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A circadian clock nanomachine that runs without transcription or translation

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The biochemical basis of circadian timekeeping is best characterized in cyanobacteria. The structures of its key molecular players, KaiA, KaiB, and KaiC are known and these proteins can reconstitute a remarkable circadian oscillation in a test tube. KaiC is rhythmically phosphorylated and its phospho-status is a marker of circadian phase that regulates ATPase activity and the oscillating assembly of a nanomachine.

Analyses of the nanomachines have revealed how their timing circuit is ratcheted to be unidirectional and how they stay in synch to ensure a robust oscillator. These insights are likely to elucidate circadian timekeeping in higher organisms, including how transcription and translation could appear to be a core circadian timer when the true pacemaker is an embedded biochemical oscillator.

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Introduction

The core mechanism of the circadian clock in eukaryotic cells is widely held to be based on a Transcription/Translation Feedback Loop (TTFL) [1,2], although there is recent evidence that this model may be incomplete or inaccurate [3,4]. In cyanobacteria, the initial evidence also supported a TTFL model [5]. However, our current understanding of the clock system in cyanobacteria is that a biochemical oscillator operates as the central ‘quartz crystal’ of the clockwork [6**], but this core pacemaker operates within (and regulates) a larger TTFL that controls outputs and replenishes the oscillator’s essential proteins [7,8**]. The remarkable discovery that this core oscillator could be transplanted as a three-protein system to oscillate *in vitro* [6**] has led some researchers to revisit the question of non-TTFL circadian

clocks in eukaryotes — a search that has recently culminated in the discovery of circadian metabolic/redox oscillations that can operate in eukaryotes in the absence of transcription [9], and which resurrects an old literature on circadian clocks in enucleated algal cells [4]. Because we know the 3D-structures of the major protein players and the oscillator can be reconstituted *in vitro*, the cyanobacterial system constitutes a unique preparation to study the biochemistry, biophysics, and structural biology of post-translational circadian timekeeping.

