Published in final edited form as:
Curr Biol. 2014 August 18; 24(16): 1836–1844. doi:10.1016/j.cub.2014.07.036.

Dynamic Localization of the Cyanobacterial Circadian Clock Proteins

Susan E. Cohen[1,2], **Marcella L. Erb**[2], **Jangir Selimkhanov**[3], **Guogang Dong**[1,2,5], **Jeff Hasty**[2,3,4], **Joe Pogliano**[2], and **Susan S. Golden**[1,2,6]

[1]Center for Circadian Biology, University of California, San Diego, La Jolla, CA 92093

[2]Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093

[3]Department of Bioengineering and San Diego Center for Systems Biology, University of California, San Diego, La Jolla, CA 92093

[4]BioCircuits Institute, University of California, San Diego, La Jolla, CA 92093

SUMMARY

Background—The cyanobacterial circadian clock system has been extensively studied and the structures, interactions and biochemical activities of the central oscillator proteins (KaiA, KaiB and KaiC) have been well elucidated. Despite this rich repository of information, little is known about the distribution of these proteins within the cell.

Results—Here we report that KaiA and KaiC localize as discrete foci near a single pole of cells in a clock-dependent fashion, with enhanced polar localization observed at night. KaiA localization is dependent on KaiC; consistent with this notion, KaiA and KaiC co-localize with each other as well as with CikA, a key input/output factor previously reported to display unipolar localization. The molecular mechanism that localizes KaiC to the poles is conserved in *Escherichia coli*, another Gram-negative rod shaped bacterium, suggesting that KaiC localization is not dependent on other clock- or cyanobacterial-specific factors. Moreover, expression of CikA mutant variants that distribute diffusely results in the striking de-localization of KaiC.

Conclusions—This work shows that the cyanobacterial circadian system undergoes a circadian orchestration of subcellular organization. We propose that the observed spatiotemporal localization pattern represents a novel layer of regulation that contributes to the robustness of the clock by facilitating protein complex formation and synchronizing the clock with environmental stimuli.

[6]*Corresponding author*: Susan S. Golden (sgolden@ucsd.edu), Division of Biological Sciences, 9500 Gilman Dr. MC0116, AP&M Annex 4721, La Jolla, CA 92093-0116. Phone: 858-246-0658, Fax: 858-534-7108.
[5]Present address: Joule Unlimited Technologies, Inc., Bedford, MA 01730

AUTHOR CONTRIBUTIONS

S.E.C., M.L.E., G.D., J.P., and S.S.G. designed research; S.E.C. and M.L.E. performed research; J.S. and J.H. contributed new analytic tools; S.E.C., M.L.E., J.P. and S.S.G. analyzed data; and S.E.C. and S.S.G. wrote the paper.

INTRODUCTION

Circadian rhythms, regulated by a 24-h biological clock, are vitally important for controlling temporal programs of cellular physiology to facilitate adaptation to daily environmental changes in diverse organisms [1]. Cyanobacteria are the simplest organisms and only prokaryotes currently known to possess a circadian clock. *Synechococcus elongatus* PCC 7942 has emerged as a premier model organism for studying the molecular details and regulation of the clock [2]. The *S. elongatus* core oscillator, encoded by the *kaiA*, *kaiB* and *kaiC* genes, regulates global patterns of gene expression [3, 4], the timing of cell division [5, 6], and compaction of the chromosome [7, 8]. Inactivation or overexpression of any of the *kai* genes stops the clock [4, 9]. KaiC is an autokinase, autophosphatase, and ATPase, whose daily rhythms of phosphorylation at residues Ser431 and Thr432 and ATPase activity are key features of the timekeeping mechanism that drives circadian rhythms in complex with KaiA and KaiB [10, 11]. KaiA binds to the C-terminal segment of KaiC, referred to as the "A-loop", promoting KaiC autokinase activity [12]. KaiB opposes KaiA's stimulatory activity by sequestering KaiA away from the A-loops, thereby promoting KaiC's intrinsic autophosphatase activity [12, 13]. These oscillations of KaiC phosphorylation can be reconstituted *in vitro* solely with purified KaiA, KaiB, KaiC and ATP [14].

These endogenously generated circadian rhythms are synchronized with the environment, through a process referred to as entrainment, via an input pathway that monitors cellular redox state. Sensing of the cellular redox environment is achieved through input components such as CikA as well as the central oscillator component KaiA. Both proteins bind quinones, whose redox states vary as a function of light intensity in photosynthetic organisms [15, 16]. Moreover, changes in the ATP:ADP ratio in the cell, which depends on photophosphorylation, can reset the phase of KaiC phosphorylation directly [17]. The oxidation of the quinone pool may act in concert with the changing levels of ATP:ADP to synchronize the circadian clock by signaling both the onset and duration of darkness [18].

While cell division is not synchronous among cells in the population or with the circadian cycle, the circadian clock "gates" the cell cycle, defining a portion of the 24-h cycle in the early night in which cell division is disallowed [5, 6, 19]. Mutations that hyperstimulate KaiC phosphorylation and/or downstream activity, including the loss of CikA or KaiB, or overexpression of KaiA, exaggerate the duration of the closed-gate phase in the circadian cycle, resulting in elongated cells [6].

The past several years have seen a revolution in our understanding of intracellular organization in bacteria. The bacterial cell is now known to possess a high degree of internal organization that is of critical importance for a wide range of cellular activities including virulence, DNA replication, chromosome segregation, cell division, chemotaxis and gene transfer, among others [reviewed in [20]]. Although it is not obvious that specific localization should be necessary for clock function, several pieces of evidence have suggested a non-random distribution of clock gene products. Input/output component CikA is localized to one pole of the cell, in a manner that depends on a specific quinone-binding domain [21], and CikA co-purifies with at least two of the Kai proteins [22]. A recent report shows that incorporation of the *in vitro* oscillator into liposomes affects circadian period,

and that KaiC associates with the membranes whereas KaiB is evenly distributed [23]. KaiC is also recovered from cells in a membrane fraction, specifically in samples collected during the subjective night [24]. Where the clock proteins are localized within the cell and how this localization may change and contribute to clock function have not been previously addressed. In eukaryotic systems subcellular localization is an important feature of timekeeping and several clock components display rhythms of nuclear localization [25, 26] and even rhythmic formation of sub-nuclear structures [27].

Here we show that the Kai complex interacts with CikA near the poles of cells in a circadian manner, where enhanced polar localization of KaiC is observed at night or subjective night. This localization is preserved in *Escherichia coli*, suggesting a conserved targeting mechanism across Gram-negative bacteria. We provide additional evidence that polar clock proteins represent a biologically active complex, as opposed to a protein aggregate targeted for degradation. Together, these results suggest that the proper timing of localization represents a previously unrecognized layer of regulation embedded into the cyanobacterial circadian system that likely contributes to robustness and synchronization of the clock.

RESULTS

KaiA and KaiC localize to the poles of cells

In order to investigate where the clock proteins are localized within the cell, we sought to create functional fluorescent fusion proteins. We identified fusions to both KaiA and KaiC that restored rhythmicity to the respective null mutants and were expressed as full-length fusion proteins, with no evidence of cleavage of the fusion protein, and at near wild-type (WT) levels as confirmed by immunoblot (Figure 1A and Figure S1A-C). Luciferase reporters confirmed that both N- and C-terminal fusions of KaiC restored rhythmicity to a *kaiC* null strain (Δ*kaiC*). N-terminal fusions to Yellow Fluorescent Protein (YFP) complemented the free running period as well as WT (Figure 1A) and were used preferentially. C-terminal fusions to either YFP or ECFP resulted in a long period phenotype, extended by ~5 h (Figure S1C) and were used only for co-localization experiments. C-terminal fusions to KaiA expressed from the native promoter (P_{kaiA}) restored rhythmicity to a *kaiA::*Tn*5* insertion strain, but displayed ~2 h period lengthening relative to WT strains (Figure 1A, Figure S3A and Figure S4A). Despite the period lengthening observed with KaiA fusions, collectively these tools allowed us to monitor the subcellular localization of the clock proteins under physiological conditions in which the clock is functional and phenotypes that are observed can be attributed to the fusion protein. Although levels of the KaiA fusion protein appeared slightly elevated (Figure S1A and Figure S4B), complementation studies confirmed that the fusions are expressed within a functional range, as overexpression of any of the Kai proteins results in apparent arrhythmia [4, 9]. We were not able to identify a fusion to KaiB, the smallest of the Kai proteins (11 KDa, about a third of the size of GFP), which satisfied our rigorous criteria.

