SUPPORTING ONLINE MATERIAL

(Materials and Methods; Text; Figs. S1-S7; Figure Legends; References)

Materials and Methods

***FH-Per1* and *Per2-FH* Mice.** The *Per1* and *Per2* loci, encompassed within BAC clones RP23-26L6 and RP23-386D12, respectively (BACPAC Resources), were modified by the addition of FH tandem affinity purification tags (*S1*) using recombineering techniques (*S2*). Targeting vectors for recombineering were generated using the selection cassette in pL451 to insert the FH tag in the *Per* 1 gene (in-frame with and directly following the start ATG codon in exon 3) or in the *Per2* gene (in-frame with and directly before the STOP codon in exon 23). The *Frt*-flanked selection cassette of pL451, placed in the preceding intron over 100 bp 5' of the exon/intron boundary in both cases, was removed from the recombineered BAC clones by expression of *Flp* recombinase. Correctly-targeted, gel-purified, circular BAC DNA was injected into pronuclei of mouse oocytes (Genome Modification Facility, Harvard University). Pups derived from pronuclear injection were genotyped by PCR to amplify tail DNA with primers flanking the FH tag coding sequence. Southern blot analyses were performed to estimate the copy number of BAC transgene integrations. We selected F1 founders carrying 1-2 copies of the integrated BAC transgene for crossing into the $Per1^{-/-}$ and/or $Per2^{-/-}$ background (129).

Tissue Collections. Mice were entrained to a 12-12-h light-dark cycle for at least 10 days and then transferred to constant darkness. Mice were euthanized under infrared light, and tissues were dissected under light and frozen immediately in liquid nitrogen. Studies were performed in accordance with the protocol approved by the Harvard Medical School Standing Committee on Animals.

Purification of PER Protein Complexes and Mass Spectrometry. Nuclei were isolated from liver extracts as described (*S3*). Nuclei were lysed in the presence of the reversible crosslinking agent DSP (Dithiobis[succimimidylpropionate], 2.5 mg/ml, Pierce), and FH-PER1

and PER2-FH complexes were extracted and affinity-purified using anti-FLAG M2 affinity agarose gel (Sigma), all as described *(S4)*. After reversal of crosslinking (95 ^{0}C, 5 min; 5% β-mercaptoethanol, 2% SDS, 10% glycerol, 6.25 mM Tris base, bromophenol blue), proteins in affinity-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 (*S5*).

Antibodies and Western Blotting. The following were obtained from the indicated suppliers: rabbit anti-PER2, rabbit anti-CRY1, rabbit anti-CRY2 (ADI); mouse anti-PSF, mouse anti-FLAG M2, mouse anti-U2AF^{65} (Sigma); rabbit anti-SIN3A, rabbit anti-HDAC1, rabbit anti-acetyl-H3K9, mouse anti-dimethyl-H3K9, rabbit anti-acetyl-H4K5, mouse anti-LaminA/C, mouse anti-Actin (Abcam); rabbit anti-CLOCK (ABR); rabbit anti-BMAL1 (Covance); HRP-conjugated secondary antibodies (GE Healthcare, 1:5000). For western blots, extracts (5-10 mg of protein) were resolved by SDS–PAGE and blotted onto nitrocellulose (Amersham) by wet transfer. Signals were detected by enhanced chemiluminescence (Amersham).

Co-Immunoprecipitation. Co-Immunoprecipitations were performed as described (*S6*), with the following modifications: approximately 150 μg of protein extract was used, the concentration of NP40 in the washes was 0.3%, and western blots were performed using TBS-0.1% Tween 20 (Sigma).

Transactivation Assays. NIH3T3 or HEK293 cells were transfected with Lipofectamine-Plus (GibcoBRL), and transactivation assays were performed essentially as described (*S6*), using 500 μg (total) pcDNA3 expression plasmid per well (Invitrogen), 15 ng of the pGL3-Per1E54bp firefly luciferase reporter (Promega), and 7.5 ng of pRL-CMV (Renilla) luciferase internal control plasmid (Promega).

Quantitative RT-PCR. Quantitative RT-PCR was performed as described (*S7*). Primer sequences are listed in Supplemental Experimental Procedures.

Chromatin Immunoprecipitation. 15μg to 20μg chromatin of tissue extract or 7.5μg chromatin of cell extract (average DNA length of 500 bp) was used for each ChIP reaction. The antibodies for ChIP are listed above, and primer sequences are listed below.

Protein depletion with shRNAs and siRNAs. Targeted RNA silencing with shRNAs and siRNAs was performed as described (*S5*).

Real-Time Monitoring of Circadian Oscillations. BLi cells 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 (*S5*).

RT-qPCR primers:

***Per1* pre-mRNA :**

Forward: 5'-ATTCTGAGGGTGTATCTGCCGCTT-3'

Reverse: 5'-TAAGGAATCACCACACCACCACCA-3'

***Dbp* pre-mRNA :**

Forward: 5'-CCCTCTTTCTTTCCTTTGGCCTCA-3'

Reverse: 5'-TCAGGCACATTCCTCATCCCTAGT-3'

***Tub* pre-mRNA:**

Forward: 5'-TTTCCCTCTTTCCACAGCGTGAGT-3'

Reverse: 5'-CCCAATGGTCTTGTCACTTGGCAT-3'

***Gapdh*:**

Forward: 5'- TTTAGCCTTGCCCTTTGAGCTTGC-3'

Reverse: 5'- TCACTCATGCCCTGGGAAGTTTGT-3'

