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Supplemental Information

Two Antagonistic Clock-Regulated Histidine Kinases Time the Activation of Circadian Gene Expression

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Inventory of Supplemental Information

The Supplemental Information file includes the following sections:

Supplemental Data
Figure S1 – provides additional data on the biochemical activities of SasA and CikA related to Fig 1. Panel A shows the relative catalytic efficiency of SasA for two substrates found in phosphotransfer profiling at 5 min (Fig. 1A). Panel B shows an extended time-incubation of phosphotransfer profiling (1 hour) of SasA~P and CikA~P. Panels C and D – exemplify how the relative phosphatase activities of SasA and CikA were obtained. These panels supports the results presented in Fig. 1C.
Figure S2 – Panel A shows the immunoblots of KaiC used to measure KaiC~P profiles in Fig 2. The Western blot shown in panel B provides the control experiment for detection for RpaA~P *in vivo*, which is the basis for obtaining the RpaA~P profiles in Fig. 2. Panels C to G show the results of quantitative western blotting for KaiC, SasA, CikA and RpaA, which was used to estimate the concentration of the *in vitro* assay components.
Figure S3 – shows autophosphorylation and phosphatase activities of SasA and CikA in the presence of aliquots obtained from partial or full clock reactions. This figure complements the results presented in Fig. 3.
Figure S4 – provides the primary data used for the quantifications shown in Fig 4. This figure also presents the detailed distribution of KaiC phosphoforms in the clock aliquots used in Fig 4.
Table S1 – Lists the response regulators of *S. elongatus* that were used for phosphotransfer profiling presented in Fig. 1 and Fig. S1B.

Supplemental Experimental Procedures
This section details the procedures used for growing the cyanobacterial cultures, the analysis of lysates by immunoblotting, and the purification of recombinant proteins utilized in kinase and phosphatase assays.

Supplemental References
This section contains the references used exclusively in the Supplemental Experimental Procedures.

