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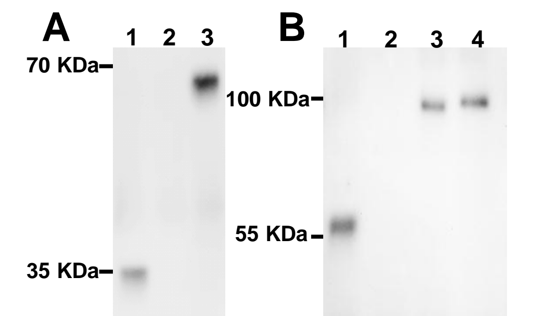
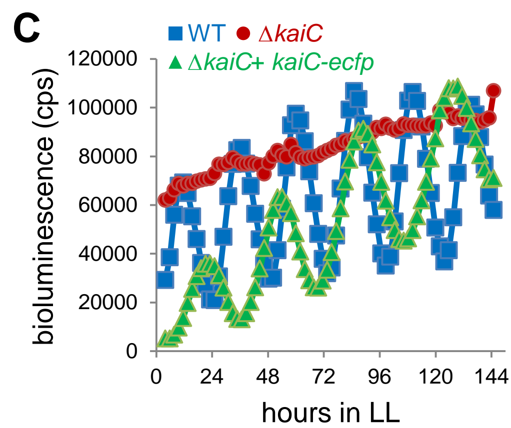
Supplemental Information

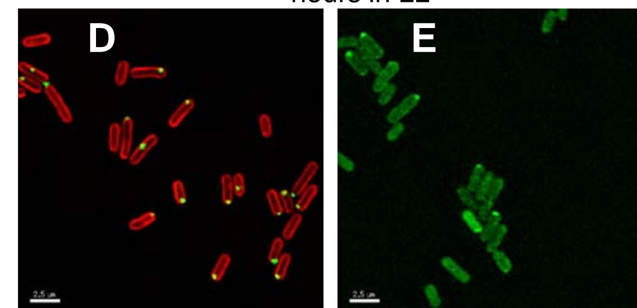
Dynamic Localization of the Cyanobacterial Circadian Clock Proteins

