Coactivation of the CLOCK–BMAL1 complex by CBP mediates resetting of the circadian clock

Yool Lee[1], Jiwon Lee[1], Ilmin Kwon[1], Yoshihiro Nakajima[2], Yoshihiro Ohmiya[2], Gi Hoon Son[1], Kun Ho Lee[3,*] and Kyungjin Kim[1,*]

[1]Department of Biological Sciences and 21st Frontier Program in Neuroscience, Seoul National University, Seoul 151-742, Korea
[2]Cell Dynamics Research Group, Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Ikeda, Osaka 563-8577, Japan
[3]Department of Marine Life Science, Chosun University, Gwangju 501-759, Korea
*Authors for correspondence (kyungjin@snu.ac.kr; leekho@chosun.ac.kr)

Accepted 25 June 2010
Journal of Cell Science 123, 3547-3557

doi:10.1242/jcs.070300

Summary

The transcription factor CLOCK–BMAL1 is a core component of the molecular clock machinery that drives circadian gene expression and physiology in mammals. Recently, we reported that this heterodimeric transcription factor functions as a signaling molecule in response to the resetting stimuli via the Ca^{2+}-dependent protein kinase C pathway. Here, we demonstrate that the CREB-binding protein (CBP) plays a key role in rapid activation of the CLOCK–BMAL1 heterodimer that leads to phase resetting of the circadian clock. Under physiological conditions, a bimolecular fluorescence complementation (BiFC) assay revealed that CLOCK and BMAL1 dimerize in the cytoplasm and subsequently translocate into the nucleus in response to serum stimuli (mean time duration was 29.2 minutes and mean velocity 0.7 μm/minute). Concomitantly, BMAL1 rapidly recruited CBP on *Per1* promoter E-box, but not p300 (a functional analog of CBP), in the discrete nuclear foci. However, recruitment of CBP by cAMP/Ca^{2+} response element-binding (CREB) protein on CRE was not markedly increased upon delivery of the resetting stimuli. Furthermore, overexpression of CBP greatly potentiated the CLOCK–BMAL1-mediated *Per1* transcription, and this effect was completely abolished by site-directed mutation of E-box elements, but not by the mutation of CRE in the *Per1* promoter. Furthermore, molecular knockdown of CBP severely dampened circadian oscillation of clock gene expression triggered by the resetting stimuli. These findings suggest that CBP recruitment by BMAL1 mediates acute transactivation of CLOCK–BMAL1, thereby inducing immediate-early *Per1* transcription and phase resetting of the circadian clock.

Key words: Circadian clock, BMAL1, CBP, PKC, Phase resetting, Bimolecular fluorescence complementation assay, BiFC

Introduction

Within a variety of living species from bacteria to mammals, the circadian clock is an intrinsic timing system that allows organisms to anticipate and prepare for periodic environmental changes represented by day and night. To coordinate their daily physiology and behavior with environmental changes, the endogenous clock is entrained to the 24 hour cycle of the earth's rotation through daily phase resetting. In mammals, the circadian rhythm is maintained by the master clock (located in the suprachiasmatic nucleus, SCN, of the hypothalamus) that can be entrained by environmental cues called *zeitgebers*, the primary one of which is the day–night cycle (Reppert and Weaver, 2002). Recently, peripheral tissues and cells, even immortalized cell lines, have been found to possess their own self-sustaining oscillators that can be entrained by neural and humoral signals from the SCN (Schibler and Sassone-Corsi, 2002). Although the master and peripheral clocks are reset by distinct external cues, both use common molecular components and signaling pathways for time adjustment. One of the prominent molecular events that cause clock resetting is the immediate-early induction of the *Per1* gene not only in SCN but also in peripheral tissues (Akashi and Nishida, 2000; Balsalobre et al., 2000a; Shigeyoshi et al., 1997; Yagita et al., 2001). Similar molecular events also occur in various cultured cells when they are reset by external stimuli such as serum shock and glucocorticoid (Balsalobre et al., 1998; Balsalobre et al., 2000a).

Thus far, intensive efforts have been made to elucidate the signal transduction pathways that lead to phase resetting of the circadian clock. These pathways include several kinase pathways such as mitogen-activated protein kinases (MAPKs), cAMP-dependent kinase (PKA), and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (Akashi and Nishida, 2000; Balsalobre et al., 2000b; Ginty et al., 1993; Obrietan et al., 1998). These kinase-mediated signaling cascades are thought to ultimately converge onto activation of cAMP/Ca^{2+} response element-binding (CREB) protein (Obrietan et al., 1998; Obrietan et al., 1999; Yokota et al., 2001). However, the rapid activation of CREB alone cannot account for the specific ability of CREB to induce the immediate-early response of the *Per* genes because CREB activation by resetting signals can induce rapid activation of only a few genes, including *fos*, *Per1* and *Per2*, despite a wide variety of genes containing functional cAMP-response elements (CREs) in their upstream regulatory regions. Even among these immediate-early genes, there are some time delays in reaching peak transcriptional levels in the SCN after the light pulse; induction of *fos* mRNA peaks within 30 minutes, whereas *Per1* and *Per2* respond more slowly and reach peak values ~1 and 2 hours after the stimulus, respectively (Albrecht et al., 1997; Crosio et al., 2000; Sherman et al., 1997; Wilsbacher et al., 2002). In addition, accumulating evidence emphasizes a pivotal role of clock-specific transcription factors in circadian clock resetting and *Per1* transcription in response to external stimuli. In *Drosophila*, activation of MAPK and CaMKII increases CLOCK/

CYCLE-dependent transcription via direct phosphorylation of CLOCK (Weber et al., 2006). In mammals, our previous studies demonstrated that acute *Per1* induction by serum stimuli is mainly attributed to rapid activation of the CLOCK/BMAL1 complex via the Ca^{2+}-dependent protein kinase C (PKC) pathway (Jung et al., 2003; Shim et al., 2007).

CLOCK and BMAL1 are members of the basic helix–loop–helix (bHLH)/PAS family of transcription factors that form a heterodimeric complex and then activate clock gene expression by binding to E-box elements located in the upstream regulatory region of target genes such as *Per1* and *Per2*. Most transcription factors that belong to the bHLH/PAS family are implicated in the signal transduction pathways activated by diverse environmental changes (Kewley et al., 2004). This family of proteins can be categorized into two subgroups on the basis of their role in the signaling process. Class I proteins typically function as sensors of environmental signals, whereas Class II proteins dimerize with Class I members and translocate into the nucleus. Heterodimerization between these two groups is an essential step, not only for functional activation but also for specifying the transcriptional output (Gu et al., 2000). Phylogenetic analysis implies that CLOCK is an environmental sensor and its partner BMAL1 is a Class II member (Gu et al., 2000; Hogenesch et al., 1998). This phylogenetic interpretation is consistent with previous findings suggesting that CLOCK acts as a bona fide signaling molecule responding to the resetting stimuli via the Ca^{2+}-dependent PKC pathway, while BMAL1 shuttles between the cytoplasm and nucleus, thereby facilitating nuclear accumulation of its partner CLOCK (Kwon et al., 2006; Shim et al., 2007).

Nevertheless, nuclear abundance of CLOCK and BMAL1 does not merely indicate transcriptional activation of target genes. A growing body of evidence suggests that transactivation of the CLOCK–BMAL1 complex requires recruitment of transcriptional coactivators such as CREB binding protein (CBP) and its close relative, p300, which mediate histone acetylation and interaction with the basal transcription factor TFIIB (Kalkhoven, 2004; Yujnovsky et al., 2006). Primarily, CBP and p300 are characterized as coactivators of CREB and can bind specifically to the phosphorylated CREB at serine 133 (Chriva et al., 1993; Parker et al., 1996); the binding of CBP to CREB has been suggested to evoke immediate-early response of the *Per1* gene during photic resetting of the circadian clock (Obrietan et al., 1998; Travinckova-Bendova et al., 2002). Recent studies, however, have demonstrated that rhythmic histone acetylation is an important regulatory mechanism underlying the clock gene activation mediated by the CLOCK–BMAL1 complex, although the coactivator responsible for the clock gene activation remains controversial (Curtis et al., 2004; Etchegaray et al., 2003; Takahata et al., 2000; Yujnovsky et al., 2006).

In the present study, we reveal the stimuli-induced protein–protein interactions that are responsible for CLOCK–BMAL1-mediated phase resetting of the circadian clock in vivo. By using a newly established bimolecular fluorescence complementation (BiFC) technique (Shyu et al., 2006), we first visualized dynamic changes in heterodimerization and nuclear translocation of CLOCK and BMAL1 in living cells, and demonstrated the kinetics of these molecular events. Then, combination of real-time measurements of complemented fluorescence and bioluminescence further showed that CBP is a key player in the rapid activation of the CLOCK–BMAL1 complex rather than of CREB in response to the resetting stimuli, and that direct interaction between CBP and BMAL1 is a crucial signaling event that leads to circadian clock resetting.

Results

BiFC using Venus is a feasible method for assessing the CLOCK–BMAL1 interactions in living cells

To investigate dynamic changes in the molecular interactions between CLOCK and BMAL1 in living cells under physiological conditions, we employed a bimolecular fluorescence complementation (BiFC) assay using an improved yellow fluorescence protein, Venus, which enables visualization of direct protein interactions in living cells under physiologically relevant temperatures (Shyu et al., 2006). To optimize BiFC conditions between CLOCK and BMAL1, we constructed various plasmids encoding complementary fragments of Venus [Venus N-terminal (VN) and Venus C-terminal (VC)] fused with the N-terminal end of full-length BMAL1 and CLOCK (VC–BMAL1 and VN–CLOCK), respectively, and then coexpressed them in COS-7 cells (Fig. 1A). Microscopic observation revealed that coexpression of VC–BMAL1 and VN–CLOCK produced a strong BiFC signal and its predominant nuclear localization (Fig. 1C, upper) was consistent with our previous findings (Kwon et al., 2006). To test whether the binding activity of CLOCK and BMAL1 is crucial for the molecular complementation between VC–BMAL1 and VN–CLOCK, we generated several BMAL1 mutants (ΔbHLH, ΔPAS1, and ΔPAS2) lacking one of the domains responsible for dimerization with CLOCK and coexpressed them with wild-type CLOCK in COS-7 cells. As shown in Fig. 1B, immunoprecipitation assay demonstrated that the ΔPAS2 mutant had the most profound defects in the interaction with wild-type CLOCK (Fig. 1B). Consistent with this finding, VC–ΔPAS2 failed to generate BiFC signal with VN–CLOCK despite the fact that its expression level and subcellular localization were comparable to those of VC–BMAL1 (Fig. 1C, lower). Furthermore, the molecular complementation between the two fragmented fluorescent probes did not affect the transcription factor activity of the BMAL1/CLOCK complex or the inhibitory effects of Cryptochrome 1 (CRY1) on its transactivation (Fig. 1D). Thus, we concluded that the BiFC assay using Venus would be a feasible approach for investigating the dynamic changes of intracellular interaction between CLOCK and BMAL1 in vivo without any significant artifacts.

