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Temporal orchestration of repressive chromatin modifiers by mammalian circadian clock PERIOD complexes

Hao A. Duong and Charles J. Weitz

Department of Neurobiology, Harvard Medical School, Boston, MA 02115 USA

Abstract

The mammalian circadian clock is built on a molecular feedback loop in which the PERIOD (PER) proteins, acting in a large, poorly-understood complex, repress CLOCK-BMAL1, the transcription factor driving their expression. We found that mouse PER complexes include the histone methyltransferase HP1 γ -Suv39H. PER proteins recruit HP1 γ -Suv39H to the *Per1* and *Per2* promoters, and HP1 γ -Suv39H proved important for circadian di- and tri-methylation of histone-3 lysine-9 (H3K9) at the *Per1* promoter, feedback transcriptional repression, and circadian clock function. HP1 γ -Suv39H was recruited to the *Per1* and *Per2* promoters ~4 hours after HDAC1, a PER-associated protein we previously implicated in clock function and H3K9 deacetylation at the *Per1* promoter. PER complexes containing HDAC1 or HP1 γ -Suv39H appeared to be physically separable. Circadian clock negative feedback by the PER complex thus involves dynamic, ordered recruitment of repressive chromatin modifiers to DNA-bound CLOCK-BMAL1.

INTRODUCTION

Circadian clocks are cell-autonomous molecular oscillators that drive daily cycles of physiology, metabolism, and behavior^{1–3}. In mammals, circadian clocks are widely distributed in different cells and tissues^{4–6}, where they locally drive rhythms of tissue-specific processes^{7–11} and ensure that a multitude of biological activities are properly coordinated with one another and advantageously timed with respect to the behavioral cycle and the daily environmental cycle¹².

The mammalian clock is built on a conserved transcriptional negative feedback loop¹ in which the three PERIOD (PER) and two CRYPTOCHROME (CRY) proteins repress the activity of CLOCK-BMAL1, the transcription factor driving their own expression. Upon entering the cell nucleus, PER and CRY proteins form one or more large protein complexes^{13,14} (PER complex) that include at least 25 distinguishable proteins¹⁴. The PER complex binds to CLOCK-BMAL1 at E-box sites on *Per*, *Cry*, and other circadian target genes, thereby repressing CLOCK-BMAL1 transcriptional activity^{15–17}. Targeted degradation of PER and CRY proteins^{18–21} enables the re-activation of CLOCK-BMAL1, initiating a new circadian transcriptional cycle with an intrinsic period of ~24 hours. An additional coupled feedback loop involving the transcription factors REV-ERB α and REV-

ERB β supports clock function^{22,23}. CLOCK-BMAL1 target genes encoding products that do not feed back into the clock mechanism serve as circadian outputs, together generating a rhythmic network of cellular functions at the transcriptional and post-transcriptional levels^{24,25}.

Although it is arguably the defining feature of the circadian oscillator, the negative feedback activity of the PER complex is poorly understood. Recent work indicates that the PER complex functions in part by recruiting the SIN3-HDAC complex²⁶, which deacetylates lysine 9 of histone 3 (H3K9) at CLOCK-BMAL1 target sites, contributing to the conversion of local chromatin to a repressive state. In addition, the PER complex recruits SETX and other helicases, thereby inhibiting termination and possibly additional post-initiation transcriptional processes¹⁴. The PER complex thus delivers multiple transcriptional regulatory proteins to DNA-bound CLOCK-BMAL1 that together inhibit transcription in an apparently complex, multi-faceted manner.

Accumulating evidence indicates that histone modifications governing transcription are dynamic and cooperative²⁷. In particular, deacetylation and methylation of H3K9 are often coupled in establishing a repressive chromatin state^{28–30}. A study of circadian chromatin modifications of the *Dbp* gene, a clock output gene, revealed circadian rhythms of H3K9 acetylation and dimethylation that were anti-phase to one another, with the peak of acetylation occurring during the phase of active transcription and the peak of dimethylation occurring during the phase of feedback repression³¹. These chromatin modifications were dependent on the binding of CLOCK-BMAL1 to the DNA, suggesting that CLOCK-BMAL1 or the PER complex (or both) delivers the relevant chromatin-modifying enzymes to the circadian target gene. Given the precedent for direct recruitment of SIN3-HDAC²⁶, an H3K9 deacetylase, by the PER complex²⁶ and evidence for coupling of H3K9 deacetylation and methylation in transcriptional repression, it seemed plausible that an unrecognized function of the PER complex might be the delivery of one or more H3K9 methyltransferases, contributing to the conversion of local chromatin to a heterochromatin-like repressive state.

