A molecular mechanism for circadian clock negative feedback

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Abstract

Circadian rhythms in mammals are generated by a feedback loop in which the three PERIOD (PER) proteins, acting in a large complex, inhibit the transcriptional activity of the CLOCK-BMAL1 dimer, repressing their own expression. Although fundamental, the mechanism of negative feedback in the mammalian clock, or any eukaryotic clock, is unknown. We analyzed protein constituents of PER complexes purified from mouse tissues and identified PSF (polypyrimidine tract binding protein-associated splicing factor). Our analysis indicates that PSF within the PER complex recruits SIN3A, a scaffold for assembly of transcriptional inhibitory complexes, and that the PER complex thereby rhythmically delivers histone deacetylases to the *Per1* promoter, repressing *Per1* transcription. These findings provide a function for the PER complex and a molecular mechanism for circadian clock negative feedback.

Circadian clocks are oscillators that drive daily rhythms of physiology and behavior (1). In mammals, circadian clocks are found in most or all tissues (2), where they play important roles in local and systemic physiology (3,4).

The mammalian clock is built at least in part on a conserved transcriptional feedback loop (1). The three PERIOD (PER) and two CRYPTOCHROME (CRY) proteins form a nuclear complex (PER complex) of ~1 megadalton (5), associate with CLOCK-BMAL1 (6,7), the dimeric transcription factor activating their expression, and repress its transcriptional activity (8–10). Turnover of PERs and CRYs (11,12) de-represses CLOCK-BMAL1 activity, initiating a new cycle.

Negative feedback is fundamental to the clock, but little is known about its mechanism. Apart from binding to CLOCK-BMAL1, PERs and CRYs have no known activity. Besides PERs and CRYs, only two constituents of a purified PER complex have been identified, NONO and WDR5 (5). NONO is a poorly understood RNA-binding protein important for clock function, and WDR5 is a histone methyltransferase subunit of plausible but uncertain relevance to the clock (5).

To search for unrecognized proteins important for circadian negative feedback, we set out to purify PER complexes from mouse tissues and identify constituent proteins by mass spectrometry. For efficient purification, we generated two mouse lines, one in which endogenous PER1 was replaced by a PER1 fusion protein tagged at the N-terminus with FLAG-Hemagglutinin (FH) (13) (FH-PER1) and another in which endogenous PER2 was replaced by a PER2 fusion protein tagged at the C-terminus (PER2-FH) (fig. S1; SOM text, in vivo epitope tag strategy). FH-PER1 and PER2-FH were similar in function to wild-type PERs in cells and in vivo in mice (fig. S1A-D). Because PER1 and PER2 are present in the

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same complexes (5), this strategy provided two ways of purifying PER complexes from mouse tissues, allowing cross-validation of results.

Tissue from littermate controls (with only wildtype PERs) was processed in parallel to that from *FH-Per1* or *Per2-FH* mice. Livers were obtained at circadian time (CT) 20, a time when nuclear PER complex represses CLOCK-BMAL1 activity. PER complexes were isolated from nuclear extracts by anti-FLAG immunoaffinity purification, and PER-associated proteins were resolved by SDS-polyacrylamide gel electrophoresis (fig. S1E). Known PER-associated clock proteins co-purified with FH-PER1 and PER2-FH (fig. S1F).

After excluding proteins detected in controls, we found by mass spectrometry that in both the FH-PER1 and PER2-FH samples, known clock proteins accounted for ten of the fifteen proteins from which the most numerous unique peptides were identified. This set consisted of PER1, PER2, PER3, CRY1, CRY2, CLOCK, BMAL1, Casein Kinase-1ε and δ, and NONO. Others were RNA-binding proteins, to be described elsewhere (14). We focused on one particular RNA-binding protein, PSF (also called PTB-associated splicing factor), a pre-mRNA splicing factor related to NONO (15) that also acts as a transcriptional co-repressor (16).

The presence of PSF in endogenous PER complexes was confirmed by co-immunoprecipitation. Antibodies to PSF co-immunoprecipitated PER2, and antibodies to PER2 co-immunoprecipitated PSF from mouse lung and liver nuclear extracts (CT18) (Fig. 1A). The abundance of *Psf* mRNA did not appear to oscillate (fig. S2A), but there was an apparent oscillation of nuclear PSF protein, peaking shortly after PER2 (fig. S2B). PSF over-expression repressed CLOCK-BMAL1 transcriptional activity, consistent with a function in the PER complex (fig. S2C,D).

