Posttranslational regulation of *Drosophila* circadian clocks

By

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ABSTRACT

Circadian clocks enable organisms to anticipate predictable environmental changes over 24-hour day-night cycles on Earth to promote health and fitness. Since the discovery of the first clock gene in *Drosophila melanogaster*, *Drosophila* has been a model organism revealing the molecular underpinnings of animal circadian clocks. The first two chapters of this thesis will investigate the regulation of molecular clocks by posttranslational mechanisms. In particular, we will investigate the functions of core clock protein phosphorylation in *Drosophila*. Despite the importance of rhythmic gene expression programs produced by the molecular clock in generating daily biological rhythms, it has now been established that phosphorylation of core clock proteins that make up the molecular clock represent central and conserved timing mechanisms across organisms. Chapter One will investigate mechanisms by which phosphorylation regulates the major circadian transcriptional activator CLOCK (CLK) of rhythmic genes in *D. melanogaster*. CLK phosphorylation states exhibit daily rhythms and regulate its abundance, subcellular localization, and transcriptional activity. We characterized the role of casein kinase 1 alpha (CK1α) as a novel CLK kinase. We identified CK1α-dependent CLK phosphorylation sites using mass spectrometry proteomics. We next characterized the function of phosphorylation at Serine 13 (S13) residue. Our results showed that CLK(S13) phosphorylation reduces the binding activity of CLK to circadian promoters, therefore reducing CLK transcriptional activity. We also showed that CK1α-CLK interaction is dependent on PERIOD (PER), the circadian transcriptional repressor that is known to inhibit CLK function. We revealed a mechanism by which repressor-dependent phosphorylation of an activator inhibits its transcriptional activity and thus closing the transcriptional-translational feedback loop (TTFL) of the molecular clock.

In Chapter Two, we focused on a comprehensive investigation of phosphorylation on TIMELESS (TIM), the heterodimeric partner of PER. We first identified phosphorylation sites on

PER-bound TIM using mass spectrometry. We found that abolishing phosphorylation at some of these residues caused altered circadian behavioral rhythms. Phosphorylation at Serine 1404 (S1404) residue promotes TIM nuclear accumulation by reducing its interaction with exportin 1 (XPO1), a nuclear export machinery. Reduced nuclear localization of TIM in S1404A nonphosphorylatable fly mutants not only resulted in lower level of nuclear PER and TIM, but also showed dampened daily rhythms in CLK phosphorylation. This is likely caused by reduced recruitment of CLK kinase by PER-TIM complex. Taking Chapter One and Two together, we provide mechanistic insights into CLK and TIM phosphorylation and how these posttranslational modifications are indispensable for the maintenance of 24-hour molecular oscillation to regulate circadian rhythms.

Chapter Three will review our current understanding of the function of *Drosophila* TIM (dTIM) and mammalian TIM (mTIM). dTIM is a cardinal clock protein, whereas the role of mTIM in regulating circadian rhythms are under debate since it was first identified over two decades ago. We will summarize the circadian and non-circadian roles of the two TIM paralogs and discuss the potential mechanisms by which mTIM regulates circadian rhythms via its non-circadian roles. We will conclude Chapter Three by summarizing recent findings about potential functional parallel between mTIM and dTIM. Overall, the research included in this thesis will provide mechanistic insights into the regulation of animal circadian clocks and contribute to future development of therapeutics for clock-related human diseases including cancer.

INTRODUCTION

The Earth rotates on its axis around the Sun with a period of ~24 hours. This gives rise to rhythmic environment on Earth, including day-night cycle, daily temperature cycle, as well as rhythms in other resources necessary for organismal survival and fitness. Since the environment changes in a relatively predictable manner, it is not surprising for organisms on Earth to evolve an endogenous timing system to anticipate these changes. This timing system, termed circadian clock, has been found in all domains of life, from bacteria to mammals (Dunlap, 1999). Circadian clocks exhibit three major properties (Pittendrigh, 1974). The first property is their abilities to align with local time, which is termed entrainment. Circadian clocks can reset everyday according to environmental time cues, such as light, temperature, and food intake. The second property is their abilities to free-run and maintain oscillation in the absence of external time cues. Even though constant environment doesn't exist in nature, this feature helps to maintain amplitude in physiology and behavior at times when environmental time cues are weak, for example on a cloudy rainy day. The third property is the resistance of temperature-induced changes in period length, termed temperature compensation. This allows a constant pace of circadian clocks regardless of fluctuation in a reasonable range of ambient and body temperature.

At the cellular level, molecular oscillators comprise of interlocked transcriptional-translational feedback loops (TTFLs). In *D. melanogaster*, the primary TTFL comprises of transcriptional activator CLOCK (CLK), CYCLE (CYC) and transcriptional repressor PERIOD (PER) and TIMELESS (TIM). The characterization of these molecular underpinnings started over half a century ago. Konopka and Benzer (1971) identified the first clock gene, *per*, in *D. melanogaster*. Given that PER protein level peaks a few hours after the peak of *per* mRNA, a negative feedback loop was proposed where PER represses its own expression (Hardin et al.,

1990). *tim* was identified as the second core clock gene that functions to promote *per* cycling (Sehgal et al., 1994; Vosshall et al., 1994). In 1998, the identification of *clk* and *cyc* closed the primary TTFL (Darlington, 1998; Bae et al., 1998; Allada et al., 1998). In the secondary TTFL, CLK drives the expression of VRILLE (VRI) and PDP1ε, which regulate *clk* expression by acting as repressor and activator, respectively (Cyran et al., 2003). Later studies showed that disrupting *pdp1ε* and *vri* leads to arrhythmic clock output pathways without compromising the oscillation of the primary TTFL (Benito et al., 2007; Zheng et al., 2009; Gunawardhana and Hardin, 2017). This suggests that the secondary TTFL in *Drosophila* is a clock output regulator, instead of core clock components.

TTFLs alone are not sufficient to generate 24-hour oscillations. Perhaps the best evidence is that fly mutants with constitutive *per* and *tim* expression still exhibit robust molecular and behavioral rhythms (Vosshall et al. 1995; Cheng and Hardin, 1998; Yang and Sehgal, 2001). In addition, TTFL-based oscillation requires addition regulatory mechanisms to maintain precise period length of 24 hours (Lev Bar-Or et al., 2000; Stewart-Ornstein et al., 2017). Several mechanisms built upon TTFLs have been shown to generate robust circadian oscillations, including but not limited to chromatin landscape, interaction with other transcription factor networks, post-transcriptional control, and posttranslational regulation (reviewed in Kojima et al., 2011; Tataroglu and Emery, 2015; Kwok et al., 2015; Mendoza-Viveros et al., 2017).

Close to three decades of research established posttranslational modification, especially phosphorylation, as the cardinal biochemical timer (Brown et al., 2012). In cyanobacteria, 24-hour rhythms in the phosphorylation state of core clock protein KaiC can be observed by putting three purified clock proteins and ATP together in a test tube (Nakajima et al., 2005), demonstrating the central roles of phosphorylation in circadian timekeeping. In *Drosophila*, phosphorylation regulates both PER, TIM and CLK functions. Rhythms in PER phosphorylation was first observed in 1994 (Edery et al., 1994). Since its hyperphosphorylation precedes PER

degradation, phosphorylation was thought to mediate PER degradation. DOUBLETIME (DBT) was the first kinase identified to phosphorylate and regulate PER accumulation (Price et al., 1998; Kloss et al., 1998). Mass spectrometry-based phosphorylation site mapping produced significant insights into phosphorylation regulation (Kivimae et al., 2008; Chiu et al., 2008). DBT-dependent PER phosphorylation at *per*-short downstream domain promotes PER repressor activity (Kivimae et al., 2008), whereas DBT-dependent phosphorylation at the N-terminal Serine 47 (S47) leads to PER degradation by increasing the binding of PER and SLIMB (Chiu et al., 2008). Interestingly, phosphorylation at S596 residue by NEMO kinase stimulates DBT-dependent phosphorylation at nearby S589 and S585 to delay S47 phosphorylation (Chiu et al., 2011), demonstrating an intricate phosphorylation-based delay mechanism to extend the period length to 24 hours. In addition to the regulation of stability, CK2-dependent and SGG-dependent phosphorylation at S151/S153 and S657 have been shown to promote nuclear entry of PER (Lin et al., 2005; Ko et al., 2010).

As the binding partner of PER, TIM also undergoes time-of-day specific phosphorylation. The function of CK2 and SGG were tested in this context. CK2 was first shown to promote nuclear entry of PER-TIM repressor complex (Akten et al., 2003). Since CK2-dependent phosphorylation sites were characterized in PER (Lin et al., 2005), PER was thought to be the major target of CK2 in regulating PER-TIM nuclear entry. However, Meissner et al. (2008) showed that CK2 cannot regulate PER in tim^0 null mutants, suggesting TIM as the major target in this context. Consistent with this notion, Top et al. (2016) revealed that CK2 and SGG collaborate to phosphorylate S297, T301, T305, S309 and S313 to promote nuclear entry of TIM. TIM phosphorylation signals nuclear entry possibly by inducing conformational change to expose the nuclear localization signal (NLS) which is recognized by Importin α1 (Imp α1) nuclear import complex (Jang et al., 2015). In addition, phosphorylation at unknown tyrosine residues regulates light-induced TIM degradation (Naidoo et al., 1999). However,

comprehensive mapping of TIM in fly tissues was not reported, which hindered further understanding the mechanisms by which phosphorylation regulates TIM function.

Similar to PER and TIM, CLK also undergoes daily rhythms in phosphorylation. Lee et al. (1998) first reported the phosphorylation of CLK and speculated its regulation in CLK abundance and transcriptional activity. CK2-dependent CLK phosphorylation was reported to stabilize and repress its transcriptional activity (Szabo et al., 2013). NEMO was also implicated to phosphorylate and destabilize CLK (Yu et al., 2011). Interestingly, CLK phosphorylation is partially dependent on PER. Yu et al. (2006) showed that CLK remains hypophosphorylated in per^0 null mutant. Since PER does not have kinase activity, a model was proposed where PER recruits kinases to phosphorylate CLK. However, the kinase(s) responsible for PER-dependent CLK phosphorylation remain(s) unknown, despite a few follow up studies (Kim and Edery, 2006; Yu et al., 2009, 2011). Recent mathematical modeling and systems analysis revealed that repressor-dependent phosphorylation on the activator is necessary for generating robust circadian oscillation (Tyler et al., 2022). Therefore, understanding how PER-dependent phosphorylation regulates CLK activity may shed light on the conserved module of animal circadian clocks.

In Chapter 1, we characterized the roles of CK1α-dependent CLK phosphorylation. Since CK1α can phosphorylate PER in mouse and *Drosophila* (Hirota et al., 2010; Lam et al., 2018), we hypothesized that CK1α is a candidate CLK kinase to modulate CLK activity. We found that CK1α is a novel CLK kinase that binds and phosphorylates CLK. We next identified CK1α-dependent CLK phosphorylation sites, including Serine 13 (S13) localized next to the DNA binding domain. Flies harboring a mutation at S13 display altered circadian locomotor activity rhythms, suggesting a functional role for S13 in circadian timekeeping. Flies expressing S13D phosphomimetic variant showed reduced CLK transcriptional activity, which is caused by compromised CLK-DNA binding. Moreover, we showed that PER is required for CK1α-CLK

interaction. Together with findings in mouse and *Neurospora*, we propose a conserved feature where repressor complexes recruit CK1 kinases to phosphorylate and quench the transcription activity of activators in fungal and animal clocks.

In Chapter 2 (Cai et al., 2021), we describe the functional characterization of PER-bound TIM phosphorylation. As mentioned above, comprehensive TIM phosphorylation site mapping in fly tissues was lacking, hindering site-specific investigation on how time-of-day function of TIM is regulated. We mapped PER-bound TIM phosphorylation sites via affinity purification of TIM from fly tissues followed by mass spectrometry proteomics. We found several phosphomutants with altered circadian locomotor phenotypes. Among them, S1404A nonphosphorylatable mutant showed reduced nuclear accumulation. TIM(S1404A) variant has increased binding to XPO1 nuclear export machinery. This action decreases nuclear PER and influences CLK phosphorylation and transcriptional activity. Significantly, we demonstrated that the phosphorylation at the repressor complex can influence the phosphorylation of the activator.

Drosophila TIM (dTIM) is a well-established clock component. Therefore, its mammalian paralog mammalian TIM (mTIM) was also thought to be a component in mammalian clocks when it was first identified. However, several lines of evidence questioned the role of mTIM as a cardinal clock component.

In Chapter 3 (Cai and Chiu, 2021), we review the function of dTIM in the *Drosophila* circadian clock, in the interface of circadian clocks and seasonal biology, and other non-circadian biological processes. We next discussed the circadian and non-circadian roles of mTIM and propose that mTIM regulates circadian clocks via its non-circadian roles. Finally, we summarized recent findings that revealed potential functional parallel between dTIM and mTIM.

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Chapter 1. CK1α-dependent phosphorylation reduces CLOCK DNA binding and transcription activity to regulate *Drosophila* circadian rhythms

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ABSTRACT

Circadian clocks regulate daily rhythms in organismal physiology and behavior to optimize health and fitness. In *Drosophila*, phosphorylation regulates time-of-day functions of core clock proteins, including the transcriptional activator CLOCK (dCLK). However, it remains unclear how dCLK phosphorylation represses and/or removes DNA-bound dCLK at circadian promoters to facilitate the closure of the negative feedback loop. In this study, we demonstrated that Casein kinase 1 alpha (CK1α) is a dCLK kinase and identified CK1α-dependent dCLK phosphorylation sites. We revealed using genetic and biochemical analyses that upon CK1α phosphorylation at dCLK(S13), dCLK occupancy at circadian promoters decreases, thereby sequestering dCLK transcriptional activity. Moreover, our results suggest that the transcriptional repressor PERIOD (PER) and the major clock kinase DOUBLETIME (DBT) facilitate CK1α-dCLK interaction. This study highlights the importance of posttranslational regulation of circadian rhythms. Finally, together with previous studies in fungi and mammals, our results suggest a conserved feature in eukaryotic clocks by which transcriptional repressors recruit CK1s to modulate the activity of transcription activators.

INTRODUCTION

Circadian clocks regulate daily rhythms on physiology and behavior to optimize health and fitness in organisms from all domains of life (Johnson et al., 2017; Dunlap and Loros, 2017; Swan et al., 2018; Cox and Takahashi, 2019; Patke et al., 2020; Creux and Harmer, 2019; Xu et al., 2022). These rhythms are driven by molecular clocks at the cellular level. A common feature for animal and fungal molecular clocks is transcription-translation feedback loops (TTFLs) (Patke et al., 2020). TTFLs, first proposed in 1990 (Hardin et al., 1990), consist of positive elements activating the transcription and translation of negative elements, which close the feedback loops by inhibiting the transcriptional outcome of positive elements. As repression is relieved, the next cycle of transcription begins. In addition, positive elements also rhythmically drive other clock-controlled genes (ccgs) in cell- and tissue-specific manner, which manifest in circadian physiology and behavior (Abruzzi et al., 2011; Zhang et al., 2014, Mure et al., 2018; Beytebiere et al., 2019). Since the discovery of negative elements in molecular clocks, a vast number of studies have investigated the mechanisms by which they confer repression activity in different model systems. Negative elements form a repressor complex and confer repression in multiple ways, which is thought to be required for a strong oscillator (Jeong et al., 2022). The repressor complexes can deliver to activators cargo corepressors, including histone modifiers (Taylor and Hardin, 2008; Duong et al., 2011; 2014; Kim et al., 2014), histone chaperones (Tartour et al., 2022) and helicases (Padmanabhan et al., 2012). Additionally, the repressor complexes can directly remove or displace the activators of circadian transcriptomes from the DNA (Menet et al., 2010; Ye et al., 2014; Xu et al., 2015; Chiou et al., 2016; Zhou et al., 2016).

Posttranslational modifications of positive elements have been shown to modulate their activity (Kondratov et al., 2003; Schafmeier et al., 2005; Kim and Edery, 2006; Weber et al., 2006; He et al., 2006; Yu et al., 2006; Huang et al., 2007; Shim et al., 2007; Yoshitane et al., 2009; Luo et al., 2012; Tamaru et al., 2015; Cho et al., 2019; Cao et al., 2021), adding

additional mechanistic insights on how activities of positive elements are repressed. Experimental evidence and mathematical modeling have established the indispensability of phosphorylation of positive elements for the generation of oscillations (Larrondo et al., 2015; Wang et al., 2019; Tyler et al., 2022). In *Drosophila*, the positive element CLOCK (dCLK) displays daily rhythms in phosphorylation level (Lee et al., 1998; Bae et al., 2000). Highly phosphorylated dCLK isoforms that appear at late night have low transcriptional activity and are gradually removed from circadian promoters, whereas dephosphorylated and newly synthesized hypophosphorylated dCLK binds DNA and exhibits high transcriptional activity after nuclear entry (Yu et al., 2006; Hung et al., 2009; Andreazza et al., 2015). Given that the onset of dCLK hyperphosphorylation occurs after nuclear translocation of the PERIOD-TIMELESS (PER-TIM) repressor complex, several studies have proposed that PER recruits dCLK kinase(s) to phosphorylate and remove dCLK from circadian promoters (Yu et al., 2006; Nawathean et al., 2007; Kim and Edery, 2006; Yu et al., 2009; Cai et al., 2021). However, it remains unclear how dCLK phosphorylation represses and/or removes DNA-bound dCLK at circadian promoters to facilitate the closure of the negative feedback loop.

To identify what kinase(s) is(are) responsible for PER-dependent dCLK phosphorylation, a few previous studies tested kinases that were previously identified in the PER-TIM repressor complex, which include DOUBLETIME (DBT) (Kloss et al., 1998; Price et al., 1998), Casein kinase 2 (CK2) (Top et al., 2016), and NEMO (Yu et al., 2011). DBT was first thought to be the dCLK kinase, since flies expressing PER (Δ), a PER variant unable to bind DBT, display constant low dCLK phosphorylation (Kim and Edery, 2006). However, flies expressing *dbt*(KR), a kinase-dead variant, exhibit dCLK hyperphosphorylation, suggesting that DBT may in fact be a scaffold for yet uncharacterized kinase(s) to phosphorylate dCLK (Yu et al., 2006, 2009). Although CK2 modulates dCLK stability and transcriptional activity, this effect still occurs in the *per* null mutant (per^0), suggesting a PER-independent regulation of CK2 on dCLK (Szabó et al.,

2013). Finally, Yu et al. (2011) showed that NEMO interacts and stabilizes dCLK, demonstrating NEMO as a candidate kinase of dCLK. However, direct evidence showing NEMO phosphorylating dCLK are lacking to date. Thus, it remains unclear which kinase(s) phosphorylate dCLK in a PER-dependent manner.

We recently reported that Casein kinase 1α (CK1α) interacts with PER (Lam et al., 2018). We therefore ask whether CK1α plays a role in the phosphorylation and regulation of dCLK. In this study, we characterize the role of CK1α in regulating dCLK-DNA binding. We found that CK1α binds and phosphorylates dCLK in *Drosophila* S2 cells. We then employed mass spectrometry-based proteomics to identify CK1α-dependent phosphorylation of dCLK expressed in and purified from S2 cells. Our results identified five CK1α-dependent and several CK1α-independent phosphorylation sites on dCLK, including Serine 13 (S13), a phosphosite adjacent to the basic helix-loop helix (bHLH): DNA binding domain of dCLK. Next, we found that mutating S13 to nonphosphorylatable alanine (A) or phosphomimetic aspartic acid (D) both resulted in altered circadian locomotor activity rhythms. By analyzing the molecular clock of S13 mutants, we provide evidence supporting the importance of dCLK(S13) phosphorylation in circadian timekeeping. Specifically, dCLK(S13) phosphorylation decreases dCLK occupancy at dCLK-target promoters, which causes reduced dCLK-target mRNA levels. Significantly, we showed that PER-DBT interaction influences CK1α-dependent downregulation of dCLK transcriptional activity. Taken together, our results suggest that CK1α is recruited by PER-DBT to modulate dCLK transcriptional activity and CK1α-dependent dCLK(S13) phosphorylation reduces dCLK-DNA binding and transcriptional activity to regulate circadian rhythms.

RESULTS

CK1α physically interacts and phosphorylates dCLK

We first sought to determine whether CK1α physically interacts with dCLK. To detect this interaction, protein extracts from Drosophila S2 cells coexpressing dCLK-V5 and CK1α-cmyc were utilized to perform coimmunoprecipitation (coIP) assays (Figures 1A-C). We detected interactions between dCLK and CK1α when using dCLK-V5 as a bait. Reciprocal coIP using CK1α-cmyc as bait also detected dCLK-V5 and resulted in the same conclusion. Control experiments were performed using extracts of S2 cells expressing either of the proteins alone, clearing showing minimal non-specific binding (Figure 1B).

Next, we determined whether CK1α phosphorylates dCLK. We analyzed dCLK in protein extracts of *Drosophila* S2 cells expressing either dCLK alone or dCLK coexpressed with CK1α. We observed slower-migrating dCLK isoforms on an SDS-PAGE gel (Figure S1), likely representing phosphorylated dCLK. Phos-Tag SDS-PAGE gel (Kinoshita et al., 2006) was used to enhance phosphorylation-dependent mobility shift (Figures 1D and 1E). In addition, to test whether CK1α catalytic activity is responsible for the observed mobility shift, we coexpressed dCLK with either CK1α(WT) or CK1α(K49R), a kinase-dead variant (Lam et al., 2018). We observed substantially more slower migrating dCLK isoforms in the presence of CK1α(WT). The abundance of slower-migrating isoforms was significantly reduced with CK1α(K49R) coexpression. These results indicate that CK1α kinase activity is required for dCLK phosphorylation. Taken together, our results show that dCLK is a substrate of CK1α.

Mass spectrometry analysis identifies CK1α-dependent dCLK phosphorylation sites

To understand the mechanisms by which CK1α regulates dCLK and circadian rhythms, we leveraged mass spectrometry (MS) to identify CK1α-dependent phosphorylation sites of dCLK expressed in *Drosophila* S2 cells. This cell culture system has been used to map physiologically relevant phosphorylation sites on *Drosophila* PER (Chiu et al., 2008; Ko et al.,

2010; Garbe et al., 2013), TIM (Kula-Eversole et al., 2020) and dCLK (Lee et al., 2014; Mahesh et al., 2014). We coexpressed dCLK tagged with FLAG epitope with either CK1α or CK1α(K49R) in S2 cells and performed FLAG affinity purifications prior to MS analysis. We identified eight phosphorylation sites on dCLK (Figure 2A and Table 1). Among them, we identified five sites that exhibited elevated phosphopeptide abundance when coexpressed with CK1α as compared to the kinase-dead K49R variant. These CK1α-dependent sites include Serine 13 (S13), which is next to the bHLH DNA binding domain (Darlington et al., 1998; Bae et al., 1998); S258 and S311 next to PAS B protein binding domain (Darlington et al., 1998; Bae et al., 1998); and T484 within the nuclear localization signal (NLS) (Hung et al., 2009). Based on the location of these CK1α-dependent phosphorylation sites, we reasoned that they may modulate dCLK-DNA binding, interaction of dCLK with other proteins, and subcellular distribution of dCLK respectively.

Flies harboring mutations at dCLK(S13) display altered circadian behavioral rhythms

The proximity of dCLK(S13) to the bHLH domain (Darlington et al., 1998; Bae et al., 1998) hints at its functionality at regulating dCLK-DNA binding and thus the role of dCLK as a positive element in the TTFL of the molecular clock. To determine if CK1α-dependent dCLK(S13) phosphorylation regulates circadian timekeeping, we generated mutant transgenic fly lines swapping S to nonphosphorylatable Alanine (A) (S13A) or S to Aspartic acid (D) which acts as a phosphomimetic (S13D). Nomenclature for the transgenic lines is p{*clk*(X)-V5} where X represents WT, S13A or S13D variants transgenes. These lines were generated and crossed into clk^{out} deletion background (Mahesh et al., 2014) to remove endogenous *clk* expression. Next, we monitored locomotor activity rhythms of *clk* transgenic flies as a robust and reliable behavioral output of the circadian clock (Chiu et al., 2010). Flies were entrained for 4 days in 12h:12h Light:Dark cycles (herein referred as LD cycle) followed by monitoring of free-running

rhythms for 7 days in constant darkness (DD). As expected, *clk*^{out} null mutant exhibited arrhythmic locomotor activity (Mahesh et al., 2014). Homozygous *clk*(WT) flies displayed robust rhythms with a ~24-hour period, indicating rescue of the arrhythmic *clk*^{out} mutation (Figure 2B and Table 2). Heterozygous *clk*(WT) flies exhibited longer periods by 0.6 hours, which is consistent with previous findings that reduced *clk*(WT) dosage lengthens circadian period (Allada et al., 1998). As compared to heterozygous *clk*(WT), heterozygous *clk*(S13A) displayed period lengthening by 1.1 hours and reduced rhythmicity. Heterozygous *clk*(S13D) also exhibited reduced rhythmicity and the severe arrhythmic phenotype made it difficult to determine if their period lengths were altered. Homozygous *clk*(S13D) flies similarly showed reduced rhythmicity with some indication of period lengthening by 1.2 hours as compared to homozygous *clk*(WT) flies. Taken together, dCLK(S13) phosphorylation is required for circadian timekeeping.

dCLK(S13) phosphorylation decreases dCLK occupancy at dCLK-target gene promoters

To determine if dCLK(S13) phosphorylation influence behavioral rhythms by modulating dCLK-DNA binding, we performed dCLK chromatin immunoprecipitation (dCLK-ChIP) followed by quantitative PCR (qPCR) using extracts from adult fly heads (Figure 3). We observed significantly lower dCLK occupancy in *clk*(S13D) mutants as compared to *clk*(WT) flies at ZT19 at *per CRS* (Figure 4A), a region within the promoter critical for generating rhythmic *per* expression (Hao et al., 1997, 1999). dCLK occupancy at multiple time points at *tim* E-box and *vri* E-box are also significantly lower in *clk*(S13D) mutants (Figures 4B and 4C). At *pdp1*ε E-box (Figure 4D), dCLK occupancy of *clk*(S13D) mutants is not significantly different to that in *clk*(WT) flies. We reasoned that the differential effect of dCLK(S13) phosphorylation on decreasing dCLK occupancy of various dCLK-target promoters tested could be due to their respective local chromatin landscape. Although not included in this thesis, our lab also has data

from *in vitro* experiments showing that dCLK(S13D) purified from *E. coli* showed reduced binding to E-box DNA as compared to dCLK(WT) (data not shown). Together with data from *in vitro* experiments, our results revealed that dCLK occupancy at dCLK-target gene promoters decreases upon dCLK(S13) phosphorylation.

dCLK(S13) phosphorylation reduces dCLK transcriptional activity

Next we sought to determine if decreases in dCLK occupancy on clock gene promoters as a result of dCLK(S13) phosphorylation result in reduction in dCLK transcriptional activity. Using *per-luciferase* (*per-luc*) transcriptional reporter assay in *Drosophila* S2 cells (Darlington et al., 1998; Nawathean and Rosbash, 2004), we examined dCLK transcriptional activity in conditions where we up- or downregulate CK1α (Figures 4A-B). We observed a significant increase in *per-luc* activity when we treated S2 cells with *ck1*α dsRNA to knock down endogenously expressed *ck1*α in the presence of dCLK (Figure 4A). On the contrary, overexpressing CK1α significantly reduced dCLK-dependent *per-luc* activity (Figure 4B). Furthermore, expression of dCLK(S13A) and dCLK(S13D) led to *per-luc* activities significantly above and below that of dCLK(WT), respectively (Figure S2). Our results suggest that CK1α reduces dCLK transcriptional activity partly through phospho-occupancy of S13.

To determine if dCLK(S13) phosphorylation-mediated downregulation in transcriptional activity translates to whole animals, we quantified known dCLK mRNA targets including core clock genes. We observed significant reduction in levels and cycling amplitude of mRNAs of canonical dCLK targets, including *per*, *tim*, *pdp1*ε and *vri*, in *clk*(S13D) mutants as compared to *clk*(WT) flies (Figures 4C-F and Table S3). The dampened mRNA oscillation in *clk*(S13D) flies is consistent with dampened behavioral rhythmicity (Figure 2B). In the case of *clk*(S13A) flies, the peak phase of dCLK-target mRNAs is delayed (Figure S3 and Table S3), which is consistent with lengthened behavioral period (Figure 2B). Interestingly, the abundance of dCLK-target

mRNAs in *clk*(S13A) mutants are comparable to that in *clk*(WT) flies, except that *pdp1ε* mRNA at ZT16 is significant higher in *clk*(S13A) mutants (Figure S3). Taken together, our results suggest that CK1α-dependent dCLK(S13) phosphorylation downregulates dCLK transcriptional activity, although there is clear discrepancy between the extent to which the *clk*(S13A) mutation impacts dCLK target gene expression in tissue culture vs in whole animals.

Finally, given that previous studies showed that phosphorylation-dependent attenuation of dCLK stability (Szabó et al., 2013) is also a plausible mechanism to reduce dCLK transcriptional activity, we wanted to rule out the possibility that CK1α can modify dCLK stability (Figure S4). To measure dCLK degradation independent of TTFLs, we performed cycloheximide (CHX) chase assays in *Drosophila* S2 cells expressing dCLK with or without CK1α. We observed similar rates of dCLK protein degradation in the absence or presence of CK1α, suggesting that CK1α does not regulate dCLK stability.

PER-DBT interaction influences CK1α-dependent downregulation of dCLK transcriptional activity

PER-TIM repressor complexes recruit yet uncharacterized kinases for timely dCLK phosphorylation to enhance repression (Yu et al., 2009; Cai et al., 2021). Since CK1α has been shown to interact with PER in both cytoplasm and nucleus (Lam et al., 2018), we hypothesized that CK1α plays a role to enhance PER repression of dCLK activity (Figure 5A). We first performed *per-luc* reporter assay to measure dCLK transcriptional activity in S2 cells expressing dCLK and PER in the absence or presence of CK1α (Figure 5B). As expected, PER expression downregulates dCLK transcriptional activity. In congruency with our model (Figure 5A), CK1α coexpression further reduced dCLK transcriptional activity, indicating an enhanced PER repression. We observed no significant difference between baseline luciferase activity and cells

expressing dCLK and PER in conjunction with CK1α, suggesting that PER and CK1α together essentially abolished dCLK transcriptional activity.

CK1α regulates PER repression activity by favoring PER nuclear entry and promoting PER-DBT interaction and phosphorylation-dependent degradation (Lam et al., 2018). We expressed NLS-tagged PER (PER-NLS) and DBT(K/R), a kinase-dead DBT variant (Muskus et al., 2007), in order to minimize the regulatory effect of CK1α on PER and more specifically examine the role of CK1α in modulating dCLK activity (Figure 5C). We observed a significant reduction in dCLK-dependent *per-luc* activity upon CK1α coexpression with PER-NLS and DBT(K/R). This suggests that in addition to the regulation of CK1α on PER nuclear entry and stability, the enhanced CK1α-dependent PER repression of dCLK is also mediated through dCLK phosphorylation by CK1α.

