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Timeless in animal circadian clocks and beyond

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Abstract

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.

Keywords

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

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Conflict of interest

The authors declare no conflict of interest.

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 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 [73–75]. 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 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 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] 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 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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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

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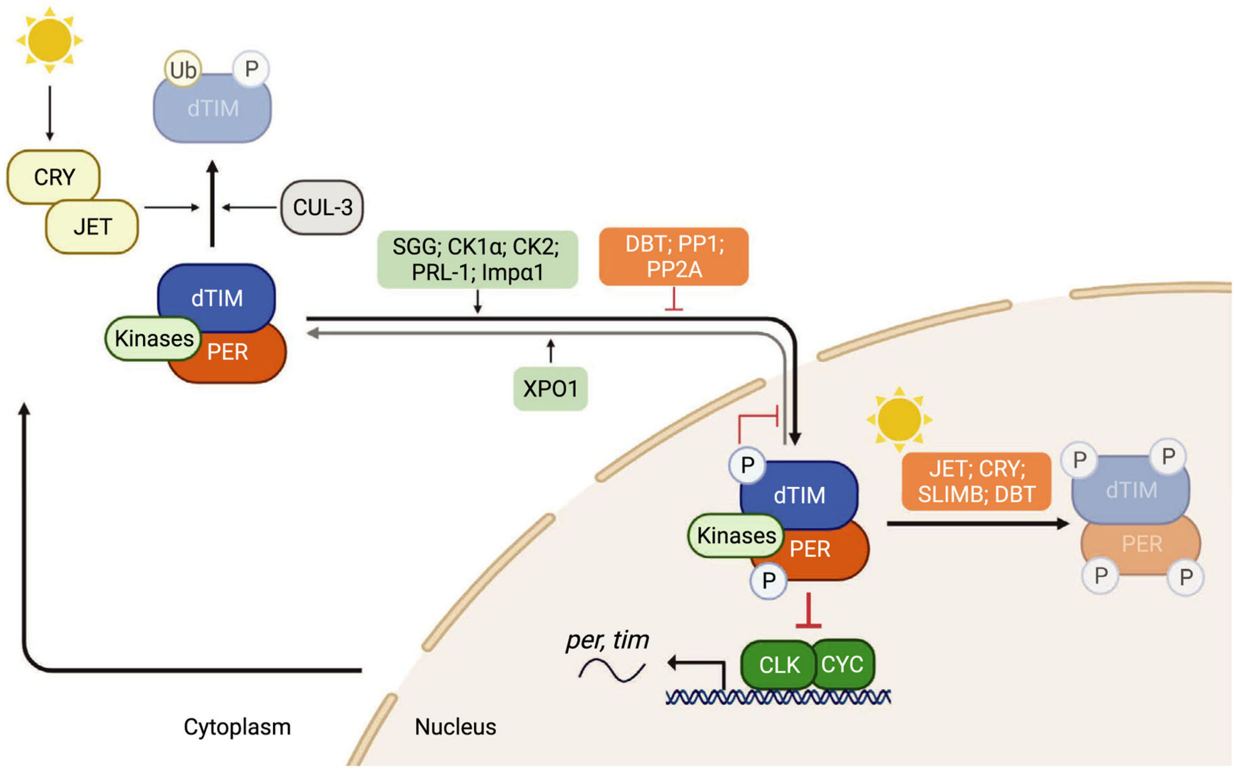

Fig. 1.

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].

