Supporting Information

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SI Text

Recombinant Protein Expression and Purification. KaiA and KaiA-HA. KaiA and KaiA-HA were expressed as N-terminal GST fusions from the pGEX-6P-1 plasmid (1) in the DH5α strain of Escherichia coli. Overnight starters were made from fresh transformants before dilution into 1-L cultures in LB medium containing 50 µg/mL carbenicillin. Cultures were then grown at 37 °C to OD₅₆₀ ∼ 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 KaiA and KaiA-HA, resuspended cell pellets were passed through an Emulsiflex-C3 homogenizer (Avestin), and soluble KaiA from the clarified lysate was bound to a GSTrap column (GE Healthcare) and washed with buffer containing 1 mM ATP. The GST tag was cleaved on-column by overnight incubation at 4 °C with Precision protease (GE Healthcare). The resulting KaiA was then purified on a Resource Q anion exchange column (GE Healthcare), concentrated to 20–30 µM in a buffer containing 10% (vol/vol) glycerol, and flash-frozen in small aliquots.

KaiB and KaiB-FLAG. KaiB and KaiB-FLAG were both expressed with N-terminal His6 tags from the pET47b(+) vector (1) in the BL21 (DE3) strain of E. coli. An overnight starter culture was 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 OD₅₆₀ ∼ 0.6, chilled to 30 °C, and induced with 100 µM IPTG. Cells were harvested by centrifugation ∼3 h later and flash-frozen in liquid nitrogen.

To purify KaiB and KaiB-FLAG, resuspended cell pellets were passed through an Emulsiflex-C3 homogenizer (Avestin), and soluble KaiB or KaiB-FLAG from the clarified lysate was purified first by Ni affinity on a HisTrap HP column (GE Healthcare). The His tag was then cleaved in solution by overnight incubation at 4 °C with HRV 3C protease (EMD Millipore) before additional purification by anion exchange chromatography on a Resource Q column (GE Healthcare).

WT KaiC and KaiC mutants. WT KaiC was expressed with an N-terminal His6 tag from the pRSET-B vector (1) in the BL21 (DE3) strain of E. coli. Multiple colonies from fresh transformants were inoculated directly into 1-L cultures in Terrific Broth with 50 µg/mL carbenicillin. Cultures were grown at 25 °C for 48 h without induction. Expression of KaiC relied on leaky expression from the T7 promoter. Cells were subsequently harvested by centrifugation and flash-frozen in liquid nitrogen.

To purify KaiC, resuspended cell pellets were passed through an Emulsiflex-C3 homogenizer (Avestin), and soluble KaiC from the clarified lysate was purified first by metal affinity on a HisTrap HP or HiTrap TALON column (GE Healthcare). The His 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.

Mutant KaiC proteins were similarly expressed and purified after constructing plasmids bearing the desired mutations to the catalytic carbonylates and/or phosphorylation sites using a QuikChange Mutagenesis kit (Stratagene).

Proteins used in ATPase measurements were additionally purified over a Resource Q (GE Healthcare) or HiTrap Q (GE Healthcare) anion exchange column.

In vitro Kai protein reactions. The KaiA-KaiC (WT KaiC or N-terminal domain CI cat’ mutant) and KaiB-KaiC (WT KaiC or CI cat’ mutant) partial reactions (Fig. 2 B and C) and the oscillating KaiABC (WT KaiC or CI cat’ mutant) reactions (Fig. 2D) were run in the following standard reaction buffer:

20mM Tris·Cl (pH 8.0), 150 mM NaCl, 5mM MgCl₂, 5mM ATP, and 10% glycerol.

The KaiA-KaiC partial reactions and full oscillatory KaiABC reactions shown in Fig. 1 B and C were run in reaction buffers containing 10 mM mixtures of ATP and ADP; 10 mM total nucleotide was used in these reactions to ensure that, even at low fractions of ATP (e.g., 25%), enough ATP would be in solution to saturate KaiC phosphorylation kinetics (in the absence of ADP).

All reactions used ∼1.5 µM KaiA, 3.5 µM KaiB, and 3.5 µM KaiC as measured by Bradford assay against a BSA standard curve. Reactions were run at 30 °C. At each time point, ∼2 µL reaction mixture were sampled either by hand from Eppendorf tubes or by a liquid-handling robot (GX-271; Gilson) from open tubes with mineral oil overlays directly into SDS-containing buffer (without glycerol in the case of robot samples) to stop the reactions. Samples acquired by robot were placed in an open-topped 96-well plate and allowed to dry.

Analysis of KaiC phosphorylation by SDS/PAGE. Before analysis, robot-acquired samples were resuspended in SDS-containing buffer with glycerol. All samples were heated briefly at 95 °C. To measure the total fraction of phosphorylated KaiC in Figs. 1 and 2, reaction samples were analyzed by SDS/PAGE on precast 7.5% Tris-HCl Criterion gels (Bio-Rad) for 110 min at a constant 125 V, fixed and stained in SimplyBlue SafeStain (Invitrogen), and then scanned on an LI-COR infrared scanner. The proportion of KaiC molecules present in each phosphorylated form was estimated by manually determining the boundaries between each band on the gel and integrating the stain intensity over the bands. To analyze the distribution of phosphorylated forms of KaiC in Fig. S1, samples were analyzed by SDS/PAGE on 10% acrylamide gels (37.5:1 acrylamide:bis-acrylamide) run for 4.5–5 h at 35 mA constant current at 12 °C.

Immunoprecipitation of KaiB•KaiC complexes. Twenty-microliter samples of the reactions in Fig. 2F were collected after incubation for 10 h at 30 °C. For the reactions in nonhydrolyzable ATP analog buffers, KaiC-EE protein was first buffer-exchanged using P-30 polyacrylamide spin columns (Bio-Rad) into the

KaiC mutants

<table>
<thead>
<tr>
<th>Description</th>
<th>Cl cat’</th>
<th>ClI cat’</th>
<th>KaiC-EE</th>
<th>Cl’ EE</th>
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<tr>
<td>E77Q; E78Q</td>
<td>E318Q</td>
<td>S431E; T432E</td>
<td>E77Q; E78Q</td>
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Cl fragment

| 248 → TAA stop codon |

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Our proposed model of the in vitro oscillator (model + and C working EE, and CII statistic (sum of squares/degrees of freedom).

