Title

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Journal

Journal of Neuroscience, 38(50)

ISSN

0270-6474

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Publication Date

2018-12-12

DOI

10.1523/jneurosci.0871-18.2018

Peer reviewed

Cellular/Molecular

CK1α Collaborates with DOUBLETIME to Regulate PERIOD Function in the *Drosophila* Circadian Clock

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The animal circadian timing system interprets environmental time cues and internal metabolic status to orchestrate circadian rhythms of physiology, allowing animals to perform necessary tasks in a time-of-day-dependent manner. Normal progression of circadian rhythms is dependent on the daily cycling of core transcriptional factors that make up cell-autonomous molecular oscillators. In *Drosophila*, PERIOD (PER), TIMELESS (TIM), CLOCK (CLK), and CYCLE (CYC) are core clock proteins that function in a transcriptional–translational feedback mechanism to regulate the circadian transcriptome. Posttranslational modifications of core clock proteins provide precise temporal control over when they are active as regulators of clock-controlled genes. In particular, phosphorylation is a key regulatory mechanism that dictates the subcellular localization, stability, and transcriptional activity of clock proteins. Previously, casein kinase 1α (CK1α) has been identified as a kinase that phosphorylates mammalian PER1 and modulates its stability, but the mechanisms by which it modulates PER protein stability is still unclear. Using *Drosophila* as a model, we show that CK1α has an overall function of speeding up PER metabolism and is required to maintain the 24 h period of circadian rhythms. Our results indicate that CK1α collaborates with the key clock kinase DOUBLETIME (DBT) in both the cytoplasm and the nucleus to regulate the timing of PER-dependent repression of the circadian transcriptome. Specifically, we observe that CK1α promotes PER nuclear localization by antagonizing the activity of DBT to inhibit PER nuclear translocation. Furthermore, CK1α enhances DBT-dependent PER phosphorylation and degradation once PER moves into the nucleus.

Key words: casein kinase 1; circadian clock; CK1alpha; *Drosophila*; phosphorylation

Significance Statement

Circadian clocks are endogenous timers that integrate environmental signals to impose temporal control over organismal physiology over the 24 h day/night cycle. To maintain the 24 h period length of circadian clocks and to ensure that circadian rhythms are in synchrony with the external environment, key proteins that make up the molecular oscillator are extensively regulated by phosphorylation to ensure that they perform proper time-of-day-specific functions. Casein kinase 1α (CK1α) has previously been identified as a kinase that phosphorylates mammalian PERIOD (PER) proteins to promote their degradation, but the mechanism by which it modulates PER stability is unclear. In this study, we characterize the mechanisms by which CK1α interacts with DOUBLETIME (DBT) to achieve the overall function of speeding up PER metabolism and to ensure proper time-keeping.

Introduction

Circadian clocks regulate molecular oscillations that manifest into physiological and behavioral rhythms in all kingdoms of life, from bacteria to mammals (Zhang and Kay, 2010; Hardin and Panda, 2013; Andreani et al., 2015). Although circadian clock genes are not highly conserved across kingdoms, the design principle of circadian oscillators in all organisms studied to date appears to be variations on the same theme. Transcriptional regulators, the daily mRNA and protein levels of which are tightly controlled, serve as key components of the molecular oscillator and operate through transcriptional–translational feedback loops to

Received April 4, 2018; revised Oct. 5, 2018; accepted Oct. 21, 2018.

Author contributions: V.H.L. wrote the first draft of the paper; V.H.L., Y.H.L., and J.C.C. edited the paper; V.H.L., Y.H.L., and J.C.C. designed research; V.H.L., Y.H.L., X.L., K.A.M., J.S.D., and J.C.C. performed research; R.S.K. and J.C.C. contributed unpublished reagents/analytic tools; V.H.L., Y.H.L., K.A.M., and J.C.C. analyzed data; V.H.L. wrote the paper.

This work was supported by the National Institutes of Health (Grant R01GM10225 to J.C.C.) and the National Science Foundation (Grant IOS1456297 to J.C.C.). We thank Dr. Michael Paddy (UC Davis) and Dr. Yong Zhang (University of Nevada, Reno) for technical advice in immunofluorescence and confocal imaging; Paul Hardin for providing the *pdf*-Gal4 fly line; and the Bloomington *Drosophila* stock center and VDRC for supplying *ck1α* RNA*i* responder and overexpressor fly lines.

The authors declare no competing financial interests.

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https://doi.org/10.1523/JNEUROSCI.0871-18.2018

 0270-6474/18/3810631-13$15.00/0

control cyclical expression of the circadian transcriptome. In *Drosophila melanogaster*, these core oscillator proteins are PERIOD (PER), TIMELESS (TIM), CLOCK (CLK), and CYCLE (CYC) (Allada and Chung, 2010; Dubowy and Sehgal, 2017). Starting in late day, CLK and CYC heterodimerize to activate the transcription of *per*, *tim*, as well as other clock-controlled genes (McDonald and Rosbash, 2001; Yu et al., 2006; Liu et al., 2017). Although *per* and *tim* mRNAs accumulate, PER and TIM protein levels remain low as PER requires TIM binding for stabilization, but TIM is degraded in the presence of light (Hunter-Ensor et al., 1996; Peschel et al., 2009; Hara et al., 2011; Jang et al., 2015). During early night, however, TIM and PER accumulate and enter the nucleus, where they repress the activity of the CLK-CYC heterodimer. In the early morning, proteasome-dependent PER degradation relieves this repression to initiate another round of CLK-CYC-mediated transcription (Grima et al., 2002; Ko et al., 2002).

The abundance, subcellular localization, and transcriptional activity of core clock proteins are critical for normal progression of circadian rhythms. To precisely control their phase-specific functions and to allow for environmental and metabolic input, core clock proteins are heavily regulated by posttranslational modifications (Hirano et al., 2016). Most notably, the progressive phosphorylation of PER over the circadian cycle has been shown to be closely linked to the speed of the circadian oscillator and the 24 h period of circadian rhythms. A number of kinases have been shown to modulate the phosphorylation status of PER, including DOUBLETIME (DBT/CK1δ/ε) (Kloss et al., 1998, 2001; Price et al., 1998), casein kinase 2 (CK2) (Smith et al., 2008; Szabó et al., 2013; Top et al., 2016), SHAGGY (SGG/GSK3β) (Martinek et al., 2001; Ko et al., 2010), and NEMO-like kinase (NLK) (Chiu et al., 2011; Yu et al., 2011). Specifically, DBT first phosphorylates PER in the cytoplasm during the day to prevent premature nuclear entry (Cyran et al., 2005; Muskus et al., 2007). Upon PER nuclear entry in the middle of the night, additional DBT-dependent phosphorylation events potentiate the transcriptional repressor activity of PER while progressively enhancing its proteasome-dependent degradation, which peaks at dawn (Nawathean and Rosbash, 2004; Kivimäe et al., 2008; Top et al., 2018). Whereas DBT-dependent PER phosphorylation at the N terminus promotes binding to the F-box protein SLIMB (β-TrCP), resulting in proteasome-dependent PER degradation (Chiu et al., 2008), phosphorylation of the per^S region by DBT creates a phospho-delay mechanism that delays N-terminal phosphorylation (Chiu et al., 2011). The phospho-delay circuit is initiated by NLK-dependent phosphorylation of PER(S596) and has the overall function of extending the PER phosphorylation cycle to 24 h. Finally, CK2 and SGG have both been shown to promote PER nuclear translocation (Lin et al., 2002, 2005; Akten et al., 2003; Ko et al., 2010).

CK1α is a member of the CK1 family along with DBT. It has been identified as one of the targets of Longdaysin, a small-molecule inhibitor that lengthens the period of zebrafish and human U2OS cells (Hirota et al., 2010). Biochemically, CK1α phosphorylates mammalian PER1 and accelerates its degradation in HEK293T cells (Hirota et al., 2010). Although the role of CK1α as a clock kinase has been established, the mechanism by which it modulates PER protein stability is still unclear. Here, we confirmed the role of CK1α in regulating the *Drosophila* clock and characterized the mechanisms by which CK1α collaborates with DBT to regulate PER function and the circadian oscillator.

Materials and Methods

Transgenic flies and locomotor activity assays. Targeted expression of *ck1α RNAi* in *tim*-expressing and *pdf*-expressing clock neurons was achieved using the UAS/Gal4 system (Brand and Perrimon, 1993). The Gal4 driver lines used here were *w;* UAS-*dicer2; tim*-UAS-Gal4 (*TUG*) (Blau and Young, 1999) and *w;*UAS-*dicer2; pdf*-Gal4 (Park et al., 2000). Four independent responder RNA*i* lines, two from the Bloomington *Drosophila* Stock Center (BDSC stock nos. 25786 and 41711) and two from the Vienna *Drosophila* Resource Center (VDRC stock nos. 110768 and 13664), were evaluated. The regions targeted by the respective responder *ck1α RNAi* lines are as follows: UAS-*ck1α RNAi*^{25786} targets *ck1α* nucleotide numbers 145–590, *UAS-ck1α RNAi*^{110768} targets *ck1α* nucleotide numbers 299–590, UAS-*ck1α* RNA*i*^{13664} targets *ck1α* nucleotide numbers 317–670, and UAS-*ck1α* RNA*i*^{41711} targets *ck1α* nucleotide numbers 1101–1121. Nucleotide numbering corresponds to Flybase transcript variant RF of *ck1α*. Regions targeted by all responder lines are present in all transcript variants of *ck1α*. Male flies from the driver lines were crossed to female flies of the UAS-RNA*i* responder lines. Male progenies of the crosses were then assayed for locomotor activity using the *Drosophila* Activity Monitoring System (DAMS, Trikinetics) as described previously (Chiu et al., 2010). Control flies for these experiments included all parental lines for the crosses and progenies of the cross between *w;* UAS-*dicer2; tim*-UAS-Gal4 (*TUG*) and the genetic background control w^{1118} in the case of *ck1α RNAi* knock-down experiments. Flies were entrained for 4 d in light/dark (LD) cycles (12 h light/12 h dark), followed by 7 d of total darkness (DD) to access their free-running rhythms. Experiments were performed at 25°C in a Percival incubator. To ensure that any changes in oscillator function were not the result of impaired neuronal development, we used *tub-Gal80ts* to repress the activity of *tim*-UAS-G*al4* in driving the RNA*i* construct until adulthood as described previously in McGuire et al. (2003) (BDSC stock no. 7108). Flies were reared at 18°C after genetic crosses. Three days before and also during behavioral assays, the flies were shifted from 18°C to 29°C, thus inactivating *tub-Gal80ts* and allowing the expression of the RNA*i* construct in clock neurons.