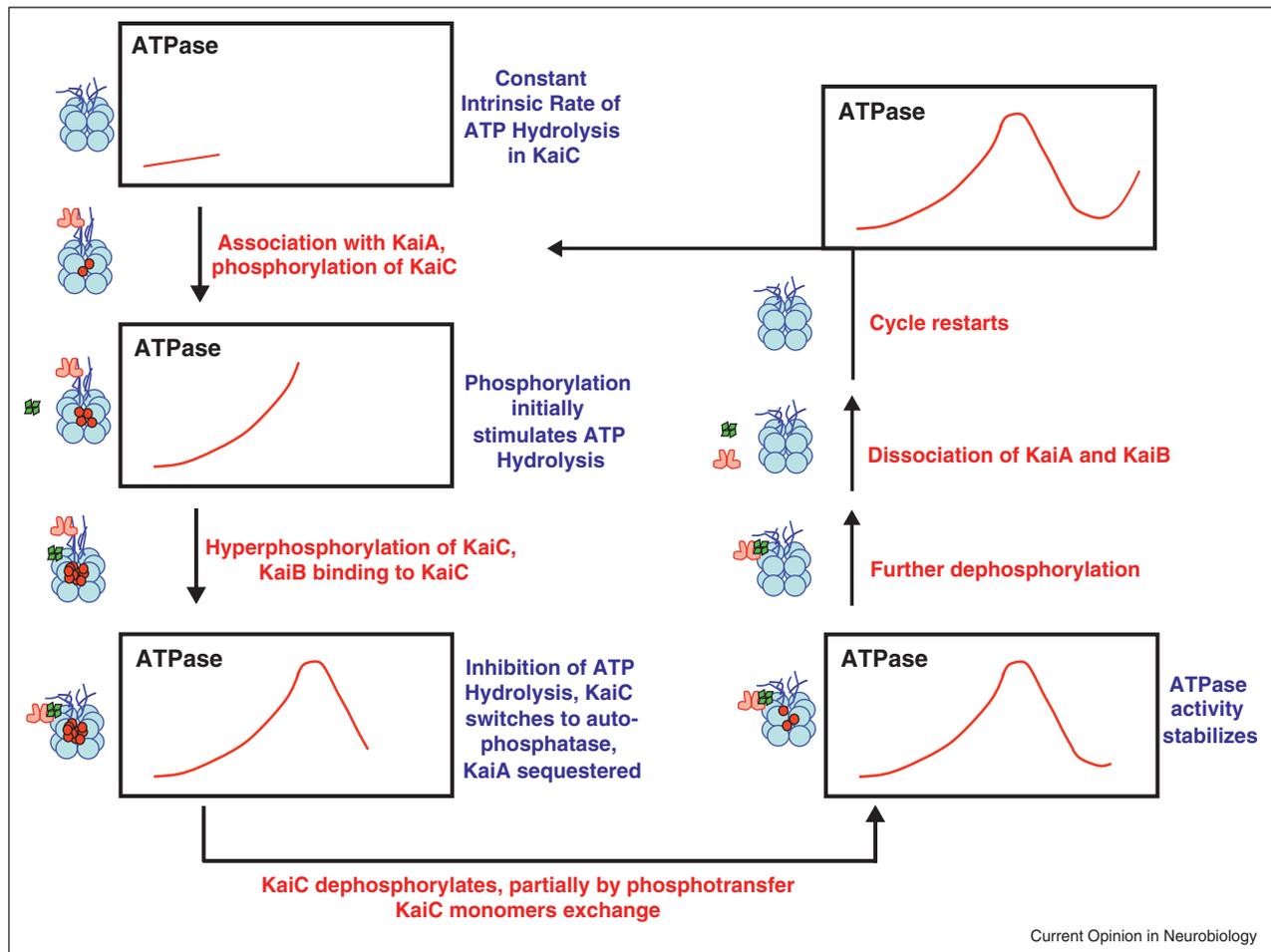
To set the stage for the information that we summarize below, KaiC hydrolyzes ATP and this reaction may be an intrinsically constant-rate timer [10] that drives KaiC phosphorylation. The status of KaiC phosphorylation feeds back to regulate first, the ATP hydrolytic rate (so that its intrinsic constant rate becomes rhythmic) and second, switching of the KaiABC nanocomplex between autokinase and autophosphatase states (Figure 1). An overly simplified analogy would be to consider KaiC ATPase activity as an ‘hourglass timer’ which is restarted each cycle (the ‘hourglass is turned over’) by KaiC’s phosphorylation status, thereby converting the hourglass timer into an oscillation. Therefore, the extent of KaiC phosphorylation is both a marker of circadian phase [11,12] and a regulator of KaiC’s myriad activities (ATPase, autokinase, autophosphatase, phosphotransferase, and nanocomplex formation).

Architecture and function of KaiC, the central player

The N-terminal and C-terminal domains (termed CI and CII, respectively) of KaiC exhibit sequence similarity with ATPases, such as the DnaB helicase and RecA recombinase. The crystal structure of *Synechococcus elongatus* KaiC resembles a double doughnut with CI and CII rings of similar height and width and a constricted waist region that is spanned by 15-amino acid linkers (Figure 2a) [13]. The conformation of the C-terminal region in CII differs considerably from the one in CI that constitutes the linker and winds along the outer surface of the CI/CII interface. Thus, CII C-terminal peptides form a crown-like arrangement of S-shaped loops (‘tentacles’), comprised of residues E487 to I497, that rim the central KaiC channel and then protrude from the outer dome-shaped surface of CII.

ATP molecules are wedged between subunits in the CI and CII rings and bound to P-loop motifs (Figure 2) [13]. The two rings possess different functions; the CI ring

Figure 1



The circadian *in vitro* oscillator that acts as a post-translational oscillator (PTO) *in vivo*. The reaction is composed of ATP hydrolytic activity and phosphorylation within KaiC, and rhythmic associations of KaiC with KaiA and KaiB to form a nanocomplex that regulates ATPase and phosphorylation activities. Cartoons depict associations among the KaiC hexamer (light blue), the KaiA dimer (pink), the KaiB tetramer (green) and phosphorylation on KaiC subunits (red dots). Rectangles show ATPase activity over time. Starting from the upper left corner, KaiA associates with unphosphorylated KaiC and stimulates autophosphorylation, which accelerates the rate of ATP hydrolysis. The stages of the reaction proceed as described in the text [41*] in a counter-clockwise direction.

