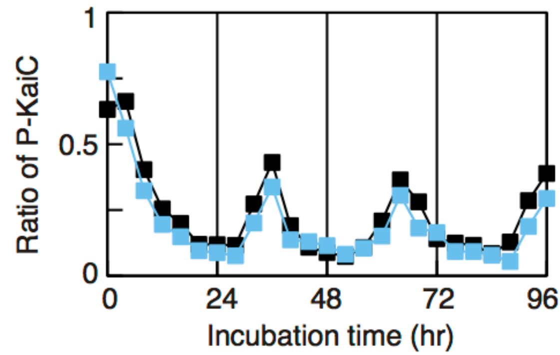
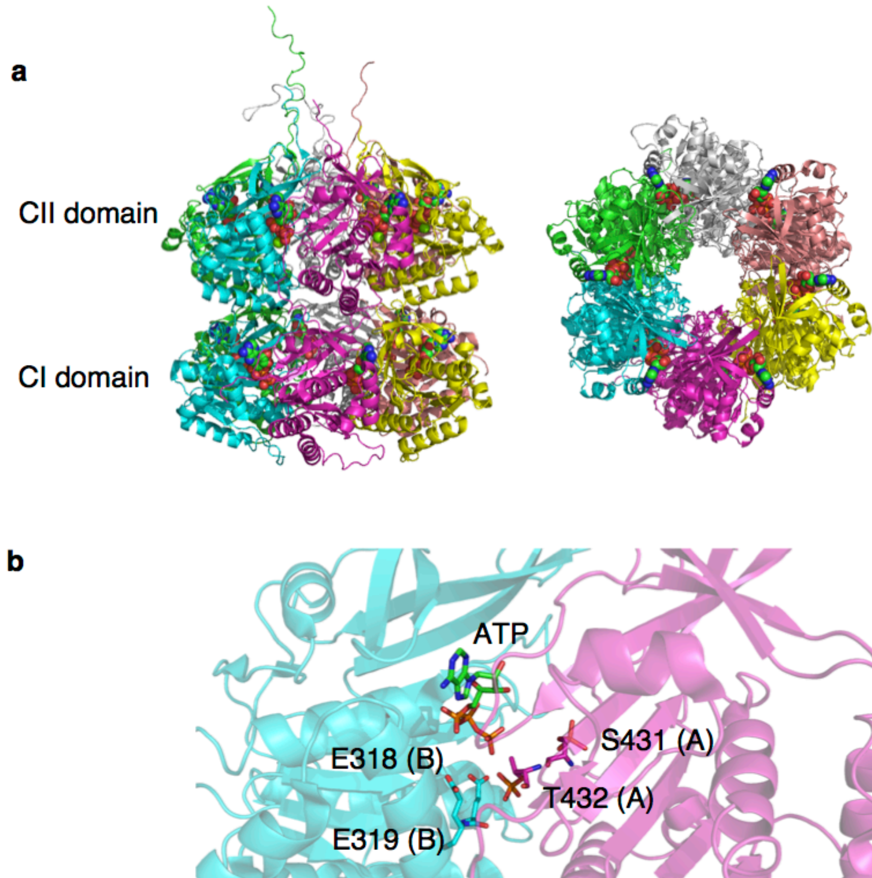
**Supplementary Figure S1**