CI fragment protein (3.5 fi × fi C k e is the maximum phosphorylation rate and K k −[S1]; was similarly measured in triplicate at the k and 3 fl C

To immunoprecipitate KaiB-FLAG, monoclonal anti-FLAG M2 antibody was coupled to Protein G Dynabeads (Invitrogen) in tris-buffered saline with Tween 20 (TBST). Beads were then washed repeatedly in the corresponding reaction buffer. Pellet beads equivalent to 30 μL Dynabead stock were mixed with the equivalent of 10 μL reaction and gently vortexed at room temperature for 10 min. Each tube was then rapidly washed two times with the corresponding reaction buffer. KaiB-FLAG complexes were eluted from the beads by incubation with 10 μL 0.25 mg/mL 3xFLAG peptide (Sigma-Aldrich) or nonreducing SDS/PAGE sample buffer for 10 min at room temperature. Eluate from the beads was analyzed by SDS/PAGE on 4–20% Tris-HCl gradient gels (BioRad) and stained with SimplyBlue SafeStain (Invitrogen). The ratio of KaiC-EE band intensity in each lane to the KaiB-FLAG band intensity was measured by densitometry.

Kinetic analysis of reactions. To measure the effect of varying the ATP/ADP ratio on the KaiA-KaiC and KaiA-CI cat’ mutant reactions shown in Figs. 1B and 3A, analysis was restricted to the first 4 h of data, when the reactions are dominated by the first phosphorylation step (phosphorylation of T432) (2). Under this initial velocity approximation, we treated the kinetics using a first-order reversible reaction scheme:

\[ P(t) = P_0 + \left( \frac{k_{\text{phos}}}{k_{\text{phos}} + k_{\text{dep}} + k_{\text{off}}} \right) \left( 1 - e^{-\left( k_{\text{dep}} + k_{\text{off}} \right)t} \right), \]

where \( k_{\text{phos}} \) is the phosphorylation rate at T432 and \( k_{\text{dep}} \) is the dephosphorylation rate.

To model how the phosphorylation rate changes as the ATP/ADP ratio is varied, the rate constants derived above were fit to a competitive inhibition scheme:

\[ k_{\text{max}} \frac{[\text{ATP}]}{[\text{ATP}] + K_i[\text{ADP}]} \]

where \( k_{\text{max}} \) is the maximum phosphorylation rate and \( K_i \) is the effective relative affinity for ADP vs. ATP. These nonlinear regressions were performed using the least-squares fitting package in GraphPad Prism.

The kinetic data shown in Fig. 2 B and C were fit to a linear model for phosphofructointerconversion (Fig. 4B) by nonlinear least-squares regression in MATLAB (MathWorks). The rate constants for each conversion process and the initial concentrations for each phosphofructo were constrained to be positive values and allowed to vary throughout the fit. The final fit was evaluated by Runge–Kutta four-based numerical integration of the linear model. To estimate uncertainty in the fits, 100 bootstrap datasets were drawn with replacement from the experimental dataset to derive SDs on the rate constants and the reduced \( \chi^2 \) statistic (sum of squares/degrees of freedom).

In fitting the dephosphorylation series in Fig. 2B, all phosphorylation processes were explicitly disallowed. The rate constant for ST → T for both KaiC and the CI cat’ mutant protein went to zero, even when allowed to vary throughout the fit.

To estimate the rate constant for KaiB•KaiC complex assembly in the data shown in Fig. 3 C and D, the measured ratios of KaiC-EE to KaiB-FLAG intensities were fit to a kinetic model of complex assembly, where KaiB is assumed to be in excess:

\[ \frac{d[B \cdot C]}{dt} = k_{\text{cat}}([C] - [B \cdot C])/[B] - k_{\text{off}} [B \cdot C], \]

where \([B]\) is the concentration of KaiB-FLAG, \([C]\) is the concentration of KaiC-EE, and \([B \cdot C]\) is the concentration of KaiB-FLAG bound to KaiC-EE. We estimated the rate constants \( k_{\text{cat}} \) and \( k_{\text{off}} \) by least-squares minimization of the difference between the measured data and numerical integration of this differential equation. To estimate uncertainty in the fits, 100 bootstrap datasets were drawn with replacement from the experimental datasets to derive SDs on each rate constant. Curves in Fig. 3C showing the hypothetical mass-action scaling of the complex assembly rate were also generated by numerically integrating this equation.

Measuring ATPase activity. CI fragment protein (3.5 μM) was incubated in various ATP/ADP buffer conditions containing 1 mM ATP at 30 °C for 48 h. Samples were taken at the beginning and end of the incubation period, flash-frozen, and stored at −80 °C. To measure ATPase activity, 60 μL thawed reaction were diluted into a total of 500 μL 5 mM Tris (pH 8) and spun down in a 10-kDa centrifugal filter device (Millipore) for 15 min at 14,000 × g at 4 °C to separate protein from nucleotides. The flow-through/ nucleotide portion of the reaction was collected and further diluted into 3 mL of 5 mM Tris (pH 8). ATP and ADP in the reaction mixtures were separated on a MonoQ 5/50 GL anion exchange column (GE Healthcare) at room temperature at a flow rate of 1.0 mL/min in a mobile phase of 50 mM NH₂H₂PO₄ (pH 4.8) and eluted with a salt gradient up to 0.75 M NH₂H₂PO₄ (pH 4.8). ATP and ADP amounts were determined as the area under the A280 absorbance for each peak on the chromatograph using Unicorn software (GE Healthcare). ATPase activity was determined as ADP produced per hour per CI fragment molecule. Spontaneous hydrolysis of ATP to ADP was negligible as measured by 48-h incubations of each ATP/ADP buffer without the CI fragment protein.

The mean rates shown in Fig. 3B are from at least three biological replicates for each condition. These additional measurements include reactions of shorter (30 h) and longer (72 h) incubation periods and reactions with increased enzyme concentration (5.25 μM CI fragment protein and 1.5x working concentration of KaiC) to assess for stability of the measurements. The measured rates were fit to the same competitive inhibition scheme as described above for kinase activity. To characterize the experimental error in this chromatography assay, we carried out triplicate technical replicates of a 40% ADP (60% ATP) mixture of pure nucleotides and obtained an SD of 1.13% ADP and an SE of 0.65% ADP.

WT, CI’ EE, and CI’’ EE mutant protein ATPase activity shown in Fig. S3C was similarly measured in triplicate at the 100% ATP condition.