Susan E. Cohen, Marcella L. Erb, Jangir Selimkhanov, Guogang Dong, Jeff Hasty, Joe Pogliano, and Susan S. Golden

Figure S1







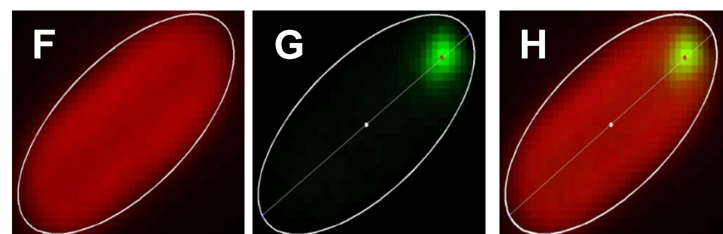
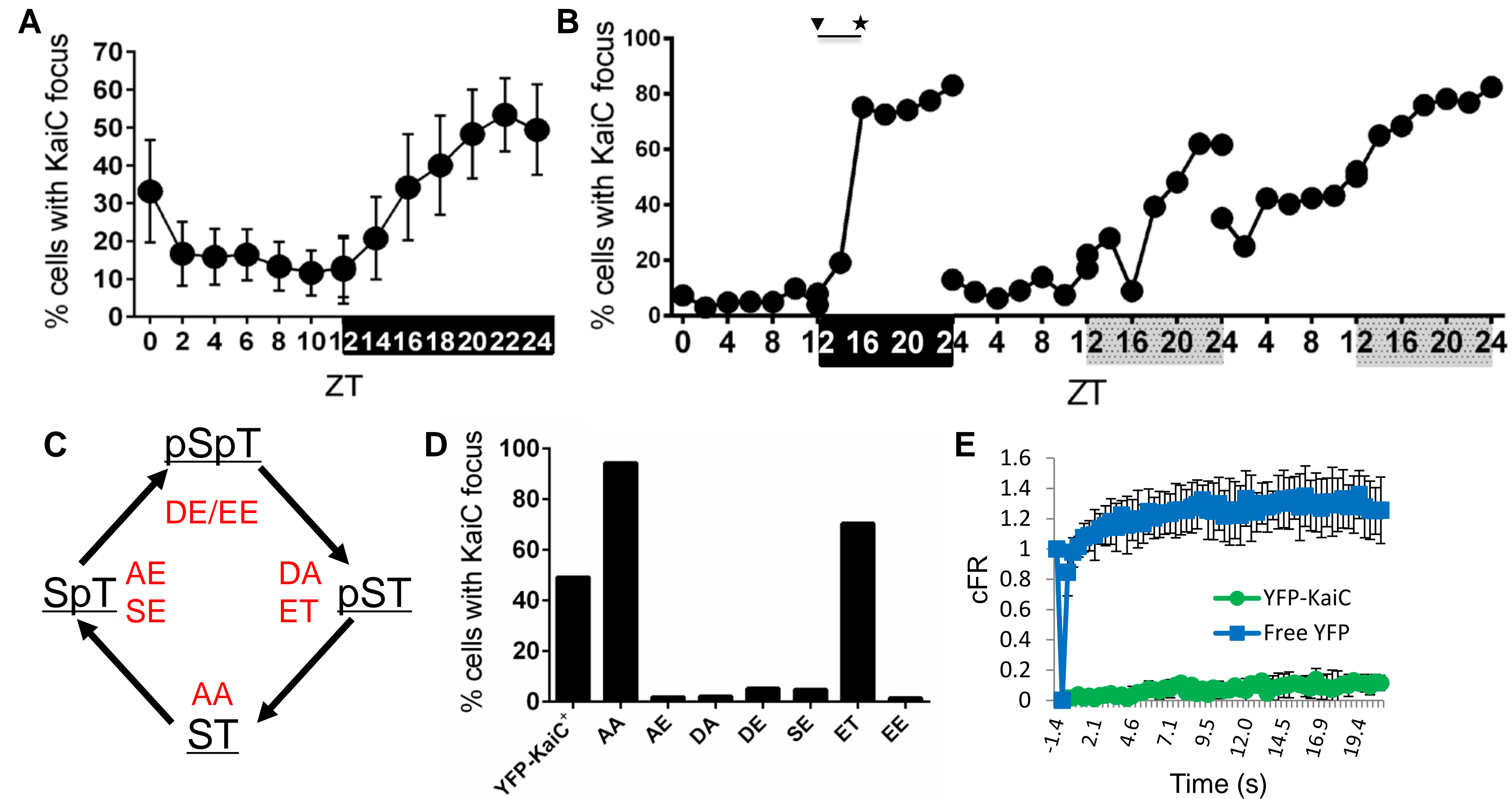


