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Cell Reports
Report

A Positive Role for PERIOD in Mammalian Circadian Gene Expression

Makoto Akashi,[1,*] Akihiko Okamoto,[1] Yoshiki Tsuchiya,[2,5] Takeshi Todo,[3] Eisuke Nishida,[2] and Koichi Node[4]
[1]The Research Institute for Time Studies, Yamaguchi University, Yamaguchi 753-8511, Japan
[2]Department of Cell and Developmental Biology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan
[3]Department of Radiation Biology and Medical Genetics, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan
[4]Department of Cardiovascular Medicine, Saga University, Saga 849-8501, Japan
[5]Present address: Department of Physiology and Systems Bioscience, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan
*Correspondence: akashima@yamaguchi-u.ac.jp
http://dx.doi.org/10.1016/j.celrep.2014.03.072

SUMMARY

In the current model of the mammalian circadian clock, PERIOD (PER) represses the activity of the circadian transcription factors BMAL1 and CLOCK, either independently or together with CRYPTOCHROME (CRY). Here, we provide evidence that PER has an entirely different function from that reported previously, namely, that PER inhibits CRY-mediated transcriptional repression through interference with CRY recruitment into the BMAL1-CLOCK complex. This indirect positive function of PER is consistent with previous data from genetic analyses using *Per*-deficient or mutant mice. Overall, our results support the hypothesis that PER plays different roles in different circadian phases: an early phase in which it suppresses CRY activity, and a later phase in which it acts as a transcriptional repressor with CRY. This buffering effect of PER on CRY might help to prolong the period of rhythmic gene expression. Additional studies are required to carefully examine the promoter- and phase-specific roles of PER.

INTRODUCTION

Circadian rhythms are essential biological processes that are found in almost all organisms, ranging from bacteria to mammals (Menaker et al., 1997; Young and Kay, 2001). The rhythmic cell-autonomous expression of clock genes generates circadian rhythms (Rosbash, 1998; Schibler and Sassone-Corsi, 2002). In mammals, the most widely accepted theory for the rhythmic expression of clock genes is that the expression of *Per* genes is driven by the CLOCK (NPAS2)-BMAL1 transcription complex (Gekakis et al., 1998). Subsequently, PER proteins, independently or together with CRY, function to negatively regulate this complex (Ko and Takahashi, 2006; Reppert and Weaver, 2001).

In vivo phenotypic approaches have limitations for elucidating clock protein functions; therefore, it is essential to support them by in vitro functional analyses. However, this approach has not been completely successful in the case of PER protein, for several reasons. *Per* knockout mice exhibit severe circadian defects (Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 1999, 2001), but transcriptional activity assays have demonstrated that the repressive effect of PER on transcription is much lower than that of CRY, implying that in vitro functional analyses of PER have not provided evidence to support the in vivo phenotypes. In contrast to *Cry*-deficient mice, *Per* knockout mice show reduced levels of *Per* and *Cry* expression, which contradicts the general model in which PER proteins are transcriptional repressors of *Per* and *Cry* (Bae et al., 2001; Zheng et al., 2001; Zheng et al., 1999). These discrepancies may indicate that PER has unknown functions. In fact, several previous reports suggested that PER2 may have a positive effect on transcription in a promoter-specific manner (Chappuis et al., 2013; Hampp et al., 2008; Kaasik and Lee, 2004).

Here, we provide evidence that PER may inhibit CRY-mediated transcriptional repression via the physical interaction of PER with CRY, suggesting that PER functions as a buffer to decelerate the transcriptional repression process. Subsequently, via a posttranslational modification of PER, such as phosphorylation, the PER-CRY complex would gradually take on an active role as a transcriptional repressor.

RESULTS

PER2 Induces Transcriptional Upregulation in a CRY-Dependent Manner

We examined the transcriptional activity of *Per2* in the presence of PER2 or CRY1 expression vectors to compare the functional roles of PER and CRY in *Per2* transcription (Figure 1A). *Per2* is one of the clock genes and its transcription is activated by the CLOCK (NPAS2)-BMAL1 complex (Akashi et al., 2006; Travnickova-Bendova et al., 2002; Yoo et al., 2005). As expected, a luciferase reporter assay revealed that transfection of only a small amount of a CRY1 expression vector caused potent inhibition of the *Per2-luc* activity in a dose-dependent manner. However, the introduction of an increasing amount of PER2 resulted in a dose-dependent mild upregulation of *Per2* transcription. The upregulation levels were not high (approximately 2-fold elevation) and the upregulation became saturated with relatively low amounts of PER2. These

