Mammalian Circadian Clock: The Roles of Transcriptional Repression and Delay

Yoichi Minami, Koji L. Ode, and Hiroki R. Ueda

Abstract The circadian clock is an endogenous oscillator with a 24-h period. Although delayed feedback repression was proposed to lie at the core of the clock more than 20 years ago, the mechanism for making delay in feedback repression in clock function has only been demonstrated recently. In the mammalian circadian clock, delayed feedback repression is mediated through E/E′-box, D-box, and RRE transcriptional *cis*-elements, which activate or repress each other through downstream transcriptional activators/repressors. Among these three types of *cis*-elements, transcriptional negative feedback mediated by E/E′-box plays a critical role for circadian rhythms. A recent study showed that a combination of D-box and RRE elements results in the delayed expression of *Cry1*, a potent transcriptional inhibitor of the E/E′-box. The overall interconnection of these *cis*-elements can be summarized as a combination of two oscillatory motifs: one is a simple delayed feedback repression where only an RRE represses an E/E′-box, and the other is a repressilator where each element inhibits another in turn (i.e., E/E′ box represses an RRE, an RRE represses a D-box, and a D-box represses an E/E′ box). Experimental verification of the roles of each motif as well as post-transcriptional regulation of the circadian oscillator will be the next challenges.

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1 Circadian Clock in Mammals

In mammals the master clock is located in the suprachiasmatic nucleus (SCN). Transcript analyses have indicated that circadian clocks are not restricted to SCN, but are found in several tissues including the liver (Yamazaki et al. 2000) and cultured cells such as rat fibroblasts Rat-1 (Balsalobre et al. 1998), mouse fibroblasts NIH3T3 (Tsuchiya et al. 2003), or human osteosarcoma U-2OS cells (Isojima et al. 2009; Vollmers et al. 2008). Therefore, circadian rhythms are driven by cell-autonomous oscillators. Studies across species have elucidated the conserved feature of molecular mechanisms underlying circadian rhythms: at the core of the clock lies a transcriptional/translational negative feedback loop. For example, in mice the transcription factors CLOCK and BMAL1 dimerize and activate transcription of the *Per* and *Cry* genes. PER and CRY proteins accumulate in the cytosol become phosphorylated and return to the nucleus where they inhibit the activity of CLOCK and BMAL1. The turnover of PER and CRY proteins leads to a new cycle of activation by CLOCK and BMAL1 via E/E′-box (Dunlap 1999; Griffin et al. 1999; Kume et al. 1999; Reppert and Weaver 2002; Young and Kay 2001). In this process, PER and CRY form a negative feedback loop that inhibits their own transcription. However, reciprocal activation of positive (CLOCK and BMAL1) and negative (PER and CRY) regulators in a negative feedback loop is not sufficient: there must be a delay or immediate self-inhibition of CRY and PER would result in the stable lower expression of these factors rather than oscillation. What molecular mechanism imposes this time delay? This chapter summarizes the transcription network of the mammalian circadian clock and provides insights into how the network together with post-translational regulation of clock proteins works as a delayed negative feedback loop.

2 Identification of the Circadian Transcriptional Network

2.1 Transcriptional Network Based on Three Clock-Controlled Elements

2.1.1 The E/E′-Box, the D-Box, and the RRE

The overall topology of mammalian circadian transcription network can be understood by the combination of three clock-controlled elements (CCEs), short consensus DNA sequences typically located near the promoter region of clock genes. These CCEs are called the E/E′-box (CACGT(T/G)) (Gekakis et al. 1998; Hogenesch et al. 1997; Ueda et al. 2005; Yoo et al. 2005), the D-box (DBP response

element) (TTATG(C/T)AA) (Falvey et al. 1996; Ueda et al. 2005), and the RRE [RevErbA response element, also called as ROR response element (RORE)] [(A/T) A(A/T)NT(A/G)GGTCA] (Harding and Lazar 1993; Preitner et al. 2002; Ueda et al. 2002, 2005).

By performing transcriptome analysis, expression of 24-h periodic genes was reported in cultured cells (Grundschober et al. 2001), the SCN (Panda et al. 2002; Ueda et al. 2002), and other tissues such as heart (Storch et al. 2002), liver (Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002), aorta (Rudic et al. 2005), adipose tissues (Zvonic et al. 2006), calvarial bone (Zvonic et al. 2007), and hair follicle (Akashi et al. 2010). Although there are differences in the rhythmicity of circadian-expressed genes in each tissue, the following mammalian clock genes most commonly have circadian oscillation: *Period1* (*Per1*), *Per2*, *Per3*, *Dec1* (*Bhlhb2*), *Dec2* (*Bhlhb3*), *Cryptochome1* (*Cry1*), *Clock*, *Npas2*, *Bmal1* (*Arntl*), *Dbp*, *E4bp4* (*Nfil3*), *RevErbAa* (*Nr1d1*), *RevErbAb* (*Nr1d2*), and *Rora*. The temporal expression of each gene is controlled by a different combination of CCEs. Evolutionary conserved E/E′-boxes are located in the noncoding regions of nine genes (*Per1*, *Per2*, *Cry1*, *Dbp*, *Rorγ*, *RevErbAa*, *RevErbAb*, *Dec1*, and *Dec2*), D-boxes are contained in eight genes (*Per1*, *Per2*, *Per3*, *Cry1*, *RevErbAa*, *RevErbAb*, *Rorα*, and *Rorβ*), and RREs in six genes (*Bmal1*, *Clock*, *Npas2*, *Cry1*, *E4bp4*, and *Rorc*). The expressed gene product positively or negatively regulates transcription activity by acting on CCEs: CCEs and these clock genes form a closed network structure (Fig. 1) as described below.

