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# Design Principles of Phosphorylation-Dependent Timekeeping in Eukaryotic Circadian Clocks

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The circadian clock in cyanobacteria employs a posttranslational oscillator composed of a sequential phosphorylation–dephosphorylation cycle of KaiC protein, in which the dynamics of protein structural changes driven by temperature-compensated KaiC's ATPase activity are critical for determining the period. On the other hand, circadian clocks in eukaryotes employ transcriptional feedback loops as a core mechanism. In this system, the dynamics of protein accumulation and degradation affect the circadian period. However, recent studies of eukaryotic circadian clocks reveal that the mechanism controlling the circadian period can be independent of the regulation of protein abundance. Instead, the circadian substrate is often phosphorylated at multiple sites at flexible protein regions to induce structural changes. The phosphorylation is catalyzed by kinases that induce sequential multisite phosphorylation such as casein kinase 1 (CK1) with temperature-compensated activity. We propose that the design principles of phosphorylation-dependent circadian-period determination in eukaryotes may share characteristics with the posttranslational oscillator in cyanobacteria.

The circadian clock produces near-24-h rhythms in physiological activities. From multicellular animals to plants, circadian clocks regulate cellular metabolic pathways and other physiological responses of the organism, such as sleep–wake cycles and immune responses (Levi and Schibler 2007; Bass and Takahashi 2010; Mohawk et al. 2012; Albrecht 2013; Greenham and McClung 2015; Lu et al. 2017; Sanchez and Kay 2017). The evolutionary advantage of a circadian clock was demonstrated by coculture experiments with prokaryotic cyanobacteria, in which strains with a circadian period matching the light–dark cycle of their environment became dominant in the population (Woelfle et al. 2004). This advantage should apply to many species; circadian rhythmicity of physiological activities is observed in evolutionarily distant species. Conserved features of the molecular mechanisms that drive circadian clocks in different phyla have been revealed through studies in a variety of organisms, including conventional models such as rodents in mammals, *Drosophila melanogaster* in insects, *Arabidopsis*

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*thaliana* in higher plants, *Neurospora crassa* in fungi, and *Synechococcus elongatus* in cyanobacteria. In all organisms, circadian rhythmicity is cell-autonomous. Even in mammals, various cell types show robust circadian rhythmicity in gene expression and physiological activity, even when cultured in vitro (Welsh et al. 1995; Yagita et al. 2001; Nagoshi et al. 2004; Yoo et al. 2004). Cell-autonomous circadian oscillation is produced by the system-level orchestration of transcription–translation feedback and/or a posttranslational interaction network (Gallego and Virshup 2007; Ukai and Ueda 2010; Millius and Ueda 2017), but the molecular components of the oscillators especially for transcriptional or translational regulators differ between species (Rosbash 2009; Brown et al. 2012; van Ooijen and Millar 2012; Takahashi 2017). However, the question arises whether a common design principle underlies the molecular components of circadian clocks in different organisms.

In most organisms, circadian molecular oscillators seem to be based on transcription–translation feedback loops (TTFLs). However, how circadian clocks maintain a robust 24-h oscillation is a mystery. The suprachiasmatic nucleus, a region of the brain central to circadian rhythm in mammals, can sustain oscillating gene expression for years when cultured in vitro (Yamazaki and Takahashi 2005). However, the periodic gene expression in artificial biological oscillators encoded as TTFLs is less stable when subjected to cell-intrinsic or cell-extrinsic changes (Elowitz and Leibler 2000; Danino et al. 2010). This observation suggests that an additional layer exists in the period-keeping mechanism of circadian cellular oscillators. Here, we review the circadian-clock features in various organisms from the perspective of TTFL-based design principles and posttranslational modifications of oscillator components. Through the comparison between a phosphorylation-based oscillator in cyanobacteria and multisite phosphorylation of clock components that can regulate the circadian period in eukaryotes, we propose that shared design principles can be found in the circadian period-determination mechanisms.

## TRANSCRIPTION NEGATIVE FEEDBACK IN EUKARYOTIC CIRCADIAN OSCILLATORS

Figure 1 summarizes the major molecular components of circadian oscillators in different phyla. Eukaryote oscillators have conserved negative feedback loops in the transcription–translation network (Fig. 1A). The PERIOD protein (PER) is a key transcriptional repressor in mammals and *Drosophila*. PER binds and inhibits a basic helix–loop–helix transcription-activator complex consisting of CLOCK and BMAL1 in mammals (Fig. 1B). In *Drosophila*, PER inhibits a complex of CLOCK (CLK) and CYCLE (CYC), a BMAL1 ortholog (Fig. 1C). The CLOCK–BMAL1 (CLK-CYC in *Drosophila*) complex activates *Per* transcription; thus, the overall transcription network creates a negative feedback loop. PER binds other partners in various organisms to act on transcription activators. In *Drosophila*, PER represses its own transcription by binding TIMELESS (TIM), the vertebrate homolog of which maintains chromosomal integrity (Chou and Elledge 2006; Tanaka et al. 2009). In contrast, mammalian PER forms a complex with CRYPTOCHROME (CRY). The cryptochrome superfamily is widely conserved from bacteria to plants, but nonmammalian cryptochrome functions as a blue-light receptor rather than as a circadian transcription repressor (Lin and Todo 2005; Ozturk et al. 2007). Phylogenetically, the closest homolog of mammalian cryptochrome is 6–4 photolyase, which catalyzes the repair of damaged DNA residues using energy from blue light. These functional divergence of TIM and CRY in maintaining DNA integrity and circadian rhythmicity may be related to the evolutionarily close relationship of DNA-damage responses and circadian clocks in an environment with daily oscillations in sunlight.