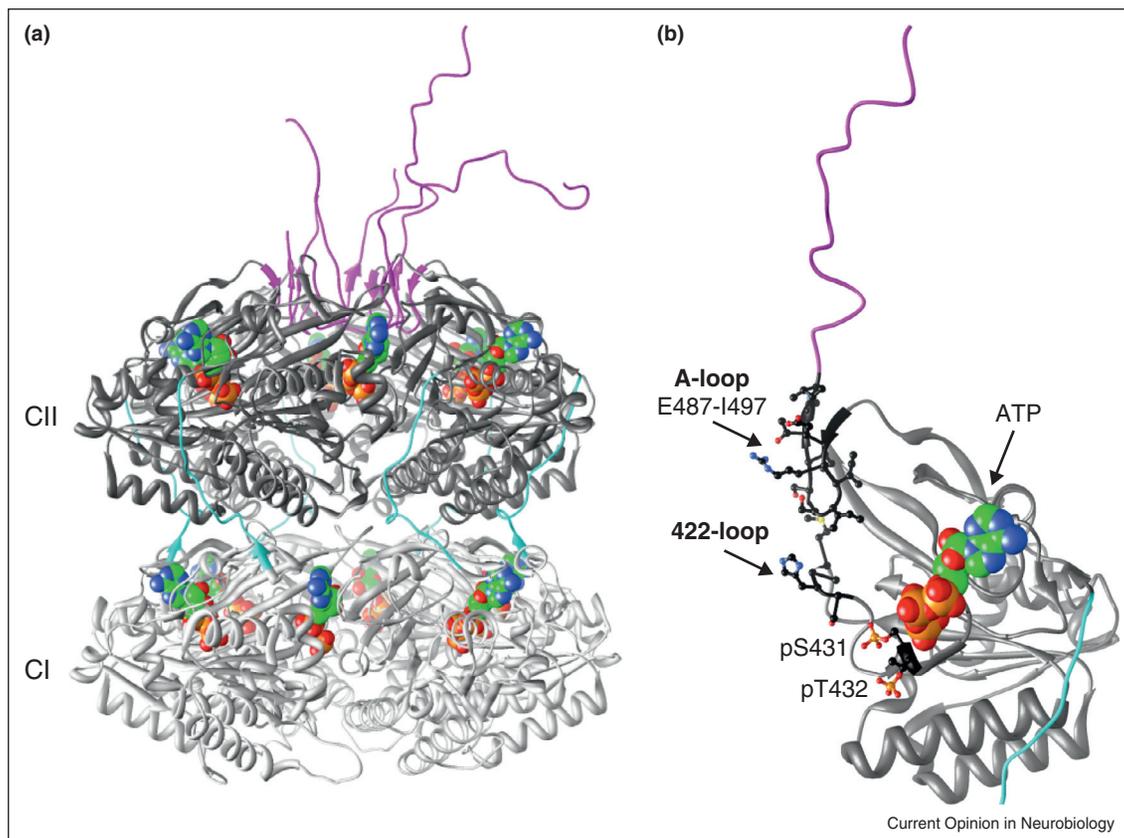
harbors ATPase activity [10] and the CII ring catalyzes rhythmic phosphorylation and dephosphorylation reactions. The kinase activity [14,15] results in phosphorylation of Thr432 and Ser431 across CII subunit interfaces [13,16]. A third residue, T426, influences phosphorylation and dephosphorylation of T432/S431, possibly by acting as a labile phosphorylation site whereby a phosphate is shuttled between T426 and S431 [16,17]. The T432 residue is phosphorylated first in the cycle, followed by phosphorylation at S431 [11,12], and this strict order of phosphorylation can be explained with a kinetically controlled kinase activity, where the ATP γ -phosphate is closest to the hydroxyl group of T432 that is therefore phosphorylated before S431 [13,16]. The dephosphorylation reaction then proceeds in the same order (pT432 is followed by pS431), such that the overall process from the

hypo-phosphorylated to the hyper-phosphorylated and back to the hypo-phosphorylated state of KaiC involves the following stages: TS \rightarrow pTS \rightarrow pTpS \rightarrow TpS \rightarrow TS [11,12]. The phosphorylation status of KaiC is therefore a marker for the phase of the *in vitro* oscillator [12].