2.1.2 Transcription Regulation via the E/E′-Box and Clock Genes

The E/E′-box is positively regulated by *Bmal1*, *Clock*, and *Npas2* and negatively regulated by *Per1–3*, *Cry1–2*, and *Dec1–2*. CRY and PER are hypothesized to autoregulate their own expression by repressing the heterodimeric complex of the basic helix–loop–helix (bHLH) PER-ARNT-SIM (PAS) domain transcriptional activators CLOCK and BMAL1, which bind to E/E′-box elements in the *Cry1* and *Per1–2* promoters. Although both positive regulators (*Bmal1*, *Npas2*, *Clock*) and negative regulators (*Per1–3* and *Cry1–2*) have circadian rhythmic expression patterns, peak time of positive regulators are antiphase to that of negative regulators (delayed negative feedback).

2.1.3 Transcription Regulation via the D-Box and Clock Genes

The D-box is positively regulated by PAR-bZIP (proline- and acidic amino acid-rich basic leucine zipper) transcription factors (*Dbp*, *Tef*, and *Hlf*) and negatively by *E4bp4*. Like the E/E′-box, an antiphase relationship of gene expression between negative and positive regulators can be observed. In the D-box case,

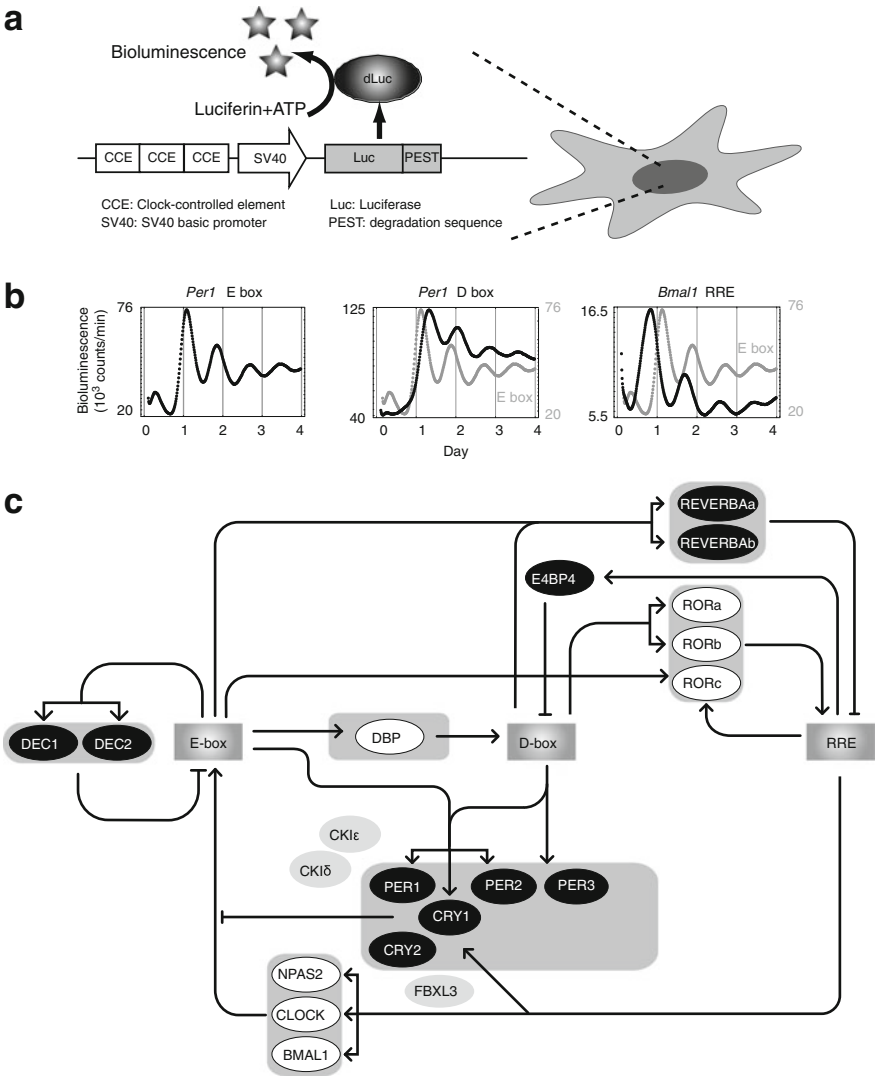

Fig. 1 Schematic representation of the transcriptional network of the mammalian circadian clock. (**a**) In vitro cycling assay. Cultured mammalian cells (Rat-1) were transfected with d*Luc* under the control of a clock-controlled element (CCE) and SV40 basic promoter (Ueda et al. 2005). (**b**) Representative circadian rhythms of bioluminescence from a wild-type *Per1* E-box CCE fused to the SV40 basic promoter driving a d*Luc* reporter (*left panel*) and compared to bioluminescence rhythms driven by a *Per1* D-box (*center panel*) and a RRE (*right panel*). Original figures are reproduced from Ueda et al. (2005). (**c**) Genes and CCEs are depicted as *ellipsoids* and *rectangles*, respectively. Transcriptional/translational activation is shown by *arrows* (→) and repression is depicted by *arrows with flat ends* (⊣)

the expression phase of the positive regulator *Dbp* is similar to that of *Per1*, whereas the expression phase of the negative regulator *E4bp4* is similar to that of *Bmal1* (Mitsui et al. 2001).

