# CELLULAR FUNCTION AND LOCALIZATION OF CIRCADIAN CLOCK PROTEINS IN CYANOBACTERIA

A Dissertation

by

GUOGANG DONG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Microbiology

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Approved by:

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|---|---|
| Chair of Committee, | Susan S. Golden |
| Committee Members, | Deborah Bell-Pedersen |
| | Andy LiWang |
| | Deborah Siegele |
| Head of Department, | Thomas D. McKnight |

December 2008

Major Subject: Microbiology

# ABSTRACT

Cellular Function and Localization of Circadian Clock Proteins in Cyanobacteria.

(December 2008)

Guogang Dong, B.S., Peking University

Chair of Advisory Committee: Dr. Susan S. Golden

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. A mutant of a clock gene in the input pathway, *cikA*, has a cell division defect, and the circadian clock inhibits the cell cycle for a short period of time during each cycle. However, the interaction between the circadian cycle and the cell cycle and the molecular mechanisms underlying it have been poorly understood. In addition, the subcellular localization of clock proteins and possible localization dynamics, which are critical in the timing circuit of eukaryotic clock systems and might also shed light on the interaction between circadian cycle and cell cycle, have

remained largely unknown. A combination of genetics, cell biology, and microscopy techniques has been employed to investigate both questions.

This work showed that the cell division defect of a *cikA* mutant is a function of the circadian clock. High ATPase activity of KaiC coincides with the inhibition of cytokinesis by the circadian clock. CikA likely represses KaiC's ATPase activity through an unknown protein, which in *cikA*'s absence stimulates both the ATPase and autokinase activities independently of KaiA or KaiB. SasA-RpaA acts as an output in the control of cell division, and the localization of FtsZ is the target, although it still remains to be seen how RpaA, directly or indirectly, inhibits FtsZ localization.

The project also showed that clock proteins are localized to the cell poles. KaiC is targeted to the cell pole in a phosphorylation-dependent manner. KaiB and CikA are also found at the poles independently of KaiC. KaiA likely only localizes to the cell pole during the dephosphorylation phase, which is dependent on both KaiB and KaiC, specifically on the phosphorylation of KaiC at S431.

Overall, significant progress was made in both areas and this project sheds light on how the circadian oscillator operates in cyanobacterial cells and interacts with another fundamental cellular function.

To My Father

Dong, Xingmin

and

My Mother

Gao, Suxia

for

their Patience and Unconditional Love.

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The course of this dissertation is also a process during which I have been learning and adjusting to a completely new culture. More than six years ago, I boarded a plane from Beijing with eleven fellow Chinese students to attend graduate school in a small town of Texas, U.S.A. It was my first time on a plane, my first trip out of the country, and the first time English was spoken to mean it. From that moment on I have received a great deal of help from many different people, some of whom later became friends and some I only got to meet once. To this day I still remember vividly the morning I landed in Houston and an airport worker dialed an international call to my parents when I asked him for locations of public pay phones to tell them I was safe. That was my first impression of the people of Houston, of Texas, and of the U.S.A., and it was great. Just like that, it would be nearly impossible here to give thanks properly to everyone who has helped me in the past, both with my life in a foreign country and with my research, but the fear of overlooking someone certainly should not prevent me from trying my best. Therefore, I will start my acknowledgement with an apology to everyone, whether mentioned below or not, because words pale in comparison to my gratitude towards them.

I would like to thank my adviser, Susan Golden, for her constant guidance, encouragement and support both academically and personally. I feel extremely fortunate to have the opportunity to work with her during these years in graduate

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# NOMENCLATURE

| | |
|---|---|
| LL | Constant light |
| LD | Light/Dark cycle |
| CT | Circadian time |
| ZT | Zeitgeber time |
| $\tau$ | Period of endogenous clock; free-running period |
| T | Period of exogenous cycle, such as LD cycle |
| SCN | Suprachiasmatic nucleus |
| IPTG | isopropyl-β-D-thiogalactopyranoside |

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# CHAPTER I

# INTRODUCTION*

Circadian rhythmicity is an endogenous biological process in which an organism's behaviors, metabolism and gene expression cycle with a periodicity of approximately 24 hours. An endogenous circadian clock 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. Despite the wide distribution of circadian clocks in eukaryotes, cyanobacteria are the only group of prokaryotes demonstrated to possess one (Ditty et al., 2003; Golden, 2003; Young and Kay, 2001).

## CIRCADIAN RHYTHMS

Several basic concepts are frequently used to describe a rhythm, such as period, phase angle (phase) and amplitude. Period is the time after which a defined phase of an oscillation recurs; phase is the instantaneous state of an oscillation within a period (for example, the timing of a peak relative to a reference point), and amplitude is the mean of an oscillation to its maximum or minimum. Three criteria must be satisfied to define a cyclic biological phenomenon as a circadian rhythm: 1) the process cycles with a period of close

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to, but usually not exactly, 24 hours under constant conditions. The persistence of the rhythm in constant light or dark conditions is critical, as it proves the rhythm to be endogenous, rather than an exogenously driven one. 2) The phase of the rhythm must be able to be synchronized to local time, a process called entrainment. In order for the circadian clock to properly time its host organism's activities, it has to be able to sense the external time and adjust accordingly, since the period of the internal clock is rarely exactly 24 hours. The two most obvious environmental cues that entrain a circadian clock are light intensity and temperature, which fluctuate during the earth's day-night cycle. 3) The period of the rhythm remains relatively stable at various ambient temperatures (is temperature compensated) in order to be an accurate time-keeper rather than a thermometer; i.e., the circadian period is buffered against temperature changes, whereas the phase of the rhythm responds to temperature shift. By convention, the period of a cycle under free-running conditions ($\tau$) is divided into 24 equal parts (circadian time, CT), so that relative phase can be compared among individuals that have different values of $\tau$ (Johnson et al., 2004).

The first evidence for an endogenous circadian clock was published in 1729, when Jean Jacques Ortous de Mairan, a French astronomer, observed that the daily rhythm of leaf movement of the plant *Mimosa pudica* persisted even when placed in constant darkness (Roenneberg and Merrow, 2005). Since then, scientists have identified circadian rhythms in almost all taxa of life ranging from bacteria to humans. In the bread mold *Neurospora crassa*, the formation of

spore-containing conidia is controlled by the circadian clock (Sargent et al., 1966). A rhythm of eclosion, which is the emergence of an adult insect from a pupal case or an insect larva from an egg, has been well documented for many insects, including the fruit fly *Drosophila melanogaster* (Myers, 2003). In mammals, wheel-running activity of mice and sleep cycles of humans are all examples of the prevalence of circadian rhythms. In cyanobacteria, the activity rhythm of nitrogenase, a key enzyme involved in nitrogen fixation, was demonstrated in *Synechococcus* sp. RF-1 to satisfy all three criteria of circadian rhythm in 1990 (Huang et al., 1990), establishing indisputable evidence for a circadian clock in a prokaryote. Recently, a rhythm of gene expression was discovered in the photosynthetic purple bacterium *Rhodobacter sphaeroides*, which shares two homologous clock genes (*kaiB* and *kaiC*) with cyanobacteria; this rhythm oscillates with about a 21-hour period under aerobic conditions, although the circadian property of the rhythm still remains to be investigated (Min et al., 2005).

The presence of circadian clocks in a wide range of living organisms has suggested the evolutionary advantage the clock imposes upon those organisms. However, systematic, rigorous experiments aimed to test the fitness advantage of the clock have not been the focus of most circadian rhythm research to date. It is worth pointing out that testing the fitness significance of circadian systems is not an easy task, as differences in the growth, development and reproduction under standard experimental conditions in clock-impaired mutants may not be

apparent, and can only be discovered in natural environments where the host organism is interacting within and across species. In one real-world test, SCN-ablated white-tailed antelope ground squirrels, whose circadian rhythms at the behavioral level were completely abolished, were put in a controlled-field environment together with SCN-intact animals. Sixty percent of the SCN-lesioned animals fell to prey to the unplanned nighttime attack of a feral cat compared to only 29% of the control group (DeCoursey et al., 1997), supporting the idea that the circadian clock may improve an organism's survival chance against competition from other species, such as their predators. Another hypothesis, which is coined "circadian resonance", states that fitness is enhanced when the free-running period ($\tau$), which is the circadian period in constant conditions, is matched closely with that of the exogenous environmental cycles (T cycle). This hypothesis was supported in higher plants when it was found that growth rate was highest when plants were placed in 24 h T cycles, compared with those with 12 h or 48 h T cycles (Highkin and Hanson, 1954). In 1972, Colin Pittendrigh and coworkers showed that the lifespan of the fruit fly *D. melanogaster* was significantly superior under natural T cycles with a 24 h period rather than those with much shorter or longer periods (Pittendrigh and Minis, 1972), although later attempts at repeating the experiment were unsuccessful, even in the original lab (Ouyang et al., 1998). The most direct evidence supporting the circadian resonance hypothesis comes from experiments in cyanobacteria. An arrhythmic mutant of *S. elongatus* was

outcompeted under LD conditions when mixed together with the wild type (WT) strain, while no difference in growth was observed when the strains were grown separately under LL or LD conditions (Woelfle et al., 2004). In another experiment, mutants with short or long periods were mixed together and grown under short or long T cycles, and in each case the strain whose period was more similar to that of the T cycle outcompeted the others (Ouyang et al., 1998). Most recently, similar work was carried out in *Arabidopsis thaliana* and showed that photosynthesis and growth are significantly enhanced when various period mutants and the WT strains are placed in T cycles that resonate with their endogenous period; furthermore, when grown together in a competing environment, the strain with a $\tau$ that is closest to T outcompetes all other strains that have a bigger deviation from the T (Dodd et al., 2005). A molecular mechanism to explain the adaptive advantage of circadian systems, however, is still elusive.

Although the molecular mechanism underlying the circadian clock is quite complex and varies from one organism to another, a simplified three-component system is still widely used to describe it: the input pathway, the central oscillator and the output pathway (Eskin, 1979). The heart of each circadian clock is the central oscillator, which is the engine that generates a close to 24-hour oscillation. Inactivation of any of the components of the central oscillator makes the circadian clock to cease function. The input pathway senses changes in environmental parameters (such as light intensity and temperature) and

transmits that information to the central oscillator; thus, the input pathway is responsible for the entrainment of the rhythm to match the external daily cycle. The output pathway feeds off the information from the central oscillator and translates it into rhythmic activities such as gene expression, metabolism and behavior.

Circadian clocks seem to have evolved at least four independent times, as clock genes from bacteria, fungi, plants and animals are not well conserved among one another. In spite of major differences in the molecular players that make up the circadian clock, autoregulatory negative feedback loops, mainly of transcription and translation, are a common theme in all the systems examined so far. Usually a positive element activates the production or activity of a negative element, which in turn blocks the production or activity of the positive element, and the decay of the negative element allows a restart of the cycle. In *N. crassa* the positive elements White collar-1 and White collar-2 form a protein complex (WCC) and act as a transcriptional activator of the *frequency* (*frq*) gene, which codes for the central oscillator protein FRQ that in turn physically interacts with the WCC and inactivates its own *frq* transcription (Loros, 1998). In circadian systems multiple feedback loops are often interconnected and thus make the clock more complex and robust. In *D. melanogaster* Clock (Clk) and Cycle (Cyc) form a heterodimer that activates the transcription of the genes *period* (*per*) and *timeless* (*tim*), whose protein products enter the nucleus with the help of Doubletime (Dbt) and shut off their own transcription by inactivating the Clk-Cyc

complex. In a separate but overlapping feedback loop, Clk-Cyc also activates the transcription of *vrille* (*vri*) and *pdp-1*ε, of which Vri is a transcription repressor that inhibits the expression of the *clk* gene (Hardin, 2005). In mice, a similar two-feedback loop system exists, though some of the genes involved are different (Hardin, 2004). In *A. thaliana* CCA1 and LHY are two transcription repressors that inhibit the expression of TOC1, which induces the expression of *CCA1* and *LHY* possibly with the help of GIGANTEA (Salome and McClung, 2004). Recently it was shown that the abundance of cyclic adenosine diphosphate ribose (cADPR) in *A. thaliana* is under the control of the circadian clock, and cADPR upregulates *GI* and downregulates *CCA1* and *LHY*, among many other genes, possibly adding another feedback loop to the system (Dodd et al., 2007). In *S. elongatus*, the central clock protein KaiC represses global gene expression when in excess, including at its own promoter, whereas KaiA seems to have a positive effect on *kaiC* transcription (Ishiura et al., 1998; Nakahira et al., 2004). However, recently it was discovered that the transcription-translation feedback loop is not essential for the circadian oscillation (Ditty et al., 2005; Tomita et al., 2005); rather, the feedback interactions among the three Kai proteins at the post-translational level appear to be crucial, which will be discussed in detail below (Nakajima et al., 2005; Rust et al., 2007).

## *SYNECHOCOCCUS ELONGATUS* PCC 7942 AS A MODEL ORGANISM

Cyanobacteria, also known as blue-green algae, are a diverse group of Gram-negative prokaryotic microorganisms capable of carrying out oxygenic photosynthesis, a process that converts solar energy into chemical energy stored in carbohydrates, while releasing oxygen into the atmosphere. Existing fossil evidence has dated cyanobacteria as early as 2.8 billion years ago (Olson, 2006). Today cyanobacteria are found in a wide variety of habitats from fresh waters to oceans, damp soils and even rocks. Different species of cyanobacteria have been used as model organisms in the study of photosynthesis, nitrogen fixation, hydrogen production, heterocyst development, light response and circadian rhythms (Berman-Frank et al., 2003; Ghirardi et al., 2007; Golden and Yoon, 2003; Golden and Canales, 2003; Nelson and Yocum, 2006; Zhang et al., 2006a).

*Synechococcus elongatus* PCC 7942 (*S. elongatus*), formerly named *Anacystis nidulans* R2, is a unicellular cyanobacterium isolated from fresh water in California, U.S.A. (Pasteur Culture Collection), and is an obligate photoautotroph. *S. elongatus* cells are rod-shaped, and measure at 3-5 μm in length and ~1 μm in diameter. The cell wall is sandwiched between an outer and an inner membrane, and layers of thylakoid membranes interconnected with one another are folded closely along the inner membrane on the cytoplasmic side, harboring photo pigments and protein complexes for light-driven reactions of photosynthesis (Nevo et al., 2007; van de Meene et al., 2006). *S. elongatus*

reproduces by binary fission, and cell division is inhibited in the dark (Asato, 1983). Unlike many cyanobacteria, *S. elongatus* does not fix nitrogen.

*S. elongatus* is the first cyanobacterium demonstrated to be reliably transformable with the addition of exogenous DNA (Shestakov and Khyen, 1970), which helped advance the development of various genetic tools (Andersson et al., 2000; Golden et al., 1987; Kuhlemeier and van Arkel, 1987). The complete genome sequence of *S. elongatus* has been released (GenBank Access No. CP000100) and functional annotation of the genome is well under way (Holtman et al., 2005). The genome consists of about 2.7 Mbps of sequence coding for ~2800 genes with relatively little redundancy, which aids in genetic analysis. The successful application of a luciferase of either a bacterial or an insect origin as a reporter has automated large-scale monitoring of gene expression in this organism, and has proven to be critical in the elucidation of circadian mechanisms in cyanobacteria (Kondo and Ishiura, 1994; Kondo et al., 1993; Kondo et al., 1994; Liu et al., 1995a). Circadian rhythms were first demonstrated in *Synechococcus* sp. RF-1 (Huang et al., 1990); however, this species of cyanobacteria is not genetically tractable and further characterization of the clock mechanism is difficult. With luciferase as a reporter, circadian rhythms of prokaryotic gene expression were first demonstrated in *S. elongatus*, and later in *Synechocystis* sp. PCC 6803 (Aoki et al., 1997) and *Thermosynechococcus elongatus* (Onai et al., 2004). Due to its relatively fast growth, small genome, robust reporter-gene expression rhythms and the many

genetic tools available, *S. elongatus* PCC 7942 is the most popular and best model organism in the study of circadian rhythm in bacteria.

## CIRCADIAN RHYTHMS IN *S. ELONGATUS* PCC 7942

Fusion of the promoter of *psbAI* gene, which codes for the D1 protein of photosystem II of photosynthesis, to the promoterless bacterial luciferase gene *luxAB* created the reporter strain of *S. elongatus* named AMC149. Circadian rhythmicity of *psbAI* promoter activity in AMC149 was successfully reported in both liquid cultures and on solid medium with automated bioluminescence monitoring systems (Kondo et al., 1993; Kondo et al., 1994). The rhythm has a period of slightly longer than 24 h at 30 °C and peaks around CT 12, which corresponds to the early subjective night. Later a random insertion of promoterless *luxAB* genes throughout the genome revealed that all promoters examined showed rhythmic gene expression (Liu et al., 1995b). Most of the promoters examined, classified as Class 1, to which *psbAI* promoter belongs, peak around CT 12; a minority group termed Class 2 promoters peak around CT 0, 12 h out of phase with Class 1 promoters. It is tempting to propose that the circadian clock coordinates incompatible biochemical events at different times of the day; however, the few Class 2 genes identified so far have only hinted at such a possibility (Liu et al., 1996; Min and Golden, 2000).

In 1994, an ethane methyl sulfonate (EMS)-mediated random mutagenesis experiment was carried out in AMC149, and various clock mutants were

obtained with phenotypes ranging from short (16 h) or long (60 h) periods to arrhythmia (Kondo et al., 1994). Four years later, the first clock genes in cyanobacteria were successfully cloned and a cluster of three genes, *kaiA*, *kaiB*, and *kaiC*, were identified, with *kai* meaning *cycle* in Japanese (Ishiura et al., 1998). Inactivation of any of these genes causes arrhythmia, and overexpression of either *kaiA* or *kaiC* abolishes the rhythm. By mapping the position of mutations in period mutants, it is noteworthy that mutations in *kaiA* tend to lengthen the period and those in *kaiB* shorten it, whereas *kaiC* mutations can lead either way (Ishiura et al., 1998; Nishimura et al., 2002). The *kaiA* gene is expressed from its own promoter, whereas *kaiBC* are arranged in an operon and thus expressed from a single promoter. Activity of both *kaiA* and *kaiBC* promoters oscillate, evidenced by both the mRNA level and the luciferase reporter assay. Based on the observations that KaiC overexpression represses its own expression, and KaiA overexpression enhances *kaiC* expression level, Ishiura et al. proposed a transcription-translation feedback loop model with KaiA being the positive element and KaiC being the negative element as the fundamental mechanism of timekeeping in cyanobacteria, similar to the ones found in *N. crassa* and *D. melanogaster* at that time (Ishiura et al., 1998). However, expression of *kai* genes ectopically from Class 2 promoters (*kai* promoters are Class 1) or from promoters originated from *Escherichia coli* restores the circadian rhythm phenotype in *kai* null backgrounds (Ditty et al., 2005; Xu et al., 2000). Furthermore, the phosphorylation rhythm of KaiC persists

when no transcription or translation is allowed in vivo (Tomita et al., 2005), suggesting that a transcription-translation feedback loop is not essential for the generation of a circadian rhythm. Amazingly, circadian oscillation of KaiC phosphorylation is reconstituted in vitro with a mixture of the three Kai proteins plus ATP (Nakajima et al., 2005), making it the simplest and the only post-translational circadian oscillator known so far.

## THE BIOCHEMICAL OSCILLATOR

KaiC is both an autokinase and autophosphatase that can be phosphorylated at two positions, serine 431 and threonine 432 (Nishiwaki et al., 2004; Xu et al., 2004). 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 1.1). In the reconstituted in vitro oscillator, the abundance of each of the four phosphoforms oscillates with a different phase, demonstrating a sequential program of phosphorylation and dephosphorylation of KaiC (Nishiwaki et al., 2007; Rust et al., 2007). 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, accumulating S-KaiC, which then becomes U-KaiC. At any particular phase, all four phosphoforms exist, and the relative composition 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

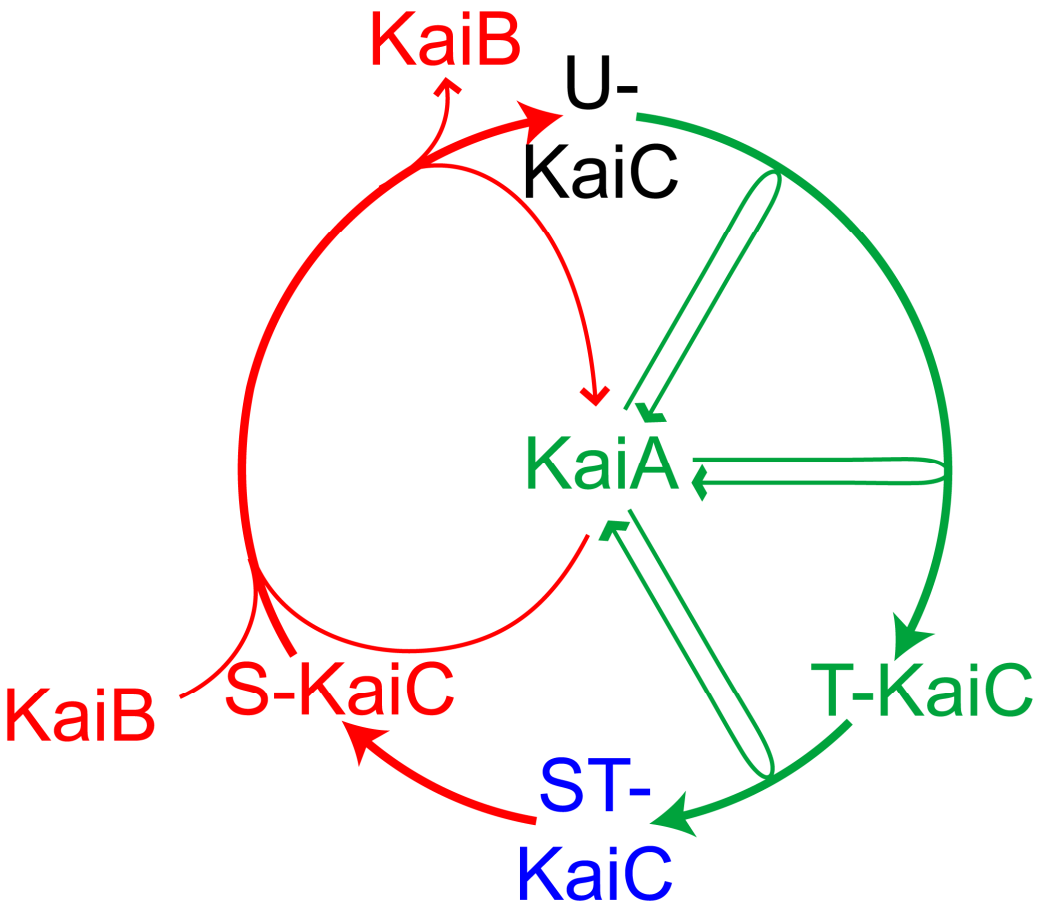


**Figure 1.1. A Model of the KaiC Phosphorylation Rhythm.**
During a circadian cycle (represented by a circle), the phosphorylation states of KaiC proceed in an orderly manner. The relative timing of the peak for each phosphoform, based on published data (Nishiwaki et al., 2007; Rust et al., 2007), is shown by its position on the circle. KaiA stimulates KaiC phosphorylation by repeated association (indicted by the green looping arrows) with KaiC. Starting from unphosphorylated KaiC (U-KaiC), KaiC is first phosphorylated at T432 (T-KaiC), which is further phosphorylated to the fully phosphorylated form (ST-KaiC); T432 residue dephosphorylates from ST-KaiC first, resulting in KaiC phosphorylated only at S431 (S-KaiC). 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.

mixture enriched in S-KaiC begins with the dephosphorylation phase (Rust et al., 2007).

When incubated alone, KaiC's autophosphatase is dominant over its autokinase activity (Kim et al., 2008). KaiA rapidly and repeatedly associates with KaiC and shifts the equilibrium to favor the autokinase activity, and KaiB negates KaiA's action (Iwasaki et al., 2002; Kageyama et al., 2006; Kitayama et al., 2003; Williams et al., 2002). Crystal structures of all three Kai proteins have been solved. KaiA is a dimer, and KaiC forms ATP-dependent hexamers. KaiB is either a dimer or tetramer, which seems to be species-specific (Garces et al., 2004; Hayashi et al., 2003; Hitomi et al., 2005; Iwase et al., 2005; Pattanayek et al., 2004; Pattanayek et al., 2008; Uzumaki et al., 2004; Vakonakis and LiWang, 2004a, b; Vakonakis et al., 2004; Williams et al., 2002; Ye et al., 2004). KaiA binds to the C-terminal peptide of KaiC, which protrudes from the double-doughnut structure of the KaiC hexamer (Akiyama et al., 2008; Pattanayek et al., 2006; Vakonakis and LiWang, 2004b). 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 (Kim et al., 2008).

