Supplementary information for

CK1δ/ε protein kinases prime the PER2 circadian phosphoswitch

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SI Materials and Methods

Plasmids and Reagents:

myc-*mPer2*, myc-*mPer2*-S659A, myc-*mPer2*-450-763, myc-*mPer*2-S662A (450-763), myc-CK1δ1, myc-CK1ε and HA-CK1ε expression plasmids were described previously (1-3). Human CK1δ2 was cloned into the same backbone of human CK1δ1 in pCS2-MT plasmid with 5x myc tag to the N-terminus of its coding sequence and cloned into the plasmid using digestion sites BamH1 and Xba1. myc-CK1δ1-Δ400 plasmid was generated by introducing a stop codon at the 400^{th} amino acid of CK1δ1 by site-directed mutagenesis. Following chemical compounds were used in this study, DMSO (Sigma Aldrich), PF670 (PF670462, Tocris Bioscience) and D4476 (Sigma Aldrich). Various forms of FASP synthetic peptides were manufactured at SABio, Singapore with 95% or more purity. CK1δ1-Active (C65-10G) and CK1ε-Active (C66-10G) recombinant proteins were purchased from Signal Chem. Anti-myc (9E10, sc-40, Santa Cruz Biotechnology), anti-CK1δ (ab85320, Abcam), anti-CK1ε (H-60, sc-25423 - Santa Cruz Biotechnology), anti-HA (sc-805, Santa Cruz Biotechnology) and anti-tubulin (ab52623, Abcam) antibodies were purchased.

Cell culture and transfection

HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM 10313, Gibco, Carlsbad, CA) supplemented with 10% FBS (Gibco), 50 units/mL penicillin, 50 μg/mL streptomycin (Invitrogen) and maintained at 37°C in a 5% CO2 environment. Cells were transfected using Lipofectamine® 2000 transfection reagent (Life Technologies) following the manufacturer's manuals. Total plasmid DNA of either 1 or 2 μg was used for each well of a 12 or 6-well-plate respectively.

SDS-PAGE and Western blotting

Either IP-*in vitro* kinase assay samples or whole cell extracts of transfected HEK293 cells lysed with cell lysis buffer were analyzed by denaturing SDS-PAGE gel, which was transferred on PVDF membrane (Immobilon, Millipore). Blot was further probed using indicated primary antibodies and appropriate secondary antibodies conjugated with HRP. Signal was detected with ECL reagents from Thermo Fisher Scientific. Densitometric analysis of WB bands were performed using the ImageJ software (National Institutes of Health) (Fig. 4C).

Gene silencing by siRNA

1x 10^5 HEK293 cells were seeded onto a 12 well plate and transfected with 100 nM of either non-targeting con siRNA (siCtrl; D-001810-0X) or human CK1δ or CK1ε or both CK1δ (50 nM) and CK1ε (50 nM) specific siRNA 12 hr later using Dharmafect Transfection Reagent (Dharmacon RNAi Technologies), in accordance with the manufacturer's instructions. The target sequences of human CK1δ and CK1ε-specific ON-TARGETplus siRNAs (Dharmacon RNAi Technologies) have been previously reported (4, 5). 48 hr after siRNA transfection, cells were

transfected with myc-*Per2* plasmid and lysed after 48 hr using the protocols above and the samples were probed with indicated antibodies for WB.

Radioactive kinase assay

Two independent reaction mixtures (50 µl) containing 200 µM of the FASP peptide in the reaction buffer (25 mM Tris pH 7.5, 7.5 mM $MgCl_2$, 1 mM DTT, 0.1 mg/mL BSA) were preincubated for 5 min with or without CK1δ ΔC (for Fig. 1B and 1C, 20 and 200 nM respectively) and the reaction was started by addition of 750 µM of ATP (Ultra pure ATP, Promega) containing 1-2 µCi of γ-^{32}p ATP (Perkin Elmer). After incubation of the reaction mix at 30℃, an 8 µl aliquot of the reaction mix was transferred to a P81 cellulose paper (Reaction Biology Corp.) at each indicated time point. The P81 cellulose paper was washed thrice with 75 mM of orthophosphoric acid and once with acetone. The air-dried paper was counted for Pi incorporation using a scintillation counter (Perkin Elmer) by Cherenkov counting (Fig. 1B & 1C).

Immunoprecipitation and *in vitro* (IP-*in vitro*) kinase assay

HEK293 cells transfected with myc-*Per2* plasmid was lysed in cell lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, and 0.5% deoxycholic acid containing Complete protease inhibitors (Roche) and PhosStop phosphatase inhibitors (Roche)). 100 µg of the protein lysate was added with 1 µg of anti-myc antibody and allowed to rotate at 4℃ for 1 hr, which was followed by addition of Protein A/G magnetic beads (Thermo Scientific) and rotation at 4℃ for 1hr. Then the beads were collected and washed thrice with lysis buffer and twice with kinase assay buffer (25 mM Tris pH 7.5, 5 mM beta glycerophosphate, 2 mM DTT and 0.1 mM sodium orthovanadate). Next, the beads were split into two and kinase buffer containing 10 mM magnesium chloride and 200 µM ATP with 500 ng of recombinant purified CK1δ1 (Signal Chem) was added to the first half, and the same was added to the second half without kinase. Beads were collected after a 30 min incubation at 30℃ and the protein was eluted from beads by adding protein loading dye and analyzed by SDS-PAGE gel for Western blotting (Fig. 2D).

In vitro kinase assay and ELISA

For ELISA, 1 µg/ml of FASP-WT and FASP-pS659 (Fig. 2A) peptide was diluted in carbonate buffer, pH 9.5 and coated onto a 96 well ELISA plate (100 µl/well) and incubated overnight at 4℃. The next day, wells were washed with wash buffer (PBS with 0.05% Tween, PBS-T), blocked with blocking buffer for 1 hr (PBS-T with 5% BSA) and incubated with the pS659 antibody for 1 hr at room temperature (RT). Next, wells were incubated with anti-rabbit antibody conjugated to Biotin and further incubated with Streptavidin-HRP for detection. For signal detection, TMB (1-Step Ultra TMB-ELISA, Thermo Scientific) was added, incubated for color development and stopped by addition of STOP solution (Thermo Scientific), which was read at 450 nm using an xMark Spectrophotometer plate reader (Biorad). Each sample was analyzed in triplicate.

