Chapter 4
Molecular Mechanism of the Circadian Clock

David Doležel

Abstract Nearly all organisms possess a circadian clock, a genetically determined device that generates endogenous oscillations with a period of approximately 24 h. From a molecular perspective, the circadian clock relies on negative transcription-translation feedback loops. In insects, the molecular and genetic basis of the circadian clock machinery has been revealed by the remarkable genetic tools available to the fruit fly *Drosophila melanogaster*. However, the dawn of reverse genetics methods applicable to nonmodel species has led to recent significant advances in our understanding of the circadian clock beyond *Drosophila*. To illustrate the molecular mechanism behind the insect circadian clock, the first section focuses primarily on *Drosophila melanogaster* as the best established and most detailed insect model. Conserved components of the insect clocks are then identified at the genetic level, and lineage-specific idiosyncrasies and variations in setup are highlighted and further discussed. Functional evidence from non-*Drosophila* insects is reviewed, and the main descriptive data from molecular biology are presented in an evolutionary context and briefly summarized.

Keywords Cryptochrome · Evolution · Negative feedback · Oscillator · Period · Transcription

4.1 Introduction

Before we dive into the details and setup of molecular machinery driving the circadian clock in various insect species, the general background should be presented. The purpose of the biological clock ticking with a period of approximately 24 h lies on the time scale quite far from the time needed for typical biochemical processes, such as transcription, translation, protein phosphorylation,

D. Doležel (✉)
Biology Centre, Institute of Entomology, Czech Academy of Sciences, České Budějovice, Czech Republic
e-mail: david.dolezel@entu.cas.cz

H. Numata, K. Tomioka (eds.), *Insect Chronobiology*, Entomology Monographs,
https://doi.org/10.1007/978-981-99-0726-7_4

dephosphorylation, and degradation. Thus, a set of multiple interlocked loops, including some or even all of the above-listed regulatory processes, are found in the circadian clocks of various systems. It is unclear whether the circadian clock has evolved independently in kingdoms several times or if one ancestral clock has been heavily modified in different lineages of organisms during their evolution. At least, it is safe to say that we can find rather differently built clocks in cyanobacteria, plants, fungi, protozoa, and bilaterian animals (reviewed in Dunlap 1999). In cyanobacteria, the elegant phosphorylation cycle among just a few kinases can stably run even in vitro. Plant, fungal, and bilaterian clocks utilize a combination of interlocked feedback loops with transcription factors (also known as positive components) in the center that drive the expression of multiple genes, including the negative elements.

In Bilateria, two excellent models shaped our knowledge when research on one synergistically supported and motivated research on the other. First, *Drosophila* genetic tools lead to seminal discoveries of the *period* (*per*) and *timeless* (*tim*) genes. Then, new clock genes were identified in flies and mice, gradually building up the picture of the conserved animal clock setup with several specific feature characteristic of the fly and feature and component characteristic of the mouse clock. However, with the growing genomic sequencing, it became clear that the picture is not that simple and even some insect species remarkably differ from *Drosophila*. As functional genetic research further expanded, we can now see that insect clocks are more colorful than might have been expected at first. After all, with more than 400 million years of evolution and with millions of species known today, I would consider insect evolution as a remarkable collection of various solutions to the same or slightly different problems, a beautiful selection experiment for which the notes are not available. To illustrate the molecular mechanism behind the insect circadian clock, I will first focus mostly on the *Drosophila melanogaster* circadian clock as the best established and most detailed insect model (although, in some cases, the comparison to other insects will be provided immediately). Then, the conserved components of the insect clocks are identified, and lineage-specific idiosyncrasies are highlighted and further discussed.

4.2 Clock Setup in *Drosophila*

Unprecedented genetic tools predispose *D. melanogaster* to be a powerful model for gene discovery and elucidation of underlying molecular mechanisms. There are several excellent and detailed reviews on the *Drosophila* circadian clock and the mechanism involved in its regulation (Ozkaya and Rosato 2012; Hardin 2011; Peschel and Helfrich-Förster 2011; Stanewsky 2003; Hall 2003; Tataroglu and Emery 2015; Lim and Allada 2013a). A seminal screen by Ronald Konopka led to the identification of the *per* gene at the beginning of the 1970s (Konopka and Benzer 1971), whereas the gene was mapped by positional cloning more than a decade later (Zehring et al. 1984; Bargiello et al. 1984). The key feature of *per* regulation was a cyclical abundance of its transcript oscillating with a period of 24 h and, as

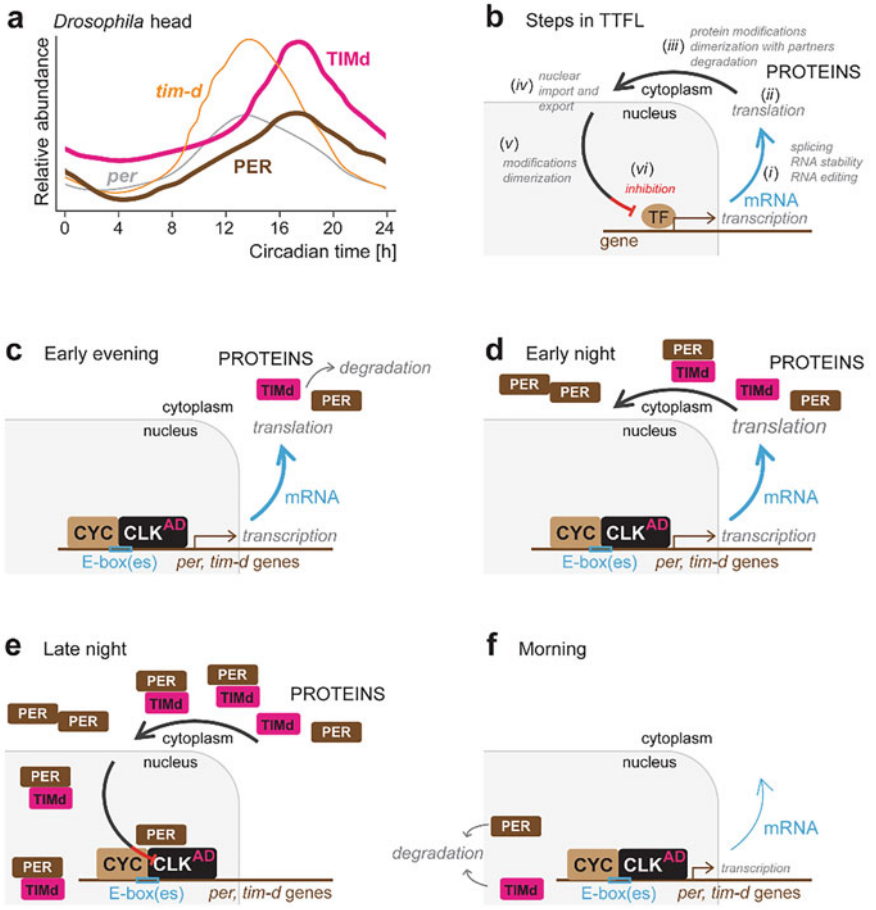

Fig. 4.1 *Drosophila*-negative transcription-translation feedback loop (TTFL). (**a**) Relative abundance of *per* and *tim-d* mRNAs compared to PER and TIMd (*Drosophila*-type TIM) proteins illustrates oscillation in abundance and a delay between the accumulation of mRNA and protein in whole head extracts (redrawn from Dunlap 1999). (**b**) Schematic depiction of TTFL with major steps and regulatory mechanisms, illustrating the activity and inhibition of a hypothetical *t*ranscription *f*actor (TF). (**c**) In the early evening, CLK-CYC drives transcription from *per* and *tim-d* genes. *A*ctivation *d*omain (AD) is located on CLK protein in *Drosophila*. The resulting mRNAs are translated, but the proteins are (mostly) degraded. (**d**) As the night progresses, PER and TIMd proteins are stabilized by heterodimerization, although PER-PER homodimers are also observed. (**e**) During the late night, PER and TIMd enter the cell nucleus, where PER molecules inhibit the transcription factor CLK. (**f**) As PER and TIMd are eventually degraded, the inhibition fades away, and a new round of transcription starts in the morning

discovered by Siwicki et al. (1988), the cyclical abundance of the PER protein, which peaked with a few-hour delay after the *per* mRNA reached its maximum. See Fig. 4.1a for an illustration of the expression pattern in the whole head extracts. The cyclical transcription implied a feedback loop (Hardin et al. 1990); however, a

simple transcription-translation feedback loop (TTFL) should produce oscillations on a scale of minutes. Thus, additional regulatory steps should be involved in TTFL participating in the circadian clock.

Figure 4.1b illustrates the general principle of the negative TTFL with major conceivable regulatory mechanisms adjusting the period of its oscillation. First, a transcription factor(s) drives the expression of mRNA coding for the negative element, which, before translation, needs to be processed, and its stability might be further regulated. To prevent degradation, the negative element protein(s) might be stabilized by modification steps such as phosphorylation, by interaction with partner protein(s) forming either the heterodimer or homodimer, and even by additional modifications (glycosylations, SUMOylation, etc.). The last step of the feedback is the interaction of the negative element with the transcription factor(s), preventing its activity. As a consequence, no mRNA coding for the negative element is transcribed. Transcription can only start once the nucleus-localized negative element protein is removed from the system. Thus, protein stability and subcellular localization are key regulatory steps of the whole process leading to 24-h oscillations.

4.2.1 *Period and* Drosophila-*Type Timeless*

In *Drosophila*, the first well-described TTFL comprises proteins PER and TIM, here referred to as *Drosophila*-type *tim-d* (TIMd for the protein) to distinguish it from its paralogous mammalian-type (*tim-m*, TIMm). Similar to *per*/PER expression, *tim-d* and TIMd oscillate in abundance in a 24-h cycle, when mRNA peaks circa 4 h before TIMd (Fig. 4.1a). Furthermore, the subcellular localization pattern of PER and TIMd changes over time, all of which led to the establishment of the following model. Let us focus on the most important clock neurons in the fly brain responsible for behavioral rhythmicity (the expression might differ in various cell types across the body; see Chap. 5). During the early evening (Fig. 4.1c), *per* and *tim-d* mRNAs are transcribed; however, the corresponding proteins are not detectable, as they are degraded. Then, during the early night, PER and TIMd are stabilized by dimerization, and both proteins are detected in the cytoplasm (Fig. 4.1d). Gradually, PER and TIMd accumulate, and then, during late night (Fig. 4.1e), both proteins are detected in the nucleus. At the same time, transcription of *per* and *tim-d* mRNAs stops because PER interacts with the transcription factor CLOCK (CLK). CLK, a basic helix-loop-helix (bHLH) Per-ARNT-Sim (PAS) protein, forms a dimer with another bHLH-PAS protein CYCLE (CYC), and together they drive expression from the so-called E-box (cis-regulatory DNA elements with motif CACGTG). E-boxes are localized in the promoters of multiple genes, including *per* and *tim-d*, and are key for their cyclical expression. The inhibition of CLK-CYC continues as long as PER is present in the nucleus. After its depletion (Fig. 4.1f), the transcription of *per* and *tim-d* mRNAs resumes again.

The above-described model has been synthesized from data gathered on mutant and wild-type flies (Hardin et al. 1990; Siwicki et al. 1988; Zerr et al. 1990; Sehgal et al. 1994; Sehgal et al. 1995; Allada et al. 1998; Rutila et al. 1998; Glossop et al. 1999). The feedback mechanism was further reconstructed in *Drosophila* Schneider 2 (S2) cell cultures (Darlington et al. 1998), and the role of the E-box was addressed both in S2 cells and in flies using elegant in vivo luciferase reporters (Brandes et al. 1996; McDonald et al. 2001). The protein domains important for PER-TIMd interaction and nuclear localization were systematically explored in S2 cells (Saez and Young 1996; Saez et al. 2011), even though real-time monitoring in S2 cells using FRET (fluorescence resonance energy transfer) revealed that the spatial organization of the dimer differs in the cytoplasm and nucleus (Meyer et al. 2006). Furthermore, functional PER-PER homodimers exist in flies, and if their mutual dimerization is impaired, molecular and behavioral rhythmicity is severely disrupted (Landskron et al. 2009). In addition to nuclear localization signals (NLS), PER and TIMd also contain nuclear export signals (NES) (Ashmore et al. 2003; Hara et al. 2011; Jang et al. 2015; Singh et al. 2019; Cai et al. 2021). For details on TIMd and TIMm biology, see a thorough review by Cai and Chiu (2021).

A great deal of data was revealed thanks to a beautiful collection of genetic mutants created in *Drosophila*, which, after all, provide the most accurate understanding of the impact on the ultimate output, the fly's behavior. A fundamental feature of the circadian clock is temperature compensation, the phenomenon when the free-running period remains constant over a physiologically acceptable range of temperatures (the meaningful temperature range for recording fly locomotor activity is approximately 15–29°C). Some of the *per* and *tim-d* mutations result in a good temperature-compensated clock of either a long or short free-running period (Rothenfluh et al. 2000a, 2000b; Hamblen et al. 1998). However, per^{L} and multiple *tim-d* mutants produce a slower clock at high temperatures (Konopka et al. 1989; Matsumoto et al. 1999; Singh et al. 2019), which can be understood as a temperature-overcompensated clock, although one might argue that the mutated proteins are simply unstable at high temperatures. Interestingly, the mutated protein TIM^{SL} shortens the free-running period when expressed in the per^{L} mutant and completely restores the temperature compensation deficiency (Rutila et al. 1996). The opposite trend, temperature undercompensated clock, is produced by per^{SLIH}, per^{S}, and per^{T} mutations (Konopka et al. 1989; Konopka et al. 1994; Hamblen et al. 1998). The last three *per* mutants were particularly instrumental in elucidating how phosphorylation influences PER stability (Chiu et al. 2008; Chiu et al. 2011).

4.2.2 Posttranslational Modifications

The key part of the negative PER-TIMd TTFL is the delay between mRNA and protein peaks. The regulation of PER and TIMd stability is tightly connected with phosphorylation and dephosphorylation performed by several kinases and phosphatases. The phosphorylation of PER can also serve as a fascinating tale illustrating the

synergistic research on the fruit fly and mammalian clocks. In 1998, two seminal papers described DOUBLETIME, a casein kinase 1 (CK1) isoform[1] type, as a key clock component in *Drosophila* (Price et al. 1998; Kloss et al. 1998). In mammals, a homologous casein kinase 1ε (CK1ε) was identified as a component of the hamster clock when the *tau* mutation was mapped by positional cloning as an arginine-to-cysteine amino acid substitution at a highly conserved position within the kinase domain (Lowrey et al. 2000). Only a year later, Toh et al. (2001) described in humans the molecular basis for familial advanced sleep phase syndrome (FASPS) as a serine-to-glycine mutation within the CK1ε binding region of human PER2[2], which results in hypophosphorylated PER2.