To determine whether PSF plays an important role in the transcriptional action of the PER complex, we introduced a small hairpin RNA construct (shRNA) into unsynchronized fibroblasts to deplete endogenous PSF. We then measured steady-state abundance of pre-mRNAs from two CLOCK-BMAL1 circadian target genes, *Per1* and *Dbp*, and an arbitrary control gene. Depletion of PSF caused an increase in the average transcription of *Per1* and *Dbp*, but it had no evident effect on transcription of the control (Fig. 1B). Thus endogenous PSF has a PER-like action in selectively repressing the transcriptional activity of CLOCK-BMAL1.

To determine whether PSF is important for clock function, we depleted PSF from reporter fibroblasts (17) and monitored circadian rhythms of bioluminescence in the synchronized cells. Three non-overlapping PSF shRNAs each caused a substantial depletion of endogenous PSF, and each caused a shortening of circadian period length that was evident in the real-time bioluminescence traces and highly significant in the group data (Fig. 1C-E; fig. S3). PSF thus plays a role in the clock mechanism.

PSF acts as a transcriptional co-repressor by recruiting the SIN3-HDAC complex (16, 18), a negative regulator of transcription (19). We therefore tested for SIN3A, the scaffold of the SIN3-HDAC complex (19), in endogenous PER complexes. An antibody to SIN3A co-immunoprecipitated PSF and PER2 from mouse lung and liver nuclear extracts (CT18) (Fig. 2A), and an antibody to PER2 co-immunoprecipitated SIN3A (fig. S4A).

To test whether PERs, CRYs, PSF, SIN3A, and HDAC1 (a histone deacetylase of the SIN3-HDAC complex) (19) are coordinately targeted to the *Per1* promoter, as expected of proteins acting in the PER complex, we performed chromatin immunoprecipitations (ChIP) from lung and liver. PER2, CRY2, PSF, SIN3A, and HDAC1 exhibited a synchronous circadian rhythm at the *Per1* proximal E-box (a CLOCK-BMAL1 binding site) (Fig. 2B; fig.

S4B). We obtained similar results from liver (fig. S5). Peak occupancy occurred at CT 10-14, a time when PERs first appear in the nucleus (14) and when *Per1* pre-mRNA begins to decline (fig. S5B). Enrichment of PER complex proteins was observed at the E-box but not other sites in the *Per1* gene (Fig. 2C, fig. S5C), mirroring BMAL1 (Fig. 2C) and CLOCK (fig. S5C). As reported for *Dbp* E-box sites (20), we observed a circadian cycle of BMAL1 at the *Per1* proximal E-box (Fig. 2B, fig. S5A). The BMAL1 cycle overlapped with the cycle of PER complex proteins, but it appeared to lead by a few hours (Fig. 2B).

We performed ChIP on lungs (CT 10) from wild-type littermates and *Per1*$^{-/-}$; *Per2*$^{-/-}$ double mutants (21). *Per1*$^{-/-}$; *Per2*$^{-/-}$ samples showed a reduction in PSF, SIN3A, and HDAC1 at the *Per1* proximal E-box but little or no change at a control promoter (Fig. 3A). We obtained similar results from liver (fig. S5D). Thus PERs are important for recruiting PSF and the SIN3-HDAC complex to the *Per1* promoter.

Depletion of PSF from unsynchronized fibroblasts caused a reduction in the average occupancies of PSF itself, SIN3A, and HDAC1 at the *Per1* promoter, but had little or no effect on SIN3A or HDAC1 at a control promoter (Fig. 3B), phenocopying *Per1*$^{-/-}$; *Per2*$^{-/-}$. Depletion of PSF did not reduce PER2 at the proximal *Per1* E-box (fig. S6). PSF is thus important for PER-dependent recruitment of the SIN3-HDAC complex to the *Per1* promoter.