For the ELISA kinase assay, 2 μg/ml of FASP-WT peptide was diluted in carbonate buffer, pH 9.5 and coated onto a 96 well plate (100 μl/well). The next day, wells were washed thrice with wash buffer and once with kinase buffer (25 mM Tris pH 7.5, 5 mM beta glycerol phosphate, 2 mM DTT and 0.1 mM sodium orthovanadate). Reaction mixture (50 μl) containing either 10, 50 or 100 ng of CK1δ or CK1ε purified protein in the kinase buffer including 10 mM magnesium chloride and 200 μM ATP was added onto each well and the plate was incubated at 30℃ for 1 hr. Next, the reaction mixture was discarded and wells were washed with wash buffer thrice and incubated with blocking buffer for 1 hr at RT. Subsequently, wells were incubated with pS659 Ab, anti-rabbit antibody conjugated to Biotin and Streptavidin-HRP for 1 hr at RT with washing step after each incubation. HRP signal was detected and read as mentioned above. For each sample, the kinase assay was performed in triplicate.

Expression and purification of recombinant proteins

Proteins were expressed from a pET22b vector in *Escherichia coli* Rosetta2 (DE3) cells. Wild-type FASP peptide (residues 642-666 of mPER2 containing an N-terminal WRKKK polybasic motif and tryptophan for UV detection) and FASP mutants each contained an N-terminal TEV-cleavable NusA tag that includes an N-terminal 6x histidine tag separated from the start of the NusA sequence by a flexible linker. S659A and S662A mutations were made in the plasmid using site-directed mutagenesis and confirmed by sequencing. Each peptide has an additional N-terminal vector artifact of 'GAMDPEF' remaining after TEV cleavage.

Cells were grown in M9 minimal medium containing 1 g/L $^{15}NH_4Cl$ for ^{15}N-labeled samples with 3 g/L [^{13}C]-glucose for ^{13}C, ^{15}N-labeled samples (Cambridge Isotope Laboratories, Inc.). Protein expression was induced with 0.5 mM IPTG when the cells reached O.D. 0.6-0.8, and expression proceeded at 18 ˚C for approximately 16 hr. Cells were lysed in 50 mM Tris pH 7.5, 500 mM NaCl, and 20 mM imidazole containing EDTA-free protease inhibitors (Pierce). HisNusA-FASP fusion proteins were purified using Ni-NTA resin (Qiagen) and eluted from the resin using 250 mM imidazole. HisNusA-FASP was buffer exchanged using an Amicon Ultra centrifugal filter (Millipore) to remove the imidazole. His-tagged TEV protease was added to cleave the HisNusA tag from FASP at 4 ˚C for approximately 16 hr. Cleaved FASP was purified from HisNusA and HisTEV using Ni-NTA resin. The FASP peptide was further purified using size exclusion chromatography on a HiLoad™ 16/600 Superdex™ 75 pg (GE Healthcare) in high salt buffer to prevent electrostatic interaction between residual HisNusA and FASP (50 mM Tris pH 7.5 or 25 mM MOPS pH 7.0, 500 mM NaCl, 1 mM EDTA, and 10% glycerol). FASP was then concentrated and buffer exchange into NMR buffer (25 mM MOPS pH 7.0, 50 mM NaCl, 2 mM b-mercaptoethanol, 1 mM EDTA, 11 mM $MgCl_2$) using an Amicon Ultra centrifugal filter (Millipore).

NMR spectroscopy

All NMR spectra were collected at 30 ˚C on a Varian INOVA 600 MHz spectrometer equipped with a ^{1}H, ^{13}C, ^{15}N triple resonance z-axis pulsed-field-gradient cryoprobe. Spectra

were processed using NMRPipe and NMRDraw and analyzed using CcpNmr Analysis(6). Backbone resonance assignments of 86% of the non-proline residues were made using standard BioPack triple resonance experiments (HNCACB, CBCA (CO)NH, HNCO, and HN (CA)CO) collected using non-uniform sampling on a sample of 0.4 mM ^{13}C, ^{15}N-labeled FASP in NMR buffer containing 10% D_2O. Non-uniform sampling reconstructions were performed using software developed and provided by the Wagner lab (6).

NMR kinase assays were performed at 30˚C with 0.2 mM ^{15}N-FASP wild-type, ^{15}N-FASP S659A, or ^{15}N-FASP S662A in NMR buffer containing 1X protease inhibitors (Pierce), 0.25 mM ATP, 5 mM b-glycerol phosphate, and 0.1 mM Na_3VO_4. CK1δΔC was added to a final concentration of 0.2 µM. At discrete time points, 7.5 nmol of FASP were removed and quenched with a final concentration of 20 mM EDTA to chelate the magnesium required for the phosphorylation reaction. The samples were stored at 4 ˚C prior to collecting the NMR data. ^{15}N-HSQC spectra were collected on each sample with identical parameters. CcpNmr Analysis was used to extract peak volumes for each time point. Relative peak volumes were calculated using equation 1 and normalized for sample variation using equation 2, as described previously (7).

Equation 1.

$$\text{Relative peak volume} = \left(\frac{V}{V_0}\right) F_{scale}$$

Where V is the peak volume at a specific time point, V_0 is the peak volume at 0 min (or 4 h for phosphorylated serines), and F_{scale} is the scaling factor used to normalize between samples.

Equation 2.

$$F_{scale} = \frac{1}{n}\sum\left(\frac{V_{ref,0}}{V_{ref}}\right)$$

Where V_{ref} is the volume of a reference peak that does not change during the reaction at the relevant time point, and $V_{ref,0}$ is the peak volume at 0 min. The values are averaged among the reference samples to calculate F_{scale}. Relative peak volume for each residue was plotted against time and fit to a one-phase exponential decay, equation 3, using GraphPad Prism 7, version 7.0c.