Figure S2



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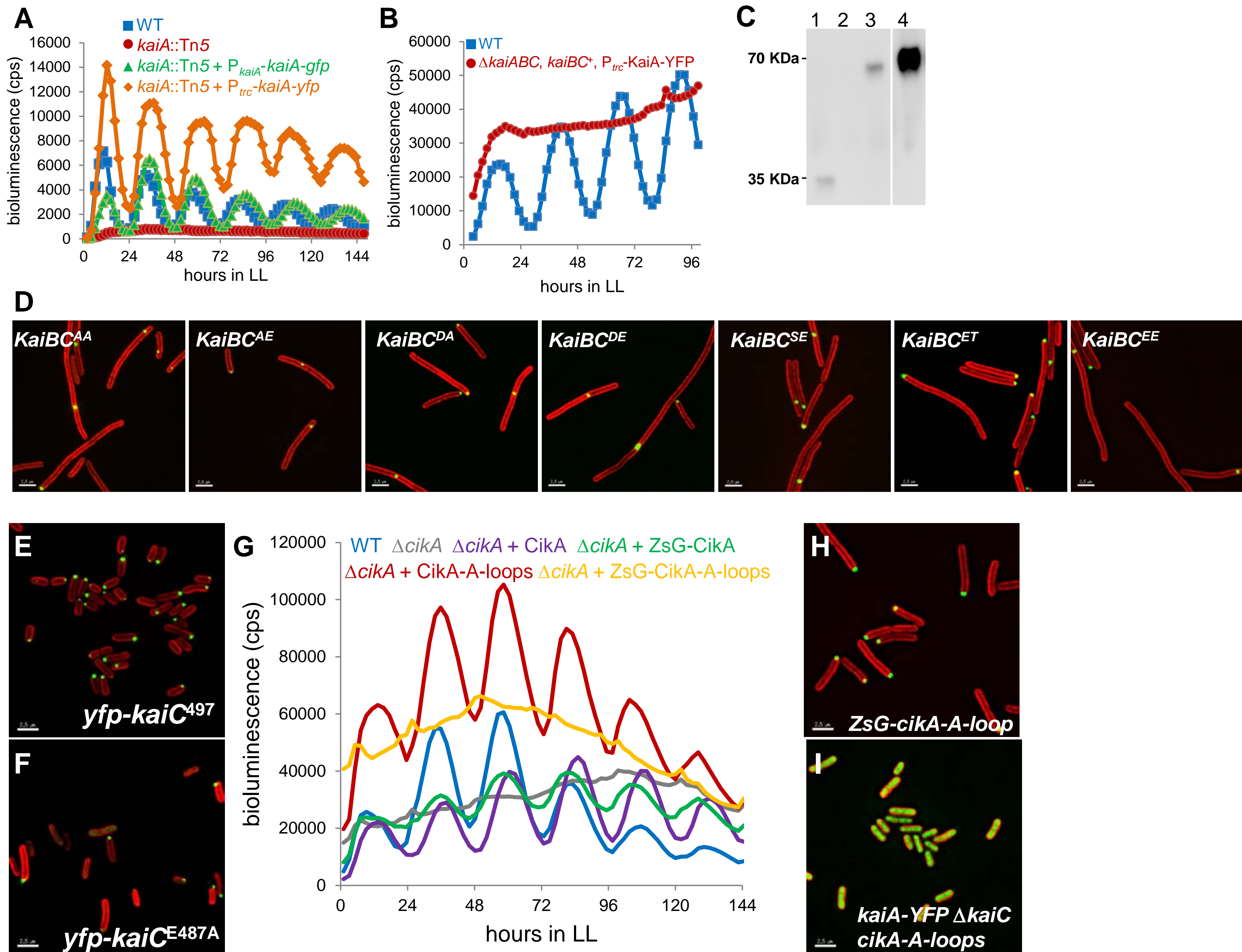
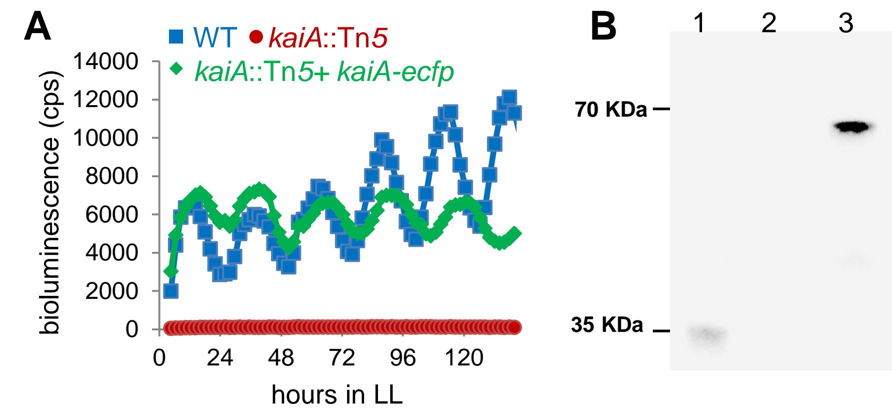