Previous studies suggest PER-DBT functions as a scaffold to recruit dCLK kinases (Yu et al., 2009). We asked whether CK1α requires DBT in complex with PER to regulate dCLK transcriptional activity. To remove DBT from PER complex, we leveraged PER(Δ), a variant lacking DBT binding domain (Nawathean et al., 2007; Kim et al., 2007). Furthermore, to only examine the scaffolding function of DBT, we continued to use the kinase-dead DBT(K/R) in these experiments. To our surprise, we observed a significant decrease in dCLK-dependent *per-luc* activity upon CK1α expression in cells coexpressing PER(Δ) and DBT(K/R) (Figure 5D), indicating PER-DBT interaction is not required for CK1α to modulate dCLK activity in the experimental condition tested. However, we noticed a significant decrease in CK1α-dependent change in *per-luc* luciferase activity in cells expressing PER(Δ)-NLS as compared to PER(WT)-NLS (Figure 5E). In sum, our results suggest PER-DBT interaction enhances CK1α-dependent regulation of dCLK transcription activity. The synergistic downregulation of dCLK transcriptional

activity by CK1α and DBT supports our model that PER-DBT acts as a scaffold to mediate CK1α-dependent phosphorylation of dCLK in the nucleus.

DISCUSSION

Phase-specific phosphorylation of clock proteins is critical to circadian timekeeping in animal clocks. *Drosophila* dCLK protein, the key transcriptional activator of the molecular clock, displays daily rhythm in phosphorylation that correlates with its transcriptional activity. However, the identity of kinase(s) responsible for reducing its transcriptional activity remains largely unknown. Here, we report that CK1α binds to and phosphorylates dCLK at multiple residues located within and close to important functional domains (Figures 1 and 2). By focusing our efforts on characterizing the site-specific function of dCLK(S13), a site close to the bHLH DNA-binding domain of dCLK, we demonstrated that CK1α-dependent S13 phosphorylation participates in circadian timekeeping (Figures 3-6). We propose the following model (Figure 6A): In the window of time around midnight, the nuclear PER-TIM repressor complex exhibits on-DNA repression in which PER complex blocks DNA-bound dCLK transcription activity. Later in the nighttime phase, CK1α in the PER complex phosphorylates the dCLK S13 residue, thereby reducing dCLK occupancy at circadian promoters. As a result, dCLK transcriptional activity is sequestered until the next circadian transcriptional cycle begins.

This study identified CK1α as a novel dCLK kinase (Figure 1). The majority of previous works have focused largely on the function of another CK1 kinase, DBT (CK1δ/ε in mammals), as the key kinase regulating circadian clocks (e.g. Price et al., 1998; Lee et al., 2001; Kim et al., 2007; Chiu et al., 2008). Together with our previous works on CK1α-PER interaction (Lam et al., 2018), we demonstrated that CK1α is another CK1 family member responsible for the regulation of circadian clocks. Interestingly, a recent paper showed that CK1δ directly phosphorylates

mammalian CLOCK (mCLOCK) (Cao et al., 2021), different from previous findings in the *Drosophila* system that DBT kinase activity is not required for dCLK phosphorylation (Yu et al., 2009). It remains possible that mammalian CK1δ also exhibits scaffolding function to deliver other dCLK kinases, likely CK1α. In addition, alternative splicing variants of CK1δ have been shown to regulate clocks (Fustin et al., 2018). Given that CK1α also has splicing isoforms with different substrate affinity (Yong et al., 2000), future investigation is needed to elucidate whether alternative splicing of CK1α contributes to circadian timekeeping.

We found a differential effect of S13 phosphorylation on dCLK occupancy at different circadian promoters (Figure 3). Daily rhythms in histone marks and RNA polymerase II occupancy at *per* and *tim* promoters display distinctive features (Taylor and Hardin, 2008). Similarly, CLOCK INTERACTING PROTEIN CIRCADIAN (CIPC) represses dCLK transcription more at *per* as compared to *tim* and *vri* (Rivas et al., 2021). Moreover, a recent study showed that during repression phase, clock-regulated genes are clustered to form foci at nuclear periphery (Xiao et al., 2021). Taken together, local chromatin environment could influence the extent of dCLK(S13) phosphorylation on dCLK occupancy at circadian promoters. Phosphorylation-dependent regulation of dCLK occupancy may collaborate with other gene/chromatin regulatory mechanisms to regulate circadian gene expression programs.

We have characterized the function of dCLK(S13) phosphorylation in downregulating dCLK transcriptional activity (Figure 4). Previous studies found that S859 phosphorylation (Mahesh et al., 2014) and a collaborative effect of phosphorylation on 15 residues (Lee et al., 2014) both downregulate dCLK transcription activity. Therefore, a progressive phosphorylation program could exist for the precise control of dCLK function, similar to PER (Reviewed in Ozkaya and Rosato, 2012; Narasimamurthy and Virshup, 2021). PP2A phosphatase can target one (or many) of these sites to reverse transcription activity of dCLK (Andreazza et al., 2015). dCLK(S13) is a likely PP2A target given the robust effect of S13 phosphorylation on dCLK

transcription activity as compared to previously characterized sites in Lee et al. (2014) and Mahesh et al. (2014). In addition, we also identified dCLK(S311) and dCLK(T484) as CK1α-dependent phosphorylation sites. Whether these sites regulate dCLK function remains unknown.

Finally, our results suggest a conserved feature of CK1-dependent repression in eukaryotic clocks (Figure 6B). In *Neurospora*, the repressor FREQUENCY (FRQ) mediates CK-1a (homolog of mammalian CK1δ/ε) phosphorylation of activators White Collar Complex (WCC) to dissociate them from DNA (Wang et al., 2019). In mammals, the repressors CRYPTOCHROME (CRY) and PER proteins recruit CK1δ to phosphorylate the activator mCLOCK and cause its dissociation from DNA (Cao et al., 2021). In addition, Marzoll et al. (2022) showed CK1-dependent phosphorylation on FRQ and PERs as conserved timing mechanisms in the fungal and animal clocks. With our findings in the *Drosophila* model showing that PER repressor complexes recruit CK1 kinases to phosphorylate and quench the transcription activity of activators, we add to this conserved pattern observed in fungal and animal clocks.

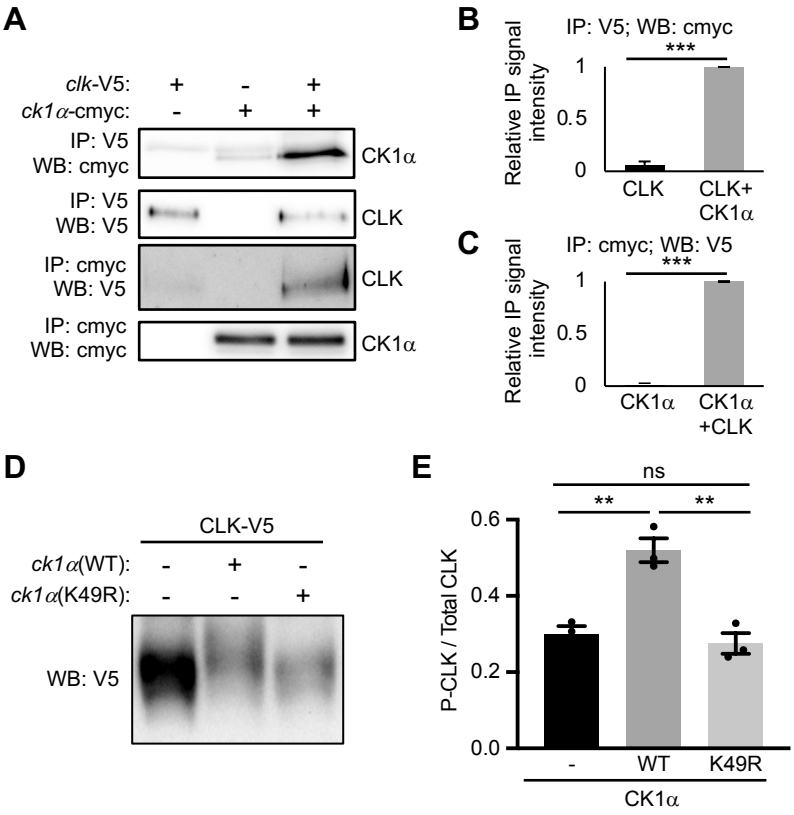

Figure 1. dCLK is a substrate of CK1α. (A) Western blots showing reciprocal coimmunoprecipitation (coIP) experiments to examine interactions of dCLK and CK1α. *Drosophila* S2 cells were cotransfected with pAc-*clk*-V5-His and pMT-*ck1α*-6xc-myc or alone. Protein extracts were divided into two equal aliquots, and each aliquot was independently incubated with either α-c-myc or α-V5 beads. Immuno-complexes were analyzed by western blotting in the presence of the indicated antibody. (B-C) Bar graphs displaying quantification of reciprocal coIPs. Values for binding are normalized to amount of bait detected in the IPs and expressed as relative signal intensity (high value = 1). Error bars indicate ± SEM (n = 3), two-tailed Student's t test. (D) Western blots showing mobility shift of dCLK on Phos-tag gel. S2 cells were transfected with pAc-*clk*-V5 together with either pMT-*ck1α*-FH, pMT-*ck1*α(K49R)-FH, or empty plasmid pMT-FH. (E) Quantification of phosphorylated/total dCLK. Error bars indicate

± SEM (n = 3); ***p<0.001; **p<0.01; one-way ANOVA and Tukey's post hoc test. The top half of the CLK signal shown middle lane is used as a reference to classify CLK isoforms as hyperphosphorylated.

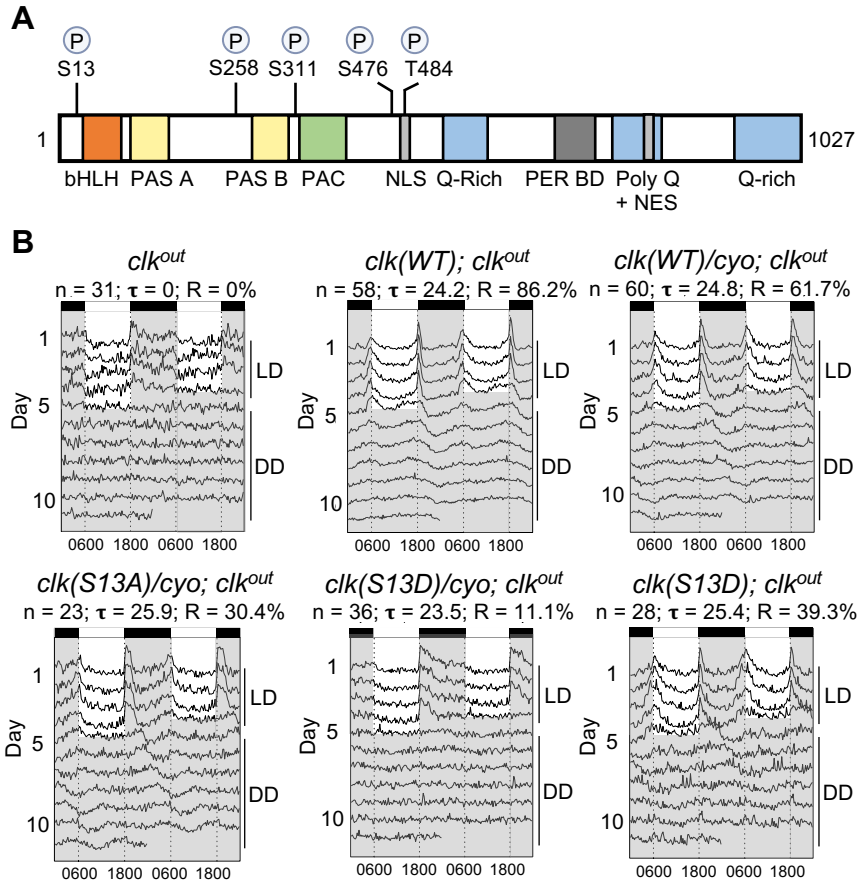

Figure 2. Circadian locomotor rhythms are altered in flies harboring mutations at CK1α-dependent dCLK phosphorylation sites. (A) Schematic diagram depicting ddCLK (amino acid 1-1027) adapted from Mahesh et al. (2014) showing CK1α-dependent ddCLK phosphorylation sites identified by mass spectrometry analysis of *Drosophila* S2 stable cell lines coexpressing dCLK tagged with 3XFLAG epitope and CK1α. Previously described dCLK domains: basic helix-loop-helix (bHLH) (aa 17-62) (Darlington et al., 1998; Bae et al., 1998); PAS-A (aa 96-144) (Darlington et al., 1998; Bae et al., 1998); PAS B (aa 264-309) (Darlington et al., 1998; Bae et al., 1998); C-terminal of PAS domain (PAC) (aa 315-379) (Bae et al.,1998); NLS (aa 480-494) (Hung et al., 2009); PER binding domain (PER BD) (aa 657-707) (Lee et al., 2016); Q-rich regions (aa 546-575, aa 957-1027), Poly-Q (aa 552-976) (Darlington et al., 1998; Bae et al., 1998) and NES (aa 840-864) (Hung et al., 2009). (B) Double-plotted actograms of flies

harboring various transgenes for ddCLK(S13), a CK1α-dependent phosphorylation site. Average activity of each genotype was plotted using FaasX. n represents the sample size; Tau (τ) represents the average period length of the indicated group of flies in constant darkness (DD). R represents the percentage of flies that are rhythmic. Flies were entrained for 4 days in 12h:12h light-dark (LD) and free-running activities were assayed for 7 days in DD.

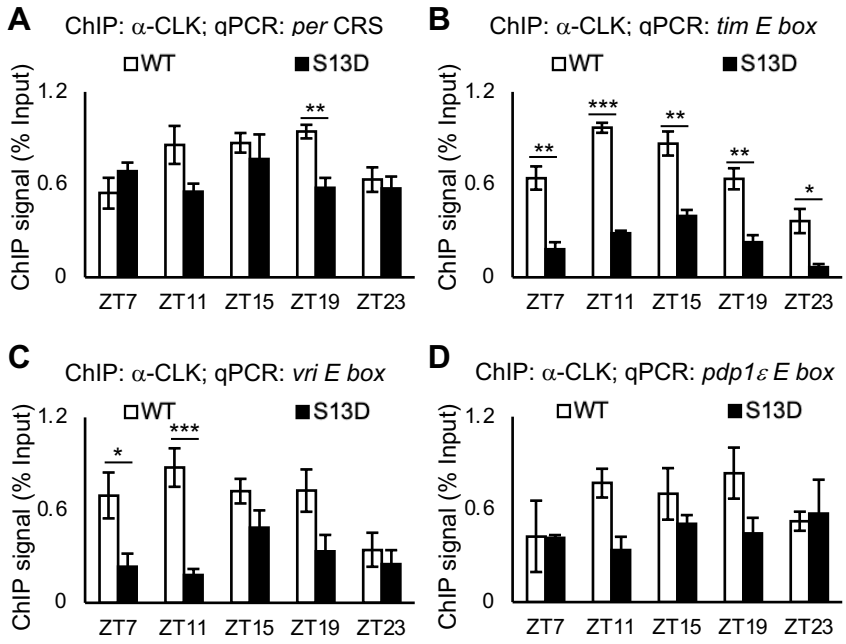

Figure 3. Phosphomimetic mutation at S13 decreases dCLK occupancy at circadian promoters. (A-D) Chromatin immunoprecipitation (ChIP) assays measuring dCLK occupancy at indicated dCLK-target promoters using head extracts from *clk*(WT) and *clk*(S13D) flies. Flies were entrained in 12h:12h light-dark (LD) cycles at 25°C and collected on LD3 at indicated time-points (ZT denotes Zeitgeber; ZT0 = lights on and ZT12 = lights off). dCLK-ChIP signals were normalized to % input. ChIP signals for two intergenic regions were used for non-specific background deduction (n=3). Error bars indicate ± SEM; ***p<0.001; **p< 0.01; *p< 0.05; two-way ANOVA and Šídák's post hoc test.

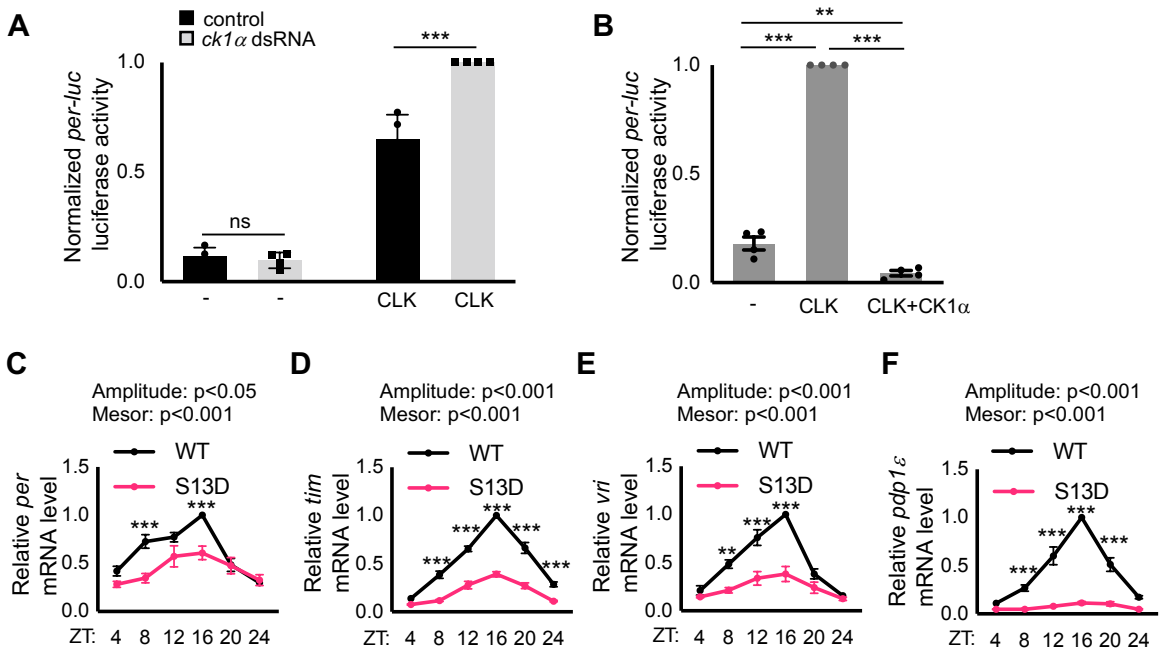

Figure 4. S13 phosphorylation reduces dCLK transcriptional activity. (A-B) *per-E-box-luciferase* (*per-luc*) transcriptional reporter assay performed in *Drosophila* S2 cells in combination with RNAi knockdown using *ck1α* dsRNA. (A-B) Luciferase activity was normalized to highest value in each data set (highest value = 1). Error bars indicate ± SEM (n=4); ***p<0.001; two-way ANOVA and Šídák's post hoc test. (C-F) Steady-state mRNA expression of *per* and *tim* in heads of *clk*(WT) and *clk*(S13D) flies. Flies were entrained in 12h:12h LD cycles at 25°C and collected on LD3 at indicated time-points (ZT). Error bars indicate ± SEM (n=4); ***p<0.001; **p<0.01; two-way ANOVA and Šídák's post hoc test.

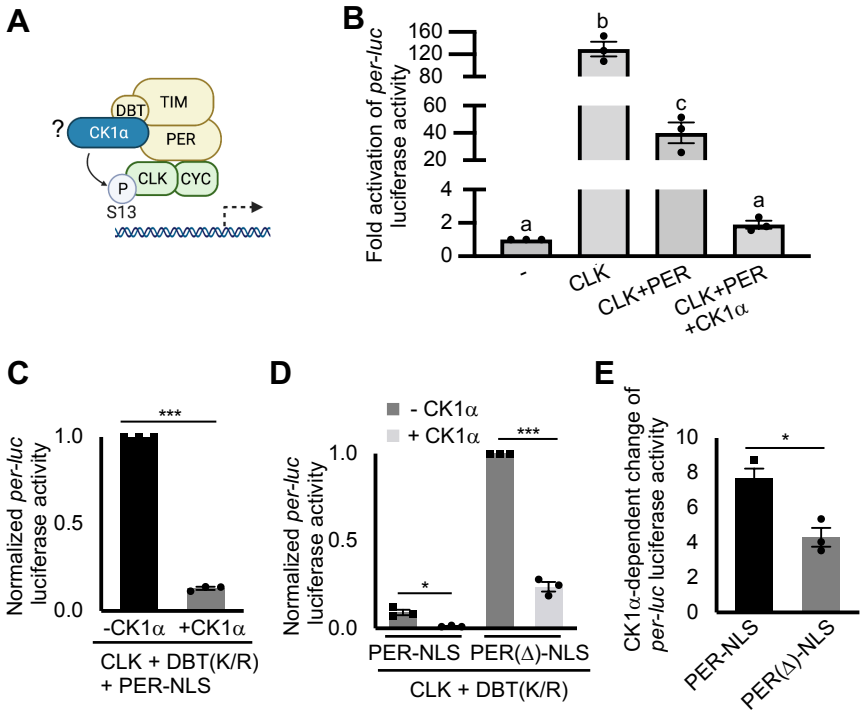

Figure 5. CK1α-dependent dCLK(S13) phosphorylation relies on PER-DBT interaction. (A) Schematic diagram illustrating the scaffolding model in which PER-DBT recruits CK1α to phosphorylate dCLK. (B-E) *per-E-box-luciferase* (*per-luc*) transcriptional reporter assay performed in *Drosophila* S2 cells. (B) Fold activation of *per-luc* were graphed with 1 representing the basal reporter activity in control cells. Error bars indicate ± SEM (n = 3), one-way ANOVA and Tukey's post hoc test. (C-D) Luciferase activity was normalized to highest value of each dataset (highest value = 1). Error bars indicate ± SEM (n = 3); ***p<0.001; *p<0.05; two-tailed Student's t test. (E) CK1α-dependent change of *per-luc* luciferase activity in (D) calculated by dividing CK1α(-) over CK1α(+). Error bars indicate ± SEM (n = 3); *p<0.05; two-tailed Student's t test.

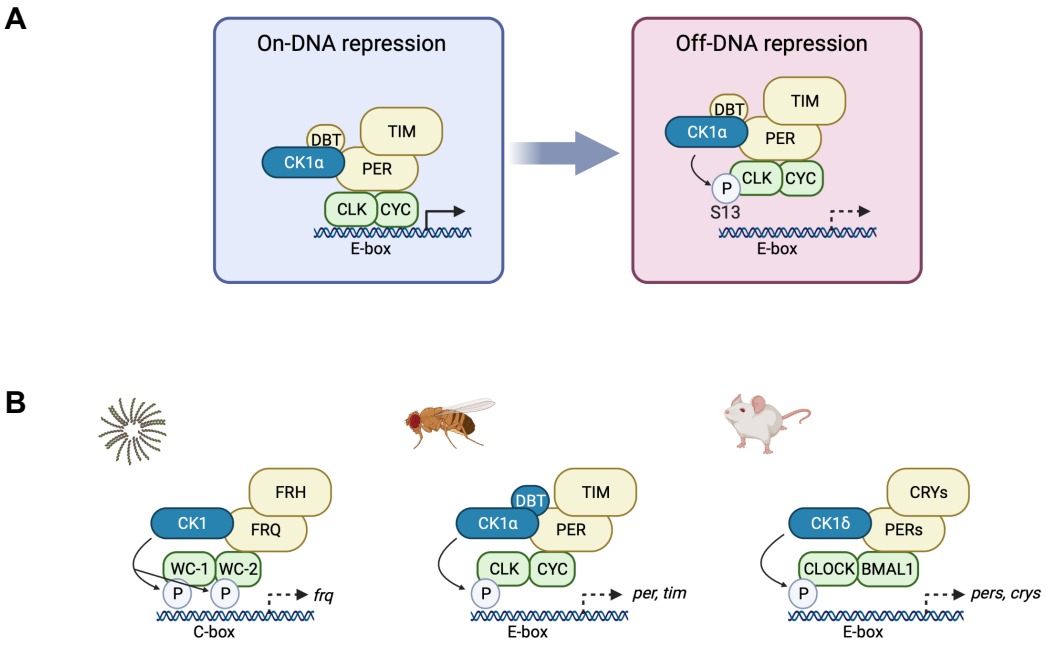

Figure 6. Model illustrating the function of dCLK(S13) phosphorylation in regulating the molecular clock. (A) PER represses dCLK-CYC transcription activity by two modes: on-DNA and off-DNA mechanisms (Menet et al., 2010). dCLK(S13) is phosphorylated upon the binding of PER-TIM repressor complex to dCLK-CYC. DBT functions as a scaffold to promote CK1α-dependent phosphorylation of dCLK, thus contributing to the transition from on-DNA to off-DNA transcriptional repression. (B) Conserved feature of CK1-dependent repression in eukaryotic clocks. In *Neurospora* (left panel), the repressors FREQUENCY (FRQ) and FRQ-interacting RNA helicase (FRH) mediates CK-1a (homolog of mammalian CK1δ/ε) phosphorylation on activators White Collar-1 (WC-1) and White Collar-2 (WC-2) to dissociate them from DNA (Wang et al., 2019). In *Drosophila* (middle panel), repressors PER and TIM recruit CK1α to phosphorylate CLK and reduce CLK-DNA binding (this paper). In mammals (right panel), the repressors CRYPTOCHROME (CRY) and PER proteins recruit CK1δ to phosphorylate the activator mCLOCK and cause its dissociation from DNA (Cao et al., 2021).

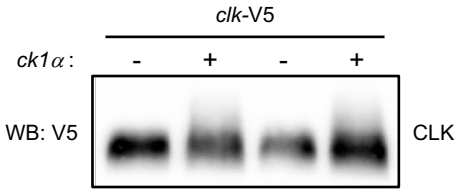

Figure S1. CK1α induces mild mobility shift of CLK on regular SDS-PAGE gel, related to Figure 1. *Drosophila* S2 cells were transfected with 0.8μg of pAc-*clk*-V5 and 0.6μg pMT-*ck1α*-3XFLAG-6XHIS. Protein extracts were analyzed on regular SDS-PAGE gel followed by western blotting. Blots of two biological replicates were shown

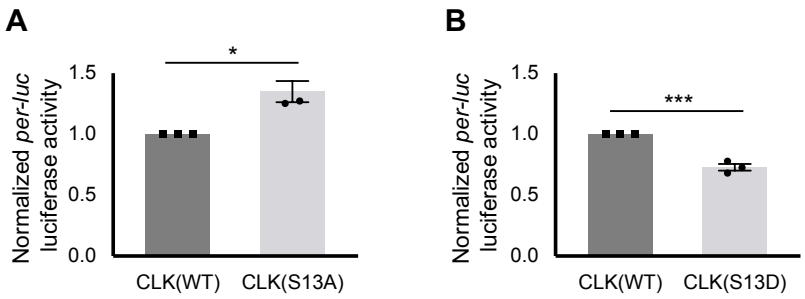

Figure S2. CLK(S13) variants exhibit altered transcriptional activity, Related to Figure 4.

(A-B) *per-E-box-luciferase* (*per-luc*) reporter assay performed in S2 cells. Luciferase activities were normalized to CLK(WT) equals to 1. Error bars indicate ± SEM (n = 3). ***p<0.001; *p<0.05, two-tailed Student's t test.

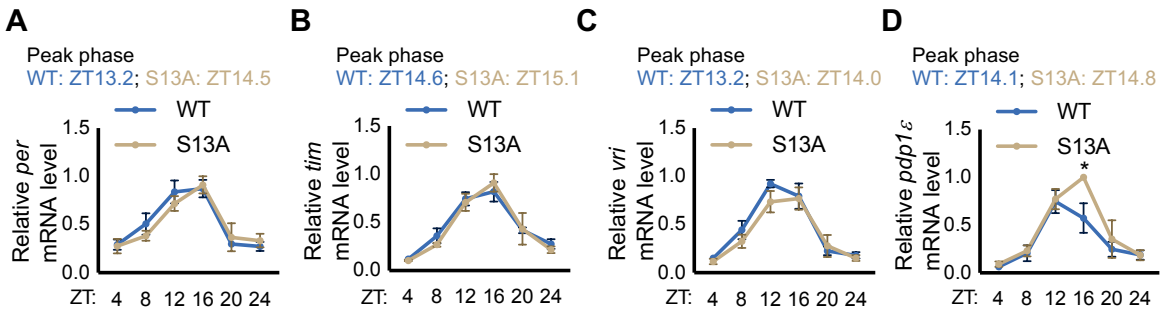

Figure S3. *clk*(S13A) flies display phase delay of CLK-target mRNAs, Related to Figure 4.

(A-D) Steady state mRNA expression in heads of *clk*(WT) and *clk*(S13A) flies. Flies were entrained in LD cycles and collected on LD3 at indicated time-points (ZT). Error bars indicate ± SEM (n=3). *p<0.05 two-way ANOVA and Šídák's post hoc test.

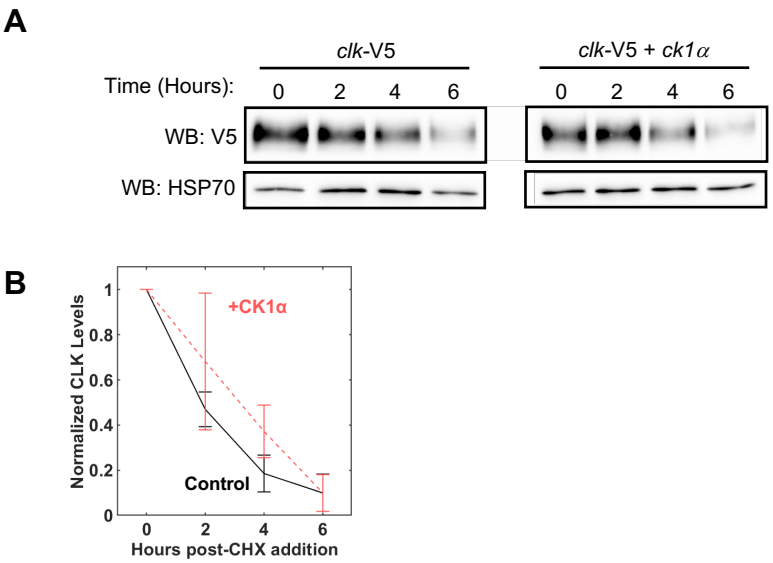

Figure S4. CK1α does not regulate CLK stability, Related to Figure 4. (A) *Drosophila* S2 cells were co-transfected with 0.8μg of pAc-*clk*-V5-His with 0.6μg of either pMT-*ck1α*-FH or pMT-FH. Following a 24-hour incubation period, kinase expression was induced with $CuSO_4$ for 16 hours. Following induction, cycloheximide was added and cells were harvested for protein extractions at the indicated times. Proteins were visualized by western blotting and detected with α-V5. (H) Western blots from (E) were quantified using ImageLab (Bio-Rad, Hercules CA) and normalized against α-HSP70. Error bars indicate ± S.E.M (n=2).

Table S1. Identification of Ck1α-dependent phosphorylation sites in *Drosophila* head tissues. Related to Figure 2.

