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Phosphorylation of DNA-binding domains of CLOCK–BMAL1 complex for PER-dependent inhibition in circadian clock of mammalian cells

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In mammals, CLOCK and BMAL1 proteins form a heterodimer that binds to E-box sequences and activates transcription of target genes, including *Period* (*Per*). Translated PER proteins then bind to the CLOCK–BMAL1 complex to inhibit its transcriptional activity. However, the molecular mechanism and the impact of this PER-dependent inhibition on the circadian clock oscillation remain elusive. We previously identified Ser38 and Ser42 in a DNA-binding domain of CLOCK as phosphorylation sites at the PER-dependent inhibition phase. In this study, knockout rescue experiments showed that nonphosphorylatable (Ala) mutations at these sites shortened circadian period, whereas their constitutive-phospho-mimetic (Asp) mutations completely abolished the circadian rhythms. Similarly, we found that nonphosphorylatable (Ala) and constitutive-phospho-mimetic (Glu) mutations at Ser78 in a DNA-binding domain of BMAL1 also shortened the circadian period and abolished the rhythms, respectively. The mathematical modeling predicted that these constitutive-phospho-mimetic mutations weaken the DNA binding of the CLOCK–BMAL1 complex and that the nonphosphorylatable mutations inhibit the PER-dependent displacement (reduction of DNA-binding ability) of the CLOCK–BMAL1 complex from DNA. Biochemical experiments supported the importance of these phosphorylation sites for displacement of the complex in the PER2-dependent inhibition. Our results provide direct evidence that phosphorylation of CLOCK–Ser38/Ser42 and BMAL1–Ser78 plays a crucial role in the PER-dependent inhibition and the determination of the circadian period.

circadian rhythm | phosphorylation | DNA binding | CRISPR-Cas9 | mathematical modeling

Significance

It has long been established that circadian clock oscillations rely on transcriptional and translational feedback loops but the specific phosphorylation sites underlying the displacement of the CLOCK–BMAL1 complex are still elusive. In this study, we explore the detailed intricate molecular mechanisms governing the circadian clock in mammals, specifically focusing on the roles of phosphorylation events within CLOCK and BMAL1 proteins and their impact on the circadian oscillation. Our research combines mathematical modeling, knockout–rescue experiments, and biochemical experiments to provide direct evidence of the critical role played by their phosphorylation in Period (PER)-dependent displacement mechanisms. These findings shed light on the hitherto elusive details of a PER-dependent displacement mechanism and demonstrate its physiological importance for the circadian clock oscillation.

The molecular mechanisms of the circadian clocks in various species consist of interlocked networks of transcriptional and translational feedback loops (TTFL) (1, 2). In mammals, the CLOCK and BMAL1 proteins form a heterodimer that binds to E-box (CACGTG and its related sequences) and activates transcription of target genes, including *Period* (*Per*) and *Cryptochrome* (*Cry*). The translated PER and CRY proteins then bind to the CLOCK–BMAL1 complex to inhibit its transcriptional activity (3, 4). This inhibition occurs through various mechanisms. For example, chromatin remodeling factors are recruited to the E-box, resulting in orchestrating histone modifications (5–7) and inducing the repositioning of E-box-containing genomic DNA to a perinuclear locale (8). Additionally, CRY can bind to the CLOCK–BMAL1 complex on the E-box to block activation of the E-box-dependent transcription (9, 10), while PER can sequester the CLOCK–BMAL1 complex from binding to the E-box (11). Furthermore, recent studies have identified PER-dependent displacement mechanisms, wherein PER removes the CLOCK–BMAL1 complex from the E-box through phosphorylation of CLOCK via PER-dependent recruitment of CK1 (9, 12, 13). This PER-dependent displacement is consistent with several previous reports showing that the CLOCK–BMAL1 complex is dissociated from the E-box at the PER-dependent suppression phase (6, 7, 14). Nevertheless, the intricate molecular details of the mechanisms and the impact of the PER-dependent displacement on the circadian clock oscillation remain elusive.

Phosphorylation of clock proteins plays a central role for the regulation of the TTFL in various species. It was previously reported that posttranslational modifications (PTMs) of mouse clock proteins regulate both the quantity and quality of the proteins (reviewed in ref. 15). PTMs of CRY proteins, including their phosphorylation, regulate their protein stability, interaction with PER, and repressor activity (16–25). Phosphorylation of PER proteins also plays a critical role in the circadian clock by regulating their protein levels (26–32) and subcellular localization (27, 28, 33, 34).

The authors declare no competing interest.

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In particular, two competing clusters of phosphorylation sites in PERs act as a phospho-switch that affects the period length by determining their degradation rate (30, 31, 35, 36). The PER–CRY complex, whose function is regulated by PTMs, interacts with the CLOCK–BMAL1 complex, thereby changing its phosphorylation state. Previously, we showed that CLOCK and BMAL1 proteins are phosphorylated in a circadian manner, and that Ser38 and Ser42 in the DNA-binding domain of CLOCK are phosphorylated at the PER-dependent suppression phase (37). These phosphorylations reduced the DNA-binding ability of the CLOCK–BMAL1 complex, which may cause inhibition of the E-box-dependent transcription (37, 38). However, the importance of these phosphorylations in the circadian clock oscillation is not understood.

In this study, to comprehensively understand the mouse circadian clock system driven by phosphorylation, we established knockout (KO)-rescue systems for CLOCK and BMAL1. These systems revealed that nonphosphorylatable (Ala) and constitutive-phospho-mimetic (Asp/Glu) mutations at the DNA-binding domains of the CLOCK–BMAL1 complex shortened the circadian period and abolished the rhythms, respectively. We analyzed the change in circadian rhythms via a combination of experimental approaches and mathematical models, and identified the importance of these phosphorylation sites for the PER-dependent displacement mechanism. The present study provides direct evidence that the "displacement" repression via phosphorylation of the DNA-binding domain in CLOCK and BMAL1 is critical for the circadian oscillation.

Results

Knockout Rescue Experiments of CLOCK and BMAL1. To evaluate the relative importance of the phosphorylation sites in CLOCK and BMAL1, we aimed to establish knockout (KO)-rescue experiments of *Clock* and *Bmal1* genes. To this end, we used CRISPR-Cas9 to knockout *Clock* gene in NIH3T3 cells (Fig. 1*A* and *SI Appendix*, Fig. S1). Western blotting analysis revealed that the full-length CLOCK protein was abolished in the mutant cells (Fig. 1*B*). When wild-type (WT) NIH3T3 cells were transiently transfected with a *Bmal1*-Luc reporter, we observed clear circadian rhythms of the bioluminescence level. These rhythms were abolished in the *Clock*-KO cells, indicating the essential role of *Clock* gene in the circadian clockwork of NIH3T3 cells (Fig. 1*C*). In the mouse behavior, the function of CLOCK can be compensated by NPAS2, a homolog of CLOCK (39, 40), but the contribution of NPAS2 is negligible in cultured cells, possibly due to undetectable expression of *Npas2* mRNA in NIH3T3 cells (*SI Appendix*, Fig. S2). It should be noted that the bioluminescence intensity in the *Clock*-KO cells was apparently higher than that in *Bmal1*-KO cells (Fig. 1*D*) that were reported in our previous study (41).

We have previously shown that *Bmal1* mRNA rhythms are not essential for the circadian oscillation, by eliminating RRE cis-elements in the 5′UTR region of *Bmal1* gene (41). Furthermore, it has been reported that constitutive overexpression of BMAL1 and CLOCK was enough to rescue the circadian oscillation in *Bmal1*-KO (42) and *Clock*-KO (43) cells, respectively. Consistently, we found that overexpression of CLOCK in the *Clock*-KO cells reduced the