The physical interaction between DBT kinase and PER is remarkably stable (Kloss et al. 2001). Interestingly and surprisingly, in vitro studies on DBT^{L} and DBT^{S} using nonphysiological substrates suggested that both mutant proteins have reduced kinase activity (Kivimaë et al. 2008; Venkatesan et al. 2019), even though the resulting behavioral phenotype of these mutants is the opposite (Price et al. 1998). The molecular mechanism behind this conundrum was first revealed for mammalian PER2 in the context of multi-kinase hierarchical activities. The current phosphoswitch model involves two competing phosphorylation sites on mouse PER2, the FASP site and the phosphodegron sites, which regulate PER2 stability in opposing ways (Zhou et al. 2015; Masuda et al. 2020). Importantly, different splicing isoforms of mammalian CK1δ, a paralog of mammalian CK1ε, have different priming phosphorylation capacities – that is, the ability to phosphorylate residues in a protein region where no other phosphorylation mark is attached. CK1δ1 and CK1ε are more active in priming phosphorylation of the FASP site, whereas CK1δ2 is more potent in priming the degron site (see the excellent review by Narasimamurthy and Virshup 2021).

In *D. melanogaster*, the intronless *dbt* gene encodes only one protein version; thus, the priming phosphorylation of PER must be regulated by a different mechanism. Similar to mammalian PER2, *Drosophila* PER contains multiple phosphoclusters (Chiu et al. 2008; Garbe et al. 2013; Kivimaë et al. 2008; Top et al. 2018). The phosphorylation of the N-terminal region (serine 47 and nearby amino acids) by DBT generates a high-affinity binding site for *supernumerary limbs* (SLIMB) (Chiu et al. 2008), an F-box protein targeting PER for ubiquitination and subsequent proteasomal degradation (Grima et al. 2002; Ko et al. 2002; see also Sect. 4.2.4). On the other hand, phosphorylation by NEMO/NLK kinase at the per-short domain, a region located in the center of PER protein (positions 585–600 in 1224-amino acid PER), stimulates additional phosphorylation of several nearby sites by DBT. This multisite phosphorylation of the central part of PER prevents N-terminal

[1] In this case, CK1 isoforms are coded by distinct genes, whereas the term isoform might also be used for variants originating from alternative splicing or alternative transcription start. See *Pdp1* and *Kay* genes in bZIP protein sections as examples of the latter.

[2] Circadian clock genes are often multiplicated in vertebrates, so there are three *per* genes, the so-called paralogs, encoding three proteins: PER1, PER2, and PER3.

phosphorylation, which is key for SLIMB binding, resulting in a time-delay phosphorylation circuit (Chiu et al. 2011). The complex phosphorylation pattern of PER seems to be one of the mechanisms behind the temperature compensation of the circadian clock in *Drosophila* (Joshi et al. 2022).

Several additional kinases were discovered as important components of the *Drosophila* clock affecting the negative TTFL with PER and TIMd. Some of them, such as NEMO (NMO), CK1α, and p38 phosphorylate PER (Chiu et al. 2011; Lam et al. 2018; Dusik et al. 2014), whereas CASEIN KINASE 2 (CK2) phosphorylates both PER and TIMd in *Drosophila* (Cai et al. 2021). SHAGGY (SGG), a kinase identified as a TIMd-phosphorylating enzyme, might also phosphorylate PER (Martinek et al. 2001; Top et al. 2016).

In addition to kinases, enzymes with the opposite role, phosphatases, participate in the regulatory loops of the circadian clock and counterbalance the impact of kinases on various TTFL components. PROTEIN PHOSPHATASE 2 (PP2), a multiunit enzyme, specifically dephosphorylates PER and regulates its abundance, PROTEIN PHOSPHATASE 1 (PP1) targets both PER and TIMd, and phosphatase of regenerating liver-1 (PRL1) selectively dephosphorylates TIMd in darkness (Sathyanarayanan et al. 2004; Fang et al. 2007; Kula-Eversole et al. 2021; Agrawal and Hardin 2016).

However, since many of the abovementioned kinases and phosphatases tend to recognize and (de)phosphorylate multiple substrates, their role in the clock has gradually become increasingly complex. For example, the transcription factor CLK is cyclically phosphorylated, with its minimum phosphorylation detected in the early night and its maximal phosphorylation in the morning (Lee et al. 1998). Its partner CYC is constitutively expressed (at least in *Drosophila* and related flies; see CLK-CYC idiosyncrasies in the lineage-specific description later in this chapter); thus, the cyclical phosphorylation of CLK seems to be the key rhythmic component of the circadian activator (Yu et al. 2006). The phase-specific hyperphosphorylation is DBT-dependent and leads to maximal repression of CLK activity. Remarkably, PP2, introduced in a previous paragraph as PER-regulating phosphatase, stabilizes CLK by counteracting the activity of DBT (Kim and Edery 2006). Furthermore, DBT-mediated degradation of CLK is further counterbalanced by CK2, which inhibits CLK degradation and reduces its activity (Szabo et al. 2013).

Not only is the phosphorylation state important to define the stability of the proteins and determine their interaction with partner proteins, but it might also specifically impact their subcellular localization, for example, the nuclear export of TIMd (Cai et al. 2021; Fang et al. 2007). An additional posttranslational modification, such as O-GlcNAcylation of PER, reduces PER interaction with CLK, a mechanism that might link the clock with the metabolic signals stemming from feeding activity (Li et al. 2019; Liu et al. 2021).

4.2.3 Entrainment by Light

The above-described machinery illustrates the molecular mechanism of the *Drosophila* clock "ticking" in constant darkness. However, the clock must be synchronized (entrained) with the external time of the surrounding environment. The most powerful cues are light and temperature. The major light-mediated synchronization involves the PER-TIMd feedback loop, although opsin-based receptors contribute as well (see Chap. 3). As was noted early with *tim-d* discovery and characterization, TIMd stabilizes PER, as there is no PER detected in the $tim\text{-}d^{01}$ mutant. At the same time, TIMd is degraded upon light illumination, which led to a model where entrainment is achieved via light-mediated degradation of TIMd, which is further relayed to PER depletion, resulting in either phase advance or phase delay of the clock (Hunter-Ensor et al. 1996; Myers et al. 1996; Zeng et al. 1996).

However, TIMd is not sensitive to light by itself; instead, the signal must be transduced from a photoreceptor, which then leads to tyrosine phosphorylation of TIMd and its subsequent degradation through a ubiquitin-proteasome mechanism (Naidoo et al. 1999). The actual photoreceptor of the light-input pathway turned out to be flavoprotein CRYPTOCHROME (Stanewsky et al. 1998; Emery et al. 1998), a member of a large protein family present in all kingdoms and including different types of photolyases (Mei and Dvornyk 2015; Xu et al. 2021). Here, we will refer to this protein as *Drosophila*-type (CRYd), although CRYd is found in the majority of Protostomia and even some basal Deuterostomia (Kotwica-Rolinska et al. 2022a). CRYd functions as a photoreceptor within clock neurons located deep in the fly brain (Emery et al. 2000a), and its mutation or complete depletion results in behavioral rhythmicity in constant light (Emery et al. 2000b; Dolezelova et al. 2007), a condition under which wild-type flies become completely arrhythmic. Simplified systems of cell cultures and yeast helped to shed light on the mechanism: S2 cell transfection experiments revealed that CRYd blocks TIMd+PER-dependent inhibition of CLK-mediated transcription under light but has no impact in darkness, and the yeast two-hybrid system identified a light-dependent interaction between CRYd and TIMd (Ceriani et al. 1999). Furthermore, Rosato et al. (2001) discovered CRYd interaction with PER. The most variable region of various CRY proteins lies in their C-terminus (C-tail), a key part necessary in *Drosophila* CRYd for regulating its interaction with TIMd upon light illumination. If the C-tail is removed from CRYd, either by a stop codon mutation or engineered in a synthetic construct, the resulting "C-tailless CRYd" is constitutively active and interacts with TIMd even in constant darkness, resulting in a long free-running period (Busza et al. 2004; Dissel et al. 2004).

The interaction between TIMd and CRYd is further affected by the N-terminal region in TIMd. The *s-tim-d* allele encodes a 1398 amino acid-long S-TIMd protein, whereas the *ls-tim-d* allele contains two alternative start codons, resulting in transcripts encoding 1398 (S-TIMd) and 1421 (L-TIMd) amino acid-long proteins, respectively (Rosato et al. 1997). The *ls-tim-d* allele originated in *D. melanogaster* in southern Italy circa 10,000 years ago and has spread in all directions (Tauber et al.

2007). L-TIMd shows a diminished interaction with CRYd (Sandrelli et al. 2007), *ls-tim-d* flies are significantly more rhythmic under continuous light than *s-tim-d* flies (Deppisch et al. 2022), and only *ls-tim-d* flies can synchronize to seminatural conditions with short "white nights" typical of high latitudes (Lamaze et al. 2022).

Constant light rhythmicity was observed in flies with mutated JETLAG (JET), an F-box protein with leucine-rich repeat (LRR) (Koh et al. 2006; Peschel et al. 2006). The jet^c mutation results in a single amino acid change in the LRR region, the part of the protein important for substrate binding. Yeast two-hybrid experiments identified an interaction between JET and CRYd and further revealed that this interaction is reduced between JET^c and CRYd. Interestingly, JET and TIMd do not bind each other in the yeast system, but their interaction was detected in S2 cells (one of several examples illustrating the limitations of yeast-based experiments for reconstructing the *Drosophila* system). The current (working) model assumes the light-dependent interaction between CRYd and JET, as well as the interaction of TIMd with JET, and both CRYd and TIMd are then degraded by the proteasome (Peschel et al. 2009).

TIMm, a protein also known as TIMEOUT or TIM2, was first identified in mice (Zylka et al. 1998) and subsequently in fruit flies (Benna et al. 2000). This protein is essential for development in both mammals and flies, which heavily complicates its functional reverse genetics research. Flies with only one *tim-m* functional allele possess reduced sensitivity to light (Benna et al. 2010). RNA-mediated silencing (RNAi) in crickets and linden bugs affects behavioral rhythmicity (Nose et al. 2017; Kotwica-Rolinska et al. 2022a), which, together with the involvement of *tim-m* in the neuronal activity rhythm of the suprachiasmatic nucleus in rats (Barnes et al. 2003) and the connection of human *tim-m* with familial advanced sleep phase syndrome (Kurien et al. 2019), suggests TIMm as a conceivable and conserved clock component. For a more detailed description of TIMd/m proteins in *Drosophila*, see the review by Cai and Chiu (2021). For a general overview of the light input into various circadian clocks, see the review by Johnsson et al. (2014).

4.2.4 Protein Degradation

The oscillation of the clock system, either in constant darkness or in a light/dark regime, depends largely on the well-regulated protein turnover. The SKP/CULLIN/F-box-containing complexes (SCF complexes) function as E3 ubiquitin ligases targeting proteins for 26S proteasomal degradation. SLIMB is an F-box/WD40-repeat protein participating in the CULLIN-1-based E3 ubiquitin ligase complex that binds phosphorylated residues in the N-terminal region of PER (Chiu et al. 2008), ubiquitinates PER, and thus stimulates its degradation. Flies with mutated *slimb* are arrhythmic, and the same phenotype is obtained when a dominant-negative form of SLIMB is expressed in clock cells (Ko et al. 2002; Grima et al. 2002). Furthermore, highly phosphorylated PER and TIMd are constitutively present in the constant darkness of *slimb* mutants. However, the cyclical oscillation of PER and TIMd abundance is maintained even in *slimb* mutants under the light/dark regime due to

the degradation pathway utilizing CRYd and JET proteins (see Sect. 4.2.3). Another ubiquitin ligase, the CULLIN-3 (CUL-3)-based complex, interacts with low-phosphorylated species of TIMd in the absence of PER. SLIMB, on the other hand, binds more phosphorylated TIMd, including PER-bound TIMd (Grima et al. 2012). CIRCADIAN TRIP (CTRIP) is an E3 ubiquitin ligase that seems to regulate the stability of both PER and CLK when *ctrip* downregulation results in a long free-running period, high CLK levels, and persistence of phosphorylated PER during the subjective day when PER is normally degraded (Lamaze et al. 2011).

A study aiming at the identification of cyclically ubiquitinated proteins, the cycling ubiquitylome, revealed a 2–2.5-fold oscillation in abundance for 52 proteins (15 % of all identified ubiquitinated proteins) (Szabo et al. 2018). A remarkable 29-fold oscillation was found for transcription of Megator (MTOR), a nuclear pore complex component. Additional cyclic ubiquitylation affects MTOR, which then feeds back to the pacemaker when it regulates the subcellular localization of the core clock proteins (Szabo et al. 2018).

4.2.5 bZIP Proteins PDP1, VRI, and KAY

The next feedback loop involves two basic leucine zipper (bZIP) transcription factors (Fig. 4.2a, b). First, *vrille* (*vri*) mRNA is cyclically transcribed by the CLK-CYC complex in a phase similar to *tim-d* expression (Blau and Young 1999; McDonald and Rosbash 2001). The second bZIP protein, PAR (proline and acidic rich) DOMAIN PROTEIN 1 (PDP1), particularly its isoform *epsilon* (PDP1ε), is cyclically translated from *Pdp1ε* mRNA, whose expression is also driven by the CLK-CYC complex from an upstream promoter in the *Pdpd1* gene. However, *Pdp1ε* peaks several hours after *vri* reached its maximum, and a similar delay was reported for the peaks of Pdp1ε and VRI proteins (Cyran et al. 2003). Even though PDP1ε and VRI remarkably differ in the organization of protein domains, both proteins contain a highly conserved DNA-binding domain. Indeed, PDP1ε and VRI bind identical cis-regulatory elements in DNA, the so-called V/P motif (also known as the D-box), but with the opposite impact (Fig. 4.2b). The early peaking VRI serves as a transcriptional repressor on the *Clk* promoter. A few hours later, PDP1ε reaches its maximum, replaces VRI, and activates *Clk* transcription. The resulting two-loop model with the negative (VRI) and positive (PDP1ε) components then explained the cyclical expression pattern of *Clk* mRNA, which runs in antiphase to the expression of *per*, *tim-d*, and *vri* mRNAs (Cyran et al. 2003). A third bZIP protein, specifically its α isoform produced from the upstream promoter in the *kayak* gene, further contributes to VRI/PDP1ε feedback. Downregulation of KAYAKα (KAYα), a homolog of mammalian FOS, in circadian pacemaker neurons prolongs period length. KAYα binds to VRI and thus inhibits its suppression of the *Clk* promoter. Surprisingly, KAYα also represses CLK activity. These opposite roles of KAYα in the two-loop model were interpreted as a mechanism bringing stability and precision to the system (Ling et al. 2012).