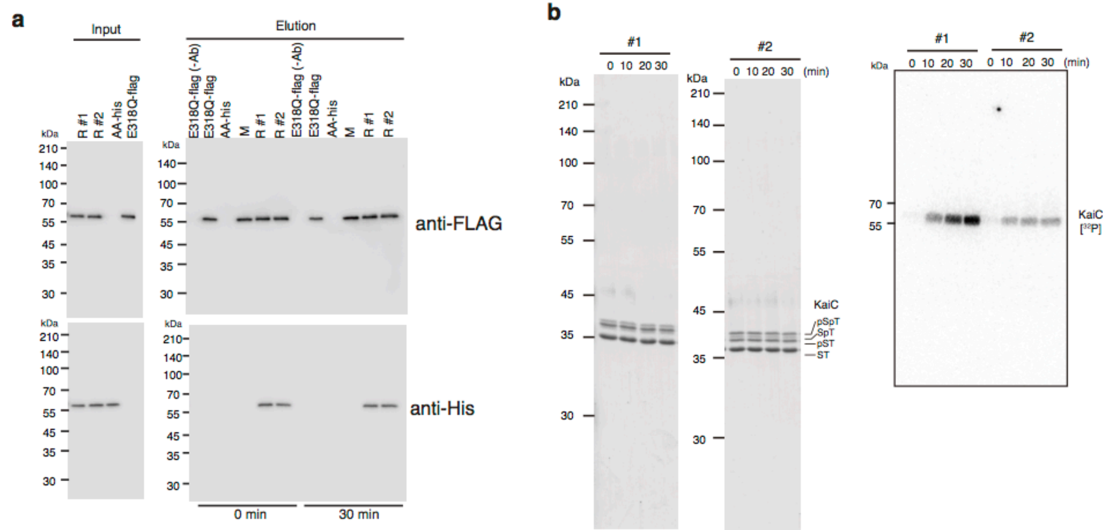

**Supplementary Fig. S1. KaiC phosphorylation rhythm of reconstituted hexamers.**
KaiA and KaiB were mixed with either FLAG-tagged KaiC-WT hexamers (black line) or FLAG-tagged KaiC-WT hexamers reconstituted from monomers (blue line). The ratio of phosphorylated KaiC to total KaiC was plotted against time.

**Supplementary Figure S2**



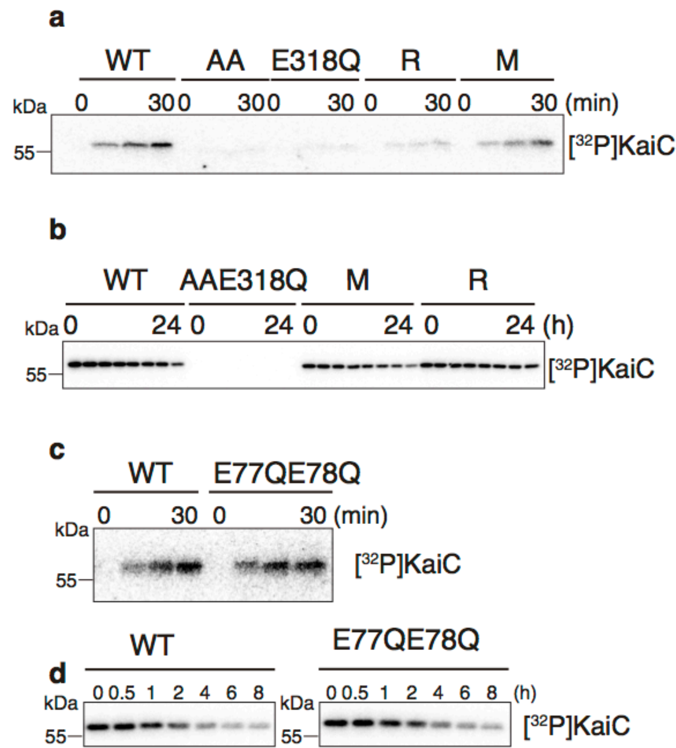

**Supplementary Fig. S2. Three-dimensional structures of KaiC hexamer.**
(**a**) Crystal structure of full-length KaiC (PDB ID 2GBL; ref. 63). The KaiC hexamer viewed from the side (left) and axial view from the CII side (right). Subunits A–F of the hexamer are colored differently, and CI and CII domains are labeled. C-terminal peptide tails were traced completely only for subunits A and F. ATP molecules are drawn in a space-filling mode. (**b**) Close-up of the conformation of subunit interface. Portions of the CII domains from adjacent subunits A and B are shown in a transparent surface representation (colored magenta and cyan, respectively). Residues of phosphorylation site of subunit A (S431 and T432) and conserved putative catalytic glutamates of subunit B (E318 and E319) are shown in stick mode.