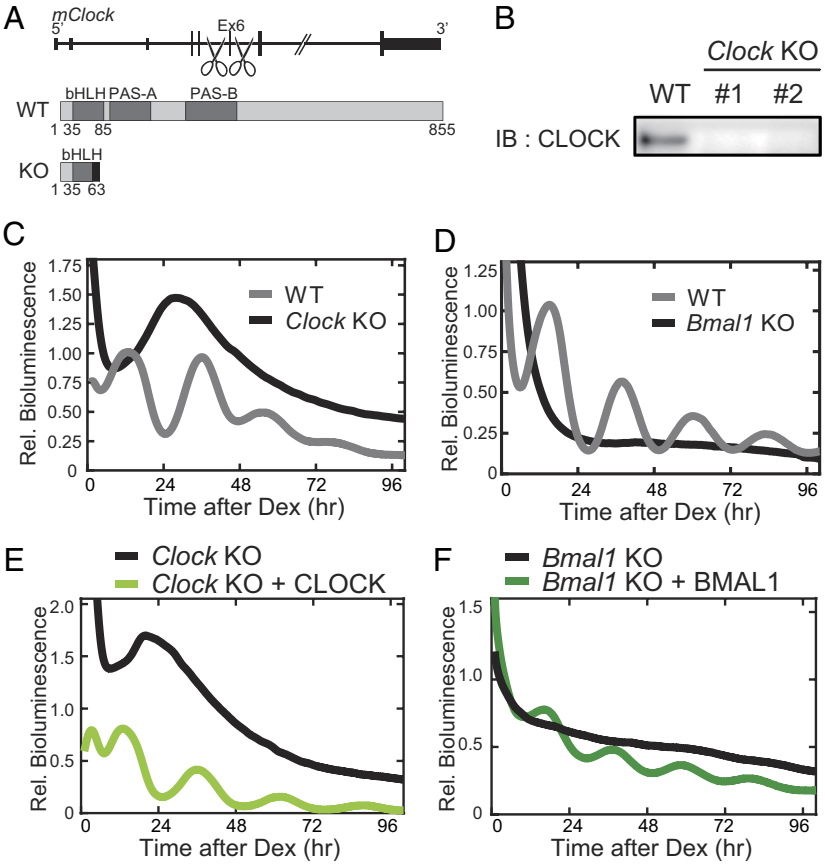

Fig. 1. KO-rescue experiments for CLOCK and BMAL1 in NIH3T3 cells. (*A*) Strategy for knocking out mouse *Clock* gene. The genome structure of mouse *Clock* gene (*Upper*) and the protein domain structure (*Lower*) are shown. The designed targeting sites of the sgRNAs in the *Clock* gene locus are indicated by scissors. (*B*) Western blotting of CLOCK protein that was immunoprecipitated from the *Clock*-KO and the control cell lines. (*C*–*F*) Representative bioluminescence rhythms from the control NIH3T3 cells (*C* and *D*; gray), the *Clock*-KO cells (*C* and *E*; black), the *Bmal1*-KO cells (*D* and *F*; black), the *Clock*-KO cells transfected with 500 ng of CLOCK–WT plasmid (*E*, green), and the *Bmal1*-KO cells transfected with 500 ng of BMAL1–WT plasmid (*F*, green). Cellular rhythms were monitored by transient transfection with the *Bmal1-luc* reporter. These cells were synchronized by 2-h pulse treatment with dexamethasone just before the bioluminescence monitoring.

abnormal high bioluminescence levels down to the normal levels and restored the circadian bioluminescence rhythms (Fig. 1*E* and *SI Appendix*, Fig. S3). Overexpression of BMAL1 also restored the rhythmicity in the *Bmal1*-KO cells (Fig. 1*F* and *SI Appendix*, Fig. S3). These results indicate that the rescue systems are suitable for investigating the role of phosphorylation at each phosphorylation site of the CLOCK–BMAL1 complex in the circadian clockwork.

In the KO-rescue experiments, intriguingly, changes in the amount of overexpressed CLOCK protein (the amount of CLOCK expressing plasmid) in the *Clock*-KO cells had no significant effect on the circadian period (Fig. 2 *A* and *B*), and similarly, those of overexpressed BMAL1 in the *Bmal1*-KO cells had marginal effects (Fig. 2 *C* and *D*). To simulate the circadian oscillation in the KO-rescue experiments, we utilized a detailed mathematical model (Kim-Forger model) that describes a mammalian circadian clock and captures changes by various clock gene mutations and pharmacological inhibition (44, 45). In this model, the transcription of clock genes is promoted and inhibited by the binding of CLOCK–BMAL1 and CRY–CLOCK–BMAL1 to E-box, respectively. Notably, the model has been updated to incorporate the displacement of CLOCK–BMAL1 complex from the E-box by PER binding, a mechanism known to play a critical role in circadian oscillation (Fig. 2*E*) (13, 46, 47). For simplicity, we did not consider the role of CK1 in promoting the displacement of CLOCK–BMAL1 from E-box by PER or PER–CRY. We eliminated CLOCK or BMAL1 in this WT model to construct the *Clock*-KO model or the *Bmal1*-KO model, respectively. Both models cannot generate rhythms (Fig. 2 *F*–*I*, black lines), consistent with observations in the cultured cells (Fig. 1 *C* and *D*). In these models, overexpression of CLOCK (Fig. 2 *F* and *G*) or BMAL1 (Fig. 2 *H* and *I*) can rescue the circadian oscillations, although the period lengths do not fully align with those observed in the cultured cells (Fig. 2 *B* and *D*). This inconsistency can be attributed to the Kim–Forger model, which was developed based on SCN exhibiting different period alteration by overexpression of CLOCK or BMAL1 protein compared to NIH3T3 cells (44). Importantly, expression levels of CLOCK or BMAL1 had only marginal effects on the circadian period (Fig. 2 *F*–*I*) as observed in the KO-rescue experiments (Fig. 2 *A*–*D*). Furthermore, overexpression of either CLOCK or BMAL1 had only minimal effects on the circadian oscillation not only in the KO models (Fig. 2 *F*–*I*) but also in the WT model (Fig. 2 *J* and *K*), whereas overexpression of both CLOCK and BMAL1 abolished the circadian oscillation (Fig. 2 *J* and *K*). The prediction by our simulation was experimentally confirmed by using WT NIH3T3 cells, in which CLOCK, BMAL1, or both was overexpressed (Fig. 2 *L* and *M*). Taken together, the level of the CLOCK–BMAL1 heterodimer should be a critical factor for the circadian oscillation, while the levels of free CLOCK or free BMAL1 proteins appear to have marginal effects on the circadian oscillation.

Ser38 and Ser42 in CLOCK and Ser78 in BMAL1 Are Critical Amino Acid Residues for Circadian Period Determination. It has been reported that phosphorylation of the CLOCK–BMAL1 complex is important for the dissociation of the complex from the E-box (13). However, the phosphorylation sites responsible for the dissociation of the complex from the E-box have not been identified. To address this question, we generated mutants, CLOCK–bHLH (1-194 S/T-all-A) and BMAL1–bHLH (1-149 S/T-all-A), where all the Ser and Thr residues (35 and 25 residues for CLOCK and BMAL1, respectively) in these N-terminal regions including their bHLH domains were replaced with Ala (i.e., nonphosphorylatable mutations) (*SI Appendix*, Fig. S4). In the KO-rescue experiments, the CLOCK–bHLH (1-194 S/T-all-A) mutant rescued the circadian oscillation in the *Clock*-KO cells, but the circadian period of the rescued cells was significantly shorter (~1.7 h) than that of the WT-rescued cells (Fig. 3 *A* and *B* magenta, *SI Appendix*, Fig. S5 *A* and *C*). In addition, BMAL1–bHLH (1-149 S/T-all-A) also had a significantly shorter (~1.6 h) period (Fig. 3 *C* and *D* magenta, *SI Appendix*, Fig. S5 *B* and *D*). These data suggest that phosphorylation in the DNA-recognition domains of the CLOCK–BMAL1 complex is important for the circadian period determination.

We previously reported that Ser38 and Ser42 of CLOCK are phosphorylated in the mouse liver and that Asp (constitutive-phospho-mimetic) mutations at these sites markedly reduce the DNA binding ability of the CLOCK–BMAL1 complex and inhibit its transcriptional activity (37). Separately, it has been reported that Ser78 of BMAL1 is an important amino acid residue for the DNA recognition and that the circadian oscillation was abolished by BMAL1–Ser78Glu (constitutive-phospho-mimetic) mutation (38). Therefore, we examined the role of these phosphorylation sites by using another mutant, CLOCK–bHLH (1-194 S/T-all-A; A38S/A42S), in which Ala38 and Ala42 of CLOCK–bHLH (1-194 S/T-all-A) were mutated back to Ser. In the KO-rescue experiments, the A38S/A42S mutation significantly attenuated the period shortening effect of CLOCK–bHLH (1-194 S/T-all-A) (Fig. 3 *A* and *B* black, *SI Appendix*, Fig. S5*C*). Similarly, back mutation of Ala78Ser on the BMAL1–bHLH (1-149 S/T-all-A) mutant almost completely abolished the period shortening effect of the all Ala mutation (Fig. 3 *C* and *D* black, *SI Appendix*, Fig. S5*D*). Taken together, the mechanism of circadian period determination by phosphorylation on the bHLH domains of CLOCK and BMAL1 is determined by a few amino acids in their DNA recognition sites.