***Per1*:**

Forward: 5'-TTCAAGCTCTCAGGACTCTG-3'

Reverse: 5'-GGCAGTTTCCTATTGGTTGG-3'

***Psf*:**

Forward: 5'-TCACAGTCAAGAAATGCAGAAACG-3'

Reverse: 5'-CGGCTCTTCCTCTCGTCTACGCCG-3'

***Hprt*:**

Forward: 5'-TGCTCGAGATGTCATGAAGG-3'

Reverse: 5'-TATGTCCCCCGTTGACTGAT-3'

ChIP qPCR primers:

***Per1* E-box:**

Forward: 5'-ATC CTG ATC GCA TTG GCT GAC TGA-3'

Reverse: 5'-TCT CTT CCT GGC ATC TGA TTG GCT-3'

***Per1* C1:**

Forward: 5'-AGCCAGGACCCAGAAAGAACTCAT-3'

Reverse: 5'-AACTCACTCACCCTGAACCTGCTT-3'

***Per1* C2:**

Forward: 5'-TCCCATTGTGAGTTAGGCAGAGCA-3'

Reverse: 5'-AGCCAGAGAAGGGCACAGTTACAT-3'

***Per1* C3:**

Forward: 5'- TCTGCCATGGAGGAAGAAGAGCAA-3'

Reverse: 5'-ACATGGTCGAAAGGAAGCCTCTCA-3'

Intergenic control:

Forward: 5'- TGC TAG ATG CTG CGG AAG AAC TGA-3'

Reverse: 5'- TCT CTA TGC TCC CAG CCA AGG TAT-3'

***Casp3* promoter:**

Forward: 5'- TGCCTCCCAAGTGCTGGGATTAAA-3'

Reverse: 5'- ACAGATGTCTACCGTTCGTTGCGT-3'

Gene silencing primers:

PSF shRNA1

Forward:

5'-

GATCCCCGCAAAGGGTTCGGGTTCATTATTCAAGAGATAATGAACCCGAACCCTTTG

CTTTTTGGAAA-3'

Reverse:

5'-

AGCTTTTCCAAAAAGCAAAGGGTTCGGGTTCATTATCTCTTGAATAATGAACCCGAA

CCCTTTGC GGG-3'

Mut-PSF shRNA1:

Forward:

5'-

GATCCCCGCAAAGGGTCGTGGTTCATTATTCAAGAGATAATGAACCACGACCCTTTG

CTTTTTGGAAA-3'

Reverse:

5'-

AGCTTTTCCAAAAAGCAAAGGGTCGTGGTTCATTATCTCTTGAATAATGAACCACGA

CCCTTTGCGGG-3'

PSF shRNA2:

Forward:

5'-

GATCCCCGGGTTCATTAAGCTTGAATCTTTCAAGAGAAGATTCAAGCTTAATGAACC

CTTTTTGGAA-3

Reverse:

5'-
AGCTTTTCCAAAAAGGGTTCATTAAGCTTGAATCTTCTCTTGAAAGATTCAAGCTTA
ATGAACCCGGG-3'

Mut-PSF shRNA2:

Forward:

5'-
GATCCCCGGGTTCATTAGACTTGAATCTTTCAAGAGAAGATTCAAGTCTAATGAACC
CTTTTTGGAAA-3'

Reverse:

5'-
AGCTTTTCCAAAAAGGGTTCATTAGACTTGAATCTTCTCTTGAAAGATTCAAGTCTA
ATGAACCCGGG-3'

PSF shRNA3:

Forward:

5'-
GATCCCCGCCAGTTTGGTCCTATTGAAATTCAAGAGATTTCAATAGGACCAAACTGG
CTTTTTGGAAA-3'

Reverse:

5'-
AGCTTTTCCAAAAAGCCAGTTTGGTCCTATTGAAATCTCTTGAATTTCAATAGGACC
AAACTGGCGGG-3'

Mut-PSF shRNA3:

Forward:

5'-
GATCCCCGCCAGTTTGGTCACTTTGAAATTCAAGAGATTTCAAAGTGACCAAACTGG
CTTTTTGGAAA-3'

Reverse:

5'-
AGCTTTTCCAAAAAGCCAGTTTGGTCACTTTGAAATCTCTTGAATTTCAAAGTGACC
AAACTGGCGGG-3'

SIN3A siRNA:

Anti-sense:

5'-rArUrCrCrArCrArUrCrCrArUrUrUrCrUrUrCrCrUrCrCrUrCrCrUrC-3'

Sense:

5'-rG rGrArG rGrArG rGrArA rGrArA rArUrG rGrArU rGrUrG rGAT-3'

Mut-SIN3A siRNA:

Anti-sense:

5'-rArUrC rCrArC rUrUrC rCrArU rUrGrC rUrUrC rCrUrC rArUrC rCrUrC-3'

Sense:

5'-rG rGrArU rGrArG rGrArA rGrCrA rArUrG rGrArA rGrUrG rGAT-3'

Text

In vivo epitpope tag strategy. To search for previously unrecognized clock proteins important for circadian negative feedback, we set out to purify PER complexes from mouse tissues in order to identify their constituent proteins by mass spectrometry. To take advantage of high-affinity monoclonal antibodies for the affinity purification of PER complexes, we chose a strategy in which we replaced endogenous PER1 or PER2 in mice with an epitope-tagged

version of the respective protein. As a first validation step, we generated cDNA expression constructs in which a cassette encoding a FLAG-Hemagglutinin (FH) tandem affinity purification tag (*S1*) was fused in-frame to the PER1 N-terminus or to the PER2 C-terminus, encoding FH-PER1 or PER2-FH, respectively. We found that FH-PER1 and PER2-FH were very similar to their wild-type counterparts in repressing CLOCK-BMAL1 transcriptional activity from *Per1* gene promoter sequences in mammalian cells (fig. S1A), indicating that FH-PER1 and PER2-FH preserve the basic negative feedback activity of native PERs.