Fluorescence microscopy of these carefully vetted fusion proteins indicated that KaiA and KaiC are localized as discrete foci at or near one pole of a subset of cells in the population (Figure 1B-C and Figure S1D-E). KaiC is an abundant protein in the cell (5,000-15,000 molecules/cell) [24] and foci were bright and easily observable, whereas KaiA expressed at

native levels is much less abundant (~500 molecules/cell) and was more difficult to image. The occurrence of 2 foci per cell, one focus at each pole, was rarely observed (~1%). Foci were occasionally found outside of the polar region of the cell; however, quantification showed that for KaiC ~94% of foci are indeed polar, with peak intensity lying within ~15% of the curved region of the cell (Figure 1D and Figure S1F-H). Together, these data are most consistent with an interaction with the thylakoid membrane system. The remaining ~6% of foci were almost exclusively localized in close proximity to the thylakoid membrane, suggesting that association with the membrane is important for KaiC polar localization.

Circadian spatiotemporal localization of KaiC

Because polar localization was observed in only a subset of cells (Figure S1D-E), we asked whether KaiC localization changes, particularly over the course of the day. Samples were entrained with 2 cycles of 12 h light followed by 12 h dark (LD) to synchronize the clocks in the population. Prior to entrainment, the samples were split and subjected to LD cycles that were 12 h out-of-phase from each other in order to collect data for a single circadian cycle in 12 h. Samples from cultures that were maintained in a diurnal cycle (LD) or had been released into conditions of continuous light (LL) were taken every two hours and fixed to preserve cellular architectures. By convention Zeitgeber Time (ZT) refers to the time relative to the light signal, such that the lights turn on at ZT0 with ZT 0-12 representing daytime and ZT 12-24 representing night-time. Under these conditions KaiC polar localization is enhanced at night (Figure 1E and Figure S2A). Persistence in continuous conditions is a fundamental property of circadian rhythms [1] and this spatiotemporal pattern of KaiC localization persists when cultures are released into LL (Figure S2B), albeit with oscillations that are not quite as striking as those observed in LD.

While KaiC is still capable of localizing to the poles in a *kaiA::Mu* strain, the occurrence of foci no longer oscillates, and instead a low constitutive level of foci is observed (Figure 1E). This YFP-KaiC is expressed from a P_{trc} promoter (Figure 1) rhythmically and in the same phase as the *kaiBC* promoter, but is not subject to feedback regulation from the clock [28, 29]. This approach allows us to distinguish the effects of KaiA on subcellular localization from the dramatic reduction in KaiC levels that *kaiA* mutation causes when *kaiBC* is expressed from its native promoter. These data demonstrate that KaiC localization is subject to spatial and temporal regulation that is circadian in nature.

The spatiotemporal pattern of KaiC localization suggests that KaiC is in a diffuse state during the phosphorylation phase in the day/subjective day and highly localized in the dephosphorylation phase at night/subjective night. In order to investigate whether a particular phosphorylation state of KaiC is required to facilitate polar localization, we generated phosphomimetic mutant variants representing each of the different phosphorylation states in the YFP-KaiC fusion construct (Figure S2C). Although these phosphomimetic variants are not perfect representations of each KaiC phosphorylation state, they can shed light onto how the phosphorylation cycle of KaiC facilitates downstream processes [6]. The results demonstrate that there is not a particular phosphorylation state of KaiC that is essential for polar localization, as KaiC localizes to some extent in all phosphomimetic mutants (Figure S2C-D). However, strains expressing either AA or ET

phosphomimetic variants have the highest incidence of polar localization, which would correlate with the dephosphorylation phase of the cycle, consistent with the time-of-day dependence in a WT strain. Phosphomimetic variants that lock the oscillator in all other states had a very low frequency of localization.

Additionally, we performed fluorescent recovery after photobleaching (FRAP) experiments to test the stability of KaiC polar complexes. Very little recovery occurred after photobleaching, even when only one focus was bleached in the rare case of a cell that possessed two foci, suggesting that polar KaiC complexes are stable when localized. In contrast, free YFP recovered rapidly after a similar photobleaching event at the pole (Figure S2E). Similarly, protein complexes in the thylakoid membrane are relatively immobile, as diffusion is slow and restricted in cyanobacterial thylakoids [30, 31].

KaiA localization is dependent on KaiC

We generated a strain in which the KaiA fusion protein could be overexpressed, as KaiA was difficult to observe when expressed at native levels. A KaiA-YFP fusion under the control of an inducible P_{trc} promoter overexpressed KaiA, as confirmed by bright polar foci, elongated cell morphology (Figure 2B), and high/arrhythmic expression from a P_{kaiB}-*luc* reporter (Figure S3B), as well as by immunoblot analysis (Figure S3C). When expressed at wild-type, non-induced levels, the KaiA-YFP fusion behaves as the GFP fusion expressed from the native promoter described in Figure 1 (Figure S3A and C). Despite overexpression, KaiA cannot localize as a discrete focus in a Δ*kaiBC* background (Figure 2A). When *kaiB* or *kaiC* is added back individually to a strain that lacks *kaiBC*, KaiA polar localization and elongated cell morphology are restored only in the strain in which KaiC is added back, indicating that KaiA polar localization is dependent on KaiC (Figure 2A-D). Additionally, KaiA localization is not dependent on a particular phosphorylation state and, when overexpressed, forms bright foci in all *kaiC* phosphomimetic-expressing strains (Figure S3D).

KaiA binds to the C-terminal A-loop tails of KaiC, an action that promotes KaiC autophosphorylation [12]. Truncation of KaiC after residue 497 results in a mutant variant, $kaiC^{497}$, which lacks the A-loop. Expression of $kaiC^{497}$ does not support KaiA polar localization, where diffuse distribution is observed (Figure 2E), although the $KaiC^{497}$ mutant itself remains localized to the pole (Figure S3E). In contrast, mutation of Glu 487 to Ala ($KaiC^{E487A}$) results in a mutant variant of KaiC with a constitutively extended A-loop [12]. Expression of $kaiC^{E487A}$ supports the localization of both KaiC and KaiA (Figure 2F and Figure S3F). These data demonstrate that the A-loops of KaiC, while not required for the localization of KaiC itself, are necessary to recruit and/or retain KaiA at the poles.

In order to address whether the A-loops are sufficient for KaiA polar localization we added the A-loop to the C-terminus of CikA, which is localized to the pole [21], and assayed for localization of KaiA. KaiA is known to bind to the A-loop peptide alone in solution [12]. Addition of the A-loops to the C-terminus of CikA with a function-preserving ZsGreen (ZsG) tag at the N-terminus to monitor localization [21] (Figure S3G), does not alter CikA's unipolar localization (Figure S3H). The CikA-A-loop fusion (without ZsG) complements a Δ*cikA* strain (Figure S3G). However, this fusion did not recruit KaiA to the pole, even when

overexpressed, in a Δ*kaiC* mutant background (Figure S3I). These results demonstrate that the A-loops are necessary, but not sufficient to promote KaiA's polar localization, and suggest that perhaps another docking site on KaiC is necessary to facilitate KaiA-KaiC interactions. Alternatively, the A-loops may not be accessible to KaiA in this particular CikA-A-loop fusion.

Co-localization of clock proteins

CikA similarly shows unipolar localization [21] and has been recovered from cyanobacterial cells in a complex with the Kai proteins [15]. Thus, we would expect CikA to co-localize with the Kai proteins *in vivo*. Using a functional KaiA-ECFP fusion (Figure S4), we investigated the pairwise localization of CikA, KaiA and KaiC using YFP/ZsG and ECFP combinations. Indeed, we observed that KaiC and KaiA co-localize with each other in essentially all cases (99%, n>90), as do CikA/KaiA and CikA/KaiC pairs (Figure 3). In contrast, KaiC does not frequently localize with the protease subunit ClpP2 (Figure 3), suggesting that the co-localization observed among CikA, KaiA and KaiC is specific. These data suggest that the clock proteins are interacting as a complex at the poles of cells in a *bona fide* biological complex.

