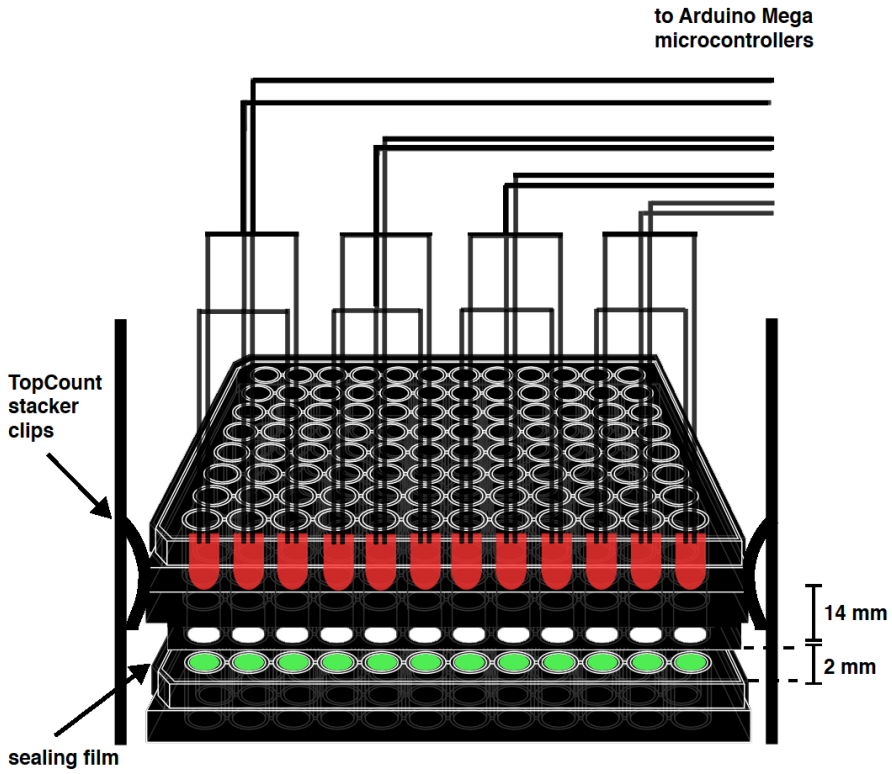
Supplemental Figures

Figure S1, related to Figure 1:

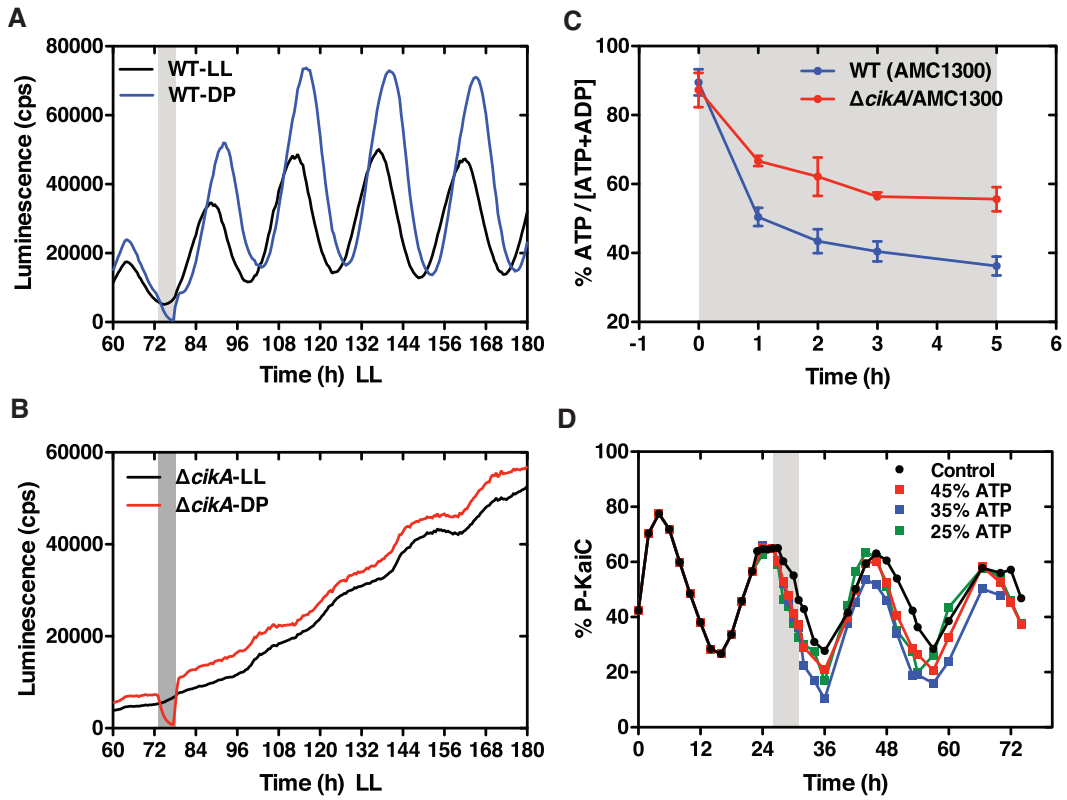


Schematic of LED illuminator used for phase-response curve measurements.

A P200 pipette tip rack was spray-painted black, aligned and glued to a drilled-out black plastic 96-well plate. The holes in the tip rack served as a mount for LEDs, and the 96-well plate acted as a baffle to ensure that each LED illuminated only the culture directly beneath it. Four LEDs were connected in series through a circuit board layer immediately above the pipette tip rack.

Only the first row of LEDs is shown for clarity. Leads from the circuit board connected the LEDs to two Arduino Mega microcontrollers that powered the illuminator via analogue outputs. The device was aligned and held above the sample using the metal clips in a TopCount plate stacker.

Figure S2, related to Figure 1:



Defective phase resetting and metabolic phenotypes of Δ*cikA*.

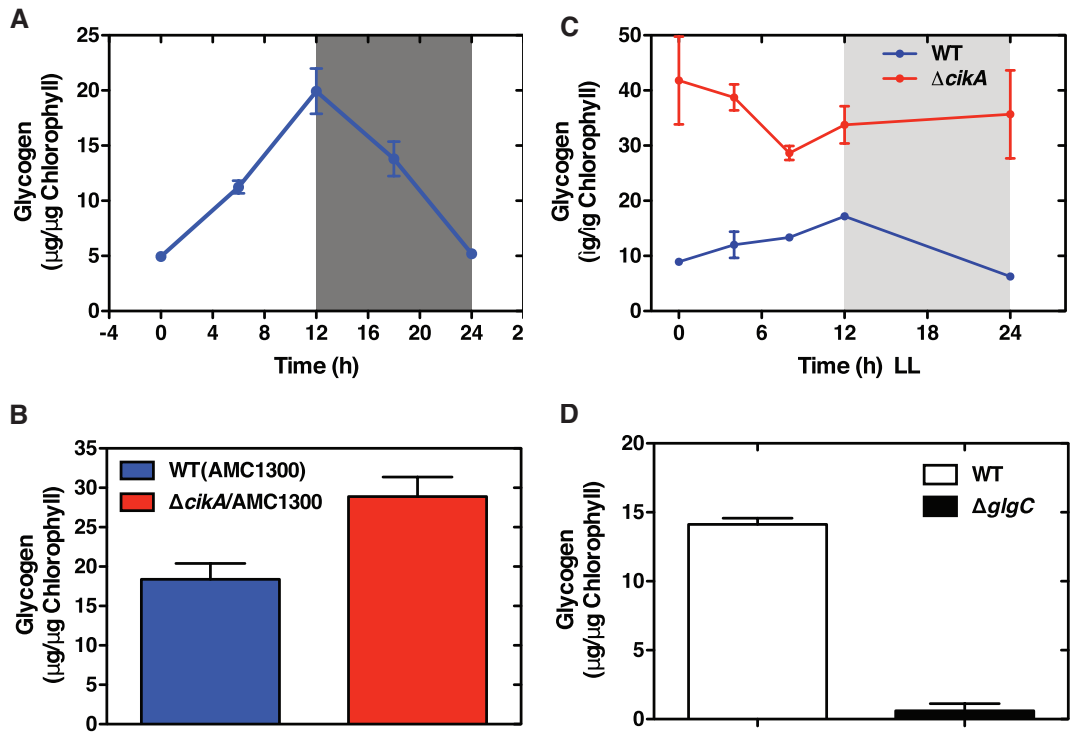
(A-B) Bioluminescence rhythms (P*kaiBC*::*luxAB*) in WT (A) and Δ*cikA* (B). Control samples (*black*) were kept in LL while experimental samples (*blue or red*) subjected to a 5-hour dark pulse (*shaded bar*). Cells were entrained by one light- dark cycle (12 h:12 h) and then released into LL.