Then, we examined the effect of Ala (nonphosphorylatable) or Asp/Glu (constitutive-phospho-mimetic) mutation on CLOCK–Ser38/Ser42 and BMAL1–Ser78. In the KO-rescue experiments, Ser38Ala and Ser42Ala mutations independently shortened the circadian period, and CLOCK–Ser38Ala/Ser42Ala double mutant had an additive effect of significantly shorter (~1.4 h) period (Fig. 3 *E*–*G* red, *SI Appendix*, Figs. S5 and S6). In addition, BMAL1–Ser78Ala mutant had also a significantly shorter (~1.0 h) period (Fig. 3 *H*–*J* red, *SI Appendix*, Fig. S5). Importantly, a CLOCK–Ser38Asp/Ser42Asp mutant and a BMAL1–Ser78Glu mutant showed arrhythmic phenotypes (Fig. 3 *E*–*J*, blue). These results indicate that these Ser residues in the bHLH domains of CLOCK and BMAL1 are important amino acids in the circadian period determination, probably by regulating the timing of the transition from the CLOCK–BMAL1 bound with E-box to the complex released from E-box, due to an increase of the negative charge by the phosphorylation at the DNA recognition sites.

Mathematical Modeling Reveals that the Phosphorylation of DNA-Binding Domains of the CLOCK–BMAL1 Complex Disrupts Its PER-Dependent Displacement from DNA. To systematically investigate how phosphorylation of CLOCK–Ser38/Ser42 and BMAL1–Ser78 modulates the circadian period, we used the *Clock*-KO and *Bmal1*-KO models, which accurately predicted the alteration of circadian periods by overexpression of CLOCK and BMAL1 in *Clock*-KO and *Bmal1*-KO cells, respectively (Fig. 2). With these models, we simulated mutations of CLOCK and BMAL1 on phosphorylation sites, based on our experimental data. Specifically, the complex of CLOCK and BMAL1 binds to the E-box in a circadian manner (6) and their mutations that mimic phosphorylation reduce its DNA-binding ability (37, 38). Furthermore, PER-dependent phosphorylation of the complex is a key step for its dissociation

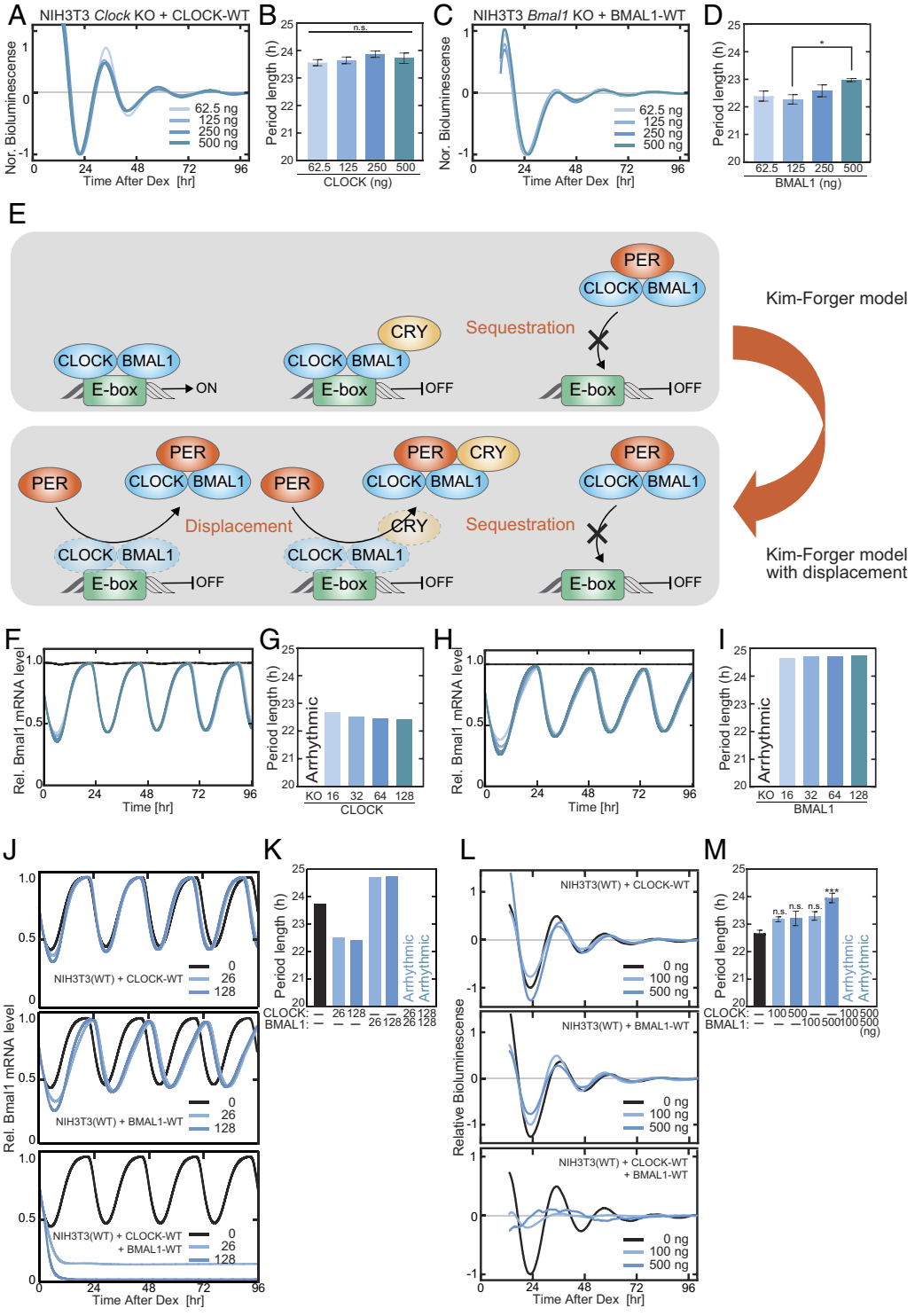

Fig. 2. Comparison of experimental data and predictions by mathematical modeling of the circadian oscillator. (*A*) Representative detrended bioluminescence rhythms from the *Clock*-KO cells transfected with indicated plasmid amounts of CLOCK–WT. (*B*) The circadian periods of the cellular rhythms in panel *A*. Data are means with SEM, n.s.; not significant ($P > 0.05$) (n = 4, Tukey–Kramer test). (*C*) Representative detrended bioluminescence rhythms from the *Bmal1*-KO cells transfected with indicated plasmid amounts of BMAL1–WT. (*D*) The circadian periods of the cellular rhythms in panel *C*. (*E*) Schematic diagram of the modified Kim-Forger model. While PER only sequesters CLOCK–BMAL1 not to bind with E-box in the original Kim-Forger model (*Upper*), it can dissociate CLOCK–BMAL1 or CRY–CLOCK–BMAL1 complex from E-box in the modified Kim-Forger model (*Lower*). (*F*) Simulated timeseries of *Bmal1* mRNA using the *Clock*-KO model when the expression level of CLOCK–WT increased from 0 (i.e., KO) to 128. (*G*) The circadian periods of the simulated rhythms in panel *F*. (*H*) Simulated timeseries of *Bmal1* mRNA using the *Bmal1*-KO model when the expression level of BMAL1–WT increased from 0 (i.e., KO) to 128. (*I*) The circadian periods of the simulated rhythms in panel *H*. (*J*) Simulated timeseries of *Bmal1* mRNA using the WT model when amounts of CLOCK–WT (*Upper*), BMAL1–WT (*Middle*), and both CLOCK–WT and BMAL1–WT (*Lower*) increased. (*K*) The circadian periods of the simulated rhythms in panel *J*. (*L*) Representative detrended bioluminescence rhythms from WT NIH3T3 transfected with indicated plasmid amounts of CLOCK–WT (*Upper*) and BMAL1–WT (*Middle*), both CLOCK–WT and BMAL1–WT (*Lower*). (*M*) The circadian periods of the cellular rhythms in panel *L*.

from the E-box (13). Collectively, we hypothesized that the mutations at the phosphorylation sites could affect the circadian period determination by altering the DNA-binding ability of the CLOCK–BMAL1 complex. To validate this hypothesis, we first simulated the Asp mutations of CLOCK by decreasing the binding ability (i.e., increasing the dissociation constant)

