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**Two antagonistic clock-regulated
histidine kinases time the activation
of circadian gene expression**

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Running title

Two antagonistic clock output enzymes control RpaA

Summary

The cyanobacterial circadian pacemaker consists of a three-protein clock – KaiA, KaiB and KaiC – that generates oscillations in the phosphorylation state of KaiC. Here we investigate how temporal information encoded in KaiC phosphorylation is transduced to RpaA, a transcription factor required for circadian gene expression. We show that phosphorylation of RpaA is regulated by two antagonistic histidine kinases, SasA and CikA, which are sequentially activated at distinct times by the Kai clock complex. SasA acts as a kinase toward RpaA, whereas CikA, previously implicated in clock input, acts as a phosphatase that dephosphorylates RpaA. CikA and SasA cooperate to generate an oscillation of RpaA activity that is distinct from that generated by either enzyme alone and offset from the rhythm of KaiC phosphorylation. Our observations reveal how circadian clocks can precisely control the timing of output pathways via the concerted action of two oppositely acting enzymes.

Highlights

- RpaA is the cognate response regulator of both SasA and CikA
- SasA is the primary kinase for RpaA and is regulated by phosphorylated KaiC
- CikA acts as phosphatase toward RpaA, and is regulated by the KaiC/KaiB complex
- RpaA phosphorylation oscillates *in vivo*, phase-advanced to KaiC phosphorylation

Introduction

Circadian clocks are endogenous oscillators found in a wide range of organisms that coordinate physiology and behavior with the diurnal cycle of the environment (Dunlap, 2004). Despite the lack of conservation of their underlying components, circadian clocks share a common set of fundamental properties – the ability to keep time with ~24 hour periodicity even in a constant environment (free run), the ability to adjust the phase to match that of environmental variation (entrainment), and a relative insensitivity of the period to temperature (temperature compensation) (Bell-Pedersen et al., 2005; Rosbash, 2009). In one of the simplest model systems known to possess a circadian clock, the cyanobacterium *Synechococcus elongatus* PCC 7942, the core pacemaker is made of the KaiA, KaiB, and KaiC proteins that interact to generate circadian oscillations in the phosphorylation state of KaiC (Nishiwaki et al., 2007; Qin et al., 2010; Rust et al., 2007). This phosphorylation cycle, which can be recapitulated *in vitro*, proceeds through sequential KaiA-promoted autophosphorylation of KaiC at two residues (threonine 432 – T-KaiC and serine 431 – S-KaiC). As more S-KaiC accumulates, KaiB binds KaiC and inhibits KaiA, switching KaiC to auto-dephosphorylation mode, and brings the system to unphosphorylated state (Rust et al., 2007). This pacemaker receives input from the environment, tuning it to the external day/night cycle, and also orchestrates output, controlling physiological processes such as gene expression (Ito et al., 2009), chromosome compaction (Smith and Williams, 2006) and the onset of cell division (Dong et al., 2010). The majority of genes in cyanobacteria are under circadian control (Ito et al., 2009; Liu et al., 1995; Vijayan et al., 2009). This

control extends to regulation of *kaiBC* operon, forming a transcription-translation feedback loop (Ishiura et al., 1998; Taniguchi et al., 2007; Taniguchi et al., 2010).

The histidine kinase SasA and the DNA-binding domain containing response regulator RpaA were shown to be essential for clock-controlled gene expression (including *kaiBC*) (Iwasaki et al., 2000; Takai et al., 2006; Taniguchi et al., 2010). SasA is capable of phosphorylating RpaA *in vitro* at a conserved aspartate residue (a process referred to as phosphotransfer), and thus these proteins were proposed to form a cognate histidine kinase-response regulator pair (Takai et al., 2006). Furthermore, SasA is bound and stimulated by KaiC (Iwasaki et al., 2000; Smith and Williams, 2006), thereby coupling the phase of the oscillator with gene expression. CikA, another clock-associated histidine kinase (Ivleva et al., 2006), also regulates *kaiBC* expression, and is thus proposed to form another clock output pathway (Taniguchi et al., 2010). However, the cognate response regulator controlled by CikA is not known. Intriguingly, CikA also has a role in clock input, as *cikA* mutants fail to entrain the phase of their clock in response to a 5 h-dark pulse (Schmitz et al., 2000).

Here we show that RpaA is the cognate response of both SasA and CikA. However, these histidine kinases exert antagonistic effects on RpaA, with SasA acting as a kinase and CikA as a phosphatase. Furthermore, we demonstrate that the Kai oscillator stimulates the activity of CikA with timing distinct from its activation of SasA. This temporal separation of two oppositely acting enzymes that converge onto the same substrate creates an RpaA~P oscillation that is phase-advanced relative to that of KaiC~P, a phenomenon that is likely important for the accurate onset of gene expression.

Results and Discussion

RpaA is the cognate response regulator of both SasA and CikA. To identify candidate cognate response regulators for CikA, we used phosphotransfer profiling (Laub and Goulian, 2007; Skerker et al., 2005) to determine which of the predicted response regulators encoded by the *S. elongatus* genome (Table S1) is preferentially phosphorylated *in vitro* by CikA~P (phosphorylated CikA). This method relies on the ability of a given phosphorylated histidine kinase to transfer a radiolabeled phosphoryl group most rapidly to its cognate response regulator(s) when incubated *in vitro* with each receiver domain-containing protein from a genome of interest (Skerker et al., 2005). We find that the only response regulator phosphorylated by CikA~P after a short incubation time is RpaA (Fig. 1A). We applied the same approach to SasA and find that RpaA is the most preferred substrate of SasA~P phosphotransfer (Fig. 1A and S1B). The response regulator *synPCC7942_1860* is also phosphorylated, but ~32-fold less efficiently than is RpaA (Fig. S1A). At longer incubation times other, likely non-cognate (Skerker et al., 2005), response regulators are phosphorylated (Fig. S1B). These results suggest that RpaA is the cognate response regulator of both SasA and CikA, indicating that it may be a key output node that integrates different circadian inputs.

Kai proteins control SasA kinase and CikA phosphatase activities. Bacterial histidine kinases can act either as kinases (through the combined processes of autophosphorylation, in which they transfer the γ -phosphoryl group of ATP to their conserved histidine, and phosphotransfer) or as phosphatases (through dephosphorylation) toward their cognate response regulators (Casino et al., 2010; Gao

and Stock, 2009; Russo and Silhavy, 1991). Either of these activities can be regulated by input stimuli to generate changes in the cellular level of phosphorylated response regulator. As both SasA and CikA have been shown to associate *in vivo* with the Kai protein complexes (Ivleva et al., 2006; Iwasaki et al., 2000) we hypothesized that the Kai oscillator may regulate the biochemical activities of these histidine kinases. We assayed SasA and CikA kinase and phosphatase activities towards RpaA and RpaA~P, using full-length proteins not modified by any tags, in the presence of different combinations of recombinant KaiA, KaiB and KaiC (Fig. 1B and Fig 1C). The kinase activity of SasA is greatly enhanced in the presence KaiC, as reported previously (Smith and Williams, 2006; Takai et al., 2006), whereas the kinase activity of CikA is modest and is largely unaffected by the presence of Kai proteins (Fig. 1B). CikA dephosphorylates RpaA~P, and this activity is greatly enhanced by KaiC and KaiB (Fig. 1C, S1C and S1D). By contrast, the phosphatase activity of SasA is modest and only slightly stimulated by the Kai proteins (Fig. 1C, S1C and S1D). Thus, CikA and SasA have opposing effects on RpaA phosphorylation that are modulated by Kai proteins.

***In vivo*, SasA promotes RpaA phosphorylation and CikA promotes**

dephosphorylation. To investigate the *in vivo* relevance of these biochemical effects, we analyzed RpaA phosphorylation in strains lacking CikA or SasA. To avoid secondary effects due to the defect in *kaiBC* expression in *sasA* and *cikA* mutants (Ivleva et al., 2006; Takai et al., 2006; Taniguchi et al., 2010), we analyzed RpaA phosphorylation in an engineered strain (referred to as the “clock-rescue”) in which *kaiBC* expression is under the control of the IPTG-inducible *P_{trc}* promoter (Murayama et al., 2008). As

previously reported (Murayama et al., 2008), addition of IPTG to the “clock-rescue” strain grown in constant light elevates KaiB and KaiC production to a level that is sufficient to restore circadian regulation of KaiC phosphorylation (Fig. 2A and S2A). In this “clock-rescue” strain we observe circadian oscillations in RpaA~P that peak 4 hours prior to the peak of KaiC~P. We then deleted *cikA* (Fig. 2B) or *sasA* (Fig. 2C) in this “clock-rescue” strain background, demonstrated that oscillations in KaiC phosphorylation are restored, and analyzed RpaA phosphorylation. In the absence of CikA, RpaA~P levels are high (Fig. 2B), and conversely, in the absence of SasA, RpaA~P is practically undetectable (Fig. 2C). Thus, in agreement with our biochemical observations, CikA and SasA have opposing actions on RpaA~P *in vivo*, with SasA promoting RpaA phosphorylation and CikA promoting accumulation of unphosphorylated RpaA. This observation is consistent with the high and low overall bioluminescence of a circadian gene reporter in *cikA*- or *sasA*-deficient strains, respectively (Taniguchi et al., 2010; Zhang et al., 2006).