KaiA stimulates KaiC phosphorylation that 'ratchets' the clockwork

KaiC can auto-phosphorylate, especially at low temperatures (e.g., 4 °C). At physiological temperatures, however, KaiA greatly stimulates the autokinase activity of KaiC [15,18]. Specifically, binding of KaiA homo-dimer to a KaiC C-terminal tail or 'tentacle' activates the kinase in the CII ring [19,20], thus triggering phosphorylation of T432 and S431 residues (Figures 2 and 3). Phosphorylation of T432 and S431/T426 leads to increased molecular

Figure 2

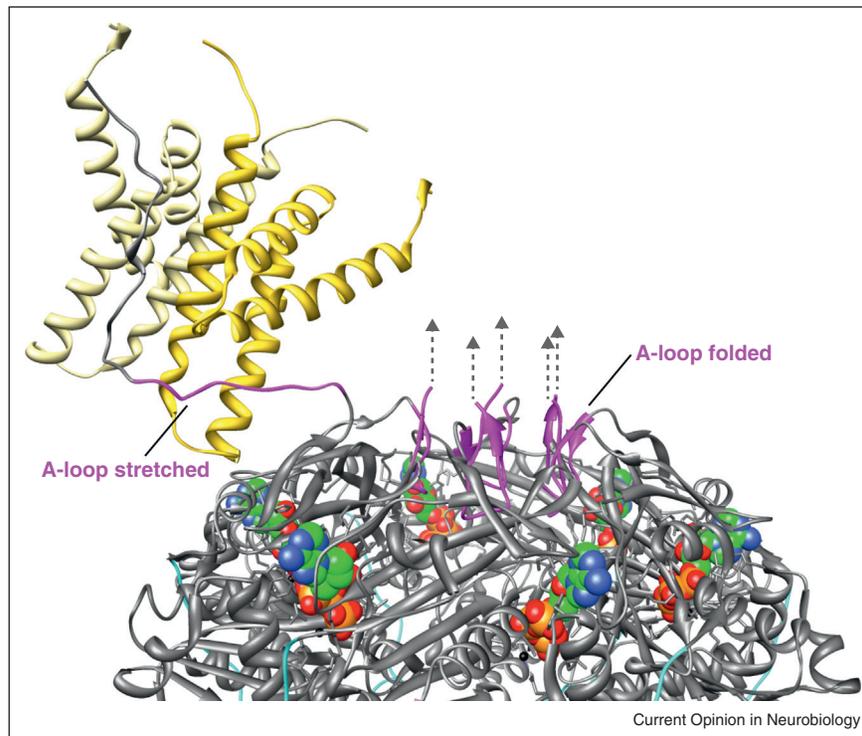


(a) Structure of the KaiC homo-hexamer (PDB ID code 3DVL) [13]. N-terminal CI and C-terminal CII domains from individual subunits are colored in light gray and dark gray, respectively, CI–CII linkers are cyan and C-terminal peptide tails are magenta. ATP molecules are shown in space filling mode with carbon, oxygen, nitrogen and phosphorus atoms colored in green, red, blue and orange, respectively. **(b)** An isolated KaiCII domain with A-loop and 422-loop as well as phosphorylation sites T432 and S431 highlighted.

interactions, salt bridges, and formation of additional hydrogen bonds [21,22,23*]. These enhanced interactions render reverse reactions unfavorable and provide a ‘ratcheting’ mechanism that can explain why time in the phosphorylation phase of the *in vitro* KaiABC reaction is unidirectional [21,24]. Therefore, ‘time doesn’t run backwards.’ Despite the presence of six C-terminal tentacles on KaiC, a single KaiA dimer is sufficient to convert KaiC from the hypo-phosphorylated to the hyper-phosphorylated form [25], thus implying an allosteric mechanism for KaiC phosphorylation [26]. On the basis of studies in which the C-terminal tentacles were deleted to various lengths, LiWang and coworkers concluded that the intramolecular action of KaiA binding to KaiC is to ‘pull’ on KaiC’s buried A-loop, which straightens and exposes it (Figures 2b, 3) [27]. This exposure of the A-loop was hypothesized to move the ATP (bound to the P-loop) closer to T432/S431, possibly mediated by a β -strand segment that lies between the A-loop and P-loop (Figure 2b) [27].