Drosophila S2 cell culture and transfection to characterize CK1α function. *Drosophila* S2 cells and DES expression medium were obtained from Life Technologies. For all cell culture experiments except immunofluorescence staining, S2 cells were seeded at 3×10^6 cells/ml. For immunofluorescence staining, S2 cells were seeded at 2×10^5 cells/ml. Transient transfection was performed using Effectene (Qiagen) according to the manufacturer's protocol. The following amounts of plasmids were used for transfection. For coimmunoprecipitation (co-IP) assays: S2 cells were cotransfected with 0.8 μg of pAc-*per*-V5-His and 0.8 μg pMT-*ck1α*-6xc-myc for detection of protein–protein interactions or transfected singly with 0.8 μg of pAc-*per*-V5-His or 0.8 μg of pMT-*ck1α*-6xc-myc in control IPs to detect nonspecific binding. For ck1α coexpression, lambda phosphatase (λ-PP), and cycloheximide (CHX) chase assays: 0.8 μg of pAc-*per*-V5-His was transfected with 0.6 μg of pMT-*ck1α-3xFLAG-6xHis* (herein referred to as pMT-ck1α-FH), 0.6 μg of *pMT-ck1α(K49R)*-FH, or an empty vector pMT-FH. For GST pull-down assay: 0.8 μg of pAc-*per(NLS)*-V5-His was transfected with 0.6 μg of pMT-*ck1α-FH*, *pMT-ck1α(K49R)*-FH, or an empty vector. pAc-*per(NLS)*-V5-His is a tissue culture expression construct in which the nuclear localization sequence (NLS) sequence of simian virus 40 large-T antigen is appended to the C terminus of the *per* coding sequence to promote nuclear localization of PER. This construct was used previously to characterize the DBT kinase binding domain on PER (Kim et al., 2007; Nawathean et al., 2007). For mobility shift assay: 0.8 μg of pAc-*per*-V5-His was transfected with either 0.6 μg of pMT-FH, 0.6 μg of pMT-*ck1α-FH*, *0.2 μg of pMT-dbt*-FH, or both 0.6 μg of pMT-*ck1α-FH* and 0.2 μg of *pMT-dbt*-FH. For immunofluorescence staining: 1.5 μg of pAc-*per*-V5-His or pAc-*per(NLS)*-V5-His was used for transfection. Expression of kinases was induced with 500 μM $CuSO_4$.

CK1α mobility shift assay. Twenty-four hours following transient transfection of *Drosophila* S2 cells, expression of kinases was induced for 16 h. For mobility shift assay, cells were harvested and lysed with EB2 (20 mM HEPES pH 7.5, 100 mM KCl, 5% glycerol, 5 mM EDTA, 1 mM DTT,

0.5 mM PMSF, 0.1% Triton X-100, 25 mM NaF, 0.01 mg/ml Aprotinin, 0.005 mg/ml Leupeptin, 0.001 mg/ml pepstatin A). Proteins were quantified using Coomassie Plus Reagent (Thermo Fisher Scientific) in combination with Biophotometer (Eppendorf) and were analyzed by Western blotting (see "Western blotting and antibodies" section).

Co-IP. Expression of *ck1α* was induced immediately following transfection (see "*Drosophila* S2 cell culture and transfection to characterize CK1α function" section for amounts of plasmids used). Following 40 h of induction, cells were harvested, washed once with 1× PBS, and lysed with modified RIPA (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate) supplemented with 1 mM EDTA, 25 mM NaF, 0.5 mM PMSF, and complete EDTA-free protease inhibitor mixture (Roche). Each IP sample was performed using 15 μl of settled beads for 4 h at 4°C. Beads were washed three times in modified RIPA with 300 mM NaCl. Reciprocal IP was performed. Immune complexes were analyzed by Western blotting (see "Western blotting and antibodies" section).

λ-PP treatment, time course mobility assay, and Phos-Tag SDS-PAGE. Expression of kinases was induced at 36 h following transfection (see "*Drosophila* S2 cell culture and transfection to characterize CK1α function" section for plasmid amount). For time course mobility assay, cells were harvested at indicated time points post kinase induction and lysed with EB2 (20 mM HEPES pH 7.5, 100 mM KCl, 5% glycerol, 5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.1% Triton X-100, 25 mM NaF, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.001 mg/ml pepstatin A). For λ-PP, after 24 h of inductions, cells were harvested and lysed with EB2 supplemented with PhosStop (Roche). Protein lysates were then subjected to IP to α-V5 beads (15 μl of settled beads per IP) for 4 h at 4°C. Beads were then washed twice in EB2 without any NaF or PhosStop (Roche) and once in λ-PP buffer (New England Biolabs) before being resuspended in 40 μl of λ-PP buffer. Control reactions were mock treated at the same temperature. Immunocomplexes were analyzed by Western blotting (see "Western blotting and antibodies" section). For Phos-Tag SDS-PAGE, after 24 h of kinase induction, CHX and MG132 were added to a final concentration of 10 μg/ml and 25 μg/ml, respectively, and incubated for 4 h. Cells were then harvested and lysed with EB2 without EDTA. Protein extracts were quantified to ensure that equal amounts were used for the analysis. Proteins were resolved using 5% SDS-PAGE supplemented with 10 μM Phos-Tag (Wako). Once resolved, gels were incubated for 10 min with gentle agitation first in transfer buffer containing 1 mM EDTA, followed by transfer buffer without EDTA. Proteins were then transferred onto PVDF membranes and visualized as described in the "Western blotting and antibodies" section.

CHX chase experiment. Twenty-four hours following transfection, *ck1α* expression was induced for 16 h. Following kinase induction, CHX was added to a final concentration of 10 μg/ml. Cells were harvested and lysed with EB2 (see section on "CK1α mobility shift assay") at the indicated times. Proteins were analyzed by Western blotting using the following antibodies: α-V5 (Life Technologies) at 1:5000 for detection of PER with α-mouse IgG-HRP secondary antibody (GE Healthcare) used at 1:2000; α-HSP70 (Sigma-Aldrich) at 1:3000 for detection of HSP 70 with α-mouse IgG-HRP (GE Healthcare) at 1:3000.

GST pull-down assays in Drosophila *S2 cells and in flies*. Pull-down assays were performed as described previously (Chiu et al., 2008). For pull-down assays in flies, prey proteins were extracted from 250 μl of fly heads per reaction.

Analysis of PER protein cycling in flies. Flies were entrained in light/dark (LD) cycles (12 h light/12 h dark) at 25°C for 3 d and collected at 6 time points over a circadian day. Fly head extracts for Western blotting were homogenized using motorized pestle using RBS (20 mM HEPES pH 7.5, 50 mM KCl, 10% glycerol, 2 mM EDTA, 1% Triton X-100, 0.4% NP-40, 1 mM DTT, 0.5 mM PMSF, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, and 0.001 mg/ml pepstatin A). Extracts were sonicated for 5 × 5 s with 10 s pauses. Proteins were resolved using 5% Criterion Tris-HCl SDS-PAGE gels (Bio-Rad) and loading was assessed using α-HSP70 (Sigma-Aldrich). To classify and quantify hypophosphorylated versus hyperphosphorylated PER isoforms by Western blotting, we used PER isoforms present at ZT8-16 in *TUG* control flies as reference because PER has been shown to be hypophosphorylated at those circadian time points (Edery et al., 1994). PER signals above the hypophosphorylated PER isoforms present at ZT20, ZT24, and ZT4 were therefore designated and quantified as hyperphosphorylated PER isoforms.

Western blotting and antibodies. Blocking reagent (5%; Bio-Rad) in 1× TBST was used for incubation with all antibodies except in the case for α-pS47, in which 2.5% blocking solution was used. Primary antibodies for Western blotting analysis were used at the following dilution: mouse α-V5 (Life Technologies) at 1:5000, mouse α-c-myc (Sigma-Aldrich) at 1:7000, mouse α-HSP70 (Sigma-Aldrich) at 1:7000, rat α-HA 3F10 (Roche) at 1:1000, α-PER (GP5620; RRID:AB_2747405) (for detection of PER in fly extracts) at 1:3000, rabbit α-pS47 (Chiu et al., 2008; RRID: AB_2747407) at 1:1000. Secondary antibodies were used as follow: α-mouse-IgG-HRP (GE Healthcare) at 1:2000, α-rat-IgG-HRP (GE Healthcare) at 1:1000, α-rabbit-IgG-HRP (GE Healthcare) at 1:2000, α-guinea pig-IgG-HRP (Sigma-Aldrich) at 1:2000.

Real-time PCR to analyze circadian gene expression and level of ck1α knock-down. Flies expressing dsRNA targeting *ck1α* and control flies (parental driver line *w*; UAS-*dicer2; tim*-UAS-Gal4; Blau and Young, 1999) were entrained for 3 d in LD cycles and collected every 4 h on the fourth day. RNA extraction was performed as described previously (Chiu et al., 2008). cDNA was generated using Thermoscript RT (Life Technologies) and real-time PCR was performed using SsoAdvanced SYBR green supermix (Bio-Rad) in a CFX96 (Bio-Rad). Primers were designed to detect these circadian transcripts (for *per:* 5′-GACCGAATCCCTGCTCAA-3′ and 5′-GTGTCATTGGCGGACTTC-3′; for *tim*: 5′-CCCTTATAC CCGAGGTGGAT-3′ and 5′-TGATCGAGTTGCAGTGCTTC-3′; for *pdp1ε*: 5′-GCGGCAACTGGTAATG-3′ and 5′-ATTTCCTGCCTGAG CT-3′ (Cyran et al., 2005); for *vrille*: 5′-ATGAACAACGTCCGGCTA TC-3′ and 5′-CTGCGGACTTATGGATCCTC-3′; for *ck1α: 5′-ATTC TGAGCGGCGGCGTTGG-3′ and 5′-ATGGATGAAGCACTTGAGAT GG-3′; for dbt*: 5′-AGCAGCACAAGGTCAATGC-3′ and 5′-TCGAGT CGTCCATGTTGAGT-3′) and *cbp20* transcripts (5′-GTCTGATTCG TGTGGACTGG-3′ and 5′-CAACAGTTTGCCATAACCCC-3′) were used for normalization. Cycling conditions were 95°C for 30 s, 40 cycles of 95°C for 5 s, followed by an annealing/extension phase for 30 s at the primer optimal annealing temperature. The reaction was concluded with a melt curve analysis going from 65°C to 95°C in 0.5°C increments at 5 s per step. Three technical replicates were performed for each biological replicate. Three biological replicates were performed for analysis of each gene. Data were analyzed using the standard ΔΔCt method and target gene mRNA expression levels were normalized to the reference gene mRNA levels (*cbp20*), which remain unchanged over a circadian day. Finally, Ct values for all time points were divided by the highest Ct value to generate a scale from 0 to 1.

