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Drosophila peptidyl-prolyl isomerase Pin1 modulates circadian rhythms via regulating levels of PERIOD

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ABSTRACT

In animal circadian clock machinery, the phosphorylation program of PERIOD (PER) leads to the spatio-temporal regulation of diverse PER functions, which are crucial for the maintenance of ~24-hr circadian rhythmicity. The peptidyl-prolyl isomerase PIN1 modulates the diverse functions of its substrates by inducing conformational changes upon recognizing specific phosphorylated residues. Here, we show that overexpression of *Drosophila pin1*, *dodo* (*dod*), lengthens the locomotor behavioral period. Using *Drosophila* S2 cells, we demonstrate that Dod associates preferentially with phosphorylated species of PER, which delays the phosphorylation-dependent degradation of PER. Consistent with this, PER protein levels are higher in flies overexpressing *dod*. Taken together, we suggest that Dod plays a role in the maintenance of circadian period by regulating PER metabolism.

1. Introduction

By means of circadian clock system, all living organisms anticipate rhythmic oscillations of their environment such as day/night and warm/cold temperatures, and manifest a broad spectrum of physiological activities and behaviors at the most advantageous times of the day [1,2]. Numerous studies using several model organisms have identified the molecular mechanisms governing cell autonomous circadian oscillators, which are conserved from lower species to higher organisms, including humans [3]. Circadian oscillators in the animal kingdom are governed by transcriptional/translational feedback loops composed of core clock proteins, most of which were identified from genetic studies using *Drosophila* [4]. In the major loop of transcriptional/translational feedback machinery, basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS)-containing transcription factors, dCLOCK (dCLK) and CYCLE (CYC), activate the transcription of *period* (*per*) and *timeless* (*tim*) in an E box (CACGTG)-dependent manner and translated PER and TIM proteins accumulate in the cytoplasm during the early night. In the late night, PER and TIM translocate to the nucleus where PER inhibits the activity of a dCLK-CYC heterodimer to down-regulate their own transcription. Timely degradation of PER and TIM during the early morning relieves the repression and allows the transcription of clock genes including *per and tim* and *clock controlled genes* (*ccgs*) to begin the next day [1,5].

Nonetheless, numerous lines of evidence indicate that post-translational modifications of clock proteins are crucial for regulating circadian clock machinery. Specifically, the timely phosphorylation of PER operates as a primary determinant for rhythm generation with ~24 h periodicity [5–8]. PER undergoes progressive phosphorylation beginning in the late night, leading to a hyper-phosphorylated state by early morning [7]. During this process, PER exhibits time-dependent changes in subcellular localization, activity, and stability [9–18]. Several kinases have been identified that phosphorylate PER among which DOUBLETIME (DBT) kinase (homolog of mammalian *casein kinase Iε*) was identified as major kinase [9,10]. Previous work, which sought to map

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the phosphorylation sites on PER induced by DBT in simplified *Drosophila* S2 cells found that several phosphorylated serine (Ser)/threonine (Thr) residues followed by proline (Pro) residues, which further suggests the role for CDK, MAPK, GSK3 and CLK family (CMGC) kinase(s) in circadian clock machinery [19].

Peptidyl-prolyl *cis/trans* isomerases (PPIases) are enzymes that facilitate intrinsic conformational protein changes by prolyl *cis-trans* isomerization [20–23]. Pro residues in proteins can exist in *cis*- or *trans*-peptide bond conformations, providing an intrinsic backbone switch yet with relatively slow inter-conversion. This slow conversion is catalyzed by four subgroups of PPIases: cyclophilins (Cyps), FK506-binding proteins (FKBPs), parvulins, and Ser/Thr phosphatase 2A activator (PTPA) [24]. Among PPIases, the evolutionarily conserved PIN1, a parvulin PPIase, is unique in that it recognizes and isomerizes specific phosphorylated Ser/Thr residues followed by Pro residues [21,25]. Numerous lines of evidence demonstrate that PIN1-induced conformational changes of substrates acts as a molecular switch to fine-tune diverse cellular processes [26]. In *Drosophila*, Dodo (Dod) was identified as mammalian PIN1 homolog and elucidated to be involved in the MAPK-mediated, phosphorylation-dependent dorso-ventral patterning of the follicular epithelium in egg chambers [27,28].