Modified Residue[a]	Peptide Sequence[b]	Localization[c]	Higher Abundance in Present of CK1α?[d]
S5[e]	MDDE**S**DDKDDTKSFLCR	1	No
S13	MDDESDDKDDTK**S**FLCR	0.996	Yes
S258[f]	EM**S**IIDPTSNEFTSK	1	Yes
S311	APPIIGYMPFEVLGTSGYDYYH FDDLD**S**IVACHEELR	0.872	Yes
S487[e,f]	TSRPASSYGNISSTGI**S**PK	0.999	No
S476[f]/487[f]	TSRPA**S**SYGNISSTGI**S**PK	0.894; 0.991	Yes
T484/S487[f]	TSRPASSYGNISS**T**GI**S**PK	0.591; 0.985	Yes
S504	GND**S**DSTSMSTDSVTSR	1	No

[a] Residues are numbered according to dCLK (amino acids 1- 1027, UniProtKB O61735).

[b] Phosphorylated residues are bolded.

[c] Probability of phosphorylation site determined by Maxquant (Cox and Mann, 2008; Kweon and Andrews, 2013).

[d] Comparison of the abundance of each phosphorylated residue between co-expression of target protein with or without CK1α.

[e] Identified in Lee et al., 2014.

[f] Identified in Mahesh et al., 2014.

Table S2. Daily locomotor activity rhythms of *clk* mutants. Related to Figure 2.

Genotype	Period (h) (mean ± SEM)	Power[a]	Rhythmicity (%)[b]	No. of flies tested	No of flies surviving[c]
w; +; clk^{out}	AR[d]	ND[e]	0	32	31
w; clk(WT)*; clk*^{out}	24.2 ± 0.10	68.7	86.2	63	58
w; clk(WT)/*Cyo; clk*^{out}	24.8 ± 0.12	59.9	61.7	64	60
w; clk(S13A)/*Cyo; clk*^{out}	25.9 ± 0.13	42.0	30.4	27	23
w; clk(S13D)/Cyo*; clk*^{out}	23.5 ± 0.20	35.5	11.1	37	36
w; clk(S13D)*; clk*^{out}	25.4 ± 0.39	50.6	39.3	32	28

[a] Measures the strength or amplitude of the locomotor activity rhythm (in arbitrary units)
[b] Percentage of flies that are rhythmic
[c] Number of flies that survived until the end of the experiment
[d] AR denotes Arrhythmic
[e] ND denotes Not Determined

Table S3. Rhythmic parameters of mRNA analysis. Related to Figures 5 and S5.

Target	WT mesor	S13D mesor	P-value for mesor difference	WT amplitude	S13D amplitude	P-value for amplitude difference	WT peak time (ZT)	S13D peak time (ZT)	P-value for difference in phase
per	0.617	0.436	***	0.310	0.174	*	13.215	15.256	*
tim	0.521	0.208	***	0.158	0.395	***	15.743	15.906	0.755
vri	0.500	0.241	***	0.402	0.131	***	13.967	14.385	0.683
pdp1ε	0.445	0.074	***	0.412	0.037	***	15.502	16.656	0.683
Target	**WT mesor**	**S13A mesor**	**P-value for mesor difference**	**WT amplitude**	**S13A amplitude**	**P-value for amplitude difference**	**WT peak time (ZT)**	**S13A peak time (ZT)**	**P-value for difference in phase**
per	0.514	0.499	0.788	0.337	0.296	0.61	13.247	14.471	0.213
tim	0.457	0.440	0.720	0.389	0.341	0.464	14.645	15.065	0.543
vri	0.454	0.397	0.272	0.410	0.358	0.470	13.165	13.956	0.283
pdp1ε	0.338	0.440	0.119	0.309	0.442	0.148	14.07	14.82	0.437

Table S4: Primers for PCR mutagenesis, ChIP analysis and RT-qPCR analysis. Related to Figures 2-5.

Primer for mutagenesis: g*clk*(S13A) F: AAG GAT GAT ACA AAA GCG TAA ATT CAC TAG ACA
Primer for mutagenesis: g*clk*(S13A) R: TGT CTA GTG AAT TTA CGC TTT TGT ATC ATC CTT
Primer for mutagenesis: g*clk*(S13D) F: AAG GAT GAT ACA AAA GAG TAA ATT CAC TAG ACA
Primer for mutagenesis: g*clk*(S13D) R: TGT CTA GTG AAT TTA CTC TTT TGT ATC ATC CTT
Primer for mutagenesis: *clk*(S13A) F: AAG GAT GAT ACA AAA GCG TTC CTT TGC AGG AAA
Primer for mutagenesis: *clk*(S13A) R: TTT CCT GCA AAG GAA CGC TTT TGT ATC ATC CTT
Primer for mutagenesis: *clk*(S13D) F: AAGGATGATACAAAAGACTTCCTTTGCAGGAAA
Primer for mutagenesis: *clk*(S13D) R: TTTCCTGCAAAGGAAGTCTTTTGTATCATCCTT
Primer for ChIP: 2R intergenic (CP023338) F: TCAGCCGGCATCATTAGCAGCCG
Primer for ChIP: 2R intergenic (CP023338) R: TCGTGTGCGGGAATCTCTGCCG
Primer for ChIP: X intergenic (FBgn0003638) F: ACTGCGTATTCAGGATACATGCC
Primer for ChIP: X intergenic (FBgn0003638) R: TGTCCACTTTAATTGATTGCGTGG
Primer for ChIP: *per* CRS F: TGCCAGTGCCAGTGCGAGTTCG
Primer for ChIP: *per* CRS R: TGCCTGGTGGGCGGCTGG
Primer for ChIP: *tim* E-box1 F: ACGTTGTGATTACACGTGAGCC
Primer for ChIP: *tim* E-box1 R: ACACTGACCGAAACACCCAC
Primer for ChIP: *vri* promoter F: AACCAGACAGTTTGGTGGCTGGG
Primer for ChIP: *vri* promoter R: CAGTGCTAGCTAACTATTTGAACTCGTC
Primer for ChIP: *pdp1*ε promoter F: TTCGAACCGTCGGCGACGTCAG
Primer for ChIP: *pdp1*ε promoter R: TCCGACGTCAGCGGCCGAG
Primer for RT-qPCR: *per* F: GACCGAATCCCTGCTCAA
Primer for RT-qPCR: *per* R: GTGTCATTGGCGGACTTC
Primer for RT-qPCR: *tim* F: CCCTTATACCCGAGGTGGAT
Primer for RT-qPCR: *tim* R: TGATCGAGTTGCAGTGCTTC
Primer for RT-qPCR: *vri* F: ATGAACAACGTCCGGCTATC
Primer for RT-qPCR: *vri* R: CTGCGGACTTATGGATCCTC
Primer for RT-qPCR: *pdp1*ε F: GCGGCAACTGGTAATG
Primer for RT-qPCR: *pdp1*ε R: ATTTCCTGCCTGAGCT
Primer for RT-qPCR: *cbp20* F: GTCTGATTCGTGTGGACTGG
Primer for RT-qPCR: *cbp20* R: CAACAGTTTGCCATAACCCC

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AUTHOR CONTRIBUTIONS

J.C.C., Y.D.C. designed research; Y.D.C. performed all research and analyzed data with contributions from G.K.C. (S2 cell coimmunoprecipitation experiment), K.C.J. (transcriptional reporter luciferase assay in S2 cells), V.H.L. (Affinity purification to identify dCLK phosphorylation sites in S2 cells), C.A.T. (primer design for ChIP experiments), H.Z. performed mass spectrometry analysis of dCLK proteins to identify phosphorylation sites. Y.D.C. interpreted data with input from J.C.C., G.K.C., V.H.L. Y.D.C. wrote the chapter with input from J.C.C.

METHODS

Material availability

All unique/stable reagents generated in this study are available from the lead contact without restriction.

Data and code availability

This manuscript includes all datasets generated or analyzed during this study. Proteomics data have been deposited into public data repository. Accession numbers are provided in Method Details.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Drosophila construct design and transformation

attB-P[acman]-*clk* (15.5kb of the genomic sequence beginning ~8kb upstream and ending ~2.5kb downstream of *clk* coding region) was kindly provided by Paul Hardin (Mahesh et al., 2014). To introduce a V5 epitope tag in the C-terminus of the *clk* coding region, a 4kb NheI-NotI *clk* fragment was subcloned into pAc-V5-His construct and sequences encoding V5 were introduced in frame by site-directed mutagenesis using Pfu Turbo Cx DNA polymerase (Agilent Technologies, SantaClara, CA) (See Table S1 for mutagenic primer sequences). The NheI-NotI *clk* fragment in *attB*-P[acman]-*clk* was then swapped with the NheI-NotI *clk*-V5 fragment in pAc-V5-His to form *attB*-P[acman]-*clk*. PhiC31-mediated transgenesis was used to generate *w*; *clk*(WT)-V5; clk^{out} (Groth et al., 2004). Plasmids were injected into w fly embryo carrying VK00018 attP sites (BestGene, Chino Hills, CA) (Venken et al., 2006). Transformants were crossed with *w*; +; clk^{out} flies (Bloomington #56754) (Mahesh et al., 2014) to remove endogenous copies of *clk* prior to behavioral and molecular analyses.

To generate flies expressing nonphosphorylatable (Serine (S) to Alanine(A)) or phosphomimetic (S to Aspartic acid (D)) *clk* mutants, a 7kb NheI-SphI *clk* fragment was subcloned into pSP72 plasmid where a NheI site were introduced to multicloning sites. After mutagenesis and confirmation by Sanger sequencing (GENEWIZ Inc, South Plainfield, NJ), the mutant variants of 7kb *clk* fragments were used to replace the corresponding WT fragment in *attB*-P[acman]-*clk*(WT)-V5.

METHOD DETAILS

Plasmids for *Drosophila* S2 cell culture

pAc-*clk*(WT)-V5 (Kim and Edery, 2006), *per-E-box-Luciferase* (Hao et al., 1999), *pCopia Renilla-luciferase* (Nawathean and Rosbash 2004), pAc-*per*(WT)-V5 (Ceriani et al., 2002), pMT-*ck1*α(WT)-c-myc and pMT-*ck1*α(K49R)-3XFLAG-6XHis (Lam et al., 2018) were previously described.

Drosophila S2 cell culture and transfection

Drosophila S2 cells and Schneider's *Drosophila* medium were obtained from Life Technologies (Carlsbad, CA). S2 cells were grown at 22˚C in Schneider's *Drosophila* medium supplemented with 10% Fetal Bovine Serum (FBS) (VWR, Radnor, PA) and 0.5% Penicillin/streptomycin (Sigma-Aldrich, ST. Louis, MO). For all cell culture experiments unless otherwise noted, S2 cells were seeded at 1 X 10^6 cells/ml in a 6-well plate and transfected using Effectene (Qiagen, Germantown, MD). For coimmunoprecipitation (coIP) assays, S2 cells were cotransfected with 0.8µg of pAc-*clk*-V5-His and 0.8µg of pMT-*ck1*α-6Xc-myc and induced with 500 µM $CuSO_4$ immediately after transfection. In control IPs to detect non-specific binding, cells were transfected alone with either pAc-*clk*-V5-His, pMT-*ck1*α-6Xc-myc, or pMT-FH. For Phos-Tag Mobility Shift assay, S2 cells were cotransfected with 0.8µg of pAc-*clk*-V5 and 0.6µg of either pMT-*ck1*α(WT)-FH, pMT-*ck1*α(K49R)-FH or pMT-FH (FH denotes 3XFLAG-6XHis). 36 hours following transfection, kinase expression was induced with $CuSO_4$ for 24 hours and treated with cycloheximide (CHX) (Sigma-Aldrich) (10µg/ml) and MG132 (Sigma-Aldrich) (25µg/ml) for 4 hours. For CHX chase assay, S2 cells were transfected with 0.8µg of pAc-*clk*(WT)-V5 and either 0.6µg of pMT-*ck1*α-FH or pMT-FH.

For luciferase reporter assay to examine the effect of dsRNA targeting *ck1*α on dCLK transcriptional activity in Figure 5A, S2 cells were cotransfected with the plasmid combination as

indicated: with 0.1μg of *per-E-box-Luciferase* (*per-luc*), 0.1μg of pCopia *Renilla-luciferase* (*ren-luc*), 8ng of pAc-*clk*(WT)-V5. S2 cells were incubated for 48 hours prior to luciferase measurement. For luciferase reporter assay to examine effect of CK1α overexpression in Figure 5B, S2 cells were cotransfected with the plasmid combination as indicated: 0.625μg of *per-luc*, 0.625μg of *ren-luc*, 0.05μg of pAc-*clk*(WT)-V5 and 0.0375μg of pMT-*ck1*α-FH. Kinase expression was induced with $CuSO_4$ 24 hours after transfection and cells were harvested 16 hours after induction. For luciferase reporter assay in Figure 6, S2 cells were cotransfected with the plasmid combination as indicated: 0.1μg of *per-E-box-Luciferase*, 0.1μg of pCopia *Renilla-luciferase*, 8ng of pAc-*clk*(WT)-V5, 8ng of pMT-*ck1*α-FH and 80ng of pAc-*per*(WT)-V5, pAc-*per*(WT)-NLS-V5 or pAc-*per*(Δ)-NLS-V5. Kinase expression was induced with $CuSO_4$ right after transfection and cells were harvested 44 hours after induction. For luciferase reporter assay to examine the effect of S13 in Figure S4, S2 cells were cotransfected with the plasmid combination as indicated: 0.025μg of *per-luc*, 0.025μg of *ren-luc* and 0.002μg of pAc-*clk*(X)-V5 where X is either WT, S13A or S13D. S2 cells were harvested 36 hours after transfection prior to reporter assay.

To generate stable S2 cell lines, 1μg of pMT-FH-*clk* and 1μg of pMT-*ck1*α(WT)-6Xc-myc or pMT-*ck1*α(K49R)-6Xc-myc in combination with 1μg of pCoHygro plasmid expressing hygromycin resistance were used for transfection using Effectene (Qiagen). Stable cell lines were established by selection with Schneider's *Drosophila* medium supplemented with 300μg/ml hygromycin (Roche, Palo Alto, CA).

Coimmunoprecipitation experiments in *Drosophila* S2 cells

CoIP experiments were performed as described previously (Lam et al., 2018) with the following modifications. Cells were harvested 40 hours after transfection, washed once with 1X PBS and lysed with modified RIPA (20mM Tris-HCl pH 7.5, 150mM NaCl, 10% glycerol, 1%

Triton X-100, 0.4% sodium deoxycholate, 0.1% SDS) supplemented with 1mM EDTA pH 8.0, 25mM NaF, 0.5mM PMSF, and SIGMAFAST EDTA-free Protease inhibitor tablet (Sigma-Aldrich). Proteins were incubated with 20µl α-V5 or α-FLAG M2 resins (Sigma-Aldrich) for 4 hours at 4˚C to pull down dCLK or CK1α, respectively. Resins were washed three times in 500µl modified RIPA buffer at 4˚C using end-over-end rotator. Immune complexes were analyzed by Western blotting. Signal intensity of interacting protein was normalized to the intensity of the bait protein. Three biological replicates were performed.

Phos-Tag gel electrophoresis and Western blotting

Cells were lysed with EB2 (20mM Hepes pH 7.5, 100mM KCl, 5% glycerol, 1mM DTT, 0.1% Triton X-100, 25mM NaF, 0.5mM PMSF, 10 µg/ml Aprotinin, 5µg/ml Leupeptin, 1µg/ml Pepstatin A). Protein extracts were resolved using 5% SDS-PAGE cast with 10µM of Phos-Tag (Wako, Richmond, VA). Once resolved, gels were incubated for 10 minutes with gentle agitation first in transfer buffer (48mM Tris, 39mM Glycine, 20% Methanol, 0.000375% SDS) containing 1mM EDTA pH 8.0 followed by transfer buffer without EDTA. Proteins were then transferred onto PVDF membranes (Bio-Rad, Hercules, CA) and visualized by western blotting. Three biological replicates were performed.

Luciferase reporter assay

For dsRNA treatment, S2 cells were seeded at 2 X 10^6 cells per well in a 6-well plate and were either treated with 30µg of *ck1α* dsRNA in serum-free media for 45 minutes at 22˚C or mock-treated at the same temperature. Cells were then supplemented with FBS (10%) and incubated for 48 hours prior to transfection. dsRNA targeting *ck1α* were synthesized using MEGAscript T7 Transcription Kit (Thermo Fisher Scientific) following the instructions of manufacturer.

Measurements were performed using the Dual-Glo Luciferase Assay System following the instructions of manufacturer (Promega, Madison, WI). Two technical replicates were performed for each biological luciferase reporter assay replicates. Three to four biological replicates were performed.

Chromatin Immunoprecipitation (ChIP)

dCLK-ChIP was performed as described previously (Kwok et al., 2015). All buffers described below, except ChIP Elution buffer, contain 1X SIGMAFAST EDTA-free protease inhibitor and 0.5 mM PMSF. Briefly, fly head tissues were homogenized using liquid nitrogen chilled mortar and pestle, mixed with Nuclear Extraction buffer (10mM Tris-HCl pH 8.0, 0.1mM EGTA pH 8.0, 10mM NaCl, 0.5mM EDTA pH 8.0, 1mM DTT, 0.5% Tergitol NP-10, 0.5mM Spermidine, 0.15mM Spermine), and lysed with a dounce homogenizer. Homogenate was transferred to a 70μm cell strainer (Thermo Fisher Scientific) prior to centrifugation at 300 g for 1 minute at 4°C. Supernatant were centrifuged at 6700 rpm for 10 minutes at 4˚C. Pellets were resuspended in NEB buffer prior to centrifugation at 11,500 rpm for 20 minutes at 4˚C on a sucrose gradient (1.6M sucrose in NEB and 0.8M sucrose in NEB). Nuclei-containing pellets were fixed with 0.3% formaldehyde in NEB and rotated at room temperature for 10 minutes. Glycine was then added at a final concentration of 0.13mM to quench crosslinking. Samples were centrifuged at 6,500 rpm for 5 minutes at 4˚C. Pellets (cross-linked chromatin) were washed twice with NEB and resuspended in Sonication buffer (10mM Tris-HCl pH 7.5, 2mM EDTA pH 8.0, 1% SDS, 0.2% Triton X-100, 0.5mM Spermidine, 0.15mM Spermine). The cross-linked chromatin was sheared by sonicator (Q80023, QSonica, Newton, Connecticut) to roughly 500 base pair fragments. Supernatant (sheared chromatin) was collected after the centrifugation at 10,000 rpm for 10 minutes. 1.5μl of dCLK antibodies (generated in this study) were incubated with 25μl of Dynabeads (Thermo Fisher Scientific) in ChIP Wash buffer (50mM Tris-HCl, 1mM

EDTA pH 8.0, 1% Triton X-100, 0.1% DOC, 10μg/ml AcBSA (Promega), 100mM KCl in 1X PBS, 150mM NaCl, 5mM EGTA pH 8.0, 0.1% SDS) at 4˚C for 2 hours. Following incubation, beads were collected using a magnet stand (Sigma-Aldrich) and incubated with sheared chromatin that were diluted 10-fold with IP buffer (50mM Tris-HCl pH 7.5, 2mM EDTA pH 8.0, 1% Triton X-100, 0.1% DOC, 150mM NaCl, 0.5mM EGTA pH 8.0) at 4˚C for 2 hours. Beads were then collected and washed twice with CW buffer for 30 minutes at 4°C, once with LiCl Wash buffer (10mM Tris-HCl pH 8.0, 250mM LiCl, 0.5% NP-40, 0.5% DOC, 1mM EDTA pH 8.0) for 30 minutes at 4˚C and once with TE buffer (1mM EDTA pH 8.0, 10mM Tris-HCl pH 8.0) for 4 minutes at 4˚C. Beads were eluted with ChIP Elution buffer (50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 1% SDS, 1mM DTT, 50mM NaCl, 4U/ml Proteinase K (NEB, Ipswich, MA), 50μg/ml RNase A (Thermo Fisher Scientific, Waltham, MA) at 37˚C for 2 hours and de-crosslinked at 65˚C overnight. Finally, DNA was purified by QIAquick PCR Purification Kit (Qiagen) and quantified by real-time qPCR. Primers for *per CRS*, *tim* E-box were described previously (Kwok et al., 2015). Primers for *pdp1*ε-E-box and *vri*-E-box were in Table S4. The average of ChIP signals for two intergenic regions, one on chromosome 2R (see Table S1) and one on the X chromosome (Kwok et al., 2015), was used for non-specific background deduction. Three technical replicates were performed for each biological ChIP replicate. Three biological ChIP replicates were performed.

Cycloheximide (CHX) chase assay

24 hours following S2 cell transfection, *ck1*α expression was induced for 16 hours prior to the treatment of CHX (10μg/ml) (Sigma-Aldrich). Cells were then harvested and lysed with EB2 supplemented with 5mM EDTA pH 8.0 at the times indicated. Protein lysates were analyzed by western blotting.

Identification of dCLK phosphorylation sites from *Drosophila* S2 cells

S2 cells were harvested by centrifuging at 4,000 rpm for 10 minutes at 4˚C. Supernatant was removed and then the cell pellet was washed once with 15ml of 50mM Hepes (pH 7.6). Cells were homogenized in lysis buffer (20mM Hepes pH 7.6, 5% glycerol, 350mM NaCl, 0.1% Triton X-100, 1mM DTT, 1mM $MgCl_2$, 0.5mM EDTA pH 8.0, 25mM NaF, supplemented with Complete EDTA-free Protease inhibitor cocktail (Sigma-Aldrich), and PhosSTOP (Roche) by using a 40 ml loose dounce homogenizer (Wheaton, Millville, NJ). Lysed cells were nutated at 4˚C for 30 minutes and then centrifuged at 15,000 rpm for 15 minutes at 4˚C. Immunoprecipitation was performed at 4˚C overnight with 120µl α-FLAG M2 beads (Sigma-Aldrich) followed by two 10-minute washes using lysis buffer without EDTA, DTT, and PhosSTOP. Bound proteins were eluted with equal bead volume (120µl) of elution buffer (30% glycerol, 3% SDS, 6 mM EDTA pH 8.0, 150 mM Tris pH 6.8) at 95˚C for 4 minutes. Eluted proteins were then reduced with 20 mM DTT at 65˚C for 20 minutes followed by alkylation at room temperature for 20 minutes with 100 mM Ioacetamide. Proteins were then analyzed by Coomassie staining on a 12% SDS-PAGE gel and dCLK containing band was excised for mass spectrometry analysis as described in Chiu et al. (2008).

Maxquant and Skyline analysis

Mass spectrometric data were processed with MaxQuant (Cox and Mann, 2008) version 1.6.1.0. MS/MS spectra were searched against the complete Uniprot *Drosophila melanogaster* protein database using the built-in Andromeda peptide search engine (Cox et al., 2011) with trypsin designated as the digestion enzyme and two missed cleavages were allowed. Oxidation, N-terminal acetylation, phosphorylation, and deamination of asparagine and glutamine were selected as variable modifications. Carbamidomethylation of cysteine was selected as fixed modification. For all other parameters, MaxQuant default values were selected. Briefly, peptide

tolerance for the initial and main search of Andromeda were specified at 20 ppm and 4.5 ppm respectively. For identification, an FDR of 0.01 was selected for peptide spectrum matches (PSM) and protein matches.

MaxQuant output data was further processed using Skyline (Schilling et al., 2012) version 4.1.0.11796. For spectral library building, a cut-off score of 0.95 was selected. For MS1 filtering, precursor with charges of 2, 3, and 4 were considered. For retention time filtering, only scans within 5 minutes of MS/MS identification were selected. Quantification of phosphorylated peptides were performed as area the curve of each identified peptide.

Locomotor activity assay

Daily locomotor activity rhythms in male flies were assayed using the *Drosophila* Activity Monitoring System (DAMS, TriKinetics, Waltham, MA) as described previously (Chiu et al., 2010).

Quantitative RT-PCR

RNA was extracted from approximately 30-50µl of fly heads using 3X volume TRI Reagent (Sigma-Aldrich). 1/5 volume of 100% chloroform (Sigma-Aldrich) was added and incubated at room temperature for 10 minutes. Upper aqueous layer was recovered after spinning down at 13,000 rpm for 15 minutes. Same volume of 100% isopropanol was added and incubated at -20C overnight to precipitate RNA. After spinning down, RNA pellet was washed with 200µl 70% ethanol once, resuspended in 20µl 1X RQ1 buffer (Promega), treated with 2µl RQ1 DNase (Promega) at 37°C for 30 minutes prior to the incubation with 2µl RQ1 DNase stop solution (Promega) at 65˚C for 10 minutes. cDNA was generated from equal amount of RNA using Superscript IV (Thermo Fisher Scientific). Real-time PCR was performed using SsoAdvanced SYBR green supermix (Bio-Rad) in a CFX96 or CFX384 (Bio-Rad). Three

technical replicates were performed for each biological qPCR replicate. Three biological replicates were performed.

Western blotting and antibodies

Western blotting and image analysis were performed as previously described (Kwok et al., 2015). Upon extraction, protein concentration was measured using Pierce Coomassie Plus Assay Reagents (Thermo Fisher Scientific). 2X SDS sample buffer was added and the mixture boiled at 95˚C for 5 minutes. Equal amounts of proteins were resolved by polyacrylamide-SDS gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Bio-Rad) using Semi-Dry Transfer Cell (Bio-Rad). Membranes were incubated in 5% Blocking Buffer (Bio-Rad) for 40 minutes, incubated with primary antibodies for 16-20 hours. Blots were then washed with 1X TBST for 1 hour, incubated with secondary antibodies for 1 hour, washed again prior to treatment of Clarity chemiluminescence ECL reagent (Bio-Rad). The following percentage of polyacrylamide-SDS gel were used: 8% for dCLK, 12% for CK1α.

Primary antibodies: α-V5 (Thermo Fisher Scientific) at 1:1000 for dCLK-V5, α-cmyc at 1:2000 for CK1α-cmyc, α-FLAG (Sigma-Aldrich) at 1:7000 for CK1α-FLAG and α-HSP70 (Sigma-Aldrich) at 1:10000. Secondary antibodies conjugated with HRP were added as follows: α-mouse IgG (Sigma-Aldrich) at 1:1000 for α-V5 detection, 1:2000 for α-c-myc detection, 1:2000 for α-FLAG detection and 1:10000 for α-HSP70 detection.

STATISTICAL ANALYSIS

RAIN, DODR, CircaCompare test were performed in R (Thaben and Westermark 2014, 2016, Parsons et al., 2020). Other statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, California). ANOVA was performed if more than two groups were compared; two-tailed Student's t test were performed if only two groups were compared.

Asterisks indicate significant differences in mean values between genotypes or conditions at indicated time-points.

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Chapter 2. CK2 inhibits TIMELESS nuclear export and modulates CLOCK transcriptional activity to regulate circadian rhythms

Yao D. Cai[1], Yongbo Xue[2], Cindy C. Truong[1], Jose Del Carmen-Li[1], Christopher Ochoa[1], Jens T. Vanselow[3], Katherine A. Murphy[1], Ying H. Li[1], Xianhui Liu[1], Ben L. Kunimoto[1], Haiyan Zheng[4], Caifeng Zhao[4], Yong Zhang[2], Andreas Schlosser[3], Joanna C. Chiu[1*]

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AUTHOR CONTRIBUTIONS

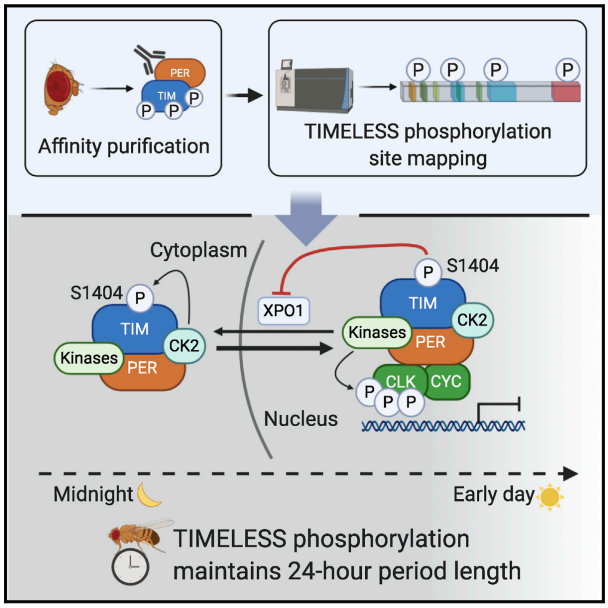
J.C.C., Y.D.C., and A.S. designed research; Y.D.C. performed all research and analyzed data with contributions from Y.X. and B.L.K. (immunofluorescence of TIM), C.C.T. (detection of pS1404 in S2 cells), J.D.C.-L. (western blotting of *tim*(S1404D) flies), C.O. (analysis of fly responses to light pulse), Y.H.L. (affinity purification to identify TIM phosphorylation sites), J.T.V., H.Z., and C.Z. performed mass spectrometry analysis of TIM proteins to identify phosphorylation sites. Y.D.C. interpreted data with input from J.C.C., Y.X., Y.Z., A.S., X.L., and K.A.M generated CLK antibody and transgenic flies and Y.D.C. wrote the chapter with input from J.C.C.

Article

Current Biology

CK2 Inhibits TIMELESS Nuclear Export and Modulates CLOCK Transcriptional Activity to Regulate Circadian Rhythms

Graphical Abstract



Highlights

- Twelve phosphorylation sites were identified in PER-bound TIM protein
- Abolishing phosphorylation of conserved TIM(S1404) alters circadian rhythms
- CK2 phosphorylates S1404 to inhibit interaction of TIM and nuclear export complex
- PER-TIM nuclear accumulation regulates the timing of CLK transcriptional activity

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In Brief

Organisms in all domains of life exhibit circadian rhythms. Cai et al. reveal that phosphorylation of TIMELESS modulates kinase accessibility to CLOCK in the nucleus. This mechanism is important in controlling daily phosphorylation rhythm of CLOCK, which is critical for its function as a key regulator of circadian rhythms.