ST-KaiC activates SasA kinase activity, and S-KaiC/KaiB complex stimulates CikA phosphatase activity. To determine which phosphorylation state of the KaiC oscillator is most potent in activating SasA and CikA, we measured the effect of adding partial clock reactions containing subsets of the Kai proteins on SasA kinase or CikA phosphatase activity. In partial clock reactions KaiC transits through four phosphorylation states: unphosphorylated KaiC (U-KaiC); KaiC phosphorylated only on threonine 432 (T-KaiC); KaiC phosphorylated on both serine 431 and threonine 432 (ST-KaiC); and KaiC phosphorylated only on serine 431 (S-KaiC) (Fig. 3A) (Rust et al., 2007). When we mix a

partial clock reaction in which KaiC is phosphorylating (KaiC mixed with KaiA) with SasA and RpaA, we find that SasA kinase activity (reflected as RpaA~P accumulation) mirrors the abundance of the ST-KaiC phosphoform (Fig. 3A), suggesting that this state of KaiC is the most potent activator of SasA kinase activity. A similar result was obtained when autophosphorylation of SasA was measured (Fig. S3A), indicating that autophosphorylation is the activity regulated by the ST-KaiC phosphoform. When we mix CikA and RpaA~P with aliquots from a partial clock reaction in which KaiC is dephosphorylating (phosphorylated KaiC mixed with KaiB), we find that CikA phosphatase activity (reflected as a reduction in RpaA~P) correlates with the abundance of S-KaiC (Fig. 3B), and this correlation is dependent on the presence of KaiB (Fig. S3B). S-KaiC and KaiB form a complex during the clock cycle, which plays a critical role in feedback that maintains clock synchrony (Kageyama et al., 2006; Nishiwaki et al., 2007; Rust et al., 2007) and in this case also functions as an activator of CikA phosphatase activity.

We then quantified the effects of the ST- and S-forms of KaiC on the kinase and phosphatase activities of SasA and CikA by adding aliquots from complete clock reactions (KaiA+KaiB+KaiC) collected when ST-KaiC and S-KaiC are at their peak levels (Rust et al., 2007). Addition of a clock reaction aliquot withdrawn when ST-KaiC is at its peak greatly enhances the initial rate of SasA kinase and autophosphorylation activity but not that of CikA (Fig. 3C and S3C). Enhancement of SasA activity by ST-KaiC was also observed in a study of KaiC phosphomimetic variants from a related cyanobacterium, *Thermosynechococcus elongatus* (Valencia S et al., 2012). Addition of a

clock reaction aliquot enriched in S-KaiC significantly increases the phosphatase activity of CikA but not that of SasA (Fig. 3D).

Clock-controlled regulation of SasA and CikA determines the timing of RpaA

phosphorylation. The timing of accumulation and decay of RpaA~P in cells is likely determined by the concerted action of SasA and CikA. Our *in vivo* measurements indicate that the peak of RpaA~P precedes the peak of KaiC~P by ~4 hours (Fig. 2A, Fig. 4A and S4A). Intriguingly, the phase of RpaA~P oscillation that would be directed by SasA kinase activity alone, as measured *in vitro* in the presence of time-resolved clock aliquots, is coincident with the phase of KaiC~P oscillation (that coincides with ST-KaiC accumulation) (Fig. 4B and S4B). On the other hand, the RpaA~P oscillation produced *in vitro* by the phosphatase action of CikA alone in the presence of clock aliquots peaks 8 h prior to the peak of KaiC~P (Fig. 4C), and its trough coincides with the increase in S-KaiC that occurs 6 h after the KaiC~P peak (Fig. S4C). The profiles of these separate activities suggest that their combined action has the potential to produce a novel phase of oscillation of RpaA~P that peaks prior to the KaiC~P peak (Fig. 4D) and would mirror the *in vivo* observations (Fig. 4A). A phase advanced-peak of RpaA~P accumulation relative to that of KaiC~P is consistent with the peak of gene expression as observed in bioluminescence and RNA abundance assays, which occurs ~4 hours prior to the peak of KaiC~P (Takai et al., 2006; Tomita et al., 2005). Such sequential convergence of two inputs that have opposite effects on a key regulator may also enhance the accuracy of transduction of temporal information in the face of cellular and environmental fluctuations.

Our biochemical observations provide insight into the functions attributed to CikA by genetic studies. Recent work implicated CikA in a clock output pathway – CikA acts as a negative regulator of *kaiBC* expression and was proposed to operate during late subjective night (Taniguchi et al., 2010), corresponding to the time when KaiC is dephosphorylating and the S-KaiC level is high. Our results enable mechanistic understanding of this role, as CikA inhibits RpaA activity (i.e. promotes its dephosphorylation), which leads to reduced *kaiBC* expression. CikA was originally identified as a component of the clock input pathway, required for entrainment of the cyanobacterial clock to changes in light availability (Schmitz et al., 2000). It is possible that CikA has a role in input unrelated to regulation of RpaA activity. Alternatively, the entrainment defect in the *cikA* mutant may be a consequence of misregulation of RpaA activity that disrupts transcriptional feedback of *kaiBC* expression. In addition to a defect in entrainment, the *cikA*- mutant also displays a cell elongation phenotype, due to its inability to properly gate the timing of cell division (Dong et al., 2010). We reason that this clock-dependent cell elongation phenotype displayed by *cikA*- and certain *kai* mutants (Dong et al., 2010) may be due to RpaA~P misregulation. For example, our observations predict that the cell elongation phenotypes of *cikA*- and *kaiB*- are due to an increased level of RpaA~P; in *cikA*- caused by the absence of CikA phosphatase activity and in *kaiB*- caused by lack of CikA activation.

Circadian clocks enable organisms to time the regulation of physiological processes to exploit the predictable variation in the earth's light/dark cycle. For example, cyanobacteria time the production of the photosynthetic apparatus to anticipate daylight (Stal and Krumbein, 1987; Vijayan et al., 2009) and also use the clock to gate the cell

division (Dong et al., 2010; Mori et al., 1996). In some cases, the appropriate timing of regulation of physiological processes may not coincide with the phase of the core oscillator. We have shown how an organism can use differentially regulated and opposing enzymes converging on a single output protein to generate a phase of output distinct from that of the clock itself, a phenomenon that is likely to be important for all circadian clocks.

Experimental procedures

Growth conditions. Wild-type, “clock-rescue” and *sasA*- and *cikA*- derivative strains of *S. elongatus* were grown in standard BG11M medium, at 30 °C, in white light with CO₂-enriched-air bubbled through cultures that were repeatedly diluted with fresh medium to maintain an OD₇₅₀ of ~0.3. Under these conditions a doubling time of 6.9 h was recorded. For circadian experiments, the cultures grown in 6 μM IPTG, were synchronized by two 12 h dark periods, spaced by 12 h in light. Sampling began 24 h after release into constant light.

Western blotting. Cells from 20 ml of culture were harvested by filtration on Whatman cellulose acetate filters and quickly frozen in liquid nitrogen. Lysates were obtained by bead-beating at 4 °C in lysis buffer (8 M urea, 20 mM Hepes-KOH pH 8, 1 mM β-mercaptoethanol (β-ME)). Total protein content was determined by Bradford assay against a BSA standard curve. KaiC immunoblotting was performed as previously described (Rust et al., 2011). For detection of RpaA~P, lysates were run at 4°C on 7% polyacrylamide gel (Hoefer SE 600 system) containing 50 μM of Phos-tag AAL-107 (Wako Chemicals) and 100 μM MnCl₂ and then transferred to a nitrocellulose membrane.

Custom-made rabbit anti-RpaA polyclonal antibodies were used to detect phosphorylated and unphosphorylated RpaA (see Supplemental Information). Quantification of the Western blot bands was performed using AlphaImager EP software (Alpha Innotech). Within each lane the lower and the upper (retarded) bands were delineated using two identically sized non-overlapping boxes, which allowed the extraction of the sum of pixel intensities corresponding to each band. In addition, a similarly sized box positioned above each band set was used for background subtraction. The extent of phosphorylation at each timepoint (lane) was estimated by calculating the upper band signal as a fraction of the sum of the intensities of both bands ($100 \times [\text{upper band signal}] / [\text{total signal of both bands}]$).

***In vitro* assays.** Purification of Kai proteins and clock reactions were largely performed as described previously (Rust et al., 2007), with the exception that the clock buffer contained an ATP-regeneration system (phosphoenolpyruvate and recombinant pyruvate kinase of *Bacillus stearothermophilus* (Sigma)). Full-length recombinant SasA, CikA and RpaA lacking a tag were prepared as described in the Supplemental Information. Except for phosphotransfer profiling, which was performed as previously described (Skerker et al., 2005), the ratios and the concentrations of the recombinant proteins used in assays were based on quantitative western blotting (Fig. S2C-S2G) and standard oscillating clock reactions (Rust et al., 2011): 3.5 μM KaiC, 3.5 μM KaiB, 1.5 μM KaiA, 2.5 μM RpaA, 0.65 μM SasA and 0.65 μM CikA. The kinase buffer used was: 20 mM Hepes-KOH pH 8, 150 mM KCl, 10% glycerol and 5 mM MgCl_2 . Unless indicated otherwise, kinase reactions were initiated with addition of ATP-containing Kai protein mixes or ATP-containing buffer (1 mM ATP final concentration) and incubated at 30°C. Aliquots

from the reaction mix were quenched with the β -ME based Laemmli buffer and run on 7% polyacrylamide gels made with the Phos-tag reagent (Wako Chemicals) as described in the Western blotting section. For estimation of kinase rates, the linear slopes of %RpaA~P accumulation over time was measured. The sampling for Fig. 2B was done for all combinations of Kai proteins in parallel at 0.5, 2 and 10 min after addition of SasA or CikA (for CikA and KaiB combination only 0.5 and 10 min time points were obtained). To capture SasA kinase activity, in Fig. 3A and Fig. 4B the reactions were done in parallel for all the partial clock and complete clock aliquots with 2 min incubation with SasA and RpaA. For Fig. 3C, the times sampled were 0.5, 2, 5, 7 and 10 min after addition of ST-KaiC enriched clock aliquot to SasA/RpaA or CikA/RpaA mixes. After electrophoresis, the gels were stained with SyproRuby (Invitrogen) and imaged with the Typhoon Trio System (GE Healthcare). Densitometry analysis was performed with ImageQuant TL7.0 (GE Healthcare).