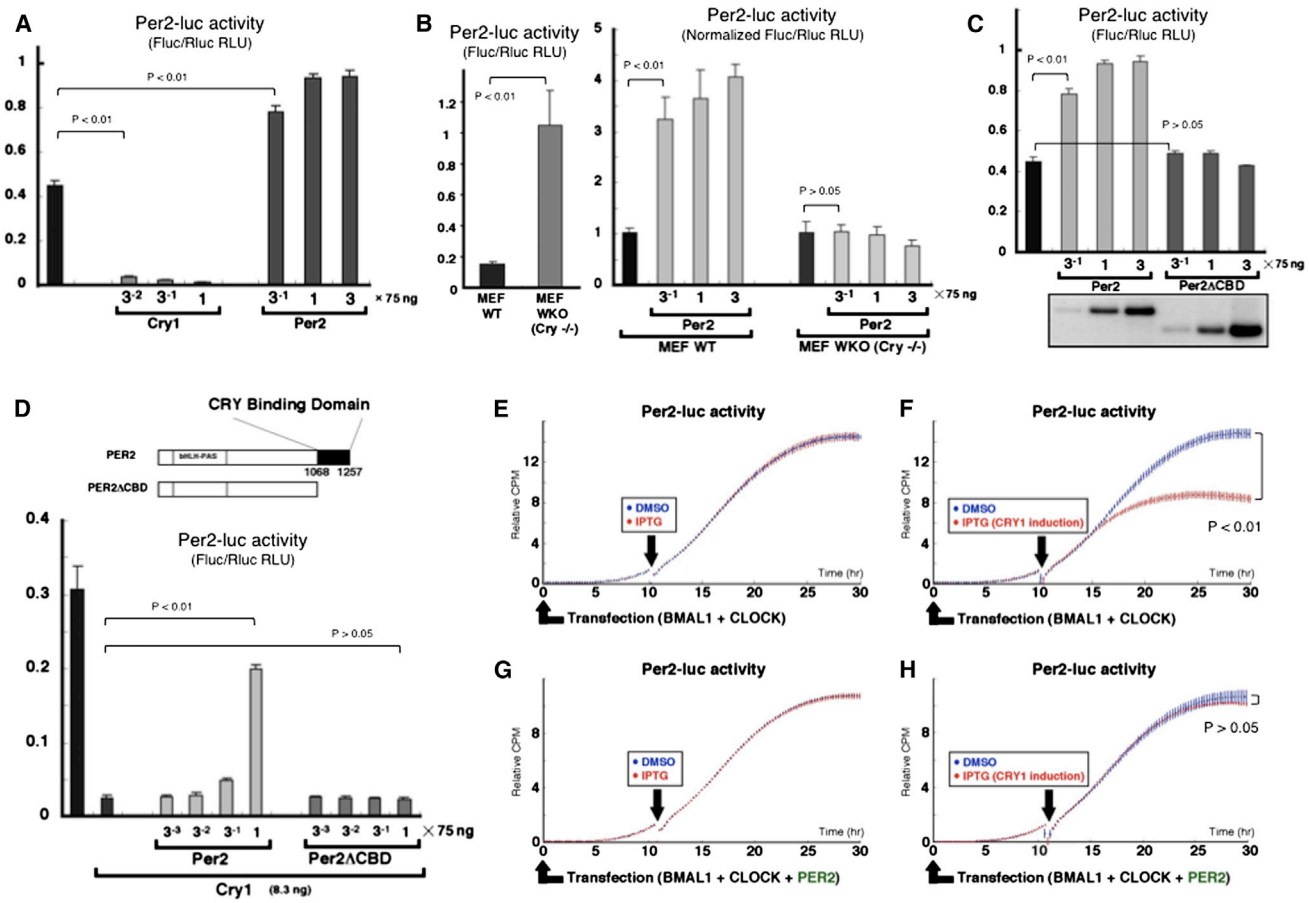

Figure 1. PER2 Counteracts CRY1 via a Physical Interaction

(A) Luciferase assay. *Per2-luc* represents the *Per2* promoter-driven firefly luciferase gene. Data (Fluc/Rluc relative light unit [RLU]) show relative firefly luciferase activity (Fluc RLU), which was normalized by Renilla luciferase activity (Rluc RLU). Data are represented as mean ± SE for triplicate samples. The numbers (under the abscissa axis) indicate the amount of DNA transfected into NIH 3T3 cells.

(B) Luciferase assay. The indicated plasmids were transfected into wild-type and *Cry*-deficient MEFs (*Cry1*/*Cry2* double knockout).

(C) Luciferase assay. Western blotting data show PER2 or PER2ΔCBD expression levels.

(D) Schematic representation of a deletion mutant of PER2 that lacked the CRY-binding domain, and luciferase assay data (NIH 3T3 cells).

(E–H) NIH 3T3 cells were transfected with the indicated expression vectors (time = 0). Immediately after transfection, BMAL1-CLOCK-activated *Per2* transcription was monitored in real time using a cell-culture-based luminescent monitoring system in the presence of luciferin. In (E)–(H), a Lac repressor-expressing vector was also introduced into cells, whereas in (F) and (H), an IPTG-inducible CRY1 expression vector was transfected simultaneously. Approximately 10 hr after transfection, 2 mM IPTG was added to the culture media to induce CRY1. To normalize for the transfection efficiency, light emission immediately before the addition of IPTG was set to one. Data are represented as mean ± SE for triplicate samples.

observations may indicate that the PER-mediated upregulation was due to an indirect effect, which was limited by an endogenous molecule(s).

How does the PER2 protein upregulate the *Per2* promoter? PER2-mediated weak activation may be associated with a physical interaction with endogenous CRY, a potent transcriptional inhibitor. PER2 interacts with CRY1 via its C-terminal domain (Akashi et al., 2002). Therefore, PER-CRY complex formation might neutralize CRY-mediated transcriptional repression. To test this hypothesis, we generated mouse embryonic fibroblasts (MEFs) carrying null mutations for the *Cry1* and *Cry2* genes (*Cry*−/−), and compared PER2-mediated upregulation in the presence and absence of endogenous CRY proteins. The basal transcriptional activity of *Per2* in the *Cry*−/− MEFs was higher than that in the wild-type MEFs, which suggests that endogenous CRY repressed *Per2* transcription (Figure 1B, left). Importantly, PER2 upregulated *Per2* transcription in the wild-type MEFs, but not in the *Cry*−/− MEFs (Figure 1B, right). These results strongly suggest that the interaction of PER2 with endogenous CRY is essential for PER2-mediated upregulation. These data also support the hypothesis that PER2-mediated mild upregulation is an indirect effect that results from the neutralization of endogenous CRY. To further validate this hypothesis, we used an expression construct of PER2 that lacked the CRY-binding domain (PER2ΔCBD, lacking 1,068–1,257). PER2ΔCBD did not upregulate *Per2* transcription (Figure 1C). The deletion of the C-terminal region had little effect on the PER2 protein levels.

PER2 Inhibits CRY1-Mediated Transcriptional Repression

The ability of PER2 to inhibit CRY-mediated transcriptional repression was verified. Interestingly, as shown in Figure 1D, PER2 counteracted the CRY1-mediated potent inhibition of *Per2* transcription. The PER2-induced effect was detectable at doses higher than those of the CRY1 expression plasmid, and it increased in a dose-dependent manner. Importantly, PER2ΔCBD did not induce any significant changes in the CRY1-mediated potent inhibition of *Per2* transcription, indicating that the PER2-induced neutralizing effect is completely dependent on the physical association with CRY1.

We constructed an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible CRY1 expression vector to obtain clear and direct evidence for the buffering effect of PER on CRY using a real-time *Per2* transcription monitoring system (Figures 1E–1H). This expression system facilitated the generation of a time lag between PER2 and CRY1 expression. In the absence of the IPTG-inducible CRY1 expression vector and independently of the presence of PER2, IPTG treatment did not affect the BMAL1-CLOCK-activated *Per2* transcription (Figures 1E and 1G). In contrast, in cells transfected with the IPTG-inducible CRY1 expression vector, IPTG-induced CRY1 gradually repressed BMAL1-CLOCK-activated *Per2* transcription (Figure 1F), whereas CRY1-mediated transcriptional repression was buffered significantly when PER2 was expressed prior to CRY1 induction (Figure 1H). These data demonstrate the buffering effect of PER on CRY function.

Specificities of PER-Mediated CRY Suppression

The buffering effect of PER2 on CRY1 was further examined in the presence of overexpressed BMAL1-CLOCK or BMAL1-NPAS2 complexes, as shown in Figure 2A. Under these conditions, CRY1-induced transcriptional repression was inhibited by the coexpression of PER2, which was completely dependent on the physical interaction between CRY1 and PER2 via the C-terminal region of PER2. These data show that PER2 had equal effects on the BMAL1-CLOCK and BMAL1-NPAS2 complexes.

The BMAL1-CLOCK complex activates transcription of not only *Per* but also *Cry*. Therefore, we performed the same analysis using the *Cry1* or *Cry2* promoter (Figure 2B). Although the effect was not strong compared with the *Per2* promoter, PER also restored the CRY-mediated inhibition of transcription from the *Cry1* and *Cry2* promoters. These results support the hypothesis that PER2 buffers CRY1-mediated transcriptional repression.