The molecular components of circadian negative feedback loops differ between fungi and plants. White Collar complex (WCC) and FREQUENCY (FRQ) are the primary drivers of *Neurospora* circadian clocks (Fig. 1D). WCC activates FRQ transcription, which in turn inhibits WCC and creates the negative feedback loop. The canonical core feedback architecture of the circadian clock in plants is more complex

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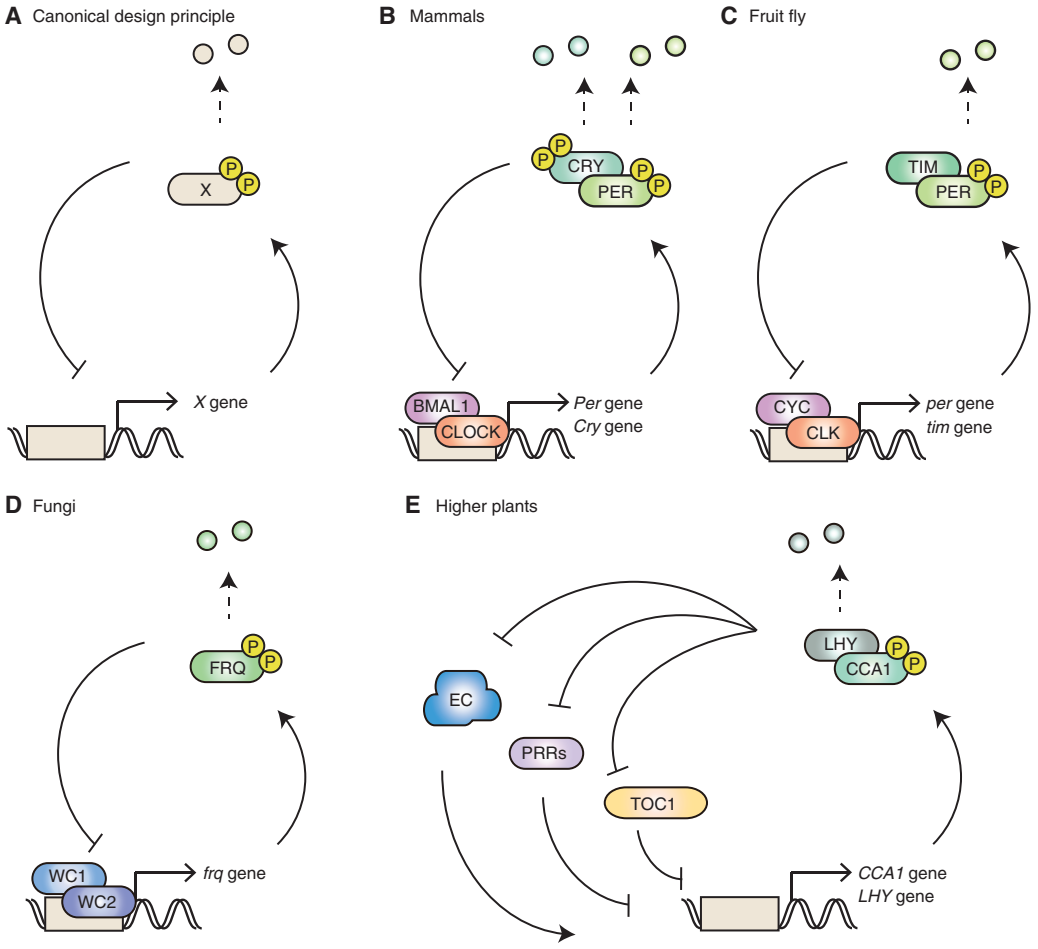


**Figure 1.** Shared structure of transcription–translation feedback loop (TTFL)-based eukaryotic circadian clocks. (*A*) Diagram showing the basic structure of a circadian negative-feedback transcription oscillator. The transcription repressor is regulated by multisite phosphorylation and proteasome-mediated proteolysis. (*B–E*) Major components of the circadian TTFL-based oscillator in each species. Note that the indicated components and pathways were selected based on the context of this review; the actual circadian systems are far more complex reaction networks involving numerous gene products. EC (evening complex) composed of ELF4, LUX, and ELF3.

(Fig. 1E). In *Arabidopsis*, the canonical model involves transcription feedback loops around two transcription repressors, CCA1 and LHY, which suppress the transcription of both activators and inhibitors of CCA1/LHY expression, creating an architecture combining positive and negative feedback.

Theoretical studies emphasize the importance of a negative feedback loop encoded as a TTFL in autonomous oscillation (Novak and Tyson 2008; Kim 2016). In a general sense, the period of TTFL-based oscillators is determined through the dynamics of protein *quantity*: the repression phase is initiated by the accumulation of transcription repressor(s) and ceases when the repressors have disappeared. Indeed, the circadian period is reported to be regulated through the delayed timing (phase) of the accumulation of mammalian transcriptional repressor *Cry* messenger RNA (mRNA) (Ukai-Tadenuma et al. 2011) or *Per* mRNA in mammals and *Drosophila* (Kadener et al. 2008; Fustin et al.

2013). These changes are regulated by the timing of transcription and the mRNA maturation process, independent of the biochemical characteristics of the CRY/PER proteins themselves. However, evidence suggests that the dynamics of mRNA abundance are less important in eliciting rhythmic transcription; noncircadian expression of the *per* gene in *Drosophila* (Ewer et al. 1988; Frisch et al. 1994; Vosshall and Young 1995; Yang and Sehgal 2001) and a constitutive supply of CRY protein in mammals can support circadian oscillation (Fan et al. 2007; Ukai-Tadenuma et al. 2011), indicating the importance of protein-level dynamics in the regulation of circadian clocks. Consistent with this view, rhythmic changes in mammalian PER levels were observed when PER was expressed under a constitutive promoter (Yamamoto et al. 2005; Fujimoto et al. 2006; Nishii et al. 2006).