Equation 3.

$$y = (y_0 - c)e^{-kt} + c$$

Where y is the peak volume at time t, y_0 is the peak volume at time 0, c is the peak volume at infinite time, and k is the rate constant expressed in units of inverse time. For peaks that did not follow exponential growth or decay, the values were fit to a linear regression.

Mathematical model of the mammalian circadian clock

The previous mathematical model of the mammalian circadian clock including the phosphoswitch(1) is modified based on the new findings. In the previous model, the translated PER2 protein has two fates, depending on the site of initial phosphorylation. Specifically, a phosphorylation by CK1 at the β-TrCP binding site leads to the β-TrCP binding and thus rapid degradation. On the other hand, a phosphorylation at another site (the FASP site) by an unknown priming kinase leads to the sequential phosphorylation at neighboring downstream sites by CK1, which stabilizes PER2 protein. In the modified model, instead of the unknown priming kinase, CK1 phosphorylates the FASP site of PER2. That is, after CK1 is bound to the translated PER2 protein, it phosphorylates either the β-TrCP binding site or the FASP site, which determines the fate of PER2 protein.

Furthermore, CK1ε and two isoforms of CK1δ, CK1δ1 and CK1δ2 with different kinetics are now included in the model (Fig. 4 D & E). Specifically, the CK_1 variable represents the concentration of CK1δ1 (Table S2). Furthermore, the CK_2 variable represents the concentration of both CK1δ2 and CK1ε (Table S2) since CK1δ2 and CK1ε have the similar phosphorylation rates (Fig. 4 C & D). We assume that the ratio of CK1ε, CK1δ1 and CK1δ2 levels is 0.1:0.45:0.45 in the cell and thus the ratio between CK_1:CK_2 is 0.45:0.55 in the model as the abundance of CK1ε is much less than that of CK1δ (8, 9).

The model is described with ordinary differential equations, which can be found in the supplementary information (Table S4). The description of variables and parameters of the mathematical model can be found in Tables S2 and S3. The parameters of the model were estimated by simulated annealing method (a stochastic global parameter-searching algorithm) (10, 11), so that the models can simulate the followings:

- The period of rhythm is ~24hr (Fig. 4D)
- The degradation kinetics of PER2 (1): three-stage decay during the accumulation phase and exponential decay during the falling phase (Fig. 4D)
- Mutation phenotypes of CK1ε-/-, CK1δ-/-, FASP (8, 12) (Fig. 4E).

To simulate the CK1ε-/-, the parameter CKt_2, which representing the concentration of CK1ε and CK1δ1 (Table S3), is reduced to 0.45/0.55 of the original value because the ratio between

CK1ε and CK1δ2 is assumed to be 0.1:0.45. To simulate CK1δ-/-, CKt_1 is reduced to 0 and CKt_2 is reduced to 0.1/0.55 of the original value. To simulate the FASP mutation, the value of priming phosphorylation rate parameters (kp_1 and kp_2) are reduced to 10% of their original values.

Computer simulation of the model

All the simulations were performed with Mathematica 11.0 (Wolfram Research). Mathematica code is provided in the supplementary information (Table S5).