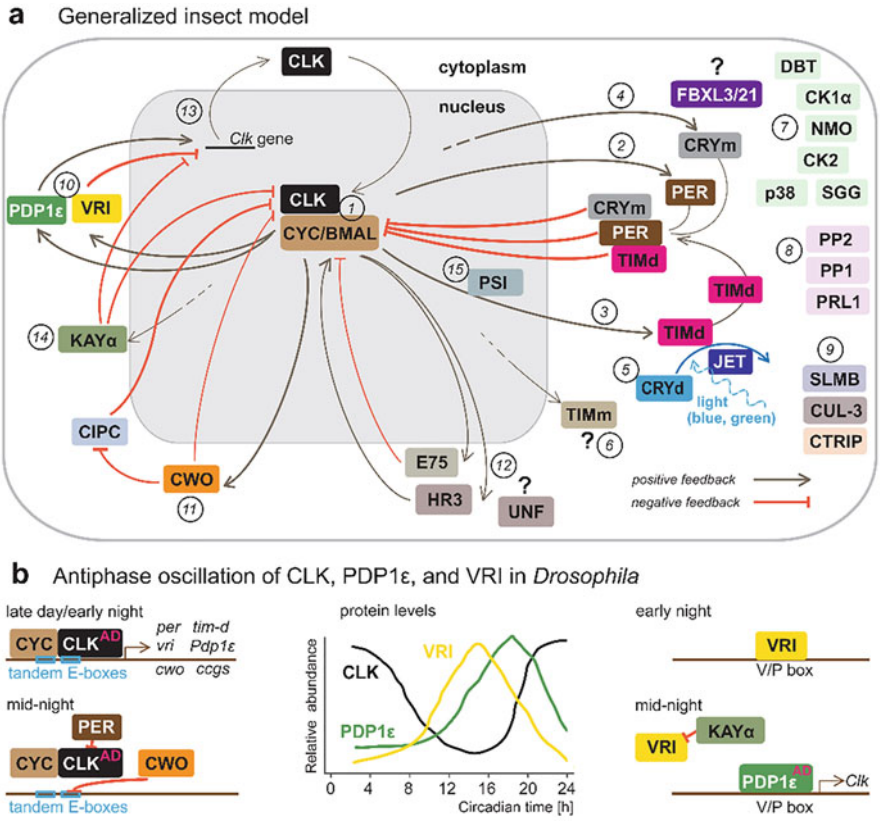

Fig. 4.2 (**a**) Major transcription-translation feedback loops (TTFL) identified in insects (note that some components might be missing in certain insect lineages). The end style and color indicate whether the loop activates the transcription factors CLK-CYC/BMAL (positive feedback) or inhibits their activity (negative feedback). Loops and key modulatory processes are numbered, and the same numbering is used in Fig. 4.3. For simplicity, promoters are not shown in panel **a**, and transcription with subsequent mRNA processing is depicted as arrows running from the nucleus to the cytoplasm. The core of system ① consists of bHLH PAS proteins CLK and CYC/BMAL that drive the expression of the majority of components, including PERIOD (PER) ②, *Drosophila*-type TIMELESS (TIMd) ③, mammalian-type CRYPTOCHROME (CRYm) ④, bZIP transcription factors VRILLE (VRI) and PAR DOMAIN PROTEIN 1 (PDP1) ⑩, bHLH protein CLOCKWORK ORANGE (CWO) ⑪, and nuclear receptors E75, HR3, and UNF ⑫. Additional components include *Drosophila*-type CRY (CRYd) ⑤, which interacts with TIMd upon light illumination and, with the involvement of F-box protein JETLAG (JET), mediates the major light entrainment of the system (see Chap. 3 for details on photic entrainment). Mammalian-type TIM (TIMm) ⑥ contributes to rhythmicity in some insects and is involved in light entrainment in *Drosophila*; however, the mechanisms behind both roles are unclear. Key regulatory steps involve posttranslational modification, such as phosphorylation by multiple kinases ⑦, dephosphorylation by phosphatases ⑧, and degradation machinery ⑨. In *Drosophila*, *Clk* is cyclically expressed ⑬, and splicing of *tim-d* mRNA is regulated by PSI ⑮ in a temperature-dependent manner. KAYAK ⑭, a bZIP protein related to mammalian FOS, contributes to rhythmicity in *Drosophila*. (**b**) Scheme illustrating the role of cis-regulatory elements in *Drosophila* promoters. "AD" in CLK and PDP1 proteins refers to the activation domain, *ccgs* stands for *clock-controled genes*. See the text for a detailed explanation

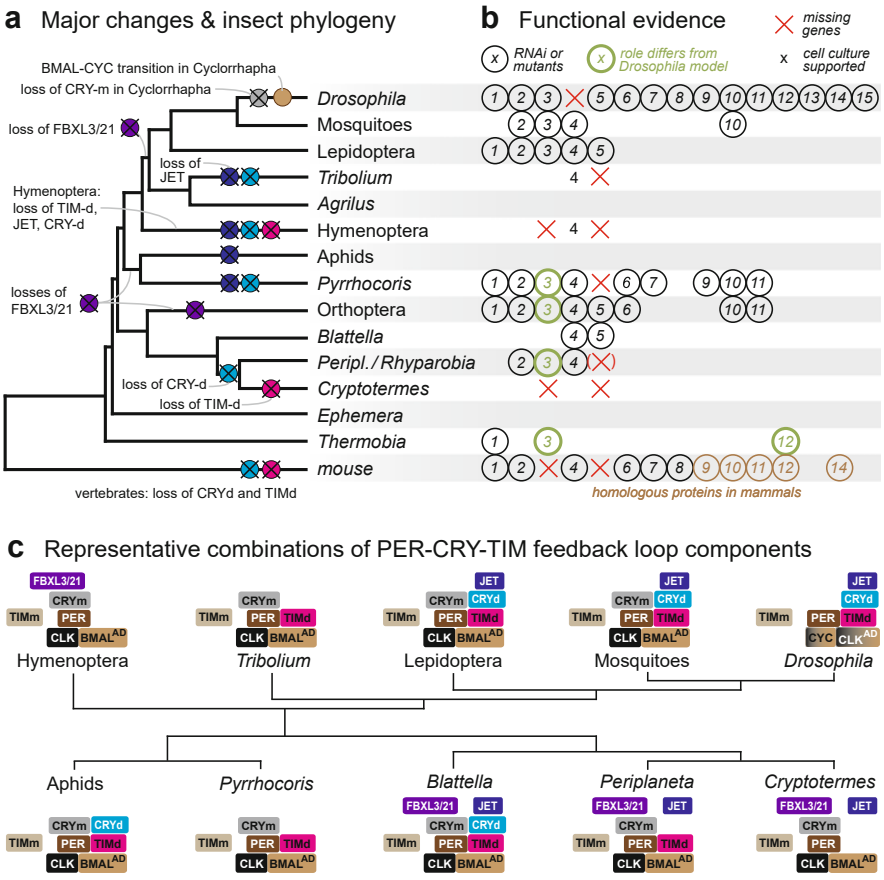

Fig. 4.3 Lineage-specific changes in the insect clock setup depicted from a genetic perspective. Colors correspond to Figs. 4.1 and 4.2. (**a**) A simplified phylogenetic tree with highlighted gene losses and the transition of BMAL to CYC. The genetic setup seems to be conserved in entire insect orders (Lepidoptera, Hymenoptera, Orthoptera) or lower taxonomic groups (aphids, mosquitoes), but in several cases, representative species are depicted. (**b**) Experimental evidence for the role of specific loops and components in particular insect lineages (numbers correspond to the scheme in Fig. 4.2a: CLK and CYC/BMAL ①; PER ②; TIMd ③; CRYm ④; CRYd ⑤; TIMm⑥; kinases ⑦; phosphatases ⑧; degradation machinery ⑨; bZIP transcription factors VRI and PDP1 ⑩; CWO ⑪; nuclear receptors E75, HR3, and UNF ⑫; cyclical expression of CLK ⑬; KAYAK ⑭; and splicing of *tim-d* mRNA regulated by PSI ⑮. If experiments were performed only in an artificial system of cell culture, the number is depicted without the circle. CRYd has been lost in *Periplaneta*, whereas its presence/absence is not fully clear in *Rhyparobia*; thus, X is depicted in parentheses. Deviation from the *Drosophila* model is highlighted by green color. As a reference, a mouse clock setup is depicted with homologous components shown in brown. (**c**) Depiction of major combinations of TTFL components in insects

4.2.6 Clockwork Orange (CWO)

Somewhat similar two-component feedback with opposite roles utilizes CWO, a protein belonging to the bHLH-ORANGE family related to mammalian DEC1 and DEC2, both of which are important for rhythmicity in mice (Honma et al. 2002). CWO was identified as a direct target of CLK (Kadener et al. 2007) and in parallel by genome-wide functional screening using an RNA interference (RNAi) system in flies (Matsumoto et al. 2007). CWO competes with the CLK-CYC dimer in binding to tandem E-boxes when the DNA-binding capacity is the highest for the CLK-CYC dimer, intermediate for CWO, and the weakest for the CLK-CYC-PER complex (Fig. 4.2b). Consequently, CWO binds E-boxes of core clock genes (*per*, *tim-d*, *vri*, *Pdp1*) in antiphase to CLK-CYC (Zhou et al. 2016). A recent study by Rivas et al. (2021) uncovered CLOCK INTERACTING PROTEIN CIRCADIAN (CIPC), an ortholog of mouse CIPC, as an additional component of the loop or more precisely a subloop within the CWO feedback (Fig. 4.2a). *Drosophila* CIPC decreases CLK-CYC-mediated transcription; however, the degree of repression is variable: the strongest for *per*, intermediate for *tim-d*, and minimal for *vri*. Flies with *Cipc* silenced by RNAi and *Cipc* null mutant flies produce a short free-running period. At the same time, the expression of *Cipc* is suppressed by CWO. Therefore, in addition to displacing CLK-CYC from tandem E-boxes and suppressing CLK-CYC transcription, CWO also indirectly activates CLK-CYC by removing CIPC repression (Rivas et al. 2021).

4.2.7 Nuclear Receptors E75, HR3, and UNF

The mammalian clock contains a feedback loop with RORα and REV-ERBα, two orphan nuclear receptors cyclically expressed by CLK-BMAL1 (*b*rain and *m*uscle *a*RNT-like *1*, a mammalian homolog of *Drosophila* CYC). Both RORα and REV-ERBα recognize and compete for the same DNA motif RORE, which is localized in the promoter of mammalian *Bmal1* and *Clk* genes. RORα serves as a transcription activator, whereas REV-ERBα suppresses transcription. The role of homologous nuclear receptors was addressed by RNAi in the basal insect *Thermobia domestica* when Kamae et al. (2014) identified E75 (homolog of REV-ERBα) and HR3 (homolog of RORα) as clock components. Both E75 and HR3 are cyclically expressed, and silencing either of them by RNAi influenced the phase of *tim-d*, *cyc*, and *Clk* expression in *Thermobia*.

Jaumouille et al. (2015) systematically analyzed all 18 nuclear receptor genes in *Drosophila* by RNAi in clock neurons. While E75 was identified as an important component of the fruit fly clock, silencing HR3 did not influence the rhythmicity. Interestingly, the silencing of another nuclear receptor, DHR51 (unfulfilled, UNF), was significant for the free-running period. S2 cell and *Drosophila* in vivo

experiments then confirmed that E75 together with UNF coregulate CLK-CYC-mediated transcription of *per* when they bind to *per* regulatory sequences.

4.2.8 *Regulation of the* Drosophila *Clock at the RNA Level*

Circadian rhythmicity is further regulated at the level of posttranscriptional RNA processing, which includes alternative splicing, polyadenylation, mRNA stability, and regulation by microRNAs (for a detailed review, see Lim and Allada 2013a). The first of them, alternative splicing, was first documented for the *per* gene, where the last exon is retained at high temperatures, while splicing is enhanced at low temperatures. Interestingly, this exon is positioned in the 3' untranslated region (3' UTR) of mRNA, and its splicing leads to an advanced phase in *per* mRNA accumulation, which in turn results in advanced evening activity at low temperatures (Majercak et al. 1999).

Alternative splicing was also reported for *tim-d*, where two cold-specific isoforms are upregulated at low temperatures. At high temperatures, another isoform, *tim-d-medium*, is produced. This isoform is characterized by retention of the intron between exons 13 and 14, resulting in a premature stop codon and an unstable, and probably nonfunctional, TIMd-medium protein (Martin Anduaga et al. 2019). Thermosensitive splicing of this *tim-d* intron requires the alternative splicing regulator *P*-element *s*omatic *i*nhibitor (PSI) (Foley et al. 2019) and additional spliceosome factors (Shakhmantsir et al. 2019).

The length of the poly adenine (A) tail heavily influences mRNA stability. The deadenylase POP2 specifically shortens the *tim-d* mRNA poly(A) tail, thus destabilizing the transcript and leading to lower TIMd levels. Interestingly, POP2 activity is inhibited by PER, the partner of TIMd (Grima et al. 2019).

However, another level in RNA metabolism, its translation, is subject to circadian clock regulation. ATAXIN-2 (ATX2), an RNA-binding protein, is necessary for PER accumulation in clock neurons. ATX2 is crucial for the functions of *twenty-four* (TYF), a key activator of PER translation, which is associated with a 5'-cap-binding complex (Lim et al. 2011). TYF and ATX2 interact with polyadenylate-binding protein (PABP) (Lim and Allada 2013b; Zhang et al. 2013b).

4.2.9 *Network Properties of the Clock*

Although the clock mechanism was first accepted as a cell-autonomous oscillatory system, circadian rhythmicity also requires intercellular communication and is, therefore, a result of the circadian network. The first evidence was indicated by the discovery of a neuropeptide *pigment-dispersing factor* (*pdf*) mutant (Renn et al. 1999). Then, specific neurons regulating the morning and evening activity were identified and genetically manipulated (Grima et al. 2004; Stoleru et al. 2004).

The study by Dissel et al. (2014) further emphasized the role of particular groups of different clock cells and their mutual communication. The interaction among different neuronal oscillators (distinct groups of neurons), each characterized by a specific neuropeptide and coupled to other oscillator groups (neurons) in the network, drives the rhythmic activity of flies (Yao and Shafer 2014). The importance of clock groups was shown in calcium imaging experiments when the Ca^{2+} rhythms displayed by a particular group of cells corresponded to the morning or evening locomotor activity peaks (Liang et al. 2016), and the key role of neuropeptide signaling in these intracellular communications was further supported by Ca^{2+} imaging (Liang et al. 2017).

4.2.10 Temperature and the Clock

Temperature is an important and variable factor in the environment that interferes with the functioning of the circadian clock. The clock is only useful for the organism if it keeps ticking at a constant (or near-constant) speed within a physiologically acceptable range of temperatures. This temperature independence, known as temperature compensation, is particularly important for poikilotherms, which are organisms without regulation of body temperature, such as insects. On the other hand, chemical reactions are strongly influenced by temperature (Arrhenius 1889). An explanation of this logical "conflict" could be a combination of reactions, where some reactions have opposite effects on the free-running period than others (Hastings and Sweeney 1957). Thus, the period-extending reactions would, for instance, prolong the free-running period even more at high temperatures. At the same time, this lengthening would be compensated by other reactions that more strongly shorten the free-running period at high temperatures. These opposing reactions could impact the stability of some negative or positive components of the feedback loops and might influence their subcellular localization (nuclear export and import), their interaction with partners, their activity, or perhaps a combination of (all) the abovementioned mechanisms. We cannot exclude the possibility that temperature compensation may be partially affected at the level of interneuronal communication. In this case, temperature compensation would also be a network property of the clock.

Although the molecular mechanism behind temperature compensation remains largely elusive, genetic and biochemical data suggest that phosphorylation of distinct regions of PER protein could be consistent with the model proposed above, even though the details of the mechanism will most likely differ between mammals and *Drosophila* (see Sect. 4.2.2.). Components of the negative feedback loop are important for temperature compensation, as illustrated by mutations specifically interfering with PER-PER homodimerization in *Drosophila* (Landskron et al. 2009) and a subset of TIMd mutations (Matsumoto et al. 1999; Singh et al. 2019; Rutila et al. 1996; see Sect. 4.2.1.).