PER1 and PER2, but not PER3, Protect Transcriptional Activity from CRY

PER1 and PER2 have nonredundant roles in the mammalian circadian clock (Bae et al., 2001; Zheng et al., 2001). We performed similar experiments using PER1 to confirm the results obtained with PER2 and to compare the effect of PER2 on CRY1-mediated transcriptional repression with that of PER1. First, we narrowed the CRY-binding domain using a deletion series of PER1 constructs. The C-terminal domain of PER1 interacted with CRY, which was also the case with PER2 (Figure S1A). Next, we examined the effect of PER1 overexpression on the basal transcriptional activity of *Per2*, which showed that PER1 mildly upregulated *Per2* transcription, which was also the case with PER2 (Figure S1B). As expected, the association of CRY1 with the C-terminal domain of PER1 was indispensable for this upregulation effect. We also examined the neutralizing effect of PER1 on CRY1-mediated transcriptional repression (Figure 2C). PER1 expression neutralized CRY1-mediated repression at doses similar to those of the PER2 expression plasmid, which was completely dependent on CRY1 binding to the C-terminal domain of PER1. Furthermore, similar experiments with CRY2-mediated transcriptional repression showed that PER2 neutralized CRY2 more potently than CRY1 (Figure 2D). Higher amounts of PER2 than CRY1 were necessary for the protection against CRY1-mediated transcriptional repression, whereas the neutralization of CRY2-mediated repression was detectable even at lower amounts of PER2 than CRY2. However, the relative amount of PER1 required to effectively counteract CRY2 was similar to that of CRY1. Overall, these results show that PER2 counteracts CRY2-mediated repression more effectively than PER1, and both PER1 and PER2 exert anti-CRY1 functions in a similar dose-dependent manner. These results confirm that PER proteins have a protective effect against CRY-mediated transcriptional repression, and demonstrate that target specificity in the PER-mediated neutralization of CRY may provide a mechanism for the distinct and nonredundant roles of PER1 and PER2 proteins.

PER3 had no effects on the basal transcriptional activity of *Per2* or CRY1-mediated transcriptional repression (Figure 2E). According to gene knockout studies (Bae et al., 2001; Shearman et al., 2000), unlike PER1 and PER2, PER3 is considered to have a minor role in the core clock and is a component required for circadian outputs. However, all three PER molecules have similar transcriptional repressor activities (Kume et al., 1999); therefore, the mechanism that underlies the functional differences among these three PER molecules remains undefined. Our present results show that there is a distinct functional difference between PER1/PER2 and PER3. Therefore, because PER3 does not have the ability to counteract CRY-mediated transcriptional repression, the role of PER3 in the core clock is distinct from that of PER1/PER2.

The restoration of transcriptional activity may be caused by the PER-induced subcellular translocation of CRY rather than the PER-mediated direct silencing of CRY activity. It is well known that overexpressed PER2 determines the intracellular localization of CRY (Albrecht et al., 2007). To investigate the effect of PER2-mediated translocation on CRY1 activity, we constructed expression vectors for PER2-NES and PER2-NLS fusion proteins to perform signal sequence-driven translocation of PER2. With increasing amounts of these expression vectors, the subcellular localization of CRY1 changed via its physical interaction with PER2 (Table S1). Nevertheless, all three PER2 molecules inhibited CRY1 activity to a similar extent (Figure 2F), suggesting that the PER2-mediated inhibition of CRY1 is independent of the subcellular localization of CRY. Thus, the PER2-CRY1 complex is functionally silenced in the cytoplasm and nucleus.

Our data might be dependent on mass action caused by overexpression. We therefore investigated the stoichiometry

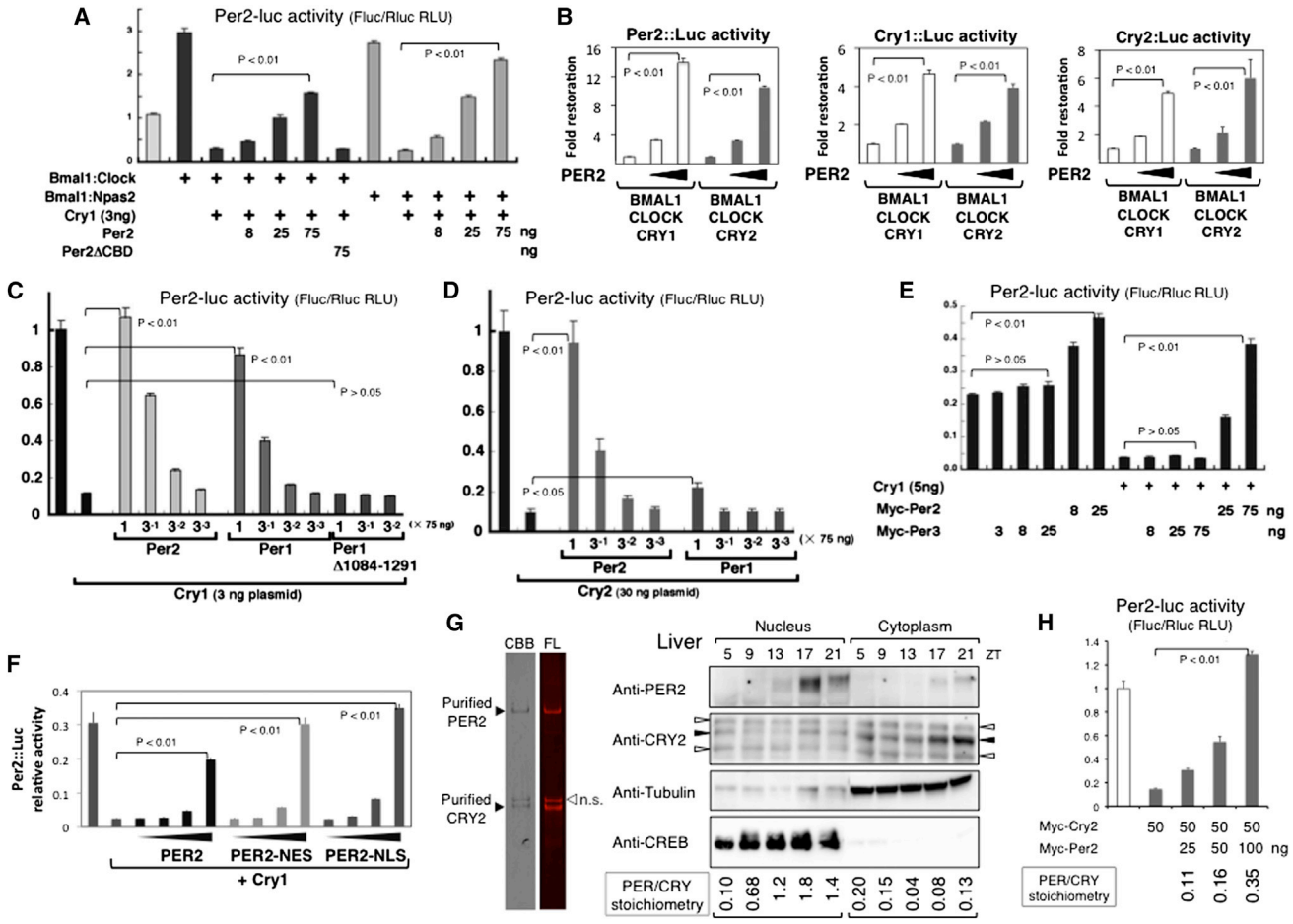

Figure 2. Target Specificity in PER-Mediated Neutralization of CRY

(A and B) Luciferase assay (NIH 3T3 cells).