Mathematical Modeling of the KaiABC in Vitro Oscillator. Description of model, including kinase inhibition by ADP and KaiB•KaiC complex formation. Our proposed model of the in vitro oscillator (model A in Fig. 4) modifies a previously proposed model (1, 2) to reflect the experimental measurements presented here and that formation of inhibitory KaiB•KaiC complexes is slow and controlled by CI ATPase activity after Ser431 phosphorylation has occurred:

\[ \frac{dU}{dt} = k_{\text{on}} T + k_{\text{phos}} S + k_{\text{dep}} SB - k_{\text{cat}} U - k_{\text{off}} U; \]
\[
\frac{dS}{dt} = k_{\text{at}}U + k_{d}D - k_{\text{ad}}S - k_{dS}S - k_{bS}S, \quad \text{[S2]}
\]

\[
\frac{dT}{dt} = k_{\text{at}}U + k_{d}D - k_{dDB}DB - k_{dT}T - k_{dT}T, \quad \text{[S3]}
\]

\[
\frac{dD}{dt} = k_{\text{at}}T + k_{dS}S - k_{dD}D - k_{bD}D, \quad \text{[S4]}
\]

\[
\frac{dB}{dt} = k_{bD}D + k_{dDB}SB - k_{dSB}SB - k_{dtB}DB, \quad \text{[S5]}
\]

\[
\frac{dSB}{dt} = k_{bS}S + k_{dSB}DB - k_{dSB}SB - k_{dtB}SB, \quad \text{[S6]}
\]

\[
k_{\text{phos}}(S) = \frac{k_{\text{phos}}[\text{KaiA]}_{\text{free}}(S)}{[\text{ATP}] + K_{I}[\text{ATP}]/K_{I}/2 + [\text{KaiA]}_{\text{free}}(S)}, \quad \text{[S7]}
\]

\[
k_{\text{dephos}}(S) = \frac{k_{\text{dephos}}[\text{KaiA]}_{\text{free}}(S)}{K_{1/2} + [\text{KaiA]}_{\text{free}}(S))}, \quad \text{[S8]}
\]

and

\[
[\text{KaiA]}_{\text{free}}(S) = \max(0, [\text{KaiA}]) - mSB - nDB, \quad \text{[S9]}
\]

where \(U\) is the concentration of unphosphorylated KaiC (U-KaiC), \(T\) is the concentration of T-KaiC (T432-P), \(S\) is the concentration of S-KaiC (S431-P), \(D\) (doubly phosphorylated) is the concentration of ST-KaiC (S431-P, T432-P), \(DB\) is the concentration of ST-KaiC in complex with KaiB, and \(SB\) is the concentration of S-KaiC in complex with KaiB. ST-KaiC and S-KaiC in complex with KaiB sequesters KaiA with high (asymptotically infinite) affinity, giving an effective free concentration of KaiA denoted as \([\text{KaiA]}_{\text{free}}(S)\) in Eq. S9. \(m\) and \(n\) are the inhibitory strengths assigned to S-KaiC and ST-KaiC, respectively. KaiB is treated implicitly and assumed to be in excess of the concentration of S-KaiC at all times.

The phosphorylation and dephosphorylation rates for each phosphorylated form of KaiC are functions of \([\text{KaiA]}_{\text{free}}\) and hence, functions of \(S\), making the differential Eqs. S1–S6 that describe the dynamics nonlinear. The phosphorylation rate constants \(k_{\text{at}}, k_{\text{ad}}, k_{dT}\), and \(k_{dS}\) are described by the general expression \(k_{\text{phos}}\) (Eq. S7), and the dephosphorylation rate constants \(k_{dS}, k_{dD}, k_{bS}\), and \(k_{bD}\) are described by \(k_{\text{dephos}}\) (Eq. S8). In particular, for each of the phosphorylation and dephosphorylation processes, there is a basal rate constant in the absence of KaiA \((k_{0}\text{phos})\), a rate constant describing the maximal effect of KaiA \((k_{0}\text{dephos})\), or \(k_{0}\text{dephos}\), and a constant \(K_{1/2}\) describing the concentration of KaiA needed to produce a half-maximal effect on KaiC’s activity. These rate constants were set experimentally from various measurements of partial reactions with and without KaiA (2).

The rate constant \(k_{d}\) describes the rate for ST-KaiC molecules in complex with KaiB to be dephosphorylated on Ser341 to produce T-KaiC. The rate constant \(k_{ad}\) describes the rate for S-KaiC molecules in complex with KaiB to be dephosphorylated on Ser431 to produce U-KaiC. We assume that these rates are the same as for ST-KaiC or S-KaiC molecules not bound to KaiB \((k_{dt}\) and \(k_{at}\), respectively) and that these dephosphorylation events induce immediate dissociation of KaiB. We also assume that ST-KaiC-bound KaiB can be dephosphorylated to produce S-KaiC–bound KaiB with rate constant \(k_{dSB} = k_{dS}\) (same as when KaiB is not bound) and that S-KaiC in complex with KaiB can be phosphorylated to produce ST-KaiC bound to KaiB with rate constant \(k_{dDB} = k_{dt}\) (same as when KaiB is not bound).

The inhibitory effect of ADP on KaiC’s kinase activity is included through the factors \([\text{ATP}]/[\text{ATP}] + K_{I}[\text{ADP}]\) modulating the phosphorylation but not the dephosphorylation rates, consistent with experimental findings (1). The rate of KaiB*KaiC complex formation is set from fits to the experimental data shown in Fig. 3C and taken as invariant to the [ATP]/[ADP] ratio as shown in Fig. 3. We set \(K_{I} = 1\) corresponding to equal affinity for ADP and ATP. With these parameters, standard concentrations of the Kai proteins, \(m = 5\) and \(n = 0.1\) (Table S4 has a list of all model parameters), that model A produces limit cycle oscillations in the phosphorylation state of KaiC with a period of \(\sim 26.9\) h.

**Phase resetting by ATP/ADP in the in vitro oscillator models.** The inclusion of a slow KaiB*KaiC binding step in model A not only allows for period robustness across a range of ATP/ADP input levels but also maintains the sensitivity of the in vitro oscillator to transient changes in ATP/ADP ratio. To simulate transient changes in ATP/ADP input, ADP levels were increased from 0% to 50% as single \(-6.5\)-h pulses at various points along the circadian cycle after the system completed one cycle. The pulse duration of 6.5 h scales with the 5-h pulses used in our previous characterization of sensitivity (1). Equal affinities for ATP and ADP were also maintained in all other simulations (\(K_{I} = 1\)).

Consistent with in vivo and in vitro data, only pulses occurring during subjective day elicited phase-shifting responses, whereas pulses occurring during subjective night are refractory (1, 3). Model A, however, is slightly less responsive to ADP pulses compared with previous in vivo (3) and in vitro data and simulations of model B with 50% ADP pulses. Specifically, oscillations in model A are maximally shifted by \(\sim 7.4\) h (after scaling and aligning to circadian period with peak phosphorylation at 12 h) compared with \(\sim 10–15\) h measured in ref. 3 and \(\sim 8.8\) h simulated in model B.