Next we used homologous recombination in bacteria to insert FH-tag coding sequences into mouse *Per1* and *Per2* bacterial artificial chromosome (BAC) genomic clones such that they encoded the FH-PER1 and PER2-FH fusion proteins, respectively (fig. S1B). Using these clones, we generated two different BAC transgenic mouse lines, one for each tagged PER. Western analysis with anti-FLAG antibodies indicated that the tagged PERs were expressed in mouse tissues and showed the expected variation in expression over the course of the day (example in fig. S1C). To determine if either tagged PER functioned in vivo in the circadian clock, we crossed the *FH-Per1* or *Per2-FH* BAC transgene into the arrhythmic $Per1^{-/-}$; $Per2^{-/-}$ double mutant background (*S8*) and monitored circadian rhythms of locomotor activity. A single copy of either the *FH-Per1* or the *Per2-FH* transgene sufficed to rescue circadian rhythms of behavior, comparable to the function of a single copy of a wild-type *Per* allele ($Per1^{-/-}$; $Per2^{-/+}$) (fig. S1D). Thus FH-PER1 or PER2-FH supports circadian clock function in vivo, demonstrating that each must form all PER complexes required for the basic operation of the clock.

For the preparative purification of PER complexes from mouse tissues, we crossed the *FH-Per1* transgene into the $Per1^{-/-}$ background and the *Per2-FH* transgene into the $Per2^{-/-}$ background, generating two lines of mice, one in which FH-PER1 replaced wild-type PER1 and one in which PER2-FH replaced wild-type PER2. Given that PER1 and PER2 are present together in the same protein complexes (*S9*), this strategy provided two ways of purifying PER complexes from mouse tissues, allowing cross-validation of purifying proteins.

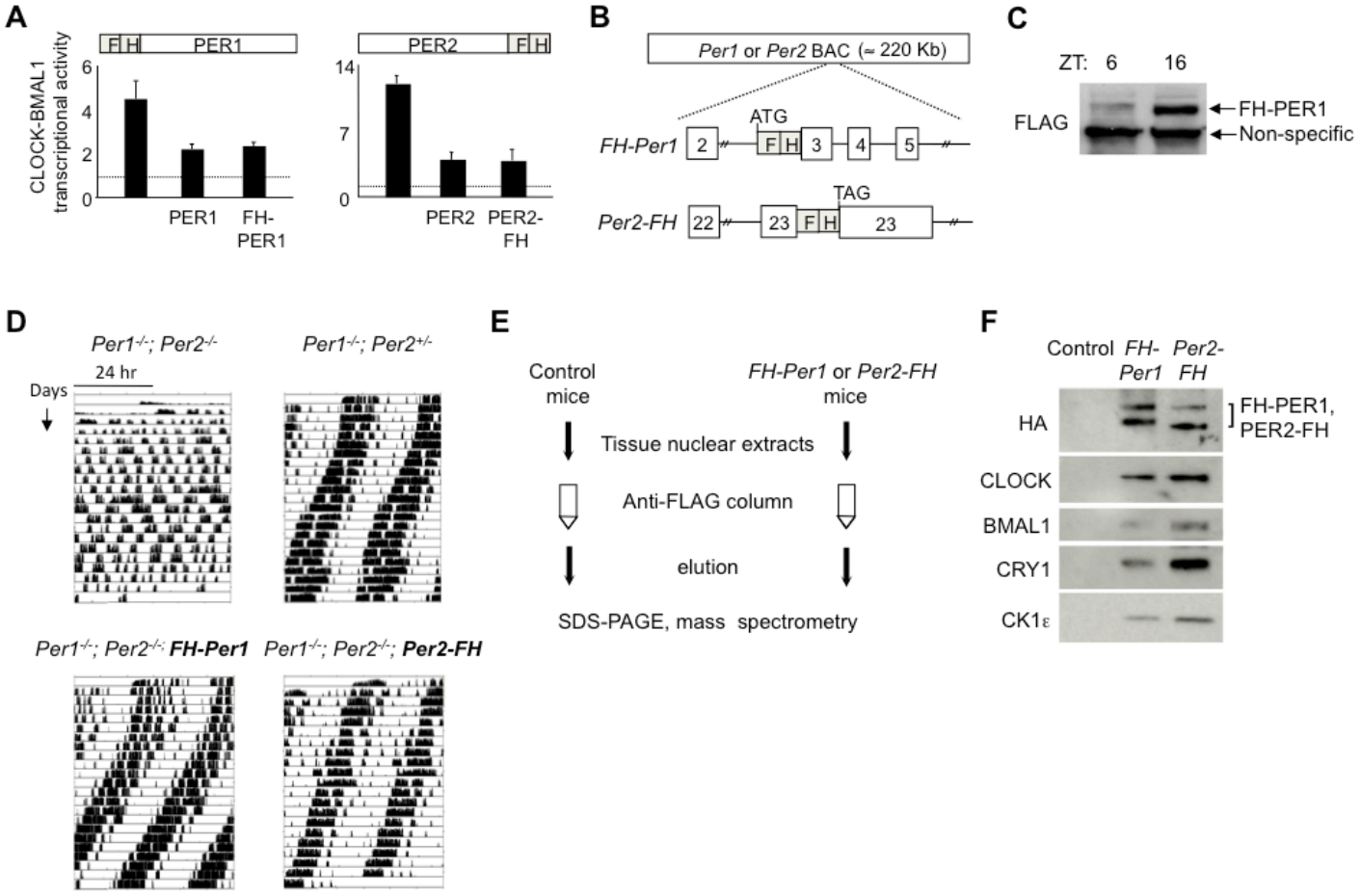
A
CLOCK-BMAL1 transcriptional activity
F H PER1
PER2 F H
PER1
FH-PER1
PER2
PER2-FH
B
Per1 or Per2 BAC (≈ 220 Kb)
ATG
FH-Per1
TAG
Per2-FH
C
ZT: 6 16
FLAG
FH-PER1
Non-specific
D
Per1-/-; Per2-/-
Per1-/-; Per2+/-
24 hr
Days
Per1-/-; Per2-/-; FH-Per1
Per1-/-; Per2-/-; Per2-FH
E
Control mice
FH-Per1 or Per2-FH mice
Tissue nuclear extracts
Anti-FLAG column
elution
SDS-PAGE, mass spectrometry
F
Control
FH-Per1
Per2-FH
HA
FH-PER1, PER2-FH
CLOCK
BMAL1
CRY1
CK1ε