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CK2 Inhibits TIMELESS Nuclear Export and Modulates CLOCK Transcriptional Activity to Regulate Circadian Rhythms

Yao D. Cai,[1] Yongbo Xue,[2] Cindy C. Truong,[1] Jose Del Carmen-Li,[1] Christopher Ochoa,[1] Jens T. Vanselow,[3] Katherine A. Murphy,[1] Ying H. Li,[1] Xianhui Liu,[1] Ben L. Kunimoto,[1] Haiyan Zheng,[4] Caifeng Zhao,[4] Yong Zhang,[2] Andreas Schlosser,[3] and Joanna C. Chiu[1,5,*]

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SUMMARY

Circadian clocks orchestrate daily rhythms in organismal physiology and behavior to promote optimal performance and fitness. In *Drosophila*, key pacemaker proteins PERIOD (PER) and TIMELESS (TIM) are progressively phosphorylated to perform phase-specific functions. Whereas PER phosphorylation has been extensively studied, systematic analysis of site-specific TIM phosphorylation is lacking. Here, we identified phosphorylation sites of PER-bound TIM by mass spectrometry, given the importance of TIM as a modulator of PER function in the pacemaker. Among the 12 TIM phosphorylation sites we identified, at least two of them are critical for circadian timekeeping as mutants expressing non-phosphorylatable mutations exhibit altered behavioral rhythms. In particular, we observed that CK2-dependent phosphorylation of TIM(S1404) promotes nuclear accumulation of PER-TIM heterodimers by inhibiting the interaction of TIM and nuclear export component, Exportin 1 (XPO1). We propose that proper level of nuclear PER-TIM accumulation is necessary to facilitate kinase recruitment for the regulation of daily phosphorylation rhythm and phase-specific transcriptional activity of CLOCK (CLK). Our results highlight the contribution of phosphorylation-dependent nuclear export of PER-TIM heterodimers to the maintenance of circadian periodicity and identify a new mechanism by which the negative elements of the circadian clock (PER-TIM) regulate the positive elements (CLK-CYC). Finally, because the molecular phenotype of *tim*(S1404A) non-phosphorylatable mutant exhibits remarkable similarity to that of a mutation in human *timeless* that underlies familial advanced sleep phase syndrome (FASPS), our results revealed an unexpected parallel between the functions of *Drosophila* and human TIM and may provide new insights into the molecular mechanisms underlying human FASPS.

INTRODUCTION

Circadian rhythms have been observed in all domains of life and are driven by a network of cellular molecular clocks in animals.[1–3] These molecular clocks are entrained by environmental time cues, such as light-dark (LD) cycles, to control daily rhythms in physiology and behavior. One conserved feature of molecular clocks within the animal kingdom is their reliance on key clock proteins that are organized in transcriptional translational feedback loops (TTFLs).[3,4] In *Drosophila*, these key pacemaker proteins are the positive elements, CLOCK (CLK) and CYCLE (CYC), and the negative elements, PERIOD (PER) and TIMELESS (TIM). During the day, CLK-CYC heterodimers activate the transcription of *per*, *tim*, and other clock-controlled genes (ccgs).[5] The accumulation of PER and TIM proteins is delayed by a number of post-transcriptional and post-translational mechanisms[6–10] until early night, when PER and TIM attain high enough levels to form heterodimeric complexes in the cytoplasm prior to nuclear entry.[11,12] Nuclear PER, likely still in complex with TIM, promotes the repression of the circadian transcriptome by inhibiting CLK-CYC transcriptional activity and removing them from clock genes before its degradation in the late night and early day.[13–17]

Although TIM itself cannot repress CLK-CYC transcriptional activity, it is essential to the molecular clock because it maintains rhythmic PER protein expression.[18–20] Constitutive cytoplasmic PER in *tim null* mutants[21] as well as in *tim* mutants with defective nuclear entry[22,23] abolishes rhythms in the molecular clock and consequently dampens behavioral rhythms. An early study

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suggested that TIM physically associates to the PER cytoplasmic localization domain (CLD) and thus blocks cytoplasmic retention.[11] A subsequent study showed that TIM actively facilitates PER nuclear entry by acting as the primary cargo of importin α1 (IMPα1)-dependent nuclear import mechanisms and cotransport PER into the nucleus.[12] Finally, TIM is suggested to counteract the effect of DOUBLETIME (DBT) kinases in preventing PER nuclear entry.[20] Taken together, TIM is proposed to promote the nuclear entry of PER.

Phosphorylation has also been implicated in regulating PER-TIM nuclear entry. The function of casein kinase 1α (CK1α), casein kinase 2 (CK2), SHAGGY (SGG), and DBT have been investigated in this context. DBT has been observed to phosphorylate PER and prevent nuclear translocation.[20] This regulatory step has recently been shown to be antagonized by CK1α-dependent phosphorylation of either PER or DBT.[24] The role of CK2 and SGG in regulating PER-TIM subcellular localization has received relatively more attention. Early studies suggest that SGG and CK2 phosphorylate both PER and TIM to promote nuclear translocation.[25–27] Subsequent studies indicate that PER may be the primary target of CK2 and SGG to control subcellular localization.[28,29] However, more recent studies indicate that perhaps CK2 regulates nuclear entry of PER-TIM by phosphorylating TIM.[30,31] In addition to kinases, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) have also been shown to influence PER-TIM nuclear accumulation.[32,33]

In addition to its role in facilitating PER nuclear entry, TIM has been observed to mediate light resetting and circadian entrainment because of its light-induced degradation.[34,35] Upon light exposure, the blue light photoreceptor CRYPTOCHROME (CRY) undergoes a conformational change and binds to TIM.[36,37] The E3 ubiquitin ligase JETLAG (JET) then collaborates with CRY to promote rapid proteasomal degradation of TIM.[36,38,39] Phosphorylation of yet uncharacterized tyrosine residues has been proposed to be required for degradation.[40] Finally, light-induced TIM degradation further promotes PER turnover, which functions to reset and entrain the molecular clock.[35]

Significant progress has been made in examining the function of site-specific PER phosphorylation, enabling in-depth mechanistic understanding of post-translational regulation of PER subcellular localization, repressor activity, and degradation to generate a 24-h rhythm.[28,29,41–45] On the other hand, the relative dearth of studies that characterize site-specific functions of TIM phosphorylation[22,31,46] remains a significant obstacle to fully understand the regulation of circadian rhythms via post-translational regulation of TIM and PER-TIM complexes. In this study, we used mass spectrometry proteomics to identify phosphorylation sites of PER-bound TIM proteins purified from *Drosophila* heads. We found that loss of phosphorylation at some of these TIM residues resulted in altered circadian behavioral rhythms. In particular, impaired CK2-dependent phosphorylation at TIM(S1404) resulted in an ~1.7-h period-shortening phenotype. By analyzing the molecular clock of non-phosphorylatable *tim*(S1404A) and phosphomimetic *tim*(S1404D) mutants, we provide evidence supporting the importance of TIM(S1404) phosphorylation in promoting TIM nuclear retention by reducing its interaction with exportin 1 (XPO1), an important component of the nuclear export machinery. Interestingly, decreased nuclear localization of TIM in *tim*(S1404A) mutant flies not only reduces the abundance of nuclear PER and TIM proteins but also dampens the daily rhythms in CLK phosphorylation. We reasoned this is caused by changes in the abundance of kinases recruited by the PER-TIM complexes to phosphorylate CLK. Consequently, this leads to phase advance of CLK occupancy rhythms at circadian promoters, which manifests into shortening of molecular and behavioral rhythms. Based upon these findings, we propose a model describing the mechanism by which CK2-dependent TIM(S1404) phosphorylation regulates PER-TIM nuclear accumulation and CLK-CYC activity to regulate circadian rhythms.

RESULTS

Mass Spectrometry Analysis Identifies TIM Phosphorylation Sites in PER-TIM Heterodimers

Comprehensive mapping of TIM phosphorylated sites has not been performed despite previous studies indicating that phosphorylation is important for the phase-specific regulation of TIM function.[25–27,31,40,46] This hinders further understanding of the mechanisms by which site-specific TIM phosphorylation regulates the molecular clock. Because TIM interacts with PER to achieve its role in circadian timekeeping, we aimed to identify phosphorylated TIM residues in the PER-TIM heterodimeric complex. Previously, our group identified PER phosphorylation sites by purifying PER from fly heads followed by mass spectrometry (MS) analysis.[47] Proteins extracted from tissues of *wper*^{0}; *p*{3XFLAG-*per*(WT)} flies were subjected to FLAG affinity purification prior to MS analysis. We observed that TIM was copurified with PER at ZT1 (ZT is defined as Zeitgeber Time), ZT3, ZT12, ZT16, ZT20, and ZT23.5 and leveraged this opportunity to identify PER-bound TIM phosphorylation sites. The MS data from multiple time points were pooled to identify TIM phosphorylation sites qualitatively. We expect that some of these phosphorylation events may be critical in regulating PER-TIM interactions and the function of PER-TIM heterodimer in the molecular clock.

We identified 12 TIM phosphorylation sites, some of which are located in previously characterized functional domains (Figure 1A; Table S1). These include S568, which is in PER binding domain 1 (PER BD1) and the NLS (nuclear localization signal);[11] S891 in PER binding domain 2 (PER BD 2);[11] and S1389, S1393, S1396, and S1404 in the cytoplasmic localization domain (CLD).[11] Based on the location of these phosphorylation sites, we reasoned that they may regulate the subcellular localization and phase-specific functions of PER-TIM heterodimers. We also identified a number of other phosphorylated residues that are located in regions of TIM proteins without characterized functions. Finally, although not the central focus of this study, we determined that TIM, like PER and CLK,[47–49] is O-GlcNAcylated at multiple residues (Table S1).

Transgenic Flies Expressing Non-phosphorylatable TIM Variants Display Altered Locomotor Activity Rhythms

To determine whether the TIM phosphorylation sites we identified play important roles in circadian timekeeping, we generated transgenic fly lines each expressing one or a cluster of non-phosphorylatable S/T to A mutations. We prioritized our efforts by

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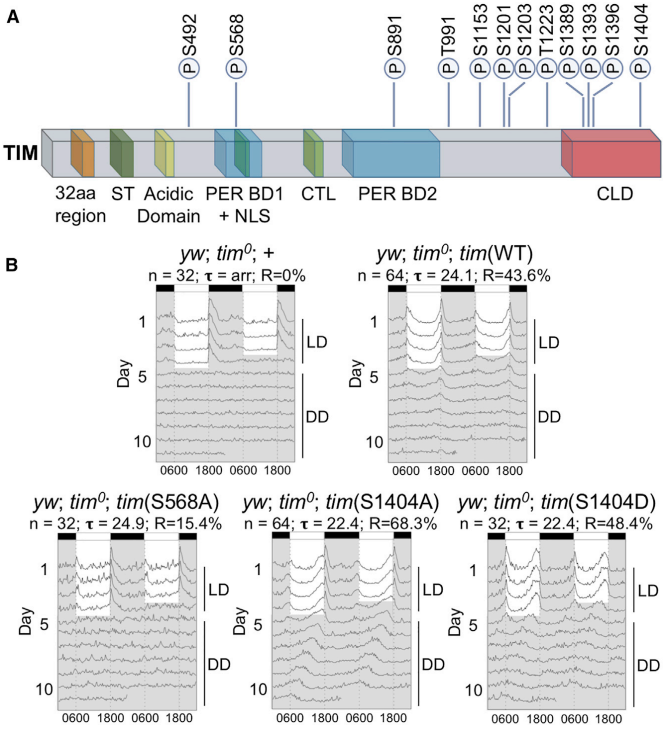

Figure 1. Daily Locomotor Activity Rhythms Are Altered in TIM Phosphorylation Site Mutants

(A) Schematic showing phosphorylation sites mapped onto TIM functional domains. All amino acid numbering is based on the L-TIM$_{1421}$ isoform.[50,51,52] Previously described domains of TIM: 32 amino acid region (amino acids [aas] 260–291),[50] also known as serine-rich domain (SRD) (aas 260–292);[30] serine/threonine (ST)-rich region (aas 293–312);[31] acidic domain (aas 383–412);[52] PER binding domain 1 (PER BD1) (aas 536–610);[11] nuclear localization sequence (NLS) (aas 558–583);[11] C-terminal tail-like sequence (CTL) (aas 640–649);[37] PER binding domain 2 (PER BD2) (aas 747–946);[11] and cytoplasmic localization domain (CLD) (aas 1,261–1,421).[11] Corresponding PEAKS Studio scores of modified peptides are shown in Table S1.

(B) Double-plotted actograms of *yw*; *tim*^{0} flies carrying transgenes for site-specific TIM phosphorylation mutations generated using FaasX. Average activity of each genotype was plotted. n represents the sample size for behavioral assay. Tau (τ) represents the average period length of the indicated group of flies in DD (SEM is shown in Table S2). R represents percentage of flies that are rhythmic. Flies were entrained for 4 days in LD and then switched to 7 days of constant darkness (DD).

See also Figures S1 and S2 and Tables S1 and S2.

focusing on sites with high MS probability score and/or those located in characterized functional domains. *p*{*tim*(X)-3XFLAG-6XHIS} transgenes (X represents the S/T to A TIM mutation or wild-type [WT] TIM) were crossed into *tim*^{0} genetic background[53] such that only transgenic *tim* was expressed. First, we evaluated daily locomotor activity rhythms of *tim* transgenic flies as activity rhythm is a reliable behavioral output of the *Drosophila* circadian clock.[54] Flies were entrained for 3 days in cycles of 12 h light and 12 h darkness (herein referred as LD cycles) followed by 7 days in constant darkness (DD) to monitor free-running rhythm. We observed that homozygous *tim*(WT) flies exhibited behavioral rhythm with an ~24-h period, indicating that the arrhythmic *tim*^{0} mutation was rescued by the *tim*(WT) transgene (Figures 1B, S1A, and S1B; Table S2). Rescue of *tim*^{0} mutants with various *tim* transgenes over the years have yielded variable results. Although the behavioral rhythmicity of *tim*(WT) in this experiment (43.6%) is somewhat lower than the extent of *tim*^{0} rescue observed in some previous studies (e.g., Top et al.[31] and Ousley et al.[50]), it is notably higher than rescue of *tim*^{0} flies by driving expression of *tim* cDNA using *tim*-Gal4.[22,54] Among the transgenic lines expressing TIM variants, *tim*(S1404A) was the only genotype that exhibited a clear period-shortening phenotype (~1.7 h shorter), although *tim*(S568A) flies were notably more arrhythmic when compared to *tim*(WT).

Among the two phosphorylation sites that resulted in changes in behavioral rhythms when mutated, we decided to proceed first with the functional characterization of TIM(S1404) as it is (1) highly conserved in animals (Figures S2A and S2B); (2) predicted to be phosphorylated by CK2, a known clock kinase; (3) also phosphorylated in the monarch butterfly TIM protein as determined by MS analysis (Figure S2C); and (4) located within TIM CLD (Figure 1A). For this reason, we generated an additional transgenic fly line expressing a phosphomimetic S1404D TIM variant to complement the analysis of *tim*(S1404A) mutants. Interestingly, *tim*(S1404D) flies also exhibited shortened period, similar to what was observed in *tim*(S1404A) flies (Figures 1B and S1B; Table S2). Although it is logical to assume that non-phosphorylatable and phosphomimetic mutations should result in opposite phenotypes (e.g., Top et al.[31] and Chiu et al.[41]), that is often not the case, as observed in previous phosphorylation studies (e.g., Lin et al.,[28] Chiu et al.,[42] and Top et al.[45]). Furthermore, although both *tim*(S1404A) and *tim*(S1404D) mutants exhibit period shortening at the behavioral level, the underlying molecular mechanisms that underlie their phenotypes may differ.

TIM(S1404) Phosphorylation Promotes TIM Nuclear Accumulation

Because TIM S1404 residue is located in the CLD, we reasoned that S1404 phosphorylation may regulate TIM nuclear accumulation. To test our hypothesis, we monitored subcellular localization of TIM in *tim*(WT), *tim*(S1404A), and *tim*(S1404D) adult brain clock neurons from early to late night using whole-mount immunocytochemistry. These experiments were performed using flies

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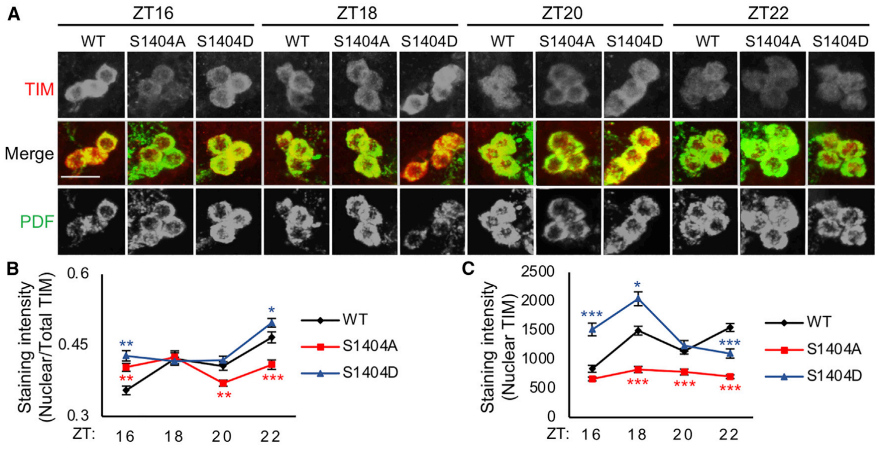

Figure 2. TIM Nuclear Accumulation Is Altered in *tim*(S1404A) and *tim*(S1404D) Mutants

(A) Representative confocal images of sLN_vs clock neurons in adult fly brains stained with α-TIM (red) and α-PDF (green). Single channels are shown in gray scale. Scale bar (merged image in WT ZT16) represents 10 μm. Flies were entrained for 4 days in LD cycles and collected at the indicated times on LD4 for fixation and immunofluorescence analysis.

(B) Line graph showing the fraction of nuclear TIM presented as nuclear TIM divided by total TIM in sLN_vs.

(C) Line graph showing nuclear TIM staining intensity in sLN_vs.

Error bars indicate SEM (n > 30); ***p < 0.001; **p < 0.01; *p < 0.05; Kruskal-Wallis test.

entrained in LD cycles to preclude phase differences between the genotypes that are caused by alterations in period length. Costaining of TIM with pigment-dispersing factor (PDF) enabled the identification of PDF+ clock neurons (small lateral ventral neurons [sLN_vs] and large lateral ventral neurons [lLN_vs]), and demarcation of nuclear versus cytoplasmic compartments as PDF is expressed in the cytoplasm.[55] In agreement with previous studies,[56] we observed that the majority of TIM was cytoplasmic at ZT16 in *tim*(WT) flies but became progressively more nuclear from early to late night (Figures 2A and 2B). In contrast, *tim*(S1404A) flies displayed significantly lower percentage of nuclear TIM (% nuclear TIM/total TIM) at late night (ZT20 and ZT22; Figure 2B), although phosphomimetic *tim*(S1404D) mutants exhibited higher percentage of nuclear TIM over *tim*(WT) flies at ZT16 and ZT22.

In addition to assessing the distribution of TIM in the nucleus versus cytoplasm, we also monitored overall nuclear TIM abundance. We observed a substantially lower abundance of nuclear TIM in *tim*(S1404A) flies between ZT18 and ZT22, although that in *tim*(S1404D) flies appeared higher than *tim*(WT) at ZT16 to ZT18 but lower at ZT22 (Figures 2A and 2C). Taken together, our results suggest that TIM(S1404) phosphorylation promotes TIM nuclear accumulation and the molecular mechanisms underlying the short-period behavioral phenotypes of *tim*(S1404A) and *tim*(S1404D) flies are likely different.

TIM(S1404) Phosphorylation Regulates TIM-XPO1 Interaction

A number of studies have established that PER-TIM nuclear accumulation is regulated by phosphorylation.[20,24,28–31,56] With the characterization of TIM nuclear import pathway[12] and functional NLS,[23] phosphorylation has been thought to influence nuclear entry. However, the evidence that nuclear PER-TIM can be translocated back to the cytoplasm[11,19] raises the possibility that phosphorylation may also regulate nuclear export to influence overall levels of nuclear accumulation. To determine whether S1404 phosphorylation regulates TIM nuclear entry or export, we first searched for potential NLS and nuclear export signal (NES) in the sequences adjacent to S1404 based on classical NLS/NES motifs.[57,58] Whereas we did not locate any sequences that resemble an NLS near S1404, we identified one putative NES (L1394-V1403) immediately adjacent to the S1404 residue (Figure 3A). Previous studies suggest that phosphorylated residue(s) within or in close proximity to an NES can regulate protein nuclear-cytoplasmic distribution by modulating the binding of cargo protein and chromosome maintenance 1 (CRM1).[59,60] CRM1 is the major export protein in mammals that facilitates the transport of proteins from the nucleus to the cytoplasm.

We therefore tested whether TIM(S1404) phosphorylation reduces the interaction between TIM and XPO1, the *Drosophila* homolog of mammalian CRM1, by performing coimmunoprecipitation (coIP) assays using *Drosophila* S2 cells coexpressing *tim*(WT)-hemagglutinin (HA) or *tim*(S1404A)-HA with *xpo1*-FLAG and *per*-V5. We observed significantly higher TIM(S1404A)-XPO1 interaction as compared to TIM(WT) when we pulled down TIM-HA and detected the presence of interacting XPO1 (Figures 3B and 3C). We also performed the reciprocal coimmunoprecipitation, which yielded the same conclusion (Figures 3B and 3D). Furthermore, we assayed the binding of TIM(S1404D) to XPO1 and observed that it was significantly

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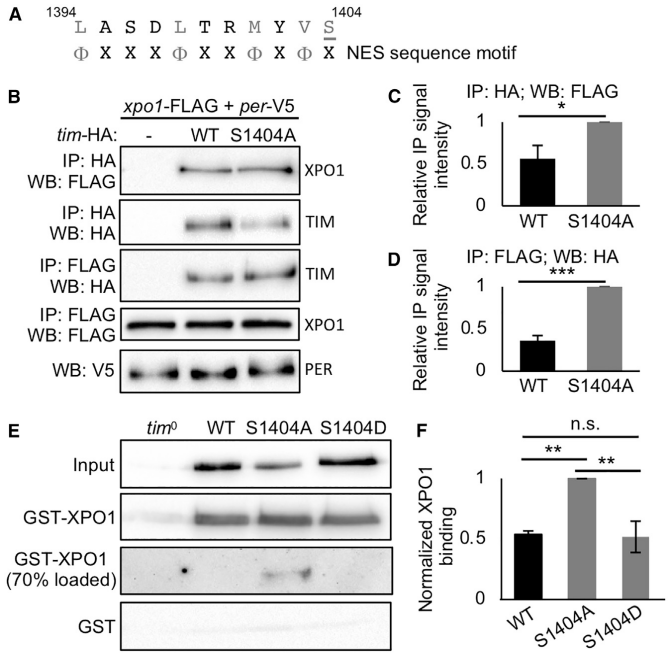

Figure 3. TIM(S1404) Phosphorylation Promotes TIM Nuclear Retention by Compromising TIM-XPO1 Interaction

(A) S1404 is located next to a putative TIM^{NES}: L1394-V1403. S1404 is underlined and shown in gray. Classical NES sequence motif is previously investigated.[58] Φ is hydrophobic amino acid (in gray): Leu; Val; Ile; Phe; or Met. X is any amino acid.

(B) Western blots showing reciprocal coimmunoprecipitations (coIPs) to examine the interactions of TIM(WT) or TIM(S1404A) to XPO1 in *Drosophila* S2 cells expressing pAc-*xpo1*-3XFLAG-6XHIS and pAc-*per*-V5 in the presence or absence of pAc-HA plasmids expressing *tim* variants. Protein extracts were directly analyzed by immunoblotting (α-V5 for PER) or immunoprecipitated with α-HA or α-FLAG resins to detect baits and interactors.

(C and D) Bar graphs displaying quantification of reciprocal coIPs. Values for binding are normalized to amount of bait detected in the IPs and expressed as relative signal intensity (high value = 1). Error bars indicate ± SEM (n = 4); ***p < 0.001; *p < 0.05; two-tailed Student's t test.

(E) Western blots showing GST-XPO1 pull-down of fly head extracts. Flies were entrained in LD cycles and collected on LD3 at ZT20. The relative amount of TIM (input) from four genotypes and that bound to GST-XPO1 or GST-bound glutathione resins were shown. To highlight the difference between genotypes, reduced amount (70%) of GST-XPO1 pull-down reactions were loaded (third panel from top).

(F) Bar graphs displaying quantification of TIM from GST-XPO1 pull-down in (E). TIMs bound to GST-XPO1 were normalized to input and expressed as relative signal intensity (high value = 1). Error bars indicate ± SEM (n = 3); **p < 0.01; one-way ANOVA. See also Figures S3 and S4.

lower when compared to that of TIM(S1404A) but similar to the levels for TIM(WT) (Figure S3). The similarity between the interactions between TIM(WT)-XPO1 and TIM(S1404D)-XPO1 can be explained by the confirmation that TIM(S1404) is phosphorylated in TIM(WT) expressed in *Drosophila* S2 cells using a pS1404 phosphospecific antibody we generated for this study (Figures S4A and S4B). The stronger interaction of XPO1 to TIM(S1404A) as compared to TIM(WT) or TIM(S1404D) was further confirmed by glutathione S-transferase (GST)-XPO1 pull-down assay using fly head extracts (Figures 3E and 3F). Taken together, our results suggest that TIM(S1404) phosphorylation inhibits the nuclear export of TIM via the XPO1-dependent pathway.

TIM(S1404) Phosphorylation Increases PER Nuclear Accumulation

Because TIM is necessary for promoting the nuclear entry of PER-TIM heterodimers,[12,22,23] we next sought to determine whether PER nuclear accumulation is also altered in *tim*(S1404A) and *tim*(S1404D) mutants. We monitored subcellular localization of PER in adult clock neurons using the same method as described for TIM. As expected, the percent of nuclear PER (% nuclear PER/total PER) gradually increased from ZT16 to ZT22 in *tim*(WT) flies (Figures 4A and 4B). In comparison, the percent of PER in the nucleus in *tim*(S1404A) mutants was significantly lower at ZT22 although that for *tim*(S1404D) mutant was significantly higher at ZT22. Furthermore, the overall abundance of nuclear PER was also significantly lower in *tim*(S1404A) mutants at ZT22 (Figures 4A and 4C). Our results therefore support that alterations in TIM subcellular localization due to phosphorylation defect at TIM(S1404) impact subcellular localization of its heterodimeric partner PER.

We next compared PER and TIM protein profiles in head extracts of WT and mutants to determine whether altered nuclear accumulation affects their daily rhythms in protein abundance and phosphorylation state. Consistent with previous studies, newly synthesized PER and TIM in *tim*(WT) flies were hypophosphorylated between ZT8 and ZT12 and became progressively more phosphorylated from early night to the following morning (Figures 4D and 4F).[24,25,61] Daily rhythms in PER and TIM protein abundance were altered in *tim*(S1404A) mutants, as determined by detection of differential rhythmicity (DODR) analysis (PER: $p < 0.05$; TIM: $p < 0.01$).[62] In congruence with the short-period phenotype of *tim*(S1404A) flies, the peak phases of both PER and TIM rhythms advanced from ZT20 in *tim*(WT) flies to ZT16 (Figures 4D and 4E) as calculated by rhythmicity analysis incorporating nonparametric methods (RAIN).[63] In addition, daily PER protein rhythmicity was dampened in *tim*(S1404A) mutants (WT: $p < 0.0001$; S1404A: $p = 0.1361$, RAIN). This is likely caused by compromised nuclear accumulation of the PER-TIM proteins, which is clearly affecting their phosphorylation programs and is

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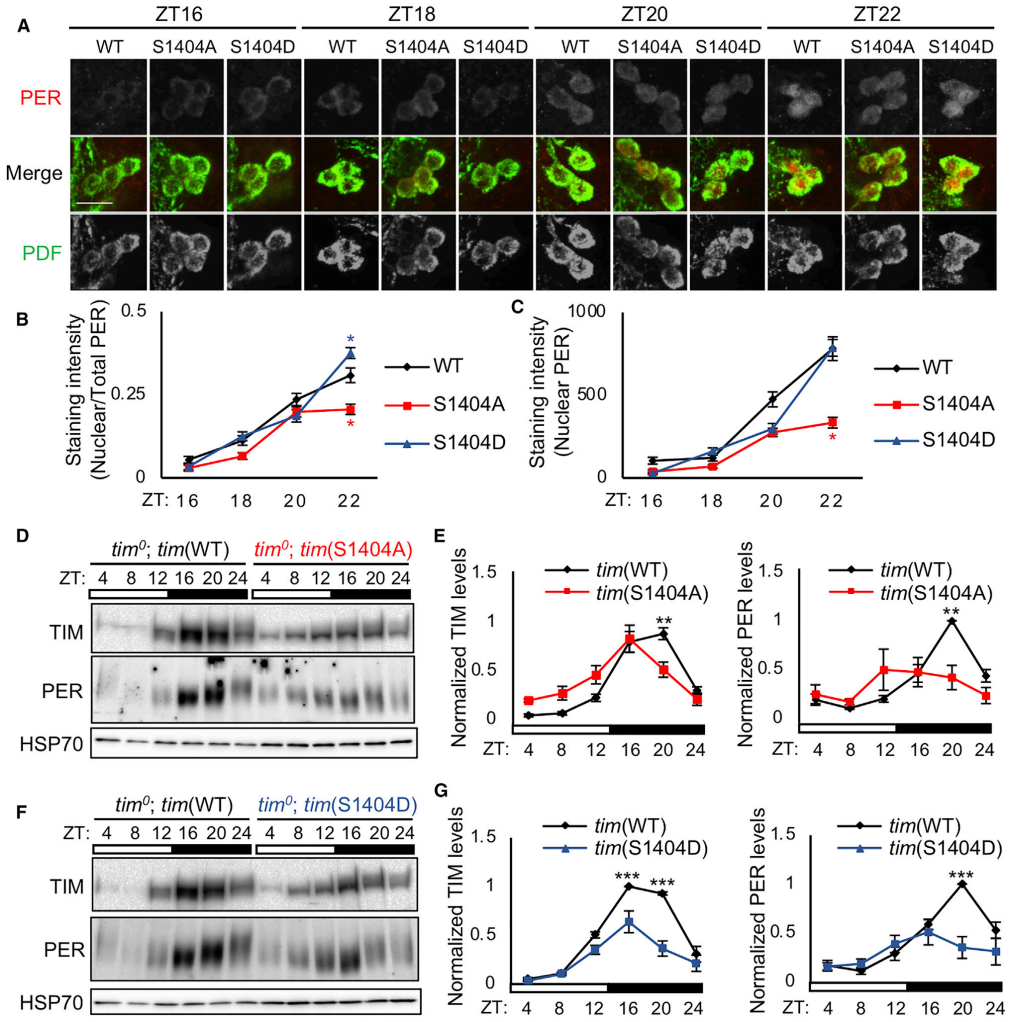

Figure 4. Altered TIM(S1404) Phosphorylation Influences PER Nuclear Accumulation

(A) Representative confocal images of sLN$_v$ clock neurons in adult fly brains stained with α-PER (red) and α-PDF (green). Single channels are shown in gray scale. Scale bar (merged image in WT ZT16) represents 10 μm. Flies were entrained as described in Figure 2A.

(B) Line graph showing the fraction of nuclear PER in sLN$_v$s presented as nuclear PER divided by total PER.

(C) Line graph showing nuclear PER staining intensity in sLN$_v$s. Error bars indicate ± SEM (n > 27); *p < 0.05; Kruskal-Wallis test.

(D and F) Western blots comparing TIM and PER profiles in heads of (D) *tim*(WT) and *tim*(S1404A) flies or (F) *tim*(WT) and *tim*(S1404D) flies. Flies were entrained in LD cycles and collected on LD3 at indicated time points (ZT). α-HSP70 was used to indicate equal loading and for normalization.

(E and G) Quantification of TIM and PER in (D) and (F). Error bars indicate ± SEM (n = 3); ***p < 0.001; **p < 0.01; two-way ANOVA.

expected to impact their phase-specific functions. Furthermore, phase advance of *per* and *tim* mRNA, discussed in the next section, is also expected to contribute to changes in PER and TIM proteins rhythms.

