Supporting Information

Espinosa et al. 10.1073/pnas.1424632112

Extended Phos-Tag Methods

To analyze RpaB and RpaA phosphorylation, protein samples were resolved using 25 μM Phos-tag acrylamide gels in combination with immunoblotting detection with polyclonal antibodies raised against RpaB and RpaA. IPTG (1 mM) was added to exponentially growing cells and at indicated times a cell aliquot was harvested by centrifugation (4 °C, 10,000 × *g*), frozen in liquid nitrogen, and stored at −20 °C. Cell extracts were prepared by mechanic cell lysis using glass beads (three rounds of 1 min using a Minibeadbeater with cooling between cycles). Unbroken cells, debris, and glass beads were pelleted down (5 min 5,500 × *g*) and the supernatant collected. Protein content was determined using the modified Lowry method (Bio-Rad). We used proteins extracts with no more than two freeze–thaw cycles as we notice that repeated cycles promote phospho-hydrolysis of RpaB~P and RpaA~P.

When necessary, cells were entrained to at least two 12:12 LD cycles: exponentially growing cells were diluted with fresh media to DO_{750} 0.3 and incubated in LD. Cells were released in LL or kept in LD and aliquots harvested at indicated timepoints and processed as described above.

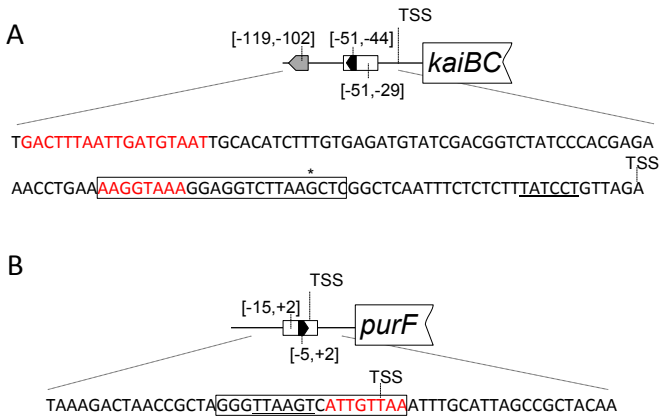

Fig. S1. Schematic representation and regulatory sequences of the *kaiBC* and *purF* promoter regions. In both A and B empty, gray and black boxes represent RpaA, RpaB and half RpaB sites, respectively, with numbers indicating their positions relative to the transcription start sites (TSS). Nucleotides, RpaA (boxed) and RpaB (in red) recognition sites are shown. The putative −10 elements are underlined. (*A*) *kaiBC* and B) *purF*. In *A* and *B*, the TSS were reported by Kutsuna et al. (1) and Vijayan et al. (2), respectively. Alternative start points were described for *kaiBC* (2) (indicated with an asterisk) and in *purF* (3) (90 nt upstream of the indicated TSS). The half RpaB motif in *kaiBC* was described by Hanaoka et al. (4). RpaB sites were predicted in silico, whereas RpaA sites correspond to nucleotides protected in footprinting assays (5).

1. Kutsuna S, Nakahira Y, Katayama M, Ishiura M, Kondo T (2005) Transcriptional regulation of the circadian clock operon *kaiBC* by upstream regions in cyanobacteria. *Mol Microbiol* 57(5):1474–1484.
2. Vijayan V, Jain IH, O'Shea EK (2011) A high resolution map of a cyanobacterial transcriptome. *Genome Biol* 12(5):R47.
3. Liu Y, Tsinoremas NF, Golden SS, Kondo T, Johnson CH (1996) Circadian expression of genes involved in the purine biosynthetic pathway of the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Mol Microbiol* 20(5):1071–1081.
4. Hanaoka M, et al. (2012) RpaB, another response regulator operating circadian clock-dependent transcriptional regulation in *Synechococcus elongatus* PCC 7942. *J Biol Chem* 287(31): 26321–26327.
5. Markson JS, Piechura JR, Puszynska AM, O'Shea EK (2013) Circadian control of global gene expression by the cyanobacterial master regulator RpaA. *Cell* 155(6):1396–1408.