**Robustness requires a relatively slow KaiB*KaiC complex formation rate that is independent of Kai protein concentration.** Model B does not require that the KaiB*KaiC complex formation rate constant (in our model, set by the CI catalytic rate) be finely tuned, but it does need to be comparable with the CII kinase rates. Limit cycle oscillations and robust circadian periods over the range of ATP/ADP conditions considered are generated even as \(k_{bc}\) is slowed to approximately one-half of the experimentally measured rate (Fig. 3C and Table S3). As \(k_{bc}\) increases above 0.3 h\(^{-1}\), oscillations at lower ATP conditions become unstable, and robustness is lost (Fig. S4A). Moreover, at these increasingly slowed rates of complex assembly, oscillations at 100% ATP, although stable, are of such low amplitude and high frequency as to be negligible. Thus, the model generates robust periodicity when the rate of CI catalysis-driven complex assembly is comparable with the CII autokinase rates. This requirement is reasonable given the homology of the two domains and supported by our experimental measurements, indicating that CI catalysis, KaiB*KaiC complex assembly, and CII autokinase all occur on the same slow timescale (Fig. 3 and Table S3).

Similarly, when all kinetic parameters were perturbed by additive Gaussian noise (SD of 10%), limit cycle oscillations were observed in 33 of 100 simulations for each ATP/ADP level considered within the 100% to 40% ATP range. The mean normalized period of these noisy simulations were also relatively stable against changing ATP/ADP conditions. This analysis also shows that the relative insensitivity of the period does not require the model parameters to be finely tuned (Fig. S4B).

Previous experimental work has shown that increasing the concentration of all three Kai proteins simultaneously does not affect the amplitude or period of oscillations in KaiC phosphorylation state—the oscillator is perfectly robust to protein concentration (2, 4). One mechanism that can create concen-
tration robustness has the assembly and disassembly rate constants for protein–protein complexes much faster than the oscillator dynamics and the protein concentrations well above the binding affinities for the complexes, which seems to be true experimentally for the KaiA–KaiC interaction (2). Such a mechanism ensures that the abundance of protein complexes will effectively be in pre-equilibrium with the slow dynamical variables (e.g., KaiC phosphorylation state). The models presented here incorporate this mechanism by treating the KaiA-KaiC interaction implicitly if the $K_{1/2}$ parameter that describes the amount of KaiA needed to cause half-maximal activation of the KaiC autokinase is interpreted as a stoichiometry that depends only on the KaiA/KaiC ratio. For example, if S-KaiC and free KaiA are normalized by total KaiC and KaiA protein concentrations, respectively, then dependence on protein concentration falls out of the equations governing their dynamics:

$$S' = \frac{[S]}{[\text{KaiC}]}$$

$$\text{KaiA}^{\text{free}}' = \frac{[\text{KaiA}^{\text{free}}]}{[\text{KaiA}]}$$

$$\frac{dS'}{dt} = k_{su}U^* + k_{ds}D^* - k_{sd}S' - k_{ud}D'^*,$$

and

$$\text{KaiA}^{\text{free}} (S) = \max\left(0, 1 - mS\frac{[\text{KaiC}]}{[\text{KaiA}]} - nD\frac{[\text{KaiC}]}{[\text{KaiA}]}, \right).$$

However, perfect robustness to protein concentration can be broken if protein–protein complex formation is slow and those rates depend on concentration according to mass action (i.e., if they are proportional to the rate of bimolecular collisions). For example, in a mass-action ordinary differential equation governing formation of KaiB–KaiC complexes, the ordinary differential equation (ODE) cannot be made dimensionless as in the case above:

$$B \cdot C' = \frac{[B \cdot C]}{[\text{KaiC}]}$$

$$\frac{d[B \cdot C]}{dt} = k_{on}[\text{KaiB}][S],$$

and

$$\frac{dB \cdot C'}{dt} = k_{on}[\text{KaiB}][S].$$

Note that the ODE retains units of concentration on the right-hand side, so that the dynamics cannot be independent of total protein concentration.

In model A, the formation of KaiB–KaiC complexes is slow, as measured experimentally, but this rate is set by the CI ATPase rate. This feature of the model causes the rate of KaiB*KaiC complex assembly to be first order in total protein concentration (because it depends only on KaiC enzymatic rates) rather than second order, consistent with our experimental measurements (Fig. 3C). Thus, perfect robustness to protein concentration is maintained in the model (Fig. S4C). If, instead, the KaiB*KaiC complex assembly rate $k_{sa}$ is made second order in protein concentration, which would occur in a simple mass-action binding scheme, perfect robustness of the oscillator is lost (Fig. S4D).

This robustness requirement for a first-order dependence of the KaiB*KaiC binding rate in the model offers additional support for the hypothesis that complex assembly is rate-limited by CI ATPase activity.

**Calculation of ADP production rates in the in vitro oscillator model.** To estimate the rates of ADP production in model A, we consider the relative contributions of both KaiC domains. Transitions into the KaiB-bound states are assumed to reflect active CI ATPase, and each kinase reaction in CII is assumed to consume ATP. We neglect possible CII ATPase activity that is not associated with phosphorylation or the possibility that some ATP is regenerated during dephosphorylation:

$$\frac{d[\text{CI ADP}]}{dt} = k_{bc}D + k_{sd}S$$

and

$$\frac{d[\text{CI ADP}]}{dt} = k_{ud}U + k_{sd}T + k_{sd}S + k_{sd}SB.$$
crease to 60%. In contrast, model B cannot generate robust circadian periods at any value of \( m \) permitting oscillatory solutions (Fig. S6B).

Model B considers the case where both serine phosphorylated forms of KaiC are inhibitory but the formation of inhibitory KaiB•KaiC complexes occurs much faster than all phosphorylation reactions (Fig. 4B):

\[
\frac{dU}{dt} = k_{in} T + k_{in} S - k_{in} U - k_{in} U, \quad [S10]
\]

\[
\frac{dS}{dt} = k_{in} U + k_{dS} D - k_{in} S - k_{dS} S, \quad [S11]
\]

\[
\frac{dT}{dt} = k_{in} U + k_{dT} D - k_{in} T - k_{dT} T, \quad [S12]
\]

\[
\frac{dD}{dt} = k_{dT} T + k_{dD} S - k_{dT} D - k_{dD} D, \quad [S13]
\]

and

\[
[KaiA]_{free} (S) = \max(0, [KaiA] - mS - nD). \quad [S14]
\]

