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Structure, function, and mechanism of the core circadian clock in cyanobacteria

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Abbreviations: TTFL, transcription-translation feedback loop; PTO, post-translational oscillator; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PsR, pseudo-receiver; HK, histidine kinase; RR, response regulator

Abstract

Circadian rhythms enable cells and organisms to coordinate their physiology with the cyclic environmental changes that come as a result of Earth's light/dark cycles. Cyanobacteria make use of a post-translational oscillator (PTO) to maintain circadian rhythms, and this elegant system has become an important model for the post-translational timing mechanisms. Comprised of three proteins, the KaiABC system undergoes an oscillatory biochemical cycle that provides timing cues to achieve a 24-hour molecular clock. Together with the input/output proteins SasA, CikA and RpaA, these six gene products account for the *timekeeping*, *entrainment* and *output signaling* functions in cyanobacterial circadian rhythms. This review summarizes the current structural, functional and mechanistic insights into the cyanobacterial circadian clock.

Form and Function of Circadian Clocks

Circadian rhythms are processes through which cells and organisms predict and adapt to the repetitive environmental changes incident to the 24-hour solar day. These rhythms originate intracellularly and persist in the absence of external cues to orchestrate temporal organization of physiology (1). To achieve this, circadian clocks must perform three distinct but necessarily

intertwined functions: *timekeeping*, *entrainment* and *output signaling*.

Timekeeping is achieved through a cycle of slow biochemical processes that together set the period of oscillation close to 24 hours. For this temporal information to be relevant, biological clocks must appropriately synchronize with their environment through *entrainment*. Temporal information runs from the timekeeping apparatus to the rest of the cell through *output signaling* mechanisms that impart circadian changes in physiology.

Timekeeping mechanisms that have been observed in circadian clocks are broadly categorized as transcription-translation feedback loops (TTFLs) or post-translational oscillators (PTOs) (2). TTFLs achieve oscillation via delayed negative feedback, where a gene product represses its own expression when its levels become high enough. This results in an oscillation in the expression level of the gene, with transitions between repression and derepression occurring at critical points in the oscillatory cycle. By contrast, PTOs utilize timekeeping mechanisms that are independent of transcription. Like TTFLs, PTOs involve a cycle of biochemical processes; but instead of being driven by changes in expression levels, their behavior is controlled by post-translational modifications, conformational

changes, protein-protein interactions and/or subcellular localization. It is important to note that while we make a distinction here between TTFLs and PTOs as general categories of negative feedback systems, many circadian clocks make use of both transcription/translation as well post-translational timing steps to constitute a biochemical oscillator.

Post-translational steps in circadian clocks present unique biochemical challenges and opportunities because they link dynamic molecular processes to biological phenotype through covalent and conformational changes in protein structure. PTOs can operate at constant protein concentrations, and are therefore amenable to *in vitro* study. In the cyanobacterial Kai (*Japanese*, 回, “cycle”) system, an entire PTO consisting of only three protein gene products (KaiA, KaiB and KaiC; or KaiABC) can reconstitute circadian biochemical oscillation *in vitro* (3). As a result, this system has become an important model for understanding how discrete biochemical steps are integrated to give rise to precise biological timing.

Circadian Biology in Cyanobacteria

Circadian rhythms are essentially ubiquitous in eukaryotes, but far fewer instances have been documented in prokaryotes (4). Cyanobacteria temporally segregate photosynthesis and nitrogen fixation, which require mutually exclusive oxidative environments (5). Evolutionary pressures such as this resulted in a cyanobacterial transcriptome in which at least one third of transcripts undergo circadian variation in accumulation levels (6) and active transcription of nearly all transcripts appears to be under circadian control (7). These changes in transcriptional output are linked to global changes in chromosome ultrastructure (8,9), but are controlled basally by a core biochemical oscillator (3,10).

The KaiABC gene cluster was originally identified in genetic screens aimed at finding TTFLs involved in cyanobacterial circadian regulation, with deletion resulting in an arrhythmic phenotype (10). It was subsequently shown that the products of this gene cluster can recapitulate circadian biochemical oscillation *in vitro* (3), and that this circadian oscillation persists *in vivo* even when transcription and translation are inhibited (11). Its ability to be reconstituted *in vitro* demonstrates that KaiABC can act as a self-

sustained PTO. *In vivo*, this PTO also functions within a TTFL framework; i.e. circadian variation of clock protein expression resulting in a more robust rhythm (4,12-14). Together with three other effector proteins, the KaiABC oscillator comprises a self-contained minimal system that is capable of integrating *timekeeping*, *entrainment* and *output signaling* processes into the cyanobacterial cell.

Domain structures of the six core clock proteins in cyanobacteria are shown in Figure 1. Genetic studies on cyanobacterial rhythms have made extensive use of the mesophilic cyanobacterium *Synechococcus elongatus*, from which these genes were originally identified (10). Biophysical studies have often taken advantage of proteins produced from homologous gene sequences in the thermophilic variant *Thermosynechococcus elongatus* for their improved solubility and *in vitro* behavior (15). The six proteins shown in Figure 1 are conserved between *S.* and *T. elongatus*, and appear to comprise a core unit capable of accounting for all of the required functions of a circadian clock. Several additional genes have been identified that play roles in the circadian rhythms of cyanobacteria (16-21), however this review will focus on the six best-characterized gene products and the mechanisms through which they mediate *timekeeping*, *entrainment* and *output signaling* functions in cyanobacterial circadian rhythms. For a broader review on circadian biology in cyanobacteria, see references (4,22).