For phosphatase assays, radiolabelled RpaA~P that was independently prepared before each experiment by utilizing a CikA-coupled resin (See Supplemental Information). Reaction conditions were similar to the ones used for kinase assays. Aliquots from reactions were stopped with the addition of Laemmli loading dye and analyzed by SDS-PAGE and autoradiography. The time points sampled in Fig. 1C and 3D were 0, 15, 30 and 60 min following addition of Kai protein mixes. Background subtracted signal intensity of each band was normalized against the band signal at time 0 (which was actually ~30 s after addition of the Kai protein mixes) in order to obtain exponential decay rates from the non-linear fits of each time series (as exemplified in Fig. S1C). As

dephosphorylation of RpaA~P appears to proceed through reverse phosphotransfer (Fig. S1C), we also included in the calculations the signal present in the histidine kinase bands, summing it with the signal of the corresponding RpaA~P band. All regressions were performed with Prism (GraphPad). The relative phosphatase activity represents the fold increase in the rate of dephosphorylation relative to the rate of RpaA~P dephosphorylation alone. In cases where the phosphatase activity was estimated from a single time point (15 min – Fig. 3B, Fig. S3B; 30 min – Fig. 4C, Fig. S4C), we reported only the fraction of RpaA~P remaining (sum of CikA~P and RpaA~P signal) relative to the signal present in the control lane (B – buffer added only). Each assay was independently performed at least twice, with similar results.

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Figure Legends

Figure 1. *In vitro* phosphorylation of RpaA by CikA and SasA.

(A) Phosphotransfer profiling of CikA and SasA against each putative receiver domain-containing protein of *S. elongatus* PCC 7942 (see also Table S1). A five-minute time point (short time) is shown. (B) Kinase activity of SasA and CikA (initial rate of %RpaA~P accumulated) in the presence or absence of recombinant clock proteins (KaiA, KaiB and KaiC). Bars show the standard errors of the linear slopes. (C) Phosphatase activity of SasA and CikA toward RpaA~P in the absence or presence of clock proteins (KaiA, KaiB and KaiC). The rate of phosphatase activity is expressed relative to the rate of RpaA~P dephosphorylation in the absence of any proteins added. Bars correspond to the standard error of the rates extracted from non-linear fits of RpaA~P dephosphorylation. See also Fig. S1.

Figure 2. Effect of *cikA* and *sasA* deletion on the level of RpaA~P *in vivo*.

(A) RpaA~P and KaiC~P profiles measured by immunoblotting of extracts from the “clock-rescue” strain grown in the presence of 6 μ M IPTG in continuous light. Lower panel – immunoblot of RpaA; graph above – quantification of RpaA~P and KaiC~P fractions. RpaA~P and KaiC~P profiles in the same genetic background but in the absence of *cikA* or *sasA* are shown in (B) and (C), respectively. See also Fig. S2A. Specific detection of RpaA and RpaA~P by immunoblotting with anti-RpaA polyclonal antibodies is shown in Fig. S2B.

Figure 3. Modulation of CikA and SasA activities by Kai proteins.

(A). Relative kinase activity of SasA in the presence of aliquots taken from partial clock reactions (U-KaiC+KaiA). Activity is expressed as fold RpaA~P change relative to the fraction of RpaA~P accumulated at time 0 h. (B) Dephosphorylation of RpaA~P by CikA in the presence of aliquots taken from KaiC auto-dephosphorylation partial clock reactions (KaiC-P+KaiB). %RpaA~P remaining is relative to the fraction of RpaA~P remaining when no clock was added. (C) Initial rates of kinase activity of SasA and CikA in the presence of a KaiA/KaiB/KaiC mix obtained from an oscillating clock reaction at the time when the ST-KaiC fraction was maximal. (D) Relative phosphatase activity of CikA and SasA in the presence of a mix obtained from the same oscillating clock reaction as in (C) at the time when S-KaiC fraction was maximal. The activity is expressed relative to the rate of RpaA~P dephosphorylation when no enzyme is added. Bars correspond to the standard error of linear (C) or non-linear rates (D). See also Fig. S3.

Figure 4. Clock-mediated changes in SasA and CikA activities coordinate to generate a distinct oscillation of RpaA~P.

(A) Circadian profile of KaiC~P and RpaA~P in synchronized wild-type cells grown in continuous light, as measured by immunoblotting (see also Fig. S4A). (B) Relative kinase activity of SasA in the presence of clock aliquots obtained from an *in vitro* oscillating reaction. Activity (fold change) is expressed relative to the trough of RpaA~P accumulation (14 h clock timepoint) (see also Fig. S4B) (C) Effect of CikA on ³²P-labeled-RpaA~P in the presence of protein aliquots obtained from an oscillating clock reaction. %RpaA~P is relative to the amount present when no clock reaction mix was

added (see also Fig. S4C). (D) Model of the differential activation and convergence of SasA and CikA activities that create phase-advanced rhythms of RpaA~P relative to the core oscillator (KaiC~P). KaiA activates the autokinase activity of KaiC which leads to ordered phosphorylation of its two sites: threonine 432 (T) and serine 431 (S) (Nishiwaki et al., 2007; Rust et al., 2007). SasA kinase activity is enhanced by ST-KaiC (doubly phosphorylated). As more S-KaiC accumulates, KaiB binds KaiC and inhibits the phosphorylation-promoting activity of KaiA, switching KaiC in auto-dephosphorylation mode. The S-KaiC-KaiB complex enhances the phosphatase activity of CikA. The integration of SasA and CikA activities generate an oscillation of RpaA~P that is phase-advanced relative to KaiC~P.