A

Peptides for kinase assay

659 662 665

mPER2-FASP **WT** peptide: RKKKTEVSAHLSSLTLPGKAE**S**VV**S**LT**S**Q

mPER2-FASP **S659A** peptide: RKKKTEVSAHLSSLTLPGKAE**A**VVSLTSQ

mPER2-FASP **S662A** peptide: RKKKTEVSAHLSSLTLPGKAESVV**A**LTSQ

mPER2-FASP **pS659** peptide: RKKKTEVSAHLSSLTLPGKAE**pS**VVSLTSQ

mPER2- aa 642-666

B

Peptide for NMR analysis

659 662 665

mPER2-FASP WTGAMDPEF**W**RKKKTEVSAHLSSLTLPGKAE**S**VV**S**LT**S**Q

Vector seq mPER2- aa 642-666

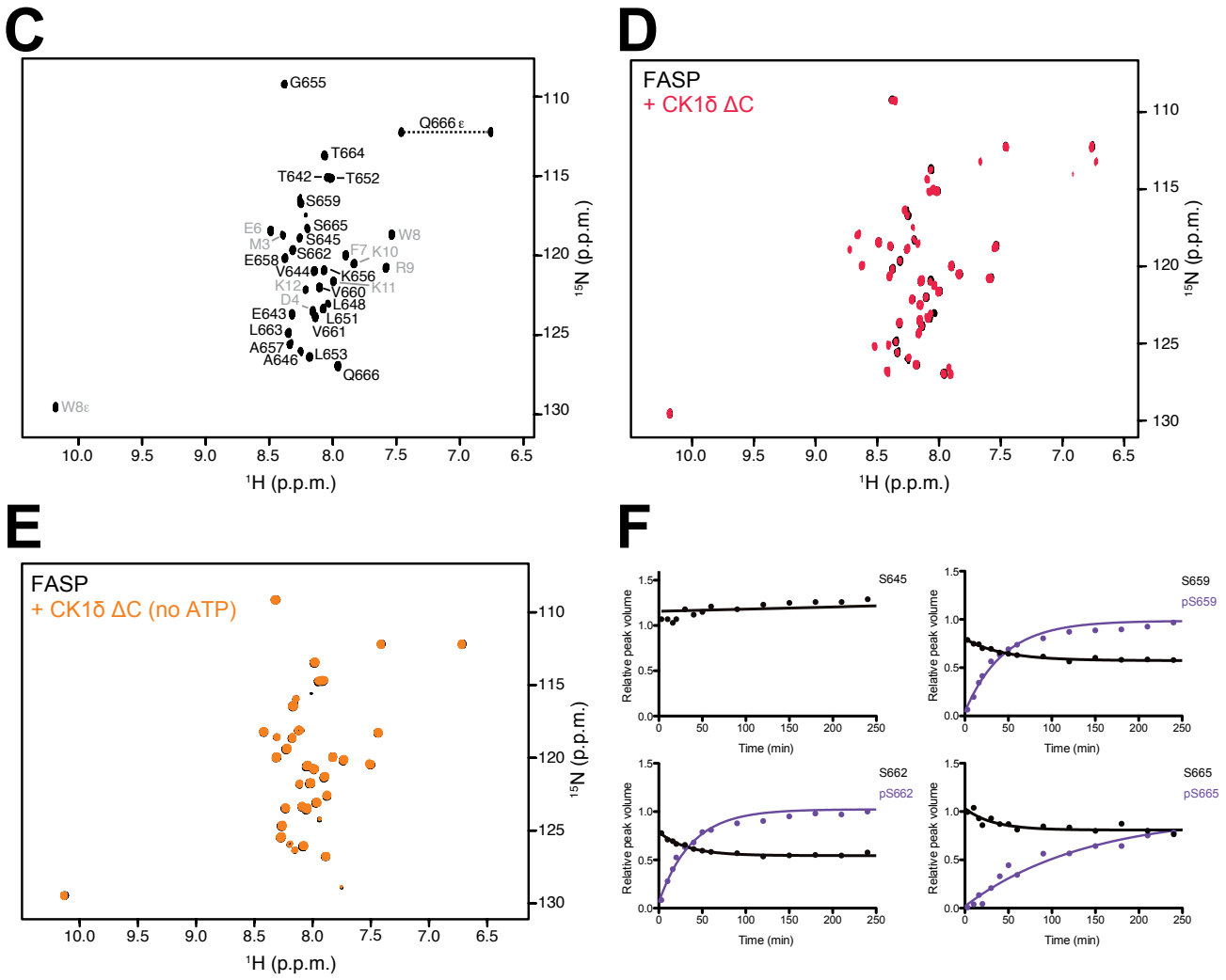

Fig. S1 Phosphorylation of S659 of mPER2 is necessary to phosphorylate the downstream serines of the FASP region.

(A) Sequences of various FASP peptides used in radioactive and ELISA-based in vitro kinase assays. Sequences from mPER2 protein are underlined and three serine residues of the FASP region are marked. RKKK polybasic motif is included to facilitate the peptide kinase assay. (B) Sequence of the wild-type peptide used in NMR-based kinase assays. In addition to residues 642 to 666 of mPER2, the peptide contains the vector artifact 'GAMDPEF', W to monitor the peptide by A280 during purification, and the basic residues included in other constructs. (C)15N/1H HSQC spectrum of 15N mPER2 FASP peptide. Peaks are annotated to show backbone amide assignments. Gray text corresponds to additional N-terminal residues not part of the native sequence, while the black text corresponds to mPER2 residues 642 to 666. (D) Full view of overlaid 115N/1H HSQC spectra of 200 μM 15N mPER2 FASP peptide alone (black) or 4 hours after addition of 200 nM CK1δ ΔC (red) at 30°C. NMR spectra were acquired after kinase reactions were quenched with 20 mM EDTA. (E) Full view of overlaid 15N/1H HSQC spectra of 200 μM 15N mPER2 FASP peptide alone (black) or 4 hours after addition of 200 nM CK1δ ΔC (orange) without ATP. (F) Quantification of peak volumes for indicated serines in the15N mPER2 FASP peptide from kinase assays monitored in real-time by NMR (unphosphorylated, black; phosphorylated, purple). Data points are fit to a one-phase exponential curve, except for S645, which is fit to a linear regression.

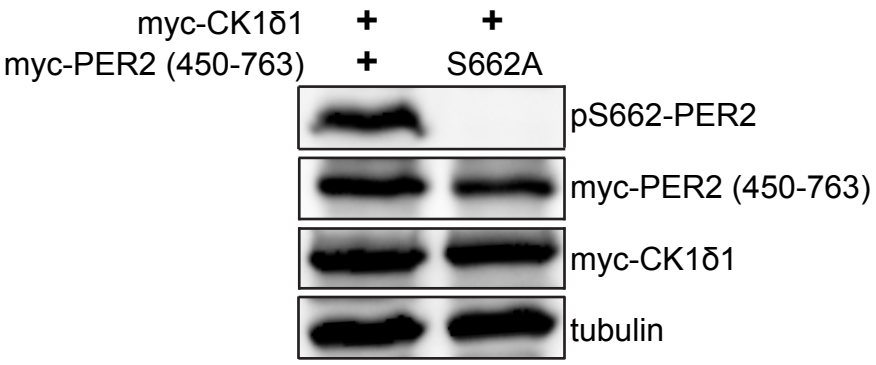

Fig. S2. Demonstration of the specificity of phospho-S662 antibody. Myc-PER2 (450-763) and myc-PER2-S662A (450-763) proteins were expressed with CK1δ1 in HEK293 cells and lysates were probed with indicated antibodies.

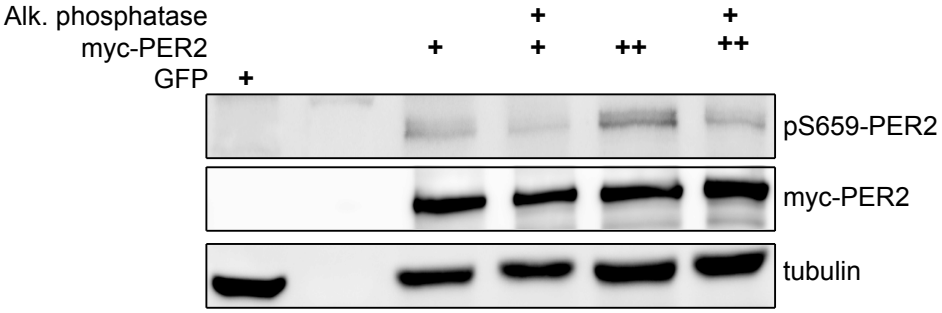

Fig. S3. Demonstration of phospho-specificity of phospho-S659 antibody.
Myc-PER2 protein was expressed in HEK293 cells and lysates were either untreated or treated with alkaline phosphatase for 1 hr before samples were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