In protein-level regulation of the circadian period, several theoretical models predict the importance of transcriptional repressor degradation rates (Gerard et al. 2009). In a simplified negative feedback TTFL model such as the Goodwin model, accelerating the degradation rate shortens the circadian period (Forger 2011), and this relationship is observed in model organisms. The degradation rate of core circadian components is fine-tuned by their phosphorylation and subsequent proteasome-dependent degradation. In mammals and *Drosophila*, the circadian period is shortened or lengthened by mutations that destabilize or stabilize PER, respectively (Vanselow et al. 2006; Chiu et al. 2008; Meng et al. 2008; Syed et al. 2011). Mammalian CRY also regulates the circadian period; genetic and pharmacological perturbations that stabilize CRY lengthen the period (Siepka et al. 2007; Hirota et al. 2012; Gao et al. 2013; Hirano et al. 2017), while destabilizing CRY shortens it (Hirano et al. 2013, 2016; Yoo et al. 2013). The causal relationship between CRY degradation and a shorter period was demonstrated by tuning the period through CRY-specific artificial proteolysis (Ode et al. 2017). In *Neurospora*, FRQ stability is also well correlated with the circadian period (Liu et al. 2000; Gorl et al. 2001; Baker et al. 2009; Tang et al. 2009).

Recent findings, however, indicate that period-determination processes can be independent of transcriptional-repressor clearance rates. Deleting a ubiquitin-ligase component leading to the proteolysis of FRQ stabilized FRQ but, surprisingly, had little effect on rhythmic FRQ expression (Larrondo et al. 2015). Several mutant alleles of mammalian CRY1 altered the circadian period but had little effect on CRY1's stability (Ode et al. 2017). One chemical biology study designed a compound that stabilizes CRY1 and shortens the circadian period (Oshima et al. 2015). These studies suggest that the regulation of protein quantity (the rates of protein production and degradation) does not tell the entire story of circadian-period determination, and a question remains about the general applicability of the TTFL model in determining circadian periods (Gallego and Virshup 2007; Blau 2008).

## POSTTRANSLATIONAL CIRCADIAN OSCILLATOR DESIGN PRINCIPLES IN CYANOBACTERIA

The prokaryotic cyanobacterial circadian clock is an example of circadian-period determination independent of protein-quantity dynamics. This clock is driven by the proteins KaiA, KaiB, and KaiC (Ishiura et al. 1998). KaiC represses transcription, and its targets include KaiC itself. Thus, it was proposed that KaiC forms a TTFL (Fig. 2A). However, a subsequent study showed that the circadian clock in cyanobacteria can operate in the absence of global transcription and translational activities (Tomita et al. 2005). Indeed, autonomous circadian rhythmicity in the phosphorylation status of purified KaiC can be reconstituted by incubating a mixture of KaiC, KaiA, and KaiB with ATP in vitro (Nakajima et al. 2005). Thus, KaiC provides a posttranslational oscillator (PTO) as well as a TTFL-based negative-feedback architecture. The coupling of PTO and TTFL-based oscillators appears to be important for cyanobacteria circadian clocks, depending on growth conditions (Kitayama et al. 2008; Teng et al. 2013). PTO rhythmicity requires fine-tuning the expression of KaiA, KaiB, and KaiC at specific ratios for oscillation in vitro (Nakajima et al. 2010). Their expression

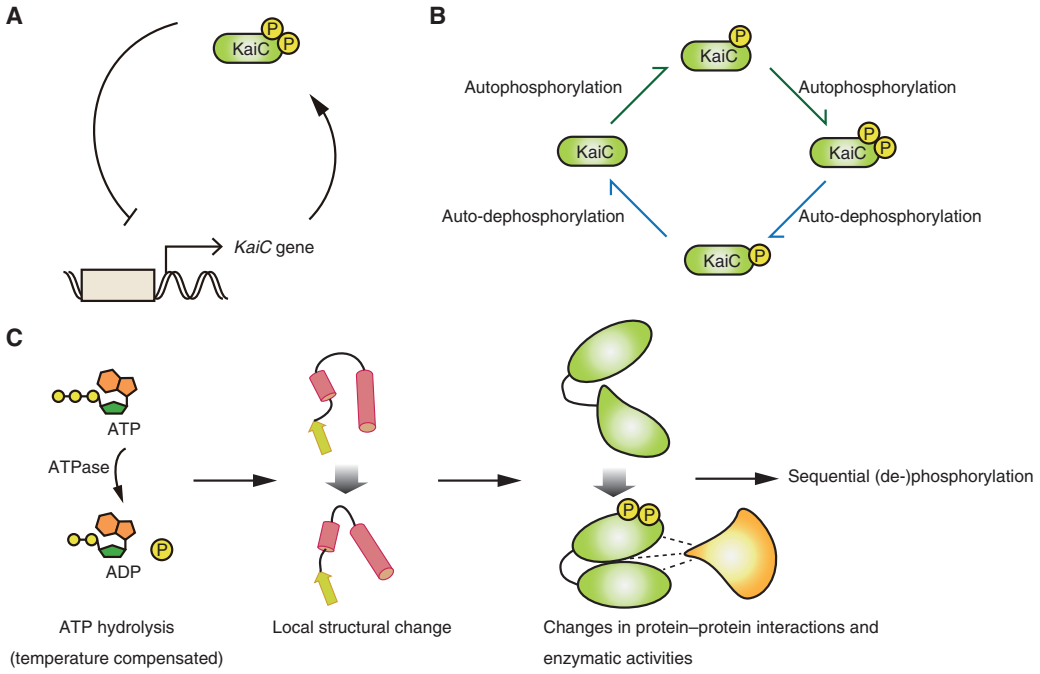


**Figure 2.** The KaiA/B/C-dependent posttranslational circadian oscillator in cyanobacteria. (*A*) KaiC represses its own transcription, and thus forms a transcriptional negative-feedback loop. Although every reaction should be reversible, biased reaction rates between antagonizing kinase/phosphatase reactions are required to create a posttranslational oscillator (PTO). (*B*) A mixture of KaiA/B/C with ATP can reconstitute a PTO in vitro. (*C*) The period-determining step of the KaiC oscillator is temperature-insensitive ATP hydrolysis, which induces a structural change in KaiC.

is also regulated by translational efficiency: nonoptimal codon usage in kaiBC genes tunes the amplitude of the circadian clock in response to growth conditions (Xu et al. 2013). However, detailed analysis of a reconstituted KaiC oscillator revealed a mechanism in which protein function itself creates the circadian oscillation and determines 24-h rhythmicity.