Clock proteins exhibit a conserved mechanism of localization

To gain insights into the mechanism of Kai protein localization we expressed each of the fusion proteins in *E. coli*, which is similar to *S. elongatus* in that it is a rod shaped Gram-negative bacterium; however, *E. coli* is not photosynthetic and is not known to possess a clock, nor does it encode homologs of *cikA* or any of the *kai* genes. Fusions to CikA, KaiA and KaiC were expressed from a P_{trc} promoter, under non-inducing conditions in which low constitutive expression occurs. Both YFP and ECFP N-terminal fusions to KaiC behaved identically in *E. coli* and were used interchangeably. Strikingly, CikA and KaiC display a conserved mechanism of localization, as they are able to independently localize to the poles of *E. coli* cells (Figure 4A), suggesting that their localization is not dependent on any photosynthetic or specific cyanobacterial factor. KaiA, on the other hand, is distributed diffusely rather than localized to the poles in *E.coli*. However, if KaiC is co-expressed with KaiA, KaiA co-localizes with KaiC at the poles (Figure 4A). While KaiC and CikA are each capable of independently localizing in *E. coli*, they co-localize when expressed in the same cell, supporting the notion that the observed polar localization in both *E. coli* and *S. elongatus* functions to facilitate interactions among the clock components.

Although KaiC displays polar localization in *E. coli*, the patterns are not regulated as in *S. elongatus*. Primarily, KaiC localization is not sensitive to changes in the day-night cycle, but rather appears to be growth-phase dependent (Figure 4B). Cells have the highest percentage of localized KaiC in stationary phase or in the lag-phase, having just emerged from stationary phase, irrespective of the lighting conditions. The growth-phase dependence may merely reflect the accumulation of KaiC in older cells. Thus, while the mechanism of localization is conserved among the two organisms, KaiC localization is subject to additional regulation in *S. elongatus*. This finding may suggest that polar localization of KaiC is the default, and mechanisms are in place in cyanobacteria to keep KaiC from the pole during the day. Alternatively, a highly conserved polar targeting factor may be required

and is subject to circadian regulation in *S. elongatus*, in which case active recruitment would occur at night.

Polar localization of KaiC is not due to formation of inclusion bodies

A major concern when expressing heterologous proteins in *E. coli* is that they may end up in inclusion bodies, which are aggregates of partially or mis-folded proteins. This possibility is especially a concern because inclusion bodies tend to display polar localization [32, 33]. In order test whether the observed polar localization of KaiC and CikA is due to inclusion body formation in *E. coli*, we co-expressed a marker of inclusion bodies, *ibpA*-mCherry [34, 35]. We found that CikA and KaiC rarely localized with IbpA (~5%), whereas a protein that forms obvious inclusion bodies, Orf39 from *Bacillus megatarium* QMB155, co-localized with IbpA (~75% of the time in *E.coli*) (Figure 4C-D). These data support the premise that the polar clock proteins represent an active biological complex.

While no such marker of inclusion bodies exists for *S. elongatus*, we performed temperature shift experiments to test whether KaiC's polar localization is exacerbated by a stress that is likely to promote unfolding and predicted to enhance inclusion body formation [36]. Strains expressing YFP-KaiC growing at 30°C were shifted from 30°C to 25°C, 30°C or 38°C and monitored for focus intensity at 1 h and 4 h post-shift. Focus intensity did not change under any conditions tested (Figure S5A-B). Moreover, the YFP-KaiC fusion retained functionality at elevated temperatures (Figure S5C). Together, the *E. coli* and *S. elongatus* data argue that the observed polar localization is not due to the formation of inclusion bodies.

KaiC is not targeted to the pole for degradation

In *Caulobacter crescentus*, the ClpXP protease is dynamically and selectively localized to the stalked cell pole in a manner that is essential for cell cycle progression [37, 38]. We hypothesized that a similar mechanism may target the clock proteins to the pole in order to be degraded by the ClpXP protease at night. Consistent with such a model, KaiC protein levels have been shown to decrease over the course of the night/subjective night [24]. The *S. elongatus* genome encodes one ClpX homolog, an ATPase required for substrate recognition, and three ClpP peptidase paralogs. ClpX, ClpP1, and ClpP2 are known to affect the circadian clock as mutant alleles of *clpX, P1* or *P2* produce long-period rhythms [39, 40]. ClpP2 is of particular interest as it is the most similar to *clpP* of *E. coli* and is organized in an operon with ClpX.

We tested this hypothesis by investigating KaiC localization in *clp* mutant strains and determined the localization of the Clp protease in *S. elongatus*. Inactivation of *clpXP* in either *E. coli* (Figure S5D) or *S. elongatus* (Figure S5E) was not sufficient to perturb localization of KaiC or CikA, although *clpX* disruption in *S. elongatus* dramatically affected cell division [39]. Although the ClpXP protease of *E. coli* was previously reported to be localized to the cell pole, recent reports have suggested that this polar localization is not biologically relevant [41].

We identified a functional fluorescent fusion to ClpP2, which is expressed as a full-length fusion protein and complements the long-period phenotype of a *clpP2::*Tn*5* strain (Figure S5F-G). Although the ClpP2-YFP fusion appears to be overexpressed by immunoblot, it complements the null strain and overexpression of *clpP2* does not have any discernable effects on rhythms of gene expression [39]. This ClpP2-YFP is localized to the pole similar to KaiC, KaiA and CikA. However, when co-expressed with KaiC-ECFP, only 12% of KaiC foci co-localize with a ClpP2 focus (Figure 3). This finding suggests that Clp-mediated proteolysis is not responsible for the localization or the function of clock proteins at the pole.

Expression of *cikA* mutant variants can de-localize KaiC

The polar localization of CikA requires its C-terminal pseudo-receiver (PsR) domain through which it binds quinones [15, 21]. We tested a set of mutant *cikA* alleles to determine how deletion or alteration of each domain would affect CikA localization. We expressed each mutant variant with an N-terminal ZsG tag in a Δ*cikA* mutant strain and checked localization patterns at either ZT 8 (day) or ZT 20 (night) time points in a diurnal cycle. Localization correlated with the ability of each variant to complement rhythmicity of a Δ*cikA* mutant. Full-length CikA and a mutant variant that lacks the cryptic N-terminus (ΔN) both fully complement rhythms and support polar localization (Figure 5A). In contrast, substitution of Ala for His in the Histidine kinase domain (HA) or deletion of the PsR domain (ΔPsR) abolishes the ability to restore these functions. Interestingly, a variant that lacks the GAF domain (ΔGAF), which does not complement the period defect of a Δ*cikA* mutant strain [21], displayed a split phenotype for localization in which strong polar localization was observed only at night and diffuse distribution was observed during the day. Thus, the Histidine kinase and PsR domains are required for proper CikA localization whereas the GAF domain is specific for daytime localization. The GAF domain of CikA is similar to that of phytochrome photoreceptors that typically bind bilin cofactors; however, the GAF of CikA lacks a conserved Cysteine or Histidine to serve as a bilin-binding site [42].

We examined the effects of CikA mis-localization on KaiC by expressing each mutant variant as the only source of CikA and testing its effect on KaiC localization. CikA is not required for the polar localization of KaiC, consistent with the fact that KaiC localizes in *E. coli*, which does not encode a *cikA* homolog (Figure 5B-C). However, we observed pervasive de-localization of KaiC in strains that express mutant variants of CikA which do not localize to the poles (HA and PsR) (Figure 5B-F), dropping from ~50% localized in WT cells to <8% localized in HA- and ΔPsR-expressing cells. These CikA variants may sequester KaiC in the cytosol, or cause changes at the pole that affect KaiC localization. Expression of the CikA(ΔGAF) variant resulted in an intermediate frequency of KaiC localization, consistent with the split localization patterns observed for that fusion. This finding favors a stoichiometric effect of CikA on KaiC localization and also supports the hypothesis that CikA and KaiC physically interact, which has previously been implied but not yet demonstrated [15]. A *cikA* mutant displays a variety of phenotypes including low amplitude and short period rhythms of gene expression, but its most striking phenotype is

the inability to reset the phase of the oscillation after receiving a dark pulse [43]. Thus, the observed localizations may contribute to mechanisms of circadian entrainment.