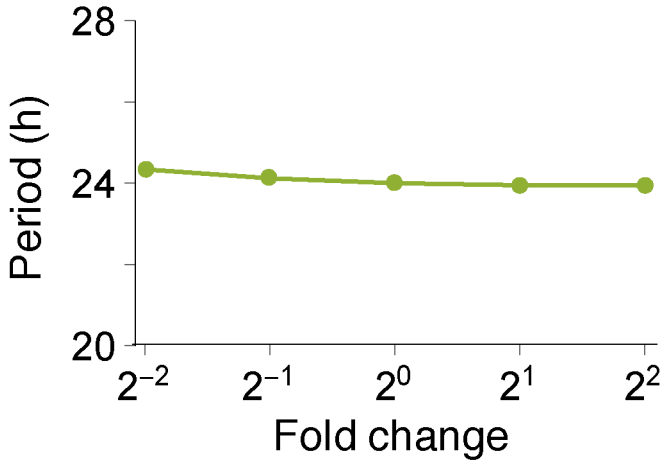

Fig. S4. Mathematical model predicts that period is robust to the simultaneous change of CK1 phosphorylation rate and dissociation constant. Here, CK1 phosphorylation rate and dissociation constant for both the priming and the β-TrCP sites (i.e. kr1, kr2, kpo, and Kcbo) are changed in the same magnitude (see Table S3 for the detailed description of parameters).

Table S1. Site-specific phosphorylation rates on peptide substrates.

Residue	Wild-type (k, min^{-1})	S659A (k, min^{-1})	S662A (k, min^{-1})
S659	0.018 ± 0.045[a]	-	0.016 ± 0.006
S662	0.015 ± 0.037	~ 0	-
S665	0.033 ± 0.012	~ 0	~ 0
pS659	0.014 ± 0.007	-	0.009 ± 0.004
pS662	0.014 ± 0.004	N.D	-
pS665	0.004 ± 0.003[b]	N.D	N.D

N.D.: no detectable peak in NMR spectra

[a] data fit to one-phase exponential decay with standard error associated with fit

[b] data fit to linear regression with standard error associated with fit

Table S2- Variables of the mathematical model

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Name	Symbol
The concentration of *Per2* mRNA	m
The concentration of unphosphorylated PER2	c_0
The concentration of unphosphorylated PER2 bound to CK1δ1/ CK1δ2	c_0CK_1/c_0CK_2
The concentration of PER2 phosphorylated at β-TrCP binding site	ct_1
The concentration of PER2 phosphorylated at β-TrCP site bound to phosphatase	ct_1PP
The concentration of PER2 phosphorylated at 1st FASP site	c_1
The concentration of PER2 phosphorylated at 1st FASP site bound to phosphatase	c_1PP
The concentration of PER2 phosphorylated at 1st FASP site bound to CK1δ1/ CK1δ2	c_1CK_1/c_1CK_2
The concentration of PER2 phosphorylated at 2nd FASP site	c_2
The concentration of PER2 phosphorylated at 2nd FASP site bound to phosphatase	c_2PP
The concentration of PER2 phosphorylated at 2nd FASP site bound to CK1δ1/ CK1δ2	c_2CK_1/c_2CK_2
The concentration of PER2 phosphorylated at 3rd FASP site	c_3
The concentration of PER2 phosphorylated at 3rd FASP site bound to phosphatase	c_3PP
The concentration of PER2 phosphorylated at 3rd FASP site bound to CK1δ1/ CK1δ2	c_3CK_1/c_3CK_2
The concentration of PER2 phosphorylated at terminal FASP site	c_4
The concentration of PER2 phosphorylated at terminal FASP site bound to phosphatase	c_4PP
The concentration of PER2 phosphorylated at terminal FASP site bound to CK1δ1/ CK1δ2	c_4CK_1/c_4CK_2
The concentration of ubiquitinated PER2	c_{ub}
The concentration of CK1δ1/ CK1δ2	CK_1/CK_2
The concentration of phosphatase	PP

While CK_2 represents the both CK1ε and CK1δ2 in the model, CK1ε is skipped in the description of variable for the simplicity.

Table S3. Parameters of the mathematical model

Parameter Description	Symbol	Value
Transcription rate constant for *Per2* mRNA	ao	59.5nM/hr
Translation rate constant for PER2	at	1/hr
Binding rate constant for CK1δ1/δ2 to unphosphorylated PER2	**kpf**	**0.032/ nM hr**
Binding rate constant for CK1δ1/δ2 to primed PER2	**kcf**	**10.77/ nM hr**
Binding rate constant for phosphatase to PER2	kppf	7.32nM/hr
Dissociation constant between PER2 and BMAL1-CLOCK	Kd	1.555×10^{-5}nM
Unbinding rate constant between CK1δ1 and unphosphorylated PER2	**Kcbo**	**6.05/hr**
Unbinding rate constant between CK1δ1/δ2 and PER2 primed at 1st & 2nd FASP sites	**Kcbt**	**1.7/hr**
Unbinding rate constant between CK1 and PER2 phosphorylated at 3rd & 4th FASP sites	**Kcbh**	**5.22×10^{-14}/hr**
Unbinding rate constant between phosphatase and PER2	Kppb	0.0694/hr
CK1δ1/δ2 phosphorylation rate constant for β-TrCP binding site	**kpo**	**287.5/hr**
CK1δ1 priming phosphorylation rate constant for PER2	**kr_1**	**6.6 /hr**
CK1δ2 priming phosphorylation rate constant for PER2	**kr_2**	**38.8 /hr**
CK1δ1/δ2 phosphorylation rate constant for primed PER2 at FASP sites	**kp**	**0.61 /hr**
Dephosphorylation rate constant for PER2 at β-TrCP binding site	kppo	28.166/hr
Dephosphorylation rate constant for PER2 at FASP sites	kpp	9.608/hr
Total CK1δ1 concentration	**CKt_1**	**166.1nM**
Total CK1δ2 concentration	**CKt_2**	**203nM**
Total phosphatase concentration	PPt	11.504nM
Total activator (BMAL1-CLOCK) in nucleus concentration	ACt	1.366nM
Degradation rate constant for *Per2* mRNA	do	0.242/hr
Degradation rate constant for unphosphorylated PER2 and phosphorylated at FAPS site of PER2	bo	6.77×10^{-4}/hr
Degradation rate constant for ubiquitinated PER2	bt	73.157/hr
Degradation rate constant for fully phosphorylated PER2 at FASP sites	bh	0.339/hr
Ubiquitination rate constant for PER2 phosphorylated at β-TrCP binding site	ub	655.9/hr

Note that CK1δ2 represents both CK1ε and CK1δ2. Newly added or modified parameters from the original model (Zhou et. al) are highlighted as bold.