Figure S4



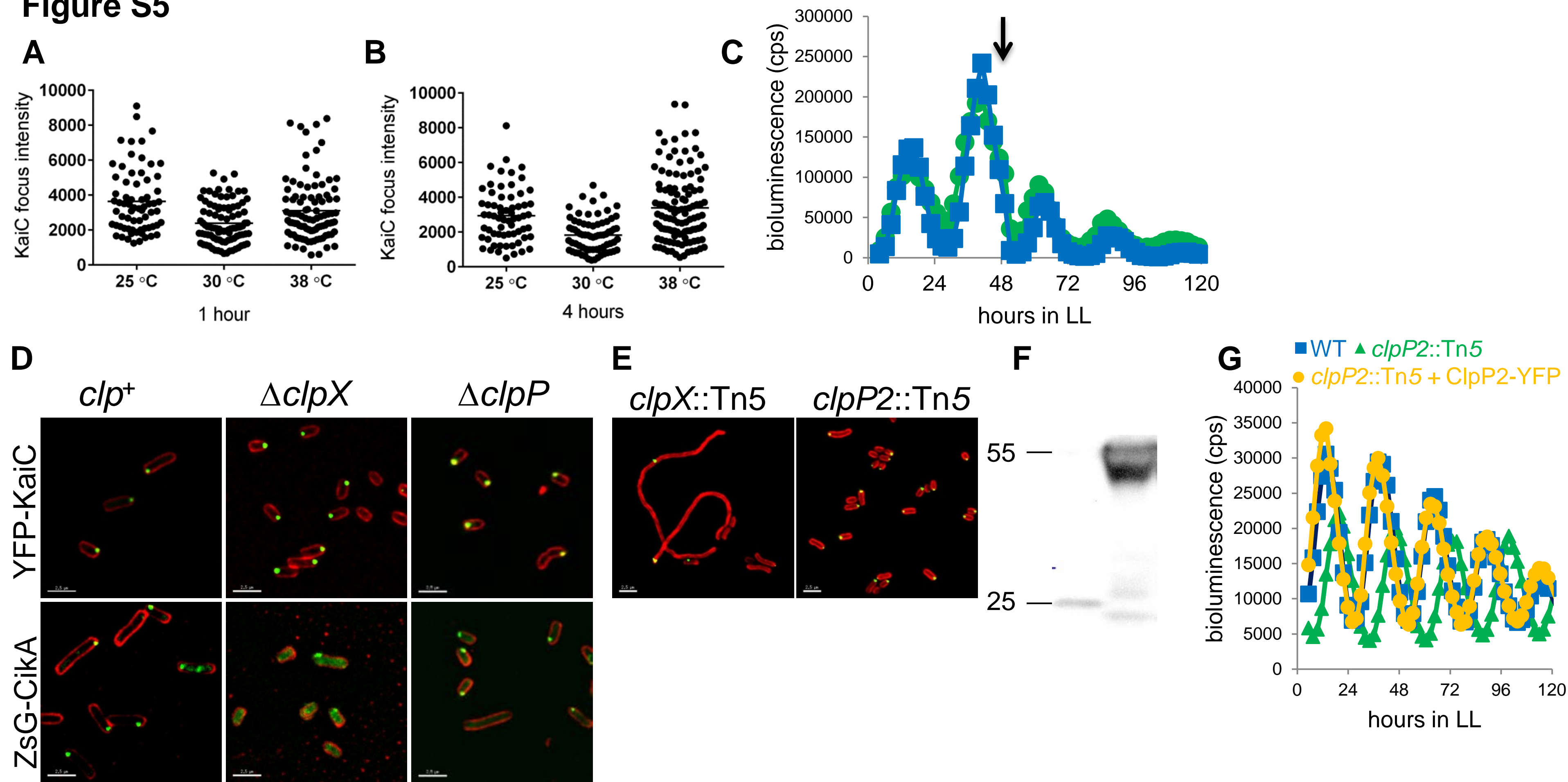


Figure S5
A
KaiC focus intensity
10000
8000
6000
4000
2000
0
25 °C
30 °C
38 °C
1 hour
B
KaiC focus intensity
10000
8000
6000
4000
2000
0
25 °C
30 °C
38 °C
4 hours
C
bioluminescence (cps)
300000
250000
200000
150000
100000
50000
0
0
24
48
72
96
120
hours in LL
D
clp+
ΔclpX
ΔclpP
YFP-KaiC
ZsG-CikA
E
clpX::Tn5
clpP2::Tn5
F
55
25
G
WT
clpP2::Tn5
clpP2::Tn5 + ClpP2-YFP
40000
35000
30000
25000
20000
15000
10000
5000
0
bioluminescence (cps)
0
24
48
72
96
120
hours in LL

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Related to Figure 1. Characterization of fluorescent Kai fusion proteins. A) Immunoblot of soluble extracts incubated with α-KaiA antiserum. Lane 1, WT; lane 2, k*aiA::*Tn*5*; lane 3, k*aiA::*Tn*5* expressing P_{kaiA}-*kaiA-gfp*. B) Immunoblot of soluble extracts from cells collected at LL 16 incubated with α-KaiC antiserum. Lane 1, WT; lane 2, Δ*kaiC*; lane 3, Δ*kaiC* expressing P_{trc}-*yfp-kaiC*; lane 4, Δ*kaiC* expressing P_{kaiB}-*kaiC-ecfp*. C) Rhythms of gene expression monitored from a P_{kaiB}-*luc* reporter strain. KaiC with ECFP fused to the C-terminus produces a long period phenotype (green, 31.4 h period) compared to WT (blue, 24.8 h period); Δ*kaiC* control strain is arrhythmic (red). D-E) Field of *S. elongatus* cells expressing D) YFP-KaiC, autofluorescence in red, and E) KaiA-GFP demonstrate that only a sub-population of cells have foci. Scale bars = 2.5 microns. F-H) Sample image used to quantify position of KaiC foci within the cell for Fig. 1D. F) An automated program draws an ellipse (white) around the cell, defined by autofluorescence. G) mid-point of the ellipse (white dot) and position of the focus (red dot) are identified. H) merged image is shown in the third panel.