(B) PER2-mediated fold restoration of *Per2*, *Cry1,* or *Cry2* transcription, the activities of which were repressed by CRY1 or CRY2, respectively.

(C–F) Luciferase assay (NIH 3T3 cells).

(G) Left: purified PER2 and CRY2 proteins were stained with Coomassie brilliant blue (CBB) and Lumitein (a red fluorescent dye; Biotium), and used to obtain a standard curve. Top right: livers were collected every 4 hr from Zeitgeber time 5 (ZT5) to ZT21, and nuclear and cytoplasmic fractions were subjected to western blot analysis. The black and white arrowheads indicate CRY2 and nonspecific signals, respectively. CREB and tubulin were used as nuclear and cytoplasmic markers, respectively. Bottom right: to examine the stoichiometry between the PER2 and CRY2 proteins, we used purified PER2 and CRY2 proteins to obtain a standard curve. Western blot signals were calibrated using the standard, and the ratio between PER2 and CRY2 signals in the nucleus and cytoplasm of the liver was semiquantitatively calculated. The reproducibility was confirmed by repeating independent experiments, and one set of representative data is presented.

(H) Luciferase assay. Bottom: whole-cell extracts were subjected to western blot analysis with anti-PER2 and -CRY2 antibodies. As mentioned above, the ratio between PER2 and CRY2 signals in luciferase assay samples was semiquantitatively calculated.

See also Figure S1 and Table S1.

between PER and CRY where PER antagonized CRY. The nuclear entry of endogenous CRY almost completely depends on PER (Lee et al., 2001), indicating that nearly all nuclear CRY forms a complex with PER. Thus, the ratio between nuclear PER and CRY molecules should be near one. To examine whether this prediction holds true for not only in vivo but also overexpression-based assays, we focused on measuring the PER2 and CRY2 ratio. The PER2-CRY2 complex seems physiologically more significant than other PER-CRY complexes, as the buffering effect of PER2 on CRY2 is much higher than that of other PER-CRY combinations. First, we semiquantitatively compared the amount of endogenous nuclear PER2 with that of CRY2 in the liver using purified PER2 and CRY2 as a standard (Figure 2G). Our findings show that the nuclear concentration of both proteins is within a similar range, as predicted. Next, we examined whether this stoichiometry holds true when PER2 inhibits CRY2 in overexpression-based reporter assays (Figure 2H). The data indicate that similar amounts of PER and CRY are needed to strongly cancel out the CRY-mediated inhibitory activity. The in vivo stoichiometry between PER2 and CRY2 is therefore consistent with that obtained in overexpression-based assays.

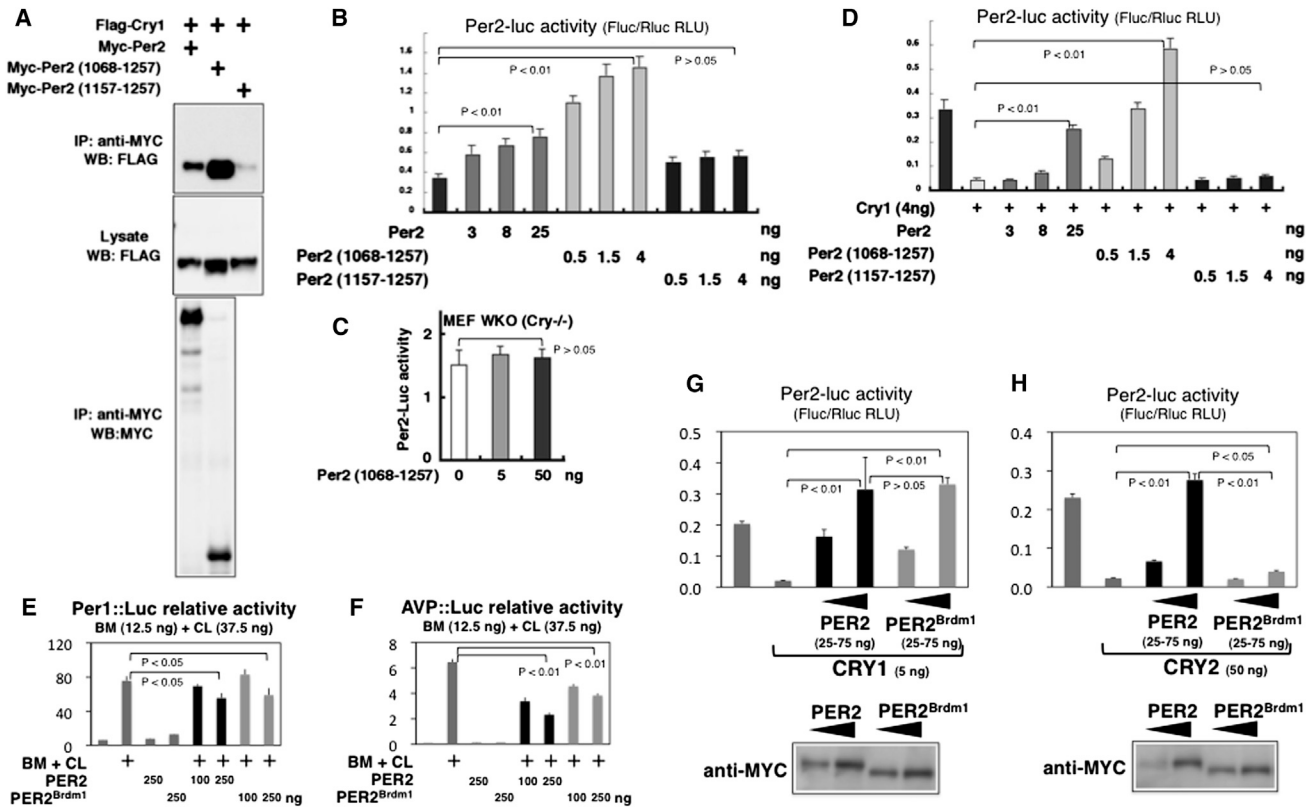

Figure 3. The C-Terminal Fragment of PER2 Potently Counteracts CRY-Mediated Transcriptional Repression, and Attenuated Anti-CRY2 Activity May Be Responsible for the Loss of Function in the PER2^{brdm1} Protein

(A) COS7 cells were transfected with the indicated expression vectors, and whole-cell extracts were subjected to immunoprecipitation (IP; anti-MYC antibody). Western blotting was performed with the indicated antibodies. Because the molecular weight of MYC-PER2 (1,157–1,257) is very low (~10 kDa), detecting this fragment via western blotting is technically difficult.