Given that the phosphorylation of PER mediates crucial functions of PER, we sought to determine whether the phosphorylation-dependent PPIase, Dod, is involved in regulating circadian clock machinery by controlling dynamics of PER. Interestingly, overexpression of *dod* in clock cells lengthened the circadian period with robust rhythmicity in flies. In *Drosophila* S2 cells, Dod preferentially associated with DBT-induced phosphorylated isoforms of PER, resulting in delayed degradation of PER. In flies overexpressing *dod*, PER levels were reproducibly higher in light/dark (LD) cycles. Taken together, these data suggest that Dod plays a role in fine-tuning the ~24-hr period by regulating PER stability.

2. Materials and methods

2.1. Plasmids and transgenic flies

To generate transgenic flies harboring *pUAST-attB-dod*, full-length *dod* cDNA was amplified by PCR using the Berkeley *Drosophila* Genome Project, *Drosophil*a Gene Collection (BDGP DGC) clone, GH25582, as a template and subcloned into *pUAST-attB* vector via the EcoRI and XbaI. PhiC31 integrase-mediated transgenesis was used to insert the *pUAST-attB-dod* plasmid into the VK00018 attP site on chromosome 2 (BestGene Inc.) [29,30]. Three independent lines of transgenic flies were obtained and labeled *UAS-dod* 1-2M, *UAS-dod* 1-4M, and *UAS-dod* 1-5M, respectively. *UAS-dod* 1–5M was primarily used for all biochemical assays. The *pAct-per* and *pMT-dbt-V5* plasmids were described previously [14,32–34]. To generate *pAct-dod*, the coding regions of *dod* were amplified by PCR from the BDGP DGC clone, GH25582, and subcloned into *pAct(B)-V5/His* (Invitrogen) using EcoRI and XbaI.

2.2. Behavioral assays

The locomotor activities of individual flies were measured as previously described using the *Drosophila* Activity Monitoring system from Trikinetics (Waltham, MA) [31]. The locomotor activity for each individual fly was analyzed using FaasX software, which was generously provided by F. Rouyer (CNRS, France). Periods were calculated for each individual fly using *chi*-square periodogram analysis and pooled to obtain a group average for each independent transgenic line or genotype. Power is a quantification of the relative strength of the rhythm during DD. Individual flies with a power $\geq$10 and a 'width' value of 2 or more (denotes number of peaks in 30-min increments above the 95% confidence line of the periodogram) were considered rhythmic. Actograms show locomotor activity throughout the behavioral experiment period. Vertical bars in the actogram represent absolute activity levels for each 30-min interval averaged for each genotype.

2.3. Immunoblotting and immunoprecipitation

Protein extracts from S2 cells and flies were prepared as previously described [35]. The cells were lysed using modified RIPA buffer (50 mM Tris–HCl [pH7.5], 150 mM NaCl, 1% IGEPAL CA-630, 0.25% sodium deoxycholate) with the addition of a protease inhibitor and a phosphatase inhibitor cocktail. Flies were collected by freezing at the indicated times in LD, and total fly head extracts were prepared using modified-RIPA buffer. 5% and 15% gels were used to resolve PER and Dod, respectively. The primary antibodies used were anti-V5 (1:5000, Invitrogen), anti-PER (1:3000, Rb1) [35], and anti-mammalian PIN1 (1:1000, 8C10, Santa Cruz). Quantification of band intensity was performed using ImageJ software (NIH).

For immunoprecipitation, cell extracts from S2 cells or fly heads were prepared and incubated with 3 μl of anti-PER (Rb1) antibody overnight at 4 °C with rotation. The following day, 20 μl of Gammabind-sepharose bead slurry (GE Healthcare) was added for additional 3-hr incubation at 4 °C. The immune complexes were eluted with 1X sample buffer without β-mercaptoethanol to reduce the elution of the immunoglobulin heavy and light chains. After elution, β-mercaptoethanol was added to the immune-complexes, which were assayed via immunoblotting.