Figure S2. Related to Figure 1. Circadian regulation of KaiC localization. A) 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”. Fraction of the population that showed KaiC polar localization in a diurnal cycle is shown for an average of 6 independent experiments in an otherwise WT background ± standard error of the mean. B) Rhythms of KaiC localization persist in continuous conditions. Cultures entrained to opposite LD cycles were sampled in diurnal conditions (dark bar represents night) and released into continuous light and sampled for an additional 2 days. Shaded bars represent subjective

night. Symbols above serve as makers to identify the phase relationships. Upside-down triangle represents peak expression of a P_{kaiBC}-*luc* reporter at ZT 12, star represents peak KaiC abundance (ZT 15-16), and the line denotes peak KaiC phosphorylation (ZT 12-16). C) Schematic of KaiC phosphorylation forms and phosphomimetics representing each phosphorylation state. ST, KaiC residues S431 and T432, both not phosphorylated; SpT, KaiC phosphorylated on T432, S431 is not phosphorylated; pSpT, phosphorylation on both S431 and T432; pST, phosphorylation on S431 only. D) Graph representing how each phosphomimetic alters KaiC polar localization demonstrates that KaiC polar localization is not dependent on, but can be modulated by, phosphorylation state. E) KaiC polar complex is stable. FRAP analysis of free YFP compared to YFP-KaiC at the poles demonstrates that, while free YFP recovers rapidly after photobleaching, the YFP-KaiC polar complex is stable, showing little recovery after photobleaching. FRAP at ZT 20/22 for Free YFP and YFP-tagged KaiC, respectively.

Figure S3. Related to Figure 2. Monitoring KaiA localization. A-D) Characterization of KaiA-YFP overexpression construct. A) When expressed at WT levels, KaiA-YFP construct complements the null mutant. WT (blue, 24.6 h period), *kaiA::*Tn*5* (red, arrhythmic), *kaiA::*Tn*5* null strain expressing *kaiA-gfp* expressed from native promoter (green, 26.1 h) or *kaiA-yfp* from uninduced P_{trc} promoter (orange, 25.7 h). B) When overexpressed, KaiA-YFP construct produces high, arrhythmic gene expression. C) Immunoblot of soluble extracts incubated with α-KaiA antiserum. Lane 1, WT; lane 2, *kaiA::*Tn*5*; lane 3, KaiA-YFP not overexpressed; lane 4, KaiA-YFP overexpressed. D) KaiA localization as monitored by KaiA-YFP overexpression in different KaiC phosphomimetic-expressing strains demonstrates that overexpressed KaiA can localize independent of KaiC phosphorylation state. E-F) Fluorescence micrographs of strains

expressing YFP-KaiC mutant variants E) $KaiC^{497}$ or F) $KaiC^{E487A}$ in a Δ*kaiC* background. G) Representative traces from strains that express CikA with and without the addition of the A-loops demonstrate that CikA remains functional when A-loops are fused to its C-terminus (red trace) but not if additionally tagged with ZsGreen at its N-terminus (yellow trace). H) Fluorescence micrographs of strains expressing CikA with the A-loops of KaiC added to the C-terminus show that the A-loops do not affect polar localization of a ZsG-CikA fusion (green). I) A-loops attached to CikA are not sufficient to recruit KaiA to the pole in a Δ*kaiC* mutant background. KaiA overexpressed from P_{trc}-KaiA-YFP (green). Scale bars = 2.5 microns.

Figure S4. Related to Figure 3. Validation of KaiA-ECFP construct. A) Rhythms of gene expression monitored from a P_{kaiB}-*luc* reporter show that expression of KaiA-ECFP from the native promoter complements a *kaiA::*Tn*5* strain. WT (blue, 24.7 h period), *kaiA::*Tn*5* (red, arrhythmic), *kaiA::*Tn*5* expressing *kaiA-ecfp* (green, 26.1 h period). F) Immunoblot of soluble extracts incubated with α-KaiA antiserum demonstrates that KaiA-ECP is expressed as a full-length fusion protein at near WT levels. Lane 1, WT; lane 2, *kaiA::*Tn*5*; lane 3, KaiA-ECFP expressed in a *kaiA::*Tn*5* disrupted background.

Supplemental Fig. 5. Related to Figure 4. A-B) KaiC focus intensity in *S. elongatus cells* at 1 h or 4 h after temperature shift to 25, 30 and 38°C. Each dot represents the average intensity of a single KaiC focus. C) Bioluminescence from P_{kaiB}-*luc* reporter after shift to 40°C (arrow) demonstrates that the YFP-KaiC fusion is functional at high temperatures. WT (blue), Δ*kaiC* + *yfp-kaiC* (green). D) Representative micrographs demonstrating that KaiC and CikA polar localization is not altered in *E. coli clpXP* null strains. FM4-64 membrane stain in red. E) KaiC

localization in *S. elongatus clpX* and *clpP2* disrupted backgrounds. Scale bars = 2.5 microns. F) Immunoblot of soluble extracts incubated with α-ClpP2. Lane 1, WT; lane 2, *clpP2*::Tn*5* + ClpP2-YFP. G) Bioluminescence from strains that carry a P_{kaiB}-*luc* reporter demonstrates that expression of ClpP2-YFP fusion complements a *clpP2*::Tn*5* strain. WT (blue, 25.4 h), *clpP2*::Tn*5* strain (green, 26.6 h), *clpP2*::Tn*5* + ClpP2-YFP (yellow, 25.4 h).

