Molecular genetic analysis of circadian timekeeping in *Drosophila*

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Abstract

A genetic screen for mutants that alter circadian rhythms in *Drosophila* identified the first clock gene - the *period* (*per*) gene. The *per* gene is a central player within a transcriptional feedback loop that represents the core mechanism for keeping circadian time in *Drosophila* and other animals. The *per* feedback loop, or core loop, is interlocked with the *Clock* (*Clk*) feedback loop, but whether the *Clk* feedback loop contributes to circadian timekeeping is not known. A series of distinct molecular events are thought to control transcriptional feedback in the core loop. The time it takes to complete these events should take much less than 24h, thus delays must be imposed at different steps within the core loop. As new clock genes are identified, the molecular mechanisms responsible for these delays have been revealed in ever-increasing detail, and provide an in depth accounting of how transcriptional feedback loops keep circadian time. The phase of these feedback loops shift to maintain synchrony with environmental cycles, the most reliable of which is light. Although a great deal is known about cell-autonomous mechanisms of light-induced phase shifting by CRYPTOCHROME (CRY), much less is known about non-cell autonomous mechanisms. CRY mediates phase shifts through an uncharacterized mechanism in certain brain oscillator neurons, and carries out a dual role as a photoreceptor and transcription factor in other tissues. Here I will review how transcriptional feedback loops function to keep time in *Drosophila*, how they impose delays to maintain a 24h cycle, and how they maintain synchrony with environmental light:dark cycles. The transcriptional feedback loops that keep time in *Drosophila* are well conserved in other animals, thus what we learn about these loops in *Drosophila* should continue to provide insight into the operation of analogous transcriptional feedback loops in other animals.

I. Introduction

Research on the fruit fly *Drosophila* melanogaster has had a profound impact on our understanding of the circadian timekeeping mechanism. Groundbreaking studies by Ron Konopka and Seymour Benzer identified the first "clock gene", *period* (*per*), in a screen for mutants with altered free-running (in constant darkness or DD) circadian periods in the rhythm of adult emergence (Konopka and Benzer, 1971). Since *per* mutants alter circadian period, *per* was thought to be integrally involved in keeping circadian time, but determining how *per* contributed to circadian timekeeping didn't take off until the *per* gene was isolated at Brandeis University in the labs of Michael Rosbash and Jeff Hall and at Rockefeller University in Michael Young's lab (Bargiello et al., 1984; Bargiello and Young, 1984; Reddy et al., 1984; Zehring et al., 1984). The PER protein sequence didn't offer many clues

about its role in the clock because its only distinguishing features were a stretch of Threonine-Glycine repeats, later shown to be involved in adapting to different thermal environments (Sawyer et al., 1997), and a region similar to a portion of the *Drosophila* SINGLE-MINDED (SIM) and mammalian aryl hydrocarbon receptor nuclear translocator (Arnt) proteins, termed the Per-Arnt-Sim (PAS) domain (Nambu et al., 1991), that mediates protein-protein interactions (Huang et al., 1993). However, the discovery that *per* mRNA and protein cycle in a circadian manner (Hardin et al., 1990; Siwicki et al., 1988), and that PER protein is required for cycling of *per* mRNA (Hardin et al., 1990), suggested that *per* contributes to circadian timekeeping via a feedback loop in which PER protein controls rhythms in *per* mRNA expression (Hardin et al., 1990). Studies demonstrating transcriptional control of *per* mRNA cycling and PER-dependent inhibition of *per* mRNA expression further refined the role of PER in this feedback loop as a transcriptional repressor (Hardin et al., 1992; Zeng et al., 1994). Subsequent studies not only support the view that this transcriptional feedback loop keeps circadian time in *Drosophila*, but show that similar feedback loops keep circadian time in diverse eukaryotic species including plants, fungi, and animals, the latter of which even shares critical feedback loop components such as *per* (for reviews see (Bell-Pedersen et al., 2005; Dunlap, 1999; Young and Kay, 2001).

Although *per* is an essential feedback loop component, many other genes are required to sustain the *per* feedback loop. In the first section of this review I will explain the roles other genes play within the *per* feedback loop, and describe how the *per* feedback loop relates to other interlocked feedback loops. Since the *per* feedback loop has many components and is inextricably linked to other feedbacks loops, I will refer to the *per* feedback loop as the 'core loop' and to the combined core and interlocked feedback loops as 'circadian feedback loops'. The different steps required to construct a transcriptional feedback loop (such as the core loop) can be completed in far less than ~24h, indicating that potent mechanisms have evolved to impart delays in feedback regulation. The second section will focus on how post-transcriptional regulation of key feedback loop components produces delays in transcriptional feedback that set the pace of the circadian oscillator. Entrainment of circadian feedback loops to environmental light-dark cycles is critical for driving physiological, metabolic and behavioral rhythms at the appropriate time of day. Unlike the case in mammals, light can directly entrain circadian feedback loops in peripheral tissues from *Drosophila*, thus there is no intermediary "master" pacemaker in the *Drosophila* brain that relays light information to the periphery. However, light can entrain the network of *Drosophila* brain pacemaker neurons via multiple mechanisms depending on which cells detect the light. Section three will discuss how light entrains circadian feedback loops in different cells and tissues.

In the last section of this review I will summarize the main conclusions from each section, point out where there are gaps in our understanding, discuss how filling these gaps may explain how these circadian feedback loops account for basic features of circadian clock such as the 24h period and entrainment to environmental cycles. The general consensus is that circadian feedback loops sit at the heart of the circadian timekeeping mechanism in eukaryotes. However, experiments demonstrating that circadian rhythms in the phosphorylation/dephosphorylation of KaiC protein in cyanobacteria can be reconstituted in

a test-tube (Nakajima et al., 2005), and rhythms in the oxidation of peroxiredoxins in a primitive eukaryotic alga *Ostrecoccus tauri* and red blood cells occurs in the absence of transcription (O'Neill and Reddy, 2011; O'Neill et al., 2011), demonstrate that other circadian timekeeping mechanisms exist in eukaryotes that don't require transcriptional feedback. I will discuss the role of circadian feedback loops in circadian timekeeping in light of these new results.

II. The circadian feedback loops of *Drosophila*

The *per* feedback loop suggested a mechanism for keeping circadian time: Once *per* transcription is initiated during mid day the levels of *per* mRNA rise until early evening, when accumulating levels of PER protein repress *per* transcription, thereby reducing the levels of *per* mRNA until the early day, when PER protein is eliminated and the next round of *per* transcription begins. Although this feedback loop provided a framework for how *per* contributes to circadian timekeeping, it raised many questions. For instance, PER feeds back to repress transcription of its own gene, but does PER achieve this by binding DNA at specific sites to repress transcription, competing with *per* activators for DNA binding sites, or binding to *per* activators to inhibit their DNA binding? Does PER feed back to repress transcription alone, or are other factors involved in insuring that feedback occurs at the correct time of day? What genes are responsible for activating *per* transcription? A combination of approaches including genetic screens, molecular interaction assays, *per* promoter analysis, and molecular searches for clock gene orthologs were used to answer these questions.

Genetic screens uncovered many additional clock genes including *timeless* (*tim*) (Sehgal et al., 1994), *Clock* (*Clk*) (Allada et al., 1998), *cycle* (*cyc*) (Rutila et al., 1998), *doubletime* (*dbt*) (Kloss et al., 1998; Price et al., 1998), *shaggy* (*sgg*) (Martinek et al., 2001), casein kinase 2 (CK2) subunits (Akten et al., 2003; Lin et al., 2002a) and *cryptochrome* (*cry*) (Stanewsky et al., 1998). A screen for proteins that interact with PER also identified *tim* (Gekakis et al., 1995), which binds to the PAS domain of PER. Dissecting the *per* promoter for transcriptional regulatory elements identified a canonical E-box element (in this case 5' CACGTG 3') that is required to activate *per* transcription (Hao et al., 1997), and was later found to be a conserved 'circadian' regulatory element for many animal genes (reviewed in (Bell-Pedersen et al., 2005; Hardin, 2004; Young and Kay, 2001)). The first genetic screen for mouse clock genes identified *Clock* (King et al., 1997a; King et al., 1997b; Vitaterna et al., 1994), which is a member of the basic-helix-loop-helix-PAS (bHLH-PAS) transcription factor family that typically binds E-box elements to activate transcription. Protein coding sequences from mouse *Clock* were used to recover the *Drosophila Clock* ortholog from a cDNA library screen (Darlington et al., 1998), thereby complementing the identification of *Clk* via genetic screening (Allada et al., 1998). The discovery of additional clock genes and regulatory sequences not only support the *per* feedback loop model, but add to its mechanistic detail. I will briefly summarize clock gene function within the core feedback loop, and refer you to a number of reviews for more detailed descriptions (Allada and Chung, 2010; Hardin, 2005; Zheng and Sehgal, 2008).