3. Results

3.1. Overexpression of dod in timeless-expressing clock cells lengthens the circadian period

To investigate whether Dod plays a role in regulating the circadian clock machinery, we sought to overexpress *dod* in all clock cells under *tim(UAS)*gal4 control via the binary UAS/GAL4 system [36]. Locomotor activity rhythms were assayed under standard conditions in which flies were maintained at 25 °C for 4 days of 12-hr light/12-hr dark (LD) cycles followed by 7 days of complete darkness (DD) to measure a free-running period. While control w^{1118} flies crossed with *tim(UAS)gal4* flies exhibited strong activity rhythms with slightly shorter circadian periods of ~23.6 h, three independent lines of *UAS-dod* transgenic flies crossed with *tim(UAS)gal4* flies [herein denoted as flies overexpressing *dod*] exhibited robust rhythms yet with periods that were about 1-hr longer (Table 1). Control flies showed bimodal locomotor activity patterns, which centered on dark-to-light transitions (morning peak) and light-to-dark transitions (evening peak) under LD cycle

Table 1
Overexpression of *dod* altered circadian behavior.[a]

Genotype	Number[b]	Tau ± S.E.M. (h)	Rhythmicity (%)[c]	Power[d]
tim > w1118	30	23.6 ± 0.06	96.7	101.9
tim > dod-1-2M	7	24.8 ± 0.12	85.7	183.2
tim > dod-1-4M	12	24.5 ± 0.14	83.3	80.1
tim > dod-1-5M	28	24.8 ± 0.06	92.9	111.4

[a] Flies were kept at 25 °C and exposed to 4 days of 12:12 LD followed by 7 days of DD.
[b] Total number of flies that survived until the end of the testing period.
[c] Percentage of flies with activity rhythms having a power value of $\geq$10 and a width value of $\geq$2.
[d] Measure of the strength or amplitude of the rhythm.

at 25 °C (Fig. 1A). In contrast, flies overexpressing *dod* showed delayed onset of evening activity in LD cycle, which is consistent with longer circadian periods (Fig. 1B). Via actogram analysis, delayed onset of both morning and evening activity was evident in flies overexpressing *dod*, as days passed under constant dark conditions (Fig. 1D). These behavioral results suggest that Dod likely plays a role in establishing proper circadian period in flies.

3.2. Dod preferentially binds to phosphorylated PER leading to increased PER stability in Drosophila S2 cells

Among PPIases, Dod is unique in that it preferentially recognizes phosphorylated Ser/Thr residues that are followed by Pro. Previous work mapped the phosphorylation sites of PER in *Drosophila* S2 cells by expressing PER singly or in combination with the kinase DBT [19]. This study demonstrated that many Ser/Thr phosphorylation sites followed by Pro residues in PER, which denote potential Dod target sites. Given that the phosphorylation of PER is critical for regulating its subcellular localization, activity, and stability, which affects circadian periods [9,11,13–15,17,18], Dod might also be engaged acting as a molecular switch to regulate the aforementioned key functions of PER. Thus, we first tested whether Dod might preferentially interact with phosphorylated PER in *Drosophila* S2 cells. S2 cells were co-transfected with *pAct-per* and *pAct-dod* in combination with or without *pMT-dbt*-V5. When DBT expression, under the control of an inducible *pMT* promoter, was induced by the addition of Cu^{2+} to the medium, phosphorylation of PER occurred as previously reported (Fig. 2A, input panel) [14]. At 24 h post-induction, we performed immunoprecipitation analysis using the PER antibody. As shown in Fig. 2A, increased amounts of Dod precipitated with phosphorylated isoforms of PER compared to un-/hypo-phosphorylated PER (Fig. 2, compare lane 2 to 4 on the top panel). Although decreased levels of phosphorylated PER were also observed in the input lane, this is likely due to the fact that hyper-phosphorylated isoforms of PER are rapidly degraded via ubiquitin-mediated proteasome degradation [14] (Fig. 2A, compare lanes 1 and 2 with 3 and 4 in the third panel). These data suggest that phosphorylated isoforms of PER serve as Dod substrates, leading to Dod-mediated conformational changes of PER.