RESULTS

PER complexes include the histone methyltransferase HP1 γ -Suv39H

As an initial test of this hypothesis, we immunoprecipitated PER complexes from mouse tissues and looked for the presence of various histone methyltransferase subunits associated with methylation of H3K9, including catalytic subunits G9a, SUV39H1, and SUV39H2 and associated subunits HP1 α , HP1 β , HP1 γ , and KAP1^{32–34}. As expected, antibodies to PER2 co-immunoprecipitated known core components of the PER complex from nuclear extracts of livers harvested during the negative feedback phase of the circadian cycle, circadian time 18 h (CT 18) (Fig. 1). G9a, HP1 α , and HP1 β were not detected in the immunoprecipitates, whereas SUV39H1, SUV39H2, HP1 γ , and KAP1 co-immunoprecipitated with PER2 (Fig. 1). In addition, antibodies to HP1 γ specifically co-immunoprecipitated PER2 and SUV39H1, and antibodies to SUV39H1 specifically co-immunoprecipitated PER2 and HP1 γ (Supplementary Figure 1a). These results indicate that an HP1 γ -SUV39H histone methyltransferase is a component of one or more PER complexes in vivo. Essentially

identical results were obtained from mouse lung nuclear extracts (Supplementary Figure 1b), indicating that the presence of these H3K9 methyltransferase components in a PER complex is not a liver-specific peculiarity, but likely reflects a general or common feature of clock architecture.

SUV39H catalytic subunits are important for clock transcriptional feedback and function

To determine if this histone methyltransferase plays a role in the transcriptional feedback actions of the PER complex, we electroporated small interfering (si) RNAs into unsynchronized cultured mouse fibroblasts (Supplementary Materials and Methods) to deplete the catalytic subunits SUV39H1 (Fig. 2a), SUV39H2 (Supplementary Figure 2a), or both (Supplementary Figure 3). We then monitored the steady-state abundance of pre-mRNAs from four CLOCK-BMAL1 target genes (subject to negative feedback regulation by the PER complex) and four arbitrary control genes. Depletion of endogenous SUV39H1, SUV39H2, or both caused highly-significant increases in the mean steady-state levels of the circadian target gene pre-mRNAs but little or no effect on control pre-mRNAs (Fig. 2b, Supplementary Figure 2b, Supplementary Figure 3, respectively). The double depletion showed an effect only slightly stronger than either single depletion. Thus SUV39H1 and SUV39H2 act selectively to repress the transcriptional activity of CLOCK-BMAL1, indicating an important role in the circadian feedback actions of the PER complex.

Because SUV39H1 and SUV39H2 contribute to circadian transcriptional feedback, the two catalytic subunits should be important for clock function. To test this prediction, we depleted endogenous SUV39H1 or SUV39H2 (as above) from circadian reporter fibroblasts³⁵ and monitored real-time circadian rhythms of bioluminescence in the synchronized cell populations. In each case, the depletion caused a shortening of circadian period length that was observed in the individual bioluminescence traces (Fig. 2c, Supplementary Figure 2c) and highly significant in the group data (Fig. 2d, Supplementary Figure 2d). In addition, when different siRNAs were used to deplete SUV39H1 or SUV39H2, in each case a comparable short-period circadian phenotype was observed (Supplementary Figure 4). These results indicate that SUV39H1 and SUV39H2 play a role in the clock mechanism. A short-period circadian phenotype is typical after depletion of negative feedback repressors of CLOCK-BMAL1 activity^{14,26,35,36}.

PER2 and HP1 γ -SUV39H show a coordinate circadian rhythm of occupancy at the *Per1* promoter

If the HP1 γ -SUV39H methyltransferase within a PER complex contributes to clock function by virtue of its known chromatin-modifying activity, then the methyltransferase should be associated with the PER complex when it interacts with CLOCK-BMAL1 on circadian target genes. To test this prediction, we first monitored the formation of this complex every four hours across a circadian cycle by co-immunoprecipitation with HP1 γ , the scaffold of the methyltransferase complex, from liver nuclear extracts. SUV39H1 and SUV39H2 specifically co-immunoprecipitated with HP1 γ equivalently at all time-points (Fig. 3a). PER2 was detectably co-immunoprecipitated only during the feedback repression phase of the circadian cycle (Fig. 3a), as expected, when PER proteins are maximally expressed. These observations suggest that a core PER complex, assembled from newly-synthesized

PER and CRY proteins at the onset of the repression phase, associates with a pre-existing HP1 γ -SUV39H methyltransferase complex.