## Supplementary Figure S3



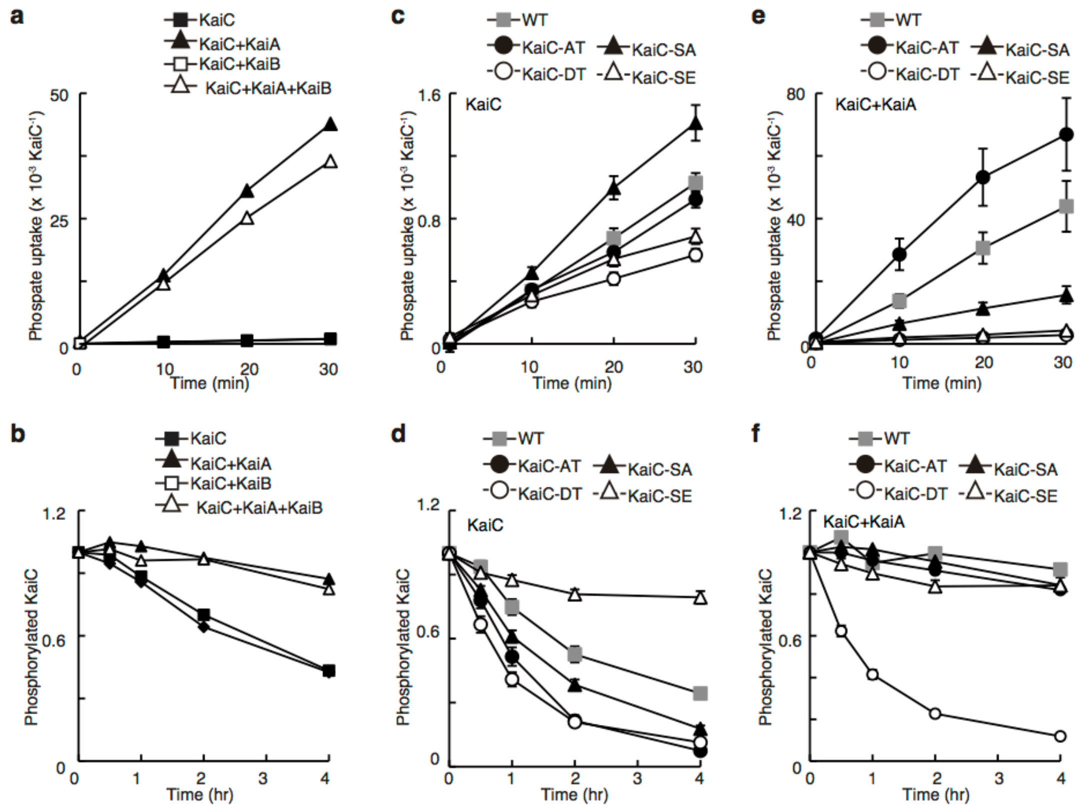

**Supplementary Fig. S3.** (**a**) Full length images of immunoblots of Figure 1c. (**b**) Full length images of Figure 3b.

**Supplementary Figure S4**




**Supplementary Fig. S4**. (**a**) Representative autoradiograph of the *in vitro* autokinase assay from Fig. 1d. "M" indicates a mixture of hexamers (AA+E318Q [M]), and "R" indicates mixed hexamers generated from monomers. (**b**) Representative autoradiograph of the *in vitro* autophosphatase assay shown in Fig. 1e. "M" indicates a mixture of hexamers (WT+AAE318Q [M]), and "R" indicates mixed hexamers generated from monomers (WT+AAE318Q [R]). (**c**) Representative autoradiograph of the *in vitro* autokinase assay from Fig. 2d. (**d**) Representative autoradiograph of the *in vitro* autophosphatase assay shown in Fig. 2e.