KaiC is biochemically unique. It functions as a homohexamer and has autophosphorylation, autodephosphorylation, and ATPase activities. KaiC has two sites that are autophosphorylated and autodephosphorylated in a defined order, such that KaiC undergoes four distinct phosphorylation states over the course of the circadian cycle (Fig. 2B) (Nishiwaki et al. 2007; Rust et al. 2007). Each phosphorylation state has a distinct affinity to KaiA and KaiB. In turn, KaiA and KaiB regulate KaiC's autophosphorylation and autodephosphorylation to autonomously switch between a "daytime" KaiC complex with autophosphorylation activity and a "nighttime" complex with autodephosphorylation activity. These switches include dynamic structural alterations in a flexible loop domain in KaiC (Chang et al. 2011; Tseng et al. 2014) and a fold switch in the KaiB structure (Chang et al. 2015).

The force that drives KaiC status switching is provided through KaiC-intrinsic ATP hydrolysis and ADP/ATP exchange. The ATP-hydrolysis rate is relatively slow (~10 ATP molecules per day), is well correlated with the circadian period in vivo for several period-modulating mutants (Terauchi et al. 2007), and is unaffected by changes in incubation temperature (Terauchi et al. 2007; Murakami et al. 2008). Thus, in cyanobacteria, temperature-insensitive ATPase activity is proposed to support temperature compensation of the circadian period, a mech-

anism for maintaining a constant circadian period irrespective of environmental temperature. The crystal structure of KaiC reveals that the ATP-hydrolysis rate is determined by the position of ATP's phosphorus atom and a water molecule (Abe et al. 2015). The unfavored position of the water molecule slows down ATP hydrolysis, which is coupled to an alternation of KaiC's structure at ATP-binding surfaces, further slowing ATP hydrolysis (Abe et al. 2015) and driving a structural change in KaiC that induces it to interact with KaiA/B (Snijder et al. 2017; Tseng et al. 2017).

Based on the KaiC oscillator mechanism, we can deduce an abstractive design principle of the posttranslational circadian oscillator (Fig. 2C): energy from temperature-compensated ATP hydrolysis induces local structural alterations that induce biochemical changes in protein–protein interactions, enzymatic activities, and modification patterns. Transitions between protein states have a significant energy barrier that determines the length of the circadian period. In this scenario, the circadian period is not determined by the dynamics of protein *quantity*, but rather by the dynamics of status change or the *quality* of each protein molecule.

## MULTISITE PHOSPHORYLATION DETERMINES THE PERIOD OF EUKARYOTIC CIRCADIAN CLOCKS

Although transcription-independent circadian oscillation marked by cellular redox states has been found in eukaryotic cells (O'Neill and Reddy 2011; O'Neill et al. 2011; Edgar et al. 2012; Reddy and Rey 2014), it is not known whether components in TTFL-based eukaryotic circadian oscillators can elicit posttranslational oscillation. If you recall, however, that the cyanobacteria circadian clock adopts a combination of PTO and TTFL-based oscillators, it may be useful to consider that some part of the design principles observed in the cyanobacteria circadian PTO might be also applicable to the regulation of eukaryotic circadian oscillators.

A PTO can theoretically be constructed using a generic kinase, phosphatase, and substrate (Jolley et al. 2012) with a similar oscillation mechanism to that proposed through the modeling of the KaiA/B/C oscillator (Fig. 3A) (Clodong et al. 2007; Rust et al. 2007; van Zon et al. 2007). A PTO constructed with a kinase, phosphatase, and substrate protein (assuming that processes are catalyzed in the Michaelis–Menten scheme) must use a substrate that is phosphorylated at multiple sites, because reversible phosphorylation at one site alone cannot produce autonomous oscillation in the substrate's phosphorylation states (Angeli and Sontag 2008; Conradi and Shiu 2015). Interestingly, various lines of evidence show that several components of the eukaryotic circadian oscillators are also phosphorylated at multiple sites (Fig. 1), and that such multisite phosphorylation is critical for determining the oscillation period. Indeed, as discussed in the following sections, several features of design principles found in cyanobacterial PTO can be also found in the eukaryotic circadian substrates of the multisite phosphorylation and kinases catalyzing the phosphorylation.

### CK1, CK2, and Glycogen Synthase Kinase (GSK)3β

Casein kinase 1 (CK1) is a conserved component that markedly regulates the circadian-clock period in mammals and *Drosophila*. CK1ε was first characterized as a period-controlling kinase coded by the *Drosophila* gene *doubletime*, mutations of which shorten or lengthen the circadian period (Kloss et al. 1998; Price et al. 1998). CK1ε was subsequently identified as the molecule responsible for the shorter circadian period found in *tau* mutant hamsters (Lowrey et al. 2000). Another CK1 isoform, CK1δ, was identified as a mutation associated with a familial advanced sleep-phase syndrome (FASPS) pedigree (Xu et al. 2005). The genetic evidence for CK1's contribution to period determination was further supported by high-throughput screening of small molecules or short interfering RNAs (siRNAs), which identified chemicals/siRNAs that lengthen the circadian period by inhibiting CK1δ/ε (Hirota et al. 2008, 2010; Isojima et al. 2009; Zhang et al. 2009; Chen et al. 2012). Screening revealed that GSK3β, casein kinase 2 (CK2), and CK1α also control the cir-