ChIP. ChIP was performed as described previously (Kwok et al., 2015).

IP to detect phosphorylated PER(S47) in flies. IP was performed essentially as described in Chiu et al. (2008) with the following modifications. Approximately 300 μl of fly heads were homogenized using liquid-nitrogen-chilled ceramic mortar and pestle, mixed with 1.5 ml of modified RIPA buffer (see "Co-IP" section for formula) supplemented with complete EDTA-free protease inhibitor mixture (Sigma-Aldrich) and PhosStop (Roche), and dounced with a glass homogenizer (Kimble Chase) for 15 strokes using the tight "B" pestle. Homogenate was then incubated with gentle rotation for 20 min at 4°C. Protein extracts were quantified using a biophotometer (Eppendorf) to ensure that an equal amount of protein extracts was used for each IP. Extracts were incubated with α-PER (GP5620) at 4°C for 4 h, followed by a 2 h incubation with gamma-bind Sepharose beads (GE Healthcare), IP complexes were then washed once with modified RIPA buffer, eluted with 2× SDS sample buffer, and resolved using a 5% Criterion Gel (Bio-Rad).

Immunofluorescence staining of Drosophila *S2 cells or clocks neurons*. *Drosophila* S2 cells were seeded onto glass coverslips coated with 1 mg/ml of the lectin concanavalin A (Con A) and allowed to adhere to coverslips for 4 h before transfection. Twenty-four hours following transfection, cells were fixed with 4% paraformaldehyde for 35 min. Fixed cells were then washed with washing solution (0.2% Triton X-100 in PBS) 6 times (3 quick washes followed by 3 washes at 10 min each) and incubated with blocking solution (5% goat serum and 0.2% Triton X-100 in PBS) at 4°C for 40 min. Mouse α-V5 (Life Technologies) was added at 1:1000 and incubated overnight at 4°C. Cells were washed with washing solution for

6 times at 20 min each and incubated overnight with α-mouse IgG Alexa Fluor 594 (Life Technologies) at 1:1000. Cells were then washed with washing solution 6 times at 20 min each, rinsed with PBS, and mounted onto slides using ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Images were collected with an EVOS FL Imaging System at 20× magnification.

Brain dissections and immunofluorescence staining procedures were as described in Fu et al. (2016). α-PDF (C7-C; DHSB) and α-PER (Rb s3968–1; RRID:AB_2747406) were added at 1:1000 and 1:500, respectively, and incubated for 16 h at 4°C. α-mouse IgG Alexa Fluor 647 (Cell Signaling Technology) and α-rabbit IgG Alexa Fluor (Life Technologies) were added at 1:1000 and 1:500, respectively. Images were collected with an inverted Zeiss Observer LSN710 confocal microscope using 40× oil-based objective and analyzed with Fiji software. Cytoplasmic and nuclear regions were manually selected using the cytoplasmic PDF marker and a nearby area was selected as background. For each neuron, the intensity of PER staining within cytoplasmic and nuclear regions was calculated and average background intensity from the PER channel was subtracted from each. Nuclear PER intensity or cytoplasmic PER intensity was divided by total PER intensity to calculate the fraction of PER in the nucleus or cytoplasm, respectively, at each time point. The nuclear/total value from each neuron was averaged and SEM was calculated for each group. The cytoplasmic/total value was computed similarly. Fifteen large ventral lateral clock neurons from 10 brains were analyzed for each group.

Statistical analysis. All numerical data described were analyzed using Excel 2016 (Microsoft). Statistical significances were determined using unpaired Student's *t* test.

Results

CK1α regulates clock-controlled locomotor activity rhythm in *Drosophila*

To characterize the role of CK1α in regulating the *Drosophila* circadian clock, we first analyzed the locomotor activity rhythms of transgenic flies expressing dsRNA against *ck1α* under the control of *tim*-UAS-Gal4 driver (*TUG*) (Blau and Young, 1999) to restrict dsRNA expression to *tim*-expressing cells and clock neurons (*TUG*>UAS-*ck1α RNAi*). Following entrainment in LD conditions at 25°C, the free-running circadian periods of parental controls and *TUG*>UAS-*ck1α RNAi* (*ck1α RNAi*) flies were assayed in DD conditions. Two independent responder *ck1α RNAi* lines from the BDSC (25786 and 41711) and two from the VDRC (110768 and 13664) were evaluated (Table 1). Compared with the parental controls, progenies expressing *ck1α* dsRNA displayed a lengthening of the endogenous period in locomotor activity rhythm by ~1.5 to 2 h (Fig. 1*A*, Table 1). The only exception is the genotype TUG>UAS-*ck1α* RNAi^{41711}, which exhibited arrhythmic locomotor activity (Table 1). However, because we observed low survival rates for these flies, we suspect that nontarget effect to overall health of the flies might have contributed to the observed arrhythmicity. We therefore opted to use *TUG*>UAS-*ck1α RNAi*^{25786} for subsequent functional characterization as described below.

We first validated that the period-lengthening effect observed in *TUG*>UAS-*ck1α RNAi*^{25786} resulted from disrupted oscillators in clock neurons by restricting dsRNA expression to only the *pdf*-positive neurons (*w;* UAS-*Dicer2; pdf*-Gal4>UAS-*ck1α RNAi*^{25786}), the principle pacemaker cells necessary for sustaining the endogenous rhythm in constant conditions (Helfrich-Förster, 1998). Restricting *ck1α dsRNA* expression to *pdf*-positive cells also lengthened the endogenous period (Table 1). The locomotor activity rhythmicities of these flies were also greatly reduced. To exclude the possibility that loss of *ck1α* function lengthened the endogenous period by altering clock cell development because CK1α is a known component of the *Wnt* and *Hh* signaling pathways (Katoh and Katoh, 2007; Chen et al., 2011), we postponed the initiation of *ck1α* dsRNA expression until animals reached adulthood using the thermogenetic Gal80 system (McGuire et al., 2003). Flies in which ck1α dsRNA was only expressed in adult *tim*-expressing cells also displayed a period-lengthening of ~2 h (Table 1). Finally, overexpression of *ck1α in tim*-expressing cells did not result in period shortening, but substantially reduced the percentage of rhythmic flies (from 52–53% to 17%), suggesting that a high amount of CK1α may also disrupt the circadian oscillator (Table 1). Together, these results not only support CK1α as a regulator of the *Drosophila* clock, but also suggested that CK1α activity normally functions to accelerate the pace of the endogenous clock in wild-type flies to maintain 24 h circadian rhythms.

Because *ck1α* shows sequence similarity to *dbt* (homolog of mammalian *ck1δ/ε*) and some mutations in *dbt*, for example, *dbt*^{L}, exhibit period lengthening in locomotor activity rhythms (Price et al., 1998; Suri et al., 2000), it is important to rule out nontarget effects of *ck1α RNAi* on *dbt*. To confirm the specificity of *ck1α RNAi* knock-down in *TUG*>UAS-*ck1α RNAi*^{25786} flies before using them for further analysis, we assayed both *ck1α and dbt* transcripts using qPCR. We determined that, whereas *ck1α* mRNA level was reduced by 40–75% in TUG>UAS-ck1α RNAi^{25786} flies compared with *TUG* parental control flies (Fig. 1*B*; ZT4: $t_{(2)} = 2.81$, $p = 0.106$; ZT8: $t_{(2)} = 6.76$, $p = 0.021$; ZT12: $t_{(2)} = 8.69$, $p = 0.013$; ZT16: $t_{(2)} = 7.56$, $p = 0.017$; ZT20: $t_{(2)} =$

Table 1. Daily locomotor activity rhythms of flies with altered ck1α expression level

Genotype[a]	Period (h) (mean ± SEM)	Power[b]	Rhythmicity (%)[c]	No. of flies tested	No. of flies surviving[d]
RNAi, *tim* driver					
w^{1118}	23.4 ± 0.06	71.1	90.3	32	31
w; UAS-*dicer2; tim*(UAS)-*Gal4(=TUG)*	23.6 ± 0.06	75	82.1	32	28
UAS-*ck1α* RNAi^{25786}	23.9 ± 0.10	54.4	60.0	32	25
UAS-*ck1α* RNAi^{110768}	22.9 ± 0.11	44.4	43.8	32	32
UAS-*ck1α* RNAi^{13664}	23.5 ± 0.04	124.6	96.9	32	32
UAS-*ck1α* RNAi^{41711}	23.7 ± 0.06	107	96.6	32	29
TUG>*w*^{1118}	23.7 ± 0.05	108.7	96.9	32	31
TUG>UAS-*ck1α* RNAi^{25786}	25.8 ± 0.19	65	66.7	32	30
TUG>UAS-*ck1α* RNAi^{110768}	24.3 ± 0.12	90.5	100	18	18
TUG>UAS-*ck1α* RNAi^{13664}	24.9 ± 0.29	88.4	96.9	32	32
TUG>UAS-*ck1α* RNAi^{41711}	AR	ND	0	32	4
RNAi, *pdf* driver					
w; UAS-dicer2; pdf-Gal4 (=pdf)	24.0 ± 0.07	83.3	89.7	32	29
UAS-*ck1α* RNAi^{25786}	23.9 ± 0.16	35	31.3	32	32
pdf>UAS-*ck1α* RNAi^{25786}	26.4 ± 0.61	47.7	19.4	32	31
RNAi, *tim* driver in adult flies					
TUG	23.4 ± 0.06	59.1	86.4	48	44
w; tub-Gal80(ts); UAS-*ck1α*RNAi^{25786}	24.0 ± 0.06	87.7	81.5	32	27
TUG>*w; tub-Gal80(ts);* UAS-*ck1α*RNAi^{25786}	25.9 ± 0.35	57.2	37.5	32	16
Overexpression, *tim* driver					
TUG(II)	24.3 ± 0.10	58.6	53.3	32	30
UAS-ck1α^{55067}	23.7 ± 0.09	37.1	51.6	32	31
TUG(II)>*UAS-ck1α*^{55067}	23.9 ± 0.29	34.8	16.7	32	30

[a]*ck1α* RNAi and overexpressor transgenic lines were identified using the stock numbers from BDSC (25786, 41711) or VDRC (13664, 110768).