(C) Drop in the ATP / (ATP + ADP) energy charge during a 5-hour dark pulse (*shaded region*) in WT (AMC1300) (*blue symbols*) and Δ*cikA*/AMC1300 (*red symbols*) cells where *cikA* is deleted in the AMC1300 background. Bars represent range (n = 2).

(D) Phase shifts in the KaiC phosphorylation rhythm in the *in vitro* KaiABC oscillator caused by changing the ATP/ADP ratio during the dephosphorylation phase. ATP / (ATP + ADP) was

lowered from 80% to various levels by addition of ADP during a 5-hour pulse (*shaded bar*) that was given after the peak phosphorylation of KaiC. The reactions were then returned to the initial buffer via desalting column.

Figure S3, related to Figure 2:



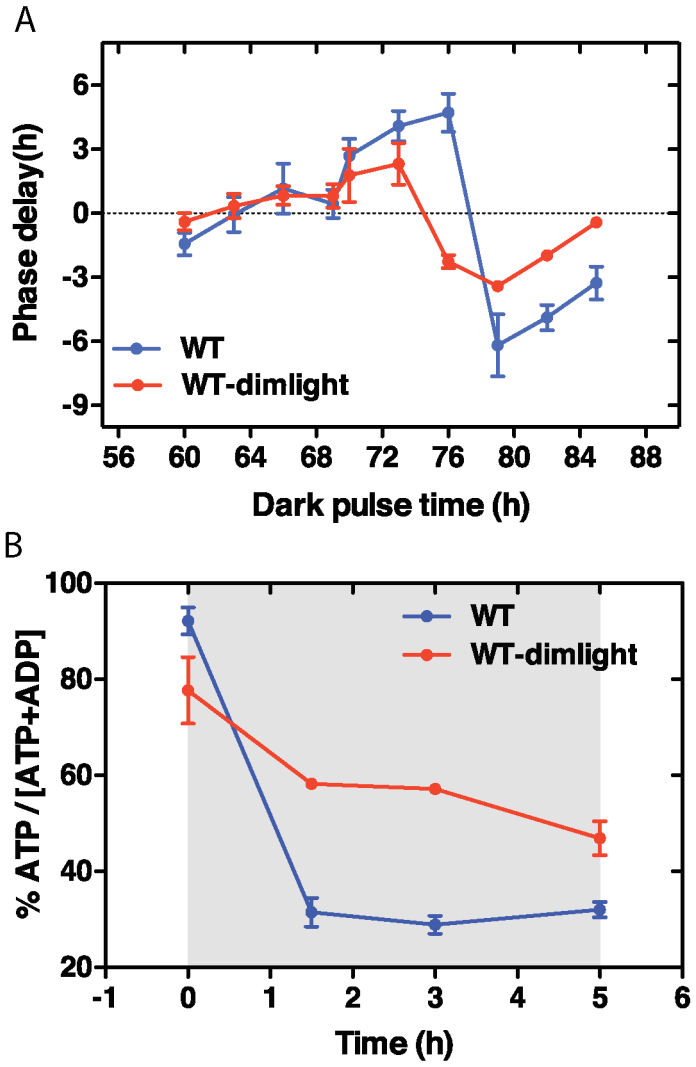
Glycogen content in wildtype (WT) and mutant strains.