In the case of the short-period *tim*(S1404D) mutant, daily rhythms in PER and TIM were significantly altered as compared to *tim*(WT) (PER: $p < 0.05$; TIM: $p < 0.05$, DODR). The peak phase of PER advanced from ZT20 to ZT16 although that for

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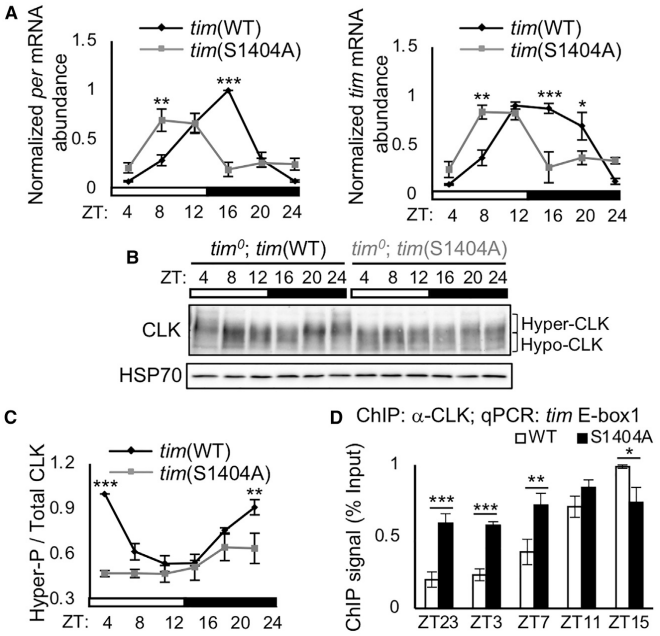

Figure 5. Reduced TIM Nuclear Retention in *tim*(S1404A) Mutant Leads to Dampening of CLK Phosphorylation Rhythm and Phase Advance in CLK-Activated Transcriptional Activation

(A) Steady-state mRNA expression of *per* and *tim* in heads of *tim*(WT) and *tim*(S1404A) flies. Flies were entrained in LD cycles and collected on LD3 at indicated time points (ZT; n = 3).

(B) Western blots comparing CLK protein profiles in heads of *tim*(WT) and *tim*(S1404A) entrained and collected as in (A). Brackets indicate hypo- and hyperphosphorylated CLK isoforms. α-HSP70 was used to indicate equal loading and for normalization.

(C) Quantification of hyperphosphorylated/total CLK. The top half of the CLK signal shown at ZT24 in *tim*(WT) flies (lane 6) is used as a reference to classify CLK isoforms as hyperphosphorylated (n = 3).

(D) ChIP assays using fly head extracts comparing CLK occupancy at *tim* promoter in *tim*(WT) and *tim*(S1404A) flies. CLK-ChIP signals were normalized to % input. ChIP signals for two intergenic regions were used for non-specific background deduction (n = 4).

Error bars indicate ± SEM; ***p < 0.001; **p < 0.01; *p < 0.05; two-way ANOVA. See also Figures S5 and S6.

TIM remained unchanged, as determined by RAIN (Figures 4F and 4G). Moreover, we observed significant dampening of the daily rhythmicity of PER proteins in *tim*(S1404D) flies (WT: $p < 0.0001$; S1404D: $p = 0.0720$, RAIN). Daily rhythmicity of TIM in *tim*(S1404D) mutants was slightly dampened as compared to *tim*(WT) although still rhythmic (WT: $p < 0.0001$; S1404D: $p < 0.0001$, RAIN). Notably, the accelerated PER and TIM turnover in *tim*(S1404D) flies at night (Figure 4G) is consistent with their increased nuclear localization at ZT22 (Figures 2B and 4B). Together, our data suggest that TIM(S1404) phosphorylation promotes PER nuclear accumulation indirectly by increasing TIM nuclear retention.

TIM(S1404) Phosphorylation Influences Rhythmic CLK Phosphorylation and Occupancy at Clock Gene Promoter

We next examined whether reduced PER-TIM nuclear accumulation in *tim*(S1404A) mutants affects the output of the molecular oscillator by assaying *per* and *tim* mRNAs. The daily rhythms in *per* and *tim* mRNAs in *tim*(WT) and *tim*(S1404A) mutants were significantly different (PER: $p < 0.001$; TIM: $p < 0.001$, DODR; Figure 5A). Given the reduction of nuclear PER to repress CLK-CYC transcriptional activity in *tim*(S1404A) flies at night, we expect *per* and *tim* mRNA levels in *tim*(S1404A) to be higher when compared to *tim*(WT) flies during the circadian repression phase (~ZT16–ZT24).[16] Surprisingly, *per* and *tim* mRNA levels were substantially lower at ZT16 and ZT20 in the *tim*(S1404A) mutant. In addition, we observed a significant phase advance in *per* mRNAs in *tim*(S1404A) flies, in congruence with the short-period phenotype of this mutant (WT: peak = ZT16; S1404A: peak = ZT12, RAIN). This resulted in significantly higher levels of *per* and *tim* mRNAs at ZT8 (Figure 5A).

Our analysis of clock gene expression clearly suggests that the short-period phenotype of *tim*(S1404A) is driven primarily by the phase advance of CLK transcriptional activity. But how does reduced nuclear accumulation of PER-TIM heterodimers lead to premature activation of CLK transcriptional activity? CLK transcriptional activity has previously been shown to correlate with CLK phosphorylation status.[15,64–69] Subsequent to nuclear translocation of PER-TIM heterodimers, kinases recruited by the PER-TIM complex have been proposed to phosphorylate CLK and inactivate its transcriptional activity.[15,69] Dephosphorylation by phosphatase then produces hypophosphorylated, transcriptionally active CLK the following morning.[70] Because TIM(S1404A) mutation reduces PER-TIM nuclear accumulation (Figures 2B and 4B), we decided to examine its impact on the daily oscillation of CLK phosphorylation (Figures 5B and 5C). We observed significant alteration in the daily rhythm of CLK phosphorylation in *tim*(S1404A) as compared to *tim*(WT) flies ($p < 0.001$, DODR), despite no significant change in CLK abundance (Figure S5A). Specifically, *tim*(S1404A) exhibited significant dampening in the daily CLK phosphorylation rhythms as compared to *tim*(WT) flies (WT: $p < 0.0001$; S1404A: $p = 0.1079$, RAIN). In particular, significantly less hyperphosphorylated CLK isoforms were detected at ZT24/0 and ZT4 in *tim*(S1404A) flies, the time when CLK is predominantly hyperphosphorylated in *tim*(WT) flies (Figures 5B and 5C). Because hypophosphorylated or intermediately phosphorylated CLK proteins have higher transcriptional activity,[15,68] our results could explain the phase advance in CLK

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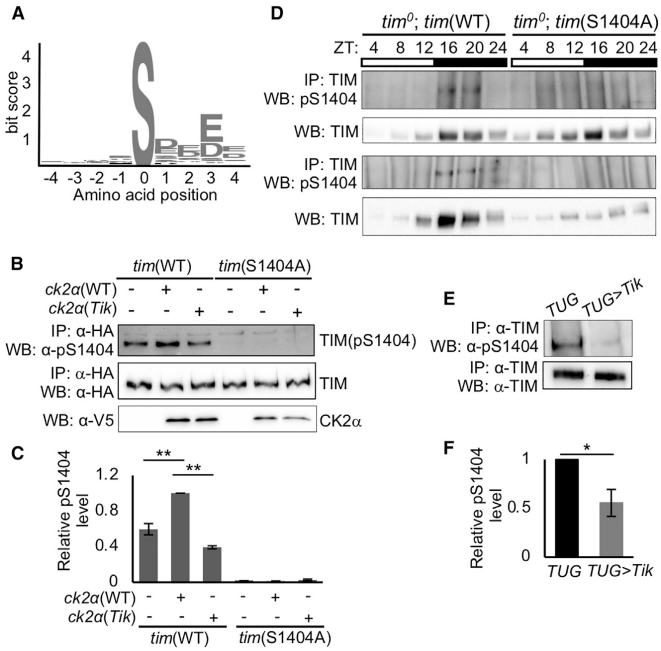

Figure 6. CK2 Phosphorylates TIM(S1404)

(A) CK2 consensus motifs generated by KinasePhos 2.0. S1404 corresponds to phosphoserine at amino acid position 0 (Support vector machine score = 0.9581).

(B) *Drosophila* S2 cells were transfected with pAc-*tim*(WT)-HA or pAc-*tim*(S1404A)-HA and co-transfected with an empty plasmid (pMT-V5-His), pMT-*ck2α*-V5, or pMT-*ck2α*(M161K E165D)-V5, referred to as *ck2α*(*tik*). Protein extracts were incubated with α-HA resin. Total TIM isoforms, TIM(pS1404), and CK2α protein levels were analyzed by western blotting with indicated antibodies.

(C) Bar graph showing relative TIM pS1404 levels in (B) normalized to total TIM isoforms. Error bars indicate ± SEM (n = 2); **p < 0.01; two-way ANOVA.

(D) Fly heads of the specified genotypes were collected at the indicated times on LD3 after 2 days of entrainment. TIM was immunoprecipitated with α-TIM prior to western blotting with α-pS1404 (top panel). Total TIM isoforms are shown in the bottom panel. 2 biological replicates are shown.

(E) Reduced pS1404 in flies overexpressing *ck2α*(*tik*) in *tim*-expressing cells (*TUG>tik*) as compared to parental control (*TUG*). Flies were entrained and collected on LD3 at ZT20. Fly head extracts were immunoprecipitated with α-TIM. TIM(pS1404) and total TIM isoforms were analyzed by western blotting.

(F) Bar graph showing relative pS1404 levels in (E), normalized to total TIM isoforms. Error bars indicate ± SEM (n = 4); *p < 0.05; two-tailed Student's t test.

See also Figure S4.

activation of *per* and *tim* expression in *tim*(S1404A) flies (Figure 5A).

We then asked whether reduction in early-morning CLK phosphorylation in *tim*(S1404A) mutants influences CLK occupancy at clock gene promoters and contributes to premature initiation of *per* and *tim* expression. We performed CLK chromatin immunoprecipitation (CLK-ChIP) followed by qPCR using extracts from adult fly heads (Figure 5D). We observed significantly higher CLK occupancy at multiple morning time points at *tim* E-box in the *tim*(S1404A) mutant as compared to that in *tim*(WT) flies. Together, our data suggest that TIM(S1404) phosphorylation can impact daily rhythms in CLK phosphorylation status and transcriptional activity to regulate circadian timekeeping.

In the case of *tim*(S1404D) flies, although daily rhythms in *per* and *tim* mRNAs were not significantly different from *tim*(WT) flies (*per*: p = 0.1742; *tim*: 0.4254, DODR), the peak phase of *per* mRNA was advanced from ZT16 to ZT12 (RAIN), and the repression of both *per* and *tim* appeared to occur earlier (Figure S6A). This may contribute to the advanced peak phase in PER protein rhythms in *tim*(S1404D) flies (Figures 4F and 4G). Because CLK protein abundance was not significantly altered in *tim*(S1404D) mutants (Figure S5B), the advanced peak phase of *per* mRNA is likely a consequence of alteration in CLK phosphorylation rhythm. In agreement with our hypothesis, the amount of hyperphosphorylated CLK in *tim*(S1404D) was significantly lower at ZT4, which is expected to result in higher CLK transcriptional activity. Finally, we observed an apparent decrease in the amplitude of daily CLK phosphorylation rhythm in *tim*(S1404D) as compared to WT, but it was not significantly altered (p = 0.3140, DODR; Figures S6B and S6C).

CK2 Kinase Phosphorylates TIM(S1404)

We next sought to identify the kinase that phosphorylates TIM(S1404). Based on KinasePhos 2.0,[71] CK2 is predicted with the highest probability to phosphorylate S1404 (Figure 6A). To confirm this *in silica* prediction, we generated a S1404 phospho-specific antibody (α-pS1404) using a phosphorylated S1404-containing peptide as antigen. We then assayed TIM(S1404) phosphorylation in protein extracts of *Drosophila* S2 cells coexpressing *tim*-HA (WT or S1404A) with either the catalytic subunit of *ck2* (*ck2α*) or a dominant-negative variant *ck2α*(*tik*).[26,30] Immunoblotting showed that TIM(pS1404) was significantly reduced when *tim*(WT)-HA was coexpressed with *ck2α*(*tik*) as compared to coexpression with *ck2α*(WT) (Figures 6B, lanes 2 to 3, and 6C). Moreover, there was little to no α-pS1404 signal detected in *tim*(S1404A) (Figures 6B, lanes 4–6, and 6C), suggesting the α-pS1404 antibody is phosphospecific. To further validate the specificity of α-pS1404, we confirmed the reduction of α-pS1404 isoforms when TIM was immunoprecipitated and phosphatase treated prior to immunoblotting (Figures S4A and S4B, lanes 1 to 2).

We proceeded to test whether downregulating CK2 activity in flies reduces TIM(S1404) phosphorylation. First, we evaluated TIM(S1404) phosphorylation over a daily cycle and observed

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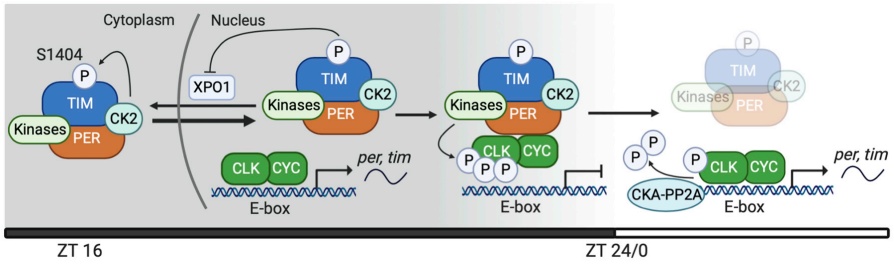

Figure 7. A Model Describing the Function of TIM(S1404) Phosphorylation in Regulating the Molecular Clock

TIM(S1404) is phosphorylated by CK2 in the cytoplasm in early night. Upon entering the nucleus, phosphorylation at S1404 inhibits interaction of TIM and XPO1 and nuclear export of PER-TIM heterodimers, thereby promoting their nuclear accumulation. This allows kinase(s) bound to PER-TIM complex to phosphorylate CLK and remove CLK from circadian promoters. The kinase(s) responsible for this step is currently unknown. CKA-PP2A then dephosphorylates CLK and promotes the onset of CLK transcriptional activity in the next cycle.[70] Other phosphorylation events on PER-TIM are not depicted for simplicity.

that phosphorylation at TIM(S1404) was detected at ZT16 and ZT20 in *tim*(WT) but absent in *tim*(S1404A) flies (Figure 6D). Next, we genetically knocked down CK2 activity by overexpressing *ck2α*(*tik*) in clock neurons using the *tim*-UAS-Gal4 driver (*TUG*).[72] Head extracts from *TUG* > UAS-*ck2α*(*tik*) flies and parental controls collected at ZT20 were probed for S1404 phosphorylation. In concordance with the results in S2 cells, we observed a significant reduction in S1404 phosphorylation in *ck2α*(*tik*)-overexpressing flies (Figures 6E and 6F). Taken together, our results suggest that CK2 phosphorylates TIM(S1404) in *tim*-expressing clock neurons.

Finally, we performed immunocytochemistry in adult brain clock neurons to investigate the subcellular localization of CK2-dependent phosphorylation of TIM(pS1404). Based on western blotting results (Figure 6D) and MS data (Table S1) showing TIM(S1404) phosphorylation at ZT16 in whole-head extracts, abundant cytoplasmic localization of CK2,[26] and previous studies indicating the role of CK2 in promoting PER-TIM nuclear import,[26,27,30,31] we hypothesize that CK2 first phosphorylates TIM at S1404 and residues important for nuclear import in the cytoplasm. This and the role of TIM(S1404) phosphorylation in inhibiting nuclear export are not mutually exclusive. In agreement with our observation from whole-head extracts, we observed prominent TIM(pS1404) signal in clock neurons at ZT16 in *tim*(WT), but not in *tim*(S1404A) mutants (Figure S4C).

DISCUSSION

To better understand the role of phosphorylation in regulating the function of the PER-TIM heterodimer, we identified multiple phosphorylation sites on PER-bound TIM proteins extracted from *Drosophila* tissues. After an initial behavioral screen of *tim* non-phosphorylatable mutants, we proceeded to characterize the function of TIM(S1404), which is located in the TIM CLD and is predicted to be phosphorylated by CK2, a known clock kinase. Leveraging the results from a series of molecular and behavioral analyses on transgenic flies expressing *tim*(S1404A) and *tim*(S1404D) mutants, we formulated a model describing the function of CK2-dependent TIM(S1404) phosphorylation in the molecular clock (Figure 7). In WT flies, TIM(S1404) is first phosphorylated by CK2 in the cytoplasm in early night. Our data do not rule out the possibility that CK2-dependent phosphorylation of TIM(S1404) continues after the entry of PER-TIM heterodimer into the nucleus around midnight. After nuclear entry, TIM(S1404) phosphorylation inhibits the interaction of TIM and the nuclear export machinery, thereby promoting nuclear accumulation of PER-TIM heterodimers. This facilitates timely CLK phosphorylation by kinases recruited by the PER-TIM complex to enhance circadian repression. The identity of kinase(s) that serve this role will need to be resolved in future investigations. Hyperphosphorylated CLK is then dephosphorylated by the CKA-PP2A complex[70] in the following morning to activate the next round of clock gene expression.

Our model is consistent with previous studies showing that PER-TIM-DBT complexes recruit as yet unknown kinases to phosphorylate CLK.[69] It is also consistent with studies proposing that CK2 regulates PER function by phosphorylating TIM.[30,31] Because the S1404A mutation results in a short-period phenotype, which is opposite to the period-lengthening effect of *ck2α*^{tik},[26] our results highlight the complex functions of CK2 in regulating the molecular clock.

In *tim*(S1404A) mutants, the nuclear entry of PER-TIM heterodimer is not affected and proceeds as normal around midnight. Once in the nucleus, TIM(S1404A) interacts with the nuclear export machinery with higher affinity as compared to TIM(WT), leading to increased nuclear export and higher percentage of PER-TIM heterodimers in the cytoplasm. As a result, CLK phosphorylation in the nucleus is reduced, as there are less PER-TIM heterodimers available to serve as scaffolds to recruit CLK kinases. In agreement with this model, we observed dampening of daily CLK phosphorylation rhythms in *tim*(S1404A) flies (Figure 5B). Specifically, there is significantly lower level of hyperphosphorylated CLK isoforms in late night to early morning. Given that CLK hyperphosphorylation is linked to reduced transcriptional activity, it is somewhat surprising that reduced CLK phosphorylation at night (ZT20–ZT24) did not significantly enhance clock gene expression (Figure 5A). This supports that other modifications, such as ubiquitination, are also important for regulating CLK transcriptional activity.[73] USP8 has been

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shown to deubiquitylate CLK at late night to facilitate repression of clock genes.

We propose that reduced CLK hyperphosphorylation represents the key driver for the short-period phenotype of *tim*(S1404A) mutant. Because CLK is not hyperphosphorylated by kinases recruited by the PER-TIM heterodimers at night, it does not have to be dephosphorylated by the CKA-PP2A complex[70] in the morning of the next cycle to activate clock gene transcription. This would explain the phase advance of CLK occupancy on circadian promoters (Figure 5D) and CLK-activated *per* and *tim* expression (Figure 5A). It is interesting to note that we did not observe diminished repression of clock genes despite the reduction of nuclear PER-TIM heterodimers at night (Figure 5A), indicating additional mechanisms, e.g., chromatin remodeling and protein modifications, that are absent during this time of the circadian cycle are necessary to activate clock gene expression, even when the level of PER repressor is reduced.

Curiously, *tim*(S1404D) mutants also exhibit a short-period phenotype in behavioral rhythms (Figure 1B). The fact that *tim*(S1404D) mutants display the opposite molecular phenotype in the context of nucleocytoplasmic localization of TIM and PER when compared to *tim*(S1404A) flies support that the S1404D mutation is phosphomimetic (Figures 2 and 4). The short-period phenotype of the *tim*(S1404D) mutant is therefore best explained by phase advance of the nuclear accumulation and subsequent degradation of PER-TIM heterodimer due to sustained inhibition of TIM-XPO1 interaction. Because of accelerated PER-TIM degradation, the time frame for PER-TIM to recruit CLK kinases is shortened. This would explain the significantly lower amount of hyperphosphorylated CLK in *tim*(S1404D) flies at ZT4 and the phase advance of *per* mRNA rhythm (Figure S6).

Previous studies suggested that TIM phosphorylation plays a role in regulating its light-dependent degradation as well as its subcellular localization. Phosphorylation of tyrosine (pY) was first proposed to precede the degradation of TIM upon light exposure.[40] We did not recover any pY residues in our MS analysis of PER-bound TIM. Nevertheless, we cannot rule out that Y phosphorylation may occur when TIM is not in complex with PER. Furthermore, it is possible that pY may result in the disassembly of the PER-TIM heterodimer or could result in very unstable TIM proteins that are difficult to purify from fly tissues. Instead of potential pY residues, we were able to identify a number of phosphorylated S/T residues that significantly impact behavioral phase shift responses to a light pulse at ZT15 or ZT21, suggesting they may be involved in mediating light-dependent TIM degradation (Figures S1C and S1D). TIM(S568) is located within a functional NLS.[23] Non-phosphorylatable *tim*(S568A) mutants exhibited arrhythmic locomotor activity (Figure 1B), which is consistent with the phenotype of mutants defective in TIM nuclear entry[22,23] and/or light entrainment.[22] Future investigations are necessary to determine whether S568 phosphorylation may regulate TIM subcellular localization and/or light responses. It will be also interesting to determine whether phosphorylation at S1389, S1393, and S1396, which are close to the NESs identified in this study, play a role in regulating TIM nuclear export. Of note, we did not observe phosphorylation at T113, S297/T301, and T305/S309/S313 residues in our MS analysis. These residues were previously suggested to promote TIM nuclear entry when phosphorylated.[22,31] It is possible that these residues are more highly phosphorylated when TIM is not bound to PER.

In summary, we describe a phosphorylation-dependent nuclear export mechanism that regulates the nuclear accumulation of PER-TIM heterodimers and consequently the phase of CLK transcriptional activity in the molecular clock. We identified an NES motif in the previously characterized TIM CLD domain and showed that S1404 phosphorylation adjacent to this NES can regulate PER-TIM nuclear export. NES has been shown repeatedly to be an important regulatory motif that modulates localization and activities of transcriptional repressor in eukaryotes.[74–78] The NES and S1404 module at the C terminus of TIM is highly conserved in drosophilids and in most species that have the *timeless* gene (Figures S2A and S2B). Together with our MS analysis showing the phosphorylation of *Danaus plexippus* TIM(S1174), the homologous site of *Drosophila melanogaster* TIM(S1404) (Figure S2C), we expect this phosphorylation-dependent mechanism that regulates TIM function to be conserved in insects. Interestingly, in *timeout/timeless 2* (homolog of mammalian *timeless*), the ancestral paralog of *tim* that has a role in circadian photoreception, but not in the oscillator itself,[79,80] this C-terminal NES is absent and serine is replaced by a glutamic acid (E) (Figure S2B). We speculate that the gain of the NES and TIM(S1404) module at some point in evolution likely enabled TIM to cycle between subcellular compartments in a phosphorylation- and phase-dependent manner over the circadian cycle. This would allow CK2-dependent TIM phosphorylation to regulate the phase-specific functions of PER-TIM heterodimers in specific lineages.

Finally, it is interesting to point out that a mutation in mammalian *timeless* that results in decreased nuclear TIM accumulation also leads to phase advance of human sleep-wake behavior, an output of the circadian clock.[81] The circadian period length of mice expressing the TIM(R1081X) mutation, which manifests into human familial advanced sleep phase syndrome (FASPS), as determined by activity rhythm, is identical to WT mice. However, proliferating embryonic fibroblasts derived from heterozygous TIM(R1081X) mutant mice as well as mammalian U2OS and HEK293 cells expressing this same mutation exhibit a significant period shortening. This suggests that decreased TIM nuclear accumulation in flies and mammals results in similar outcomes in the context of the molecular clock. Our results highlight an unexpected parallel between the functions of *Drosophila* and mammalian *timeless* in the molecular clock, even though the exact mechanisms and sequence motifs regulating their functions might have diverged. Analysis of *Drosophila tim* mutants could provide insights into the mechanisms that regulate the nuclear accumulation of mammalian TIM and further elucidate its functions in the mammalian clock. For instance, similar to what we deduced from *Drosophila tim*(S1404A) mutant, the advanced sleep phenotype in human FASPS R1081X patients could be the result of altered phosphorylation profile of BMAL1-CLOCK, leading to phase advance in circadian transcriptional activation.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.10.061.

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AUTHOR CONTRIBUTIONS

J.C.C., Y.D.C., and A.S. designed research; Y.D.C., Y.X., C.C.T., J.D.C.-L., C.O., K.A.M., Y.H.L., B.L.K., J.T.V., H.Z., and C.Z. performed research and analyzed data; Y.D.C., J.C.C., Y.X., Y.Z., and A.S. contributed to critical interpretation of the data; X.L. and K.A.M. generated reagents; and Y.D.C., A.S., and J.C.C. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Guinea pig polyclonal anti-DpPER	This paper	RRID: AB_2832970
Rat monoclonal anti-HA (clone 3F10)	Roche	Cat#12013819001
Mouse monoclonal anti-V5	Thermo Fisher Scientific	Car#R960-25
Mouse monoclonal anti-FLAG M2	Sigma-Aldrich	Cat#M3165
Goat polyclonal anti-GST	GE Healthcare	Cat#GE27-4577-01
Rat polyclonal anti-TIM (R5839)	Abrieux et al.[82]	RRID: AB_2782953
Rabbit polyclonal anti-pS1404 (RB S4602)	This paper	RRID: AB_2814716
Guinea pig polyclonal anti-CLK (GP 6139)	This paper	RRID: AB_2827523
Guinea pig polyclonal anti-PER (GP5620)	Kwok et al.[83]	RRID: AB_2747405
Mouse monoclonal anti-HSP70 (clone BRM-22)	Sigma-Aldrich	Cat#SAB4200714
Goat anti-mouse IgG-HRP	GE Healthcare	Cat#NA931
Mouse anti-goat IgG-HRP	Santa Cruz Biotechnology	Cat#sc-2354
Goat anti-guinea pig IgG-HRP	Sigma-Aldrich	Cat#A7289
Donkey anti-rabbit IgG-HRP	GE Healthcare	Cat#NA934
Goat anti-rat IgG-HRP	GE Healthcare	Cat#NA935
Rabbit polyclonal anti-PER	Zeng et al.[35]	N/A
Guinea pig polyclonal anti-TIM	Rakshit et al.[84]	N/A
Mouse monoclonal anti-PDF (clone C7-C)	Developmental Studies Hybridoma Bank	RRID: AB_760350 and AB_2315084
Cy3 AffiniPure donkey anti-rat IgG (H+L)	Jackson Immunoresearch	Cat#712-165-153RRID: AB_2340667
Alexa Fluor 488 AffiniPure donkey anti-rabbit-IgG (H+L)	Jackson Immunoresearch	Cat#711-545-152; RRID: AB_2313584
Cy5 AffiniPure donkey anti-mouse IgG (H+L)	Jackson Immunoresearch	Cat#715-175-150; RRID: AB_2340819
Bacterial and Virus Strains		
DH10BAC *E. coli*	Thermo Fisher Scientific	Cat#10361012
Sf9 cells	Thermo Fisher Scientific	Cat#11496015
BL21(DE3) *E. coli*	Sigma-Aldrich	Cat#70954
Chemicals, Peptides, and Recombinant Proteins		
Pfu Turbo Cx Hotstart DNA polymerase	Agilent	Cat#600410-51
Bacto Agar	BD Biosciences	Cat#90000-767
Grace's Insect Medium	Thermo Fisher Scientific	Cat#11605-094
Schneider's *Drosophila* Medium	Thermo Fisher Scientific	Cat#21720-024
Fetal Bovine Serum	VWR	Cat# 97068-085
Penicillin/streptomycin	Thermo Fisher Scientific	Cat#15-140-148
Trypsin/EDTA	Thermo Fisher Scientific	Cat#25300-062
TRI regent	Sigma-Aldrich	Cat#T9424
MG132	Sigma-Aldrich	Cat#C2211
Aprotinin	Sigma-Aldrich	Cat#A1153
Leupeptin	Sigma-Aldrich	Cat#L2884
Pepstatin A	Sigma-Aldrich	Cat#P5318
5ml IMAC Nickle column	Bio-Rad	Cat #7800811
Ni-NTA Superflow nickle-charged resin	QIAGEN	Cat#30761
SIGMAFAST Protease Inhibitor Cocktail, EDTA-FREE	Sigma-Aldrich	Cat#S8830

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
anti-HA Affinity Matrix	Sigma-Aldrich	Cat#11815016001
ANTI-FLAG M2 Affinity Gel	Sigma-Aldrich	Cat#A2220
Glutathione Sepharose 4B	GE Healthcare	Cat#GE17-0756-01
PhosSTOP	Sigma-Aldrich	Cat#4906845001
GammaBind Plus Sepharose antibody purification resin	GE Healthcare	Cat#17088601
Normal Goat Serum	Jackson Immunoresearch	Cat#005-000-121
λ-Phosphatase	NEB	Cat#P0753S
RQ1 DNase	Promega	Cat#M6101
SsoAdvanced SYBR green supermix	Bio-Rad	Cat#1725270
Peptide: DLTRMYVpSDEDDRLE; where p = phosphate	This paper	N/A
IGEPAL CA-630	Sigma-Aldrich	Cat#8896
Tergitol NP-40	Sigma-Aldrich	Cat#NP40S
Tergitol NP-10	Sigma-Aldrich	Cat#NP10
AcBSA	Promega	Cat#R3961
RNase A	Thermo Fisher Scientific	Cat#EN0531
Proteinase K	NEB	Cat#P8107S
Dynabeads Protein G	Thermo Fisher Scientific	Cat#10003D
Critical Commercial Assays		
Superscript IV	Thermo Fisher Scientific	Cat#18091050
Effectene Transfection Reagents	QIAGEN	Cat#301425
Pierce Coomassie Plus Assay Reagents	Thermo Fisher Scientific	Cat#1856210
XtremeGENE 9	Sigma-Aldrich	Cat#6365779001
QIAquick PCR Purification Kit	QIAGEN	Cat#28106
Deposited Data		
D. melanogaster MS data	This paper	Chorus repository: project ID 1424
Dannaus plexippus DpN1 MS data	This paper	ProteomeXchange: MSV000085748
Experimental Models: Cell Lines		
D. melanogaster: Cell line S2: S2-DRSC	Thermo Fisher Scientific	Cat#R69007
Dannaus plexippus: Cell line DpN1	Zhu et al.[85]	N/A
Experimental Models: Fly Lines		
D. melanogaster: w; UAS-dicer2; tim-UAS-Gal4	Wong et al.[71]	N/A
D. melanogaster: w; UAS-ck2α^{tik}	Bloomington Drosophila Stock Center	#24624
D. melanogaster: yw; *tim*^{0}	Myers et al.[53]	N/A
D. melanogaster: w; *clk*^{out}	Szabó et al.[67]	N/A
D. melanogaster: w^{1118}	Bloomington Drosophila Stock Center	B#3605
D. melanogaster: yw; tim^{0}*; tim*(WT)	This paper	N/A
D. melanogaster: yw; tim^{0}*; tim*(S492A)	This paper	N/A
D. melanogaster: yw; tim^{0}*; tim*(S568A)	This paper	N/A
D. melanogaster: yw; tim^{0}*; tim*(S891A)	This paper	N/A
D. melanogaster: yw; tim^{0}*; tim*(S1153A)	This paper	N/A
D. melanogaster: yw; tim^{0}*; tim*(T1200-1205A)	This paper	N/A
D. melanogaster: yw; tim^{0}*; tim*(S1404A)	This paper	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
D. melanogaster: yw; tim^0; tim(S1404D)	This paper	N/A
Oligonucleotides		
See Table S3 for primers for PCR mutagenesis, ChIP analysis and qPCR analysis.	This paper	N/A
Recombinant DNA		
Plasmid: pAc-*per*-V5-HIS	Thaben and Westermark[83]	N/A
Plasmid: pAc-3XFLAG-6XHIS	Chiu et al.[42]	N/A
Plasmid: pAc-*xpo1*-3XFLAG-6XHIS	This paper	N/A
Plasmid: pAc-*tim*(WT)-HA	Ko et al.[14]	N/A
Plasmid: pAc-*tim*(S1404A)-HA	This paper	N/A
Plasmid: pAc-*tim*(S1404D)-HA	This paper	N/A
Plasmid: pMT-*ck2α*-V5	This paper	N/A
Plasmid: pMT-*ck2α*(Tik)-V5	This paper	N/A
Plasmid: pMT-V5	Thermo Fisher	Cat#V412020
Plasmid: pFastBac1-6XHIS	Fraser et al.[86]	N/A
Plasmid: pFastBac1-6XHIS-d*clk*(1-1770)	This paper	N/A
Plasmid: pBA-*dpper*-FLAG	Zhu et al.[85]	N/A
Plasmid: pHis::Parallel1	Gustafson et al.[87]	N/A
Software and Algorithms		
Fiji ImageJ (used for analysis of immunofluorescence microscopy images)	NIH Image	https://fiji.sc
GraphPad Prism 8 for Mac OS X	GraphPad Software	https://www.graphpad.com
FaasX	Laboratory of F. Rouyer; https://neuropsi.cnrs.fr/en/cnn-home/francois-rouyer/faasx-software/#thematique	Kit version: 1.22
Excel	Microsoft	Version 16.41

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Joanna C. Chiu (jcchiu@ucdavis.edu).