A. The Core Feedback Loop

In the core feedback loop (Fig. 1), *per* and *tim* transcription are activated from ~ZT4 to ~ZT18 (Zeitgeber Time, or ZT, refers to time in hours during a light-dark cycle, where ZT0 is lights on and ZT12 is lights off), when CLK and its heterodimeric bHLH-PAS partner CYC bind E-boxes in the *per* and *tim* promoters (Allada et al., 1998; Darlington et al., 1998; Hao et al., 1997; Rutila et al., 1998). PER and TIM proteins start accumulating in the cytoplasm at ~ZT12, about 6–8hr after their respective mRNAs. This lag in PER and TIM protein accumulation is thought to be caused by the combined effects of phosphorylation-dependent degradation of PER by DBT, a homolog of mammalian casein kinase 1ε (Kloss et al., 1998; Kloss et al., 2001; Price et al., 1998), and stabilization of PER-DBT complexes by TIM (Price et al., 1995), which enables cytoplasmic accumulation of DBT-PER-TIM complexes (Curtin et al., 1995; Gekakis et al., 1995; Zeng et al., 1996). However, recent data (described below) suggest that translational regulation may mediate the delay in PER cytoplasmic accumulation rather than DBT-dependent destabilization of PER (Lim et al., 2011; Chiu et al., 2008). Phosphorylation of PER by CK2 and TIM by SGG, a homolog of mammalian Glycogen Synthase Kinase 3, promotes nuclear localization of PER-DBT and TIM (Akten et al., 2003; Lin et al., 2002a; Martinek et al., 2001). PER and TIM phosphorylation are counterbalanced by protein phosphatase 2a (PP2a) and protein phosphatase 1 (PP1) mediated dephosphorylation, respectively, which stabilize PER and TIM and alter their nuclear localization (Fang et al., 2007; Sathyanarayanan et al., 2004). Once in the nucleus, PER-DBT complexes (and/or PER-TIM-DBT complexes) then bind CLK, promote CLK phosphorylation, and release CLK-CYC from E-boxes to inhibit transcription from ~ZT18 to ~ZT4 (Bae et al., 2000; Lee et al., 1998, 1999; Menet et al., 2010; Yu et al., 2006). At ZT0 lights turn on and induce TIM degradation (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996), thus 'deprotecting' PER. Progressive phosphorylation of PER by DBT in the nucleus ultimately triggers binding of the E3 ubiquitin ligase SLIMB, which targets PER for degradation in the proteasome by ~ZT4 (Grima et al., 2002; Kloss et al., 2001; Ko et al., 2002). Once PER is degraded, hypophosphorylated CLK accumulates and CLK-CYC binds E-boxes to initiate another cycle of *per* and *tim* transcription.

The core feedback loop nicely explains the regulation of cycling mRNAs that peak in abundance during the early evening. However, not all cycling gene expression peaks in the early evening. The first example of this in flies was *Clk* mRNA, which peaks during the early morning (Bae et al., 1998; Darlington et al., 1998). Rhythms in *Clk* mRNA levels are also dependent on the core feedback loop as these rhythms were abolished in *per*[01] and *tim*[01] null mutants (Bae et al., 1998). How these rhythms were abolished was intriguing; whereas *per* and *tim* mRNAs remain at relatively high levels in *per*[01] and *tim*[01] mutants (Hardin et al., 1990; Sehgal et al., 1995), *Clk* mRNA falls to constant low levels (Bae et al., 1998). Although PER and TIM function to repress *per* and *tim* transcription (Sehgal et al., 1994; Zeng et al., 1994), the low levels of *Clk* mRNA in *per*[01] and *tim*[01] flies suggested that PER and TIM somehow activate *Clk* transcription (Bae et al., 1998). Since *per* and *tim* expression is abolished in severe loss of function *Clk*[Jrk] and *cyc*[01] mutants (Allada et al., 1998; Rutila et al., 1998), *Clk* mRNA levels were expected to also be low in *Clk*[Jrk] and *cyc*[01] flies due to the loss of PER and TIM. However, *Clk* mRNA was expressed at peak levels in *Clk*[Jrk] and *cyc*[01]

mutants, which suggested that CLK-CYC somehow represses *Clk* expression, in contrast to its traditional role as a transcription activator (Glossop et al., 1999). A model was developed to explain these puzzling results that invoked a second '*Clk* feedback loop' that interlocked with the core feedback loop (Glossop et al., 1999). The subsequent identification of the PAR transcription factors *vrille* (*vri*) and PAR domain protein 1 ε and δ (*Pdp1ε/δ*) as components of the *Clk* feedback loop strongly support the interlocked feedback loop model (Cyran et al., 2003; Glossop et al., 2003; Zheng et al., 2009). I will briefly outline the important features of the *Clk* feedback loop, and refer you to other reviews for a more detailed description (Allada and Chung, 2010; Hardin, 2005; Zheng and Sehgal, 2008).

B. Interlocked Feedback Loops

In the *Clk* loop (Fig. 2), CLK-CYC binds E-boxes to activate *vri* transcription between ~ZT4 and ZT16 (Blau and Young, 1999; Cyran et al., 2003). VRI protein accumulates in phase with *vri* mRNA, ultimately peaking in abundance at ~ZT14. As VRI levels rise, VRI binds VRI/PDP1-boxes (V/P-boxes) in the *Clk* promoter, thereby repressing *Clk* transcription (Cyran et al., 2003; Glossop et al., 2003). As PER-DBT and PER-TIM-DBT complexes feed back to inhibit CLK-CYC dependent transcription from ~ZT16 to ZT4, *vri* mRNA and protein decline to low levels, thus permitting activation of *Clk* transcription. Mutants that disrupt CLK-CYC transcriptional activity (e.g. *Clk*[Jrk], *cyc*[01]) exhibit constant high levels of *Clk* mRNA (Glossop et al., 1999), indicating that *Clk* is constitutively activated independent of circadian oscillator function. However, another PAR transcription factor, PDP1ε/δ, also plays a role in *Clk* activation (Benito et al., 2007; Cyran et al., 2003; Zheng et al., 2009). CLK-CYC binds E-boxes to activate *Pdp1ε/δ* between ZT4 and ZT16 (Cyran et al., 2003). PDP1ε/δ rises to peak levels ~ZT18, several hours after VRI levels peak, and binds V/P-boxes to activate *Clk* transcription (Cyran et al., 2003). The extent to which *Pdp1ε/δ* activates *Clk* transcription is debatable, as altering *Pdp1ε/δ* levels via RNA interference and overexpression has mild effects on *Clk* mRNA levels compared to mutants that eliminate *Pdp1ε/δ* specifically or all *Pdp1* isoforms (Benito et al., 2007; Cyran et al., 2003; Zheng et al., 2009). Perhaps *vri* and *Pdp1ε/δ* function to enhance *Clk* mRNA amplitude once developmental activators establish the *Clk* expression pattern (Houl et al., 2008). Once PER complexes are degraded during mid-day, the next cycle of *vri* transcription and VRI-dependent repression is initiated.

In addition to the *per* and *Clk* feedback loops, another feedback loop that is controlled by the bHLH-orange transcription inhibitor CLOCKWORK ORANGE (CWO) has been proposed (Fig. 2). In this feedback loop, CLK-CYC binds E-boxes to activate *cwo* transcription, and CWO then feeds back to inhibit CLK-CYC transcription (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007). Inhibition of CLK-CYC occurs through competition for binding E-boxes (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007), and thus independently reinforces inhibition by PER complexes. This model for CWO function is primarily based on *in vitro* and *Drosophila* Schneider 2 (S2) cell culture data, which contrasts with *cwo* mutant data showing period lengthening and lower levels of *per*, *tim*, *vri* and *Pdp1ε/δ* levels (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008). This *in vivo* data implies that *cwo* is necessary for high-level transcription of

CLK-CYC activated genes. Additional studies that document the phase of CWO protein cycling and DNA binding will help to resolve the role of CWO in the circadian oscillator.

Although the *per* and *Clk* feedback loops control mRNA cycling in opposite phases of the circadian cycle, they are not equally important for circadian oscillator function (Hardin, 2006). The *per* loop is required for *Clk* loop function since CLK-CYC dependent activation and PER-TIM-DBT dependent repression control rhythms in *vri* transcription. However, the *Clk* loop is not necessary for *per* loop function since reversing *Clk* mRNA cycling has little affect on molecular or behavioral rhythms (Kim et al., 2002), and rhythms in *Clk* mRNA levels don't drive rhythms in CLK levels or transcriptional activity (Yu et al., 2006). If *Clk* mRNA rhythms are not important for core feedback loop function, then what is the importance of the *Clk* feedback loop? Microarray studies show that ~10% of *Drosophila* head mRNAs are rhythmically expressed (Ceriani et al., 2002; Claridge-Chang et al., 2001; Keegan et al., 2007; Lin et al., 2002b; McDonald and Rosbash, 2001; Ueda et al., 2002; Wijnen et al., 2006), and a large fraction of those are expressed with *Clk*-like peak times near dawn. Reducing, eliminating or increasing *Pdp1ε/δ* expression abolishes behavioral rhythms (Benito et al., 2007; Zheng et al., 2009), which suggests that *Pdp1ε/δ* controls output gene expression. Indeed, *Pdp1ε/δ* controls rhythmic expression of *takeout* (*to*), which regulates courtship behavior (Dauwalder et al., 2002), but such regulation appears to be indirect as no canonical V/P-boxes are present near the *to* promoter (Benito et al., 2010). Although *vri* overexpression abolishes oscillator function due to repression of *Clk* transcription (Cyran et al., 2003; Glossop et al., 2003), it has not been possible to test whether *vri* is required for oscillator function because *vri* null mutants are lethal (Cyran et al., 2003; George and Terracol, 1997). Perhaps *vri*, like *Pdp1ε/δ*, also is required to control rhythmic transcription of output genes that peak near dawn. Given the importance of the core loop, it is imperative that the mechanisms governing rhythmic transcription within this loop are defined.