[b]Measures the strength or amplitude of the locomotor activity rhythm (in arbitrary units).

[c]Percentage of flies that are rhythmic.

[d]Number of flies that survived until the end of the experiment.

AR, Arrhythmic; ND, not determined.

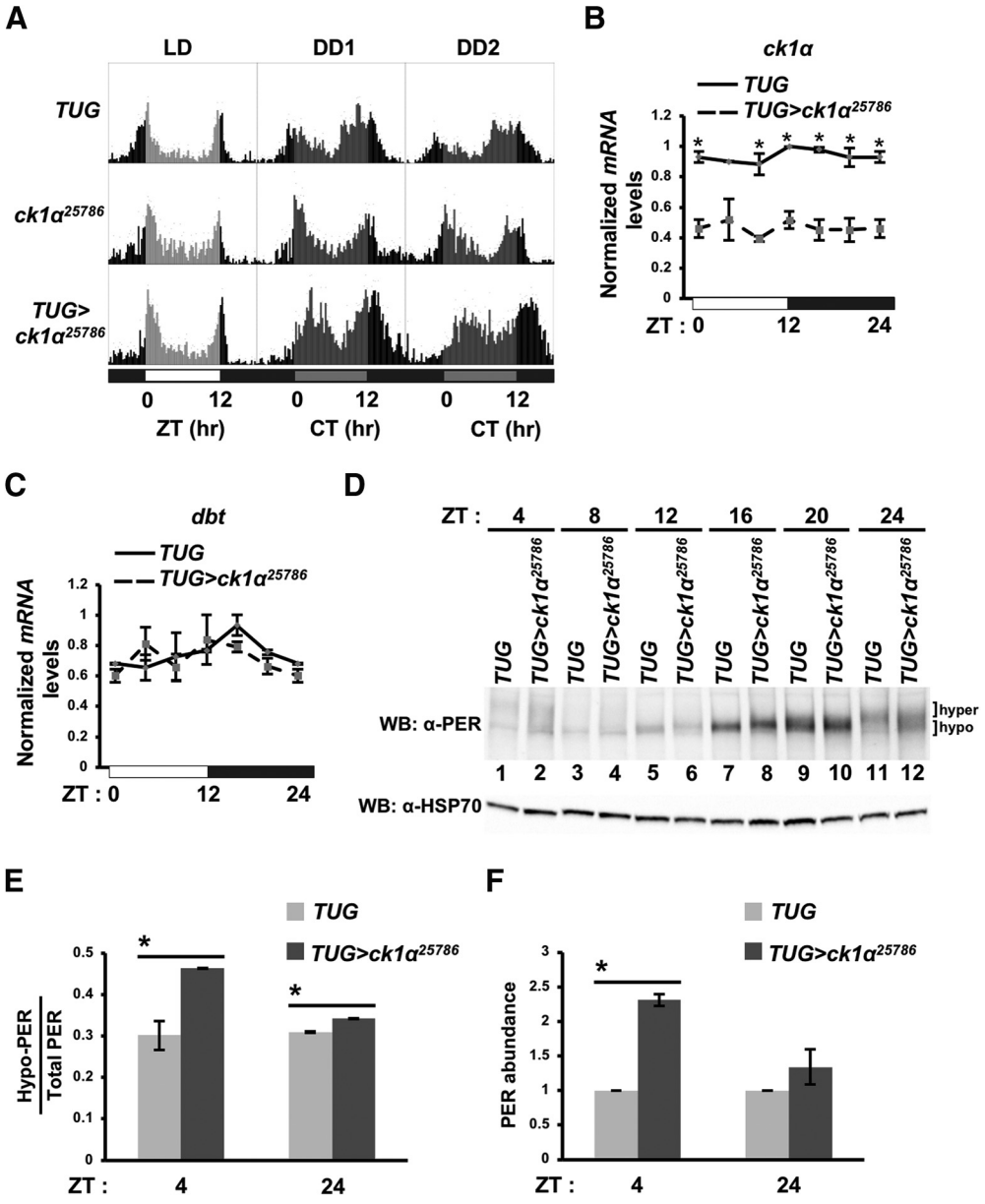

Figure 1. Locomotor activity rhythms and temporal profile of PER phosphorylation are altered in *ck1α RNAi* flies. ***A***, Flies were entrained for 4 d in LD followed by 7 d in DD (complete darkness). Shown are eduction graphs for LD3, DD1, and DD2 for *TUG>ck1α RNAi*^{25786} and parental controls. ***B***, ***C***, Steady-state mRNA levels of *ck1α* (***B***) or *dbt* (***C***) of *TUG* control (solid) and *TUG>ck1α RNAi*^{25786} (dashed) flies. mRNA level at each time point is normalized to be relative to the maximum value over the time course, which is set to 1. White and black horizontal bars below the *x*-axis represent periods of lights on and off, respectively. Error bars indicate SEM from two biological replicates. ZT24 data point was plotted twice as ZT0 and 24 to facilitate viewing of daily cycling. *$p <$ 0.05. ***D***, Flies were collected at the indicated times (ZT) on the third day of LD entrainment. Head extracts were analyzed by immunoblotting in the presence of α-PER (GP5620) (top). Equal loading was verified using α-HSP70 (bottom). Hyper and hypo, Hyperphosphorylated and hypophosphorylated PER isoforms, respectively. ***E***, Quantification of hypophosphorylated PER shown in ***D*** using ImageLab (Bio-Rad). Following quantification, the amounts of hypophosphorylated PER at ZT4 and ZT24 were presented as a fraction of total PER isoforms. Error bars indicate SEM from two biological replicates. *$p < 0.05$. ***F***, Total PER abundance at ZT4 and 24 were quantified using ImageLab (Bio-Rad). Error bars indicate SEM from two biological replicates. $p < 0.05$.

4.88, $p = 0.039$; ZT24 or ZT0: $t_{(2)} = 6.74$, $p = 0.021$), *dbt* mRNA level was not affected (Fig. 1*C*; ZT4: $t_{(2)} = 1.13$, $p = 0.377$; ZT8: $t_{(2)} = 0.38$, $p = 0.74$; ZT12: $t_{(2)} = 0.45$, $p = 0.698$; ZT16: $t_{(2)} = 1.84$, $p = 0.207$; ZT20: $t_{(2)} = 1.61$, $p = 0.248$; ZT24 or ZT0: $t_{(2)} = 1.79$, $p = 0.215$).

Temporal profile of PER protein is affected in flies in which ck1α is knocked down

Because CK1α regulates PER1 stability in mammalian cells, we investigated whether the period-lengthening phenotype observed in flies could occur through modulating PER phosphorylation and metabolism over the circadian cycle. We examined the temporal profile of PER extracted from whole heads of *TUG* (parental control) and *TUG*>UAS-*ck1α RNAi*^{25786} (*ck1α RNAi*) flies (Fig. 1*D*). In control flies, PER showed daily oscillations in abundance and phosphorylation that are consistent with published data (Edery et al., 1994), with newly synthesized hypophosphorylated species appearing at ~ZT8, peaking in abundance at ~ZT16 to ~ZT20, attaining maximal phosphorylation at ~ZT24 to ~ZT4 and finally by a rapid decrease in protein levels (Fig. 1*D*, top). In comparison, although PER in *ck1α RNAi* flies displayed oscillations in abundance and phosphorylation, there are some clear observable differences. First, PER in *ck1α RNAi* flies exhibited a delay in hyperphosphorylation and degradation

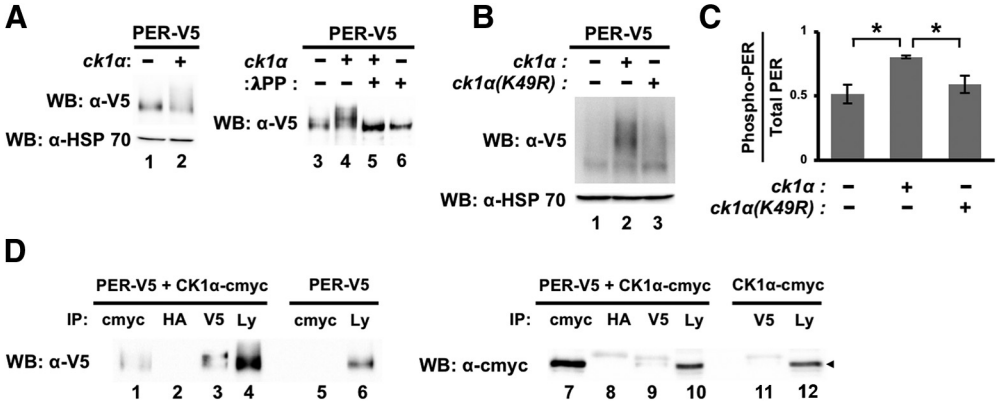

Figure 2. PER is a substrate of CK1α. ***A***, ***B***, *Drosophila* S2 cells were transfected with pAc-*per*-V5-6XHis together with an empty plasmid (pMT-FH; FH denotes 3XFLAG-6XHis), pMT-*ck1α-FH, or pMT-ck1α(K49R)*-FH. Protein extracts were either analyzed directly by Western blotting (***A***, left), with detection of HSP70 as loading control, or subjected to lambda phosphatase treatment before Western blotting analysis (***A***, right), or visualized using Phos-Tag SDS-PAGE (***B***). ***C***, Quantification of phosphorylated PER as shown in ***B*** using ImageLab (Bio-Rad). Following quantification, the amount of phosphorylated PER for each condition was presented as a fraction of the total PER isoforms present. Error bars indicate SEM from three biological replicates. $*p < 0.05$. ***D***, S2 cells were either co-transfected with pAc-*per*-V5-6XHis and pMT-*ck1α-6Xcmyc* or singly transfected with *pAc-per*-V5-6XHis or pMT-*ck1α*-6Xcmyc. Protein extracts were divided into equal aliquots and each aliquot was independently incubated with α-cmyc beads, α-HA beads, or α-V5 beads (Sigma-Aldrich). Immunocomplexes were analyzed by Western blotting in the presence of the indicated antibody. The inputs (Ly) were also assayed. Arrow on the right panel indicates CK1α. Empty, unloaded lanes are present between lanes 4 and 5 and between lanes 10 and 11.