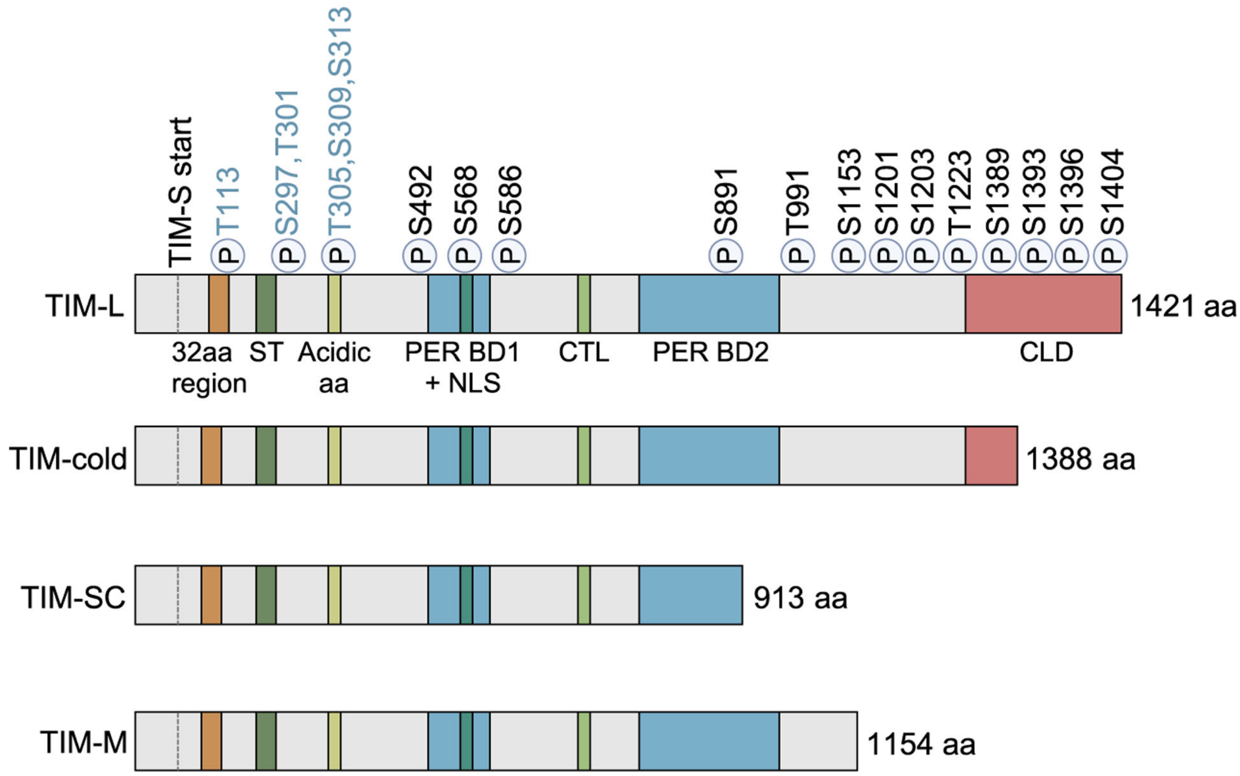

Fig. 2.

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].

