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Correlated evolution between CK1δ Protein and the Serine-rich Motif Contributes to Regulating the Mammalian Circadian Clock*[S]

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Understanding the mechanism underlying the physiological divergence of species is a long-standing issue in evolutionary biology. The circadian clock is a highly conserved system existing in almost all organisms that regulates a wide range of physiological and behavioral events to adapt to the day-night cycle. Here, the interactions between hCK1ε/δ/DBT (*Drosophila* ortholog of CK1δ/ε) and serine-rich (SR) motifs from hPER2 (ortholog of *Drosophila per*) were reconstructed in a *Drosophila* circadian system. The results indicated that in *Drosophila*, the SR mutant form hPER2^{S662G} does not recapitulate the mouse or human mutant phenotype. However, introducing hCK1δ (but not DBT) shortened the circadian period and restored the SR motif function. We found that hCK1δ is catalytically more efficient than DBT in phosphorylating the SR motif, which demonstrates that the evolution of CK1δ activity is required for SR motif modulation. Moreover, an abundance of phosphorylatable SR motifs and the striking emergence of putative SR motifs in vertebrate proteins were observed, which provides further evidence that the correlated evolution between kinase activity and its substrates set the stage for functional diversity in vertebrates. It is possible that such correlated evolution may serve as a biomarker associated with the adaptive benefits of diverse organisms. These results also provide a concrete example of how functional synthesis can be achieved through introducing evolutionary partners *in vivo*.

The assumption of conserved function between orthologs has been supported even between relatively distant species. However, orthologs are increasingly found to diverge in sequence, in expression, and even in knock-out phenotypes (1). As a kinase phosphorylates tens to hundreds of substrates, if we can catalog the genetic and functional differences of orthologous kinases and pick out relevant changes to adaptive functional regions in their substrates, then we can better understand the impact of orthologs on evolutionary novelty.

In *Drosophila*, DBT, the single *Drosophila* ortholog of CK1δ/ε, binds to and phosphorylates PER, and DBT then regulates the subcellular localization of PER and signals the rapid degradation of PER by the proteasome (2–4). The mammalian orthologs of DBT are the CK1δ/ε proteins, which mediate the phosphorylation and degradation of mPER2 and facilitate its entry into the nucleus (5). Thus, the CK1δ/ε/DBT/PER relationship is likely conserved between *Drosophila* and mammals. Interestingly, a mutation in CK1δ or its substrate PER2 causes familial advanced sleep phase syndrome (FASPS)[3] (6, 7). Moreover, the introduction of hPER2^{S662G} (the first serine in the SR motif is mutated) into mice faithfully recapitulates the human phenotype, whereas hPER2^{S662D} (which mimics the phosphorylated status) lengthened the circadian period (7). Correspondingly, a mutation in CK1δ (hCK1δ^{T44A}) also causes FASPS and hCK1δ^{T44A} expression in mice, thus shortening the circadian period (6). These studies suggest that PER2 SR phosphorylation is conserved by CK1δ in the mammalian circadian clock. In *Drosophila*, however, overexpressing hCK1δ^{T44A} lengthened the circadian period without mimicking mouse or human phenotypes (6), suggesting that CK1δ and/or its substrate PER2 may have diverged.

CK1δ and CK1ε are close paralog of the CK1 family, which is found in most vertebrates (8). One consensus phosphorylation target for CK1 is (pS/pT)*XX*(S/T) (pS/pT, phospho-serine/threonine; *X*, any amino acid) (9, 10). Interestingly, many sequential phosphorylation examples in SR motifs by CK1δ/ε have been described to play important roles in various biological processes. Phosphorylation of the SR motif in the Yes-associated protein (YAP) by CK1δ/ε was reported to promote YAP degradation and therefore suppress its oncogenic function (11). A homologous mutation in the hPER1 SR motif (hPER1^{S714G}) caused an advanced feeding rhythm in mice and disrupted their

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[3] The abbreviations used are: FASPS, familial advanced sleep phase syndrome; SR, serine-rich; YAP, Yes-associated protein; Cre, cAMP-response element; LD, light-dark; DD, dark-dark; ZT, Zeitgeber time; h, human; m, mouse; d, *Drosophila*.

metabolic oscillation (12). These well recorded examples suggest critical functions for the SR motifs in the mammalian physiology. However, the apparent complexity of CK1δ/ϵ and SR motifs in mammals has hampered efforts to catalog the genetic differences and link them to specific phenotypic consequences.

Here, we improved the available strategies for assessing circadian rhythms by reconstructing a kinase-substrate system in *Drosophila* and using a reliable circadian period as a readout together with a biochemistry assay and bioinformatics analysis to determine their correlation. We demonstrated that despite the highly conserved enzyme domains and sequences between *Drosophila* and vertebrates, CK1δ has acquired novel regulatory features that may interact with a newly emerging SR motif in their vertebrate substrates. We suggest that divergences between orthologs effectively facilitate the evolution of new functions in vertebrates. Identifying the underlying intrinsic mechanisms that drive the differences among different species can provide valuable information for determining when and how new functions occur in the species and therefore realize functional synthesis *in vivo*.

Results

Incompatibility of the hPER2 SR Motif in the Drosophila Circadian System—The maximum likelihood indicates that the difference in CK1δ in the circadian period of mammals and *Drosophila* may be related to different substrates and/or different kinase-substrate interactions. To clarify the functional differences, we compared the protein sequences of human and mouse PER1 and PER2 with those of *Drosophila* PER (dPER). A sequence alignment analysis shows that dPER does not contain the hPER2 SR motif conserved sequence (Fig. 1*A* and supplemental Fig. S1). Because the phosphorylation status of PER2 SR motif is correlated with the period length in mice, we can use point mutation-mediated period changes to quantitatively analyze whether the SR motif is compatible with the *Drosophila* system (*e.g.* $PER2^{S662G}$ abolishes sequential phosphorylation for a short period and $PER2^{S662D}$ mimics phosphorylation for a long period). In mice, however, the expression of the period paralogous $hPER1^{S714G}$ (12) or $hPER3^{S630G}$,[4] the first serine-directed mutation in the SR motif, failed to change the locomotor period as observed in the $hPER2^{S662G}$ mice, suggesting that PER2 is an optimal candidate for quantitative analysis of locomotor period changes. Therefore, we introduced $hPER2^{S662}$ (wild type), $hPER2^{S662G}$, and $hPER2^{S662D}$ into *Drosophila*. Driven by *timeless*-GAL4 (*tim*-GAL4), the overexpression of $hPER2^{S662}$, $hPER2^{S662G}$, and $hPER2^{S662D}$ all lengthened the *Drosophila* circadian period (Fig. 1*B*, *upper row*), indicating that hPER2 can regulate the circadian period in *Drosophila*. However, SR motif-dependent circadian period changes were not apparent in *Drosophila*. The period lengths from three genotypes of hPER2 transgenic flies were positively correlated with hPER2 expression but not with mutant types (Fig. 1*B*, *bottom row*). These results indicated that the SR motif of hPER2 is incompatible with the *Drosophila* circadian system. To test whether the incompatibility of the SR motif with the *Drosophila* circadian system occurs because the SR motif cannot be modified or because the *Drosophila* circadian system cannot respond to the phosphorylated SR motifs, we examined the hPER2 proteins in transgenic flies. As compared with the SR motif-dependent sequential phosphorylation of PER2 proteins in mice (Fig. 1*C*) (7), $hPER2^{S662}$, $hPER2^{S662G}$, and $hPER2^{S662D}$ showed a similar phosphorylation pattern in *Drosophila* (Fig. 1*D*), which may explain why the period lengths are not associated with their genotypes (Fig. 1*B*). These data suggest that the SR motif is not recognized by the kinases in the *Drosophila* background.