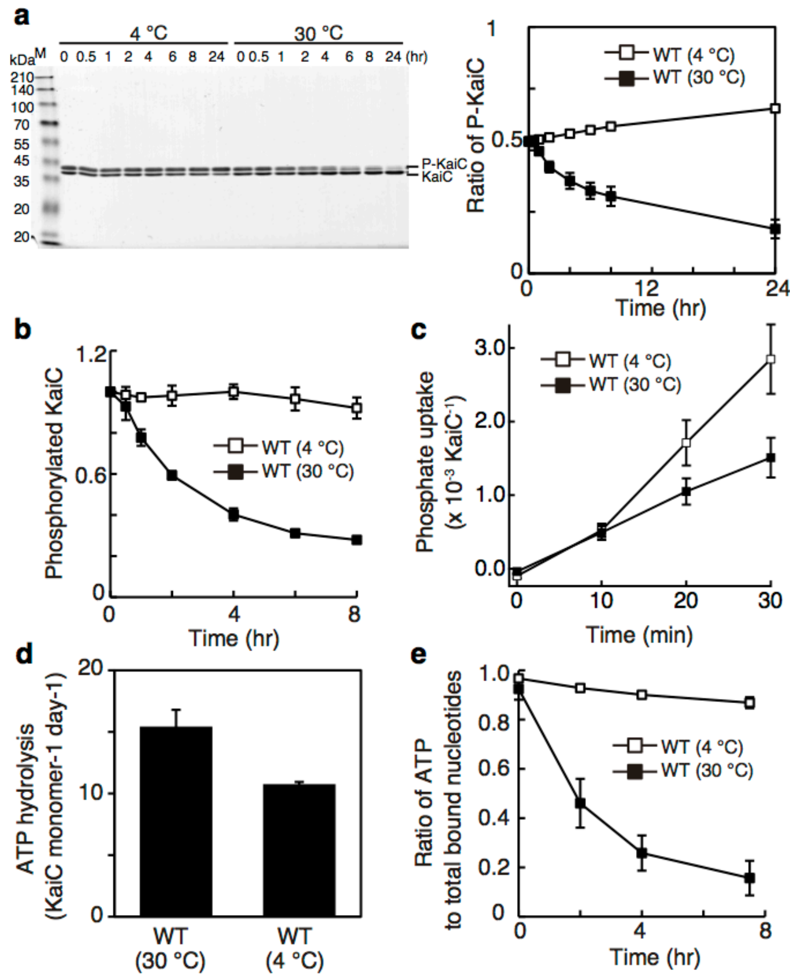
**Supplementary Figure S5**




**Supplementary Fig. S5. Measurement of autokinase and autophosphatase activities of KaiC.**

One of the KaiC phosphorylation sites was substituted with alanine, which mimics a constitutively dephosphorylated state, or with negatively charged aspartic acid or glutamic acid, which mimics a constitutively phosphorylated state. Proteins containing the S431A, S431D, T432A, and T432E mutations were designated KaiC-AT, KaiC-DT, KaiC-SA, and KaiC-SE, respectively. **(a)** **(c)** **(e)** Measurement of kinase activity. Phosphate uptake per KaiC molecule is plotted. **(b)** **(d)** **(f)** Measurement of phosphatase activity. Data were normalized to the level of phosphorylated KaiC at 0 h. **(a)** **(b)** Kinase and phosphatase activity of KaiC-WT without KaiA and KaiB (closed squares); with KaiA (closed triangles); with KaiB (open squares); and with KaiA and KaiB (open triangles). Kinase and phosphatase activity of KaiC-WT and phosphorylation mutants in the presence **(e)** **(f)** or absence **(c)** **(d)** of KaiA. KaiC-WT (closed gray squares); KaiC-AT (closed circles); KaiC-DT (open circles); KaiC-SA (closed triangles); KaiC-SE (open triangles). Data are presented as means ± s.e.m. (n=7–12).