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 1.1) (Rust et al., 2007). It has been proposed that KaiB competes with KaiA for binding to KaiC (Garces et al., 2004). 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 (Akiyama et al., 2008; Kageyama et al., 2006; Mori et al., 2007; Nishiwaki et al., 2007). KaiB preferentially binds to phosphorylated KaiC (Kageyama et al., 2006). Furthermore, pull-down assays using either FLAG-tagged KaiB or KaiC show that KaiABC complex formation coincides with the accumulation of S-KaiC, indicating that KaiB has a higher affinity for S-KaiC than other KaiC phosphoforms, which in turn traps and inactivates KaiA (Nishiwaki et al., 2007; Rust et al., 2007). The N-terminal domain of KaiA, which adopts a conformation like that of a receiver domain but lacks the conserved aspartate residue, is likely to play a role in the inhibition of KaiA activity by KaiB because C-terminal KaiA is responsible for directly stimulating KaiC phosphorylation and is insensitive to KaiB in the phosphorylation assay (Williams et al., 2002).

The assembly and disassembly of the clock protein complex in vivo, termed the periodosome, follows a circadian pattern (Golden, 2004; Kageyama et al., 2003). Recent findings in vitro are consistent with these results. The amount of KaiA-KaiC complex remains constant (Mori et al., 2007) or oscillates very weakly (Kageyama et al., 2006) 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 (Akiyama et al., 2008; Kageyama et al., 2006; Mori et al., 2007). 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 (Mori et al., 2007). 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. Keeping the molar ratio among Kai proteins constant, a stable oscillation is sustained with protein concentrations that vary over a 50-fold range, although changes in period and amplitude do occur (Kageyama et al., 2006). In addition, reducing the ratio of KaiA or KaiB to KaiC is tolerated down to 30% of the standard condition (Kageyama et al., 2006). Remarkably, mixture of six in vitro oscillations reactions that are at different phases in the cycle results in a new oscillation with a synchronized phase; specifically, KaiC that is in the phosphorylation phase will change the direction of its reaction and start to dephosphorylate until the different KaiC populations reach synchrony (Ito et al., 2007). Rust and colleagues have supported the hypothesis that the relative composition of the four KaiC phosphoforms determines the reaction direction (Rust et al., 2007). The same hypothesis could explain the phase synchronization results. An equal mixture of KaiC samples with opposite phases

may have enough S-KaiC to inactivate all KaiA molecules, starting the new oscillation from the dephosphorylation phase. This means the direction of reaction should be dose-dependent, which is supported by the fact that at least 30% of the mixture needs to be from samples in the dephosphorylation phase in order to synchronize KaiC proteins in other samples (Ito et al., 2007). The critical values of the phosphoform distributions that determine the direction of reaction have not been established, although rough estimations are available (Rust et al., 2007).

Emberly and Wingreen proposed that monomer shuffling among KaiC hexamers could account for the robustness and resilience of the circadian clock (Emberly and Wingreen, 2006). Kageyama et al. demonstrated monomer shuffling by pull-down methods, and showed that KaiA inhibits monomer exhange while KaiB has no effect (Kageyama et al., 2006). Mori and colleagues confirmed the shuffling using fluorescence resonance energy transfer (FRET); however, they detected no inhibition by either KaiA or KaiB (Mori et al., 2007). Differences in the phosphorylation state of KaiC may account for these differing results, as Ito and colleagues discovered that monomer shuffling is limited to a four-hour window during the initial phases of KaiC dephosphorylation (Ito et al., 2007). A KaiC variant that mimics the unphosphorylated state does not exchange monomers, whereas one that mimics fully phosphorylated KaiC does, further supporting the phosphorylation-dependent monomer shuffling hypothesis. Structural and gel filtration experiments have failed to detect the

presence of KaiC monomers at any phase, suggesting that the shuffling process is likely very fast (Akiyama et al., 2008; Kageyama et al., 2006; Mori et al., 2007). 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 (Mori et al., 2007). Others, however, have modeled robust oscillations of KaiC phosphorylation without considering monomer shuffling (Akiyama et al., 2008; Rust et al., 2007; van Zon et al., 2007).

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 (Terauchi et al., 2007). 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 (Nishiwaki et al., 2000). ATP binding, rather than its hydrolysis, is critical for the hexamerization of KaiC, with the N-terminal domain of KaiC contributing more to hexamerization than its C-terminal one (Hayashi et al., 2004; Hayashi et al., 2003; Nishiwaki et al., 2000). The consumption of ATP during a circadian cycle 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 (Figure 1.1). In an oscillating mixture with KaiA and KaiB, one KaiC monomer consumes ~16 molecules of ATP per day, which is orders of magnitude lower than well-characterized ATPases in the same family (Terauchi et al., 2007). 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 (Terauchi et al., 2007).

## 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 (Bell-Pedersen et al., 2005). In *S. elongatus* the phosphorylation cycle of KaiC continues in the absence of transcription or translation in vivo (Tomita et al., 2005) and can be reconstituted in vitro (Nakajima et al., 2005). However, the abundance of KaiB and KaiC does oscillate robustly in vivo (Xu et al., 2000), 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 ℃, but the clock still operates in vivo under the same condition, suggesting that the clock is more stable in vivo than in vitro (Kitayama et al., 2008). Furthermore, a transcription/translation rhythm persists in KaiC mutants that either abolish or mimic KaiC

phosphorylation or when KaiC is constitutively phosphorylated by mildly overexpressing KaiA, although it is weakened and still dependent on the presence of KaiC (Kitayama et al., 2008). KaiC overexpression represses global gene expression (Nakahira et al., 2004), and transcriptional activity is correlated with the phosphorylation state of KaiC (Murayama et al., 2008; Nishiwaki et al., 2004; Xu et al., 2004). 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. The mechanism that underlies the transcription/translation rhythm in the absence of phosphorylation rhythm is unknown, but it is notable that transcriptional activity is correlated to the phosphorylation state of KaiC (Murayama et al., 2008). Thus, it is possible that KaiC locked in a specific conformation would negatively feed back, directly or indirectly, on its own transcription, which would result in a decrease in KaiC abundance, relieving the repression and forming a cycle. It is also possible that the oscillation of KaiC ATPase activity drives the transcription/translation rhythm (Kitayama et al., 2008).

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 (Kim et al., 2008; Xu et al., 2003). Depletion of the Clp proteases lengthens circadian period, although it is not known whether the Kai proteins are direct Clp substrates (Holtman et al., 2005). The turnover rate of

unphosphorylated KaiC differs significantly from phosphorylated KaiC, suggesting it may be regulated (Xu et al., 2003). Transcriptional control of at least KaiA is targeted by the input system of the clock. The Pex protein, which is upregulated in the dark (Takai et al., 2006a), binds to the promoter region of *kaiA*, suppresses its transcription (Figure 1.2), and acts to extend the circadian period (Arita et al., 2007; Kutsuna et al., 1998; Kutsuna et al., 2007). The redox state of the cell, which feeds into the input pathway of the clock, also affects KaiA abundance (Figure 1.2) through an unknown mechanism (Ivleva et al., 2005).

## 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 (Dunlap and Loros, 2004; Hardin, 2005; Lin and Todo, 2005). In *S. elongatus*, however, no photoreceptors have been found to affect the 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 (Mackey et al., in press). 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 1.2), which is a function of light intensity (Ivleva et al., 2005; Zhang et al., 2006b). Other redox-active proteins that affect the clock have been identified as potential interaction partners of CikA (Mackey

et al., 2008). 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 (Schmitz et al., 2000). CikA is unlikely to act as a direct photoreceptor (Mutsuda et al., 2003). CikA autokinase activity is regulated positively and negatively by adjacent GAF (so named because it is found in: cGMP-phosphodiesterases, adenylyl cyclases and FhlA, the formate hydrogen lyase transcriptional activator) and pseudo-receiver (PsR) domains, respectively (Mutsuda et al., 2003; Zhang et al., 2006b). Structural analysis suggests that the PsR domain interacts with the HPK in a manner similar to a *bona fide* receiver, effectively suppressing kinase activity (Gao et al., 2007). The PsR domain is essential for CikA localization to the cell pole and may be an interaction domain with other proteins (Mackey et al., 2008; Zhang et al., 2006b). CikA's abundance is regulated by LdpA (Ivleva et al., 2005) and is sensitive to the plastoquinone analog 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) (Figure 1.2). 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 (Ivleva et al., 2006).

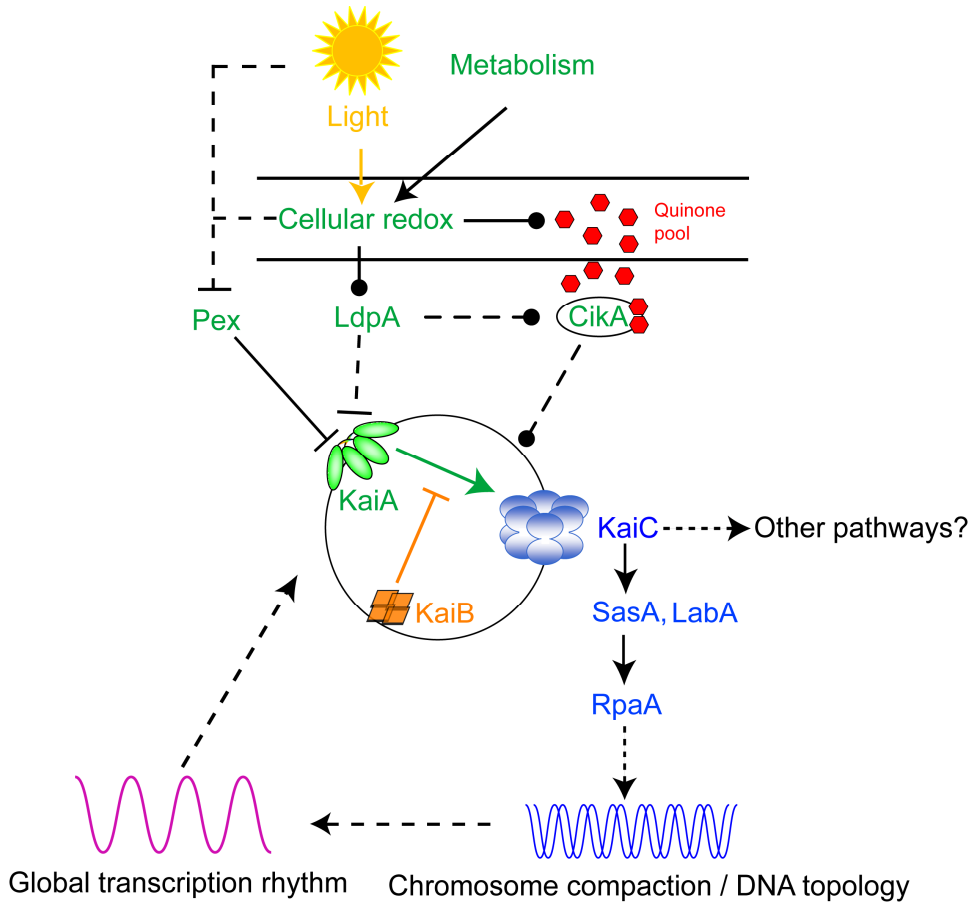


**Figure 1.2. An Overview of the Molecular Mechanism of the Circadian Clock in *S. elongatus*.**
The central oscillator is composed of KaiA, KaiB and KaiC. KaiA stimulates KaiC phosphorylation, and KaiB inactivates KaiA when KaiC reaches a certain phosphorylation state (see Figure 1.1 for details). In 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: solid line indicates a direct effect whereas 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.

LdpA is an iron-sulfur protein that adjusts circadian periods according to the light intensity (Katayama et al., 2003). Disruption of LdpA shortens the period and overexpression lengthens it (Ivleva et al., 2005; Katayama et al., 2003). Protein stability of LdpA is sensitive to the redox state of the cell (Figure 1.2). Consequently, LdpA affects the abundance of CikA and KaiA through an unknown mechanism (Ivleva et al., 2005). LdpA copurifies with clock proteins such as KaiA, CikA and SasA in a pull-down assay, and forms protein complex with them in a circadian manner in vivo. These data suggest a close physical coupling of input pathway and oscillator components in the cyanobacterial clock.

**A GLOBAL OUTPUT MECHANISM**

All promoters examined in *S. elongatus* are rhythmically expressed, even those that originate from *E. coli* (Min et al., 2004). Several lines of evidence suggest that DNA topology is involved in effecting rhythmicity of gene expression (Iwasaki and Kondo, 2004; Min et al., 2004; Mori and Johnson, 2001a). For example, in a failed attempt to identify possible *cis*-elements that determine the phasing of a Class 2 promoter, Min et al. found instead the topology-dependent *fis* promoter from *E. coli* acts as a Class 2 promoter (Min et al., 2004). 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 (Smith and Williams, 2006). 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 (Woelfle et al., 2007). Overall, it is plausible that the clock controls the global transcription rhythm by regulating DNA topology.

A key player in the output pathway, SasA, is an HPK that physically interacts with KaiC (Iwasaki et al., 2000). The chromosome compaction and gene expression rhythms persist in a *sasA* mutant, although with a damped amplitude compared to the WT (Smith and Williams, 2006). 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 (Smith and Williams, 2006). The cognate response regulator of SasA has been identified as RpaA (Figure 1.2), which carries a receiver and DNA-binding domains (Takai et al., 2006b). RpaA does not bind to the promoter region of *kaiBC*, and its target remains to be identified (Takai et al., 2006b). 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 (Taniguchi et al., 2007).

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 (Mori et al., 2002). Moreover, it is possible that certain events stimulate KaiC ATPase activity sufficiently to power mechanical motion.

## DISSERTATION OVERVIEW

The chapters of this dissertation report my contributions to our understanding of the cyanobacterial circadian clock. A portion of this Introduction chapter, which I wrote as a review article for *Current Opinion in Microbiology* (G. Dong and S. S. Golden, in press), is in press. Both Chapter II and Chapter III represent collaborative work between our lab and that of Alexander van Oudenaarden at Massachusetts Institute of Technology, who developed YFP as a reporter in *S. elongatus* and provides continuous monitoring of single cells using time-lapse microscopy and subsequent quantitative analysis. The P*trc*::*yfp*, P*trc*::*kaiA-yfp* and P*trc*::*kaiB-yfp* constructs used in Chapter III were made by Dr. van Oudenaarden. Prashant Luitel performed the time-lapse microscopy on all three YFP-fused Kai proteins, the data of which were mentioned but not shown in Chapter III, and analyzed the distribution of KaiA-YFP foci presented in the figure on page 70. Qiong Yang performed and analyzed the data of gating of cell division in WT and the effect of KaiA-YFP overexpression and decay on cell

division, which are not shown. The subcellular localization of CikA and its variants is a result of collaboration with Xiaofan Zhang, a graduate student of our lab at the time, and the work was published in *Molecular Microbiology* (Zhang et al., 2006b). Chapter IV is the result of collaboration with the lab of Andy LiWang, and has been published in *Proceedings of the National Academy of Sciences* earlier this year (Kim et al., 2008). I performed the in vivo work which complemented and supported their hypothesis, and a summary of my work is written in Chapter IV.

# CHAPTER II

# SPECIFIC STATES OF KAIC CONTROL THE CIRCADIAN GATE THAT REGULATES CELL DIVISION IN *S. ELONGATUS*

## INTRODUCTION

In a wide range of organisms, from microscopic cyanobacteria and fungi to plants and animals, endogenous genetically programmed daily cycles, known as circadian rhythms, are evident in various aspects of physiology and behavior (Bell-Pedersen et al., 2005). In the cyanobacterium *S. elongatus*, the timing of cell division (Mori et al., 1996), expression of genes on a global genomic scale (Liu et al., 1995b), and chromosome compaction (Smith and Williams, 2006) are all controlled by a circadian biological clock that exhibits the same properties as the clocks of eukaryotic organisms (Mackey and Golden, 2007). However, extensive studies in the cyanobacterium *S. elongatus* PCC 7942 have revealed details of a clock that is distinct in mechanism, protein components, and evolutionary history from those of eukaryotic clock model systems (Mackey and Golden, 2007).

In *S. elongatus*, three neighboring genes, *kaiA*, *kaiB*, and *kaiC*, code for proteins of the circadian central oscillator. Inactivation of any of the three genes abolishes the circadian rhythm, as does the overexpression of either KaiA or

KaiC (Ishiura et al., 1998). KaiC is an ATPase, autokinase, and autophosphatase; in concert with KaiA and KaiB, the kinase activity causes a daily rhythm of autophosphorylation of KaiC at serine 431 and threonine 432 residues (Nishiwaki et al., 2004; Xu et al., 2004) both in vivo and in vitro (Nakajima et al., 2005; Tomita et al., 2005). KaiA stimulates KaiC phosphorylation and KaiB blocks KaiA's activity at a point later in the oscillation to allow KaiC dephosphorylation (Iwasaki et al., 2002; Kim et al., 2008; Rust et al., 2007; Williams et al., 2002). The phosphorylation-dephosphorylation cycle of KaiC follows a stepwise manner, with T432 phosphorylated first followed by phosphorylation at S431, and T432 dephosphorylated first followed by S431 (Nishiwaki et al., 2007; Rust et al., 2007). The oscillation of KaiC phosphorylation in vitro in a simple mixture of the three Kai proteins and ATP (Nakajima et al., 2005) prompted the idea that the KaiC phosphorylation cycle is the fundamental time-keeping mechanism in cyanobacteria. However, the weak ATPase activity of KaiC also oscillates in a circadian manner, is temperature compensated even when KaiC is incubated alone, and correlates inversely with circadian period, suggestive of a fundamental role in timekeeping independent of the phosphorylation cycle (Terauchi et al., 2007). More recently, a transcription/translation rhythm was shown to persist in the absence of a KaiC phosphorylation rhythm (Kitayama et al., 2008), suggesting that other aspects of KaiC, such as the ATPase activity, could underlie the basic timing mechanism instead of, or in addition to, KaiC phosphorylation.

In the cyanobacterium, temporal information from the oscillator is broadcast to downstream genes via the histidine protein kinase SasA, whose physical interaction with KaiC (Iwasaki et al., 2000) stimulates SasA autophosphorylation (Smith and Williams, 2006). SasA then transfers the phosphoryl group to RpaA, a response regulator with a DNA binding domain. Disruption of either *sasA* or *rpaA* results in a severely damped rhythm (Iwasaki et al., 2000; Takai et al., 2006b).

In the input pathway of the circadian clock, which relays environmental information to the oscillator to synchronize it with the solar day, lies the histidine protein kinase CikA. Both CikA and an FeS protein, LdpA, appear to sense light indirectly through cofactors that perceive changes in celluar redox state that vary with photosynthetic activity (Ivleva et al., 2005; Ivleva et al., 2006; Schmitz et al., 2000). CikA copurifies with LdpA, KaiA, KaiC, and SasA in vivo, although no direct biochemical interaction has been detected between CikA and the central oscillator proteins. A null mutant of *cikA* has a circadian period shortened by about two hours, exhibits low amplitude of gene expression rhythms, and fails to reset the phase of the rhythms after an environmental cue. In addition to these notable circadian phenotypes, a *cikA* null mutant has elongated cells (Miyagishima et al., 2005), indicating a defect in cell division.

Cell division is a cyclic biological event that is tightly regulated by and coordinated with other cellular events. Only a handful of studies so far have focused on the interaction between the cell cycle and the circadian cycle, with

few molecular details. In regenerating liver cells of mice, circadian clock proteins directly control the expression of Wee1 protein, a kinase that inhibits the entry into mitosis; in contrast, the circadian clock oscillates independently of the cell cycle at the single cell level (Matsuo et al., 2003). Similarly in *S. elongatus*, the cell-division cycle is gated by the circadian clock, i.e., there are circadian phases in which cell division is inhibited and others when it is allowed, whereas the circadian cycle appears uninfluenced by the cell cycle, e.g., the circadian cycle is the same in cells with vastly different doubling times, and when cell division is quiescent (Mori et al., 1996; Mori and Johnson, 2001b). Additionally, the rate of DNA synthesis remains constant throughout the circadian cycle, suggesting that cytokinesis is the clock-regulated event (Mori et al., 1996). The molecular mechanism that underlies the circadian gating of the cell cycle has remained completely unknown in the face of rich details of the cyanobacterial circadian clock. Here, we demonstrate that the cell division defect in a *cikA* mutant is a function of the clock, and that high ATPase activity of KaiC correlates with the closing of the gate on cell division. Furthermore, we show that the gating of cell division acts through the SasA-RpaA clock output pathway, and that the localization of the FtsZ protein is the likely the target of regulation. This work also revealed the presence of an unknown activity that stimulates KaiC phosphorylation independently of KaiA.

## RESULTS

### *kaiC* Is Epistatic to *cikA* in the Regulation of Cell Division

The pleiotropic effects of a *cikA* null mutation on circadian rhythms and cell length could be explained by a model in which CikA is involved in two pathways that independently govern circadian rhythms and cell division; alternatively, the function of CikA in the circadian input pathway may be important for clock regulation of cell division. In order to differentiate between these hypotheses, we created double knockout mutants in which *cikA* is null as well as *kaiA*, *kaiB*, or *kaiC*. The prediction is that if any of these double null mutants shows no defect in cell division, then *cikA* mutant's cell division defect is related to its clock function. Cell length of the single and double mutants, which were grown under the same conditions at the same time, was examined and compared (Table 2.1). Agreeing with previous reports (Miyagishima et al., 2005; Zhang et al., 2006b), *cikA* mutant cells are significantly elongated compared to the WT (Figure 2.1). Remarkably, the *ΔkaiCΔcikA* double mutant displays cell length that is indistinguishable from that of the WT, i.e., the absence of KaiC suppresses the cell division defect of a *cikA* mutant. However, *ΔkaiAΔcikA* and *ΔkaiBΔcikA* mutants are still elongated. These data support the hypothesis that the *cikA* cell division phenotype is related to its role in the clock, and suggests that KaiC, but not KaiA or KaiB, lies downstream of CikA in this signal transduction pathway. The diameter of the cells in this and all subsequent experiments in this study did not vary significantly, nor did other aspects of the shape of cells.

**Table 2.1. Summary of Cell Length Phenotype of Clock Mutants.**

| Genetic background | Cell elongation? | Genetic background | Cell elongation? |
|---|---|---|---|
| ***ΔkaiA*** | No | ***ΔkaiAΔcikA*** | Yes |
| ***ΔkaiB*** | Yes | ***ΔkaiBΔcikA*** | Yes |
| ***ΔkaiC*** | No | ***ΔkaiCΔcikA*** | No |
| ***ΔcikA*** | Yes | ***ΔkaiBΔkaiC*** | No |
| ***ΔldpA*** | No | ***ΔldpAΔcikA*** | No |
| ***ΔsasA*** | No | ***ΔkaiBΔsasA*** | No |
| ***ΔrpaA*** | No | ***ΔkaiBΔrpaA*** | No |
| | | ***ΔcikAΔrpaA*** | No |

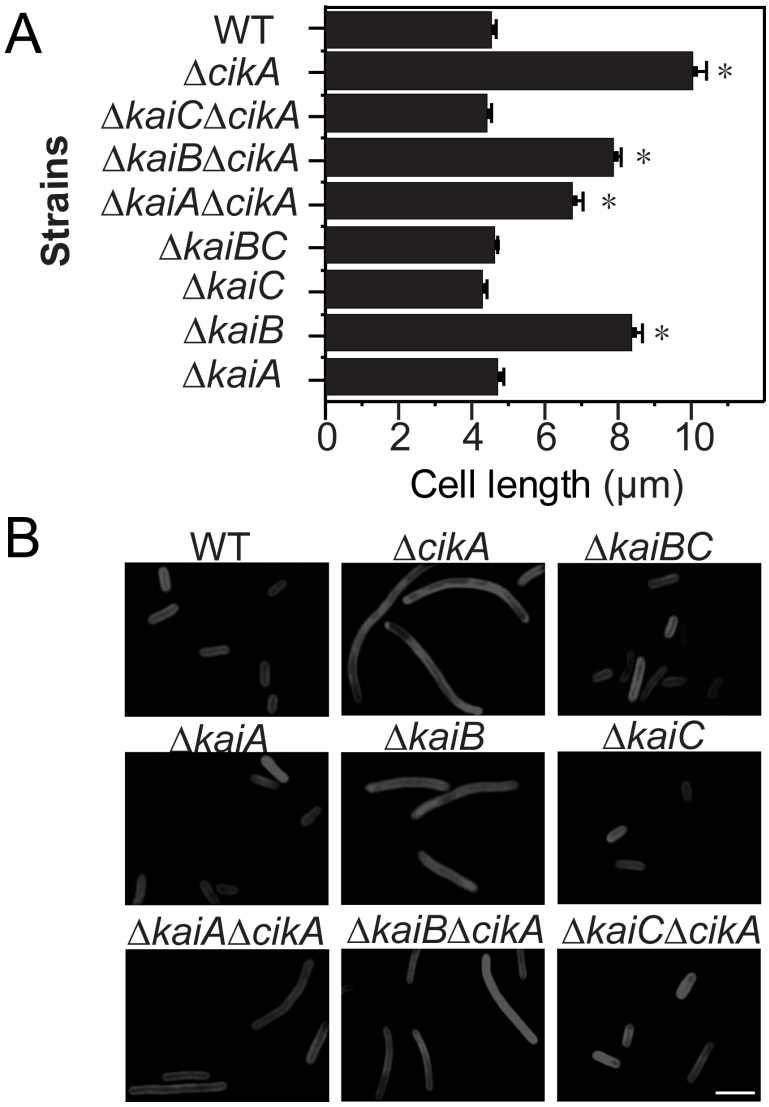


**Figure 2.1. Comparison of Cell Lengths Among Various Clock Mutants.**
(A) Single and double clock mutants are examined for cell length differences and epistasis relationships among clock genes in the control of cell division. An asterisk means the difference of cell length is statistically significant compared to that of WT. More than 150 cells are counted per sample. $p < 0.001$. Error bar = ±1 standard error of the mean (S.E.M.).
(B) Representative micrographs of cells from (A). Autofluorescence from photosynthetic pigments distributed along the peripherals of cells is captured using a fluorescence microscope. Scale bar = 5 μm.
All subsequent figures have the same settings unless otherwise noted.