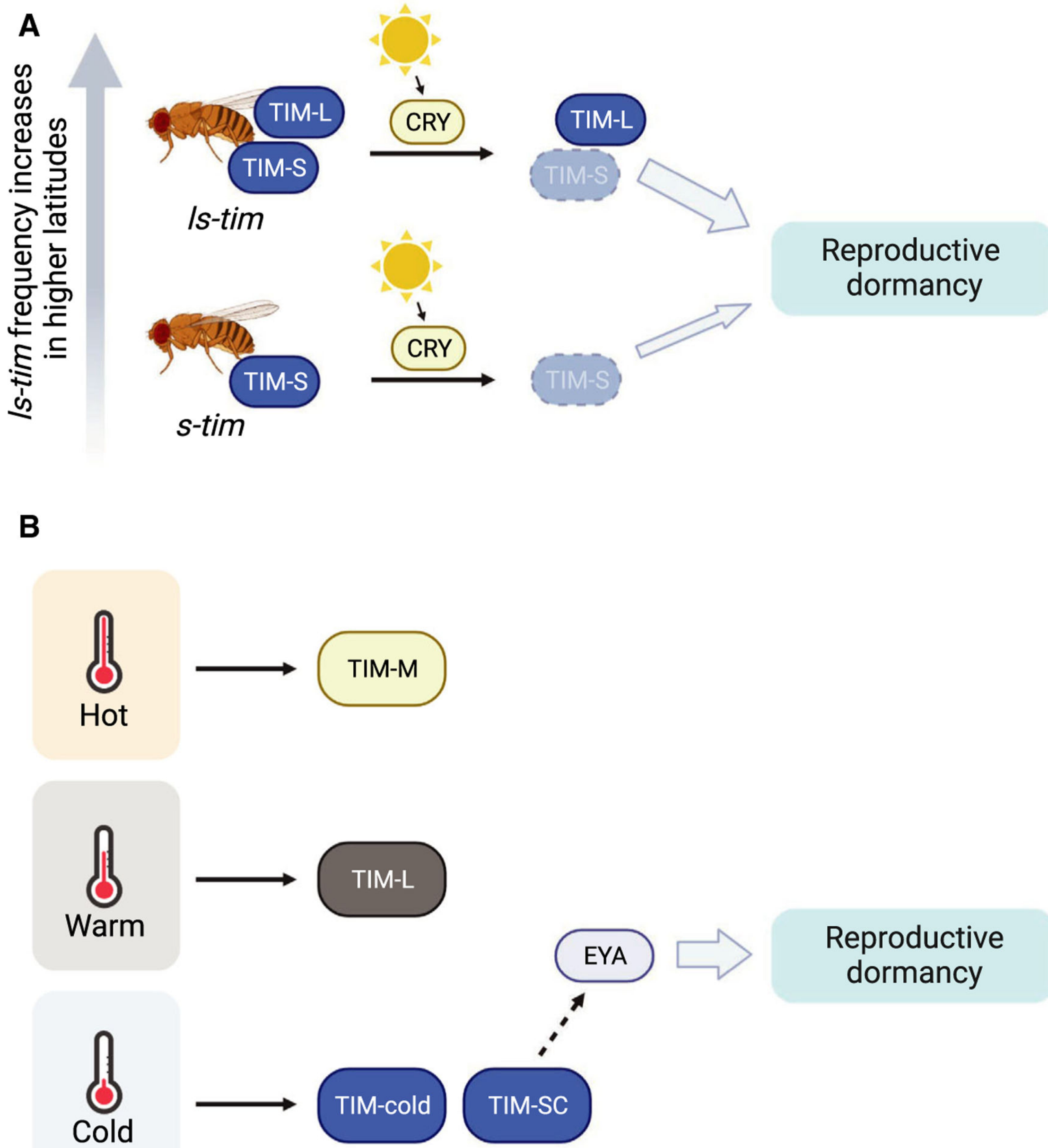

Fig. 3.
Role of *Drosophila* TIM in regulating seasonal biology. (A) Flies carrying *s-tim* allele express TIM-S, whereas flies carrying *ls-tim* allele express both TIM-L and TIM-S. Sampling of flies in North America [105] and on the eastern side of the Iberian Peninsula [101] showed that *ls-tim* allele 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].