DISCUSSION

Here we provide evidence that the spatiotemporal localization of KaiA and KaiC represents a previously unrecognized layer of regulation in the cyanobacterial circadian system. Cellular localization is not fundamental to the timekeeping mechanism itself because circadian oscillation of KaiC phosphorylation can be reconstituted *in vitro*. However, the system is more complex *in vivo* because the central oscillator must integrate input and output activities in order to regulate cellular functions. Spatial distribution of the clock components and the co-localization of factors that vary in space and time contribute to this complexity. We propose that the dynamic changes in KaiC protein localization patterns function to facilitate interactions with metabolites of photosynthesis and protein complex assembly, which together contribute to the synchronization and robustness of the circadian clock as well as its integration with other cellular processes (Figure 6).

Why does KaiC localize to the bacterial cell pole? Photosynthesis in *S. elongatus* occurs in thylakoid membranes that are arranged peripherally and concentrically inside the inner plasma membrane [44]. The regulation of clock components by metabolites of photosynthesis that change with the light-dark cycle – KaiA and CikA by oxidized quinone [15, 16] and KaiC by the ratio of ATP:ADP [17] – provides a rationale for enrichment of clock protein complexes at a specific membrane location at night. The interaction of KaiA and CikA with quinones, in particular, would require close proximity to the thylakoid, where a quinone-binding site of the photosynthetic cytochrome *b6f* complex is located close to the membrane-water interface, where protons are transferred to the aqueous phase [45]. Within the thylakoid membrane system two key respiratory electron donors move from an even distribution in high light to discrete patches with an overrepresentation at the poles in low light conditions [30]. Although the precise relationship between photosynthetic and respiratory electron transport chains in cyanobacteria remains unclear, some elements are shared, including the pool of quinone electron carriers. Thus, the quinone pool and potentially other metabolites that feed into the clock may be enriched at the poles at night.

Maintaining the appropriate stoichiometries among the Kai proteins is critically important for sustained rhythmicity *in vivo* and *in vitro* [46]. While KaiB and KaiC protein levels have been found to change ~3-fold over the circadian cycle, KaiA protein levels remain constant or vary with low amplitude [24]. Enhanced polar localization is observed at a time when KaiBC protein levels are decreasing; in this case random diffusion may not be sufficient to ensure proper complex formation (Figure 6). Others have previously reported the enhanced association of KaiA and KaiB in complex with KaiC at night/subjective night [15, 47], consistent with our observed spatiotemporal localization patterns. In another study, cellular fractionation experiments recovered KaiB in association with membrane at all circadian times tested [24], suggesting a possible constitutive membrane localization. This finding is consistent with the premise that KaiB and KaiC would need to interact at night while KaiC is dephosphorylating, according to KaiB's role in the cycle. Thus, the observed polar

reorganization of clock complexes may represent a mechanism to promote appropriate stoichiometry.

KaiC undergoes “monomer shuffling”, in which monomers are exchanged among KaiC hexamers. Monomer shuffling occurs during the dephosphorylation phase of the cycle and contributes to synchronization and stability of the Kai oscillator [48, 49]. The aggregation of KaiC hexamers at the pole during the dephosphorylation phase of the cycle may also facilitate monomer shuffling. Taken together, polarity may serve as a general feature to facilitate protein association and assembly at given times during the circadian cycle.

How does KaiC localize to the bacterial cell pole? While a wide variety of cellular components in diverse bacteria are targeted to cell poles, the mechanisms that establish polar recognition are not well understood [reviewed in [50, 51]]. In known cases the majority of proteins are recruited to the pole by specific interactions with a complex already at the pole, by a defined diffusion-and-capture mechanism. The dependence of KaiA localization on KaiC is consistent with such a model, and unknown polar elements may capture KaiC and CikA, neither of which has intrinsic membrane-associating regions. Because KaiC and CikA localize to the poles in *E. coli*, their polar targeting mechanisms are not dependent on any photosynthetic, cyanobacterial or circadian-specific factor. The polar determinant would likely be an essential protein in *E. coli*, because knocking out non-essential polar landmark proteins and performing exhaustive random mutagenesis have not yet identified such a factor. Components of the electron transport chain that are shared between respiration and photosynthesis would satisfy these criteria in that they represent membrane proteins with a high degree of conservation. Moreover, these proteins would facilitate interactions with the quinone pool or other metabolites synthesized or localized to the membrane that could feed into the clock.

Cell curvature is a feature of the bacterial cell poles that distinguishes them from the rest of the cell. Specifically, DivIVA of *B. subitilis* localizes to parts of the cell where negative curvature is the strongest [52, 53]. Because *E. coli* and *S. elongatus* are both rod shaped cells, cell curvature represents a potentially conserved mechanism to recruit KaiC to the poles. However, other mechanisms would need to be in play because both CikA and KaiC localize to the same single pole. In cases where poles could be distinguished, KaiC localized to the old cell pole ~65% of the time (n=316), suggesting that KaiC does not distinguish between the old and new cell pole.

Expression of CikA variants that display diffuse distribution can sequester KaiC in the cytosol, suggesting that either CikA can keep KaiC from the pole directly or that these variants can cause changes at the pole that affect KaiC localization. CikA variants that have altered kinase [42] or phosphatase activities may modify a scaffolding protein such that it can no longer support KaiC polar localization. The identification of polar determinants for localizing KaiC would provide insights not only into how the clock proteins are specifically targeted to the pole but also the consequences that underlie polar localization and how they affect downstream targets of the clock.

This work shows that the cyanobacterial circadian system undergoes a circadian orchestration of subcellular organization in addition to the known levels of regulation through protein abundance and phosphorylation. Thus, the central oscillator proteins may also need to be at the right place at the right time for the clock to function properly. We propose that these dynamic changes in subcellular localization function in facilitating interactions with other clock components and metabolites of photosynthesis, contributing to the robustness of the circadian clock.

EXPERIMENTAL PROCEDURES

Strains and plasmids used in this study are listed in Supplemental Tables S1-3. A detailed description of procedures including circadian bioluminescence monitoring, immunoblot analysis, fluorescence microscopy and image analysis can be found in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank B. Trial, A. Taton and S. Zou for technical support, A. Ma and J. Bordowitz for critical reading of the manuscript, and Alan Derman for sharing unpublished results and pOrf39-GFP. We also thank Adrian Clarke for the gift of ClpP2 antiserum, Iris Fung for contribution to the graphical abstract, and Jennifer Santini for help with the OMX. The OMX and Light Microscopy Facility at UCSD is funded by grant NS047101. This work was supported by NIH grant GM100116 to J. P. and S. S. G. J. S. is supported by San Diego Center for Systems Biology NIH grant GM085764 and a UCSD Chancellor's Collaboratories Program for Interdisciplinary Scholarship to J. H. and S. S. G. S. E. C. is supported by American Cancer Society Postdoctoral Fellowship PF-12-262-01-MPC.