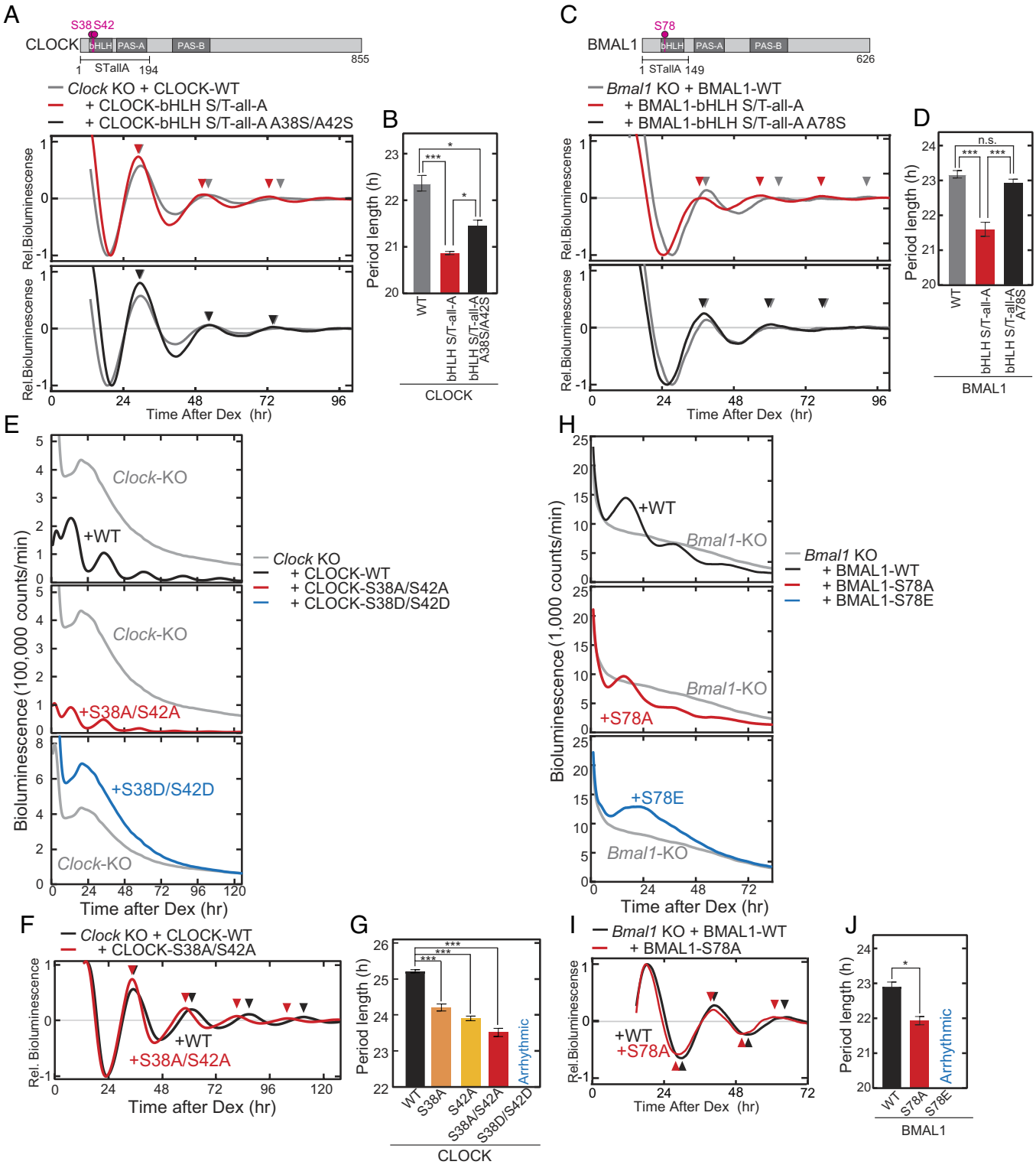

Fig. 3. Ser38 and Ser42 in CLOCK and Ser78 in BMAL1 are important amino acid residues for period determination. (*A*) Representative detrended bioluminescence rhythms from the *Clock*-KO cells transfected with 500 ng of CLOCK–WT (gray), CLOCK–bHLH (1-194 S/T-all-A) (magenta), CLOCK–bHLH (1-194 S/T-all-A A38S A42S) (black) plasmids. (*B*) The circadian period of the cellular rhythms in panel *A*. Data are means with SEM, $*P < 0.05$, $***P < 0.001$ (n = 3; Tukey–Kramer test). (*C*) Representative detrended bioluminescence rhythms from the *Bmal1*-KO cells transfected with 500 ng of BMAL1–WT (gray), BMAL1–bHLH (1-149 S/T-all-A) (magenta), BMAL1–bHLH (1–149 S/T-all-A A78S) (black) plasmids. (*D*) The circadian periods of the cellular rhythms in panel *C*. Data are means with SEM, n.s.; not significant ($P > 0.05$), $***P < 0.001$ (n = 3; Tukey–Kramer test). (*E* and *F*) Representative bioluminescence rhythms from the *Clock*-KO cells transfected with 500 ng of CLOCK–WT (black) and CLOCK–Ser38Ala/Ser42Ala (red), CLOCK–Ser38Asp/Ser42Asp (blue). (*G*) The circadian periods of the cellular rhythms in panel *F* and *SI Appendix*, Fig. S6. (*H* and *I*) Representative bioluminescence rhythms from the *Bmal1*-KO cells transfected with 500 ng of BMAL1–WT (black) and BMAL1–Ser78Ala (red), BMAL1–Ser78Glu (blue) plasmids. (*J*) The circadian periods of the cellular rhythms in panel *I*.

between the CLOCK–BMAL1 heterodimer and DNA (37) after overexpressing CLOCK in the *Clock*-KO model. This modification resulted in an arrhythmic phenotype (Fig. 4*A*), consistent with our experimental results of the Asp mutations (Fig. 3 *E* and *G*, blue line). Similarly, when we simulated the Glu (constitutive-phospho-mimetic) mutations of BMAL1