**Loss of kaiB Leads to Increased Cell Length, Dependent on KaiC**

To address the roles of clock genes in cell length control, single mutants of various known clock genes were examined (Table 2.1). Cells of a *kaiB* mutant are elongated like those of a *cikA* mutant, with cell sizes averaging almost twice as long as the WT, whereas single mutants of *kaiA*, *kaiC*, or *ldpA* have no effect on cell length (Figure 2.1 and data not shown). Because KaiB and KaiC are both involved in the control of cell length, we reasoned that they probably function in the same pathway given the extensive evidence of their interaction in the generation of circadian rhythms. Therefore, we measured the cell length of a *kaiBC* double deletion mutant and found that it is not statistically different from that of the WT or *kaiC* mutant; these data indicate that the absence of *kaiC* suppresses the *kaiB* mutant cell length phenotype, just as it did to the *cikA* mutant (Figure 2.1). We conclude that the role of KaiB in determining cell length is likely to be the same as its function in modifying KaiC phosphorylation state.

**Constitutive Phosphorylation of KaiC Has an Inhibitory Effect on Cell Division**

The only known function of KaiB is to trap KaiA on phosphorylated KaiC, which allows KaiC to dephosphorylate (Pattanayek et al., 2008; Rust et al., 2007). Loss of *kaiB* abolishes circadian rhythms (Ishiura et al., 1998) and results in constitutively phosphorylated KaiC both in vivo (Figure 2.2C) and in vitro

(Iwasaki et al., 2002; Kitayama et al., 2003; Williams et al., 2002). CikA is also reported to affect the phosphorylation state of KaiC, although the molecular mechanism remains elusive (Ivleva et al., 2006). We sought to understand whether constitutively hyper-phosphorylated KaiC inhibits cell division, as the result from the *kaiA* null mutant already indicated that constitutive hypo-phosphorylation of KaiC has no effect on cell length (Figure 2.1). We overexpressed KaiA in the WT background, in which it would stimulate KaiC phosphorylation, or in the *kaiC* null background, to determine if KaiA has a cell division influence that is KaiC independent. As shown in Figure 2.2A and 2.2B, cells overexpressing KaiA in the presence of KaiC are significantly longer than those in the *kaiC* null background and WT cells after IPTG induction, whereas no difference was observed among the three strains before induction. Note that, during the 5-day induction period, WT cells lengthened; this degree of lengthening in denser cultures, as a result of reduced light penetration, is normal and not related to IPTG (Figure 2.3). We confirmed KaiC phosphorylation status after induction, showing that it remains highly phosphorylated under KaiA-overexpressing conditions (Figure 2.2C).

Recently, two KaiC truncation mutants were shown to have opposite phosphorylation states independent of either KaiA or KaiB (Kim et al., 2008). KaiC487, which is missing the last 32 amino acids, is constitutively phosphorylated whereas KaiC497 is never phosphorylated even though it retains the S431 and T432 residues. Another KaiC mutant, KaiCE444D, is also

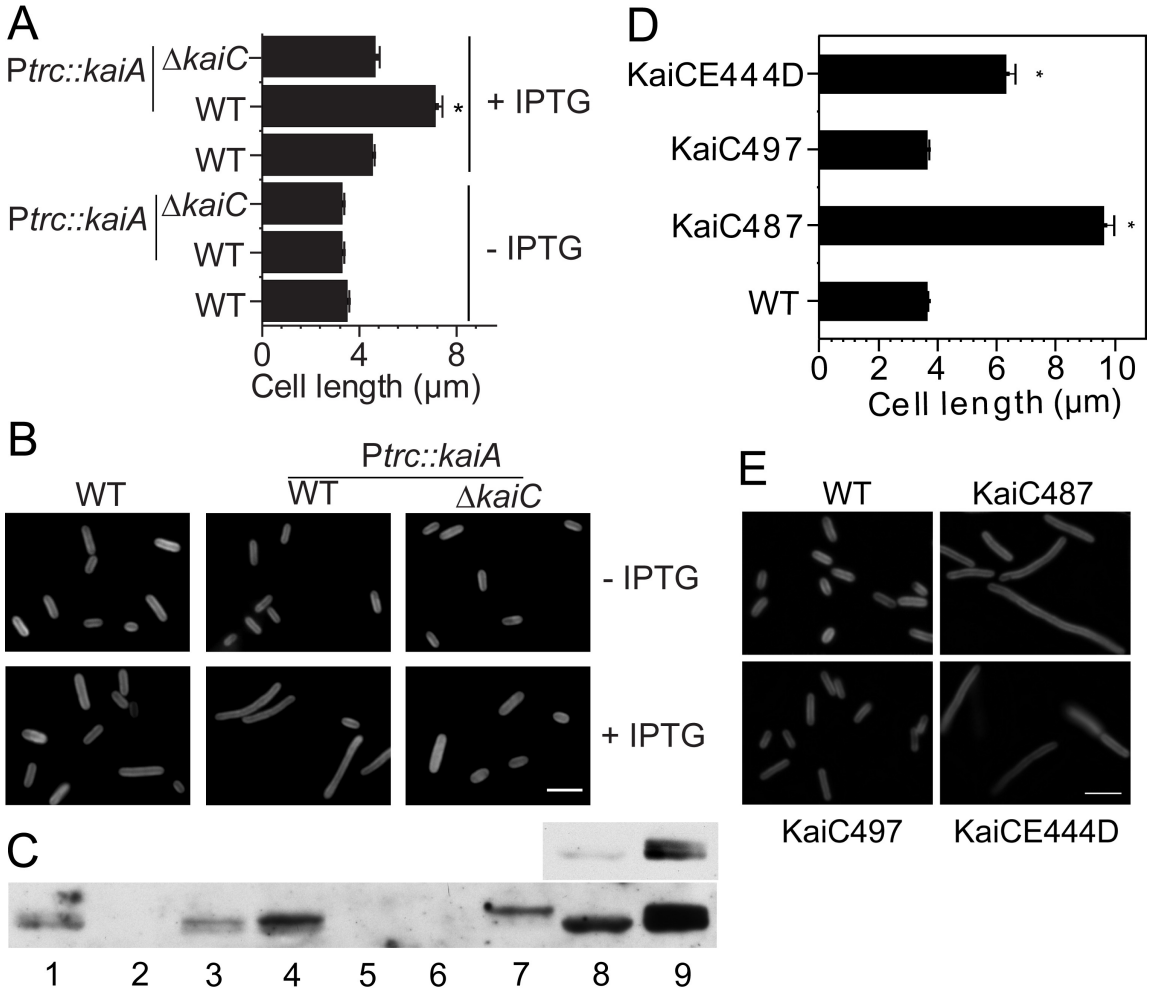


**Figure 2.2. Constitutive Phosphorylation of KaiC Inhibits Cell Division.**
(A) Overexpression of KaiA causes cell elongation in the WT background but not in a *kaiC* null background.
(B) Representative micrographs of cells from (A)
(C) Western blots of various strains showing KaiC phosphorylation states. Lane 1: WT; 2: *ΔkaiC*; 3 and 4: P*trc::kaiA* expressed in the WT background in the absence (3) or presence (4) of 1 mM IPTG; 5 and 6: P*trc::kaiA* expressed in *ΔkaiC* background in the absence (5) or presence (6) of IPTG; 7: *ΔkaiB*; 8: *ΔkaiA*; 9: *ΔkaiAΔcikA*. A less exposed picture of lane 8 and 9 is shown above them to show that KaiC in lane 9 contains a phosphorylated form.
(D) KaiC487 and KaiCE444D mutants cause cell elongation whereas KaiC497 does not.
(E) Representative micrographs of cells from (D).

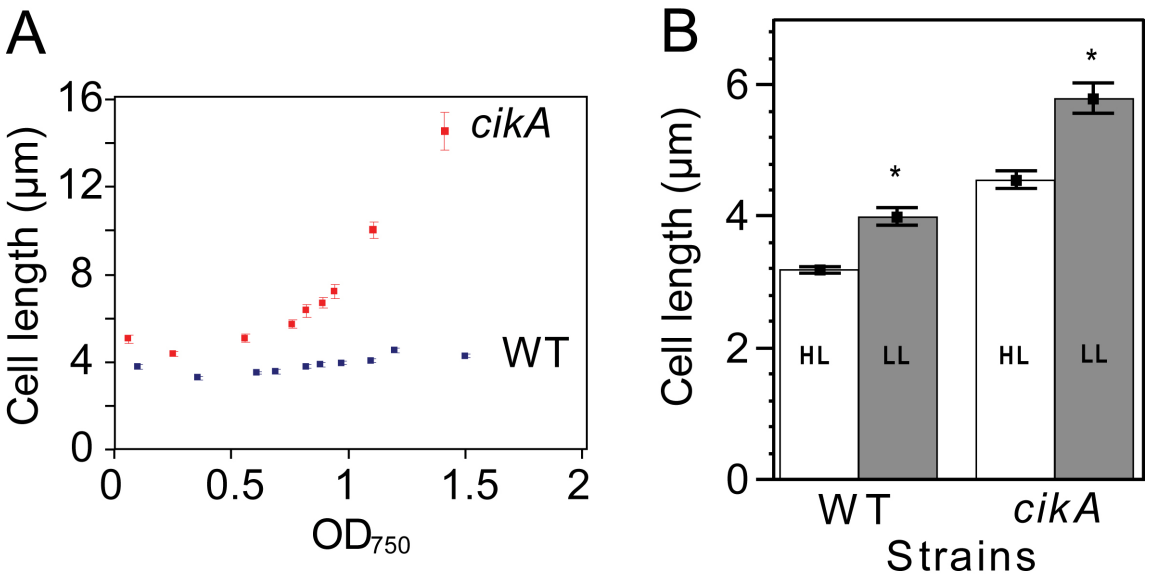


**Figure 2.3. Cell Length Is a Function of Light Intensity.**
(A) Cell length of both the WT and *cikA* mutant strains increases as the cultures grow denser.
(B) Cells of WT and *cikA* mutant grown under low light (10 μE $m^{-2}$ $s^{-1}$) coditions are significantly longer on average than those grown under the high light (100 μE $m^{-2}$ $s^{-1}$) conditions.

reported to be constitutively phosphorylated and tested here. Consistent with the hypothesis that phosphorylated KaiC is an inhibitor of cell division, KaiC487 and KaiCE444D expressed in a *kaiC* null background cause cell elongation whereas KaiC497 has no effect (Figure 2.2D and 2.2E).

Overall, the data shown above support the hypothesis that constitutively phosphorylated KaiC inhibits cell division; in the scenario of a simple switch, nonphosphorylated KaiC would have no effect on cell division.

**KaiC Can Be Phosphorylated Independent of KaiA In Vivo**

KaiA is the only protein that is known to stimulate KaiC phosphorylation. In the absence of KaiA, KaiC remains constitutively nonphosphorylated both in vivo and in vitro, regardless of whether KaiB is present. However, as shown above, *ΔkaiAΔcikA* double mutant cells are elongated (Figure 2.1). We analyzed the phosphorylation states of KaiC in this strain and surprisingly, KaiC is partially phosphorylated (Figure 2.2C). We confirmed that KaiC in the *ΔkaiA* strain from which *ΔkaiAΔcikA* is made is ~100% nonphosphorylated (Figure 2.2C). These data suggest that an unknown activity is able to stimulate KaiC phosphorylation independently of KaiA, and that this cryptic activity is inhibited by CikA. However, the data also suggest that KaiC does not need to be close to 100% phosphorylated to inhibit cell division. Either the absolute amount of phosphorylated KaiC is the determinant, or some other activity of KaiC instead of phosphorylation is the underlying mechanism.

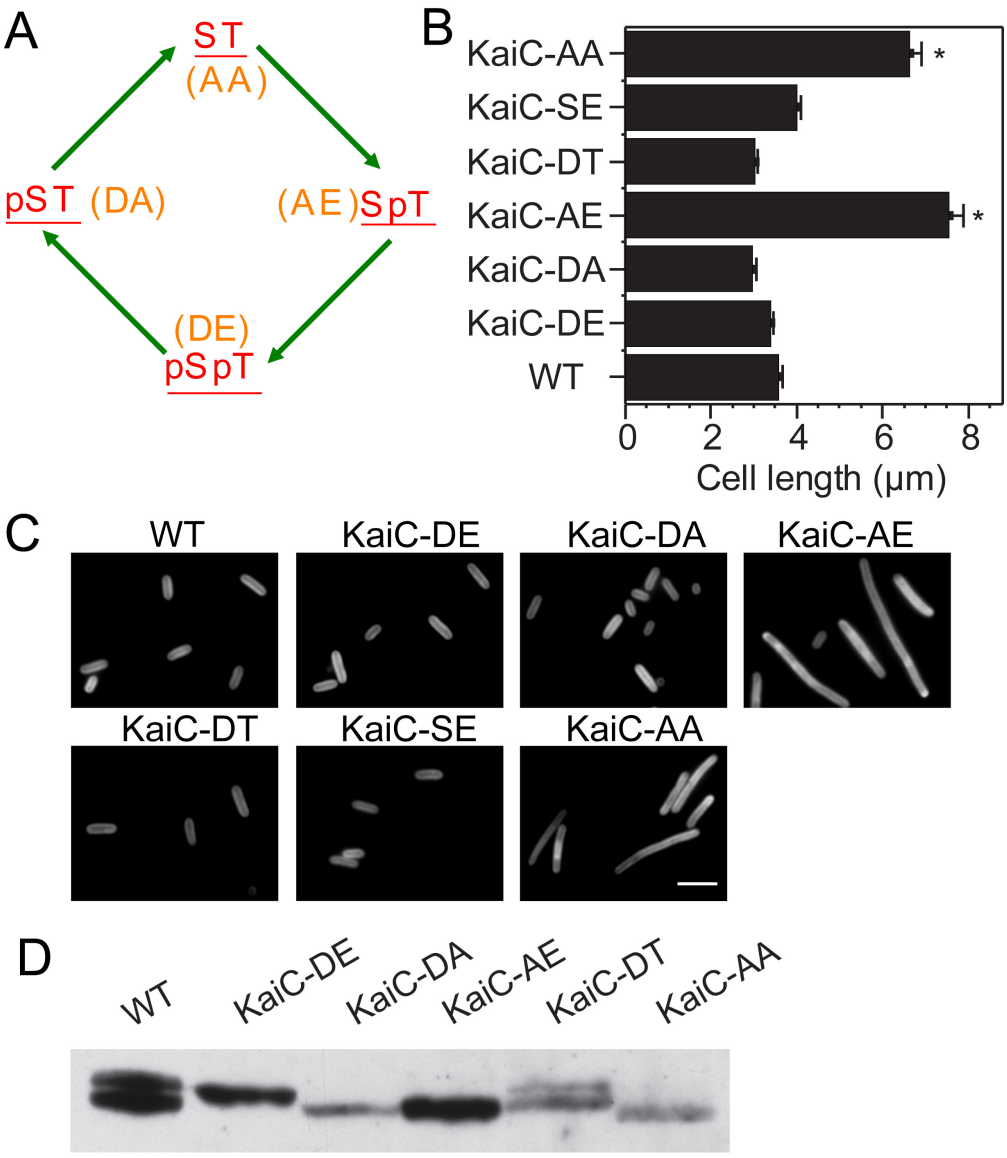


**Figure 2.4. Constitutive KaiC Phosphorylation Is Not Required for Cell Elongation.**
(A) Ordered phosphorylation steps of KaiC and amino acid substitutions that mimic each phosphoform.
(B) Effects of KaiC phosphomimetics on cell length. KaiC-AA and KaiC-AE causes cell elongation. All other mutants show WT cell length.
(C) Representative micrographs of cells shown in (B).
(D) Western blot of KaiC demonstrates the expression of KaiC phosphomimetics. KaiC-SE was undetectable and thus not shown here.

**Phosphorylation of KaiC Is Not Essential for Cell Elongation**

KaiC phosphorylation is a dynamic process and, due to the presence of distinct phosphorylation sites at serine 431 and threonine 432, there are four possible forms of KaiC that cycle in a stepwise fashion (Figure 2.4A) (Nishiwaki et al., 2007; Rust et al., 2007). Using different amino acid replacements at the two phosphorylation sites, Nishiwaki et al. (2007) successfully mimicked different phospho-forms of KaiC to characterize various biochemical properties of the Kai oscillator in vitro, such as the interaction profile with KaiA and KaiB proteins. We hypothesized that the KaiCS431D, T432E (KaiC-DE) mutant, which mimicked fully phosphorylated KaiC in the previous assays, would inhibit cell division while KaiC-AA (KaiCS431A, T432A), which cannot be phosphorylated, would have WT cell length. We included several other phosphomimetics to derive the aspects of KaiC phosphorylation state that have an effect on cell division. Surprisingly, the results were almost completely opposite to our predictions. As shown in Figure 2.4B and 2.4C, the strain expressing KaiC-DE has a normal cell length, but those expressing either KaiC-AA or KaiC-AE show inhibition of cell division. Immunoblot analysis showed that all mutant proteins were expressed at levels comparable to WT (Figure 2.4D) except KaiC-SE (data not shown), which was not detected, ruling out the possibility that KaiC-DE abundance is insufficient to inhibit cell division and rendering the KaiC-SE-expressing strain as non-informative. These data demonstrate that these phosphomimetics of KaiC are not completely faithful representations of the different KaiC phospho-forms,

although they have been useful in studying Kai protein interaction dynamics (Nishiwaki et al., 2007). In particular, KaiC-AA inhibits cell division whereas naturally non-phosphorylated KaiC, as is present in a *kaiA* null mutant, does not. To ensure that the lack of cell elongation in the *kaiA* null is due to the change in KaiC phosphorylation state and not simply because of a decrease in KaiC abundance when KaiA is absent (Ditty et al., 2005), we measured the cell length of a different *kaiA* null strain (AMC702) in which *kaiC* is constitutively highly expressed and nonphosphosrylated (Ditty et al., 2005) and confirmed that it had WT cell length (data not shown). Thus, KaiC-AA does not fully mimic non-phosphorylated KaiC; it cannot be phosphorylated, yet still has an inhibitory effect on cell division, which suggests that it is possible to separate KaiC phosphorylation from the cell division defect observed.

**Elevated ATPase Activity of KaiC Correlates with Increased Cell Length**

If phosphorylation of KaiC is not the fundamental property that inhibits cell division, it must be a state or activity of KaiC that is closely related to, but also functionally separable from, KaiC phosphorylation that represses cell division. Aside from the autokinase and autophosphatase activities, KaiC possesses an unusually low but significant ATPase activity that fits this description (Murakami et al., 2008; Terauchi et al., 2007). KaiC has two typical Walker's A motifs that are located at the N- and C-terminus of the protein (Figure 2.5A). Mutational analyses have shown that the N-terminal Walker's A motif is important in the

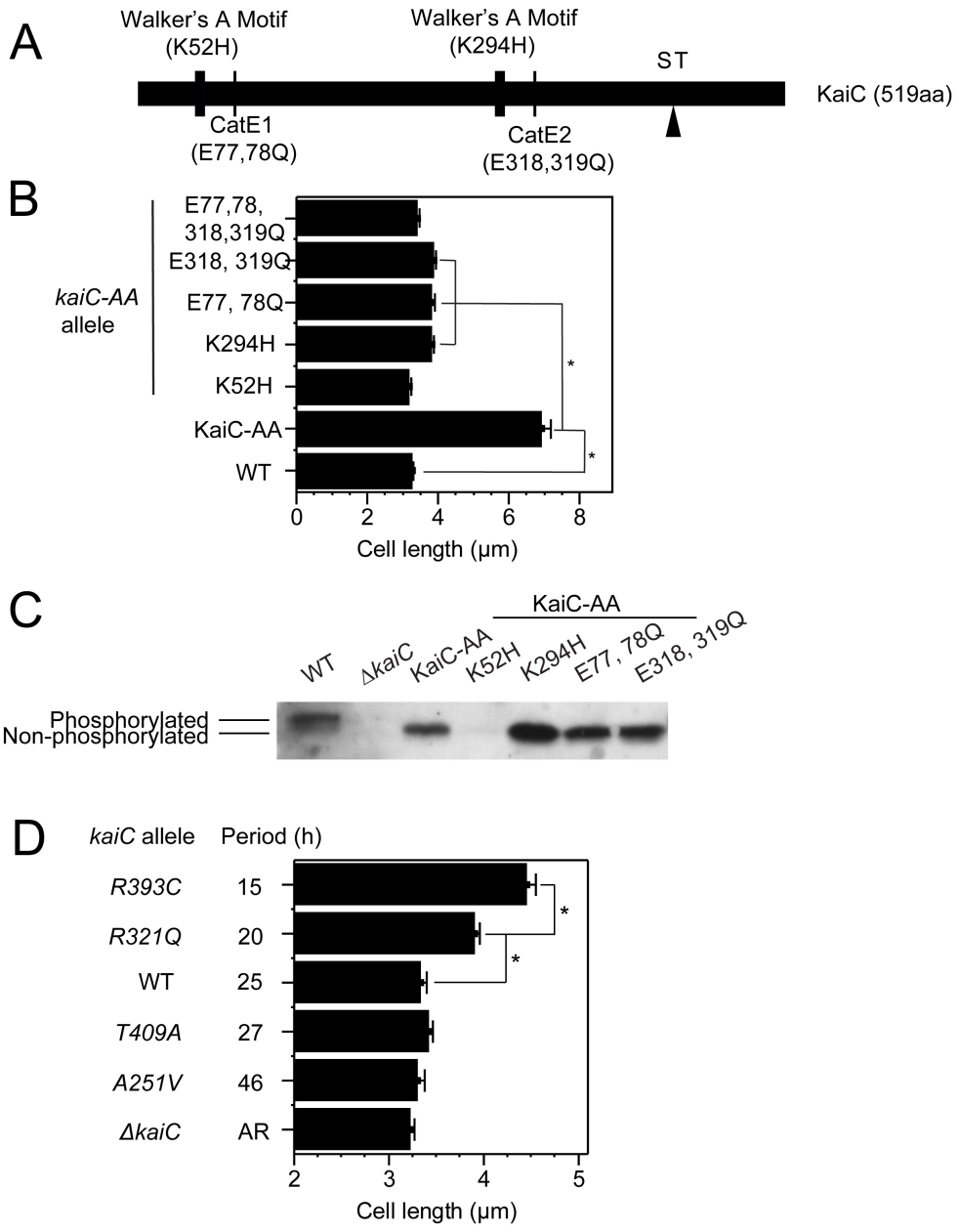


**Figure 2.5. High ATPase Activity of KaiC Coincides with Cell Elongation.**
(A) Illustration of Walker's A motifs identified in KaiC and point mutations used to disrupt them.
(B) Point mutations that disrupt Walker's A motifs significantly reduce cell length in the KaiC-AA mutant. An asterisk means that the difference of cell length of the strains connected by the lines is statistically significant.
(C) Western blot of KaiC reveals the expression level of KaiC ATPase mutants.
(D) Correlation of circadian period and cell length. Strains with circadian periods shorter than the WT are significantly elongated compared to the WT and long period mutants, and the mutant with 15-h period is also significantly longer than that with the 20-h period.

hexamerization of the protein, and that the C-terminal motif is essential for autophosphorylation at the S431 and T432 residues (Hayashi et al., 2004; Hayashi et al., 2006). Recently, it was demonstrated that KaiC hydrolyzes ATP in a circadian manner, and that phosphorylation of KaiC accounts for only a negligible portion of the total ATP consumed (Terauchi et al., 2007). WT KaiC hydrolyzes 14.5±2 ATP molecules per KaiC monomer per day when incubated alone in solution, and KaiA enhances KaiC's ATPase activity by about two fold. The KaiC-AA mutant has a constitutively high ATPase activity that is also about twice that of WT KaiC, while KaiC-DE has a slightly lower ATPase activity compared to the WT (Terauchi et al., 2007). These data prompted us to hypothesize that high ATPase activity, rather than phosphorylation of KaiC, underlies the protein's inhibitory effect on cell division. To test this hypothesis, we chose KaiC-AA and made four different ATPase mutants based on previous reports (Hayashi et al., 2004; Hayashi et al., 2003; Nishiwaki et al., 2000). KaiC$^{K52H}$-AA and KaiC$^{catE1}$-AA should inactivate the ATPase activity of N-terminal KaiC, and KaiC$^{K294H}$-AA and KaiC$^{catE2}$-AA should inactivate that of the C-terminal domain. As shown in Figure 2.5B, all ATPase mutants have significantly reduced cell size compared to KaiC-AA, even though KaiC$^{catE1}$-AA, KaiC$^{K294H}$-AA, and KaiC$^{catE2}$-AA mutants still show a slight but significant elongation of cells compared to the WT. These results suggest that ATPase activities from both domains are involved in controlling cell length. Therefore, we attempted to express a variant in which the catalytic glutamatic acid residues

from both domains are changed to glutamine (KaiC$^{catE1catE2}$-AA) in a *kaiC* null background. The cell length of this mutant does not deviate significantly from the WT. However, immunoblot analysis showed that neither KaiC$^{K52H}$-AA nor KaiC$^{catE1catE2}$-AA is detectable, whereas the other three mutants are expressed at levels comparable to KaiC-AA (Figure 2.5C). Overall, we concluded that high ATPase activity of KaiC causes cell elongation, and that both Walker's A motifs contribute to the regulation.