that can be clearly observed at ZT24 to ZT4 (Fig. 1*D*, cf. lane 11 with lane 12 and lane 1 with lane 2). Quantification of PER isoforms at ZT24 and ZT4 showed significantly higher levels of hypophosphorylated PER in *ck1α RNAi* flies compared with that in control flies (Fig. 1*E*: ZT4: $t_{(2)} = 4.65$, $p = 0.043$; ZT24: $t_{(2)} = 18.93$, $p = 0.0028$). Furthermore, quantification of total PER isoforms also showed more abundant PER at ZT4 in *ck1α RNAi* flies (Fig. 1*F*: ZT4: $t_{(2)} = 14.74$, $p = 0.0023$; ZT24: $t_{(2)} = 1.33$, $p = 0.31$). Because DBT is the primary kinase that contributes to PER phosphorylation and mobility shift, as observed in Western blotting (Price et al., 1998; Ko et al., 2010), our results suggest that wild-type CK1α activity normally enhances DBT-dependent PER phosphorylation at these time points when PER is nuclear, thus promoting PER degradation. This is consistent with higher abundance of total PER at ZT24 and ZT4 (Fig. 1*D*) and the period-lengthening phenotype of *ck1α RNAi* flies.

Interestingly, upon *de novo* synthesis of PER in the daytime (ZT12) and progression of the PER protein cycle into the early night (ZT16) when PER is still localized in the cytoplasm, PER isoforms in *ck1α RNAi* flies exhibited slower mobility (Fig. 1*D*, cf. lane 5 with lane 6 and lane 7 with lane 8), suggesting that CK1α activity is normally required for inhibiting PER phosphorylation by other kinases in the cytoplasm. As mentioned earlier, because DBT has been identified as the main kinase that phosphorylates PER and leads to an observable mobility shift on Western blots, our results point to the possibility that CK1α may antagonize DBT-dependent PER phosphorylation in the cytoplasm. The loss of *ck1α* function eliciting opposite effects on PER phosphorylation status that coincides with times in which PER alters its subcellular distribution (Curtin et al., 1995) suggests that CK1α performs distinct regulatory functions on PER depending on its subcellular localization.

CK1α phosphorylates PER in *Drosophila* S2 cells

To confirm that PER is a CK1α substrate, we coexpressed PER and CK1α in *Drosophila* S2 cells and observed slower-migrating PER isoforms that likely represent phosphorylated isoforms (Fig. 2*A*,*B*). The slower-migrating isoforms disappeared after treatment with lambda phosphatase, thus confirming that they are indeed phosphorylated isoforms (Fig. 2*A*, cf. lanes 4–5). To determine whether CK1α kinase activity is required for the observed PER phosphorylation, we analyzed extracts of PER coexpressed with either CK1α or CK1α(K49R), a catalytically "inactive" mutant that is predicted by sequence analysis to have reduced kinase activity. Here, we used Phos-Tag SDS-PAGE to enhance the phosphorylation-dependent mobility shift (Kinoshita et al., 2004, 2006) and, once again, observed prominent slower-migrating PER isoforms in the presence of CK1α (Fig. 2*B*,*C*). The amount of slower-migrating PER isoforms was clearly reduced when PER was coexpressed with CK1α(K49R) instead of CK1α(WT) (Fig. 2*B*,*C*; PER vs PER+CK1α: $t_{(4)} = 3.95$, $p = 0.0168$; PER+CK1α vs PER+ CK1α(K49R): $t_{(4)} = 3.16$, $p = 0.034$; PER vs PER+CK1α(K49R): $t_{(4)} = 0.795$; $p = 0.471$). Although protein–protein interaction is not a requirement for a kinase to phosphorylate a substrate, we were able to detect interaction of PER and CK1α by reciprocal co-IP when both proteins were coexpressed in S2 cells (Fig. 2*D*).

CK1α regulates PER stability through DBT

Because we observed a delay in PER degradation in *ck1α RNAi* flies (Fig. 1*D*), we investigated whether CK1α can directly regulate PER stability. We performed CHX chase assays in S2 cells and examined PER degradation rate in the presence or absence of CK1α coexpression (Fig. 3*A*). Our results indicate that this does not appear to be the case because PER degradation rate is similar in the absence or presence of CK1α without coexpression of other clock kinases (Fig. 3*B*).

However, because we observed more hypophosphorylated and abundant PER in *ck1α RNAi* flies at ZT24 and ZT4, times at which PER is in the nucleus and is targeted for degradation by DBT, we hypothesized that CK1α may promote DBT-dependent PER phosphorylation, leading to PER degradation, even though CK1α does not promote PER degradation on its own. First, we examined DBT-PER interactions by incubating glutathione resin bound with GST-DBT with extracts harvested from S2 cells coexpressing PER-NLS (PER fused to nuclear localization sequence of simian virus 40 large-T antigen) with either CK1α or CK1α(K49R). PER-NLS was used for this experiment to target nuclear-localized PER isoforms. Previous experiments have shown that expression of *per* in S2 cells without the addition of

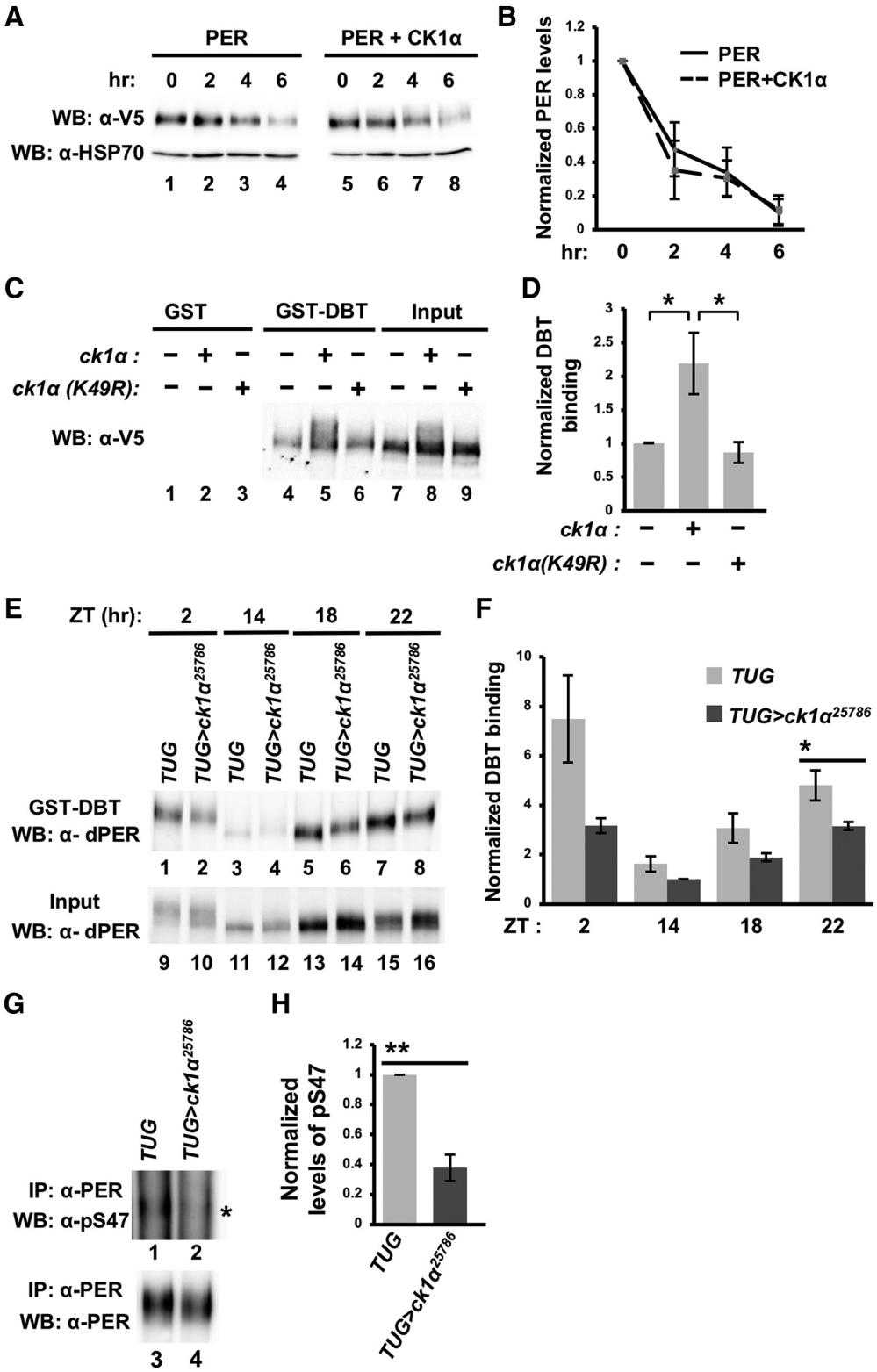

Figure 3. CK1α promotes PER-DBT interactions to regulate PER stability. ***A***, *Drosophila* S2 cells were cotransfected with pAc-*per*-V5-6XHis together with either an empty plasmid (pMT-FH; FH denotes 3XFLAG-6XHis) or pMT-*ck1α*-FH. Following a 24 h incubation period, kinase expression was induced with $CuSO_4$ for 16 h. Following induction, CHX was added and cells were harvested for protein extractions at the indicated times (in hours). Proteins were visualized by Western blotting and detected with α-V5. ***B***, Western blots were quantified using ImageLab (Bio-Rad) and normalized against α-HSP70. Error bars indicate SEM from two biological replicates. ***C***, S2 cells were transfected with pAc-*per(NLS)*-V5-His together with an empty plasmid (pMT-FH), pMT-*ck1α(WT)*-FH, or pMT-*ck1α(K49R)*-FH. Equal amounts of the extracted proteins were then incubated with glutathione resin bound with GST or GST-DBT. Bound PER (lanes 4 – 6) was eluted with 2× SDS loading buffer and visualized via Western blotting in the presence of α-V5. ***D***, Western blots from ***C*** were quantified using ImageLab (Bio-Rad) and normalized against input (lanes 7–9). Values were normalized against the PER-NLS sample (without expression of the kinase), which was set as 1. Error bars indicate SEM from four biological replicates. *$p < 0.05$. ***E***, Head extracts from *TUG* control or *TUG>ck1α RNAi* ^{25786} flies were prepared and aliquots containing equal amount of total protein for each sample were incubated with glutathione beads bound with GST-DBT. The amount of PER in total head extracts (input) and those that are bound to GST-DBT were visualized by immunoblotting in the presence of α-PER (GP5620). ***F***, Quantification of GST-DBT pull-down assays presented in ***E***. Error bars indicate SEM from four biological replicates. *$p < 0.05$. ***G***, Head extracts from *TUG* control or *TUG>ck1α RNAi* flies were immunoprecipitated (*Figure legend continues.*)