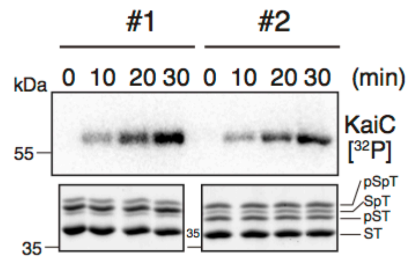
**Supplementary Figure S6**




**Supplementary Fig. S6. The effect of low temperature on KaiC activities.**

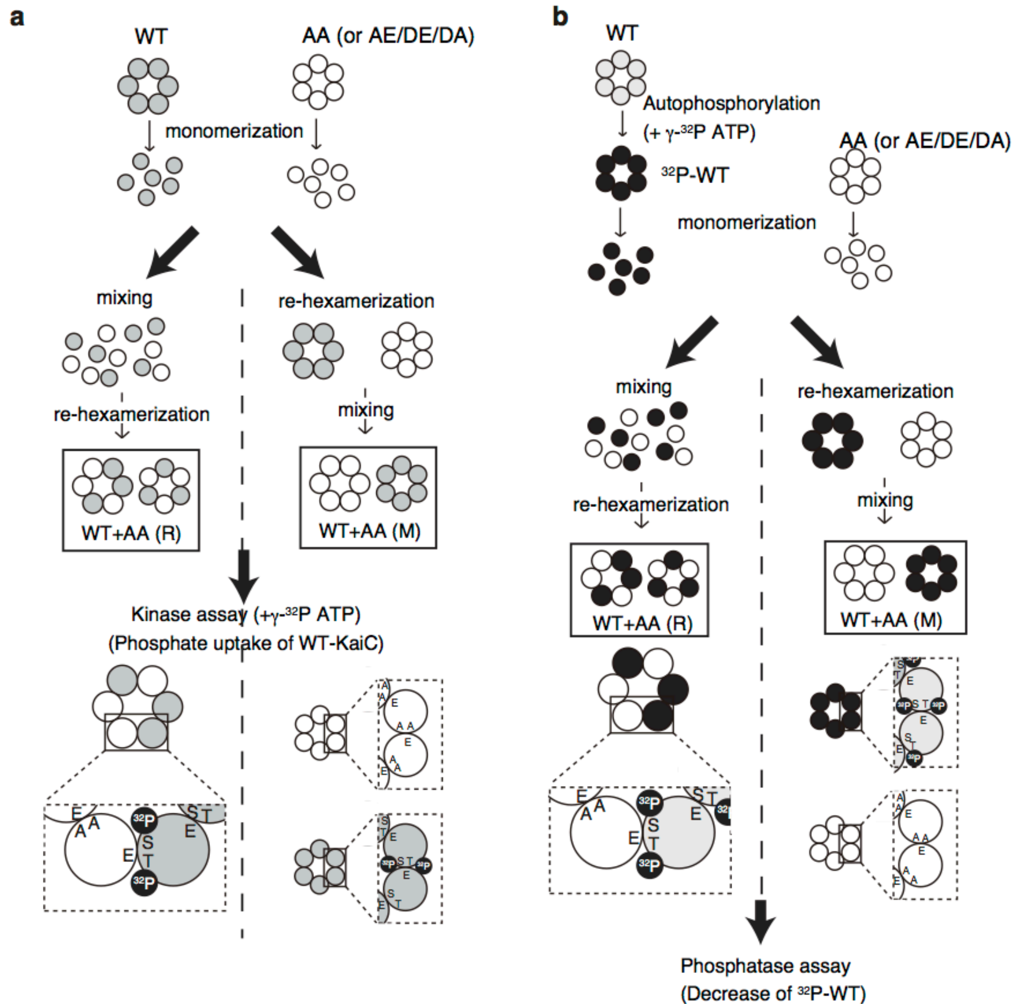
**(a)** (Left) Phosphorylation patterns of KaiC at 4°C or 30°C were determined by Coomassie brilliant blue staining. P-KaiC and KaiC designate phosphorylated and unphosphorylated KaiC, respectively. (Right) Densitometry of the bands shown in the left panel. The ratio of the amount of P-KaiC to the total amount of KaiC is plotted against the incubation time. Data represent means ± s.d. (n=4). (**b**) Dephosphorylation of KaiC at 30°C (closed square) and 4°C (open square). KaiC was autophosphorylated with [γ-$^{32}$P]ATP, and then allowed to dephosphorylate. Following SDS-PAGE, $^{32}$P-labeled KaiC was detected by autoradiography. Phosphatase activity was determined by decrease of the $^{32}$P-label band. Data are presented as means ± s.d. (n=4). (**c**) Kinase activity of KaiC at 30°C (closed square) and 4°C (open square) was determined by phosphate-uptake assay. Data are presented as means ± SEM (n=4). (**d**) ATPase activity of KaiC at 30°C (closed square) and 4°C (open square). Data are presented as means ± s.d. (n=3). (**e**) Characterization and quantitation of KaiC-bound nucleotides. The ratio of ATP relative to total nucleotides bound to KaiC at 30°C (closed squares) or 4°C (open squares) was plotted against time. Data represent means ± s.d. (n=4).