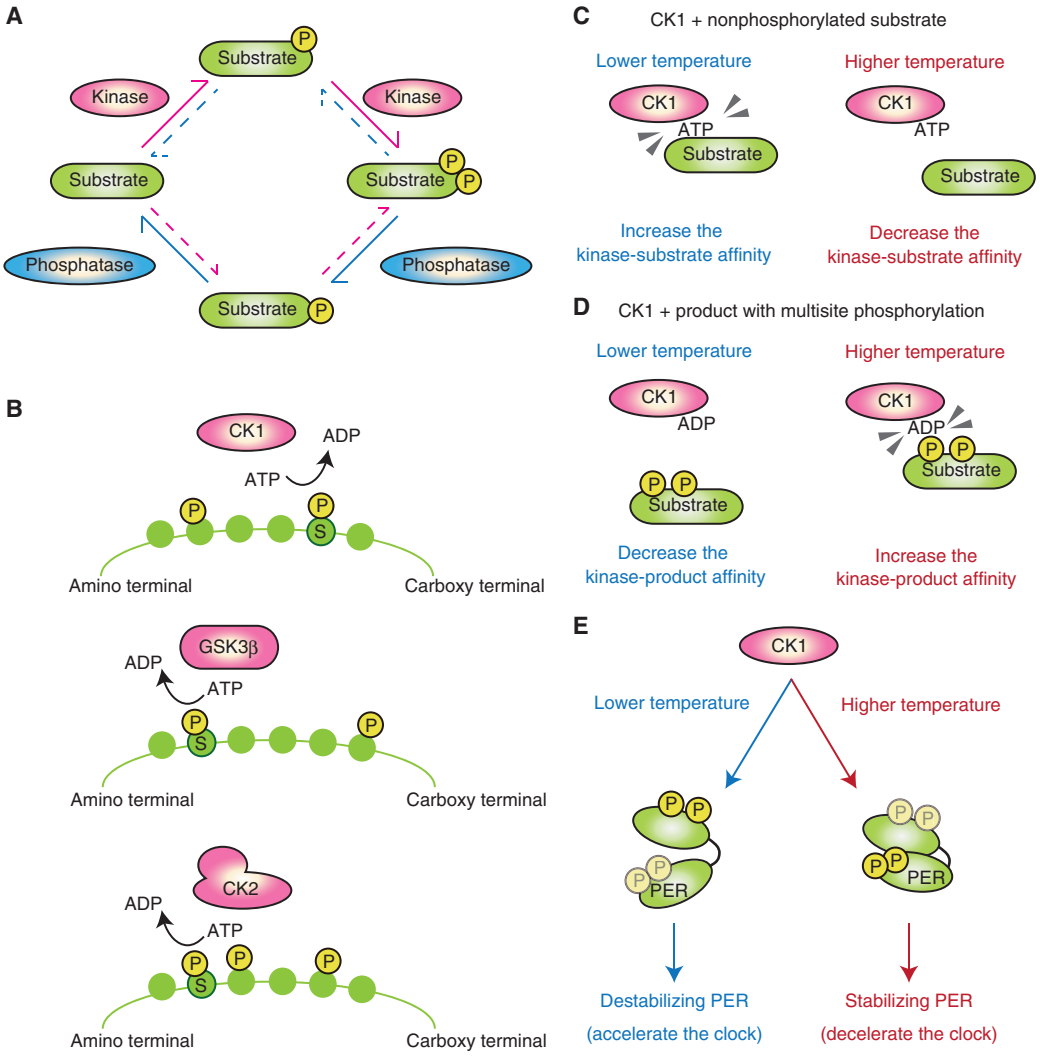


**Figure 3.** Multisite phosphorylation is the core mechanism of period determination in eukaryotic circadian clocks. (*A*) Multisite phosphorylation is a prerequisite for forming a posttranslational oscillator (PTO) from a generic kinase, a phosphatase, and a substrate. (*B*) The kinases responsible for period determination preferentially promote sequential phosphorylation. "S" denotes the target residues for phosphorylation. (*C–D*) Ueda and his colleagues found that raising the temperature decreases casein kinase 1 (CK1)'s affinity for the substrate (Shinohara et al. 2017). Raising the temperature also prompts CK1 to bind the phosphorylated product. These effects decrease the turnover number and thus decelerate the enzyme's reaction rate. (*E*) Virshup, Forger, and their colleagues (2015) found that raising the temperature promotes PERIOD protein (PER) phosphorylation by CK1, which stabilizes PER and thereby lengthens the period (Zhou et al. 2015).

cadian period (Hirota et al. 2008, 2010; Maier et al. 2009; Zhang et al. 2009). Although other kinases such as AMP-activated protein kinase (AMPK), calmodulin-dependent protein kinase II (CaMKII), and mitogen-activated protein kinase (MAPK) regulate circadian clocks by incorporating various cellular and environmental signals (Gallego and Virshup 2007; Reischl and Kramer 2011), the importance of CK1, CK2, and GSK3β in period determination is highlighted both by screening studies and in the conservation of their roles across species. Among

these kinases, CK1 may have one of the most prominent effects on the circadian period because the inhibition of CK1δ/ε can double the period up to 48 h (Isojima et al. 2009) and because CK1δ/ε-dependent phosphorylation in vitro and CK1δ/ε-dependent degradation in cellulo are both temperature-compensated (Isojima et al. 2009).

Kinases can phosphorylate multiple sites on a substrate in a random manner, phosphorylating each site with a random access, or in a sequential manner in which the kinase phosphorylates the target sites in a specific order (Yamada and Forger 2010). Intriguingly, CK1, GSK3β, and CK2 undergo sequential phosphorylation (Fig. 3B). CK1's substrate consensus sequence is pS/T-X-X-S/T (pS/T denotes phosphorylated serine or threonine, X denotes any amino acids, and S/T denotes the target residue). The CK1 kinase preferentially phosphorylates residues downstream of prephosphorylated residues (Flotow et al. 1990). The consensus sequence of GSK3β is S/T-X-X-X-pS/T (Frame and Cohen 2001), and that of CK2 is S/T-X-X-D/E, where substituting pS into the +1 and +3 positions optimize the phosphorylation efficiency (St-Denis et al. 2015). Thus, all three of these important period-controlling kinases preferentially phosphorylate residues near prephosphorylated residues. When a kinase with this property acts on multiple and clustered target residues, phosphorylation events preferentially follow a defined order. For instance, CK1 preferentially phosphorylates sites sequentially, from the peptide amino terminus toward the carboxy terminus. This sequential phosphorylation also makes it easy to enrich local protein regions with phosphorylated residues.