Structure and Function of KaiC

The core cyanobacterial clock is centered around the hexameric ATPase KaiC, which achieves *timekeeping* through circadian oscillations in phosphorylation that are intertwined with oscillations between the quaternary structures depicted in Figure 2. The primary sequence of KaiC is a concatenation of two P-loop ATPase domains (10), termed CI and CII. In solution, KaiC binds adenosine triphosphate (ATP) to form a hexamer through interactions at the CI subunit interfaces (23-25). Homologous ATPase active sites are present at the subunit interfaces of both CI and CII, however, these active sites catalyze distinct chemical transformations. CI transfers phosphate from ATP to water, and CII catalyzes transfer of phosphate between ATP and the sidechain hydroxyl groups of residues S431 and T432 (26,27).

Phosphorylation at these sites oscillates circadianly in concert with interactions between KaiC and the other core clock proteins KaiA and KaiB (28,29). These interactions are regulated by the phosphorylation state of KaiC, and take place on non-homologous regions of the CI and CII domains, known as the respective B and A-loops, where they in turn regulate the enzymatic activities on KaiC in a negative feedback loop.

During the day, KaiA is bound to the A-loops on CII where it promotes autophosphorylation (30-32). In the evening, KaiB binds to phosphorylated KaiC at the B-loops of the CI domain (33) through a slow conformational selection mechanism (34), adding a third stacked toroid to the KaiC double-doughnut (35) (Figure 2). At this point, KaiA is recruited to the KaiB-CI complex where it is inactivated, sequestering it from CII throughout the night (33,35). In the absence of KaiA, enzymatic equilibria at the CII active sites favor the autodephosphorylation of T432 and S431 (31). Around dawn, CII phosphorylation is at a minimum and affinity for KaiB is lost (30). The repressive complex is released, freeing KaiA to bind to the A-loops and begin the cycle anew.

Post-translational modification at residues S431 and T432 of KaiC occurs in a specific order, with T432 acting as a substrate in the first reaction of both the phosphorylation and dephosphorylation arms of the cycle (29,36). The order of the KaiC phosphorylation states is denoted in this review using the shorthand: $S/T \rightarrow S/pT \rightarrow pS/pT \rightarrow pS/T$. The S/T and S/pT states are dominant during the daytime phosphorylation arm of the oscillatory cycle, characterized by binding of KaiA at CII^{26,28}. Accumulation of pS/pT initiates the nighttime state (28,30,35), characterized by the formation of the KaiABC complex, which persists through the pS/T state until phosphorylation reaches a minimum and KaiA is recruited back to CII.

Sustained oscillation requires a power source, and KaiABC appears to achieve this through ATPase activity of the CI domain, whose enzymatic activity is correlated with period both *in vitro* and *in vivo* (37,38). CI ATPase activity oscillates circadianly, with the unphosphorylated forms of KaiC consuming ATP approximately twice as fast as the phosphorylated form (37). This enzymatic activity shows a relatively modest rate enhancement of ATP hydrolysis, with an apparent

k_{cat}/K_m on the order of $10^{-4} \text{ M}^{-1}\text{s}^{-1}$, about six orders of magnitude slower than the structurally similar F_1 ATPase (38). High-resolution structures have been obtained for the isolated CI ring in the pre-ATP and post-ATP hydrolysis states (38) resulting in the three specific structural changes depicted in Figure 3A (38). Together with studies showing that catalytically dead KaiC is unable to interact with KaiB (26), one emerging model is that CI ATPase functions in timing the recruitment of KaiB and resultant formation of the nighttime repressive complex, though no specific structural or biochemical information exists yet on how these two processes might be related.

A direct structural connection between CII phosphorylation and the interactions of KaiC with other clock proteins has also proven difficult to pin down. High-resolution crystal structures exist of different phosphorylated and phosphomimetic states (39), but these structures overlay almost perfectly. This is unexpected given the well-established functional differences between the phosphostates of KaiC. One possible explanation for this structural convergence is that crystallization of KaiC variants in these structures was performed in the presence of non-hydrolyzable ATP analogue (24,39), and structural changes associated with CII phosphorylation could be masked in the pre-ATP hydrolysis state. Alternatively, phosphorylation may influence dynamic structural equilibria that are not observable within a crystal lattice.

The latter idea is supported by lower-resolution studies on the overall shape and dynamic structural properties of KaiC. Time-resolved small angle X-ray scattering data that show an increase in the apparent radius of gyration in the pS/T phosphostate (40), providing evidence of a change in shape for KaiC in the night. Interactions between isolated CI and CII domains increase across the phosphorylation cycle with a minimum at the ST state and a maximum in the pS/T state (41), and NMR line-shape analysis indicates a more rigid KaiC structure in these states as well (41). These structural changes, known collectively as “ring-ring stacking,” presumably affect the accessibility of the B-loops to regulate binding of thioredoxin domains.