**Supplementary Figure S7**



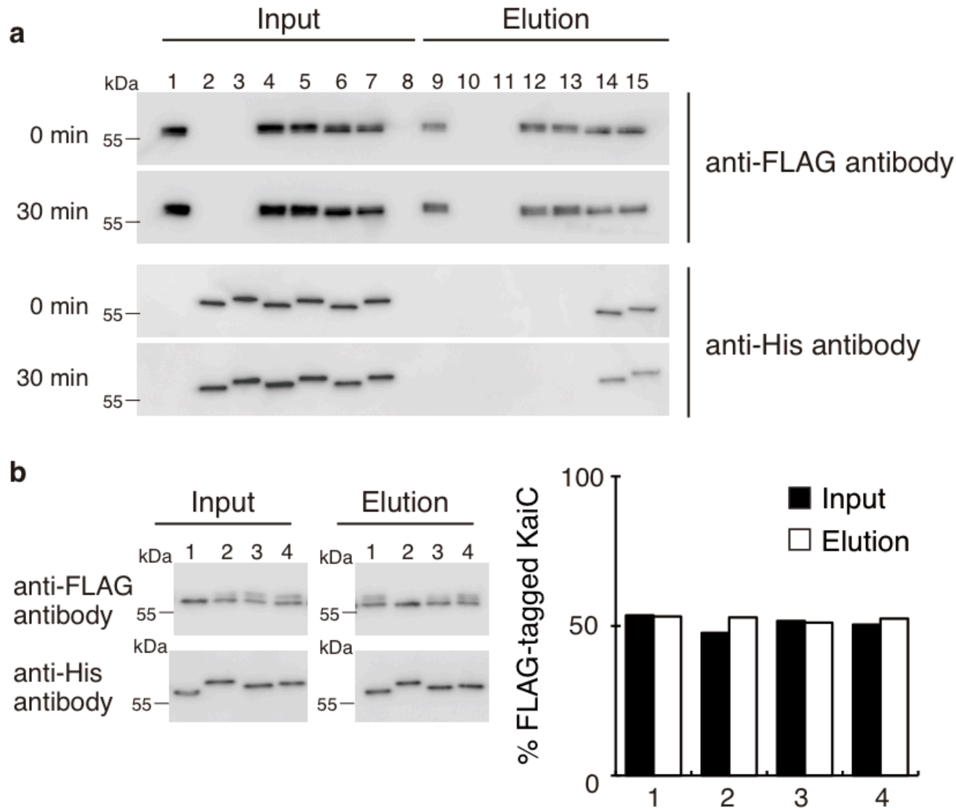

**Supplementary Fig. S7. The effect of hexamer compositions in the presence of KaiA.** Kinase activity of KaiC (#1, KaiC enriched in pT; #2, KaiC enriched in pS) was determined by phosphate-uptake assay. (Upper) Autoradiograph of $^{32}$P-labeled KaiC. (Lower) Coomassie brilliant blue–stained gel. Four bands with different mobilities were detected. From most to least mobile, the bands represent the S/T, pS/T, S/pT, and pS/pT forms of KaiC (ref. 14).

## Supplementary Figure S8




**Supplementary Fig. S8. (a)** Diagram of experimental procedures for Figure 3c and 3d. KaiC-WT and phosphorylation-mutant hexamers (KaiC-AA, -AE, -DE, -DA) were dissociated into monomers, and then reconstituted by mixing equal amounts of KaiC-WT and mutant monomers. Reconstituted mixed hexamers (WT+MT [R]) are expected to have half the WT number of intact interfaces, and should therefore exhibit half the phosphorylation of WT if there is no intersubunit communication. KaiC-WT and KaiC-mutant–only hexamers were also reconstituted and mixed to prepare the mixture of hexamers (WT+MT [M]). The autokinase activities of “R” and “M” hexamers were determined by phosphate-uptake assay. **(b)** Diagram of detailed procedures used for Figure 3e. KaiC-WT was labeled with [$\gamma$-$^{32}$P]ATP by autophosphorylation and dissociated into monomers. Equal amounts of $^{32}$P-labeled KaiC-WT and KaiC-mutant monomers were mixed to prepare reconstituted mixed hexamers (WT+MT [R]). KaiC-WT and KaiC-mutant–only hexamers were also reconstituted and mixed to prepare the mixture of hexamers (WT+MT [M]). The autophosphatase activities of “R” and “M” hexamers were determined by monitoring the decrease of $^{32}$P-labeled KaiC-WT.