Supplemental model E assumes that only S-KaiC is inhibitory (\( n = 0 \)), but as in model A and consistent with our experimental findings, the formation of KaiB•KaiC complexes is slow and controlled by CI ATPase (Fig. S6E). We again assume that dephosphorylation of KaiB bound S-KaiC to U-KaiC or D-KaiC results in immediate dissociation of KaiB:

\[
\frac{dU}{dt} = k_{in} T + k_{in} S + k_{dUB} S - k_{in} U - k_{in} U, \quad [S15]
\]

\[
\frac{dS}{dt} = k_{in} U + k_{dS} D - k_{in} S - k_{bc} S, \quad [S16]
\]

\[
\frac{dT}{dt} = k_{in} U + k_{dT} D - k_{in} T - k_{dT} T, \quad [S17]
\]

\[
\frac{dD}{dt} = k_{dT} T + k_{dD} S + k_{dDB} S - k_{dT} D - k_{dD} D, \quad [S18]
\]

\[
\frac{dSB}{dt} = k_{bc} S - k_{dSB} S - k_{dSB} S, \quad [S19]
\]

and

\[
[KaiA]_{free} (S) = \max(0, [KaiA] - mS). \quad [S20]
\]

Supplemental model F assumes that only S-KaiC is inhibitory, but like in model B, the formation of KaiB•KaiC complexes is faster than the phosphorylation reactions (Fig. S6F) (2):

\[
\frac{dU}{dt} = k_{in} T + k_{in} S - k_{in} U - k_{in} U, \quad [S21]
\]

\[
\frac{dS}{dt} = k_{in} U + k_{dS} D - k_{in} S - k_{dS} S, \quad [S22]
\]

\[
\frac{dT}{dt} = k_{in} U + k_{dT} D - k_{in} T - k_{dT} T, \quad [S23]
\]

\[
\frac{dD}{dt} = k_{dT} T + k_{dD} S - k_{dT} D - k_{dD} D, \quad [S24]
\]

\[
[KaiA]_{free} (S) = \max(0, [KaiA] - mS). \quad [S25]
\]

The rate constants for all phosphorylation and dephosphorylation reactions for both models E and F are as defined in Eqs. S6 and S7. In model E, we also take the transitions from S-KaiC to inhibitory KaiB•KaiC complexes as occurring with the rate constant \( k_{bc} \). \( k_{bc} \) is the same rate constant used in model A and obtained from fitting the kinetics of KaiC-EE association with KaiB-FLAG (Fig. 3 and Table S3). Both models E and F are able to generate oscillatory solutions over a range of \( m \) values (Fig. S6G).

**Robustness diminishes if CI is made sensitive to ATP/ADP input.** Our experimental data indicate that the CI ATPase (Fig. 3B) and the assembly kinetics of KaiB•KaiC-EE complexes are not sensitive to ATP/ADP under our buffer conditions. To assess the importance of this insensitivity, we studied a model where KaiB•KaiC binding is slow but has a rate that can be modulated by ATP/ADP analogously to the CII kinase rates:

\[
k_{bc} = \frac{[ATP]}{[ATP] + K_c [ADP]}.\]

This model has improved robustness properties relative to the model without the CI ATPase step (compare Fig. 4B) and oscillates stably over a wider range of conditions. However, the period of oscillations increases markedly at low ATP/ADP conditions relative to the model with an invariant KaiB binding step (compare Fig. 4A with Fig. S7A and B).

**Robustness of models with explicit treatment of KaiB-KaiC complex formation after activation by CI ATPase.** Model A assumes that KaiB is in excess, and therefore, KaiB•KaiC complex formation is set only by CI ATPase activity. However, we have also analyzed a modified model explicitly incorporating the effects of KaiB concentration (Fig. S7A). We assume that CI ATPase activity produces activated states of S-KaiC denoted by \( S^* \) and \( D^* \) and that KaiB can encounter activated KaiC with a typical macro-molecular association rate to produce KaiB•KaiC complexes. We hypothesize that these activated forms of KaiC are associated with CI bound to the ATPase reaction products (ADP and P_i). We suppose that ATP can reload into these states, returning them to a binding-incompetent form with a rate \( k_{ADP \rightarrow ATP} \):

\[
\frac{dU}{dt} = k_{in} T + k_{in} S + k_{dUB} S - k_{in} U - k_{in} U, \quad [S26]
\]

\[
\frac{dS}{dt} = k_{in} U + k_{dS} D + k_{ADP \rightarrow ATP} S^* - k_{in} S - k_{dS} S, \quad [S27]
\]

\[
\frac{dT}{dt} = k_{in} U + k_{dS} D + k_{ADP \rightarrow ATP} D^* - k_{in} T - k_{dT} T, \quad [S28]
\]

\[
\frac{dD}{dt} = k_{dT} T + k_{dS} D + k_{ADP \rightarrow ATP} D^* - k_{dT} D - k_{dD} D, \quad [S29]
\]

\[
\frac{dS^*}{dt} = k_{bc} S + k_{dSB} S - k_{dSB} S, \quad [S30]
\]
\[
\frac{dDB}{dt} = k_{on}D'\text{KaiB} + k_{ad}SB - k_{off}DB - k_{db}DB - k_{ds}SB, \quad \text{[S31]}
\]
\[
\frac{dS'}{dt} = k_{cb}S + k_{off}SB + k_{ds}D' - k_{on}S'\text{KaiB} - k_{ad}S' - k_{ADP\rightarrow ATP}S', \quad \text{[S32]}
\]
\[
\frac{dSB}{dt} = k_{on}S'\text{KaiB} + k_{db}DB - k_{off}SB - k_{ds}SB - k_{db}SB, \quad \text{[S33]}
\]
\[
d\text{KaiB} = k_{off}SB + k_{db}DB + k_{ad}SB - k_{db}DB - k_{on}S'\text{KaiB} - k_{ds}D'\text{KaiB}. \quad \text{[S34]}
\]

An association rate of \( k_{on} = 10^5 \text{ M}^{-1}\text{s}^{-1} \) and an activated state lifetime of \( k_{ADP\rightarrow ATP} = 1 \text{ m} \) were assumed. Then, for eqimolar concentrations of KaiB and KaiC (\([\text{KaiB}] = [\text{KaiC}] = 3.4 \text{ mM} \)), the hypothetical affinity \( K_d \) of the KaiB\textbullet KaiC complex was varied in the above model. For \( K_d << 1 \text{ mM} \), the model reduces to model A (Fig. S7B).