To determine the downstream effects of Dod associating with phosphorylated PER, we examined PER metabolism upon DBT expression in the presence or absence of Dod. Consistent with previous reports [14], progressive phosphorylation and rapid degradation of PER were observed after DBT induction (Fig. 3, top panel, lanes 1, 3, 5, and 7). At 8 h post-DBT induction, there were no significant differences in PER metabolism with or without Dod. However, at 16 and 24 h post-DBT induction, the band intensity of hyper-phosphorylated isoforms of PER was stronger when Dod was co-expressed (Fig. 3, top panel, compare lane 5–6, and lane 7–8).

To directly measure the stability of hyper-phosphorylated PER, we inhibited the translation of PER by adding cycloheximide (CHX) at 18 h post-DBT induction and measured PER levels. When Dod was co-expressed, the decrease in PER levels was significantly slower (Fig. 3A and C). However, in the absence of DBT, similar stabilities of un/hypo-phosphorylated PER were observed with or without co-expressed Dod (Fig. 3B and D). This result is consistent with the observation that Dod did not preferentially associate with un/hypo-phosphorylated PER (Fig. 2A). Taken together, these data suggest that Dod recognizes and associates with PER at phosphorylated Ser/Thr-Pro residue(s), which results in a conformational change that leads to slowed PER degradation.

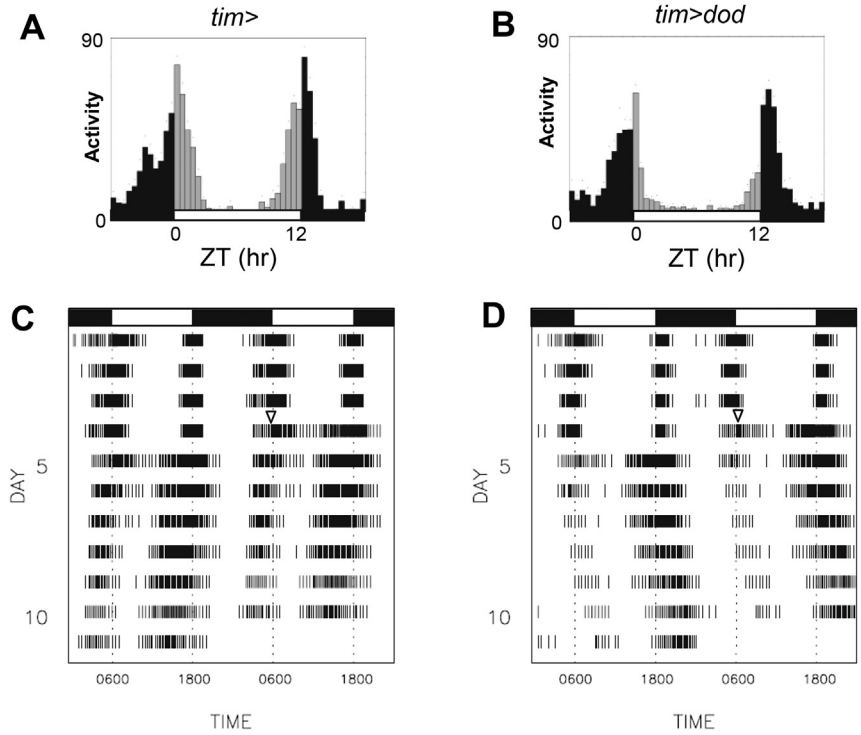

Fig. 1. Daily locomotor activities of flies overexpressing *dod*. Adult male flies for a given genotype were entrained in 12:12 LD for 4 days followed by 7 days of constant dark condition. White and black horizontal bars indicate light and dark phases, respectively. (A, B) Each panel represents the average activity of male flies for a given genotype during the last day of entrainment. Each vertical bar represents relative activity levels during a 30-min bin (gray bar, activity of light-on period; black bar, activity of light-off period). ZT indicates Zeitgeber time where ZT0 is light on time. (C, D) The vertical black bars on each row of the actogram depict fly activity (measured in 30 min intervals). To better visualize rhythmic behavior, each row of an actogram was double plotted. Arrow head indicates when the constant dark condition started.