(B–D) Luciferase assay (B and D: NIH 3T3 cells; C: *Cry*-deficient MEFs).

(E and F) NIH 3T3 cells were transfected, and *Per1* or *AVP* transcription was evaluated using a luciferase assay.

(G and H) Luciferase assay (NIH 3T3 cells). The western blotting data show PER2 and PER2^{brdm1} expression levels.

See also Figure S2.

The C-Terminal Region of PER2 Contains a Domain that Facilitates the Potent Protection of Transcription from CRY

The C-terminal region of PER, including the CRY-binding domain, must be essential for the PER-mediated protection of transcription. Therefore, we performed further experiments to investigate whether the C-terminal region alone is sufficient to protect transcription. An expression vector was constructed for the C-terminal region of PER2 (PER2 [1,068–1,257]). First, the ability of this region to bind to CRY1 was examined in an immunoprecipitation study (Figure 3A). Because the expression level of PER2 (1,068–1,257) was similar to that of full-length PER2, this C-terminal fragment physically interacted with CRY1 more strongly than full-length PER2. This result suggests the hypothesis that the C-terminal region, including the CRY-binding domain, is masked in the full-length protein and the accessibility to CRY1 is therefore limited.

Interestingly, PER2 (1,068–1,257) not only promoted *Per2* transcription much more strongly than the full-length protein (Figure 3B) but also resulted in a very potent inhibition of CRY-mediated transcriptional repression (Figure 3D). These effects may have been dependent on the strong affinity between CRY and the unmasked C-terminal region of PER, as shown in Figure 3A. Similar to what was observed for the full-length PER2, the PER2 (1,068–1,257)-mediated activation of transcription was dependent on endogenous CRYs, because the activation was not detected in MEFs (*Cry*−/−; Figure 3C), thus indicating that the transcriptional activation was caused mainly by the PER2 (1,068–1,257)-mediated potent neutralization of endogenous CRY function. On the other hand, PER2 (1,157–1,257) with an incomplete CRY-binding domain had no obvious effect on *Per2* transcription.

The PER2^{brdm1} Protein Causes Dysfunctional Anti-CRY2 Activity

Although *Per2^{brdm1}* is considered a loss-of-function allele, it remains unclear why the deletion of 87 amino acids causes functional defects. First, we examined the differences in transcriptional repression activity between the wild-type PER2 and PER2^{brdm1} protein (Figures 3E and 3F). Because transcriptional

repression activity was not detectable with the *Per2* promoter, as shown in Figure 1A, we used *Per1* and the *Avp* (arginine vasopressin) promoter in the evaluation. The results showed that although both proteins had a mild transcriptional repression activity, an obvious difference between these two proteins was not detectable.

Furthermore, we performed a comparison between the wild-type PER2 and PER2^{brdm1} proteins on the basis of their physical affinities to BMAL1, CLOCK, CRY1, and CRY2 (Figure S2). As shown in previous studies, PER2 is able to bind to all of them (Langmesser et al., 2008). As expected, both PER2 proteins had similar affinities to CRY1 and CRY2, because the C-terminal region of the mutant was intact. The deleted region contained part of the PAS-B domain, but PER2^{brdm1} did not exhibit any defects in its ability to bind to BMAL1 and CLOCK (Figure S2A). The affinities were semiquantitatively confirmed, and the results show that the affinity of PER2 to BMAL1-CLOCK is much lower than that to CRY (Figure S2B). Importantly, a previous report demonstrated that endogenous BMAL1-CLOCK does not bind to posttranslationally unmodified PER (Lee et al., 2001), which is consistent with our present findings because most PER exists in a posttranslationally unmodified form when overexpressed in cultured cells. Thus, the binding of PER to BMAL1-CLOCK observed in Figure S2A might be physiologically insignificant, suggesting that the PER-CRY complex does not bind to the BMAL1-CLOCK complex when posttranslationally unmodified PER inhibits CRY.

Next, we examined the difference between the wild-type PER2 and PER2^{brdm1} on the basis of their abilities to suppress CRY-induced transcriptional repression (Figures 3G and 3H). Interestingly, both of the PER proteins had a similar neutralizing effect on CRY1, whereas PER2^{brdm1} had a severely reduced effect on CRY2. The data obtained in the present study suggest that the reduced anti-CRY2 activity of PER2^{brdm1} provides a mechanistic explanation for the loss of function with the *Per2^{brdm1}* allele.

Consistent with numerous previous reports, PER2 exerted a low inhibitory activity on the *Per1* promoter (Figure 3E) and moderately repressed the transcriptional activity of the *AVP* promoter (Figure 3F), although we needed to use a much higher amount of a PER2 expression vector (~100 ng) than when PER2 activated *Per2* transcription (~10 ng), as shown in our findings. PER2 could not inhibit the *Per2* promoter at all, and instead activated it via inhibition of CRY, indicating that PER2 may play different roles in a promoter-specific manner. For further validation of the promoter-specific effect of PER2, we investigated the change in expression levels of BMAL1-CLOCK-controlled genes (output genes) in *Per2*-deficient mice (Table S2). We used two data sets: one from a comprehensive analysis of gene-expression levels in *Per2*-deficient mice (Grimaldi et al., 2010), and one including genes whose expression is under the direct control of BMAL1-CLOCK (Koike et al., 2012). We extracted genes common to both data sets, and the results demonstrate that expression is upregulated in some genes but downregulated in others, supporting the idea that PER2 may play different roles in a promoter-specific manner.

PER Blocks CRY Recruitment into the BMAL1-CLOCK Complex

Next, we investigated the formation of the clock protein complex on an E-box consensus sequence, using a purified protein assay system to elucidate the mechanism by which PER protects transcription from CRY. To exclude the possibility that the BMAL1 or CLOCK monomer is structurally unstable, BMAL1 and CLOCK were coexpressed in HEK293A cells and purified as a dimer (Figure 4A). CLOCK expression levels were much lower than BMAL1 expression levels, and a considerable proportion of BMAL1 was immunoprecipitated as a monomer when an antibody against BMAL1 was used. To reduce monomer contamination, we purified BMAL1-saturated CLOCK using an antibody against CLOCK. The purified BMAL1-CLOCK complex was incubated with a probe containing an E-box sequence, which was applied to nondenaturing polyacrylamide gels (Figure 4B). We confirmed that the electrophoretic mobility of the probe was shifted by the physical interaction with BMAL1-CLOCK. Antibodies against each protein were used to validate that the BMAL1-CLOCK-bound probe was supershifted. As expected, CRY did not bind directly to the probe independently of or together with PER. Moreover, we confirmed CRY recruitment into the BMAL1-CLOCK-bound E-box in an electrophoretic mobility shift assay (EMSA) (Figure 4C). Incubation of the BMAL1-CLOCK-bound probe with purified CRY1 caused an additional slight electrophoretic mobility shift of the probe. We found that incubation with an antibody against purified CRY1 was highly effective at enhancing the mobility shift. In the experiments described below, we used the same assay procedure to evaluate the effect of PER on CRY recruitment into the BMAL1-CLOCK complex.