2.1.4 Transcription Regulation via the RRE and Clock Genes

RRE is positively regulated by *Rora*, *Rorb*, and *Rorc* and negatively regulated by *RevErbAa* and *RevErbAb*. In the SCN, *Rora* and *Rorb* have circadian rhythms but not *Rorc* (Ueda et al. 2002). Liu et al. reported that *RevErbAa* and *RevErbAb* are functionally redundant and necessary for oscillation of the RRE-regulated gene *Bmal1*. By contrast, *Ror*s contribute to *Bmal1* amplitude, but are not required for generating oscillation (Liu et al. 2008).

2.1.5 Timing of Each CCE

The circadian timing at which each element becomes active for transcription can be monitored with an in vitro cell culture system in which a destabilized firefly luciferase (d*Luc*) reporter is driven by different clock-controlled promoters. After cells are synchronized (i.e., with dexamethasone, forskolin, or serum), oscillations in gene expression are recorded by bioluminescence (Nagoshi et al. 2004; Ueda et al. 2002, 2005; Welsh et al. 2004). Using this in vitro cycling assay, the "phase" of each CCE can be determined (Ueda et al. 2002, 2005) (Fig. 1). Note that the term "phase" used in this chapter represents relative peak timing of each circadian gene expression within single circadian cycle. Each CCE is responsible for the gene expression at distinct circadian times: the peak time of E/E′-box-driven expression is followed by D-box-driven expression after an interval of ~5 h. Then, RRE-driven expression follows D-box expression after ~8 h. E/E′-box-driven expression begins to appear again ~11 h after RRE-driven expression. In the case of the SCN, the subjective time drawn by each CCE can be illustrated as "morning-time" for the E/E′-box, "evening-time" for the D-box, and "nighttime" for the RRE (Ueda et al. 2005).

2.2 Importance of Gene Regulation via the E/E′-Box

2.2.1 Circadian Clock Perturbation via CCEs

The three CCEs have different impacts on cellular circadian rhythms: perturbation of E/E′-box regulation abolishes circadian rhythms; perturbation of RRE regulation has an intermediate but significant effect; and D-box disruption has almost no effect.

A study using Rat-1 cell showed this by overexpressing regulatory genes with repressive activity to different CCEs (Ueda et al. 2005) (Fig. 2). The *Per2* promoter is regulated via an E/E′-box and a D-box, and the *Bmal1* promoter is regulated via

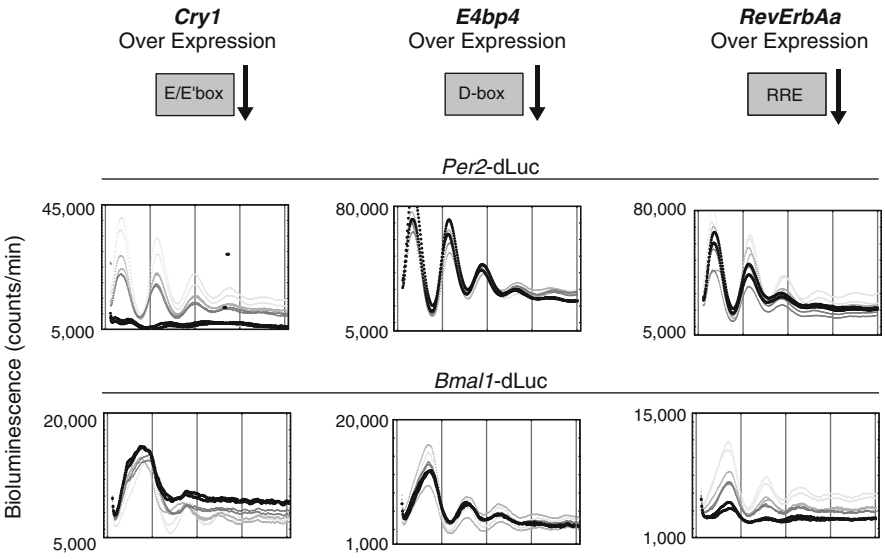

Fig. 2 Importance of the E/E′-box. Effect of repression on each CCE. The E/E′-boxes, D-box, and RRE were repressed by overproduction of CRY1, E4BP4, and REVERBAa, respectively. The consequences of those repressions were monitored by bioluminescence from *Per2* and *Bmal1* promoter driving a destabilized luciferase (*Per2*-dLuc and *Bmail1*-dLuc). Original figures are reproduced from Ueda et al. (2005). The *different shades of gray* in the plot indicate different amounts of transfected vector

an RRE. When E/E′-box activity is perturbed by overexpression of the *Cry1* gene, both *Per2*-promoter-driven reporter gene (*Per2*-dLuc) and *Bmal1*-promoter-driven reporter gene (*Bmal1*-dLuc) lose circadian rhythms. When an RRE is perturbed through *RevErbAa* overexpression, *Bmal1*-dLuc loses circadian rhythms and the amplitude of *Per2*-dLuc rhythmic expression is decreased. The impact of RRE perturbation through *RevErbAa* overexpression appeared to be more significant in mice liver. Kornmann et al. showed that liver-specific overexpression of *RevErbAa* abolishes the rhythmicity of PER2::Luc expression in liver explants (Kornmann et al. 2007). Contrary to the case of E/E′-box and RRE, D-box perturbation through *E4bp4* overexpression causes both *Per2*-dLuc and *Bmal1*-dLuc transcriptional activity to have normal circadian rhythms (Ueda et al. 2005). These varying effects are difficult to explain by mere quantitative differences in the strength of the three repressors, which suggests that there is some qualitative difference between E/E′-box, D-box, and RRE regulation in circadian rhythmicity.