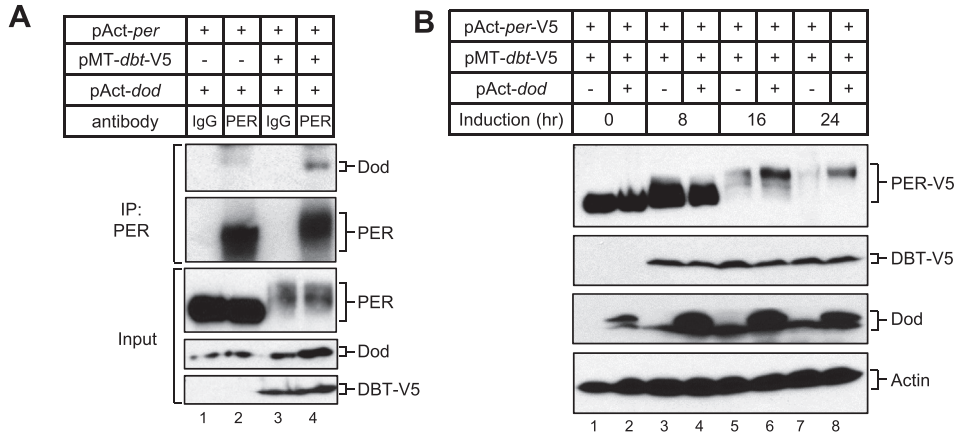

Fig. 2. Interaction between DOD and phosphorylated PER in *Drosophila* S2 cells. (A) S2 cells were transiently transfected with 600 ng of *pAct-per* and 600 ng of *pAct-dod* in the presence (+) or absence (−) of 200 ng of *pMT-dbt*-V5. Expression of DBT was induced 24 h after transfection by adding 500 μM $CuSO_4$ to the medium. Cells were harvested at 24 h post DBT induction, and protein extracts were subjected to immunoprecipitation (IP) with PER Ab or immunoblotting (Input). Immune complexes were analyzed for Dod or PER. (B) S2 cells were transiently transfected with 600 ng of *pAct-per*-V5 and 200 ng of *pMT-dbt*-V5 in the presence (+) or absence (−) of 600 ng of *pAct-dod*. At the indicated times post-DBT induction, cells were harvested, and protein extracts were subjected to immunoblotting. Actin was used as loading control.

3.3. Levels of PER were higher in flies overexpressing dod

To further examine whether overexpression of *dod* also protects PER from degradation *in vivo*, we measured daily profiles of PER levels. Both control flies carrying the *UAS-dod* transgene and flies overexpressing *dod* were entrained with 12:12 LD cycles, and on the third day, flies were collected at indicated Zeitgeber time (ZT). In flies overexpressing *dod*, increased expression of Dod was confirmed by immunoblotting for endogenous Dod (Fig. 4A, middle panel). In flies overexpressing *dod*, PER levels were reproducibly higher and exhibited stable interactions with Dod (Fig. 4A and B). These results suggest that PER is stabilized by *dod* overexpression *in vivo*.

4. Discussion

For animal circadian clock systems, timely phosphorylation of PER, which leads to precise spatio-temporal regulation of its stability, nuclear entry, and activity, is crucial for maintaining proper rhythmicity and periodicity [9,11,13–15,17,18]. Numerous studies have found that the mammalian peptidyl-prolyl isomerase, PIN1, acts as a key molecular switch to modulate phosphorylated signaling molecules and control diverse cellular processes by isomerizing specific phosphorylated Ser/Thr-Pro bonds, which leads to conformational protein changes [26]. We sought to examine how *Drosophila* Pin1, Dod might be involved in circadian clock regulation by inducing post-phosphorylation modification of