Table S4- Differential equation to describe the mathematical model

$$\frac{d[m]}{dt} = \frac{ao(1-([c_4]+[c_4PP]+[c_4CK_1]+[c_4CK_2]))-Kd+\sqrt{(1-([c_4]+[c_4PP]+[c_4CK_1]+[c_4CK_2]))-Kd)^2+4Kd)}}{2ACt} - do[m]$$

$$\frac{d[c_0]}{dt} = at[m]-kpf[c_0]([CK_1]+[CK_2])+Kpb_1[c_0CK_1]+Kpb_2[c_0CK_2]+kpp[c_1PP]+kppo[ct_1PP]-bo[c_0]$$

$$\frac{d[c_0CK_i]}{dt} = kpf[c_0][CK_i]-(Kpb_i+kr_i+kpo+bo)[c_0CK_i] \ (i=1,2)$$

$$\frac{d[ct_1]}{dt} = kpo([c_0CK_1]+[c_0CK_2])-kppf[ct_1][PP]+Kppb[ct_1PP]-(ub+bo)[ct_1]$$

$$\frac{d[ct_1PP]}{dt} = kppf[ct_1][PP]-(Kppb+ub+bo+kppo)[ct_1PP]$$

$$\frac{d[c_1]}{dt} = kr_1[c_0CK_1]+kr_2[c_0CK_{d2}]-kppf[c_1][PP]+Kppb[c_1PP]-kcf[c_1]([CK_1]+[CK_2])+Kcbt([c_1CK_1]+[c_1CK_2])-bo[c_1]+kpp[c_2PP]$$

$$\frac{d[c_1PP]}{dt} = kppf[c_1][PP]-(Kppb+bo+kpp)[c_1PP]$$

$$\frac{d[c_1CK_i]}{dt} = kcf[c_1][CK_i]-(Kcbt+bo+kp)[c_1CK_i] \ (i=1,2)$$

$$\frac{d[c_2]}{dt} = kp([c_1CK_1]+[c_1CK_2])-kppf[c_2][PP]+Kppb[c_2PP]-kcf[c_2]([CK_1]+[CK_2])+Kcbt([c_2CK_1]+[c_2CK_2])-bo[c_2]+kpp[c_3PP]$$

$$\frac{d[c_2PP]}{dt} = kppf[c_2][PP]-(Kppb+bo+kpp)[c_2PP]$$

$$\frac{d[c_2CK_i]}{dt} = kcf[c_2]([CK_i])-(Kcbt+bo+kp)[c_2CK_i] \ (i=1,2)$$

$$\frac{d[c_3]}{dt} = kp([c_2CK_1]+[c_2CK_2])-kppf[c_3][PP]+Kppb[c_3PP]-kcf[c_3]([CK_1]+[CK_2])+Kcbh([c_3CK_1]+[c_3CK_2])-bo[c_3]+kpp[c_4PP]$$

$$\frac{d[c_3PP]}{dt} = kppf[c_3][PP]-(Kppb+bo+kpp)[c_3PP]$$

$$\frac{d[c_3CK_i]}{dt} = kcf[c_3][CK_i]-(Kcbh+bo+kp)[c_3CK_i] \ (i=1,2)$$

$$\frac{d[c_4]}{dt} = kp([c_3CK_1]+[c_3CK_2])-kppf[c_4][PP]+Kppb[c_4PP]-kcf[c_4]([CK_1]+[CK_2])+Kcbh([c_4CK_1]+[c_4CK_2])-bh[c_4]$$

$$\frac{d[c_4PP]}{dt} = kppf[c_4][PP]-(Kppb+bh+kpp)[c_4PP]$$

$$\frac{d[c_4CK_i]}{dt} = kcf[c_4][CK_i]-(Kcbh+bh)[c_4CK_i] \ (i=1,2)$$

$$\frac{d[c_{ub}]}{dt} = ub([ct_1]+[ct_1PP])-bt[c_{ub}]$$

$$\frac{d[CK_i]}{dt} = -kcf[CK_i]([c_1]+[c_2]+[c_3]+[c_4])-kpf[c_0][CK_i]+(kp+bo)([c_1CK_i]+[c_2CK_i]+[c_3CK_i])+bh[c_4CK_i]$$
$$+(Kpb_i+kr_i+bo+kpo)[c_0CK_i]+Kcbt([c_1CK_i]+[c_2CK_i])+Kcbh([c_3CK_i]+[c_4CK_i]) \ (i=1,2)$$

$$\frac{d[PP]}{dt} = -kppf[PP]([ct_1]+[c_1]+[c_2]+[c_3]+[c_4])+(Kppb+kpp+bo)([c_1PP]+[c_2PP]+[c_3PP])$$
$$+(Kppb+kppo+bo)[ct_1PP]+(Kppb+kpp+bh)[c_4PP]+ub[ct_1PP]$$