Function of NEMO/NLK in Setting the Clock Speed Is Not Conserved—The recent discovery of phosphorylation by the NEMO/NLK kinase at the "per-short" domain (Fig. 1*A*) on dPER stimulates a hierarchical phosphorylation by DBT to set the circadian period in the *Drosophila* (13, 14), strikingly similar to the PER2 SR motif in mammals (a potential SR motif on dPER). To estimate the conservation of this kinase and its potential role as PER1 and PER2 kinase within a more functional context in mice, we generated *Nlk* knock-out mice with targeted embryonic stem cells obtained from the International Mouse Mutagenesis Program (Fig. 2*A*). Because *Nlk*-deficient mice died within 2 days after birth, we tried to examine the function of NLK in the central clock by examining the circadian phenotype of the *Nestin-cre*;$Nlk^{f/f}$ mice, in which *Nlk* is knocked out in central and peripheral neuron cells. However, as with *Nlk* conventional knock-out mice, most *Nestin-cre*;$Nlk^{f/f}$ mice died after birth. We only generated one *Nestin-cre*;$Nlk^{f/f}$ mouse that survived. Tested by wheel running, the circadian period of this mouse is 23.29 h, as compared with the 23.44 h of the control mice (Fig. 2*B*). Given the limited number of *Nestin-cre*;$Nlk^{f/f}$ mice, we cannot come to a confident conclusion of the function of *NLK* in the central clock. Because *Nlk* expressed at a high level in the liver (data not shown), *Alb-Cre* (*Cre* recombinase transcription under the control of the albumin promoter) mice were crossed with $Nlk^{f/f}$ mice to generate liver-specific *Nlk* knock-out mice (*Alb*;$Nlk^{f/f}$) (Fig. 2*C*) (15, 16). To determine the role of NLK in clock function, we crossed the *Alb*;$Nlk^{f/-}$ mice to the $Nlk^{f/f}$;$mPER2^{Luc}$ knock-in reporter mice (17) and monitored *Per2*-luciferase oscillation in liver tissues. The circadian period of PER2::LUC expression from the liver tissues was comparable in the WT and *Alb*;$Nlk^{f/f}$ mice (Fig. 2*D*). Furthermore, we found that the discernible expression changes of the core clock genes such as *Per2* and *Cry1*, and time-dependent changes in phosphorylated state of PER1 and PER2 relative to the WT and *Alb*;$Nlk^{f/f}$ mice, did not occur (Fig. 2, *E* and *F*). These data indicate that the phosphorylation function of NEMO/NLK on the SR-like or SR motif was not conserved in *Drosophila* and mammals. hCK1δ, hPER2, and NEMO/NLK cannot recapitulate the function in *Drosophila* or mammals, which suggests that divergences may have occurred in both substrates and kinases.

CK1δ Restores the Function of the SR Motif in the Drosophila Circadian System—The mechanisms by which new functions evolved can be identified by introducing new components into ancestral backgrounds and characterizing the effects of their interactions (18, 19). We attempted to restore the SR motif function by reconstructing $hPER2^{S662}$, $hPER2^{S662G}$, and $hPER2^{S662D}$ expression in hCK1ϵ, hCK1δ, or DBT backgrounds

[4] Z. Liu and Y. Xu, unpublished data.