2.2.2 Circadian Feedback Repression: Heart of the Circadian Transcriptional Network

PER and CRY play key roles in the circadian clock transcriptional network by closing the negative feedback loop of E/E′-box regulation. CRY has stronger

repressor activity than PER (Kume et al. 1999). Sato et al. reported that interference of CRY1's repressor activity on E/E′-box-mediated transcription can abolish circadian transcriptional oscillations. They screened both human CLOCK and BMAL1 alleles that were insensitive to CRY1 repression but maintained normal transcriptional activity. Selected clones have normal transcriptional activities similar to wild type in the absence of CRY1, but have greater reporter activity in the presence of CRY1. By analyzing either *Per2*-dLuc or *Bmal1*-dLuc, they observed that cotransfection of either CLOCK or BMAL1 mutant alleles resulted in substantial impairment of circadian rhythmicity after one or two cycles of oscillation; cotransfection of both CRY-insensitive mutant CLOCK and BMAL1 together resulted in the loss of circadian promoter activity. This suggests that transcriptional repression of CLOCK/BMAL1 by CRY1 is required for circadian regulation via both an E/E′-box and an RRE (Sato et al. 2006) (Fig. 3).

3 Minimal Circuit of the Mammalian Circadian Clock

3.1 Two Delayed Negative Feedback Loops

How is the negative feedback to an E/E′-box delayed? Although there is an E′-box and an E-box in *Cry1*'s regulatory region (Fustin et al. 2009; Ueda et al. 2005), the peak of *Cry1* expression is evening-time, which is substantially delayed relative to other genes with an E/E′-box (Fustin et al. 2009; Ueda et al. 2005). *Cry1* has two functional RREs in one of its introns (Ueda et al. 2005) and also D-box in its promoter region (Ukai-Tadenuma et al. 2011). Ukai-Tadenuma et al. experimentally confirmed that the combination of daytime elements (D-box) and nighttime elements (RREs) within its intronic enhancer gives rise to *Cry1*'s delayed evening-time expression. Interestingly, the observed delayed expression was well explained by a simple phase-vector model that enabled artificially designed delayed expressions (Ukai-Tadenuma et al. 2011) (Fig. 4).

Based on this simple phase-vector model (Fig. 4), they generated an array of *Cry1* constructs that have different phases and used these in a genetic complementation assay to restore circadian oscillation in arrhythmic $Cry1^{-/-}$:$Cry2^{-/-}$ cells established from $Cry1^{-/-}$:$Cry2^{-/-}$ double-knockout mice (van der Horst et al. 1999). These experiments reveal that substantial delay of *Cry1* expression is required to restore single-cell-level rhythmicity and that prolonged delay of *Cry1* expression can slow circadian oscillations (Fig. 5). These results suggest that phase delay in *Cry1* transcription is required for mammalian clock function and these results provide formal proof that the design principle of the mammalian circadian clock transcriptional network is negative feedback with delay (Ukai-Tadenuma et al. 2011).

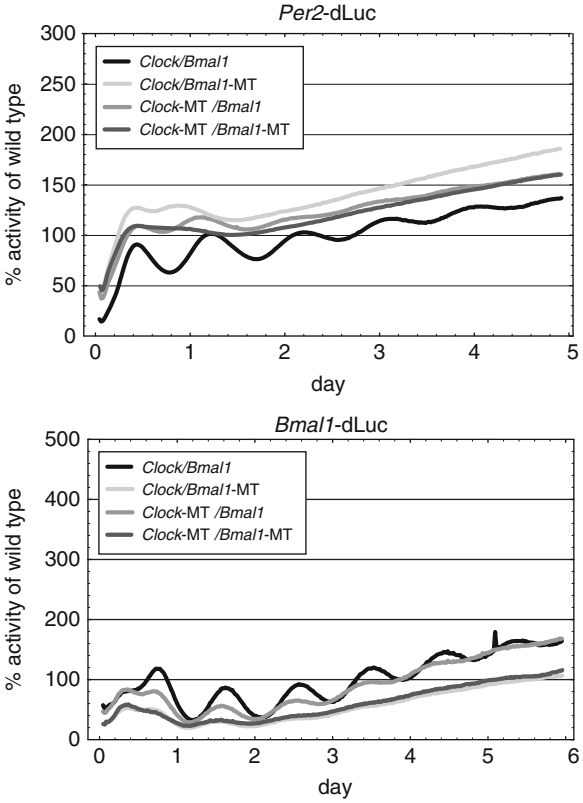

Fig. 3 The impairment of CRY-mediated repression. Coexpression of CLOCK/BMAL1 mutant heterodimers that are insensitive to CRY repression ablates circadian E-box and RRE activities in NIH3T3 cells. Plasmids expressing Flag-tagged CLOCK and BMAL1 alleles were transiently cotransfected with the *Per2*-dLuc (*upper panel*) or *Bmal1*-dLuc reporter plasmid into NIH3T3 cells (*lower panel*). *Per2* or *Bmal1* promoter activities in NIH3T3 cells transfected with single or double CRY1-insensitive CLOCK, and BMAL1 mutants (MT) were monitored over 5 (*upper panel*) or 6 days (*lower panel*). All reporter activities were normalized such that the median wild-type luciferase activity over the time course was 100 %. Original figures are reproduced from Sato et al. (2006)

Based on these results, they hypothesized that the transcriptional network can be simplified into a model consisting of two transcriptional activations and four transcriptional repressions on three regulatory DNA elements (Fig. 6). Notably, this diagram can be envisaged as a composite of two distinct oscillatory network motifs (1) a repressilator, which is composed of three repressions, and (2) a delayed negative feedback loop, which is composed of two activations and one repression. Both oscillatory network motifs include delayed feedback repression and can generate autonomous oscillations independently (Elowitz and Leibler 2000; Stricker et al. 2008).