**Approximate model for input compensation in the circadian oscillator.** Here, we formulate an extremely simplified graphical model that captures the essence of the compensatory mechanism that allows the oscillator period to remain robust, even as kinase rates change. We suppose that, at any moment in time, the KaiABC system exists exclusively in either an autocine mode (characterized by the kinase activity in CII, \( k_{CI} \)) or a phosphatase mode (characterized by a dephosphorylation rate, \( k_d \)). We assume that the oscillation occurs between two phosphorylation levels \( A \) and \( B \), where the system switches from a kinase mode to a KaiB-interacting phosphatase mode. To describe the role of input signaling in modulating CII kinase activity, we describe the kinase rate as \( \eta_{\text{CI}} \), where \( \eta \) is an input parameter (e.g., the ATP/ADP ratio).

**Approximate model B.** We first characterize the performance of a model that does not consider the CI domain in controlling the kinetics of KaiB\textbullet KaiC complex assembly. Consider the oscillation beginning from the trough level of phosphorylation \( A \). The approach to the turning point \( B \) takes time

\[
t_{\text{rising}} = \frac{1}{\eta_{\text{CI}}} \log \left( \frac{1-A}{1-B} \right).
\]

In model B, KaiB-mediated inhibition of KaiA occurs instantaneously, and the system immediately enters the dephosphorylation phase. Return to the trough level of phosphorylation \( A \) takes time

\[
t_{\text{falling}} = \frac{1}{k_d} \log \frac{B}{A}.
\]

The period of the oscillator is then

\[
T = t_{\text{rising}} + t_{\text{falling}} = \frac{1}{\eta_{\text{CI}}} \log \left( \frac{1-A}{1-B} \right) + \frac{1}{k_d} \log \frac{B}{A},
\]

which varies like \( 1/\eta \) and is not robust (Fig. S8).

**Approximate model A.** We now consider an approximate model that includes a CI catalytic step required to form the KaiB\textbullet KaiC inhibitory complex. Again, beginning from a trough phosphorylation level \( A \), the system takes a time \( t_{\text{rising}} \) to reach phosphorylation level \( B \), where enough phosphorylation has accumulated to inhibit KaiA by KaiB\textbullet KaiC. However, in this model, KaiC must then go through a catalytic step in CI before these complexes form. This takes time

\[
t_{\text{CI}} = \frac{1}{k_{\text{CI}}},
\]

During this CI-mediated delay, phosphorylation in CII continues. The system accumulates overshoot phosphorylation to a level

\[
P_{\text{overshoot}} = 1 + (B-1)e^{-\eta_{\text{CI}}/k_{\text{CI}}},
\]

Then, the system enters the dephosphorylation phase. The phosphorylation accumulated during the CI-controlled overshoot must be removed. This takes time

\[
t_{\text{overshoot}} = \frac{1}{k_d} \log \frac{P_{\text{overshoot}}}{B} = \frac{1}{k_d} \log \left( \frac{1 + (B-1)e^{-\eta_{\text{CI}}/k_{\text{CI}}}}{B} \right).
\]

The oscillator period in model A is, therefore,

\[
T = t_{\text{rising}} + t_{\text{CI}} + t_{\text{overshoot}} + t_{\text{falling}} = \frac{1}{\eta_{\text{CI}}} \log \left( \frac{1-A}{1-B} \right) + \frac{1}{k_d} \log \frac{B}{A}
\]

For realistic values of the parameters \( A, B, k_{\text{CI}}, k_{\text{CI}}, \) and \( k_d \), this approximate model gives a period that depends only weakly on the input parameter \( \eta \) (Fig. S8).

**In vitro oscillator model predicts that period mutants can arise from coordinated changes in CII kinase and phosphatase rates.** We have shown experimentally that CII kinase activity is attenuated by the ATP/ADP ratio, but neither CII phosphatase nor CI ATPase activities are strongly affected. This balance in sensitivities permits period robustness against nucleotide conditions in our in vitro oscillator model. However, if both the rates of CII kinase and phosphatase activities are similarly affected, a strong effect on the oscillator period can be observed regardless of CI ATPase insensitivity (Fig. S8C). Given that the kinase and phosphatase reactions occur in the same active site, we speculate that known period mutants in the in vitro oscillator, some of which are point mutants in CII, may function in this manner (7).

**Fig. S1.** Additional characterization of the KaiABC in vitro oscillator at various ATP/ADP levels. (A) KaiC autophosphorylation in KaiA-KaiC reactions at various ATP/ADP conditions (colored symbols); all buffers have 1 mM ATP. (B–E) Multisite phosphorylation states of KaiABC in vitro oscillator reactions at various ATP/ADP conditions. Shown are the KaiABC in vitro oscillator reactions from Fig. 1C. Black squares indicate total phosphorylation, green triangles indicate Thr432 phosphorylation (T-KaiC), blue circles indicate Thr432 and Ser431 phosphorylation (ST-KaiC), and inverted red triangles indicate Ser431 phosphorylation (S-KaiC).
Fig. S2. Comparison of KaiC and Cl cat™ mutant protein phosphoform kinetics and sensitivity with KaiA in partial reactions. (A) KaiC and (B) Cl cat™ mutant protein phosphorylation in the presence of KaiA. Autodephosphorylation of (C) KaiC and (D) Cl cat™ mutant protein. Solid lines indicate least-squares fits to a four-state linear interconversion model (model B without KaiB-mediated negative feedback). Best-fit rates are compiled in Table S2. Total KaiC phosphorylation is shown over time for reactions of (E) 3.5 μM KaiC or (F) 3.5 μM Cl cat™ mutant protein with different concentrations of KaiA (colored symbols).
Fig. S3. KaiB/KaiC binding requires catalytic activity in the CI domain; the CI ATPase is relatively insensitive to ATP/ADP. 

(A) Representative SDS/PAGE analysis of proteins coimmunoprecipitating with KaiB-FLAG after 8-h incubations in standard reaction buffer at 30 °C. Results are ordered on the gels as an input (I) of KaiC or the indicated mutant protein with or without KaiA, supernatant (S) and eluate (E). 

(B) Same as in A, but KaiC-EE was first buffer-exchanged into reaction buffer containing 2 mM indicated ATP analog and preincubated for 96 h to allow nucleotide exchange before the addition of KaiB-FLAG. 

(C) ATPase activity, measured as ADP produced per day per protein, for WT KaiC (n = 3), CI cat EE (n = 3), CII cat EE (n = 3), and CI fragment (n = 7) proteins. Measurements of the CI fragment are the same as those measurements shown in Fig. 3B at the 100% ATP condition. 