To test the role of the SIN3-HDAC complex in PER function, we monitored acetylation of HDAC1 histone target residues (22) in the *Per1* promoter in lung (CT14) in wild-type littermates and *Per1*$^{-/-}$; *Per2*$^{-/-}$ mutants. The *Per1* promoter of *Per1*$^{-/-}$; *Per2*$^{-/-}$ mice, but not a control promoter, exhibited increased acetylation of histone 3, lysine 9 in histone 3 (H3K9), and, to a lesser extent, lysine 5 in histone 4 (H4K5) (Fig. 3C). Similar results were obtained in fibroblasts after depletion of PSF (Fig. 3D). Thus the PER complex promotes histone deacetylation at the *Per1* promoter by the PSF-dependent recruitment of the SIN3-HDAC complex.

We tested the role of the SIN3-HDAC complex in circadian negative feedback and clock function. Depletion of SIN3A from unsynchronized fibroblasts by a small interfering RNA (siRNA) caused an increase in the average transcription of the CLOCK-BMAL1 target genes *Per1* and *Dbp* but had little or no effect on control gene transcription (Fig. 4A). Depletion of SIN3A from synchronized fibroblasts caused a shortening of circadian period length that was evident in the real-time bioluminescence traces and highly significant in the group data (Fig. 4BD). Thus SIN3A depletion mimicked the effects of PSF depletion on both *Per1* transcription and clock function.

These results establish a specific function for the PER proteins and a molecular mechanism for circadian clock negative feedback, central aspects of clock function that have long been poorly understood. Our analysis indicates that a PER complex rhythmically associates with DNA-bound CLOCK-BMAL1 at the *Per1* promoter and, by virtue of its constituent PSF, recruits the SIN3-HDAC complex, thereby deacetylating histones 3 and 4 and repressing transcription. CLOCK preferentially acetylates H3K9 (23), so PER- and PSF- dependent recruitment of the SIN3-HDAC complex could serve to reverse modifications produced by CLOCK, generating a circadian rhythm of H3K9 acetylation. Our ongoing work indicates that a PER complex has an additional role in negative feedback, acting to repress *Per* and *Cry* transcriptional elongation (14).

The function of the SIN3-HDAC complex in transcriptional repression is conserved across virtually all eukaryotes (19). Circadian cycles of histone 3 acetylation have been observed at the promoters of clock genes in extraordinarily diverse organisms, including mammals (20,

24–26), insects (27), plants (28), and fungi (29). Thus it is conceivable that the role of the SIN3-HDAC complex in circadian negative feedback dates back to the evolutionary origins of eukaryotic circadian clocks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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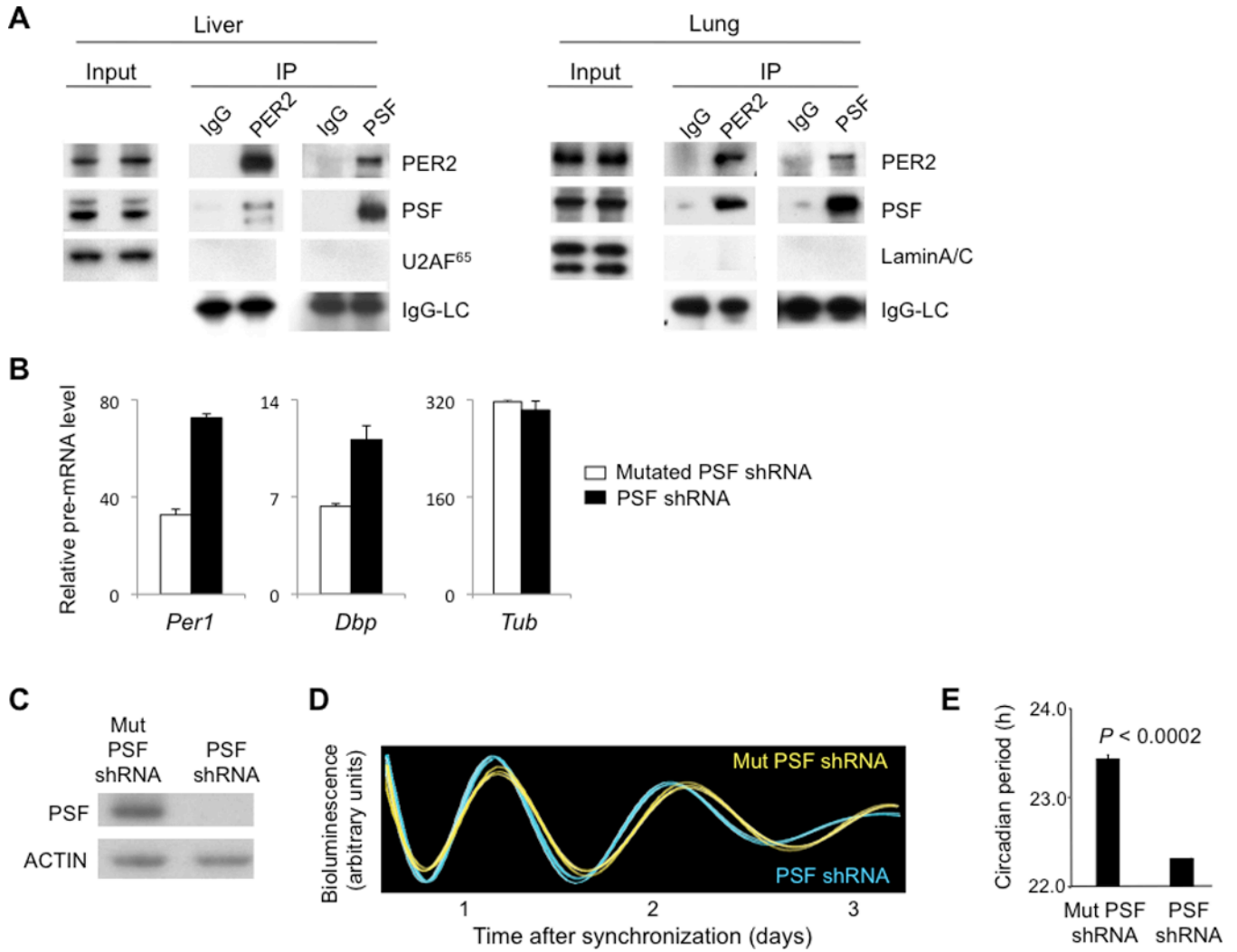29. Belden WJ, Loros JJ, Dunlap JC. Mol Cell. 2007; 25:587. [PubMed: 17317630]