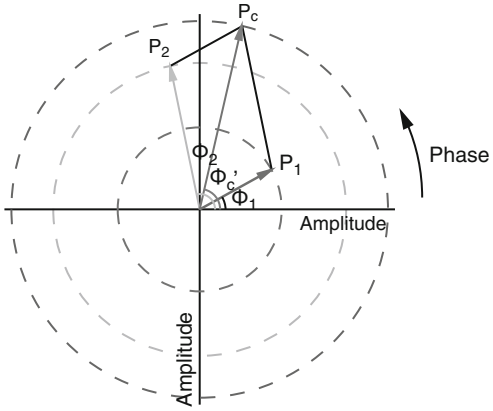

Fig. 4 Phase-vector model. A new phase results from the combinatorial synthesis of two transcriptional regulators or two clock-controlled DNA elements, which can be illustrated to a first-order approximation by a phase-vector model. This combinatorial regulatory mechanism for generating new circadian phases of transcription represents a general design principle underpinning complex system behavior. Assume wave function $f_x(t) = A_x \cos(\theta(t) + \phi_x)$. The amplitude of wave A is represented by the length of a phase vector P, and the phase of wave ϕ is represented by the angle of P. The component waves f_1 and f_2 are displayed by phase vectors P_1 and P_2. P_c is the summed phase vector of P_1 and P_2. Original graph is reproduced from Ukai-Tadenuma et al. (2011)

3.2 Genetic Evidence for the Importance of CCEs

The minimal circuit model implies that all of three CCEs have substantial importance for the circadian oscillator. The importance of an E/E′-box-mediated regulation is manifested by the phenotypes of several clock gene-knockout mice. The circadian clock governs physiological phenomena like day–night variation of activity, so changes in behavioral rhythms reflect differences in the endogenous clock of mutant mice. Accordingly, disruption of *Bmal1*, a positive regulator of E/E′-box-mediated regulation, directly results in the loss behavioral rhythms in mice (Bunger et al. 2000; Shi et al. 2010). Disruption of *Clock* gene did not result in loss of behavioral rhythms (DeBruyne et al. 2007) probably because *Clock* and another gene *Npas2* have redundant roles: *Clock* and *Npas2* double-knockout mice have arrhythmic behavioral patterns (DeBruyne et al. 2007), while *Npas2*-disrupted mice have normal behavioral rhythms (Dudley et al. 2003). Loss of negative regulator of E/E′-box-mediated transcription also results in arrhythmic phenotypes. Both *Per1* and *Per2* disrupted mice have loss of circadian rhythmicity of behavioral activity (Bae et al. 2001; Zheng et al. 2001), and $Cry1^{-/-}$:$Cry2^{-/-}$ mice have arrhythmic behavioral patterns (van der Horst et al. 1999; Vitaterna et al. 1999).

The minimal structure shown in Fig. 6 implies that D-box and RRE also play an essential role to maintain the delay time for negative feedback. For example, the double knockout of *RevErbAa* and *RevErbAb* mice has arrhythmic