Fig. S1

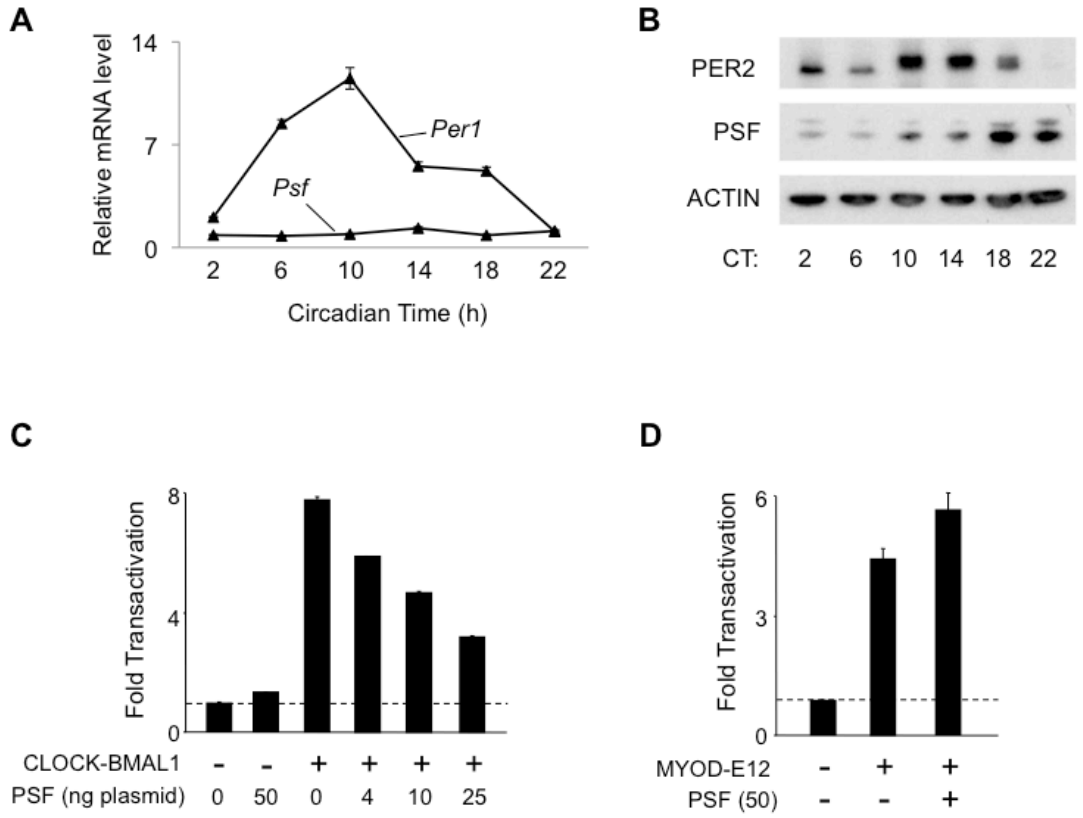
A
Relative mRNA level
14
7
0
Per1
Psf
2
6
10
14
18
22
Circadian Time (h)
B
PER2
PSF
ACTIN
CT: 2 6 10 14 18 22
C
Fold Transactivation
8
4
0
CLOCK-BMAL1 - - + + + +
PSF (ng plasmid) 0 50 0 4 10 25
D
Fold Transactivation
6
3
0
MYOD-E12 - + +
PSF (50) - - +