One of the most surprising observations related to the enzymology of CII is that free radioactive ATP accumulates transiently in dephosphorylation reactions of ³²P-labeled pS/pT KaiC (42), suggesting that dephosphorylation

occurs by transfer of phosphate from sidechains on KaiC to the β -phosphate of an adenosine diphosphate (ADP) nucleophile. The surprising ability of KaiC to catalyze this energetically expensive reaction has garnered speculation of an evolutionary relationship with F_1F_0 ATP synthase (43), which also takes on a double-doughnut-like structure (44). Because phosphorylation and dephosphorylation reactions on KaiC-CII appear to occur via the same chemistry going in opposite directions, the equilibrium can be regulated by substrate availability and ground state interactions without major restructuring of the active-site. This model is consistent with the apparent mechanisms by which KaiA regulates CII phosphorylation, as described below.

KaiA: Primary Regulator of KaiC Phosphorylation

KaiA acts as the primary regulator of enzymatic activity on the CII domain of KaiC, and therefore, the phosphorylation cycle in general. Throughout the day, KaiA promotes phosphorylation of CII via two distinct mechanisms: 1) structural rearrangement of the CII catalytic core (32,45) and 2) exchange of active-site ADP molecules for ATP (46). The enzymatic equilibrium of CII favors phosphatase activity when KaiC is in isolation (31), making KaiA occupancy the primary determinant of enzymatic equilibrium on CII.

Without KaiA bound, the A-loop residues project into the central cavity of KaiC (32). KaiA competes away interactions in this position, binding the loops and causing them to take on an α -helical conformation (47). When these loops are deleted, CII is in constitutive phosphorylation mode (32), indicating that the interaction of the A-loops with the cavity of KaiC abrogates CII phosphorylation and that KaiA disrupts those interactions. Additionally, KaiA has been shown to act as a nucleotide exchange factor, controlling the equilibrium of phosphorylation on CII at the level of substrate availability (46). KaiA increases the rate of ATP/ADP exchange on KaiC as well as the ratio of bound ATP at equilibrium (46). Hence, KaiA promotes CII phosphorylation by increasing the local concentration of ATP, as well as by direct perturbation of the active site.

Inactivation of KaiA during the night occurs through a conformational change that

abrogates its A-loop binding activity (Figure 3B). In the daytime state, the A-loops of KaiC form a helical bundle with the KaiA dimerization interface, flanked on either side by its N-terminal pseudo-receiver (PsR) domains. In this conformation, each β -strand from the interdomain linker crosses close to the other to form an intermolecular β -sheet, followed by a helical linker connecting the PsR domain. A high-resolution crystal structure of KaiA in the nighttime state was recently obtained by crystallizing its C-terminal dimerization domain and interdomain linker as part of a complex with KaiB and an engineered monomeric form of the KaiC-CI domain (35). KaiA docks onto an exposed β -sheet on KaiB in the KaiBC complex using the β -strand of its interdomain linker, which repositions the helical linker to dock into its own A-loop binding site. This intramolecular interaction between the α -helix of the interdomain linker and the dimerization interface precludes binding to an A-loop peptide from another KaiC hexamer, which demonstrates that binding to the KaiBC complex stabilizes an autoinhibited form of KaiA at night. The PsR domains of KaiA are not needed to bind KaiBC (48) and were omitted for this structure. This structure of an isolated CI-KaiB-KaiA complex agrees with a lower-resolution model obtained by cryo-electron microscopy using full-length wild-type KaiA, KaiB and hexameric KaiC fully assembled in the nighttime state (49). Although the PsR domains were included here, they did not give sufficient density to be built into the model, suggesting that they are flexibly tethered to the complex.

KaiB: Attenuator of KaiC Phosphorylation

The dephosphorylation arm of the KaiABC oscillatory cycle is initiated by the binding of KaiB at the CI domain of KaiC, followed by inactivation of KaiA. Historically, some discord has existed surrounding the location of KaiB binding on KaiC and how this leads to a shift of phosphorylation equilibrium that lasts through the night. Recently, a series of corroborative structural and biochemical studies (33-35,49,50) have put this controversy to rest. Our understanding of the role of KaiB in the clock has expanded immensely through the discovery of a major conformational rearrangement that occurs between the free and KaiC-bound states of KaiB. In the ground state, KaiB takes on the

unique fold depicted in Figure 3C and oligomerizes to form a dimer of dimers (51-53). When bound to the CI domain of KaiC, KaiB is in a monomeric thioredoxin fold (34), referred to as a ‘fold-switched’ state because it comprises a major conformational shift involving reassignment of secondary and tertiary structure in roughly 50 residues comprising the C-terminal half of KaiB.

Binding of KaiB to KaiC occurs remarkably slowly, taking hours to go to completion *in vitro* (33,53). This binding is quickened by mutations that favor the dimeric or thioredoxin-like monomeric states of KaiB (34,53). Native mass-spectrometry experiments have shown that binding occurs through a monomeric form of KaiB (54), and appears to be cooperative, as masses were obtained for KaiC bound to either one KaiB molecule or six, but none of the subsaturated stoichiometries in between (54). This is consistent with the observation of electrostatic complementarity between KaiB subunits in the recent structures of the KaiBC complex (35,49).