Supplemental Data

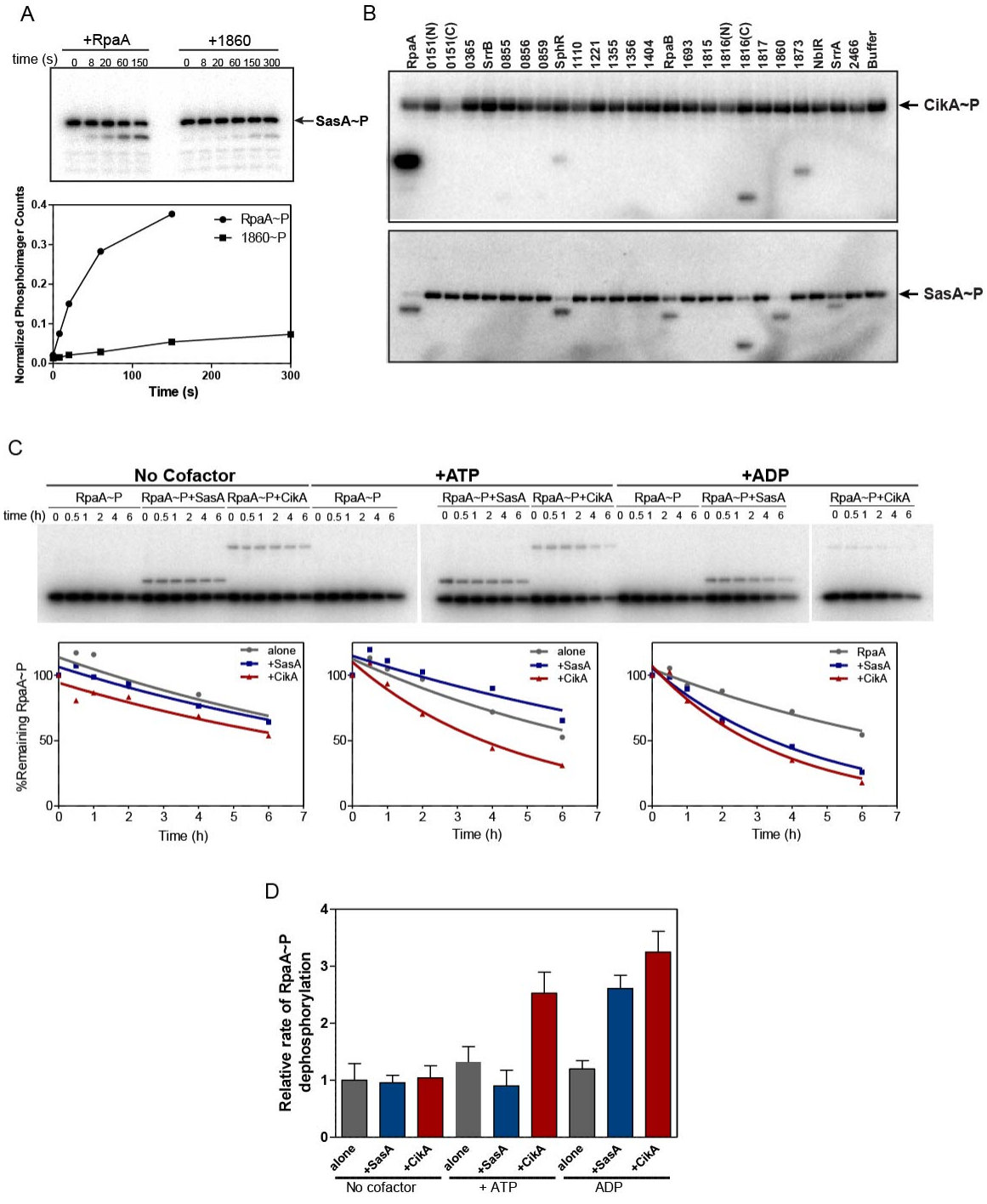
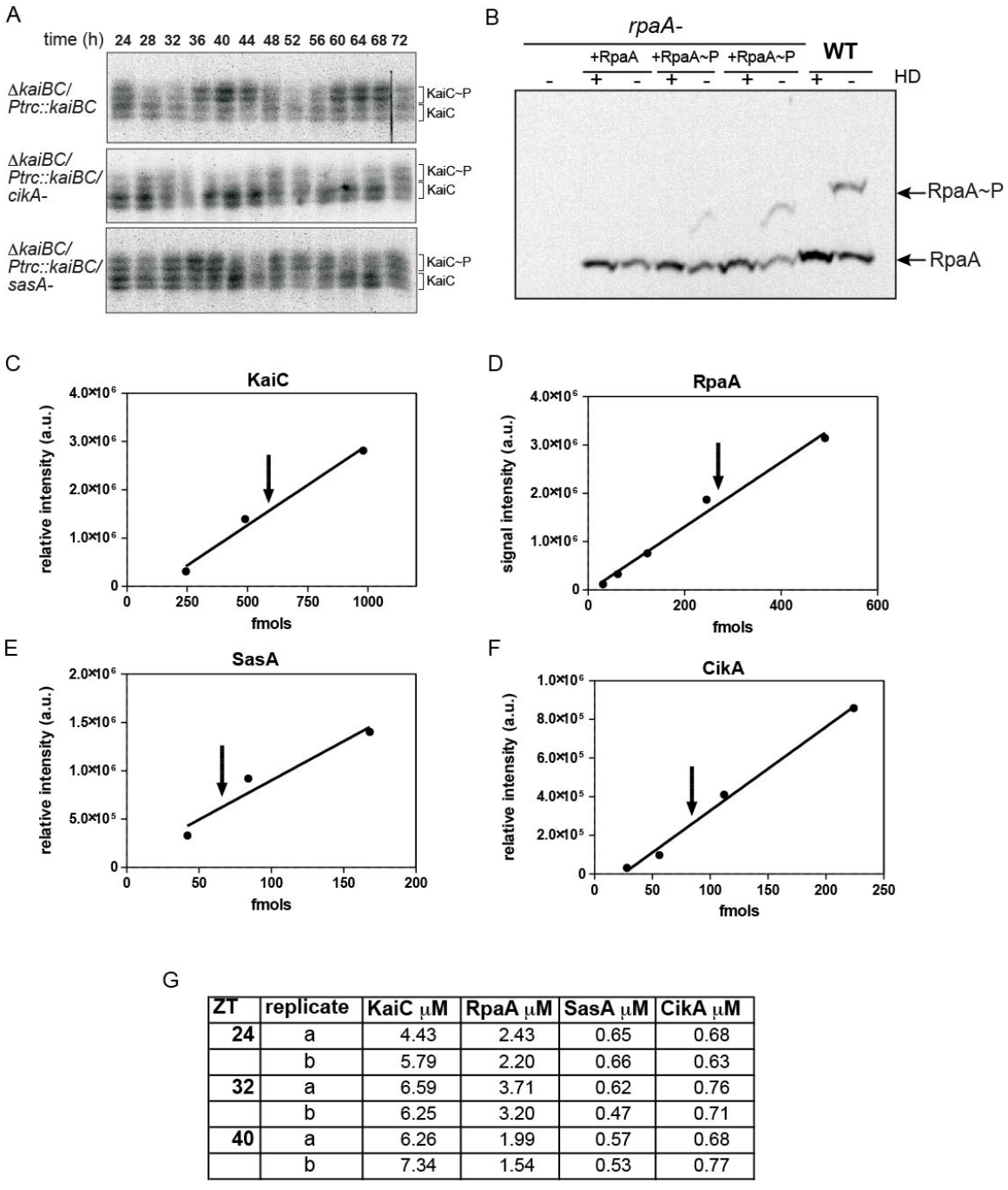