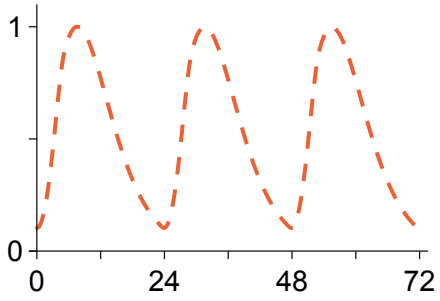
Table S5 Mathematica code

Mathematica notebook file is available upon request (Jae Kyoung Kim, KAIST, jaekkim@kaist.ac.kr)

```
endt = 300; (*Simulation time*)

(*Parameters. See Table S3 for details*)
{ao, at, kpf, kcf, kppf, Kd, Kcbo, Kcbt, Kcbh, Kppb, kpo,
   kr1, kr2, kp, kppo, kpp, CKt1, CKt2, PPt, ACt, do, bo, bt, bh, ub} =
  {59.5, 1, 0.032, 10.77, 7.32, 1.555 * 10^-5, 6.05, 1.7, 5.22 * 10^-14,
   0.0694, 287.5, 6.6, 38.8, 0.61, 28.166, 9.608, 166.1, 203,
   11.504, 1.366, 0.242, 6.77 * 10^-4, 73.157, 0.339, 655.9};

(*Model equations. See Table S1 and S2 for details*)
aone =
  {m'[t] == ao * (1 - (c4[t] + c4PP[t] + c4CK1[t] + c4CK2[t]) / ACt - Kd / ACt +
         Sqrt[(1 - (c4[t] + c4PP[t] + c4CK1[t] + c4CK2[t]) / ACt - Kd / ACt) ^ 2 +
           4 * Kd / ACt]) / 2 - do * m[t],
   c0'[t] == at * m[t] - kpf * c0[t] * (CK1[t] + CK2[t]) + Kcbo * c0CK1[t] +
     Kcbo * c0CK2[t] + kpp * c1PP[t] + kppo * ct1PP[t] - bo * c0[t],
   c0CK1'[t] == kpf * c0[t] * CK1[t] - (Kcbo + kr1 + bo + kpo) * c0CK1[t],
   c0CK2'[t] == kpf * c0[t] * CK2[t] - (Kcbo + kr2 + bo + kpo) * c0CK2[t],
   ct1'[t] == kpo * c0CK1[t] + kpo * c0CK2[t] - kppf * ct1[t] * PP[t] +
     Kppb * ct1PP[t] - (ub + bo) * ct1[t],
   ct1PP'[t] == kppf * ct1[t] * PP[t] - (Kppb + ub + bo + kppo) * ct1PP[t],
   c1'[t] == kr1 * c0CK1[t] + kr2 * c0CK2[t] -
     kppf * c1[t] * PP[t] + Kppb * c1PP[t] - kcf * c1[t] * (CK1[t] + CK2[t]) +
     Kcbt * c1CK1[t] + Kcbt * c1CK2[t] - bo * c1[t] + kpp * c2PP[t],
   c1PP'[t] == kppf * c1[t] * PP[t] - (Kppb + bo + kpp) * c1PP[t],
   c1CK1'[t] == kcf * c1[t] * CK1[t] - (Kcbt + kp + bo) * c1CK1[t],
   c1CK2'[t] == kcf * c1[t] * CK2[t] - (Kcbt + kp + bo) * c1CK2[t],
   c2'[t] == kp * (c1CK1[t] + c1CK2[t]) -
     kppf * c2[t] * PP[t] + Kppb * c2PP[t] - kcf * c2[t] * (CK1[t] + CK2[t]) +
     Kcbt * c2CK1[t] + Kcbt * c2CK2[t] - bo * c2[t] + kpp * c3PP[t],
   c2PP'[t] == kppf * c2[t] * PP[t] - (Kppb + bo + kpp) * c2PP[t],
   c2CK1'[t] == kcf * c2[t] * CK1[t] - (Kcbt + kp + bo) * c2CK1[t],
   c2CK2'[t] == kcf * c2[t] * CK2[t] - (Kcbt + kp + bo) * c2CK2[t],
   c3'[t] == kp * (c2CK1[t] + c2CK2[t]) -
     kppf * c3[t] * PP[t] + Kppb * c3PP[t] - kcf * c3[t] * (CK1[t] + CK2[t]) +
     Kcbh * c3CK1[t] + Kcbh * c3CK2[t] - bo * c3[t] + kpp * c4PP[t],
   c3PP'[t] == kppf * c3[t] * PP[t] - (Kppb + bo + kpp) * c3PP[t],
   c3CK1'[t] == kcf * c3[t] * (CK1[t]) - (Kcbh + kp + bo) * c3CK1[t],
   c3CK2'[t] == kcf * c3[t] * (CK2[t]) - (Kcbh + kp + bo) * c3CK2[t],
```

```
    c4'[t] == kp * (c3CK1[t] + c3CK2[t]) - kppf * c4[t] * PP[t] + Kppb * c4PP[t] -
      kcf * c4[t] * (CK1[t] + CK2[t]) + Kcbh * c4CK1[t] + Kcbh * c4CK2[t] - bh * c4[t],
    c4PP'[t] == kppf * c4[t] * PP[t] - (Kppb + bh + kpp) * c4PP[t],
    c4CK1'[t] == kcf * c4[t] * CK1[t] - (Kcbh + bh) * c4CK1[t],
    c4CK2'[t] == kcf * c4[t] * CK2[t] - (Kcbh + bh) * c4CK2[t],
    cub'[t] == ub * (ct1[t] + ct1PP[t]) - bt * cub[t],
    CK1'[t] == -kcf * CK1[t] * (c1[t] + c2[t] + c3[t] + c4[t]) - kpf * c0[t] * CK1[t] +
      (kp + bo) * (c1CK1[t] + c2CK1[t] + c3CK1[t]) + bh * c4CK1[t] + (Kcbo + kr1 + bo + kpo) *
       (c0CK1[t]) + Kcbt * (c1CK1[t] + c2CK1[t]) + Kcbh * (c3CK1[t] + c4CK1[t]),
    CK2'[t] == -kcf * CK2[t] * (c1[t] + c2[t] + c3[t] + c4[t]) - kpf * c0[t] * CK2[t] +
      (kp + bo) * (c1CK2[t] + c2CK2[t] + c3CK2[t]) + bh * c4CK2[t] + (Kcbo + kr2 + bo + kpo) *
       (c0CK2[t]) + Kcbt * (c1CK2[t] + c2CK2[t]) + Kcbh * (c3CK2[t] + c4CK2[t]),
    PP'[t] == -kppf * PP[t] * (ct1[t] + c1[t] + c2[t] + c3[t] + c4[t]) +
      (Kppb + kpp + bo) * (c1PP[t] + c2PP[t] + c3PP[t]) +
      (Kppb + kppo + bo) * ct1PP[t] + (Kppb + kpp + bh) * c4PP[t] + ub * ct1PP[t]};
atwo = {m[0] == 0, c0[0] == 0, c0CK1[0] == 0, c0CK2[0] == 0, ct1[0] == 0,
    ct1PP[0] == 0, c1[0] == 0, c1PP[0] == 0, c1CK1[0] == 0, c1CK2[0] == 0,
    c2[0] == 0, c2PP[0] == 0, c2CK1[0] == 0, c2CK2[0] == 0, c3[0] == 0, c3PP[0] == 0,
    c3CK1[0] == 0, c3CK2[0] == 0, c4[0] == 0, c4PP[0] == 0, c4CK1[0] == 0,
    c4CK2[0] == 0, cub[0] == 0, CK1[0] == CKt1, CK2[0] == CKt2, PP[0] == PPt};
afive = {m, c0, c0CK1, c0CK2, ct1, ct1PP, c1, c1PP, c1CK1, c1CK2, c2, c2PP, c2CK1,
    c2CK2, c3, c3PP, c3CK1, c3CK2, c4, c4PP, c4CK1, c4CK2, cub, CK1, CK2, PP};
qt = NDSolve[Join[aone, atwo], afive, {t, 0, endt},
    MaxSteps → 10 000 000, MaxStepSize → 0.01];
```

```
(*Simulated PER trajectory*)

k[t_] := c0[t] + c0CK1[t] + c0CK2[t] + ct1[t] + ct1PP[t] + c1[t] + c1PP[t] +
    c1CK1[t] + c1CK2[t] + c2[t] + c2PP[t] + c2CK1[t] + c2CK2[t] + c3[t] + c3PP[t] +
    c3CK1[t] + c3CK2[t] + c4[t] + c4PP[t] + c4CK1[t] + c4CK2[t] + cub[t] ;

xqt = qt;
po = FindPeaks[Table[-k[t] /. xqt[[1]], {t, 0, endt, 0.1}]][[3]][[1]];
wX = Table[k[t] /. xqt[[1]], {t, po * 0.1, po * 0.1 + 24 * 3, 0.1}];
wX = wX / Max[wX];
ytick = {0, 23 / 2, 23} / 23;
ytickl = {0, "", 1};
xtick = {0, 3, 6, 9, 12, 15, 18, 21, 24} * 40;
xtickl = {0, "", 24, "", 48, "", 72, "", 24};
is = 72 × 1.55 * 3 / 1.5;
ta = 0.006 / 2;(*Thickness of axis*)
ts = 0.02; (*length of thick*)
ft = 15;
top = 1.1;
ListLinePlot[{wX},
 Ticks -> {Table[{xtick[[jj]], xtickl[[jj]], {0, ts}}, {jj, 1, Length[xtick]}],
   Table[{ytick[[jj]], ytickl[[jj]], {0, ts}}, {jj, 1, Length[ytick]}]},
 AxesStyle → Directive[Thickness[ta], FontSize → ft, Black],
 PlotRange → {0, top}, ImageSize → is,
 PlotStyle -> Join[{{ColorData[97, "ColorList"][[4]], AbsoluteDashing[{7,
       10}], Thickness[0.01]}},
   Table[ColorData[97, "ColorList"][[j]], {j, {3, 2, 1}}]],
 PlotStyle → Table[ColorData[97, "ColorList"][[j]], {j, {4, 2, 3, 1}}]]
```