The current biochemical data suggest that KaiB binds through a conformational selection mechanism, in which the ground state dimeric and tetrameric forms of KaiB act as a thermodynamic sink to decrease the concentration of KaiC binding-competent KaiB monomer and limiting the rate of binding. Several different mutations have been identified that stabilize the KaiB monomer, each of which take on the fold-switched conformation (34,35). In addition to increasing the rate of binding to KaiC *in vitro*, these mutations halt circadian oscillation *in vitro* and *in vivo*, resulting in a dominant arrhythmic phenotype that locks KaiC in the unphosphorylated state (34). In the fold-switched form, KaiB forms a ternary complex with KaiA and the CI domain of KaiC (34,35), thus rendering KaiA inactive and mediating the dephosphorylation arm of the cycle. Together, these studies paint a unified picture of the repressive state of the cyanobacterial oscillator, and highlight the clarifying role of structural biology, which together with genetic and biochemical studies have demonstrated how the three proteins of the cyanobacterial circadian PTO work in concert with one another to sustain biochemical oscillation.

Output Signaling: SasA, CikA and RpaA

Output signaling from the cyanobacterial PTO takes place through a two-component

regulatory system (55). Two-component systems are pervasive in bacterial sensing and signal transduction (56). In the archetypal form, they consist of a sensor histidine kinase (HK) and a response regulator (RR) (57,58). There is often overlap between cognate RRs and HKs; for example, an HK may activate multiple RRs, or an RR may be regulated by multiple HKs (59). Moreover, the relevant active domains of the components may reside within a single protein that has both kinase and substrate functions. Cross-talk between regulatory networks increases dramatically from prokaryotes to eukaryotes (60), and cyanobacteria appear to fall somewhere in the middle of this complexity. The circadian signaling network in cyanobacteria is not yet fully understood, and new components are still being identified (18,19).

Despite the general complexity of regulatory networks in cyanobacteria, a two-component system consisting of two HKs and one RR stands out as being centrally involved in output signaling from the KaiABC oscillator (55,61). RpaA is an RR that governs transcription from a locus encoding additional transcription factors that mediate the global changes in gene expression associated with circadian rhythms (62). This regulon includes the cotranscribed KaiBC genes, thus integrating the PTO into a TTFL (63).

RpaA is regulated by two cognate HKs, SasA and CikA (61). The sensor domain of SasA adopts a thioredoxin fold that is almost identical in structure to the fold-switched form of KaiB (34,64) and binds to the pS/pT form of KaiC (34,61,65). Activation of SasA follows a canonical HK signaling mechanism. The sensor domains on SasA recognizes and binds to phosphorylated KaiC, resulting in autophosphorylation of H161 in the SasA kinase domain (61) and subsequent transfer of that phosphate to D53 on RpaA, rendering the transcription factor competent for DNA-binding (63). SasA has been shown by analytical ultracentrifugation to form a trimer in solution (65), and gel filtration experiments support a stoichiometry of one SasA trimer per KaiC hexamer. HKs usually oligomerize as dimers, but in rare cases have been found to function as higher order oligomers (66) or monomers (67). It is yet unknown whether this unusual oligomeric state for SasA is linked to its sensory function.

At the biochemical level, SasA competes with KaiB for the B-loops on the CI domain of phosphorylated KaiC (48). Binding of SasA is faster than that of KaiB, presumably due to the refolding requirement of KaiB and its conformational selection mechanism of binding (34,65). This difference suggests an evolutionary selection for the metamorphic properties of KaiB in controlling the temporal window of SasA signaling, in addition to providing a timing cue for the oscillatory cycle, though more evidence is needed to support either of these models definitively. KaiA also plays a role in the competition between KaiB and SasA for the CI domain of KaiC. While the presence of KaiA does not affect k_{obs} for binding of KaiB to KaiC, it does make a difference in competitive KaiB binding when KaiC is pre-bound with SasA (48). This property implicates a role for KaiA in active eviction of SasA by KaiB, though no physical model for this currently exists.

The activating effect of SasA on RpaA is reversed by CikA, a multi-domain protein that, in addition to its role in dephosphorylating RpaA, ties the clock to subcellular localization and entrainment functions (68-71). CikA is incorporated into the Night Complex through the interactions of its pseudo-receiver (PsR) domain with fold-switched KaiB at an interface that overlaps with the KaiA binding site (Figure 2). Consistent with a potential competitive mechanism, inclusion of the CikA PsR domain in an *in vitro* oscillation reaction results in shortened period and decreased amplitude (34). Combined with the observation that CikA knockout results in a relatively severe gene expression phenotype (72), these data suggest that CikA has a more integral role in the *timekeeping* functions *in vivo* than suggested by its known roles in *entrainment* and *output signaling*. The integrated roles of CikA and the other core clock proteins are summarized in Figure 4.

Metabolic of Entrainment of the Clock

The cyanobacterial circadian clock achieves *entrainment* by linking the oscillatory cycle to photosynthesis through metabolic processes (73), rather than direct entrainment by light via sensory photoreceptors, as is commonly found in eukaryotic organisms (74). This metabolic entrainment of the cyanobacterial clock occurs in two ways: through sensitivity of the

phosphorylation cycle to ATP/ADP ratios within the cell (75) and through the presence of nighttime-associated photosynthetic metabolites (71,72,76-78). The former entrainment cue occurs as a result of enzymatic sensitivity of the core oscillator to ATP/ADP ratios⁷², causing it to align with the maximal photosynthetic period at midday when this ratio is highest (75,79).