III. Post-Transriptional Regulation of Rhythmic Transcription

To keep circadian time, the various molecular events within the core feedback loop must be completed in ~24h. However, the transcriptional activation and elongation, transcript processing and transport to the cytosol, protein synthesis and accumulation, nuclear localization, transcriptional repression, and repressor degradation that mediate feedback loop function collectively take much less than 24h to complete. Consequently, delays must be imposed at one or more steps in the core loop to achieve a 24h cycle. As described in the last section, there is good evidence that PER stability, nuclear localization, and transcriptional repression is controlled at the post-transcriptional level by TIM binding and kinases and phosphatases that control the phosphorylation state of PER and TIM. In this section I will review recent studies that begin to reveal the molecular consequences of PER-TIM interactions and site specific PER phosphorylation by different kinases. In addition, I will discuss the regulation and function of rhythms in CLK phosphorylation and other forms of post-transcriptional regulation that govern timekeeping by the core loop.

A. PER Phosphorylation and Translational Control

PER phosphorylation increases as PER accumulates during the night, and peaks as PER is degraded in the proteasome a few hours after dawn (Edery et al., 1994; Naidoo et al., 1999). DBT binds to PER and promotes PER degradation (Kim et al., 2007; Kloss et al., 1998; Kloss et al., 2001; Price et al., 1998), whereas TIM binds to PER and prevents PER degradation (Kloss et al., 1998; Kloss et al., 2001; Ko et al., 2002; Price et al., 1998). Much progress has been made in understanding the regulation of PER degradation after light-induced removal of TIM (Fig. 3c). At this time of the circadian cycle, PER is complexed with DBT and CLK-CYC to repress transcription (Kloss et al., 2001; Lee et al., 1999; Yu and Hardin, 2006). PER is progressively phosphorylated during this timeframe, but its SLIMB F-box protein (homolog of mammalian B-TrCP) induced degradation is delayed for several hours even in the absence of TIM (Grima et al., 2002; Ko et al., 2002). The reason for this delay is that DBT phosphoryation at PER serine 47 (S47) is the final step in a series of DBT phosphorylation events that produce an atypical SLIMB binding site (Chiu et al., 2008). Once SLIMB binds, PER is ubiquitinated and rapidly degraded (Chiu et al., 2008), thereby releasing transcriptional repression.

When DBT is co-expressed with PER in S2 cells, DBT phosphorylates PER at many sites before the ultimate phosphorylation event on S47 (Chiu et al., 2008; Kivimae et al., 2008). However, PER is phosphorylated by other kinases in the presence or absence of DBT in S2 cells (Chiu et al., 2008; Kivimae et al., 2008). For instance, PER is phosphorylated by CK2 at S149, S151 and S153 to promote nuclear localization of PER complexes (Chiu et al., 2008; Lin et al., 2005) (Fig. 3b). PER is also phosphorylated at multiple consensus proline-directed kinase target sites (Chiu et al., 2008), in which a proline follows the phosphorylated serine or threonine. One such proline-directed site at S661 is phosphorylated by an as yet unidentified kinase, but rather than promoting PER degradation, S661 phosphorylation primes phosphorylation of S657 by SGG to promote PER nuclear localization (Ko et al., 2010). SGG was thought to promote nuclear localization of PER complexes by phosphorylating TIM (Martinek et al., 2001), but SGG directly interacts with and stabilizes CRY, which in turn stabilizes TIM (Stoleru et al., 2007), and consequently PER, thus providing increased CK2 and SGG substrate to effect PER nuclear localization. Moreover, CK2 and SGG are found predominantly in the cytoplasm (Lin et al., 2002a; Yuan et al., 2005), consistent with their role in promoting PER nuclear localization. SGG phosphorylation promotes PER nuclear localization but not PER degradation (Ko et al., 2010), which implies that these nuclear localization and degradation are programmed by independent phosphorylation cascades. The (or at least a) lynchpin for PER nuclear localization is the proline-directed kinase that acts in the cytoplasm to prime SGG phosphorylation by phosphorylating S661. Identifying this proline-directed kinase and determining the relationship between SGG and CK2 dependent phosphorylation will provide a clearer picture of how PER nuclear localization is regulated.

PER is phosphorylated at S661 and several additional consensus proline-directed kinase sites (Chiu et al., 2008; Kivimae et al., 2008). One such site at S596 is situated within the "short period domain" of PER, a region spanning amino acids S585 to Y601 that contains mutants that shorten circadian period including per^S and per^T (Baylies et al., 1987; Baylies

et al., 1992; Konopka et al., 1994; Rutila et al., 1992; Yu et al., 1987). Disrupting the only proline-directed phosphorylation site in this region by mutating Y597 results in short period rhythms (Baylies et al., 1992), suggesting that phosphorylation at this site normally acts to lengthen (or delay) the circadian cycle. Indeed, recent work demonstrates that NEMO (NMO) kinase phosphorylates S596 (Chiu et al., 2011), and *nmo* mutant and *nmo* RNAi knockdown flies show short period rhythms (Chiu et al., 2011; Yu et al., 2011) (Fig. 3d). Mutating S595 to A also shortens circadian period, and gates the phosphorylation of nearby residues S589 (the original *per*[S] mutant site), S595 and S593 by DBT (Chiu et al., 2011). Phosphorylation of PER by NMO at S596 delays the phosphorylation of S47 by DBT (Chiu et al., 2011), and thus PER degradation, consistent with a delay in PER degradation in the morning when NMO is overexpressed (Yu et al., 2011). A model proposed by Edery and colleagues postulates that phosphorylation of PER by NMO and DBT within the "short period domain" alters the conformation of PER, thereby inhibiting the phosphorylation of sites required for SLIMB binding and delaying PER degradation (Chiu et al., 2011).

In addition to the delay in PER degradation, PER protein accumulation lags 6–8h behind *per* mRNA accumulation. This lag was thought to be produced by DBT induced PER degradation and protection by TIM. However, PER is not phosphorylated at the key S47 site until after dawn (Chiu et al., 2008), thus arguing against the same SLIMB-dependent mechanism functioning in the cytoplasm. The delayed accumulation of PER in the cytoplasm could be achieved by accelerating PER degradation or impeding PER synthesis (Fig. 3a). DBT associates with PER as PER accumulates in the cytoplasm, thus it is possible that cytoplasmically localized F-box proteins could bind phosphorylated PER and promote degradation. Alternatively, there may be a delay in the translation of *per* mRNA. Recent work on the *twenty-four* (*tyf*) gene suggests that PER accumulation is translationally controlled (Lim et al., 2011). TYF associates with the 5' cap binding complex, polyA binding protein and *per* mRNA, and loss of *tyf* reduces PER accumulation in ventralateral neurons (LNvs) in the fly brain, which are necessary and sufficient for locomotor activity rhythms (Frisch et al., 1994; Grima et al., 2004; Renn et al., 1999; Stoleru et al., 2004), suggesting that TYF promotes PER translation in LNvs (Lim et al., 2011). This loss of *tyf* function result implies that *per* mRNA is poorly translated and/or under active translational repression, and that TYF enhances translation efficiency or removes the translation block. The extent to which regulation of *per* mRNA translation contributes to the delay in PER cytoplasmic accumulation is not known, but such regulation could play a major role in determining the pace of the circadian oscillator. TYF appears to function only in LNvs (Lim et al., 2011), thus translational regulation by different factors or other post-transcriptional regulatory processes act to delay the cytoplasmic accumulation of PER in other oscillator cells. Translational regulation of other oscillator components is mediated by microRNAs. The microRNA *bantam* targets sequences in the 3'UTR of *Clk* mRNA, and mutating these *bantam* target sites greatly decreases rescue of behavioral rhythms by *Clk* transgenes (Kadener et al., 2009). Translation of *vri* and *cwo* mRNAs are also thought to be regulated by microRNAs (Kadener et al., 2009), and a number of microRNAs are rhythmically expressed (Yang et al., 2008).

As PER accumulates it is phosphorylated and enters the nucleus, where TIM plays an important role in stabilizing phosphorylated PER (Fig. 3b). TIM likely stabilizes PER by inhibiting phosphorylation at sites that promote SLIMB binding and degradation. How TIM inhibits PER phosphorylation at these sites is not well characterized, but recent analysis suggests that TIM may inhibit PER phosphorylation by delivering protein phosphatase 1 (PP1) to PER (Fang et al., 2007). PER is also dephosphorylated by protein phosphatase 2a (PP2a) (Sathyanarayanan et al., 2004), but dephosphorylation of PER by PP2a is not mediated by TIM (Fang et al., 2007). Nevertheless, PP2a dependent dephosphorylation of PER would further delay PER phosphorylation and degradation (Sathyanarayanan et al., 2004). Since phosphorylation enhances PER's ability to repress transcription (Nawathean and Rosbash, 2004), TIM-dependent stabilization of phosphorylated PER likely plays a key role in effectively repressing CLK-CYC transcription. It is not clear how PER phosphorylation enhances transcriptional repression, but it doesn't appear to act by enabling PER-CLK binding (Yu et al., 2009). Thus, delays in the synthesis, nuclear localization and degradation of PER contribute to the determination of circadian period.