The PKC pathway mediates the serum-evoked heterodimerization of CLOCK and BMAL1

Previously, we demonstrated that CLOCK phosphorylation by Ca^{2+}-dependent PKC is essential for transactivation of the CLOCK–BMAL1 complex during the serum-evoked phase resetting of the clock (Shim et al., 2007). To examine the effects of the resetting stimuli on the dimerization of CLOCK and BMAL1 in vivo, we performed the BiFC assay between VN–CLOCK and VC–BMAL1 in living cells after serum shock. Within 1 hour of serum shock, fluorescence complementation between VN–CLOCK and VC–BMAL1 was drastically increased, predominantly in the nucleus, although tiny speckles were distributed in the cytoplasm in COS7 and NIH3T3 cells (Fig. 2A; supplementary material Fig. S1A). In addition, quantitative analysis demonstrated that the number of cells with nuclear fluorescent signals was augmented more than fourfold after the brief serum shock (Fig. 2B). These effects returned to control level by pretreatment with the PKC inhibitor GF109203X (GF), but not by pretreatment with cyclohexamide and other kinase inhibitors, including the PKA inhibitor KT5720

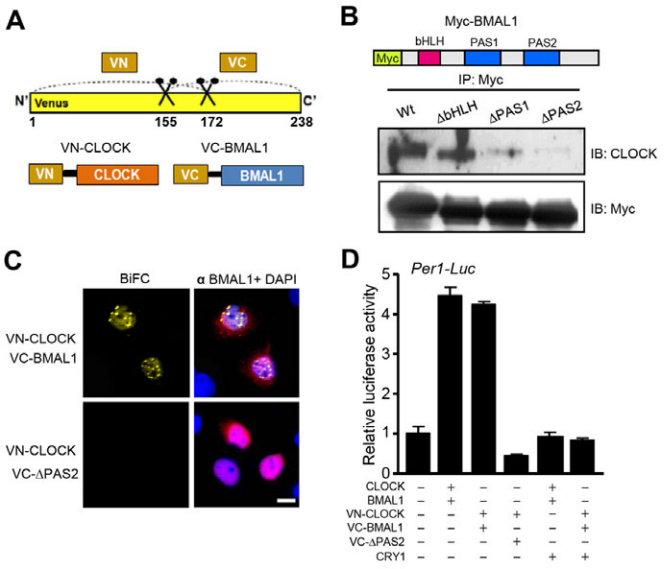

Fig. 1. Establishment of optimum conditions for BiFC analysis of CLOCK–BMAL1 dimerization. (**A**) Schematic representation of CLOCK–BMAL1 BiFC plasmid construction. The sequences encoding amino acid residues 1–172 or 155–238 of Venus (VN or VC, respectively) were fused to the 5′ ends of the coding regions for either CLOCK or BMAL1 to generate VC–BMAL1 and VN–CLOCK, respectively. (**B**) Schematic representation of Myc-tagged BMAL1 (Myc–BMAL1) and immunoprecipitation analysis for CLOCK–BMAL1 interaction with intact and internally deleted mutants; Myc-tagged wild-type BMAL1 (Wt), Myc–BMAL1 Δ71–140 (ΔbHLH), Myc–BMAL1 Δ210–320 (ΔPAS1), and Myc–BMAL1 Δ350–480 (ΔPAS2). Western blot analysis was carried out with anti-CLOCK antibody for immunoprecipitated Myc–BMAL1 and its mutated derivatives. (**C**) Examination of the CLOCK–BMAL1 complex-induced BiFC signal specificity using the VC–BMAL1 and VC–ΔPAS2; Venus C-terminal fragment (VC) was fused to the N-terminal end of the BMAL1 mutant with a deleted PAS2 domain to generate VC–ΔPAS2. At 12 hours post-transfection of the plasmids encoding VN–CLOCK, as well as either VC–BMAL1 or VC–ΔPAS2, into COS-7 cells, the cells were fixed and immunostained with anti-BMAL1 antibody (red) and DAPI (blue). The images were captured by fluorescence imaging microscopy using specific filter sets for YFP and RFP. Scale bar: 10 μm. (**D**) Examination of normal transcriptional activity of CLOCK–BMAL1 BiFC fusion proteins for *Per1* expression. COS-7 cells were transiently transfected with the *Per1* promoter-driven luciferase reporter construct and the indicated factors. At 24 hours post-transfection, *Per1* luciferase activities in cell extracts were analyzed and normalized by co-transfected pRL-TK activity in each sample. The data are shown as means ± s.e.m. of three independent experiments.

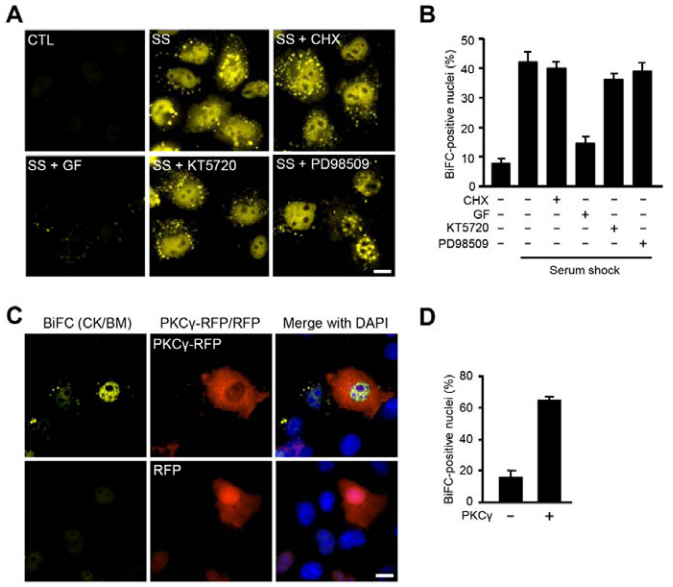

Fig. 2. The PKC pathway mediates serum-shock-induced dimerization and nuclear translocation of CLOCK and BMAL1. (**A**) Identification of the specific kinase signaling pathway responsible for the serum-stimulated interaction of CLOCK and BMAL1. At 12 hours post-transfection with VN–CLOCK and VC–BMAL1, cells were serum starved for 12 hours and treated with 5 μM cycloheximide, 5 μM GF109203X (GF), 0.5 μM KT5720 and 10 μM PD98509 2 hours before serum shock. Scale bar: 10 μm. (**B**) Quantitative analyses of serum shock or kinase inhibitor effects on CLOCK–BMAL1 interactions. Cells showing strong nuclear BiFC signals were counted among the whole cells after serum shock in the presence or absence of the indicated inhibitors. (**C**) Enhancing effect of PKCγ overexpression on CLOCK–BMAL1 dimerization and nuclear localization. PKCγ-tagged with red fluorescence protein (PKCγ–RFP) was coexpressed with CLOCK–BMAL1 (CK–BM) BiFC fusion proteins in COS-7 cells. The cells were visualized with a fluorescence imaging microscope using RFP- and YFP-selective filters. Scale bar: 10 μm. (**D**) Quantitative analysis of PKCγ-mediated increase in CLOCK–BMAL1 BiFC-positive cell nuclei. Cells showing strong BiFC signals in nuclei were counted in RFP or PKCγ–RFP-expressing cells and the percentage of BiFC-positive nulcei presented as means ± s.e.m.

and the MAPK inhibitor PD98509 (Fig. 2A,B; supplementary material Fig. S1A and Fig. S2). Consistently, treatment with phorbol 12-myristate 13-acetate (PMA), a PKC kinase activator, markedly induced CLOCK–BMAL1 BiFC complexes in the cells, as did serum shock, which was also inhibited by pretreatment with GF. However, addition of other activators, including forskolin (FSK), PKA activator, epidermal growth factor (EGF) and MAPK activator, did not elicit the stimuli-induced effect. (supplementary material Fig. S2). To further verify the role of PKC in heterodimerization of CLOCK and BMAL1, red fluorescence protein (RFP)-tagged PKCγ (PKCγ–RFP) was coexpressed with VC–BMAL1 and VN–CLOCK in COS-7 cells. As expected, coexpression of PKCγ–RFP markedly increased the BiFC signals of CLOCK and BMAL1 in COS-7 cells compared to those in cells expressing the BiFC-competent proteins alone (Fig. 2C). In addition, more BiFC-positive nuclei were found in PKCγ–RFP-transfected cells than in control RFP-expressing ones (Fig. 2D). These observations indicate that Ca^{2+}-dependent PKC stimulates the protein–protein interaction between CLOCK and BMAL1 and enhances the nuclear localization of these proteins.