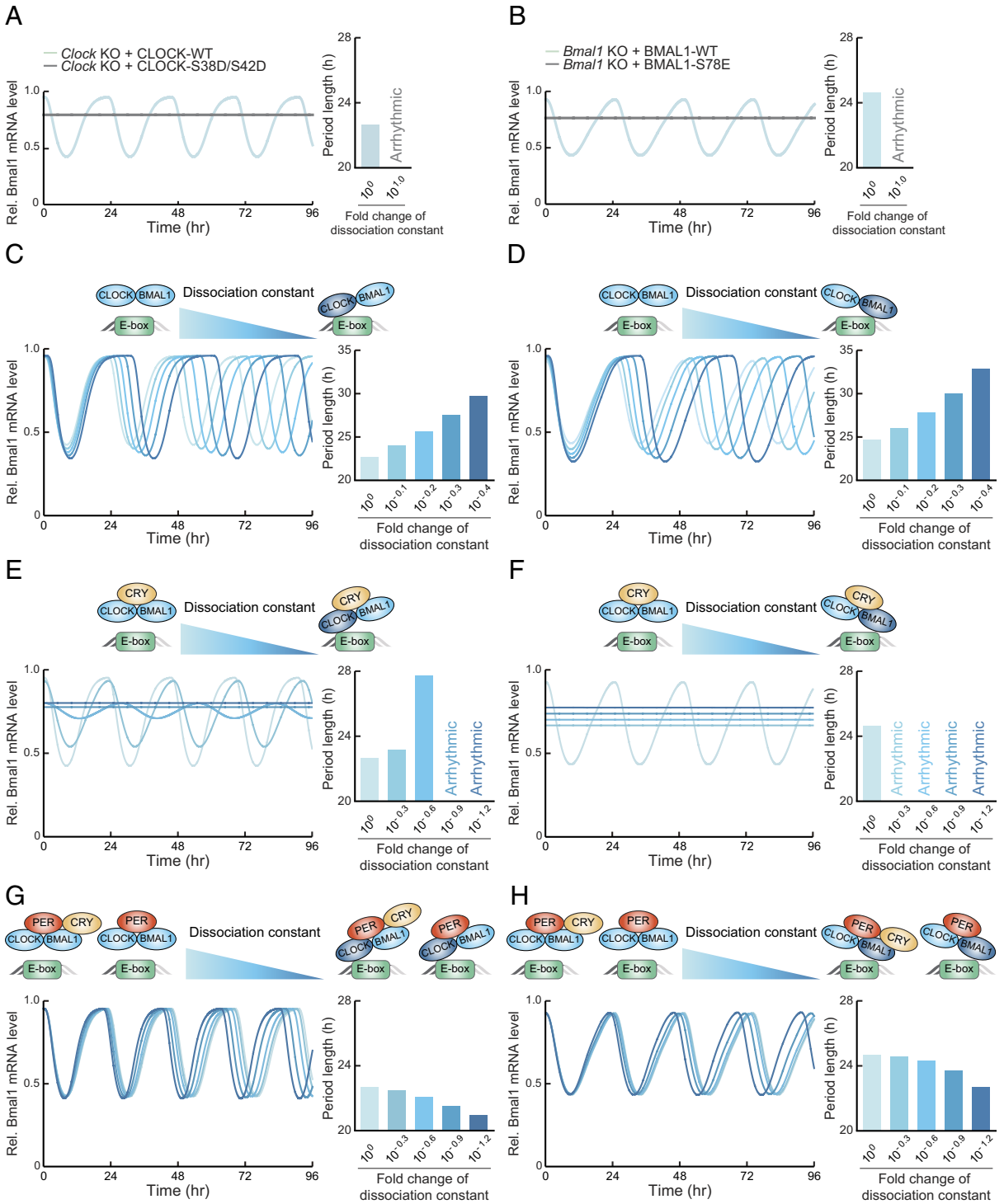

Fig. 4. Mathematical modeling reveals that the phosphorylation of DNA-binding domains of CLOCK–BMAL1 complex causes its PER-dependent displacement and determines the circadian period. (*A*) Simulated timeseries of *Bmal1* mRNA after overexpressing CLOCK in the Clock-KO model when the dissociation constant between CLOCK–BMAL1 complex and E-box decreased, and their circadian periods. (*B*) Simulated timeseries of *Bmal1* mRNA after overexpressing BMAL1 in the *Bmal1*-KO model when the dissociation constant between CLOCK–BMAL1 complex and E-box decreased, and their circadian periods. (*C*) Simulated timeseries of *Bmal1* mRNA after overexpressing CLOCK in the *Clock*-KO model when the dissociation constant between CLOCK–BMAL1 complex and E-box increased, and their circadian periods. (*D*) Simulated timeseries of *Bmal1* mRNA after overexpressing BMAL1 in the *Bmal1*-KO model when the dissociation constant between CLOCK–BMAL1 complex and E-box increased, and their circadian periods. (*E*) Simulated timeseries of *Bmal1* mRNA after overexpressing CLOCK in the *Clock*-KO model when the dissociation constant between CRY–CLOCK–BMAL1 complex and E-box increased, and their circadian periods. (*F*) Simulated timeseries of *Bmal1* mRNA after overexpressing BMAL1 in the *Bmal1*-KO model when the dissociation constant between CRY–CLOCK–BMAL1 complex and E-box increased, and their circadian periods. (*G*) Simulated timeseries of *Bmal1* mRNA after overexpressing CLOCK in the *Clock*-KO model when the dissociation constant between both CRY-CLOCK–BMAL1 and PER–CRY–CLOCK–BMAL1 complexes and E-box increased, and their circadian periods. (*H*) Simulated timeseries of *Bmal1* mRNA after overexpressing BMAL1 in the *Bmal1*-KO model when the dissociation constant between both CRY–CLOCK–BMAL1 and PER–CRY–CLOCK–BMAL1 complexes and E-box increased, and their circadian periods.

by decreasing its binding ability to DNA after overexpressing BMAL1 in the *Bmal1*-KO model, an arrhythmicity was observed in the model (Fig. 4*B*). This indicates that the arrhythmicity of these Asp/Glu (constitutive-phospho-mimetic) mutations appears to stem from the reduced binding of the CLOCK–BMAL1 complex to DNA.

Next, to assess the effects of the Ala (nonphosphorylatable) mutations, disrupting the phosphorylation of CLOCK or BMAL1, we investigated the impact of decreasing the dissociation constant (i.e., enhancing the binding affinity) between the CLOCK–BMAL1 complex and DNA. This led to a lengthening of the circadian period in the simulated rhythms (Fig. 4 *C* and *D*). On the other hand, we showed that the phospho-deficient mutation of these sites shortened the circadian period of the cellular clock (Fig. 3 *G* and *J*), while the phospho-mimetic mutation reduced the binding affinity against E-box (Fig. 5*B*; see below), similar to the previous results by ref. 13. These suggest that phosphorylatable but not phosphorylated serine residues of CLOCK and BMAL1 should positively influence E-box affinity and have a period-shortening effect. However, this is inconsistent with our simulations in Fig. 4 *C* and *D*. To address this inconsistency, we examined whether Ala (nonphosphorylatable) mutation affects the binding of CRY–CLOCK–BMAL1 and both PER–CLOCK–BMAL1 and PER–CRY–CLOCK–BMAL1 on DNA. While the decreased dissociation constant (i.e., the enhanced binding affinity) of CRY–CLOCK–BMAL1 with DNA lengthened the period (Fig. 4 *E* and *F*), the decreased dissociation constant of both PER–CLOCK–BMAL1 and PER–CRY–CLOCK–BMAL1 with DNA shortened the periods (Fig. 4 *G* and *H*) as observed in our experimental results (Fig. 3). This indicates that the Ala mutation may disrupt the displacement of the CLOCK–BMAL1 complex from DNA, induced by PER.

Ser38 and Ser42 in CLOCK and Ser78 in BMAL1 Contribute to the Inhibitory Effect of PER2. To confirm the model prediction, we examined whether phosphorylations of CLOCK–Ser38/Ser42 and BMAL1–Ser78 contribute to the PER2-dependent suppression on the E-box activity. In a dual-luciferase reporter assay using HEK293 cells, CLOCK–Ser38Ala/Ser42Ala and BMAL1–Ser78Ala activated the E-box-dependent transcription, and the transcriptional activity was significantly higher than that of WT CLOCK–BMAL1 complex (Fig. 5 *A*, *Upper*, black). Coexpression of PER2 caused a significant inhibition of the E-box activity, and the inhibitory effect of PER2 on the E-box activity was significantly attenuated by the Ala (nonphosphorylatable)