Table S1. Cyanobacterial strains used in this study

Strain	Genotype	Antibiotics	Source
AMC06	WT *Synechococcus elongatus* PCC 7942		Lab collection
AMC1825	P_{kaiB}-*luc* reporter in NS1	SpSm	This work
AMC541	P_{kaiB}-*luc* reporter in NS2	Cm	[S1]
AMC2036	P_{kaiB}-*luc* reporter in NS3	Cm	This work
AMC2158	P_{kaiB}-*luc* reporter in NS3	Nt	This work
AMC704	Δ*kaiC* in-frame deletion in AMC541	Cm	[S2]
AMC1161	Insertional mutation of *kaiA* in AMC541	Km, Cm	[S2]
AMC571	AMC541(P_{kaiB}-*luc*) Δ*cikA*::Gm	Cm, Gm	Lab collection
AMC2254	AMC2036 transformed with pAM4252 (Δ*kaiABC*::Km)	Cm, Km	This work
AMC2295	AMC2254 (Δ*kaiABC*) expressing P_{trc}-*kaiA-yfp* from NS1	Cm, Km, SpSm	This work
AMC571	AMC541(P_{kaiB}-*luc*) Δ*cikA*::Gm	Cm, Gm	Lab collection
AMC2296	AMC1825 (P_{kaiB}-*luc*) and Δ*cikA*::Gm	SpSm, Gm	This work
AMC2297	AMC1161(*kaiA*::Tn*5*) and Δ*cikA*::Gm	Cm, Km, Gm	This work
AMC2298	AMC704 (Δ*kaiC*) and Δ*cikA*::Gm	Cm, Gm	This work
AMC2299	AMC704 (Δ*kaiC*) and P_{trc}-*yfp-kaiC* in NS2 and Δ*cikA*::Gm	Km, Gm	This work
AMC2300	AMC704 (Δ*kaiC*) and Δ*cikA*::Gm and P_{trc}-*kaiA-yfp* in NS1	Cm, Gm, SpSm	This work

Table S2. *E. coli* strains used in this study

Strain	Genotype	Source
MG1655	F^- λ^- *ilvG- rfb-50 rph-1*	Lab collection
GL60	MG1655 and *ibpA-mCherry* expressed from native locus, Km^R	[S3]
BW25113	F^- Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) λ^- *rph-1*Δ*(rhaD-rhaB)568 hsdR514*	Lab collection
Δ*clpX*	BW25113 and Δ*clpX724::frt*	[S4]
Δ*clpP*	BW25113 and Δ*clpP723::frt*	[S4]