Time-lapse BiFC imaging demonstrates rapid nuclear translocation of the CLOCK–BMAL1 complex in living cells

As shown in Fig. 2C, Ca^{2+}-dependent PKC was predominantly localized in the cytoplasm, but the BiFC signal of CLOCK–BMAL1 heterodimer was concentrated in the nucleus. These observations raise the possibility that the two clock proteins dimerize in the cytoplasm and then translocate into the nucleus. To explore the nuclear translocation of the CLOCK–BMAL1 complex in response to the resetting stimuli, we conducted time-lapse imaging experiments in COS-7 and NIH3T3 cells after serum shock. In these experiments, cells were expressed with VC–BMAL1 and VN–CLOCK, and the BiFC signal of the cells was then imaged under normal culture conditions. As shown in the upper panels of

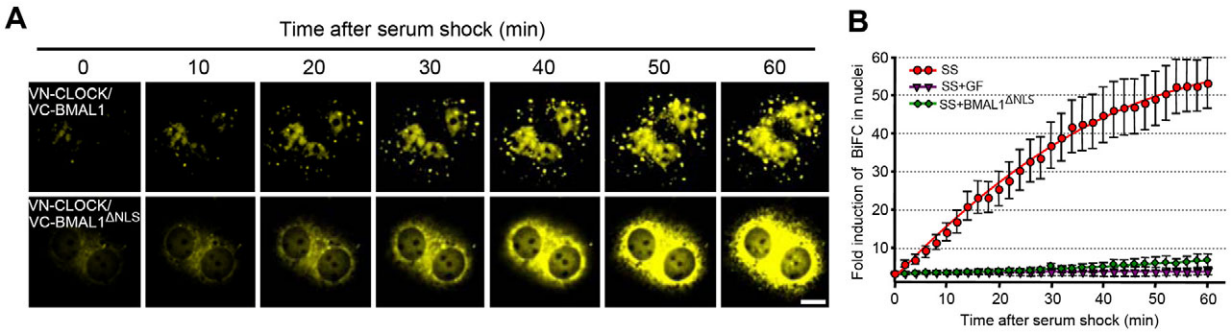

Fig. 3. Time-lapse imaging of serum-shock-stimulated interaction and nuclear localization of CLOCK and BMAL1 in living cells. (**A**) After either the combination of VN–CLOCK/VC–BMAL1 (upper panel) or of VN–CLOCK/VC–BMAL1 ΔNLS (lower panel) was expressed in COS-7 cells, time lapse fluorescence images were taken every 1 or 2 minutes for 1 hour after serum shock in living cells. Left to right, images illustrate 7 time points in every 10-minute interval out of the total acquired images for 1 hour. Scale bar: 10 μm. (**B**) Temporal changes of CLOCK–BMAL1 or CLOCK–BMAL1 ΔNLS BiFC signal intensities were measured in single cell nuclei of over 50 cells after either serum shock alone (SS, red; SS + BMAL1 ΔNLS, green) or together with pretreatment of 5 μM GF109203X (SS + GF, violet). The changes in BiFC signal intensities were represented by a statistical regression graph as a function of mean fluorescence intensity values.

Fig. 3A, the complemented fluorescence signal rapidly emerged in both the cytoplasm and nucleus after serum treatment. More specifically, the cytosolic fluorescence was abruptly speckled and appeared to move into the nucleus, whereas the nuclear signal was gradually enhanced throughout the nucleoplasm and was saturated within 1 hour of serum shock (Fig. 3B; supplementary material Fig. S1B and Movie 1). However, when the cells were transfected with the plasmid encoding a BMAL1 mutant that lacked a functional nuclear localization signal (VC–BMAL1 ΔNLS) (Kwon et al., 2006) instead of wild-type BMAL1, the fluorescence signal rapidly accumulated in the cytoplasm but failed to increase in the nucleus in response to the resetting stimuli (Fig. 3A lower panels; Fig. 3B). All these effects were abolished by pretreatment of the cells with a PKC inhibitor (Fig. 3B; supplementary material Fig. S1B,C). These data suggest that the rapid nuclear accumulation of the BiFC signal would be mainly attributed to its cytosolic dimerization in a PKC-dependent manner upon serum stimuli.

To further clarify the nuclear translocation of the cytoplasmic CLOCK–BMAL1 complex, we randomly selected 13 fluorescent speckles in the cytoplasm and characterized their migration by using image analysis software (supplementary material Fig. S3 and Movie 2). All the cytosolic speckles rapidly moved into the nucleus (mean time duration: 29.2 minutes; mean velocity: 0.7 μm/minute) with concomitant increase in nuclear fluorescence, although the particles did not move straight toward the nucleus and the parameters of their migration varied according to each speckle (Table 1; supplementary material Movie 2). Therefore, these findings clearly demonstrate that the resetting stimuli rapidly induce dimerization and nuclear translocation of the cytosolic CLOCK and BMAL1. Moreover, these signaling processes provide a good explanation for the kinetics of immediate-early *Per1* expression, which peaked within 90 minutes of delivery of the resetting stimuli (Shigeyoshi et al., 1997).

Rapid nuclear translocation of CLOCK–BMAL1 is essential for resetting of the circadian clock

Although individual cells intrinsically express circadian genes with ~24-hour period, the cells gradually lose coherence at the population level and, hence, the circadian amplitude is damped (Nagoshi et al., 2004; Welsh et al., 2004). Therefore, without the synchronization of individual cellular clocks by resetting stimuli, clock genes in cultured cells exhibit constant expression profiles. To assess the physiological relevance of the rapid nuclear translocation of CLOCK–BMAL1 to circadian clock synchronization, we conducted real-time bioluminescence assays in living cells by using a destabilized luciferase reporter gene (*dLuc*) controlled by the *Per2* promoter (*Per2 Pro-dLuc*), which drives robust circadian gene expression (Kwon et al., 2006).

Table 1. Temporospatial analysis of CLOCK–BMAL1 BiFC complex particle dynamics

Target signal	Displacement (μm)	Duration (min)	Length (μm)	Velocity (μm/min)
1	21.7	17	34.7	1.3
2	22.5	41	27.9	0.6
3	20.5	37	52.8	0.6
4	27.9	34	43.4	0.8
5	16.8	25	24.6	0.7
6	9.7	15	17.3	0.6
7	9.6	46	30.9	0.2
8	12.2	35	32	0.3
9	17.7	40	42.6	0.4
10	18.1	36	44.8	0.5
11	14.8	11	20.6	1.3
12	13.4	14	22.3	0.9
13	24.1	28	37.3	0.8
Mean	17.6	29.2	33.2	0.7

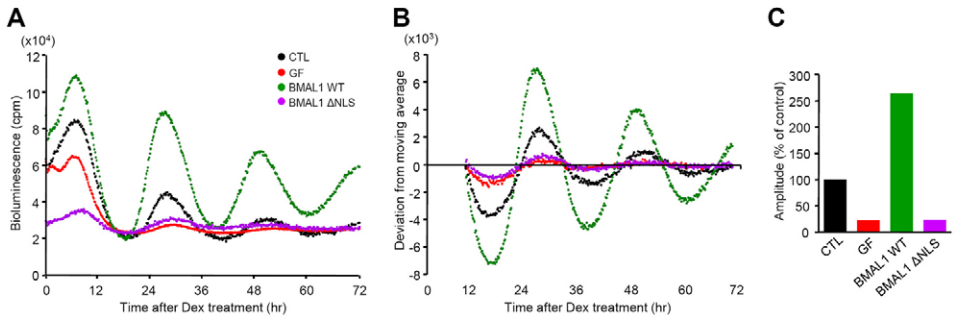

Fig. 4. Rapid nuclear translocation of CLOCK–BMAL1 is required for stimuli-induced circadian oscillation of *Per2* promoter activity. (**A**) NIH3T3 cells were transiently transfected with 1 μg/plate *Per2-Pro-dLuc* reporter plasmid along with 500 ng pcDNA 3.1 empty vector (CTL, black), 500 ng pcDNA 3.1 BMAL1 (BMAL1 WT, green), and 500 ng pcDNA 3.1 BMAL1 NLS mutant (BMAL1 ΔNLS, purple). The cells were then synchronized by addition of 100 nM Dex and the bioluminescence recorded using photomultiplier tubes (c.p.m., counts per minute). 5 μM GF109203X (GF) was administered 2 hours before Dex treatment (GF, red). The representative raw data were taken from three independent experiments that gave similar results. (**B**) The detrended data is shown with the date sets detrended by subtracting 24-hour running means (moving average) from the raw data. (**C**) The amplitudes of the bioluminescence rhythms of the indicated samples were calculated from the detrended data and plotted as relative percentage.

Cells were co-transfected with vectors carrying *Per2 Pro-dLuc* and the indicated genes, following which their bioluminescence was measured continuously for 72 hours after administration of the resetting stimuli, such as dexamethasone (Dex), a synthetic glucocorticoid (Fig. 4). Time-course changes in the bioluminescent signal exhibited a circadian rhythm in the cells transfected with *Per2 Pro-dLuc* alone (Fig. 4A,B, black). Intriguingly, the circadian oscillation of cellular bioluminescence was more robust, displaying significantly higher amplitude when cells were coexpressed with wild-type BMAL1 (Fig. 4, green). The oscillation and amplitude of the reporter activity were severely dampened by pretreatment with a PKC inhibitor (Fig. 4, red). More importantly, the cells coexpressed with mutant BMAL1 lacking NLS (BMAL1 ΔNLS) also markedly decreased the amplitude of the rhythmic expression of the reporter gene (Fig. 4, violet). These results suggest that both the nuclear localizing activity of BMAL1 and its dimerization with CLOCK in a PKC-dependent manner are essential for the rhythmic gene expression in the given cell population.