Materials Availability

All unique/stable reagents generated in this study are available from the lead contact without restriction.

Data and Code Availability

This manuscript includes all datasets generated or analyzed during this study. Proteomics data have been deposited into public data repository. Accession numbers are provided in Method Details.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila construct design and transformation

A *p*{*tim*(WT)-*luc*} transgene, containing 4.1kb of the *tim* promoter, *tim* full-length cDNA (*ls-tim* allele), and a luciferase reporter in the *pattB* vector, was kindly provided by Patrick Emery. The luciferase reporter was removed using MluI/XhoI restriction sites, and a 3XFLAG-6XHIS epitope was added in frame to the C terminus of the *tim* coding region. PhiC31 site-directed recombination[88] was used for transgenesis to generate *yw*; *tim*^{0}; *tim*(WT) (also described in[82]). Plasmids were injected into *yw* fly embryos carrying *attP* sites on chromosome 3 (*attP2*) (BestGene, Chino Hills, CA). Transformants were crossed with *yw*; *tim^0* flies[53] to remove endogenous copies of *tim* prior to behavioral and molecular analyses. To generate flies expressing non-phosphorylatable (Serine/Threonine (S/T) to Alanine (A)) or phosphomimetic (S/T to Aspartic acid (D)) *tim* mutants, pAc-*ls-tim*-HA was used as the template for site-directed mutagenesis using Pfu Turbo Cx DNA polymerase (Agilent Technologies, Santa Clara, CA) (see Table S3 for mutagenic primer

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sequences). After mutagenesis and confirmation by Sanger sequencing (UC Davis DNA Sequencing Facility), the mutant variants of 2.8 kb MluI-XbaI *tim* subfragments were used to replace the corresponding WT fragments in *pattB-p{tim*(WT)-3XFLAG-6XHIS}.

Targeted expression of *ck2α* dsRNA in *tim*-expressing neurons was achieved via the UAS/Gal4 system[89]. The Gal4 driver line, *w; UAS-dicer2; tim-UAS-Gal4* (*TUG*)[72], was used to drive expression in *tim* expressing clock neurons. *UAS-ck2α*^{tik} responder line (B24624) from the Bloomington *Drosophila* Stock Center was used to reduce endogenous function of CK2α.

METHOD DETAILS

Identification of TIM PTM sites from fly tissues

PER-bound TIM phosphorylation and O-GlcNAcylation sites were identified from the label-free mass spectrometry (MS) proteomics experiments as previously described[47]. Procedures for immunoprecipitation of PER-TIM complexes and mass spectrometry (MS) were previously described[47]. Epitope-tagged PER proteins were pulled down using α-FLAG and PER-bound TIM proteins were pulled down simultaneously and subjected to MS analysis.

Mass spectrometric data was analyzed with PEAKS Studio X+ (Bioinformatics Solutions Inc., Canada). Raw data refinement was performed with the following settings: Merge Options: no merge, Precursor Options: corrected, Charge Options: 1-6, Filter Options: no filter, Process: true, Default: true, Associate Chimera: yes. *De novo* sequencing and database searching were performed with a Parent Mass Error Tolerance of 10 ppm. Fragment Mass Error Tolerance was set to 0.02 Da, and Enzyme was set to none. The following variable modifications were applied: Oxidation (M), pyro-Glu from Q (N-term Q), phosphorylation (STY), acetylation (protein N-terminal) and HexNAc (STNY). Carbamidomethylation (C) was set as fixed modification. A maximum of 5 variable PTMs were allowed per peptide. A custom database of appropriate size (550 protein sequences) containing TIMELESS protein sequence (UniProt ID A0A1W5PW00) from UniProt was used for database searching. Database search results were filtered to 1% PSM-FDR. Phosphosite localization was validated by inspecting fragment ion spectra of all phosphopeptides. Identification of GlcNAc-modified peptides was confirmed by inspecting the corresponding fragment ion spectra for the presence of characteristic fragment ions (m/z 168.07, 186.08, 204.09). The *Drosophila* MS data has been deposited into Chorus repository (project ID 1424): (https://chorusproject.org/anonymous/download/experiment/e47a30f7f2c749aba438652d7d88ef04) and (https://chorusproject.org/anonymous/download/experiment/e6d6163b31bf40288606f827c6f18371)

Danaus plexippus DpN1 cell culture

Monarch butterfly DpN1 cells[85], kindly provided by Steven Reppert and Christine Merlin, were grown at 28°C in Grace's medium (Thermo Fisher Scientific, Waltham, MA), supplemented with 10% Fetal Bovine Serum (FBS) (VWR, Radnor, PA) and 1X penicillin/streptomycin (Thermo Fisher Scientific). Cells were passed every 7 days. Old medium was removed and cells were washed with cell culture grade 1XPBS (Thermo Fisher Scientific) once before treating with Trypsin/EDTA (Thermo Fisher Scientific) for at least two minutes at 28°C. To halt trypsinization, FBS-containing Grace's medium was added to the cells. Cell suspensions were then passed into new tissue culture flask with FBS-containing Grace's medium.

AP-MS of *Danaus plexippus* PER and TIM

For each time-point, roughly 2.5g of cell pellet in Lysis Buffer (20mM HEPES pH 7.5, 1mM DTT, 0.5mM PMSF and SIGMAFAST EDTA-free protease inhibitor cocktail (Sigma) were dounced prior to centrifugation at 800xg for 15 minutes at 4°C to separate nuclear and cytoplasmic fractions. The nuclear fraction (pellet) was washed twice with Lysis Buffer prior to resuspension in Nuclear Extraction Buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 0.5mM EDTA, 1mM DTT, 1mM $MgCl_2$, 1% Triton X-100, 0.4% sodium deoxycholate, 0.5mM PMSF, 10mM NaF, 10% glycerol and SIGMAFAST EDTA-free protease inhibitor cocktail) and dounced with tight pestle supplemented with MG132 (Sigma) and DNase I (Promega, Madison, WI). The cytoplasmic fraction (supernatant) was supplemented with 150mM NaCl, 1mM $MgCl_2$, 0.5mM EDTA, 1% Triton X-100, 0.4% sodium deoxycholate, 10mM NaF, 10% glycerol and SIGMAFAST EDTA-free protease inhibitor cocktail. After 30 minutes incubation at 4°C, nuclear fraction was diluted to 0.1% SDS with concentration of other content unchanged. Nuclear and cytoplasmic fractions were then centrifuged at 15,000 rpm for 15 minutes. Supernatants were incubated with gammabind Sepharose beads (GE Healthcare, Piscataway, NJ) for 30 minutes at 4°C to reduce nonspecific binding prior to overnight incubation with α-DpPER (GP5913, RRID: AB_2832970). On the second day, samples were incubated with gammabind Sepharose beads (GE Healthcare). Beads were washed three times with Wash buffer (20mM HEPES, 150mM NaCl, 1mM $MgCl_2$, 1mM DTT, 0.5mM EDTA, 1% Triton X-100, 0.4% sodium deoxycholate, 10mM NaF, 0.5mM PMSF, 10% glycerol) and subsequently eluted in 200ul 2X SDS sample buffer at 95°C for 4 minutes. After resolving eluates on a Tris-Tricine gel, excised gel containing eluates was digested with protease and followed by mass spectrometry as previously described[47]. DpTIM was copurified with DpPER and analyzed by MS. DpN1 MS data has been deposited into ProteomeXchange (ftp://massive.ucsd.edu/MSV000085748/).

Locomotor activity assay

Daily locomotor activity rhythms in male flies was assayed using the *Drosophila* Activity Monitoring System (DAMS, Trikinetics, Waltham, MA) as described previously[90]. Young adult male flies (~3-5-day old) were entrained for 4 days in light/dark (LD) cycles (12h light/12h dark), followed by 7 days of constant darkness (DD) to assess their free-running rhythms in incubators at 25 C. Each fly was kept in 5mm glass tube that contains fly food, 5% Sucrose, 2% Bacto Agar (BD Biosciences, San Jose, CA), at one end and plugged

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with yarn at the other end. The locomotor activity data from individual flies were analyzed using the FaasX software (Fly Activity Analysis Suite for Mac OSX). Periods of each fly were calculated using the chi-square periodogram analysis and pooled for a composite for each genotype.

Assaying responses to light pulse

Male flies were entrained for 4 days in LD cycle (12h light/12h dark). In the dark phase on LD4, the light-pulsed (LP) flies were given a 10-minute pulse of light at ZT 15 or ZT 21 before being placed in 7 days of DD, while the non-light pulsed (NLP) flies were not exposed to light pulse treatments. Activity rhythms were measured using the DAMS and analyzed as using FaasX as described in the locomotor activity assay section. Peaks in activity rhythms were restricted to between ZT 6 and ZT 18 and converted into a value in degrees using Excel (Microsoft, Redmond, WA) (24 hours = 360°). LD3 data was used to normalize the DD1 data of each fly by subtracting the LD3 from DD1, within each respective genotype. *tim*(WT) degree values were then subtracted from each mutant to determine the phase shift as a result of the light pulse. The difference in degrees was then converted back to hours using Excel (Microsoft).

Plasmids for *Drosophila* S2 cell culture

Exportin 1 (*xpo1*) ORF was amplified from cDNA that was reverse transcribed (Superscript IV, Thermo Fisher Scientific) from total RNA extracted from fly heads using TRI Reagent (Sigma, St. Louis, MO). The PCR product was cloned into pAc-3XFLAG-6XHIS[42] and pMT-*gst*[41].

Drosophila S2 cell culture and transfection

Drosophila S2 cells and *Schneider's Drosophila* medium were obtained from Life Technologies (Carlsbad, CA). For all cell culture experiments, S2 cells were seeded at 1 X 10^6 cells/ml in a 6-well plate and transfected using Effectene (QIAGEN, Germantown, MD). For coimmunoprecipitation (coIP) assays, S2 cells were co-transfected with 0.8 μg of pAc-*per*-V5-HIS (herein referred to as pAc-*per*-V5), 0.8 μg of pAc-*xpo1*-3XFLAG-6XHIS (herein referred to as pAc-*xpo1*-FH) and 0.8 μg of pAc-*tim*(X)-HA, where X is either WT or S1404A to detect protein-protein interactions. In control IPs to detect non-specific binding, cells were co-transfected with pAc-*per*-V5-His and pAc-*xpo1*-FH without pAc-*tim*(X)-HA. S2 cells were harvested 40 hours after transfection. For GST pull-down assays, S2 cells were transfected with either 0.8 μg of pMT-*gst*-*xpo1* or 0.8 μg of pMT-*gst* and induced with 500 μM $CuSO_4$ immediately after transfection and cells were harvested 44 hours after induction. For IP to detect TIM(pS1404), S2 cells were co-transfected with 0.8 μg of pAc-*tim*(X)-HA and either 0.2 μg of pMT-*ck2α*-V5 or 0.2 μg pMT-V5-His as empty control. Expression of *ck2α* was induced with 500 μM $CuSO_4$ immediately after transfection and cells were harvested 40 hours after kinase induction.

Coimmunoprecipitation in *Drosophila* S2 cells

CoIP experiments were performed as described previously[10] with the following modifications. Cells were harvested 40 hours after transfection, washed once with 1X PBS and lysed with modified RIPA (20mM Tris-HCl pH 7.5, 150mM NaCl, 10% glycerol, 1% Triton X-100, 0.4% sodium deoxycholate, 0.1% SDS, 1mM EDTA) supplemented with 25mM NaF, 0.5mM PMSF, and SIGMAFAST EDTA-free protease inhibitor cocktail. Proteins were incubated with 20 μL α-HA or α-FLAG M2 resins (Sigma) for 4 hours at 4°C to pull down TIM or XPO1, respectively. Resins were washed three times in modified RIPA buffer. Immune complexes were analyzed by western blotting. Signal intensity of interacting protein was normalized to the intensity of the bait protein.

GST pull-down assays using fly head extracts

GST pull-down assays were performed as previously described[41] with the following modifications. GST Lysis Buffer (20mM Tris-HCl pH 7.5, 0.05% IGEPAL CA-630 (Sigma), 1mM EDTA, 5mM DTT, 150mM NaCl, 25mM NaF) was used for protein extractions from S2 cells. Extracts containing GST or GST-XPO1 were incubated with 25 μL glutathione Sepharose beads (GE healthcare) for 6 hours at 4°C. Beads were washed two times in GST Lysis Buffer and once with modified RIPA buffer. Prey proteins were extracted from 500 μL of fly heads per reaction with modified RIPA buffer and incubated with GST- or GST-XPO1-bound beads for 6 hours. Beads were washed three times in modified RIPA buffer. Input and bound TIM were analyzed by western blotting.

Western blotting and antibodies

Protein extractions from *Drosophila* S2 cells and adult fly heads, western blotting, and image analysis was performed as previously described[41,83]. RBS buffer (20mM HEPES pH7.5, 50mM KCl, 10% glycerol, 2mM EDTA, 1mM DTT, 1% Triton X-100, 0.4% NP-40, 10 μg/ml Aprotinin, 5 μg/ml Leupeptin, 1 μg/ml Pepstatin, 0.5mM PMSF, 25mM NaF) was used for protein extractions from fly heads. Protein concentration was measured using Pierce Coomassie Plus Assay Reagents (Thermo Fisher Scientific). 2X SDS sample buffer was added and the mixture boiled at 95°C for 5 minutes. Equal amounts of proteins were resolved by polyacrylamide-SDS gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) using Semi-Dry Transfer Cell (Bio-Rad). Membranes were incubated in 5% Blocking Buffer (Bio-Rad) for 40 minutes, incubated with primary antibodies for 16-20 hours. Blots were then washed with 1X TBST for 1 hour, incubated with secondary antibodies for 1 hour, washed again prior to treatment of chemiluminescence ECL reagent (Bio-Rad). The following percentage of polyacrylamide-SDS gel were used: 6% for PER, TIM; 8% for CLK and XPO1, 10% for HSP70 and 12% for CK2α.

Primary antibodies: α-HA 3F10 (Roche, Indianapolis, IN) at 1:2000 for TIM-HA, α-V5 (Thermo Fisher Scientific) at 1:3000 for PER-V5, α-FLAG (Sigma) at 1:7000 for XPO1-FLAG, α-GST (GE Healthcare) at 1:5000 for GST and GST-XPO1, α-TIM (R5839,

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RRID:AB_2782953) at 1:1000 for TIM[82], α-pS1404 (RB S4602-2, RRID:AB_2814716) at 1:2000 for TIM(pS1404) isoforms, α-CLK (GP6139, RRID:AB_2827523) at 1:2000 for CLK, α-PER (GP5620; RRID:AB_2747405) at 1:2000 for PER and α-HSP70 (Sigma) at 1:10000 was used for to indicate equal loading and for normalization. Secondary antibodies conjugated with HRP were added as follows: α-mouse IgG (Sigma) at 1:2000 for α-V5 detection, 1:2000 for α-FLAG detection, or 1:10,000 for α-HSP70 detection, α-goat IgG (Santa Cruz Biotechnology) at 1:3000 for α-GST detection, α-guinea pig IgG (Sigma) at 1:1000 for α-PER detection, α-rabbit IgG (Sigma) at 1:2000 for α-pS1404 detection, and α-rat IgG (Sigma) at 1:1000 for detecting α-HA and α-TIM.

Generating *Drosophila* CLOCK antibodies

The first 1770 nucleotides of *Drosophila clk* cDNA (Flybase: FBpp0099478) was subcloned into a modified His-tagged pFastBac1 vector (Invitrogen, Carlsbad, CA) as previously described[86]. The recombined construct, pFastBac1-6XHis-d*clk* (1-1770), was transformed into DH10BAC *E. coli* (Invitrogen) and bacmid DNA was then purified. To generate viral stock, bacmid DNA was transfected into Sf9 cells using XtremeGENE 9 transfection reagent (Sigma) and media is collected according to the manufacturer's protocol. Viral stock was used to infect Sf9 cells (Thermo Fisher Scientific) for large-scale expression of CLK antigen. As the CLK antigen is insoluble in extraction buffer (20mM HEPES pH 7.5, 400mM KCl, 5mM imidazole, 10% glycerol, 10mM β-mercaptoethanol) supplemented with 1X SIGMAFAST EDTA-free protease inhibitor cocktail, we collected the insoluble cell pellet after extraction and dissolved it in denaturing solution (50mM Na_3PO_4, 1% SDS) by boiling for 10 minutes. SDS in the sample was diluted to 0.05% before purification using 5ml of Ni-NTA Superflow nickle-charged resin (QIAGEN). CLK antigen was sent to Covance Inc. (Princeton, NJ) for antibody production in guinea pigs. Antibody specificity was confirmed by comparing signals in WT and Clk^{out} flies (Figure S5C).

Generating *Danaus plexippus* PERIOD antibodies

Danaus plexippus per (*dpper*) cDNA sequence that encodes amino acid 898-1095 was amplified from pBA-*dpper*-FLAG[85] and subcloned into the pHis::Parallel1 bacterial expression vector kindly provided by Carrie Partch. The BL21 (DE3) *E. coli* strain containing plasmids with *dpper* fragment were grown to an OD_{600} of ~0.7-0.8 in the presence of ampicillin (125 μg/ml) and protein expression was induced as described previously[87]. Protein expression was induced with 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 16-20 hours at 18°C in Luria Broth (Sigma). Cells were lysed in with microfluidizer (Microfluidics, West Wood, MA). Affinity purification was performed using NGC system (Bio-Rad): the soluble fraction of lysate was passed over a 5ml IMAC Nickle column, washed thoroughly, and eluted with 250mM imidazole. Fractions of interest were buffer exchanged into lysis buffer using 3 kDa molecular weight cutoff filters (EMD Millipore, Burlington, MA). Purified dpPER fragment was used as immunogen in guinea pigs (Covance). This antibody has been deposited into the Antibody registry (RRID: AB_2832970).

Generating TIM(S1404) phosphospecific antibodies

Phosphospecific antibodies were generated by ProteinTech Group, Inc (Rosemont, IL). Rabbits were immunized with a 15-amino-acid peptide (amino acid 1397-DLTRMYVpSDEDDRLE-amino acid 1411; where pS = phosphoserine). The resulting rabbit sera was further affinity-purified using the pS1404 phosphopeptide.

Detecting pS1404 in S2 cells and fly extracts

IP and λ-PP treatment were performed as described previously[41]. TIM proteins from S2 cells were extracted using EB2 (20mM HEPES pH 7.5, 100mM KCl, 5% Glycerol, 5mM EDTA, 0.1% Triton X-100, 0.5mM PMSF, 1mM DTT, 10 μg/ml Aprotinin, 5 μg/ml Leupeptin, 1 μg/ml Pepstatin) supplemented with 1X PhosSTOP (Roche) and 25mM NaF, and were pulled down using 20 μL of α-HA resin per IP reaction. IP with fly protein lysates (extracted with RBS supplemented with 1X PhosSTOP) were performed using 4 μL α-TIM and 20ul gammabind Sepharose beads (GE Healthcare) per reaction.

For λ-PP treatment, resin was washed three times with EB2 (without NaF and PhosSTOP), and once with λ-PP buffer (50mM Tris-HCl pH 7.5, 0.1mM EDTA, 5mM DTT, 0,01% Triton X-100, 2mM $MnCl_2$, and 0.1mg/ml BSA). Resin was then split into two and re-suspended in 40 μL λ-PP buffer, with half treated with 1 μL λ-PP (NEB) for 30 minutes at 30°C and the other half mock-treated at the same temperature. Immune complexes were then analyzed by western blotting as described above.

Immunofluorescence and confocal imaging

Brain dissections and immunofluorescence staining procedures were performed as described previously[91]. 3-5 day-old flies were entrained for 4 days and fixed with 4% paraformaldehyde for 40 minutes at 2-hour intervals between ZT16 and ZT22. Brains were washed three times in 1XPBST (0.1% Triton X-100 in PBS), blocked with 10% Normal Goat Serum (Jackson Immunoresearch, West Grove, PA) in PBST for 90 minutes and incubated with primary antibodies overnight. Primary antibodies against PDF, PER, and TIM were used at the following dilutions: 1:1500 rabbit α-PER (Gift from Dr. Patrick Emery)[35], 1:100 GP α-TIM (Gift from Dr. Patrick Emery)[84] and 1:400 mouse α-PDF (C7-C; Developmental Studies Hybridoma Bank, Iowa City, IA). Brains were then washed and probed with secondary antibodies at a 1:200 dilution for α-rat-cy3 (Jackson Immunoresearch, 706-165-148), α-rabbit IgG Alexa Fluor 488 (Jackson Immunoresearch, 711-545-152) and α-mouse-cy5 (Jackson Immunoresearch, 715-175-150). PDF staining was used to label the sLNvs and as a cytoplasmic marker. Total neuron and nuclear TIM staining was quantified based on a single layer of confocal image with the highest TIM signal. Nuclear TIM was determined by the non-overlapping portion of TIM between TIM and PDF signal in each neuron. Eight to ten fly brains for each genotype were dissected and imaged. Representative images are shown. Fiji software was used for image analysis[92].

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Quantitative RT-PCR

RNA was extracted from approximately 50 μL of fly heads using 3X volume TRI Reagent (Sigma). 1/5 volume of 100% chloroform (Sigma) was added and incubated at room temperature for 10 minutes. Upper aqueous layer was recovered after spinning down. Same volume of 100% isopropanol was added and incubated at −20C overnight to precipitate RNA. After spinning down, RNA pellet was washed with 70% ethanol once, resuspended in 20 μL 1X RQ1 buffer (Promega), treated with 2 μL RQ1 DNase (Promega) at 37C for 30 minutes prior to the incubation with 2 μL RQ1 DNase stop solution (Promega) at 65C for 10 minutes. cDNA was generated from equal amount of RNA using Superscript IV (Thermo Fisher Scientific). Real-time PCR was performed using SsoAdvanced SYBR green supermix (Bio-Rad) in a CFX96 (Bio-Rad). Three technical replicates were performed for each biological qPCR replicate. Three biological replicates were performed for each experiment.

Chromatin Immunoprecipitation (ChIP)

CLK-ChIP was performed as described previously[83]. All buffers described below, except ChIP Elution buffer, contain 1X SIGMAFAST EDTA-free protease inhibitor cocktail and 0.5 mM PMSF. Briefly, fly head tissues were homogenized using liquid nitrogen chilled mortar and pestle, mixed with Nuclear Extraction buffer (10mM Tris-HCl pH 8.0, 0.1mM EGTA pH 8.0, 10mM NaCl, 0.5mM EDTA pH 8.0, 1mM DTT, 0.5% Tergitol NP-10, 0.5mM Spermidine, 0.15mM Spermine), and lysed with a dounce homogenizer. Homogenate was transferred to a 70 μm cell strainer prior to centrifugation at 300 g for 1 minute at 4°C. Supernatant were centrifuged at 6700 rpm for 10 minutes at 4°C. Pellets were resuspended in NEB buffer prior to centrifugation at 11,500 rpm for 20 minutes at 4°C on a sucrose gradient (1.6M sucrose in NEB and 0.8M sucrose in NEB). Nuclei-containing pellets were fixed with 0.3% formaldehyde in NEB and rotated at room temperature for 10 minutes. Glycine was then added at a final concentration of 0.13mM to quench crosslinking. Samples were centrifuged at 6,500 rpm for 5 minutes at at 4°C. Pellets (cross-linked chromatin) were washed twice with NEB and resuspended in Sonication buffer (10mM Tris-HCl pH 7.5, 2mM EDTA pH 8.0, 1% SDS, 0.2% Triton X-100, 0.5mM Spermidine, 0.15mM Spermine). The cross-linked chromatin was sheared by sonicator (Q80023, QSonica, Newton, Connecticut) to roughly 500 base pair fragments. Supernatant (sheared chromatin) was collected after the centrifugation at 10,000 rpm for 10 minutes. 1.5 μL of CLK antibodies (generated in this study) were incubated with 25 μL of Dynabeads in ChIP Wash buffer (50mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 1% Triton X-100, 0.1% DOC, 10 μg/ml AcBSA (Promega), 100mM KCl in 1X PBS, 150mM NaCl, 5mM EGTA pH 8.0, 0.1% SDS) at 4°C for 2 hours. Following incubation, beads were collected and incubated with sheared chromatin that were diluted 10-fold with IP buffer (50mM Tris-HCl pH 7.5, 2mM EDTA pH 8.0, 1% Triton X-100, 0.1% DOC, 150mM NaCl, 0.5mM EGTA pH 8.0) at 4°C for 2 hours. Beads were then collected and washed twice with CW buffer for 30 minutes at 4°C, once with LiCl Wash buffer (10mM Tris-HCl pH 8.0, 250mM LiCl, 0.5% NP-40, 0.5% DOC, 1mM EDTA pH 8.0) for 30 minutes at 4°C and once with TE buffer (1mM EDTA pH 8.0, 10mM Tris-HCl pH 8.0) for 4 minutes at 4°C. Beads were eluted with ChIP Elution buffer (50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 1% SDS, 1mM DTT, 50mM NaCl, 4U/ml Proteinase K (NEB), 50 μg/ml RNase A (Thermo Fisher Scientific)) at 37°C for 2 hours and de-crosslinked at 65°C overnight. Finally, DNA was purified by QIAquick PCR Purification Kit (QIAGEN) and quantified by real-time qPCR. Primers for *tim* E-box were described previously[83]. The average of ChIP signals for two intergenic regions, one on chromosome 2R (see Key Resources Table) and one on the X chromosome[83], was used for non-specific background deduction. Three technical qPCR replicates were performed for each biological ChIP replicate. Four biological ChIP replicates were performed.

QUANTIFICATION AND STATISTICAL ANALYSIS

RAIN, DODR, Rayleigh test, Shapiro-Wilk normality test and Watson Williams test were performed in R[62,63,93]. Other statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, California). In the case of normally distributed data (Shapiro-Wilk normality test, $p > 0.05$), ANOVA was performed if more than two groups were compared; two-tailed Student's t test were performed if only two groups were compared. If data were not normally distributed, non-parametric Kruskall-Wallis test was applied. Asterisks indicate significant differences in mean values between genotypes or conditions at indicated time-points.

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Supplemental Information

CK2 Inhibits TIMELESS Nuclear Export and Modulates CLOCK Transcriptional Activity to Regulate Circadian Rhythms

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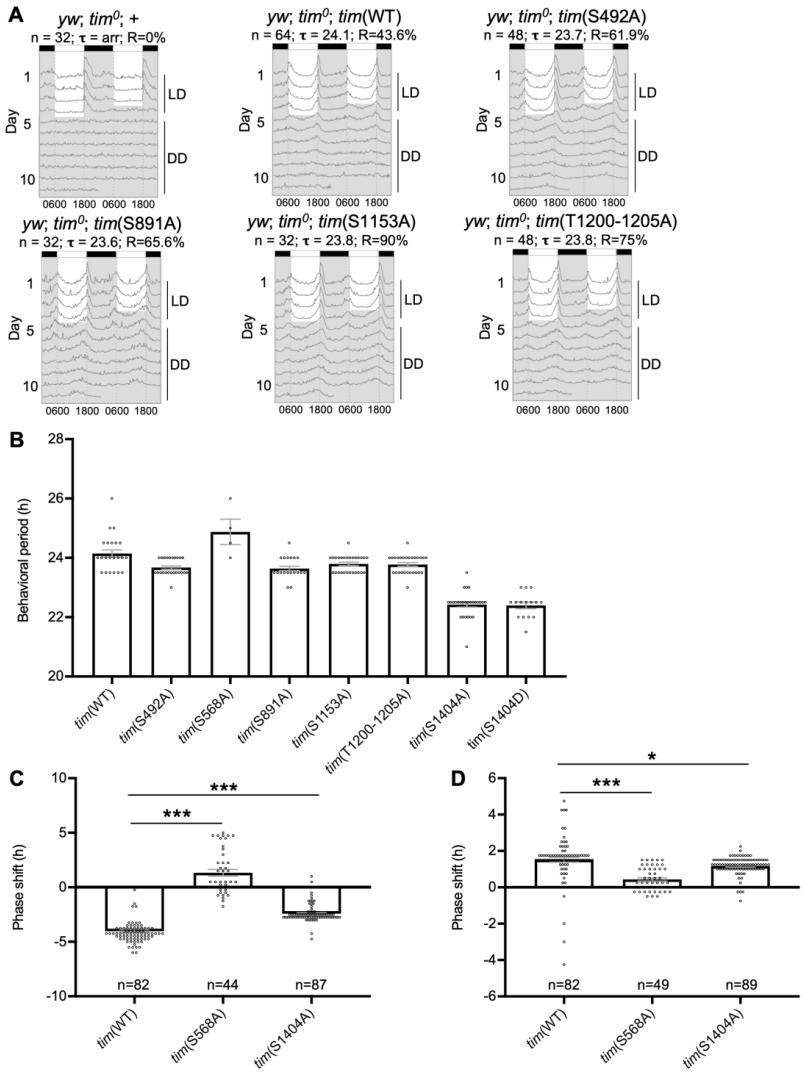

Figure S1. Daily locomotor activity rhythms and responses to light pulse are altered in TIM phosphorylation site mutants, Related to Figure 1. (A) Double-plotted actograms of *yw*; *tim*^{0} flies carrying transgenes for site-specific TIM phosphorylation mutation. n represents the sample size for behavioral assay. Average activity of each genotype was plotted. Tau (τ) represents the average period

length of the group of flies in DD. R represents percentage of flies that are rhythmic. Flies were entrained for 4 days in LD cycles and then switched to 7 days of constant darkness, DD. (B) Bar graphs showing average period length (h) of control flies and *tim* mutants in (A) and in Figure 1B. Data points for individual flies are included to illustrate variance. *yw*; tim^0 flies were not included because of their arrhythmicity. (C-D) Bar graphs showing the phase shift of *tim*(WT) and *tim* mutants in response to light pulse at (C) ZT15 and (D) ZT21, respectively. Error bars indicate SEM; ***p<0.001, *p<0.05, as compared to *tim*(WT), two-tailed Student's t test. Sample size (n) is shown. Data points for individual flies are included to illustrate variance. The Rayleigh test was used to confirm significant synchronization of *tim*(WT) and *tim*(S1404A) fly populations on LD3 and DD1 (p<0.0001). Behavioral arrhythmicity of *tim*(S568A) was confirmed by Rayleigh test (p=0.6105). The Watson-Williams test was used to confirm a significant phase shift (p<0.01) for *tim*(S1404A) mutants.