B. CLK Phosphorylation

CLK is phosphorylated coincident with the entry of PER repression complexes into the nucleus (Yu et al., 2006). CLK phosphorylation coincides with transcriptional repression (Kim and Edery, 2006; Menet et al., 2010; Yu et al., 2006; Yu et al., 2009), but whether CLK phosphorylation is required for repression is not known (Fig. 3c). PER carries DBT into the nucleus (Kloss et al., 2001), and DBT is required for CLK phosphorylation (Kim and Edery, 2006; Yu et al., 2006; Yu et al., 2009). However, DBT doesn't phosphorylate CLK directly, though DBT (whether catalytically active or inactive) must be present in the PER repression complex to mediate CLK phosphorylation by one or more other kinases (Yu et al., 2009). The kinase(s) responsible for most CLK phosphorylation has not been identified, but one kinase implicated in CLK phosphorylation is NMO (Fig. 3d). Loss of *nmo* function increases CLK levels and shortens circadian period, whereas increasing *nmo* function decreases CLK levels and lengthens circadian period (Yu et al., 2011), consistent with period changes associated with increased or decreased CLK copy number (Kadener et al., 2008). These results suggest that NMO promotes CLK degradation to slow the pace of the circadian cycle (Yu et al., 2011), but whether NMO lowers CLK levels directly by phosphorylating CLK or indirectly by phosphorylating PER is not known.

Once PER is degraded, the levels of hypophosphorylated CLK increase along with CLK-CYC transcription. Since overall CLK levels are essentially constant (Yu et al., 2006), the increase in hypophosphorylated CLK levels could be due to the replacement of degraded hyperphosphoylated CLK with newly synthesized CLK, the dephosphorylation of hyperphosphorylated CLK, or both. Although *Clk* mRNA levels peak around dawn (Bae et al., 1998), *Clk* mRNA levels only vary ~3-fold over the circadian cycle, thus increased synthesis is less likely to account for the almost complete conversion of hyperphosphorylated to hypophosphorylated CLK over 3–6h. Phosphatases that would dephosphorylate CLK have not been identified, though PP1 and PP2a function within the core oscillator and PP2a regulatory subunit mRNAs cycle in abundance (Fang et al., 2007; Sathyanarayanan et al., 2004). Dephosphorylation of CLK after PER degradation may

impose another delay within the feedback loop that is necessary to convert CLK-CYC to a transcriptionally active form. This is likely the case for CLOCK in mammals since CLK is phosphorylated upon interaction with PER-CRY repressor complexes and returned to a dephosphorylated form when CLOCK-BMAL1 activates transcription (Lee et al., 2001; Spengler et al., 2009; Yoshitane et al., 2009).

IV. Light entrainment of the *Drosophila* circadian feedback loops

In animals, environmental cycles of light, temperature and/or social cues set the phase of (i.e. entrain) circadian oscillators so that overt rhythms in physiology, metabolism and behavior occur at the appropriate time of day. The most potent and reliable environmental cue is light, which mediates entrainment via different mechanisms depending on tissue type and species. In mammals, light is detected by melanopsin in retinal ganglion cells in the eye, which transmit signals to the "master clock" in the suprachiasmatic nucleus (SCN) through the retinohypothalamic tract (reviewed in (Golombek and Rosenstein, 2010)). These signals entrain circadian oscillators in the SCN, which relay information about their new phase via humoral signals to entrain oscillators in peripheral tissues (reviewed in (Dibner et al., 2010)). This two-stage entrainment process (one stage for the SCN and a second for peripheral oscillators) differs from that in *Drosophila* because almost all *Drosophila* oscillator cells either detect light directly or receive light information from photoreceptor cells. The cell-autonomous nature of circadian light entrainment is best illustrated in flies that use a *per*-promoter driven luciferase reporter gene (*per*-luc) to drive rhythms in bioluminescence in isolated wings, and antennae and probosci that can be entrained by light (Plautz et al., 1997). Thus, there is no "master clock" in *Drosophila* that entrains peripheral oscillators (and no closed circulatory system to efficiently send entrainment signals through even if there were a master clock).

Nevertheless, like the SCN clock, pacemaker cells in the *Drosophila* brain control locomotor activity rhythms, which can be monitored with great precision to detect light-induced shifts in oscillator phase. Light alters the phase of activity differently depending on when it is detected during the circadian cycle (Pittendrigh and Minis, 1964). A light pulse applied during the early night delays the phase of activity by up to 4h, a light pulse applied during the day (or subjective day if the flies are in kept in constant darkness) has little effect on activity phase, and a light pulse applied during the late night advances the phase of activity up to 3h (Myers et al., 1996; Saunders et al., 1994). Light induces phase shifts by delaying or advancing the phase of the core feedback loop. This section will focus primarily on the molecular mechanism that governs light-induced phase shifts, but will also discuss how light integrates with other environmental cues to shift circadian phase.

A. TIM Degradation, CRY and Other Circadian Photoreceptors

In 1996 three groups discovered that light induces the degradation of TIM in fly heads (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). Light drastically reduces TIM levels within 30 minutes, thereby destabilizing PER and shifting the phase of the core feedback loop. Although light's effect on TIM levels is unidirectional, how reduced levels of TIM are interpreted by the clock depends on when the light pulse is applied. Light-induced degradation of TIM during the early evening produces a phase delay because *tim* mRNA

levels are high, and new synthesis can replenish TIM levels within a few hours. During late night, light-induced degradation of TIM produces a phase advance because *tim* mRNA levels are low, thus premature loss of TIM resets the core loop to its normal state near dawn. Little or no phase shifts are seen during the actual or subjective day because TIM levels are normally very low and can't be further reduced. Thus, light-dependent degradation of TIM initiates a phase shift, and the levels of *tim* mRNA and protein determine the direction of the phase shift through their effect on the core loop. Light induces TIM degradation in the proteasome (Naidoo et al., 1999). Upon light exposure, TIM is phosphorylated by a tyrosine kinase based on pharmacological experiments (Naidoo et al., 1999), but this kinase has not been identified.

Though TIM is rapidly degraded after light exposure, TIM is not itself a photoreceptor. Loss of external eyes and ocelli or eliminating opsin-based photoreception via vitamin A depletion or phototransduction mutants reduces light sensitivity of the clock but doesn't abolish entrainment of locomotor activity rhythms by light (Blaschke et al., 1996; Hu et al., 1978; Ohata et al., 1998; Pearn et al., 1996), which suggests that other photoreceptors function to entrain circadian oscillators in brain pacemaker cells. A screen for mutants that disrupted rhythms in bioluminescence produced by *per*-luc identified CRYPTOCHROME (CRY) (Emery et al., 1998; Stanewsky et al., 1998), which is an ortholog of blue light photoreceptor cryptochromes in plants (Ahmad and Cashmore, 1993; Lin et al., 1998). The severely hypomorphic cry^b mutant abolishes bioluminescence rhythms in whole flies during and after entrainment in 12h light:12h dark (LD) cycles, but not after entrainment to temperature cycles, suggesting a defect in the light entrainment pathway (Stanewsky et al., 1998). Despite the loss of bioluminescence rhythms, cry^b flies are behaviorally rhythmic during and after LD entrainment, indicating that pacemaker cells continue to receive light information. However, cry^b flies can't be phase shifted by brief (e.g. 10 minute) pulses of light (Stanewsky et al., 1998), and remain rhythmic in constant light after entrainment in LD cycles (Emery et al., 2000a), in contrast to wild type flies that become arrhythmic in constant light (Konopka et al., 1989). Defects in behavioral rhythms due to cry^b can be rescued by expressing CRY in brain pacemaker neurons, demonstrating that CRY functions cell-autonomously (Emery et al., 2000b). Taken as a whole, these studies demonstrate that CRY is a cell-autonomous photoreceptor that resets the phase of behavioral rhythms in *Drosophila* to short pulses of light, but is not required for light entrainment to LD cycles.

Loss of phototransduction in external eyes (i.e. compound eyes and ocelli) by removing the NORP-A phosholipase C together with CRY further weakens, but doesn't abolish, light entrainment of behavioral rhythms (Stanewsky et al., 1998), indicating that other photoreceptors and/or phototransduction cascades function to entrain locomotor activity rhythms to light. The remaining photoreceptors capable of mediating light entrainment of locomotor activity are a pair of extraretinal eyes called the Hofbauer-Buchner (H-B) eyelet; eliminating all external photoreceptors, the H-B eyelet and CRY renders flies unentrainable to light (Helfrich-Forster et al., 2001). Eliminating phototransduction in external eyes along with CRY abolishes core feedback loop function in all brain clock neurons except the LNvs and dorsal neuron 1s (DN1s), which implies that the H-B eyelet relays light information to this subset of clock neurons to mediate behavioral rhythms (Helfrich-Forster et al., 2001).

Thus, behavioral rhythms can be entrained by independent light input pathways in external eyes, the H-B eyelet, and CRY, but light resetting to short pulses is mediated by CRY.