Further support for this allosteric model and the general importance of the A-loop region for phosphorylation of KaiC comes from an analysis of the structure and dynamics of the KaiC A422V mutant. An alanine for valine exchange is a very conservative change, and yet this mutation has rather drastic consequences for function that include abnormal phase-resetting responses to dark pulses, abolished rhythms of KaiC accumulation, and a reduced amplitude of the KaiC phosphorylation rhythm [28]. Close inspection of the KaiC crystal structure reveals that the 422-loop is buried underneath the A-loop and that apical residues of the two loops are in direct contact. Therefore removal of the A-loop alters the constraints of the 422-loop and the region connecting A/V422 to the T432 and S431 phosphorylation sites (Figure 2b) [29]. Molecular dynamics (MD) simulations support the notion that the KaiA-mediated pulling of an A-loop alters the dynamics of KaiCII, triggering increased mobility and facilitating phosphorylation [29]. The consensus that emerges is that KaiA stimulates the KaiC kinase via a

Figure 3



KaiA dimer (only C-terminal domains are depicted) interacting with a KaiCII C-terminal tentacle by pulling out the A-loop (highlighted in magenta) and unraveling it. The model is a fusion of the KaiC crystal structure [13] and the NMR structure of the KaiA–KaiC peptide complex [19]. See Figure 2 for KaiC color code.

concerted allosteric mechanism [27,29] and that no large-scale conformational changes are required in either the CI or CII halves (as evidenced by the MD work) to move from the ST to the pTS and ultimately the pTPS state, using the aforementioned ratcheting that favors the forward reaction [21].

KaiC dephosphorylates by regenerating ATP

KaiC dephosphorylation has been generally assumed to involve a phosphatase activity that might require a considerable conformational adjustment to move pT432 and pS431 away from the kinase active site and reposition them at a putative phosphatase active site [21]. Those assumptions are probably incorrect. First, a number of studies using multiple techniques indicate that a large conformational change of KaiC is unlikely [13,16,20,22,30,31,32^{*}]. Second, signature motifs of common types of phosphatases [23^{*}] are missing in the KaiC sequence. Finally, two independent studies have confirmed that dephosphorylation of T432 and S431 proceeds — at least partially — via a phosphotransferase reaction that results in the regeneration of ATP from the phosphates at T432/S431 and the ADP bound at the P-loop (Figure 2) [23^{*},33]. This phosphotransfer activity complicates accounting for the number of ATP molecules hydrolyzed over the daily clock cycle. In particular, the

reported amount of 15 ATPs consumed per day per KaiC monomer [10] has to be considered a net balance because the absolute numbers of ATP molecules hydrolyzed and then regenerated in a 24-hour interval is not known. What is the basis of dephosphorylating by regenerating ATP? Possibly this is a mechanism to conserve ATP when its cellular concentration declines as a result of cells not being able to photosynthesize [34^{*}], thereby allowing the clock to keep running under extended dark exposure [35,36].

Phosphorylation state mimics: a useful but potentially misleading tool

It is common practice to replace serine and threonine with aspartic or glutamic acid residues in proteins to mimic phospho-serine and phospho-threonine and many studies of the structure and function of the KaiABC post-translational oscillator (PTO) have relied on this approach (e.g. the recent Refs. [8^{**},10,22,27,37,38,39^{*}]). However, it is important to keep in mind when using this mimicry that Asp and Glu are not perfect replacements of pSer or pThr. This is not just due to different sterics but also deviating negative charges (i.e. -1 for Asp and Glu and -2 for pSer and pThr at neutral pH). Crystal structures even at relatively low resolutions of around 3.5 Å can easily distinguish between unphosphorylated and phosphorylated Ser and

