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How a cyanobacterium tells time

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Summary of recent advances

The cyanobacterium *Synechococcus elongatus* builds a circadian clock on an oscillator comprised of three proteins, KaiA, KaiB, and KaiC, which can recapitulate a circadian rhythm of KaiC phosphorylation *in vitro*. The molecular structures of all three proteins are known, and the phosphorylation steps of KaiC, the interaction dynamics among the three Kai proteins, and a weak ATPase activity of KaiC have all been characterized. An input pathway of redox-sensitive proteins uses photosynthetic function to relay light/dark information to the oscillator, and signal transduction proteins of well-known families broadcast temporal information to the genome, where global changes in transcription and a compaction of the chromosome are clock regulated.

Introduction

Studies with cyanobacteria have shown that some prokaryotes have an internal biological clock for measuring daily time. An endogenous circadian clock—a clock that measures 24-h time—enables an organism to anticipate and adjust to the predictable light-dark alternation and temperature variation that accompany the day-night cycle, rather than to simply respond acutely to challenges, incessantly, until the end of its life. Despite the wide distribution of circadian clocks in eukaryotes, cyanobacteria comprise the only group of prokaryotes demonstrated to possess one. In *Synechococcus elongatus* PCC 7942, the cyanobacterium whose clock is best studied, the central oscillator is composed of only three proteins: KaiA, KaiB, and KaiC. Inactivation of any of the *kai* genes results in arrhythmia [1]. Amazingly, a nearly 24-h oscillation of KaiC phosphorylation can be reconstituted *in vitro* with a mixture of the three Kai proteins plus ATP [2], making this system the simplest and the only post-translational circadian oscillator known so far. This “clock-in-a-test-tube” has enabled great advances in understanding the biochemical properties of a minimal circadian oscillator with unsurpassed resolution. Additional studies *in vivo* have revealed a clock that integrates the biochemical oscillator with transcription, translation, metabolism, and chromosome dynamics. Highlighting literature from the past three years, we will first summarize the recent findings of the biochemical oscillator, such as the interaction dynamics among Kai proteins and the mechanism that leads to sequential phosphorylation and dephosphorylation of KaiC. We will then focus on how the circadian clock functions within the cyanobacterial cell.

The biochemical oscillator

KaiC is both an autokinase and autophosphatase that can be phosphorylated at two positions, serine 431 and threonine 432 [3,4]. There are four possible KaiC phosphorylation states: fully phosphorylated (ST-KaiC); phosphorylated at S431 only (S-KaiC); phosphorylated at T432 only (T-KaiC); and unphosphorylated (U-KaiC) (Figure 1). In the reconstituted *in vitro* oscillator, the abundance of each of the four phosphoforms oscillates with a different phase,

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demonstrating a sequential program of phosphorylation and dephosphorylation of KaiC [5,6]. It is now clear that, starting from U-KaiC, KaiC is first phosphorylated at T432, followed by phosphorylation at S431, creating ST-KaiC; during dephosphorylation, T432 is dephosphorylated first, leading to accumulation of S-KaiC, which then becomes U-KaiC. At any particular phase, all four phosphoforms exist, and the relative ratios of the phosphoforms likely determines the phase of the oscillation; i.e., a mixture enriched in T-KaiC starts the oscillation in the phosphorylation phase, whereas a mixture enriched in S-KaiC begins with the dephosphorylation phase [6].

When incubated alone, KaiC's autophosphatase is dominant over its autokinase activity. KaiA rapidly and repeatedly associates with KaiC and shifts the equilibrium to favor the autokinase activity, and KaiB negates KaiA's action [7–10]. KaiA binds to the C-terminal peptide of KaiC, which protrudes from the double-doughnut structure of the KaiC hexamer [11–13]. The binding of KaiA to the KaiC tail likely stabilizes the exposed conformation of the tail's neighboring residues 488–497 (termed the "A-loop"), which without KaiA is proposed to flip between buried and exposed conformations. Consequently, exposed A-loops may move the ATP-binding pocket closer to the phosphorylation site, resulting in enhanced KaiC phosphorylation [14]. KaiB does not interact with the A-loop directly and does not affect KaiC phosphorylation by itself. Rather, KaiB acts by inactivating KaiA, shifting the equilibrium back to favor KaiC autophosphatase activity (Figure 1) [6]. It has been proposed that KaiB competes with KaiA for binding to KaiC [15]. However, both structural and biochemical data strongly suggest that KaiA and KaiB work together in a KaiABC ternary complex to initiate the dephosphorylation phase of KaiC by an unknown mechanism [5,10,13,16].