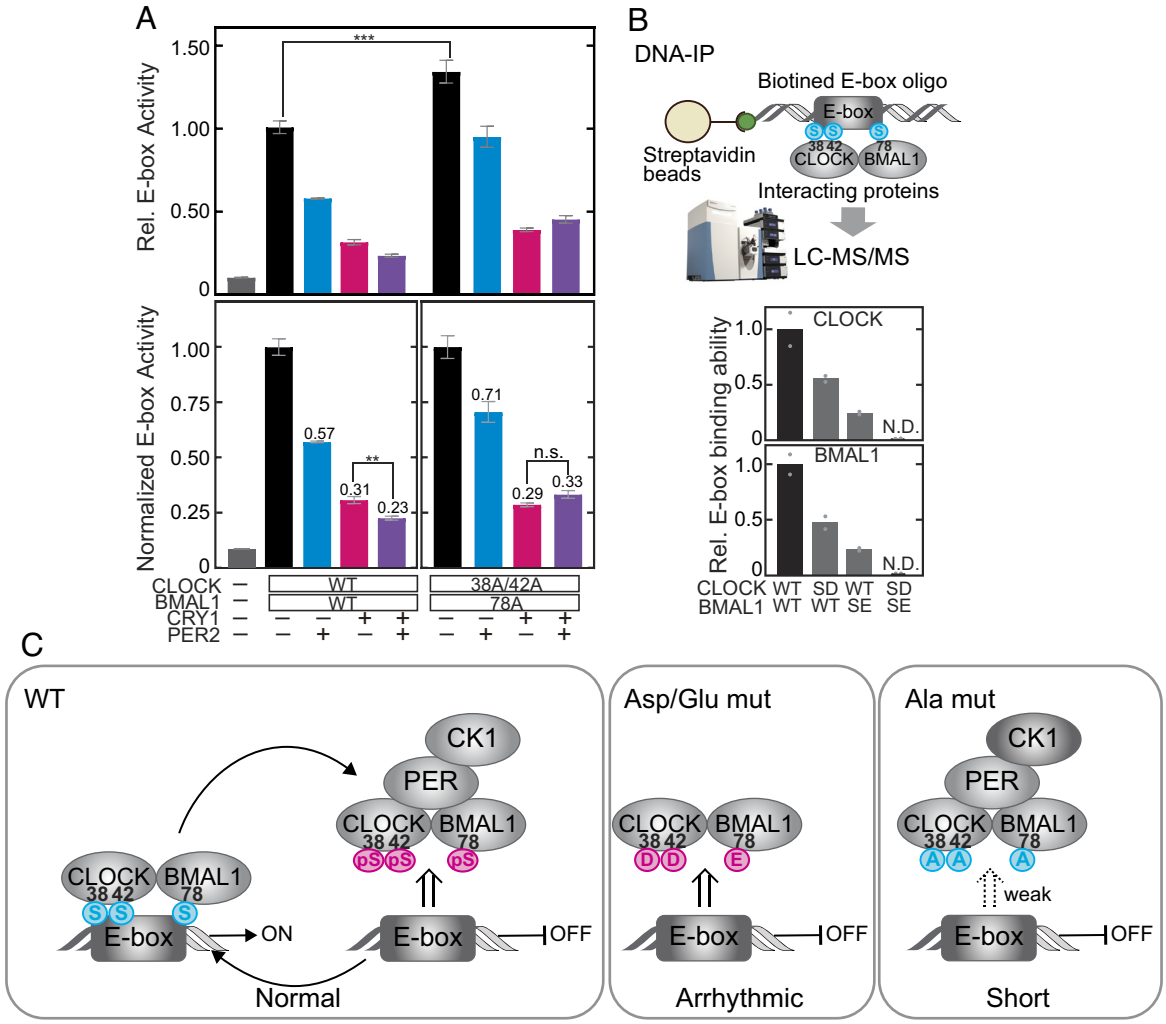

Fig. 5. The inhibitory effect of PER2 is mediated by Ser38 and Ser42 in CLOCK and Ser78 in BMAL1. (*A*) Inhibitory effects of PER2 (blue), CRY1 (red), or PER2/CRY1 (purple) on the E-box-dependent transcription by CLOCK and BMAL1 were examined by a dual luciferase reporter assay. The values of the E-box activity are shown as ratios of bioluminescence signals from firefly luciferase (E-box activity) relative to those from renilla luciferase (internal control). The mean of the signal ratios by CLOCK–WT and BMAL1–WT in the absence of PER2 and CRY1 was set to 1 (*Upper*). The means of the signal ratios in the absence of PER2 and CRY1 were set to 1 (*Lower*). Data are means with SEM, n.s.; not significant ($P > 0.05$), $^{**}P < 0.01$, $^{***}P < 0.001$ (n = 3; Tukey–Kramer test). (*B*) E-box binding abilities of CLOCK–WT and BMAL1–WT, CLOCK–Ser38Aps/Ser42Asp and BMAL1–WT, CLOCK–WT and BMAL1–Ser78Glu, and CLOCK–Ser38Asp/Ser42Asp and BMAL1–Ser78Glu were examined by a DNA pull down assay. N.D., Not Detected. (*C*) Model showing the role of the negative charge of phosphorylation at CLOCK–Ser38/Ser42 and BMAL1–Ser78 in the mammalian circadian clock (*Left*). The Asp/Glu mutations disrupt the DNA-binding ability of the complex and cause the arrhythmic phenotype (*Middle*). The Ala mutations weaken the dissociation of the complex from the E-box at the PER-dependent repression phase and shorten the circadian period (*Right*).

mutations of the complex (Fig. 5*A*, blue). It should be noted that an inhibitory effect of CRY1 was not significantly altered by the mutation (Fig. 5*A*, red). In the presence of CRY1, coexpression of PER2 additively inhibited the transcriptional activity of the WT complex (Fig. 5 *A*, *Left*, red vs. purple), but the additive inhibitory effect was not observed when the E-box was activated by the SA mutants (Fig. 5 *A*, *Right*, red vs. purple). This implies that the SA mutants disrupt the PER2-dependent inhibition, while the CRY1-dependent inhibition remains. This is mainly due to that CRY1 is also involved in multiple types of repressions including the displacement type of repression (48).

Next, to examine the effect of the phospho-mimic mutations on the E-box-binding ability of the CLOCK–BMAL1 complex, we performed a DNA pull-down assay, which quantitatively determines protein levels of CLOCK and BMAL1 that bind to a biotinylated E-box-containing DNA oligo. The DNA pull-down assay revealed that CLOCK–Ser38Asp/Ser42Asp and BMAL1–Ser78Glu mutations as mimicking phosphorylation additively attenuated the E-box-binding ability of the complex (Fig. 5*B*). These results indicate that the phosphorylations of both CLOCK–Ser38/Ser42 and BMAL1–Ser78 play a crucial role in the PER2-dependent displacement repression.

Finally, to investigate how the Ala (nonphosphorylatable) mutations of the CLOCK–BMAL1 complex affect the circadian phase, we simulated the mutations under a standard 12:12 light-dark (LD) condition as well as under the constant dark (DD) condition (*SI Appendix*, Fig. S7). Specifically, we simulated a decrease in the dissociation constants of both PER–CLOCK–BMAL1 and PER–CRY–CLOCK–BMAL1 with DNA after overexpressing CLOCK and BMAL1 in the *Clock*-KO (*SI Appendix*, Fig. S7*A*) and *Bmal1*-KO models (*SI Appendix*, Fig. S7*B*), respectively. We found that the displacement rate exhibited the circadian rhythm, with its peak time similar to that of the nuclear PER rhythm and PER–(CRY)–CLOCK–BMAL1 rhythm (*SI Appendix*, Fig. S7*A*). Importantly, the Ala (nonphosphorylatable) mutations reduced the displacement rate and caused about 1 h advance in the circadian phase (*SI Appendix*, Fig. S7 *A* and *B*, *Left* column), consistent with the short-period phenotypes (*SI Appendix*, Fig. S7 *A* and *B*, *Right*). Collectively, these findings suggest that PER proteins recruit kinases including CKI to the CLOCK–BMAL1 complex, inducing phosphorylations of CLOCK–Ser38/Ser42 and BMAL1–Ser78 (Fig. 5*C*). As one of the inhibitory mechanisms on the E-box-dependent transcription, these phosphorylations inhibit the E-box-dependent transcription by displacing the CLOCK–BMAL1 complex from the E-box to determine the circadian period.

Discussion

PER protein is generally considered as a state variable of TTFLs, and its protein level and functions regulate the speed of the circadian period. A recent study suggested that the PER2-dependent-recruitment of CKI to the CLOCK–BMAL1 complex caused dissociation of the complex from the E-box (13). However, it has remained unclear how the displacement mechanisms governed by the state variable PER has an impact on the circadian oscillation. In the present study, a combination of mathematical modeling and biochemical experiments revealed that the PER-dependent-displacement of the complex from the E-box is a critical step for the circadian clock. Specifically, we adopted an experimental strategy of replacing Ser and Thr residues of transcription factors with Ala and selectively reintroducing phosphorylatable residues into the Ala mutants. Especially, we focused on the DNA-binding domains of CLOCK and BMAL1, because phosphorylation of these domains possibly promotes the dissociation of the complex from DNA due to increase of the negative charges. Through this approach, we identified CLOCK–Ser38/Ser42 and BMAL1–Ser78 as the functional phosphorylation sites. Furthermore, with a combination of mathematical modeling incorporating displacement-type repression (Fig. 2*E*), we found that these sites are responsible for the period-shortening effect (Figs. 3 and 4) and the PER-dependent repression (Figs. 4 and 5).

Our strategy also works as a screening system for other transcription factors to identify a key phosphorylation site(s). In general, phosphorylation plays a key role in regulatory mechanisms for transcription factors (e.g., CLOCK, BMAL1, PER, and CRY) by altering not only their protein abundance but also their functions, such as subcellular localization, protein–protein interaction, and DNA-binding ability (15, 49, 50). Furthermore, these phosphorylations can shorten or lengthen the circadian period, depending on the phosphorylation sites. Notably, one well-known example is the phospho-switch of PER, where phosphorylations of two serine clusters change protein stability in opposite directions (30, 50). Subsequently, the stabilization and degradation of PER protein leads to lengthening and shortening of the circadian period, respectively. In the present study, we conclude that phosphorylations of the DNA-binding domains of the CLOCK–BMAL1 complex promote the dissociation of the complex from the E-box (Fig. 5*B*) and contribute to determining the circadian period (Fig. 3). Our findings are supported by a previous study in *Neurospora*, which showed that its circadian rhythms were not abolished by mutations at phosphorylation sites of WC-1 alone but by those of both WC-1 and WC-2 (51). The essential role of the phosphorylation in the fungi clock is consistent with our idea that triple mutations at the phosphorylation sites in CLOCK and BMAL1 proteins also blunt the mammalian clock.

Many kinases, such as GSK3β (52), CK2α (53), S6K1 (54), CDK5 (55), AKT (56), and CK1 (4), are known to be responsible for phosphorylating the CLOCK–BMAL1 heterodimer. However, a kinase(s) responsible for the phosphorylations at CLOCK–Ser38/Ser42 and BMAL1–Ser78 remains unclear. According to Kinase Library, a substrate motif analysis based on human serine/threonine kinome datasets (57), CK1 (probably isoforms δ and/or ε) is one of the strong candidate kinases for phosphorylation of CLOCK–Ser38/Ser42 and BMAL1–Ser78. CK1δ/ε are reported to form a complex including CLOCK, BMAL1, PERs, and CRYs (4, 58). CK1δ in the large complex phosphorylates CLOCK protein, resulting in dissociation of the complex from the E-box (13). This model of displacement repression where CK1 governs the dissociation of the transactivators is conserved in not only mammals but also *Neurospora* and *Drosophila* (51, 59, 60). To our knowledge, however, no reports have identified the CK1-dependent phosphorylation sites of CLOCK. In the present study, we found that the phosphorylations at CLOCK–Ser38/Ser42 and BMAL1–Ser78 were associated with the PER2-dependent displacement (Fig. 5*A*). Based on these results, it is possible that CK1 is the kinase responsible for these phosphorylation sites, but we could not exclude the contribution of other kinases.