We next performed chromatin immunoprecipitation (ChIP) assays on mouse liver nuclear extracts to monitor the occupancy of the proteins on the *Per1* promoter across the circadian cycle. HP1 γ and SUV39H1 showed a circadian rhythm at the *Per1* proximal E-box that was synchronous with the rhythm of PER2, with the peak of the rhythm occurring in the feedback repression phase of the cycle (Fig. 3b). This result is consistent with the interaction of a PER-HP1 γ -SUV39H holocomplex with DNA-bound CLOCK-BMAL1, resulting in the targeted delivery of the histone methyltransferase to chromatin of circadian target genes.

PER proteins are required for recruitment of HP1 γ -SUV39H to the *Per1* promoter

If a PER complex delivers HP1 γ -SUV39H to chromatin at CLOCK-BMAL1 circadian target genes, then PER loss-of-function mutations should result in reduced occupancies of HP1 γ -SUV39H at circadian target sites but not irrelevant sites. To examine this possibility, we performed ChIP assays on samples from livers of *Per1*^{-/-}; *Per2*^{-/-} double mutant mice and wildtype littermates obtained at CT18, during the phase of circadian feedback repression. Chromatin from *Per1*^{-/-}; *Per2*^{-/-} mice showed no reduction in the occupancies of HP1 γ or SUV39H1 at a non-circadian control promoter (Fig. 4a), indicating that the PER proteins have no general role in the association of HP1 γ -SUV39H with chromatin. In contrast, the samples from *Per1*^{-/-}; *Per2*^{-/-} mice showed dramatic reductions of both proteins at the *Per1* proximal E-box (Fig. 4b). Thus the recruitment of HP1 γ -SUV39H to the *Per1* E-box site is dependent on PER protein function.

HP1 γ links chromatin-bound PER complex to SUV39H1

In at least some contexts, HP1 γ serves as a scaffold that recruits SUV39H1 to sites of histone methyltransferase action³². If this is the case in the clock mechanism, then depletion of HP1 γ should reduce the recruitment of SUV39H1 to circadian target sites but have little or no effect on the occupancy of PER proteins. Furthermore, depletion of SUV39H1 should not cause a reduced occupancy of PER proteins or HP1 γ at the site.

To test the first prediction, we depleted HP1 γ from mouse fibroblasts (Fig. 4c) and performed ChIP assays to monitor the occupancies of PER2, HP1 γ , and SUV39H1 at the proximal *Per1* E-box. As predicted, depletion of HP1 γ caused a dramatic reduction in the occupancy of SUV39H1, but not PER2 (Fig. 4d). A similar result was observed at two additional CLOCK-BMAL1 circadian target sites, a *Per2* E-box and the *Per1* distal E-box (Supplementary Figure 5a, b).

We next depleted SUV39H1 (Fig. 4e) and performed ChIP assays as above. As predicted, depletion of SUV39H1 did not reduce the occupancy of either PER2 or HP1 γ at the *Per1* promoter (Fig. 4f). Unexpectedly, HP1 γ occupancy was increased, apparently as a consequence of a compensatory increase in total nuclear HP1 γ following SUV39H1 depletion (Supplementary Figure 5c). These results indicate that a PER complex delivers HP1 γ -SUV39H to circadian target genes and that HP1 γ serves to link SUV39H to the chromatin-associated PER complex.

PER proteins and SUV39H1 are important for di- and tri-methylation of H3K9 at the *Per1* promoter

SUV39H proteins prefer mono- and di-methylated H3K9 as substrates in vitro and appear to be involved primarily in di- and tri-methylation of H3K9 in vivo^{37,38}. If the transcriptional repression activity of HP1 γ -SUV39H delivered by a PER complex results from its histone methyltransferase activity, then there should be a circadian rhythm of H3K9 di- and tri-methylation (H3K9-Me₂ and H3K9-Me₃, respectively) on the *Per1* promoter that mirrors the rhythm of occupancy of the catalytic subunit SUV39H1. To test this prediction, we performed ChIP assays on mouse liver samples collected every four hours across a circadian cycle. We observed a robust circadian rhythm of both H3K9-Me₂ and H3K9-Me₃ at the *Per1* promoter, peaking at approximately CT16 (Fig. 5a), essentially identical to the rhythm of SUV39H1 occupancy (Fig. 3b). This rhythm resembles the cycle of H3K9-Me₂ previously observed on the *Dbp* gene³¹.