(A) Diurnal changes of glycogen content in WT cells. Cultures were grown for two days in light-dark cycles (12 h:12 h). After the end of the 2nd dark cycle (t = 0), samples were harvested every 6 hours for the next 24 hours. Shaded bar indicates darkness. Error bars represent standard deviations (n = 3).

(B-C) Glycogen content in WT (AMC1300, *blue bar*) and Δ*cikA*/AMC1300 (*red bar*) cells (B), where *cikA* is deleted in the AMC1300 background, and glycogen timecourse in WT (*blue symbols*) and Δ*cikA* (*red symbols*) cells (C). Cultures were entrained by a 12 h:12 h light-dark cycle and subsequently released into LL (t = 0). Samples were harvested at t = 12 h (B) and then at 4 h intervals (C). Shaded region represents the subjective nights. Error bars represent standard deviations (n = 4).

(D) Glycogen content in WT and Δ*glgC* cells. Cultures were entrained by a 12 h:12 h light-dark cycle and subsequently released into LL (t = 0). Samples were harvested at t = 12 h. Bars represent range (n = 2).

Figure S4, related to Figure 4



Phase resetting curve and metabolic changes in cultures under dim illumination.

(**A**) Phase delay of the bioluminescence rhythm (P*kaiBC*::*luxAB*) in response to 5-hour dark pulses at the indicated time. Wild-type (WT; *blue symbols*) cells were entrained by one light-dark cycle (12 h:12 h) and then released into constant light (LL; light intensity is ~75 μmol photons m^{-2} s^{-1}). Similarly, for dim light illumination, WT cells (WT-dimlight; *red symbols*) were entrained by one light- dark cycle (12 h:12 h) and then released into constant light, where the light intensity was held at ~15-20 μmol photons m^{-2} s^{-1}. Phase delays relative to an untreated control were estimated by fitting the time series to a sinusoid. Error bars represent standard deviations (n = 8).

(**B**) Drop in the ATP / (ATP + ADP) energy charge during a 5-hour dark pulse (begins at *shaded region*). Wild-type (WT) cells were entrained by one light- dark cycle (12 h:12 h) and then released into constant light (~90 μmol photons m^{-2} s^{-1} for WT: *blue symbols,* and ~10 μmol photons m^{-2} s^{-1} for WT-dimlight: red symbols, respectively). After 25 h of growth in constant light, cultures were exposed to dark and samples were harvested at selected time points for ATP/(ATP+ADP) measurement. Bars represent standard errors (n = 4) for t=0 before the dark pulse and range of two replicates for later time points.

Supplemental Table 1

See attached spreadsheet.

Up- and down-regulated genes in Δ*cikA*.

Columns indicate the JGI identifier for each gene, the gene name, circadian phase measured out of 360 degrees as determined by Vijayan *et al.* [S7], and Z score for mean expression relative to wildtype. Genes that are up- or down-regulated with $p < 0.01$ by two-sided *t* test are included in the table.

