttacgtta-3’

**Cry2 pre-mRNA**

5’ set, for ChRIP
Forward: 5’-gtctctggtgcactggtc-3’
Reverse: 5’-cagagtggcgacggctggagt-3’

3’ set
Forward: 5’-gacttgccagctttggca-3’
Reverse: 5’-cagacgaagggagata-3’

**Kpnb1 pre-mRNA**

Forward: 5’-aggtggcagagttgcagctg-3’
Reverse: 5’-aggattggacagtcacttacat-3’

**Rpl37 pre-mRNA**

Forward: 5’-taccaccttcagaagttgct-3’
Reverse: 5’-ctcagccaaacccacacct-3’

**Tubα pre-mRNA**

Forward: 5’-tttcccctttccacagcgtg-3’
Reverse: 5’-ccccatggttctttcactggtc-3’

**Rev-erbα pre-mRNA**

Forward: 5’-aagacatgaagccctggac-3’
Reverse: 5’-atgeggtttagcattcc-3’
**Dbp pre-mRNA (2 sets)**

5’ set
Forward: 5’-ctgaggagcttctgcagggaa-3’
Reverse: 5’-cccttaggccctecagggtca-3’

3’ set
Forward: 5’-gacccctgagacaccegtctctc-3’
Reverse: 5’-cagaagccctcatgactgtatgct-3’

**Bmal1**
Forward: 5’-tggtcttttggtaccaacatgc-3’
Reverse: 5’-agcctgagcttcatctctca-3’

**Hprt**
Forward: 5’-tgctcagatgtcatgaagg-3’
Reverse: 5’-tatgtccccctgtgactgat-3’

**Readthrough primers:**

**Per1**
Forward: 5’-acaggagatcctgggttcg-3’
Reverse: 5’-gctgctctagatgtcaaata-3’

**Cry2**
Forward: 5’-gtggctgagacgtgatgatgatgat-3’
Reverse: 5’-atccccaggaggtcagaatggat-3’

**Cypa**
Forward: 5’-accaagegtgtgatgag-3’
Reverse: 5’-gcatgaagaacctgtcgtcc-3’

3’ Pause site (termination) primers

**Per1**
Forward: 5’-tatagctccacggcacaag-3’
Reverse: 5’-actttcgetaatcgagcaga-3’

**Cry2**
Forward: 5’-ttaccaggctgtcttg-3’
Reverse: 5’-gaaagagggagggaggaaga-3’

**Cypa**
Forward: 5’-agctgtttggagatctga-3’
Reverse: 5’-ttccatacacacaggttca-3’

**Bmal1**
Forward: 5’-tgcccagttaaaggtcagt-3’
Reverse: 5’-cccaaggagatgtggcaca-3’

**RNAi constructs:**

**DDX5 #1**
Sense- 5’-GAAGTCTACTTGCATCTATTT-3’

**Mutant control for DDX5 #1**
Sense- 5’-GAAGTCTGTTTATGCCTATTT-3’
DDX5 #2
Sense- 5’-GGGACTTACCAGAACGGTTTT-3’

Point mutant control for DDX5 #2
Sense- 5’-GGGACTTGCAGGAACGGTTTT-3’

DHX9#1
Sense-5’-CCGGGCGCTTCTAATAATACATTGAACTCGAGTTCAATGTATTTAAG
AAGCGCTTTTTG-3’

Point mutant control for DHX9 #1
Sense-5’-CCGGTCCGTCTTAGATACATTGAACTCGAGTTCAATGTAATCTAAG
ACGGACTTTTTTG-3’

DHX9#2
Sense-5’-CCGGCGAAAGTGTGGTGGATCCAGTACTCGAGTACTGGATCAAACA
CTTTGCGTTTTTG-3’