Since TIM is degraded in response to short pulses of light, photodetection by CRY must initiate a cell-autonomous pathway leading to TIM degradation (Fig. 4). Indeed, CRY binds directly to TIM in a light-dependent manner, which irreversibly commits TIM to degradation in the proteasome (Busza et al., 2004; Ceriani et al., 1999; Dissel et al., 2004; Naidoo et al., 1999). CRY is also degraded in the proteasome upon activation by light, but CRY degradation occurs more slowly than TIM degradation (Busza et al., 2004; Dissel et al., 2004; Lin et al., 2001). Although light-induced TIM and CRY degradation were thought to be controlled independently (Busza et al., 2004; Sathyanarayanan et al., 2008), more recent evidence suggests that they are linked (Peschel et al., 2009). Light-dependent degradation of both TIM and CRY is mediated by the F-box protein JETLAG (JET) (Koh et al., 2006; Peschel et al., 2009; Peschel et al., 2006). However, TIM and CRY are degraded sequentially due to a higher affinity of JET for TIM than CRY (Peschel et al., 2009). Thus, when CRY is activated by light it becomes a substrate for JET binding, and binding of activated CRY to TIM makes TIM an even higher affinity substrate for JET than CRY. Light-induced degradation of TIM requires the COP9 signalosome (Knowles et al., 2009), which apparently acts downstream of JET to promote TIM (and maybe CRY) degradation in the proteasome. Mutants in *cry*, *jet* and the COP9 signalosome are all rhythmic in constant light (Emery et al., 2000a; Knowles et al., 2009; Koh et al., 2006; Peschel et al., 2006), which suggests that these genes function in the same pathway to mediate light-dependent resetting of circadian oscillators in pacemaker cells that control rhythmic activity.

In contrast to CRY mediated phase resetting to light, the molecular and cellular mechanisms by which the H-B eyelet and external photoreceptors in the compound eye and ocelli have only just begun to be characterized. The H-B eyelet projects to the large subset of ventrolateral neurons (lLNvs), which are necessary for light-induced phase resetting at dawn and increase their firing rate when stimulated by light (Shang et al., 2008; Sheeba et al., 2008). The lLNvs project throughout the optic lobe, including the vicinity of the small subset of ventrolateral neurons (sLNvs) (Helfrich-Forster et al., 2007), suggesting that light information from the H-B eyelet may be sent to lLNvs, and then on to the sLNvs to mediate phase resetting near dawn (Shang et al., 2008; Sheeba et al., 2010). How compound eyes and ocelli contribute to phase resetting is not known.

B. Resetting Behavioral Rhythms – Cellular and Sensory Integration

Since TIM is rapidly degraded in fly heads after light exposure, it has been assumed that light leads to TIM degradation uniformly in all oscillator cells including the LNv pacemaker neurons. Although TIM is degraded in all brain oscillator neurons including the LNvs when phase advancing light pulses are applied at ZT21, TIM is not degraded in LNvs (but is degraded in dorsal brain oscillator neurons) when phase delaying light pulses are applied at ZT15 (Tang et al., 2010). Overexpressing JET in the LNvs of a *jet* null mutant enables light-induced TIM degradation at ZT15, but doesn't produce a phase delay in behavior (Tang et al., 2010), thus TIM degradation in LNvs is neither necessary nor sufficient for phase delays. If CRY expression is knocked down in only LNvs, light-induced phase delays and

advances are both blocked, indicating that LNvs are necessary for behavioral phase shifts even though TIM is not degraded by a light pulse at ZT15 in these cells (Tang et al., 2010).

These results suggest that light-sensitive dorsal brain oscillator neurons signal through LNvs to promote light-induced phase delays. A good candidate for dorsal neurons that initiate light-induced phase delays are the DN1s because a subset of these neurons are CRY positive (Benito et al., 2008; Yoshii et al., 2008), light pulses at ZT15 consistently induce TIM degradation in these neurons (Tang et al., 2010), and LNvs signal to DN1s via PDF while DN1s may signal LNvs via projections to LNv cell bodies (Helfrich-Forster et al., 2007; Kaneko and Hall, 2000; Zhang et al., 2010a). Whether DN1s or other dorsal oscillator neurons mediate light-dependent phase delays via LNvs, it is clear that the standard cell-autonomous CRY-dependent degradation of TIM paradigm for light-induced phase resetting doesn't explain how light induces phase delays in locomotor activity.

During LD conditions *Drosophila* display morning and evening peaks in activity that anticipate the lights-on and lights-off transitions, respectively. These activity peaks are controlled by separate sets of brain oscillator neurons, where morning activity is controlled by LNvs that express the PDF neuropeptide and evening activity is controlled by LNds and the PDF negative 5th sLNv (Grima et al., 2004; Stoleru et al., 2004). The phase of these morning and evening activity peaks adjusts to seasonal differences in photoperiod and temperature. When photoperiod is long and temperature is high in the summer, flies are more active in before dawn and after dusk, but when photoperiod is short and temperatures are low, flies are more active after dawn and before dusk (Majercak et al., 1999). This behavioral plasticity is influenced by molecular mechanisms that act to adjust oscillator phase including temperature sensitive splicing of *per* intron 8 (Collins et al., 2004; Low et al., 2008; Majercak et al., 2004; Majercak et al., 1999), temperature sensitive splicing of the last *tim* intron (Boothroyd et al., 2007), and light-induced transcription of *tim* only at low temperatures (Chen et al., 2006). Communication between morning cells that serve as dominant clocks in the dark and evening cells that serve as dominants clocks in light (along with a subset of DN1s) is also important for controlling seasonal differences in activity (Cusumano et al., 2009; Murad et al., 2007; Stoleru et al., 2007; Zhang et al., 2009). Integration of light and temperature information was recently shown to occur in the DN1s, which alter the phase and amplitude of morning and evening activity peaks depending on temperature and light intensity (Zhang et al., 2010a; Zhang et al., 2010b). How temperature and light information is integrated in DN1s to modulate morning and evening activity will no doubt be the subject of future studies.

Social interactions among flies also alters locomotor activity rhythms. Flies entrained to a particular circadian phase influence the activity of flies entrained to a different phase (Levine et al., 2002b), and courtship interactions between males and females shift activity to the night and away from dusk (Fujii et al., 2007). In both of these cases olfactory cues are responsible for the activity changes (Fujii et al., 2007; Krupp et al., 2008; Levine et al., 2002b), which indicates that olfactory signaling, which is also under circadian control (Krishnan et al., 1999; Krishnan et al., 2008; Tanoue et al., 2008; Tanoue et al., 2004), is likely integrated with light and temperature to determine the timing of activity. Several excellent reviews have been published that provide a more in-depth description of the

discovery, function and modulation of morning and evening oscillators (Allada and Chung, 2010; Choi and Nitabach, 2010; Dubruille and Emery, 2008), and insight into the mechanisms by which temperature entrains circadian oscillators to effect seasonal adaptation (Chen et al., 2007; Dubruille and Emery, 2008; Glaser and Stanewsky, 2007).

C. Other Functions of *Drosophila* CRY

Bioluminescence rhythms in *cry*[b] flies bearing *per*-luc can be entrained by temperature cycles, but not LD cycles (Stanewsky et al., 1998). Since the vast majority of *per*-luc expression is from peripheral oscillators, this result implies that CRY is also required for light entrainment of circadian oscillators in peripheral tissues. While this may be the case, CRY is also required for oscillator function *per se* in at least some peripheral tissues including the eye, the antenna, Malpighian tubules (the fly kidney equivalent), and forelegs (Collins et al., 2006; Ivanchenko et al., 2001; Krishnan et al., 2001; Levine et al., 2002a). The evidence that CRY is required for oscillator function differs depending on the peripheral tissue. In antenna, rhythmic output in the form of rhythmic electroantennagram (EAG) responses to odors is severely impaired or abolished in *cry*[b] mutants during and after entrainment to LD or temperature cycles (Krishnan et al., 2001). This loss of EAG rhythms in *cry*[b] flies is due to the disruption of rhythmic expression within the core feedback loop, as reported by *per*-luc and *tim*-luc in antennae cultured during and after LD entrainment and after temperature entrainment (Krishnan et al., 2001; Levine et al., 2002a). This result is reminiscent of the arrhythmicity seen in mice lacking mCRY1 and mCRY2, which repress CLOCK-BMAL1 mediated transcription (Griffin et al., 1999; Kume et al., 1999; Lee et al., 2001; Preitner et al., 2002).

These experiments show that CRY is required for oscillator function in antennae, but it is difficult to know whether CRY also serves to entrain antennae to light cycles since EAGs and gene expression in *cry*[b] flies were arrhythmic during LD. Loss of rhythmic *per*-luc and *tim*-luc expression in forelegs from *cry*[b] flies during and after LD entrainment implies that CRY also functions within the oscillator, in contrast to the situation in wing where *per*-luc and *tim*-luc expression is rhythmic in *cry*[b] flies during and after LD entrainment (Levine et al., 2002a). In Malpighian tubules from *cry*[b] flies, loss of TIM degradation to light pulses suggests that CRY is required for phase resetting, but cycling of PER and TIM levels during LD cycles suggests that entrainment occurs through some other mechanism (Ivanchenko et al., 2001). Rhythms in *tim*-luc and PER and TIM protein cycling were abolished in *cry*[b] mutants during DD, demonstrating that CRY is necessary for oscillator function (Ivanchenko et al., 2001). The role CRY plays within fly peripheral oscillators was investigated in fly heads, where >80% of clock gene expression is from peripheral oscillators in the eye (Glossop and Hardin, 2002). In *cry*[b] flies, expression of CLK-CYC activated genes was constantly near peak levels, suggesting that CRY represses CLK-CYC transcription (Collins et al., 2006). Consistent with this result, CRY collaborates with PER to inhibit CLK-CYC transcription in eyes and S2 cells, but not in sLNvs (Collins et al., 2006). If CRY is indeed required for rhythmic transcription within peripheral oscillators, it is difficult to understand how oscillator function persists in *cry*[b] fly heads after temperature entrainment (Stanewsky et al., 1998). Despite this inconsistency, CRY and PER dependent repression of CLK-CYC transcription is completely in line with mCRY1 and mCRY2

function in mammals, which repress CLOCK-BMAL1 transcription in collaboration with mPER2 (Griffin et al., 1999; Kume et al., 1999; Lee et al., 2001; Preitner et al., 2002). How *Drosophila* CRY carries out independent functions in brain pacemaker cells and peripheral oscillators is not known, though other insects (e.g. Monarch butterflies, mosquito, moth) carry out photoreceptor and transcriptional repressor function using two independent *cry* genes (Yuan et al., 2007; Zhu et al., 2008).