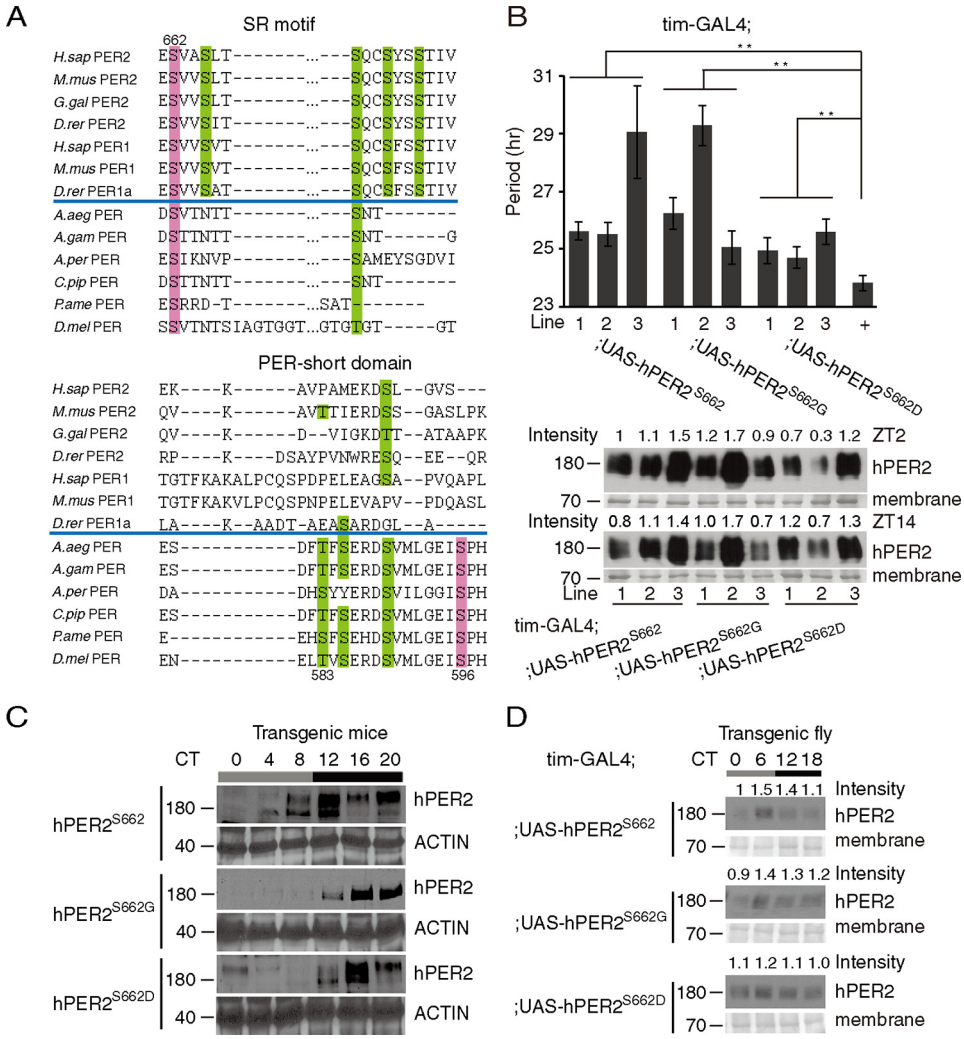


FIGURE 1. **Response of the hPER2 SR motif in the *Drosophila* circadian system.** *A*, sequence alignments of PER1, PER2, and insect PER protein sequences around the SR motif and the PER-short domain. The prime phosphorylation sites are marked in *pink*, and residues phosphorylated by CK1δ or DBT are marked in *green*. Species above the *blue line* are vertebrates, and species below the *blue line* are insects. The species are as follows: *H. sap* (*Homo sapiens*), *M. mus* (*Mus musculus*), *G. gal* (*Gallus gallus*), *D. rer* (*Danio rerio*), *A. aeg* (*Aedes aegypti*), *A. gam* (*Anopheles gambiae*), *A. per* (*Antheraea pernyi*), *C. pip* (*Culex pipiens*), *P. ame* (*Periplaneta americana*), and *D. mel* (*Drosophila melanogaster*). The UniProt IDs of these PER proteins are as follows: *H. sap* PER2 (O15055), *M. mus* PER2 (O54943), *G. gal* PER2 (Q8QGQ8), *D. rer* PER2 (B3DJN7), *H. sap* PER1 (O35973), *M. mus* PER1 (O35973), *D. rer* PER1a (B3DJN2), *A. aeg* PER (Q16ZM1), *A. gam* PER(Q7PYB3), *A. per* PER (Q17062), *C. pip* PER (A0A0A0V9X0), *P. ame* PER (Q25637), and *D. mel* PER (P07663). *B*, the locomotor activity analysis for flies overexpressing hPER2^{S662}, hPER2^{S662G}, and hPER2^{S662D} and driven by *tim*-GAL4 consisted of a free-running period under constant dark conditions (*upper panel*). A bar diagram of the average periods of rhythmic flies from more than 20 transgenic flies for each line is shown; the values are expressed as the mean ± S.E. Significance was calculated using Student's *t* test. **, $p < 0.01$. *Lower panel*, Western blotting analysis of hPER2 protein from three lines of *tim*-GAL4:hPER2^{S662}; *tim*-GAL4:hPER2^{S662G}; and *tim*-GAL4:hPER2^{S662D} transgenic flies. The proteins were extracted from 100 fly heads at ZT2 and ZT14. The values are representative of three repeats. Because the size of the PER2 protein is more than 130 kDa and the fly head samples are small, we used the same stained membrane for the loading control instead of actin staining. The relative intensities are labeled in the *top lane* and thereafter. *C*, Western blotting analysis of the hPER2 protein from hPER2^{S662}, hPER2^{S662G}, and hPER2^{S662D} transgenic mouse liver extracts at different circadian time points as described in a previous study (7). CT, Circadian time. *D*, Western blotting analysis of the hPER2 protein in *tim*-GAL4:hPER2^{S662}; *tim*-GAL4:hPER2^{S662G}; *tim*-GAL4:hPER2^{S662D} transgenic fly heads at ZT0, ZT6, ZT12, and ZT18. The values are representative of the WB of three independent repeats.

in *Drosophila*. First, we introduced DBT, hCK1ϵ, or hCK1δ into *Drosophila* and fused a V5 tag to their C terminus to compare the expression levels among different lines and different proteins. Consistent with previous studies (6), the transgenic lines expressing hCK1δ, hCK1ϵ, or DBT driven by *tim*-GAL4 all showed a significantly prolonged circadian period (Table 1), indicating that CK1ϵ and CK1δ are able to regulate components for circadian function in a *Drosophila* genetic background. Although the expression levels of DBT, hCK1ϵ, and hCK1δ in transgenic flies are comparable among different lines (Fig. 3*A*), the overexpression of hCK1ϵ and hCK1δ produced a longer circadian period than DBT (Table 1), suggesting that hCK1ϵ and hCK1δ may display different activity and/or interact with different substrates than DBT in *Drosophila*. Interestingly, when hCK1δ was coexpressed with hPER2^{S662}, hPER2^{S662G}, or hPER2^{S662D} in *Drosophila* under *tim*-GAL4 driver, hPER2^{S662G}::CK1δ double transgenic flies showed a significantly shorter period relative to hPER2^{S662}::CK1δ and hPER2^{S662D}::CK1δ flies (Fig. 3*B*), thus recapitulating the mouse function (7). However, the expression of hPER2^{S662}, hPER2^{S662D}, or hPER2^{S662G} from CK1ϵ- or DBT-overexpressed background produced weak or no effects on the circadian period (Fig. 3*B*). These phenotypes suggest that hCK1δ preferably (or at least partially) interacts with the SR motif to generate

