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

Received for publication, August 1, 2016, and in revised form, November 21, 2016 Published, JBC Papers in Press, November 22, 2016, DOI 10.1074/jbc.M116.751214

Lijuan Xing1, Yang An2, Guanseng Shi1, Jie Yan3, Pancheng Xie9, Zhipeng Qu2, Zhihui Zhang1, Zhiwei Liu1, Dejing Pan1, and Ying Xu1§2

From the4 Cambridge-Suda Genomic Resource Center, Soochow University, 199 Renai Road, Suzhou 215123 and the 9 MOE Key Laboratory of Model Animal for Disease Study, Model Animal Research Center, Nanjing University, 12 Xuefu Road, Pukou District, Nanjing 210061, China

Edited by Ruma Banerjee

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). 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) (6, 7). Moreover, the introduction of hPER2S662G (the first serine in the SR motif is mutated) into mice faithfully recapitulates the human phenotype, whereas hPER2S662D (which mimics the phosphorylated status) lengthened the circadian period (7). Correspondingly, a mutation in CK1δ (hCK1δT44A) also causes FASPS and hCK1δT44A 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 hCK1δT44A 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 (hPER1S714G) caused an advanced feeding rhythm in mice and disrupted their

* This work was supported by grants from the National Science Foundation of China (31230049) (to Y. X), Royal Society-Newton Advanced Fellowship (NA150373) (to Y. X), and a project funded by the Priority Academic Development Program of the Jiangsu Higher Education Institutions. The authors declare that they have no conflicts of interest with the contents of this article.

§ This article contains supplemental Figs. 1 and 2 and supplemental Table S. To whom correspondence may be addressed. Tel.: 86-51265883781; Fax: 86-51265883562; E-mail: pandejing@suda.edu.cn.

‡ To whom correspondence may be addressed. Tel.: 86-51265883781; Fax: 86-51265883562; E-mail: yingxu@suda.edu.cn.

© 2017 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
metabolic oscillation (12). These well recorded examples suggest critical functions for the SR motifs in the mammalian physiology. However, the apparent complexity of CK1δ/e 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. 1A 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. PER2S662D; aboliishes sequential phosphorylation for a short period and PER2S662G mimics phosphorylation for a long period). In mice, however, the expression of the period paralogous hPER1S714G (12) or hPER3S630G,4 the first serine-directed mutation in the SR motif, failed to change the locomotor period as observed in the hPER2S662G mice, suggesting that PER2 is an optimal candidate for quantitative analysis of locomotor period changes. Therefore, we introduced hPER2S662D (wild type), hPER2S662G, and hPER2S662D into *Drosophila*. Driven by *timeless-GAL4 (tim-GAL4)*, the overexpression of hPER2S662, hPER2S662G, and hPER2S662D all lengthened the *Drosophila* circadian period (Fig. 1B, 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 the 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. 1C, 7), hPER2S662D, hPER2S662G, and hPER2S662D showed a similar phosphorylation pattern in *Drosophila* (Fig. 1D), which may explain why the period lengths are not associated with their genotypes (Fig. 1B). 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. 1A) 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. 2A). 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−/−* 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−/−* mice died after birth. We only generated one *Nestin-cre;Nlk−/−* 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. 2B). Given the limited number of *Nestin-cre;Nlk−/−* 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−/− mice to generate liver-specific Nlk knock-out mice (*Alb;Nlk−/−*) (Fig. 2C) (15, 16). To determine the role of Nlk in clock function, we crossed the *Alb;Nlk−/−* mice to the Nlk−/−;*mPER2Dcr* 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−/−* mice (Fig. 2D). 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−/−* 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 hPER2S662, hPER2S662G, and hPER2S662D expression in hCK1δ, hCK1δ, or DBT backgrounds.