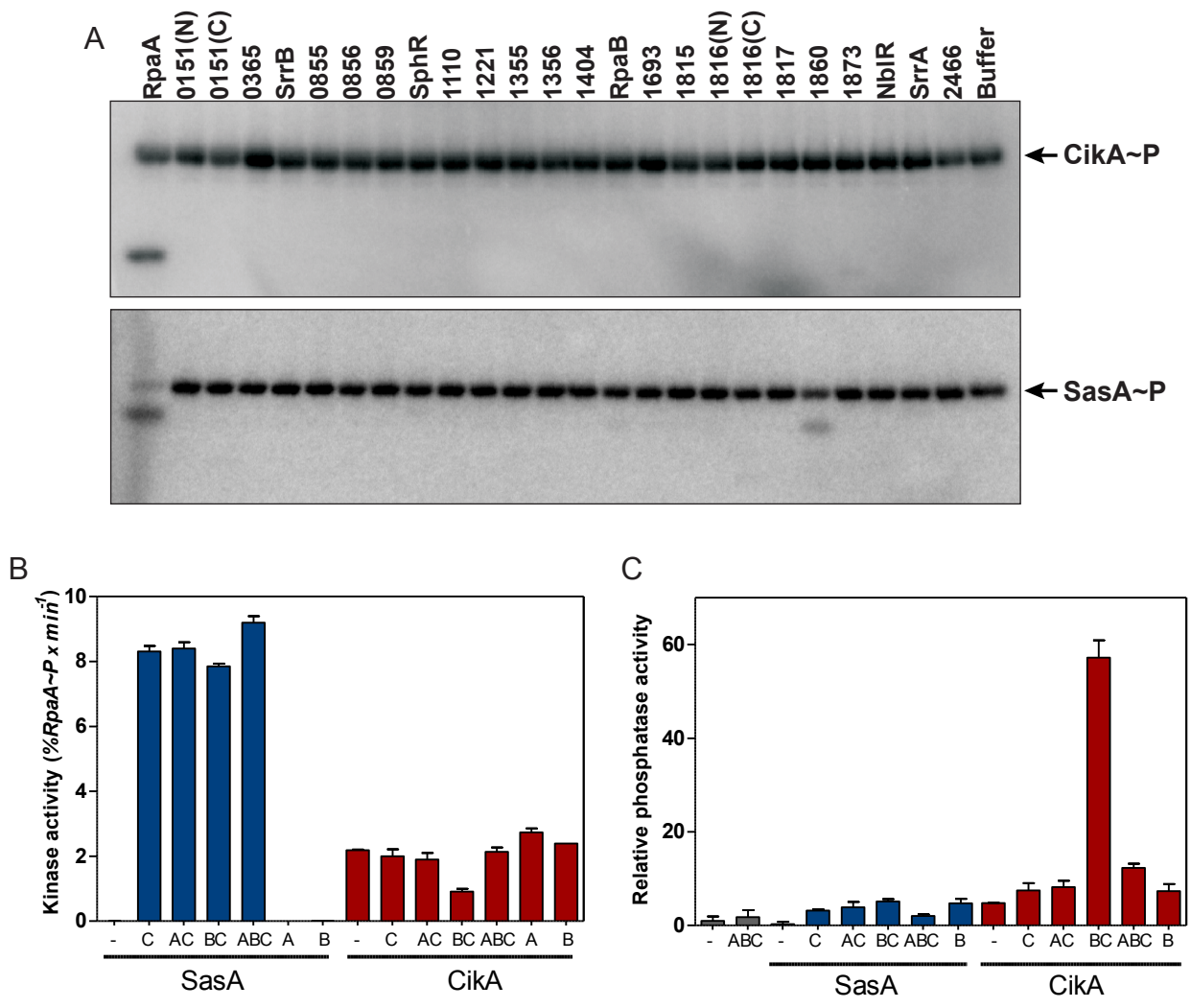


Figure1

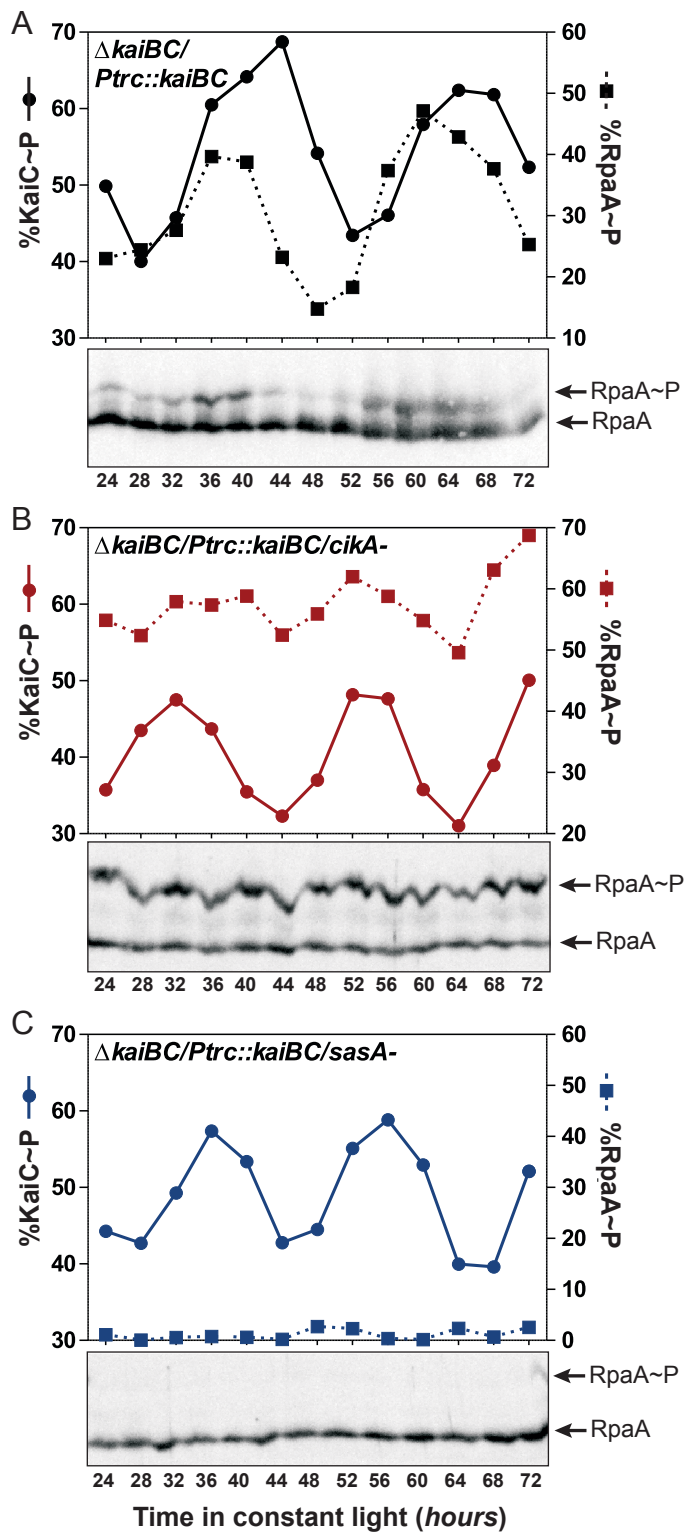


Figure 2

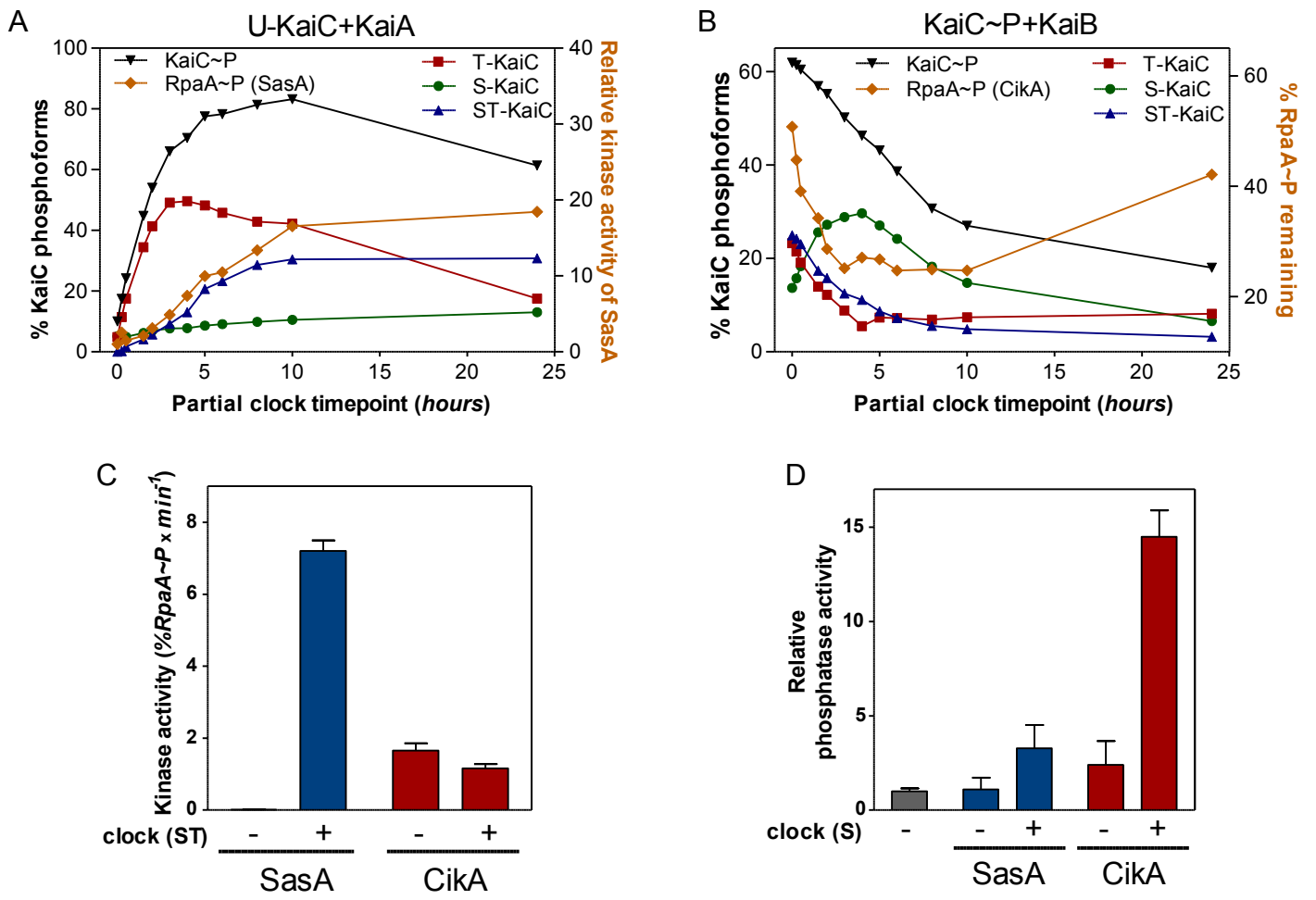


Figure 3

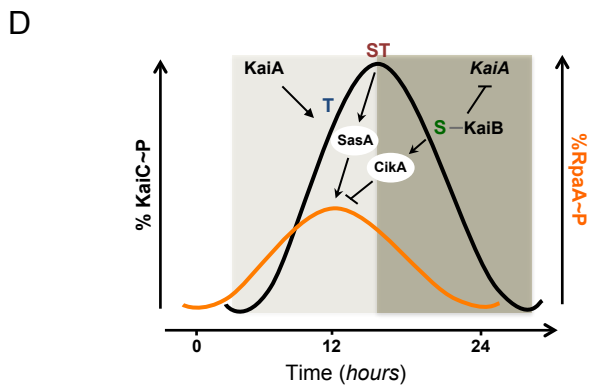
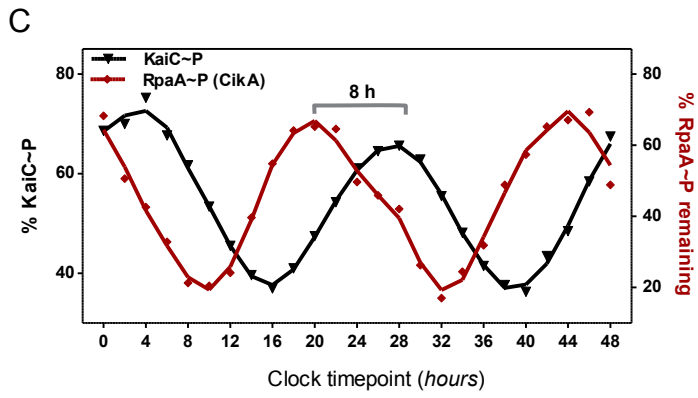
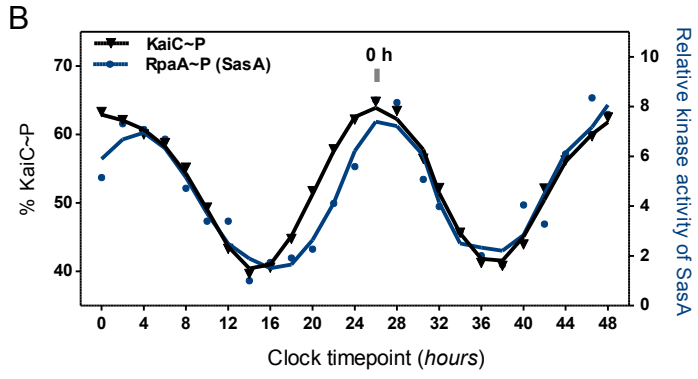
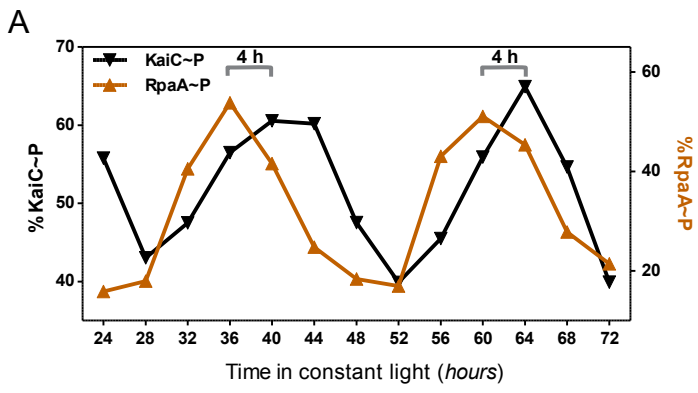


Figure 4

Supplemental Information for:

Two antagonistic clock-regulated histidine kinases time the activation of circadian gene expression