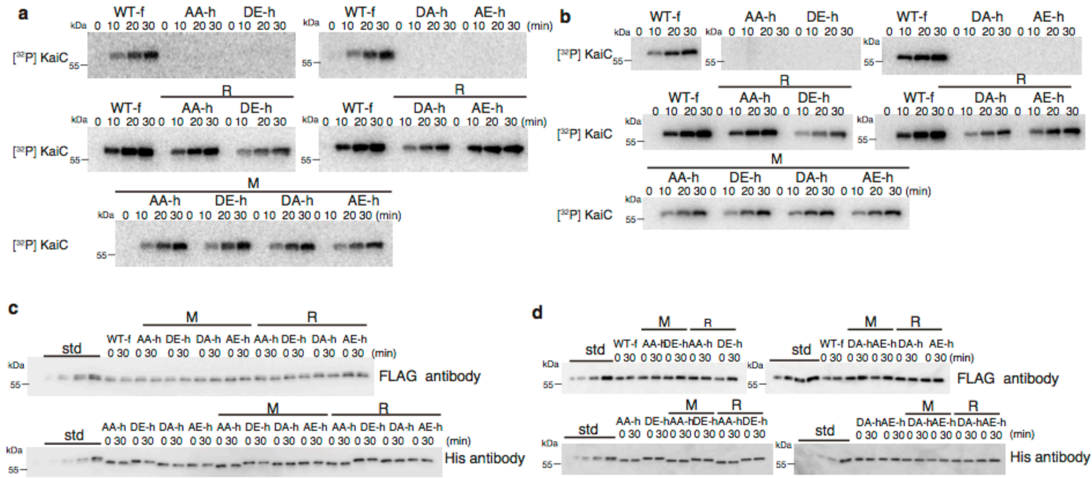
**Supplementary Figure S9**




**Supplementary Fig. S9. Characterization of hexamer composition.**

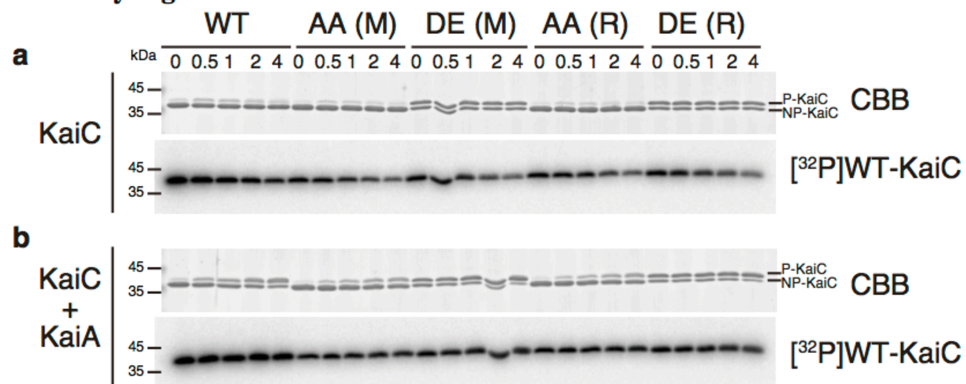
(**a**) Reconstituted hexamers of FLAG- and His-tagged KaiC ("R") and a mixture of FLAG- and His-tagged hexamers ("M") were analyzed by immunoprecipitation with anti-FLAG antibody. Input and immunoprecipitated proteins were detected by immunoblotting using anti-FLAG or anti-$His_6$ antibody. Input samples were loaded in lanes 1–7, and eluates were loaded in lanes 8–15. Lanes: 1 and 9, FLAG-tagged KaiC-WT; 2 and 10, His-tagged-KaiC-AA; 3 and 11, His-tagged-KaiC-DE; 4 and 12, FLAG-tagged KaiC-WT+His-tagged-KaiC-AA (M); 5 and 13, KaiC-WT+His-tagged-KaiC-DE (M); 6 and 14, KaiC-WT+His-tagged-KaiC-AA (R); 7 and 15, KaiC-WT+His-tagged-KaiC-DE (R); 8, FLAG-tagged KaiC-WT without anti-FLAG antibody (negative control). (**b**) Quantitation of FLAG-tagged KaiC-WT in reconstituted hexamers. Input and precipitated samples were analyzed by immunoblotting, and the percentage of FLAG-tagged KaiC in reconstituted hexamers was estimated by comparison of Western-blot signals to serial dilutions of known amounts of FLAG-tagged KaiC and His-tagged KaiC. 1, FLAG-tagged KaiC-WT+His-tagged KaiC-AA; 2, FLAG-tagged KaiC-WT+His-tagged KaiC-DE; 3, FLAG-tagged KaiC-WT+His-tagged KaiC-DA; 4, FLAG-tagged KaiC-WT+His-tagged KaiC-AE.