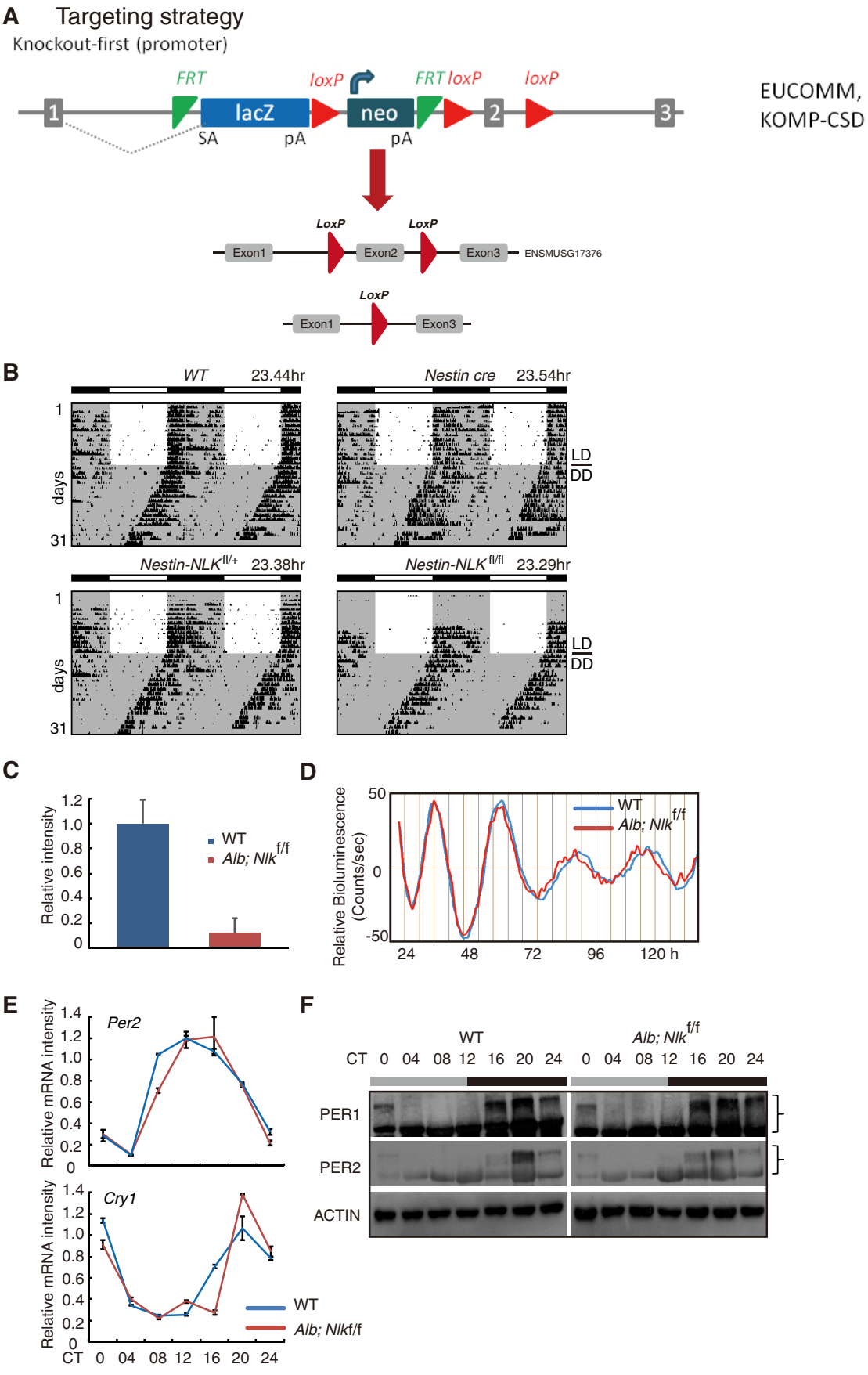

A
Targeting strategy
Knockout-first (promoter)
FRT
lacZ
loxP
neo
FRT loxP
loxP
1
2
3
SA
pA
pA
EUCOMM,
KOMP-CSD
LoxP
LoxP
Exon1
Exon2
Exon3
ENSMUSG17376
LoxP
Exon1
Exon3
B
WT
23.44hr
Nestin cre
23.54hr
Nestin-NLK fl/+
23.38hr
Nestin-NLK fl/fl
23.29hr
days
1
31
LD
DD
C
Relative intensity
WT
Alb; Nlk f/f
D
Relative Bioluminescence (Counts/sec)
WT
Alb; Nlk f/f
24
48
72
96
120 h
E
Per2
Cry1
Relative mRNA intensity
WT
Alb; Nlkf/f
CT 0 04 08 12 16 20 24
F
WT
Alb; Nlk f/f
CT 0 04 08 12 16 20 24 0 04 08 12 16 20 24
PER1
PER2
ACTIN

TABLE 1
Period length of DBT and CK1δ/ϵ in transgenic flies

	Line	Period ± S.E.	% of rhythmic (Rhy/Tested)
		h	
tim-GAL4/UAS-DBT	1	25.7 ± 0.3	77 (30/39)
	2	25.3 ± 0.4	71 (32/45)
	3	25.3 ± 0.3	87 (20/23)
tim-GAL4/UAS-hCK1δ	1	26.4 ± 0.4	71 (22/31)
	2	26.6 ± 0.5	71 (15/21)
	3	26.9 ± 0.5	86 (18/21)
tim-GAL4/UAS-hCK1ϵ	1	27.1 ± 0.5	91 (21/23)
	2	27.0 ± ± 0.4	95 (39/41)
	3	27.4 ± 0.5	72 (28/39)
tim-GAL4/UAS-hCK1δT44A	1	26.2 ± 0.5	73 (22/30)
	2	25.7 ± 0.5	83 (25/30)
	3	26.1 ± 0.4	82 (23/28)
UAS-DBT/+	1	23.9 ± 0.2	96 (26/27)
	2	23.7 ± 0.3	89 (17/19)
	3	23.7 ± 0.4	94 (32/34)
UAS-hCK1δ/+	1	23.6 ± 0.3	84 (21/25)
	2	23.6 ± 0.4	74 (23/31)
	3	23.7 ± 0.3	100 (29/29)
UAS-hCK1ϵ/+	1	23.8 ± 0.3	88 (22/25)
	2	23.6 ± 0.4	92 (23/25)
	3	23.7 ± 0.3	97 (29/30)
UAS-hCK1δT44A/+	1	23.8 ± 0.3	88 (23/26)
	2	23.7 ± 0.2	94 (30/32)
	3	23.6 ± 0.3	92 (24/26)
tim-GAL4/+		23.8 ± 0.3	88 (21/24)

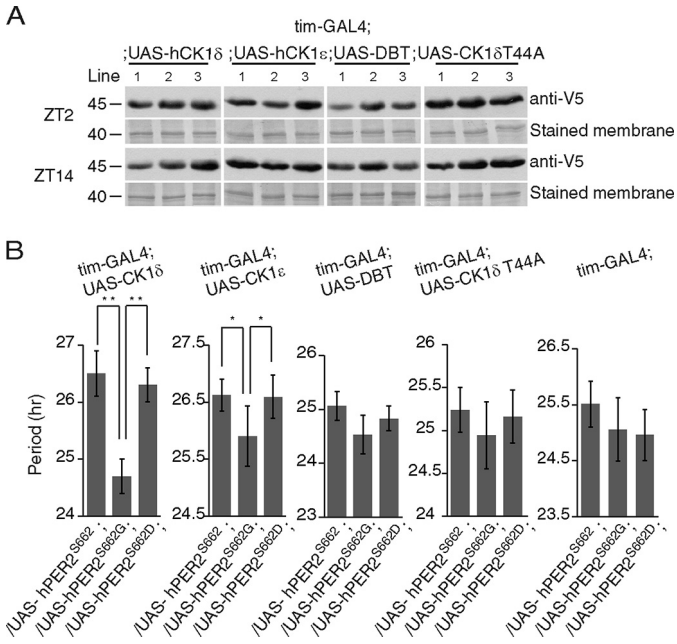


FIGURE 3. **CK1δ restores the function of the SR motif in the *Drosophila* circadian system.** *A*, Western blotting analysis of the protein levels of hCK1δ, hCK1ϵ, DBT, and hCK1δT44A from transgenic fly heads. *B*, locomotor activity analysis for hCK1δ/hCK1ϵ/DBT/hCK1δT44A and hPER2^{S662}/hPER2^{S662G}/hPER2^{S662D} double transgenic flies, which was calculated by a free-running period under DD conditions. The period is the average of more than 20 flies for each line. Values are expressed as the mean ± S.E. Significance was calculated using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

a behavioral response in the *Drosophila* circadian clock. In other words, hCK1δ (rather than CK1ϵ or DBT) is the preferred kinase for the PER2 SR motif.

Different Kinase Activity of DBT and hCK1δ on the SR Motif—To provide more direct evidence to support this conclusion, we examined hPER2 protein levels from different kinase backgrounds and found that hCK1δ has differential effects on PER2^{S662}, PER2^{S662G}, and PER2^{S662D} protein levels, leading to varied and distinct protein oscillation profiles (Fig. 4*A*). In contrast, DBT has a uniform destabilizing effect on these very different PER2 mutants (Fig. 4*A*). The comparable interaction of DBT and hCK1δ with hPER2 (Fig. 4*B*) suggests that DBT phosphorylates other sites in PER2. Although the mechanisms by which the hPER2^{S662G}/hPER2^{S662}/hPER2^{S662D} proteins were affected and how PER2 protein change was linked to a period event are not well understood, we speculate that the kinase activity of hCK1δ increased during evolution. To test this hypothesis, we generated transgenic flies expressing hCK1δ^{T44A}, which reduced the kinase activity on PER2 (6). Similar to DBT and CK1ϵ, hCK1δ^{T44A} also lengthened the *Drosophila* circadian period like DBT and CK1ϵ (Table 1), and the expression levels among these kinases are comparable (Fig. 3*A*). Interestingly, hCK1δ^{T44A} has an impaired ability to shorten period length in hPER2^{S662G} transgenic flies (Fig. 3*B*), suggesting that the kinase activity of hCK1δ is critical for the SR motif.