Thr residues, and indeed crystallographic studies revealed important differences in the structure, phosphorylation states and electrostatic surface potentials (ESPs) of KaiC phosphorylation state mimics that do not seem to be generally considered when interpreting functional data employing KaiC mutants comprising E or D in place of the phosphorylation site residues T432 and/or S431. Moreover, functional studies uncovered a difference between the T432A/S431A mutant and authentic unphosphorylated KaiC [40^{*}]. X-ray crystallography revealed the following phosphorylation patterns in the structures of KaiC mutants [16,32^{*}]: T432E/S431A (pTS mimic) has T426 phosphorylated in four subunits, in S431D (TpS mimic) three out of six T432 residues carried a phosphate, and T432E/S431E (pTpS mimic) exhibited a new phosphorylation site at S320 in two subunits and its ESP on the CII dome surface matched neither that of hyper-phosphorylated nor that of hypo-phosphorylated KaiC.

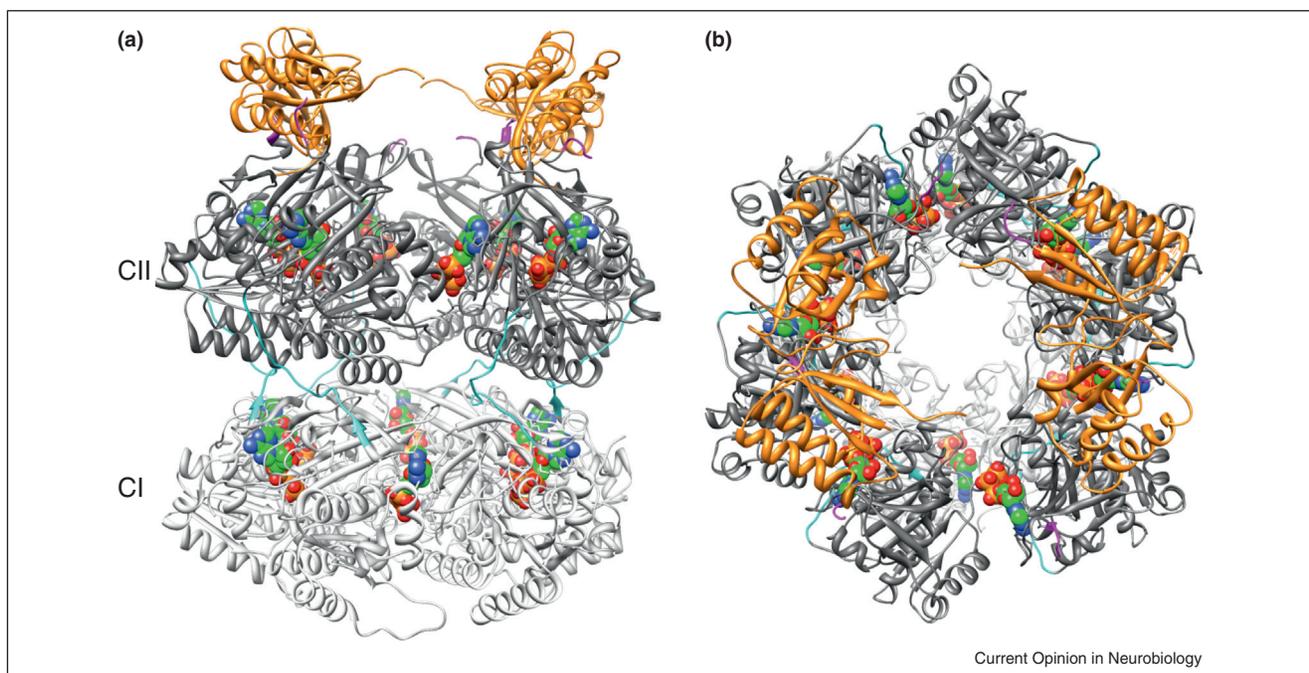
Maintaining synchronous KaiC hexamers and robust rhythmicity