The assembly and disassembly of the clock protein complex, termed the periodosome, follows a circadian pattern [17,18]. The amount of KaiA-KaiC complex remains constant [16] or oscillates very weakly [10] throughout the circadian cycle, with about 10–15% of total KaiC complexed with KaiA. KaiB-KaiC and KaiA-KaiB-KaiC complexes oscillate robustly and peak during the dephosphorylation phase of KaiC [10,13,16]. Each of these three Kai complexes exists at any given phase, suggesting that not all KaiC molecules go through all forms and complexes during a circadian cycle [16]. The predominant form of KaiC at any phase, however, is free KaiC hexamer, which is estimated to be 40–60% of total KaiC.

The circadian clock must endure perturbations such as cell division, protein synthesis and turnover, and ambient temperature variations, and still be able to tell time accurately. Studies of the *in vitro* oscillator have shed light on how resilience is achieved. Two explanations that could contribute to synchrony have been put forward. Rust and colleagues have supported the hypothesis that the relative composition of the four KaiC phosphoforms determines the reaction direction [6]. Consistent with this model, the mixing of six *in vitro* oscillation reactions that are at different phases in the cycle results in a new oscillation with a synchronized phase; furthermore, the reaction direction of a mixture between two oscillations seem to be dose-dependent. [19]. Another model was put forward by Emberly and Wingreen, who proposed that monomer shuffling among KaiC hexamers could account for the robustness and resilience of the circadian clock [20]. Monomer shuffling has been demonstrated biochemically by different groups [10,16]. Mori and colleagues modeled a robust oscillation by assuming that monomer shuffling occurs preferentially between KaiC hexamers in the same conformation, and the model rhythm quickly damps out without monomer shuffling [16]. Others, however, have modeled robust oscillations of KaiC phosphorylation without considering monomer shuffling [6,13,21].

While much attention has been paid to the phosphorylation rhythm of KaiC, it was recently discovered that KaiC also possesses a very weak ATPase activity that oscillates in a circadian manner in phase with the autokinase activity [22]. Both N- and C- terminal domains of KaiC,

each of which contains a typical Walker's motif, hydrolyze ATP. Mutations in the Walker's motifs, with the exception of K294H, completely abolish circadian rhythms *in vivo* [23]. The consumption of ATP during a circadian cycle far exceeds the quantity that is calculated to be required for phosphorylation of KaiC residues, indicating that the majority of ATP is accounted for by KaiC ATPase activity. KaiA stimulates KaiC's ATPase activity, and KaiB slightly inhibits it directly, whereas its activity in stimulating autophosphatase activity acts through KaiA. In an oscillating mixture with KaiA and KaiB, one KaiC monomer consumes ~16 molecules of ATP per day through the combined ATPase and kinase activities, which is orders of magnitude lower than other well-characterized ATPases in the RecA superfamily [22]. Circadian period correlates with ATPase activity; e.g., KaiC mutants that produce short-period phenotypes display elevated ATPase activity *in vitro*. The ATPase activity of KaiC, even in the absence of a phosphorylation rhythm, is temperature compensated. Thus, the ATPase may be the basic mechanism that underlies the timing circuit. [22].