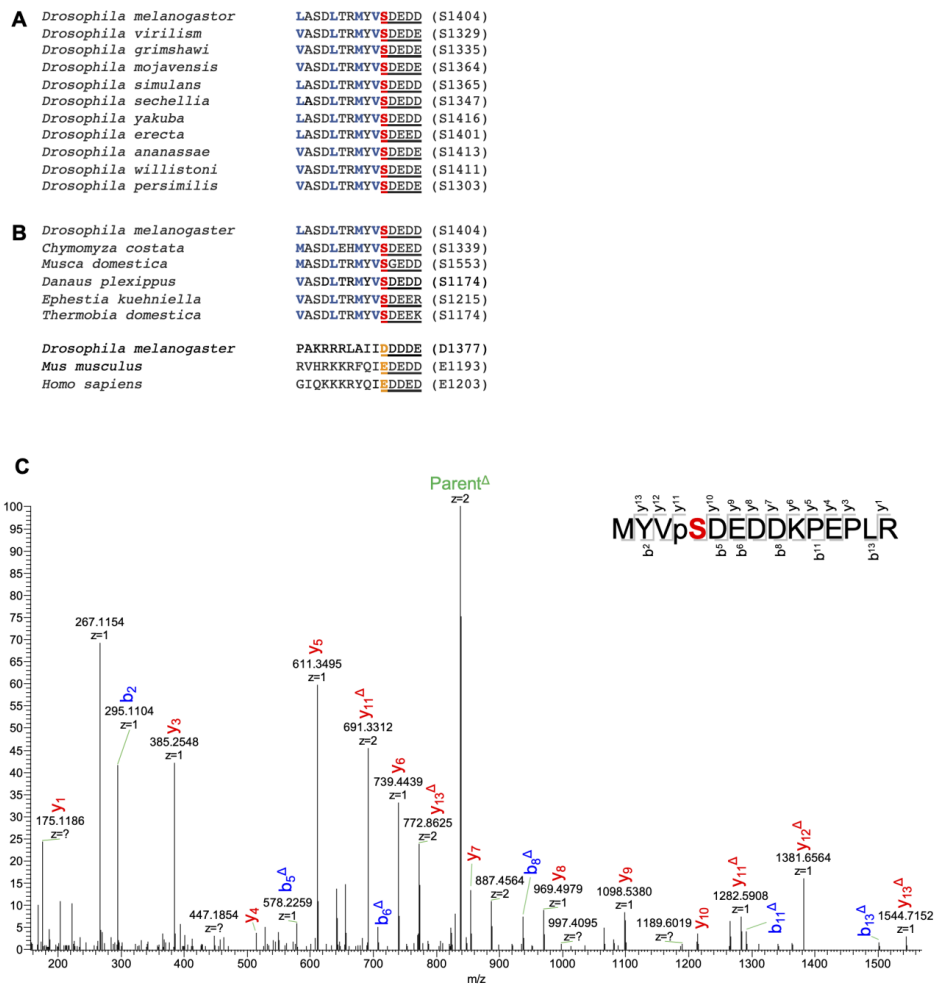

Figure S2. TIM(S1404) phosphorylation is conserved in insects, Related to Figure 1. (A) Amino acid sequences surrounding TIM(S1404) are conserved in *Drosophila* species. *D. pseudoobscura* is not included because it is missing the S1404 region. S1404 is shown in red. Critical hydrophobic amino acids within NES sequence motif are shown in blue. Consensus CK2 site is underlined. (B) Alignment of *D. melanogaster* TIM(S1404) region to the corresponding homologous sequence in indicated insect species, mouse (*Mus musculus*), and human (*Homo sapiens*). Glutamic acid or aspartic acids in *D. melanogaster*

TIMEOUT/TIMELESS2, mouse and human TIMELESS sequences that potentially replaces TIM(S1404) in *D. melanogaster* TIMELESS are in orange. (C) Phosphorylation at S1404 homologous residue in *Danaus plexippus*, DpTIM(S1174), is detected at ZT16, ZT20 and ZT24 in DpN1 cells by mass spectrometry proteomics. DpTIM was copurified with DpPER at these time-points. Representative HPLC/MS/MS spectrum showing phosphopeptide 1171-MYVpSDEDDKPEPLR-1184, where pS= phosphoserine. Parent$^{\Delta}$ denotes neutral loss ion. The notations b_n^{Δ} or y_n^{Δ} denotes the corresponding b_n or y_n ions with neutral loss.

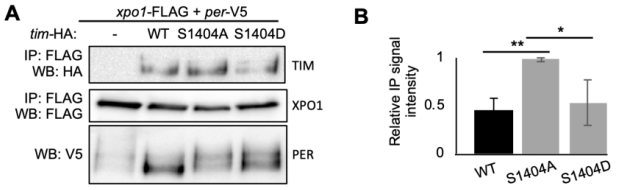

Figure S3. TIM(S1404D) compromises TIM-XPO1 interaction, Related to Figure 3. (A) Western blots showing coimmunoprecipitation (coIP) to examine the interactions of TIM(WT), TIM(S1404A) or TIM(S1404D) to XPO1 in *Drosophila* S2 cells expressing pAc-*xpo1*-3XFLAG-6XHIS and pAc-*per*-V5 in the presence or absence of pAc-HA plasmids expressing *tim* variants. Protein extracts were directly analyzed by immunoblotting (α-V5 for PER) or immunoprecipitated with α-FLAG resins and analyzed by immunoblotting to detect baits and interactors. (B) Bar graph displaying quantification of (A). Values for binding are normalized to amount of bait detected in the IPs and expressed as relative signal intensity (high value = 1). Error bars indicate ± SEM (n=3), **p<0.01, *p<0.05, one-way ANOVA.

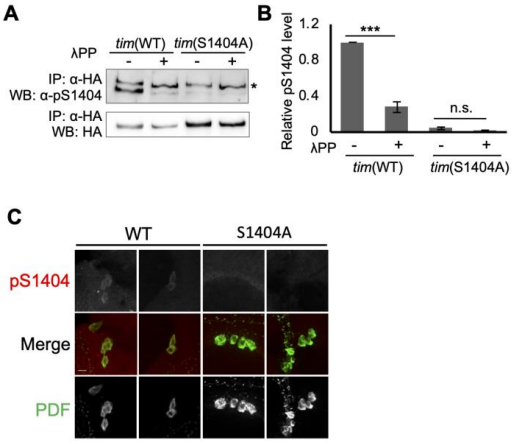

Figure S4. TIM(S1404) is phosphorylated in TIM(WT) but not in TIM(S1404A) mutant, Related to Figure 3 and Figure 6. (A) *Drosophila* S2 cells were transfected with pAc-*tim*(WT)-HA or pAc-*tim*(S1404A)-HA. Protein extracts were incubated with α-HA resins. Half of the immunocomplexes received lambda phosphatase (λPP) treatment while the other received sham treatment. TIM(pS1404) and TIM protein levels were analyzed by Western Blotting. Asterisk (*) indicates nonspecific signal (upper band). (B) Quantification of TIM(pS1404) was normalized to total TIM isoforms. Error bars indicate ± SEM (n=2), ***p<0.001, one-way ANOVA. (C) Representative confocal images of LN_vs clock neurons in adult fly brains stained with α-TIM(pS1404) (red) and α-PDF (green). Single channels are shown in grey scale. Scale bar represents 10μm.Flies were entrained for 2 days in LD cycles and collected at ZT16 on LD3, fixed and analyzed by immunofluorescence and confocal imaging.

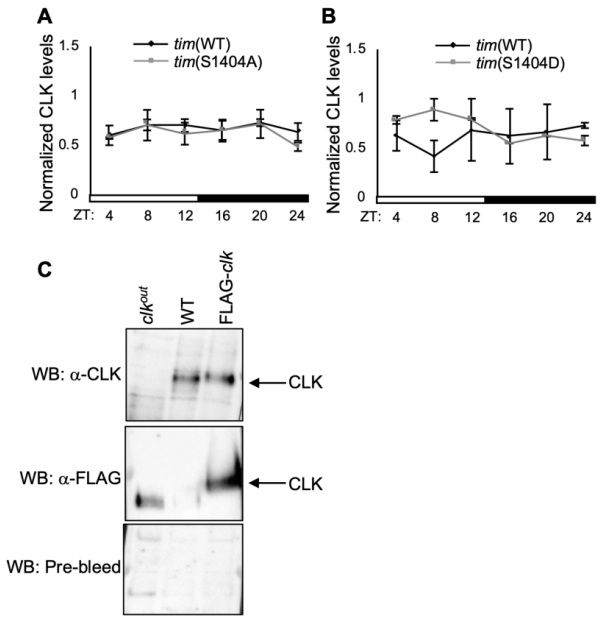

Figure S5. *tim*(S1404) mutations do not alter daily rhythms in CLK protein abundance in fly heads, Related to Figure 5. (A) Quantification of CLK abundance presented in Figure 5B; (B) Quantification of CLK in Figure S6B. Error bars indicate ± SEM (n=3), $p>0.05$ at all ZTs, two-way ANOVA. (C) Western blots showing that CLK was detected in the head extracts of WT (w^{1118}) and *w*; FLAG-*clk*; clk^{out} but not clk^{out} flies (Top panel). FLAG tag was detected to confirm the size of FLAG-CLK (middle panel). Pre-bleed was used to detect the nonspecific signals (bottom panel), since pre-bleed was collected before the injection of CLK antigen.

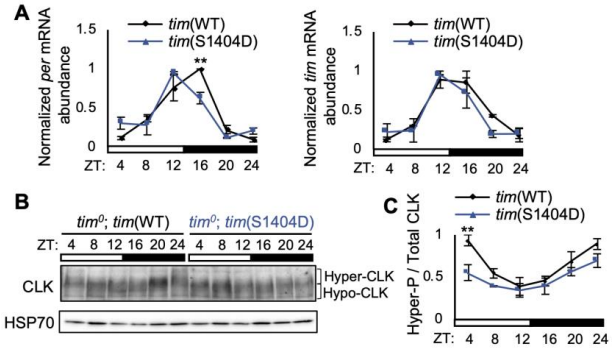

Figure S6. Increased TIM nuclear retention in *tim*(S1404D) mutant leads to shortened molecular rhythms, Related to Figure 5. (A) Steady state mRNA expression of *per* and *tim* in heads of *tim*(WT) and *tim*(S1404D) flies, entrained in LD cycles and assayed on LD3 (n=3). (B) Western blots comparing CLK profiles in heads of *tim*(WT) and *tim*(S1404D) entrained and collected as in (A). Brackets indicate hypo- and hyperphosphorylated CLK isoforms. α-HSP70 was used to indicate equal loading and for normalization. (C) Quantification of hyperphosphorylated/total CLK ratios. The top half of the CLK signal shown at ZT24 in *tim*(WT) flies (lane 6) was used as a reference to denote hyperphosphorylated CLK isoforms. Error bars indicate ± SEM (n=3). **p<0.01, two-way ANOVA.

Sequence (best peptide)	Fragmentation	Score[a] (-10lgP)	Site	PTM[b]	Zeitgeber (ZT)[c]
LGQKS(+79.97)PHAGQLQLTK	ETD[d]	69.3	S-492	p[e]	1, 3, 16, 20, 23.5
TIMS(+79.97)PMDKK	HCD[f]	44.5	S-568	p	16
FM(+15.99)NTPPKS(+79.97)PLTI PTTSLTEM(+15.99)TK	HCD	60.0	S-891	p	1, 3, 12, 16, 20, 23.5
TLLTT(+79.97)PTSATTESGIEIK	HCD	65.6	T-991	p	16, 20
TLLT(+203.08)TPTSATTESGIEIK	HCD	61.5	[T-987; T-990; T-991; T-993; S-994; T-996; T-997; S-999][g]	h[h]	1, 16, 20
FDASELEDATASS(+79.97)PSR	HCD	84.9	S-1153	p	1, 3, 12, 16, 20, 23.5
AHAM(+15.99)AST(+79.97)PS(+79.97)PSEIFAVPK	HCD	77.6	S-1201 & S-1203	2p	1, 16, 20
AHAM(+15.99)ASTPS(+203.08)PSEIFAVPK	HCD	42.7	[S-1200; T-1201; S-1203; S-1205] [g]	h	1, 16, 20
IIRYT(+79.97)PDPTPPVPNW	HCD	40.1	T-1223	p	1, 12, 16, 20, 23.5
ELNS(+79.97)DNVSLASDLTR	HCD	68.7	S-1389	p	1, 3, 16, 20
ELNSDNVS(+79.97)LASDLTR	HCD	63.1	S-1393	p	1, 3, 16, 20, 23.5
ELNSDNVSLAS(+79.97)DLTR	HCD	57.9	S-1396	p	1, 3, 16, 20
MYVS(+79.97)DEDDRLER	HCD	78.2	S-1404	p	1, 3, 12, 16, 20, 23.5

[a] Scores computed using PEAKS Studio X+
[b] PTM = Post-translational modification
[c] ZT = time-point(s) at which PTM is detected (presence or absence); does not represent occupancy
[d] ETD = Electron Transfer Dissociation
[e] p = phosphorylation
[f] HCD = Higher-energy C-trap Dissociation
[g] Only one of these sites is modified
[h] h = HexNAc (O-GlcNAcylation)

Table S1. Identification of TIM phosphorylation and O-GlcNAcylation sites in *Drosophila* head tissues. Related to Figure 1.

Genotype	Period (h) (mean ± SEM)	Power[a]	Rhythmicity (%)[b]	No. of flies tested	No of flies surviving[c]
yw; tim^0; +	AR[d]	ND[e]	0	32	31
yw; tim^0; tim(WT)	24.1 ± 0.12	56.4	43.6	64	55
yw; tim^0; tim(S492A)	23.7 ± 0.06	88.6	61.9	48	42
yw; tim^0; tim(S568A)	24.9 ± 0.43	63.3	15.4	32	26
yw; tim^0; tim(S891A)	23.6 ± 0.08	88.2	65.6	32	32
yw; tim^0; tim(S1153A)	23.8 ± 0.06	99.9	90	32	30
yw; tim^0; tim(T1200-1205A)	23.8 ± 0.06	89.9	75	48	36
yw; tim^0; tim(S1404A)	22.4 ± 0.06	83.2	68.3	64	60
yw; tim^0; tim(S1404D)	22.4 ± 0.11	47.5	48.4	32	31

[a] Measures the strength or amplitude of the locomotor activity rhythm (in arbitrary units)
[b] Percentage of flies that are rhythmic
[c] Number of flies that survived until the end of the experiment
[d] AR denotes Arrhythmic
[e] ND denotes Not Determined

Table S2. Daily locomotor activity rhythms of *tim* mutants. Related to Figure 1.

Primer for mutagenesis: *tim*(S492A) F: AAATTGGGCCAAAAGGCCCCACATGCCGGCCAG
Primer for mutagenesis: *tim*(S492A) R: CTGGCCGGCATGTGGGGCCTTTTGGCCCAATTT
Primer for mutagenesis: *tim*(S568A) F: CCACGGACGATTATGGCGCCAATGGACAAAAAG
Primer for mutagenesis: *tim*(S568A) R: CTTTTTGTCCATTGGCGCCATAATCGTCCGTGG
Primer for mutagenesis: *tim*(S891A) F: AATACTCCTCCGAAGGCGCCACTCACCATTCCT
Primer for mutagenesis: *tim*(S891A) R: AGGAATGGTGAGTGGCGCCTTCGGAGGAGTATT
Primer for mutagenesis: *tim*(S1153A) F: GATGCGACGGCGTCGGCTCCGTCGCGTTACCAC
Primer for mutagenesis: *tim*(S1153A) R: GTGGTAACGCGACGGAGCCGACGCCGTCGCATC
Primer for mutagenesis: *tim*(S1200-05A) F: GCACACGCCATGGCAGCCGCGGCAGCGGCCGCCGAGATTTTCGCGGTT
Primer for mutagenesis: *tim*(S1200-05A) R: AACCGCGAAAATCTCGGCGGCCGCTGCCGCGGCTGCCATGGCGTGTGC
Primer for mutagenesis: *tim*(S1404A) F: ACCAGAATGTATGTGGCCGATGAGGACGATCGA
Primer for mutagenesis: *tim*(S1404A) R: TCGATCGTCCTCATCGGCCACATACATTCTGGT
Primer for mutagenesis: *tim*(S1404D) F: ACCAGAATGTATGTGGACGATGAGGACGATCGA
Primer for mutagenesis: *tim*(S1404D) R: TCGATCGTCCTCATCGTCCACATACATTCTGGT
Primer for ChIP: 2R intergenic (CP023338) F: TCAGCCGGCATCATTAGCAGCCG
Primer for ChIP: 2R intergenic (CP023338) R: TCGTGTGCGGGAATCTCTGCCG
Primer for ChIP: X intergenic (FBgn0003638) F: ACTGCGTATTCAGGATACATGCC
Primer for ChIP: X intergenic (FBgn0003638) R: TGTCCACTTTAATTGATTGCGTGG
Primer for ChIP: *tim* E-box1 F: ACGTTGTGATTACACGTGAGCC
Primer for ChIP: *tim* E-box1 F: ACACTGACCGAAACACCCAC
Primer for RT-qPCR: *per* F: GACCGAATCCCTGCTCAA
Primer for RT-qPCR: *per* R: GTGTCATTGGCGGACTTC
Primer for RT-qPCR: *tim* F: CCCTTATACCCGAGGTGGAT
Primer for RT-qPCR: *tim* R: TGATCGAGTTGCAGTGCTTC
Primer for RT-qPCR: *cbp20* F: GTCTGATTCGTGTGGACTGG
Primer for RT-qPCR: *cbp20* R: CAACAGTTTGCCATAACCCC

Table S3: Primers for PCR mutagenesis, ChIP analysis and RT-qPCR analysis. Related to Figures 1 and 5.

Chapter 3. *Timeless* in animal circadian clocks and beyond

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STATE-OF-THE-ART REVIEW

Timeless in animal circadian clocks and beyond

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Keywords
cell cycle; circadian clock; DNA replication; *Drosophila timeless*; mammalian *timeless*; seasonal biology; *timeout*

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TIMELESS (TIM) was first identified as a molecular cog in the *Drosophila* circadian clock. Almost three decades of investigations have resulted in an insightful model describing the critical role of *Drosophila* TIM (dTIM) in circadian timekeeping in insects, including its function in mediating light entrainment and temperature compensation of the molecular clock. Furthermore, exciting discoveries on its sequence polymorphism and thermosensitive alternative RNA splicing have also established its role in regulating seasonal biology. Although mammalian TIM (mTIM), its mammalian paralog, was first identified as a potential circadian clock component in 1990s due to sequence similarity to dTIM, its role in clock regulation has been more controversial. Mammalian TIM has now been characterized as a DNA replication fork component and has been shown to promote fork progression and participate in cell cycle checkpoint signaling in response to DNA damage. Despite defective circadian rhythms displayed by *mtim* mutants, it remains controversial whether the regulation of circadian clocks by mTIM is direct, especially given the interconnection between the cell cycle and circadian clocks. In this review, we provide a historical perspective on the identification of animal *tim* genes, summarize the roles of TIM proteins in biological timing and genomic stability, and draw parallels between dTIM and mTIM despite apparent functional divergence.

Introduction

Circadian rhythms are common features in all domains of life and are driven by molecular clockworks [1–6]. Molecular clocks incorporate a range of environmental time cues, such as light–dark and temperature signals, and metabolic signals to orchestrate daily rhythms in physiology and behavior [4,6,7]. This allows organisms to synchronize their biology to their external environment, thereby promoting organismal health and fitness [8–11]. The animal circadian clock is powered by cell-autonomous interlocked transcription–translation feedback loops (TTFLs) [6]. In the primary TTFL in *Drosophila*, which relies heavily on *Drosophila* TIM (dTIM) function, transcription factors CLOCK (CLK) (ortholog of mammalian CLOCK) and CYCLE (CYC) (ortholog of mammalian BMAL1) are positive elements that heterodimerize and activate the expression of negative elements, PERIOD (PER) (ortholog of mammalian PER1, PER2, and PER3) and dTIM (functionally replaced by CRYPTOCHROMEs (CRYs) in mammalian clockworks). In addition to core clock components, CLK-CYC also activates the transcription of other clock-controlled output genes [12–14], often in tissue-specific manner [15–17]. To complete the TTFL, PER, and dTIM form a repressor complex that enters the nucleus in a time-of-day-dependent manner [18–22] to repress CLK-CYC

Abbreviations
CLK, CLOCK; CRY, CRYPTOCHROME; CYC, CYCLE; dTIM, *Drosophila* TIM; mTIM, mammalian TIM; PER, PERIOD; PTM, posttranslational modification; TIM, TIMELESS; TTFL, transcription–translation feedback loop.

transcription activity [23–25]. This repression is relieved when both PER and dTIM are degraded in a proteasome-dependent manner [26–30]. In addition to its role within the molecular clock, thermosensitive alternative splicing of *dtim* RNA [31–34] and light sensitivity [35–38] of TIM protein are key features that allow dTIM to function at the interface between circadian and seasonal timing.

In the mammalian clock, CRYs replace TIM to partner with PERs to maintain circadian rhythms [39–44]. Whether mammalian TIM (mTIM) is a key component of the mammalian clock has been heavily debated since it was first characterized [45–47]. On the other hand, evidence supporting the role of mTIM in DNA replication and DNA damage response is strong. We will discuss the controversial role of mTIM in timekeeping below.

This review summarizes the various roles played by dTIM in *Drosophila* circadian clocks, in the regulation of seasonal biology, and other non-circadian processes. We will then discuss the circadian and non-circadian functions of mTIM, highlighting data that either support its role in circadian timekeeping or are in conflict with the notion. Finally, we conclude the review by summarizing recent findings on the potential functional parallel between dTIM and mTIM.

Drosophila TIM plays critical roles in circadian timekeeping

Drosophila TIM in the molecular clock

Circadian timekeeping relies on cycling genes and proteins that maintain a free-running period of approximately 24 h. Investigations to elucidate the inner workings of the molecular clockwork started around 50 years ago, when Konopka and Benzer [48] isolated the first three clock mutants in *Drosophila melanogaster* via genetic screening. The mutations were all located in the same loci, which were later confirmed as the key clock gene, *period (per)* [49–53]. Hardin *et al.* [54] suggested that PER may feedback to repress its own mRNA expression to establish molecular oscillations that manifest into behavioral and physiology rhythms. In the next few years, taking advantage of high throughput genetic screening in *Drosophila*, Sehgal *et al.* [55] identified *dtim* as the second clock gene. This gene encodes a protein with novel structure at the time and the only recognizable sequence feature the authors highlighted was a stretch of acidic residues [56]. The arrhythmic PER nuclear localization as well as locomotor activity in *dtim* null mutants has led to the model illustrating how the coordination of *per* and *dtim* may generate 24-h free-running period via negative feedback: (a) transcriptional activation of *per* and *dtim* in midday due to the absence of nuclear PER; (b) PER and dTIM heterodimerize and enter the nucleus at dusk; (c) increasing amount of nuclear PER blocks *per* and *dtim* mRNA transcription and accumulation at night; (d) nuclear PER and dTIM decline because of inhibited mRNA production and subsequent protein turnover in late night to early morning (Fig. 1) [57]. This model was eventually expanded to incorporate CLK [58,59] and CYC [60] after their characterization, thereby establishing the TTFL model of the *Drosophila* clock.

As a negative component in the molecular clockwork, dTIM does not have intrinsic repression activity. Instead, it is essential in maintaining rhythmic PER expression and activity (Fig. 1). This is strongly supported by observations that PER rhythmic expression and behavioral rhythmicity are abolished in *dtim* null mutant [18] and mutants that are defective in TIM nuclear entry [61,62]. Early studies suggest that dTIM binds to and blocks the cytoplasmic localization domain (CLD) of PER and thus reduces PER cytoplasmic retention [63]. Another study described a mechanism by which dTIM antagonizes the activity of DOUBLETIME (DBT, homolog of mammalian casein kinase 1 delta/epsilon) in inhibiting PER nuclear entry [22]. dTIM also acts as the major cargo recognized by the Importin-α1 (IMPα1) nuclear entry machinery, thus transporting PER into the nucleus [64]. Saez *et al.* [61] identified a functional nuclear localization signal (NLS) that is potentially recognized by IMPα1 (Fig. 2). Once in the nucleus, dTIM appears to be bound to PER constitutively and facilitates PER repression [25,65]. Sun *et al.* [66] suggested that dTIM may act as a scaffold to promote PER-CLK interaction. Alternatively, dTIM may facilitate yet-to-be-characterized CLK kinase(s) [23,24,67] in the PER-dTIM repressor complex to phosphorylate CLK and inactivate transcriptional activity.

dTIM function is extensively regulated by posttranslational modifications (PTMs). Notably, phosphorylation is the best-studied protein modification to achieve dTIM time-of-day specific functions. Casein kinase 2 (CK2) and SHAGGY [SGG, homolog of mammalian glycogen synthase kinase-3β (GSK3β)] have been shown to phosphorylate both PER and dTIM and promote nuclear entry [68–72] (Fig. 1). Interestingly, once in the nucleus, PER-dTIM complexes are subjected to phosphorylation-dependent nuclear export, providing an additional means to control nuclear accumulation [21,67]. Protein phosphatases also participate in regulating PER-dTIM nuclear accumulation [73–

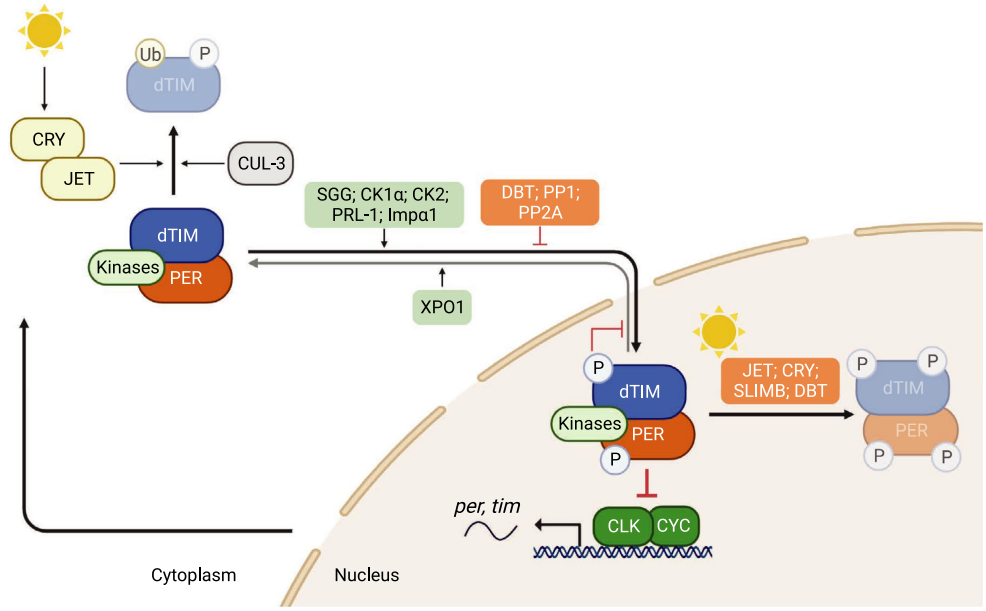

Fig. 1. *Drosophila* TIM (dTIM) is a core component of the molecular oscillator. During the day, CLK-CYC heterodimers activate the transcription of rhythmic genes, including *per* and *tim* in the nucleus [6]. In the cytoplasm, dTIM undergoes proteasomal degradation mediated by CRYPTOCHROME (CRY) [35–38] and JETLAG (JET) [28,88] upon light exposure. CULLIN-3 (CUL-3) has also been observed to mediate dTIM degradation in a light-independent manner [27]. Early in the night, SHAGGY (SGG) [68], casein kinase 1α (CK1α) [187], casein kinase 2 (CK2) [69,70], Importin-α1 (Impα1) [64] and phosphatase of regenerating liver-1 (PRL-1) [75] promote nuclear accumulation of PER-dTIM complex. This is antagonized by DOUBLETIME (DBT) [25], protein phosphatase 1 (PP1) [74] and protein phosphatase 2A (PP2A) [73]. Once PER-dTIM complex is in the nucleus, CK2-dependent phosphorylation of dTIM (S1404) inhibits PER-dTIM nuclear export by exportin 1 (XPO1) complex, retaining PER-dTIM complex in the nucleus [67]. At midnight, nuclear PER–dTIM complex interacts with CLK-CYC and represses their transcriptional activity [23,25]. From late night to early morning, CRY and JET mediate light-dependent TIM degradation [28,88], whereas DBT and SUPERNUMERARY LIMBS (SLIMB) mediate PER degradation [26,29]. There have also been reports suggesting the involvement of SLIMB in TIM degradation [27].