To investigate whether PER inhibited CRY1 recruitment, we purified PER proteins using a method similar to that described for BMAL1-CLOCK purification (Figure 4D). Because PER may be structurally unstable as a monomer, especially in the PER2 deletion mutant proteins, we purified PER as a complex with CRY by immunoprecipitation using an antibody against CRY. PER2ΔCBD severely lacked any affinity to CRY1, and the amount of the purified protein was therefore greatly reduced when it was coimmunoprecipitated with CRY1. In an EMSA experiment, we found that PER2 interfered with CRY recruitment into the BMAL1-CLOCK complex, as expected (Figure 4E). When a higher amount of CRY1-PER2 was added, CRY1 was recruited into the BMAL1-CLOCK complex, suggesting two possibilities: (1) a fraction of CRY1 was purified as the PER2-unbound monomer, which caused the mobility shift of the probe as the concentration increased, or (2) the BMAL1-CLOCK complex associated kinetically with CRY1 in competition with PER2, and CRY1-BMAL1-CLOCK increased with the CRY1-PER2 concentration. Interestingly, PER3 did not interfere with CRY1 recruitment, and this was also explained by PER3's dispensability as a component of the clock machinery on the basis of in vivo phenotypic analyses.

Next, we performed the same EMSA experiment using PER2 mutant proteins. PER2ΔCBD did not affect the formation of the CRY1-BMAL1-CLOCK complex on the probe. As expected, PER2 (1,068–1,257), the CRY-binding domain, inhibited CRY1 recruitment. Unlike the full-length PER2 protein, CRY1 recruitment was blocked almost completely, even in the presence of a higher amount of CRY1-PER2 (1,068–1,257). As shown in

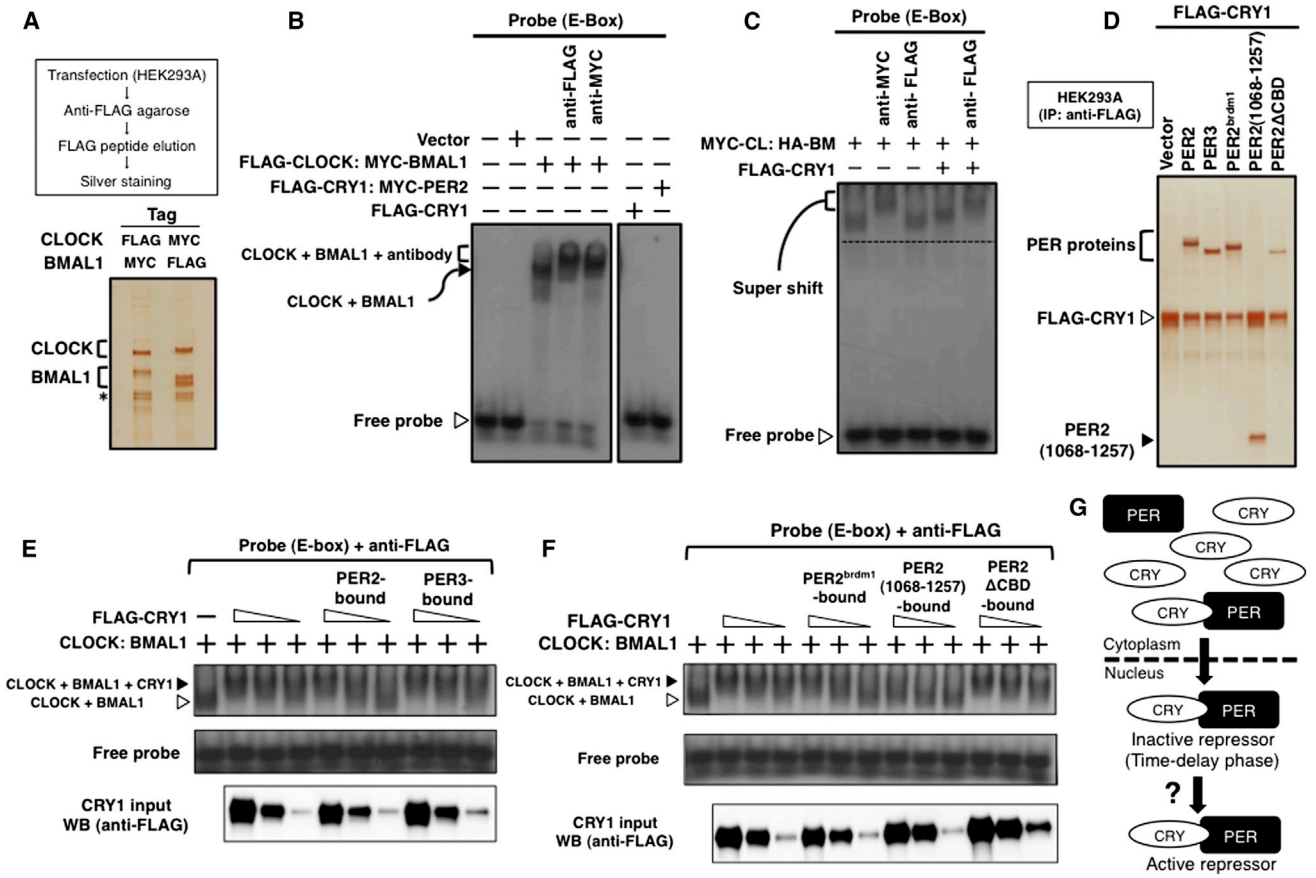

Figure 4. PER Interferes with CRY Recruitment into the BMAL1-CLOCK Complex

(A) Silver staining of SDS-PAGE gels. HEK293A cells were cotransfected and the BMAL1-CLOCK complex was purified with anti-FLAG antibody-conjugated agarose beads. The brackets show the purified CLOCK and BMAL1 proteins. The asterisk shows a nonspecific contaminant.

(B) EMSA. A ^{32}P-labeled double-stranded oligonucleotide that contained an E-box consensus sequence was used as a probe. The BMAL1-CLOCK-bound probe is indicated with an arrowed line. The brackets show that the BMAL1-CLOCK-bound probe was supershifted by the addition of antibodies. The free probe is indicated with an open arrowhead.

(C) EMSA. The same probe was incubated with BMAL1-CLOCK in the presence or absence of FLAG-CRY1. The dashed line indicates the leading edge of the BMAL1-CLOCK-bound probe.

(D) Silver staining of SDS-PAGE gels. HEK293A cells were cotransfected with the indicated plasmids, and PER-bound FLAG-CRY1 was purified with anti-FLAG antibody-conjugated agarose beads.