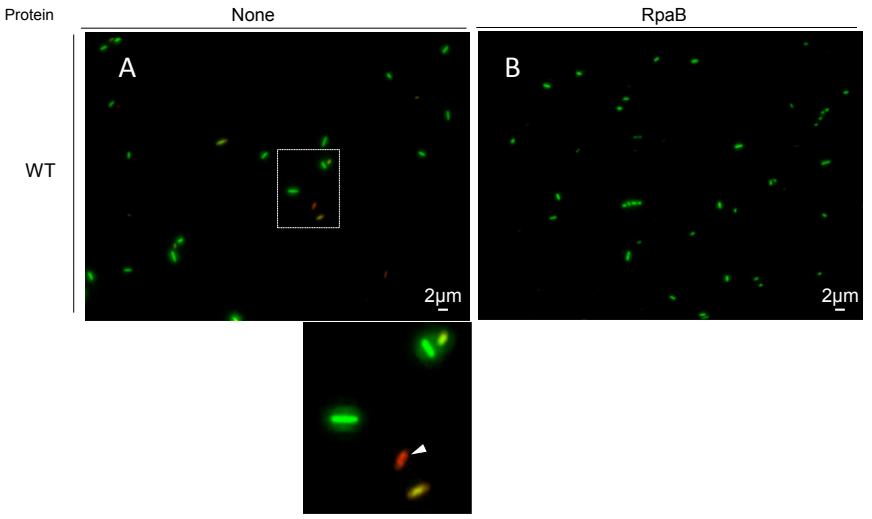

Fig. S2. Cell viability test on the basis of membrane integrity after 96 h of overexpression of RpaB and staining with "LIVE/DEAD BacLight kit" following the manufacturer's instructions. After staining with a mixture of the SYTO 9 and propidium iodide stains, cells with intact membranes fluoresce in green, whereas those with damaged membranes fluoresce in red (because of cyanobacterial pigments, autofluorescence, and propidium iodide emission spectrum overlap). Fluorescence was recorded with a Leica fluorescence microscope at a magnification of 1,000×. The dashed box was magnified 3× to show an example of a cell with nucleic acids stained in red by propidium iodide (white arrowhead) that would be classified as dead. WT background without and with RpaB-overexpression are shown in *A* and *B*, respectively.

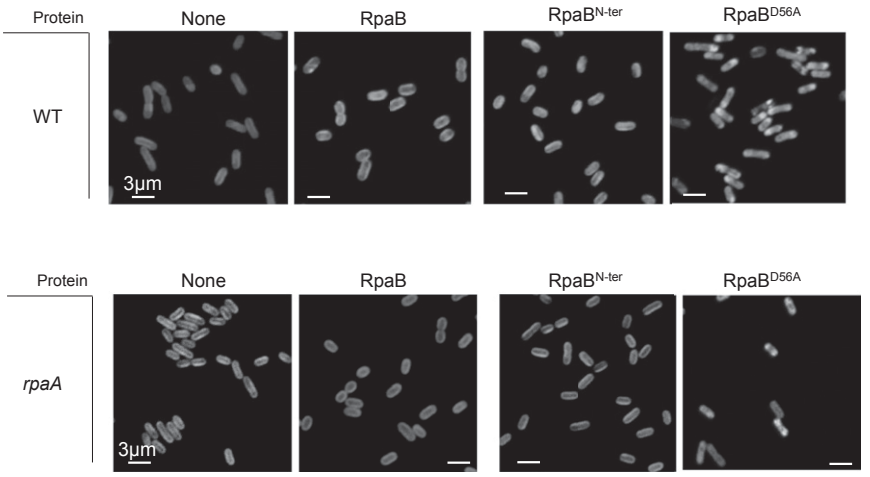

Fig. S3. Cell appearance under confocal microscope of the indicated strains after 72 h of IPTG induction. Note that 1Ptrc-rpaB^{D56A} shows atypical autofluorescence distribution.

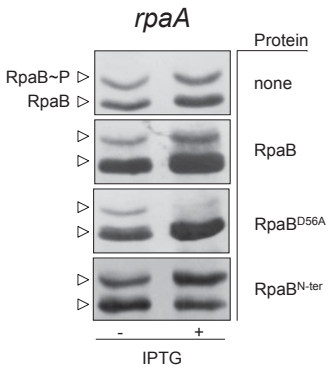

Fig. S4. Immunodetection of RpaB~P and RpaB by Phos-tag in the *rpaA*-null mutant that overexpresses RpaB, RpaB^{D56A}, or RpaB$^{N\text{-}ter}$. Midexponential phase cells were collected before inducer addition (−IPTG) and after 4 h of incubation (+IPTG). Immunodetected bands are indicated at the left. Note that RpaB levels are elevated even in the absence of inducer, as is common for Ptrc-expressed genes in *Synechococcus elongatus* (1).