NLS largely resulted in cytoplasmic-localized PER proteins (Chang and Reppert, 2003; Kim et al., 2007; Nawathean et al., 2007). We observed an enhanced DBT-PER-NLS interaction in the presence of CK1α, suggesting that CK1α-dependent phosphorylation of PER-NLS strengthens the interaction between DBT and PER-NLS (Fig. 3*C*,*D*; PER vs PER+CK1α: $t_{(6)} = 2.6$, $p = 0.041$; PER+CK1α vs PER+CK1α(K49R): $t_{(6)} = 2.73$, $p = 0.034$; PER vs PER+CK1α(K49R): $t_{(6)} = 0.85$, $p = 0.43$). We then validated our finding in animals using head extracts of control *TUG* and *ck1α RNAi* flies (Fig. 3*E*,*F*). Compared with control, *ck1α RNAi* flies displayed significantly reduced DBT-PER interactions at ZT22 (ZT2: $t_{(6)} = 2.41$, $p = 0.052$; ZT14: $t_{(6)} = 1.96$, $p = 0.098$; ZT18: $t_{(6)} = 1.92$, $p = 0.10$; ZT22: $t_{(6)} = 2.64$, $p = 0.039$) even though PER level is higher in *ck1α RNAi* flies at this time point (Fig. 3*E*, cf. lanes 15–16).

To determine whether the lower levels of nuclear PER-DBT binding in *ck1α RNAi* flies correlate to more stable PER, we next compared the phospho-occupancy of serine 47 (pS47) of PER between control and *ck1α RNAi* flies. DBT-dependent phosphorylation of PER(S47) is a key determinant that regulates its degradation through the proteasome pathway, with phosphorylated S47 acting as a recognition signal for SLIMB, a component of the E3 ubiquitin ligase complex (Chiu et al., 2008). We observed significantly less PER(pS47) signal in *ck1α RNAi* flies at ZT24 compared with control flies (Fig. 3*G*,*H*; $t_{(4)} = 7.18$, $p = 0.002$). The increased in PER stability in response to the loss of *ck1α* function can thereby be attributed to a reduction in nuclear PER-DBT interaction, leading to a decrease in PER(S47) phosphorylation. Together, our data suggest that CK1α phosphorylates PER to promote PER-DBT interactions, leading to DBT-dependent modification of the phosphodegron and subsequent degradation of PER.

Knock-down of ck1α increases PER-dependent repression of clock genes

Because *CK1α* collaborates with DBT to modulate PER stability in the nucleus, we expect that knock-down of ck1α should result in reduced clock gene expression as PER is the key repressor of CLK-CYC activity. A comparison of steady-state mRNA levels of clock genes (*per*, *tim*, *vri*, and *pdp1ε*) between control and *ck1α RNAi* flies revealed that *ck1α RNAi* flies displayed lower levels of circadian transcripts during the transcriptional activation phase of the circadian cycle (Fig. 4*A*–*D*) (Fig. 4*A*; ZT4: $t_{(4)}$ 3.33, $p = 0.029$; ZT16: $t_{(4)} = 4.74$, $p = 0.009$; Fig. 4*C*; ZT16: $t_{(4)} = 5.1$, $p = 0.007$; Fig. 4*D*; ZT16: $t_{(4)} = 5.98$, $p = 0.0039$). This is likely due to the persistence of PER proteins and repression of CLK-CYC activity because of increased PER stability. This conclusion is supported by increased PER occupancy observed at *per* and *tim* promoters in *ck1α RNAi* flies as assayed by ChIP-qPCR (Fig. 4*E*; ZT4: $t_{(4)} = 1.47$, $p = 0.215$; ZT10: $t_{(4)} = 4.35$, $p = 0.012$; ZT16: $t_{(4)} = 3.2$, $p = 0.033$; ZT22: $t_{(4)} = 4.02$, $p = 0.016$) and (Fig. 4*F*; ZT4: $t_{(2)} = 1.03$, $p = 0.411$; ZT10: $t_{(2)} = 4.44$, $p = 0.047$; ZT16: $t_{(4)} = 7.04$, $p = 0.02$; ZT22: $t_{(2)} = 0.602$, $p = 0.608$).

CK1α antagonizes DBT-dependent phosphorylation of PER in the cytoplasm

Next, we sought to understand the potential regulatory function of CK1α on cytoplasmic PER because loss of *ck1α* function in flies appeared to slow down PER mobility at ZT12 and ZT16 (Fig. 1*D*, cf. lane 5 with lane 6 and lane 7 with lane 8). Because PER mobility shift by SDS-PAGE is predominately influenced by DBT activity, we investigated whether CK1α normally antagonizes DBT activity in the cytoplasm. This could explain the slower-migrating PER isoforms in *ck1α RNAi* flies that are prominently observed at ZT12 and ZT16. We coexpressed PER with CK1α, DBT, or both kinases in *Drosophila* S2 cells and monitored PER phosphorylation status following kinase induction (Fig. 5*A*,*B*). PER lacking NLS will largely localize to the cytoplasm upon induction in S2 cells, allowing us to examine the effect of CK1α on cytoplasmic PER (Fig. 5*C*,*D*) (Saez and Young, 1996; Chang and Reppert, 2003; Nawathean and Rosbash, 2004; Cyran et al., 2005; Kim et al., 2007; Nawathean et al., 2007). In the presence of CK1α, cytoplasmic PER exhibited a delay in DBT-dependent phosphorylation that was significant at 6 and 12 h after kinase induction (Fig. 5*A*, cf. bottom two panels) (Fig. 5*B*, 0 h: $t_{(6)} = 0.27$, $p = 0.795$; 6 h: $t_{(6)} = 2.57$, $p = 0.042$; 12 h: $t_{(6)} = 3.07$, $p = 0.022$; 24 h: $t_{(6)} = 1.31$, $p = 0.237$). Our results support the hypothesis that CK1α antagonizes DBT kinase activity on PER in the cytoplasm. CK1α inhibition of DBT-dependent PER phosphorylation could be the result of a kinase cascade on PER or a reduction of DBT activity due to CK1α phosphorylation of DBT. Our current data cannot rule out either scenario at this time.

CK1α promotes nuclear entry of PER

Given that DBT was shown to prevent premature PER nuclear entry in flies (Cyran et al., 2005; Muskus et al., 2007) and our data suggest that CK1α can antagonize DBT-dependent PER phosphorylation in the cytoplasm, we proceeded to determine whether knock-down of *ck1α in tim*-expressing cells can slow down PER nuclear translocation. This could also contribute to the period-lengthening phenotype of *ck1α RNAi* flies. Immunostaining of clock neurons (l-LNvs) revealed that nuclear translocation of PER was indeed delayed in *ck1α RNAi* flies compared with control (*TUG*) flies (Fig. 6*A*); we observed a significantly smaller fraction of PER in the nucleus in *ck1α RNAi* flies at ZT16 (~20%) compared with control flies at the same time point (~46%) (Fig. 6*B*; ZT16: $t_{(28)} = 5.65$, $p = 0.0000046$; ZT18: $t_{(28)} = 2.32$, $p = 0.03$; ZT20: $t_{(28)} = 2.7$, $p = 0.01$). This difference in nuclear PER localization between the two genotypes was diminished at ZT18 and ZT20, suggesting that CK1α kinase is involved in regulating the timing of nuclear PER translocation. The fraction of PER located in clock neuron cytoplasm was also computed and the results supported the same conclusion (Fig. 6*C*; ZT16: $t_{(28)} = 5.65$, $p = 0.0000046$; ZT18: $t_{(28)} = 2.32$, $p = 0.03$; ZT20: $t_{(28)} = 2.7$, $p = 0.01$). Finally, we investigated the amount of nuclear PER, expressed as pixel intensity, and observed that it was significantly higher in *ck1α RNAi* flies at ZT20 even though our results indicated that PER nuclear translocation was delayed in these flies compared with control flies (Fig. 6*D*; ZT16: $t_{(28)} = 2.39$, $p = 0.02$; ZT18: $t_{(28)} = 0.72$, $p = 0.48$; ZT20: $t_{(28)} = 2.2$, $p = 0.04$). This can be explained by our observation that PER in *ck1α RNAi* flies is more stable than in control flies upon entry into the nucleus. Together, our findings support that CK1α promotes the nuclear entry of cytoplasmic PER and degradation of nuclear PER and therefore has an overall function of speeding up the circadian oscillator.

←

(*Figure legend continued.*) with α-PER (GP5620) before Western blotting analysis in the presence of α-PER(pS47) (top) and α-PER (GP5620) (bottom). Asterisk indicates the majority of PER(pS47)-containing isoforms (top). ***H***, Western blots from ***G*** were quantified using ImageLab (Bio-Rad) and normalized against the amount of immunoprecipitated total PER. Levels of PER(pS47)-containing isoforms in *TUG* control were set at 1. Error bars indicate SEM from three biological replicates. $^{**}p < 0.005$.