Circadian period length correlates with the ATPase activity of KaiC. KaiC mutants with higher ATPase activities exhibit shorter periods, whereas those with higher activity have longer periods (Terauchi et al., 2007). We propose that KaiC mutant strains with circadian periods shorter than WT would have longer cells, and those with longer periods would have normal cell lengths because low ATPase activity or the absence of KaiC does not cause cells to be significantly shorter than the WT. We picked two KaiC mutants whose ATPase activities have been quantified previously—KaiCR393C with a 15 h period and KaiCA251V with a period of 46 h (Terauchi et al., 2007)—as well as two other KaiC period mutants—KaiCR321Q (22 h) and KaiCT409A (27 h)—to compare their cell lengths. As predicted, the short-period mutants showed longer cells compared to the WT, including KaiCR321Q, whose ATPase activity had not been previously measured but is expected to be high (Figure 2.5D). No significant difference was observed among the long period mutants and the WT (Figure 2.5D).

**SasA-RpaA Output Pathway Is Epistatic to CikA and KaiC in the Control of Cell Division**

The two component signal transduction pathway composed of SasA and RpaA has been characterized as the key protein pair of the output pathway to circadian rhythms (Iwasaki et al., 2000; Takai et al., 2006b). Loss of either gene greatly suppresses rhythmic gene expression and the chromosome compaction cycle (Smith and Williams, 2006), and mutants of *sasA* or *rpaA* do not have cell elongation phenotypes (Figure 2.6A, 2.6B and data not shown). In order to test whether the same output pathway is utilized for the control of cell division by the circadian clock, cell lengths of double inactivation mutants of *cikA* with *sasA* (*ΔcikAΔsasA*) and *kaiB* with *sasA* (*ΔkaiBΔsasA*) or *rpaA* (*ΔkaiBΔrpaA*) were compared to the WT and single mutants (Table 2.1). In all cases, cell size was restored to WT length (Figure 2.6A and 2.6B). It has been shown that the expression level of *kaiBC* is significantly reduced in *sasA* mutants (Iwasaki et al., 2000); thus, it was possible the suppression phenotype observed was merely a result of a reduction in KaiC's protein level, rather than disruption of the SasA-RpaA output pathway. To test this hypothesis, we introduced a copy of WT *kaiC* under the control of P*trc* into the *ΔkaiBΔsasA* background and elevated KaiC protein to different levels by adjusting the concentration of IPTG. Cells maintained an average WT length regardless of KaiC abundance (Figure 2.6C and 2.6D), suggesting that the SasA-RpaA pathway is indeed downstream of KaiC in the control of cell division.

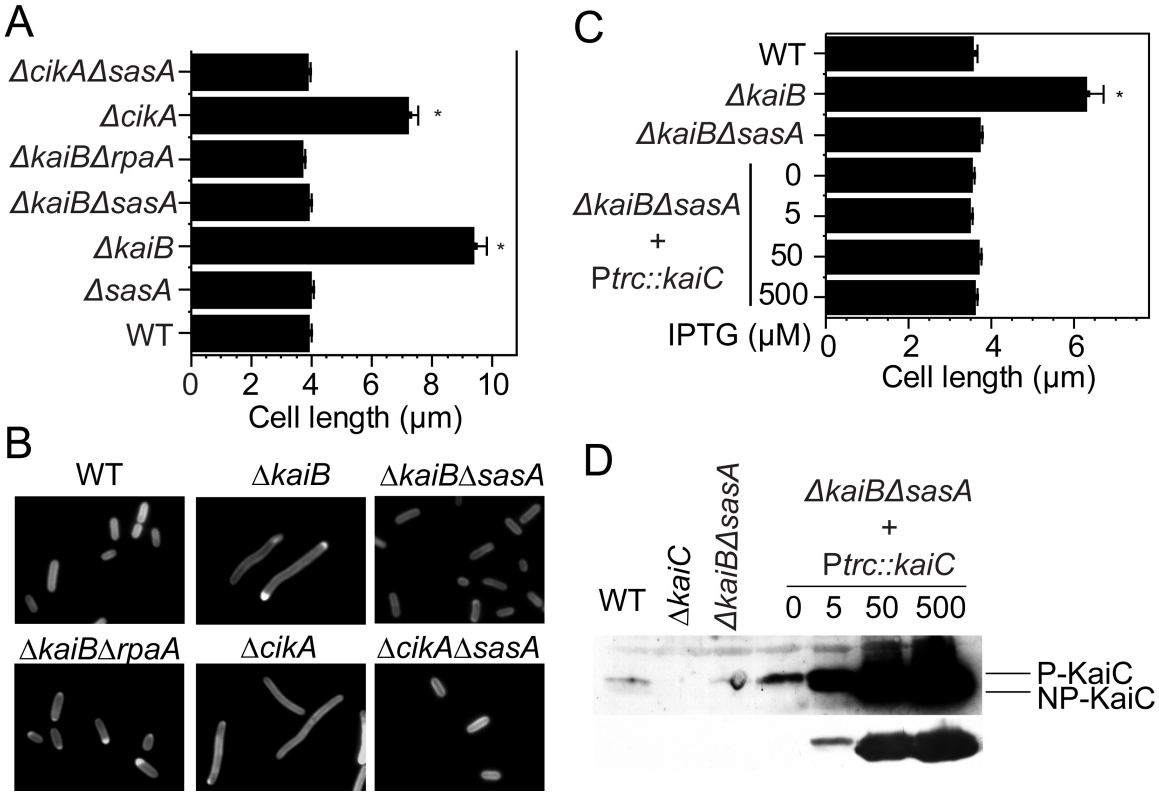


**Figure 2.6. SasA and RpaA Are Downstream of the Central Oscillator and CikA in the Control of Cell Division.**
(A) Knockout of *sasA* or *rpaA* from *cikA* or *kaiB* null strains release inhibition of cell division.
(B) Representative micrographs of cells from (A).
(C) Elevated level of KaiC does not restore the cell elongation phenotype to the *ΔkaiBΔsasA* strain.
(D) Western blot shows abundance of KaiC in strains measured in (C). The lower panel is a result of less exposure compared to the upper panel. KaiC level is lower in *ΔkaiBΔsasA* strain than in the WT as expected, and expression of P*trc::kaiC* in this background elevated KaiC level to different degrees, depending on the concentration of the inducer, IPTG. Even without IPTG or with only 5 μM IPTG, KaiC's level is higher than in the WT and is predominantly in the phosphorylated form.

**FtsZ Suppresses Cell Division Defect of Clock Mutants**

Mori et al. (1996) found that circadian gating of cell division occurs at the cytokinesis step, and that DNA replication is not affected. Consistent with this result, in the *cikA* mutant, the “ring” localization at the septation site of the FtsZ protein, a conserved bacterial homolog of tubulin, is reduced although the total amount of FtsZ is unaffected (Miyagishima et al., 2005). Targeting of FtsZ as a key step in the regulation of cell size has been a common theme in the bacterial world (Kawai et al., 2003; Weart et al., 2007). We reasoned that if the circadian clock blocks FtsZ localization at the septation site to gate cell division, an increase in the FtsZ level may overwhelm the inhibition and thus permit cells to divide. Thus, we expressed *ftsZ* from P*trc* in the *cikA*, *kaiB*, and *kaiC-AA* mutant backgrounds. In all cases, cell size was significantly reduced even in the absence of IPTG (Figure 2.7A), suggesting that an elevated level of FtsZ suppresses the cell division defect caused by the clock mutants. Immunoblot results confirmed that the level of FtsZ was the same among the WT and mutants with elongated cells, but was elevated by about two fold in mutants with an extra copy of the *ftsZ* gene (Figure 2.7B). The addition of IPTG, further elevating FtsZ, forced cells to divide and resulted in minicell formation (data not shown).

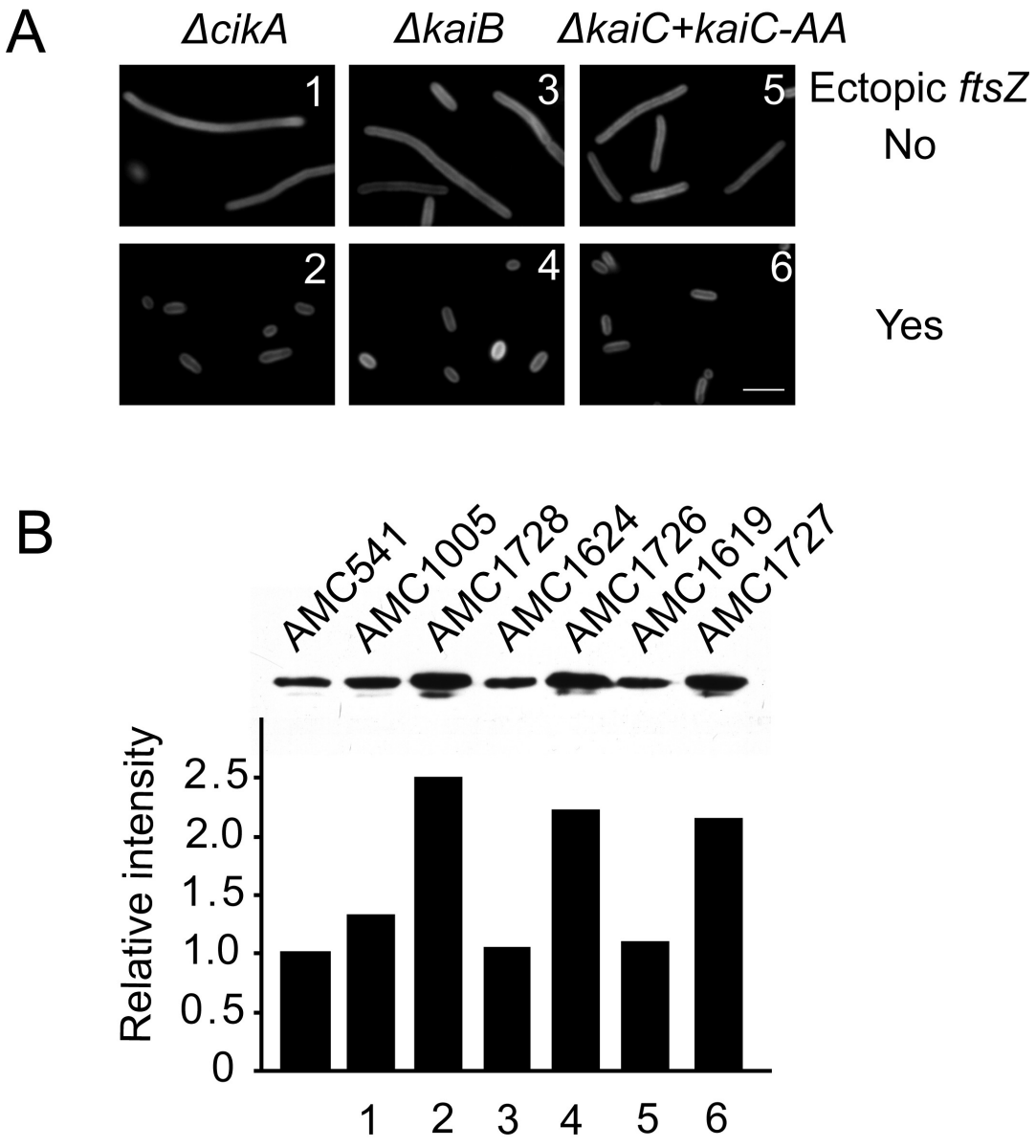


**Figure 2.7. FtsZ Suppresses the Cell Division Defect in Clock Mutants.**
(A) Ectopic expression of *ftsZ* in clock mutants that cause cell elongation reduces cell size.
(B) Comparison of FtsZ levels in the WT and strains measured in (A). Top: western blot using FtsZ antiserum. Bottom: Quantification of the relative levels of FtsZ shown on top. Level of FtsZ in the WT is set at 1.

## DISCUSSION

### KaiC Is the Gatekeeper

In *S. elongatus*, cell division is gated by the circadian clock such that it is prohibited within a 4-hour window during the subjective day-night transition (Mori et al., 1996). We confirmed independently the presence of such a gate by monitoring single cells continuously (data not shown). Furthermore, our work provides a molecular explanation for the gating phenomenon. The circadian clock has an active negative effect on cell division, i.e., the absence of a functional clock does not affect cell division. Based on previous studies and our own findings, we propose a model in which the default state of the gate is open so that cell division operates independently of the circadian clock; when a critical phase of the circadian clock is reached, which is marked by a specific state of KaiC that in turn transmits the information through the SasA-RpaA two-component system to target FtsZ localization, cytokinesis of cell division is blocked and thus the gate is closed. In this model, only the timing of cell division is gated. Because the length of a closed gate (~4 hours) is shorter than the fastest doubling time (~6 hours) for *S. elongatus* cells, the frequency of the cell cycle is unaffected by the clock. This explains why no difference in cell length is observed between WT and clock-less cells.

Our data show that elevated ATPase activity of KaiC correlates with the closing of the gate, and we propose that a threshold level of activity is

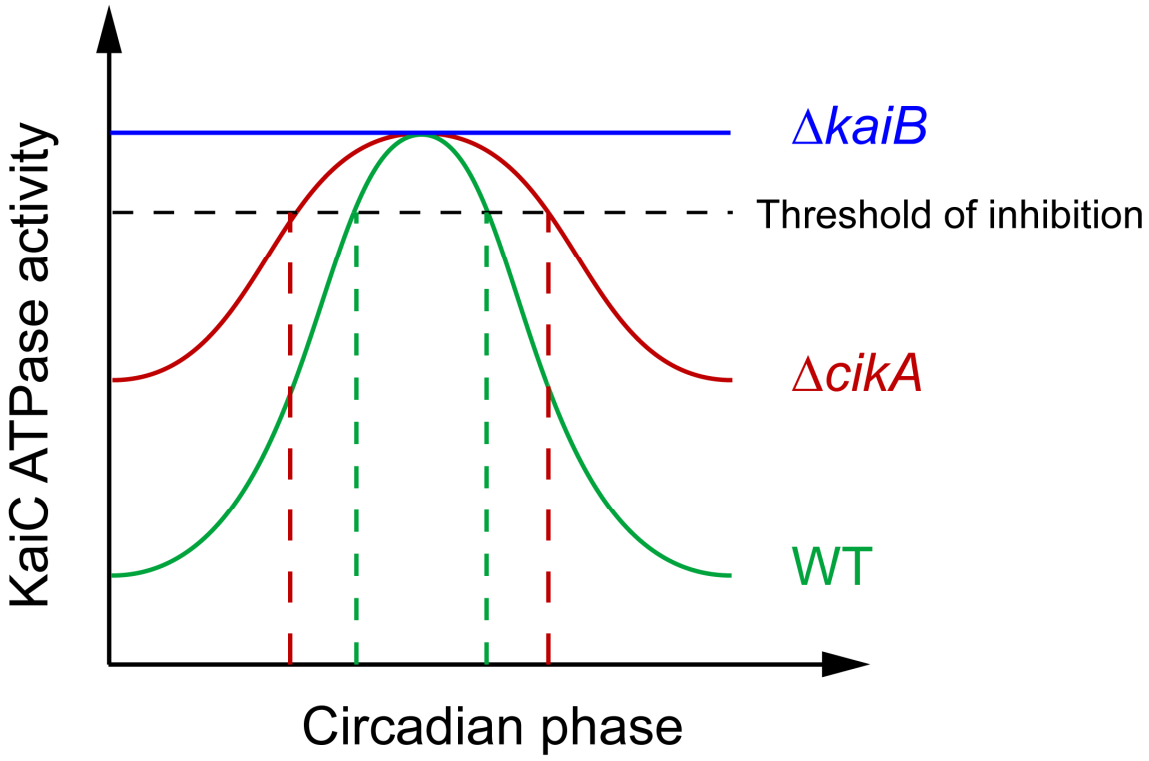


**Figure 2.8. A Model of Inhibition of Cell Division by High ATPase Activity of KaiC.**
When the ATPase activity of KaiC reaches a certain threshold, the gate on cell division is closed and no cell division is allowed until it drops below the threshold value. In *ΔkaiB* mutant or under *kaiA* overexpression conditions, KaiC is locked at the high ATPase activity and thus cell division is constitutively inhibited which was observed using time-lapse microscopy (data not shown). In *ΔcikA* mutant, ATPase activity still oscillates, but the basal level is elevated due to the stimulation from an unknown factor, which results in a wider window of inhibition of cell division every day, and thus the larger cell size. The low amplitude at relatively high level could potentially explain a similar phenotype of gene expression rhythm observed in the *ΔcikA* mutant.

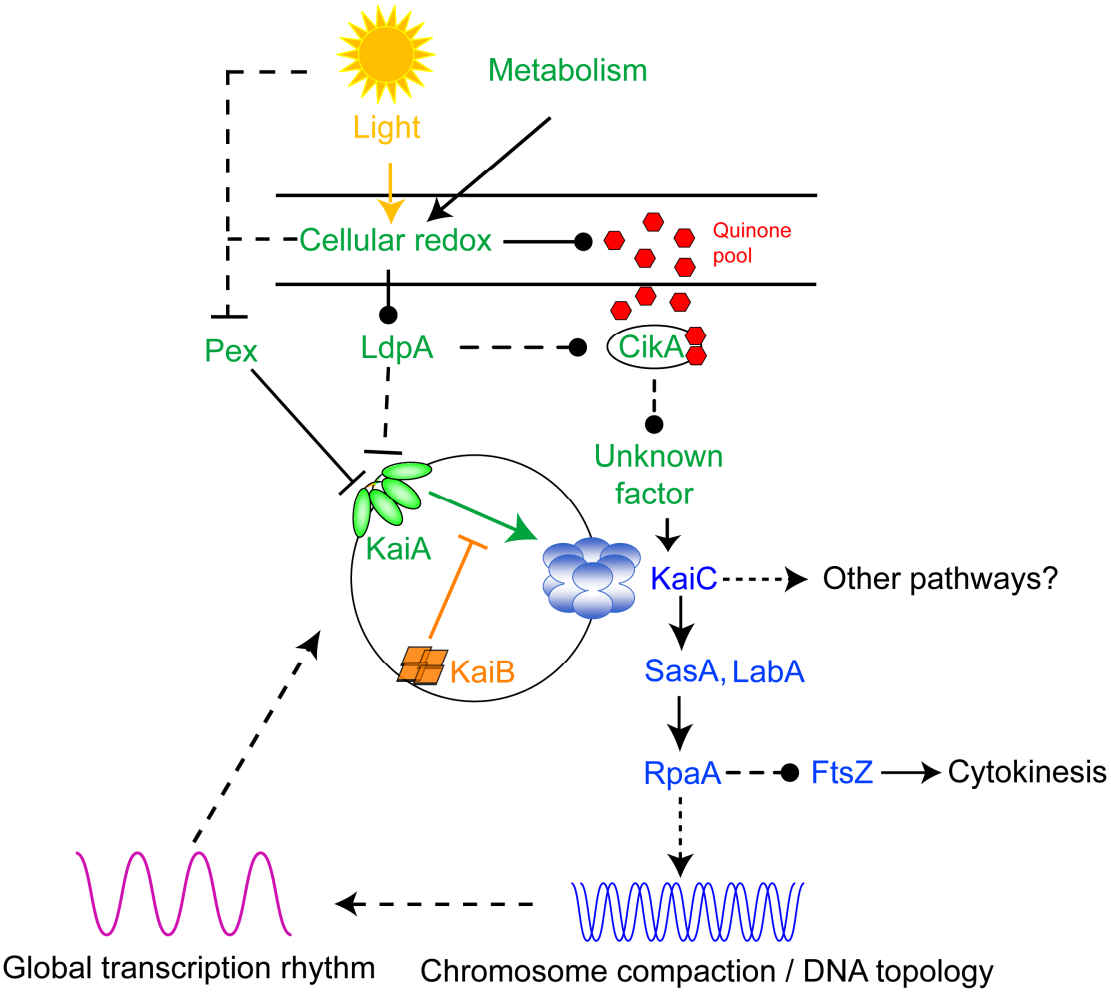


**Figure 2.9. A Revised Comprehensive Model of the Molecular Mechanism of Circadian Clock in *S. elongatus*.**
One major addition to the in the input pathway is that CikA acts to inhibit KaiC ATPase/autokinase activities through an unknown factor. There are two parallel pathways that function in the input pathway: a KaiA-dependent pathway and a CikA-dependent pathway that converges onto KaiC. These two pathways are also connected by LdpA, which regulates the abundance of both KaiA and CikA through an unknown mechanism. The second change is in the output pathway: the gating of cell division functions through the SasA-RpaA two-component system. Localization of FtsZ is targeted, which results in an inhibition of cytokinesis. For detailed description of the other aspects of the model, refer to Figure 1.2 of this dissertation.

responsible for gating (Figure 2.8). As the ATPase activity of KaiC goes above a critical value in a circadian oscillation, it closes the gate until the activity drops below the critical value. KaiC mutants with higher average ATPase activities would spend more time per day above the critical value, resulting in longer inhibition of cell division and thus longer cells. Even though we have shown that a nonphosphorylable KaiC mutant still causes cell elongation, likely due to its high ATPase activity, we cannot rule out the possibility that kinase activity of KaiC works synergistically with ATPase activity to gate cell division, because in WT situations these two activities are closely related with each other (Terauchi et al., 2007). In fact, it is likely that the conformation of KaiC that favors ATPase activity also favors the kinase activity that leads to KaiC phosphorylation; this specific conformation of KaiC, rather than either enzymatic activity, may mediate the gating of cell division. This model might explain the observation that cells are still slightly elongated in ATPase mutants based on KaiC-AA, where both phosphorylation and ATP hydrolysis are defective.

**How Is CikA Involved?**

Until this work it was unclear whether the *cikA* mutant's circadian rhythm and cell division phenotypes were related. We presented evidence that all known *cikA* phenotypes are a function of the circadian clock. Specifically, we propose that CikA indirectly suppresses the ATPase activity of KaiC (Figure 2.9), which is consistent with the cell elongation and shorter period phenotypes observed in

the *cikA* mutant. The lower amplitude, at a relatively high level, of the transcription rhythm could be explained by the oscillation of KaiC ATPase activity at a constitutively high level with low amplitude (Figure 2.8). Most significantly, the model predicts that CikA resets the clock by modulating ATPase activity of KaiC. During a dark pulse, CikA abundance goes up (Ivleva et al., 2005; Ivleva et al., 2006), which in turn would suppress the ATPase activity of KaiC. At the same time, abundance of Pex protein, a period-extending protein placed in the input pathway, also goes up, resulting in a reduction of KaiA expression and thus less stimulation of KaiC ATPase activity (Figure 2.9). We do not believe that these events are coincidental; rather, they work synergistically to achieve a delay in KaiC phosphorylation or an advance in KaiC dephosphorylation, with the CikA-modulated pathway playing a dominant role in resetting.

The discovery of a KaiA-independent, but CikA-repressed factor that stimulates KaiC ATPase and kinase activities suggests that there are two separate pathways regulating KaiC activities in vivo: a KaiA-KaiB-KaiC loop, which is responsible for generating the KaiC phosphorylation rhythm, and a CikA-dependent pathway. These two pathways converge on KaiC. We propose that the CikA-mediated pathway is a part of the regulatory mechanism for gene expression rhythms even though they persist in a *cikA* null mutant, because overexpression of CikA results in arrhythmia (Zhang et al., 2006b). This CikA-mediated pathway might also underlie the transcription/translation rhythm that is

observed in the absence of a KaiC phosphorylation rhythm (Kitayama et al., 2008). We predict that eliminating CikA from strains that lack the phosphorylation rhythm would destabilize or even abolish the residual transcription rhythm. We propose that the interlocking of these two pathways enhances the robustness and resilience of the circadian clock. We do not know at this point what the unknown factor is that stimulates KaiC ATPase and kinase activities. Given that a kinase mutant of CikA phenocopies that of a *cikA* null, it is likely that the unknown factor is a protein whose activity is regulated by CikA kinase activity.