Fig. S1. Biochemical activities of SasA and CikA (related to Fig. 1).
(A) Relative catalytic efficiency of SasA~P towards RpaA and *synpcc7942_1860* response regulators that were identified as potential phosphotransfer targets (see Fig. 1A). 5 µM SasA was first autophosphorylated, and then mixed with 0.25 µM 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. ^{32}P-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 ^{32}P 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.



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ZT	replicate	KaiC μM	RpaA μM	SasA μM	CikA μM
24	a	4.43	2.43	0.65	0.68
	b	5.79	2.20	0.66	0.63
32	a	6.59	3.71	0.62	0.76
	b	6.25	3.20	0.47	0.71
40	a	6.26	1.99	0.57	0.68
	b	7.34	1.54	0.53	0.77

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} ~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 µg 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.

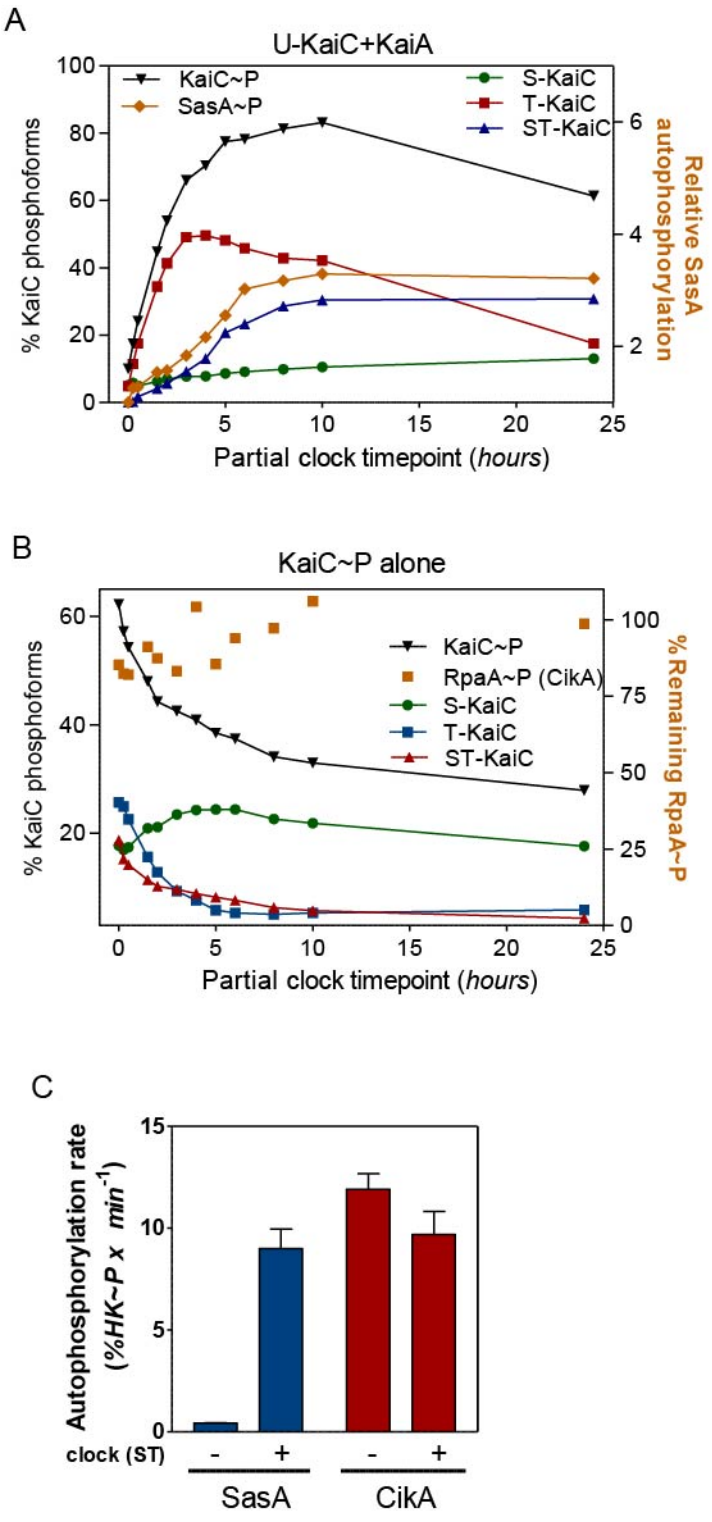

Fig S3. Modulation of SasA and CikA activities by Kai proteins mixes obtained from partial clock reactions (related to Fig 3).
(A) Autophosphorylation activity of SasA in the presence of Kai proteins obtained from partial clock reactions of KaiC phosphorylation (U-KaiC+KaiA). To measure

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.

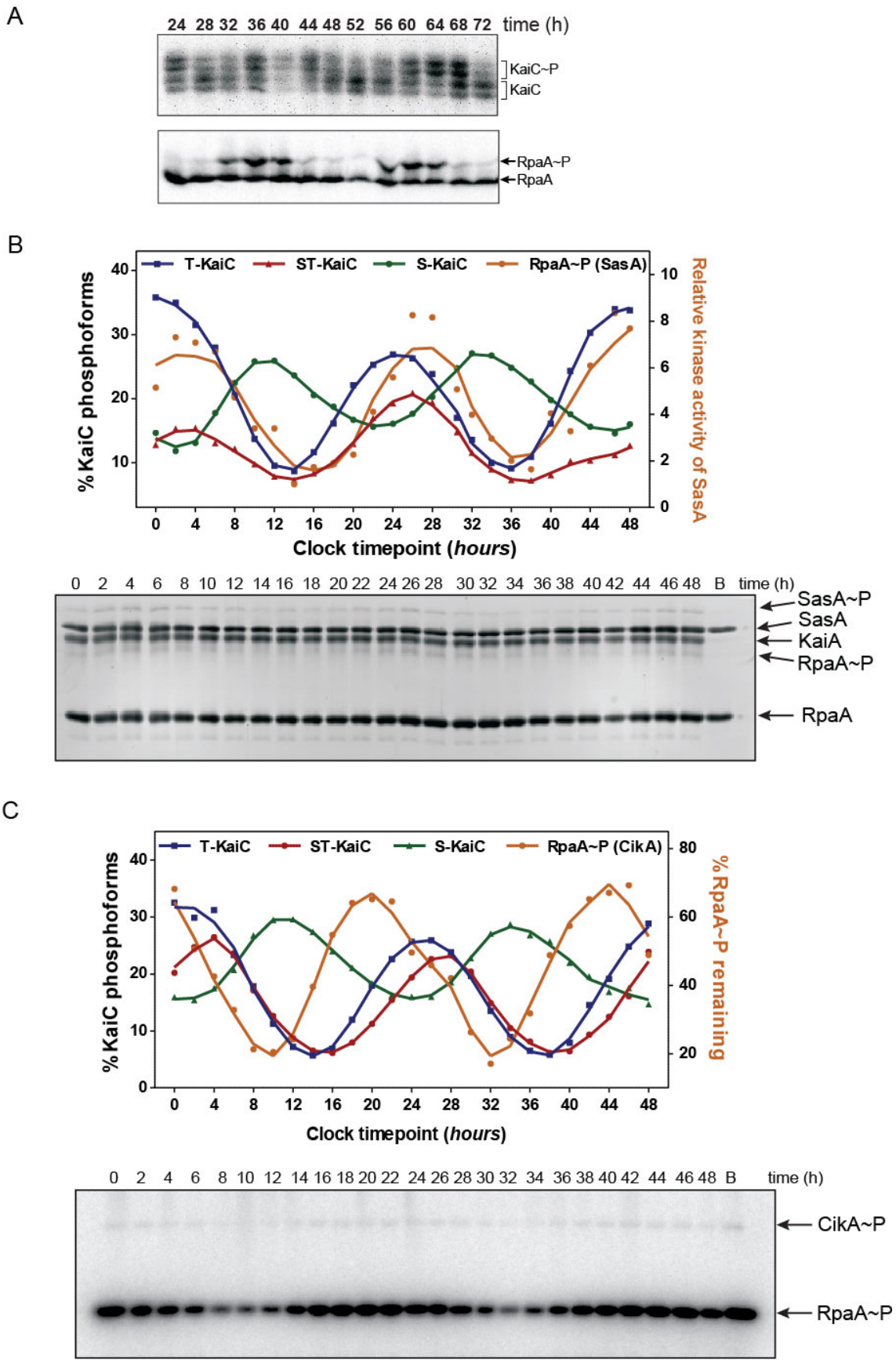
A
24 28 32 36 40 44 48 52 56 60 64 68 72 time (h)
KaiC~P
KaiC
RpaA~P
RpaA
B
T-KaiC
ST-KaiC
S-KaiC
RpaA~P (SasA)
% KaiC phosphoforms
Relative kinase activity of SasA
Clock timepoint (hours)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 B time (h)
SasA~P
SasA
KaiA
RpaA~P
RpaA
C
T-KaiC
ST-KaiC
S-KaiC
RpaA~P (CikA)
% KaiC phosphoforms
% RpaA~P remaining
Clock timepoint (hours)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 B time (h)
CikA~P
RpaA~P