CBP activates the nuclear CLOCK–BMAL1 complex through direct binding to BMAL1

As described above, nuclear translocation of the CLOCK–BMAL1 complex is essential for cellular clock resetting by the extracellular stimuli. Nonetheless, it is important to note that nuclear abundance of CLOCK and BMAL1 during the circadian cycle does not correlate with their transcriptional activity (Kondratov et al., 2003; Kwon et al., 2006; Lee et al., 2001). This paradoxical feature is mediated by potent negative regulators of the CLOCK–BMAL1 heterodimer, including PERs and CRYs. In particular, the binding of CRY to the heterodimeric transcription factor facilitates the nuclear accumulation of this complex by suppressing its degradation and transactivation (Kwon et al., 2006; Lee et al., 2001). Indeed, coexpression of CRY1 with the fusion proteins VC–BMAL1 and VN–CLOCK noticeably enhanced the BiFC signal in the nucleus without the resetting stimuli (supplementary material Fig. S4). These observations raise the possibility that there is another regulatory mechanism underlying the transcriptional activation of the CLOCK–BMAL1 heterodimer. In addition, growing evidence emphasizes a pivotal role of histone acetylation in circadian gene activation, although the key player responsible for this enzyme modification is still elusive (Doi et al., 2006; Etchegaray et al., 2003). This prompted us to investigate the potential role of the transcriptional coactivators CBP and p300, which are known histone acetyltransferases (HATs) that bind to the CLOCK–BMAL1 complex, in circadian clock resetting. As shown in Fig. 5A, coexpression of either CBP or p300 with CLOCK–BMAL1 greatly increased CLOCK–BMAL1-dependent *Per1* transcription. Subsequent immunoprecipitation assay demonstrated that both CBP and p300 can interact with the CLOCK–BMAL1 complex (Fig. 5B). However, the results from the immunoprecipitation experiment do not provide details about how one interacts specifically with the other within the protein complex. Hence, to further dissect the protein–protein interactions between the heterodimeric complex and the coactivators, we developed BiFC constructs for CBP and p300 and analyzed their interaction with either CLOCK or BMAL1 in living cells by using all possible combinations with the BiFC constructs of CLOCK and BMAL1 (Fig. 5C; supplementary material Fig. S5). Interestingly, BMAL1 was capable of interacting with both CBP and p300, but CLOCK failed to interact with CBP. All these fluorescence signals were strongly detected as discrete foci distributed throughout the nucleus.

Next, to examine the effects of the resetting stimuli on the interaction between the coactivators and the CLOCK–BMAL1 complex, we monitored consecutive changes in Venus fluorescence in living cells expressing possible combinations of the BiFC fusion proteins after serum stimulation. The time-lapse imaging data revealed that the BiFC signal between CBP and BMAL1 was dramatically enhanced in the nucleus shortly after delivery of the resetting stimuli and was saturated within 2 hours (Fig. 5D upper panels; see supplementary material Movie 3). The mean value of the fluorescence intensity of 50 nuclei further confirmed the kinetics of the interaction between CBP and BMAL1 after treatment with the extracellular stimuli (Fig. 5E). In sharp contrast, there were no significant changes in the BiFC signal produced by either VC–p300/VN–BMAL1 or VC–p300/VN–CLOCK (Fig. 5D middle and lower panels, Fig. 5E; see supplementary material Movies 4 and 5). It is therefore likely that immediate recruitment of CBP to BMAL1 after nuclear translocation of the CLOCK–BMAL1 complex is a key molecular event in the activation of transcription of target genes.

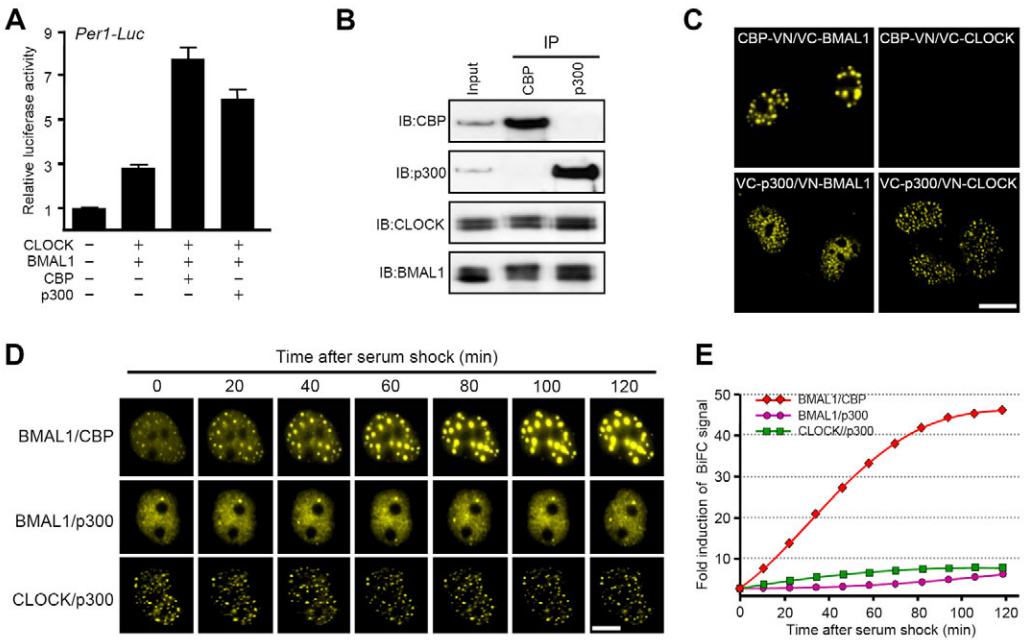

Fig. 5. CBP, not p300, rapidly associates with BMAL1 in the nucleus in response to serum shock. (**A**) COS-7 cells were transiently transfected with *Per1-Luc* reporter construct alone or co-transfected with plasmids expressing CLOCK, BMAL1, CBP and p300 in the indicated combinations. (**B**) Nuclear extracts from cells coexpressed with CBP, p300, CLOCK and/or BMAL1 were immunoprecipitated with anti-CBP or anti-p300, and subjected to immunoblotting using anti-CLOCK or anti-BMAL1. (**C**) At 12 hours post-transfection of BiFC plasmids expressing CBP–VN, VC–p300, VN (or VC)–CLOCK and VN (or VC)–BMAL1, fluorescent images were captured upon serum shock using a specific YFP filter set. Scale bar: 10 µm. (**D**) After CBP, p300, CLOCK and BMAL1 BiFC fusion proteins were expressed as indicated, time lapse images with a 2-minute interval were captured from single cell up to 2 hours after serum shock. Scale bar: 5 µm. (**E**) For quantification of increasing BiFC signal of BMAL1/CBP (red), BMAL1/p300 (purple) and CLOCK/p300 (green), fluorescence intensities in the cell nuclei of more than 50 individual fluorescent cells were measured using an automated intensity recognition feature of the imaging analysis system. The graph was produced by statistical regression of BiFC signal changes in single cell nuclei as a function of mean values.

Recruitment of CBP, not by CREB but by BMAL1, is crucial for clock gene induction

CBP has been identified as a coactivator for the transcription factor CREB and binds when CREB is phosphorylated (Kwok et al., 1994; Mayr and Montminy, 2001). In addition, several previous reports have suggested that CREB acts as a major effector molecule of multiple signaling pathways that lead to immediate-early response of *Per1* gene transcription during resetting of the circadian clock (Gau et al., 2002; Ginty et al., 1993; Travinckova-Bendova et al., 2002). Therefore, we assessed the possibility that CBP plays a substantial role in CREB activation, thereby inducing the immediate-early *Per1* gene expression. First, we analyzed whether the resetting stimuli promote CBP binding to CREB in COS-7 cells by using a BiFC assay. The CREB protein fused with the C-terminal fragment of Venus (CREB-VC) successfully produced moderate fluorescence exclusively in the nucleus when coexpressed with CBP–VN (data not shown). However, the BiFC signal of CREB and CBP did not increase to a great extent after serum stimuli when compared to that of BMAL1 and CBP (Fig. 6A,B; see supplementary material Movie 6). Next, to investigate the functional relevance of CBP recruitment by BMAL1 and CREB to *Per1* gene induction, cells were co-transfected with a *Per1* promoter reporter and various combinations of plasmids expressing CBP, CLOCK, BMAL1 and CREB. As shown in Fig. 6C, both CLOCK–BMAL1 and CREB induced *Per1* promoter activation at comparable levels, but the synergistic effect of CBP with CLOCK–BMAL1 was much greater than that combined with CREB on *Per1* promoter activation. These results are consistent with the BiFC data demonstrating that CBP binding to BMAL1 is more efficient than binding to CREB (Fig. 6A,B).

To further assess the role of CBP in the *Per1* gene activation induced by CLOCK–BMAL1 and/or CREB, we generated site-directed mutants of the *Per1* promoter lacking functional CRE or E-box elements (the binding site of CLOCK–BMAL1) and conducted promoter reporter assays using these mutant promoters under the same experimental conditions as given in Fig. 6C (Fig. 6D,E). Unexpectedly, the mutant promoter lacking CRE was activated with a pattern similar to that of the intact *Per1* promoter, although the overall promoter activity was slightly decreased (Fig. 6D). However, the transcriptional enhancing effect of CBP on CLOCK–BMAL1 was completely abolished when the consensus E-boxes were destroyed (Fig. 6E). More importantly, chromatin immunoprecipitation (ChIP) assay demonstrated that CBP recruitment to the first proximal E-box of *Per1* promoter was more significantly increased than its recruitment to CRE shortly after serum shock; however, p300 binding to both the E-box element and the CRE were not significantly altered after serum shock, although its binding activity was comparable to that of CBP (Fig. 7). Thus, CBP appears to play a crucial role in the rapid induction of *Per1* gene transcription, not by binding to CREB but by binding to BMAL1.