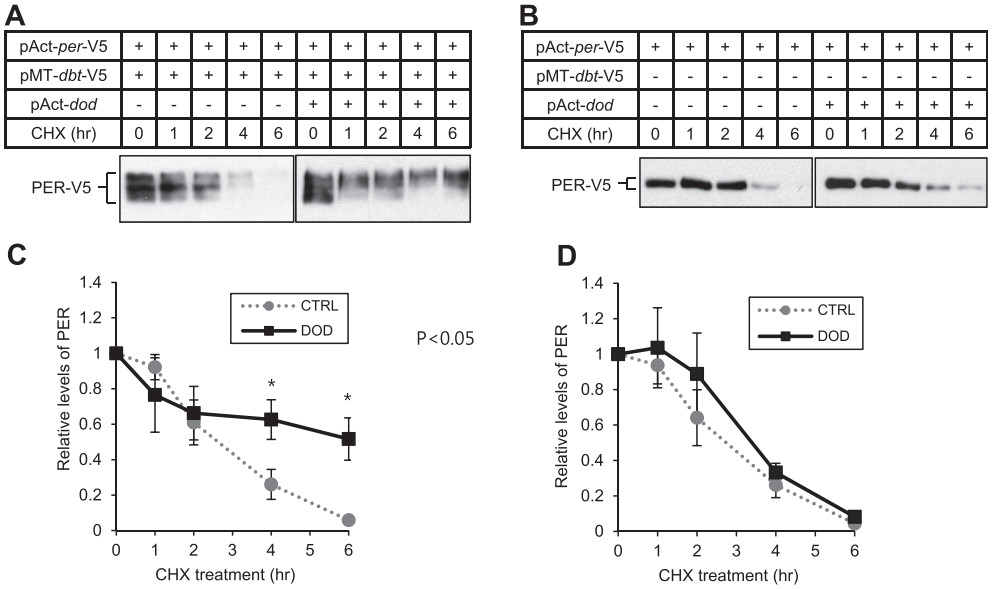

Fig. 3. Overexpression of Dod protects DBT-dependent phosphorylated PER, but not un/hypo-phosphorylated PER, from degradation. S2 cells were transiently transfected with 600 ng of *pAct-per*-V5 in the presence (+) or absence (−) of 200 ng of *pMT-dbt-V5* or 600 ng of *pAct-dod* as indicated at the top of each panel. At 24 h post-DBT induction (A) or 36 h post-transfection (B), a final concentration of 10 μg/ml cycloheximide (CHX) was added to the medium. Cells were harvested at the indicated times after CHX treatment, and protein extracts were subjected to immunoblotting. (C, D) Quantification of PER levels shown in A and B in addition to three other independent experiments was performed using ImageJ software (*$p < 0.05$, error bars denote standard error of the mean [S.E.M.]).

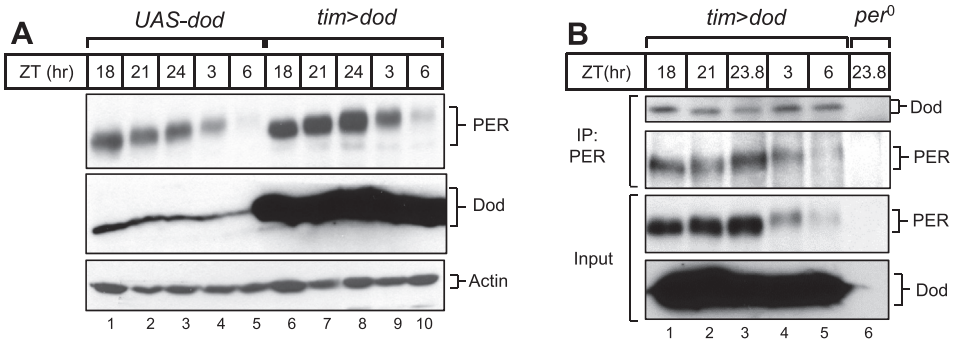

Fig. 4. PER levels were increased in flies overexpressing *dod*. Adult control flies (*UAS-dod*) and flies overexpressing *dod* (*tim > dod*) were entrained in 12:12 LD entrainment. per^0 flies, which do not express PER, were used as negative controls. On the third day of entrainment, flies were collected, and protein extracts were obtained for direct immunoblotting (A) and immunoprecipitation (B) analysis.

core clock proteins. In this study, we demonstrate that the overexpression of *dod* in *tim*-expressing clock cells lengthened the circadian locomotor period in *Drosophila*, which suggests a novel role for Dod in fine-tuning circadian period.