REFERENCES

1. Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat Rev Genet. 2005; 6:544–556. [PubMed: 15951747]
2. Kondo T, Strayer CA, Kulkarni RD, Taylor W, Ishiura M, Golden SS, Johnson CH. Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. Proc Natl Acad Sci U S A. 1993; 90:5672–5676. [PubMed: 8516317]
3. Kondo T, Tsinoremas NF, Golden SS, Johnson CH, Kutsuna S, Ishiura M. Circadian clock mutants of cyanobacteria. Science. 1994; 266:1233–1236. [PubMed: 7973706]
4. Ishiura M, Kutsuna S, Aoki S, Iwasaki H, Andersson CR, Tanabe A, Golden SS, Johnson CH, Kondo T. Expression of a gene cluster *kaiABC* as a circadian feedback process in cyanobacteria. Science. 1998; 281:1519–1523. [PubMed: 9727980]
5. Mori T, Binder B, Johnson CH. Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. Proc Natl Acad Sci U S A. 1996; 93:10183–10188. [PubMed: 8816773]
6. Dong G, Yang Q, Wang Q, Kim YI, Wood T, Osteryoung KW, van Oudenaarden A, Golden SS. Elevated ATPase activity of KaiC constitutes a circadian checkpoint of cell division in *Synechococcus elongatus*. Cell. 2010; 140:529–539. [PubMed: 20178745]
7. Smith RM, Williams SB. Circadian rhythms in gene transcription imparted by chromosome compaction in the cyanobacterium *Synechococcus elongatus*. Proc Natl Acad Sci U S A. 2006; 103:8564–8569. [PubMed: 16707582]

8. Woelfle MA, Xu Y, Qin X, Johnson CH. Circadian rhythms of superhelical status of DNA in cyanobacteria. Proc Natl Acad Sci U S A. 2007; 104:18819–18824. [PubMed: 18000054]
9. Xu Y, Ma P, Shah P, Rokas A, Liu Y, Johnson CH. Non-optimal codon usage is a mechanism to achieve circadian clock conditionality. Nature. 2013; 495:116–120. [PubMed: 23417065]
10. Xu Y, Mori T, Pattanayek R, Pattanayek S, Egli M, Johnson CH. Identification of key phosphorylation sites in the circadian clock protein KaiC by crystallographic and mutagenetic analyses. Proc Natl Acad Sci U S A. 2004; 101:13933–13938. [PubMed: 15347809]
11. Nishiwaki T, Satomi Y, Nakajima M, Lee C, Kiyohara R, Kageyama H, Kitayama Y, Temamoto M, Yamaguchi A, Hijikata A, et al. Role of KaiC phosphorylation in the circadian clock system of *Synechococcus elongatus* PCC 7942. Proc Natl Acad Sci U S A. 2004; 101:13927–13932. [PubMed: 15347812]
12. Kim YI, Dong G, Carruthers CW Jr. Golden SS, LiWang A. The day/night switch in KaiC, a central oscillator component of the circadian clock of cyanobacteria. Proc Natl Acad Sci U S A. 2008; 105:12825–12830. [PubMed: 18728181]
13. Chang YG, Tseng R, Kuo NW, LiWang A. Rhythmic ring-ring stacking drives the circadian oscillator clockwise. Proc Natl Acad Sci U S A. 2012; 109:16847–16851. [PubMed: 22967510]
14. Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, Oyama T, Kondo T. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. Science. 2005; 308:414–415. [PubMed: 15831759]
15. Ivleva NB, Gao T, LiWang AC, Golden SS. Quinone sensing by the circadian input kinase of the cyanobacterial circadian clock. Proc Natl Acad Sci U S A. 2006; 103:17468–17473. [PubMed: 17088557]
16. Wood TL, Bridwell-Rabb J, Kim YI, Gao T, Chang YG, LiWang A, Barondeau DP, Golden SS. The KaiA protein of the cyanobacterial circadian oscillator is modulated by a redox-active cofactor. Proc Natl Acad Sci U S A. 2010; 107:5804–5809. [PubMed: 20231482]
17. Rust MJ, Golden SS, O'Shea EK. Light-driven changes in energy metabolism directly entrain the cyanobacterial circadian oscillator. Science. 2011; 331:220–223. [PubMed: 21233390]
18. Kim YI, Vinyard DJ, Ananyev GM, Dismukes GC, Golden SS. Oxidized quinones signal onset of darkness directly to the cyanobacterial circadian oscillator. Proc Natl Acad Sci U S A. 2012; 109:17765–17769. [PubMed: 23071342]
19. Yang Q, Pando BF, Dong G, Golden SS, van Oudenaarden A. Circadian gating of the cell cycle revealed in single cyanobacterial cells. Science. 2010; 327:1522–1526. [PubMed: 20299597]
20. Rudner DZ, Losick R. Protein subcellular localization in bacteria. Cold Spring Harb Perspect Biol. 2:a000307. [PubMed: 20452938]
21. Zhang X, Dong G, Golden SS. The pseudo-receiver domain of CikA regulates the cyanobacterial circadian input pathway. Mol Microbiol. 2006; 60:658–668. [PubMed: 16629668]
22. Ivleva NB, Bramlett MR, Lindahl PA, Golden SS. LdpA: a component of the circadian clock senses redox state of the cell. EMBO J. 2005; 24:1202–1210. [PubMed: 15775978]
23. Kojima, M.; Nakajima, M.; Takiguchi, K.; Homma, M.; Kondo, T.; Fukuda, T. Evaluation of Biolgical Clock Activity Capsulated by Lipid-mono-layer.. IEEE/RSJ International Conference on Intelligent Robots and Systems; San Francisco, CA. September 25-30; 2011. p. 1-6.
24. Kitayama Y, Iwasaki H, Nishiwaki T, Kondo T. KaiB functions as an attenuator of KaiC phosphorylation in the cyanobacterial circadian clock system. EMBO J. 2003; 22:2127–2134. [PubMed: 12727879]
25. Kondratov RV, Chernov MV, Kondratova AA, Gorbacheva VY, Gudkov AV, Antoch MP. BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. Genes Dev. 2003; 17:1921–1932. [PubMed: 12897057]
26. Saez L, Meyer P, Young MW. A PER/TIM/DBT interval timer for *Drosophila's* circadian clock. Cold Spring Harb Symp Quant Biol. 2007; 72:69–74. [PubMed: 18419263]
27. Kim Y, Lim J, Yeom M, Kim H, Kim J, Wang L, Kim WY, Somers DE, Nam HG. ELF4 regulates GIGANTEA chromatin access through subnuclear sequestration. Cell Rep. 2013; 3:671–677. [PubMed: 23523352]