CBP is a key player in the CLOCK–BMAL1-mediated circadian clock resetting

Despite the essential role of CBP in rapid *Per1* induction, its relevance to circadian physiology remains obscure. To evaluate

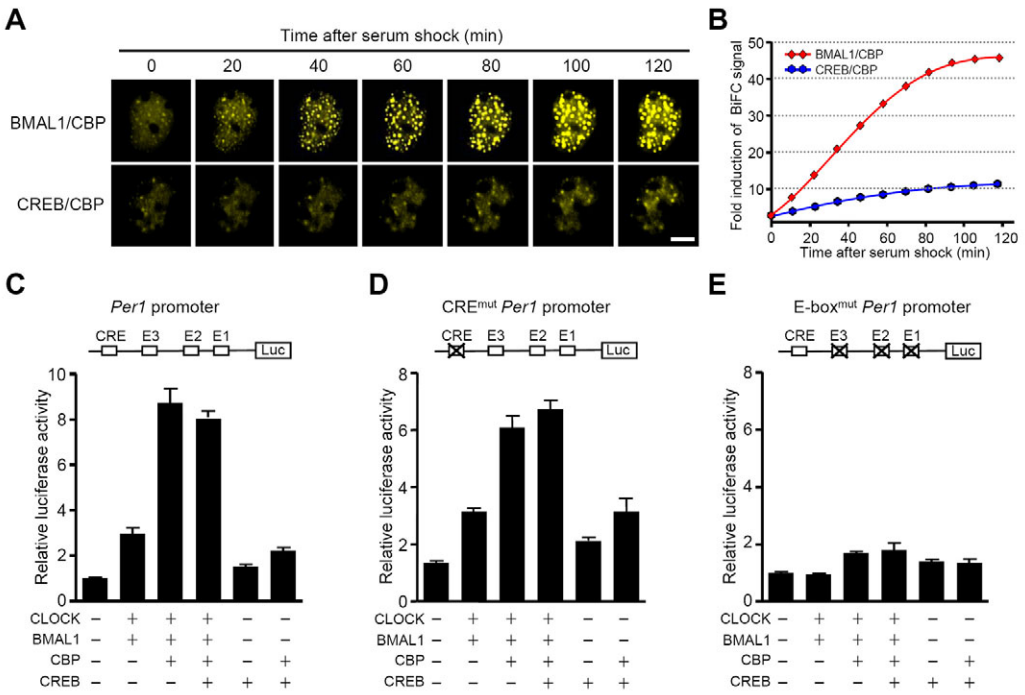

Fig. 6. CBP recruitment to BMAL1, not CREB, is required for transcriptional potentiation of *Per1* induction via E-box elements. (**A**) Time lapse images with a 2 minute interval were captured for 2 hours after serum shock in cells expressing CBP, BMAL1 and/or CREB BiFC fusion proteins. Scale bar: 5 μm. (**B**) For quantitative analyses of BMAL1/CBP (red) and CREB/CBP (blue) interactions, fluorescence intensities in the nuclei of more than 50 individual fluorescent cells were measured and represented by statistical regression of BiFC signal changes as a function of mean values. COS-7 cells were transfected with (**C**) intact *Per1-Luc* reporter construct (*Per1* promoter), (**D**) CRE mutated *Per1-Luc* reporter construct (CRE^{mut} *Per1* promoter), or (**E**) E-box mutated *Per1* reporter construct (E-box^{mut} *Per1* promoter), either alone or together with CLOCK, BMAL1, CREB and/or CBP expression plasmids, as indicated. The total DNA amount was kept constant by adding empty vectors as required. At 24 hours post-transfection, *Per1* promoter-driven luciferase activities were measured and normalized with pRL-TK activity. The data are shown as means ± s.e.m. of three independent experiments.

the physiological relevance of CBP to the CLOCK–BMAL1-mediated clock resetting, we employed an siRNA technique to knockdown CBP and other related molecules in living cells. The synthesized siRNAs specific to CBP, p300 and CREB effectively blocked the target gene expression without any significant effect on cell morphology and expressions of other genes (Fig. 8A and data not shown). We then analyzed the responsiveness of these knockdown cells to the resetting stimuli by a real-time bioluminescence assay. To this end, cells were co-transfected with *Per2* promoter-driven luciferase reporter plasmid and specific siRNA against the indicated genes (Fig. 8B), and were then exposed to the resetting stimuli. A long-time measurement of bioluminescence in the living cells demonstrated a pivotal role of CBP in producing circadian oscillation in gene expression. Depletion of CBP severely dampened circadian oscillation of reporter gene expression (Fig. 8B). By contrast, siRNA against p300, CREB or both did not significantly affect the periodic *Per2* gene expression in response to the resetting stimuli. These findings strongly suggest that CBP is essential not only for the CLOCK–BMAL1-mediated clock gene transcription but also for circadian clock resetting.

Discussion

Rapid signaling events via protein–protein interactions in response to extracellular stimuli are widely required for triggering timely physiological responses to environmental changes. By performing real-time analysis of molecular interactions in living cells with BiFC assay, we demonstrated that rapid recruitment of CBP to BMAL1 concomitant with nuclear translocation of the CLOCK–BMAL1 heterodimer is a key molecular event that leads to the synchronization of individual cellular clocks in the presence of entrainment stimuli (Fig. 9).

Accumulating evidence suggests that a number of transcription factors can interact with their binding partners to functionally mediate various signaling events in a phosphorylation-dependent manner (Lassot et al., 2001; Nateri et al., 2005). Considering our previous report demonstrating the rapid activation of CLOCK by PKC-mediated phosphorylation (Shim et al., 2007), the present data using BiFC analysis suggests that phosphorylated CLOCK acquires the ability to interact with BMAL1 to form a transactivation complex. Our BiFC data account for previous results that PKC activation by treatment with PMA promoted the binding of both CLOCK and BMAL1 to E-box elements of the *Per1* promoter, *Per1* mRNA expression, and eventually the phase shifts of the circadian rhythm in cultured fibroblasts (Shim et al., 2007). Moreover, the real-time bioluminescence data revealed that inhibition of PKC virtually abolished immediate-early induction and rhythmic oscillation of the *Per2* gene upon treatment with Dex (a potent clock resetting agent), further emphasizing the physiological significance of the role of PKC in molecular clock resetting (Fig. 4). Consistent with these results, PKC activation in the SCN caused advanced circadian rhythm in neurons, whereas inhibition of the PKC pathway eliminated the phase shift of SCN activity induced by melatonin and neuropeptide Y (Biello et al.,

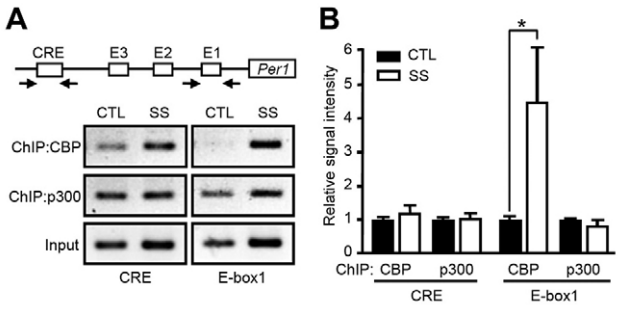

Fig. 7. CBP is rapidly recruited to *Per1* promoter E-box in response to serum shock. (**A**) Scheme of *Per1* promoter and the sites of the amplified DNA fragments. NIH3T3 cells grown to confluence in a growth medium (10% FBS) were incubated with serum-free medium for 24 hours and then treated with 50% horse serum for 1 hour. Formaldehyde-crosslinked chromatin samples from the 50% serum-treated (SS) or control (CTL) cells were incubated with anti-CBP antibody and anti-p300 antibody as indicated. PCR analysis was performed using a primer set flanking either CRE or the proximal E-box of the *Per1* promoter, and the PCR products were analyzed by electrophoresis on 1% agarose gel (see Materials and Methods). PCR data of the cell lysates (input) are shown as controls for the total amount of protein–DNA complex in each lane. (**B**) For quantitative analysis, the level of enrichment of the PCR products was determined and plotted relative to the total amount of input DNA, measured using a densiometer. Means ± s.e.m. (n=3) are shown; *P<0.05 (Student's t-test).

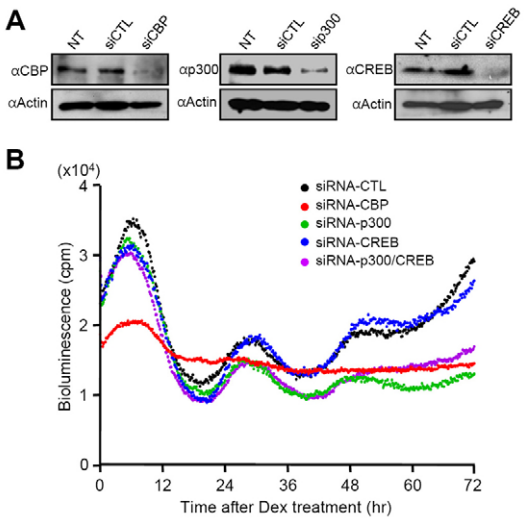

Fig. 8. CBP is required for the robust induction and oscillation of *Per2* promoter activity. (**A**) NIH3T3 cells were transfected with siRNA directed against CBP, p300, CREB or a control. At 48 hours post-transfection, cell extracts were prepared, and immunoblot analyses were performed with specific antibodies against CBP, p300, CREB and actin. (**B**) NIH3T3 cells were co-transfected with *Per2 Pro-dLuc* reporter and indicated siRNAs. After 48 hours of transfection, the cells were synchronized by 100 nM Dex treatment, and the induced circadian oscillation of the reporter was monitored.

1997; McArther et al., 1997; Schak and Harrington, 1999). Taken together, our BiFC data unveiled the functional relevance of PKC activation for CLOCK–BMAL1-mediated phase resetting of central and peripheral clocks.

Given that CLOCK acts as a signaling molecule via PKC-mediated phosphorylation, initially it was predicted that CLOCK might be a transcription factor responsible for signal-induced CBP recruitment, as in the case of phosphorylation-dependent coactivator recruitment of several transcription factors in response to various stimuli (Chriva et al., 1993; Foulds et al., 2004). However, BiFC analyses after treatment with the resetting stimuli revealed that BMAL1 rapidly interacted with CBP after nuclear entry, but CLOCK did not (Fig. 5C,D). In line with these observations, a previous study suggested that CBP can interact with a transcriptional activation domain (TAD) at the C-terminal region of BMAL1 when it is heterodimerized with CLOCK (Takahata et al., 2000). Indeed, deletion of the PAS2 domain of BMAL1, which eliminated the interaction with CLOCK (Fig. 1D), substantially weakened the BiFC between BMAL1 and CBP, whereas overexpression of CLOCK strengthened the signal (Y.L., unpublished data). Intriguingly, the BMAL1 C-terminal domain was found to also associate with CRY1, a potent repressor of the CLOCK–BMAL1 heterodimer, thereby suppressing its target gene transcription (Kiyohara et al., 2006; Sato et al., 2006). These findings present the possibility that the BMAL1 C-terminal has antagonistic dual functions and serves as a molecular switch for the gene transcription controlled by the CLOCK–BMAL1 heterodimer.