The significance of transcription and translation in the circadian clock

The basic timing mechanism in eukaryotic circadian systems appears to be built on multiple intertwined transcription/translation feedback loops [24]. In *S. elongatus* the phosphorylation cycle of KaiC continues in the absence of transcription or translation *in vivo* [25] and can be reconstituted *in vitro* [2]. However, the abundance of KaiB and KaiC does oscillate robustly *in vivo* [26], and a transcription and translation rhythm of *kaiBC* likely contributes to the resilience of the circadian clock. The *in vitro* oscillator stops working at 20 °C, but the clock still operates *in vivo* under the same condition, suggesting the clock is more stable *in vivo* than *in vitro* [27]. Furthermore, a transcription/translation rhythm persists when KaiC is constitutively phosphorylated, although it is weakened and still dependent on the presence of KaiC [27]. KaiC overexpression represses global gene expression [28], and transcriptional activity is correlated with the phosphorylation state of KaiC [29]. If we regard the phosphorylation rhythm of KaiC as an oscillation of feedbacks among Kai proteins, and the transcription/translation rhythm as another oscillation, the intertwining of the these two circuits connected by KaiC resembles the basic themes found in the eukaryotic systems. It is also possible that the oscillation of KaiC ATPase activity drives the transcription/translation rhythm [27].

Transcription and translation are also involved in the regulation of other aspects of the circadian clock. Changes in the abundance of Kai proteins affect circadian period [14,30]. Depletion of the Clp proteases lengthens circadian period, although it is not known whether the Kai proteins are direct Clp substrates [31]. Transcriptional control of at least KaiA is targeted by the input system of the clock. The Pex protein, which is upregulated in the dark [32], binds to the promoter region of *kaiA*, suppresses its transcription, and acts to extend the circadian period [33–35]. The redox state of the cell, which feeds into the input pathway of the clock, also affects KaiA abundance (Figure 2) through an unknown mechanism [36].

Transcription and translation pose complications to the oscillator that do not exist *in vitro*. For example, the KaiC$^{S431A, T432A}$ (KaiC-AA) mutant, which is used to mimic unphosphorylated KaiC, does not participate in monomer exchange and addition of it to an *in vitro* oscillation has no effect [19]. However, when KaiC-AA is expressed in a WT background, it causes a dominant negative effect on the circadian rhythm of gene expression and KaiC phosphorylation [14]. The data suggest that hexamers form upon *de novo* synthesis and can be poisoned by incorporating non-functional subunits, even if they do not exchange with these subunits after they are made.

A redox input into the central oscillator

In eukaryotic circadian systems, the input pathway is usually composed of at least one photoreceptor that directly relays light information to the central oscillator [37–39]. In *S. elongatus*, however, no photoreceptors have been found to affect resetting of the clock. Experiments aimed to inactivate each locus that encodes predicted light-sensing domains and test circadian phase resetting have returned only negative results [40]. On the other hand, two of the three known proteins involved in the input pathway, LdpA and CikA, are sensitive to the redox state of the cell (Figure 2), which is a function of light intensity [36,41]. Other redox-active proteins that affect the clock have been identified as potential interaction partners of CikA [42]. Taken together, the data suggest that the *S. elongatus* clock likely senses the cellular redox state, rather than light intensity, to keep track of external time.

The CikA histidine protein kinase (HPK) is a major player in resetting the clock, and in the absence of CikA the circadian clock is blind to a 5-h dark pulse that usually resets the phase of rhythms by up to 8 h [43]. CikA is unlikely to act as a direct photoreceptor [44]. CikA autokinase activity is regulated positively and negatively by adjacent GAF and pseudo-receiver (PsR) domains, respectively [41,44]. Structural analysis suggests that the PsR domain interacts with the HPK in a manner similar to a *bona fide* receiver, effectively suppressing kinase activity [45]. The PsR domain is essential for CikA localization to the cell pole and may be an interaction domain with other proteins [41,42]. CikA's abundance is regulated by LdpA [36] and is sensitive to the plastoquinone analog 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) (Figure 2). The PsR domain of CikA binds DBMIB directly, revealing a new function for PsR domains, which are present in plant clock proteins as well, where their activities are unknown [46].

A global output mechanism

All promoters examined in *S. elongatus* are rhythmically expressed, even those that originate from *Escherichia coli* [47]. Several lines of evidence suggest that DNA topology is involved in effecting rhythmicity of gene expression [47–49]. Smith and Williams showed that the chromosome slowly compacts during the subjective day and decompacts during the subjective night, and the compaction rhythm continues in constant conditions and is dependent on KaiC [50]. Woelfle and colleagues discovered that promoters inserted in a plasmid that replicates independently in *S. elongatus* are also expressed rhythmically in a Kai-dependent manner, and the supercoiling status of the plasmid changes during the circadian cycle [51]. Overall, it is plausible that the clock controls the global transcription rhythm by regulating DNA topology (Figure 2).