(E and F) EMSA. The same probe was incubated with BMAL1-CLOCK, and FLAG-CRY1 or PER-bound FLAG-CRY1 was added. All samples were incubated with an anti-FLAG antibody to detect CRY1 recruitment into the BMAL1-CLOCK-bound probe. The open arrowhead shows the BMAL1-CLOCK-bound probe. The filled arrowhead shows the CRY1-BMAL1-CLOCK-bound probe. The amount of FLAG-CRY1 used was confirmed by western blotting (bottom column).

(G) A schematic model of the PER-mediated time delay during circadian transcriptional repression. Previous in vivo reports suggested that the amount of CRY is much higher than that of PER, while PER is essential for the nuclear entry of CRY (Lee et al., 2001), which indicates that nuclear CRY is saturated with PER. PER-bound CRY does not produce immediate potent transcriptional repression because of the PER-induced silencing effect. The PER-mediated neutralization of CRY causes a time delay in CRY-mediated transcriptional repression, which may help to extend the transcriptional oscillation period.

See also Table S2.

Figure 3A, PER2 (1,068–1,257) bound to CRY1 more strongly than the full-length PER2 protein, which may be the mechanism that underlies the results obtained in the EMSA experiment, as well as the luciferase assay data. In agreement with the results of the luciferase assay shown in Figure 3, PER2^{brdm1} had almost the same effect on CRY1 recruitment as the full-length PER2. Because the anti-CRY2 activity of the mutant protein was remarkably decreased, as shown in the luciferase assay described above, we attempted to perform an EMSA experiment using purified CRY2. However, the purified protein appeared to be nonfunctional in vitro, and thus we were unable to perform the assay. Overall, the purified protein-based assays showed that the molecular mechanism that allows PER to protect transcription from CRY might be PER-induced interference with CRY recruitment into the BMAL1-CLOCK complex.

DISCUSSION

In vivo phenotypic approaches are necessary but insufficient for obtaining a thorough understanding of the functions of

clock components at the molecular level, and detailed in vitro analyses can therefore provide powerful support for in vivo phenotypic evidence. In the case of PER, however, although the in vivo phenotypic data obtained mainly from knockout mice have provided definitive proof that PER is an essential component of the clock machinery, in vitro functional analyses have not fully elucidated the indispensable roles of PER. Our present results support the hypothesis that PER might function as a buffer in CRY-mediated transcriptional repression, and Figure 4G shows the significance of our findings in the circadian gene-expression model. According to previous reports, the nuclear entry of CRY is almost completely dependent on PER, indicating that all of the endogenous nuclear CRY proteins form a complex with PER (Lee et al., 2001). If the nuclear PER-CRY complex were to act immediately as a potent negative regulator against the BMAL1-CLOCK complex, it would be difficult to generate a circadian period in the transcriptional negative feedback loop. The present study shows that the PER-CRY negative regulatory complex might be temporally inactivated in the nuclei, thereby leading to a time delay in the feedback machinery. Consistent with this interpretation, numerous observations have indicated that levels of *Per2* mRNA remain high even during the phase in which the PER-CRY complex highly accumulates in the nucleus, indicating that the complex is not fully active as a transcriptional repressor. Subsequently, the PER-CRY complex would become active as a transcriptional repressor via an unknown factor(s) or simply by a gradual increase in the PER-CRY concentration in nuclei. This buffering effect of PER may play a role in generating the stable and long-duration transcriptional feedback.

Interestingly, the function of PER identified in the present study appears to be highly consistent with previous in vivo phenotypic data obtained using mainly knockout mice. Indeed, the phenotypes observed in $Per2^{brdm1}$ mutant mice and *Per*-deficient mice are supported by the in vitro functional data obtained in the present study. The $PER2^{brdm1}$ protein lacks 87 amino acids that overlap with part of the PAS-B domain. Phenotypic analyses have suggested that $PER2^{brdm1}$ is a loss-of-function protein, but in vitro functional analyses have not provided evidence for the mechanism by which the deletion causes functional defects. We confirmed that there was little difference between the wild-type PER2 and $PER2^{brdm1}$ proteins in terms of their transcriptional repression activities. In contrast, we found that the $PER2^{brdm1}$ protein severely lacked a buffering effect on CRY2 activity, which might be the mechanism that underlies the loss of function observed with the $PER2^{brdm1}$ protein. The PER1 protein has a redundant role with PER2 in anti-CRY1 activity, but not in anti-CRY2 activity, as shown in Figure 2D. However, further studies are required to elucidate why the $Per2^{brdm1}$ mutation causes a defect only in anti-CRY2 activity and not in anti-CRY1 activity, and how the mutant protein causes the functional defect despite the existence of an intact CRY-binding domain. In addition to this mechanistic issue, another possible explanation for the $Per2^{brdm1}$ phenotype is that the $PER2^{brdm1}$ protein is simply less stable or its translation efficiency is lower than that of wild-type PER2 in vivo. In the overexpression experiments shown here, it is difficult to investigate these possibilities, and thus our data cannot lead to a definitive conclusion about why this mutation results in loss of function.

Importantly, the relationships between the types of PER molecules and the patterns of protection against CRY are highly consistent with previous studies of *Per* knockout mice. First, PER3 had no protective effect on transcription, which is consistent with the fact that the clock functions normally in *Per3* knockout mice (Bae et al., 2001; Shearman et al., 2000). Second, although PER1 and PER2 have a similar protective effect against CRY1-mediated transcriptional repression, PER2 provides more potent protection of transcription from CRY2 than PER1. Thus, PER2 could overcome the loss of PER1-mediated anti-CRY activity. This indicates that PER2 has a more essential role in the core clock, which is also consistent with animal studies in which *Per2* knockout caused more severe circadian dysfunctions than *Per1* knockout (Bae et al., 2001; Zheng et al., 2001). Furthermore, an interesting report showed that *Cry2* disruption restored circadian rhythmicity in *Per2* mutant mice (Oster et al., 2002). The present study results may explain the molecular mechanism in the following manner: PER2 strongly buffers CRY2-mediated transcriptional repression, and circadian gene expression is disrupted by an excess of free CRY2 in *Per2* mutant mice. *Cry2* inactivation in *Per2* mutant mice may reinstate the quantitative balance between PER and CRY proteins, suggesting that this could be the restorative mechanism in *Per2*/*Cry2* double-knockout mice.

PER might have a bimodal function. First, previous reports have shown that, in addition to its low activity as a transcriptional repressor, PER has an indispensable role in the nuclear entry of CRY, which suggests that PER is involved in the negative limb in the circadian transcriptional feedback loop. Second, we obtained in vitro evidence that PER could protect transcription from CRY via the physical interaction of PER with CRY, suggesting that PER functions as a buffer that decelerates the transcriptional repression process. Thus, PER could be classified as a modulator rather than a component of the negative limb. Future studies will be required to reveal how PER exerts promoter- and phase-specific roles in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

In this study, NIH 3T3 fibroblasts were used in all of the cell-culture-based assays, except that COS7 or HEK293A were used in special cases that required relatively high levels of protein expression. NIH 3T3 cells are a well-used cell line and are known to contain a functional, cell-autonomous circadian clock system (Akashi and Nishida, 2000). Cells were grown in Dulbecco's modified Eagle's medium (supplemented with antibiotics and 10% fetal bovine serum) and cultured in 5% CO_2. Transfection of plasmid DNA into cells was performed with Lipofectamine PLUS (Invitrogen) as described previously (Akashi et al., 2002).