In addition to single amino acid mutant variants isolated in genetic screens, even certain *Drosophila per* alleles occurring abundantly in nature show altered temperature compensation. *Drosophila* PER contains a series of threonine-glycine (Thr-Gly) repeats that are polymorphic in length. The most common variants consist of 14, 17, 20, or 23 Thr-Gly pairs, and their frequencies in naturally occurring European populations display a clear latitudinal cline (Costa et al. 1992). Furthermore, a detailed analysis involving transgenic flies in which the Thr-Gly repeats and flanking regions were modified revealed the role of this PER in temperature compensation (Sawyer et al. 1997).

Although the free-running period remains constant (compensated) in a certain temperature range, the daily distribution of activity is often affected by temperature. Furthermore, circadian clock genes impact daily activity patterns under light-dark regimes. In *D. melanogaster*, morning and evening activity peaks are separated by midday siesta. At high temperatures, the morning peak is earlier, and the evening peak is delayed, whereas, at low temperatures, the trend is reversed. The temporal regulation of the morning and evening peaks includes alternative splicing of *per* mRNA in its 3' UTR and complex alternative splicing of *tim-d* transcripts (see Sect. 4.2.8; Majercak et al. 1999; Martin Anduaga et al. 2019; Foley et al. 2019).

4.3 Lineage-Specific Variations in the Clock Setup

The remarkable progress in genomics and transcriptomics during the last decade resolved the phylogeny of insects with reasonable precision and dated the separation of all major insect lineages (Misof et al. 2014; Johnson et al. 2018; Kawahara et al. 2019; McKenna et al. 2019; Wipfler et al. 2019). At the same time, these phylogenomics-oriented studies produced a remarkable wealth of data in which clock genes can be identified. In the case of nonmodel insects, the transcriptomes are particularly valuable, as the complete coding sequences are often retrieved and gene paralogs can be reliably defined. Thus, the presence of different circadian clock genes in the insect phylogeny could be usefully mapped and important gene changes and losses identified. Pinpointing gene loss is a nontrivial endeavor. If the gene in question is well conserved across insects (let us say at the level of a protein it encodes) yet is absent in some species, we may claim that we are not able to identify it in a genome of certain insects[3]. However, genome assemblies of nonmodel insects are often fragmented; moreover, some genomes are remarkably large. For example, the genome of the migratory locust is more than twice the size of a human genome (Wang et al. 2014). Therefore, the absence of a gene in an individual species must be interpreted with extreme caution. Nevertheless, if the genomes and transcriptomes of

[3] The automatic gene annotation is prone to artifacts, and therefore careful phylogenetic analysis is often needed to assign a gene/protein to a particular type (especially when multiple paralogs exist, such as in the case of cryptochromes).

all species within a specific monophyletic group (i.e., a group of organisms with a common ancestor) do not contain a particular gene, and if the quality of these genomes/transcriptomes is reasonably good, then the gene loss becomes the most parsimonious explanation.

This part of the chapter aspires to compare the circadian clock across insects using *D. melanogaster* as the reference model. Here, it is important to keep in mind that the depth of our knowledge remarkably varies among different insect groups. For some, only genomic/transcriptomic evidence is available, whereas, in others, the circadian clock was functionally studied by gene silencing using RNAi and, in some species, stable genetic mutations were introduced. Another set of insights includes temporal expression analyses and immunohistochemistry, valuable data that might support mechanistic explanation, even though functional reverse genetic evidence is not available.

We will start with the closest relatives of *D. melanogaster* and gradually focus on the major changes in the circadian clock setup. The key TTFL loops and modification enzymes, such as kinases and phosphatases, depicted in a generalized insect clock model (Fig. 4.2a) are first approached from a phylogenetic perspective in Fig. 4.3, where panel "a" defines major gene losses and changes during insect evolution, whereas panel "b" summarizes the experimental evidence for each loop (using the numbers in circles) in a particular lineage. The description of the circadian clock at the RNA and protein levels is summarized separately in Table 4.1.

The *D. melanogaster* clock setup seems to be, at least according to available descriptive data, conserved in cyclorrhaphan flies, including the housefly *Musca domestica* (Codd et al. 2007; Bazalova and Dolezel 2017), the olive fly (Bertolini et al. 2018), and the Medfly *Ceratitis capitata* (Kotwica-Rolinska et al. 2022a). The *tim-d* mutant of *Chymomyza costata*, a drosophilid fly living in temperate regions, not only confirmed the role of *tim-d* in its molecular oscillator (Kobelkova et al. 2010) but also supported the involvement of the circadian machinery in seasonality (Pavelka et al. 2003; Stehlik et al. 2008).

4.3.1 *Unique Features of the* Drosophila *(Cyclorrhaphan) Clock*

Two or perhaps three major and probably connected features are unique to *Drosophila* and related fly species. First, *Drosophila* CLK contains a transactivation domain at its C-terminus, and a similar pattern is found in cyclorrhaphan Diptera (including *Musca*, the olive fly, and the medfly). Perhaps connected to that, *Clk* mRNA is cyclically expressed in these species, whereas the *cyc* level is constant (Cyran et al. 2003; Codd et al. 2007; Bertolini et al. 2018). BMAL1, the mammalian homolog of *Drosophila* CYC, is characterized by cyclical *bmal1* expression in mice, and the transactivation domain, the so-called the BMAL1 C-terminal region

Table 4.1 Overview of descriptive analyses characterizing circadian clock factors

	CLK BMAL*	PER	TIMd	CRYm	CRYd	TIMm	KIN.	PHOS.	SLMB	PDP1 VRI	CWO	E75 HR3**	PDF
Drosophila	*mRNA* IHC, G4	*mRNA* IHC, G4	*mRNA* IHC, G4	X	*mRNA* IHC, G4	*mRNA*	*mRNA* IHC	*mRNA* IHC	*(mRNA)*	*mRNA* IHC	*mRNA* IHC	*(mRNA)*	*mRNA* IHC, G4
Mosquitoes	*mRNA*	*mRNA* IHC	*mRNA*	*mRNA*	*mRNA*					*mRNA*			IHC
Lepidoptera	IHC	*mRNA* IHC	*mRNA* IHC	*mRNA*	*mRNA* IHC		*mRNA* IHC						IHC
Coleoptera***		IHC			(X)								
Hymenoptera	*mRNA*	*mRNA* IHC	X	*mRNA*	X	*mRNA*							IHC
Aphids	*mRNA*	*mRNA* IHC	*mRNA*	*mRNA*	*mRNA* IHC					*mRNA*			X
Heteroptera***	*mRNA*	*mRNA* IHC	*mRNA* IHC	*mRNA* (IHC)	(X)					*mRNA*			IHC
Orthoptera	*mRNA*	*mRNA* IHC	*mRNA*	*mRNA*	*mRNA* IHC	*mRNA*	IHC			*mRNA*	*mRNA*	*mRNA*	IHC
Blattella		IHC											IHC
Peripl./Rhyparobia		*mRNA*	*mRNA*	*mRNA*	X								IHC
Termites***			(X)		X								IHC
Ephemeroptera		IHC											
Thermobia	*mRNA*	IHC	*mRNA*									*mRNA*	

The table summarizes the description at the level of mRNA (either by in situ hybridization or by addressing the expression level by RNase protection assay, quantitative real-time polymerase reaction, microarray analysis, or GAL4 transgenic reporters, indicated as G4) and protein (IHC, immunohistochemistry) in representative insect groups. This summary is a synthesis of publications cited in the mechanistic descriptions of this chapter and is further supported by studies on *Drosophila* (Houl et al. 2006; Richier et al. 2008), mosquitoes (Baik et al. 2020; Rund et al. 2011), Lepidoptera (Iwai et al. 2008; Sauman et al. 2005; Sehadova et al. 2004; Kobelkova et al. 2015; Zavodska et al. 2012; Zhu et al. 2008), Coleoptera (Frisch et al. 1996), Hymenoptera (Fuchikawa et al. 2017; Beer et al. 2018), aphids (Barbera et al. 2017; Barbera et al. 2022; Colizzi et al. 2021), Heteroptera (Dolezel et al. 2008; Ikeno et al. 2008; Kotwica-Rolinska et al. 2017; Stroppa and Garcia 2019; Vafopoulou and Steel 2012; Koide et al. 2021; Kotwica-Rolinska et al. 2022b), Orthoptera mostly represented by crickets (Sehadova et al. 2003; Shao et al. 2006), *Blattella* (Wen and Lee 2008), *Periplaneta* and *Rhyparobia* (Gestrich et al. 2018), termites (Zavodska et al. 2008), Ephemeroptera (Zavodska et al. 2003a), and *Thermobia* (Zavodska et al. 2003b)

*CYC in *Drosophila*. **In *Drosophila*, UNF participates in this negative feedback loop ***Some circadian clock genes were lost only in a subset of certain orders (*cry-d* in some Coleoptera and Heteroptera, *tim-d* in the majority of termites), and the loss of CRYd is unclear for *Rhyparobia*. For details on clock setups, see Kotwica-Rolinska et al. 2022a)

(BCTR), lies in the C-terminus[4]. The same protein architecture was identified in the BMAL of the silk moth (Chang et al. 2003) and even sand flies (Meireles-Filho et al. 2006), indicating the unique and relatively recent rearrangement of the positive components in the *Drosophila* clock (Fig. 4.3a, c). The role of CLK-BMAL as positive regulators of the circadian machinery was experimentally confirmed by RNAi in Heteroptera, Orthoptera, and *Thermobia* (Kotwica-Rolinska et al. 2022a; Uryu et al. 2013; Moriyama et al. 2012; Kamae et al. 2014) (Fig. 4.3b). In

[4]Some authors use the term CYC in all insect species, whereas others distinguish between BMAL (activation domain is present) and CYC (activation domain has been lost). See also Fig. 4.3c.

Lepidoptera, stable genetic mutants were created for both CLK and BMAL (Markert et al. 2016; Zhang et al. 2017) in addition to the reconstruction of the feedback in cell culture (Chang et al. 2003). Furthermore, the *bmal* transcript is cyclically expressed in numerous (and phylogenetically distant) insects, including sand flies (Meireles-Filho et al. 2006), crickets (Uryu et al. 2013), and *Thermobia* (Kamae et al. 2014).

The second unique feature of the *Drosophila* setup is the absence of mammalian-type CRYPTOCHROME (CRYm), also known as CRY2, CRYII, or mCRY (Kotwica-Rolinska et al. 2022a). In mammals, two paralogous mammalian-type CRYs are key components of the negative TTFL (Kume et al. 1999; van der Horst et al. 1999). A similar transcriptional repressive function was confirmed in cell cultures for CRYm from mosquitoes, Lepidoptera, Coleoptera, and Hymenoptera (Yuan et al. 2007). Functionally, CRYm was identified as a clock component by RNAi in mosquitoes, Heteroptera, crickets, and cockroaches (Meuti et al. 2015; Ikeno et al. 2011a; Tokuoka et al. 2017; Bazalova et al. 2016; Werckenthin et al. 2020). Stable genetic mutants were created in the linden bug *Pyrrhocoris apterus* and the monarch butterfly *Danaus plexippus* (Kotwica-Rolinska et al. 2022a; Zhang et al. 2017). The latter model was instrumental in explaining that monarch CRYm regulates circadian repression of BMAL through the activation domain (AD) via two independent mechanisms (Zhang et al. 2017). The perfect correlation of CRYm loss with the absence of AD in BMAL/CYC of certain dipteran insects implies that once CRYm was lost, probably in an ancestor of Cyclorrhapha, repression of AD on BMAL was no longer used. The activation domain is localized on CLK in insects that have lost CRYm (Thakkar et al. 2022).

The third specificity of *Drosophila* is the involvement of the nuclear receptor UNF (Jaumouille et al. 2015), whereas basal insect *Thermobia* utilizes HR3, a homolog of the well-established mammalian component RORα (Kamae et al. 2014) (see Sect. 4.2.7 also). It is tempting to speculate that UNF recruitment to the circadian machinery might be connected to the shift of the activation domain from BMAL to CLK because the oscillating expression of *Clk* and noncyclical expression of *cyc*, characteristic only for cyclorrhaphan flies, imply evolutionary changes in the promoters of both genes. It will be interesting to see if HR3 and/or UNF participate in the circadian clock of other insect groups.

4.3.2 Losses of CRYd and TIMd

The mammalian clock setup is characterized by the absence of CRYd and TIMd. Therefore, it was a remarkable surprise to see a comparable situation in Hymenoptera when the genome of the honey bee *Apis mellifera* was analyzed (Rubin et al. 2006). A recent detailed inspection confirmed that TIMd and CRYd were lost in all Hymenoptera. Furthermore, TIMd has been lost in the animal kingdom at least three times, in Hymenoptera, vertebrates, and the majority of termites, and these three losses always correlate with the loss of CRYd (Kotwica-Rolinska et al. 2022a; Fig. 4.3a).

However, in several lineages, CRYd has disappeared and TIMd is still present. We can see a particularly nice gradient of gene losses in cockroaches and termites (which are from a phylogenetic point of view a subset of cockroaches): the basal cockroaches (such as *Blattella germanica*) possess the complete toolkit, *Periplaneta* lost CRYd, and termites also lost TIMd (except for just one basal termite species *Porotermes*, where a portion of *tim-d* transcript was found). It will be extremely interesting to see how the light entrainment pathway functions in these three types of clocks detected in cockroaches/termites.

In Orthoptera, a sister group to cockroaches, the role of both *cryptochrome* types was tested by RNAi applied to the cricket *Gryllus bimaculatus*, leading to a new model with a CRYm-CRYd oscillatory loop independent of PER-TIMd (Tokuoka et al. 2017). Furthermore, light input seems to require c-FOS, a bZIP protein participating in the mammalian entrainment pathway (Kornhauser et al. 1990). In the proposed cricket model, the light signal is perceived by the compound eyes, from which the information is transmitted to the clock neurons to stimulate *c-fos* mRNA expression, which is finally relayed on the CRYm-CRYd loop that feeds back on CLK-CYC/BMAL (Kutaragi et al. 2018).

Interestingly, RNAi silencing of *Gryllus tim-d* did not result in behavioral arrhythmicity; instead, the free-running period was significantly shorter (Danbara et al. 2010). A similar short free-running period was observed after *tim-d* silencing in the firebrat *T. domestica* (Kamae and Tomioka 2012). These findings contrast with the fundamental role of *tim-d* in the circadian clock of *Drosophila* but also in Lepidoptera (Nartey et al. 2021). The role of *tim-d* was rigorously addressed in the linden bug *P. apterus* (Heteroptera), an insect that does not have *cry-d* and instead possesses *cry-m* (Bajgar et al. 2013). Genetically created null mutant in *tim-d* showed a free-running period of their locomotor activity shortened by more than 1 h, but the rhythmicity was robust. These *tim-d* phenotypes provided a possible explanation for evolutionary changes in the clock setup within animals in general and insects in particular (Kotwica-Rolinska et al. 2022a): “The dispensability of TIMd in *P. apterus* suggests a scenario of transition between clock architectures relying on distinct components of their negative feedback loops. The proposed model implies that the clock would be functional in each step of the transition from the ancestral state to the PER & CRYm system known today in vertebrates. A similar clock gene combination in Hymenoptera (PER & CRYm) indicates a convergent evolution of the circadian system, although functional evidence from this insect group is not yet available. The circadian clock observed in *P. apterus* could then correspond to an early clock setup that facilitates *tim-d* loss without a complete collapse of circadian cycling. However, the timing and causality of the proposed events might have been lineage-specific, where either the loss of cry-d triggered the transition of TIMd to its modulatory role, or alternatively, the loss of JET or change in TIMd properties compromised its interaction with CRYd, which in turn was subsequently lost.”