Since PER proteins are required for the recruitment of HP1 γ -SUV39H to the *Per1* promoter, loss of PER proteins should result in a decrease of H3K9-Me₂, H3K9-Me₃, or both at the site. We therefore performed ChIP to compare the levels of H3K9-Me₂ and H3K9-Me₃ at the *Per1* promoter during the negative feedback phase in livers of wildtype mice and littermate *Per1*^{-/-}; *Per2*^{-/-} double mutants. Both H3K9-Me₂ and H3K9-Me₃ were substantially reduced in the *Per1*^{-/-}; *Per2*^{-/-} mutants at the *Per1* promoter, but there was no significant difference between genotypes for either modification at a non-circadian control promoter (Fig. 5b). Depletion of SUV39H1 from mouse fibroblasts caused a similar reduction of both H3K9-Me₂ and H3K9-Me₃ at the *Per1* promoter (Fig. 5c). This depletion had no detectable effect on H3K9-Ac levels at *Per1* E-box sites (Supplementary Figure 6), indicating that regulation of H3K9 acetylation at these sites is not coupled to di- or tri-methylation.

If PER-associated HP1 γ -SUV39H acts specifically to catalyze the formation of H3K9-Me₂ and H3K9-Me₃ (but not H3K9-Me₁), then depletion of SUV39H1 should cause an increase of H3K9-Me₁³⁷ at the *Per1* promoter, representing accumulation of unconverted substrate. Alternatively, if it also catalyzes H3K9 mono-methylation, then SUV39H1 depletion should cause a decrease of H3K9-Me₁ at the *Per1* promoter. Liver samples from *Per1*^{-/-}; *Per2*^{-/-} double mutants showed a substantial reduction in H3K9-Me₁ at the *Per1* promoter (Fig. 5d), indicating that all three H3K9 methylation reactions depend on PER function. In contrast, depletion of SUV39H1 from mouse fibroblasts caused a dramatic increase in H3K9-Me₁ at the *Per1* promoter (Fig. 5e). Thus HP1 γ -SUV39H recruited by a PER complex preferentially di- and tri-methylates H3K9 at the *Per1* promoter, exhibiting little or no activity on an unmethylated substrate.

Temporally distinct recruitment of HDAC1 and SUV39H1 to *Per* promoters

Because many examples of transcriptional repression suggest that de-acetylation and methylation of H3K9 are tightly coupled^{28,30,39}, we initially expected that a chromatin-associated PER complex would include both HDAC1²⁶ and HP1 γ -SUV39H, which would act together in a concerted process, seemingly simultaneous on a circadian time-scale. However, we observed circadian recruitment of SUV39H1 to the *Per1* promoter a few hours

after HDAC1 (Fig. 6a). To examine this phenomenon more closely, we obtained livers from mice at 1-h intervals throughout the circadian negative feedback phase (CT12–22) and performed ChIP assays to monitor the two enzymes on the *Per1* and *Per2* promoters. For both genes, the circadian profile of SUV39H1 binding lagged behind that of HDAC1 binding by approximately 4 h, with little or no overlap between the two peaks (Fig. 6b, c). As expected, a decrease in H3K9-Ac followed the early recruitment of HDAC1, and an increase in H3K9-Me₃ followed the late recruitment of SUV39H1 (Fig. 6b, c). Unexpectedly, at both sites the peak binding of PER2 lagged behind that of PER1 by about 5 hours, with the PER2 profile resembling that of SUV39H1 and the PER1 profile resembling that of HDAC1 (Fig. 6b, c). Similar results were obtained at a third E-box site (Supplementary Figure 7).