**SasA-RpaA Mediate KaiC-Dependent Gating of Cell Division**

SasA and RpaA belong to the family of bacterial two-component systems, which are often employed in signal transduction. Circadian rhythms of gene expression and chromosome compaction have been shown to be controlled by the SasA-mediated output pathway (Iwasaki et al., 2000; Smith and Williams, 2006). Our data show that gating of cell division is also regulated through the same output pathway. We propose that SasA phosphorylation is controlled by specific states of KaiC, and RpaA reads information from SasA to target the localization of FtsZ through a yet-unknown protein (Figure 2.9). Some available data show that the presence of KaiC stimulates SasA phosphorylation (Smith and Williams, 2006); however, the detailed molecular mechanism is still missing, in part because SasA protein is difficult to purify and loses activity quickly in vitro. The target site

for RpaA's DNA binding domain will shed light on which proteins are involved. A tantalizing possibility is that RpaA, which is widely conserved in cyanobacteria, binds to the Hip1 DNA repeat element that is also common in this group, and that this combination is part of the chromosome compaction mechanism.

**How Faithful Are KaiC Phosphomimetics?**

Aspartic acid and glutamic acid have many times been successfully used to mimic phosphorylated serine and threonine, respectively, because of their similarities in shape and charge (Dutta et al., 2008; Gutierrez et al., 2008). Using these phosphomimetics, kinetics of KaiC phosphorylation and dephosphorylation were characterized previously (Nishiwaki et al., 2007), as was the binding affinity among Kai proteins (Nishiwaki et al., 2007), which was later supported by an independent study (Rust et al., 2007). However, there were no assays to test whether these mimetic proteins are genuine representations of the different KaiC phosphoforms. The cell division defect caused by clock mutants in this study makes such an assay possible. We found that KaiC-AA does not fully mimic unphosphorylated KaiC, as the former interferes with cell division and the latter does not. Similarly, we concluded that KaiC-DE is unlikely to perfectly mimic constitutively phosphorylated KaiC as these two KaiC variants, too, have opposite effects on cell division. In both cases, it appears that the mimetics do not have the same ATPase activities as the originals. Additional evidence comes from the fact that both KaiC-DE and

KaiC-EE have been used to mimic fully phosphorylated KaiC, but they differ significantly from each other as KaiC-DE fails to restore circadian rhythmicity to a *kaiC* null strain whereas KaiC-EE does (Kitayama et al., 2008). The difference between them, and evidence to establish which one of them is a better mimic, remains unknown. Thus, phosphomimetics can represent only certain aspects of the protein but not others, and caution should be taken when conclusions are drawn based on the use of phosphomimetics.

**Why Does the Circadian Clock Gate Cell Division?**

The biological advantage of cell division gating by the circadian clock is unknown. One hypothesis is that a closed gate protects the circadian clock from malfunctioning, e.g., clock protein complexes may have an asymmetric cellular localization or are in the process of monomer shuffling, and cell division would result in uneven distribution of clock protein complexes in the daughter cells, which would cause phase difference among the same population. It is known that the *S. elongatus* clock is inherited with exceptional fidelity (Mihalcescu et al., 2004), and it stands to reason that a checkpoint exists to safeguard this accuracy. Alternatively, the circadian clock gates cell division to protect other cellular events from damage that might result from cytokinesis at a vulnerable time. It could prevent damage to the chromosome at a particular topology, which is also controlled by the circadian clock. To test these hypotheses, one could break the gate, while keeping a functional clock, and look for defects in either

the circadian clock or other aspects of the cells, such as shape and physiology. We predict that our *ftsZ* construct, which forces cells to divide when overexpressed, could be used to break the gate and test these hypotheses. Mori et al. stopped cell division by overexpressing FtsZ. We do not know what exactly accounts for their observation of the opposite phenotype than we report; however, it has been reported in *E. coli* that overproduction of FtsZ by two to seven fold resulted in induction of the minicell phenotype, whereas overexpression of FtsZ beyond this range inhibits cell division (Ward and Lutkenhaus, 1985). We reason that Mori et al. may have overexpressed FtsZ to a very high level, resulting in aggregation of FtsZ that disrupted its function, whereas our overexpression still produces functional FtsZ polymers that force cells to divide prematurely.

Overall, our work provides a mechanistic insight to the gating of cell division and sheds light on multiple aspects of the circadian clock. Moreover, this study provides evidence for an unprecedented linear flow of information from known components of the input (CikA), oscillator (KaiC), and output (SasA/RpaA) divisions of the clock. Future studies on the gating mechanism will likely deepen our knowledge of the interaction between the circadian clock and critical cellular events, such as the cell cycle and metabolism.

**Table 2.2. Plasmids Used in Chapter II.**

| Plasmid | Characteristics | Antibiotic resistance | Source or reference |
|---|---|---|---|
| **8S15-E11** | Tn*5* transposon inserted after codon #11 of *rpaA* | Km | (Holtman et al., 2005) |
| **pAM1303** | Cloning vector with NS I integration sequence | Sp, Sm | (Andersson et al., 2000) |
| **pAM1579** | Cloning vector with NS II integration sequence | Km | Lab collection |
| **pAM2152** | $Gm^R$ Ω-cassette inserted in partially deleted *cikA* | Gm, Ap | (Mutsuda et al., 2003) |
| **pAM2176** | $Gm^R$ Ω-cassette inserted in SmaI site of *sasA* | Gm, Ap | Lab collection |
| **pAM2255** | *E. coli* cloning vector with *trc* promoter | Ap | (Mutsuda et al., 2003) |
| **pAM2302** | P*kaiBC::kaiC* in NS I vector | Sp, Sm | (Ditty et al., 2005) |
| **pAM2314** | Multiple cloning sites introduced in pAM1303 | Sp, Sm | (Ditty et al., 2005) |
| **pAM3110** | Gateway cloning vector based on pAM2314 | Sp, Sm | (Ditty et al., 2005) |
| **pAM3685** | P*trc::kaiA-yfp* cloned into pAM1303 | Sp, Sm | This study |
| **pAM3842** | P*trc::kaiA* based on pAM3685 | Sp, Sm | This study |
| **pAM3868** | P*kaiBC::kaiC487* based on pAM2302 | Sp, Sm | (Kim et al., 2008) |
| **pAM3870** | P*kaiBC::kaiC-AA* based on pAM2302 | Sp, Sm | (Kim et al., 2008) |
| **pAM3875** | Gateway cloning vector based on pAM1579 | Km, Cm | This study |
| **pAM3876** | P*kaiBC::kaiC* recombined into pAM3875 | Km | This study |
| **pAM3910** | P*kaiBC::kaiC497* based on pAM2302 | Sp, Sm | (Kim et al., 2008) |
| **pAM4039** | P*kaiBC::kaiC-DE* based on pAM3876 | Km | This study |
| **pAM4040** | P*kaiBC::kaiC-DT* based on pAM3876 | Km | This study |
| **pAM4041** | P*kaiBC::kaiC-AE* based on pAM3876 | Km | This study |
| **pAM4042** | P*kaiBC::kaiC-SE* based on pAM3876 | Km | This study |
| **pAM4043** | P*kaiBC::kaiC-DA* based on pAM3876 | Km | This study |
| **pAM4131** | P*trc::ftsZ* cloned into pAM1303 | Sp, Sm | This study |
| **pAM4238** | P*kaiBC::kaiCR393C* from pAM2302 | Sp, Sm | This study |
| **pAM4239** | P*kaiBC::kaiC-A251V* from pAM2302 | Sp, Sm | This study |
| **pAM4245** | P*kaiBC::kaiC-AA-K52H* based on pAM3870 | Sp, Sm | This study |
| **pAM4246** | P*kaiBC::kaiC-AA-K294H* based on pAM3870 | Sp, Sm | This study |
| **pAM4247** | P*kaiBC::kaiC-AA-E77,78Q* based on pAM3870 | Sp, Sm | This study |
| **pAM4248** | P*kaiBC::kaiC-AA-E318,319Q* based on pAM3870 | Sp, Sm | This study |
| **pAM4249** | P*kaiBC::kaiC-AA-E77,78,318,319Q* based on pAM3870 | Sp, Sm | This study |

**Table 2.3. Cyanobacterial Strains Used in Chapter II.**

| Strains | Genetic characteristics | Antibiotic resistance | Source or reference |
|---|---|---|---|
| **AMC541** | WT | Cm | (Ditty et al., 2003) |
| **AMC702** | *kaiA* in-frame deletion | Cm | (Ditty et al., 2005) |
| **AMC704** | *kaiC* in-frame deletion | Cm | (Ditty et al., 2005) |
| **AMC705** | *kaiBC* in-frame deletion | Cm | (Ditty et al., 2005) |
| **AMC1005** | *cikA* null by $Gm^R$ Ω-cassette insertion | Gm, Km, Cm | (Mutsuda et al., 2003) |
| **AMC1161** | *kaiA* null by $Km^R$ Ω-cassette insertion | Km, Cm | (Ditty et al., 2005) |
| **AMC1275** | *sasA* in-frame deletion | Cm | Lab collection |
| **AMC1548** | pAM3685 in AMC1161 | Km, Sp, Sm | This study |
| **AMC1619** | pAM3870 in AMC704 | Cm, Sp, Sm | (Kim et al., 2008) |
| **AMC1621** | pAM3868 in AMC704 | Cm, Sp, Sm | (Kim et al., 2008) |
| **AMC1623** | pAM3910 in AMC704 | Cm, Sp, Sm | (Kim et al., 2008) |
| **AMC1624** | *kaiB* in-frame deletion | Sp, Sm, Cm | This study |
| **AMC1627** | pAM2152 in AMC704 | Gm, Sp, Sm | This study |
| **AMC1628** | pAM3842 in AMC704 | Cm, Sp, Sm | This study |
| **AMC1670** | pAM4039 in AMC704 | Km | This study |
| **AMC1671** | pAM4040 in AMC704 | Km | This study |
| **AMC1672** | pAM4041 in AMC704 | Km | This study |
| **AMC1673** | pAM4042 in AMC704 | Km | This study |
| **AMC1674** | pAM4043 in AMC704 | Km | This study |
| **AMC1675** | pAM3842 in AMC541 | Km | This study |
| **AMC1679** | pAM2152 in AMC702 | Gm, Cm | This study |
| **AMC1700** | kaiCR393C in AMC704 | Cm, Sp, Sm | This study |
| **AMC1701** | KaiCA251V in AMC704 | Cm, Sp, Sm | This study |
| **AMC1708** | pAM2176 in AMC1624 | Sp, Sm, Gm, Cm | This study |
| **AMC1709** | 8S15-E11 in AMC1624 | Sp, Sm, Km, Cm | This study |
| **AMC1710** | pAM2595 in AMC1708 | Sp, Sm, Gm, Km | This study |
| **AMC1713** | pAM2152 in AMC1275 | Gm, Cm | This study |
| **AMC1714** | pAM2152 in AMC1161 | Km, Gm, Cm | This study |
| **AMC1722** | 8S15-E11 in AMC541 | Km, Cm | This study |
| **AMC1735** | pAM4245 in AMC704 | Sp, Sm, Cm | This study |
| **AMC1736** | pAM4246 in AMC704 | Sp, Sm, Cm | This study |
| **AMC1737** | pAM4247 in AMC704 | Sp, Sm, Cm | This study |
| **AMC1738** | pAM4248 in AMC704 | Sp, Sm, Cm | This study |
| **AMC1739** | pAM4249 in AMC704 | Sp, Sm, Cm | This study |

## EXPERIMENTAL PROCEDURES

### Bacterial Cultures, Growth Conditions and DNA Manipulations

WT *S. elongatus* PCC 7942 strain and its genetic variants were propagated in BG-11 medium with constant shaking at 250 rpm and at 30 ℃. Antibiotics, when needed, were added at concentrations previously described (Clerico et al., 2007; Ditty et al., 2003; Mackey et al., 2007), except that when multiple antibiotics were needed, concentrations of gentamycin and kanamycin were halved. Basic molecular cloning was performed according to standard lab protocols (Ivleva et al., 2005). Mutant alleles of *kaiC* were made by the QuickChange (Stratagene, La Jolla, CA) site-directed mutagenesis protocol. All clones were verified by DNA sequencing. Plasmids were propagated in *E. coli* DH10B. A complete list of plasmid constructs and cyanobacterial strains used in this study is detailed in Tables 2.2 and 2.3.

### Sample Preparation and Epifluorescence Microscopy

All experiments were performed on strains growing in liquid medium in a shaking incubator at a light intensity of 45 μE $m^{-2}$ $s^{-1}$. To minimize variations of cell length due to light intensity, growth phase, etc., the following growth routine was followed: 5 mL of liquid culture were subcultured into 100 mL fresh BG-11 media with appropriate antibiotics, allowed to grow for 3 days, and further subcultured into 100 mL BG-11 media with a 1:100 dilution factor. Measurement of cell

length was taken at the same time for all strains after 3-4 days growth. Cells from 1 mL cyanobacterial culture were collected and resuspended in 100 µL sterile water; from thus suspension 2 µL were loaded on a glass slide, followed by 5 µL of 1% low-melting agarose dissolved in BG-11 medium, and covered with a cover glass. Autofluorescence images of cyanobacterial cells were taken using an Olympus IX 70 inverted microscope with a 100X oil-immersion objective.

**Image Processing and Statistical Analysis**

Micrographs of cyanobacterial cells were saved as 8-bit gray images. Subsequent analysis was performed with the free software ImageJ (http://rsbweb.nih.gov/ij/). The distance between pixels was first calibrated using a stage micrometer image taken under exactly the same conditions as the image. Multiple cells that were touching each other were manually separated by electronic addition of a black line with a 5 pt width. Images were segmented using the “Otsu Thresholding 8bit” plugin and cell length was acquired by applying the “Measure Roi Curve” plugin function, with default settings, to the segmented images. Cell length data were then imported into SPSS 14.0 and compared using Dunnett’s T3 algorithm in One-way ANOVA analysis with $p < 0.001$. All experiments were repeated at least three times.

**Western Blot**

Western blots were performed essentially as described (Ivleva et al., 2005), except that KaiC antiserum was used at 1:2000 dilution.

# CHAPTER III

# SUBCELLULAR LOCALIZATION OF CIRCADIAN CLOCK PROTEINS IN *S. ELONGATUS* PCC 7942[†]

## INTRODUCTION

In eukaryotic circadian clock systems, the specific subcellular localization and shuttling of clock proteins play a key role in the generation and maintenance of circadian rhythms. Deletion of the nuclear localization signal (NLS) from FREQUENCY, a central circadian oscillator protein of *N. crassa*, prevents its nuclear entry and results in arrhythmia (Luo et al., 1998). In *D. melanogaster*, PERIOD (PER) and TIMELESS (TIM) are both required for rhythmicity and they negatively regulate their own expression by inhibiting the function of dCLOCK and CYCLE, which are basic helix-loop-helix transcription factors that activate *per* and *tim* genes (Glossop et al., 1999; Lee et al., 1999; Rutila et al., 1998). The nucleocytoplasmic shuttling of PER and TIM is tightly regulated. In cultured *D. melanogaster* S2 cells the formation of heterodimeric PER/TIM complexes promotes their efficient nuclear entry (Curtin et al., 1995), although monomeric PER or TIM is able to enter the nucleus independently of the other (Ashmore et

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al., 2003; Shafer et al., 2004; Shafer et al., 2002). DOUBLE-TIME (DBT), a Casein Kinase 1ε (CK1ε) homolog, destabilizes cytoplasmic PER and prevents its premature nuclear accumulation when the TIM level is low. As more TIM accumulates in the cytoplasm, the stable PER/TIM complex is resistant to phosphorylation by DBT and enters the nucleus (Cyran et al., 2005; Price et al., 1998; Suri et al., 2000). In the mammalian clock system, similar translocation is also seen. Co-expression of mPER proteins with the mCRYPTOCHROMEs (mCRY) leads to their efficient nuclear entry, although it was later shown that mCRY is not necessary for the translocation (Kume et al., 1999; Shearman et al., 2000; Yagita et al., 2000). In the plant *A. thaliana*, Phytochrome B is the primary high-intensity red light photoreceptor for circadian control and it translocates from the cytoplasm to the nucleus in a light-dependent manner (Chen et al., 2005; Somers et al., 1998; Yamamoto and Deng, 1999).

Prokaryotes are usually tiny in size (several micrometers in length) and lack membrane-bound subcellular organelles in contrast to eukaryotes. However, specific subcellular localization of many, if not most, proteins in prokaryotes is crucial for proper cellular function. For example, the prokaryotic homolog of tubulin, FtsZ, polymerizes at the site of septum formation and forms a ring-like structure during cell division. Failure of the appropriate Z-ring formation or false localization results in aberrant cell division and elongated daughter cells (Errington et al., 2003). For clock proteins in *S. elongatus*, their localization within the cell is likely to play an important role in clock function, and information

on localization may also serve as a probe for pursuing their biological functions, as little is known about them. The shuttling of clock proteins may provide a delay in the post-translational timing loop. Kitayama et al. have investigated the subcellular localization of Kai proteins using cellular fractionation followed by western-blot analysis to detect the distribution of Kai proteins. They report that KaiA and KaiC localize to the cytoplasm all the time, and KaiB is found mainly in the membrane fraction and enters the cytoplasm at specific circadian times (Kitayama et al., 2003). However, as the authors partitioned the cells into only two fractions, the pellet, which the authors claimed to be the membrane fraction, may very likely contain other components such as large macromolecular complexes. Moreover, others in our lab have found that KaiA fractionates into a particulate fraction when particular methods of cell breakage are employed (N.B. Ivleva and S.S. Golden, personal communication). Thus, further studies with a more careful fractionation technique or a method that is more likely to reflect the in vivo situation are necessary.

In this study we successfully used two fluorescent proteins, a yellow fluorescent protein (YFP) from the jellyfish *Aequorea victoria* and a reef coral fluorescent protein named ZsGreen (ZsG) from the coral *Zoanthus sp.*, as tags that report the subcellular localization of circadian clock proteins. We found that KaiA is localized to the cell pole in a dose-dependent manner, and that the polar localization is dependent on the presence of KaiB and KaiC. More specifically, the phosphorylation states of KaiC affect KaiA localization. Localization of KaiB,

KaiC and CikA were also determined and a model to explain the relationship of localization and function is proposed.

## RESULTS

### KaiA Is Uniformly Localized in the Cytoplasm

To investigate the localization of KaiA, we tethered YFP to the C-terminus of KaiA and expressed it from either the native *kaiA* promoter or an IPTG inducible *trc* promoter (P*trc*) ectopically in the *S. elongatus* chromosome. As a control we expressed YFP alone from the same promoters. As is shown in Figure 3.1A, KaiA-YFP expressed from both promoters was able to restore rhythmicity to the *kaiA* null mutant, suggesting that tagging KaiA with YFP does not interfere with its clock function. Immunobolot analysis confirmed that YFP had not been cleaved from KaiA (data not shown). Note that no IPTG was present when KaiA-YFP was expressed from P*trc*, indicating that basal level of expression is sufficient for complementation. However, expression of KaiA-YFP at this level resulted in a long circadian period compared to the WT background. Expressing YFP alone had no effect on circadian rhythms, regardless of expression levels (data not shown). We then recorded the intracellular localization of KaiA-YFP using a fluorescence microscope (Figure 3.1C and 3.1D). Fluorescence from KaiA-YFP was visible but extremely weak, and the signal was distributed throughout the cytoplasm, suggesting a homogenous localization of KaiA. As a

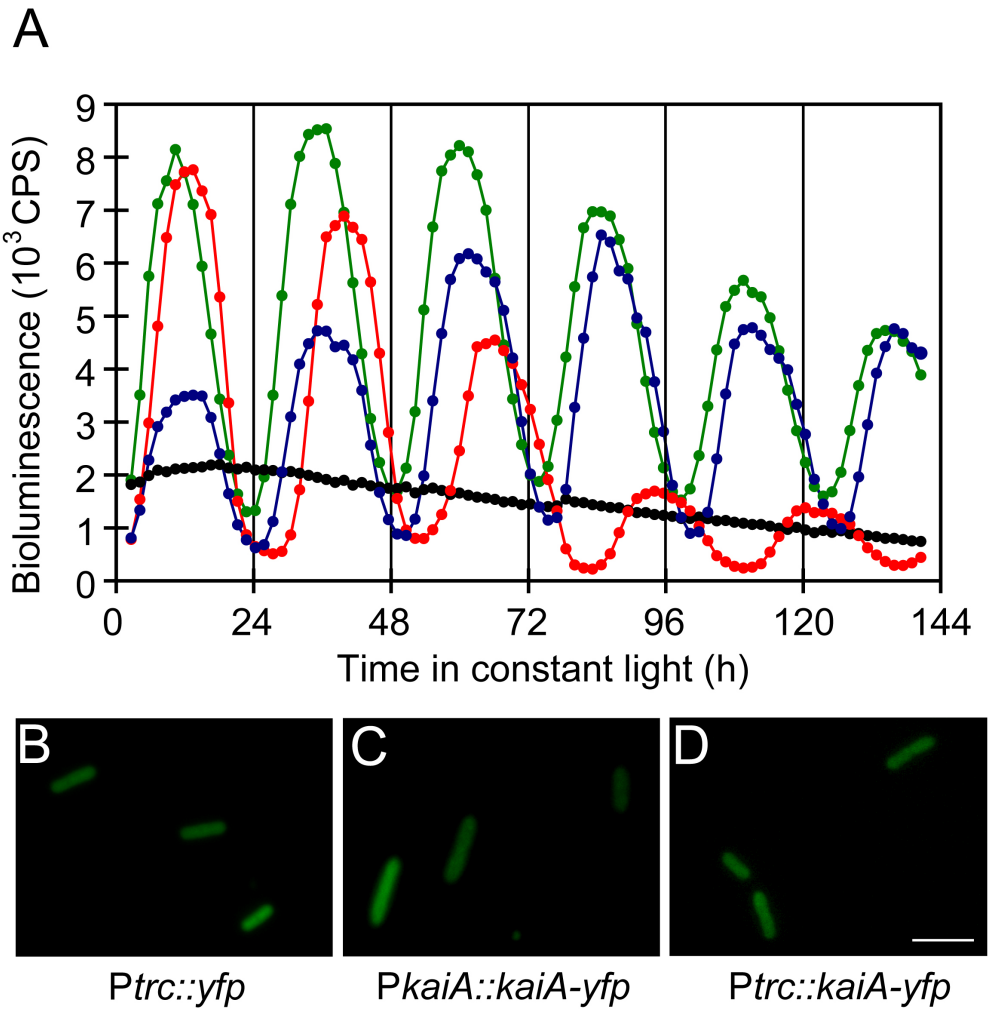


**Figure 3.1. Subcellular Localization of KaiA at Native Levels.**
(A) Complementation of gene expression rhythm in a *kaiA* null strain (black) by *kaiA-yfp* under the control of either *kaiA*'s native promoter (blue) or an IPTG inducible-*trc* promoter (red) without the addition of IPTG. All strains have a P*kaiBC::luc* as a reporter of gene expression. WT is shown in green. X axe represents the time cyanobacterial cells are incubated at constant light conditions, and Y axe is the intensity of bioluminescence read by the TopCount instrument.
(B-D) Localization of YFP alone (B), KaiA-YFP with native promoter (C) or P*trc* promoter without IPTG (D). Scale bar = 5 μm unless otherwise stated.

control, localization of YFP was also uniform and showed no bias towards any part of the cell (Figure 3.1B). In order to test whether subcellular localization of KaiA is dynamic, cultures of KaiA-YFP strains were entrained by two light/dark cycles and released in constant light conditions and samples were examined every two hours. No changes in the KaiA-YFP localization pattern were observed. A group of cells was also continuously monitored using time-lapse microscopy and similar results were acquired. Expression of KaiA-YFP in a WT background showed similar distribution (data not shown).

**KaiA Is Localized to the Cell Pole in a Dose-Dependent Manner**

Because the KaiA-YFP fluorescence is very weak when expressed at native levels, we asked whether a localization pattern would reveal itself when the expression level was elevated and the fluorescence signal increased. We first overexpressed YFP by itself in either a WT background or a *kaiA*-null strain, and found it to show no bias in localization at elevated levels and to have no effect on the circadian rhythm (Figure 3.2A and data not shown). Next, we overexpressed KaiA-YFP in a WT or *kaiA* null background and, surprisingly, together with elevated signal in the cytoplasm, a bright, polar localization was observed (Figure 3.2B). Only one foci was seen in most of the cells, and the majority of these cells localize KaiA-YFP at or near one of the two poles, whereas in a small population of cells localization of KaiA-YFP was away from the poles (Figure 3.2F).