75]. Over the past 10 years, site-specific functions of dTIM phosphorylation have been characterized in a few studies (Fig. 2). *In vivo* functional analysis leveraging mutagenesis of dTIM protein revealed that T113 is critical for rhythmic dTIM expression [62]. Mutating T113 to non-phosphorylatable alanine (A) abolishes dTIM nuclear entry, whereas mutations at a nearby proline (P115) produce similar defects. Combining genetic and biochemical studies, Top *et al.* [72] showed that SGG and CK2 phosphorylate five residues at ST region (S297, T301, T305, S309, and S313) to promote dTIM nuclear accumulation. Interestingly, SGG and CK2 appear to regulate PER-dTIM only in a subset of clock neurons, which may contribute to the divergent functions of specific neuronal groups within the circadian neuronal circuitry. This could potentially explain how alteration in TIM phosphorylation in flies carrying tim^{blind} allele (A1128V, L1131M) results in lengthened locomotor activity rhythms but normal eclosion rhythms [76]. Activity and eclosion rhythms are two well characterized output of the *Drosophila* clock and are normally altered to the same extent in most fly mutants, including the three *per* mutants Konopka and Benzer identified in 1971 [48]. The mechanisms by which kinases phosphorylate PER-dTIM in specific neurons remain unclear. Since alternative pre-mRNA splicing patterns were observed in different clock neurons including for *sgg* mRNAs [77], we speculate that this may result in cell-type-specific posttranslational modification programs for key clock proteins, including dTIM.

Recently, two studies harnessed mass spectrometry proteomics to identify dTIM phosphorylation sites [67,75] (Fig. 2). Kula-Eversole *et al.* [75] identified five dTIM

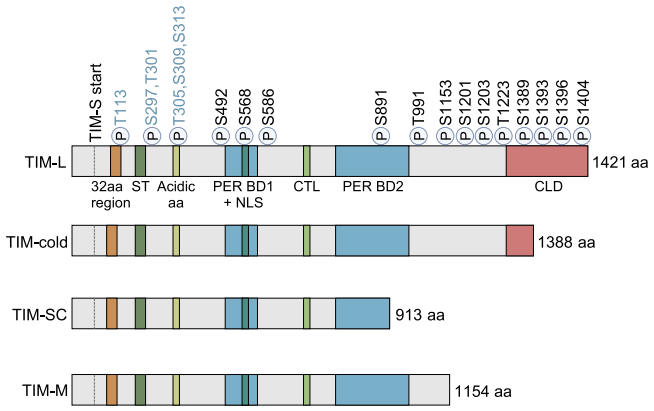

Fig. 2. Schematic illustrating domain structure of TIM isoforms generated from alternative splicing. All amino acid numbering is based on the TIM-L_{1421} isoform. 'TIM-S start' denotes alternative translation start site for TIM-S. Previously described domains of TIM: 32 amino acid region (amino acid [aa] 260–291) [188], also known as serine-rich domain (SRD) (aa 260–292) [71]; serine/threonine (ST)-rich region (aa 293–312) [72]; a stretch of acidic amino acid residues (acidic aa) (aa 383–412) [56]; PER binding domain 1 (PER BD1) (aa 536–610) [61]; nuclear localization sequence (NLS) (aa 558–593) [61]; C-terminal tail-like sequence (CTL) (aa 640–649) [87]; PER binding domain 2 (PER BD2) (aa 747–946) [61]; and cytoplasmic localization domain (CLD) (aa 1261–1421) [61]. P = phosphorylation sites [62,67,72,75]. Phosphorylation sites in black = identified via mass spectrometry; blue = identified via *in vivo* functional analysis but have not been validated by mass spectrometry or phospho-specific antibodies. TIM-cold, TIM-SC, TIM-M isoforms are based on Shakhmantsir *et al.*, Foley *et al.*, Martin Anduaga *et al.* [31–33].

phosphorylation sites in *Drosophila* $S2R^+$ cells coexpressing dTIM and relevant kinases (SGG and CK2). S586 and T991 are shown to be dephosphorylated by Phosphatase of Regenerating Liver-1 (PRL-1), which in turn promotes dTIM nuclear accumulation. In Cai *et al.* [67], we identified 12 phosphorylation sites in PER-bound dTIM from *Drosophila* tissues. In particular, we showed that S1404 phosphorylation inhibits the interaction between dTIM and the nuclear export complex, thereby promoting dTIM nuclear accumulation. S1404 phosphorylation status in fly tissues was confirmed using phospho-specific antibody.

In addition to nuclear accumulation, phosphorylation also regulates dTIM protein turnover. CULLIN-3 (CUL-3) and SKP1-CUL1-F-box-protein/SUPERNUMERARY LIMB complex (SCF/SLIMB) differentially facilitates dTIM degradation depending on its phosphorylation status [27,78], thus fine-tuning dTIM phase-specific functions (Fig. 1). Besides phosphorylation, O-GlcNAcylation at multiple residues on dTIM was also identified [67]. Since O-GlcNAcylation modifies serine/threonine residues and regulates the function of many proteins including PER and CLK [79–82], it will be interesting to determine how the two types of PTMs coordinate to regulate dTIM phase-specific functions. Given O-GlcNAcylation is nutrient-sensitive, this could be a mechanism by which metabolic signals can integrate with time-of-day environmental signals to promote robust circadian rhythms.

Finally, besides PTMs, *dtim* expression is regulated by posttranscriptional mechanisms. Carbon catabolite repression-negative on TATA-less deadenylation complex (CCR4-NOT) has been shown to regulate *dtim* mRNA stability to support phase-specific dTIM function [83]. *Drosophila tim* also exhibits alternative splicing pattern in response to environmental conditions, which will be described later.

Drosophila TIM and light entrainment of circadian rhythms

To confer fitness, a circadian clock must be synchronized to local time. Environmental time cues such as daily light–dark or temperature cycles entrain the circadian clock [84]. Identification of clock genes paved the way to investigations on molecular components that mediate clock entrainment. Two years after the identification of *dtim* in 1994, four exciting papers showed that dTIM displays light sensitivity, thus coupling the molecular clockwork to photic input from the environment [35–38] (Fig. 1). CRY is the major photoreceptor that mediates TIM light-dependent

degradation [85–87]. Light induces CRY conformational change, thus enabling CRY to bind to dTIM. Thereafter, E3 ubiquitin ligase JETLAG (JET) along with CRY promotes rapid TIM proteasomal degradation [28,87,88] upon yet uncharacterized TIM tyrosine phosphorylation [89]. QUASIMODO (QSM), a light-responsive protein expressing predominantly in CRY-negative clock neurons, also trigger dTIM degradation upon light exposure [90]. dTIM degradation promotes PER turnover, thus resetting the circadian clock [37].

Drosophila TIM and temperature compensation of the circadian clock

Whereas rates of chemical reactions are often temperature-dependent on a molecular level, a clock is only meaningful if its period length stays constant over a wide range of temperatures. The circadian clock has the property of temperature compensation; its pace is stable over a wide range of temperatures [84]. PER was first identified to participate in this process. A repetitive threonine-glycine (Thr-Gly) tract in PER exhibits more flexible conformation in higher temperature [91], which correlates with the observation that flies expressing PER with a deletion in the Thr–Gly tract display impaired temperature compensation of the circadian clock [92]. In wild *D. melanogaster* populations, the Thr–Gly tract is polymorphic in length; this is adaptive and enables flies to maintain the pace of the clock in environments with different range of temperatures [93].

dtim has also been demonstrated to contribute to temperature compensation of the clock. At the posttranscriptional level, manipulating *dtim* thermosensitive splicing results in defective temperature compensation [32,33]. Elucidating the function of each *dtim* isoform under different temperatures could help understand how they regulate temperature compensation in future studies. At the posttranslational level, mutant lines bearing a number of amino acid substitutions, tim^{rit} (P1116A) and tim^{blind}, exhibit impaired temperature compensation [94,95]. The mechanism by which dTIM regulates temperature compensation remains unclear. One possibility is that temperature directly modulates PER-dTIM interaction. Another possibility is that temperature may indirectly modulate site-specific phosphorylation to regulate phase-specific functions of PER-dTIM and achieve temperature compensation. In mammalian systems, temperature has been shown to determine the priority of competing phosphorylation sites to regulate PER2 turnover rate [96,97]. Therefore, mass spectrometry-based phosphorylation site mapping in combination with molecular genetics may further expand our understanding of how dTIM phosphorylation confers temperature compensation in flies.

Sequence polymorphism and alternative splicing of *Drosophila tim* regulates seasonal biology

To prepare for seasonal changes, plants and animals rely on internal photoperiodic timers, allowing them to undergo physiological and behavioral changes to survive unfavorable times [98–100]. Genetic analysis of wild *D. melanogaster* populations as well as molecular studies revealed that polymorphism at the *dtim* locus facilitates seasonal adaptation (Fig. 3A). *ls-tim* is a derived *dtim* allele that evolved 300–3000 years ago in Europe [101] and has a G nucleotide insertion upstream of the original ATG translational start site [102,103]. This generates an extra ATG 23 amino acids upstream of the TIM-S start codon. *ls-tim* allele thus generates two protein isoforms: TIM-S and a 23-aa longer TIM-L (Fig. 2) (TIM-S and TIM-L were originally named S-TIM and L-TIM but we are renaming them to follow the convention used in more recent publications describing other TIM protein isoforms resulting from alternative pre-mRNA splicing). TIM-L displays reduced light sensitivity, largely due to its reduced binding affinity to CRY [88]. Since light-dependent degradation of dTIM is critical to the resetting of the clock, reduced light sensitivity is thought to keep the molecular clockwork rhythmic in long summer days [104]. Furthermore, in anticipation of the onset of winter, flies carrying *ls-tim* alleles enter reproductive dormancy earlier in autumn as compared with flies carrying only *s-tim* alleles [103]. This is expected to be adaptive for flies inhabiting higher latitudes where harsh conditions are common in winter. For this reason, it was surprising that Tauber *et al*. [103] initially found the highest *ls-tim* allele frequency in southeastern Italy and decrease of *ls-tim* as the sampling distance increases both northward and southward. Subsequent analysis now suggests that this derived allele is only 300–3000 years old; it is still under selection and has not yet achieved fixation [101]. In fact, more extensive sampling in Spain [101] and in North America [105] reported a strong latitudinal cline where *ls-tim* allele increases in frequency as latitude increases.

In addition to sequence polymorphism at the *dtim* locus, *dtim* displays thermosensitive alternative splicing. This has been proposed to be a temperature sensing mechanism to regulate *D. melanogaster* seasonal biology. In response to temperature changes, *dtim* produces four splice variants: *tim-cold*, *tim-short and cold* (*tim-sc*), *tim-M* (also called *tim-tiny*), and *tim-L* (full-length isoform) (Fig. 2). At moderate temperature (25 °C), constitutively spliced *tim-L* is the major

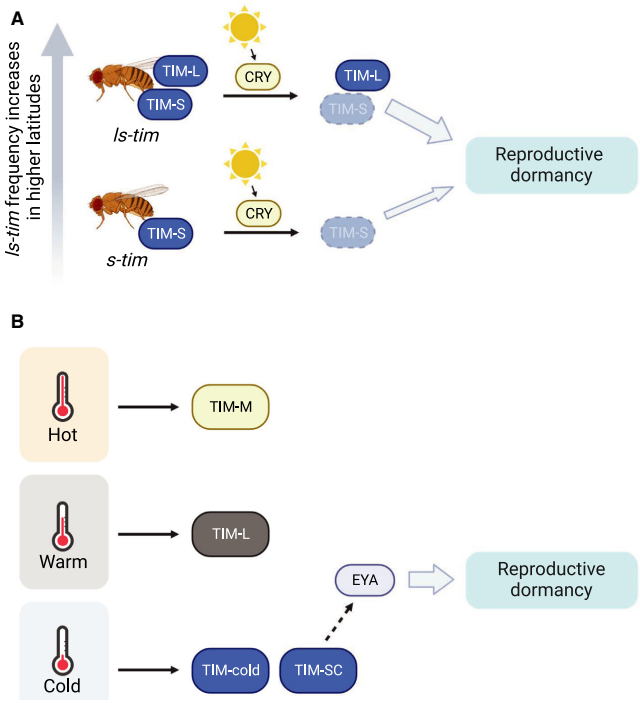

Fig. 3. Role of *Drosophila* TIM in regulating seasonal biology. (A) Flies carrying *s-tim* allele express TIM-S, whereas flies carrying *ls-tim* allele express both TIM-L and TIM-S. Sampling of flies in North America [105] and on the eastern side of the Iberian Peninsula [101] showed that *ls-tim* allele frequency exhibits a latitudinal cline and increases with latitude. Since TIM-L is less susceptible to light-activated CRY-dependent degradation, flies carrying *ls-tim* allele interpret light signal differently and have higher inducibility of reproductive dormancy at the onset of winter to survive harsh conditions [103]. (B) High temperature promotes accumulation of TIM-M isoform [31]. TIM-L is the major isoform at warm temperature [32]. Cold temperature promotes the accumulation of TIM-SC and TIM-cold isoforms [32,33]. TIM-SC can potentially stabilize EYES ABSENT (EYA) to promote reproductive dormancy [34].

isoform and produces full-length TIM [32] (Fig. 3B). *tim-cold* and *tim-sc* are major isoforms in colder temperatures (10–18 °C) [32–34,106], whereas *tim-tiny* intron is retained in higher temperatures, resulting in high levels of *tim-M* isoform (29–35 °C) [31,32,107]. Thermosensitive alternative splicing is also observed in three other *Drosophila* species, indicating this could be a conserved mechanism across the genus [32]. Less is known regarding the functional divergence of each *dtim* splice variant and how the pattern of splicing modulates the circadian clock in different seasonal conditions. Since some splicing events generate truncated TIM proteins, they could differentially affect TIM function in the circadian clock. For example, the TIM-SC protein lacks the C-terminal CLD and part of PER-binding domain, which may compromise nuclear accumulation of the PER-dTIM complex. Further functional studies on TIM isoforms are required to test this hypothesis.

There has been a substantial amount of evidence to support the role of *dtim* in regulating seasonal biology in addition to the studies mentioned above. They include the observed correlation between *tim* alleles and photoperiodic diapause in *D. triauraria* [108], changes in *tim* expression levels in response to photoperiod in several insect species [109,110], and differential photosensitive alternative splicing of *tim* observed in cold-adapted *D. montana* populations collected in a wide latitudinal range [111]. We recently provided evidence supporting the role of dTIM in seasonal physiology in *D. melanogaster* [34] (Fig. 3B). We showed that *dtim* null mutants fail to enter reproductive dormancy in simulated winter condition, while flies overexpressing *dtim* exhibit higher incidence of reproductive dormancy. We report evidence indicating that the cold-induced and light-insensitive isoform TIM-SC facilitates the accumulation of EYES ABSENT (EYA) protein in winter condition, an event that is sufficient to promote reproductive dormancy. It remains unclear why TIM-SC is not subjected to light-dependent degradation and how it interacts with EYA. One possibility is that the truncated protein reduces the binding affinity to CRY and/or JET, and somehow stabilizes EYA via yet unknown mechanisms. A temperature-dependent alternative splicing event is also observed in *frequency* (*frq*), a key repressor in the

Neurospora clockwork [112–114]. It is possible that this temperature-regulated event also contributes to *Neurospora* seasonal adaptation.

What is the mechanism by which temperature regulates *dtim* alternative splicing? So far, splicing regulator P-element somatic inhibitor (PSI) [33] and triple small nuclear ribonuclearprotein (tri-snRNP) spliceosome [31] have been shown to regulate *dtim* splicing. Temperature is known to modulate alternative splicing at multiple levels, including the expression of splicing-related genes [115,116], PTMs [117], spliceosome assembly [118], and spliceosome localization [119,120].

Non-circadian roles of *Drosophila* TIM

The fact that dTIM is expressed and differentially regulated in non-clock cells has led to the investigation of non-circadian roles of dTIM. A few studies revealed unexpected results regarding dTIM circadian expression pattern and light sensitivity in non-clock cells. dTIM and its binding partner PER remain constitutively cytoplasmic in the fly ovary, which is known to lack intracellular molecular clocks [121–123]. This is unlike the subcellular shuttling of PER-dTIM observed consistently in clock neurons. Furthermore, dTIM in the follicle cells is not susceptible to light-induced degradation [123,124]. It is noteworthy that egg-laying rhythms persist under constant light, in contrast to the arrhythmic eclosion and locomotor activity rhythms in the same condition [125]. Whether the peculiar PER-dTIM behavior in ovaries relates to rhythmic egg laying under constant light remains unclear. Although *dtim* null mutants display reduced fitness in terms of female fertility and fecundity [123], it has been proposed that this is likely due to the overall loss of the circadian clock [11]. To examine non-circadian roles of *dtim*, it is necessary to manipulate *dtim* specifically in target cells/tissues. One possibility is that *dtim* expressed in non-clock cells has a residual role in maintaining chromosome integrity inferred from its ancestral paralog dTIMEOUT, the homolog of mTIM [126] (Fig. 4A). The non-circadian function of mTIM will be discussed below.

Debate on mammalian TIM function in circadian timekeeping

Evidence supporting the role of mammalian TIM in the circadian clock

Whether mTIM is a core component in the mammalian clock has been controversial. Due to their sequence similarity, mTIM was first identified as the homolog of dTIM in late 1990s [127–130]. Because of its rhythmic mRNA expression in the mammalian brain [127,131] and physical interaction to core clock proteins mPER1/2/3 [130,132] and CRY1/2 [133–136], mTIM was implicated as a clock protein. In addition, short-term mTIM knockdown causes phase resetting, whereas long-term knockdown of mTIM disrupts circadian neuronal activity rhythms [132]. Recently, Kurien *et al.* [137] reported a mutation in human TIM (hTIM) that causes familial advanced sleep phase syndrome (FASPS), reviving the discussion of the potential role of mTIM in mammalian clockworks. This mutation inhibits TIM nuclear accumulation and destabilizes PER/CRY2 repressor complex at the molecular level.

Evidence contradicting a direct role of mammalian TIM in regulating circadian rhythms

Multiple lines of evidence argue against a direct role of mTIM in the molecular clock. Homozygous *mTim* mutant mice are lethal in embryonic stage, whereas other homozygous clock mutants remain viable, suggesting a critical non-circadian role of mTIM [45]. The binding of mTIM to CRY1/2 does not necessarily support a circadian role of mTIM given that CRY1/2 also participates in non-circadian processes. CRY1 and CRY2 are known to modulate DNA damage response [138] and cell proliferation [139], and the interaction of mTIM-CRY1 and mTIM-CRY2 are critical for checkpoint activation [140,141]. Furthermore, phylogenetic analysis revealed that mTIM is an ortholog of dTIMEOUT [142]. *Drosophila* TIMEOUT is the widely conserved ancestral paralog of dTIM among eukaryotes that originated from gene duplication at the time of Cambrian Explosion [45,46,143]. Unlike dTIM, dTIMEOUT is an essential gene in *Drosophila* development and maintenance of chromosome integrity [126].

Non-circadian roles of mammalian TIM

There have been extensive investigations focusing on non-circadian roles of mTIM (Fig. 4B,C). Similar to its yeast homolog topoisomerase 1-associated factor 1 (*tof1*) [144], mTIM and its evolutionally conserved partner Tim-interacting protein (TIPIN) maintain replisome stability [145,146] and promote fork progression through hard-to-replicate regions [147–151]. In response to DNA damage, mTIM collaborates with cardinal signaling kinases ataxia telangiectasia-mutated checkpoint kinase 1 (ATR-CHK1) [140,152], ataxia telangiectasia and Rad3-related checkpoint kinase 2 (ATM-CHK2) [153], and poly [ADP-ribose]

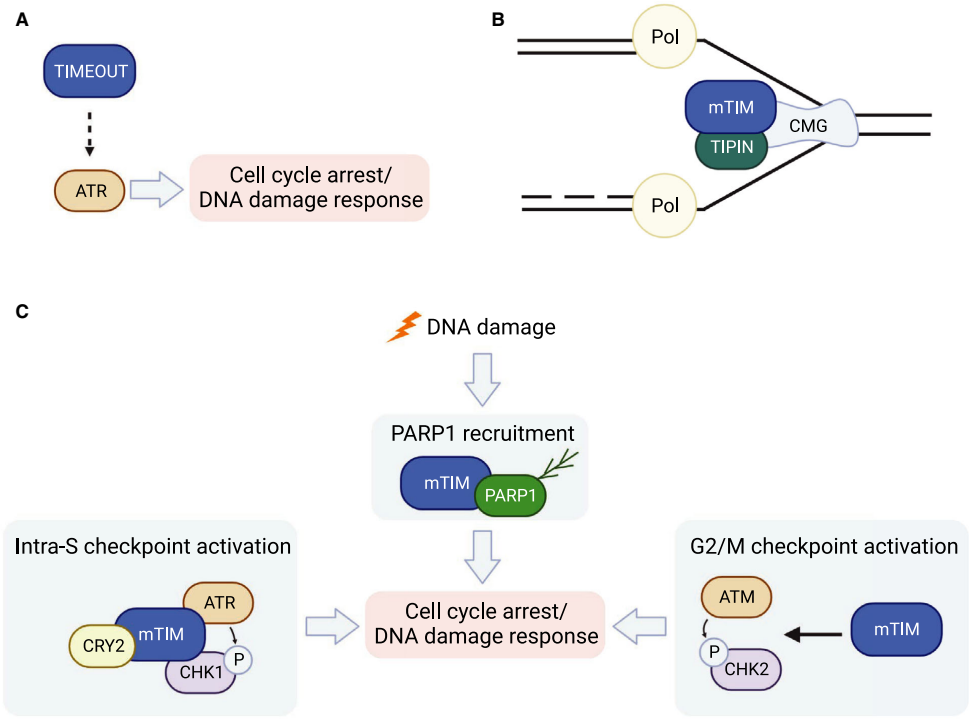

Fig. 4. *Drosophila* TIMEOUT and mammalian TIMELESS in genome maintenance. (A) *Drosophila* TIMEOUT interacts with Ataxia telangiectasia and Rad3-related (ATR) (genetically) to maintain genomic stability [126]. (B) mTIM and Tim-interacting protein (TIPIN) couple replicative DNA helicase CMG (CDC45, MCM2-7, GINS) and DNA polymerase (Pol) [145,146] in progressing replication fork. (C) In response to DNA damage, mTIM physically interacts with and recruits poly [ADP-ribose] polymerase 1 (PARP1) to damaged sites [153,154]. ATR and ataxia telangiectasia mutated (ATM) can both sense DNA damage and phosphorylate checkpoint kinase 1/2 (CHK1/2) [189]. This is dependent on a number of partner proteins including mTIM [140,152,153].

polymerase 1 (PARP1) [154,155] to facilitate proper checkpoint control and DNA repair [156–158]. Because of its role in genome maintenance, it is not surprising that mTIM dysregulation is commonly found in many cancer types [153,159,160]. Specifically, mTIM promotes cancer development by protecting cancer cells from replication stress and cell cycle arrest [153,161,162]. Thus, mTIM appears to be a promising target for anticancer treatment. However, given its ability to influence the circadian clock, the side effect of clock disruption needs to be considered, as clock disruption has been linked to increased risks of many diseases including metabolic disorders and cancers [163,164].

Considering the role of mTIM discussed in this section, it is noteworthy that the period shortening phenotype on the molecular clock resulting from the mTIM(R1081X) mutation is limited to proliferative cells [137]. Since the circadian clock ticks regardless of cell proliferation status, why was the period shortening phenotype only observed in proliferating cells? We speculate that mTIM modulates the circadian clock through its role in other cellular processes occurring only in proliferating cells. Specifically, its elevated expression in proliferative tissues such as spleen and thymus are consistent with its cell cycle-related function [137,165]. DNA damage has been shown to induce a circadian phase shift [166–168], with mTIM downregulation attenuating this effect [165]. Interestingly, the FASPS mutation found in hTIM lacks the C-terminal domain critical for mTIM-mediated DNA repair and checkpoint activation through replication stress response regulator SDE2 and PARP1 binding, respectively [153,154,162]. Taken together, it is

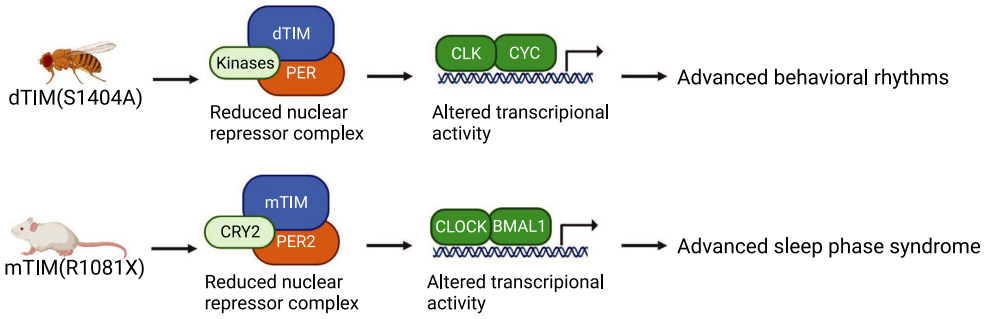

Fig. 5. Functional parallel between *Drosophila* and mammalian TIM. *Drosophila* TIM(S1404A) elevates PER-dTIM nuclear export [67]. The reduced abundance of nuclear PER-dTIM repressor complex leads to altered phosphorylation status of CLK and transcriptional activity of CLK-CYC, resulting in advanced behavioral rhythms. Mammalian TIM (R1081X) results in reduced nuclear mTIM [137], similar to the phenotype observed in dTIM(S1404A). This promotes destabilization of PER2-CRY2 repressor complex, thus altering transcriptional activity of CLOCK-BMAL1 and resulting in advanced sleep phase syndrome. Phosphorylation status of CLOCK or BMAL1 was not examined in [137].

plausible that the period shortening effect in proliferating cells can be attributed to a non-circadian role of mTIM.

Despite functional divergence of mTIM and dTIM, there are still some parallels. *Drosophila* TIMEOUT is expressed in the optic lobe of adult *Drosophila* and contributes to light entrainment, analogous to light sensitivity of dTIM [126]. Decreased dTIM and mTIM nuclear accumulation in *Drosophila* and mammals respectively both lead to similar outcome in circadian rhythms at the molecular and behavioral levels [67,137] (Fig. 5). This highlights an unexpected functional parallel between mTIM and dTIM in circadian regulation.

Conclusion and perspectives

The very name of the *timeless* gene hints at its critical function in biological timing. Since its discovery, almost three decades ago in *D. melanogaster*, a large body of work have uncovered the role of dTIM as a cardinal clock protein necessary to maintain circadian timekeeping, mediate light entrainment, and modulate temperature compensation. Thermosensitive splicing of *tim* mRNA in combination with the light sensitivity of dTIM protein enables its role in regulating seasonal physiology. Its ancestral paralog *timeout* (mTIM in mammals) surprisingly plays a distinct role in the maintenance of genomic stability. An important unanswered question regarding the role of dTIM in biological rhythms is how splice variants affect dTIM protein function in response to thermal and photic cues. The answer would clarify how the circadian clock interplays with seasonal timing. Another area of interest is to elucidate how mTIM regulates the molecular clockwork and potentially sits at the intersection between circadian clocks and cell cycle regulation. This would further shed light on the functional similarity and divergence of the two TIM paralogs. More importantly, this would extend our understanding of the interconnection between the circadian clock and the cell cycle. Circadian regulation of the cell cycle has been found in all domains of life [169–178], and the cell cycle also influences the phase and amplitude of circadian rhythms [166,179,180]. Given the accumulating evidence on circadian regulation of the cell cycle in the context of cancer and tissue regeneration upon injury [181–186], understanding the interaction of the circadian clock and the cell cycle could pave the way for innovative therapeutics for cancer and improved recovery of patients who suffered injuries.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

YDC wrote the initial draft of the manuscript with input from JCC. JCC edited the manuscript for submission.

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CONCLUSION

Over five decades of investigation have revealed the molecular mechanisms by which circadian clocks perceive environmental time cues, maintain the pace of the clock and keep time, and manifest daily rhythms in physiology and behavior. Despite different sets of clock genes involved in transcriptional-translational feedback loops (TTFLs) in diverse organisms from bacteria to mammals (Dunlap, 1999, Patke et al., 2020), phosphorylation-based timing mechanisms remain a conserved feature (Brown et al., 2012).

Chapters 1 and 2 expanded the understanding of the functional roles of phosphorylation in core clock proteins. Our findings revealed a mechanism by which phosphorylation closes the TTFLs in *Drosophila*: upon nuclear accumulation, repressor complexes recruit CK1α to phosphorylate and sequester the transcription activity of CLK. We demonstrated how the phosphorylation of clock repressors can regulate the phosphorylation of clock activators in the molecular clock. Given that similar mechanisms were found in *Neurospora* and mammalian clocks (Wang et al., 2019; Cao et al., 2021), our findings highlight this as a conserved feature of circadian timekeeping. Moreover, our findings contribute to the understanding of the molecular underpinnings of a human familial advanced sleep phase syndrome (FASPS) caused by a mutation in mammalian TIMELESS (Kurien et al., 2019).

Crosstalk of posttranslational modifications (PTMs) has become an additional layer of regulation in biological processes (Venne et al., 2014). Multiple types of PTMs exist on the same protein (e.g. Zaborowska et al., 2016; Gates et al., 2017; Limorenko and Lashuel, 2021) to regulate its structure and interaction with other molecules such as DNA, cofactors, and other proteins. In addition to phosphorylation, O-GlcNAcylation, proline isomerization, and acetylation have been found in mammalian and *Drosophila* clock proteins (e.g. Asher et al., 2008; Kim et al., 2012; Gustafson et al., 2017). Very few studies investigate the interaction of more than one PTM in the regulation of clock proteins. Therefore, future studies looking into the collaborative

regulation of different PTMs on clock proteins would further elucidate the circadian timing mechanisms.

Cell-type specific phosphorylation of core clock proteins remains to be further investigated. A kinase-dependent control of TIM nuclear entry is restricted to a subset of neurons in *Drosophila* clocks, showcasing potential broad diversity of cell-type specific phosphorylation (Top et al., 2016). Similarly, the impact of a mutation that influences CLK phosphorylation is found to be neuron-dependent (Lee et al., 2016). Research tools such as proximal ligation assay using a phosphospecific antibody (Gullberg and Andersson, 2009) would help to localize a phosphorylated residue of interest. Moreover, omics analysis at the single cell level can also be powerful. For example, single cell analysis of alternative splicing (Wang et al., 2018) revealed differential splicing of kinases in clock neurons. We expect that applying single-cell proteomics (Schoof et al., 2021; Brunner et al., 2022) in this context would yield profound insights.

Generalizing the common features of circadian timing mechanisms would help understand the design and evolution of clocks. On one hand, clock mechanisms need be expanded to non-model organisms in a systematic way. On the other hand, comparative analysis with the aid of mathematical modeling (e.g. Jeong et al., 2022; Tyler et al., 2022) needs to move forward in parallel.

Chapter 3 provided an update on the debate on the circadian role of mammalian TIM. We summarized roughly three decades of investigations on the circadian and non-circadian functions of *Drosophila* TIM (dTIM) and mammalian TIM (mTIM). We suggest mTIM as a non-clock component that influences circadian timing. Functional characterization of dTIM splicing isoforms would help reveal the role of dTIM regulating seasonal biology. Moreover, future investigations on the mechanisms by which mTIM influences circadian clocks would further shed light on the interconnection between cell cycle and circadian clocks.

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