We found that the mechanism for the PER-dependent displacement is critical in generating circadian rhythms. Interestingly, as well as the displacement, two additional repression mechanisms, blocking and sequestration, are employed together for the transcriptional negative feedback loop in the circadian clock of mammals and *Drosophila* (9, 10, 13, 61). For instance, in the mammalian circadian clock, CRY binds to the CLOCK–BMAL1 complex on E-box to block the transcription, while PER sequesters the CLOCK–BMAL1 complex not to bind with E-box and displaces the CLOCK–BMAL1 complex bound to E-box. When

these three mechanisms for seemingly redundant repressions are used together, circadian rhythms with high amplitude (62) and robust signal to noise can be generated (63). The PER-dependent displacement contributes to not only generating strong rhythms but also the mobility of the CLOCK–BMAL1 complex to its target sites (46). This is reminiscent of the fact that the displacement of NF-κB by IκB plays an important role in generating coherent oscillation of NF-κB across cells with different binding affinities between NF-κB and IκB (64).

Collectively, we conclude that a highly conserved mechanism of the displacement repression in the circadian clockwork is mediated by phosphorylation at DNA-binding domains of the transactivators and is essential for the normal clock oscillation.

Materials and Methods

Cell Culture. The NIH3T3 and HEK293T/17 cells were cultured and passaged under 5% CO_2 in Dulbecco's modified Eagle's medium (D5796; Sigma or 08459-64; Nacalai Tesuque) containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum.

Genome Editing with the CRISPR-Cas9 System. For knockout of CLOCK protein, targeting sequences were determined by CRISPRdirect (https://crispr.dbcls.jp) (65). pX459 plasmid containing a chimeric guide RNA and SpCas9 expression cassette (Cat. No. 42230; Addgene) (66, 67) was digested by BbsI restriction enzyme, and the targeting sequences were inserted. NIH3T3 cells were transfected with the plasmid by Lipofectamine3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. After the transfection, cells were seeded at a density of one cell per well in 96-well plates. For isolating cell lines, wells containing a single colony were selected by CloneSelect Imager (Molecular Device). The genome DNA sequences of the target site were read by direct Sanger sequencing.

Real-Time Monitoring of Cellular Rhythms. Cellular bioluminescence rhythms were monitored as described previously (68) with minor modifications. Briefly, NIH3T3 (WT, *Clock*-KO, or *Bmal1*-KO cells) plated on 35-mm dishes were transiently transfected with *Bmal1*-luc/pGL4.12 and indicated plasmids by Lipofectamine3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. After 24 h, the cells were treated with 0.1 μM (final) dexamethasone for 2 h, and then, the media were replaced by recording media (phenol-red free Dulbecco's modified Eagle's medium [Sigma] supplemented with 10% fetal bovine serum, 3.5 g/L glucose, 25 U/mL penicillin, 25 μg/mL streptomycin, 0.1 mM luciferin, and 10 mM HEPES-NaOH; pH 7.0). The bioluminescence signals of the cultured cells were continuously recorded for 5 to 10 d at 37 °C in air with Dish Type Luminescencer, Lumi-Cycle (Actimetrics).

For the determination of the circadian period, the raw data of bioluminescence rhythms were detrended by subtracting their 24-h moving averages and were smoothed by their 2-h moving averages. The circadian period was calculated from the slope of a regression line fitted to time points of consecutive peaks and troughs of cellular rhythms by the least-squares method. At least 5 time points of peaks and troughs of cellular rhythms were selected for the fitting. We observed that the calculated circadian periods of the cultured cells have small variations among cultured dishes in a single experiment but large variations between experiments performed on different days (*SI Appendix*, Figs. S3 and S5). Therefore, in this study, we compared the circadian periods between the WT-rescued cells and the mutant-rescued cells in a single-day experiment.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*. The Mathematica codes to simulate the mathematical models are available at https://github.com/Mathbiomed/The-mammalian-circadian-clock-with-PER-dependent-displacement (69).

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1. J. C. Dunlap, Molecular bases for circadian clocks. *Cell* **96**, 271–290 (1999).
2. S. L. Harmer, S. Panda, S. A. Kay, Molecular bases of circadian rhythms. *Annu. Rev. Cell Dev. Biol.* **17**, 215–253 (2001).
3. C. Lee, J. P. Etchegaray, F. R. Cagampang, A. S. Loudon, S. M. Reppert, Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107**, 855–867 (2001).
4. R. P. Aryal *et al.*, Macromolecular assemblies of the mammalian circadian clock. *Mol. Cell* **67**, 770–782.e6 (2017).
5. C. Crosio, N. Cermakian, C. D. Allis, P. Sassone-Corsi, Light induces chromatin modification in cells of the mammalian circadian clock. *Nat. Neurosci.* **3**, 1241–1247 (2000).
6. J. A. Ripperger, U. Schibler, Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. *Nat. Genet.* **38**, 369–374 (2006).
7. N. Koike *et al.*, Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science* **338**, 349–354 (2012).
8. L. Aguilar-Arnal *et al.*, Cycles in spatial and temporal chromosomal organization driven by the circadian clock. *Nat. Struct. Mol. Biol.* **20**, 1206–1213 (2013).
9. R. Ye *et al.*, Dual modes of CLOCK:BMAL1 inhibition mediated by Cryptochrome and Period proteins in the mammalian circadian clock. *Genes Dev.* **28**, 1989–1998 (2014).
10. Y.-Y. Chiou *et al.*, Mammalian period represses and de-represses transcription by displacing CLOCK-BMAL1 from promoters in a Cryptochrome-dependent manner. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E6072–E6079 (2016).
11. C. Rosensweig, C. B. Green, Periodicity, repression, and the molecular architecture of the mammalian circadian clock. *Eur. J. Neurosci.* **51**, 139–165 (2020).
12. R. Ye, C. P. Selby, N. Ozturk, Y. Annayev, A. Sancar, Biochemical analysis of the canonical model for the mammalian circadian clock. *J. Biol. Chem.* **286**, 25891–25902 (2011).
13. X. Cao, Y. Yang, C. P. Selby, Z. Liu, A. Sancar, Molecular mechanism of the repressive phase of the mammalian circadian clock. *Proc. Natl. Acad. Sci. U.S.A.* **118**, e2021174118 (2021).
14. H. Yoshitane *et al.*, CLOCK-controlled polyphonic regulation of circadian rhythms through canonical and noncanonical E-boxes. *Mol. Cell. Biol.* **34**, 1776–1787 (2014).
15. A. Hirano, Y.-H. Fu, L. J. Ptáček, The intricate dance of post-translational modifications in the rhythm of life. *Nat. Struct. Mol. Biol.* **23**, 1053–1060 (2016).
16. Y. Harada, M. Sakai, N. Kurabayashi, T. Hirota, Y. Fukada, Ser-557-phosphorylated mCRY2 is degraded upon synergistic phosphorylation by glycogen synthase kinase-3 beta. *J. Biol. Chem.* **280**, 31714–31721 (2005).
17. N. Kurabayashi, T. Hirota, M. Sakai, K. Sanada, Y. Fukada, DYRK1A and glycogen synthase kinase 3beta, a dual-kinase mechanism directing proteasomal degradation of CRY2 for circadian timekeeping. *Mol. Cell. Biol.* **30**, 1757–1768 (2010).
18. A. Hirano *et al.*, In vivo role of phosphorylation of cryptochrome 2 in the mouse circadian clock. *Mol. Cell. Biol.* **34**, 4464–4473 (2014).
19. S. M. Siepka *et al.*, Circadian mutant overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. *Cell* **129**, 1011–1023 (2007).
20. S. I. H. Godinho *et al.*, The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. *Science* **316**, 897–900 (2007).
21. L. Busino *et al.*, SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science* **316**, 900–904 (2007).
22. A. Hirano *et al.*, FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. *Cell* **152**, 1106–1118 (2013).
23. S.-H. Yoo *et al.*, Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm. *Cell* **152**, 1091–1105 (2013).
24. A. Hirano *et al.*, USP7 and TDP-43: Pleiotropic regulation of cryptochrome protein stability paces the oscillation of the mammalian circadian clock. *PLoS One* **11**, e0154263 (2016).
25. K. L. Ode *et al.*, Knockout-rescue embryonic stem cell-derived mouse reveals circadian-period control by quality and quantity of CRY1. *Mol. Cell* **65**, 176–190 (2017).
26. K. L. Toh *et al.*, An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* **291**, 1040–1043 (2001).
27. K. Vanselow *et al.*, Differential effects of PER2 phosphorylation: Molecular basis for the human familial advanced sleep phase syndrome (FASPS). *Genes Dev.* **20**, 2660–2672 (2006).