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Supplemental Data

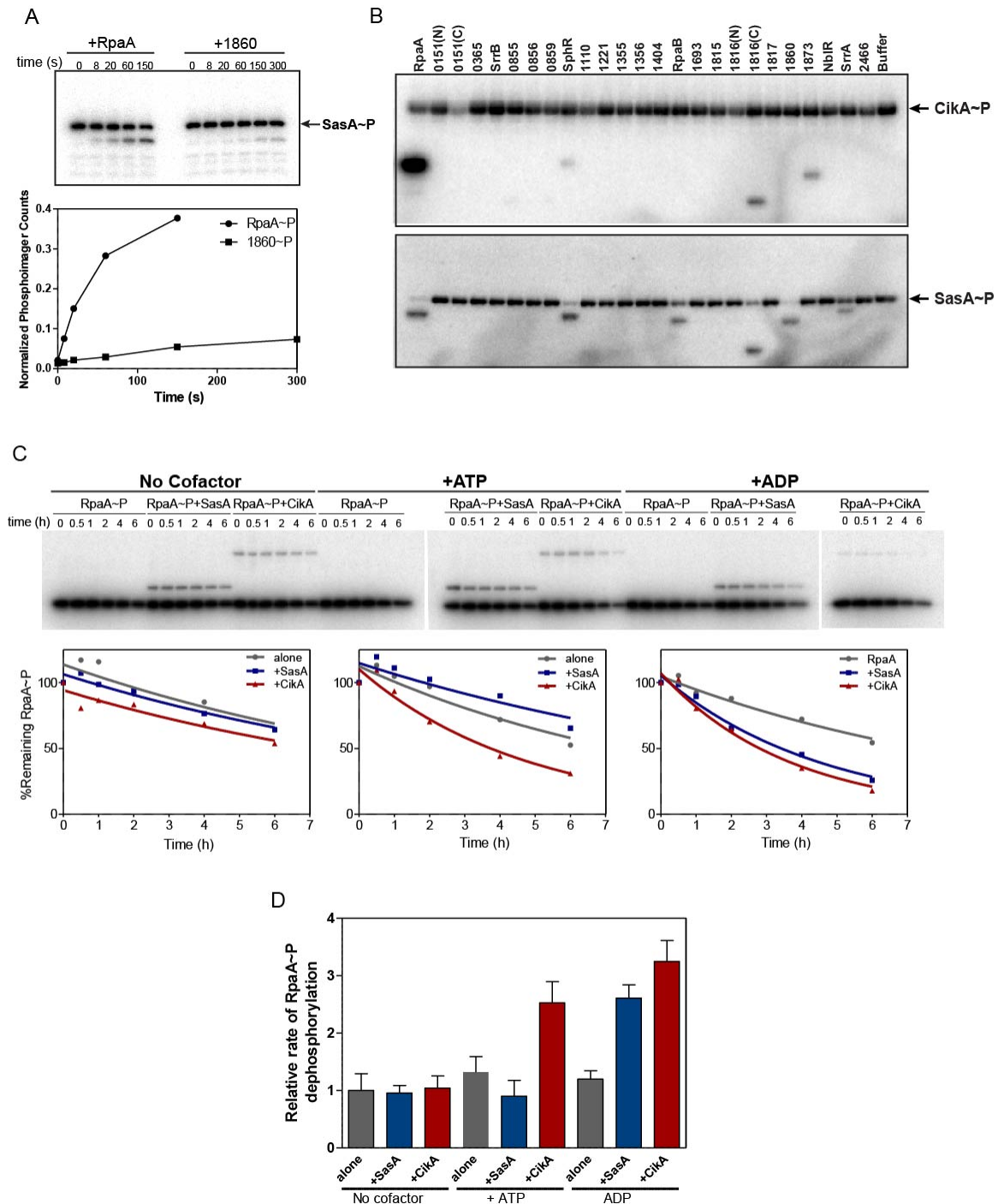


Fig. S1. Biochemical activities of SasA and CikA (related to Fig. 1).

(A) Relative catalytic efficiency of SasA~P towards RpaA and *synpcc7942_1860* response regulators that were identified as potential phosphotransfer targets (see Fig. 1A). 5 μ M SasA was first autophosphorylated, and then mixed with 0.25 μ M response regulator and at the indicated times, aliquots from the mix were taken and analyzed for

phosphotransfer. Given the limiting substrate concentration, and comparing the same concentration of the enzyme and substrate, estimation of the initial velocities of the phosphotransfer reaction is an acceptable way to compare the relative catalytic efficiency of the enzyme (Skerker et al., 2005). Quantification of phosphorylated response regulator is reported as the fraction of SasA~P signal at time 0. The initial rate of RpaA~P accumulation (0 to 20 s) is ~32 times greater than the rate of 1860~P accumulation (0 to 150 s). (B) One-hour time point for phosphotransfer profiling of CikA and SasA against each putative receiver domain-containing protein of *S. elongatus* PCC 7942 (see Table S1). Phosphorylated histidine kinases were mixed in an equal molar amount with each of the response regulators for 60 min at 22.5°C and the reactions were stopped with Laemmli denaturing loading dye. Each mix was then analyzed for phosphotransfer by SDS-PAGE and autoradiography. N and C add-ons represent the N- or C- terminal position of the receiver domain when more than one was present within the predicted protein product.

(C) Phosphatase activities of SasA and CikA. Upper panel: Radiographs of SDS-PAGE gels used to measure relative RpaA~P dephosphorylation. ³²P-labeled RpaA~P (2.5 μM total RpaA) was incubated alone, with SasA (0.65 μM) or with CikA (0.65 μM). The assays were performed at 30°C in the presence of 1 mM ATP or 1 mM ADP, as no influence of SasA or CikA was observed without these cofactors. Lower panel: Corresponding ³²P signal quantification of each time series relative to the time point 0 h. (D) Relative phosphatase rates that were normalized by the rate of RpaA~P dephosphorylation in the absence of cofactor (set to 1). Bars show the standard errors of the nonlinear fits.

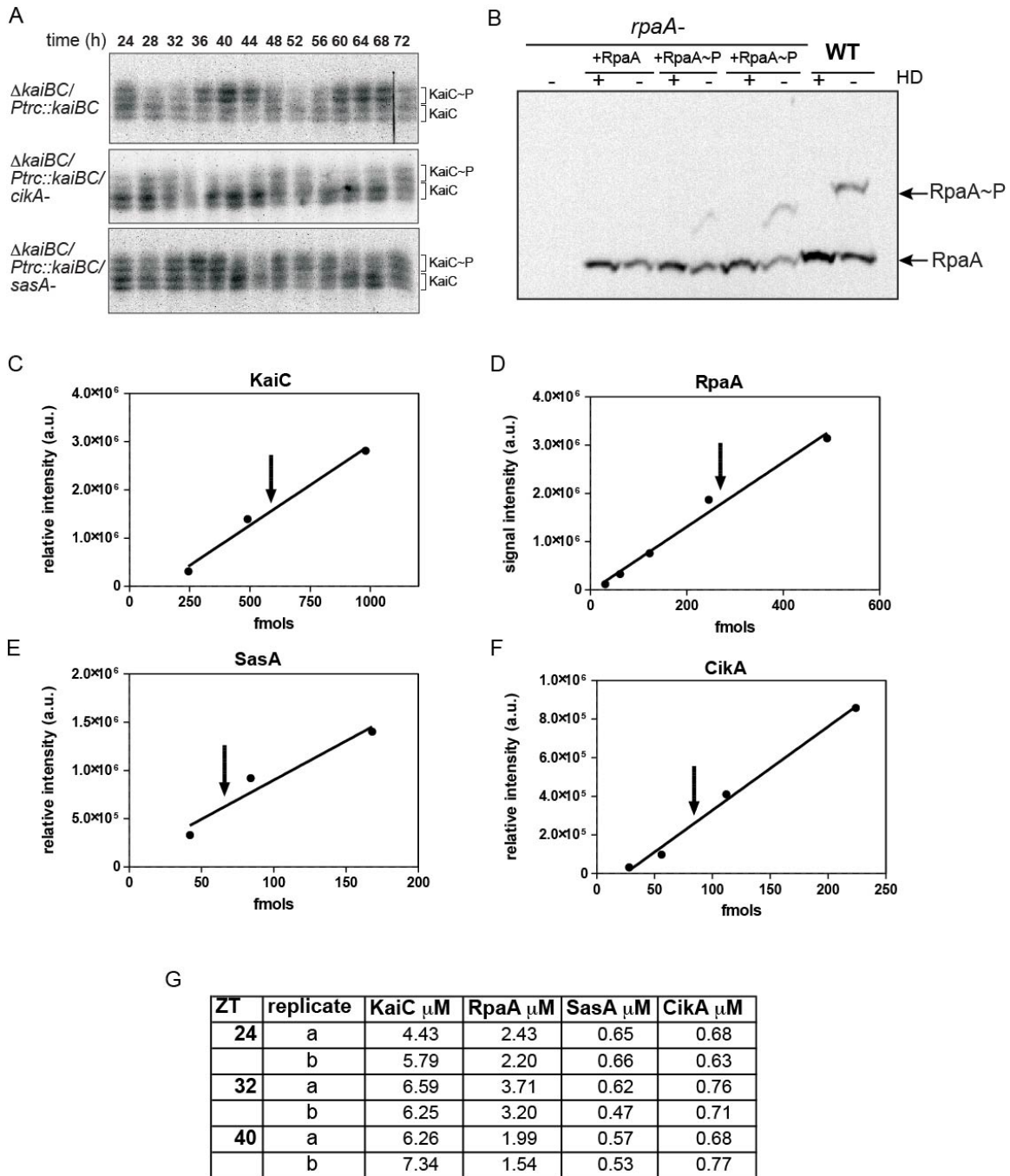


Fig S2. Western blot analysis of KaiC, RpaA, SasA and CikA (related to Fig. 2). (A) *In vivo* KaiC phosphorylation as assessed by Western blotting in lysates obtained from the “clock-rescue” strain (Δ *kaiBC*/*Ptrc::kaiBC*) (top blot), and the derived *cikA*- (middle blot) and *sasA*- (bottom blot) strains. Polyclonal anti-KaiC antibodies were used for detection. The top two bands were assigned as KaiC~P and the bottom two as unphosphorylated KaiC, based on previously published observations (Rust et al., 2011). Samples presented are from Fig. 2. (B) Detection of RpaA phosphorylation *in vivo* using SDS-PAGE with Phos-tag reagent. Control experiment showing a Western blot with lysates obtained from *rpaA*- and wild-type strains and probed with polyclonal antibodies

generated against RpaA. Unphosphorylated or *in vitro* phosphorylated RpaA (~30% and ~50% phosphorylation achieved with the addition of SasA~P) was added to lysates from the *rpaA*- strain. HD denotes heat-denaturation of samples (5 min at 95°C) prior to electrophoresis, which is sufficient to hydrolyze the phosphoaspartate. (C-G) Estimation of cellular concentrations of KaiC, RpaA, CikA, and SasA by quantitative Western blotting. Samples obtained from two independent wild-type cultures (replicates a and b) grown in continuous light in semi-chemostatic conditions ($OD_{750} \sim 0.3$, corresponding to 7.57×10^7 cells/ml as assessed with a Beckman Multisizer3 Coulter Counter) were lysed in a urea-based buffer and analyzed (10 μ g per lane) by Western blotting with polyclonal antibodies generated against each of the indicated proteins (see Western blotting section in the Supplemental Experimental Procedure). Arrows indicate the approximate signal level of each target in cell lysates. Lysis was assumed to be 100% and the volume of the cyanobacterial cell used in calculations was 4 fl. ZT – refers to the Zeitgeber time at which the samples were collected.