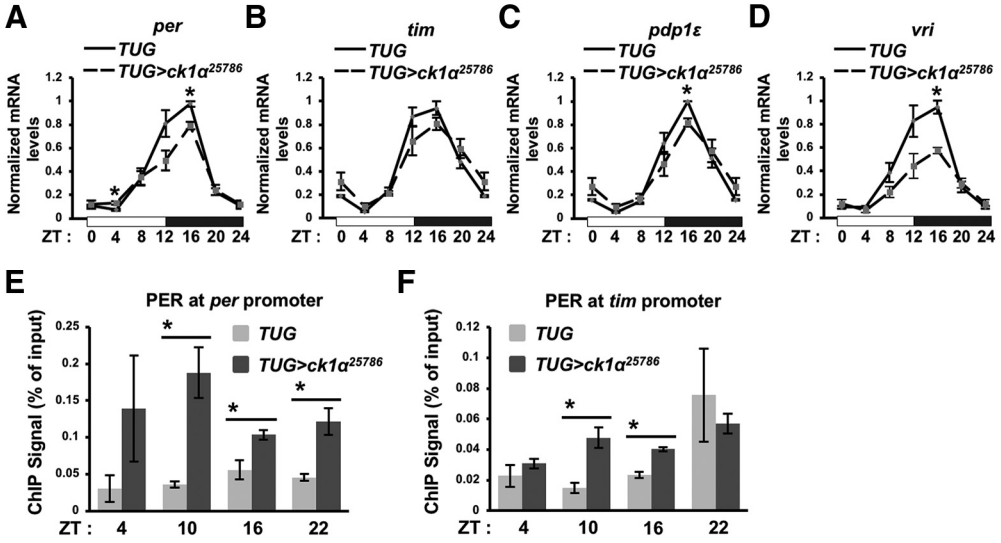

Figure 4. *ck1α* knock-down lowers clock gene transcription through increased PER occupancy at clock gene promoters. ***A–D***, Clock gene expression was quantified by real-time PCR and normalized to noncycling *cbp20* expression. Values were processed so that the mRNA level at each time point is relative to the maximum value over the time course, which is set at 1. White and black horizontal bars on the *x*-axis represent lights on and off, respectively. Error bars indicate SEM from three biological triplicates. ZT24 data point was plotted twice as ZT0 and ZT24 to facilitate viewing of daily cycling. Solid and dashed lines represent *TUG* control and *TUG>ck1α RNAi*^{25786} respectively. *$p < 0.05$. ***E***, ***F***, Chromatin IP analysis of PER occupancy in *TUG* control and *TUG>ck1α RNAi*^{25786}. Shown is the amount of ChIP signal on *per* and *tim* promoter as measured by qPCR relative to the input at the indicated time points. Error bars indicate SEM from biological replicates ($n = 3$ for *per* promoter, $n = 2$ for *tim* promoter). *$p < 0.05$.

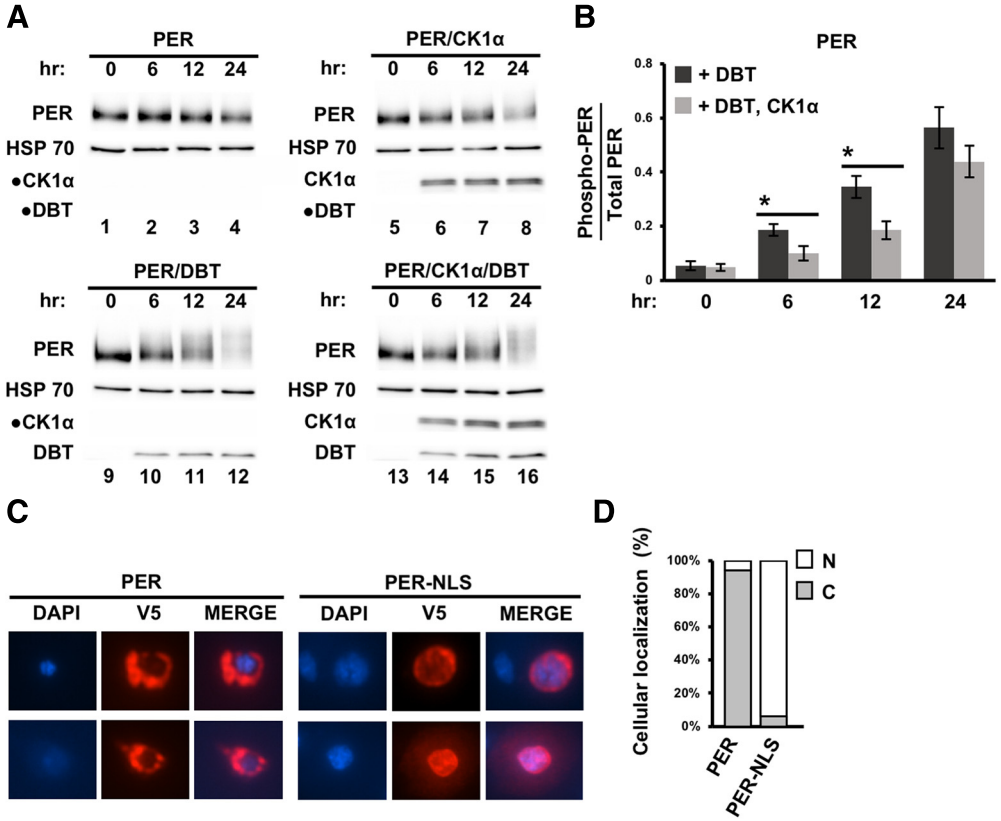

Figure 5. CK1α antagonizes DBT activity on cytoplasmic PER. ***A***, *Drosophila* S2 cells were transfected with pAc-*per*-V5-His together with one of the following: no kinase plasmids; pMT-*ck1α-FH* only, FH denotes *3XFLAG-6XHis*; *pMT-dbt*-V5–6XHis only; or pMT-*ck1α-FH* and pMT-*dbt*-V5–6XHis. Cells were harvested and proteins extracted at the indicated times (in hours) post kinase induction. PER, DBT, and CK1α proteins were visualized by Western blotting in the presence of α-V5 or α-FLAG antibodies. Detection of α-HSP70 represents loading control. Circular dots indicate blots without any signal detected as expected. ***B***, Quantification of phosphorylated PER (phospho-PER) using ImageLab (Bio-Rad). Following quantification, the number of phospho-PER isoforms for each time point was presented as a fraction of total PER isoforms present. Error bars indicate SEM from four biological replicates. *$p < 0.05$. ***C***, Immunofluorescence of PER in S2 cells. Left, Nuclear staining using DAPI. Middle, PER staining using α-V5; (right) merged image showing localization of PER or PER-NLS. Top and bottom, Representative images of different S2 cells. ***D***, The subcellular localization of PER and PER-NLS were determined and expressed as the percentage of total number of cells analyzed per construct ($n = 34$). N, Nuclear; C, cytoplasmic localization.

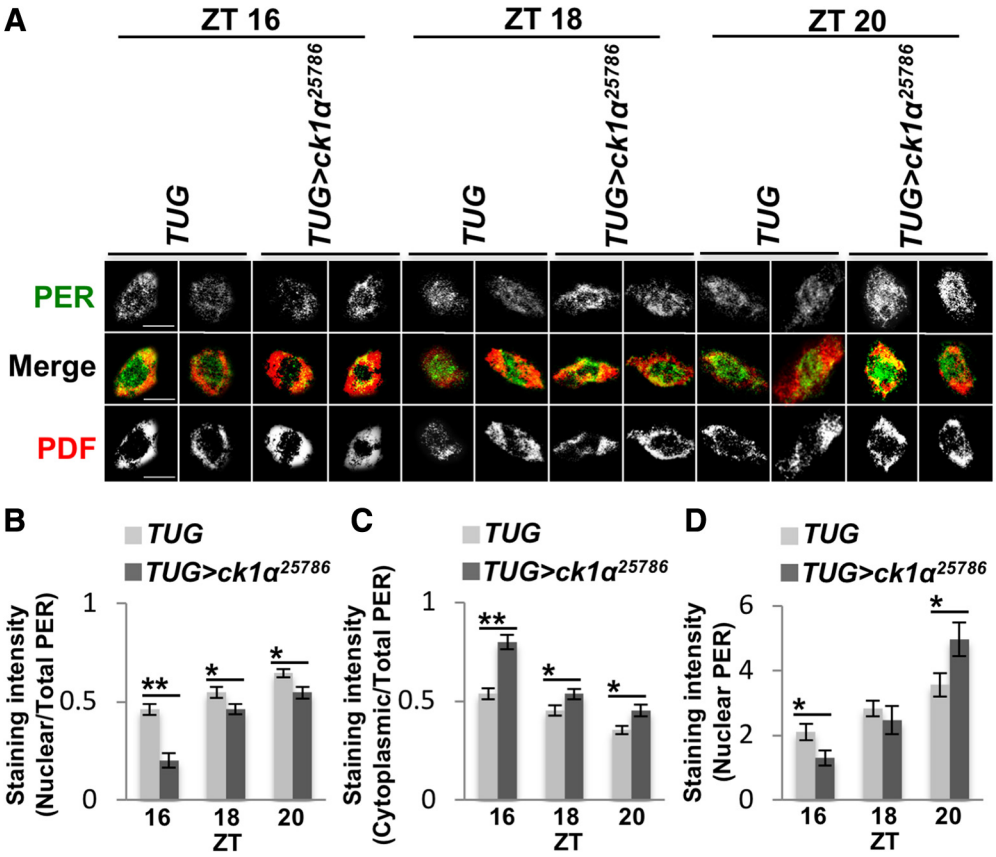

Figure 6. CK1α promotes nuclear entry of PER. ***A***, Confocal images of brains from adult *TUG* control and *ck1α RNAi*^{25786} flies stained with α-PER Rb s3968-1 (green) and α-PDF (red) in l-LNv clock neurons. Scale bars are shown on the left-most panels only and indicate 10 μm. Flies were entrained for 3 d in LD cycles and collected at the indicated times on LD3 for fixation and immunostaining. Duplicate panels are shown for each condition. ***B***, Bar graph displaying the fraction of PER in the nucleus presented as nuclear PER intensity divided by total intensity of PER. ***C***, Bar graph displaying the fraction of PER in the cytoplasm presented as cytoplasmic PER intensity divided by total intensity of PER. ***D***, Bar graph showing the amount of nuclear PER expressed as pixel intensity (in thousands). Error bars indicate SEM of 15 neurons from 10 brains. $*p < 0.05$, $**p < 0.005$.