Cyanobacteria utilize quinone pools as intramembrane electron shuttles, reducing them during the process of photosynthesis (80). As a result, the concentration of oxidized quinones rises rapidly but briefly when photosynthesis shuts down with the onset of darkness before other pathways restore the redox steady state (76). Ectopic introduction of oxidized quinones can entrain the clock both *in vivo* and *in vitro*, and both KaiA and CikA play roles related to entrainment through binding of their PsR domains to oxidized quinones (71,72,76-78). The aggregation state and/or stability of the proteins is affected by quinone binding *in vivo* (71,77). Still, the precise mechanism by which quinone binding influences the PTO is not currently well-understood.

Applications in Synthetic Biology

There is considerable interest in synthetic biology to control temporally regulated gene expression (81,82). However, even the most robust synthetic oscillators are far less effective than the fully integrated oscillation of gene expression in cyanobacteria (83,84). Because of its ability to function at constant protein concentrations and provide relatively stable temporal regulation at different temperatures, it could be enticing to reverse engineer synthetic oscillators from the cyanobacterial PTO. This has been achieved, on a rudimentary level, in a transplantation study where the core oscillator was introduced into *Escherichia coli*, resulting in weak but measurable *in vivo* circadian rhythms in KaiC phosphorylation in a natively non-circadian organism (85). In this study, the clock was linked to transcriptional output signaling through a two-hybrid approach, though the researchers were unable to show circadian oscillation of gene expression originating from KaiABC. Thus, a great deal of opportunity still exists to integrate the cyanobacterial PTO more deeply into the physiology of the host organism and achieve clock-driven oscillations in gene expression. Undeniably, these efforts benefit from

the mechanistic advances in our understanding of the cyanobacterial PTO. Furthermore, a great deal of work has gone into computational modeling of the cyanobacterial PTO (36,86-89). These studies can inform efforts to re-engineer the oscillatory period of the PTO, leading to future technologies in

which the cyanobacterial PTO may be used as a starting point to introduce a flexible range of temporal routines of gene expression into target organisms.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions

J.A.S.: Conceptualization, visualization, writing original draft, writing-review, editing.

S.S.G.: Funding acquisition, writing-review, editing.

A.L.: Funding acquisition, writing-review, editing.

C.L.P.: Funding acquisition, writing-review, editing.

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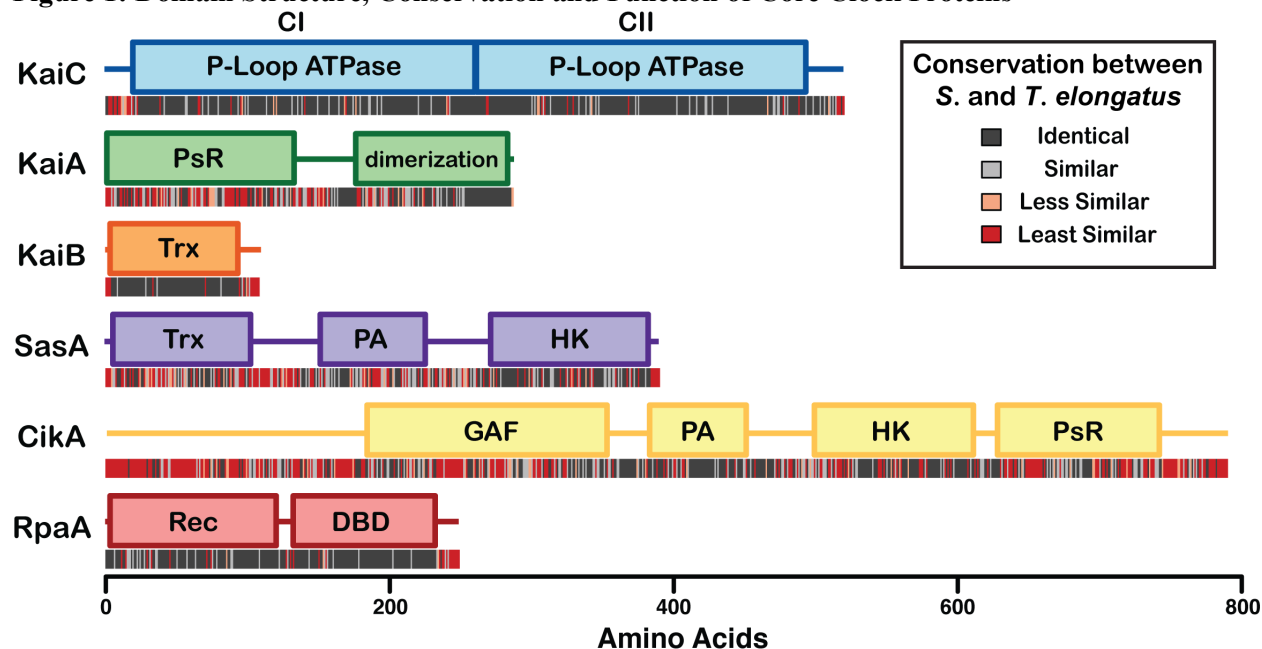
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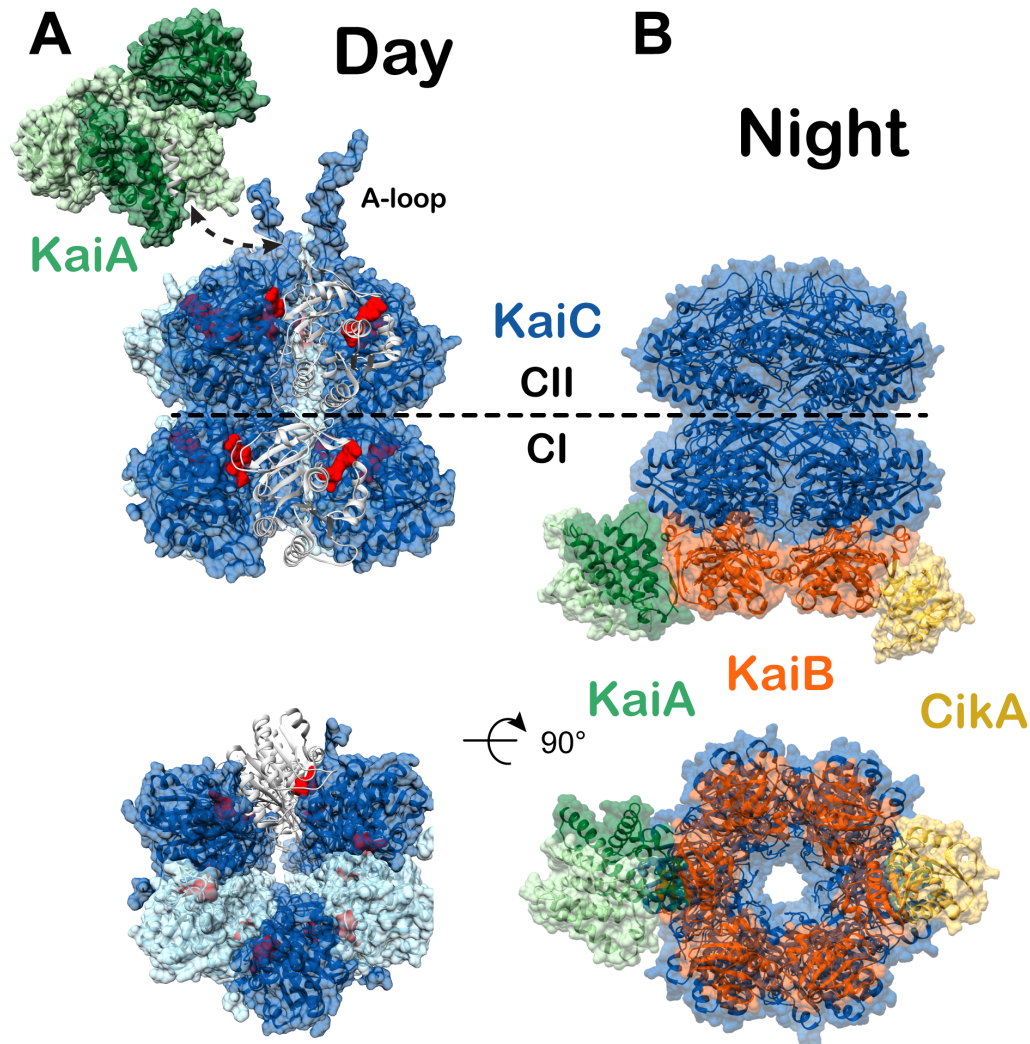
Figure 1: Domain Structure, Conservation and Function of Core Clock Proteins