V. Summary and Conclusions

In this review I have covered several topics related to the interlocked feedback loops that keep circadian time in *Drosophila*. There are several take home points from this review. First, many feedback loop components have been identified in *Drosophila* that carry out specific roles in regulating time-dependent transcription including the CLK and CYC activators and PER and TIM repressors. Although the *per* and *Clk* feedback loops drive rhythmic transcription in opposite phases of the circadian cycle, the *per* loop controls the *Clk* loop and functions to keep circadian time. Second, time delays are built into multiple steps of the core feedback loop to achieve a period of ~24h. These delays are controlled post-transcriptionally via regulated synthesis, accumulation, nuclear localization, degradation, and activity of transcriptional activators and repressors. Third, light shifts the *Drosophila* circadian oscillator to a new circadian phase by promoting TIM degradation. In most oscillator cells CRY acts as a cell-autonomous photoreceptor that binds TIM to induce JET-dependent degradation of both TIM and CRY in the proteasome after light exposure, but also functions as a transcriptional repressor to support core feedback loop function in many peripheral oscillator cells. Retinal photoreceptors, extraretinal photoreceptors and CRY all contribute to the entrainment of locomotor activity pacemaker cells to light-dark cycles, but other environmental factors such as temperature and olfactory cues also integrate with light information to modulate fly activity according to the season and social context. Understanding how the circadian clock is entrained by environmental cues and maintains a ~24h period under constant conditions in a simple model organism such as *Drosophila* will no doubt continue to provide important insights into the basic mechanisms that control circadian clock function in mammals.

Despite the progress that has been made in identifying components of the circadian feedback loops, major gaps in our understanding of how these components contribute to circadian timekeeping remain. The *Clk* feedback loop controls rhythmic transcription that peaks near dawn, but is dependent on the core loop. Though rhythmic outputs including locomotor activity are dependent on *Pdp1ε/δ* within the *Clk* loop (Benito et al., 2008; Benito et al., 2010; Zheng et al., 2009), we don't know if this feedback loop contributes to circadian timekeeping. The *Neurospora* interlocked feedback loop contributes to oscillator stability (Cheng et al., 2001), whereas the mammalian interlocked loop influences period length and phase shifting to light (Preitner et al., 2002). Manipulating components of the *Clk* loop such as *vri* and *Clk* activators will provide the tools to test whether the *Clk* loop contributes to timekeeping function. Identifying *Clk* activators would fill a gap in our understanding of the *Clk* loop since we would be able to determine whether these activators both determine clock cell identity and maintain *Clk* transcription in adults. Likewise, building tools to inducibly manipulate *vri* expression in adult flies would enable experiments to test whether *vri* is

required for circadian timekeeping and/or output rhythms. The traditional role for *cwo* as a repressor of CLK-CYC transcription is based mainly on *in vitro* and cell culture data, and doesn't jibe with *in vivo* data suggesting that *cwo* contributes to activating CLK-CYC transcription (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008). Better tools for CWO detection and biochemistry are needed to determine how this gene contributes to the circadian timekeeping mechanism.

A major question in circadian biology is how circadian feedback loops maintain periods of ~24h. While it is clear that delays are incorporated into various steps of the feedback loop, we are now beginning to understand the mechanisms that control these delays. The delayed accumulation of PER repressor complexes in the cytoplasm (at least in the LNvs) involves regulated translation by *tyf* (Lim et al., 2011), but it is not as clear how much PER phosphorylation by DBT and PER stabilization by TIM and PP1 and PP2a phosphatases contribute to slowing the rate of PER accumulation in the cytoplasm (Fang et al., 2007; Kloss et al., 1998; Kloss et al., 2001; Ko et al., 2002; Price et al., 1998; Sathyanarayanan et al., 2004). Determining whether additional kinases, phosphatases, E3 ubiquitin ligases, or other factors promote PER degradation in the cytoplasm, and whether PER translation is regulated in other oscillator tissues, will be essential for understanding the extent to which these processes contribute to delays in PER cytoplasmic accumulation. SGG, CK2 and an as yet unidentified proline directed kinase phosphorylates PER and/or TIM to promote their nuclear localization (Akten et al., 2003; Ko et al., 2010; Lin et al., 2002a; Martinek et al., 2001), thus enabling these proteins to bind CLK-CYC and inhibit transcription. Movement of PER-TIM repressor complexes into the nucleus coincides with CLK phosphorylation, transcriptional repression, and the removal of CLK-CYC from E-boxes (Kim et al., 2007; Menet et al., 2010; Yu et al., 2006; Yu et al., 2009). The kinases that phosphorylate CLK have not been identified, though it is possible that NMO phosphorylates CLK to promote CLK degradation (Yu et al., 2011). To understand how PER complexes repress transcription, it is important to determine which kinases phosphorylate CLK, where CLK is phosphorylated, and how such phosphorylation represses transcription and induces CLK-CYC release from E-boxes. Kinases also contribute to light-induced and clock-dependent TIM degradation (Naidoo et al., 1999), but the identity of these kinases and their target sites on TIM haven't been determined, but are essential to understanding how the clock maintains a ~24h period since the loss of TIM promotes PER degradation. Great strides have been made to understand how PER degradation is regulated in the nucleus. NMO phosphorylation of PER at S596 primes further phosphorylation by DBT to alter PER conformation, thus delaying phosphorylation of other DBT target sites including S47, which triggers SLIMB binding and PER degradation (Chiu et al., 2011). Determining the temporal profile of DBT phosphorylation sites on PER and the requirements for phosphorylation at specific sites would provide an unprecedented understanding of a process that is essential for setting circadian period. Once PER is degraded, CLK is returned to a dephosphorylated state that enables E-box binding and transcriptional activation by CLK-CYC (Kim and Edery, 2006; Yu et al., 2006; Yu et al., 2009). Identifying phosphatases that act on CLK during this phase of the circadian cycle will be key to understanding the extent to which CLK dephosphorylation contributes to period determination.

Although the basic molecular events that control light-induced phase shifting by CRY have been characterized, it is apparent that CRY mediates phase delays in locomotor activity independent of TIM degradation. Where light is detected to induce phase delays and how CRY acts to shift the LNv clock represent major gaps in our understanding. A subset of DN1s send projections to LNvs (Helfrich-Forster et al., 2007; Kaneko and Hall, 2000), and certain DN1s also express CRY (Benito et al., 2008; Yoshii et al., 2008). If the CRY positive DN1s also projection to LNvs, these cells could receive light and signal the LNvs just as the H-B eyelet receives light and signals the LNvs (Helfrich-Forster et al., 2001). CRY is required for light shifting in LNvs in the absence of TIM degradation (Tang et al., 2010), which suggests that CRY induces phase delays in LNvs by altering the level or activity of some other core feedback loop component. Determining how LNvs receive light signals during the early evening and shift the oscillator in a CRY-dependent manner is critical for a comprehensive understanding of behavioral phase resetting to light. However, CRY is not the only photoreceptor capable of entraining behavioral rhythms to LD cycles. H-B eyelet neurons may effect light-induced entrainment of behavior by signaling lLNvs, which then relay this information to the sLNvs (Shang et al., 2008; Sheeba et al., 2010). Additional studies are needed to solidify this pathway, define the cellular pathways through which photoreceptors in the compound eyes and ocelli effect light entrainment, and determine how light information from other photoreceptor cells effect shifts in the sLNv molecular oscillator. Lastly, recent work shows that light and temperature information is integrated in DN1s to modulate the pattern of locomotor activity rhythms during LD conditions (Zhang et al., 2010a; Zhang et al., 2010b). How temperature information is received by DN1s, integrated with CRY-dependent light signals, and sent to LNvs to modify behavioral activity are important challenges for future studies, as is the integration of olfactory-based social cues that also modulate behavioral activity.