4.3.3 JET and FBXL3/21 Proteins

JET, a protein essential for the interaction between CRYd and TIMd in *Drosophila*, which results in their degradation, is found in numerous protostomian lineages but is also independently lost at least six times in insects (Kotwica-Rolinska et al. 2022a). Thus, various combinations of JET with TIMd and CRYd exist in insects (Fig. 4.3c): JET, TIMd, and CRYd (Diptera, Lepidoptera, some Coleoptera, *Blatella*); JET, TIMd, and no CRYd (*Periplaneta*); and JET, no TIMd, and no CRYd (*Cryptotermes*). Similarly, JET was lost, but TIMd and CRYd are present in aphids (Cortés et al. 2010). In *Pyrrhocoris*, JET was lost and only TIMd is present, whereas all these three genes were lost in Hymenoptera.

In mammals, CRYm interacts with FBXL3 and FBXL21, two closely related paralogs also known as *overtime* and *after-hours*, respectively (Godinho et al. 2007; Siepka et al. 2007; Hirano et al. 2013). In protostomes and basal deuterostomes, only the ancestral protein FBXL3 is found. Similar to JET, FBXL3 has been lost multiple times in insects and is present in four lineages: Ephemeroptera, Blattodea (cockroaches + termites), Thysanoptera, and Hymenoptera (Kotwica-Rolinska et al. 2022a). Thus, FBXL3 is not necessary for CRYm function in some systems, such as the circadian clock of Lepidoptera or *Pyrrhocoris*. Nonetheless, it will be very interesting to see whether FBXL3 participates in CRYm regulation in Hymenoptera, a group of insects with a clock setup remarkably similar to that of mammals.

4.3.4 Conserved Components of Insect Clocks

PER, an iconic circadian clock protein found in all insects, seems to participate in the rhythmicity in all tested species, albeit to a different extent. It is absolutely necessary for *Drosophila*, but in *Pyrrhocoris*, approximately one-third of genetic mutants are still robustly rhythmic (Kotwica-Rolinska et al. 2022a). Similarly, RNAi silencing of *per* in the Madeira cockroach resulted in only a partial phenotype (Werckenthin et al. 2020). It is possible that in the abovementioned (and many other) insect species, CRYm serves as the most important negative element, whereas the PER contribution to the negative feedback is smaller than that in *Drosophila*. However, depletion of PER in the silk moth *Bombyx mori* leads to arrhythmicity (Ikeda et al. 2019), even though the lepidopteran clock relies on CRYm (Zhang et al. 2017).

The role of bZIP proteins VRI and PDP1 was tested in *Pyrrhocoris*, where *vri* silencing nonsignificantly shortened the free-running period, while *Pdp1* knockdown resulted in arrhythmicity (Kotwica-Rolinska et al. 2022a). In crickets, however, cosilencing of *cyc* was necessary to obtain *vri*- and *Pdp1*-dependent changes in the free-running period (Narasaki-Funo et al. 2020). In the northern house mosquito *Culex pipiens*, both *vri* and *Pdp1* were tested for their role in diapause, and their cyclical expression was confirmed (Chang and Meuti 2020).

CWO, a bHLH protein whose depletion slows down the clock in *Drosophila*, was silenced with a comparable 1-h extension of periodicity in *Pyrrhocoris* (Kotwica-Rolinska et al. 2022a) and crickets (Tomiyama et al. 2020).

The role of components working at the protein level, i.e., kinases and F-box proteins, was minimally tested outside *Drosophila*. Silencing *dbt* robustly extended the free-running period in *Pyrrhocoris*, and a less extreme yet remarkable phenotype was observed after *slibm* knockdown, whereas *nmo* knockdown marginally sped up the clock (Kotwica-Rolinska et al. 2022a).

4.3.5 Descriptive Studies

The majority of the mechanisms described in this chapter were elaborated in *D. melanogaster* and further challenged and expanded in several insect models. Although an occasional spontaneous mutation or a variant is mapped in a nonmodel organism, these examples are relatively rare for chronobiology outside of *D. melanogaster* (Pavelka et al. 2003; Kozak et al. 2019). Therefore, reverse genetic tools have been key for the analysis of nonmodel organisms. One of the most powerful tools is RNAi, which, if spread in the organism systemically, can be a cost-effective and fast approach for chronobiological studies. However, the target transcript might only be partially reduced; thus, the data interpretation becomes, at least in some specific cases, nontrivial. Therefore, stable genetic modifications are more attractive from an interpretation point of view, but these techniques are also quite laborious and time demanding. Nevertheless, some insect models have become accessible to stable modification and are successfully used in chronobiology (Markert et al. 2016; Zhang et al. 2017; Kotwica-Rolinska et al. 2019; Ikeda et al. 2019).

In some groups, however, no reverse genetics data are available. However, many of these insect species are extremely interesting for their biology and even for their role as agricultural pests or for their ability to transmit pathogens. Thus, even descriptive evidence might provide important and valuable hints about the molecular mechanism behind their circadian clocks. Therefore, Table 4.1 briefly summarizes the evidence obtained at the RNA and protein levels across selected insect groups. For the anatomy of the insect circadian clock, see Chap. 5.

4.4 Conclusion and Future Perspective

The genetic architecture of the above-described circadian clocks observed in various insect lineages invites us to speculate why and how this diversity has originated. Insects are a large group with more than 400 million years of evolution (Misof et al. 2014). Some lineages, such as aphids, underwent noteworthy gene expansion, resulting in >35 thousand genes, which is remarkably more than the gene count in

D. melanogaster (~13.5 thousand) or even in the body louse *Pediculus humanus* (~11 thousand) (Thomas et al. 2020).

A possible mechanistic explanation for the various clock setups might be provided by mammalian research when Pett et al. (2018) showed that the importance of individual feedback loops differs in a tissue-specific manner. In mammals, this mechanistic flexibility may account for organ-specific differences in clock gene expression and allow for the hierarchical organization of the mammalian clock. A somewhat similar tissue-specific clock architecture exists in *D. melanogaster*, where CRYd serves as a light photoreceptor in clock neurons (Emery et al. 2000a), whereas CRYd is a CLK/CYC repressor in the periphery (Collins et al. 2006). Indeed, the role in the peripheral clock allowed the identification of *cry-d*^{b} mutation in a luciferase reporter-based screen (Stanewsky et al. 1998).

In insects, we can assume that similar flexibility in clock setup allows for gene loss, which has some impact on rhythmicity, albeit not detrimental. Indeed, this is the case for the *tim-d* gene in *P. apterus* and probably other insect species, where TIMd participates only as a modulator of the free-running period (Kotwica-Rolinska et al. 2022a; Danbara et al. 2010). Thus, modifications of a certain loop may, for example, impact the light entrainment capacities of the system, whereas the clock remains either fully functional or with only a mild impact on its properties. Whether the combination of these changes is beneficial or disadvantageous for the particular organism depends on the life strategy in a specific environment. For example, organisms living in high geographical latitudes face multiple challenges, including extreme photoperiods (day-to-night ratio). Under such conditions, the less light-sensitive clock might be an advantage.

Another conceivable selection pressure might be the role of circadian clock genes in different time-measuring systems than the circadian clock itself. The possible role of (at least a subset of) clock genes was suggested in photoperiodic timing (Ikeno et al. 2010, 2011b, 2013; Kotwica-Rolinska et al. 2017, 2022b; Sakamoto et al. 2009) but not for the tidal and lunar rhythms (Takekata et al. 2014; Zhang et al. 2013a). Therefore, components recruited by a different time-measuring system (s) might be, in addition to their role in the circadian clock, under selection pressure connected to the properties of the noncircadian system.

Taken together, during the last two decades, we could see remarkable progress in understanding the circadian clock mechanism at the molecular level. Research on *D. melanogaster* has been dominating insect molecular chronobiology for decades, and the fruit fly will always be an excellent model with unparalleled tools and opportunities. However, emerging reverse genetic tools available to nonmodel organisms will facilitate research on genetically interesting clock setups and chronobiological, ecological, and physiological phenomena unavailable, or weakly expressed, in *D. melanogaster*.

References

Agrawal P, Hardin PE (2016) An RNAi screen to identify protein phosphatases that function within the *Drosophila* circadian clock. G3 6:4227–4238. https://doi.org/10.1534/g3.116.035345

Allada R, White NE, So WV, Hall J, Rosbash M (1998) A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell 93:791–804. https://doi.org/10.1016/S0092-8674(00)81440-3

Arrhenius S (1889) Über die reaktionsgeschwindigkeit bei der inversion von Rohrzucker durch Saeuren. Z Phys Chem 4:226–248. https://doi.org/10.1515/zpch-1889-0416

Ashmore LJ, Sathyanarayanan S, Silvestre DW, Emerson MM, Schotland P, Sehgal A (2003) Novel insights into the regulation of the timeless protein. J Neurosci 23:7810–7819. https://doi.org/10.1523/jneurosci.23-21-07810.2003

Baik LS, Nave C, Au DD, Guda T, Chevez JA, Ray A et al (2020) Circadian regulation of light-evoked attraction and avoidance behaviors in daytime- versus nighttime-biting mosquitoes. Curr Biol 30:3252–3259. https://doi.org/10.1016/j.cub.2020.06.010

Bajgar A, Jindra M, Dolezel D (2013) Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc Natl Acad Sci USA 110:4416–4421. https://doi.org/10.1073/pnas.1217060110

Barbera M, Collantes-Alegre JM, Martinez-Torres D (2017) Characterisation, analysis of expression and localisation of circadian clock genes from the perspective of photoperiodism in the aphid *Acyrthosiphon pisum*. Insect Biochem Mol Biol 83:54–67. https://doi.org/10.1016/j.ibmb.2017.02.006

Barbera M, Collantes-Alegre JM, Martinez-Torres D (2022) Mapping and quantification of cryptochrome expression in the brain of the pea aphid *Acyrthosiphon pisum*. Insect Mol Biol 31:159–169. https://doi.org/10.1111/imb.12747

Bargiello TA, Jackson FR, Young MW (1984) Restoration of circadian behavioral rhythms by gene transfer in *Drosophila*. Nature 312:752–754. https://doi.org/10.1038/312752a0

Barnes JW, Tischkau SA, Barnes JA, Mitchell JW, Burgoon PW, Hickok JR et al (2003) Requirement of mammalian *timeless* for circadian rhythmicity. Science 302:439–442. https://doi.org/10.1126/science.1086593

Bazalova O, Dolezel D (2017) Daily activity of the housefly, *Musca domestica*, is influenced by temperature independent of 3′ UTR *period* gene splicing. G3 7:2637–2649. https://doi.org/10.1534/g3.117.042374

Bazalova O, Kvicalova M, Valkova T, Slaby P, Bartos P, Netusil R et al (2016) Cryptochrome 2 mediates directional magnetoreception in cockroaches. Proc Natl Acad Sci USA 113:1660–1665. https://doi.org/10.1073/pnas.1518622113

Beer K, Kolbe E, Kahana NB, Yayon N, Weiss R, Menegazzi P et al (2018) Pigment-dispersing factor-expressing neurons convey circadian information in the honey bee brain. Open Biol 8:170224. https://doi.org/10.1098/rsob.170224

Benna C, Bonaccorsi S, Wülbeck C, Helfrich-Förster C, Gatti M, Kyriacou CP et al (2010) *Drosophila timeless2* is required for chromosome stability and circadian photoreception. Curr Biol 20:346–352. https://doi.org/10.1016/j.cub.2009.12.048

Benna C, Scannapieco P, Piccin A, Sandrelli F, Zordan F, Rosato E et al (2000) A second *timeless* gene in *Drosophila* shares greater sequence similarity with mammalian *tim*. Curr Biol 10:R512–R513. https://doi.org/10.1016/s0960-9822(00)00594-7

Bertolini E, Kistenpfennig C, Menegazzi P, Keller A, Koukidou M, Helfrich-Förster C (2018) The characterization of the circadian clock in the olive fly *Bactrocera oleae* (Diptera:Tephritidae) reveals a *Drosophila*-like organization. Sci Rep 8:816. https://doi.org/10.1038/s41598-018-19255-8

Blau J, Young MW (1999) Cycling *vrille* expression is required for a functional *Drosophila* clock. Cell 99:661–671. https://doi.org/10.1016/S0092-8674(00)81554-8

Brandes C, Plautz JD, Stanewsky R, Jamison CF, Straume M, Wood KV et al (1996) Novel features of *Drosophila period* transcription revealed by real-time luciferase reporting. Neuron 16:687–692. https://doi.org/10.1016/S0896-6273(00)80088-4

Busza A, Emery-Le M, Rosbash M, Emery P (2004) Roles of the two *Drosophila* CRYPTOCHROME structural domains in circadian photoreception. Science 304:1503–1506. https://doi.org/10.1126/science.1096973

Cai YD, Chiu JC (2021) Timeless in animal circadian clocks and beyond. FEBS J 289:6559. https://doi.org/10.1111/febs.16253

Cai YD, Xue Y, Truong CC, Del Carmen-Li J, Ochoa C, Vanselow JT et al (2021) CK2 inhibits TIMELESS nuclear export and modulates CLOCK transcriptional activity to regulate circadian rhythms. Curr Biol 31(502-514):e507. https://doi.org/10.1016/j.cub.2020.10.061

Ceriani MF, Darlington TK, Staknis D, Mas P, Petti AA, Weitz CJ et al (1999) Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science 285:553–556. https://doi.org/10.1126/science.285.5427.553

Chang DC, McWatters HG, Williams JA (2003) Constructing a feedback loop with circadian clock molecules from the silkmoth, *Antheraea pernyi*. J Biol Chem 278:38149–38158. https://doi.org/10.1074/jbc.M306937200

Chang V, Meuti ME (2020) Circadian transcription factors differentially regulate features of the adult overwintering diapause in the Northern house mosquito, *Culex pipiens*. Insect Biochem Mol Biol 121:103365. https://doi.org/10.1016/j.ibmb.2020.103365

Chiu JC, Ko HW, Edery I (2011) NEMO/NLK phosphorylates PERIOD to initiate a time delay phosphorylation circuit that sets circadian clock speed. Cell 145:357–370. https://doi.org/10.1016/j.cell.2011.04.002

Chiu JC, Vanselow JT, Kramer A, Edery I (2008) The phospho-occupancy of an atypical SLIMB-binding site on PERIOD that is phosphorylated by DOUBLETIME controls the pace of the clock. Genes Dev 22:1758–1772. https://doi.org/10.1101/gad.1682708

Codd V, Dolezel D, Stehlik J, Piccin A, Garner KJ, Racey SN et al (2007) Circadian rhythm gene regulation in the housefly *Musca domestica*. Genetics 177:1539–1751. https://doi.org/10.1534/genetics.107.079160

Colizzi FS, Beer K, Cuti P, Deppisch P, Martinez Torres D, Yoshii T et al (2021) Antibodies against the clock proteins period and cryptochrome reveal the neuronal organization of the circadian clock in the pea aphid. Front Physiol 12:705048. https://doi.org/10.3389/fphys.2021.705048

Collins B, Mazzoni EO, Stanewsky R, Blau J (2006) *Drosophila* CRYPTOCHROME is a circadian transcriptional repressor. Curr Biol 16:441–449. https://doi.org/10.1016/j.cub.2006.01.034