Supplemental Experimental Procedures

Cyanobacterial Strains

The reporter strain AMC1300 carries P*kaiBC::luxAB and* P*psbAI::luxCDE,* so that the cultures autonomously produce the enzyme luciferase and the long-chain aldehyde substrate for luciferase, respectively [S1]. In AMC1300, P*kaiBC::luxAB* is integrated at NS1 with a spectinomycin resistance cassette and P*psbAI::luxCDE* is integrated at NS2 with a kanamycin resistance cassette [S1]. In the previously generated AMC1005 (Δ*cikA*) strain, P*kaiBC::luxAB* is integrated at NS2 with a kanamycin resistance cassette and P*psbAI::luxCDE* is integrated at NS2.2 with a chloramphenicol resistance cassette [S2]. AMC1005 and AMC1300 were kind gifts of Susan S. Golden (University of California, San Diego). The other strains used in this study i.e., *cikA*-null (MRC1001), *kaiBC*-null (MRC1002), *sasA*-null (MRC1003), and *glgC*-null (MRC1004) are derivatives of the AMC1300 background.

Molecular Cloning and Construction of Mutant Strains

Basic DNA manipulation was performed by standard procedures. *S. elongatus* transformations were performed as described earlier [S3]. A BssHII and SphI digested 1.13-kb DNA fragment carrying the gentamycin resistance cassette (Gm^r) was taken from pAM3558 [S4] end-filled and inserted into SmaI-digested pBluescript II SK^+ to create the plasmid pMR0089.

To disrupt the *cikA* gene from the Synechococcus genome, DNA fragments were PCR amplified from the genomic DNA using two pairs of oligonucleotide primers, 5'-GGAAGCTTATGCTGGCACCATCCTCAAA-3' (cikAko1) and 5'-GGGAATTCTAGCGCTGAATACTGTCAGC-3' (cikAko2) and, 5'-GGTCTAGACAGCTACGGTCGTCTGTGAA-3' (cikAko3) and 5'-

GGGAGCTCTGCAAGGTCGTCAACAGCAG-3' (cikAko4), respectively. The DNA fragment (301 bp) amplified by cikAko1/cikAko2 primers was digested with Hind III and EcoRI (underlined) and inserted into pMR0089 plasmid at the respective restriction enzyme sites, to create the pMR0089.1 plasmid. The DNA fragment (299 bp) amplified by cikAko3/cikAko4 primers was digested with XbaI and SacI (underlined) and cloned into the pMR0089.1 plasmid at the respective restriction sites, resulting in the pΔ*cikA::Gm*^{r} plasmid (pMR0090). AMC1300 was then transformed with the pMR0090 plasmid to generate the *cikA* null strain (Δ*cikA*; MRC1001).

Similarly, to disrupt the *kaiBC* from genome, DNA fragments were amplified by PCR from the genomic DNA using two pairs of oligonucleotide primers, 5'-GGAAGCTTAAAGGTAAAGGAGGTCTTA-3' (BCko1) and 5'-GGGAATTCCACCTTGAAATTCAACTTCG-3' (BCko2) and, 5'-GGACTAGTGCAACAATGCCTTCCGCCAA-3' (BCko3) and 5'-GGGAGCTCCTTGAACGCCGCGCACAATT-3' (BCko4), respectively. The DNA fragment (201 bp) amplified by BCko1/BCko2 primers was digested with HindIII and EcoRI (underlined) and inserted into pMR0089 plasmid at the respective restriction enzyme sites, creating the pMR0089.2 plasmid. The DNA fragment (378 bp) amplified by BCko3/BCko4 primers was digested with SpeI and SacI (underlined) and cloned into the pMR0089.2 plasmid at the respective restriction sites resulting in the pΔ*kaiBC::Gm*^{r} plasmid (pMR0091). AMC1300 was then transformed with the pMR0091 plasmid was used to generate the *kaiBC* null strain (Δ*kaiBC*; MRC1002).

We replaced the kanamycin resistance cassette with the Gm^r cassette in the plasmid pSasA-Nina (kindly provided by Carl H. Johnson, Vanderbilt University). The BssHII and SphI

digested Gm^r cassette from pAM3558 plasmid was end filled and inserted into DraIII and SmaI digested and blunted pSasA-Nina to generate the p∆*sasA::Gm*^{r} plasmid (pMR0092). AMC1300 was transformed with pMR0092 plasmid was used to make the *sasA* null strain (∆*sasA*; MRC1003).