References

1. Zhou M, Kim JK, Eng GWL, Forger DB, Virshup DM (2015) A Period2 Phosphoswitch Regulates and Temperature Compensates Circadian Period. *Molecular Cell* 60(1):77–88.

2. Eide EJ, et al. (2005) Control of Mammalian Circadian Rhythm by CKI -Regulated Proteasome-Mediated PER2 Degradation. *Molecular and Cellular Biology* 25(7):2795–2807.

3. Eng GWL, Edison, Virshup DM (2017) Site-specific phosphorylation of casein kinase 1 δ (CK1δ) regulates its activity towards the circadian regulator PER2. *PLoS ONE* 12(5):e0177834–17.

4. Cheong JK, et al. (2015) Casein kinase 1α-dependent feedback loop controls autophagy in RAS-driven cancers. *J Clin Invest* 125(4):1401–1418.

5. Cheong JK, et al. (2011) IC261 induces cell cycle arrest and apoptosis of human cancer cells via CK1δ/ε and Wnt/β-catenin independent inhibition of mitotic spindle formation. *Oncogene* 30(22):2558–2569.

6. Delaglio F, et al. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 6(3):277–293.

7. Theillet F-X, et al. (2013) Site-specific NMR mapping and time-resolved monitoring of serine and threonine phosphorylation in reconstituted kinase reactions and mammalian cell extracts. *Nat Protoc* 8(7):1416–1432.

8. Lee H, Chen R, Lee Y, Yoo S, Lee C (2009) Essential roles of CKIdelta and CKIepsilon in the mammalian circadian clock. *Proc Natl Acad Sci USA* 106(50):21359–21364.

9. Shinohara Y, et al. (2017) Temperature-Sensitive Substrate and Product Binding Underlie Temperature-Compensated Phosphorylation in the Clock. *Molecular Cell* 67(5):783–798.e20.

10. González JR, et al. (2007) SNPassoc: an R package to perform whole genome association studies. *Bioinformatics* 23(5):644–645.

11. Kim JK, Forger DB (2012) A mechanism for robust circadian timekeeping via stoichiometric balance. *Mol Syst Biol* 8(1):630.

12. Xu Y, et al. (2007) Modeling of a Human Circadian Mutation Yields Insights into Clock Regulation by PER2. *Cell* 128(1):59–70