More importantly, we found that CBP was rapidly recruited to BMAL1 on *Per1* promoter E-box shortly after stimulation, whereas p300, a closely related protein, failed to do so. Despite their functional overlap, accumulating evidence emphasizes that CBP and p300 have distinct roles in various physiological settings such as cell proliferation, differentiation and apoptosis (Vo and Goodman, 2001). In particular, it has been reported that the transcriptional activity of CBP and p300 can be differentially regulated by post-translational modifications such as phosphorylation. For instance, in vivo phosphorylation at specific sites of CBP (sites that p300 lacks) by PKC and CaMKIV controls CBP recruitment to the related transcription complexes and subsequent transcriptional activation of these elements (Impey et al., 2002; Zanger et al., 2001). Furthermore, the present real-time bioluminescence data demonstrate that specific knockdown of CBP, but not p300, disrupted circadian gene transcription (Fig. 8). This notion is further supported by a recent study suggesting that CBP is phosphorylated and recruited to the CLOCK–BMAL1 complex via D2R receptor signaling, thereby potentiating *Per1* gene expression in cultured retinal neurons (Yujnovsky et al., 2006). By contrast, co-immunoprecipitation experiments using mouse liver and HeLa cells showed that p300, but not CBP, interacts with CLOCK in a time-dependent manner to activate CLOCK–BMAL1-mediated circadian transcription through rhythmic histone acetylation (Curtis et al., 2004; Etchegaray et al., 2003). Accordingly, unlike p300, CBP seems to have unique regulatory properties for signal-regulated transcriptional activation mostly via phosphorylation-dependent interaction with related transcription factor complexes.

Recently, we demonstrated that BMAL1 can be modified by SUMO2/3 and that this modification localizes BMAL1 exclusively to the promyelocytic leukemia (PML) nuclear body and potentiates its transcription factor activity (Lee et al., 2008). In fact, the PML nuclear body has been reported to serve as a discrete nuclear compartment in which a number of transcription factors and their co-regulators such as p53, CBP and RNA polymerase II (Pol II) reside (Zhong et al., 2000). Moreover, CBP is associated with RNA Pol II and PML in a subset of nuclear bodies (von Mikecz et al., 2000). It was reported that the prompt formation of nuclear

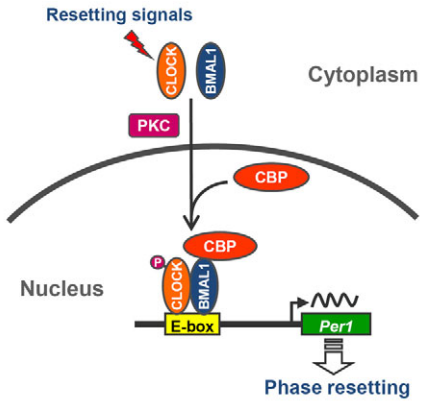

Fig. 9. Proposed mechanism of CLOCK–BMAL1-mediated phase resetting of the circadian clock via CBP recruitment. Rapid CLOCK–BMAL1 heterodimerization and nuclear translocation via PKC and subsequent CBP recruitment to BMAL1 in the nucleus are responsible for acute *Per1* or *Per2* gene expression in response to environmental signals, thereby leading to the phase resetting of the circadian clock.

bodies upon serum stimulation coincides with the immediate-early induction of the *fos* gene in cultured fibroblasts (Matsuzaki et al., 2003). Consistent with previous findings, our results from BiFC analysis revealed that binding of CBP to BMAL1 predominantly occurs at discrete nuclear foci (Fig. 5C,D), and that the BiFC signal colocalizes with immunostaining of the PML protein (Y.L., K.H.L. and K.K., unpublished data). These observations raise the possibility that formation of nuclear bodies containing the BMAL1/CBP complex after stimulation provides transcriptionally active sites of downstream target genes, including *Per1* and *Per2*.

We have shown that CREB exhibits much less efficiency than BMAL1 for CBP recruitment in response to serum stimuli (Fig. 6A,B). CREB is generally known as a central mediator in light-induced clock resetting, on the basis of the observation that CREB was phosphorylated at serine 133 in vivo in response to photic stimulation (Gau et al., 2002; Ginty et al., 1993). However, several lines of evidence have shown that formation of the CREB–CBP complex was readily induced by a cAMP agonist, but not well promoted or even inhibited in cells exposed to a variety of mitogenic or stress signals, including epidermal growth factor, PMA and other growth factors, despite the comparable levels of CREB phosphorylation (Mayr et al., 2001; Nakajima et al., 1996; Sun et al., 1994; Parker et al., 1998; Wagner et al., 2000; Xing et al., 1996). This could be a possible explanation for the minor increase in the CREB/CBP complex-induced BiFC signal after serum shock, which includes diverse stimuli (Fig. 6A,B). Consistent with the BiFC data, our transcriptional assay showed that CBP-potentiated upregulation of *Per1* promoter activity by CREB was relatively far less than by CLOCK–BMAL1 (Fig. 6C). Even in ChIP analysis, CBP was recruited on the proximal E-box of *Per1* promoter far more effectively than on the CRE region in response to serum shock stimuli. (Fig. 7). Moreover, specific knockdown of CREB by siRNA did not efficiently abrogate rhythmic *Per2* expression (Fig. 8). In fact, diverse extracellular stimuli, including fibroblast growth factor, platelet-derived growth factor, serum and PMA, have been shown to induce the immediate-early expression of *Per1* or *Per2*, resulting in a robust circadian oscillation of gene expression (Akashi et al., 2000). Taken together, it appears that without significant interference by the other signaling cascades, CBP recruitment to BMAL1, rather than to CREB, can serve as a major signaling route to elicit rapid transcriptional response upon entrainment stimuli. This notion is further supported by the data showing that the upregulation of *Per1* expression by CBP coactivation of CLOCK–BMAL1 or CREB was substantially inhibited by the disrupted E-boxes, but was conserved even in the absence of the CREB responsive element (Fig. 6C,E).

Materials and Methods

Cell culture, transfection, and luciferase assay

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Invitrogen) at 37°C under 9% CO_2. For luciferase reporter assays, COS-7 cells were transfected using Lipofectamine PLUS reagents (Invitrogen). At 24 hours after transfection, the cells were lysed, and luciferase activities in the cell extracts were analyzed by a Dual-Luciferase reporter assay system (Promega).

Plasmids

PKCγ–RFP was kindly provided by Dineke S. Verbeek (University of Amsterdam Academic Medical Center, Amsterdam, The Netherlands). pcDNA 3.1-mouse CBP and pcDNA 3.1-mouse p300 were gifted by Ronald Cohen (University of Chicago Medical School, Chicago, IL). RSV–rat CREB was kindly provided by Jea Bum Kim (Seoul National University, School of Biological Sciences, Seoul, Korea). CFP–CRY1 was constructed by subcloning the cDNA of cyan fluorescence protein (CFP) into the upstream region of mouse CRY1 cDNA, which was cloned in pFLAG–CMV2 (Sigma). The pGL3–*mPer1-Luc* promoter was previously described (Travinckova-Bendova et al., 2002). The CRE-defective mouse *Per1* (*mPer1*) promoter was generated by mutation of 4 nt (5′-TGACGTCA→AAAAAAAA-3′). E-boxes within the *mPer1* promoter were abrogated by mutation of 2 nt (5′-CACGTG→AGCGTG-3′). A subset of Myc–BMAL1 expression clones was previously described (Kwon et al., 2006). Internal deletions of BMAL1 produced Myc–BMAL1 Δ71–140 (ΔbHLH), Myc–BMAL1 Δ210–320 (ΔPAS1) and Myc–BMAL1 Δ350–480 (ΔPAS2), respectively.

Immunoblot analysis

Proteins were resolved on 6 or 8% sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore). Target proteins were detected with anti-BMAL1 (Kwon et al., 2006), anti-CLOCK (S-19; Santa Cruz Biotechnology), anti-CBP (A22; Santa Cruz Biotechnology), anti-p300 (N-15; Santa Cruz Biotechnology) and anti-CREB (48HE; Cell Signaling Technology). The immune complexes were visualized with an ECL detection kit (Pierce).

Preparation of nuclear extracts and immunoprecipitation

NIH3T3 cells were transfected with the indicated plasmids. At 36 hours post-transfection, the cells were washed twice in phosphate-buffered saline (PBS) and scraped in hypotonic lysis buffer A (50 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1% NP-40). After centrifugation for 10 minutes at 4000 ***g*** (4°C), the supernatant was discarded and the crude nuclear pellet was resuspended in hypotonic buffer C [50 mM HEPES pH 7.6, 1.5 mM $MgCl_2$, 450 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, protease inhibitor cocktail (Sigma)], vortex-mixed for 5 seconds, incubated on ice for 30 minutes, and centrifuged for 30 minutes at 12,000 ***g*** (4°C). The supernatant was transferred to a fresh tube and diluted in two volumes of hypotonic buffer C without NaCl before immunoprecipitation. For immunoprecipitation, whole cell lysates or nuclear extracts were incubated with 2 μg of precipitating antibodies, as indicated, for 1.5 hours at 4°C and then added to protein A/G-Sepharose bead slurry (Amersham). The final immune complexes were analyzed by immunoblotting as indicated above.

Chromatin immunoprecipitation assay

ChIP assays were performed using a commercial ChIP assay kit (Upstate Biotech) according to the manufacturer's instructions. The pre-cleared chromatin was immunoprecipitated for 2 hours at 4°C by agitating with 2 μg of anti-CBP antibody and anti-p300 antibody. The cell lysates without incubation of antibody were used for input control. Immune complexes were collected by incubation with protein-A Sepharose beads and sheared salmon sperm DNA. The primer sets used for ChIP assays were as follows: 1st (proximal) *mPer1* E-Box (E-box1) forward primer, 5′-CCCTCACTTCCCTTTCATTATTGACG-3′ and reverse E-box1 primer, 5′-TGCATAATGCCAGGCCCTGCCCCTCATTGG-3′ (amplified 250 bp) (Lee et al., 2001); the *mPer1* CRE forward primer was 5′-CAGCTGCCTCGCCCCGCCTC-3′, and reverse CRE primer, 5′-CCCAAGCAGCCATTGCTCGC-3′ (amplified 215 bp).