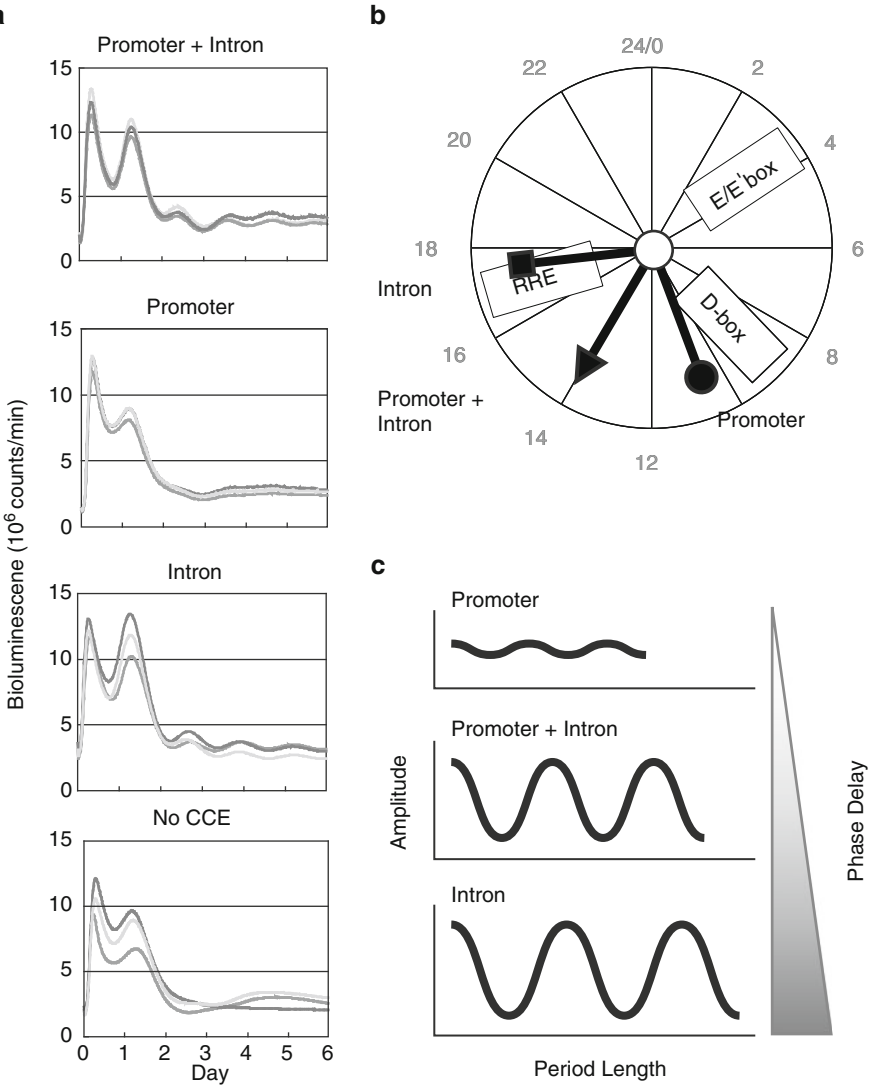

Fig. 5 The biological relevance of delayed *Cry1* expression in circadian clock function. (**a**) *Per2*-dLuc bioluminescence levels in transfected *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ cells. The *Per2*-dLuc reporter and a *Cry1* expression construct were cotransfected into *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ cells. (**b**) *Cry1* expression phases under different promoters (Ueda et al. 2005; Ukai-Tadenuma et al. 2011) that contain either a D-box (*Cry1* promoter), RRE (*Cry1* intron), or both (promoter + intron). (**c**) Substantial delay in feedback repression is required for mammalian clock function. The decreased delay dampens the amplitude of circadian oscillations (*top panel*), and the prolonged delay in feedback repression slows the frequency of circadian oscillations (*bottom panel*) compared to wild type (*middle panel*). Original figures are reproduced from Ukai-Tadenuma et al. (2011). *Different trace shades* represent results from triplicated experiments

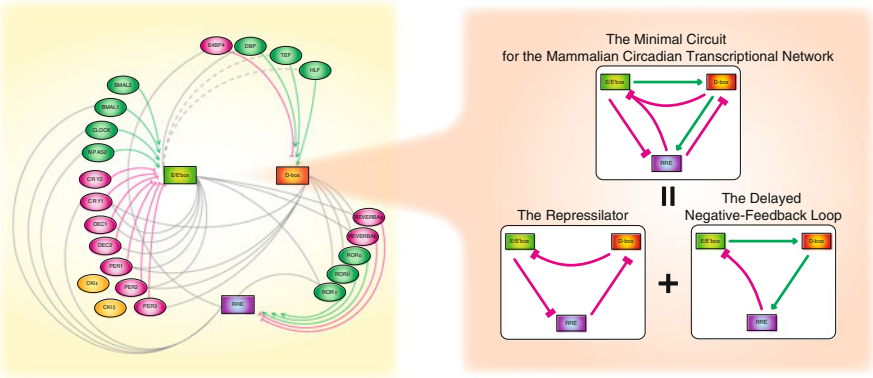

Fig. 6 The minimal circuit for the mammalian circadian transcriptional network. (**a**) The transcription network of the mammalian circadian clock (Ueda et al. 2005; Ukai-Tadenuma et al. 2011). (**b**) The minimal circuit (*top panel*) can be illustrated as a composite of two distinct oscillatory network motifs: a repressilator (*bottom left panel*) and a delayed negative feedback loop (*bottom right panel*). Transcriptional activation (*arrows*); transcriptional repression (*arrows with flat ends*); regulatory DNA elements (*rectangles*; E/E′-box, morning; D-box, daytime; RRE, nighttime). Original graph is reproduced from Ukai-Tadenuma et al. (2011)

behavioral phenotypes and arrhythmic clock gene expression (Bugge et al. 2012; Cho et al. 2012).

The importance of D-box transcriptional regulators is still unclear because no report shows that dysfunctional mice for D-box regulators have completely arrhythmic behavioral patterns. Lopez-Molina et al. reported that *Dbp* knockout mice have normal behavioral rhythms compared to wild type (Lopez-Molina et al. 1997). *Hlf* or *Tef* disrupted mice also have almost normal behavioral rhythms (Gachon et al. 2004). Even triple knockout of PAR-bZIP transcriptional factor mice have almost normal behavior rhythms (Gachon et al. 2004). Although *E4bp4* knockout mice was constructed (Gascoyne et al. 2009), behavioral rhythms of the mice were not reported.

3.3 Generation of Various Phases by the Combination of CCEs

From DNA microarray data, more than 10 % of expressed genes have circadian rhythms with a wide range of peak timings (Delaunay and Laudet 2002); the distribution of peak timing is not limited to three circadian times corresponding to the expression timing of each CCE. How do these "intermediate" expression timings arise? One possibility is that the combination of three CCEs generates various circadian phases.