KaiB associates with KaiC during dephosphorylation and this association correlates with the phase of KaiC subunit exchange, suggesting a functional link between KaiB's binding to KaiC and its monomer exchange (Figure 1) [41^{*}]. This subunit monomer exchange is important in terms of maintaining synchrony of the phosphorylation status among all the KaiC hexamers in the population of hexamers present in the *in vitro* reaction (and presumably

in vivo as well) [41^{*},42]. By maintaining synchrony among the KaiC hexamers, robust rhythmicity is achieved, which has been experimentally demonstrated to be a property of the *in vitro* reaction for at least 10 days [42]. An equally important factor for maintaining synchrony among KaiC hexamers is the sequestration and progressive inactivation of KaiA dimers during the dephosphorylation phase [12,26,37]. Therefore, it appears that there are at least two mechanisms for maintaining KaiC phosphosynchrony and robust rhythmicity: KaiC monomer exchange/KaiB binding and KaiA sequestration, both of which are primarily occurring during the KaiC dephosphorylation phase.

The timing and impact of the binding of KaiA and KaiB to KaiC are critical for maintaining synchrony among KaiC hexamers, thereby promoting high-amplitude oscillations that persist for many cycles. But where do KaiA and KaiB bind to KaiC? As mentioned above, at the beginning of the phosphorylation phase, KaiA associates with the C-terminal tentacles of KaiC (Figures 2 and 3); at the end of the phosphorylation phase, KaiA is sequestered by KaiC (or by a KaiB–KaiC complex, Figure 4) to an unknown site, but it might be near the waist/linker region of KaiC [32^{*},37]. The identity of the binding site(s) of KaiB to KaiC has recently become contested. In 2008, we presented a three-dimensional model of the KaiBC complex based on negative stain and cryo EM that features two KaiB dimers bound to the dome-shaped surface of KaiCII

Figure 4



EM-based model of the KaiB–KaiC complex [30], viewed (a) from the side and (b) rotated by 90° around the horizontal and viewed along the central KaiC channel from the CII side. KaiB dimers are colored in orange and the coloring code for KaiC matches that in Figure 2. A new cryo-EM structure of the KaiBC complex at 16 Å resolution revealed six KaiB monomers bound to a KaiC hexamer [61].

(Figure 4) [30]. LiWang and coworkers recently used solution NMR and gel filtration chromatography to study the interactions between KaiB and separate CI and CII rings as well as monomeric CI and CII domains and arrived at the conclusion that KaiB binds to the CI ring [38]. However, EM studies of complexes between KaiB and KaiC S431D mutant hexamers carrying C-terminal His₆ tags labeled with nickel(II) nitrilotriacetic acid gold nanoparticles (Ni-NTA-Nanogold) revealed that gold is located on the same side as KaiB, thus providing unequivocal evidence that B is bound to CII [43].

Entraining the PTO pacemaker embedded within a TTFL slave oscillator

Thus, the underlying circadian system in cyanobacterial cells appears to comprise two subcomponents: a PTO and a TTFL [7,8^{**}]. The TTFL drives global gene expression and the PTO is the core ‘quartz crystal’ of the timekeeper. Modeling indicates a mechanism by which the TTFL can feed into the PTO such that new synthesis of clock proteins can phase-shift or entrain the core PTO pacemaker. This prediction was experimentally tested and confirmed by entraining the *in vivo* circadian system with cycles of new clock protein synthesis that modulate the phosphorylation status of the clock proteins in the PTO [8^{**}]. Consequently the TTFL can provide entraining input into the PTO.

But what are the metabolic input signals and how are they related to the daily light/dark cycle that entrains the clock? Four hour dark pulses readily reset the phase of cyanobacterial cells that are free-running in LL [28]. Insight into phase-shifting has recently been gained by way of two sources that were identified to feed into the KaiABC oscillator and thus allow cyanobacteria to keep track of the daily light/dark cycle. Rust *et al.* demonstrated that KaiC phosphorylation is directly influenced by the cellular ATP/ADP ratio that is itself dependent on photophosphorylation [34^{*}]. Interestingly, the ATP/ADP ratio also affects KaiC phosphorylation in the *in vitro* KaiABC PTO and was demonstrated to be sufficient to reset the phase of phosphorylation. In addition to the ATP/ADP ratio, the phase of the KaiABC PTO is influenced by the oxidation state of the plastoquinone (PQ) pool [44]. PQ appears to interfere with KaiA-stimulated KaiC phosphorylation in at least two ways. Thus, in its oxidized but not in the reduced form, it causes KaiA aggregation by preferably binding to the N-terminal receiver domain, as was demonstrated with dibromothymoquinone, a water soluble PQ analog [44]. Using gel-based and crystallographic assays with the KaiA-dibromothymoquinone complex, we established that the PQ analog interferes with KaiA binding to the KaiC C-terminus, thereby hampering KaiC phosphorylation [45^{*}]. Both cues, ATP/ADP ratio and the pool of oxidized PQ, affect KaiC phosphorylation and operate in tandem [44]. The ATP/ADP ratio slowly declines during the night and thus