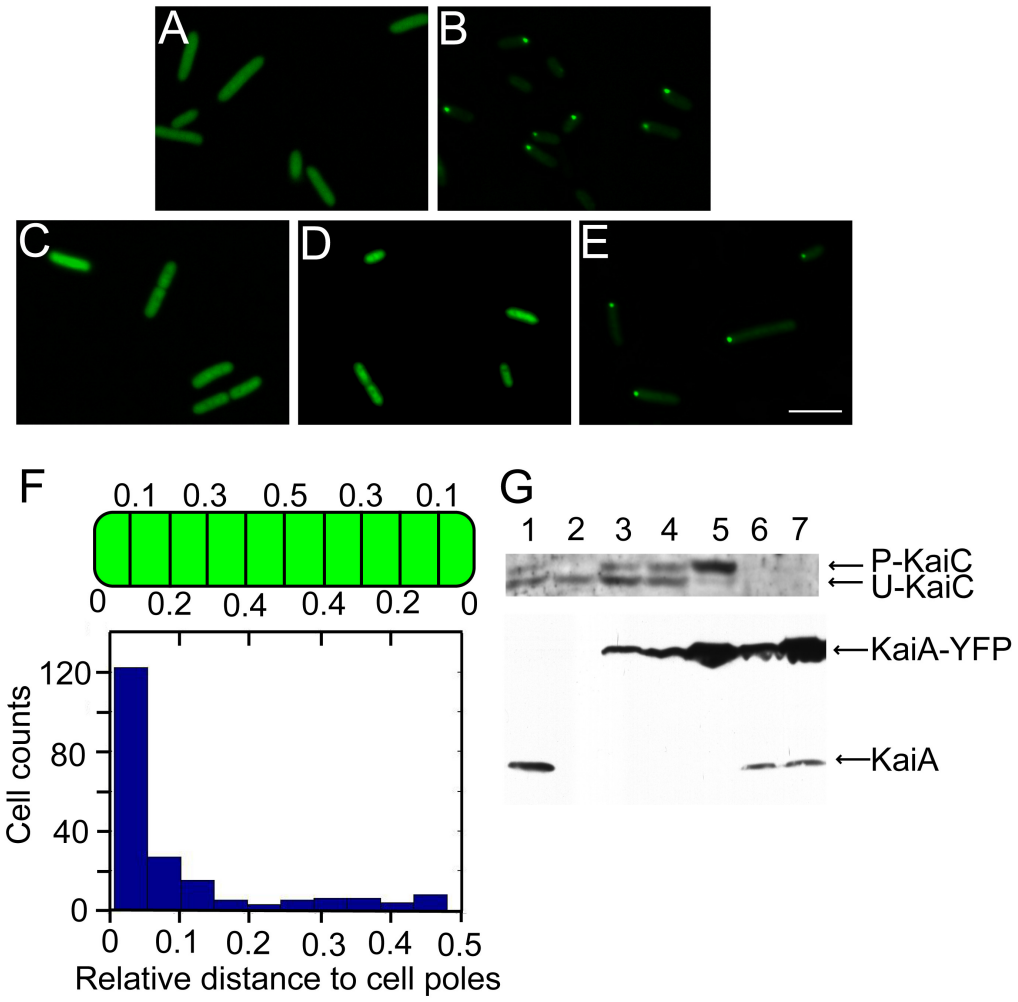


**Figure 3.2. Elevated KaiA Targets Cell Poles Dependent on KaiB and KaiC.**
(A) Uniform localization of YFP alone when it is overexpressed in a WT background.
(B-E) Localization of KaiA-YFP after IPTG induction in the WT (B), *kaiB* null (C), *kaiC* null (D) or *kaiC* null that carries an ectopic copy of *kaiC* (E) backgrounds.
(F) Distribution of localization of KaiA-YFP at overexpressing conditions in the WT cells. The distance between cell poles and mid-cell is divided equally into 5 parts (see upper panel), and the number of localized foci that fell into each part is counted. More than 200 cells with localized foci are counted.
(G) Immunoblots of KaiA-YFP and KaiC. 1: WT; 2: *kaiA* null; 3: P*kaiA::kaiA-yfp* expressed in *kaiA* null background; 4 and 5: P*trc::kaiA-yfp* expressed in *kaiA* null background in the absence (4) or the presence (5) of IPTG; 6 and 7: P*trc::kaiA-yfp* in *kaiC* null background in the absence (6) or the presence (7) of IPTG.

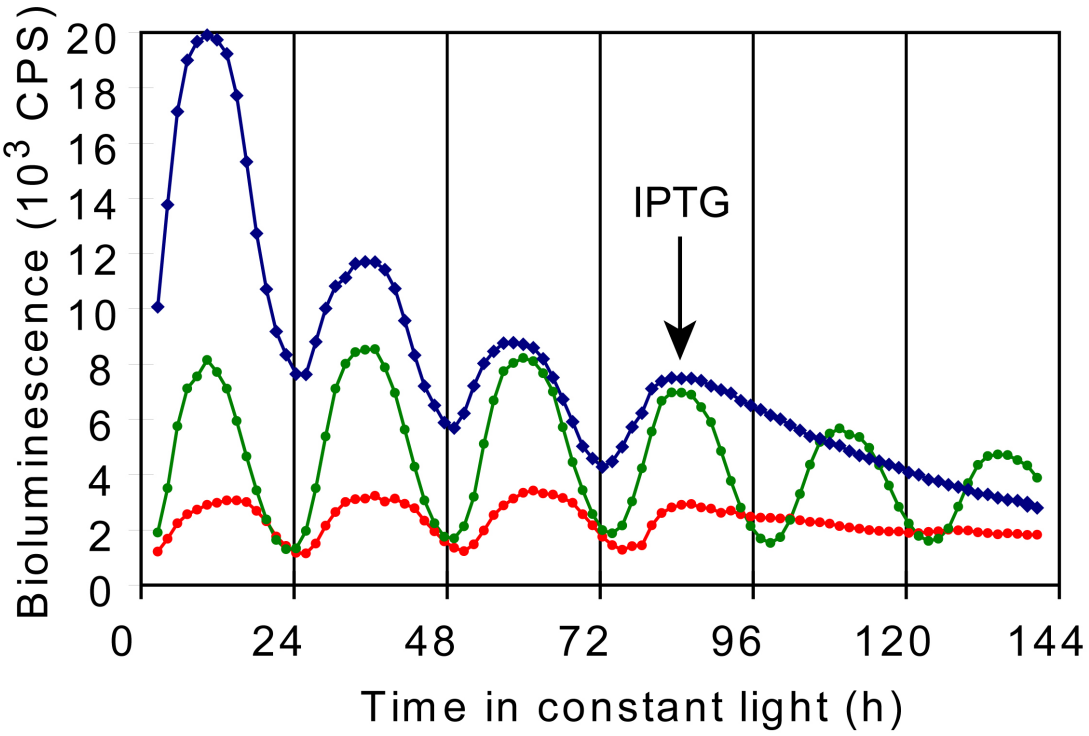


**Figure 3.3. KaiA-YFP Overexpression Abolishes Circadian Rhythm Similar to KaiA Overexpression.**
WT strain (green) and strains of WT background expressing either KaiA (blue) or KaiA-YFP (red) were synchronized by two light/dark cycles before being released into constant light. IPTG (1 mM final concentration) was added to all strains at the time indicated by an arrow.

Overexpression of KaiA-YFP abolished the circadian rhythm of gene expressions, as does overexpression of KaiA (Figure 3.3). Therefore, we were unable to determine whether the localization is dynamically regulated by the clock.

**Polar Localization of KaiA Is Dependent on Both KaiB and KaiC**

The polar localization pattern for KaiA-YFP could be biologically meaningful, or it could result from an artifactual aggregation due to the production of too much protein. We reasoned that if random aggregation was indeed the cause, then this localization pattern should be dependent only on the abundance of KaiA-YFP. Therefore, we overexpressed KaiA-YFP using the same construct in various clock mutant backgrounds to test for dependency. As is shown in Figure 3.2C and 3.2D, the absence of either KaiB or KaiC resulted in uniform distribution of KaiA-YFP signals. Immunoblots confirmed that KaiA-YFP was overexpressed to similar levels in all backgrounds tested, ruling out the possibility that it was insufficiently expressed in *kaiB* or *kaiC* null mutants (data not shown). We then re-introduced KaiC into the *kaiC* null, *kaiA-yfp* overexpression strain, and the polar localization of KaiA-YFP was rescued when its expression was induced with 1 mM IPTG (Figure 3.2E). We concluded from these observations that the polar localization of KaiA-YFP is most likely biologically meaningful and an aspect of clock function. Both KaiB and KaiC are necessary for the polar localization of KaiA. Phosphorylation states of KaiC and

abundance of KaiA-YFP were all confirmed by immunoblot analysis (Figure 3.2G).

KaiA has two domains, an N-terminal pseudo-receiver domain (KaiA135N) and a C-terminal domain (KaiA180C) that stimulates KaiC phosphorylation (Williams et al., 2002). In an attempt to test which domain of KaiA is responsible for its localization, we fused separately its N- and C-terminal domains to YFP and overexpressed them in a *kaiA* null strain. KaiA135N, which is known to be monomeric and which does not interact with KaiC (Williams et al., 2002), was successfully overexpressed and its localization was uniform under these conditions. Unfortunately, KaiA180C failed to overexpress, thus it remains unclear whether it is the C-terminus of KaiA that determines its localization (data not shown).

**Phosphorylation States of KaiC Determine KaiA Localization**

KaiA stimulates KaiC phosphorylation by binding to its C-terminal tail and stabilizing the conformation that favors the autokinase activity (Kim et al., 2008). When KaiA-YFP was overexpressed, KaiC was constitutively phosphorylated (Figure 3.2G). We hypothesized that phosphorylation states of KaiC regulate localization of KaiA. KaiC has two neighboring residues that can be phosphorylated, S431 and T432. Four possible KaiC phosphoforms exist and the relative composition of them likely determines circadian phase (Rust et al., 2007). Phosphorylation and dephosphorylation at the two residues follows a

linear order, with T432 phosphorylated first and S431 second; fully phosphorylated KaiC then dephosphorylates at T432 first, which is followed by S431, finishing a full cycle (Chapter I, Figure 1.1). Different phosphorylation states of KaiC could be mimicked by using aspartate (D) and glutamate (E) to mimic phosphorylated serine and threonine residues, respectively, and alanine to mimic non-phosphorylated residues (Nishiwaki et al., 2007). We introduced these KaiC phosphomimetics into the *kaiC* null background that overexpresses KaiA-YFP to test their effects on KaiA localization. KaiC-DE (Figure 3.4A), KaiC-DA (Figure 3.4B), KaiC-DT (Figure 3.4C) and KaiC-SE (Figure 3.4D) were all able to rescue the polar localization of KaiA-YFP at overexpression conditions, whereas KaiC-AE (Figure 3.4E) and KaiC-AA (Figure 3.4F) did not. Immunoblot analysis showed that all of the KaiC phosphomimetics were expressed at similar levels (data not shown). Thus, we concluded that the phosphorylation status at residue S431 is critical for KaiA localization: phosphorylated S431 causes excess KaiA localization to the cell pole and lack of phosphorylation at this residue causes KaiA to be uniformly distributed, which is consistent with our hypothesis that KaiC phosphorylation states determine KaiA localization.

**Subcellular Localization of KaiB**

Similarly to KaiA-YFP, KaiB was tethered to the N-terminus of YFP and expressed under the control of P*trc*. Because our *kaiB* null strain also fails to express KaiC (Ditty et al., 2005), we expressed KaiC from its native promoter in

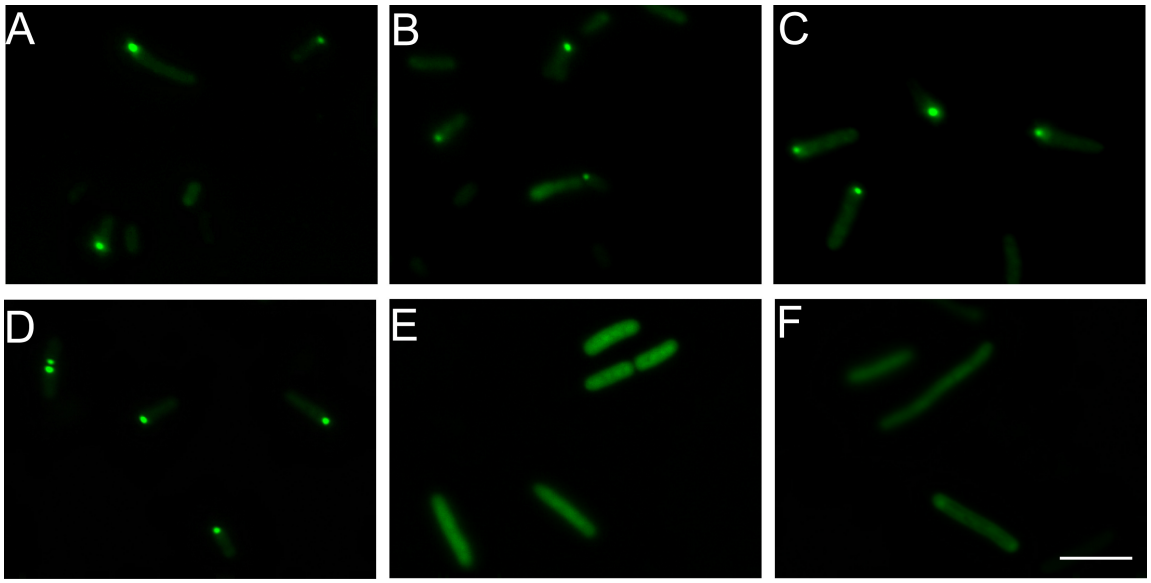


**Figure 3.4. Polar Localization of KaiA-YFP Is Dependent on KaiC Phosphorylation at S431.**
KaiA-YFP induced with 1 mM IPTG in strains expressing (A) KaiC-DE; (B) KaiC-DA; (C) KaiC-DT; (D) KaiC-SE; (E) KaiC-AE and (F) KaiC-AA.

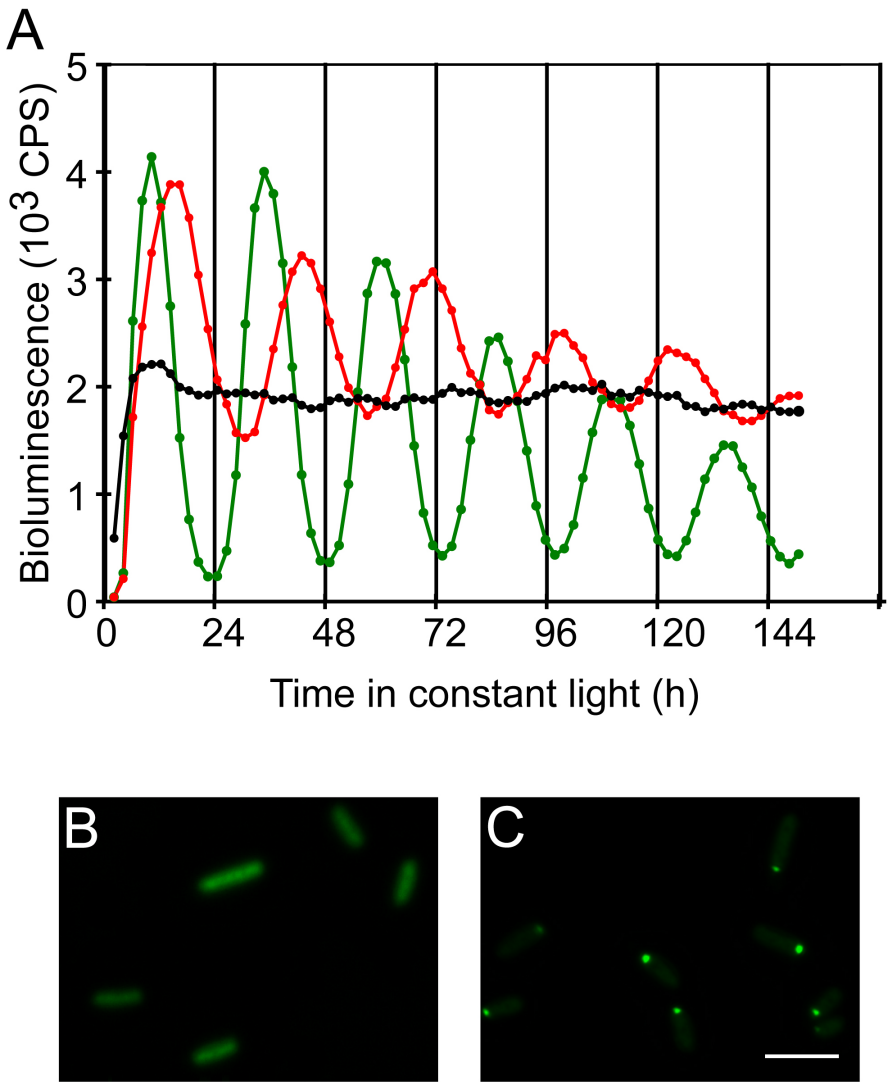


**Figure 3.5. Subcellular Localization of KaiB.**
(A) Expression of KaiB-YFP from the P*trc* promoter and KaiC from its native promoter in the same construct restores circadian rhythmicity to a *kaiBC* knockout strain (red). Gene expression activity of the WT (green) and *kaiBC* null (black) strains are also shown. All strains have a P*kaiBC::luc* reporter gene integrated at NS II of the chromosome.
(B and C) Localization of KaiB-YFP expressed from the P*trc* promoter with (C) or without (B) IPTG.

the same construct with KaiB-YFP. As shown in Figure 3.5A, this construct complemented the circadian rhythm of the *kaiBC* double knockout strain without IPTG, although the circadian period is longer than in WT. Localization of KaiB-YFP was uniform and no dynamic changes were detected during a circadian cycle (Figure 3.5B and data not shown). Overexpression of KaiB-YFP drove its localization to the cell poles (Figure 3.5C), similarly to KaiA-YFP. A circadian rhythm of gene expression persisted under this condition, with a period that was shortened by ~2 hours. To confirm that this phenotype was not an artifact due to an alteration of function of KaiB fused to YFP, we overexpressed KaiB by itself in a WT background and a similar period-shortening phenotype was observed (data not shown). Unlike KaiA, the polar localization of KaiB-YFP was independent of KaiC, and the absence of CikA or KaiA had no effect on its localization (data not shown).

**Polar Localization of KaiC Is Mediated by Its Phosphorylation States**

Because KaiA physically interacts with KaiC, and its localization is dependent on the phosphorylation states of KaiC, we hypothesized that KaiC is also localized to the cell poles in a phosphorylation-dependent manner. A fusion of YFP-KaiC was constructed using P*trc* to drive its expression. This fusion protein, when expressed at basal levels without IPTG induction, was able to restore circadian rhythms to a *kaiC* null strain (Figure 3.6A); however, the period is shorter, and the amplitude is less robust in constant conditions than in WT rhythms,

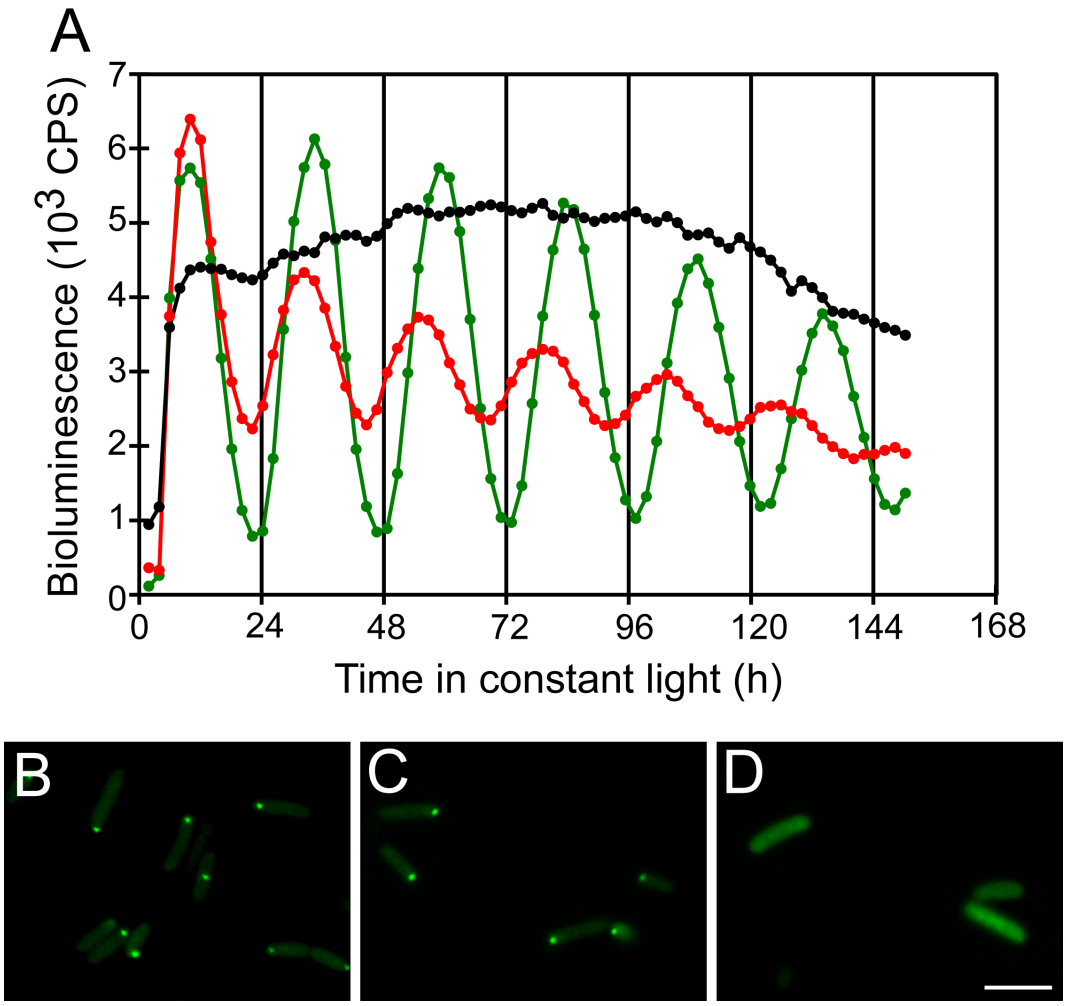


**Figure 3.6. Subcellular Localization of KaiC.**
(A) YFP-KaiC expressed from the native *kaiBC* promoter (red) complements circadian rhythmicity to the *kaiC* null strain. Gene expression activity of the WT (green) and *kaiC* null (black) strains are also shown. All strains have P*kaiBC::luc* reporter gene integrated at NS II of the chromosome.
(B) Localization of WT KaiC.
(C) Localization of YFP-KaiC-DE.
(D) Localization of YFP-KaiC-AA.

**Figure 3.7. CikA Shows Polar Localization But Loses This Pattern without PsR.**
(A, C, E and G) DIC images are shown for the WT (AMC1004, panel A) and *cikA* null (AMC1005, panel G) strains. Ectopically-expressed CikA variants that do (TH-CikA, panel C) or do not (ZsG-TH-CikAΔP, panel E) correct the mild cell division defect are shown.
(B, D, F and H) Fluorescence images are shown for AMC1004 and AMC1005 transformed with ectopically-expressed alleles that encode: unfused ZsG (panels B and H, respectively); AMC1005 expressing ZsG-TH-CikA (panel D) or ZsG-TH-CikAΔP (panel F). Panels D and F are shown at slightly higher magnification to clarify distribution of the ZsG-CikA variants. Green fluorescence indicates the fusion protein; red fluorescence arises from autofluorescence of chlorophyll in the photosynthetic apparatus and marks the cell periphery where thylakoid membranes are located. The difference in cell length between panels D and F is meaningful, as the full-length ZsG-TH-CikA allele complements all phenotypes of *cikA*, including the cell division defect, whereas the PsR-truncated allele does not. Expression of the ZsG-encoding alleles was induced with 100 μM IPTG to facilitate imaging; the same localization was observed by eye without induction in each case.


A. AMC1004
B. AMC1004-ZsG
C. AMC1005-THCikA
D. AMC1005-ZsG-THCikA
E. AMC1005-THCikAΔP
F. AMC1005-ZsG-THCikAΔP
G. AMC1005
H. AMC1005-ZsG

suggesting that YFP may hinder the function of KaiC slightly. Nevertheless, YFP-KaiC was able to maintain a functional clock for more than 6 days. Fluorescence microscopy results showed that KaiC was localized in the cytoplasm as well as at the cell poles throughout the circadian cycle (Figure 3.6B and data not shown). KaiC-AA (Figure 3.6D), which mimics non-phosphorylatable KaiC, was uniformly distributed whereas KaiC-DE (Figure 3.6C) was still localized at the poles. These data are consistent with our hypothesis that phosphorylated KaiC is localized to the cell pole, which also matches the findings of KaiA-YFP localization. The polar localization of KaiC was not affected when it was expressed in *kaiA*, *kaiB* or *cikA* null mutants (data not shown).