Although interlocked transcriptional feedback loops function to keep circadian time in eukaryotes, this is not the case in cyanobacteria. The core timekeeping mechanism in cyanobacteria does not require transcription, but instead consists of a ~24h phosphorylation/ dephosphorylation cycle mediated by the KaiA, KaiB and KaiC proteins. When KaiA, KaiB, KaiC are incubated with ATP, KaiA stimulates KaiC autophosphorylation and KaiB blocks KaiA to stimulate KaiC dephosphorylation, which results in a KaiC phosphorylation cycle with a ~24h period (Nakajima et al., 2005). Although this cycle necessarily occurs independent of transcription, the Kai oscillator drives rhythms in virtually all transcripts by periodically altering chromosome compaction (Smith and Williams, 2006; Woelfle and Johnson, 2006; Woelfle et al., 2007). Although the core KaiC phosphorylation oscillator functions under most conditions *in vitro*, transcriptional rhythms contribute to the stability and entrainment of the Kai oscillator *in vivo* (see review by (Dong and Golden, 2008)). Recent studies show that circadian clocks in some eukaryotic organisms and cell types can operate in the absence of rhythmic transcription. Red blood cells lack nuclei and mitochondria, and thus no transcription can occur. However, the dimerization of peroxyredoxin enzymes, which function to reduce reactive oxygen species, cycles with a circadian rhythm that is temperature entrainable (O'Neill and Reddy, 2011). In fact, binding of reactive oxygen species is what catalyzes the formation of peroxyredoxin dimers, which are then converted back to monomers via reduction by thioredoxin (O'Neill and Reddy,

2011). A similar phenomenon has been observed in the unicellular eukaryotic algae *Ostreococcus tauri*, which cease transcriptional activity in the dark, yet circadian oscillations in peroxyredoxin dimerization persist (O'Neill et al., 2011). Rhythms in peroxyredoxin dimerization also occur under conditions when the transcriptional feedback loops are operating in *Ostreococcus* and mouse embryonic fibroblasts, thus both circadian oscillators function simultaneously in the same cell (O'Neill and Reddy, 2011; O'Neill et al., 2011). These oscillators apparently interact at some level as mutant mouse embryonic fibroblasts that abolish transcriptional feedback loops lengthen the period of peroxyredoxin dimerization rhythms (O'Neill and Reddy, 2011). Given that peroxyredoxin dimerization rhythms are seen in mouse embryonic fibroblasts, rhythms in peroxyredoxin dimerization likely occur in *Drosophila*. Once the oscillator that drives peroxyredoxin dimerization rhythms is identified, it will be fascinating to determine the extent to which this oscillator influences transcription-based oscillators.