Cortés T, Ortiz-Rivas B, Martínez-Torres D (2010) Identification and characterization of circadian clock genes in the pea aphid *Acyrthosiphon pisum*. Insect Mol Biol 19:123–139. https://doi.org/10.1111/j.1365-2583.2009.00931.x

Costa R, Peixoto AA, Barbujani G, Kyriacou CP (1992) A latitudinal cline in a *Drosophila* clock gene. Proc Biol Sci 250:43–49. https://doi.org/10.1098/rspb.1992.0128

Cyran SA, Buchsbaum AM, Reddy KL, Lin MC, Glossop NRJ, Hardin PE et al (2003) *vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. Cell 112:329–341. https://doi.org/10.1016/S0092-8674(03)00074-6

Danbara Y, Sakamoto T, Uryu O, Tomioka K (2010) RNA interference of *timeless* gene does not disrupt circadian locomotor rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 56:1738–1745. https://doi.org/10.1016/j.jinsphys.2010.07.002

Darlington TK, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N, Steeves TDL et al (1998) Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. Science 280:1599–1603. https://doi.org/10.1126/science.280.5369.1599

Deppisch P, Prutscher JM, Pegoraro M, Tauber E, Wegener C, Helfrich-Forster C (2022) Adaptation of *Drosophila melanogaster* to long photoperiods of high-latitude summers is facilitated by the *ls-timeless* allele. J Biol Rhythms 37:185–201. https://doi.org/10.1177/07487304221082448

Dissel S, Codd V, Fedic R, Garner KJ, Costa R, Kyriacou CP et al (2004) A constitutively active cryptochrome in *Drosophila melanogaster*. Nat Neurosci 7:834–840. https://doi.org/10.1038/nn1285

Dissel S, Hansen CN, Ozkaya O, Hemsley M, Kyriacou CP, Rosato E (2014) The logic of circadian organization in *Drosophila*. Curr Biol 24:2257–2266. https://doi.org/10.1016/j.cub.2014.08.023

Dolezel D, Zdechovanova L, Sauman I, Hodkova M (2008) Endocrine-dependent expression of circadian clock genes in insects. Cell Mol Life Sci 65:964–969. https://doi.org/10.1007/s00018-008-7506-7

Dolezelova E, Dolezel D, Hall JC (2007) Rhythm defects caused by newly engineered null mutations in *Drosophila*'s *cryptochrome* gene. Genetics 177:329–345. https://doi.org/10.1534/genetics.107.076513

Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271–290. https://doi.org/10.1016/s0092-8674(00)80566-8

Dusik V, Senthilan PR, Mentzel B, Hartlieb H, Wulbeck C, Yoshii T et al (2014) The MAP kinase p38 is part of *Drosophila melanogaster's* circadian clock. PLoS Genet 10:e1004565. https://doi.org/10.1371/journal.pgen.1004565

Emery P, So WV, Kaneko M, Hall JC, Rosbash M (1998) CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell 95:669–679. https://doi.org/10.1016/S0092-8674(00)81637-2

Emery P, Stanewsky R, Hall JC, Rosbash M (2000b) *Drosophila* cryptochromes - a unique circadian-rhythm photoreceptor. Nature 404:456–457. https://doi.org/10.1038/35006558

Emery P, Stanewsky R, Helfrich-Forster C, Emery-Le M, Hall JC, Rosbash M (2000a) *Drosophila* CRY is a deep brain circadian photoreceptor. Neuron 26:493–504. https://doi.org/10.1016/S0896-6273(00)81181-2

Fang Y, Sathyanarayanan S, Sehgal A (2007) Post-translational regulation of the *Drosophila* circadian clock requires protein phosphatase 1 (PP1). Genes Dev 21:1506–1518. https://doi.org/10.1101/gad.1541607

Foley LE, Ling J, Joshi R, Evantal N, Kadener S, Emery P (2019) *Drosophila* PSI controls circadian period and the phase of circadian behavior under temperature cycle via *tim* splicing. eLife 8:e50063. https://doi.org/10.7554/eLife.50063

Frisch B, Fleissner G, Fleissner G, Brandes C, Hall JC (1996) Staining in the brain of *Pachymorpha sexguttata* mediated by an antibody against a *Drosophila* clock gene product: Labeling of cells with possible importance for the beetle's circadian rhythms. Cell Tissue Res 286:411–429. https://doi.org/10.1007/s004410050711

Fuchikawa T, Beer K, Linke-Winnebeck C, Ben-David R, Kotowoy A, Tsang VWK et al (2017) Neuronal circadian clock protein oscillations are similar in behaviourally rhythmic forager honeybees and in arrhythmic nurses. Open Biol 7:170047. https://doi.org/10.1098/rsob.170047

Garbe DS, Fang Y, Zheng XZ, Sowcik M, Anjum R, Gygi SP et al (2013) Cooperative interaction between phosphorylation sites on PERIOD maintains circadian period in *Drosophila*. PLoS Genetics 9:e1003749. https://doi.org/10.1371/journal.pgen.1003749

Gestrich J, Giese M, Shen W, Zhang Y, Voss A, Popov C et al (2018) Sensitivity to Pigment-Dispersing Factor (PDF) is cell-type specific among PDF-expressing circadian clock neurons in the Madeira cockroach. J Biol Rhythms 33:35–51. https://doi.org/10.1177/0748730417739471

Glossop NR, Lyons LC, Hardin PE (1999) Interlocked feedback loops within the *Drosophila* circadian oscillator. Science 286:766–768. https://doi.org/10.1126/science.286.5440.766

Godinho SI, Maywood ES, Shaw L, Tucci V, Barnard AR, Busino L et al (2007) The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. Science 316:897–900. https://doi.org/10.1126/science.1141138

Grima B, Chelot E, Xia R, Rouyer F (2004) Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. Nature 431:869–873. https://doi.org/10.1038/nature02935

Grima B, Dognon A, Lamouroux A, Chelot E, Rouyer F (2012) CULLIN-3 controls TIMELESS oscillations in the *Drosophila* circadian clock. PLoS Biol 10:e1001367. https://doi.org/10.1371/journal.pbio.1001367

Grima B, Lamouroux A, Chelot E, Papin C, Limbourg-Bouchon B, Rouyer F (2002) The F-box protein slimb controls the levels of clock proteins period and timeless. Nature 420:178–182. https://doi.org/10.1038/nature01122

Grima B, Papin C, Martin B, Chelot E, Ponien P, Jacquet E et al (2019) PERIOD-controlled deadenylation of the *timeless* transcript in the *Drosophila* circadian clock. Proc Natl Acad Sci U S A 116:5721–5726. https://doi.org/10.1073/pnas.1814418116

Hall JC (2003) Genetics and molecular biology of rhythms in *Drosophila* and other insects. Adv Genet 48:1–280. https://doi.org/10.1016/s0065-2660(03)48000-0

Hamblen MJ, White NE, Emery PTJ, Kaiser K, Hall JC (1998) Molecular and behavioral analysis of four *period* mutants in *Drosophila melanogaster* encompassing extreme short, novel long, and unorthodox arrhythmic types. Genetics 149:165–178. https://doi.org/10.1093/genetics/149.1.165

Hara T, Koh K, Combs DJ, Sehgal A (2011) Post-translational regulation and nuclear entry of TIMELESS and PERIOD are affected in new *timeless* mutant. J Neurosci 31:9982–9990. https://doi.org/10.1523/JNEUROSCI.0993-11.2011

Hardin PE (2011) Molecular genetic analysis of circadian timekeeping in *Drosophila*. Adv Genet 48(74):141–173. https://doi.org/10.1016/B978-0-12-387690-4.00005-2

Hardin PE, Hall JC, Rosbash M (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature 343:536–540. https://doi.org/10.1038/343536a0

Hastings JW, Sweeney BM (1957) On the mechanism of temperature independence in a biological clock. Proc Natl Acad Sci U S A 43:804–811. https://doi.org/10.1073/pnas.43.9.804

Hirano A, Yumimoto K, Tsunematsu R, Matsumoto M, Oyama M, Kozuka-Hata H et al (2013) FBXL21 regulates oscillation of the circadian clock through ubiquitination and stabilization of cryptochromes. Cell 152:1106–1118. https://doi.org/10.1016/j.cell.2013.01.054

Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M et al (2002) *Dec1* and *Dec2* are regulators of the mammalian molecular clock. Nature 419:841–844. https://doi.org/10.1038/nature01123

van der Horst GT, Muijtjens M, Kobayashi K, Takano R, Kanno S, Takao M et al (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627–630. https://doi.org/10.1038/19323

Houl JH, Yu W, Dudek SM, Hardin PE (2006) *Drosophila* CLOCK is constitutively expressed in circadian oscillator and non-oscillator cells. J Biol Rhythms 21:93–103. https://doi.org/10.1177/0748730405283697

Hunter-Ensor M, Ousley A, Sehgal A (1996) Regulation of the *Drosophila* protein timeless suggests a mechanism for resetting the circadian clock by light. Cell 84:677–685. https://doi.org/10.1016/s0092-8674(00)81046-6

Ikeda K, Daimon T, Sezutsu H, Udaka H, Numata H (2019) Involvement of the clock gene *period* in the circadian rhythm of the silkmoth *Bombyx mori*. J Biol Rhythms 34:283–292. https://doi.org/10.1177/0748730419841185

Ikeno T, Ishikawa K, Numata H, Goto SG (2013) Circadian clock gene *Clock* is involved in the photoperiodic response of the bean bug *Riptortus pedestris*. Physiol Entomol 38:157–162. https://doi.org/10.1111/phen.12013

Ikeno T, Katagiri C, Numata H, Goto SG (2011a) Causal involvement of mammalian-type *cryptochrome* in the circadian cuticle deposition rhythm in the bean bug *Riptortus pedestris*. Insect Mol Biol 20:409–415. https://doi.org/10.1111/j.1365-2583.2011.01075.x

Ikeno T, Numata H, Goto SG (2008) Molecular characterization of the circadian clock genes in the bean bug, *Riptortus pedestris*, and their expression patterns under long- and short-day conditions. Gene 419:56–61. https://doi.org/10.1016/j.gene.2008.05.002

Ikeno T, Numata H, Goto SG (2011b) Photoperiodic response requires mammalian-type *cryptochrome* in the bean bug *Riptortus pedestris*. Biochem Biophys Res Commun 410:394–397. https://doi.org/10.1016/j.bbrc.2011.05.142

Ikeno T, Tanaka SI, Numata H, Goto SG (2010) Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biol 8:116. https://doi.org/10.1186/1741-7007-8-116

Iwai S, Trang LTD, Sehadova H, Takeda M (2008) Expression analyses of *casein kinase 2α* and *casein kinase 2β* in the silkmoth, *Bombyx mori*. Comp Biochem Physiol B 149:38–46. https://doi.org/10.1016/j.cbpb.2007.08.004

Jang AR, Moravcevic K, Saez L, Young MW, Sehgal A (2015) *Drosophila* TIM binds importin α1, and acts as an adapter to transport PER to the nucleus. PLoS Genet 11:e1005205. https://doi.org/10.1371/journal.pgen.1004974

Jaumouille E, Machado Almeida P, Stahli P, Koch R, Nagoshi E (2015) Transcriptional regulation via nuclear receptor crosstalk required for the *Drosophila* circadian clock. Curr Biol 25:1502–1508. https://doi.org/10.1016/j.cub.2015.04.017

Johnson KP, Dietrich CH, Friedrich F, Beutel RG, Wipfler B, Peters RS et al (2018) Phylogenomics and the evolution of hemipteroid insects. Proc Natl Acad Sci U S A 115:12775–12780. https://doi.org/10.1073/pnas.1815820115

Johnsson A, Helfrich-Förster C, Engelmann W (2014) How light resets circadian clocks. In: Björn L (ed) Photobiology. Springer, New York, pp 243–297. https://doi.org/10.1007/978-1-4939-1468-5_18

Joshi R, Cai YD, Xia Y, Chiu J, Emery P (2022) PERIOD phosphoclusters control temperature compensation of the *Drosophila* circadian clock. Front Physiol 13:888262. https://doi.org/10.1101/2021.12.23.474078

Kadener S, Stoleru D, McDonald M et al (2007) *Clockwork Orange* is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. Genes Dev 21:1675–1686. https://doi.org/10.1101/Gad.1552607

Kamae Y, Tomioka K (2012) *timeless* is an essential component of the circadian clock in a primitive insect, the firebrat *Thermobia domestica*. J Biol Rhythms 27:126–134. https://doi.org/10.1177/0748730411435997

Kamae Y, Uryu O, Miki T, Tomioka K (2014) The nuclear receptor genes HR3 and E75 are required for the circadian rhythm in a primitive insect. PLoS One 9:e114899. https://doi.org/10.1371/journal.pone.0114899

Kawahara AY, Plotkin D, Espeland M, Meusemann K, Toussaint EFA, Donath A et al (2019) Phylogenomics reveals the evolutionary timing and pattern of butterflies and moths. Proc Natl Acad Sci U S A 116:22657–22663. https://doi.org/10.1073/pnas.1907847116

Kim EY, Edery I (2006) Balance between DBT/CKIε kinase and protein phosphatase activities regulate phosphorylation and stability of *Drosophila* CLOCK protein. Proc Natl Acad Sci U S A 103:6178–6183. https://doi.org/10.1073/pnas.0511215103

Kivimaë S, Saez L, Young MW (2008) Activating PER repressor through a DBT-directed phosphorylation switch. PLoS Biol 6:1570–1583. https://doi.org/10.1371/journal.pbio.0060183

Kloss B, Price JL, Saez L (1998) The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iε. Cell 94:97–107. https://doi.org/10.1016/S0092-8674(00)81225-8

Kloss B, Rothenfluh A, Young MW, Saez L (2001) Phosphorylation of PERIOD is influenced by cycling physical associations of DOUBLE-TIME, PERIOD, and TIMELES in the *Drosophila* clock. Neuron 30:699–706. https://doi.org/10.1016/S0896-6273(01)00320-8

Ko HW, Jiang J, Edery I (2002) A role for Slimb in the degradation of *Drosophila* PERIOD protein phosphorylated by DOUBLETIME. Nature 420:673–678. https://doi.org/10.1038/Nature01272

Kobelkova A, Bajgar A, Dolezel D (2010) Functional molecular analysis of a circadian clock gene *timeless* promoter from the drosophilid fly *Chymomyza costata*. J Biol Rhythms 25:399–409. https://doi.org/10.1177/0748730410385283

Kobelkova A, Zavodska R, Sauman I, Bazalova O, Dolezel D (2015) Expression of clock genes *period* and *timeless* in the central nervous system of the Mediterranean flour moth, *Ephestia kuehniella*. J Biol Rhythms 30:104–116. https://doi.org/10.1177/0748730414568430

Koh K, Zheng X, Sehgal A (2006) JETLAG resets the *Drosophila* circadian clock by promoting light-induced degradation of TIMELESS. Science 312:1809–1812. https://doi.org/10.1126/science.1124951

Koide R, Xi J, Hamanaka Y, Shiga S (2021) Mapping PERIOD-immunoreactive cells with neurons relevant to photoperiodic response in the bean bug *Riptortus pedestris*. Cell Tissue Res 385: 571–583. https://doi.org/10.1007/s00441-021-03451-6

Konopka RJ, Benzer S (1971) Clock mutants of *Drosophila melanogaster*. Proc Nat Acad Sci U S A 68:2112–2116. https://doi.org/10.1073/pnas.68.9.2112