28. Markson JS, Piechura JR, Puszynska AM, O'Shea EK. Circadian control of global gene expression by the cyanobacterial master regulator RpaA. Cell. 2013; 155:1396–1408. [PubMed: 24315105]
29. Xu Y, Weyman PD, Umetani M, Xiong J, Qin X, Xu Q, Iwasaki H, Johnson CH. Circadian yin-yang regulation and its manipulation to globally reprogram gene expression. Curr Biol. 2013; 23:2365–2374. [PubMed: 24210617]
30. Liu LN, Bryan SJ, Huang F, Yu J, Nixon PJ, Rich PR, Mullineaux CW. Control of electron transport routes through redox-regulated redistribution of respiratory complexes. Proc Natl Acad Sci U S A. 2012; 109:11431–11436. [PubMed: 22733774]
31. Sarcina M, Tobin MJ, Mullineaux CW. Diffusion of phycobilisomes on the thylakoid membranes of the cyanobacterium *Synechococcus* 7942. Effects of phycobilisome size, temperature, and membrane lipid composition. J Biol Chem. 2001; 276:46830–46834. [PubMed: 11590154]
32. Coquel AS, Jacob JP, Primet M, Demarez A, Dimiccoli M, Julou T, Moisan L, Lindner AB, Berry H. Localization of protein aggregation in *Escherichia coli* is governed by diffusion and nucleoid macromolecular crowding effect. PLoS Comput Biol. 2013; 9:e1003038. [PubMed: 23633942]
33. Winkler J, Seybert A, Konig L, Pruggnaller S, Haselmann U, Sourjik V, Weiss M, Frangakis AS, Mogk A, Bukau B. Quantitative and spatio-temporal features of protein aggregation in *Escherichia coli* and consequences on protein quality control and cellular ageing. EMBO J. 2010; 29:910–923. [PubMed: 20094032]
34. Li G, Young KD. Isolation and identification of new inner membrane-associated proteins that localize to cell poles in *Escherichia coli*. Mol Microbiol. 2012; 84:276–295. [PubMed: 22380631]
35. Reyes-Lamothe R, Possoz C, Danilova O, Sherratt DJ. Independent positioning and action of *Escherichia coli* replisomes in live cells. Cell. 2008; 133:90–102. [PubMed: 18394992]
36. Haase-Pettingell CA, King J. Formation of aggregates from a thermolabile in vivo folding intermediate in P22 tailspike maturation. A model for inclusion body formation. J Biol Chem. 1988; 263:4977–4983. [PubMed: 2965152]
37. McGrath PT, Iniesta AA, Ryan KR, Shapiro L, McAdams HH. A dynamically localized protease complex and a polar specificity factor control a cell cycle master regulator. Cell. 2006; 124:535–547. [PubMed: 16469700]
38. Iniesta AA, McGrath PT, Reisenauer A, McAdams HH, Shapiro L. A phospho-signaling pathway controls the localization and activity of a protease complex critical for bacterial cell cycle progression. Proc Natl Acad Sci U S A. 2006; 103:10935–10940. [PubMed: 16829582]
39. Imai K, Kitayama Y, Kondo T. Elucidation of the role of clp protease components in circadian rhythm by genetic deletion and overexpression in cyanobacteria. J Bacteriol. 2013; 195:4517–4526. [PubMed: 23913328]
40. Holtman CK, Chen Y, Sandoval P, Gonzales A, Nalty MS, Thomas TL, Youderian P, Golden SS. High-throughput functional analysis of the *Synechococcus elongatus* PCC 7942 genome. DNA Res. 2005; 12:103–115. [PubMed: 16303742]
41. Landgraf D, Okumus B, Chien P, Baker TA, Paulsson J. Segregation of molecules at cell division reveals native protein localization. Nat Methods. 2012; 9:480–482. [PubMed: 22484850]
42. Mutsuda M, Michel KP, Zhang X, Montgomery BL, Golden SS. Biochemical properties of CikA, an unusual phytochrome-like histidine protein kinase that resets the circadian clock in *Synechococcus elongatus* PCC 7942. J Biol Chem. 2003; 278:19102–19110. [PubMed: 12626498]
43. Schmitz O, Katayama M, Williams SB, Kondo T, Golden SS. CikA, a bacteriophytochrome that resets the cyanobacterial circadian clock. Science. 2000; 289:765–768. [PubMed: 10926536]
44. Sherman DM, Troyan TA, Sherman LA. Localization of Membrane Proteins in the Cyanobacterium *Synechococcus* sp. PCC7942 (Radial Asymmetry in the Photosynthetic Complexes). Plant Physiol. 1994; 106:251–262. [PubMed: 12232325]
45. Hasan SS, Yamashita E, Baniulis D, Cramer WA. Quinone-dependent proton transfer pathways in the photosynthetic cytochrome *b6f* complex. Proc Natl Acad Sci U S A. 2013; 110:4297–4302. [PubMed: 23440205]
46. Nakajima M, Ito H, Kondo T. In vitro regulation of circadian phosphorylation rhythm of cyanobacterial clock protein KaiC by KaiA and KaiB. FEBS Lett. 2010; 584:898–902. [PubMed: 20079736]

47. Kageyama H, Kondo T, Iwasaki H. Circadian formation of clock protein complexes by KaiA, KaiB, KaiC, and SasA in cyanobacteria. J Biol Chem. 2003; 278:2388–2395. [PubMed: 12441347]

48. Kageyama H, Nishiwaki T, Nakajima M, Iwasaki H, Oyama T, Kondo T. Cyanobacterial circadian pacemaker: Kai protein complex dynamics in the KaiC phosphorylation cycle in vitro. Mol Cell. 2006; 23:161–171. [PubMed: 16857583]

49. Ito H, Kageyama H, Mutsuda M, Nakajima M, Oyama T, Kondo T. Autonomous synchronization of the circadian KaiC phosphorylation rhythm. Nat Struct Mol Biol. 2007; 14:1084–1088. [PubMed: 17965725]

50. Davis BM, Waldor MK. Establishing polar identity in Gram-negative rods. Curr Opin Microbiol. 2013; 16:752–759. [PubMed: 24029491]

51. Laloux G, Jacobs-Wagner C. How do bacteria localize proteins to the cell pole? J Cell Sci. 127:11–19. [PubMed: 24345373]

52. Eswaramoorthy P, Erb ML, Gregory JA, Silverman J, Pogliano K, Pogliano J, Ramamurthi KS. Cellular architecture mediates DivIVA ultrastructure and regulates min activity in *Bacillus subtilis*. MBio. 2011; 2

53. Ramamurthi KS, Losick R. Negative membrane curvature as a cue for subcellular localization of a bacterial protein. Proc Natl Acad Sci U S A. 2009; 106:13541–13545. [PubMed: 19666580]

HIGHLIGHTS

- KaiA and KaiC localize to a single pole at night
- Oscillation is clock dependent
- KaiA and KaiC co-localize with each other as well as with CikA
- Mechanism of localization is conserved in *Escherichia coli*
- Kai protein localization is not due to inclusion bodies or Clp-based proteolysis
- CikA mutants that distribute diffusely throughout the cell can de-localize KaiC

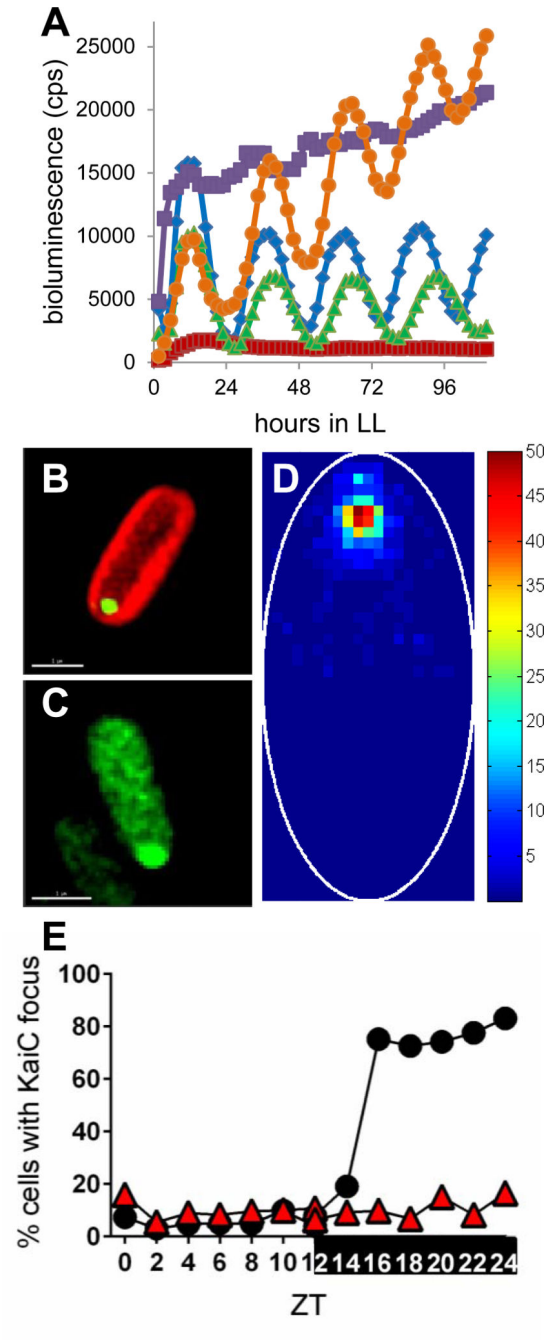

Figure 1.

Spatiotemporal localization of circadian clock proteins. A) Bioluminescence from strains that carry a P_{kaiB}-*luc* reporter for circadian rhythmicity. WT (*kaiA*$^{+}$*kaiC*$^{+}$), blue/25 h period; Δ*kaiC*, purple/arrhythmic; *kaiA::*Tn*5*, red/arrhythmic; Δ*kaiC* expressing P_{trc}-*yfp-kaiC*, orange/25 h period; *kaiA::*Tn*5* expressing P_{kaiA}-*kaiA-gfp*, green/27 h period. B) 3 Dimensional-Structured Illumination Microscopy (3D-SIM) micrograph of an individual representative cell expressing YFP-KaiC in green, autofluorescence in red C) Deconvolution fluorescence micrograph of a cell expressing KaiA-GFP, for which autofluorescence was omitted to improve visualization of KaiA. Scale bars = 1 micron. D) Heat map of KaiC

localization. The positions of >800 foci were determined relative to an ellipse drawn to fit the cell based on autofluorescence. Colors represent number of foci present at each cellular position as indicated. E) Strains that express YFP-KaiC were entrained to opposite light-dark cycles and sampled every 2 hours in either light (ZT 0-12) or dark (ZT 12-24). Zeitgeber time (ZT) refers to the time relative to “lights on”. The fraction of the population that showed KaiC polar localization is plotted for WT (dark circles) or *kaiA*-disrupted cells (red triangles) in a diurnal cycle for a representative experiment.