Acknowledgments

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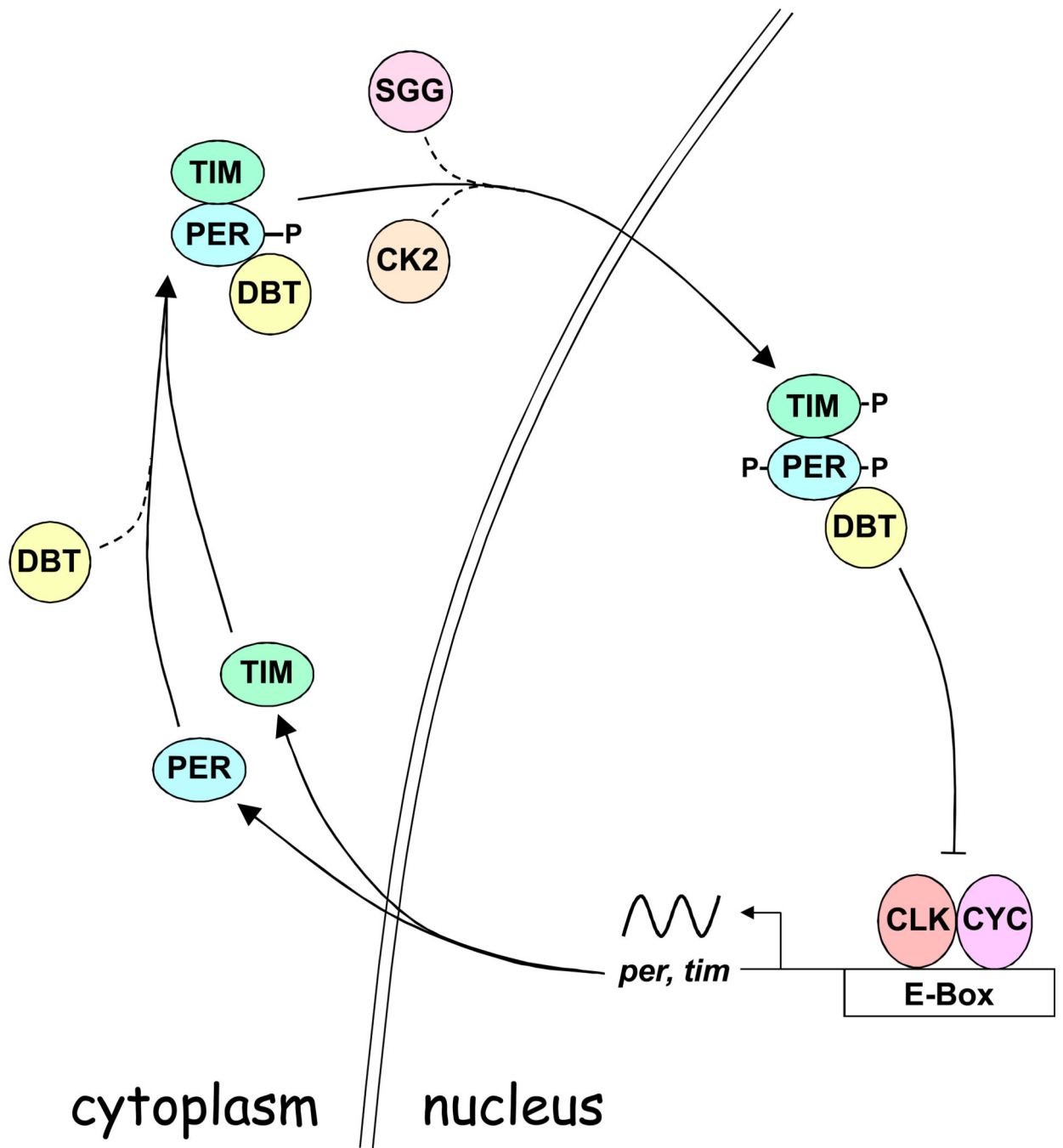
Figure 1.
The core feedback loop. All gene, regulatory element, and protein names are as defined in the text. Double line, nuclear envelope; sinusoidal line, mRNA rhythm; solid arrows, steps in the pathway; blocked line, repression; dashed line, step employing protein activity; P, phosphorylation. See text for detailed description.
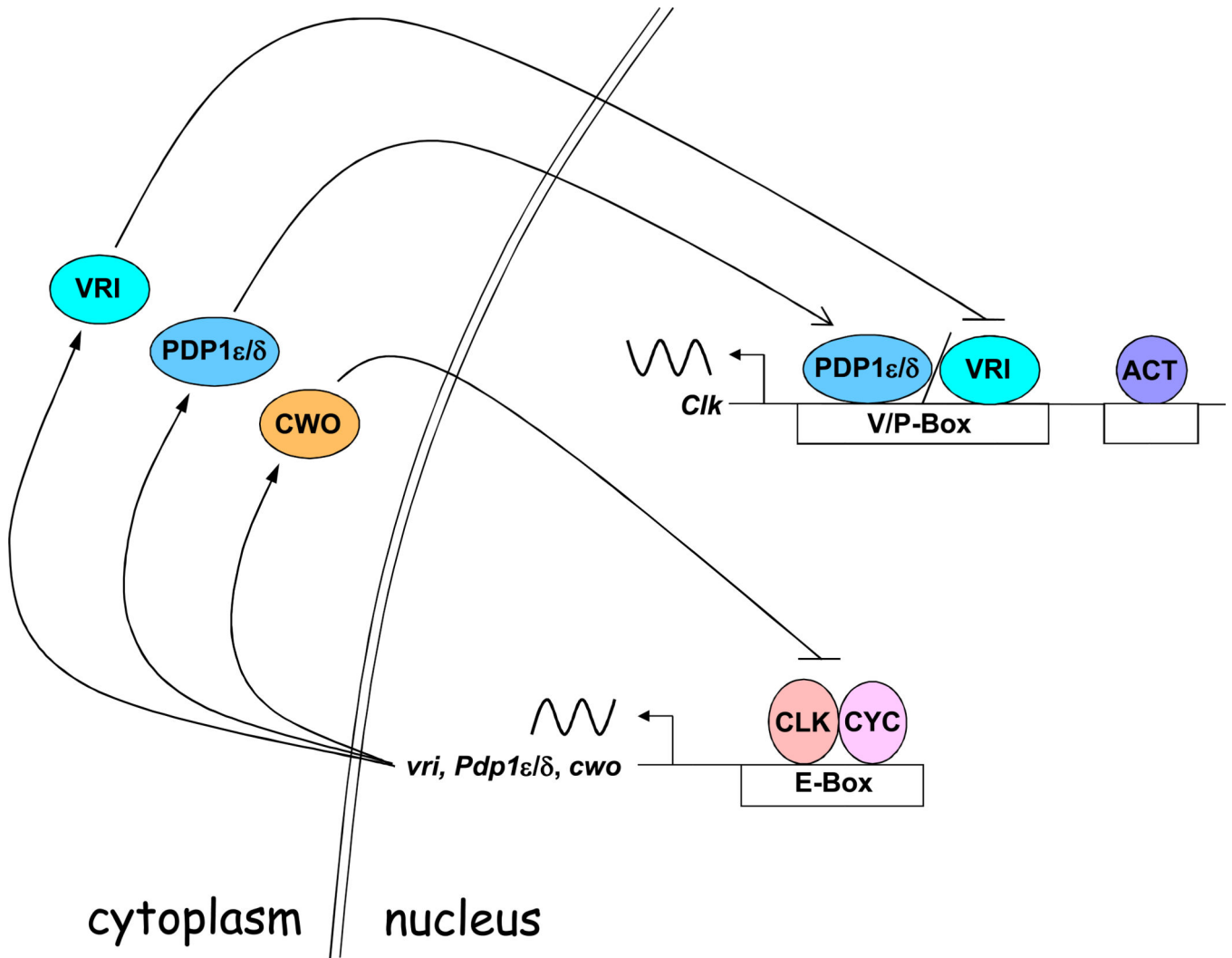
Figure 2.
The *Clk* feedback loop. All gene, regulatory element, and protein names are as defined in the text. All symbols are as defined in Figure 1. ACT, *Clk* activator; open arrow, transcription activation; antiphase sinusoidal line, antiphase mRNA rhythm; backslash, binding by one or the other protein. See text for detailed description.
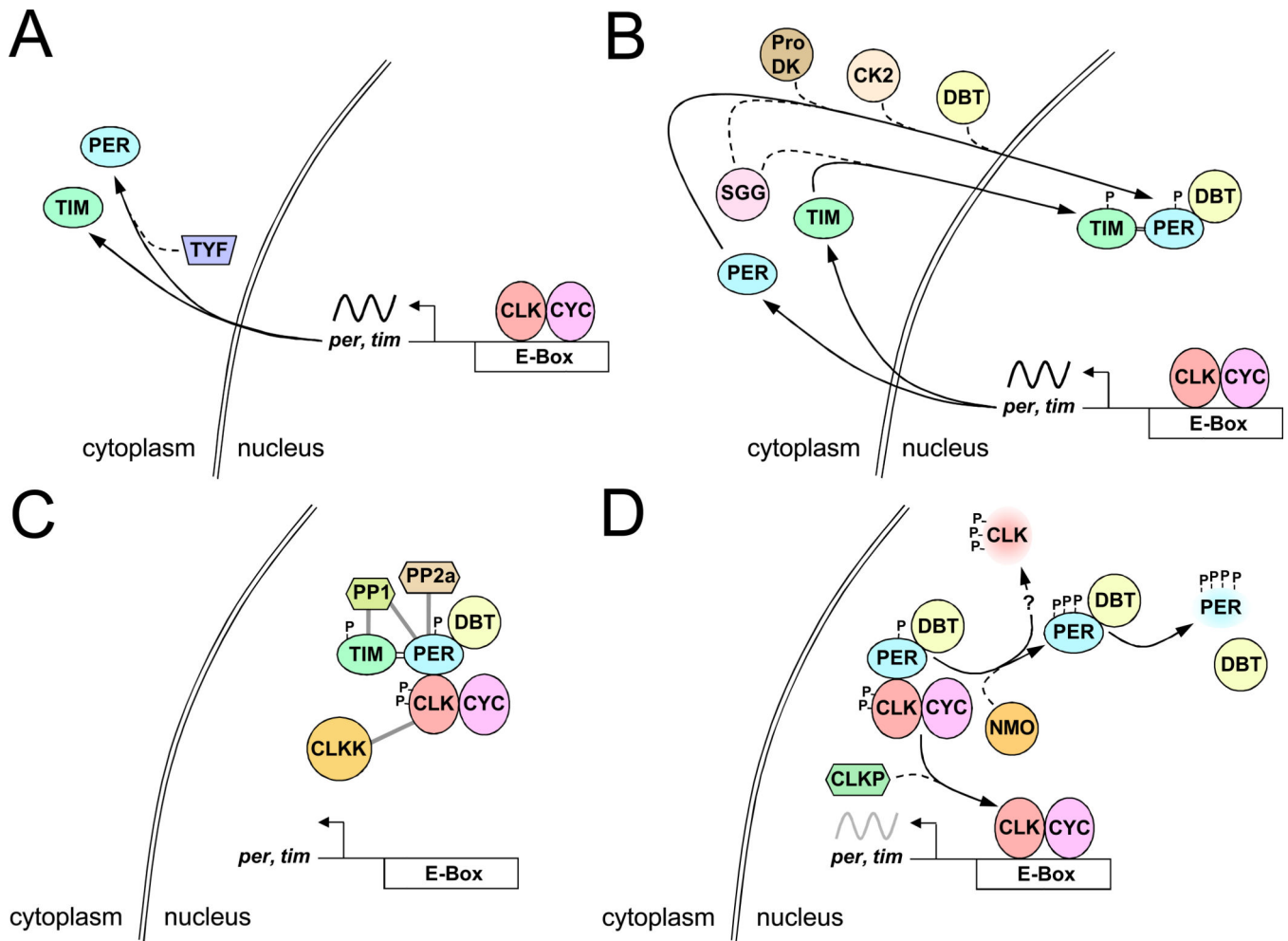
Figure 3.
Post-transcriptional regulatory steps within the core feedback loop. All gene, regulatory element, and protein names are as defined in the text. All symbols are as defined in Figure 1. A. Delay in PER synthesis from ~ZT6-ZT12. B. Movement of PER and TIM into the nucleus from ~ZT16-20. C. Stabilization of nuclear PER as PER complexes repress CLK-CYC transcription from ~ZT16-ZT0. D. Release of PER repression and reactivation of CLK-CYC transcription from ~ZT3-ZT6. Double bar, stabilizing activity; CLKK, CLK kinase; ProDK, proline-directed kinase; gray bars, kinase and phosphatase targets; faded protein symbols, protein degradation; ?, putative direct effect; gray sinusoidal line, initiation of transcription. See text for detailed description.
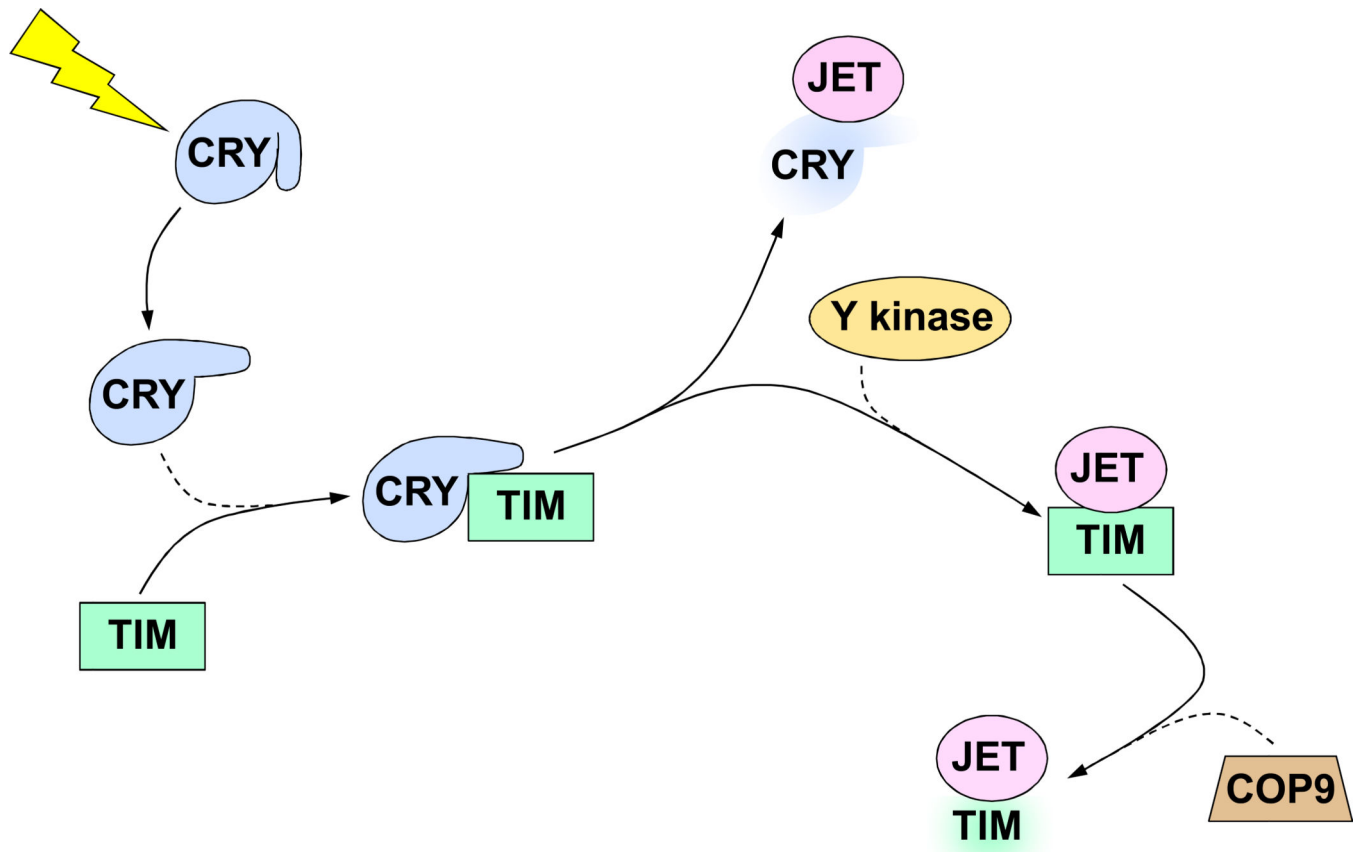
Figure 4.
Light-induced phase resetting mechanism. All gene, regulatory element, and protein names
are as defined in the text. All symbols are as defined in Figures 1 and 3. Y kinase, tyrosine
kinase. See text for detailed description.