Fig. S2

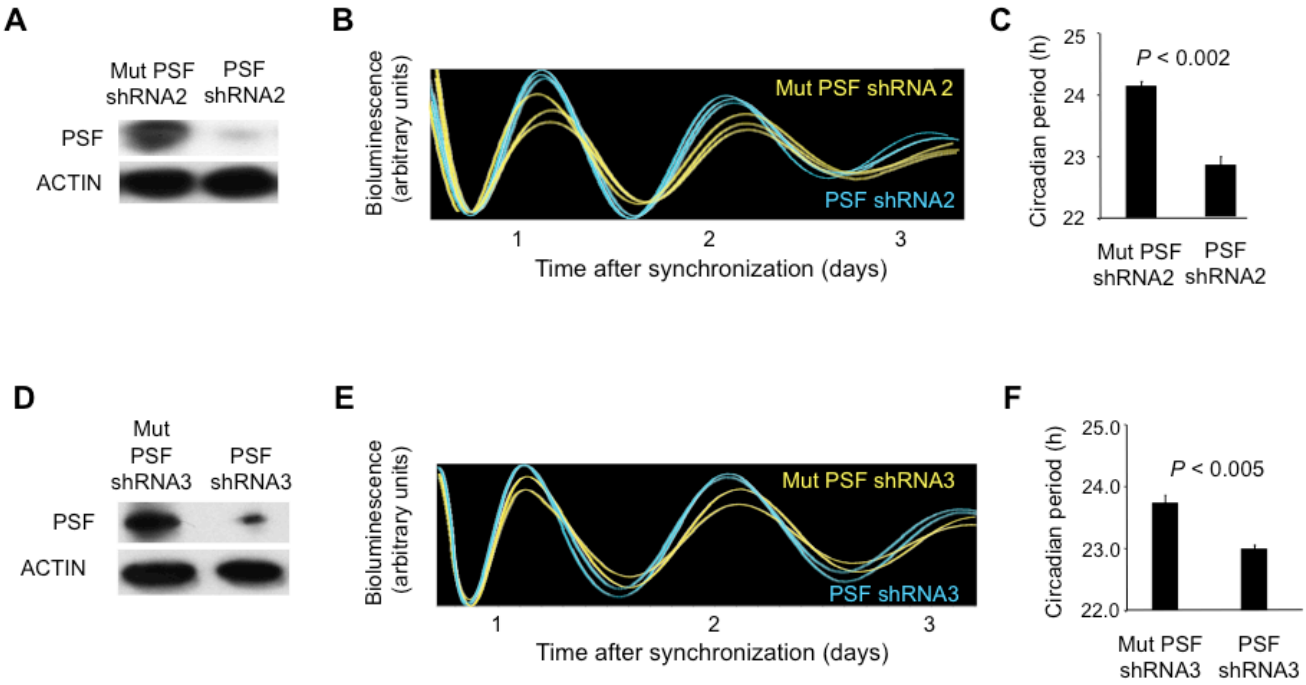
A
Mut PSF shRNA2
PSF shRNA2
PSF
ACTIN
B
Bioluminescence (arbitrary units)
Mut PSF shRNA 2
PSF shRNA2
1
2
3
Time after synchronization (days)
C
Circadian period (h)
25
24
23
22
P < 0.002
Mut PSF shRNA2
PSF shRNA2
D
Mut PSF shRNA3
PSF shRNA3
PSF
ACTIN
E
Bioluminescence (arbitrary units)
Mut PSF shRNA3
PSF shRNA3
1
2
3
Time after synchronization (days)
F
Circadian period (h)
25.0
24.0
23.0
22.0
P < 0.005
Mut PSF shRNA3
PSF shRNA3

Fig. S3

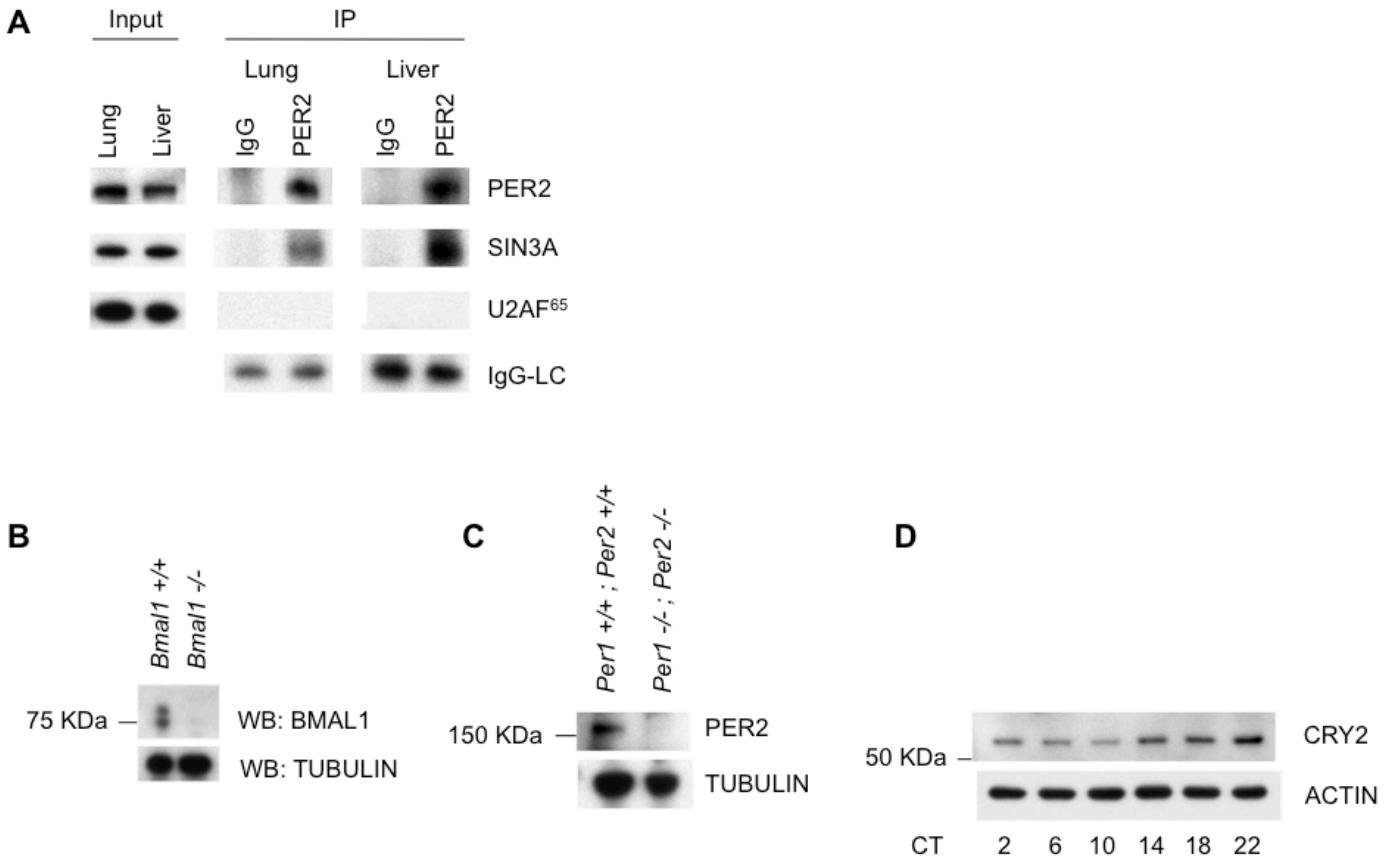
A
Input
IP
Lung
Liver
Lung
Liver
IgG
PER2
IgG
PER2
PER2
SIN3A
U2AF65
IgG-LC
B
Bmal1 +/+
Bmal1 -/-
75 KDa
WB: BMAL1
WB: TUBULIN
C
Per1 +/+ ; Per2 +/+
Per1 -/- ; Per2 -/-
150 KDa
PER2
TUBULIN
D
50 KDa
CRY2
ACTIN
CT
2
6
10
14
18
22