To investigate the effects of Dod on core clock proteins, which play a key role in setting circadian period, we analyzed PER metabolism in the presence or absence of co-expressed Dod in *Drosophila* S2 cells. Interestingly, co-expression of Dod spared the hyper-phosphorylated isoforms of PER (Fig. 2B) in the mechanism of preferential interaction of Dod with phosphorylated PER species (Fig. 2A). These results suggest that Dod likely delays the degradation kinetics of phosphorylated PER by altering its conformation. It is well established that hyper-phosphorylated PER is degraded through the ubiquitin-proteasome pathway via recognition of Ser47 phosphorylation by SCF complex, which contains the ubiquitin ligase, *slimb* [13,14,19]. It is interesting that changes in protein stability are the most common consequences of mammalian PIN1-mediated conformational changes [reviewed in Ref. [37]]. PIN1 either stabilizes or destabilizes proteins by inducing *trans* to *cis* or *cis* to *trans* conformational changes, depending on the specific phospho-Ser/Thr-Pro residue in the target substrates [reviewed in Ref. [37]]. For example, PIN1 protects Cyclin D1, β-catenin, p53, and other proteins from degradation by the ubiquitin-proteasome pathway. In contrast, cMyc, PML, and TAU become destabilized upon PIN1-mediated conformational changes. Interestingly, PIN1 stabilizes early mitotic inhibitor 1 (Emi1) by attenuating its association with the SCF complex containing β-TrCP, a mammalian *Slimb* homolog [38]. From structural studies, it has been shown that the SCF^{cdc4} ubiquitin ligase complex preferentially binds to *trans* conformations of the phospho-Ser/Thr-Pro motif, suggesting that prolyl isomerase-induced conformational changes could be pivotal determinants in the regulation of ubiquitin-proteasome pathway-mediated protein degradation [39].

Consistent with S2 cell results, PER levels were reproducibly higher in flies overexpressing *dod*, and PER exhibited stable interactions with Dod (Fig. 3A and B). Although the amount of Dod that precipitated with the PER antibody was similar at all time points tested (Fig. 3B), we suspect that increased interactions between Dod and PER occurred during the early morning (Fig. 3B, e.g. from ZT3 to ZT6) given that the amount of hyper-phosphorylated PER was decreased at these times due to hyper-phosphorylation-dependent degradation (Fig. 3B, second panel from the top, compare lanes 1–3 to 4–5). Nonetheless, these results do not rule out the possibility that increased PER levels might have arisen by an indirect mechanism in which Dod changes the activity of other substrate(s), which affect PER metabolism, such as kinase(s) and/or phosphatase(s). Additionally, previous studies demonstrated that NEMO, which belongs to the Nemo-like kinase (Nlk) family of proline-directed Ser/Thr kinases, phosphorylates PER at Ser596 [6]. This delays the degradation of PER by inducing the DBT-mediated phosphorylation of nearby Ser/Thr clusters, which results in a more electrostatic-repulsive conformational change such that Slimb has restricted access to Ser47 containing degron on PER [6]. Future work will be required to determine the precise mechanism that results in increased PER levels in flies overexpressing *dod*.

In summary, our work suggests that phosphorylation-dependent prolyl isomerase functions in regulating circadian clock machinery. It is intriguing to note that mammalian PIN1 is upregulated in most human cancers [40–42]. Given that disruption of circadian rhythm occurs in more than 50% of metastatic cancers, leading to poor prognosis [reviewed in Ref. [43]], it is plausible that up-regulation of PIN1 might affect cancer progression partly by disrupting circadian clocks, in addition to affecting cell cycle progression.

Conflict of interest

None.

Acknowledgments

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