The temporally distinct profiles suggested that the two H3K9-modifying enzymes might be components of different PER complexes. To test this prediction, we performed sequential immunoprecipitations from nuclear extracts of mouse livers obtained at CT18, a phase when HDAC1 binding at *Per* promoters has returned to baseline and SUV39H1 binding has reached its maximum. First, we immunoprecipitated PER complexes with an antibody against PER2, then eluted the complexes with a peptide corresponding to the PER2 epitope. The eluted material included core PER complex component CRY1, HP1 γ -SUV39H1, and HDAC1, but not an irrelevant nuclear protein (Fig. 6d, **left panels**). Thus a PER complex containing HDAC1 is present in the nucleus at CT18, even though it no longer detectably associates with chromatin at this time. Because both enzymes co-immunoprecipitated with PER2, both must be in PER complexes that include PER2, so the temporally-distinct ChIP profiles for PER1 and PER2 (Fig. 6b, c) presumably reflect PER complexes of differing PER protein stoichiometry or post-translational modification, not complexes mutually exclusive for PER1 or PER2, as might be initially imagined.

Next, we immunoprecipitated the eluted material with an antibody against HP1 γ and monitored the presence of the same proteins by immunoblot. We detected CRY1 and HP1 γ -SUV39H1, but no signal was detected for HDAC1 (Fig. 6d, **right panels**) even though PER complexes containing HDAC1 were present in the sample. Thus HP1 γ -SUV39H1 is a component of PER complexes that do not detectably include HDAC1, suggesting that physically distinct PER complexes deliver HDAC1 and HP1 γ -SUV39H1 to catalyze sequential modifications of H3K9 at distinct circadian phases.

DISCUSSION

The results described here reveal new aspects of PER complex action in the core circadian clock feedback loop. Circadian rhythms of histone modification have been observed at the promoters of core clock genes in multiple organisms^{25,31,40–42}, but their mechanistic basis and relationship to components of the core clock machinery was unclear. Recent work and the results described here indicate that in mammals at least three of these rhythmic epigenetic modifications, H3K9 deacetylation²⁶ and H3K9 di- and tri-methylation, result from direct negative feedback actions of the PER complex. Our analysis indicates that a PER complex, by virtue of association with HP1 γ , recruits an HP1 γ -SUV39H1/H2 histone methyltransferase to DNA-bound CLOCK-BMAL1 to di- and tri-methylate H3K9, thereby

establishing a local repressive chromatin state important for circadian clock negative feedback and clock function.

An unrelated histone methyltransferase subunit, WDR5, was previously identified as a PER-associated protein and shown to be important for H3K4 tri-methylation and H3K9 dimethylation at the *Rev-Erba* promoter, although it did not appear to be important for clock function¹³. That study did not assess chromatin modifications at *Per* genes, making a direct comparison with our findings difficult. Our data show that PER complexes act in the sequential modification of H3K9 at *Per1* and *Per2* genes of the core feedback loop. We do not yet know if the same PER complexes act broadly at circadian target genes or if there are PER complex variants selectively recruited to CLOCK-BMAL1 at different target genes.

The finding of HP1 γ rather than HP1 α or HP1 β in a PER-associated SUV39H histone methyltransferase is at first glance somewhat unexpected. It is the HP1 α and HP1 β isoforms, working in conjunction with H3K9 methylation, that are most commonly associated with transcriptional repression, albeit characteristically in the setting of heterochromatic gene silencing³³. These actions are mediated by the dual abilities of HP1 α and HP1 β to bind to methylated H3K9 and to recruit histone methyltransferases to methylate H3K9 on neighboring nucleosomes, thereby leading to additional HP1 α or HP1 β binding, H3K9 methylation, and propagation of a repressive chromatin state along the chromosome^{32,33}.

In contrast, HP1 γ is known in at least some contexts to function as a euchromatic transcriptional activator by virtue of enhancing transcriptional elongation and possibly pre-mRNA splicing and termination^{43–45}. However, HP1 γ is also known to function in association with SUV39H1 as a gene-specific euchromatic transcriptional repressor^{46,47}, acting in a manner that requires interaction with a transcription factor for specific gene targeting but not with methylated histones⁴⁷. The transcriptional repression in these cases is highly labile or readily reversible^{46,47}. Our data indicate a similar repressive role for PER-associated HP1 γ SUV39H in the clock feedback loop, a network that depends on reversal of feedback transcriptional repression each circadian cycle. These observations do not exclude a possible role for HP1 α accumulation at methylated H3K9 sites in circadian transcriptional repression, as observed at the *Dbp* locus³¹.