## PER

Transcription repressors in the eukaryotic circadian oscillator are regulated through multisite phosphorylation, catalyzed at least partly by the above-mentioned kinases. PER in fly and mammals is among the best-understood substrates of CK1δ/ε (DBT in *Drosophila*). PER is progressively phosphorylated as the circadian cycle progresses (Edery et al. 1994; Lee et al. 2001). The mammalian PER sequence contains a repeat of the CK1 motif. Once the amino-terminal side of the S-X-X-S repeat is phosphorylated (i.e., priming phosphorylation), CK1 can efficiently phosphorylate the downstream serine cluster (Vanselow et al. 2006). A mutation on the priming phosphorylation site, found in a FASPS pedigree (Toh et al. 2001), destabilizes the PER protein (Vanselow et al. 2006), indicating that phosphorylation at this site helps stabilize PER and regulate the circadian period. The kinase responsible for the priming site is unclear, but CK1δ/ε may be able to act on the nonphosphorylated PER-derived sequence (Isojima et al. 2009). Another PER phosphorylation domain is important for proteasome-dependent PER degradation (Eide et al. 2005; Shirogane et al. 2005; Shanware et al. 2011; Zhou et al. 2015). CK2 also controls the period of the mammalian circadian clock by phosphorylating PER (Tsuchiya et al. 2009). In *Drosophila*, DBT phosphorylates PER at multiple sites (Chiu et al. 2008). DBT-dependent multisite phosphorylation is primed by NEMO kinase (Chiu et al. 2011; Yu et al. 2011). In addition, CK2 (Lin et al. 2002; Akten et al. 2003; Nawathean and Rosbash 2004) and GSK3β (Martinek et al. 2001; Fang et al. 2007; Ko et al. 2010; Hara et al. 2011) regulate the action of *Drosophila* PER, although the effect is probably mediated partly through the phosphorylation of TIM bound with PER (Top et al. 2016).

## CRY

Mammalian CRY also has multiple phosphorylation sites (Lamia et al. 2009; Gao et al. 2013; Ode et al. 2017) targeted by various kinases, including CK1δ/ε (Eide et al. 2002; Qin et al. 2015; Ode et al. 2017) and GSK3β (Kurabayashi et al. 2010). The multisite phosphorylation of CRY plays a critical role in period determination since combinatorial mutations in the phosphorylation sites results in the twofold change of the period length from 19 to 38 h (Ode et al. 2017). CRY phosphorylation sites appear to be concentrated in the P-loop and carboxy terminus, and mutations in several phosphorylation sites in these regions significantly affect the circadian period (Gao et al. 2013; Liu and Zhang 2016;

Ode et al. 2017). Furthermore, a recent study identified a FASPS pedigree CRY mutation in which an alanine in the P-loop region is replaced by threonine (Hirano et al. 2016). This substitution might create a de novo phosphorylation site around the P-loop that modulates the circadian period. Indeed, the threonine-substitution phenotype can be recapitulated by aspartic-acid substitution, which mimics the phosphorylated residue (Hirano et al. 2016).

### FRQ

Like PER, FRQ is progressively phosphorylated during the circadian cycle (Garceau et al. 1997) at multiple sites, many of which are presumably phosphorylated by CK1 and CK2 (Baker et al. 2009; Tang et al. 2009). CK1 stably binds FRQ and is responsible for its clustered phosphorylation (He et al. 2006; Baker et al. 2009; Querfurth et al. 2011). CK2 also directly phosphorylates FRQ (Yang et al. 2003; Mehra et al. 2009).

### CCA1

In *Arabidopsis*, CCA1 is phosphorylated by CK2 to obtain the proper circadian period (Sugano et al. 1998, 1999; Daniel et al. 2004). Depleting CK2 or increasing its activity lengthens or shortens the circadian period, respectively (Sugano et al. 1999; Lu et al. 2011). Although the kinases responsible for phosphorylating CCA1 are not as well understood as those in other conventional model organisms, several circadian proteins exhibit circadian variations in their phosphorylation state (Fujiwara et al. 2008), suggesting that multisite phosphorylation is critical in regulating the circadian period in plants (Seo and Mas 2014). A study using the unicellular plant *Ostreococcus tauri* revealed that pharmacological inhibition of CK1 markedly lengthens the period over 8 h, suggesting that the central role of CK1 in determining the circadian period is conserved in the plant kingdom (van Ooijen et al. 2013).

## Temperature Compensation

CK1 and CK2 and their substrates are important for the temperature compensation that preserves the circadian period. Mammalian CK1 is linked to temperature compensation (Tosini and Menaker 1998; Isojima et al. 2009; Zhou et al. 2015), and CK2 is at least partly responsible for the temperature compensation in *Neurospora* and higher plants (Mehra et al. 2009; Portoles and Mas 2010).

In general, increased temperature accelerates biochemical reactions such as phosphorylation and protein degradation, thus raising the temperature should accelerate clock speed. However, a previous study indicated that CK1δ/ε kinase activity markedly influences the speed of mammalian circadian clocks and is largely insensitive to temperature changes in vitro (Isojima et al. 2009). A subsequent study revealed that temperature-insensitive kinase activity is achieved through CK1δ/ε's ability to bind substrate and phosphorylated products in a temperature-dependent manner (Shinohara et al. 2017). When CK1 acts on a substrate with a single phosphorylation site, which may be similar to the reaction step for catalyzing the priming phosphorylation site, raising the temperature decreases the affinity between the kinase and the substrate to be phosphorylated (Fig. 3C). In addition, when CK1 acts on a substrate with multiple phosphorylation sites, raising the temperature increases the affinity between the kinase and the phosphorylated product at multiple sites in the presence of ADP, reducing the turnover number of CK1 action (Fig. 3D). These temperature-sensitive affinity alterations reduce substrate binding and product release at higher temperatures. Therefore, the net kinase-reaction rate stays nearly constant despite temperature changes. Ueda and his colleagues successfully conferred temperature insensitivity on another temperature-sensitive kinase protein by introducing an evolutionarily conserved CK1-specific domain that seems to bind to substrates and phosphorylated product.