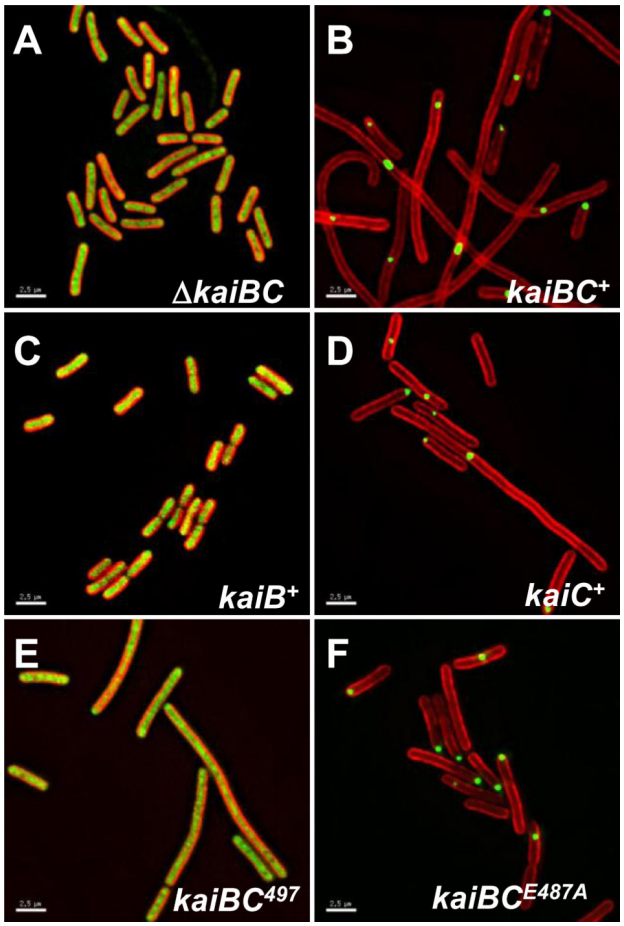

Figure 2.

KaiA polar localization is dependent on KaiC. Fluorescence micrographs of overexpressed KaiA-YFP (green) as the only source of KaiA overlaid onto autofluorescence (red) in A) Δ*kaiBC* background, B) Δ*kaiBC* background complemented with *kaiBC*$^{+}$. C-F) KaiA-YFP overexpression in a Δ*kaiBC* background expressing C) *kaiB*$^{+}$ only, D) *kaiC*$^{+}$ only, E) *kaiBC*^{497} mutant allele, truncated to not express A-loops, F) *kaiBC*^{E487A} mutant allele, which expresses KaiC in which the A-loops are constitutively extended. Scale bars = 2.5 microns.

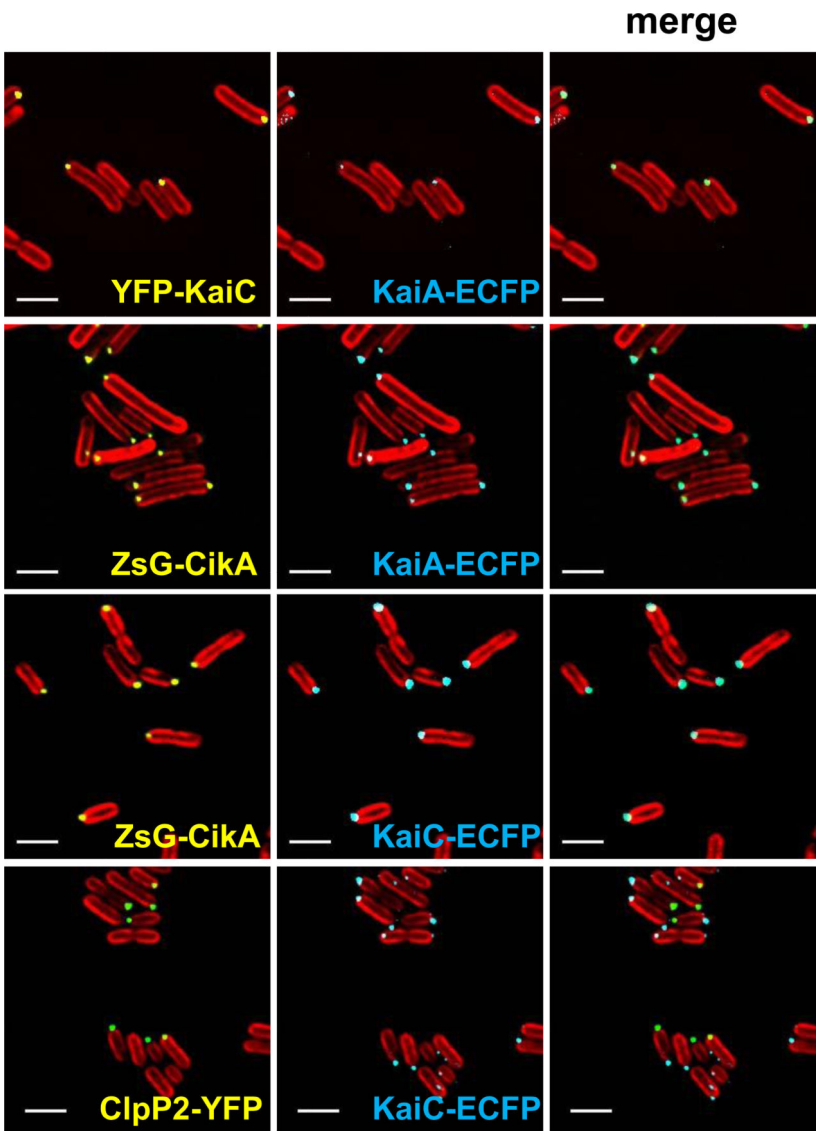

Figure 3.

Co-localization of clock components in *S. elongatus*. Fluorescence micrographs demonstrating co-localization of Row 1: YFP-KaiC and KaiA-ECFP; Row 2: ZsG-CikA and KaiA-ECFP; Row 3: ZsG-CikA and KaiC-ECFP. For pairs among KaiC, KaiA and CikA, co-localization is observed in essentially all cases (99%, n>90). In contrast, KaiC does not show a high degree of co-localization with ClpP2. Row 4: ClpP2-YFP and KaiC-ECFP. Only 11.8% of KaiC foci were co-localized with ClpP2 focus n >224 foci. Autofluorescence in red. Scale bar = 2.5 microns.