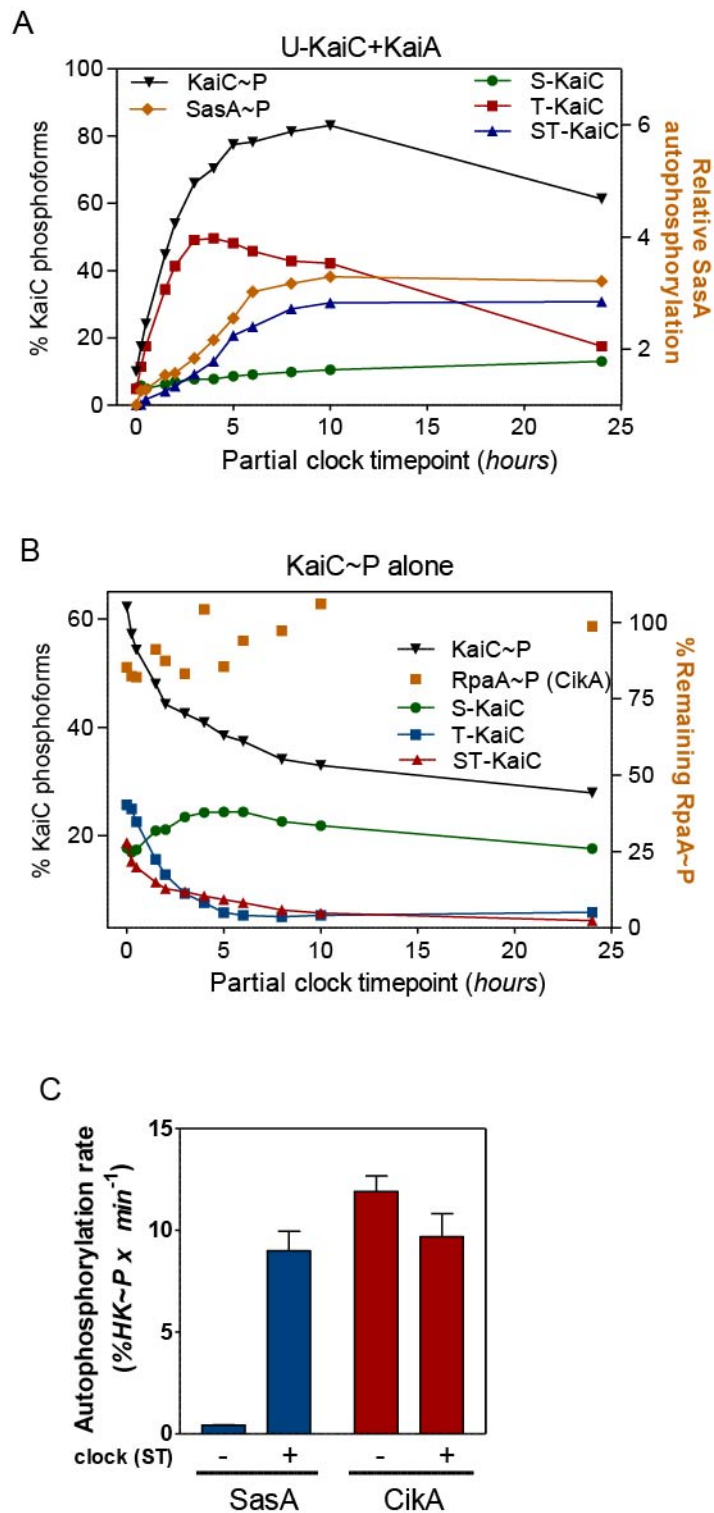
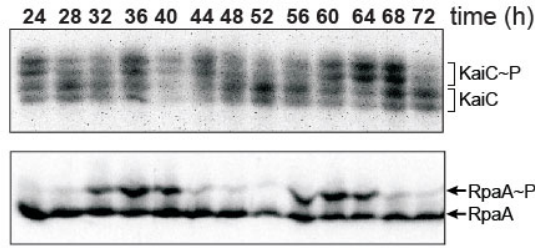


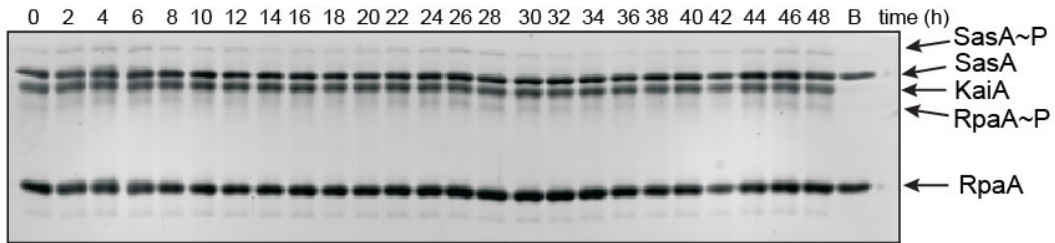
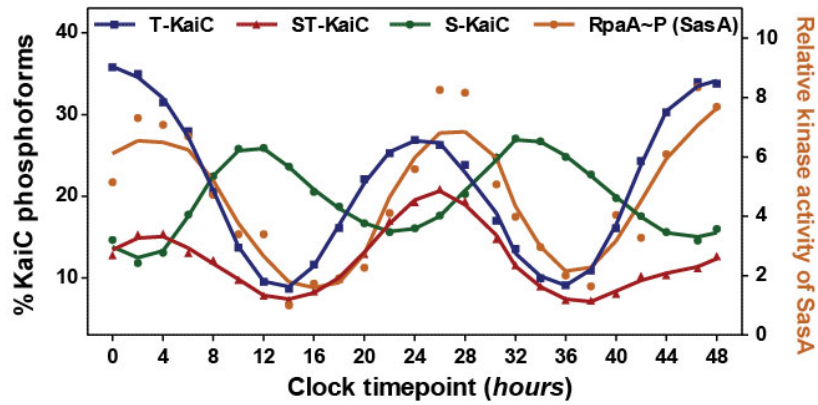
Fig S3. Modulation of SasA and CikA activities by Kai proteins mixes obtained from partial clock reactions (related to Fig 3).
 (A) Autophosphorylation activity of SasA in the presence of Kai proteins obtained from partial clock reactions of KaiC phosphorylation (U-KaiC+KaiA). To measure

autophosphorylation, SasA was added to each ATP-containing partial time-clock aliquot for a period of 2 min and then denatured and separated by SDS-PAGE with Phos-tag to estimate the fraction of SasA~P. The fold increase in SasA~P was obtained by normalizing each value by the SasA~P fraction obtained when no Kai proteins were added. The fractions of KaiC phosphoforms were obtained from the same gel used for SasA~P analysis. (B) Dephosphorylation of ^{32}P -labeled-RpaA~P by CikA in the presence aliquots obtained from a partial clock reaction where KaiC~P is dephosphorylating (no KaiB added). To estimate the phosphatase activity of CikA, ^{32}P -labeled RpaA~P was incubated with CikA and each ATP-containing partial clock aliquot for a period of 15 min. Samples were then denatured, separated by SDS-PAGE, and autoradiographed to extract signal intensities. We report the fraction of RpaA~P remaining relative to no partial clock reaction added. KaiC phosphoforms profiles were estimated from a parallel set of partial clock aliquots. (C) Autophosphorylation activity of SasA and CikA measured in the presence of a clock aliquot (KaiA+KaiB+KaiC) obtained from an *in vitro* oscillating reaction when the ST-KaiC fraction was maximal (the same used in Fig. 3C). The initial rates were obtained from linear slopes of phosphorylated fractions accumulation at 0.5, 1, 2 and 4 min after the addition of ATP-containing aliquots. %HK~P denote %SasA~P or %CikA~P. Bars show the standard error of the linear fits.

A



B



C

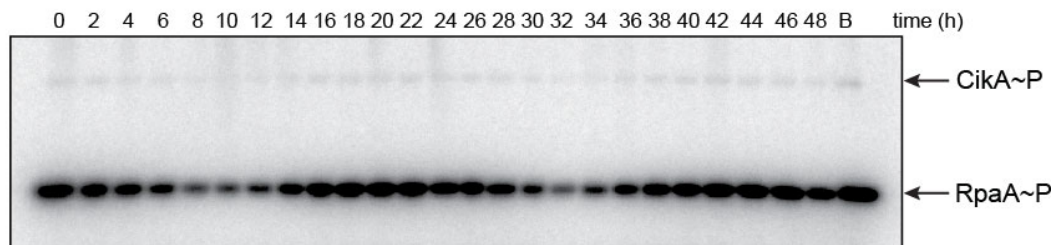
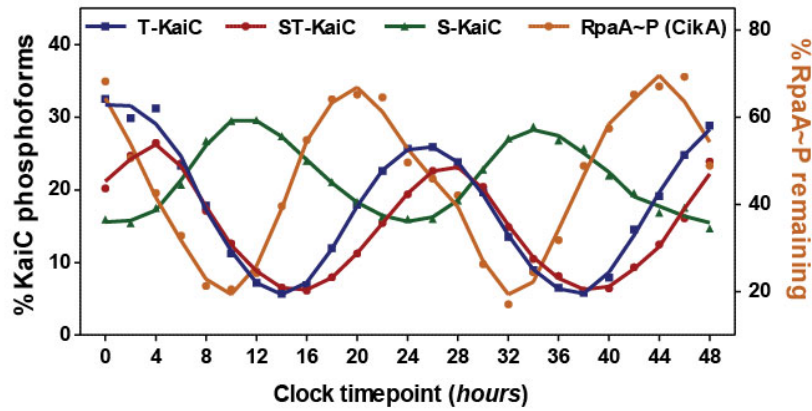


Fig S4. Phase-advanced oscillation of RpaA~P is controlled by the clock through SasA and CikA (related to Fig 4).