---

4 Z. Liu and Y. Xu, unpublished data.
Expands Function of Substrates with Serine-rich Motifs

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. sapiens (Mus musculus), G. gallus (Gallus gallus), D. rerio (Danio rerio), A. aegypti (Aedes aegypti), A. pernyi (Periplaneta americana), and D. mel (Drosophila melanogaster). The UniProt IDs of these PER proteins are as follows: H. sapiens PER2 (Q15055), M. mus PER2 (O54943), G. gallus PER2 (Q09Q08), D. rerio PER2 (B3DJN7), H. sapiens PER1 (Q55973), D. rerio PER1A (B3DJN2), A. aegypti PER (Q1Z6M1), A. pernyi PER (Q17062), C. pipiens PER1 (Q26537), and D. mel PER (P07663). B, the locomotor activity analysis for flies overexpressing hPER2S662, hPER2S662G, and hPER2S662D 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 the hPER2 protein from three lines of tim-GAL4:hPER2S662; tim-GAL4:hPER2S662G; and tim-GAL4:hPER2S662D 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 hPER2S662, hPER2S662G, and hPER2S662D 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:hPER2S662; tim-GAL4:hPER2S662G; and tim-GAL4:hPER2S662D 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, hCK1δ, 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. 3A), 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 hPER2S662, hPER2S662G, or hPER2S662D in Drosophila under tim-GAL4 driver, hPER2S662D:CK1δ double transgenic flies showed a significantly shorter period relative to hPER2S662::CK1δ and hPER2S662D::CK1δ flies (Fig. 3B), thus recapitulating the mouse function (7). However, the expression of hPER2S662, hPER2S662G, or hPER2S662D from CK1ε- or DBT-overexpressed background produced weak or no effects on the circadian period (Fig. 3B). These phenotypes suggest that hCK1δ preferably (or at least partially) interacts with the SR motif to generate
**Targeting strategy**

Knockout-first (promoter)

A

![Diagram of Targeting strategy]

B

|        | WT       | Nestin cre | Nestin-NLK <sup>f/f</sup> |
|--------|----------|------------|---------------------------|
| LD     | 23.44hr  | 23.54hr    | 23.38hr                   |
| DD     | 23.54hr  | 23.29hr    | 23.29hr                   |

C

|        | Relative intensity |
|--------|--------------------|
| WT     |                     |
| Alb; Nlk<sup>f/f</sup> |               |

D

![Graph of Relative Bioluminescence](Counts/sec)

E

|        | Relative mRNA intensity |
|--------|------------------------|
| Per2   |                        |
| Cry1   |                        |

F

|        | WT | Alb: Nlk<sup>f/f</sup> |
|--------|----|------------------------|
| CT 0   |     |                        |
| CT 24  |     |                        |

**CK1δ Expands Function of Substrates with Serine-rich Motifs**
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 PER2S662, PER2S662G, and PER2S662D protein levels, leading to varied and distinct protein oscillation profiles (Fig. 4A). In contrast, DBT has a uniform destabilizing effect on these very different PER2 mutants (Fig. 4A). The comparable interaction of DBT and hCK1δ with hPER2 (Fig. 4B) suggests that DBT phosphorylates other sites in PER2. Although the mechanisms by which the hPER2S662/hPER2S662G/hPER2S662D 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 hCK1δT44A, which reduced the kinase activity on PER2 (6). Similar to DBT and CK1ε, hCK1δT44A also lengthened the Drosophila circadian period like DBT and CK1ε (Table 1), and the expression levels among these kinases are comparable (Fig. 3A). Interestingly, hCK1δT44A has an impaired ability to shorten period length in hPER2S662G transgenic flies (Fig. 3B), 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 SR motif 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.

**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 cells 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 light and dark bars indicate the LD cycles during entrainment prior to release in DD. Circadian periods are indicated (in hours).

**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 hPER2S662G/hPER2S662D 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.