28. S. Reischl *et al.*, Beta-TrCP1-mediated degradation of PERIOD2 is essential for circadian dynamics. *J. Biol. Rhythms* **22**, 375–386 (2007).
29. Y. Xu *et al.*, Modeling of a human circadian mutation yields insights into clock regulation by PER2. *Cell* **128**, 59–70 (2007).
30. M. Zhou, J. K. Kim, G. W. L. Eng, D. B. Forger, D. M. Virshup, A period2 phosphoswitch regulates and temperature compensates circadian period. *Mol. Cell* **60**, 77–88 (2015).
31. R. Narasimamurthy *et al.*, CK1δ/ε protein kinase primes the PER2 circadian phosphoswitch. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 5986–5991 (2018).
32. J. M. Philpott *et al.*, Casein kinase 1 dynamics underlie substrate selectivity and the PER2 circadian phosphoswitch. *Elife* **9**, e52343 (2020).
33. S. Beesley *et al.*, Wake-sleep cycles are severely disrupted by diseases affecting cytoplasmic homeostasis. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 28402–28411 (2020).
34. S. J. Chae, D. W. Kim, S. Lee, J. K. Kim, Spatially coordinated collective phosphorylation filters spatiotemporal noises for precise circadian timekeeping. *iScience* **26**, 106554 (2023).
35. S. Masuda *et al.*, Mutation of a PER2 phosphodegron perturbs the circadian phosphoswitch. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 10888–10896 (2020).
36. J. M. Philpott *et al.*, PERIOD phosphorylation leads to feedback inhibition of CK1 activity to control circadian period. *Mol. Cell* **83**, 1677–1692.e8 (2023).
37. H. Yoshitane *et al.*, Roles of CLOCK phosphorylation in suppression of E-box-dependent transcription. *Mol. Cell. Biol.* **29**, 3675–3686 (2009).
38. Z. Wang, Y. Wu, L. Li, X.-D. Su, Intermolecular recognition revealed by the complex structure of human CLOCK-BMAL1 basic helix-loop-helix domains with E-box DNA. *Cell Res.* **23**, 213–224 (2013).
39. J. P. Debruyne *et al.*, A clock shock: Mouse CLOCK is not required for circadian oscillator function. *Neuron* **50**, 465–477 (2006).
40. J. P. DeBruyne, D. R. Weaver, S. M. Reppert, CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nat. Neurosci.* **10**, 543–545 (2007).
41. Y. O. Abe *et al.*, Rhythmic transcription of Bmal1 stabilizes the circadian timekeeping system in mammals. *Nat. Commun.* **13**, 4652 (2022).
42. A. C. Liu *et al.*, Redundant function of REV-ERBalpha and beta and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. *PLoS Genet.* **4**, e1000023 (2008).
43. A. N. Abdo, C. Rintisch, C. H. Gabriel, A. Kramer, Mutational scanning identified amino acids of the CLOCK exon 19-domain essential for circadian rhythms. *Acta Physiol.* **234**, e13794 (2022).
44. J. K. Kim, D. B. Forger, A mechanism for robust circadian timekeeping via stoichiometric balance. *Mol. Syst. Biol.* **8**, 630 (2012).
45. D. W. Kim *et al.*, Systems approach reveals photosensitivity and PER2 level as determinants of clock-modulator efficacy. *Mol. Syst. Biol.* **15**, e8838 (2019).
46. A. A. Koch *et al.*, Quantification of protein abundance and interaction defines a mechanism for operation of the circadian clock. *Elife* **11**, e73976 (2022).
47. E. M. Jeong *et al.*, Systematic modeling-driven experiments identify distinct molecular clockworks underlying hierarchically organized pacemaker neurons. *Proc. Natl. Acad. Sci. U.S.A.* **119**, e2113403119 (2022).
48. Y. Naruse *et al.*, Circadian and light-induced transcription of clock gene Per1 depends on histone acetylation and deacetylation. *Mol. Cell. Biol.* **24**, 6278–6287 (2004).
49. A. Brenna, U. Albrecht, Phosphorylation and circadian molecular timing. *Front. Physiol.* **11**, 612510 (2020).
50. R. Narasimamurthy, D. M. Virshup, The phosphorylation switch that regulates ticking of the circadian clock. *Mol. Cell* **81**, 1133–1146 (2021).
51. B. Wang, A. N. Kettenbach, X. Zhou, J. J. Loros, J. C. Dunlap, The phospho-code determining circadian feedback loop closure and output in neurospora. *Mol. Cell* **74**, 771–784.e3 (2019).
52. M. L. Spengler, K. K. Kuropatwinski, M. Schumer, M. P. Antoch, A serine cluster mediates BMAL1-dependent CLOCK phosphorylation and degradation. *Cell Cycle* **8**, 4138–4146 (2009).
53. T. Oshima *et al.*, Cell-based screen identifies a new potent and highly selective CK2 inhibitor for modulation of circadian rhythms and cancer cell growth. *Sci. Adv.* **5**, eaau9060 (2019).
54. J. O. Lipton *et al.*, The circadian protein BMAL1 regulates translation in response to S6K1-mediated phosphorylation. *Cell* **161**, 1138–1151 (2015).
55. Y. Kwak *et al.*, Cyclin-dependent kinase 5 (Cdk5) regulates the function of CLOCK protein by direct phosphorylation. *J. Biol. Chem.* **288**, 36878–36889 (2013).
56. H. Yoshitane *et al.*, mTOR-AKT signaling in cellular clock resetting triggered by osmotic stress. *Antioxid. Redox Signal.* **37**, 631–646 (2022).
57. J. L. Johnson *et al.*, An atlas of substrate specificities for the human serine/threonine kinome. *Nature* **613**, 759–766 (2023).
58. C. Lee, D. R. Weaver, S. M. Reppert, Direct association between mouse PERIOD and CKIε is critical for a functioning circadian clock. *Mol. Cell. Biol.* **24**, 584–594 (2004).
59. W. Yu, H. Zheng, J. H. Houl, B. Dauwalder, P. E. Hardin, PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes Dev.* **20**, 723–733 (2006).
60. E. Y. Kim, H. W. Ko, W. Yu, P. E. Hardin, I. Edery, A DOUBLETIME kinase binding domain on the Drosophila PERIOD protein is essential for its hyperphosphorylation, transcriptional repression, and circadian clock function. *Mol. Cell. Biol.* **27**, 5014–5028 (2007).
61. J. S. Menet, K. C. Abruzzi, J. Desrochers, J. Rodriguez, M. Rosbash, Dynamic PER repression mechanisms in the Drosophila circadian clock: From on-DNA to off-DNA. *Genes Dev.* **24**, 358–367 (2010).
62. E. M. Jeong, Y. M. Song, J. K. Kim, Combined multiple transcriptional repression mechanisms generate ultrasensitivity and oscillations. *Interface Focus* **12**, 20210084 (2022).
63. E. M. Jeong, J. K. Kim, A robust ultrasensitive transcriptional switch in noisy cellular environments. *NPJ Syst. Biol. Appl.* **10**, 30 (2024).
64. Z. Wang, D. A. Potoyan, P. G. Wolynes, Molecular stripping, targets and decoys as modulators of oscillations in the NF-κB/IκBα/DNA genetic network. *J. R. Soc. Interface* **13**, 20160606 (2016).
65. Y. Naito, K. Hino, H. Bono, K. Ui-Tei, CRISPRdirect: Software for designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics* **31**, 1120–1123 (2015).
66. L. Cong *et al.*, Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013).
67. F. A. Ran *et al.*, Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).
68. K. Imamura *et al.*, ASK family kinases mediate cellular stress and redox signaling to circadian clock. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 3646–3651 (2018).
69. Y. Otobe *et al.*, The-mammalian-circadian-clock-with-PER-dependent-displacement. Github. https://github.com/Mathbiomed/The-mammalian-circadian-clock-with-PER-dependent-displacement. Deposited 14 May 2024.