(A) Western blots used for measurement of KaiC and RpaA phosphorylation for samples shown in Fig. 4A. (B) Kinase activity of SasA. Upper panel: distribution of the T-, ST- and S-KaiC phosphoforms from the clock aliquots used to estimate the relative kinase activity of SasA presented in Fig. 4B. The lines represent 2nd order smoothing of an average of three neighboring points. Lower panel: portion of the polyacrylamide-Phos-tag gel used for quantification of %RpaA~P. It shows the separation of RpaA and RpaA~P, as well as SasA and SasA~P in the kinase reaction after 2 min incubation with each ATP-containing clock aliquots. B – refers to the addition of clock buffer only (no Kai proteins). (C) Phosphatase activity of CikA. Upper panel: distribution of T-, ST- and S-KaiC phosphoforms in clock aliquots used to estimate the relative phosphatase activity of CikA presented in Fig 4C. The lines represent 2nd order smoothing of an average of three neighboring points. Lower panel: gel image showing the relative amount of ³²P-labeled-RpaA~P after incubation with CikA and each ATP-containing time-resolved clock aliquot for 30 min. Quantification of this signal (which is a proxy of CikA phosphatase activity) is presented in panel above and Fig. 4C. B – refers to addition of clock buffer only (no Kai proteins).

Table S1. List of *S. elongatus* PCC 7942 response regulators and receiver domain containing proteins used in phosphotransfer profiling of SasA and CikA (related to Fig 1A and Fig S1B).

GeneID 7942_	PCC 6301 GeneID (slr)	Name/type	Predicted #aa of full length ORF	Genome Colocalization	Fusion construct kDa
0095	1409	RpaA	249	Alone	45
0151(N) ¹	1354	PsfR	796	Alone	29.8
0151(C)	1354	PsfR	796	Alone	30.3
0365	1148	Unclassified	209	Alone	38.6
0556	0965	SrrB	237	Alone	43
0855	0685	Unclassified	417	Next to 0856/0859	29.7
0856	0684	CheY-like	120	Next to 0855/0859	29.5
0859	0681	Hybrid His-kinase	859	Next to 0855/0856	29.9
1012	0533	SphR	257	Next to 1011	45.2
1014 ²	0532	Hybrid His-kinase	928	Alone	
1110	0439	Unclassified	208	Alone	69.8
1221	0329	CheY-like	125	Alone	30.1
1355	0200	Hybrid His-kinase	929	Next to 1356/1357	30.2
1356	0199	CheY-like	151	Next to 1355/1356	33.3
1404	0151	OmpR-like	229	Alone	42.1
1453	0104	RpaB	244	Alone	43.6
1693	2397	CheY-like	133	Alone	30.8
1815	2279	CheY-like	411	Next to 1816	29.9
1816(N)	2278	Hybrid His-Kinase	977	Next to 1815/1817	29.9
1816(C)	2278	Hybrid His-Kinase	977	Next to 1815/1817	30
1817	2277	LtnA	121	Next to 1816	29.8
1860	2234	LuxR/NarL-like	235	Alone	42.6
1873	2221	OmpR-like	226	Next to 1872	42.6
2305	1796	NblR	228	Alone	42.4
2416	1690	SrrA	255	Alone	44.7
2466	1638	OmpR-like	235	Alone	42.4
B2647 ²		OmpR-like	224	Next to B2646	

¹ Denotes the N or C- terminal position of the receiver domain when more than one was present within the predicted protein product.

² Receiver domain of 7942_1014 and PCC7942_B2647 response regulator were not included in this analysis (See Phosphotransfer profiling section in the Supplemental Experimental Procedures).

Supplemental Experimental Procedures

Strains and culturing conditions

The wild-type strain of *S. elongatus* PCC 7942 used in this study (EOC113) is a derivative of AMC395 (Min and Golden, 2000) and has the bioluminescent reporter system (*PpsbA1::luxCDE* with a spectinomycin resistance cassette in neutral site 1 and *PkaiBC::luxAB* with a chloramphenicol resistance cassette in neutral site 2). The “clock-rescue” strain (EOC75) has *Ptrc::kaiBC* in neutral site 1 (the *kaiBC* coding sequence was cloned into pAM2991 (Ivleva et al., 2005) downstream of the *Ptrc* promoter), and the native *kaiBC* genes were partially replaced with the kanamycin resistance gene (+1 to +1818 relative to the *kaiB* translation start). The *cikA*- strain was made using the pAM2152 plasmid (Mutsuda et al., 2003). The strain lacking *sasA* was made using the pBR322 plasmid carrying the gentamycin resistance cassette flanked by ~1.1 kb of DNA from upstream and downstream of *sasA*. The coordinates of these DNA regions were selected based on a previous published construct of *sasA*- (Iwasaki et al., 2000). The *rpaA*- strain was made using pDrpaA construct (Takai et al., 2006). All genetic manipulations of *S. elongatus* were performed as previously described (Clerico et al., 2007).

The strains were cultured in BG11M medium with 10 mM Hepes-KOH pH 8 and with appropriate antibiotic concentrations (5 µg/ml kanamycin, 10 µg/ml chloramphenicol, 5 µg/ml spectinomycin, 2 µg/ml gentamycin). Cultures (350 ml) were grown at 30°C under ~ 100 µE/m²/s cool white fluorescent lights (Phillips) (as measured with a LI-COR LI-190A quantum sensor connected to LI-250A light meter) while being bubbled with air premixed with 1% CO₂ (~100 ml/min). Based on OD₇₅₀, the doubling time recorded under these conditions was 6.9 h. For circadian experiments, cells were inoculated to an initial OD₇₅₀ of 0.01. 24 hours after inoculation, the cultures were synchronized with two 12 hour dark pulses spaced by a 12 h light period and then allowed to grow in free-running constant light conditions while keeping a relatively constant OD₇₅₀ of 0.3 (ranging between 0.25 and 0.35) by manual dilution with fresh medium every 4 hours. Cell sampling began 24 h after release into constant light.

Western blotting

To prepare total proteins samples, 20 ml of culture were collected on nitrocellulose acetate filters (Whatman), quickly frozen in liquid nitrogen, and kept at -80°C (for up to a week) until ready for processing. For cell breakage, cells were resuspended in 300 µl of lysis buffer (8 M urea with 20 mM Hepes-KOH, pH 8 and 1 mM β-mercaptoethanol (β-ME)) in precooled 2 ml screw-cap tubes containing 0.1 mm glass beads and subjected to bead-beating at 4°C. After 10 x 30 s cycles of bead-beating spaced by 2 min cooling periods on ice, lysates were collected and centrifuged for 10 min at 20000 x g to sediment the remaining beads and cell debris and the supernatants transferred to a new tube. The protein content in lysates was determined with the Bradford assay against a BSA (Bio-Rad) standard curve. Lysates were aliquoted and stored at -80°C.

KaiC immunoblotting was performed as previously described (Rust et al., 2011) using rabbit polyclonal anti-KaiC serum that was generated against full-length recombinant KaiC at Cocalico Biologicals (Reamstown, PA).

For detection of RpaA phosphorylation, 15 µg of total protein lysate was loaded on a 7% polyacrylamide gel (29:1 acrylamide:bisacrylamide) made with 50 µM of Phos-tag AAL-107 reagent (Wako Chemicals) and 100 µM MnCl₂. Gels were run at 4°C at 35 mA constant current (Hoefer SE 600 system), until the bromophenol blue loading dye front reached the bottom edge of the gel (~2.5 h). After electrophoresis the gel was gently washed two times for 10 min in transfer buffer (40 mM glycine, 50 mM Tris, 0.063 % SDS, 20 % v/v methanol). Buffer in the first wash contained 1 mM EDTA necessary to chelate the Mn²⁺. The protein was then transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad), and processed using a standard Western blotting procedure. Rabbit polyclonal anti-RpaA serum was used at 1:1000 dilution and was then probed with the HRP-conjugated goat-anti-IgG-rabbit secondary antibodies. The signal was detected by chemiluminescence (Pierce Femto kit) on Alpha Innotech Imaging station and measured using its accompanying AlphaImager EP software.

CikA and SasA-specific antibodies were generated and purified from rabbit sera which were obtained by immunization with the full-length recombinant CikA (fusion construct with the maltose binding protein tag) or SasA at Cocalico Biologicals (Reamstown, PA). First, CikA and SasA-Affigel-conjugated beads (~0.5 ml) were prepared using 2-3 mg of purified proteins (see below) and Affigel 10/15 resin (Bio-Rad) following the manufacturer's instructions. Then, 10 ml of each serum was incubated with the corresponding resin prep for 2 hours at room temperature. After washing extensively with Tris-HCl pH 7.5 and Tris-HCl, pH 7.5/0.5 M NaCl buffers, the antibodies were eluted with 0.1 mM Glycine (pH 2.5) that was quickly neutralized in Tris-HCl pH 8. The final protein concentration of the eluted, pooled and concentrated fractions was ~1 mg/ml as determined by absorption at 280 nm. Aliquots of purified antibodies (containing 10% glycerol and 0.03% NaN₃) were flash-frozen and stored at -80°C. Antibody specificity was assessed using cell lysates from wild type and *sasA*- or *cikA*- strains, along with the purified recombinant proteins. Primary anti-SasA and anti-CikA antibodies were diluted 1:1000 for use in Western blotting. In the case of anti-CikA, several smaller size cross-reacting bands were detected in both the *cikA*- and WT lysates at this dilution.