The presence of a temperature-insensitive kinase such as CK1 can provide another layer of regulation to achieve the circadian temperature compensation. Detailed analysis of the degradation kinetics of mammalian PER showed that raising the temperature stabilizes PER, which were proposed by the combination of

temperature-insensitive and temperature-sensitive kinases (Zhou et al. 2015). At higher temperatures, CK1δ/ε preferentially phosphorylates PER on the PER-stabilizing domain (which contains the site of the mutation found in the FASPS pedigree), while at lower temperatures, the CK1δ/ε preferentially phosphorylates PER on the PER-destabilizing domain (Fig. 3E). Thus, increasing the temperature decelerates the PER degradation, slowing down the pace of the circadian clock. The similar temperature-dependent stability mechanism was also proposed in *Neurospora* FRQ. A study proposed that increasing the temperature accelerates FRQ stabilization and counteracts any temperature-dependent acceleration of the circadian clock (Mehra et al. 2009). This balance can be modulated by mutations of either CK2 or the CK2-phophorylation site on FRQ. These studies indicated that temperature-dependent kinase activity counteracts the canonical acceleration of clock speed in response to a rise in temperature by modulating the substrate degradation rate.

To summarize, the CK1δ/ε kinase reaction itself can be insensitive to temperature changes, providing the mechanism of temperature compensation at the systems' component level. This temperature-insensitive kinase together with other temperature-sensitive kinases (or probably phosphatases) may provide the system-level tuning of FRQ/PER proteolysis, which further compensates the effect of temperature changes. These mechanisms are not mutually exclusive in circadian clocks (Hogenesch and Ueda 2011); however, the former model suggests that temperature-insensitive reactions are not exclusive to the circadian substrate. Rather, if the affinity of CK1δ/ε for phosphorylated products is important for temperature-insensitive kinase reactions, such temperature-compensated reactions may also function in other biological processes. In a study of temperature-compensated budding yeast metabolic oscillation, the oscillation period was sensitive to CK1 and GSK3β (Causton et al. 2015). Indeed, a budding yeast homologue of CK1 also preserves temperature-compensated kinase activity (Shinohara et al. 2017). Therefore, the CK1 enzyme might confer robust biochemical reaction rates on circadian clock and other systems to protect against environmental temperature fluctuations.

## MULTISITE PHOSPHORYLATION AT FLEXIBLE PROTEIN REGIONS

The multisite phosphorylation process itself may contribute to period determination independent of the regulation of protein degradation. In a recent study in *Neurospora*, mutations in FRQ phosphorylation sites strongly affected the circadian period even in a genetic background in which FRQ's stability was unchanged (Larrondo et al. 2015). In another study, a series of phosphorylation-site mutants of mammalian CRY severely affected the circadian period, even though CRY's stability was only marginally affected (Ode et al. 2017). Furthermore, a deletion-mutant CRY found in delayed sleep-phase disorder extends the circadian period without a detectable change in the stability of the CRY protein (Patke et al. 2017). In plants, CK2 phosphorylation regulates CCA1 transcription without affecting the amount of CCA1 protein (Portoles and Mas 2010).

KaiC studies strongly suggest that circadian clock speed is encoded as the time necessary for changes in protein structure. On the other hand, a common feature of other circadian-period regulatory processes is that the period is largely determined by multisite phosphorylation by kinases that elicit clustered and sequential phosphorylation, although the events downstream of the phosphorylation may vary among circadian systems. Are there any links connecting the design principles of the KaiC oscillator and multisite phosphorylation-based oscillation? Serial enzymatic reactions such as multisite phosphorylation can produce cooperative responses and a sufficient delay to produce fully modified substrates, and can potentially determine period length (Salazar and Hofer 2009; Salazar et al. 2010; Ferrell and Ha 2014). Studies suggest that even apart from its time-related characteristics, multisite and clustered phosphorylation might be able to strongly modulate protein structure.

Multisite phosphorylation often affects protein structure and function by acting on intrin-

sically disordered regions (IDRs) of substrate proteins (Valk et al. 2014; Wright and Dyson 2015). In IDRs, the polypeptide structure is highly flexible, not rigid as in an α-helical conformation. IDRs tend to be located at the protein surface where they are accessible to kinases. Indeed, statistical analyses of proteomics data indicated that phosphorylation sites and that IDRs are located close together (Iakoucheva et al. 2004; Xie et al. 2007), and IDRs are often regulated by phosphorylation (Holt et al. 2009; Tyanova et al. 2013; Sharma et al. 2014). Multiple phosphorylations on IDRs dramatically alter the bulk electrostatic potential of the region and may induce dynamic structural changes (Fig. 4A) (Theillet et al. 2014). When the flexibility of an IDR strongly affects intramolecular interactions, multisite phosphorylation can change the overall structure of the proteins themselves. If the IDR is located at the interface of protein–protein interactions, such structural changes could affect binding affinity.

Multisite phosphorylations on IDRs may have a critical role for circadian-period control. It has been suggested that FRQ is an intrinsically disordered protein (IDP) (Hurley et al. 2013). Multisite phosphorylation changes the structure of FRQ (Querfurth et al. 2011), and the nonphosphorylated FRQ is proposed to have a compact form through an interaction between the basic amino-terminal domain and the acidic carboxy-terminal domain. Multisite phosphorylation of the amino-terminal domain loosens the interaction, and the FRQ becomes more sensitive to limited protease digestion. The multisite phosphorylation on PER appears to be similar to FRQ. Although PER contains functionally important structured domains, such as Par-Arnt-Sim (PAS) domains and the CRY-binding domain (Hennig et al. 2009; Kucera