To disrupt the AGPase gene (*glgC*), the genomic regions flanking the *glgC* were amplified by PCR using two pairs of oligonucleotide primers, 5'-GGAAGCTTAGATGATGTCAGCCCCTGCTTT -3' (glgCko1) and 5'-GAGAATTCCAAGTCAAGCGGCGCTGAGA-3' (glgCko2) for the upstream region and 5'-GGGGATCCGTGGTCAAAGGGGCGGTTATT-3' (glgCko3) and 5'-A GGGAGCTCATCTTCATTATTGATGGTAGTTG-3' (glgCko4) for the downstream region, respectively. The upstream fragment (460 bp) was restriction digested with Hind III and EcoRI (underlined) and inserted into pMR0089 plasmid at the respective restriction enzyme sites resulting in pMR0089.3 plasmid. The downstream fragment (894 bp) was digested with BamHI and SacI (underlined) and cloned into pMR0089.3 plasmid that generated the p∆*glgC::Gm*^{r} plasmid (pMR0093). AMC1300 was transformed with the pMR0093 plasmid was used to make the *glgC* null strain (∆*glgC*; MRC1004).

All the mutant strains were allowed to segregate completely by growing them repeatedly in BG-11M liquid medium supplemented with appropriate antibiotics followed by genomic DNA extraction and confirmation of the absence of the wild-type allele or presence of the mutant allele by PCR analysis.

Circadian Bioluminescence Measurements

We used luciferase reporter strains and a PerkinElmer TopCount luminometer to assay bioluminescence rhythms. For TopCount experiments 30 μL of culture samples (OD_{750} ~0.4) were inoculated onto 250 μL BG-11M agar pads in a 96-well black plastic plate. The plate was sealed with adhesive film and a hole was punched for aeration over each well. Plates were placed under a custom-made LED illuminator consisting of red LEDs (628 nm peak emission, 8° viewing angle, Super Bright LEDs, Inc.) mounted in a black 96-well plate glued to a second, drilled-out 96-well plate to act as a baffle (see Fig. S1). Arduino Mega microcontrollers supplied power (1 V across each LED) according to a pre-programmed schedule to groups of four LEDs connected in series. The LED array provided the equivalent of ~75 μmol photons m^{-2} s^{-1} of red light to each well. To assay bioluminescence rhythms under dim illumination, the voltage applied to the LED arrays was reduced, so that the arrays provided the equivalent of 15-20 μmol photons m^{-2} s^{-1} of red light to each well. This device was mounted in the TopCount plate stacker so that it illuminated the 96-well plate being studied (see Figure. S1).

To synchronize the circadian clock, the cultures were acclimated to one light-dark cycle (12 h:12 h) and the released into LL. While growing in LL, cells were received a 5- hour dark pulse at the specified time and were then returned to LL to monitor the circadian rhythm. Untreated control cultures (no dark pulse) were grown side-by-side on the same plate for comparison. We collected bioluminescence measurements every 30 min. The bioluminescence from each well was integrated over 5 seconds.

To measure phasing and period of the rhythms, we log-transformed the bioluminescence data, then fit the resulting time series to a sinusoid with linear offset using least-squares regression. For experiments with dark pulses, we selected a region beginning >24 h after the end of the dark pulse to use to fit the data. Phase and period of the circadian rhythms were extracted

from the best-fit parameters. Phase differences were measured as the phase difference between experimental samples and the average of the constant light controls.