Fig. S4

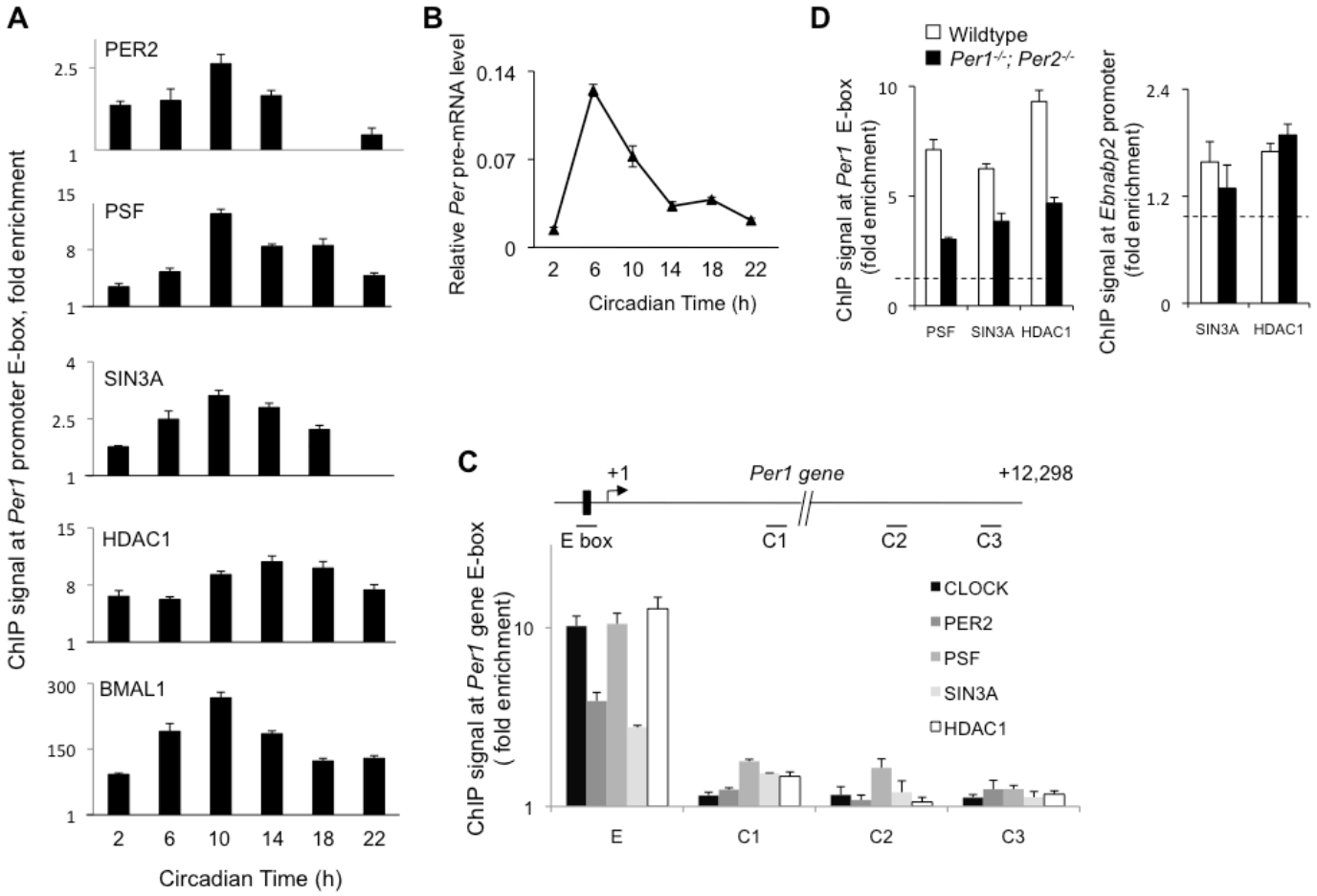
A
PER2
PSF
SIN3A
HDAC1
BMAL1
ChIP signal at Per1 promoter E-box, fold enrichment
Circadian Time (h)
B
Relative Per pre-mRNA level
Circadian Time (h)
C
+1
Per1 gene
+12,298
E box
C1
C2
C3
CLOCK
PER2
PSF
SIN3A
HDAC1
ChIP signal at Per1 gene E-box (fold enrichment)
D
Wildtype
Per1-/-; Per2-/-
ChIP signal at Per1 E-box (fold enrichment)
ChIP signal at Ebnabp2 promoter (fold enrichment)