Table S3. Plasmids used in this study

Plasmid	Description	Antibiotic Resistance	Source
pAM4252	Km^R Ω-cassette replacing most of *kaiA* and all of *kaiBC*	Km	Lab collection
pAM2969	Km^R Ω insertional mutation of *kaiA*	Km	[S2]
pAM2151	Gm^R Ω-casette replacing most of *cikA*	Gm	Lab collection
pAM2945	Tn*5* insertional mutation of *clpP2*	Km	[S5]
7G3-M2	*Mu* insertional mutation into *kaiA*	Cm	[S5]
8S13-P3	Tn*5* insertional mutation into *clpX* of *S. elongatus*	Km	[S5]
pAM5080	P_{trc}-*yfp-kaiC* expressed in NS2	Km	This work
pAM5081	P_{trc}-*yfp-kaiC* expressed in NS1	SpSm	This work
pAM2914	P_{kaiA}-*kaiA-gfp* expressed in NS1	SpSm	This work
pAM3685	P_{trc}-*kaiA-yfp* expressed in NS1	SpSm	[S6]
pAM5082	P_{kaiB}-*kaiC-ecfp* expressed in NS2	Gm	This work
pAM5083	P_{kaiB}-*kaiC-ecfp* expressed in NS2	Km	This work
pAM5084	P_{trc}-*ecfp-kaiC* cloned into pACYC177	Ap	This work
pAM5085	P_{kaiA}-*kaiA-ecfp* expressed from NS1	SpSm	This work
pAM5086	P_{kaiA}-*kaiA-ecfp* expressed from NS2	Nt	This work
pAM4334	P_{trc}-*clpP2-yfp* expressed from NS1	SpSm	This work
pORF39	ORF39 from large plasmid pMB400 of *Bacillus megataruim* QMB155 expressed from $pCNB^-$ low copy number plasmid	Ap	This work
pAM5087	P_{trc}-*yfp* expressed from NS2	Km	This work
pAM4645	P_{kaiB}-$kaiBC^+$ expressed from NS2	Nt	This work
pAM5089	P_{kaiB}-$kaiB^+$ expressed from NS2	Nt	This work
pAM5090	P_{trc}-$kaiC^+$ expressed from NS2	Nt	This work
pAM5091	P_{kaiB}-$kaiBC^{497}$ expressed from NS2, modified from pAM4645	Nt	This work
pAM5092	P_{kaiB}-$kaiBC^{E487A}$ expressed from NS2, modified from pAM4645	Nt	This work
pAM4648	P_{kaiB}-$kaiBC^{AA}$ expressed from NS2, modified from pAM4645	Nt	This work
pAM4650	P_{kaiB}-$kaiBC^{AE}$ expressed from NS2, modified from pAM4645	Nt	This work
pAM4649	P_{kaiB}-$kaiBC^{DA}$ expressed from NS2, modified from pAM4645	Nt	This work
pAM4651	P_{kaiB}-$kaiBC^{DE}$ expressed from NS2, modified from pAM4645	Nt	This work
pAM4733	P_{kaiB}-$kaiBC^{SE}$ expressed from NS2, modified from pAM4645	Nt	This work
pAM4730	P_{kaiB}-$kaiBC^{ET}$ expressed from NS2, modified from pAM4645	Nt	This work

pAM4734	P_{kaiB}-*kaiBC*^{EE} expressed from NS2, modified from pAM4645	Nt	This work
pAM5093	P_{trc}-*yfp-kaiC*^{497} expressed from NS2, modified from pAM5080	Km	This work
pAM5094	P_{trc}-*yfp-kaiC*^{E487A} expressed from NS2, modified from pAM5080	Km	This work
pAM5095	P_{trc}-*yfp-kaiC*^{AA} expressed from NS2, modified from pAM5080	Km	This work
pAM5096	P_{trc}-*yfp-kaiC*^{AE} expressed from NS2, modified from pAM5080	Km	This work
pAM5097	P_{trc}-*yfp-kaiC*^{DA} expressed from NS2, modified from pAM5080	Km	This work
pAM5098	P_{trc}-*yfp-kaiC*^{DE} expressed from NS2, modified from pAM5080	Km	This work
pAM5099	P_{trc}-*yfp-kaiC*^{SE} expressed from NS2, modified from pAM5080	Km	This work
pAM5100	P_{trc}-*yfp-kaiC*^{ET} expressed from NS2, modified from pAM5080	Km	This work
pAM5101	P_{trc}-*yfp-kaiC*^{EE} expressed from NS2, modified from pAM5080	Km	This work
pAM3389	P_{trc}-Trx-His-*cikA* expressed from NS1	SpSm	[S7]
pAM3645	P_{trc}-ZsGreen-*cikA* expressed from NS1	SpSm	[S7]
pAM5102	P_{trc}-*ZsG-cikA-A-loops* expressed from NS2	Nt	This work
pAM5103	P_{trc}-*cikA-A-loops* expressed from NS1	Nt	This work
pAM3732	P_{trc}-ZsG-*cikA*$^{\Delta N}$ expressed from NS1	SpSm	This work
pAM3733	P_{trc}-ZsG-*cikA*$^{\Delta GAF}$ expressed from NS1	SpSm	This work
pAM3734	P_{trc}-ZsG-*cikA*^{HA} expressed from NS1	SpSm	This work
pAM3646	P_{trc}-ZsG-*cikA*$^{\Delta PsR}$ expressed from NS1	SpSm	[S7]
pAM3390	P_{trc}-Trx-His-*cikA*^{HA} expressed from NS1	SpSm	[S7]
pAM3391	P_{trc}-Trx-His-*cikA*$^{\Delta PsR}$ expressed from NS1	SpSm	[S7]
pAM3392	P_{trc}-Trx-His-*cikA*$^{\Delta N}$ expressed from NS1	SpSm	[S7]
pAM2293	P_{trc}-Trx-His-*cikA*$^{\Delta GAF}$ expressed from NS1	SpSm	[S7]

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions and DNA Manipulations

Plasmids, *E. coli* and *S. elongatus* PCC 7942 strains are described in Tables S1-3. All *S. elongatus* strains were grown as previously described [S8, 9]. Plasmids were constructed using the GeneArt Seamless Cloning and Assembly Kit (Life Technologies) as described elsewhere [S10] and propagated in *E. coli* DH5α with antibiotics as needed. Mutant alleles were made using the QuickChange (Stratagene) protocol and clones were verified by DNA sequencing. Under our laboratory conditions homogenous segregation of alleles was confirmed by PCR for all knock-out and disruption alleles used.