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 ^{32}P-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 (P*psbA1::luxCDE* with a spectinomycin resistance cassette in neutral site 1 and P*kaiBC*::*luxAB* with a chloramphenicol resistance cassette in neutral site 2). The "clock-rescue" strain (EOC75) has P*trc::kaiBC* in neutral site 1 (the *kaiBC* coding sequence was cloned into pAM2991 (Ivleva et al., 2005) downstream of the P*trc* 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 μm/ml kanamycin, 10 μm/ml chloramphenicol, 5 μm/ml spectinomycin, 2 μm/ml gentamycin). Cultures (350 ml) were grown at 30°C under ~ 100 $\mu E/m^2/s$ 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% CO_2 (~100 ml/min). Based on $OD_{750,}$ the doubling time recorded under these conditions was 6.9 h. For circadian experiments, cells were inoculated to an initial OD_{750} 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 OD_{750} 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).

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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 $MnCl_2$. 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 Mn^{2+}. 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% NaN_3) 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 intro 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 OD_{600} 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 $MgCl_2$, 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 -80C. 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 OD_{600} 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 Explored 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 μM. 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 μM. 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 μM and that of RpaA was 2.5 μM, 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 μM KaiC, 3.5 uM KaiB and 1.5 μM KaiA). The kinase buffer used

was: 20 mM Hepes-KOH pH 8, 150 mM KCl, 10% glycerol and 5 mM $MgCl_2$. 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 µl of resin was mixed with 100 µl of kinase buffer containing 50 µM RpaA and 125 µCi ^{32}P-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 $MgCl_2$, 10 mM ATP, 10 mM phosphoenolpyruvate, 10 U/ml of pyruvate kinase of *Bacillus stearothermophilus* (Sigma), 50 µg/ml kanamycin and 10% glycerol. The final concentrations of Kai proteins as measured by Bradford against a BSA standard curve were: 3.5 µM KaiC, 3.5 µM KaiB, and 1.5 µM of KaiA. Reactions were incubated at 30°C. 20 µl 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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