1. Zhang X, Dong G, Golden SS (2006) The pseudo-receiver domain of CikA regulates the cyanobacterial circadian input pathway. *Mol Microbiol* 60(3):658–668.

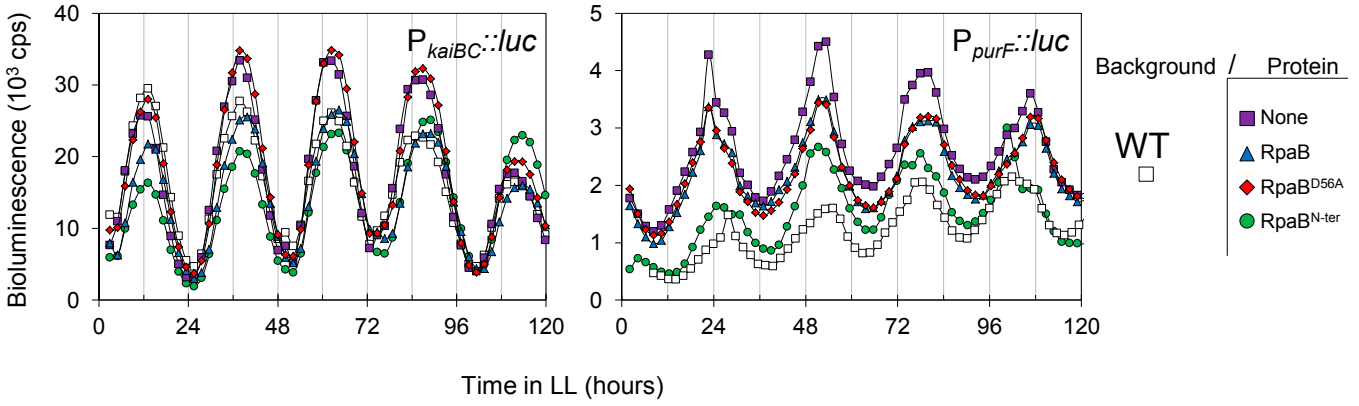

Fig. S5. Bioluminescence patterns from P$_{kaiBC}$::*luc* and P$_{purF}$::*luc* fusions in the indicated strains without IPTG.