serves as a measure for the duration of darkness, eventually inducing a phase shift. Conversely, diminishing light leads to the rapid oxidation of the PQ pool and signals impending darkness.

Coupling of PTO and TTFL to orchestrate global circadian regulation

The clock-controlled output pathway that governs transcriptional oscillation of clock-regulated genes involves the histone kinase SasA and the transcription factors RpaA and RpaB that may constitute a classic two-component system, whereby SasA senses the phosphorylation state of KaiC, autophosphorylates and subsequently effects phosphorylation of the transcription factors that bind to the *kaiBC* promoter [46–48]. As in the case of KaiB discussed above, two different models of the interaction between KaiC and SasA have been proposed, one which proposes SasA to bind to the CII dome of KaiC [32^{*}] and the other to the CI domain [49,50]. The end result of this SasA-mediated coupling between PTO and TTFL is to globally regulate [51–53] via circadian changes in chromosomal topology [54] and/or the transcriptional factors RpaA/RpaB [47,48,55] the expression of output genes but also to replenish the central clock components KaiA, KaiB, and KaiC, thereby completing a transcription and translation loop [8^{**}].

Conclusion and implications for eukaryotic clocks

A system composed of a mass-action biochemical pacemaker embedded within a transcription/translation loop has important implications. Biochemical reactions that involve small numbers of molecules (e.g., transcription factors) are intrinsically noisy, being dominated by large concentration fluctuations. However, a PTO that is rooted in the phosphorylation status of thousands of molecules would be expected to be robust in the face of noise, as we modeled for the cyanobacterial system [8^{**}]. Not only is the circadian system resilient within the cyanobacterial cell, but it is robust among a population of cells [56]. Resilience of the daily timekeeper is particularly important for cells that must keep accurate track of time in the face of cell division, when a TTFL might become perturbed because the ratio of DNA to transcriptional factors can change during replication and when DNA can become less accessible during chromosomal condensation in preparation for division [4]. The cyanobacterial clock does an excellent job of maintaining a consistent circadian period while simultaneously controlling the timing of cell division [40,56,57].

Can the studies in cyanobacteria lead us to new insights into the eukaryotic clockwork, which is currently thought to have a TTFL as the core timing circuit? Early evidence for a TTFL as the core pacemaker in the cyanobacterial system came from numerous studies that showed the same phenomena which has been used to support a TTFL

model in eukaryotes [5,8^{**},35]. However, the data summarized here indicate that the central TTFL dogma has been replaced by a PTO/TTFL system in cyanobacteria [6^{**},7,8^{**},58]. Could self-sustained biochemical core oscillators underlie eukaryotic clocks? The introductory paragraph of this paper briefly mentioned some of the studies that are beginning a re-evaluation of the core-TTFL model for eukaryotic clocks. The advantages that accrue to the cyanobacterial system by having a post-translational mechanism at its core are also relevant to eukaryotic clocks [4,21]. For example, individual mammalian fibroblasts express cell-autonomous, self-sustained circadian oscillations of gene expression that are largely unperturbed by cell division [59,60] in a fashion reminiscent of cyanobacteria [4,56,57]. Our perspective (admittedly a biased viewpoint) is that the results with cyanobacteria have important implications for eukaryotic clock systems in that they can explain how a TTFL could appear to be a core circadian clockwork when in fact the true pacemaker is an embedded biochemical oscillator [4,8^{**},21].

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