To further compare the kinase activity among DBT, CK1ϵ, and CK1δ on the SR motif, we immunoprecipitated DBT, CK1ϵ, and CK1δ from transgenic *Drosophila* heads with a V5 antibody because DBT purified from *Escherichia coli* is enzymatically inactive (21–24). Replacing the first serine with an aspartate residue in the PER2 SR motif could facilitate the sequential phosphorylation (7, 25). We thus substituted the first serine with an aspartate residue in the SR motif (DVASLTSQCSYSS) (termed SD) to test the phosphorylation reactions *in vitro*. GST was linked to SD (GST-SD: GST-DVASLTSQCSYSS), and this peptide was expressed in *E. coli* by induction with isopropyl β-D-1-thiogalactopyranoside and purified with a glutathione-Sepharose affinity resin. The *in vitro* kinase assay showed that CK1δ had the greatest activity on SD (Fig. 4*C*). Because the level of DBT auto-phosphorylation was much lower than that of CK1δ, we reasoned that the difference in kinase activity over the SR motif was not caused by auto-phosphorylation because auto-phosphorylation likely inhibits the activity of kinases (26–28). Finally, we compared

FIGURE 2. **The function of NEMO/NLK is not conserved for circadian clock.** *A*, schematic illustration of the targeting strategy for *Nlk3*-floxed mice. Targeted embryonic stem colonies obtained from the Knockout Mouse Project (KOMP) were directly used for blastocyst injections. The mice that retained the *loxP* sites flanking exon 3 at the endogenous *Nlk* locus were bred to *Alb*-Cre mice in a C57BL/6N background to generate liver-specific knock-out mice. *EUCOMM*, European Conditional Mouse Mutagenesis Program; *CSD*, CHORI/Sanger/UC Davis. *B*, locomotor activity recordings of representative mice. Alternating *white* and *dark bars* indicate the LD cycles during entrainment prior to release in DD. Circadian periods are indicated (in hours). *C*, quantitative PCR shows the depletion of the *Nlk* expression in *Alb;Nlk$^{f/f}$* liver tissues. *D*, the panels show representative bioluminescence signals from *Nlk$^{f/f}$;Per2^{luc}* (*blue*) and *Alb;Nlk$^{f/f}$;Per2^{luc}* (*red*) mice. *E*, the expression levels of the *Per2* and *Cry1* in the WT (*blue*) and *Alb;Nlk$^{f/f}$* (*red*) liver tissues were measured by quantitative RT-PCR and were normalized to *Gapdh* expression. The expression levels were plotted as arbitrary units, and the highest value in the sample was set to 1.0. Each value represents the mean ± S.D. ($n = 3$) from 3 mice. *CT*, circadian time. *F*, protein profiles of PER1 and PER2 in total extracts from the WT and *Alb;Nlk$^{f/f}$* liver tissues of 6–8-week-old mice. Actin was used as the loading control. Each image shows a representative example from three independent experiments. All of the tissues were collected at 4-h intervals during the first day in DD. The samples and blots were processed in parallel in paired experiments.

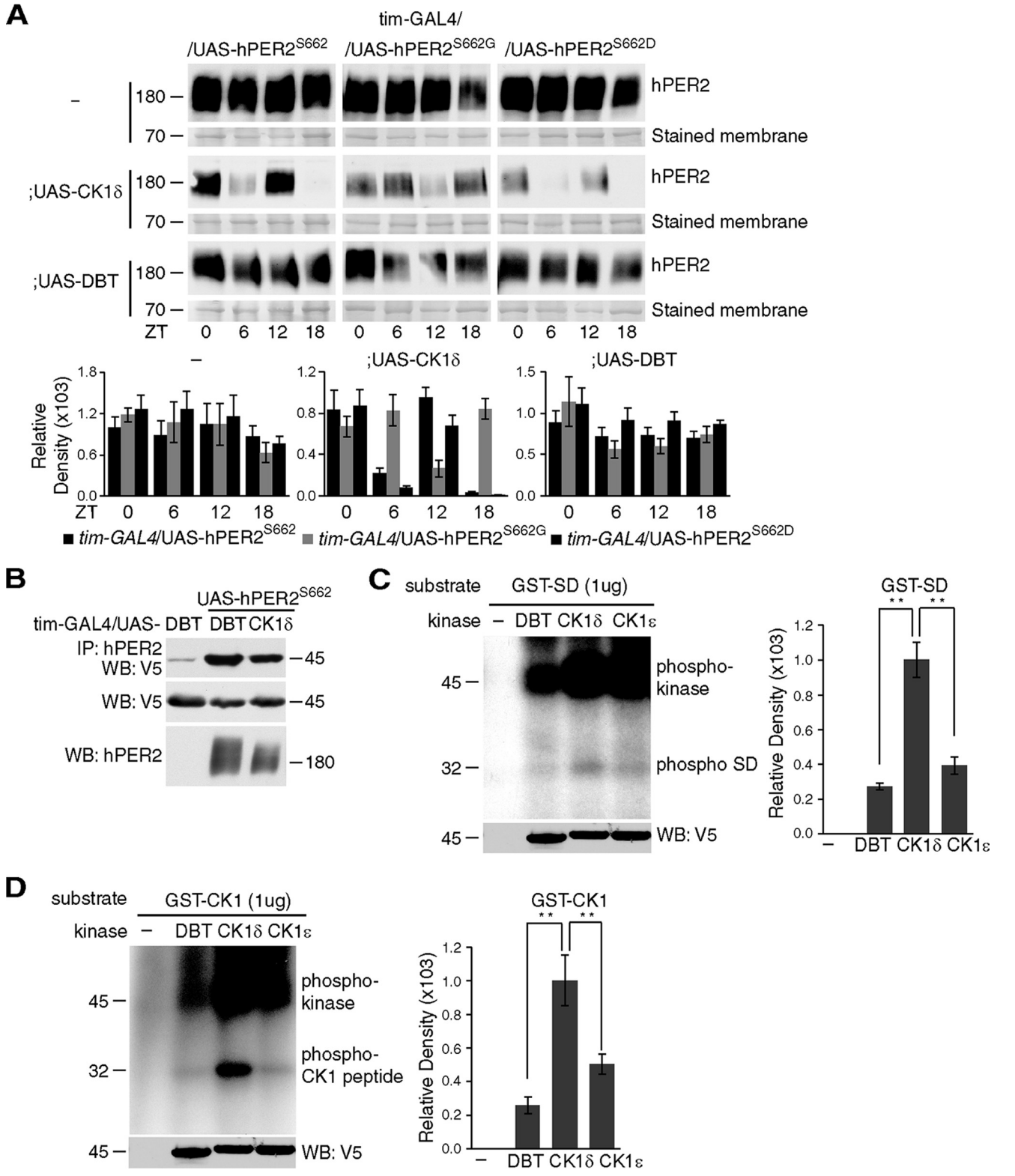


FIGURE 4. **Effects of DBT/CK1δ/ϵ on SR motifs.** *A*, representative Western blot of the hPER2 protein from double transgenic fly head extracts at different ZTs as described above. Intensities of different genotypes are shown in the *bottom row*. Values are expressed as the mean ± S.E. *B*, interaction of hPER2 protein with DBT and CK1δ. Cell lysates of transgenic fly heads were immunoprecipitated with antibody to hPER2 and immunoblotted with V5 antibody. *IP*, antibodies used for immunoprecipitation; *WB*, antibodies used for immunoblotting. *C* and *D*, *in vitro* kinase assay for DBT, hCK1δ, and hCK1ϵ on SD (*C*) and CK1-peptide (*D*). Kinases were immunoprecipitated from the corresponding transgenic fly heads collected at ZT20. w^{1118} flies were used as a negative control. These results are representative for three repeats for each reaction. The kinases in *B* and *C* were immunoprecipitated from the same fly head lysates, and therefore, the kinase input is the same. Values are expressed as the mean ± S.E. **, $p < 0.01$.

the effect of these kinases on the generalized CK1 peptide (GST-RRKDLHDDEEDEAMSITA) (29), where serine phosphorylation is primed by a series of aspartic or glutamic acids. Consistent with the effect on the SR motif, hCK1δ phosphorylated the CK1 peptide much more efficiently than DBT (Fig. 4*D*). Altogether, these data suggest that hCK1δ strongly enhanced phosphorylation at the SR motif and did so more efficiently than DBT and CK1ϵ.