Ukai-Tadenuma, Kasukawa et al. adopted a synthetic approach to physically simulate the correlation between CCEs combinations and the peak timing of

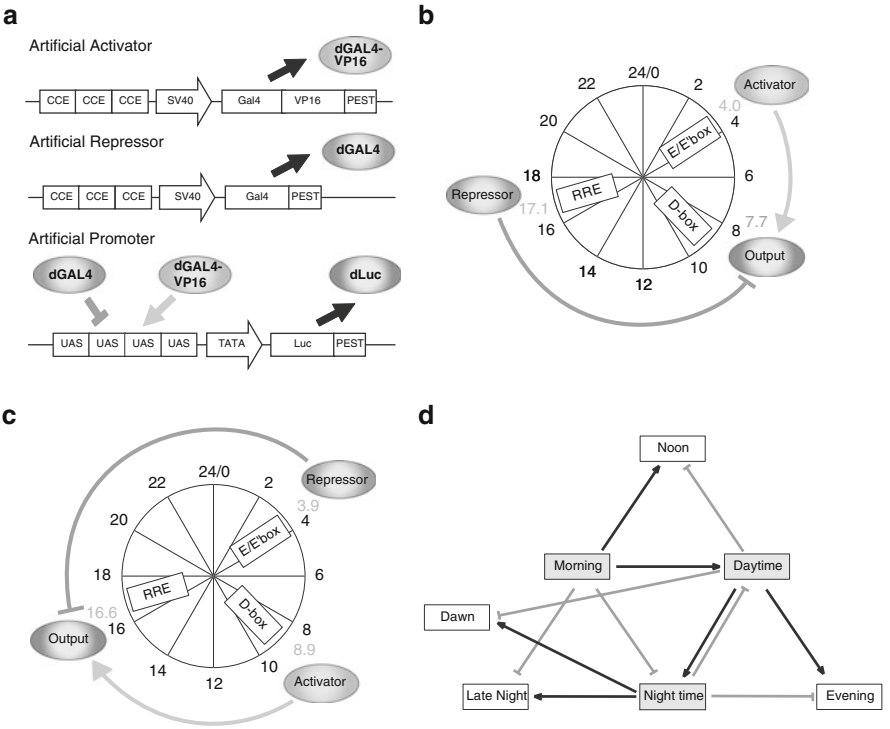

Fig. 7 Combinatorial regulation of circadian phases by a synthetic system. (**a**) The artificial transcription system. Activator and repressor are driven under clock-controlled elements (CCEs). Details are described in main text. (**b**, **c**) Promoter activities of an activator, repressor, and output in different artificial transcriptional circuits. The schemes summarize the representative promoter activities of each artificial circuit monitored by bioluminescence from NIH3T3 cells, where an activator, repressor, and output phases are indicated with their peak time (*gray numbers*). Morning activator under E′-box control and nighttime repressor under RRE control and (**b**) daytime activator under D-box control and morning repressor under E′-box control (**c**). (**d**) The relationship of the expression timings of the transcription factors and output. Various expression timing is generated from three basic phases (morning, daytime, and nighttime). *Black lines* indicate activation (*arrows*) and *gray lines* repression (*arrows with flat ends*). Original figures and graphs are reproduced from Ukai-Tadenuma et al. (2008)

expression. They used three components: an artificial activator (dGAL4-VP16), an artificial repressor (dGAL4), and a dGAL4-VP16-driven reporter gene (d*Luc*) as an output (Fig. 7a). If the expression of artificial activator and repressor are controlled by different CCEs, then the output may vary according to a combination of the various peak timings of each CCE. By taking the peak expression timing of clock gene expression in mouse liver, phase of each CCE-driven gene expression can be related with subjective circadian time: E/E′-box-driven expression peak timing as "morning," RRE-driven expression peak timing as "night," and D-box-driven peak timing as "daytime." They created "daytime" expression by the combination of E/E′-box (morning)-driven activator and RRE (night)-driven repressor (Fig. 7b). This

is similar to transcriptional regulation via D-box control; D-box is activated by E/E′-box-controlled *Dbp* and repressed by RRE-controlled *E4bp4*, and output phase is "daytime." Next, they created "night" by the combination of a D-box-driven activator and an E/E′-box-driven repressor (Fig. 7c). This is similar to an RRE with output phase "night": RRE is regulated by D-box-driven activator (*Rora*) and E/E′-box-driven repressor (*RevErbAa*), though *RevErbAa* is also controlled by D-box. By combining these CCEs in different arrangements, Ukai-Tadenuma, Kasukawa et al. also generated additional phases (Fig. 7d), which are not identical to any of the original CCE timings (Ukai-Tadenuma et al. 2008).

4 Post-Translational Regulation, Another Layer of Delay or Another Oscillator?

4.1 Phosphorylation of PER

As we discussed above, accumulating evidence indicates that Cry1-mediated delayed negative feedback plays a critical role in the circadian transcription network. If so, is the network structure of transcription activator/inhibitor relationship sufficient for generating mammalian circadian properties? If we replace all transcription factors with artificial ones [such as GAL4-VP16 used in Ukai-Tadenuma et al. (2008)] but keep the network structure, could we reproduce a robust circadian system? Natural circadian systems, however, seem to be more complex than the transcription-translation network; post-translational regulation is also critical for circadian function (Gallego and Virshup 2007). In particular, phosphorylation of PERs by CKIδ/ε is one of the determinants of circadian period length (Lowrey et al. 2000; Toh et al., 2001; Xu et al., 2005). The first circadian mutant identified in mammal was the *tau*-mutant hamster, which has a shorter behavioral period length compared to a normal hamster (Ralph and Menaker 1988). Takahashi's group identified the *tau* mutation in the *CKIe* gene and found that PER phosphorylation is lower in *tau*-mutant hamsters (Lowrey et al. 2000). The importance of PER phosphorylation by CKIδ/ε for circadian rhythms is also true in humans. Toh et al. discovered that familial advanced sleep-phase syndrome (FASPS) is caused by a mutation in the CKIδ/ε binding site of PER2 (Toh et al. 2001). Likewise, Xu et al. found that a mutation in CKIδ can also cause FASPS by modulating PER stability (Xu et al. 2005). Additionally, chemical biology approaches identified several compounds that shorten or lengthen circadian period (Chen et al. 2012; Hirota et al. 2008; Isojima et al. 2009). One remarkable example is a series of CKIδ/ε inhibitors, which can lengthen molecular clock period from 24 h to 48 h at the cellular level (Isojima et al. 2009).