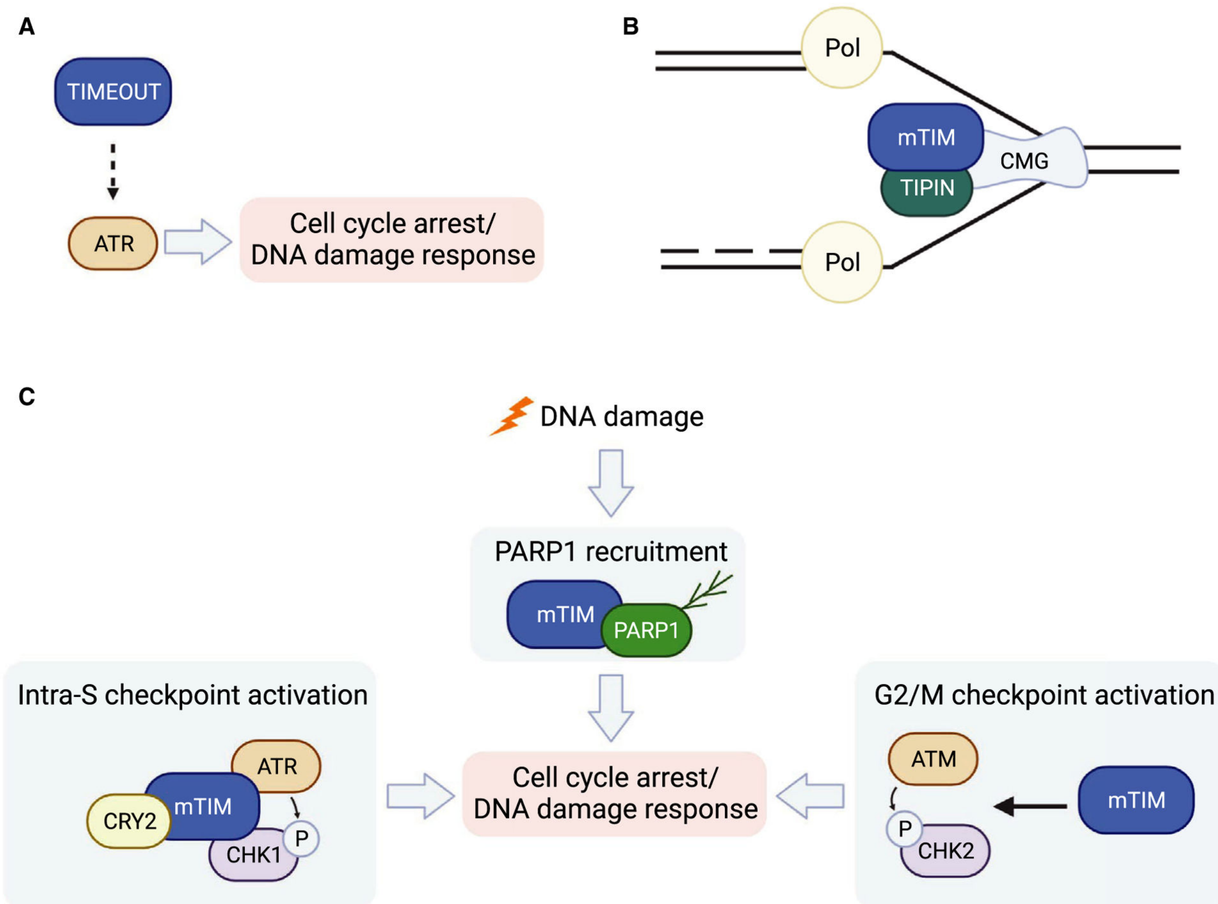

Fig. 4.

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].

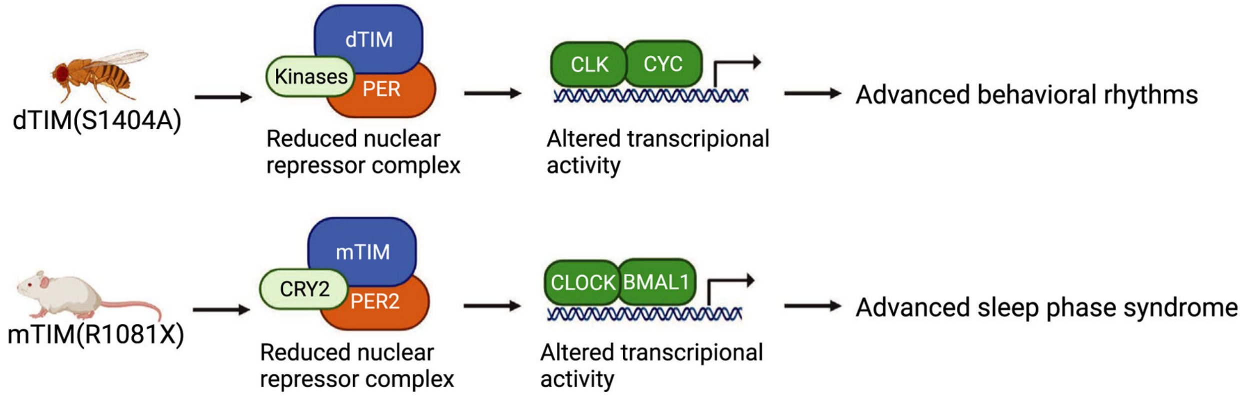

Fig. 5.

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].