## Supplementary Figure S10



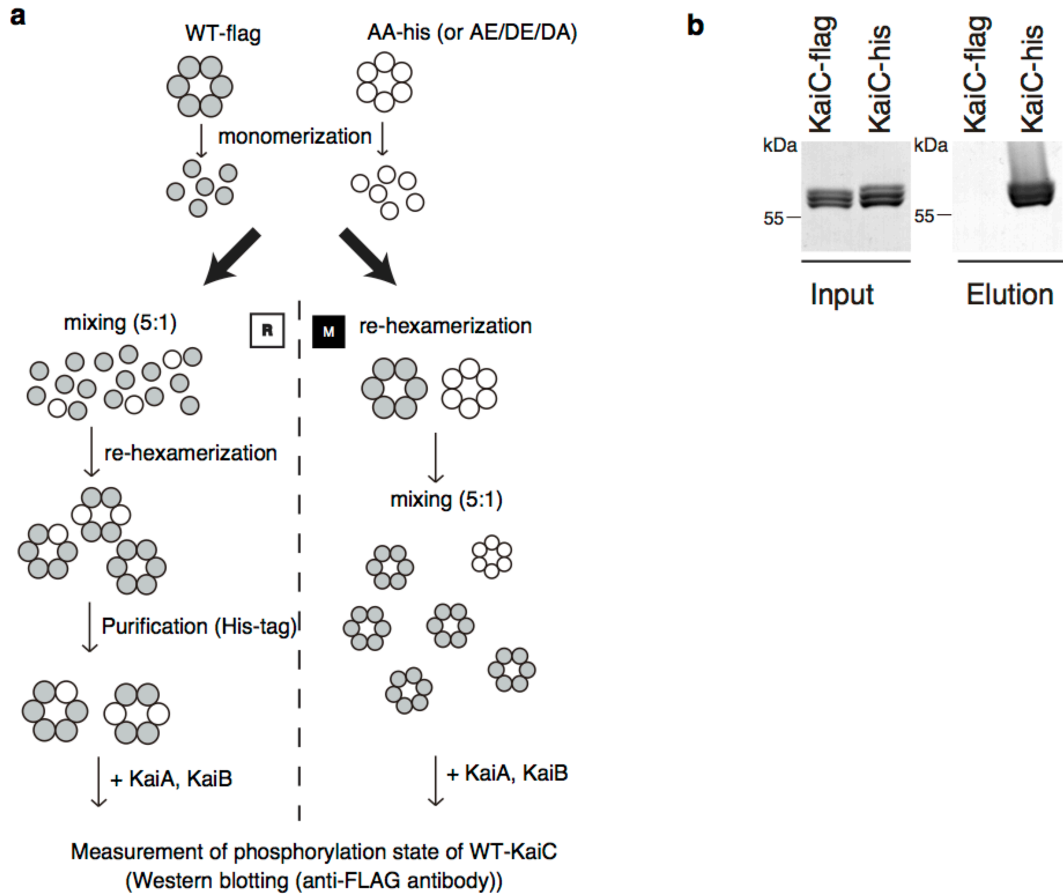

**Supplementary Fig. S10**. (**a**) (**b**) Representative autoradiographs of the *in vitro* autokinase assay from Fig. 3c and 3d without KaiA (**a**) and with KaiA (**b**). Upper panel: reconstituted pure KaiC-WT and mutant proteins. Middle panel, reconstituted mixed hexamers. Lower panel, 1:1 mixture of WT and mutant hexamers. "M" indicates a mixture of WT and mutant hexamers, and "R" indicates mixed hexamers generated from WT and mutant monomers. (**c**) (**d**) Immunoblot of samples corresponding to those in Fig. 3c and 3d, without KaiA (**c**) or with KaiA (**d**). Samples were subjected to SDS-PAGE, followed by immunoblotting with anti-FLAG and anti-$His_6$ antibodies. KaiC-WT was detected with anti-FLAG antibody, and KaiC mutants were detected with anti-$His_6$ antibody.

**Supplementary Figure S11**




**Supplementary Fig. S11**. Representative Coomassie brilliant blue–stained gel (upper panel) and autoradiograph (lower panel) of the *in vitro* autophosphatase assay shown in Fig. 3e, without KaiA (**a**) or with KaiA (**b**). The upper and lower bands correspond to phosphorylated KaiC (P-KaiC) and unphosphorylated KaiC (NP-KaiC), respectively.

**Supplementary Figure S12**




**Supplementary Fig. S12.** (**a**) Diagram of detailed procedures used for Figure 4. FLAG-tagged KaiC-WT and His-tagged phosphorylation mutant hexamers (KaiC-AA, -AE, -DE, -DA) were dissociated into monomers, and then reconstituted by mixing a 5:1 ratio of KaiC-WT and mutant monomers (WT+MT [R]). Hexamers containing His-tagged KaiC were collected by Ni-affinity chromatography. KaiC-WT and KaiC-mutant–only hexamers were also reconstituted and mixed to prepare the mixture of hexamers (WT+MT [M]). KaiA and KaiB were mixed with either "R" or "M" hexamers. The phosphorylation pattern of FLAG-tagged KaiC-WT was determined by Western blotting using anti-FLAG antibody. (**b**) Validation of the purification process. FLAG-tagged–only and His-tagged–only KaiC hexamers were subjected to purification by Ni-affinity chromatography. Samples were analyzed by immunoblotting using anti-FLAG or anti-His antibody. FLAG-tagged KaiC–only hexamers were not purified by this procedure.

**Supplementary Table S1 Primers for QuikChange mutagenesis of plasmids**

| Mutant | Primers |
|---|---|
| E318Q | 5'-ATTCTGTTCGCTTATCAAGAGTCACGAGCTCAGCTG-3' |
| | 5'-CAGCTGAGCTCGTGACTCTTGATAAGCGAACAGAAT-3' |
| S431A/T432E | 5'-TCCCATATCGCAGAAATTACGGAT-3' |
| | 5'-ATCCGTAATTTCTGCGATATGGGA-3' |
| S431D/T432A | 5'-TCCCATATCGATGCAATTACGGATT-3' |
| | 5'-ATCCGTAATTGCATCGATATGGGA-3' |
| E77QE78Q | 5'-GTTTTCGTTACTTTCCAACAAACCCCGCAAGATATC-3' |
| | 5'-GATATCTTGCGGGGTTTGTTGGAAAGTAACGAAAAC-3' |

**Supplementary References**

63. Pattanayek, R. *et al.* Analysis of KaiA–KaiC protein interactions in the cyanobacterial circadian clock using hybrid structural methods. *EMBO J.* **25**, 2017-2028 (2006).