**Polar Localization of CikA Is a Function of Its Pseudo-receiver (PsR) Domain**

The PsR domain of CikA regulates CikA kinase activity (Mutsuda et al., 2003), and genetic experiments suggested that this domain is responsible for interaction with cellular partners. We proposed that the PsR domain might localize CikA and provide spatial regulation of the kinase. If this hypothesis is correct, we would predict that the localization of CikA in the cell would be different with and without the PsR domain. The commonly used Green Fluorescent Protein (GFP) from *A. victoria*, which works well in other cyanobacteria (Wu et al., 2004), does not fluoresce in *S. elongatus* although we

can detect its expression immunologically (N.B. Ivleva and S.S. Golden, unpublished data). ZsGreen (ZsG), a green fluorescent protein from a *Zoanthus* sp. coral, was successfully used to determine the cellular localization of CikA in *S. elongatus*. Genes that encode ZsG-tagged Thioredoxin-$His_6$-CikA (TH-CikA) and TH-CikAΔPsR were constructed and transferred into *S. elongatus*. The TH tag was necessary for solubility of CikA variants that are not full length, and does not affect CikA activity when fused to the N terminus of WT CikA (Zhang et al., 2006b). The *zsg-TH-cikA* allele was able to complement the *cikA* null strain and its overexpression phenotype is the same as for TH-CikA; likewise, ZsG-TH-CikAΔPsR functions like TH-CikAΔPsR (data not shown). As shown in Figure 3.7B and 3.7H, unfused ZsG is globally distributed in the cell. ZsG-TH-CikA showed a polar localization pattern, with one or two foci per cell (Figure 3.7D). ZsG-TH-CikAΔPsR, in which the PsR domain is missing, showed a global distribution pattern similar to that of unfused ZsG (Figure 3.7F), which supports the hypothesis that PsR is an interaction module that is crucial for the proper localization of CikA. Localization of CikA is unaffected by the absence of any of the three Kai proteins (data not shown).

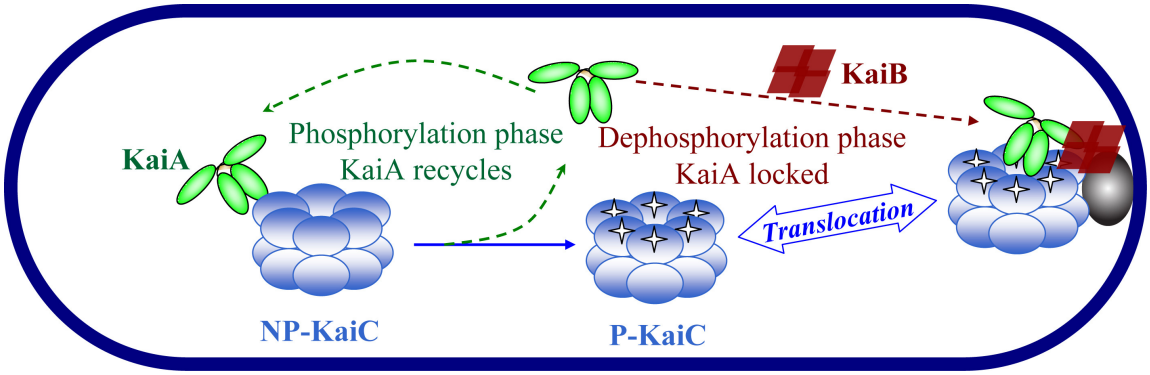


**Figure 3.8. A Model of Dynamic Localization of Clock Proteins.**
Phosphorylation states of KaiC regulate its subcellular localization. Nonphosphorylated KaiC localizes uniformly in the cytoplasm. Phosphorylated KaiC, however, has an affinity towards the cell poles. KaiA associates and dissociates with KaiC quickly and repeatedly during the phosphorylation phase and remains in the cytoplasm. During the dephosphorylation phase, KaiB and S431 phosphorylated-KaiC work together to lock KaiA at the cell poles and thus inhibits its activity. CikA is also polarly localized dependent on its PsR domain and interacts with the periodosome (not shown in the model). Localization of KaiB, KaiC and CikA is not dependent upon one another, thus a common scaffold factor (grey oval) is proposed to recruit them at the cell poles.

## DISCUSSION

### A Model of Dynamic Protein Localizations

Based on previous data and our own findings in this paper, we propose a model in which the localization of Kai proteins is determined by KaiC phosphorylation states and thus oscillates during a circadian cycle (Figure 3.8). Fully phosphorylated KaiC and KaiC phosphorylated only at S431 are localized to the cell pole. Unphosphorylated KaiC, such as newly synthesized KaiC, is uniformly distributed. KaiA is evenly localized in the cytoplasm during the phosphorylation phase of KaiC, so that it can interact with the uniformly localized unphosphorylated KaiC and stimulate its phosphorylation. As phosphorylated KaiC accumulates, KaiB locks KaiA onto KaiC to inactivate KaiA (Nishiwaki et al., 2007; Pattanayek et al., 2008; Rust et al., 2007), resulting in polar localization of both KaiA and KaiB during the dephosphorylation phase. This model could explain the polar localization data for KaiA at overexpression levels. Because all four phosphoforms of KaiC exist at any phase during the circadian cycle, both cytoplamic and polar localization of KaiC are observed at all times. There could be changes in the quantitative distribution of the localization during a circadian cycle; however, possibly due to technical limitations, we were unable to detect such changes. We are currently working on improving the functionality of the YFP-KaiC fusion so that it resembles more closely WT KaiC itself. The absence of polar localization of KaiA-YFP at any time at native expression levels

could be explained by the relatively low abundance of KaiA-YFP as evidenced by the extremely low fluorescence level. It has been estimated that only ~500 molecules of KaiA are present in a single cell at any time (Kitayama et al., 2003). Another factor that could also play a role is the relatively fast turnover rate of phosphorylated KaiC compared to unphosphorylated KaiC (Xu et al., 2003). The combination of low levels of KaiA and relatively fast turnover rate of phosphorylated KaiC could explain the absence of KaiA polar localization during the time-lapse microscopy. We predict that if KaiA-YFP could be expressed at a higher level without abolishing the function of the circadian clock, a dynamic localization pattern would be seen. We also attribute the failure to observe KaiB-YFP at the pole under native conditions to the short half-life of phosphorylated KaiC, with which KaiB interacts (Nishiwaki et al., 2007).

**A Common Scaffold for Clock Proteins**

Among the four proteins whose localizations were investigated, only KaiA showed any dependence on other proteins. KaiB, KaiC and CikA, all of which localize to the cell poles at least in certain conditions, did not affect one another's localization pattern. In vivo data suggest that they do form a complex, termed the periodosome, at certain times of the circadian cycle (Kageyama et al., 2003). None of these proteins has trans-membrane domains; if localization is dynamic, one would expect that membrane association would be peripheral. These data suggest that there is a common scaffold that is localized at the cell

poles that interacts with these clock proteins and is responsible for their polar localization (Figure 3.8).

**How Does KaiB Affect KaiA Localization?**

Existing evidence suggests that KaiA does not interact with KaiB directly. However, our results showed that polar localization of KaiA is dependent on the presence of KaiB. KaiA stimulates KaiC phosphorylation by associating and dissociating with KaiC quickly and repeatedly (Kageyama et al., 2006), and KaiB inhibits this process by inactivating KaiA (Rust et al., 2007). One of the proposed models suggests that as a specific KaiC phosphoform accumulates, KaiB locks KaiA onto KaiC to form a KaiABC complex, thus allowing KaiC to dephosphorylate. This model could explain the dependence of KaiA polar localization of KaiB and KaiC. Without KaiB locking KaiA, KaiA is able to associate and dissociate with KaiC repeatedly regardless of KaiC's phosphorylation state; without KaiC, there is no anchor for KaiA to be locked onto. Thus, both KaiB and KaiC are required for the polar localization of KaiA. Our data here support the model that KaiB inactivates KaiA by locking it on KaiC.

**Why Do Clock Proteins Target the Cell Poles?**

The significance of polar localization for clock proteins remains unknown and may be revealed by finding and knocking out the proposed common scaffold, which would presumably affect localization of clock proteins. The co-localization of clock proteins would increase their local concentration, which could facilitate the interactions among them and increase the efficiency of signal transduction. There might be multiple reasons that together account for the polar localization. For example, the PsR domain of CikA recently was found to bind a quinone analog that affects CikA's stability. Quinones are water-insoluble and thus cannot permeate into the cytoplasm. The localization of CikA at the cell pole, which is rich in membrane exposures, makes binding of quinones by the PsR domain possible. Most protein topology prediction tools classify CikA as a membrane protein, although it is readily expressed and isolated from *E. coli* in soluble form. These data are consistent with a dynamic localization of CikA at the periphery of the membrane. Localization of other proteins important for clock function at the poles may also be a factor. Depletion of the ClpP or ClpX protease components, for example, lengthens circadian period (Holtman et al., 2005). It has been shown in *Caulobacter* that ClpXP protease is localized at the cell poles (McGrath et al., 2006). Thus, clock proteins may go the cell poles, where the Clp proteases are localized, to be turned over. Many cell division and cytoskeleton proteins are also found at the cell poles in other bacteria (Howard, 2004; Shapiro et al., 2002). As discussed in the previous chapter, the circadian

clock gates cell division. Thus, it is possible that the polar localization of clock proteins plays a role in the regulation of cell division. Conversely, co-localization of clock proteins with cell division and cytoskeleton proteins may make it easier to segregate clock proteins into two daughter cells evenly, although we do not know yet whether clock proteins are actively segregated or not.

## EXPERIMENTAL PROCEDURES

### Bacterial Strains and Culture Conditions

The WT cyanobacterial strain *S. elongatus* PCC 7942 and its derivatives were propagated at 30 ℃ in BG-11 medium (Bustos and Golden, 1991) under a light intensity of 50 μE $m^{-2}$ $s^{-1}$. Antibiotics were added as needed for selection as reported previously (Mutsuda et al., 2003). All bioluminescent reporter strains either carry a *kaiB::luc* (integrated at neutral site II, NS2) construct and luciferin substrate was added externally (Ditty et al., 2005), or *kaiB::luxAB* (integrated at neutral site II, NS2, pAM2857, $Km^r$) and *psbAI::luxCDE* constructs.

For expression of the fluorescent protein ZsGreen, the open reading frame (ORF) of its gene *zsg* was amplified from the plasmid pZsGreen1-N1 (BD Clontech) and cloned immediately after the P*trc* promoter in a Gateway pDONR (Clontech) NS1 targeting vector (pAM3110), creating pAM3516. The strain transformed by this plasmid was used as a control to view the subcellular localization of unfused ZsG. Alleles that encode TH-CikA and TH-CikAΔP fused

to the C-terminus of ZsG were constructed with a 7-aa flexible linker (GSGSGSG) in between. These constructs were introduced into NS1 of AMC1005 by transformation with pAM3645 (TH-CikA) or pAM3646 (TH-CikAΔP). All other constructs and cyanobacterial strains are listed in Tables 3.1 and 3.2.

*E. coli* strain DH10B was the host for plasmid propagation. These strains were cultured in LB medium with the appropriate antibiotics at concentrations described previously (Mutsuda et al., 2003).

**Bioluminescence Assays of Circadian Rhythmicity**

Bioluminescence assays were performed using a Packard TopCount scintillation counter (PerkinElmer) as described previously (Mutsuda et al., 2003). Data were analyzed using I&A with FFT-NLLS (available from S.A. Kay, University of California at San Diego, La Jolla, CA) (Plautz et al., 1997) and BRASS (available from A. Millar and P. Brown, University of Edinburgh, Edinburgh UK, http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm) software packages to calculate circadian periods. Cyanobacterial strains were synchronized with two cycles of 12 h light: 12 h dark, then released to continuous light.

**Table 3.1. Plasmids Used in Chapter III.**

| Plasmid | Characteristics | Antibiotic resistance | Source or reference |
|---|---|---|---|
| **pAM1303** | Cloning vector with NS I integration sequence | Sp, Sm | (Andersson et al., 2000) |
| **pAM1579** | Cloning vector with NS II integration sequence | Km | Lab collection |
| **pAM2255** | *E. coli* cloning vector with *trc* promoter | Ap | (Mutsuda et al., 2003) |
| **pAM2314** | Multiple cloning sites introduced in pAM1303 | Sp, Sm | (Ditty et al., 2005) |
| **pAM3110** | Gateway cloning vector based on pAM2314 | Sp, Sm | (Ditty et al., 2005) |
| **pAM3516** | P*trc::zsGreen* cloned into pAM3110 | Sp, Sm | This study |
| **pAM3525** | *zsGreen* gene cloned into pAM2255 | Ap | This study |
| **pAM3684** | P*trc::yfp* cloned into pAM1303 | Sp, Sm | This study |
| **pAM3685** | P*trc::kaiA-yfp* cloned into pAM1303 | Sp, Sm | This study |
| **pAM3686** | P*trc::kaiB-yfp* cloned into pAM1303 | Sp, Sm | This study |
| **pAM3700** | *yfp* cloned into pAM2255 | Ap | This study |
| **pAM3813** | P*kaiA::kaiA-yfp* cloned into pAM2314 | Sp, Sm | This study |
| **pAM3842** | P*trc::kaiA* based on pAM3685 | Sp, Sm | This study |
| **pAM3843** | P*trc::kaiB* based on pAM3686 | Sp, Sm | This study |
| **pAM3875** | *attP* cassette from pDONR21 cloned into pAM1579; Gateway cloning vector | Km, Cm | This study |
| **pAM3876** | P*kaiBC::kaiC* recombined into pAM3875 | Km | This study |
| **pAM3890** | P*kaiBC::kaiC* cloned into pAM3686 | Sp, Sm | This study |
| **pAM4024** | P*kaiBC::yfp-kaiC* cloned into pAM2314 | Sp, Sm | This study |
| **pAM4039** | P*kaiBC::kaiC-DE* based on pAM3876 | Km | This study |
| **pAM4040** | P*kaiBC::kaiC-DT* based on pAM3876 | Km | This study |
| **pAM4041** | P*kaiBC::kaiC-AE* based on pAM3876 | Km | This study |
| **pAM4042** | P*kaiBC::kaiC-SE* based on pAM3876 | Km | This study |
| **pAM4043** | P*kaiBC::kaiC-DA* based on pAM3876 | Km | This study |
| **pAM4049** | P*kaiBC::kaiC-AA* based on pAM3876 | Km | This study |
| **pAM4081** | P*trc::NkaiA-yfp* (coding for first 170 residues of KaiA) cloned into pAM1303 | Sp, Sm | This study |
| **pAM4082** | P*trc::CkaiA-yfp* (coding for residues 140-284 of KaiA)cloned into pAM1303 | Sp, Sm | This study |
| **pAM4197** | P*kaiBC::yfp-kaiC-DE* based on pAM4024 | Sp, Sm | This study |
| **pAM4198** | P*kaiBC::yfp-kaiC-AA* based on pAM4024 | Sp, Sm | This study |

**Table 3.2. Cyanobacterial Strains Used in Chapter III.**

| Strains | Genetic characteristics | Antibiotic resistance | Source or reference |
|---|---|---|---|
| **AMC06** | WT | No | Lab collection |
| **AMC541** | WT | Cm | (Ditty et al., 2003) |
| **AMC704** | *kaiC* in-frame deletion | Cm | (Ditty et al., 2005) |
| **AMC705** | *kaiBC* in-frame deletion | Cm | (Ditty et al., 2005) |
| **AMC1004** | WT | Km, Cm | (Zhang et al., 2006b) |
| **AMC1005** | *cikA* null by $Gm^R$ Ω-cassette insertion | Gm, Km, Cm | (Mutsuda et al., 2003) |
| **AMC1161** | *kaiA* null by $Km^R$ Ω-cassette insertion | Km, Cm | (Ditty et al., 2005) |
| **AMC1409** | pAM3516 in AMC06 | Sp, Sm | This study |
| **AMC1442** | pAM3686 in AMC704 | Sp, Sm, Cm | This study |
| **AMC1446** | pAM3685 in AMC704 | Sp, Sm, Cm | This study |
| **AMC1448** | pAM3685 in AMC541 | Sp, Sm, Cm | This study |
| **AMC** | | | |
| **AMC1541** | pAM3842 in AMC1004 | Sp, Sm, Cm, Km | This study |
| **AMC1544** | pAM3843 in AMC1004 | Sp, Sm, Cm, Km | This study |
| **AMC1548** | pAM3685 in AMC1161 | Km, Sp, Sm | This study |
| **AMC1551** | Tn*5* insertion in *kaiC* in AMC541 | Km, Cm | This study |
| **AMC1580** | pAM3684 in AMC704 | Cm, Sp, Sm | This study |
| **AMC1581** | pAM3684 in AMC1161 | Km, Cm, Sp, Sm | This study |
| **AMC1629** | pAM3876 in AMC1446 | Sp, Sm, Km | This study |
| **AMC1631** | pAM4018 in AMC705 | Sp, Sm, Cm | This study |
| **AMC1651** | pAM4025 in AMC1551 | Sp, Sm, Cm, Km | This study |
| **AMC1716** | pAM4039 in AMC1446 | Sp, Sm, Km | This study |
| **AMC1717** | pAM4040 in AMC1446 | Sp, Sm, Km | This study |
| **AMC1718** | pAM4041 in AMC1446 | Sp, Sm, Km | This study |
| **AMC1719** | pAM4042 in AMC1446 | Sp, Sm, Km | This study |
| **AMC1720** | pAM4043 in AMC1446 | Sp, Sm, Km | This study |
| **AMC1721** | pAM4049 in AMC1446 | Sp, Sm, Km | This study |

**Visualization of Intracellular Localization Using YFP and ZsGreen**

Fluorescence from YFP-derived constructs was observed with an Olympus IX70 inverted microscope equipped with a cooled-CCD camera using 100x oil-immersion lens. Exposure time was set at 1 s. To immobilize the cells, cultures were concentrated 10 times before 2 μL samples were loaded on a glass slide, which was then covered by 5 μL 1% low-melting agarose followed by a cover slip.

Uninduced cultures that carry ZsG-TH-CikA complement the null phenotype of AMC1005, indicating function of the fusion protein under those conditions. Uninduced samples were compared to induced samples visually, and no change in localization was detected after IPTG induction. To facilitate imaging, ZsG proteins were induced by adding a final concentration of 1 mM of IPTG to fresh cultures of reporter strains and fluorescence was observed about 10 h later. Samples (2 μL) were directly loaded on a slide treated with poly-lysine, covered with a coverslip and sealed with nail polish. Differential Interference Contrast (DIC) images were acquired with a Zeiss Axioplan2 microscope and Hamamatsu C5810 3CCD camera. Exposure time was 1 s for fluorescence images and 0.33 s for DIC. Green and red fluorescence were acquired separately using standard filter sets for fluoroscein isothiocyanate and Texas Red, respectively, and the images were merged.

All images were processed with the publicly-available program ImageJ (http://rsb.info.nih.gov/ij). A convolution filter with default settings was applied to the red autofluorescence image arising from chlorophyll to achieve apparent sectioning of the cell and reveal the peripheral localization of the photosynthetic apparatus.

**Immunoblot Assays**

Immunoblot analysis was carried out as previously described (Ditty et al., 2005). In detail, cyanobacterial cells from 10 mL of culture with an $OD_{750}$ of 0.6 were collected by centrifugation and the pellet was resuspended in 200 μL IA lysis buffer (Ivleva and Golden, 2007). The mixture was then transferred to a 1.5 mL tube with ~100 mg of glass beads. Cells were broken by shaking vigorously on a bead-beater for two 1.5 min-cycles. 100 μL of the supernatant fraction was transferred to a sterile 1.5 ml tube and 20 μL of 5x protein loading dye was added. After separation on a PAGE gel, proteins were transferred to nitrocellulose membrane using the semi-dry method (Bio-Rad, Hercules, California). The membrane was then blocked for an hour with 2% (w/v) non-fat milk in washing buffer (1x TBS with 0.1% Tween-20), another hour with primary antibody (KaiA antiserum is diluted 1:5,000, and KaiC antiserum is diluted 1:2,000) in the blocking buffer, and then another hour with secondary antibody (1:5,000) conjugated with horse peroxidase, with 3 washings using the washing

buffer between each step. Substrates were incubated with the membrane briefly, which was then exposed to X-ray film for various periods of time depending on the strength of the signal.

# CHAPTER IV

# THE DAY/NIGHT SWITCH IN KAIC, A CENTRAL OSCILLATOR COMPONENT OF THE CIRCADIAN CLOCK OF CYANOBACTERIA[‡]

## INTRODUCTION AND SUMMARY OF IN VITRO FINDINGS

This work is a collaboration between our lab and the LiWang lab from the University of California at Merced, and has been published in the September 2 issue of *Proceedings of the National Academy of Sciences* this year (Kim et al., 2008); I am the second author of this manuscript. I will summarize the findings from this study here as an introduction and then present the experiments that I performed in the Results section.

Because the phosphorylation cycle of the *S. elongatus* KaiC protein is apparently intrinsic to the timekeeping of the cyanobacterial circadian oscillator, we set out to investigate how KaiA and KaiB shift the relative autokinase and autophosphatase rates in KaiC. In this study, we present evidence that a segment of residues near the C terminus of each KaiC subunit determines which activity is dominant. We propose that when these "A-loops" are buried in the protein, KaiC is an autophosphatase. However, when the A-loops are exposed

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or removed, KaiC is an autokinase. Consistent with this hypothesis, a truncation mutant that lacks the A-loop (KaiC487) is constitutively hyperphosphorylated independently of KaiA or KaiB and a mutant missing only the tail to which KaiA binds (KaiC497) is constitutively hypophosphorylated. Several other point mutations which should destabilize the interaction of the A-loop with its neighboring residues and lead it to be exposed are also hyperphosphorylated constitutively. We suggest that there is a dynamic equilibrium between the buried and exposed states of the A-loops, so that an ensemble of KaiC molecules exhibits both activities simultaneously, but in varying ratios. In the absence of other proteins, KaiC is both an autokinase and autophosphatase, with the latter activity dominant over the former (Iwasaki et al., 2002; Williams et al., 2002; Xu et al., 2003). In this case, according to our model the dynamic equilibrium favors the buried state of the A-loops. We propose that KaiA stabilizes the exposed state, thereby increasing the autokinase rate relative to that of the autophosphatase. We think that KaiB acts by preventing this KaiA-mediated stabilization.

## RESULTS AND DISCUSSION

KaiC487 and KaiC497 proteins purified from *E. coli* are constitutively hyper- and hypophosphorylated. We confirmed these in vitro findings by expressing the proteins in *S. elongatus* and performing immunoblot analysis and λ phosphatase assays. The data showed that KaiC487 and KaiC497 are respectively hyper-

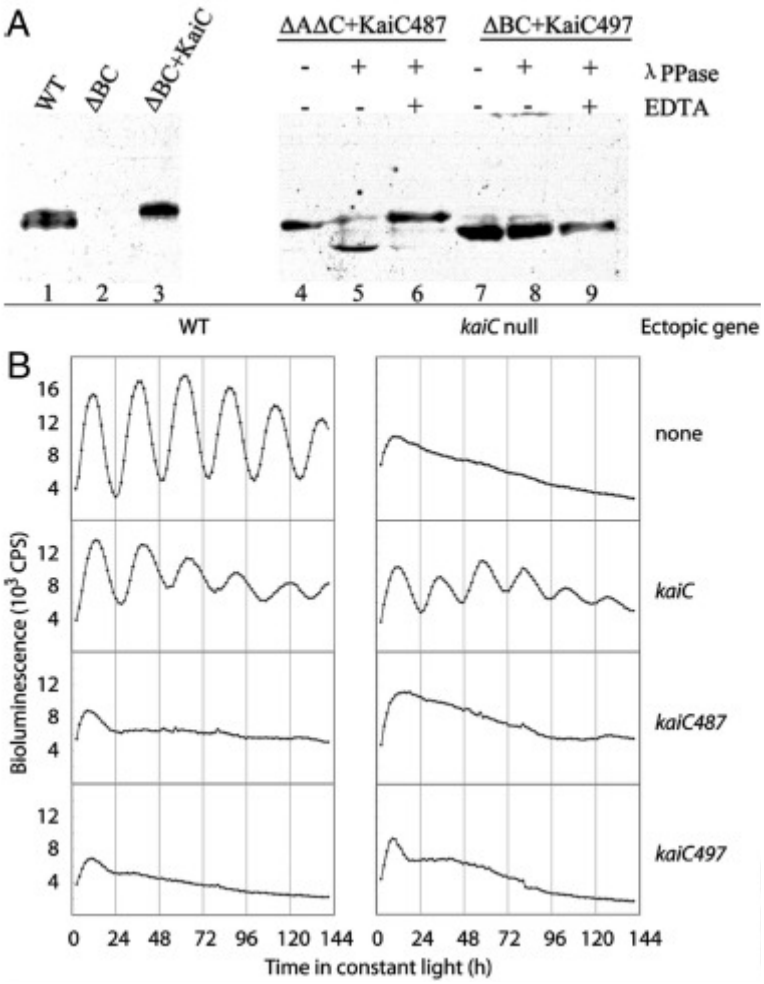


**Figure 4.1. Phosphorylation States and Functional Analysis of KaiC487 and KaiC497 In Vivo.**

(A) KaiC487 and KaiC497 are, respectively, constitutively hyper- and hypophosphorylated independently of KaiA in *S. elongatus*. Immunoblots of KaiC and its truncated variants were detected in soluble protein extracts from strains that express: lane 1, WT KaiC; lane 2, no KaiB or KaiC; lane 3, WT KaiC from an ectopic site in a *kaiBC* null background (KaiA is present); lanes 4–6, KaiC487 from an ectopic site in a *kaiA kaiC*-null background (KaiB is present); lanes 7–9, KaiC497 in a *kaiBC*-null background (KaiA is present). The phosphorylation status of KaiC variants, whose mobility differs from WT KaiC, was determined by λ phosphatase treatment with (lanes 6 and 9) or without (lanes 5 and 8) the inhibitor EDTA.