(D) Sample chromatographs from measurements of CI fragment ATPase activity at a 25% ADP condition. Shown are the preincubation (Left) and postincubation (Right) chromatographs for two different measurements. ADP elutes at ∼25.3 mS/cm, and ATP elutes at ∼41.8 mS/cm.
Robustness of the in vitro oscillator model requires a slow CI catalytic rate that is independent of Kai protein concentration. (A) Effects of varying the CI catalytic rate parameter $k_{bc}$ (colored symbols) on the in vitro oscillator model period at various ATP/ADP conditions. (B) Model A is robust to noise in kinetic rate constants; 33 of 100 simulations in which all rate constants were perturbed with 10% Gaussian noise resulted in stable limit cycle oscillations across all ATP/ADP conditions considered within the 100% to 40% ATP range. Shown are the mean normalized periods (gray squares) and SDs (error bars) compared with simulations with measured, unperturbed rate constants (red symbols) (compare Fig. 4A). Periods are normalized to the period of the 100% ATP condition in A and B. (C) Numerical integration of model A (Fig. 4A) as protein concentrations are increased proportionately (colored lines; $1 \times = 3.4 \mu M$ KaiC and $1.3 \mu M$ KaiA, $2 \times = 6.8 \mu M$ KaiC and $2.6 \mu M$ KaiA, etc.). Curves for all conditions overlap. (D) Same as C but where $k_{bc}$ depends on KaiB concentration, following the law of mass action.
Fig. S5. ADP production in the in vitro oscillator model. (A) ADP produced by KaiC CI domain in model A assuming that transitions into KaiB-bound states reflect activation of the CI ATPase. (B) ADP produced by KaiC CII domain in model A assuming that ATP is consumed only during kinase steps. (C) Total ADP produced per KaiC per day as the sum of (A) KaiC CI and (B) CII domain contributions (red curve, left y axis) compared with KaiC phosphorylation cycle (black curve, right y axis). Note that the total ADP production rate peaks before phosphorylation.
Inclusion of the Cl ATPase step in the in vitro oscillator model enhances robustness to the assumed inhibitory strength of S-KaiC (m) and ST-KaiC (n). (A and B) The range of oscillatory values for the parameters m and n that generate stable oscillatory solutions for models A and B presented in Fig. 4 and their corresponding periods as indicated in the color maps. (C and D) Average peak (C) and trough heights (D) of the in vitro oscillations shown in Fig. 1C (black circles) compared with peak and trough heights from model A (red symbols) and model B (blue symbols). Error bars indicate SDs from fits to 3 d after the first trough. Dashed lines indicate linear regressions of the data to show trend. (E and F) Models in which only S-KaiC forms inhibitory KaiB•KaiC complexes through either a Cl ATPase-mediated slow binding step (E; same as choosing n = 0 in A) or a fast binding step (F; same as choosing n = 0 in B). (G) The range of values of the parameter m that generate stable oscillatory solutions for models E (black circles) and F (blue squares).
Fig. S7. Robustness of the in vitro oscillator model if CI catalysis-driven KaiB•KaiC complex assembly is sensitive to ATP/ADP; robustness of the in vitro oscillator model with an explicit treatment of KaiB binding with finite affinity. (A) Numerical integration of a modified reaction network in which KaiB•KaiC complex formation is sensitive to ATP/ADP input. (B) Comparison of period robustness in a CI ATPase sensitive model (gray symbols), model A (red symbols), and experimental data (black circles). (C) Modified reaction network in which CI ATPase activity initiates KaiB•KaiC complex formation, but the binding step is determined by mass action. (D) Comparison of robustness of the modified reaction network shown in C at various affinities $K = k_{off}/k_{on}$ with simulations of model A. Periods in B and D have been normalized to the period of the 100% ATP condition.
Fig. S8. An approximate graphical model for input compensation in the KaiABC in vitro oscillator. (A) Sketches of two systems at different input levels $\eta_1$ (solid lines) and $\eta_2$ (dashed lines) are shown, with $\eta_1 > \eta_2$. Robust periods in model A occur through gated entry into a CI ATPase-mediated input invariant delay (red trace). In kinase mode (black trace), CII-driven phosphorylation drives the system past some threshold before a CI catalytic step occurs. However, phosphorylation continues during the CI step, resulting in an overshoot that is proportional to $\eta$ and that must be removed after switching to phosphatase mode (gray trace). System $\eta_2$’s entry into the CI step is slower but with less overshoot, thus requiring less time to remove during the dephosphorylation phase. The small overshoot effectively compensates for the slower approach, so that system $\eta_2$’s period equals that of system $\eta_1$. (B) Normalized periods of approximate models A (includes roles of CI and CII; black curve) and B (considers only CII; red curve) with respect to kinase rate. Parameters, as described in SI Text, are $A = 0.1$, $B = 0.4$, $k_d = 0.2 \text{ hr}^{-1}$, $k_{CI} = 0.3 \text{ hr}^{-1}$, and $k_{CII} = 0.3 \text{ hr}^{-1}$. (C) Normalized periods from simulations of model A (Fig. 4A), in which kinase and phosphatase rate parameters are perturbed together from the measured rates by a common scaling factor (gray squares) compared with simulations in which kinase rates are perturbed alone (black circles).

Table S1. KaiC and CII cat$^-$ mutant CII autokinase rates at varying ATP/ADP conditions from fits of the first 4 h of data shown in Figs. 1B and 3A, respectively, to a first-order reversible reaction (SI Text)

<table>
<thead>
<tr>
<th>Percent ADP</th>
<th>KaiC 10 mM ATP $[k_{\text{phos}} (\text{h}^{-1}) \pm \text{SE}]$</th>
<th>KaiC 1 mM ATP $[k_{\text{phos}} (\text{h}^{-1}) \pm \text{SE}]$</th>
<th>CII cat$^-$ mutant 1 mM ATP $[k_{\text{phos}} (\text{h}^{-1}) \pm \text{SE}]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.41 ± 0.02</td>
<td>0.41 ± 0.03</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>14</td>
<td>—</td>
<td>0.35 ± 0.02</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>25</td>
<td>0.33 ± 0.07</td>
<td>0.32 ± 0.02</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>40</td>
<td>0.31 ± 0.03</td>
<td>0.27 ± 0.02</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>50</td>
<td>0.21 ± 0.01</td>
<td>0.24 ± 0.02</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>60</td>
<td>0.19 ± 0.07</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>75</td>
<td>0.15 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>0.15 ± 0.01</td>
</tr>
</tbody>
</table>
Table S2. Phosphoform interconversion rates determined from fitting the data in Fig. 2 B and C to model B (Fig. 4B)