Fig. S5

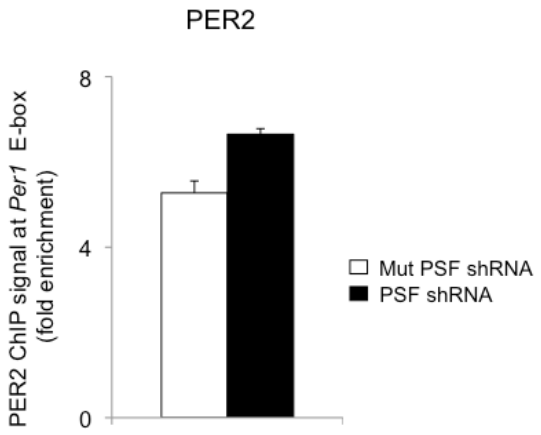
PER2
PER2 ChIP signal at *Per1* E-box
(fold enrichment)
8
4
0
Mut PSF shRNA
PSF shRNA

Fig. S6

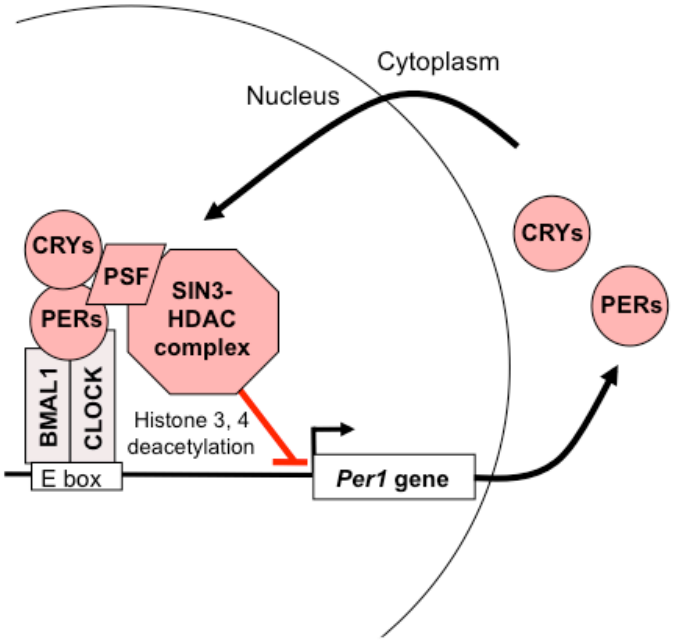
Cytoplasm
Nucleus
CRYs
PSF
SIN3-
HDAC
complex
PERs
BMAL1
CLOCK
Histone 3, 4
deacetylation
E box
Per1 gene
CRYs
PERs

Fig. S7

Supplementary Figure Legends (Figs. S1-S7)

Fig. S1 Affinity purification of PER complexes from mouse tissues. (**A**) Similarity of FH-PER1 and PER2-FH to respective wildtype PERs in repressing transcriptional activity of CLOCK-BMAL1. Top, diagram of FH-PER1 (left) and PER2-FH (right), in which the FLAG-Hemagglutinin (FH) tandem affinity purification tag has been fused to the PER1 N-terminus or PER2 C-terminus, respectively. Effect of the indicated PERs on CLOCK-BMAL1 transactivation of a luciferase reporter from *Per1* E-boxes in HEK293 cells (mean +/- SD; N ≥ 3). Mean luciferase activity after transfection of expression vector without inserts has been set to 1 (dashed line). (**B**) Structures of the mouse bacterial artificial chromosome (BAC) clones engineered to encode FH-PER1 or PER2-FH, as labeled. Numbered boxes indicate exons; ATG, start codon; TAG, stop codon. (**C**) Western blot of liver extracts from *FH-Per1* BAC transgenic mice probed with anti-FLAG antibody. Livers were harvested at the indicated times of day. ZT, Zeitgeber time. (**D**) Double-plotted actograms showing running-wheel activity of individual mice of the indicated genotypes in constant darkness. (**E**) Diagram summarizing the control and affinity purification of PER complexes from mouse tissues. (**F**) Western blot of samples from the control and affinity purifications of PER complexes (labeled at top) from mouse livers and probed with the antibodies indicated at left.