Domain	Clock Protein	Function
P-Loop ATPase	KaiC	CI: pacemaking ATPase, KaiB binding
		CII: oscillation in PO ₄ -ylation, KaiA binding
pseudo-receiver (PsR)	KaiA	quinone recognition
	CikA	
dimerization	KaiA	KaiC binding, upregulation of KaiC PO ₄ -ylation
thioredoxin (Trx)	KaiB	Sensing of KaiC PO ₄ -ylation
	SasA	
phosphoacceptor (PA)	SasA	trimerization, activation of RpaA
	CikA	oligomerization, deactivation of RpaA
histidine kinase (HK)	SasA	PO ₄ -ylation of PA domains
	CikA	
GAF	CikA	CikA HK regulation
receiver (Rec)	RpaA	regulation by cognate HKs SasA and CikA
DNA-binding (DBD)	RpaA	transcription initiation

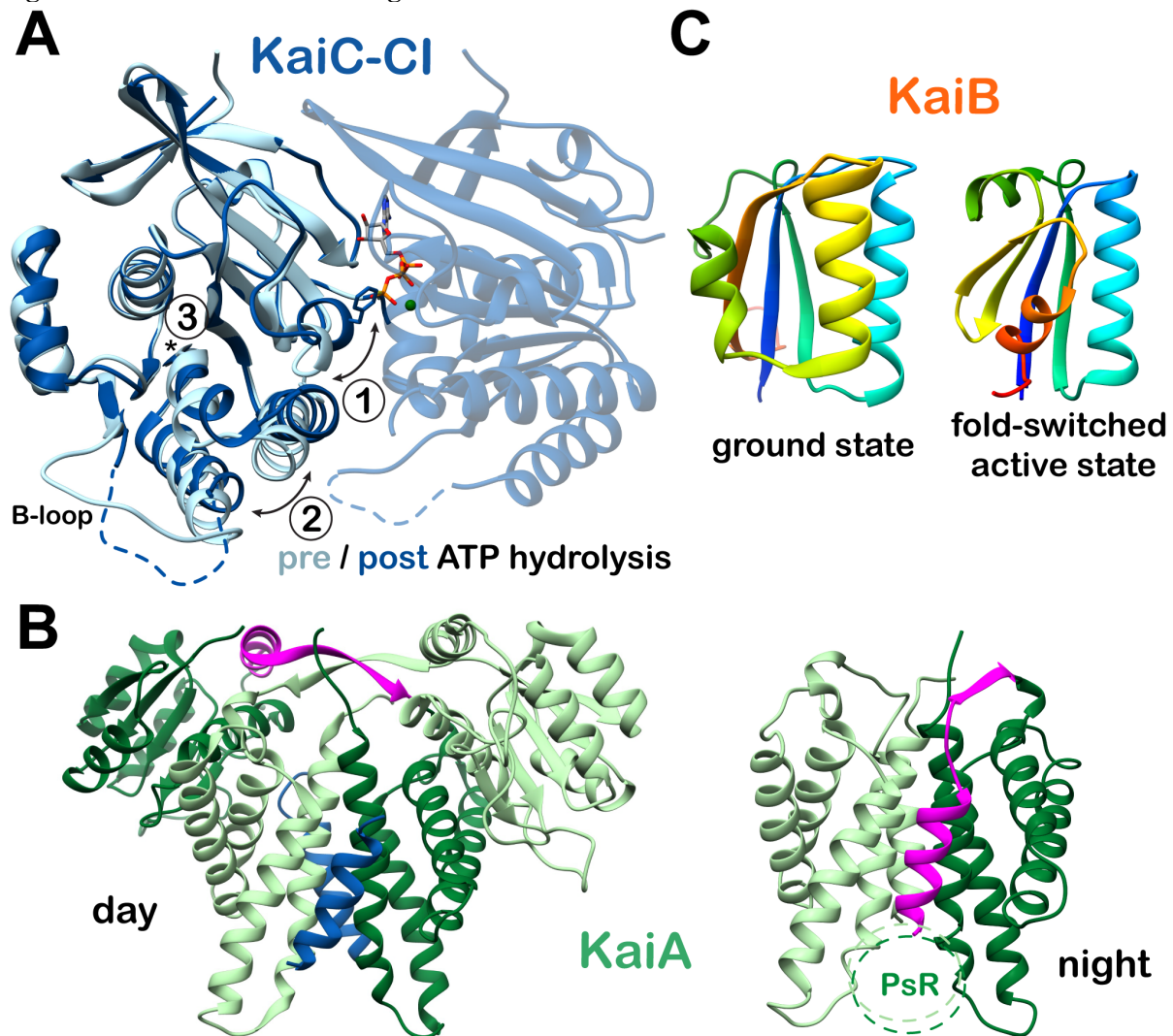
Domain structures of the six core clock proteins from *S. elongatus* are depicted as colored bars using boundaries and annotated functions from the Interpro database (90). Sequences were aligned with homologs from the thermophilic variant *T. elongatus* using ClustalΩ (91). Identity scores at each position in the alignment are indicated below the domain maps as color-coded bars. Observed function for each of the core clock protein domains are summarized below.