Preparation of recombinant proteins

Response regulators

To streamline the process of cloning and one step purification of the proteins used in phosphotransfer profiling, we used the same pipeline employed by Laub and coworkers (Laub et al., 2007). Briefly, the genes predicted to encode predicted two-component domains (as identified with PFAM) were PCR amplified and cloned into pENTR-TA vectors (Invitrogen). In the case of large multi-domain proteins, only the receiver domain was cloned. After confirmation of their identities through sequencing, the cloned fragments were shuttled into a thioredoxin-his-tag-based Gateway destination vector (except *cikA* and 7942_1011 for which a his-maltose-binding-tag vector was used instead) and used to transform BL21 (DE3) Tuner cells (Laub et al., 2007; Skerker et al., 2005). Induction and the one-step purification procedure of each construct was performed as previously described (Laub et al., 2007), yielding between 0.5 and 10 mg total protein

(as measured by Bradford against a BSA standard) and with an estimated purity of 50 to 90% as estimated by densitometric analysis on 4-20% Novex Tris-Glycine gels.

Kai proteins

Expression and purification of Kai proteins was performed as previously described (Rust et al., 2011; Rust et al., 2007), except that the final storage buffer contained 20 mM Hepes-KOH (pH 8) and 150 mM KCl, instead of the 20 mM Tris-HCl (pH 8) and 150 mM NaCl.

RpaA

The RpaA purification procedure was performed in a manner similar to that previously reported (Takai et al., 2006). A pGEX-6P-1 (GE Healthcare) plasmid containing GST-RpaA was transformed into BL21(DE3) *E. coli* cells. Several colonies from the transformation were inoculated into 1 liter of LB medium (with 50 µg/ml carbenicillin) and grown at 37°C to an OD₆₀₀ of 0.5, at which time 1 mM IPTG was added and the culture was allowed to grow at 22.5°C for another 3.5 h. Cell pellets were obtained through centrifugation and flash-frozen in liquid nitrogen. All subsequent steps were done at 4°C. The pellets were re-suspended in 15 ml cold lysis buffer (50 mM Tris-HCl (pH 8), 300 mM NaCl, 1 mM EDTA, 1 mM MgCl₂, 1 mM dithiothreitol (DTT), one tablet of EDTA-free protease inhibitor (Roche), 1 mg/ml lysozyme and 50 U/ml benzonase nuclease (EMD Chemicals)) and passed two times through French press cell at 16,000 PSI. The lysate was cleared through centrifugation and after filtration with a syringe through a 0.45 µm Millex PDVF filter (Millipore) the supernatant was loaded on GSTrap column on an AKTA Explorer FPLC (GE Healthcare). RpaA was cleaved from the GST tag overnight on-column with PreScission Protease (GE Healthcare). The RpaA eluate was then further purified by cation exchange chromatography using a Resource Q column (GE Healthcare). The pooled fractions of RpaA were buffer exchanged and brought to ~100 µM RpaA in a storage buffer (10% glycerol, 20 mM Hepes-KOH pH 8, 150 mM KCl and 1 mM DTT), flash-frozen and kept at -80°C. Typical yield was ~3 mg per 1 liter of *E. coli* culture.

SasA

pET32a (Novagen) carrying Trx-His-SasA was transformed into BL21(DE3) Tuner *E. coli* cells. Several colonies from a fresh transformation were used to inoculate a 1 liter culture of LB (50 µg/ml carbenicillin) and grown to OD₆₀₀ 0.5 at 37°C. After addition of 80 µM IPTG, cells were grown at 17°C for 16 h. The cells pellets obtained through centrifugation were flash-frozen and maintained at -80°C until used for lysis. The frozen cells were re-suspended in 10 ml chilled lysis buffer (50 mM Tris-HCl pH 8, 250 mM NaCl, 20 mM imidazole pH 8, 1 mM β-ME, 10% glycerol, 10 unit/ml benzonase nuclease and 1 tablet EDTA-free protease inhibitor) and passed through the French press cell (16,000 PSI). After clearing of the lysate, the filtered supernatant was loaded onto a pre-equilibrated 1 ml HisTrap column (GE Healthcare) on an AKTA Explorer FPLC system (GE Healthcare). The Trx-His-SasA fusion protein was eluted from the column with a gradient of imidazole made with the use of two buffers: A (50 mM Tris-HCl pH 8, 250 mM NaCl, 20 mM imidazole pH 8, 1 mM β-ME, 10% glycerol) and B (50 mM Tris-HCl pH 8, 250 mM NaCl, 500 mM imidazole pH 8, 1 mM β-ME, 10% glycerol). Eluted

fractions that were enriched in Trx-His-SasA were pooled, dialyzed overnight into buffer A and treated with 100 U of HRV 3C protease (GE Healthcare). The HRV 3C cleavage site was introduced in front of the initiator methionine in the SasA coding sequence during the cloning steps. The cleaved SasA was separated from the Trx-His tag by passing the protein prep through another HisTrap column, which retained the Trx-His tag. The protein was buffer exchanged and further purified by gel filtration chromatography on a HiPrep 16/60 S-300 column (GE Healthcare). The final storage buffer was the same as that used for RpaA. The amount of SasA was measured by Bradford against a BSA standard curve and concentrated to ~40 μ M. SasA was estimated to be 95% pure by densitometric analysis of bands on 4-20% Novex Tris-Glycine gels (Invitrogen). Aliquots were flash-frozen and kept at -80°C. The typical final yield was 1.5 – 2 mg protein per 1 liter of *E. coli* culture.

CikA

A variant of the destination vector carrying the His-MBP-CikA construct used in phosphotransfer profiling was made in which the HRV 3C cleavage site was introduced in front of the CikA coding sequence. Induction and purification conditions were similar to those employed for SasA, with the exception that 500 U of HRV 3C protease was used and instead of gel filtration, cation exchange chromatography (Resource Q column (GE Healthcare)) was used. Typical yield was 1-1.5 mg protein per 1 liter of culture. Purity of CikA was estimated at 99%.

Phosphotransfer profiling

The assay was performed largely as previously described (Laub et al., 2007). We first determined the autophosphorylation activity of SasA and CikA constructs and found that its maximal level (peak radiolabel accumulation) occurs at 10 min for His-MBP-CikA and 2 hours for Trx-His-SasA at 22.5°C. (His-MBP-SasA construct was found to be less active than its TRX-based variant). Then, to each response regulator we added the phosphorylated histidine kinase mix and incubated for 5 min or 1 h at 22.5°C. The final concentration of each component was 2.5 μ M. The reactions were stopped with the addition of Laemmli loading dye and each mix was directly loaded and run on Novex 4-20% Tris-Glycine gels (Invitrogen). Following electrophoresis, the gels were dried onto Whatman filter paper, and scanned with Typhoon Trio Imaging System (GE Healthcare) (Fig 1A and Fig. S1B). Two predicted receiver domain-containing proteins of *S. elongatus* PCC7942 were not included in this analysis. PCC7942_1014 (encoded as part of a hybrid histidine kinase) was not used because of a poor protein yield; and PCC7942_B2647 – a plasmid-borne response regulator co-encoded next to its likely cognate histidine kinase, PCC7942_B2647 – was not analyzed because it is absent in the closely related strain, *S. elongatus* PCC6301.

Kinase assays

The concentration of SasA and CikA used was 0.65 μ M and that of RpaA was 2.5 μ M, similar to the values measured *in vivo* (Fig S2). As the measured *in vivo* concentration of KaiC (Fig S2) was found to be similar to what was reported previously (Kitayama et al., 2003), we decided to use the concentrations of Kai proteins used in standard oscillating clock reactions (3.5 μ M KaiC, 3.5 μ M KaiB and 1.5 μ M KaiA). The kinase buffer used

was: 20 mM HEPES-KOH pH 8, 150 mM KCl, 10% glycerol and 5 mM MgCl₂. Phosphorylation was estimated from SDS-PAGE gels made with Phostag reagent, which was optimized to separate the phosphorylated and non-phosphorylated species (see Western blotting section). The extent of phosphorylation was calculated by measuring the fraction of the retarded band signal divided by the sum of a given protein's total signal.

Phosphatase assays

Phosphorylated RpaA was prepared using a CikA-conjugated Affigel 10/15 resin prep, similar to that used for purification of CikA antibodies (see Western blotting section). Approximately 100 μ l of resin was mixed with 100 μ l of kinase buffer containing 50 μ M RpaA and 125 μ Ci ³²P- γ -ATP (Perkin Elmer) and incubated for 30 min at 22.5°C. RpaA~P was subsequently washed off the resin with 5 ml of kinase buffer and concentrated and exchanged into fresh kinase buffer with aid of a Nanosep 10k Omega column (Pall) that removed the unincorporated ATP. The RpaA~P concentration was measured by Bradford assay against an unphosphorylated RpaA standard curve. Except where indicated, reaction conditions and concentrations of the proteins used in phosphatase assays were the same as in kinase assays. The reactions were stopped with the addition of Laemmli loading dye and analyzed by SDS-PAGE as described in the phosphotransfer profiling section. The analysis of relative phosphatase activity was performed as shown in Fig. S1C and S1D and reported in the main Experimental Procedure section.

Clock reactions

In vitro clock reactions were performed as previously described (Rust et al., 2011) with minor modifications such as the inclusion of an ATP regeneration system to maintain a constant level of ATP over the course of sampling. The clock reaction buffer contained: 20 mM HEPES-KOH pH 8, 150 mM KCl, 5 mM MgCl₂, 10 mM ATP, 10 mM phosphoenolpyruvate, 10 U/ml of pyruvate kinase of *Bacillus stearothermophilus* (Sigma), 50 μ g/ml kanamycin and 10% glycerol. The final concentrations of Kai proteins as measured by Bradford against a BSA standard curve were: 3.5 μ M KaiC, 3.5 μ M KaiB, and 1.5 μ M of KaiA. Reactions were incubated at 30°C. 20 μ l aliquots were sampled every 2 hours and flash-frozen in liquid nitrogen and kept at -80°C until further use. Along with the clock reactions, a sample with reaction buffer only was maintained in the same conditions and flash-frozen at the end of the experiment to be used as buffer only control in SasA and CikA kinase and phosphatase assays. The distribution of phosphoforms in each clock aliquot was measured on phosphoform gels (Rust et al., 2007) or extracted from the same Phostag gels used in kinase assays.

Supplemental References

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