Fig. 1.
PSF is a constituent of endogenous PER complexes and is important for clock function. (**A**) Co-immunoprecipitation of PSF and PER2. Nuclear extracts (CT18) from liver or lung (input) and immunoprecipitates (IP, antibodies at top) from the extracts were probed with antibodies at right. U2AF^{65} and LaminA/C, negative controls; IgG-LC (light chain), positive control. (**B**) Depletion of PSF increases *Per1* transcription. Quantitative RT-PCR assays showing steady-state abundance of indicated pre-mRNAs (normalized to *Gapdh* mRNA) in mouse fibroblasts after introduction of point-mutant control PSF shRNA (white) or after depletion of PSF by PSF shRNA (black). Shown are mean +/− SEM of triplicate experiments; representative of 3 experiments. (**C-E**) Short circadian period length caused by depletion of endogenous PSF from fibroblasts. (**C**) Western blot showing the effect of point-mutant control (Mut) shRNA or PSF shRNA on steady-state level of endogenous PSF. ACTIN, loading control. (**D**) Circadian oscillations of bioluminescence in synchronized reporter fibroblasts after delivery of control Mut PSF shRNA (yellow) or PSF shRNA (blue). Traces from three independent cultures are shown for each. (**E**) Circadian periods of fibroblasts in (D) (mean +/− SEM; N = 3 for each condition; *t*-test, two-tailed).
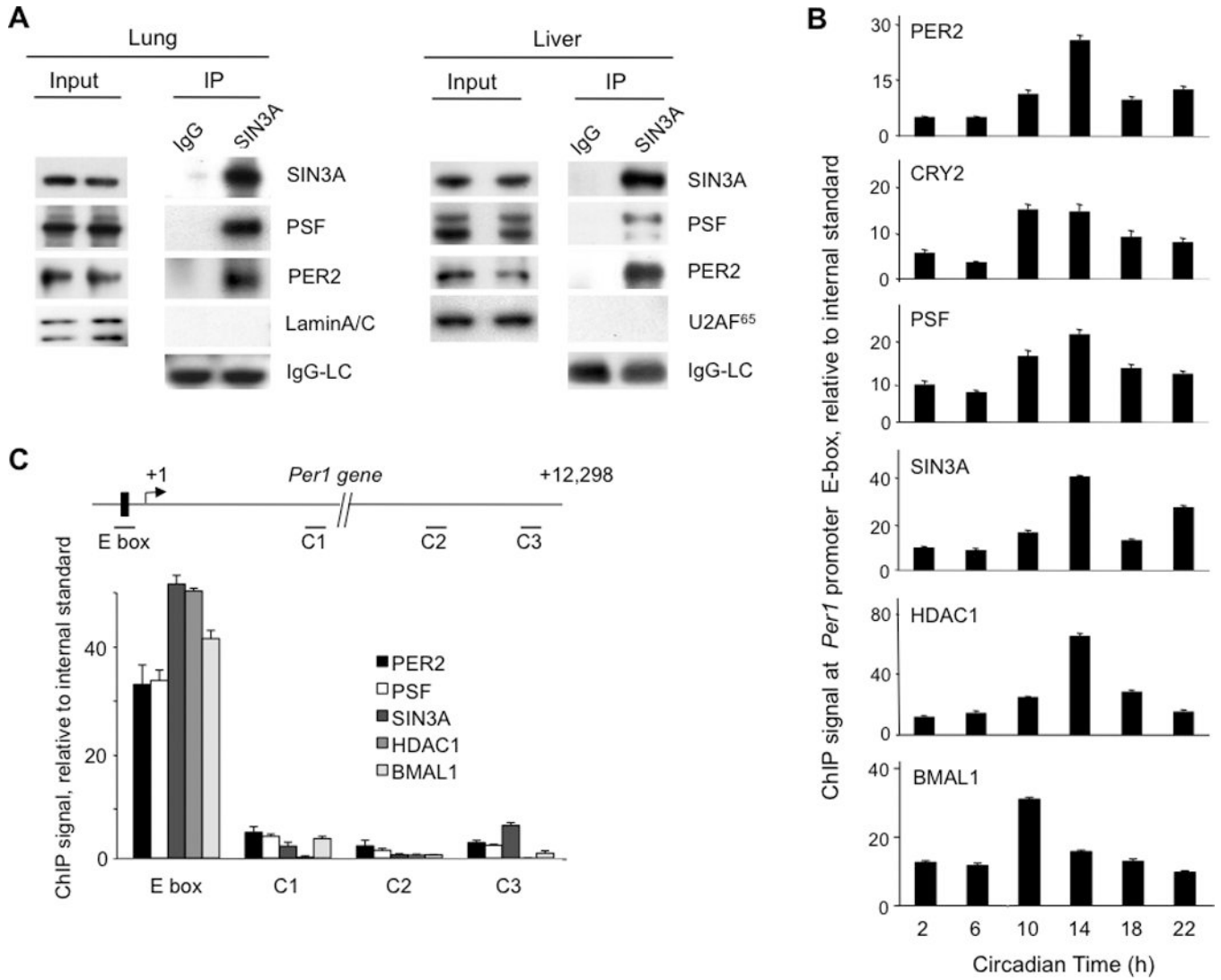
Fig. 2.
PERs, PSF, and the SIN3-HDAC complex. (**A**) Co-immunoprecipitation of endogenous PER2 and PSF with SIN3A. Nuclear extracts (CT18) from liver or lung (input) and immunoprecipitates (IP, antibodies at top) from the extracts were probed with antibodies at right. U2AF^{65} and LaminA/C, negative controls; IgG-LC (light chain), positive control. (**B**) Synchronous circadian cycle of PER2, CRY2, PSF, SIN3A, and HDAC1 at *Per1* proximal E-box site. ChIP assays from lungs sampled across a circadian cycle (bottom) performed with antibodies at the top left of each panel. ChIP values are relative to the signal at an arbitrary internal control genomic region (SOM methods). (**C**) Top, diagram of mouse *Per1* gene showing positions of proximal E-box and control sites C1, C2, and C3. Bottom, ChIP assays from lungs harvested at CT10-14, performed with antibodies against the indicated proteins. ChIP data show mean +/− SEM of triplicate experiments; representative of 2-4 experiments.