Consistent with previous studies of SUV39H1, we found that PER-associated SUV39H specifically catalyzed H3K9 di- and tri-methylation at the *Per1* promoter, with little or no activity on an unmethylated H3K9 substrate. The PER-dependence of all three methylation steps strongly suggests that a PER complex recruits an as-yet unidentified histone methyltransferase that catalyzes the addition of a single methyl group to unmethylated H3K9 after the de-acetylation action of PER-associated HDAC1 and prior to the action of PER-associated HP1 γ -SUV39H.

The unexpectedly large 4-hour time difference between PER complex recruitment of HDAC1 and SUV39H1 to *Per* promoters indicates that the assembly or stability of particular PER complexes or their interaction with DNA-bound CLOCK-BMAL1 is under precise temporal control on the time-scale of a few hours. This regulation appears to insure that repressive chromatin modifying enzymes acting successively on H3K9 are recruited in

the appropriate sequence. We do not know the mechanisms underlying this process. It seems plausible that clock-dependent rhythms of post-translational modifications of CLOCK-BMAL1, PER proteins, or components of PER complexes, if set at appropriate phases, could generate an interval timer governing the temporal orchestration of chromatin modifiers within the circadian clock feedback loop.

ONLINE METHODS

Methods and associated references are available in Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

HP1γ-SUV39H histone methyltransferase is a constituent of a PER complex in vivo. Nuclear extract (CT18) of mouse liver (input) and immunoprecipitates (IP) from the extract (antibodies at top) were probed with antibodies to the proteins indicated at right. The abundant nuclear protein U2AF65 (65-kD subunit of U2 small nuclear ribonucleoprotein particle auxiliary factor) served as negative control.

Figure 2.

SUV39H1 histone methyltransferase catalytic subunit is important for circadian feedback transcriptional repression and circadian clock function. **(a)** Western blot showing the effect of point-mutant control *Suv39h1* siRNA or matched *Suv39h1* siRNA on the steady-state abundance of endogenous SUV39H1 in cultured mouse fibroblasts. Non-specific upper band serves as loading control. **(b)** Depletion of SUV39H1 from mouse fibroblasts causes a specific increase in transcription of CLOCK-BMAL1 circadian target genes. Quantitative RT-PCR assays showing the steady-state abundance of the indicated pre-mRNAs in mouse fibroblasts after introduction of point-mutant control siRNA (black) or after depletion of SUV39H1 by *Suv39h1* siRNA (gray; data normalized to control condition). Shown are mean $\pm$ SEM of triplicate experiment; representative of 3 experiments. Increased transcription of CLOCK-BMAL1 circadian target genes as a class was highly significant compared to control genes as a class after depletion of SUV39H1 ($N = 3$, t -test, one-tailed). **(c)** Circadian oscillations of bioluminescence in synchronized reporter fibroblasts after delivery of point-mutant control siRNA (blue) or *Suv39h1* siRNA (red). Traces from three independent cultures are shown for each. **(d)** Circadian periods of fibroblasts in (c) (mean $\pm$ SEM; $N = 3$ for each condition; t -test, one-tailed).

Figure 3.

Coordinate circadian rhythm of PER2 and HP1 γ -SUV39H at the *Per1* promoter. **(a)** Circadian profiles of co-immunoprecipitation of the proteins indicated at right with control IgG (left) or HP1 γ IgG (right) from mouse liver nuclear extracts. U2AF65 served as negative control, IgG-HC (heavy chain) as positive control. Circadian times (CT) shown at bottom. **(b)** Circadian cycle of PER2, HP1 γ , and SUV39H1 at *Per1* proximal E-box site. ChIP assays from mouse livers sampled across a circadian cycle (bottom) performed with antibodies at the top left of each panel. ChIP values are normalized to the signal from a parallel IgG control ChIP (dashed line); data are displayed as mean $\pm$ SEM of triplicate experiment and are representative of three independent experiments. Diagrams showing the sites of primers for ChIP assays of *Per* gene E-box sites can be found in Supplementary Figure 5b.

Figure 4.