Figure 2: Day and Night States of the Cyanobacterial Clock



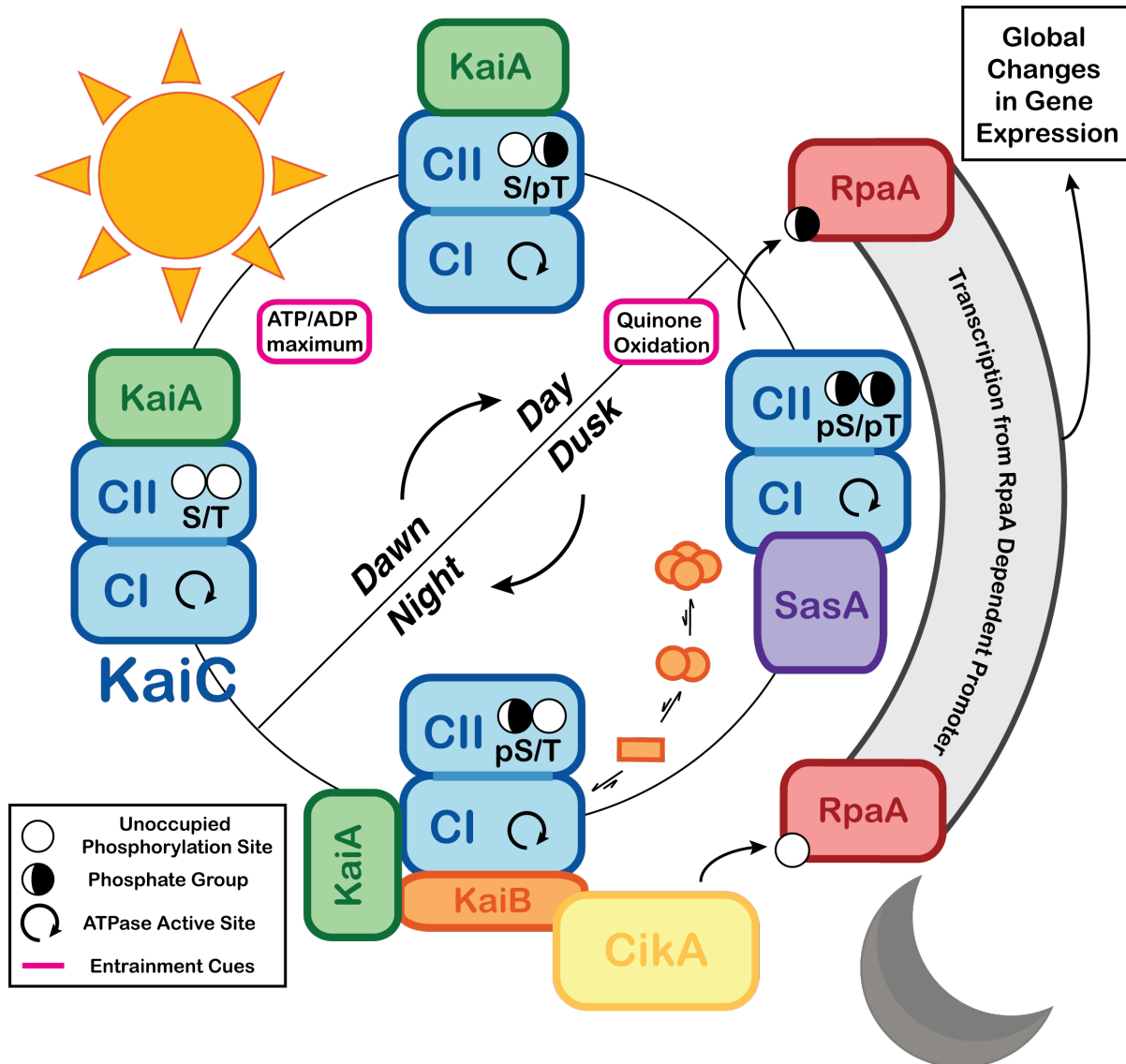
High resolution models of the day and nighttime states of the core oscillator are compared. Coloring scheme is the same as in Figure 1. A) **The Day Complex.** The CI and CII hexamers form stacked doughnuts (alternating subunits shown in light and dark blue for contrast, PDB:3K0C). One subunit is shown in cartoon mode to highlight the nucleotide binding sites at the subunit interfaces (red). Phosphorylation of KaiC residues T426 and S432 takes place near the CII nucleotide binding sites. The KaiA dimer (light and dark green for contrast) binds to C-terminal A-loops of KaiC (white, shown in complex with KaiA PDB:5C5E). The dashed arrow represents the point of connection between the KaiA-CII loop structure and the CII A-loop extensions shown on the right. B) **The Night Complex.** The intermediate-resolution cryo-EM model (PDB:5NYT) is combined with higher resolution models from studies on independent subcomplexes. The S431E phosphomimetic of the pS/T state of KaiC binds six molecules of KaiB (PDB:5JWQ). This results in the recruitment and sequestration of KaiA near the KaiB-CI interface (PDB:5JWR). Output signaling occurs through interactions between KaiB and the C-terminal PsR domain of CikA (PDB:5JYV), as well as through interactions between KaiC and SasA at dusk, though no high-resolution structure exists for the latter.

Figure 3: Conformational Changes in the Core Oscillator



A) CI ATP hydrolysis. High-resolution crystal structures of the CI domain of KaiC in the ATP-bound (PDB:4LTA) and ADP-bound states (PDB:4LT9, chain C) are overlaid. Three major structural differences are noted upon ATP hydrolysis: 1) The helix bearing F199 is flipped into an alternate conformation, 2) the $\alpha 6$ - $\alpha 7$ / $\alpha 8$ helices are repositioned away from the nucleotide binding site, and 3) the *cis* peptide bond between D145 and S146 in the ATP-bound state is found in the *trans* isomer in the ADP-bound state. **B) KaiA inactivation.** The daytime and nighttime states of KaiA are compared. During the day, the A-loops of KaiC (blue) are bound at the dimer interface, and the interdomain linker (pink) crosses over the complex. In the Night Complex, the linker shifts to occupy the A-loop binding site. **C) KaiB fold-switching.** KaiB undergoes a major structural reorganization between its free and KaiC-bound forms, involving changes in both secondary and tertiary structure for the C-terminal half of the protein. Both structures are represented with rainbow coloring, starting with dark blue at the N-terminus. Note that the first half of the two proteins is identical (dark blue through light green), while the C-terminal halves are completely different. This interconversion is thought to happen spontaneously resulting in a conformational selection mechanism for formation of the KaiBC complex.

Figure 4: Integrated *timekeeping*, *entrainment* and *output signaling* functions of the cyanobacterial clock.



Timekeeping, *entrainment* and *output signaling* functions are highlighted within the oscillatory cycle of the cyanobacterial clock. Powered by ATPase activity on its CI domain, KaiC cycles through a series of phosphorylation states that is interdependent on its quaternary structure. KaiA is bound to the CII domain of KaiC during the day, stimulating phosphorylation. This process is sensitive to the ATP/ADP ratio, which peaks at midday, providing an entrainment cue. At dusk, levels of oxidized quinones rise in the cell, and the clock is entrained by this as well. Around this time, KaiC reaches the pS/pT state, and SasA binds to the CI domain to activate RpaA. CI-bound SasA is eventually competed away by KaiB. Binding of KaiB is slowed by its intrinsically unfavorable equilibrium that sequesters it in inactive states. Accumulation of KaiB in its KaiC-bound form recruits and inactivates KaiA, allowing CII dephosphorylation. The input/output protein CikA also interacts with the fold-switched form of KaiB, causing CikA to dephosphorylate RpaA, inactivating it.