(B) Ectopic expression of KaiC487 or KaiC497 abolishes circadian rhythmicity of gene expression in WT *S. elongatus*. WT KaiC, KaiC487, and KaiC497 are expressed from the native *kaiBC* promoter in a WT (*left panel*) or *kaiC* null (*right panel*) background. All strains harbor a bioluminescence reporter gene driven by the *kaiBC* promoter.

and hypophosphorylated as well (Figure 4.1A). Moreover, KaiC487 remains hyperphosphorylated even when KaiA is absent and KaiB is present (Figure 4.1A, lanes 4-6), a situation that causes constitutive dephosphorylation in the WT protein (Ditty et al., 2005). Likewise, KaiC497 remains hypophosphorylated when KaiA is present and KaiB is absent (lanes 7-9), which would lead to constitutive phosphorylation in WT KaiC. All of the in vivo data were consistent with the in vitro findings, and with the hypothesis that KaiA stimulates KaiC phosphorylation by stabilizing the A-loop in the extended conformation and thereby switching KaiC into the kinase mode.

Ectopic expression of either KaiC487 or KaiC497 in a WT *S. elongatus* background has a dominant negative effect, abolishing circadian rhythmicity of gene expression (Figure 4.1B), even though they have opposite states of phosphorylation. In contrast, rhythmicity is preserved when WT KaiC is expressed in a WT background, with a slightly longer free-running period than normal. The correlation between circadian period length and KaiC abundance is consistent: the more KaiC, the longer the period (Figure 4.2). Also, KaiC487 and KaiC497 are unable to restore rhythmicity to a *kaiC* null strain (Figure 4.1B). These results suggest that the circadian cycle cannot occur in vivo when the A-loop is either always pulled out or permanently tucked inside for all or a fraction of the KaiC population.

The dominant negative effect of KaiC487 and KaiC497 on circadian rhythms could stem from their poisoning the ternary KaiC complex which serves as the

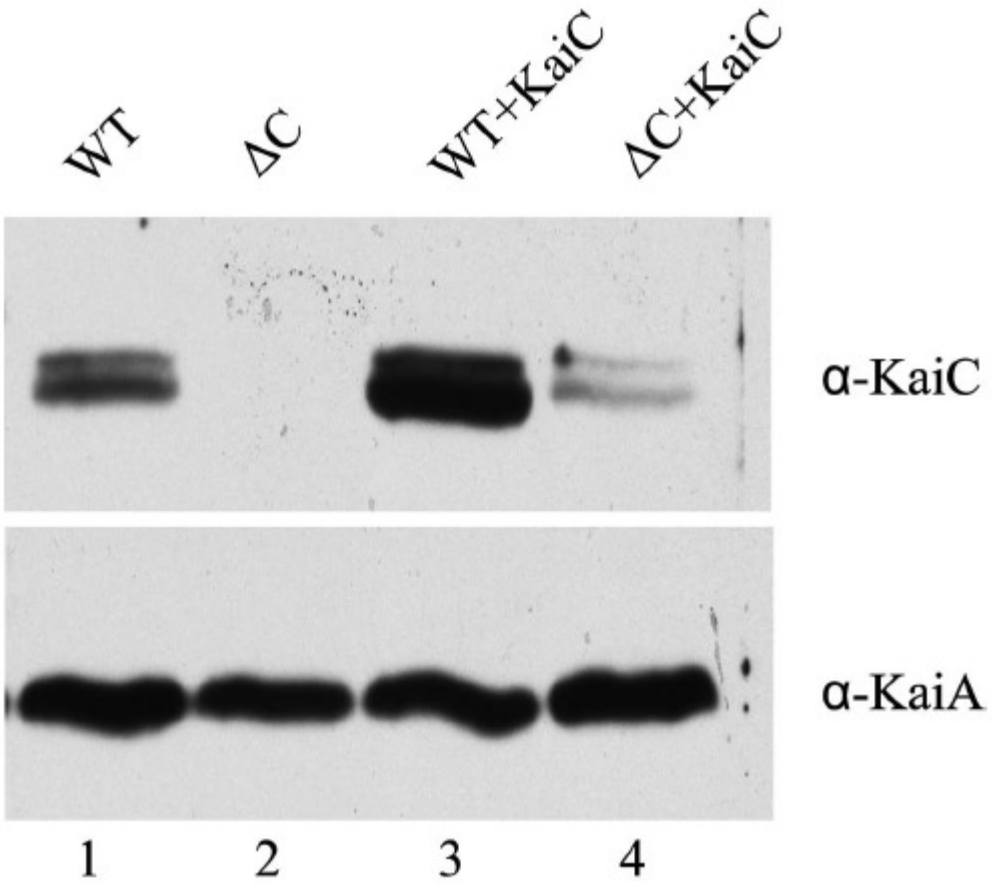


**Figure 4.2. The Circadian Period of the Gene Expression Rhythm Correlates with Intracellular KaiC Abundance in *S. elongatus*.**
Immunoblots of KaiC (*upper panel*) detected in soluble protein extracts from strains that express KaiC in the following backgrounds (with period values, $n$ = 12): 1, WT KaiC (25.02 ± 0.05 h); 2, no KaiC (arrhythmic); 3, WT KaiC from an ectopic site in a WT background (25.43 ± 0.07 h); 4, WT KaiC from an ectopic site in a *kaiC* null (23.33 ± 0.09 h). KaiA (*lower panel*) was detected in the same samples as an internal loading control.

central oscillator. However, it has been shown in vitro that KaiC-AA has no effect when it is added to a mixture with ongoing circadian oscillation of KaiC phosphorylation (Ito et al., 2007). The authors reasoned that because KaiC-AA cannot exchange monomers it cannot affect the central oscillator. In this in vitro situation non-oscillating defective hexamers and fully functional WT KaiC hexamers co-exist in the reaction in a non-interacting manner.

Given the in vitro data which suggested a lack of ternary interaction between functional and non-functional KaiC subunits, we reasoned that the data could also be explained by a scenario in which the central oscillator continues to function while the output pathway is inhibited by the non-functional KaiC subunits. Because phosphorylated WT KaiC has a different mobility on a SDS-PAGE gel compared with KaiC487 and KaiC497, it would be possible to see an oscillation in the phosphorylation status of WT KaiC, if it occurs, when KaiC487 or KaiC497 is expressed in a WT background. Immunoblots using samples taken every 4 hours from synchronized cultures showed that no such oscillation was present (data not shown), suggesting that the central oscillator is disrupted. As a control, we also expressed KaiC-AA, a nonphosphorylatable mutant of KaiC that was previously shown to not exchange monomers with KaiC in an in vitro reaction, in the WT background; this non-cycling KaiC variant also had a dominant negative effect on circadian rhythms of gene expression. Western blots showed that the central oscillator is disrupted similar to the findings for

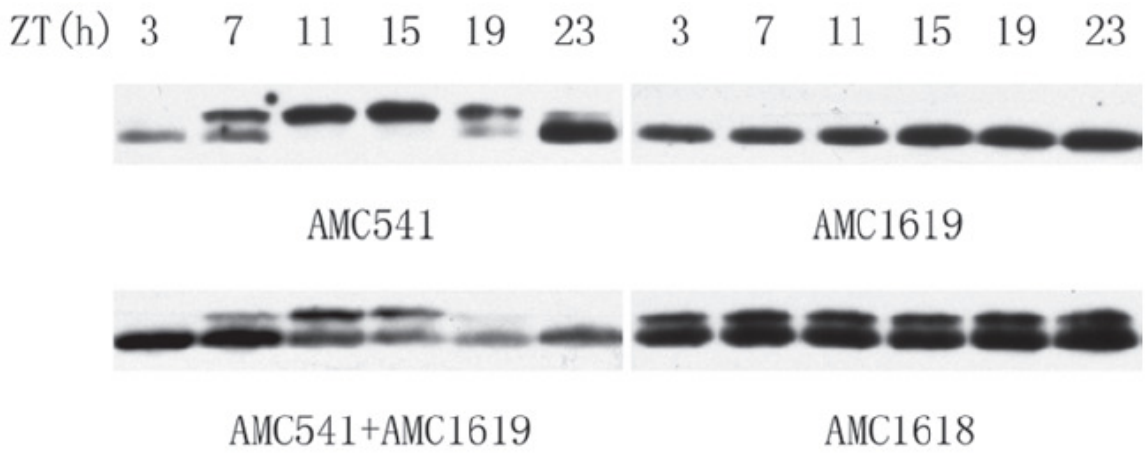


**Figure 4.3. Expression of *kaiC-AA* in a WT Background Stops the Phosphorylation Cycle of KaiC.**
A WT strain (AMC541) and strains that carry *kaiC-AA* expressed in either the WT background (AMC1618) or Δ*kaiC* background (AMC1619) were subjected to two synchronizing light/dark cycles before being released in constant light. Samples were taken every 4 h and analyzed by immunoblot using a KaiC antiserum. The KaiC phosphorylation state oscillates in AMC541, and no rhythm is observed with AMC1619, which expresses only the nonoscillating KaiC-AA. Oscillation of KaiC phosphorylation is still observable when AMC541 and AMC1619 samples are equally mixed in vitro, even though nonphosphorylated KaiC and KaiC-AA run as a single band. This figure demonstrates that oscillating phospho-KaiC would be observable above the background of nonphosphorylated KaiC in AMC1618 if WT KaiC continued to oscillate in the presence of KaiC-AA. AMC1618 shows no rhythm in KaiC phosphorylation, indicating that expression of KaiC-AA in the WT background has a dominant negative effect on the central oscillator.

KaiC487 and KaiC497 (Figure 4.3). The discrepancy suggests that hexamers of KaiC form from de novo synthesis and can be poisoned by incorporating non-functional subunits (in vivo situation) even when they might not exchange with these subunits after they are made (in vitro situation).

## EXPERIMENTAL PROCEDURES

### Cyanobacterial Strains, Culture Conditions and Bioluminescence Assays

The WT strain of *S. elongatus* PCC 7942 and its derivatives were propagated in BG-11 medium with appropriate antibiotics at 30 ℃ as previously described (Clerico et al., 2007; Mackey et al., 2007). Ectopic alleles of various KaiC constructs, described in Table 4.1, were introduced into neutral site I (NS1, GenBank accession no. U30252) of the *S. elongatus* chromosome. Bioluminescence assays of these strains were performed on a Packard TopCount scintillation counter (PerkinElmer Life Sciences) according to a previous protocol (Mackey et al., 2007).

### Immunoblot Analysis and λ Phosphatase Assay

Total cell extracts from 10 mL of $OD_{750}$ = 0.5 cyanobacterial cultures were prepared and further analyzed by immunoblotting as previously described (Ivleva and Golden, 2007). Polyclonal KaiC antiserum (Ditty et al., 2005) was

used at 1:2,000 dilution. Treatment with λ phosphatase was performed according to the manufacture's recommendation (New England BioLabs). Briefly, 100 µg of total protein was incubated with 400 U of λ phosphatase in 50 µL of total volume at 30 ℃ for 30 minutes. EDTA was used at a final concentration of 50 mM to inhibit the phosphatase reaction.

**Table 4.1. Constructs and Cyanobacterial Strains Used in Chapter IV.**

| Plasmid | Characteristics | Source or reference |
|---|---|---|
| **pAM2302** | WT *kaiC* driven by *kaiBC* promoter | (Ditty et al., 2005) |
| **pAM2596** | WT *kaiC* driven by an IPTG-inducible *trc* promoter | (Xu et al., 2003) |
| **pAM2969** | $Km^R$-Ω cassette in the BamHI site of *kaiA* | (Ditty et al., 2005) |
| **pAM3868** | kaiC487 (based on pAM2302) | This study |
| **pAM3871** | kaiC487 (based on pAM2596) | This study |
| **pAM3910** | kaiC497 (based on pAM2302) | This study |
| **pAM4047** | kaiC497 (based on pAM2596) | This study |

| ***S. elongatus* strain*** | **Genetic background** | **Ectopic *kai* plasmid** | **Source or reference** |
|---|---|---|---|
| **AMC541** | WT | none | (Ditty et al., 2003) |
| **AMC704** | *kaiC* deletion | none | (Ditty et al., 2005) |
| **AMC705** | *kaiB* and *kaiC* deletion | none | (Ditty et al., 2005) |
| **AMC1617** | *kaiA* insertion and *kaiC* deletion | none | This study |
| **AMC1276** | WT | pAM2302 | (Ditty et al., 2005) |
| **AMC1274** | *kaiC* deletion | pAM2302 | (Ditty et al., 2005) |
| **AMC1620** | WT | pAM3868 | This study |
| **AMC1621** | *kaiC* deletion | pAM3868 | This study |
| **AMC1622** | WT | pAM3910 | This study |
| **AMC1623** | *kaiC* deletion | pAM3910 | This study |
| **AMC1624** | *kaiB* and *kaiC* deletion | pAM2302 | This study |
| **AMC1625** | *kaiB* and *kaiC* deletion | pAM4047 | This study |
| **AMC1626** | *kaiA* insertion and *kaiC* deletion | pAM3871 | This study |

* All *S. elongatus* strains in this table carry a firefly luciferase gene (*luc*) driven by the *kaiBC* promoter.

# CHAPTER V

# CONCLUSIONS AND DISCUSSION

## THE CELL BIOLOGY OF CIRCADIAN CLOCK IN *S. ELONGATUS*

*S. elongatus* is an excellent microorganism for genetic studies. The powerful genetic tools available have helped in the identification of the clock genes that are currently known to us, as well as in figuring out the signal transduction pathways among the clock proteins. Recently, it was discovered that a circadian oscillation of KaiC phosphorylation could be reconstituted in a test tube outside the cellular system with a surprisingly simple mixture of only three Kai proteins and an energy source (ATP) (Nakajima et al., 2005), which justifiably has shifted much of the focus of the field to the biochemical characterization and mathematical modeling of the in vitro oscillator. The beauty of science many times lies in the simplistic nature of the truth underlying an intricate phenomenon. However, scientists are often inevitably reductionists, isolating complex systems into separable modules so that the number of variables is manageable. Thus, the simple in vitro oscillator discovered could be the truth that underlies the circadian clock, or, more likely, it is a key module that has been successfully isolated and characterized from an otherwise complex biological system. The finding that a rhythm of transcription and translation persists under conditions in which the KaiC phosphorylation rhythm is abolished (Kitayama et al., 2008)

supports the idea that the basic mechanism of circadian clock is more than just a rhythm of KaiC phosphorylation. The in vitro oscillator could likely be a minimal oscillator that is self-autonomous, but it is unlikely the optimal oscillator needed in vivo that is robust enough to deal with all the cellular and environmental perturbations. Throughout this dissertation I have used mainly molecular genetics and cell biology techniques to investigate the working of the circadian clock within the cellular system, and have revealed a key factor in the molecular mechanism of circadian gating of cell division, as well as subcellular localization dynamics of Kai proteins, which would be impossible to investigate in vitro. The in vivo work has also confirmed some findings from in vitro assays, such as interaction characteristics among the three Kai proteins. Therefore, employment of cell biology techniques has both complemented nicely the biochemical and biophysical techniques used in the in vitro work, and advanced the understanding of the biology of circadian rhythms. By comparing and applying the in vitro findings to the in vivo ones, I have also consistently found that the cellular clock is more complex than the in vitro oscillator.

**A NOVEL FACTOR THAT STIMULATES KAIC PHOSPHORYLATION**

Circadian rhythms and the cell cycle are the two major cyclic events that coexist in the unicellular organism, and the circadian clock gates cell division. I have uncovered the backbone of the molecular mechanism of this gating phenomenon. It is clear now that KaiC activities, whose information is passed

through the two-component signal transduction system of SasA-RpaA, determine the opening and closing of the gate. The observed cell division defect of a *cikA* mutant is also attributable to its affect on KaiC activity. Therefore, all three major components of the circadian clock, the input, the central oscillator, and the output, are involved in the regulation of cell division and the information flows strictly from input to the central oscillator then to the output. From this work, the presence of an unknown factor that could stimulate KaiC ATPase and kinase activity is inferred.

The unknown KaiC-stimulatory factor, most likely a protein, depends on the autokinase activity of CikA, as *cikA* mutants lacking the kinase activity show the same phenotypes as a *cikA* null. Thus, it is tempting to propose that the unknown factor is a response regulator protein, which has been putatively named CikR, that receives a phosphoryl group from CikA. However, extensive work from our lab has failed to identify CikR using candidates that are predicted response regulators with no known kinase partners (S. Mackey and S. S. Golden, personal communication). Thus, the unknown factor is either a response regulator that has more than one kinase partner, or it is not a response regulator at all (thus not CikR).

The subcellular localization data of CikA, together with genetic analysis of CikA variants, strongly suggest the PsR domain of CikA is an interaction partner with other proteins. Therefore direct protein-protein interactions may be employed in the signal transduction instead of phosphoryl transfers. Because

this unknown factor stimulates KaiC autokinase and ATPase activities like KaiA does, I reasoned that it may share some sequence similarity with the C-terminus of KaiA. A BLAST search using KaiA C-terminal sequence against the *S. elongatus* genome, however, did not return any meaningful result. Using yeast-two hybrid methods, nine candidates for CikA interaction partners have been identified, four of which are further characterized and published (Mackey et al., 2008). None of these has a null phenotype consistent with being the KaiC-stimulating factor. Future work using reverse genetics can be done focusing on the nine candidates to test whether they are directly involved in the regulation of KaiC activities. Several forward genetic screens in both the Golden and Kondo laboratories have failed to identify true resetting mutants other than additional alleles of *cikA* and an allele of *kaiC* that does not exhibit robust phosphorylation cycling (M. Katayama and S. S. Golden, personal communication; Kiyohara et al., 2005). A resetting screen of the entire *S. elongatus* genomic insertion clone set would allow each locus to be assayed for involvement in this process. As a result of the research presented here, a new forward screen for mutants that suppress the cell elongation phenotype of *cikA*- and *kaiA*-null mutant based on screening colony morphology can be designed, because colonies of long cells have visibly fuzzy edges, whereas those of WT cells are smooth. A mutant hunt based on this phenotype may better home in on the desired mutants, without the background of period mutants that come through resetting assays (Katayama et al., 2003), and with a simpler protocol.

## SIMILARITIES OF CIRCADIAN CLOCKS BETWEEN PROKARYOTIC AND EUKARYOTIC SYSTEMS

The most basic properties of circadian rhythms apply to both prokaryotic and eukaryotic systems. However, at the molecular level, clock proteins from these two domains of life do not share any sequence similarities, suggesting that the prokaryotic circadian system evolved independently from those of eukaryotes. In addition, the transcription/translation feedback loop (TTFL), which is a common theme that underlies the models for eukaryotic circadian clocks (Bell-Pedersen et al., 2005), is deemed nonessential for the cyanobacterial clock (Nakajima et al., 2005; Tomita et al., 2005). How much similarity do the two types of systems share? In this dissertation, I have found that the two systems are similar at many levels, even at the fundamental mechanistic level. As I have discussed in Chapter II, there seem to be two independent pathways that regulate KaiC activities: the well-characterized KaiA-KaiB pathway and the newly discovered CikA-unknown factor pathway. The KaiA-KaiB-KaiC system can be regarded as a negative feedback loop at the post-translational level, with KaiA negatively affecting its own activity by stimulating KaiC phosphorylation, which results in a KaiBC complex that inactivates KaiA. It is not known whether the CikA-mediated pathway forms any kind of feedback loop with KaiC, either transcriptionally or post-transcriptionally. I have proposed that the CikA-mediated pathway may underlie the transcription/translation rhythm observed in the absence of a phosphorylation rhythm (Kitayama et al., 2008). Thus, the intertwining of these

two oscillation circuits closely resembles those found in a eukaryotic circadian system. Additionally, both prokaryotic and eukaryotic clocks regulate cell division, whereas the cell cycle has no effect on circadian rhythms in either system. Subcellular localization of central oscillator proteins, which in eukaryotes are tightly regulated and are part of the timing circuit, are also likely to be dynamic in cyanobacteria, although the significance of such localizations for the circadian clock has not been established.

## IS SUBCELLULAR LOCALIZATION OF CLOCK PROTEINS INVOLVED IN THE GATING OF CELL DIVISION?

Although it is known that the circadian clock gates cell division in *S. elongatus*, it remains unknown why the gate exists. My work on the localization of clock proteins may shed light on this issue. The gate closes for about 4 hours each circadian cycle during the subjective day-night transition, which is consistent with the timing of high ATPase activity of KaiC. The phosphorylation level of KaiC is high, and according to my observation and model, phosphorylated KaiC is localized to one of the two cell poles. A cell division event during this time would result in the uneven distribution of at least KaiC, which would likely cause the two daughter cells to be out of phase with each other. For an organism that can divide up to four times a day, such events would render the clock essentially useless, as the clock would lose track of time after each cell division. Experimental data have shown that the circadian clock in *S. elongatus* is

amazingly precise without apparent intercellular coupling. Daughter cells derived from the same mother cells are kept in the same phase after cell division (Mihalcescu et al., 2004). Therefore, I propose that the gating of cell division serves to protect the circadian clock, and specifically to prevent clock proteins from being unevenly partitioned in the daughter cells. The fact that no apparent cell defects are observed in clockless strains, in which there is presumably no gating of cell division, supports the idea that gating of cell division is more likely to protect the circadian clock itself than other aspects of the cells. One way to test this hypothesis is to break the gate in a still rhythmic strain and monitor the property of its circadian rhythms over an extended period of time. The ability to do so requires identification of other components of the gate, or of a partially functional allele of a known component that provides the needed phenotype to conduct the test.

**REMAINING QUESTIONS**

Even though *S. elongatus* possesses a circadian clock that seems less complex than those of eukaryotic systems, and with excellent genetic tools and years of dedicated research, many significant questions still remain. At the molecular level, a lot of pathways still have missing links; e.g., what is the unknown factor between CikA and KaiC? What is the target of RpaA? What targets the localization of FtsZ? And what is the scaffold that recruits clock proteins to the cell poles?

Mechanistically, the role of LdpA in the input pathway is still not completely clear. It is also not known whether temperature-dependent entrainment works through the same input pathway as the light dark cycle. Temperature compensation of the circadian rhythm is still not fully understood, although it is proposed to lie in the ATPase activity of KaiC. In the output pathway, it remains to be seen whether SasA-RpaA is the only pathway, and how the output pathway controls DNA topology and global gene expression. The integration of the post-translational KaiC phosphorylation rhythm with transcription/translation will also remain a focus of the field in the foreseeable future.

Evolutionarily, the advantage of possessing an autonomous circadian clock is largely unknown, although competitive advantage has been shown previously in a few cases (Ouyang et al., 1998). If the circadian clock is used to coordinate incompatible cellular events so that they occur at different times, one would expect the lack of such a clock would put the organism at a disadvantage even when grown without competition, which does not happen (Ouyang et al., 1998). Thus, either the condition tested does not resemble that which *S. elongatus* faces in its natural habitat, or the circadian clock only increases fitness in a competition. The molecular mechanism of the competitive advantage observed is completely unknown. Given that a defect in the growth rate of the outcompeted strain is only seen at competing conditions, the easiest explanation is that cell-cell signaling is involved. It will thus be exciting to see what type of cell-cell communication is responsible in this process. However, such

communication has been reported as insignificant in the generation and maintenance of a highly precise circadian rhythm among a population of cells derived from the same mother cell (Mihalcescu et al., 2004).

The availability of the whole-genome sequence of *S. elongatus*, coupled with powerful genetic, biochemical and proteomics tools, will advance the field of circadian rhythms research at an unsurpassed pace. The clock of this organism promises to be the first that we will understand with clarity. Undoubtedly, that knowledge will open our eyes to commonalities that have been overlooked in eukaryotic organisms as well.

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# VITA

Name: Guogang Dong

Address: 3258 TAMU
Department of Biology
Texas A&M University
College Station, TX 77843-3258

Email Address: guogang.dong@gmail.com

Education: Ph.D., Microbiology, Texas A&M University, College Station, TX, U.S.A.

B.S., Biochemistry and Molecular Biology, Peking University, Beijing, China