Nucleotide Analysis

Perchloric Acid Extraction

Cultures were exposed to a 12-h dark pulse to synchronize the circadian clock and then returned to LL (~60 μmol photons m^{-2} s^{-1}) conditions in a Percival incubator to free run. Synchronizing cultures were grown in LL for 50 h and then transferred to dark for 5 h. For nucleotide measurement under dim illumination, cultures were entrained by one light-dark cycle (12 h:12 h) and then released into constant light (~10 μmol photons m^{-2} s^{-1}); the reference bright illumination culture was kept at (~90 μmol photons m^{-2} s^{-1}). After 25 h of growth in constant light, cultures were exposed to dark and samples were harvested at desired time points. We took special care to not alter the light exposure of the culture in the time between sampling and destruction of cells by injection into perchloric acid. Samples (3 mL) were harvested after 25 hours and 50 hours of growth in LL (t = 0 h) and each hour after dark incubation. 3 mL of liquid cultures (OD_{750} ~ 0.3) were added rapidly to 0.75 mL of ice cold 3 M perchloric acid with 77 mM EDTA, vortexed immediately and incubated on ice for 5 minutes. The mixture was then neutralized with 2 mL of 1 M KOH, 0.5 M Tris, 0.5 M KCl to give pH ~7 by pH paper and centrifuged (4000 rpm) for 20 min at 4°C. The supernatant from the neutralization reaction was filtered through a 10 kDa centrifugal filter (Amicon Ultra-4, Merck Millipore Ltd.) and stored at –80°C.

Luciferase Assay for ATP and ADP

Samples from -80°C were thawed on ice and centrifuged at 4°C. Supernatants were taken for nucleotide measurement. For [ATP] measurement, extracts were diluted 2.6x in a buffer containing 25 mM KCl, 50 mM $MgSO_4$ and 100 mM HEPES pH 7.4 (LK buffer) along with 1 mM phosphoenolpyruvate (PEP). To measure [ADP + ATP], we diluted extracts as above except that we also added 3 U / mL type II pyruvate kinase from rabbit muscle (Sigma-Aldrich) to catalyze the conversion of ADP to ATP. Extracts were incubated for 33 minutes at 37°C and then were incubated at 90°C for 11 min to heat inactivate pyruvate kinase. After heat inactivation, the samples were allowed to cool to room temperature. We added 260 μL of these extracts to individual 1.5 mL microcentrifuge tubes preloaded with 0, 25, 50 or 75 pmol of ATP as internal standards and 260 μL of LK buffer. We rapidly added 30 μL of a solution containing 35 μg / mL firefly luciferase (Sigma-Aldrich) and 1 mM luciferin (Sigma-Aldrich) in LK buffer to the microcentrifuge tubes one by one, mixed by pipetting and then incubated in a luminometer (Glomax, Promega) in the dark for 30 sec at 25°C before recording the luminescence signal from each sample. We determined [ATP] and total nucleotide [ADP + ATP] content in each tube by linear regression from the four internal standards used for each samples.

Kai Protein Expression and Purification

Recombinant KaiC was expressed as an N-terminal GST fusion from the pET41b(+) vector in the BL-21 (DE3) strain of *E. coli*. Overnight starters were made from fresh transformants before dilution into 1-L cultures in LB medium containing 50 μg/mL kanamycin. Cultures were then grown at 37°C to $OD_{600} \approx 0.6$, chilled to 16°C, and induced overnight with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were harvested the next morning by centrifugation and flash-frozen in liquid nitrogen. To purify KaiC, resuspended cell pellets were passed through a

chilled Emusiflex-C3 homogenizer (Avestin). Soluble KaiC from the clarified lysate was bound to a GSTrap column (GE Healthcare) that was washed with buffer containing 5 mM ATP, before elution with 40 mM reduced L-glutathione (Sigma Aldrich). The GST tag was then cleaved by overnight incubation at 4°C with HRV 3C protease (EMD Millipore) before passage through a HiPrep 16/60 S-300 (GE Healthcare) size-exclusion column. Fractions consistent with the expected molecular weight of a KaiC hexamer were pooled, concentrated to 40–55 μM using a 30-kDa centrifugal filter (Millipore), and flash-frozen in small aliquots. Recombinant KaiA and KaiB proteins were expressed and purified as described previously [S5].