Konopka RJ, Hamblencoyle MJ, Jamison CF, Hall JC (1994) An ultrashort clock mutation at the *period* locus of *Drosophila melanogaster* that reveals some new features of the fly's circadian system. J Biol Rhythms 9:189–216. https://doi.org/10.1177/074873049400900303

Konopka RJ, Pittendrigh C, Orr D (1989) Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. J Neurogenet 6:1–10. https://doi.org/10.3109/01677068909107096

Kornhauser JM, Nelson DE, Mayo KE, Takahashi JS (1990) Photic and circadian regulation of *c-fos* gene expression in hamster suprachiasmatic nucleus. Neuron 5:127–134. https://doi.org/10.1016/0896-6273(90)90303-W

Kotwica-Rolinska J, Chodáková L, Chvalova D, Pauchova L, Provaznik J, Hejnikova M et al (2019) CRISPR/Cas9 genome editing introduction and optimization in the non-model insect *Pyrrhocoris apterus*. Front Physiol 10:891. https://doi.org/10.3389/fphys.2019.00891

Kotwica-Rolinska J, Chodáková L, Smykal V, Damulewicz M, Provaznik J, Wu BC et al (2022a) Loss of *timeless* underlies an evolutionary transition within the circadian clock. Mol Biol Evol 39:msab346. https://doi.org/10.1093/molbev/msab346

Kotwica-Rolinska J, Damulewicz M, Chodáková L, Krištofová L, Dolezel D (2022b) Pigment Dispersing Factor is a circadian clock output and regulates photoperiodic response in the linden bug, *Pyrrhocoris apterus*. Front Physiol 13:884909. https://doi.org/10.3389/fphys.2022.884909

Kotwica-Rolinska J, Pivarciova L, Vaneckova DD (2017) The role of circadian clock genes in the photoperiodic timer of the linden bug *Pyrrhocoris apterus* during the nymphal stage. Physiol Entomol 42:266–273. https://doi.org/10.1111/phen.12197

Kozak GM, Wadsworth CB, Kahne SC, Bogdanowicz SM, Harrison RG, Coates BS et al (2019) Genomic basis of circannual rhythm in the European corn borer moth. Curr Biol 29:3501–3509. https://doi.org/10.1016/j.cub.2019.08.053

Kula-Eversole E, Lee DH, Samba I, Yildirim E, Levine DC, Hong HK et al (2021) Phosphatase of regenerating Liver-1 selectively times circadian behavior in darkness via function in PDF neurons and dephosphorylation of TIMELESS. Curr Biol 31:138–149. https://doi.org/10.1016/j.cub.2020.10.013

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin XW et al (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell 98:193–205. https://doi.org/10.1016/S0092-8674(00)81014-4

Kurien P, Hsu PK, Leon J, Wu D, McMahon T, Shi G et al (2019) TIMELESS mutation alters phase responsiveness and causes advanced sleep phase. Proc Natl Acad Sci U S A 116:12045–12053. https://doi.org/10.1073/pnas.1819110116

Kutaragi Y, Tokuoka A, Tomiyama Y, Nose M, Watanabe T, Bando T et al (2018) A novel photic entrainment mechanism for the circadian clock in an insect: involvement of *c-fos* and *cryptochromes*. Zool Lett 4:26. https://doi.org/10.1186/s40851-018-0109-8

Lam VH, Li YH, Liu X, Murphy KA, Diehl JS, Kwok RS et al (2018) CK1α collaborates with DOUBLETIME to regulate PERIOD function in the *Drosophila* circadian clock. J Neurosci 38: 10631–10643. https://doi.org/10.1523/JNEUROSCI.0871-18.2018

Lamaze A, Chen C, Leleux S, Xu M, George R, Stanewsky R (2022) A natural *timeless* polymorphism allowing circadian clock synchronization in "white nights". Nat Commun 13:1724. https://doi.org/10.1038/s41467-022-29293-6

Lamaze A, Lamouroux A, Vias C, Hung HC, Weber F, Rouyer F (2011) The E3 ubiquitin ligase CTRIP controls CLOCK levels and PERIOD oscillations in *Drosophila*. EMBO Rep 12:549–557. https://doi.org/10.1038/embor.2011.64

Landskron J, Chen KF, Wolf E, Stanewsky R (2009) A role for the PERIOD:PERIOD homodimer in the *Drosophila* circadian clock. PLoS Biol 7:820–835. https://doi.org/10.1371/journal.pbio.1000003

Lee C, Bae K, Edery I (1998) The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER-TIM complex. Neuron 21:857–867. https://doi.org/10.1016/S0896-6273(00)80601-7

Li YH, Liu X, Vanselow JT, Zheng H, Schlosser A, Chiu JC (2019) O-GlcNAcylation of PERIOD regulates its interaction with CLOCK and timing of circadian transcriptional repression. PLoS Genet 15:1007953. https://doi.org/10.1371/journal.pgen.1007953

Liang X, Holy TE, Taghert PH (2016) Synchronous *Drosophila* circadian pacemakers display nonsynchronous Ca^{2+} rhythms *in vivo*. Science 351:976–981. https://doi.org/10.1126/science.aad3997

Liang X, Holy TE, Taghert PH (2017) A series of suppressive signals within the *Drosophila* circadian neural circuit generates sequential daily outputs. Neuron 94:1173–1189. https://doi.org/10.1016/j.neuron.2017.05.007

Lim C, Allada R (2013a) Emerging roles for post-transcriptional regulation in circadian clocks. Nature Neurosci 16:1544–1550. https://doi.org/10.1038/nn.3543

Lim C, Allada R (2013b) ATAXIN-2 activates PERIOD translation to sustain circadian rhythms in *Drosophila*. Science 340:875–879. https://doi.org/10.1126/science.1234785

Lim C, Lee J, Choi C, Kilman VL, Kim J, Park SM et al (2011) The novel gene twenty-four defines a critical translational step in the *Drosophila* clock. Nature 470:399–403. https://doi.org/10.1038/nature09728

Ling J, Dubruille R, Emery P (2012) KAYAK-α modulates circadian transcriptional feedback loops in *Drosophila* pacemaker neurons. J Neurosci 32:16959–16970. https://doi.org/10.1523/JNEUROSCI.1888-12.2012

Liu X, Blazenovic I, Contreras AJ, Pham TM, Tabuloc CA, Li YH et al (2021) Hexosamine biosynthetic pathway and O-GlcNAc-processing enzymes regulate daily rhythms in protein O-GlcNAcylation. Nat Commun 12:4173. https://doi.org/10.1038/s41467-021-24301-7

Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR et al (2000) Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science 288:483–491. https://doi.org/10.1126/science.288.5465.483

Majercak J, Sidote D, Hardin PE, Edery I (1999) How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron 24:219–230. https://doi.org/10.1016/S0896-6273(00)80834-X

Markert MJ, Zhang Y, Enuameh MS, Reppert SM, Wolfe SA, Merlin C (2016) Genomic access to monarch migration using TALEN and CRISPR/Cas9-mediated targeted mutagenesis. G3 6:905–915. https://doi.org/10.1534/g3.116.027029

Martin Anduaga A, Evantal N, Patop IL, Bartok O, Weiss R, Kadener S (2019) Thermosensitive alternative splicing senses and mediates temperature adaptation in *Drosophila*. eLife 8:e44642. https://doi.org/10.7554/eLife.44642

Martinek S, Inonog S, Manoukian AS, Young MW (2001) A role for the segment polarity gene shaggy/GSK-3 in the *Drosophila* circadian clock. Cell 105:769–779. https://doi.org/10.1016/S0092-8674(01)00383-X

Masuda S, Narasimamurthy R, Yoshitane H, Kim JK, Fukada Y, Virshup DM (2020) Mutation of a PER2 phosphodegron perturbs the circadian phosphoswitch. Proc Nat Acad Sci U S A 117:10888–10896. https://doi.org/10.1073/pnas.2000266117

Matsumoto A, Tomioka K, Chiba Y, Tanimura T (1999) *tim*^{rit} lengthens circadian period in a temperature-dependent manner through suppression of PERIOD protein cycling and nuclear localization. Mol Cell Biol 19:4343-4354. https://doi.org/10.1128/MCB.19.6.4343

Matsumoto A, Ukai-Tadenuma M, Yamada RG, Houl J, Uno KD, Kasukawa T et al (2007) A functional genomics strategy reveals clockwork orange as a transcriptional regulator in the *Drosophila* circadian clock. Genes Dev 21:1687–1700. https://doi.org/10.1101/Gad.1552207

McDonald MJ, Rosbash M (2001) Microarray analysis and organization of circadian gene expression in *Drosophila*. Cell 107:567–578

McDonald MJ, Rosbash M, Emery P (2001) Wild-type circadian rhythmicity is dependent on closely spaced E boxes in the *Drosophila timeless* promoter. Mol Cell Biol 21:1207–1217. https://doi.org/10.1128/Mcb.21.4.1207-1217.2001

McKenna DD, Shin S, Ahrens D, Balke M, Beza-Beza C, Clarke DJ et al (2019) The evolution and genomic basis of beetle diversity. Proc Natl Acad Sci U S A 116:24729–24737. https://doi.org/10.1073/pnas.1909655116

Mei Q, Dvornyk V (2015) Evolutionary history of the photolyase/cryptochrome superfamily in eukaryotes. PLoS One 10:e0135940. https://doi.org/10.1038/s41598-021-89345-7

Meireles-Filho AC, Amoretty PR, Souza NA, Kyriacou CP, Peixoto AA (2006) Rhythmic expression of the cycle gene in a hematophagous insect vector. BMC Mol Biol 7:38. https://doi.org/10.1186/1471-2199-7-38

Meuti ME, Stone M, Ikeno T, Denlinger DL (2015) Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, *Culex pipiens*. J Exp Biol 218:412–422. https://doi.org/10.1242/jeb.113233

Meyer P, Saez L, Young MW (2006) PER-TIM interactions in living *Drosophila* cells: an interval timer for the circadian clock. Science 311:226–229. https://doi.org/10.1126/science.1118126

Misof B, Liu S, Meusemann K, Peters RS, Donath A, Mayer C et al (2014) Phylogenomics resolves the timing and pattern of insect evolution. Science 346:763–767. https://doi.org/10.1126/science.1257570

Moriyama Y, Kamae Y, Uryu O, Tomioka K (2012) *Gb'clock* is expressed in the optic lobe and is required for the circadian clock in the cricket *Gryllus bimaculatus*. J Biol Rhythms 27:467–477. https://doi.org/10.1177/0748730412462207

Myers MP, WagerSmith K, Rothenfluh-Hilfiker A, Young MW (1996) Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. Science 271:1736–1740. https://doi.org/10.1126/science.271.5256.1736

Naidoo N, Song W, Hunter-Ensor M, Sehgal A (1999) A role for the proteasome in the light response of the timeless clock protein. Science 285:1737–1741. https://doi.org/10.1126/science.285.5434.1737

Narasaki-Funo Y, Tomiyama Y, Nose M, Bando T, Tomioka K (2020) Functional analysis of *Pdp1* and *vrille* in the circadian system of a cricket. J Insect Physiol 127:104156. https://doi.org/10.1016/j.jinsphys.2020.104156

Narasimamurthy R, Virshup DM (2021) The phosphorylation switch that regulates ticking of the circadian clock. Mol Cell 81:1133–1146. https://doi.org/10.1016/j.molcel.2021.01.006

Nartey MA, Sun X, Qin S, Hou CX, Li MW (2021) CRISPR/Cas9-based knockout reveals that the clock gene *timeless* is indispensable for regulating circadian behavioral rhythms in *Bombyx mori*. Insect Sci 28:1414–1425. https://doi.org/10.1111/1744-7917.12864

Nose M, Tokuoka A, Bando T, Tomioka K (2017) *timeless2* plays an important role in reproduction and circadian rhythms in the cricket *Gryllus bimaculatus*. J Insect Physiol 105:9. https://doi.org/10.1016/j.jinsphys.2017.12.007

Ozkaya O, Rosato E (2012) The circadian clock of the fly: a neurogenetics journey through time. Adv Genet 77:79–123. https://doi.org/10.1016/B978-0-12-387687-4.00004-0

Pavelka J, Shimada K, Kostal V (2003) TIMELESS: a link between fly's circadian and photoperiodic clocks? Eur J Entomol 100:255–265. https://doi.org/10.14411/eje.2003.041

Peschel N, Chen KF, Szabo G, Stanewsky R (2009) Light-dependent interactions between the *Drosophila* circadian clock factors cryptochrome, jetlag, and timeless. Curr Biol 19:241–247. https://doi.org/10.1016/j.cub.2008.12.042

Peschel N, Helfrich-Förster C (2011) Setting the clock - by nature: circadian rhythm in the fruitfly *Drosophila melanogaster*. FEBS Lett 585:1435–1442. https://doi.org/10.1016/j.febslet.2011.02.028

Peschel N, Veleri S, Stanewsky R (2006) *Veela* defines a molecular link between cryptochrome and timeless in the light-input pathway to *Drosophila's* circadian clock. Proc Nat Acad Sci U S A 103:17313–17318. https://doi.org/10.1073/pnas.0606675103

Pett JP, Kondoff M, Bordyugov G, Kramer A, Herzel H et al (2018) Co-existing feedback loops generate tissue-specific circadian rhythms. Life Sci Alliance 1:e201800078. https://doi.org/10.26508/lsa.201800078

Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW (1998) *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell 94:83–95. https://doi.org/10.1016/S0092-8674(00)81224-6

Renn SCP, Park JH, Rosbash M, Hall JC, Taghert PH (1999) A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. Cell 99:791–802. https://doi.org/10.1016/S0092-8674(00)81676-1

Richier B, Michard-Vanhee C, Lamouroux A, Papin C, Rouyer F (2008) The clockwork orange *Drosophila* protein functions as both an activator and a repressor of clock gene expression. J Biol Rhythms 23:103–116. https://doi.org/10.1177/0748730407313817

Rivas GBS, Zhou J, Merlin C, Hardin PE (2021) CLOCKWORK ORANGE promotes CLOCK-CYCLE activation via the putative *Drosophila* ortholog of CLOCK INTERACTING PROTEIN CIRCADIAN. Curr Biol 31:4207–4218.e4. https://doi.org/10.1016/j.cub.2021.07.017

Rosato E, Codd V, Mazzotta G, Piccin A, Zordan M, Costa R et al (2001) Light-dependent interaction between *Drosophila* CRY and the clock protein PER mediated by the carboxy terminus of CRY. Curr Biol 11:909–917. https://doi.org/10.1016/s0960-9822(01)00259-7

Rosato E, Trevisan A, Sandrelli F, Zordan M, Kyriacou CP, Costa R (1997) Conceptual translation of *timeless* reveals alternative initiating methionines in *Drosophila*. Nucleic Acids Res 25:455–458. https://doi.org/10.1093/nar/25.3.455

Rothenfluh A, Abodeely M, Price JL, Young MW (2000b) Isolation and analysis of six *timeless* alleles that cause short- or long-period circadian rhythms in *Drosophila*. Genetics 156:665–675. https://doi.org/10.1093/genetics/156.2.665

Rothenfluh A, Young MW, Saez L (2000a) A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. Neuron 26:505–514. https://doi.org/10.1016/s0896-6273(00)81182-4