**TABLE 1**

| Line                 | Period ± S.E. | % of rhythmic (Rhy/Tested) |
|----------------------|---------------|---------------------------|
| tim-GAL4/UAS-DBT     | 25.7 ± 0.3    | 77 (30/39)                |
| tim-GAL4/UAS-hCK1δ   | 26.4 ± 0.5    | 71 (32/45)                |
| tim-GAL4/UAS-hCK1ε   | 27.1 ± 0.5    | 87 (20/23)                |
| tim-GAL4/UAS-hCK1δT44A | 26.2 ± 0.5 | 73 (22/30)                |
| UAS-DBT/+            | 23.9 ± 0.2    | 96 (26/27)                |
| UAS-hCK1δ/+          | 23.6 ± 0.3    | 83 (25/30)                |
| UAS-hCK1ε/+          | 23.6 ± 0.4    | 82 (23/28)                |
| UAS-hCK1δT44A/+      | 23.8 ± 0.3    | 88 (22/25)                |
| tim-GAL4/+           | 23.8 ± 0.3    | 88 (21/24)                |
FIGURE 4. Effects of DBT/CK1δ 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. w1118 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-RKDLHDDEDEAMSITA) (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. 4D). 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 PGC1α and YAP1 (7, 11, 12, 31–34). These SR motifs are well conserved in vertebrates (Fig. 5A), which is consistent with the highly conserved vertebrate CK1δ/ε protein sequence (supplemental Fig. S2 and Fig. 5B). 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. 5C). However, the SR motifs in PER1 and PER2 are well conserved in different vertebrates (Fig. 1A). 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 SXSSXSSXXSXXS (5S) motif with their human orthologs and compared them with proteins containing other conserved similar pattern motifs such as AXAXAXAXAXXX (Fig. 5D, 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. 5D, 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. 5D, left side). An almost identical trend was also observed in proteins containing the 4S motif (Fig. 5E), although additional proteins contained the 4S motif rather than the 5S motif (compare Fig. 5E with Fig. 5D). 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 hPER2S662Δ/Δ hPER2S662Δ/Δ hPER2S662Δ/Δ 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 vitro 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...
CK1δ Expands Function of Substrates with Serine-rich Motifs

A

H. sapiens APC
M. musculus APC
G. gallus APC
D. rerio APC
S. kowai APC
S. purpureus APC
D. melanogaster APC
A. melas APC

H. sapiens NFATC1
D. rerio NFATC1
H. sapiens NFATC2
D. rerio NFATC2
H. sapiens NFATC3
D. rerio NFATC3
B. frogs NFATC
S. kowai NFATL
S. purpureus NFAT
D. melanogaster NFAT
A. melas NFAT

B

Mus musculus CK15
Homo sapiens CK15
Gallus gallus CK15
Xenopus tropicalis CK15
Danio rerio CK1b
Danio rerio CK1a
Mus musculus CK1c
Homo sapiens CK1c
Gallus gallus CK1c
Xenopus tropicalis CK1c
Danio rerio CK1c
Clona intestinalis ENSCNI0000002874
Clona savignyi ENSCSAVG0000007837
Drosophila melanogaster DBT
Caenorhabditis elegans Kin-20

C

Pan troglodytes PER3
Gorilla gorilla Q5Q666_9PRIM
Homo sapiens PER3
Pongo pygmaeus Q5R8B0
Macaca mulatta PER3
No SXSS motif
Tarsius syrichta PER3
Mus musculus PER3
1s1S-G
Otolemur garnetti PER3
Tipaisa belangi PER3
4p1S-N
Mus musculus PER3
Rattus norvegicus PER3
Dipodomys ordi PER3
Spermophilus tridecemlineatus PER3
Canis porcinus PER3
2t0G & 2t0S lost
Pteropus vampyrus PER3
Myotis lucifugus PER3
Tursiops truncatus PER3
Bos taurus IP00730814.3
Equus ferus caballus PER3
Canis familiaris PER3
Felis catus PER3
Loxodonta africana PER3
Procavia capensis PER3
1s1S-N
Desacylcnoica PER3
Cholepus rufus PER3
Maus domestica PER3

D

Number of proteins containing conserved 5A
motif with H. sapiens orthologs

E

Number of proteins containing conserved 5A
motif with H. sapiens orthologs
CK1δ Expands Function of Substrates with Serine-rich Motifs