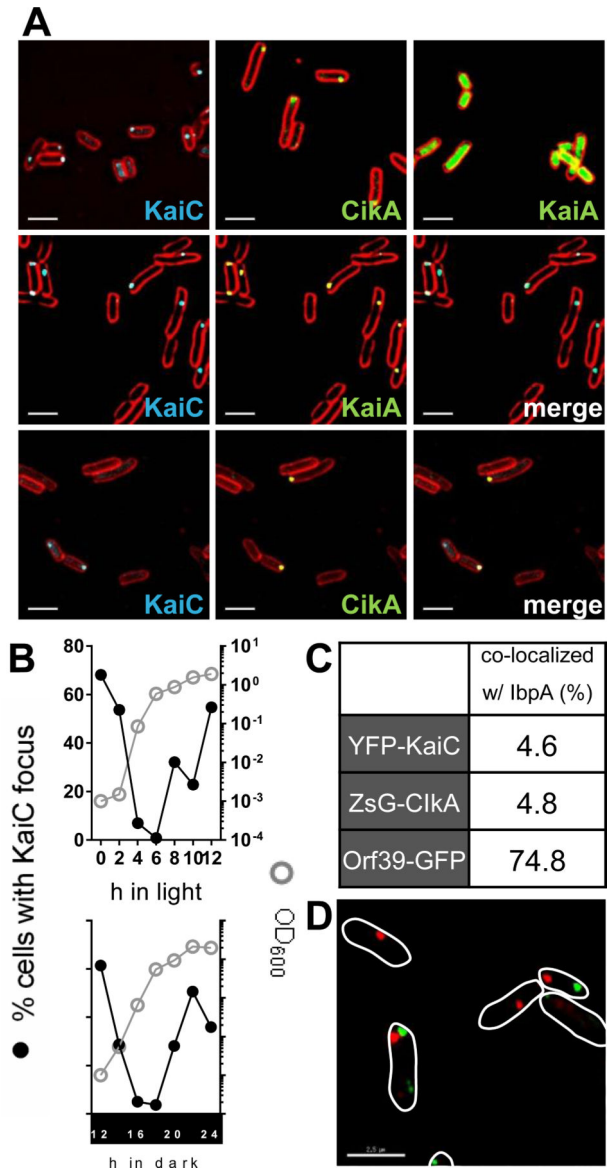

Figure 4.

Clock protein localization is conserved between *S. elongatus* and *E. coli*. A) Fluorescence micrographs of *E. coli* MG1655 stained with vital membrane stain FM4-64 (red) expressing Row 1: only fluorescently labeled KaiC, CikA or KaiA. Row 2: both ECFP-KaiC and KaiA-YFP, showing that KaiA polar localization in *E. coli* is KaiC dependent. Row 3: both ECFP-KaiC and ZsG-CikA, demonstrating that they co-localize when expressed in the same cell. B) KaiC localization in *E. coli* is not sensitive to a light-dark cycle. Closed symbols represent percentage of the population that possesses a KaiC focus (left Y-axis) and open symbols represent OD_{600} (right Y-axis). C) Percentage of KaiC or CikA foci that co-localized with a marker of inclusion bodies (IbpA-mCherry) compared to an inclusion body positive control, ORF39 from large plasmid pMB400 of *Bacillus megataruim* QMB155. n > 100 foci were counted for IpbA co-localization. D) Representative micrograph of cells expressing both YFP-KaiC and IbpA-mCherry, cell outlines drawn in white.

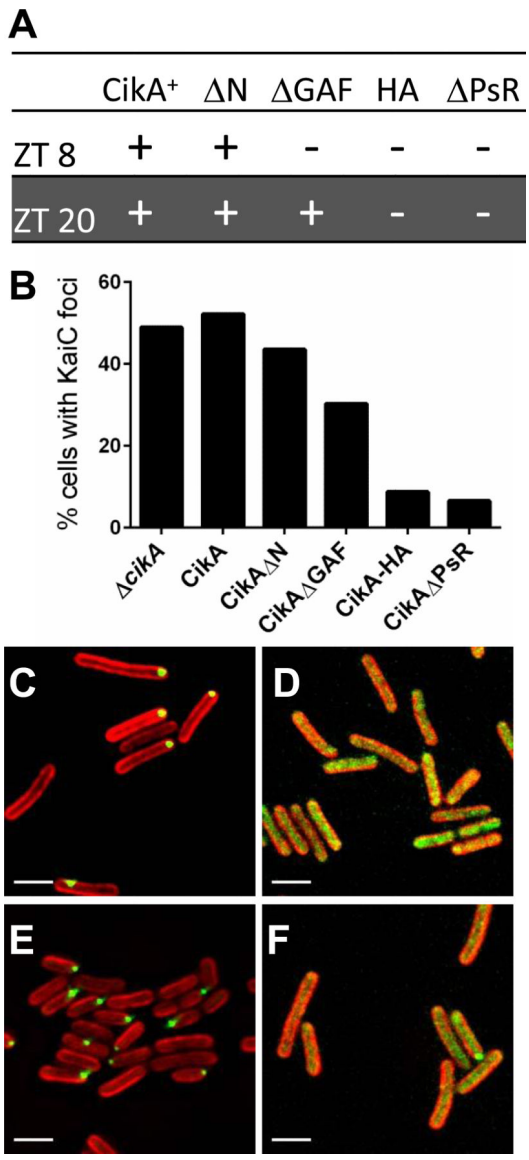

Figure 5.

CikA alters, but is not required for KaiC localization. A) Table representing how deletion or alteration of each CikA domain affects its localization, compared to the full-length (FL) protein. Symbols indicate whether polar localization was observed (+) or fluorescence was diffuse throughout all cells observed (−), n>100. Cells were sampled during the day (ZT 8) and night (ZT 20). B-D) CikA can affect KaiC localization. B) Graph demonstrating that reduced YFP-KaiC polar localization is observed when mutant variants of CikA that display diffuse distribution are expressed. Representative micrographs of YFP-KaiC fusion expressed in C) Δ*cikA* mutant background, D) Δ*cikA* + *cikA*Δ*PsR*, E) Δ*cikA* + *cikA*Δ*N*, or F) Δ*cikA* + *cikA-HA*. Autofluorescence in red. Scale bar = 2.5 microns.

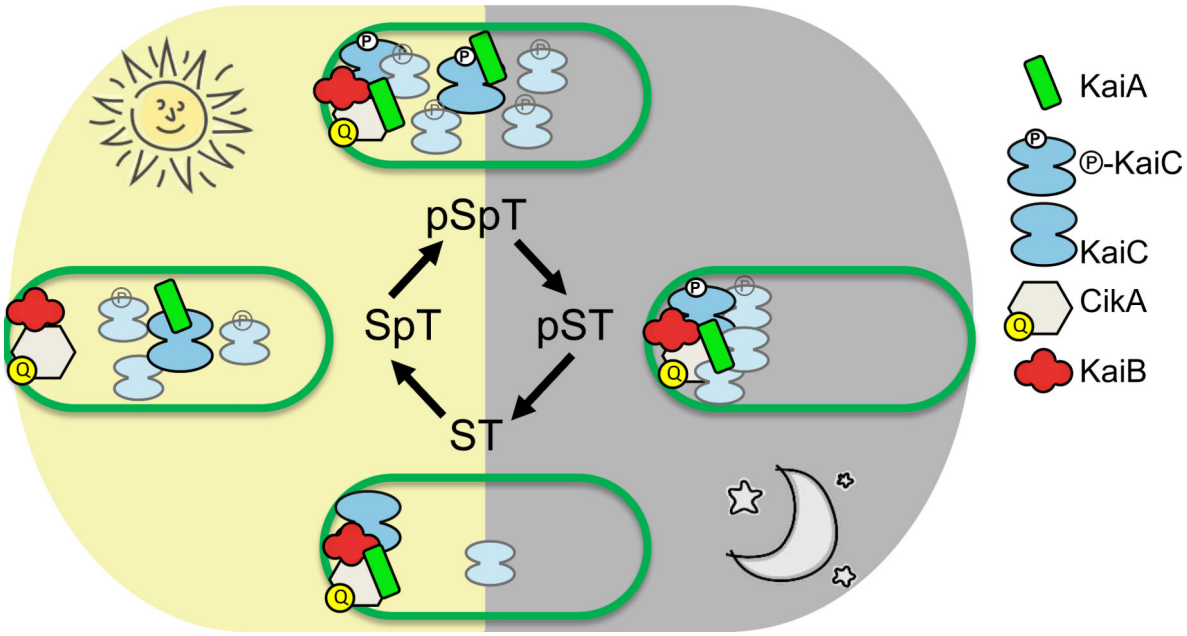

Figure 6.

Graphic representation of subcellular localization patterns of the clock proteins. KaiC and KaiA are predominantly distributed in the cytosol during the day (yellow shaded region), a time that corresponds to the phosphorylation phase of the cycle. During the night or subjective night (grey shaded region) Kai proteins co-localize with CikA at the poles of the cells in order to facilitate protein associations and interactions with metabolites of photosynthesis. KaiC protein abundance rhythms are depicted as numbers of KaiC molecules present in the cell.