Table S1. Strains and plasmids used in this work

Strain/plasmid	Genotype or relevant characteristics	Source
WT	Wild-type *Synechococcus elongatus* PCC 7942	Pasteur culture collection
AMC541	WT P*kaiBC*::*luc* NS2, Cm^r	(1)
AMC601	WT P*purF*::*luc* NS2, Km^r	(2)
1P*trc**,†	P*trc* NS1, Sm^r	Present work
1P*trc*-rpaB*,†	Φ(P*trc*::*rpaB*) NS1, Sm^r	Present work
1P*trc*-rpaB$^{N\text{-}ter}$*,†	Φ(P*trc*::*rpaB*$^{1\text{-}131}$) NS1, Sm^r	Present work
1P*trc*-rpaB^{D56A}*,†	Φ(P*trc*::*rpaB*^{D56A}) NS1, Sm^r	Present work
rpaA†	*rpaA*::Gm, Gm^r	(3)
1P*trc*-rpaB *rpaA*†	*rpaA*::Gm, Φ(P*trc*::*rpaB*) NS1, Sm^r Gm^r	Present work
*cikA**,†	*cikA*::Gm, Gm^r	(4)
1P*trc cikA**,†	*cikA*::Gm, P*trc* NS1, Sm^r Gm^r	Present work
1P*trc*-rpaB *cikA**,†	*cikA*::Gm, Φ(P*trc*::*rpaB*) NS1, Sm^r Gm^r	Present work
1P*trc*-rpaB$^{N\text{-}ter}$ *cikA**,†	*cikA*::Gm, Φ(P*trc*:: *rpaB*$^{1\text{-}131}$) NS1, Sm^r Gm^r	Present work
1P*trc*-rpaB^{D56A} *cikA**,†	*cikA*::Gm, Φ(P*trc*::*rpaB*^{D56A}) NS1, Sm^r Gm^r	Present work
sasA	Gm cassette cloned into *sasA*, Gm^r	(5)
kaiC	Ω-cassette inserted into *kaiC*, Km^r	(3)
rpaA	Gm cassette cloned into *rpaA*, Gm^r	(3)
1P*trc*	P*trc* NS1, Sm^r	(6)
1P*trc*-rpaB	Φ(P*trc*::*rpaB*) NS1, Sm^r	(6)
1P*trc*-rpaB$^{N\text{-}ter}$	Φ(P*trc*::*rpaB*$^{1\text{-}131}$) NS1, Sm^r	Present work
1P*trc*-rpaB^{D56A}	Φ(P*trc*::*rpaB*^{D56A}) NS1, Sm^r	Present work
1P*trc rpaA*	P*trc* NS1, *rpaA*::Gm, Sm^r	Present work
1P*trc*-rpaB *rpaA*	Φ(P*trc*::*rpaB*) NS1, *rpaA*::Gm (heteroallelic), Sm^r	Present work
1P*trc*-rpaB$^{N\text{-}ter}$ *rpaA*	Φ(P*trc*::*rpaB*$^{1\text{-}131}$) NS1, *rpaA*::Gm, Sm^r	Present work
1P*trc*-rpaB^{D56A} *rpaA*	Φ(P*trc*::*rpaB*^{D56A}) NS1, *rpaA*::Gm, Sm^r Gm^r	Present work
pAM2152	*cikA*::Gm, Gm^r	(4)
pAM2176	*sasA*::Gm, Gm^r	(5)
pAM4523	*rpaA*::Gm, Gm^r Ap^r	(3)
pUAGC758	Φ(C.K1(+)-*rpaB*), Ap^r Sm^r	(6)
pUAGC763	Φ(C.S3(+)-*rpaB*), Ap^r Sm^r	(6)
pUAGC280	C.S3 *lacI*^{q} and P*trc*, into NS1, Ap^r Sm^r	(6)
pUAGC282	pUAGC280 with P*trc*-*rpaB*, Ap^r Sm^r	(6)
pUAGC283	pUAGC280 with P*trc*::*rpaB*$^{N\text{-}ter}$, Ap^r Sm^r	Present work
pUAGC284	pUAGC280 with P*trc*::*rpaB*^{D56A}, Ap^r Sm^r	Present work

*In reporter strain AMC541.
†In reporter strain AMC601.

1. Ditty JL, Williams SB, Golden SS (2003) A cyanobacterial circadian timing mechanism. *Annu Rev Genet* 37:513–543.
2. Ditty JL, Canales SR, Anderson BE, Williams SB, Golden SS (2005) Stability of the *Synechococcus elongatus* PCC 7942 circadian clock under directed anti-phase expression of the *kai* genes. *Microbiology* 151(Pt 8):2605–2613.
3. Paddock ML, Boyd JS, Adin DM, Golden SS (2013) Active output state of the *Synechococcus* Kai circadian oscillator. *Proc Natl Acad Sci USA* 110(40):E3849–E3857.
4. Zhang X, Dong G, Golden SS (2006) The pseudo-receiver domain of CikA regulates the cyanobacterial circadian input pathway. *Mol Microbiol* 60(3):658–668.
5. Dong G, et al. (2010) Elevated ATPase activity of KaiC applies a circadian checkpoint on cell division in *Synechococcus elongatus*. *Cell* 140(4):529–539.
6. Moronta-Barrios F, Espinosa J, Contreras A (2013) Negative control of cell size in the cyanobacterium *Synechococcus elongatus* PCC 7942 by the essential response regulator RpaB. *FEBS Lett* 587(5):504–509.

Table S2. Oligonucleotides used in this work

Oligo name	Sequence
RpaB-ptrc-1F	5′-AGAGGGAATTCTTGGAAAATCGCAAG-3′
RpaB-HCN-1R	5′-GATCGGATCCGGCGCTGGCTGCTCTAAC-3′
RpaB-HCN-2R	5′-GTCGGGATCCTAGCTGTTGATCTGGATG-3′