Experimental Procedures

Generation of Transgenic Flies and Behavioral Analyses—The coding sequences for hPER2S662D, hPER2S662G, and hPER2S662I 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 (Pfeifferberger 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 homogeneous 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 MgCl2, 0.5 mM DTT, 200 mM ATP, 50 mg/ml BSA, and 100 μM [γ-32P]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-32P 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 blue. The second serines/threonines are marked pink, and the following serines/threonines are marked in green. The species are as follows: H. sapiens (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 floridum). S. kow (Saccoglossus kowalevskii), S. pur (Strongylocentrotus purpuratus), A. mel (Apsis 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, Anostraca, Caenorrhabditis 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.
CKI\(a\) Expands Function of Substrates with Serine-rich Motifs

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.

Acknowledgments—We thank members of the Xu laboratory for their assistance.

References

1. Studer, R. A., and Robinson-Rechavi, M. (2009) How confident can we be that orthologs are similar, but paralogs differ? Trends Genet. 25, 210–216
2. Cyran, S. A., Yiannoulos, G., Buchsbaum, A. M., Saez, L., Young, M. W., and Blau, J. (2005) The DOUBLE-TIME protein kinase regulates the subcellular localization of the Drosophila clock protein PERIOD. J. Neurosci. 25, 543–549
3. Kloss, B., Price, J. L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C. S., and Ptácek, L. J. (2007) Modeling of a human circadian mutation causing familial advanced sleep phase syndrome. Nature 434, 640–644
4. Xu, Y., Padiath, Q. S., Shapiro, R. E., Jones, C. R., Wu, S. C., Saitoh, N., Saitoh, K., Ptácek, L. J., and Fu, Y. H. (2005) Functional consequences of a CKI\(a\) mutation causing familial advanced sleep phase syndrome. J. Biol. Chem. 280, 555–565
5. Chen, J. C., Ko, H. W., and Edery, I. (2011) NEMO/NLK phosphorylates core period determination by slowing the pace of the Drosophila circadian oscillator. Mol. Cell Biol. 31, 2288–2297
6. Liu, Z., Huang, M., Wu, X., Shi, G., Xing, L., Dong, Z., Qu, Z., Yan, J., Yang, L., Panda, S., and Xu, Y. (2014) PER1 phosphorylation specifies feeding rhythm in mice. Cell Rep. 7, 1509–1520
7. Chiu, J. C., Ko, H. W., and Edery, I. (2011) NEMO/NLK phosphorylates PERIOD to initiate a time-delay phosphorylation circuit that sets circadian clock speed. J. Biol. Rhythms 23, 511–521
8. Gross, S. D., and Anderson, R. A. (1998) Casein kinase I: spatial organization and positioning of a multifunctional protein kinase family. Cell. Sig. 9, 57–67
9. Flotow, H., and Roach, P. J. (1989) Synergistic phosphorylation of rabbit muscle glycogen synthase by cyclic AMP-dependent protein kinase and casein kinase I: implications for hormonal regulation of glycogen synthase. J. Biol. Chem. 264, 9126–9128
10. Flotow, H., Graves, P. R., Wang, A. Q., Fiol, C. L., Roese, R. W., and Roach, P. J. (1990) Phosphate groups as substrate determinants for casein kinase I action. J. Biol. Chem. 265, 14264–14269
11. Zhao, B., Li, L., Tumaneng, K., Wang, C.-Y., and Guan, K.-L. (2000) A coordinated phosphorylation by Lats and CK1 regulates YAP stability and its role in carcinogenesis. EMBO J. 19, 398–405
12. Takahashi, J. S. (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc. Natl. Acad. Sci. U.S.A. 101, 5339–5346
13. Dean, A. M., and Thornton, J. W. (2007) Mechanistic approaches to the study of evolution: the functional synthesis. Nat. Rev. Genet. 8, 675–688
14. Yokoyama, S., Tada, T., Zhang, H., and Britt, L. (2008) Elucidation of phenotypic adaptations: molecular analyses of dim-light vision proteins in vertebrates. Proc. Natl. Acad. Sci. U.S.A. 105, 13480–13485
15. Yu, W., Zheng, H., Price, J. L., and Hardin, P. E. (2009) DOUBLETIME plays a noncatalytic role to mediate CLOCK phosphorylation and represses CLOCK-dependent transcription within the Drosophila circadian clock. Mol. Cell Biol. 29, 1452–1458
16. Peuss, F., Fan, J.-Y., Kalive, M., Bao, S., Schuenemann, E., Bjes, E. S., and Price, J. L. (2004) Drosophila doubletime mutations which either shorten or lengthen the period of circadian rhythms decrease the protein kinase activity of casein kinase I. Mol. Cell Biol. 24, 886–898
17. Liu, Z., Huang, M., Wu, X., Shi, G., Xing, L., Dong, Z., Qu, Z., Yan, J., Yang, L., Panda, S., and Xu, Y. (2014) PER1 phosphorylation specifies feeding rhythm in mice. Cell Rep. 7, 1509–1520
18. Chiu, J. C., Ko, H. W., and Edery, I. (2011) NEMO/NLK phosphorylates core period determination by slowing the pace of the Drosophila circadian oscillator. Mol. Cell Biol. 31, 2288–2297
19. Chen, J. C., Ko, H. W., and Edery, I. (2011) NEMO/NLK phosphorylates PERIOD to initiate a time-delay phosphorylation circuit that sets circadian clock speed. J. Biol. Rhythms 23, 511–521
20. Beltrán, P., Trinidad, J. C., Fiedler, D., Roguev, A., Lim, W. A., Shokat, K. M., Burlingame, A. L., and Krokan, N. J. (2009) Evolution of phosphorylation: comparison of phosphorylation patterns across yeast species. PLoS Biol. 7, e1000134
21. Tan, C. S., Pasculescu, A., Lim, W. A., Pawson, T., Bader, G. D., and Linding, R. (2009) Positive selection of tyrosine loss in metazoan evolution. Science 325, 1686–1688
22. Vanselow, K., Vanselow, J. T., Westermak, P. O., Reischl, S., Maier, B., Korte, T., Herrmann, A., Herzel, H., Schlosser, A., and Kramer, A. (2006)