Rubin EB, Shemesh Y, Cohen M, Elgavish S, Robertson HM, Bloch G (2006) Molecular and phylogenetic analyses reveal mammalian-like clockwork in the honey bee (*Apis mellifera*) and shed new light on the molecular evolution of the circadian clock. Genome Res 16:1352–1365. https://doi.org/10.1101/gr.5094806

Rund SS, Hou TY, Ward SM, Collins FH, Duffield GE (2011) Genome-wide profiling of diel and circadian gene expression in the malaria vector *Anopheles gambiae*. Proc Natl Acad Sci U S A 108:E421–E430. https://doi.org/10.1073/pnas.1100584108

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell 93:805–814. https://doi.org/10.1016/S0092-8674(00)81441-5

Rutila JE, Zeng H, Le M, Curtin KD, Hall JC, Rosbash M (1996) The tim^{SL} mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. Neuron 17:921–929. https://doi.org/10.1016/s0896-6273(00)80223-8

Saez L, Derasmo M, Meyer P, Stieglitz J, Young MW (2011) A key temporal delay in the circadian cycle of *Drosophila* is mediated by a nuclear localization signal in the Timeless protein. Genetics 188:591–U166. https://doi.org/10.1534/genetics.111.127225

Saez L, Young MW (1996) Regulation of nuclear entry of the *Drosophila* clock proteins period and timeless. Neuron 17:911–920. https://doi.org/10.1016/S0896-6273(00)80222-6

Sakamoto T, Uryu O, Tomioka K (2009) The clock gene *period* plays an essential role in photoperiodic control of nymphal development in the cricket *Modicogryllus siamensis*. J Biol Rhythms 24:379–390. https://doi.org/10.1177/0748730409341523

Sandrelli F, Tauber E, Pegoraro M, Mazzotta G, Cisotto P, Landskron J et al (2007) A molecular basis for natural selection at the *timeless* locus in *Drosophila melanogaster*. Science 316:1898–1900. https://doi.org/10.1126/science.1138426

Sathyanarayanan S, Zheng X, Xiao R, Sehgal A (2004) Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. Cell 116:603–615. https://doi.org/10.1016/s0092-8674(04)00128-x

Sauman I, Briscoe AD, Zhu HS, Shi DD, Froy O, Stalleicken J et al (2005) Connecting the navigational clock to sun compass input in monarch butterfly brain. Neuron 46:457–467. https://doi.org/10.1016/j.neuron.2005.03.014

Sawyer LA, Hennessy JM, Peixoto AA, Rosato E, Parkinson H, Costa R et al (1997) Natural variation in a *Drosophila* clock gene and temperature compensation. Science 278:2117–2120. https://doi.org/10.1534/genetics.106.058792

Sehadova H, Markova EP, Sehnal FS, Takeda M (2004) Distribution of circadian clock-related proteins in the cephalic nervous system of the silkworm, *Bombyx mori*. J Biol Rhythms 19:466–482. https://doi.org/10.1177/0748730404269153

Sehadova H, Sauman I, Sehnal F (2003) Immunocytochemical distribution of pigment-dispersing hormone in the cephalic ganglia of polyneopteran insects. Cell Tissue Res 312:113–125. https://doi.org/10.1007/s00441-003-0705-5

Sehgal A, Price JL, Man B, Young MW (1994) Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant *timeless*. Science 263:1603–1606. https://doi.org/10.1126/science.8128246

Sehgal A, Rothenfluhhilfiker A, Hunterensor M, Chen YF, Myers MP, Young MW (1995) Rhythmic expression of *timeless* – a basis for promoting circadian cycles in *period* gene autoregulation. Science 270:808–810. https://doi.org/10.1126/science.270.5237.808

Shakhmantsir I, Nayak S, Grant GR, Sehgal A (2019) Spliceosome factors target *timeless* (*tim*) mRNA to control clock protein accumulation and circadian behavior in *Drosophila*. eLife 7: e39821. https://doi.org/10.7554/eLife.39821

Shao QM, Sehadova H, Ichihara N, Sehnal FS, Takeda M (2006) Immunoreactivities to three circadian clock proteins in two ground crickets suggest interspecific diversity of the circadian clock structure. J Biol Rhythms 21:118–131. https://doi.org/10.1177/0748730405283660

Siepka SM, Yoo SH, Park J, Song WM, Kumar V, Hu YN et al (2007) Circadian mutant overtime reveals F-box protein FBXL3 regulation of *cryptochrome* and *period* gene expression. Cell 129: 1011–1023. https://doi.org/10.1016/j.cell.2007.04.030

Singh S, Giesecke A, Damulewicz M, Fexova S, Mazzotta GM, Stanewsky R et al (2019) New *Drosophila* circadian clock mutants affecting temperature compensation induced by targeted mutagenesis of *timeless*. Front Physiol 10:1442. https://doi.org/10.3389/fphys.2019.01442

Siwicki KK, Eastman C, Petersen G, Rosbash M, Hall JC (1988) Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. Neuron 1:141–150. https://doi.org/10.1016/0896-6273(88)90198-5

Stanewsky R (2003) Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. J Neurobiol 54:111–147. https://doi.org/10.1002/Neu.10164

Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA et al (1998) The cry^b mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell 95:681–692. https://doi.org/10.1016/S0092-8674(00)81638-4

Stehlik J, Zavodska R, Shimada K, Sauman I, Kostal V (2008) Photoperiodic induction of diapause requires regulated transcription of *timeless* in the larval brain of *Chymomyza costata*. J Biol Rhythms 23:129–139. https://doi.org/10.1177/0748730407313364

Stoleru D, Peng Y, Agosto J, Rosbash M (2004) Coupled oscillators control morning and evening locomotor behaviour of *Drosophila*. Nature 431:862–868. https://doi.org/10.1038/nature02926

Stroppa MM, Garcia BA (2019) Clock gene *timeless* in the chagas disease vector *Triatoma infestans* (Hemiptera: Reduviidae). Am J Trop Med Hyg 101:1369–1372. https://doi.org/10.4269/ajtmh.19-0169

Szabo A, Papin C, Cornu D, Chelot E, Lipinszki Z, Udvardy A et al (2018) Ubiquitylation dynamics of the clock cell proteome and TIMELESS during a circadian cycle. Cell Rep 23:2273–2282. https://doi.org/10.1016/j.celrep.2018.04.064

Szabo A, Papin C, Zorn D, Ponien P, Weber F, Raabe T et al (2013) The CK2 kinase stabilizes CLOCK and represses its activity in the *Drosophila* circadian oscillator. PloS Biol 11:e1001645. https://doi.org/10.1371/journal.pbio.1001645

Takekata H, Numata H, Shiga S, Goto SG (2014) Silencing the circadian clock gene *Clock* using RNAi reveals dissociation of the circatidal clock from the circadian clock in the mangrove cricket. J Insect Physiol 68:16–22. https://doi.org/10.1016/j.jinsphys.2014.06.012

Tataroglu O, Emery P (2015) The molecular ticks of the *Drosophila* circadian clock. Curr Opin Insect Sci 7:51–57. https://doi.org/10.1016/j.cois.2015.01.002

Tauber E, Zordan M, Sandrelli F, Pegoraro M, Osterwalder N, Breda C et al (2007) Natural selection favors a newly derived *timeless* allele in *Drosophila melanogaster*. Science 316:1895–1898. https://doi.org/10.1126/science.1138412

Thakkar N, Giesecke A, Bazalova O, Martinek J, Smykal V, Stanewsky R et al (2022) Evolution of casein kinase 1 and functional analysis of new *doubletime* mutants in *Drosophila*. Front Physiol 13:1062632. https://doi.org/10.3389/fphys.2022.10626

Thomas GWC, Dohmen E, Hughes DST, Murali SC, Poelchau M, Glastad K et al (2020) Gene content evolution in the arthropods. Genome Biol 21:15. https://doi.org/10.1186/s13059-019-1925-7

Toh KL, Jones CR, He Y, Eide EJ, Hinz WA, Virshup DM et al (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science 291:1040–1043. https://doi.org/10.1126/science.1057499

Tokuoka A, Itoh TQ, Hori S, Uryu O, Danbara Y, Nose M et al (2017) cryptochrome genes form an oscillatory loop independent of the *per/tim* loop in the circadian clockwork of the cricket *Gryllus bimaculatus*. Zoological Lett 3:5. https://doi.org/10.1186/s40851-017-0066-7

Tomiyama Y, Shinohara T, Matsuka M, Bando T, Mito T, Tomioka K (2020) The role of *clockwork orange* in the circadian clock of the cricket *Gryllus bimaculatus*. Zoological Lett 6:12. https://doi.org/10.1186/s40851-020-00166-4

Top D, Harms E, Syed S, Adams EL, Saez L (2016) GSK-3 and CK2 kinases converge on Timeless to regulate the master clock. Cell Rep 16:357–367. https://doi.org/10.1016/j.celrep.2016.06.005

Top D, O'Neil JL, Merz GE, Dusad K, Crane BR, Young MW (2018) CK1/Doubletime activity delays transcription activation in the circadian clock. eLife 7:e32679. https://doi.org/10.7554/eLife.32679

Uryu O, Karpova SG, Tomioka K (2013) The clock gene cycle plays an important role in the circadian clock of the cricket *Gryllus bimaculatus*. J Insect Physiol 59:697–704. https://doi.org/10.1016/j.jinsphys.2013.04.011

Vafopoulou X, Steel CG (2012) Metamorphosis of a clock: remodeling of the circadian timing system in the brain of *Rhodnius prolixus* (Hemiptera) during larval-adult development. J Comp Neurol 520:1146–1164. https://doi.org/10.1002/cne.22743

Venkatesan A, Fan JY, Bouyain S, Price JL (2019) The circadian tau mutation in casein kinase 1 is part of a larger domain that can be mutated to shorten circadian period. Int J Mol Sci 20:4. https://doi.org/10.3390/ijms20040813

Wang X, Fang X, Yang P, Jiang X, Jiang F, Zhao D et al (2014) The locust genome provides insight into swarm formation and long-distance flight. Nat Commun 5:2957. https://doi.org/10.1038/ncomms3957

Wen CJ, Lee HJ (2008) Mapping the cellular network of the circadian clock in two cockroach species. Arch Insect Biochem Physiol 68:215–231. https://doi.org/10.1002/arch.20236

Werckenthin A, Huber J, Arnold T, Koziarek S, Plath MJA, Plath JA et al (2020) Neither *per*, nor *tim1*, nor *cry2* alone are essential components of the molecular circadian clockwork in the Madeira cockroach. PLoS One 15:e0235930. https://doi.org/10.1371/journal.pone.0235930

Wipfler B, Letsch H, Frandsen PB, Kapli P, Mayer C, Bartel D et al (2019) Evolutionary history of Polyneoptera and its implications for our understanding of early winged insects. Proc Natl Acad Sci U S A 116:3024–3029. https://doi.org/10.1073/pnas.1817794116

Xu L, Chen S, Wen B, Shi H, Chi C, Liu C et al (2021) Identification of a novel class of photolyases as possible ancestors of their family. Mol Biol Evol 38:4505–4519. https://doi.org/10.1093/molbev/msab191

Yao Z, Shafer OT (2014) The *Drosophila* circadian clock is a variably coupled network of multiple peptidergic units. Science 343:1516–1520. https://doi.org/10.1126/science.1251285

Yu W, Zheng H, Houl JH, Dauwalder B, Hardin PE (2006) PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. Genes Dev 20:723–733. https://doi.org/10.1101/gad.1404406

Yuan Q, Metterville D, Briscoe AD, Reppert SM (2007) Insect cryptochromes: Gene duplication and loss define diverse ways to construct insect circadian clocks. Mol Biol Evol 24:948–955. https://doi.org/10.1093/molbev/msm011

Zavodska R, Fexova S, von Wowern G, Han GB, Dolezel D, Sauman I (2012) Is the sex communication of two pyralid moths, *Plodia interpunctella* and *Ephestia kuehniella*, under circadian clock regulation? J Biol Rhythms 27:206–216. https://doi.org/10.1177/0748730412440689

Zavodska R, Sauman I, Sehnal F (2003a) Distribution of PER protein, pigment-dispersing hormone, prothoracicotropic hormone, and eclosion hormone in the cephalic nervous system of insects. J Biol Rhythms 18:106–122. https://doi.org/10.1177/0748730403251711

Zavodska R, Sauman I, Sehnal F (2003b) The cycling and distribution of PER-like antigen in relation to neurons recognized by the antisera to PTTH and EH in *Thermobia domestica*. Insect Biochem Mol Biol 33:1227–1238. https://doi.org/10.1016/j.ibmb.2003.06.009

Zavodska R, Wen CJ, Hrdy I, Sauman I, Lee HJ, Sehnal F (2008) Distribution of corazonin and pigment-dispersing factor in the cephalic ganglia of termites. Arthropod Struct Dev 37:273–286. https://doi.org/10.1016/j.asd.2008.01.005

Zehring WA, Wheeler DA, Reddy P, Konopka RJ, Kyriacou CP, Rosbash M et al (1984) P-element transformation with *period* locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. Cell 39:369–376. https://doi.org/10.1016/0092-8674(84)90015-1

Zeng HK, Qian ZW, Myers MP, Rosbash M (1996) A light-entrainment mechanism for the *Drosophila* circadian clock. Nature 380:129–135. https://doi.org/10.1038/380129a0

Zerr DM, Hall JC, Rosbash M, Siwicki KK (1990) Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. J Neurosci 10:2749–2762. https://doi.org/10.1523/JNEUROSCI.10-08-02749.1990

Zhang L, Hastings MH, Green EW, Tauber E, Sladek M, Webster SG et al (2013a) Dissociation of circadian and circatidal timekeeping in the marine crustacean *Eurydice pulchra*. Curr Biol 23:1863–1873. https://doi.org/10.1016/j.cub.2013.08.038

Zhang Y, Ling J, Yuan C, Dubruille R, Emery P (2013b) A role for *Drosophila* ATX2 in activation of PER translation and circadian behavior. Science 340:879–882. https://doi.org/10.1126/science.1234746

Zhang Y, Markert MJ, Groves SC, Hardin PE, Merlin C (2017) Vertebrate-like CRYPTOCHROME 2 from monarch regulates circadian transcription via independent repression of CLOCK and BMAL1 activity. Proc Natl Acad Sci U S A 114:E7516–E7525. https://doi.org/10.1073/pnas.1702014114

Zhou J, Yu W, Hardin PE (2016) CLOCKWORK ORANGE enhances PERIOD mediated rhythms in transcriptional repression by antagonizing E-box binding by CLOCK-CYCLE. PLoS Genet 12:e1006430. https://doi.org/10.1371/journal.pgen.1006430

Zhou M, Kim JK, Eng GW, Forger DB, Virshup DM (2015) A Period2 phosphoswitch regulates and temperature compensates circadian period. Mol Cell 60:77–88. https://doi.org/10.1016/j.molcel.2015.08.022

Zhu HS, Sauman I, Yuan Q, Casselman A, Emery-Le M, Emery P et al (2008) Cryptochromes define a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. PLoS Biol 6:138–155. https://doi.org/10.1371/journal.pbio.0060004

Zylka MJ, Shearman LP, Levine JD, Jin XW, Weaver DR, Reppert SM (1998) Molecular analysis of mammalian *timeless*. Neuron 21:1115–1122. https://doi.org/10.1016/S0896-6273(00)80628-5