Discussion

CK1α was previously identified as a clock kinase in mammalian cells via a high-throughput chemical screen (Hirota et al., 2010) and was shown to regulate the clock through modulation of PER1 stability. However, the mechanisms by which CK1α regulates PER metabolism and in turn the circadian oscillator remain unclear. Using the *Drosophila* model, we provided further support for a role of CK1α in animal clocks and, more importantly, provided additional insights into the mechanisms by which CK1α acts in coordination with DBT in both the cytoplasm and the nucleus to regulate the function of the molecular oscillator. First, our analysis using *Drosophila* S2 cell culture demonstrated the ability of CK1α to phosphorylate PER. This was corroborated in whole animals where RNA*i* knock-down of *ck1α in tim*-expressing cells affected the phosphorylation program of PER *in vivo*. Knock-down of *ck1α by RNAi* led to period-lengthening of locomotor activity rhythms in flies, strongly suggesting that CK1α functions to speed up the clock in wild-type animals to maintain 24 h rhythms.

Similar to DBT (CK1δ/ε), another CK1 isoform, our results suggested that CK1α plays multiple regulatory roles in the molecular oscillator. Due to the period-lengthening phenotype of *ck1α RNAi* flies, we initially hypothesized that CK1α promotes PER degradation to accelerate the pace of the clock in wild-type animals independently of DBT. Indeed, reducing *ck1α* expression in flies using RNAi knock-down seemed to produce more stable PER at ZT24 to ZT4, when PER should be degrading rapidly. Further analysis using both tissue culture cells and fly extracts revealed that the increase in PER stability upon reduction of CK1α activity resulted primarily from weakened interactions between PER and DBT. This consequently led to the reduction of PER(S47) phosphorylation, which is known to promote proteasomal-dependent PER degradation. Our data are consistent with previous results demonstrating a negative correlation between the interaction of PER and DBT and the stability of PER (Kim et al., 2007; Nawathean et al., 2007). PER proteins lacking the DBT-binding domain remain hypophosphorylated and at constant high levels over the circadian cycle. Interestingly, CK1α was observed to elicit these effects only during the latter part of the circadian cycle, suggesting that CK1α regulates the turnover of nuclear PER.

In addition to its role on PER degradation, we also demonstrated that CK1α has a role in the cytoplasm to promote PER nuclear localization by antagonizing the activity of DBT. Although DBT has been proposed to play a role in regulating PER nuclear translocation since *dbt* mutants were first characterized (Price et al., 1998) and the gene that encodes the kinase was first cloned (Kloss et al., 1998), whether its role is stimulatory or inhibitory is not always unequivocal. Evidence that supports the stimulatory role of DBT with regard to PER nuclear translocation mostly come from experiments using *Drosophila* S2 tissue culture (Nawathean and Rosbash, 2004; Nawathean et al., 2007). Nawathean and Rosbash (2004) knocked down endogenous *dbt* in S2 cells using dsRNA and observed a significant reduction in

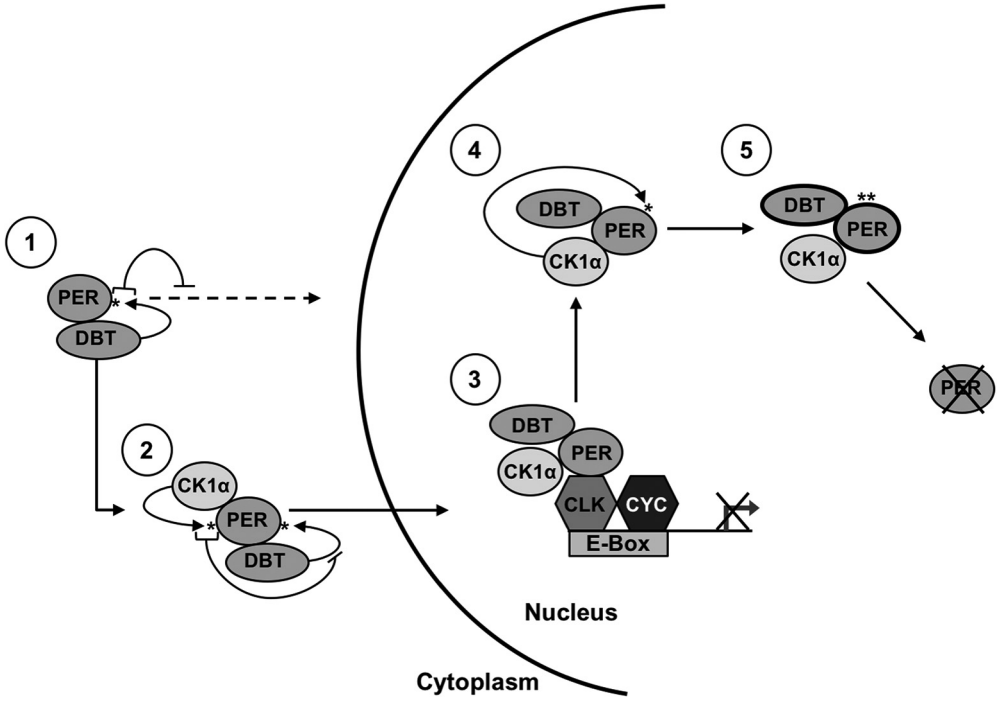

Figure 7. Model describing the proposed function of CK1α in the circadian oscillator. Step 1: In the cytoplasm, DBT phosphorylates PER to prevent premature nuclear entry of PER. Step 2: During times of nuclear translocation, CK1α phosphorylates PER to inhibit its DBT-dependent phosphorylation, therefore favoring nuclear entry of PER. Step 3: Upon entry into the nucleus, PER in complex with multiple kinases and then interacts with and represses the transcriptional activity of CLK-CYC heterodimer on circadian promoters. Not all kinases in the complex are shown. Steps 4 and 5: Finally, in preparation for the new circadian cycle, nuclear PER is phosphorylated by CK1α, which enhances/stabilizes PER-DBT interaction to promote phosphorylation of PER in the phosphodegron and its subsequent degradation. Line width and asterisks denote strength of interactions and phosphorylation events, respectively.

the nuclear accumulation of PER and a substantial decrease in transcriptional repressor activity. This prompted them to suggest that DBT may promote PER nuclear localization, although they proposed that DBT likely plays a greater role in activating the repressor function of PER, whereas its regulation on PER nuclear translocation may be an indirect consequence of the association of active PER and DNA. In a follow-up study, Nawathean et al. (2007) characterized a PER variant containing a deletion in what is now termed the DBT binding domain of PER (PERΔ). They observed that this PER variant is resistant to DBT-dependent phosphorylation and is highly stable, but exhibited a low level of nuclear accumulation and repressor activity when expressed in S2 cells. Although they performed rescue experiments in flies and determined that PERΔ was not able to rescue the locomotor activity rhythm of per^0 mutants, they did not examine PER subcellular localization in PERΔ fly brains. Nevertheless, they regarded this as further support for their previous conclusion in linking DBT-dependent phosphorylation to promotion of PER repressor activity. Their additional studies on the PERΔ also provided them with more confidence to suggest that DBT actively phosphorylates PER in the cytoplasm to promote nuclear translocation.

Conversely, evidence supporting the inhibitory role of DBT on PER nuclear entry mostly originated from *in vivo* fly studies in which various *dbt* mutants were characterized. First, analysis of the strongly hypomorphic dbt^P mutant containing a P-element insertion showed that significant reduction in DBT activity resulted in a lack of daily rhythmic PER nucleocytoplasmic shuttling, with PER appearing to be constitutively nuclear in clock neurons (Kloss et al., 1998; Price et al., 1998). Second, characterization of dbt^{AR}, another hypomorphic *dbt* mutant with a histidine to tyrosine point mutation at amino acid 126 (Rothenfluh et al., 2000), demonstrated that PER can translocate to the nucleus even in the absence of TIM (i.e., in tim^0 mutant), but only when DBT activity is severely dampened (Cyran et al., 2005). This suggest that perhaps DBT acts to prevent premature nuclear entry of PER in wild-type flies in the early part of the circadian cycle before the accumulation of TIM proteins. Finally, nuclear PER was observed in mutant flies expressing kinase-inactive DBT ($dbt^{K/R}$) during periods in which PER would be cytoplasmic (e.g., early nights), thus supporting an inhibitory function of DBT on PER nuclear localization (Muskus et al., 2007). Given that S2 cell culture does not possess a cycling molecular oscillator or fully recapitulate *in vivo* physiological condition, it is perhaps logical to put more weight on the inhibitory role of DBT supported by fly studies.

Significantly, our study adds new supporting data to the inhibitory role of DBT activity on PER nuclear translocation. Integrating our results with published data regarding the effects of DBT-dependent PER phosphorylation, we propose the following model (Fig. 7). Starting in late day, PER levels in the cytoplasm begin to accumulate in response to heterodimerization with, and stabilization by TIM (Kloss et al., 1998; Price et al., 1998; Ko et al., 2002). DBT-dependent phosphorylation temporarily retains PER in the cytoplasm to prevent premature entry of PER into the nucleus to initiate transcription repression of circadian genes (Cyran et al., 2005). During early night, CK1α phosphorylates PER to antagonize DBT-dependent PER phosphorylation, thereby promoting PER nuclear entry. At the moment, we cannot rule out the alternative hypothesis in which CK1α phosphorylates DBT to reduce its kinase activity and phosphorylation of PER. Once localized to the nucleus, PER initiates the repression phase of circadian transcription by inhibiting CLK-CYC activity. Toward late night transitioning into early day, CK1α phosphorylates nuclear PER, which promotes or strengthens interactions

between PER and DBT. This eventually leads to DBT-dependent phosphorylation of PER(S47), which promotes PER-SLIMB interaction and PER degradation via the proteasome pathway.

Future experiments to determine whether the various regulatory roles of CK1α are conserved in mammalian clock will be important to understand the full extent of the effects of the CK1α-targeting Longdaysin (Hirota et al., 2010), especially if this chemical compound were to be considered as a therapeutic option for circadian disorders. Our results highlighting the multiple roles of CK1α and its coordination with DBT in regulating PER proteins are in congruence with the observation that small molecules such as Longdaysin that target multiple kinases of the same substrate due to their similarities in active sites often produce the largest period effects and may represent the most effective therapeutic compounds (Hirota et al., 2010).

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