Glycogen Measurement

Cultures were exposed to a 12-h dark pulse to synchronize the circadian clock and then returned to LL conditions to free run. After 24 h in LL, cultures corresponding to 4.5 mL at an OD_{750} ~ 0.3 were collected at 4 h intervals for the next 48 h and cell palettes were frozen at -80°C for glycogen analysis. Cell palettes from -80°C were resuspended thoroughly in 1 mL of methanol and centrifuged at 15,000 rpm for 5 min at 4°C. The chlorophyll content in the supernatant was determined [S6]. Glycogen content in each sample was normalized to the chlorophyll content. Cultures were held semi-turbidostatically at OD_{750} ~ 0.3 by manual dilution with buffered BG11-M every 3-4 hours.

To determine the cellular glycogen contents, the pellets were resuspended in 600 μL of 40% KOH followed by incubation at 95°C for 1.5 h. 1.2 mL of 100% ethanol was added to each extracts and the samples were incubated at -20°C for 16 h to precipitate the glycogen. Then, samples were centrifuged for 1 h at 4°C, pellets were washed two times with 100% ethanol, then resuspended in 200 μL of 2 N HCL and incubated at 95°C for 30 min. 200 μl of 2 N NaOH and

100 μL 1 M phosphate buffer, pH 7, and 500 μL of distilled water were added to each sample. Briefly, 75 μl of sample extract was mixed with 200 μl of enzyme solution in a 96-well plate (Costar, ultraviolet light proof) and incubated for 20 min at 25°C. The resulting generation of NADPH was measured at 340 nm using a microplate reader (Tecan Safire 2). Glycogen from bovine liver Type IX (Sigma) was used as a standard.

Microarray Expression Analysis

60 ng of total RNA for each time point was mixed with appropriate amount of One-Color Spike Mix (Agilent, Inc). Total RNA (plus spike-ins) was converted to cDNA by using the cDNA master mix and amplified into cyanine 3-CTP labeled cRNA using Low Input Quick Amp WT Labeling kit according to Agilent One Color Microarray Based Exon Analysis (Agilent, Inc). Each array was hybridized with 600 ng of Cyanine 3-labeled cRNA and incubated with rotation (10 rpm) at 65°C for 17 h in an Agilent hybridization oven. Arrays were subsequently washed in wash buffer I and II provided by Agilent and scanned immediately at wavelength 532 nm to extract Tiff image files using GenePix 4000B scanner (GenePix pro 6.0 software) at 5-μm resolution with PMT voltage setting to 690 and power (%) to 100 for Cy3 channel (Axon instrument, Inc). The data was extracted by Agilent Feature Extraction Software v9.5 from the image files.

To analyze the microarray time courses, we first preprocessed the data by selecting the genes that were identified as confidently rhythmic in the Vijayan *et al*. 2009 data set [S7]. For each array, we log-transformed the data, then calculated a calibration curve using the RNA spike-ins that fell in the linear part of the array's dynamic range, and normalized the accordingly. For each time point, strain, and each gene, we then calculated the median of the normalized data

out of the four probes designed for that ORF, and used that statistic as an overall expression estimate. Using this pre-processed data, we then computed a Spearman rank-order correlation coefficient between all time points and both strains, shown as a color scale matrix plot in Fig. 3B.

To produce the clustered heat map in Fig. 3A, we first normalized all of the log-transformed expression data for a given circadian gene to give zero mean and unit amplitude for the WT time course (log maximum – log minimum = 1). We then sorted the heatmap based on an estimate of wildtype circadian phasing [S7].

To create a list of upregulated and downregulated genes, we computed the mean and variance of the log-transformed data for each gene over the time course. To call circadian genes as upregulated or downregulated in Δ*cikA* vs. WT we performed a two-sided *t* test using the estimated mean and variance, taking genes with $|Z| > 2.58$ ($p < 0.01$, assuming normality). From this list of genes, we used the estimated circadian phase from data set [S7] to produce the double-plotted histograms in Fig 3C.

Supplemental References

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