Correlated Evolution of CK1δ and SR Motifs—If CK1δ had evolved to obtain the ability to catalyze the SR motif in the PER2 protein, it should be able to perform similar reactions on the SR motif in other substrates or utilize SR motifs in other protein to expand the repertoire of its enzymatic activities and result in functional adaptation (30). We found that an increased number of studies have suggested that CK1δ/ϵ phosphorylate SR motifs in many proteins including PER2, PER1, APC, NFATC, and PGC1a and YAP1 (7, 11, 12, 31–34). These SR motifs are well conserved in vertebrates (Fig. 5*A*), which is consistent with the highly conserved vertebrate CK1δ/ϵ protein sequence (supplemental Fig. S2 and Fig. 5*B*). In addition, we found that SR motifs are divergent among 48% (13/27) of PER3 proteins lacking one or two serine residues at SR motifs (Fig. 5*C*). However, the SR motifs in PER1 and PER2 are well conserved in different vertebrates (Fig. 1*A*). Interestingly, mouse PER3 does not show interaction with CK1ϵ/δ, which is inconsistent with PER1 and PER2 (35). Both observations suggest that the functional evolution of CK1δ kinase activity is likely correlated with the evolution of the SR motif and/or that this activity is likely to preserve SR motifs in CK1δ substrates.

Finally, a statistical analysis was performed of the conserved SR motifs in various species using protein sequence data retrieved from the OrthoMcl database. We searched for proteins in each species that contain a conserved S*XX*S*XX*S*XX*S*XX*S (5S) motif with their human orthologs and compared them with proteins containing other conserved similar pattern motifs such as A*XX*A*XX*A*XX*A*XX*A (Fig. 5*D*, A is an any amino acid). Interestingly, the number of conserved 5S motif-containing proteins was higher relative to other conserved 5A motif-containing proteins in vertebrates (Fig. 5*D*, *right side*, and supplemental Table S). Due to the evolutionary distance with humans, the number of conserved 5S and 5A motif-containing proteins in non-vertebrate species was much lower than that in vertebrates, and the number of conserved 5S motif-containing proteins was average (Fig. 5*D*, *left side*). An almost identical trend was also observed in proteins containing the 4S motif (Fig. 5*E*), although additional proteins contained the 4S motif rather than the 5S motif (compare Fig. 5*E* with Fig. 5*D*). As described above, the emergence of a conserved SR motif in vertebrates suggests that the critical function of the SR motif might be evolutionarily correlated with the kinase activity of CK1δ/ϵ.

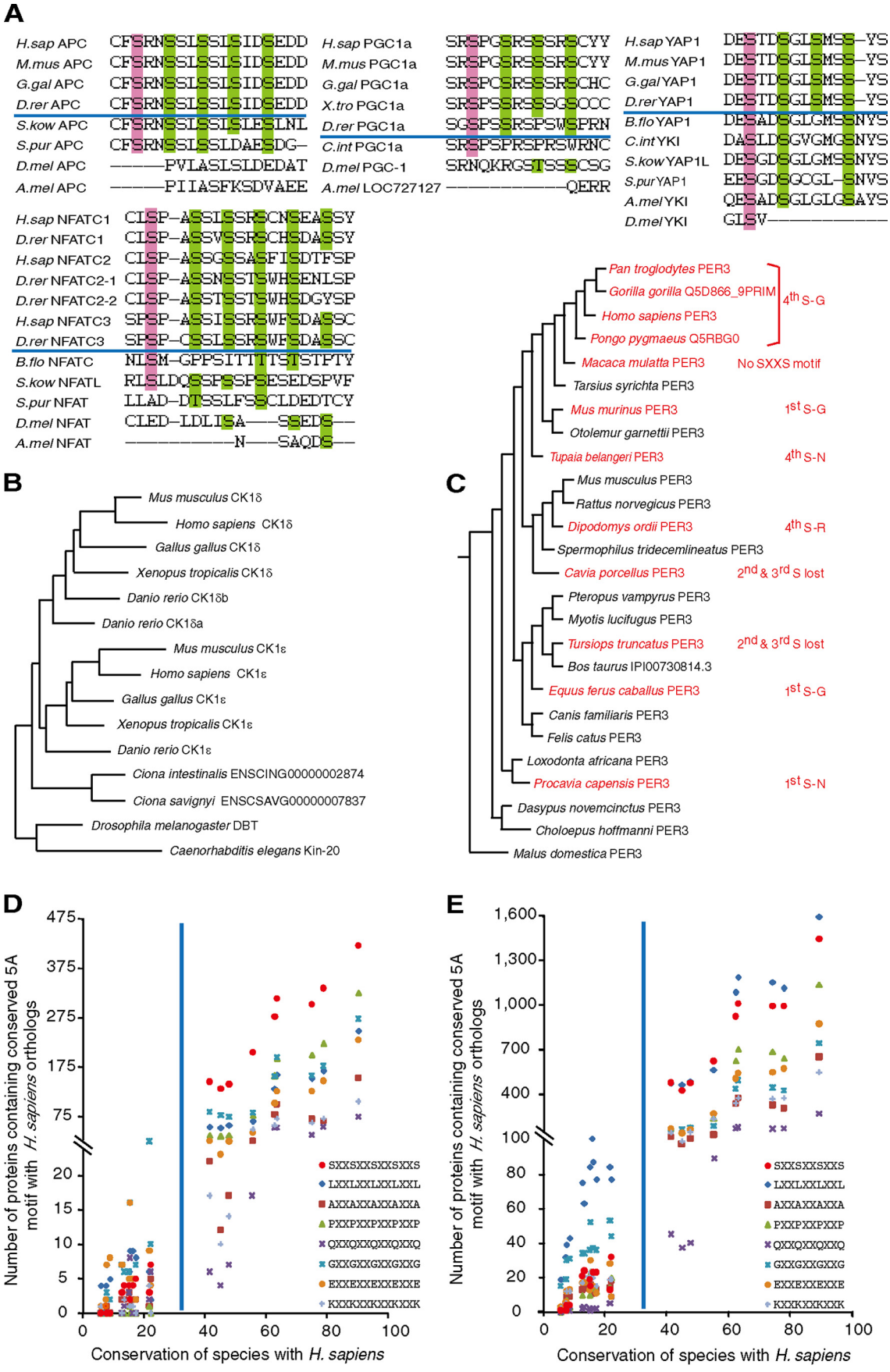
Discussion

The different phenotypes induced by CK1δ mutations in mouse and *Drosophila* circadian system challenged the notion that the CK1δ-PER interaction is functionally conserved (6). This conclusion is supported by Sekine *et al.* (36), in which they introduced hCK1ϵ into *Drosophila* and compared the function of DBT and hCK1ϵ on fly circadian rhythm and PER phosphorylation. They found that hCK1ϵ cannot replace the function of DBT in the fly, suggesting the evolution of kinase substrate interaction (36).