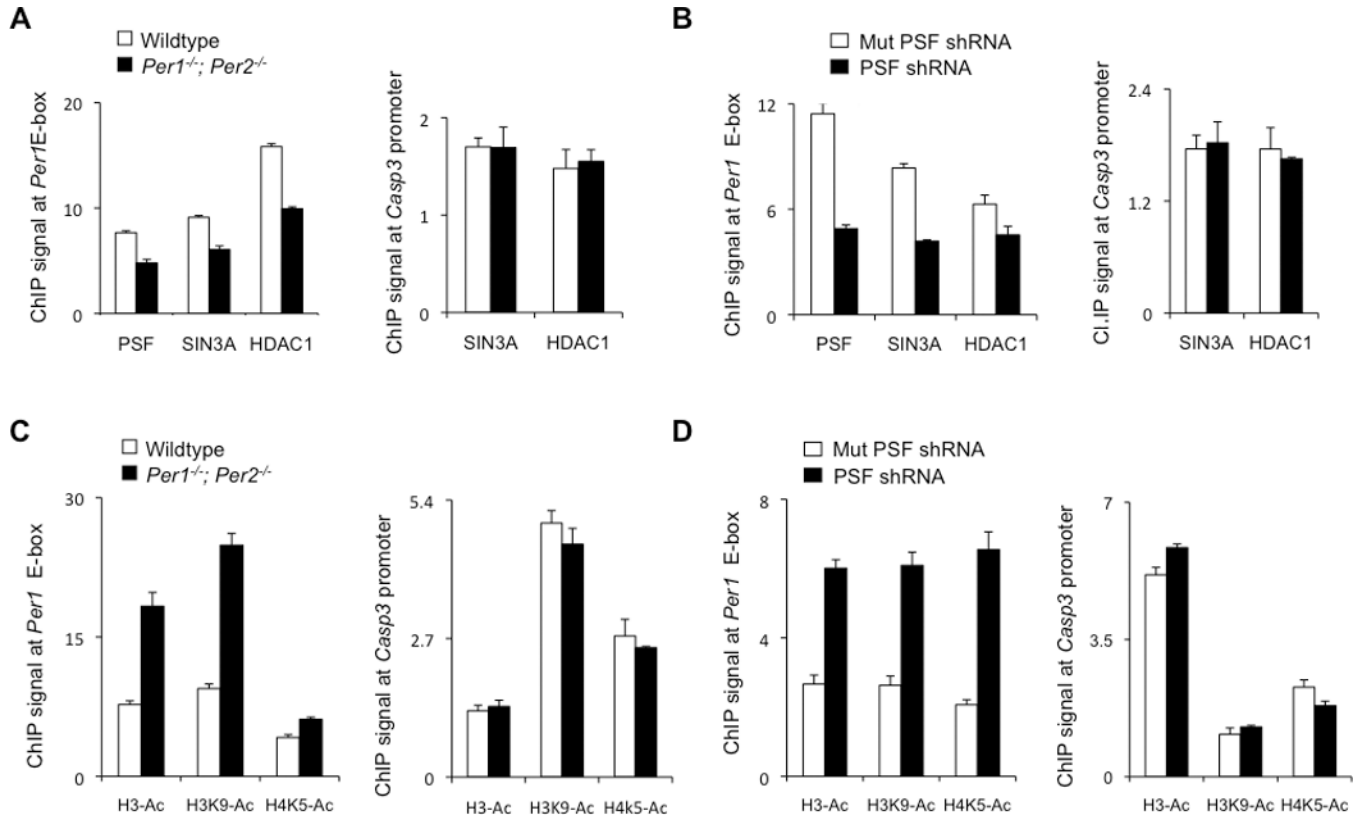
Fig. 3.
Presence of SIN3-HDAC1 at *Per1* promoter depends on PER and PSF. (**A**) ChIP assays from lung (CT 10) comparing PSF, SIN3A, and HDAC1 at the *Per1* proximal E-box or control promoter in wild-type littermates (white) and *Per1*$^{-/-}$; *Per2*$^{-/-}$ mice (black). (**B**) ChIP assays, as in (A), from mouse fibroblasts after introduction of point-mutant control PSF shRNA (white) or after depletion of PSF by effective PSF shRNA (black). (**C**) ChIP assays, as in (A), from lung (CT14) comparing acetylation of HDAC1 targets (at bottom) at the *Per1* E-box site or control promoter in wild-type littermates (white) and *Per1*$^{-/-}$; *Per2*$^{-/-}$ mice (black). (**D**) ChIP assays, as in (C), from fibroblasts after introduction of point-mutant control PSF shRNA (white) or after depletion of PSF by effective PSF shRNA (black). ChIP values are plotted relative to the signal at an arbitrary internal control genomic region (SOM methods). All show mean +/− SEM of triplicate experiments and are representative of 2-4 experiments.