A key player in the output pathway, SasA, is an HPK that physically interacts with KaiC [52]. The chromosome compaction and gene expression rhythms persist in a *sasA* mutant, although with a damped amplitude compared to the WT [50]. The bioluminescence rhythms from reporter genes in a *sasA* mutant have a short circadian period and are evident only under low light conditions, suggesting the existence of an independent output pathway that is light sensitive. The autokinase activity of SasA is greatly enhanced by KaiC, and presumably the phosphorylation state of KaiC is important in relaying information [50]. The cognate response regulator of SasA has been identified as RpaA (Figure 2), which carries a receiver and DNA-binding domains [53]. RpaA does not bind to the promoter region of *kaiBC*, and its target remains to be identified [53]. Another gene in the temporal output pathway is *labA*; in its absence, global transcription repression caused by KaiC overexpression is suppressed, and elevated trough levels of gene expression are observed. Genetic analysis suggests that LabA functions upstream of RpaA, but parallel to SasA; thus, SasA and LabA converge positively and negatively, respectively, on RpaA to affect gene expression [54].

Despite these exciting details of cyanobacterial clock mechanism, we do not know how the circadian clock controls DNA topology. Identification of the target of RpaA may yield some clues. Alternatively, an unidentified output pathway, or even KaiC itself, could be directly involved, as KaiC binds to forked DNA *in vitro* [55]. Moreover, it is possible that certain events stimulate KaiC ATPase activity sufficiently to power mechanical motion.

Conclusions

The cyanobacterial circadian clock research community has made major strides in the past several years. The cyanobacterial clock is composed of a novel post-translational oscillator coupled with a reinforcing transcription-translation oscillation, a redox-sensing input pathway, and a global output pathway that controls the genome through changes in DNA topology. Future studies will likely reveal in exquisite detail how this simple oscillator tells time accurately and precisely and coordinates the many life events of a unicellular cell to exploit a predictable, but ever-changing, environment.

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•• of outstanding interest

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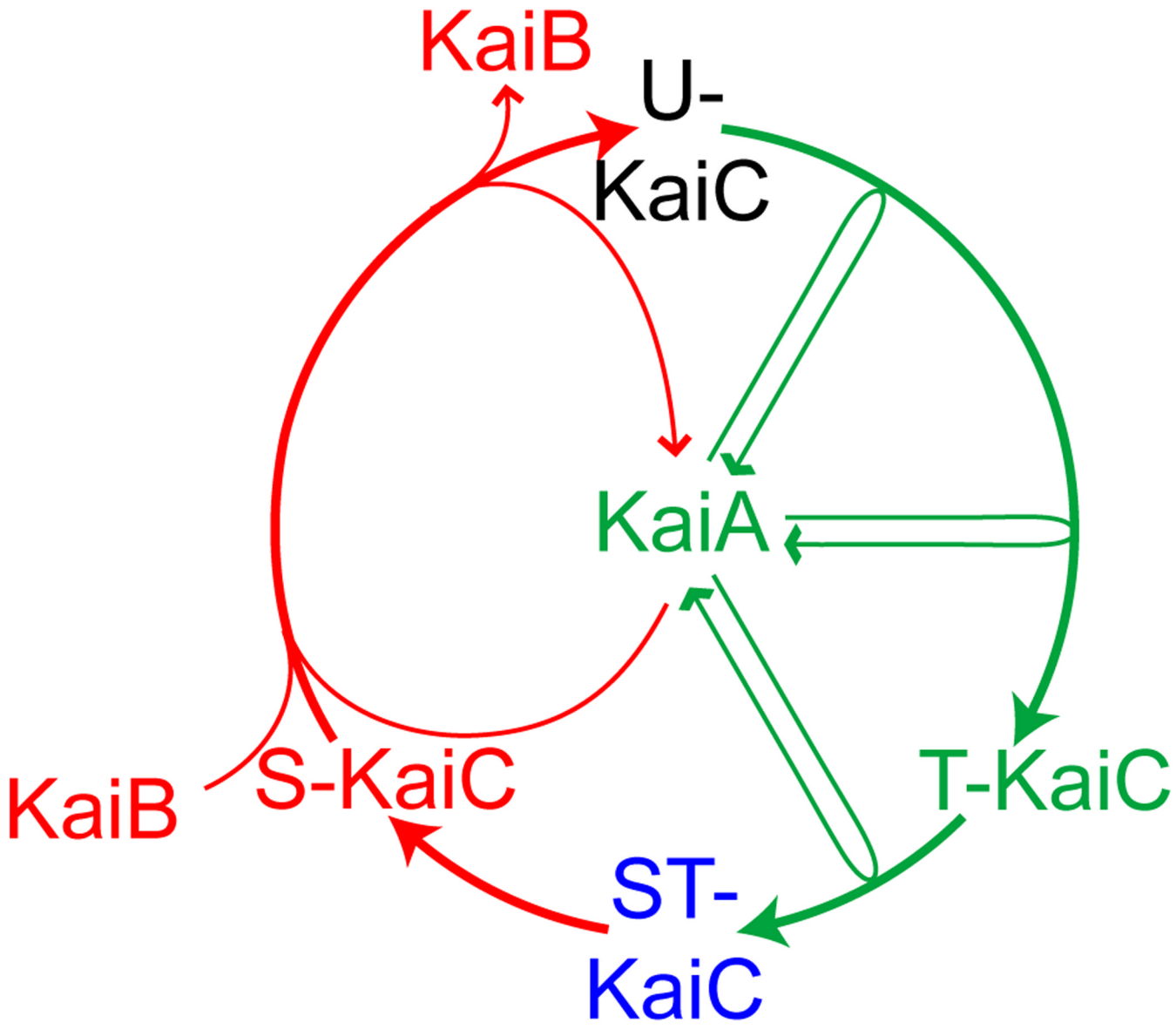