170 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 292 • NUMBER 1 • JANUARY 6, 2017

ASMB
Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS). *Genes Dev.* **20**, 2660–2672

40. Roach, P. J. (1991) Multisite and hierarchical protein phosphorylation. *J. Biol. Chem.* **266**, 14139–14142

41. Chiu, J. C., Vanselow, J. T., Kramer, A., and Edery, I. (2008) The phospho-occupancy of an atypical SLIMB-binding site on PERIOD that is phosphorylated by DOUBLETIME controls the pace of the clock. *Genes Dev.* **22**, 1758–1772

42. Eide, E. J., Woolf, M. F., Kang, H., Woolf, P., Hurst, W., Camacho, F., Vielhaber, E. L., Giovanni, A., and Virshup, D. M. (2005) Control of mammalian circadian rhythm by CKI\(^\text{\textregistered}/\text{H9254}\)-regulated proteasome-mediated PER2 degradation. *Mol. Cell. Biol.* **25**, 2795–2807

43. Schlosser, A., Vanselow, J. T., and Kramer, A. (2005) Mapping of phosphorylation sites by a multi-protease approach with specific phosphopeptide enrichment and NanoLC-MS/MS analysis. *Anal. Chem.* **77**, 5243–5250

44. Emery, P. (2007) Protein extraction from *Drosophila* heads. *Methods Mol. Biol.* **362**, 375–377

45. Emery, P., So, W. V., Kaneko, M., Hall, J. C., and Rosbash, M. (1998) CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* **95**, 669–679

46. Pfeiffenberger, C., Lear, B. C., Keegan, K. P., and Allada, R. (2010) Processing circadian data collected from the *Drosophila* Activity Monitoring (DAM) System. *Cold Spring Harb. Protoc.* 2010, 10.1101/pdb.prot5519

**CK1δ Expands Function of Substrates with Serine-rich Motifs**