Circadian Bioluminescence Monitoring

Measurement of bioluminescence from *S. elongatus* strains expressing a P_{kaiBC}-*luc* reporter was monitored at 30 °C under LL conditions after two cycles of 12 h light: 12 h dark to synchronize the population as described previously [S9]. Under our conditions the WT strains show bioluminescence rhythms with a period of 25 ± .4 h. Data were analyzed with the Biological Rhythms Analysis Software System import and analysis program using Microsoft Excel (http://millar.bio.ed.ac.uk/pebrown/brass/brasspage.htm).

Immunoblot analysis

Whole-cell extract preparation and immunoblot analysis were performed as previously described [S11]. Equal amounts of total protein from whole-cell extracts (1μg for KaiA or KaiC, 10μg for ClpP2) were separated by SDS-PAGE (10%), transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with 2.5% w/v nonfat dry milk /Tris Buffered Saline + 0.1 % Tween-20 (TBS-T). Membranes were probed with α-KaiC, α-KaiA [S12] or α-ClpP2 [S13], at 1:10,000 in 2.5% milk in TBS-T, followed by several washes in TBS-T and finally probed with horseradish

peroxidase (HRP)-conjugated goat anti-chicken (Aves Labs) for KaiC or HRP-conjugated goat anti rabbit (Calbiochem) for KaiA and ClpP2, both at 1:10,000 in 2.5% milk in TBS-T. Chemiluminescent detection was performed using Pierce SuperSignal West Femto detection reagents (Thermo Scientific).

Fluorescence Microscopy and Image Analysis

Cells were placed on a pad of 1.2% agarose in BG-11 medium and covered with a coverslip. Microscopy was performed with a DeltaVision Core system (Applied Precision) with a WeatherStation attached to an Olympus IX71 inverted microscope and an Olympus Plan Apochromat 100× objective at 30 °C with the following filter settings: tetramethyl rhodamine isocyanate (TRITC) (EX555/EM617), YFP (EX500/EM535), GFP(EX470/EM515), CFP (EX436/EM470). Images were captured using a CoolSnap HQ CCD camera (Photometrics) and deconvolved using the SoftWorx imaging program (Applied Precision). Exposure times are limited to conditions where we do not observe fluorescence from WT strains in either the GFP or YFP channels to limit bleed-through from thylakoid fluorescence. For 3D-SIM, cells were imaged using a Delta Vision OMX version 3 prototype (Applied Precision). For analysis of KaiC localization in time course experiments, aliquots of cells were taken at designated time points and fixed directly in growth medium (BG11) with a final concentration of 2.4% (vol/vol) paraformaldehyde (Electron Microscopy Sciences) in 30 mM $NaPO_4$ buffer (pH 7.5) for 20 min at room temperature before they were moved to 4 °C. For imaging *E. coli* strains aliquots of cells were stained with the membrane dye FM4-64 (240 ng/ml-1 µg/ml; Molecular Probes). Images were colorized in SoftWorx and then transferred to Photoshop (Adobe) for figure assembly. To track the number and location of KaiC foci per cell, we developed an algorithm to analyze fluorescent images in MATLAB® using Image Toolbox ™. To identify cell and foci objects, we

first subtracted background from the cell and KaiC images and then filtered them using median filter. We generated preliminary masks of the cell and foci objects with otsu threshold algorithm. The mask was further refined by user input parameters such as cell width, solidity, area, and length-to-width ratio, which were used to break apart clumped cell objects. Foci objects were assigned to each cell based on the amount of overlap between cell and object masks. To identify cells with foci, we calculated the coefficient of variation (CV) of KaiC fluorescence inside the cell object. Cells whose KaiC fluorescence CV was above a user-defined threshold, were deemed to contain KaiC foci. For cells with foci, we fit an ellipse to the cell with the regionprops.m function and then calculated foci centroid position relative to the center of the fitted ellipse (scaled by the dimensions of the ellipse). Fluorescence Recovery after Photobleaching (FRAP) was performed and analyzed as previously described [S14]. Briefly, a small portion of a *S. elongatus* cell containing a focus or diffuse YFP was photobleached using a 488-nm argon laser (QLM module, API) for 0.01 s for free YFP or 0.05 s for YFP-KaiC at 70% power and then followed with time lapse imaging recorded every 0.3 s.

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