How PER phosphorylation controls circadian period is still mysterious, but phosphorylation affects PER stability. PER protein is degraded by proteasome-mediated proteolysis when phosphorylation of PER triggers recruitment of βTrCP,

a subunit of the SCF ubiquitin ligase (Eide et al. 2005; Shirogane et al. 2005). However, the FASPS mutation site is different from the region involved in βTrCP recognition of PER (Eide et al. 2005). Furthermore, several results imply that phosphorylation on FASPS-mutated site stabilizes PER protein (Shanware et al. 2011; Vanselow et al. 2006; Xu et al. 2007). Therefore, phosphorylation may regulate the stability of PER in multiple ways. Recent studies of *Drosophila melanogaster* PER and *Neurospora crassa* FRQ (a functional counterpart of PER) show that multisite phosphorylation induces conformational changes in these proteins (Chiu et al. 2011; Querfurth et al. 2011). A similar case might also be true for mammalian PER: phosphorylation may control the stability of mammalian PER by changing its global structure, not just by creating a recognition site for βTrCP at a specific location.

The stability control of PER also may contribute to delay for transcriptional negative feedback. Unlike other clock genes, the expression peak of *Per1* and *Per2* mRNA is ~4 h earlier than PER1/PER2 proteins (Pace-Schott and Hobson 2002). This delay between mRNA and protein may be one of the determinants of period length.

4.2 Stability Control of CRY in Circadian Oscillations

Recently, researchers noticed that not only PER but also CRY stability is important for clock period. In 2007, two lines of ENU-mutant mice with long behavioral rhythms were reported from different groups—*Overtime* (Siepka et al. 2007) and *Afterhours* (Godinho et al. 2007). Both the *Ovt* and *Afh* mutations are located in the same gene *Fbxl3*. *Fbxl3* encodes an ubiquitin ligase E3 and controls CRY stability by inducing CRY protein ubiquitination and degradation (Godinho et al. 2007; Siepka et al. 2007). Delayed expression of CRY1 could be caused by the combinatorial effect of delayed transcription activation and active degradation. These data suggest that temporal control of clock gene products (like PER and CRY) is also important for generating circadian rhythms. Effects of the $CKI\varepsilon^{tau}$ and $Fbxl3^{Afh}$ mutations are additive and independently contribute to circadian period (Maywood et al. 2011).

4.3 Post-Translational Oscillation of the Mammalian Circadian Clock

Phosphorylation-dependent degradation may be directly related to PER oscillation. Two reports showed that PER2 protein translated from constitutively expressed mRNA undergoes circadian oscillation (Fujimoto et al. 2006; Nishii et al. 2006). These studies imply that a layer of post-translational control blankets the

transcription-translation circadian machinery. Consistent with this idea, several studies have shown that circadian rhythmicity is robust against fluctuations in oscillating transcriptional activity. For example, the expression pattern of *Bmal1* and *Clock* can be constant throughout the circadian cycle (von Gall et al. 2003). Reducing the overall transcriptional activity only modestly affects the period length of circadian rhythms in cultured cells (Dibner et al. 2009). Even in for CRY, rhythmic expression is dispensable for circadian oscillation to a certain extent; weak circadian oscillations can be observed in $Cry1^{-/-}$:$Cry2^{-/-}$cells rescued by *Cry1* under constant expression (Ukai-Tadenuma et al. 2011) or a constant supply of CRY proteins (Fan et al. 2007). Genetic studies in *Drosophila* show that flies with constant expression of PER maintain circadian rhythmicity (Ewer et al. 1988; Frisch et al. 1994; Vosshall and Young 1995; Yang and Sehgal 2001). Taken together, these results suggest that circadian oscillations do not necessarily depend solely on the transcriptional activity in the E/E′-box feedback loop, because post-translational control of clock proteins can compensate for loss of transcriptional rhythms.

A post-translational circadian oscillator was also found in the cyanobacterium circadian clock. Oscillations occur in the phosphorylation state of KaiC, a central component of cyanobacterial circadian clock, even after the termination of global transcriptional activity (Tomita et al. 2005). This KaiC-phosphorylation rhythm can be reconstituted in vitro by mixing KaiC and its regulatory factors KaiB and KaiC together with ATP (Nakajima et al. 2005). In mammals, a recent study discovered the presence of the circadian oscillations in the redox state of enucleated human red blood cells (O'Neill and Reddy 2011). The circadian oscillation in redox status of peroxiredoxin proteins is conserved from prokaryotes to eukaryotes (Edgar et al. 2012) and can regulate the neuronal activity of SCN (Wang et al. 2012). Although a core post-translational circadian oscillator in mammals remains to be identified, cooperation of transcription-translation oscillator and post-transcriptional oscillator would provide a more robust circadian timekeeping system. The investigation of compatible interactions between delayed negative feedback loops mediated by the CCEs and yet-unknown core post-translational oscillators will lead to a new understanding of mammalian circadian clocks.

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