EMSA

The binding buffer contained 15 mM Tris-HCl (pH 7.5), 15 mM NaCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 7.5% glycerol, 0.3% NP-40, and 1 μg/ml BSA. The radiolabeled probe was added at 0.5 nM, and the purified clock proteins were mixed and incubated for 10 min. Finally, the antibodies were added to perform supershift experiments. All of the reactions were incubated at 25°C. The reactions were loaded onto 4% nondenaturing polyacrylamide gels and resolved at 150 V for 2 hr at 4°C. The gels were dried and exposed to an X-ray film.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.072.

ACKNOWLEDGMENTS

We thank Tomoko Ishikawa (Osaka University) for sharing reagents and Rie Ichiyama, Ritsuko Matsumura, Rie Tashiro, and Rumiko Imaizumi for expert technical assistance. The *Per2-luciferase* knockin mice were kindly provided by Joseph Takahashi. We also thank Michael Rosbash (HHMI, Brandeis University) for a critical reading of the manuscript and Hiroshi Matsuno (Yamaguchi University) for general support. We acknowledge the support of fellowships from the Nakajima Foundation, the Naito Foundation, the Takeda Science Foundation, and the Japan Society for the Promotion of Science.

Received: September 20, 2013
Revised: February 5, 2014
Accepted: March 28, 2014
Published: May 1, 2014

REFERENCES

Akashi, M., and Nishida, E. (2000). Involvement of the MAP kinase cascade in resetting of the mammalian circadian clock. Genes Dev. *14*, 645–649.

Akashi, M., Tsuchiya, Y., Yoshino, T., and Nishida, E. (2002). Control of intracellular dynamics of mammalian period proteins by casein kinase I epsilon (CKIepsilon) and CKIdelta in cultured cells. Mol. Cell. Biol. *22*, 1693–1703.

Akashi, M., Ichise, T., Mamine, T., and Takumi, T. (2006). Molecular mechanism of cell-autonomous circadian gene expression of Period2, a crucial regulator of the mammalian circadian clock. Mol. Biol. Cell *17*, 555–565.

Albrecht, U., Bordon, A., Schmutz, I., and Ripperger, J. (2007). The multiple facets of Per2. Cold Spring Harb. Symp. Quant. Biol. *72*, 95–104.

Bae, K., Jin, X., Maywood, E.S., Hastings, M.H., Reppert, S.M., and Weaver, D.R. (2001). Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron *30*, 525–536.

Cermakian, N., Monaco, L., Pando, M.P., Dierich, A., and Sassone-Corsi, P. (2001). Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene. EMBO J. *20*, 3967–3974.

Chappuis, S., Ripperger, J.A., Schnell, A., Rando, G., Jud, C., Wahli, W., and Albrecht, U. (2013). Role of the circadian clock gene Per2 in adaptation to cold temperature. Mol. Metab. *2*, 184–193.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564–1569.

Grimaldi, B., Bellet, M.M., Katada, S., Astarita, G., Hirayama, J., Amin, R.H., Granneman, J.G., Piomelli, D., Leff, T., and Sassone-Corsi, P. (2010). PER2 controls lipid metabolism by direct regulation of PPARγ. Cell Metab. *12*, 509–520.

Hampp, G., Ripperger, J.A., Houben, T., Schmutz, I., Blex, C., Perreau-Lenz, S., Brunk, I., Spanagel, R., Ahnert-Hilger, G., Meijer, J.H., and Albrecht, U. (2008). Regulation of monoamine oxidase A by circadian-clock components implies clock influence on mood. Curr. Biol. *18*, 678–683.

Kaasik, K., and Lee, C.C. (2004). Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. Nature *430*, 467–471.

Ko, C.H., and Takahashi, J.S. (2006). Molecular components of the mammalian circadian clock. Hum. Mol. Genet. *15* (Spec No 2), R271–R277.

Koike, N., Yoo, S.H., Huang, H.C., Kumar, V., Lee, C., Kim, T.K., and Takahashi, J.S. (2012). Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science *338*, 349–354.

Kume, K., Zylka, M.J., Sriram, S., Shearman, L.P., Weaver, D.R., Jin, X., Maywood, E.S., Hastings, M.H., and Reppert, S.M. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell *98*, 193–205.

Langmesser, S., Tallone, T., Bordon, A., Rusconi, S., and Albrecht, U. (2008). Interaction of circadian clock proteins PER2 and CRY with BMAL1 and CLOCK. BMC Mol. Biol. *9*, 41.

Lee, C., Etchegaray, J.P., Cagampang, F.R., Loudon, A.S., and Reppert, S.M. (2001). Posttranslational mechanisms regulate the mammalian circadian clock. Cell *107*, 855–867.

Menaker, M., Moreira, L.F., and Tosini, G. (1997). Evolution of circadian organization in vertebrates. Braz. J. Med. Biol. Res. *30*, 305–313.

Oster, H., Yasui, A., van der Horst, G.T., and Albrecht, U. (2002). Disruption of mCry2 restores circadian rhythmicity in mPer2 mutant mice. Genes Dev. *16*, 2633–2638.

Reppert, S.M., and Weaver, D.R. (2001). Molecular analysis of mammalian circadian rhythms. Annu. Rev. Physiol. *63*, 647–676.

Rosbash, M. (1998). Why the rat-1 fibroblast should replace the SCN as the in vitro model of choice. Cell *93*, 917–919.

Schibler, U., and Sassone-Corsi, P. (2002). A web of circadian pacemakers. Cell *111*, 919–922.

Shearman, L.P., Jin, X., Lee, C., Reppert, S.M., and Weaver, D.R. (2000). Targeted disruption of the mPer3 gene: subtle effects on circadian clock function. Mol. Cell. Biol. *20*, 6269–6275.

Travnickova-Bendova, Z., Cermakian, N., Reppert, S.M., and Sassone-Corsi, P. (2002). Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. Proc. Natl. Acad. Sci. USA *99*, 7728–7733.

Yoo, S.H., Ko, C.H., Lowrey, P.L., Buhr, E.D., Song, E.J., Chang, S., Yoo, O.J., Yamazaki, S., Lee, C., and Takahashi, J.S. (2005). A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. Proc. Natl. Acad. Sci. USA *102*, 2608–2613.

Young, M.W., and Kay, S.A. (2001). Time zones: a comparative genetics of circadian clocks. Nat. Rev. Genet. *2*, 702–715.

Zheng, B., Larkin, D.W., Albrecht, U., Sun, Z.S., Sage, M., Eichele, G., Lee, C.C., and Bradley, A. (1999). The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature *400*, 169–173.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z.S., Eichele, G., Bradley, A., and Lee, C.C. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell *105*, 683–694.