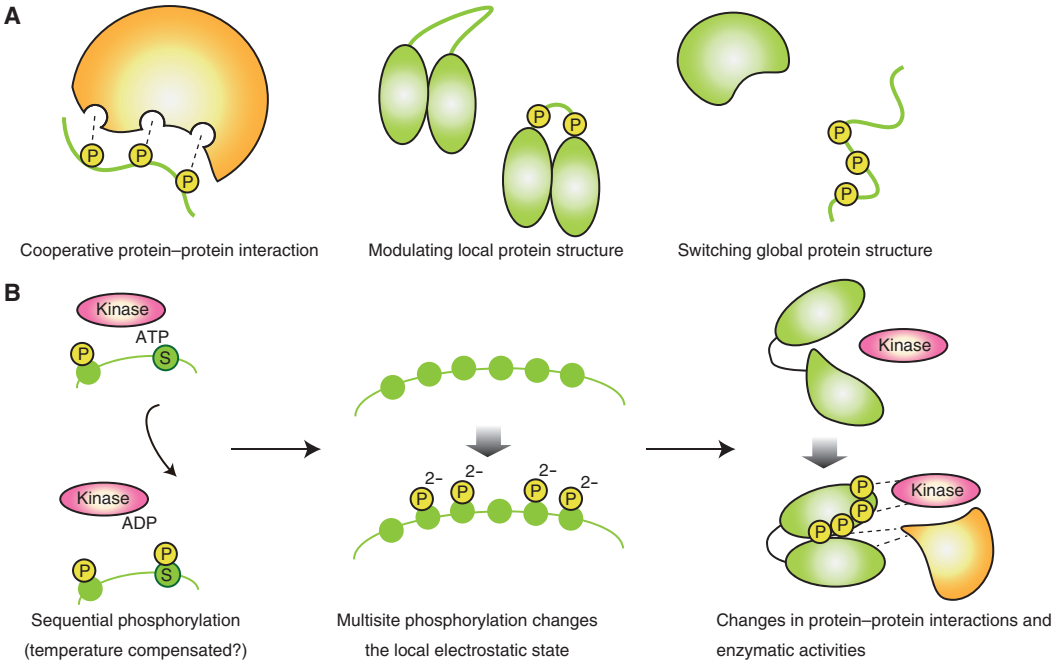


Figure 4. Multisite phosphorylation affects protein structure and function. (*A*) Multisite phosphorylation, particularly around the flexible protein regions, affects protein–protein interactions and local/global protein structure. (*B*) The multisite phosphorylation process could be a period-determining step in eukaryotic circadian clocks. Reaction rates of the kinases responsible for these events can be temperature-insensitive. This abstractive principle may be compatible with the design principle of cyanobacterial circadian-period determination (see Fig. 2C).

et al. 2012; Nangle et al. 2014), most of the other regions are predicted to be disordered (Gustafson and Partch 2015). In both mammalian and *Drosophila* PER, phosphorylation states control the PER global structure, as indicated by an altered susceptibility to protease treatment (Chiu et al. 2011; Zhou et al. 2015). In contrast to FRQ and PER, the structure of the photolyase homology domain of cryptochrome superfamily proteins is well conserved, indicating that CRY is not an IDP. Nonetheless, clustered phosphorylation sites that are critical for controlling the circadian period are located near the flexible P-loop (Hirano et al. 2016; Liu and Zhang 2016; Ode et al. 2017). Multisite phosphorylation appears to additively regulate the circadian period, and the number of phosphorylation sites around this loop domain may serve as a cumulative timer for the circadian period (Ode et al. 2017). These lines of evidence suggest that structural changes in flexible regions, elicited by multisite phosphorylation, are a critical step in determining the circadian period.

Although the detailed mechanisms of period-determination remain unknown, the outcome of phosphorylation-dependent protein structural change could be coupled to TTFL-based architecture via proteolysis (Chiu et al. 2011; Zhou et al. 2015). Changes in the multiple phosphorylation state would also affect the protein–protein interaction. We further point out that the substrate with multiple phosphorylation sites can reciprocally regulate the action of kinases such as CK1. For example, PER protein alters the CK1's substrate preference (Qin et al. 2015). Anionic small molecules and phosphorylated peptides can bind to the allosteric site of CK1 and thereby modulate the CK1's enzymatic activity (Shinohara et al. 2017). These substrate–CK1 interactions may imply that phosphorylation events in a stable protein complex, including CK1, PER, and CRY, should not be interpreted as a simple mixture of kinases and substrates. Instead, the components in a kinase–substrate complex cooperatively regulate the process of multiple phosphorylation, providing the temperature-compensated and accurate period-determining information for the eukaryotic circadian clocks.

## CONCLUDING REMARKS

The importance of multisite phosphorylation and structural changes in eukaryotic circadian-period control remind us of the unsolved questions: What is the source of the 24-h period? What are the rate-limiting steps? Why is the circadian period is so slow (a scale of minutes) compared with biochemical events in general (a scale of seconds), but still accurate (Akiyama 2012). Studies of the KaiC oscillator revealed a structural basis for slow kinetics (Abe et al. 2015). In contrast, a structural basis for its accuracy remains unknown. Although transcription–translation dynamics are at the core of noncyanobacterial circadian clocks, we may well find phosphorylation-dependent structural changes at the core of timekeeping mechanisms in these organisms. In addition, a recent study proposed that the slow *cis*/*trans* isomerization of specific peptide bonding in BMAL1 is a timekeeping mechanism in mammalian circadian clocks (Gustafson et al. 2017). Such rate-limiting events, encoded as protein structures, could be induced directly (via control of the local electrostatic state) or indirectly (via protein–protein interactions) through multisite phosphorylation. Furthermore, the temperature-compensation property of reaction speed could be encoded via multisite phosphorylation by CK1 (Isojima et al. 2009; Shinohara et al. 2017).

From this perspective, the circadian timekeeping mechanism in the KaiC protein oscillator and eukaryotic circadian oscillator may converge as the progressive regulation of protein structure powered by temperature-compensated enzymatic activities—KaiC's ATPase activity and CK1's phosphorylation activity (Fig. 4B), thus encoding circadian-period determination as a *quality* rather than *quantity* of protein dynamics (Larrondo et al. 2015; Ode et al. 2017). The rate-limiting step should be encoded as a protein structural property to keep the period length constant regardless of fluctuations in expression (Dibner et al. 2009), especially for low-abundance circadian-transcription factors (Forger and Peskin 2005; Narumi et al. 2016). To evaluate this perspective, we must execute a structure-based analysis of the clock proteins,

and reconstruct and design circadian timekeeping steps in eukaryotic circadian systems.

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