Point mutant control for DHX9 #2
Sense-5’-CCGGCGAGTACTGTCTGATGCAGTACTCGAGTACTGCATCAGACA
GTACCTCGTTTTTG-3’
fig. S1 Depletion of endogenous DDX5 (A-C) or DHX9 (D-F) causes increased endogenous *Per1* transcription and short circadian period. (A) Left, immunoblot showing effect of point-mutated control siRNA (Mut DDX5) or DDX5 siRNA on steady-state level of endogenous DDX5 in mouse fibroblasts. α-TUBULIN, loading control. Right, effect of DDX5 depletion on average transcription of the *Per1* gene or a control gene (assessed by quantitative RT-PCR of the respective pre-mRNAs relative to *Hprt* pre-mRNA). (B) Circadian oscillations of bioluminescence in synchronized circadian reporter fibroblasts after introduction of siRNA against DDX5 (blue) or control siRNA (yellow). Traces from three independent cultures are shown for each. (C) Group data for circadian periods of fibroblasts after depletion of DDX5 (mean +/- SEM; N = 3 for each; *t*-test, two-tailed). (D-F) Similar results after depletion of DHX9 by shRNA.
fig. S2  Confirmation of short circadian period length caused by depletion of endogenous DDX5 or endogenous DHX9. Shown are data following electroporation of (A) an additional, non-overlapping siRNA against DDX5 and its matched point-mutated control or (B) an additional non-overlapping shRNA against DHX9 and its point-mutated matched control. Left, immunoblots showing the reduction of steady-state levels of DDX5 or DHX9, respectively, relative to controls in mouse fibroblasts. α-TUBULIN, loading control. Middle, circadian oscillations of bioluminescence in synchronized circadian reporter fibroblasts after depletion of DDX5 or DHX9 (yellow traces) relative to controls (respective blue traces). Traces from three independent cultures are shown for each. Right, group data for circadian period lengths of fibroblasts after depletion of DDX5 (top) or DHX9 (bottom). Shown are mean +/- SEM; N = 3 for each; t-test, two-tailed.
**Fig. S3** RNA Polymerase II and PER complex. (A) Co-immunoprecipitation of endogenous PER2 with RNA Polymerase II large subunit phosphorylated on Ser-2 or Ser-5 of the C-terminal domain, markers of the activated polymerase. Proteins immunoprecipitated (IP) from nuclear extracts of liver (CT20) with control IgG, RNA Polymerase II-Phospho-Ser2, or RNA Polymerase II-Phospho-Ser5 antibody were probed with PER2 or IgG light chain antibodies, as indicated. (B and C) Control experiments for optiprep density gradient analysis of affinity-purified PER complexes from mouse liver nuclei. (B) Immunoblot of gradient fractions (10-50% Optiprep, left-to-right; larger complexes toward right) of FLAG affinity purification sample from liver of wildtype control mouse with only native (untagged) PER2. None of the PER complex components were detected. Position of marker protein indicated at top. (C) Same experiment performed on crude liver nuclear extract (not affinity-purified). There was no co-sedimentation of the proteins, as expected if only a small fraction of the proteins is in PER complexes. Note that some RNA Polymerase II complexes sediment at a much larger size than purified PER complexes, indicating that the PER complexes shown in Fig. 2C represent resolved complexes, not unresolved high-mass material.
**fig. S4**  ChIP-seq profiles from mouse livers obtained at CT10 or CT22, the approximate peak and trough, respectively, of *Per1* transcription, showing RNA Polymerase II occupancy at “low magnification” for 1 Mb encompassing the *Per1* gene. Note that peaks of RNA Polymerase II are tightly associated with transcription units along the chromosome and that the signals show little change overall between the two opposite circadian phases. Even at this scale, the circadian modulation of the polymerase on the *Per1* gene is apparent, and the accumulation of RNA Polymerase II at the *Per1* 3’ termination site at CT22 stands out (arrow). At bottom, thick blocks represent exons and thin blocks untranslated regions of the annotated genes. DNA scale bar is at lower right.
fig. S5  Circadian patterns of RNA Polymerase II or SETX occupancy on selected clock genes or arbitrary control genes.  (A) Quantification from ChIP-seq tag counts of RNA Polymerase II binding near the 5’ start and 3’ termination sites of the indicated genes across circadian time. Note accumulations at 3’ site of Per1, Per2, and Cry2 genes at CT22. Little or no signal is seen at 3’ region of Bmal1 at any time. Note that this experiment tabulates RNA Polymerase II occupancy over a 1-kb segment, whereas signals from the ChIP experiment in Fig. 3D are obtained from shorter sequences.  (B) Two examples of representative control genes: ChIP-seq profiles showing RNA Polymerase II occupancy (vertical traces) along the genes (5’ to 3’, left to right) at the six indicated circadian times. Little or no polymerase detectably accumulates near the 3’ termination site at any circadian time. DNA scale, lower right. At bottom of each profile, thick blocks represent exons; thin blocks, untranslated regions. 5’ transcriptional start site and 3’ termination site are centered within gray bars, as indicated.  (C) Accumulation of RNA Polymerase II at 3’ sites of Per1 and Cry2 genes is unusual. Graph shows quantification of RNA Polymerase II occupancy (normalized ChIP sequence tag counts) at CT22 near 3’ termination sites relative to 5’ start sites of Per1, Cry2, and 19 control genes scattered throughout the genome (Bmal1 plus 18 arbitrarily-selected genes: vcpip1, ncoa2, arid5a, mobkl2b, nfx1, tnn1, cited2, ddit2, cdc215, zscan12, ann1, eif2a, fbxo, six5, gadd45a, itpr1, magi3, myc). For the control genes, the value is 0.072 +/- 0.020 (SEM), with the 99% confidence limit maximum = 0.132. Per1 = 1.19; Cry2 = 0.91.  (D) ChIP assays showing circadian rhythm of SETX at or near Per1 and Cry2 3’ termination sites in mouse liver (relative to control IgG ChIP). SETX appears to mirror RNA Polymerase II, including accumulation at CT22.
References and Notes


10. Materials and Methods are available as Supplementary Materials on *Science* Online.


19. In ongoing experiments, we have identified both RNA polymerase II large subunit and SETX in purified PER complexes by mass spectrometry.