<table>
<thead>
<tr>
<th>Process</th>
<th>Rate (h⁻¹) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>KaiC</td>
<td></td>
</tr>
<tr>
<td>U → T</td>
<td>0.36 ± 0.08</td>
</tr>
<tr>
<td>T → ST</td>
<td>0.15 ± 0.09</td>
</tr>
<tr>
<td>S → ST</td>
<td>0.00 ± 0.35</td>
</tr>
<tr>
<td>U → S</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>T → U</td>
<td>0.28 ± 0.07</td>
</tr>
<tr>
<td>ST → T</td>
<td>0.00 ± 0.12</td>
</tr>
<tr>
<td>ST → S</td>
<td>0.39 ± 0.04</td>
</tr>
<tr>
<td>S → U</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>χ²</td>
<td>1.47 ± 2.16</td>
</tr>
</tbody>
</table>

Cl cat⁻ mutant

<table>
<thead>
<tr>
<th>Process</th>
<th>Rate (h⁻¹) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>U → T</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td>T → ST</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>S → ST</td>
<td>0.45 ± 0.42</td>
</tr>
<tr>
<td>U → S</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>T → U</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td>ST → T</td>
<td>0.00 ± 0</td>
</tr>
<tr>
<td>ST → S</td>
<td>0.37 ± 0.06</td>
</tr>
<tr>
<td>S → U</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>χ²</td>
<td>2.51 ± 2.57</td>
</tr>
</tbody>
</table>

Bootstrap analysis was used to compute SDs and χ² statistics (SI Text).

Table S3. Rates of KaiB•KaiC complex assembly approximated from fits to the data in Fig. 3 B and C

<table>
<thead>
<tr>
<th>Process</th>
<th>k_on (h⁻¹) ± SD</th>
<th>k_off (h⁻¹) ± SD</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 3C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5x</td>
<td>0.12 ± 0.03</td>
<td>0.02 ± 0.00</td>
<td>0.74</td>
</tr>
<tr>
<td>1.0x</td>
<td>0.13 ± 0.02</td>
<td>0.02 ± 0.00</td>
<td>0.31</td>
</tr>
<tr>
<td>2.0x</td>
<td>0.14 ± 0.01</td>
<td>0.01 ± 0.00</td>
<td>0.16</td>
</tr>
<tr>
<td>4.0x</td>
<td>0.14 ± 0.02</td>
<td>0.00 ± 0.00</td>
<td>0.83</td>
</tr>
<tr>
<td>Fig. 3D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0% ADP</td>
<td>0.18 ± 0.03</td>
<td>0.00 ± 0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>50% ADP</td>
<td>0.29 ± 0.05</td>
<td>0.00 ± 0.02</td>
<td>1.99</td>
</tr>
<tr>
<td>75% ADP</td>
<td>0.24 ± 0.05</td>
<td>0.01 ± 0.01</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Bootstrap analysis was used to compute SDs and χ² statistics (SI Text).

Table S4. Comparison of KaiABC in vitro oscillator and simulated model periods at various ATP conditions

<table>
<thead>
<tr>
<th>Percent ATP</th>
<th>Normalized period (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro oscillator (Fig. 1C)</td>
</tr>
<tr>
<td>100</td>
<td>23.08</td>
</tr>
<tr>
<td>75</td>
<td>22.72</td>
</tr>
<tr>
<td>50</td>
<td>22.37</td>
</tr>
<tr>
<td>25</td>
<td>23.22</td>
</tr>
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</table>
Table S5. Parameters used in the simulations of various models of the KaiABC in vitro oscillator

<table>
<thead>
<tr>
<th>Process</th>
<th>Parameter name</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal rates (no KaiA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T → U</td>
<td>$k_{U0}$</td>
<td>0.21 h$^{-1}$</td>
</tr>
<tr>
<td>ST → S</td>
<td>$k_{S0}$</td>
<td>0 h$^{-1}$</td>
</tr>
<tr>
<td>ST → U</td>
<td>$k_{U2}$</td>
<td>0.31 h$^{-1}$</td>
</tr>
<tr>
<td>S → U</td>
<td>$k_{U3}$</td>
<td>0.11 h$^{-1}$</td>
</tr>
<tr>
<td>Maximal effect of KaiA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U → T</td>
<td>$k_{U4}$</td>
<td>0.479 h$^{-1}$</td>
</tr>
<tr>
<td>T → ST</td>
<td>$k_{ST4}$</td>
<td>0.213 h$^{-1}$</td>
</tr>
<tr>
<td>S → ST</td>
<td>$k_{ST5}$</td>
<td>0.5057 h$^{-1}$</td>
</tr>
<tr>
<td>U → S</td>
<td>$k_{S6}$</td>
<td>0.0532 h$^{-1}$</td>
</tr>
<tr>
<td>T → U</td>
<td>$k_{U7}$</td>
<td>0.0798 h$^{-1}$</td>
</tr>
<tr>
<td>ST → T</td>
<td>$k_{ST8}$</td>
<td>0.173 h$^{-1}$</td>
</tr>
<tr>
<td>ST → S</td>
<td>$k_{S9}$</td>
<td>−0.32 h$^{-1}$</td>
</tr>
<tr>
<td>S → U</td>
<td>$k_{U10}$</td>
<td>−0.13 h$^{-1}$</td>
</tr>
<tr>
<td>KaiB•KaiC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S → SB</td>
<td>$k_{bc}$</td>
<td>0.133 h$^{-1}$</td>
</tr>
<tr>
<td>SB → STB</td>
<td>$k_{sb}$</td>
<td>0.5057 h$^{-1}$</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration of KaiA causing half-maximal effect on KaiC</td>
<td>$K_{1/2}$</td>
<td>0.43 μM</td>
</tr>
<tr>
<td>Concentration of KaiA</td>
<td>[KaiA]</td>
<td>1.3 μM</td>
</tr>
<tr>
<td>Concentration of KaiC</td>
<td>[KaiC]</td>
<td>3.4 μM</td>
</tr>
<tr>
<td>Inhibitory strength of S-KaiB</td>
<td>M</td>
<td>5, variable</td>
</tr>
<tr>
<td>Inhibitory strength of ST-KaiB</td>
<td>N</td>
<td>0.1, variable</td>
</tr>
<tr>
<td>Effect of ATP/ADP</td>
<td></td>
<td></td>
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<tr>
<td>Effective affinity for ADP vs. ATP in kinase reactions</td>
<td>$K_J$</td>
<td>1.0</td>
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<tr>
<td>Initial conditions</td>
<td></td>
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<tr>
<td>[T-KaiC]</td>
<td>$T_0$</td>
<td>0.68 μM</td>
</tr>
<tr>
<td>[ST-KaiC]</td>
<td>$D_0$</td>
<td>1.36 μM</td>
</tr>
<tr>
<td>[S-KaiC]</td>
<td>$S_0$</td>
<td>0.34 μM</td>
</tr>
<tr>
<td>[KaiB•KaiC]</td>
<td>$SB_0$, $DB_0$</td>
<td>0 μM</td>
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