Transfection of siRNA

siRNAs targeting mouse CBP or mouse p300 and a non-targeting control siRNA were commercially available (Santa Cruz Biotechnology). These siRNAs are composed of a pool of three or four individual siRNAs designed to different regions

of their respective target mRNA. Double-stranded RNAi oligonucleotides for mouse CREB were designed and synthesized by Shanghai GenePharma (Shanghai, China). The sequence (forward: 5′-CAGCCACAGATTGCCACATTA-3′, reverse: 5′-TAATGTGGCAATCTGTGGCTG-3′) targets mouse CREB (NM_009952, 313 to 333). Transfection of 100 pmole siRNAs per well in NIH3T3 cells was conducted with Lipofectamine 2000 (Invitrogen) in 12-well plates according to the manufacturer's instructions. Immunoblotting was performed 48 hours after transfection.

Real-time measurement of bioluminescence and data analysis

NIH3T3 cells were plated in a 35-mm dish and transfected with the *Per2* promoter fused with destabilized firefly luciferase (*Per2 Pro-dLuc*) alone or co-transfected with pcDNA BMAL1 or pcDNA BMAL1 NLSΔ (Fig. 4) and siRNAs targeting mouse CBP, mouse p300 or mouse CREB as indicated (Fig. 7). The cells were pre-incubated in serum-free DMEM and synchronized with 100 nM Dex (Sigma) for 2 hours, and the medium was replaced with a recording medium [DMEM supplemented with 10% FBS and 0.1 mM D-luciferin (Promega)], as previously described (Kwon et al., 2006). GF109203X (5 μM) was added 1 hour before Dex treatment. Bioluminescence was measured with photomultiplier tube detector assemblies (AB-2550 Kronos-Dio; ATTO, Tokyo, Japan). For the detrended data, rhythms were plotted following a standard detrending procedure whereby the 24-hour running mean was subtracted from the raw data to remove the baseline drift that can mask the circadian rhythm, as previously described (Tsuchiya et al., 2009). Quantitative and statistical analysis of amplitudes from the bioluminescence rhythms were performed using cosinor periodogram provided by Roberto Refinetti (University of South Carolina, Salkehatchie, SC).

BiFC and immunofluorescence analyses

For BiFC assay, cDNAs encoding N-terminal residues 1–172 (VN173) and C-terminal residues 155–238 (VC155) of Venus were fused upstream of sequences encoding BMAL1 using linker sequences for 5′-GGGGSGGGGS-3′, and the chimeric coding regions were cloned into pcDNA 3.1 (Invitrogen) to produce VN–BMAL1 and VC–BMAL1 respectively. For BMAL1–VN and BMAL1–VC, cDNA encoding BMAL1 was subcloned into pFLAG–VN173 and pHA–VC155, respectively, which were kindly provided by Chang-Deng Hu (Purdue University School of Pharmacy, West Lafayette, IN) (Shyu et al., 2006). For VC–ΔPAS 2, cDNA encoding BMAL1 Δ350–480 (ΔPAS2) was subcloned in place of wild-type BMAL1 in VC–BMAL1. VC–BMAL1 ΔNLS was generated by fusing VC fragment upstream of BMAL1 ΔNLS, which lacks a functional nuclear localization signal (Kwon et al., 2006). For CLOCK BiFC plasmids, VN173 and VC155 were fused upstream of sequences encoding CLOCK using a linker for 5′-ANSSIDL-ISVPVEYPYDVPDYASR-3′, and the chimeric coding regions were cloned into pFLAG–CMV2 (Sigma) to produce VN–CLOCK and VC–CLOCK, respectively. For CLOCK–VN and CLOCK–VC, CLOCK cDNA was subcloned to pFLAG–VN173 and pHA–VC155. For BiFC analysis of CBP recruitment, cDNA encoding full-length mouse CBP was cloned into pFLAG–VN173. For VC–p300, VC155 was fused upstream of mouse p300 cDNA in pcDNA 3.1 expression vector. rCREB–VC was generated by subcloning cDNA encoding full-length rCREB into pHA–VC155.

For BiFC analyses, COS-7 cells were cultivated in 24-well plates overnight and then co-transfected with expression vectors as indicated in each experiment (200 ng each) using Lipofectamine Plus reagents (Invitrogen). At 12 hours post-transfection, the cells were placed in 1% serum media for 12–16 hours of serum starvation followed by 1–2 hours exposure to media containing 50% horse serum or various kinase activators, including 1 μM PMA (Sigma), 1 μM forskolin (Calbiochem) and 10 ng/ml EGF (Calbiochem). Various inhibitors, including 5 μM cycloheximide (Sigma), 0.5 μM KT5720 (Calbiochem), 10 μM PD98509 (Calbiochem) and 5 μM GF109203X (Calbiochem), were added 2 hours before the addition of 50% horse serum or the indicated kinase activators. The samples were examined under a fluorescence microscope, and fluorescent images were captured using a YFP filter set (EX=500 nm, DM=515 nm, EM=535 nm). For quantitative analyses, the cells showing nuclear signals were counted among BiFC signal-positive cells after serum shock in the presence or absence of the indicated inhibitors.

For immunofluorescence analyses, cells were fixed with 3.75% paraformaldehyde in PBS and incubated with anti-BMAL1, anti-CLOCK, anti-c-Fos (PC38; Calbiochem), anti-phospho-RORαSer35 (Lee et al., 2010), anti-phospho-CREB (#9191; Cell Signaling Technology) and anti-phospho-pERK (#9101: Cell Signaling Technology) antibodies. The cells were visualized with secondary antibodies conjugated with TRITC (Jackson ImmunoResearch Laboratories).

Time lapse imaging analyses/deconvolution microscopy

Cells were plated in a live cell chamber (Live Cell Instrument, Seoul, South Korea) in 10% FBS-containing growth medium. For serum shock experiments, the transfected cells, as indicated above, were incubated in a humidified chamber at 37°C and at ambient CO_2 levels for 12 hours and starved in medium containing 1% FBS for 12–16 hours before 50% horse serum treatment. Images were collected using a Delta Vision deconvolution microscope (Applied Precision, Isaquah, WA) at 37°C. At every time point, a fluorescence image was taken with specific filter sets for YFP. For quantification of changes in BiFC signals, fluorescence intensity in the nuclei of more than 50 individual fluorescent cells was measured using an automated intensity recognition feature of the Delta Vision imaging analysis system. Four-dimensional particle tracking analyses of BiFC signal in cells were conducted using Imeris imaging analysis software provided by Applied Precision.

We thank Chang-Deng Hu, (Purdue University School of Pharmacy, West Lafayette, IN) for generously providing the BiFC plasmids. Anti-phospho-RORαSer35 antibody was kindly provided by Sung Hee Baek (Department of Biological Sciences, Seoul National University, Seoul, Korea). This work was supported by the Brain Research Center of the 21st Century Frontier Program in Neuroscience from the Korea Ministry of Education, Science and Technology (MEST), National Research Foundation of Korea (2009-0070022), and the BioImaging Research Center at GIST. Y.L., J.L. and I.K. are supported by the Brain Korea 21 Research Fellowships from MEST.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/20/????/DC1

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.

Albrecht, U., Sun, Z. S., Eichele, G. and Lee, C. C. (1997). A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. *Cell* **91**, 1055-1064.

Balsalobre, A., Damiola, F. and Schibler, U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**, 929-937.

Balsalobre, A., Brown, S. A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H. M., Schutz, G. and Schibler, U. (2000a). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* **289**, 2344-2347.

Balsalobre, A., Marcacci, L. and Schibler, U. (2000b). Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr. Biol.* **10**, 1291-1294.

Biello, S. M., Golombek, D. A., Schak, K. M. and Harrington, M. E. (1997). Circadian phase shifts to neuropeptide Y in vitro: cellular communication and signal transduction. *J. Neurosci.* **17**, 8468-8475.

Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R. and Goodman, R. H. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855-859.

Crosio, C., Cermakian, N., Allis, C. D. and Sassone-Corsi, P. (2000). Light induces chromatin modification in cells of the mammalian circadian clock. *Nat. Neurosci.* **3**, 1241-1247.

Curtis, A. M., Seo, S. B., Westgate, E. J., Rudic, R. D., Smyth, E. M., Chakravarti, D., FitzGerald, G. A. and McNamara, P. (2004). Histone acetyltransferase-dependent chromatin remodeling and the Vascular Clock. *J. Biol. Chem.* **279**, 7091-7097.

Doi, M., Hirayama, J. and Sassone-Corsi, P. (2006). Circadian regulator CLOCK is a histone acetyltransferase. *Cell* **125**, 497-508.

Etchegaray, J. P., Lee, C., Wade, P. A. and Reppert, S. M. (2003). Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature.* **421**, 177-182.

Foulds, C. E., Nelson, M. L., Blaszczak, A. G. and Graves, B. J. (2004). Ras/mitogen-activated protein kinase signaling activates Ets-1 and Ets-2 by CBP/p300 recruitment. *Mol. Cell. Biol.* **24**, 10954-10964.

Gau, D., Lemberger, T., von Gall, C., Kretz, O., Le Minh, N., Gass, P., Schmid, W., Schibler, U., Korf, H. W. and Schütz, G. (2002). Phosphorylation of CREB Ser142 regulates light-induced phase shifts of the circadian clock. *Neuron* **34**, 245-253.

Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Takahashi, J. S. and Greenberg, M. E. (1993). Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science* **260**, 238-241.

Gu, Y. Z., Hogenesch, J. B. and Bradfield, C. A. (2000). The PAS superfamily: sensors of environmental and developmental signals. *Annu. Rev. Pharmacol. Toxicol.* **40**, 519-556.

Hogenesch, J. B., Gu, Y. X., Jain, S. and Bradfield, C. A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc. Natl. Acad. Sci. USA* **95**, 5474-5479.