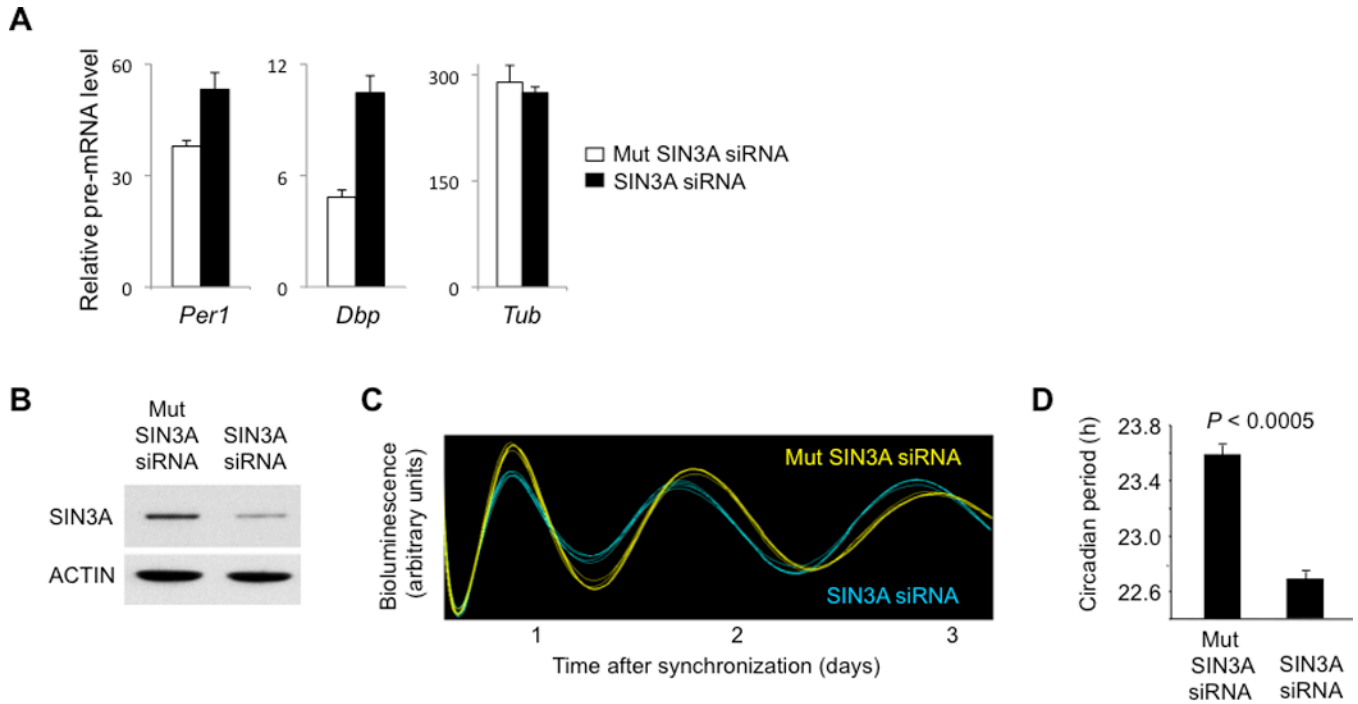
Fig. 4.
Depletion of SIN3A increases *Per1* transcription and shortens circadian period length. (**A**) Quantitative RT-PCR assays, as in Fig. 1B, showing steady-state abundance of indicated pre-mRNAs in fibroblasts after introduction of point-mutant control SIN3A shRNA (white) or after depletion of SIN3A by an effective SIN3A shRNA (black). Shown are means +/− of triplicate experiment; representative of 3 experiments. (**B-D**) Short circadian period length caused by depletion of endogenous SIN3A from fibroblasts. (**B**) Western blot showing the effect of mutated control (Mut) siRNA or SIN3A siRNA on steady-state level of endogenous SIN3A. ACTIN, loading control. (**C**) Circadian oscillations of bioluminescence in synchronized circadian reporter fibroblasts after delivery of control Mut SIN3A siRNA (yellow) or SIN3A siRNA (blue). Traces from three independent cultures are shown for each condition. (**D**) Circadian period of fibroblasts with control Mut SIN3A siRNA or SIN3A siRNA (mean +/− SEM; N = 3; *t*-test, two-tailed).