PER proteins and HP1 γ are required for recruitment of SUV39H to the *Per1* promoter. (a) Control ChIP assays, displayed as in Fig. 3b, monitoring the occupancies of HP1 γ (left) and SUV39H1 (right) at the promoter of non-circadian gene *Tpt1* in livers (CT18) of wild-type littermates (black) or *Per1*^{-/-}; *Per2*^{-/-} double mutant mice (white). No significant differences were detected. (b) ChIP assays as in (a) showing the occupancies of PER2 (left), HP1 γ (middle), and SUV39H1 (right) at the *Per1* proximal E-box site in wildtype and *Per1*^{-/-}; *Per2*^{-/-} mutant mice. (c) Western blot showing the effect of point-mutant control *HP1γ* siRNA or matched *HP1γ* siRNA on the abundance of endogenous HP1 γ in cultured unsynchronized mouse fibroblasts. Non-specific band serves as loading control. (d) ChIP assays, as in (b), showing the mean occupancies of PER2, HP1 γ , and SUV39H1 at the *Per1* proximal E-box site in unsynchronized mouse fibroblasts after introduction of point-mutant control *HP1γ* siRNA (black) or after depletion of HP1 γ by effective *HP1γ* siRNA (gray). (e) Western blot showing the effect of point-mutant control *Suv39h1* siRNA or matched *Suv39h1* siRNA on the abundance of endogenous SUV39H1 in cultured unsynchronized mouse fibroblasts. Non-specific band serves as loading control. (f) ChIP assays, as in (d), showing the mean occupancies of PER2, HP1 γ , and SUV39H1 at the *Per1* proximal E-box

site in unsynchronized mouse fibroblasts after introduction of point-mutant control *Suv39h1* siRNA (black) or after depletion of SUV39H1 by effective *Suv39h1* siRNA (gray). All panels show mean $\pm$ SEM of triplicate experiments and are representative of 3 independent experiments.

Figure 5.

PER proteins and SUV39H1 are important for di- and tri-methylation of H3K9 at the *Per1* promoter. **(a)** Circadian cycle of di-methylated (top) and tri-methylated (bottom) H3K9 at *Per1* proximal E-box site. ChIP assays are from mouse livers sampled across a circadian cycle (bottom). ChIP values are relative to the signal from parallel IgG control. **(b)** ChIP assays showing di-methylated (top) and tri-methylated (bottom) H3K9 at *Per1* proximal E-box site in livers (CT18) of wild-type littermates (black) or *Per1*^{-/-}; *Per2*^{-/-} double mutant mice (white). **(c)** ChIP assays showing di-methylated (top) and tri-methylated (bottom) H3K9 at *Per1* proximal E-box site in unsynchronized mouse fibroblasts after introduction of point-mutant control *Suv39h1* siRNA (black) or after depletion of SUV39H1 by effective *Suv39h1* siRNA (gray). **(d)** ChIP assays showing mono-methylated H3K9 at *Per1* proximal E-box site in livers (CT18) of wild-type littermates (black) or *Per1*^{-/-}; *Per2*^{-/-} mutants (white). **(e)** ChIP assays showing mono-methylated H3K9 at *Per1* proximal E-box site in unsynchronized mouse fibroblasts after introduction of point-mutant control *Suv39h1* siRNA (black) or after depletion of SUV39H1 by effective *Suv39h1* siRNA (gray; normalized to control condition). All data are displayed as mean $\pm$ SEM of triplicate experiments; panels

(a)–(c) are representative of three independent experiments and (d) and (e) are representative of two independent experiments.

Figure 6.

Distinct and ordered recruitment of HDAC1 and SUV39H1 to *Per* promoters. **(a)** Circadian cycle of occupancies of HDAC1 (top) and SUV39H1 (bottom) at *Per1* proximal E-box site. ChIP assays are from mouse livers sampled across a circadian cycle (bottom). Shown are mean $\pm$ SEM of triplicate experiments. **(b)** ChIP assays showing temporal profiles of HDAC1, SUV39H1, H3K9-Ac, H3K9-Me₃, PER1, and PER2 (as indicated at top left of each panel) at *Per1* E-box site during the phase of circadian feedback transcriptional repression at 1-h time resolution. Shown are mean $\pm$ SEM of triplicate experiments; representative of three independent ChIP assays from the 11 mice. **(c)** ChIP assay as in **(b)** but showing temporal profiles at *Per2* E-box site. Shown are mean $\pm$ SEM of triplicate experiments; representative of two independent ChIP assays from the 11 mice. **(d)** PER complexes containing HP1 γ -SUV39H1 do not detectably include HDAC1. Nuclear extracts (Input) of mouse livers (CT18) were first immunoprecipitated with control IgG or PER2 IgG and probed for the indicated proteins (left panels). PER complexes were then eluted from

PER2 IgG by competing epitope peptide, immunoprecipitated with control IgG or HP1 γ IgG, and probed for the same indicated proteins (right panels). U2AF65, negative control.