Impey, S., Fong, A. L., Wang, Y., Cardinaux, J. R., Fass, D. M., Obrietan, K., Wayman, G. A., Storm, D. R., Soderling, T. R. and Goodman, R. H. (2002). Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV. *Neuron* **34**, 235-244.

Jung, H., Choe, Y., Kim, H., Park, N., Son, G. H., Khang, I. and Kim, K. (2003). Involvement of CLOCK:BMAL1 heterodimer in serum-responsive mPer1 induction. *NeuroReport* **14**, 15-19.

Kalkhoven, E. (2004). CBP and p300: HATs for different occasions. *Biochem. Pharmacol.* **68**, 1145-1155.

Kewley, R. J., Whitelaw, M. L. and Chapman-Smith, A. (2004). The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int. J. Biochem. Cell. Biol.* **36**, 189-204.

Kiyohara, Y. B., Tagao, S., Tamanini, F., Morita, A., Sugisawa, Y., Yasuda, M., Yamanaka, I., Ueda, H. R., van der Horst, G. T., Kondo, T. et al. (2006). The BMAL1 C terminus regulates the circadian transcription feedback loop. *Proc. Natl. Acad. Sci. USA* **103**, 10074-10079.

Kondratov, R. V., Chernov, M. V., Kondratova, A. A., Gorbacheva, V. Y., Gudkov, A. V. and Antoch, M. P. (2003). BMAL1-dependent circadian oscillation of nuclear

CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. *Genes Dev.* **17**, 1921-1932.

Kwok, R. P., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bächinger, H. P., Brennan, R. G., Roberts, S. G., Green, M. R. and Goodman, R. H. (1994). Nuclear protein CBP is a co-activator for the transcription factor CREB. *Nature* **370**, 223-226.

Kwon, I., Lee, J., Chang, S. H., Jung, N. C., Lee, B. J., Son, G. H., Kim, K. and Lee, K. H. (2006). BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer. *Mol. Cell. Biol.* **26**, 7318-7330.

Lassot, I., Ségéral, E., Berlioz-Torrent, C., Durand, H., Groussin, L., Hai, T., Benarous, R. and Margottin-Goguet, F. (2001). ATF4 degradation relies on a phosphorylation-dependent interaction with the SCF(betaTrCP) ubiquitin ligase. *Mol. Cell. Biol.* **21**, 2192-2202.

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.

Lee, J., Lee, Y., Lee, M. J., Park, E., Kang, S. H., Chung, C. H., Lee, K. H. and Kim, K. (2008). Dual modification of BMAL1 by SUMO2/3 and ubiquitin promotes circadian activation of the CLOCK/BMAL1 complex. *Mol. Cell. Biol.* **28**, 6056-6065.

Lee, J. M., Kim, I. S., Kim, H., Lee, J. S., Kim, K., Yim, H. Y., Jeong, J., Kim, J. H., Kim, J. Y., Lee, H. et al. (2010). RORalpha attenuates Wnt/beta-Catenin signaling by PKCalpha-dependent phosphorylation in colon cancer. *Mol. Cell* **37**, 183-195.

Matsuzaki, K., Minami, T., Tojo, M., Honda, Y., Saitoh, N., Nagahiro, S., Saya, H. and Nakao, M. (2003). PML-nuclear bodies are involved in cellular serum response. *Genes Cells* **8**, 275-286.

Mayr, B. M. and Montminy, M. R. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell Biol.* **2**, 599-609.

Mayr, B. M., Canettieri, G. and Montminy, M. R. (2001). Distinct effects of cAMP and mitogenic signals on CREB-binding protein recruitment impart specificity to target gene activation via CREB. *Proc. Natl. Acad. Sci. USA* **98**, 10936-10941.

McArthur, A. J., Hunt, A. E. and Gillette, M. U. (1997). Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. *Endocrinology* **138**, 627-634.

Nagoshi, E., Saini, C., Bauer, C., Laroche, T., Naef, F. and Schibler, U. (2004). Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* **119**, 693-705.

Nakajima, T., Fukamizu, A., Takahashi, J., Gage, F. H., Fisher, T., Blenis, J. and Montminy, M. R. (1996). The signal-dependent coactivator CBP is a nuclear target for pp90RSK. *Cell* **86**, 465-474.

Nateri, A. S., Spencer-Dene, B. and Behrens, A. (2005). Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. *Nature* **437**, 281-285.

Obrietan, K., Impey, S. and Storm, D. R. (1998). Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. *Nat. Neurosci.* **1**, 693-700.

Obrietan, K., Impey, S., Smith, D., Athos, J. and Storm, D. R. (1999). Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nuclei. *J. Biol. Chem.* **274**, 17748-17756.

Parker, D., Ferreri, K., Nakajima, T., LaMorte, V. J., Evans, R., Koerber, S. C., Hoeger, C. and Montminy, M. R. (1996). Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Mol. Cell. Biol.* **16**, 694-703.

Parker, D., Jhala, U., Radhakrishnan, I., Yaffe, M., Reyes, C., Shulman, A., Cantley, L., Wright, P. and Montminy, M. (1998). Analysis of an activator:coactivator complex reveals an essential role for secondary structure in transcriptional activation. *Mol. Cell* **2**, 353-359.

Reppert, S. M. and Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature* **418**, 935-941.

Sato, T. K., Yamada, R. G., Ukai, H., Baggs, J. E., Miraglia, L. J., Kobayashi, T. J., Welsh, D. K., Kay, S. A., Ueda, H. R. and Hogenesch, J. B. (2006). Feedback repression is required for mammalian circadian clock function. *Nat. Genet.* **38**, 312-319.

Schak, K. M. and Harrington, M. E. (1999). Protein kinase C inhibition and activation phase advances the hamster circadian clock. *Brain Res.* **840**, 158-161.

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

Shearman, L. P., Zylka, M. J., Weaver. D. R., Kolakowski, L. F., Jr and Reppert, S. M. (1997). Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* **19**, 1261-1269.

Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., Moriya, T., Shibata, S., Loros, J. J., Dunlap, J. C. et al. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell* **91**, 1043-1053.

Shim, H. S., Kim, H., Lee, J., Son, G. H., Cho, S., Oh, T. H., Kang, S. H., Seen, D. S., Lee, K. H. and Kim, K. (2007). Rapid activation of CLOCK by Ca^{2+}-dependent protein kinase C mediates resetting of the mammalian circadian clock. *EMBO Rep.* **8**, 366-371.

Shyu, Y. J., Liu, H., Deng, X. and Hu, C. D. (2006). Identification of new fluorescent protein fragments for bimolecular fluorescence complementation analysis under physiological conditions. *Biotechniques* **40**, 61-66.

Sun, P., Enslen, H., Myung, P. and Maurer, R. (1994). Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinase type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes. Dev.* **8**, 2527-2539.

Takahata, S., Ozaki, T., Mimura, J., Kikuchi, Y., Sogawa, K. and Fujii-Kuriyama, Y. (2000). Transactivation mechanisms of mouse clock transcription factors, mClock and mArnt3. *Genes Cells* **5**, 739-747.

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.

Tsuchiya, Y., Akashi, M., Matsuda, M., Goto, K., Miyata, Y., Node, K. and Nishida, E. (2009). Involvement of the protein kinase CK2 in the regulation of mammalian circadian rhythms. *Sci. Signal.* **2**, ra26.

Vo, N. and Goodman, R. H. (2001). CREB-binding protein and p300 in transcriptional regulation. *J. Biol. Chem.* **276**, 13505-13508.

von Mikecz, A., Zhang, S., Montminy, M., Tan, E. M. and Hemmerich, P. (2000). CREB-binding protein (CBP)/P300 and RNA polymerase II colocalize in transcriptionally active domains in the nucleus. *J. Cell Biol.* **150**, 265-273.

Wagner, B., Bauer, A., Schutz, G. and Montminy, M. (2000). Stimulus-specific interaction between activator-coactivator cognates revealed with a novel complex-specific antiserum. *J. Biol. Chem.* **275**, 8263-8266.

Weber, F., Hung, H. C., Maurer, C. and Kay, S. A. (2006). Second messenger and Ras/MAPK signalling pathways regulate CLOCK/CYCLE dependent transcription. *J. Neurochem.* **98**, 248-257.

Welsh, D. K., Yoo, S. H., Liu, A. C., Takahashi, J. S. and Kay, S. A. (2004). Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr. Biol.* **14**, 2289-2295.

Wilsbacher, L. D., Yamazaki, S., Herzog, E. D., Song, E. J., Radcliffe, L. A., Abe, M., Block, G., Spitznagel, E., Menaker, M. and Takahashi, J. S. (2002). Photic and circadian expression of luciferase in mPeriod1-luc transgenic mice in vivo. *Proc. Natl. Acad. Sci. USA* **99**, 489-494.

Xing, J., Ginty, D. and Greenberg, M. (1996). Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273**, 959-963.

Yagita, K., Tamanini, F., van Der Horst, G. T. and Okamura, H. (2001). Molecular mechanisms of the biological clock in cultured fibroblasts. *Science* **292**, 278-281.

Yokota, S., Yamamoto, M., Moriya, T., Akiyama, M., Fukunaga, K., Miyamoto, E. and Shibata, S. (2001). Involvement of calcium-calmodulin protein kinase but not mitogen-activated protein kinase in light-induced phase delays and Per gene expression in the suprachiasmatic nucleus of the hamster. *J. Neurochem.* **77**, 618-627.

Yujnovsky, I., Hirayama, J., Doi, M., Borrelli, E. and Sassone-Corsi, P. (2006). Signaling mediated by the dopamine D2 receptor potentiates circadian regulation by CLOCK:BMAL1. *Proc. Natl. Acad. Sci. USA* **103**, 6386-6391.

Zanger, K., Radovick, S. and Wondisford, F. E. (2001). CREB binding protein recruitment to the transcription complex requires growth factor-dependent phosphorylation of its GF box. *Mol. Cell* **7**, 551-558.

Zhong, S., Salomoni, P. and Pandolfi, P. P. (2000). The transcriptional role of PML and the nuclear body. *Nat. Cell Biol.* **2**, E85-E90.