Fig. S2 PSF expression and transcriptional properties. **(A)** Quantitative reverse-transcriptase PCR assay showing the temporal expression profiles of *Psf* mRNA in the livers of wildtype mice harvested at six times across a circadian cycle (indicated at bottom). *Per1* mRNA, measured in the same samples, serves as a positive control for circadian oscillation. Shown are mean +/- SEM; N = 3. Data were normalized to *Hprt* mRNA expression, and the minimum value of each transcript profile has been set to 1. (**B**) Western blot showing representative temporal profile of PSF protein abundance in mouse liver nuclear extract. ACTIN served as loading control and PER2 as positive control for circadian regulation. CT, circadian time. (**C**) Potent inhibitory effect of PSF on CLOCK-BMAL1 transactivation of a luciferase reporter from *Per1* E-boxes in

NIH3T3 cells (mean +/- SD; N ≥ 3). Mean luciferase activity after transfection of expression vector without inserts has been set to 1 (dashed line). –, empty vector ; +, vectors with indicated inserts. (**D**) No inhibitory effect of PSF (50 ng of expression plasmid) on MyoD-E12 transactivation of a luciferase reporter from *Mck* gene E-boxes. Data displayed as in (B). (**E**) Greater inhibition of CLOCK-BMAL1 transcriptional activity from *Per1* E-boxes by sub-maximal expression of PSF plus sub-maximal PER1 than with either alone. Displayed as in (A).

Fig. S3 Depletion of PSF shortens circadian period length: evidence from two additional non-overlapping PSF shRNAs. (**A, D**) Western blot showing the effect of mutated control shRNA (Mut PSF shRNA, three point-mutations in 22-nt core sequence) or PSF shRNA on steady-state level of endogenous PSF. ACTIN, loading control. (**B, E**) Real-time circadian oscillations of bioluminescence in synchronized circadian reporter fibroblasts after delivery of control Mut PSF shRNA (yellow) or PSF shRNA (blue). Traces from three independent cultures are shown for each shRNA. (**C, F**) Circadian period of fibroblasts expressing the indicated control Mut PSF shRNA or PSF shRNA (mean +/- SEM; N = 3 for each; *t*-test, two-tailed).

Fig. S4 Co-immunoprecipitation of SIN3A with PER2 and validation of antibodies to circadian clock proteins used in ChIP analysis. (**A**) SIN3A forms a complex with PER2 in vivo. Co-immunoprecipitation of SIN3A with PER2 in wild-type mice. Immunoprecipitates (IP, antibodies indicated at top) from nuclear extracts from liver or lung (CT18) were probed with antibodies indicated at right. $U2AF^{65}$, negative control. IgG-LC, IgG light chain positive control for immunoprecipitation. (**B**, **C**) Western blots of liver nuclear extracts from mice of the genotypes indicated at the top were probed with the antibodies to proteins indicated at right of panel. (**D**) Western blots of wild-type mouse liver nuclear extracts obtained across a circadian cycle (circadian times at bottom) probed with antibodies to CRY or ACTIN (loading control). Single band of correct size shows a circadian rhythm of abundance with a phase appropriate for CRY2.

Fig. S5 PERs, PSF, and the SIN3-HDAC complex in the liver. (**A**) Synchronous circadian cycle of PER2, PSF, SIN3A, and HDAC1 at proximal E-box site of the *Per1* gene. Chromatin immunoprecipitation (ChIP) assays from livers harvested at six times across a circadian cycle (bottom) performed with the antibodies against the proteins marked at the top left of each panel. PCR product is centered on the *Per1* proximal E-box, a binding site for CLOCK-BMAL1. Background, set to 1, was determined from internal control amplification of an arbitrary intergenic region. Although not obvious here, the BMAL1 cycle appears to lead the cycle of the PER complex proteins by a few hours (see Figure 4B). Shown are mean +/- SEM of triplicate experiment; representative of 2-4 experiments for each antibody. (**B**) Circadian profile in the liver of *Per1* pre-mRNA, reflection circadian transcription of the *Per1* gene, assayed by quantitative reverse-transcriptase PCR. Shown are mean +/- SEM; N = 3. Data were normalized to *Hprt* mRNA expression. *Per1* pre-mRNA begins to decline at about the time of peak occupancy of PER complex proteins at the *Per1* proximal E-box site (panel A above; Figure 4C). (**C**) Top, diagram of the mouse *Per1* gene showing the position of the proximal E-box and sites amplified by three sets of control primers (C1, C2, C3 respectively). Bottom, ChIP assays from livers harvested at circadian time 10 performed with antibodies against the indicated proteins. All showed selective occupancy at the proximal E-box site, as did CLOCK, as expected. Background, set to 1, was determined from internal control amplification of an arbitrary intergenic region. Shown are mean +/- SEM of triplicate experiment; representative of 2-4 experiments for each antibody. (**D**) Presence of SIN3-HDAC1 at *Per1* promoter depends on PSF—confirmation in liver. ChIP assays from liver (CT 10) comparing occupancies of PSF, SIN3A, and HDAC1 (indicated at bottom) at the *Per1* proximal E-box or control promoter in wildtype littermates (white) and $Per1^{-/-}$; $Per2^{-/-}$ mice (black). Background (dashed line) as in Fig. 2. Shown are mean +/- SEM of triplicate experiment; representative of 2-4 experiments for each antibody.

Fig. S6 Depletion of PSF does not reduce PER2 occupancy at *Per1* promoter E-box. PER2 ChIP assays (PCR product centered on the *Per1* proximal E-box) from mouse fibroblasts after introduction of point-mutant control PSF shRNA (white) or after depletion of PSF by effective PSF shRNA (black). Shown are mean +/- SEM of triplicate experiment; representative of 3 independent experiments.

Fig. S7 Model for a mechanism of negative feedback in the mammalian circadian clock.

References

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S2. P. Liu, N. A. Jenkins, N. G. Copeland, *Genome Res*. **13**, 476 (2003).

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S6. W. N. Zhao *et al*., *Nat. Cell Biol*. **9**, 268 (2007).

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S9. S. A. Brown *et al*., *Science* **308**, 693 (2005).