Figure 1. A model of the KaiC phosphorylation rhythm

During a circadian cycle (represented by a circle), the phosphorylation states of KaiC proceed in an orderly manner. The relative timing of the peak for each phosphoform, based on published data [5,6], is shown by its position on the circle. KaiA stimulates KaiC phosphorylation by repeated association with KaiC. Starting from unphosphorylated KaiC (U); KaiC is first phosphorylated at T432 (T), which is further phosphorylated to the fully phosphorylated form (ST); T432 residue dephosphorylates from ST-KaiC first, resulting in KaiC phosphorylated only at S431 (S). KaiB preferentially binds S-KaiC, which forms a ternary complex with KaiA and, presumably, inactivates it and allows KaiC to return to the unphosphorylated state. The phosphorylation phase is represented in green and the dephosphorylation phase in red.

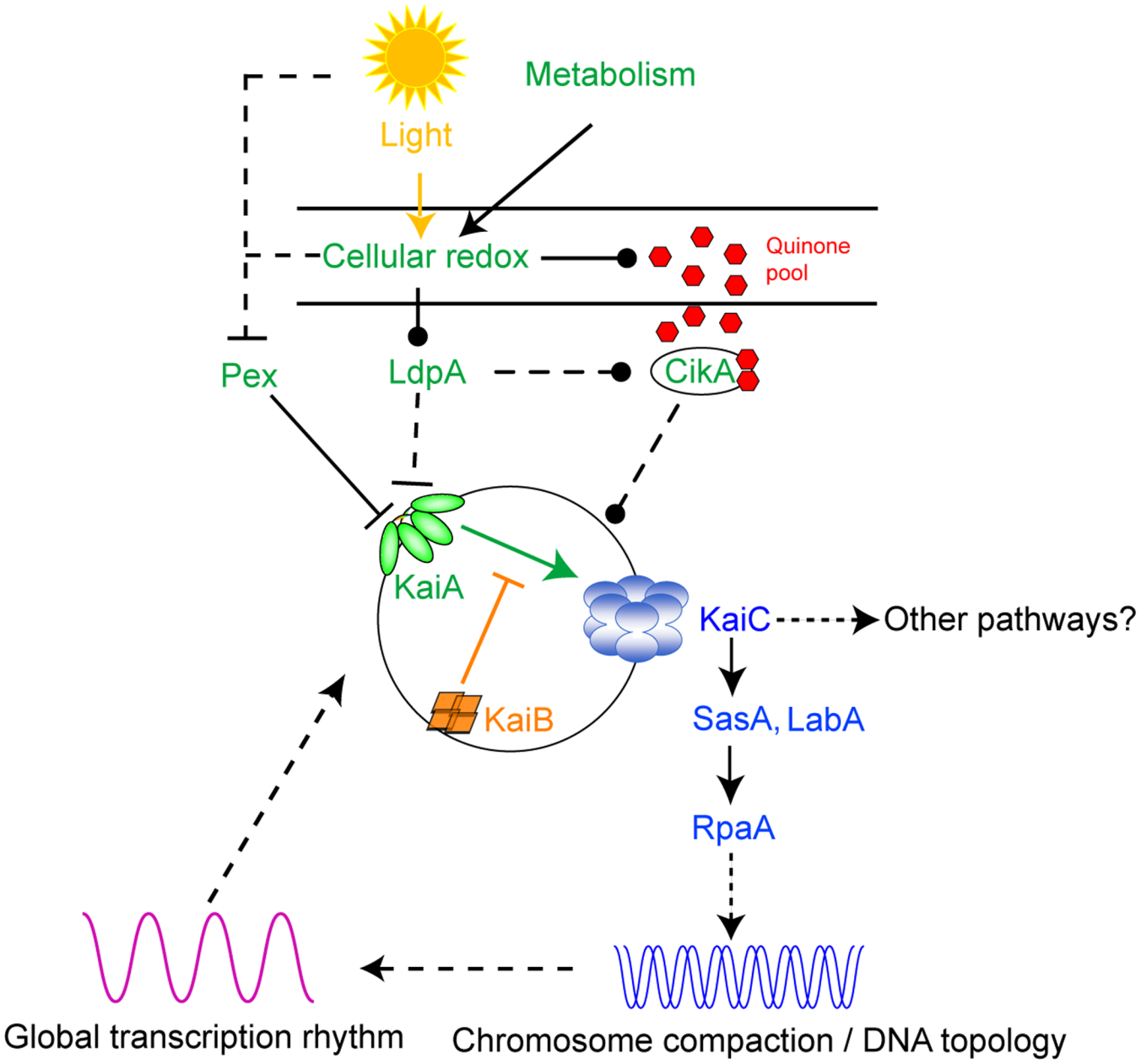

Figure 2. An Overview of the Molecular Mechanism of the Circadian Clock in *S. elongatus*

The central oscillator is composed of KaiA, KaiB and KaiC. KaiA stimulates KaiC phosphorylation, and KaiB inactivates KaiA when KaiC reaches a certain phosphorylation state (see Figure 1 for details). In the input pathway, both LdpA and CikA sense the cellular redox state, which is regulated by light and cell metabolism. LdpA affects the stability of CikA and KaiA through an unknown mechanism. Through its PsR domain, CikA binds quinone molecules directly, which destabilizes CikA. CikA affects phosphorylaton states of KaiC, but where and how it works in the signal transduction pathway is unknown. Pex is a transcriptional repressor of KaiA, and its abundance is sensitive to light, but it is not clear whether the pathway that regulates *pex* senses light directly or does it through cellular redox. In the output pathway, SasA interacts physically with KaiC and autophosphorylates, and then transfers the phosphoryl group to RpaA, a response regulator with a DNA binding domain. The target of RpaA has not

been identified. LabA works upstream of RpaA and downstream of KaiC, but its exact function is not clear. A SasA- and RpaA- independent output pathway might exist. The output pathway controls DNA topology, which is proposed to regulate global gene expression. A transcription/translation rhythm could interact with and reinforce the post-translational rhythm of KaiC activities. Figure legends: a solid line indicates a direct effect whereas a dotted line indicates an indirect effect or an effect whose mechanism is unknown. Arrows indicate the direction of the information flow or a stimulation of activity or both. Blunt-ends represent an inhibition of protein activity or abundance, whereas an end with a filled circle suggests a regulation of unspecified direction.