We have tried to directly examine the activity of DBT and CK1δ/ϵ on hPER2^{S662}/hPER2^{S662G}/hPER2^{S662D} *in vitro*. However, we failed to find a proper *in vitro* system to perform the test. When expressed in mammalian cell culture, *Drosophila* DBT seems enzymatically inactive. It can barely phosphorylate its own substrate PER in human embryonic kidney 293 cells. When overexpressed in the *Drosophila* S2 cell, hPER2 proteins were easily degraded, especially when overexpressed together with kinases (data not shown), which makes the detection of phospho-hPER2s very difficult.

By reconstructing the interactions of DBT/hCK1δ/ϵ and SR motif mutated hPER2s in the *Drosophila* circadian system, we can speculate on the function of DBT and CK1δ/ϵ on hPER2 SR motif by comparing the phenotype of different double transgenic flies. We discovered that hCK1δ is required for the function of the SR motif, which is not observed in *Drosophila* PER. One limitation of our *in vivo* experimental design is that we examined the activity of CK1δ/ϵ on hPER2 in the presence of endogenous DBT. Although the overexpressed CK1δ/ϵ is much higher than the endogenous DBT, it is still possible that the endogenous DBT complicated the activity of CK1δ/ϵ on hPER2s. Given the fact that CK1ϵ cannot rescue the circadian arrhythmicity of DBT mutant fly (36), we did not repeat the experiment in DBT-deficient background. By *in vitro* kinase assay using kinases immunoprecipitated from transgenic flies, we directly demonstrated varied kinase activity of DBT and CK1δ on the SR motif. Although more work is needed to address the mechanistic details of their coevolution, we have shown that DBT is less effective than hCK1δ in phosphorylating the SR motif, which suggests that the co-variation between kinase and substrates may be responsible for the functional diversification of the interaction and therefore may play a role in the phenotypic evolution of organisms. This study also provides an example of the correlated evolution of orthologous kinase and their substrates, and shows their contribution to physiological evolution. These results are consistent those of other studies in which protein kinases and phosphorylation have been reported to provide important sources of phenotypic diversity (37, 38).

Despite a pivotal role in several biological processes, the specific function of SR motifs is poorly understood. The phosphorylation of SR motifs may play different regulatory roles in various proteins. Vanselow *et al.* (39) reported that the hPER2 S662G mutation destabilizes the PER2 protein. Phosphorylation of YAP and PGC1α SR motifs was reported to facilitate the degradation of proteins (11, 31). For NFATC, phosphorylation of the SR motif increases the localization of NFATC to the nucleus (32). APC is phosphorylated by the mutual priming of CK1ϵ and GSK-3β and then competes with axin for binding to β-catenin, which leads to the degradation of the complex (33). These studies indicate that the conserved SR identified in each species' proteome would be versatile. Generally, the multiple phosphorylation targets in SR motifs enable the hierarchical regulation of the protein by different kinases and the formation


A
H.sap APC CFSRNSSLSSLSIDSEDD
M.mus APC CFSRNSSLSSLSIDSEDD
G.gal APC CFSRNSSLSSLSIDSEDD
D.rer APC CFSRNSSLSSLSIDSEDD
S.kow APC CFSRNSSLSSLSLESLNL
S.pur APC CFSRNSSLSSLDAESDG–
D.mel APC ———PVLASLSLDEDAT
A.mel APC ———PIIASFKSDVAEE
H.sap PGC1a SRSPGSRSSSRSCYY
M.mus PGC1a SRSPGSRSSSRSCYY
G.gal PGC1a SRSPCSRSSSRSCHC
X.tro PGC1a SRSPSSRSSSGSCCC
D.rer PGC1a SGSPSSRSPSWSPRN
C.int PGC1a SRSPSPRSPRSWRNC
D.mel PGC-1 SRNQKRGSTSSSCSG
A.mel LOC727127 ——————————QERR
H.sap YAP1 DESTDSGLSMSS–YS
M.mus YAP1 DESTDSGLSMSS–YS
G.gal YAP1 DESTDSGLSMSS–YS
D.rer YAP1 DESTDSGLSMSS–YS
B.flo YAP1 DESADSGLGMSSNYS
C.int YKI DASLDSGVGMGSNYS
S.kow YAP1L DESGDSGLGMSSNYS
S.pur YAP1 EESGDSGCGL–SNVS
A.mel YKI QESADSGLGLGSAYS
D.mel YKI GLSV————————
H.sap NFATC1 CLSP–ASSLSSRSCNSEASSY
D.rer NFATC1 CLSP–ASSVSSRSCHSDASSY
H.sap NFATC2 CLSP–ASSGSSASFISDTFSP
D.rer NFATC2-1 CLSP–ASSNSSTSWHSENLSP
D.rer NFATC2-2 CLSP–ASSTSSTSWHSDGYSP
H.sap NFATC3 SPSP–ASSISSRSWFSDASSC
D.rer NFATC3 SPSP–CSSLSSRSWFSDASSC
B.flo NFATC NLSM–GPPSITTTTSTSTPTY
S.kow NFATL RLSLDQSSPSSPSESEDSPVF
S.pur NFAT LLAD–DTSSLFSSCLDEDTCY
D.mel NFAT CLED–LDLISA———SSEDS——
A.mel NFAT ——————N———SAQDS——
B
Mus musculus CK1δ
Homo sapiens CK1δ
Gallus gallus CK1δ
Xenopus tropicalis CK1δ
Danio rerio CK1δb
Danio rerio CK1δa
Mus musculus CK1ε
Homo sapiens CK1ε
Gallus gallus CK1ε
Xenopus tropicalis CK1ε
Danio rerio CK1ε
Ciona intestinalis ENSCING00000002874
Ciona savignyi ENSCSAVG00000007837
Drosophila melanogaster DBT
Caenorhabditis elegans Kin-20
C
Pan troglodytes PER3
Gorilla gorilla Q5D866_9PRIM
Homo sapiens PER3
Pongo pygmaeus Q5RBG0
4th S-G
Macaca mulatta PER3
No SXXS motif
Tarsius syrichta PER3
Mus murinus PER3
1st S-G
Otolemur garnettii PER3
Tupaia belangeri PER3
4th S-N
Mus musculus PER3
Rattus norvegicus PER3
Dipodomys ordii PER3
4th S-R
Spermophilus tridecemlineatus PER3
Cavia porcellus PER3
2nd & 3rd S lost
Pteropus vampyrus PER3
Myotis lucifugus PER3
Tursiops truncatus PER3
2nd & 3rd S lost
Bos taurus IPI00730814.3
Equus ferus caballus PER3
1st S-G
Canis familiaris PER3
Felis catus PER3
Loxodonta africana PER3
Procavia capensis PER3
1st S-N
Dasypus novemcinctus PER3
Choloepus hoffmanni PER3
Malus domestica PER3
D
Number of proteins containing conserved 5A motif with H. sapiens orthologs
Conservation of species with H. sapiens
SXXSXXSXXSXXS
LXXLXXLXXLXXL
AXXAXXAXXAXXA
PXXPXXPXXPXXP
QXXQXXQXXQXXQ
GXXGXXGXXGXXG
EXXEXXEXXEXXE
KXXKXXKXXKXXK
E
Number of proteins containing conserved 5A motif with H. sapiens orthologs
Conservation of species with H. sapiens
SXXSXXSXXS
LXXLXXLXXL
AXXAXXAXXA
PXXPXXPXXP
QXXQXXQXXQ
GXXGXXGXXG
EXXEXXEXXE
KXXKXXKXXK

of different complexes. Moreover, various conformations would be elicited by hierarchical phosphorylation that may allow the protein to serve multiple functions (40). Here, we searched a collection of proteins containing SR motifs, most of which remain unidentified to date. Thus, future studies are needed to obtain additional data regarding the specific substrates and functions of the motifs in each protein and further understand how new functions are developed in vertebrates. Our observations also suggest that with genetic synthesis, phenotypes caused by single or additive co-evolution changes can likely be recapitulated in other species.

Besides the SR motif in PER2 and per-short domain in dPER (Fig. 1*A*), other CK1 phosphorylation sites have been identified in PER proteins. Ser-47 of dPER (supplemental Fig. S1, marked in *blue*) was reported to be phosphorylated by DBT, providing a binding site for the F-box protein, SLIMB (ortholog of βTrCP) and therefore mediating the degradation of dPER (41). Sequence alignment showed that Ser-47 is well conserved among PER proteins from different species (supplemental Fig. S1). In mammals, mutations in Ser-477 and Gly-479 in mPER2 abolished the CK1ε-dependent βTrCP binding, indicating that Ser-477 (supplemental Fig. S1, marked in *yellow*) might be the phosphorylation target of CK1ε, which recruits βTrCP and mediates the degradation of mPER2 (42). Using mass spectrometric analysis, multiple phosphorylation sites have been identified in dPER and mPER2 (24, 39, 41, 43). Among these phospho-peptides, another SR motif of mPER2 (Ser-525, Ser-528, Ser-531) (supplemental Fig. S1, marked in *brown*) was identified by two separate groups (39, 43). This SR motif is conserved between mouse and human but not present in other species, indicating that it is a newly emerged CK1δ target (supplemental Fig. S1). One SR motif (perSD domain) was found to be phosphorylated by DBT in dPER (Ser-604, Ser-607, Thr-610, Ser-613, Ser-629) (supplemental Fig. S1, marked in *red*) (24), which was shown to be important for the transcriptional repression activity of dPER (24). Despite the similar pattern with the PER2 SR motif, they are not evolutionarily conserved domains (supplemental Fig. S1). Kivimae *et al.* (24) suggest that the function of perSD domain is tightly regulated by the phosphorylation of Ser-589, which is 15 residues N-terminal to perSD domain, suggesting different regulation of perSD domain with hPER2SR motif. Thus, it is possible that the weak activity of DBT on the SR motif might be sufficient to regulate the activity of perSD domain.

Experimental Procedures

Generation of Transgenic Flies and Behavioral Analyses—The coding sequences for hPER2^{S662}, hPER2^{S662G}, and hPER2^{S662D} were inserted into the pUAST vector. The V5 tag coding sequence was added to the C terminus of the DBT, hCK1δ, and CK1δT44A coding sequences. DBT-V5/hCK1δ-V5/CK1δT44A-V5-pUAST vectors were constructed, and transgenic flies were generated by injecting each vector into w^{1118} *Drosophila* embryos using a standard protocol. Three independent lines for each genotype were selected and then crossed to *tim*-GAL4 (45) and w^{1118} (control) flies.

Drosophila were reared on standard cornmeal/agar medium and maintained under 12:12 light-dark (LD) cycles at 25 °C. The circadian periods of the flies were analyzed using the ClockLab (Actimetrics) software as described previously (Pfeiffenberger *et al.* (46)). For each *Drosophila* line, the locomotor activity tests were repeated in at least two trials. The results were consistent between trials.

Western Blotting Analysis—One hundred fly heads of the specified genotype were collected at the indicated time points after 3 days of rearing under LD conditions. Western blots were conducted as described previously (20, 44). The hPER2 proteins were probed with homemade hPER2 antibody (7) with 1:1000 dilution.

In Vitro Kinase Assay—One hundred heads from w^{1118}, DBT, and hCK1δ transgenic flies were collected at ZT20. The tissue lysates were incubated with an anti-V5 monoclonal antibody (Sigma-Aldrich) followed by incubation with 60 μl of a 50% slurry of protein G-Sepharose (BioVision). Sepharose was washed three times with lysis buffer and then subjected to a kinase assay in a 20-μl reaction mix (30 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.5 M DTT, 200 mM ATP, 50 mg/ml BSA, and 100 μM [γ-^{32}P]ATP (0.5 μCi) (PerkinElmer)) with 1 μg of substrate (GST-SD or GST-CK1 peptide) for 30 min at 30 °C. The reaction was terminated by adding 20 μl of 4× sample buffer. The products were resolved on 12% SDS-PAGE gels, and γ-^{32}P incorporation was detected in the dried gels by exposure of X-ray films (Kodak) at −80 °C for 3 days.

Bioinformatic Analysis of SR Motifs—The protein sequences for different species were retrieved from the OrthoMCL DB database and then screened to identify the 5A or 4A motifs. The proteins containing 5A or 4A motifs were aligned with their human orthologs. The number of proteins containing conserved 5A or 4A motifs with human orthologs was counted for each species.

FIGURE 5. **Expansion of the SR motif in vertebrates.** *A*, alignment of sequences around SR motifs in CK1δ/ε substrates. Species above the *blue lines* are vertebrates. The first serines/threonines of the SR motifs are marked *pink*, and the following serines/threonines are marked in green. The species are as follows: *H. sap* (*Homo sapiens*), *M. mus* (*Mus musculus*), *G. gal* (*Gallus gallus*), *X. tro* (*Xenopus tropicalis*), *D. rer* (*Danio rerio*), *C. int* (*Ciona intestinalis*), *B. flo* (*Branchiostoma floridae*), *S. kow* (*Saccoglossus kowalevskii*), *S. pur* (*Strongylocentrotus purpuratus*), *A. mel* (*Apis mellifera*), and *D. mel* (*Drosophila melanogaster*). *B*, phylogenetic tree for CK1ε and CK1δ retrieved from ensemble database. CK1ε and CK1δ exist in most vertebrates. *C*, phylogenetic tree of PER3 derived from the Ensembl database. The PER3 proteins that do not contain the integrated SR motif are shown in *red*. Variations of SR motifs in different species are illustrated. *D* and *E*, number of proteins containing conserved 5A (*D*) and 4A (*E*) motifs with *Homo sapiens* orthologs. The species included in this study from the distal to the proximal *x* axis are as follows: *Pan troglodytes*, *Equus caballus*, *Canis lupus familiaris*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Takifugu rubripes*, *Tetraodon nigroviridis*, *Danio rerio*, *Acyrthosiphon pisum*, *Ciona intestinalis*, *Anopheles gambiae* str. PEST, *Aedes aegypti*, *Nematostella vectensis*, *Drosophila melanogaster*, *Ixodes scapularis*, *Culex pipiens*, *Caenorhabditis elegans*, *Caenorhabditis briggsae AF16*, *Chlamydomonas reinhardtii*, and *Neospora caninum*. The species on the *right side* of the *blue line* are vertebrates. The *numbers* on the *x* axis represent the conservation of each species as compared with humans, which is calculated by multiplying the percentage of proteins that have human orthologs by the orthologous sequence identity. From the 19 control motifs, we selected 7 motifs for the actograms. For the other 12 motifs, the number of 5A motif-containing proteins was less than 100 in most species and the conservation values were extremely low.

Author Contributions—L. X. and Y. X. contributed to the experimental design, work, and data analysis. Y. A. generated mouse models and provided Nlk knockout mouse data. J. Y. performed bioinformatics assay. P. X., Z. L., Z. Z., Z. Q., and G. S. contributed to vector constructs and fly work. D. P. provided intellectual input and data analysis. L. X. and Y. X. wrote the manuscript.

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