

# The Circadian Timekeeping System of *Drosophila* Review

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Daily rhythms in behavior, physiology and metabolism are controlled by endogenous circadian clocks. At the heart of these clocks is a circadian oscillator that keeps circadian time, is entrained by environmental cues such as light and activates rhythmic outputs at the appropriate time of day. Genetic and molecular analyses in *Drosophila* have revealed important insights into the molecules and mechanisms underlying circadian oscillator function in all organisms. In this review I will describe the intracellular feedback loops that form the core of the *Drosophila* circadian oscillator and consider how they are entrained by environmental light cycles, where they operate within the fly and how they are thought to control overt rhythms in physiology and behavior. I will also discuss where work remains to be done to give a comprehensive picture of the circadian clock in *Drosophila* and likely many other organisms.

## Introduction

Circadian clocks regulate rhythmic phenomena in animals, plants, fungi and even some prokaryotes. In *Drosophila*, these clocks control a number of rhythmic outputs, including adult emergence (eclosion), locomotor activity and olfactory physiology. The molecular nature of the *Drosophila* clock is being elucidated at a rapid pace, and serves as a good model for clocks in other animals given that many of the components have been conserved. Moreover, clocks enable an organism to adapt to daily environmental cycles by mechanisms that are starting to be uncovered in *Drosophila*. After an initial description of *Drosophila* clock components, I will focus on the intracellular feedback loops that lie at the center of the circadian oscillator and how they are entrained by light. I will then describe where these oscillators operate in adult flies, the rhythmic outputs they are known or suspected to control, and how these rhythms are proposed to be controlled, before concluding with a perspective on the direction and significance of future work on the *Drosophila* clock.

## Components of the *Drosophila* Circadian Clock

Genetic analysis has revealed a number of 'clock' genes that are critical for clock function in *Drosophila* (reviewed in [1]). These genes can be divided up roughly according to the molecular nature of their

products, in particular, whether they are thought to activate transcription, to repress transcription, to alter protein stability or subcellular localization, or to degrade proteins. In the transcriptional activator category are two basic-helix-loop-helix/PAS domain transcription factors, Clock (Clk) and Cycle (Cyc), which form heterodimers to activate transcription [2–4], and one basic-leucine zipper transcription factor, Par domain protein 1 $\epsilon$  (PDP1 $\epsilon$ ) [5]. The transcriptional repressor category includes Period (Per), another PAS domain protein, and Timeless (Tim), which function as heterodimers to inhibit Clk–Cyc function [4], and the basic-leucine zipper transcriptional repressor Vri [5–7].

The category of proteins that alter protein stability and subcellular localization includes kinases that destabilize proteins that control clock gene transcription: the mammalian casein kinase 1 $\epsilon$  (CK1 $\epsilon$ ) homolog Doubletime (Dbt), also known as Discs overgrown or Dco, destabilizes Per [8,9]; casein kinase 2 (CK2), which has  $\alpha$  and  $\beta$  subunits, destabilizes Per and also affects its nuclear localization [10,11]; and the glucose synthase kinase 3 (GSK3) homolog Shaggy (Sgg) phosphorylates Tim to promote nuclear localization of Per–Tim heterodimers [12]. In contrast to the destabilizing effects of the protein kinases, protein phosphatase 2a (PP2a), which has regulatory subunits Twins (Tws) and Widerborst (Wdb), stabilizes Per via dephosphorylation [13]. The protein degradation category includes the F-Box/WD40 protein Slimb (Slmb), which targets phosphorylated Per for degradation in the proteasome [14,15].

Having introduced the key components of the *Drosophila* clock, in the next section I will focus on their roles within the circadian oscillator.

## Molecular Circuitry of the *Drosophila* Circadian Oscillator

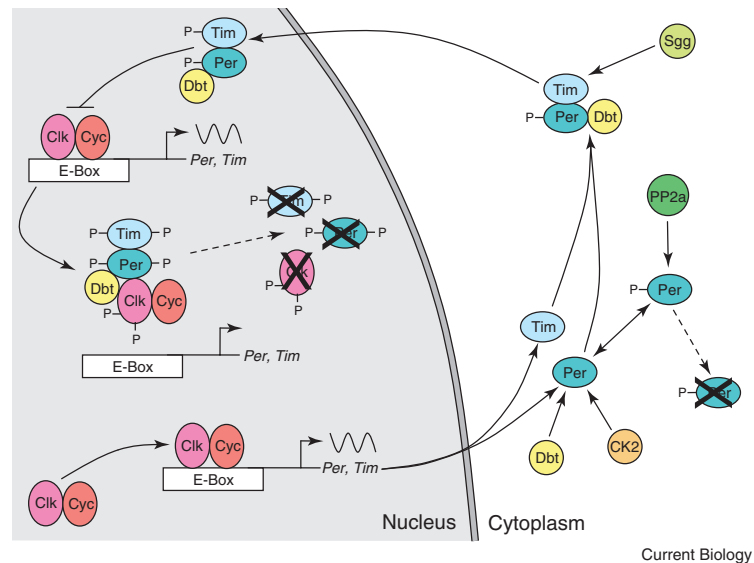
The *Drosophila* circadian oscillator is composed of two intracellular feedback loops in gene expression: a *Per/Tim* loop and a *Clk* loop [16,17]. Within these feedback loops, rhythmic transcription of particular clock genes is controlled via feedback from their own protein products. Post-translational mechanisms control the levels and subcellular localization of clock proteins so that transcriptional feedback occurs at the appropriate time of day. These feedback loops use different mechanisms to regulate transcription in different phases of the circadian cycle, yet are interlocked by their requirement for Clk–Cyc dependent transcription. The *Per/Tim* loop is required for the function of both loops, and will thus be described first.

### The *Per/Tim* Feedback Loop

To initiate the *Per/Tim* feedback loop, Clk–Cyc heterodimers bind E-box regulatory elements from mid-day through early night, thereby activating

Figure 1. Model of the *Per/Tim* feedback loop.

Clk–Cyc heterodimers bind to E-boxes and activate transcription of *Per* and *Tim*. As *Per* is produced it is phosphorylated by Dbt and CK2, which leads to its degradation. *Tim* binds to, and stabilizes, phosphorylated *Per*, which remains bound to Dbt. *Per* is also stabilized by PP2a, which removes phosphates that were added to *Per*. The *Tim–Per–Dbt* complexes are phosphorylated by Sgg which, in concert with phosphorylation by CK2, promotes their transport into the nucleus. The *Tim–Per–Dbt* complexes then bind to Clk–Cyc, thereby removing Clk–Cyc from the E-box and inhibiting *Per* and *Tim* transcription. *Per* and Clk are then destabilized, via Dbt phosphorylation, and degraded, whereas *Tim* degradation (at least in response to light) is triggered by tyrosine phosphorylation. The accumulation of non-phosphorylated (or hypophosphorylated) Clk



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leads to heterodimerization with *Cyc* and another cycle of *Per* and *Tim* transcription. Solid lines with arrow, sequential steps in the feedback loop; blocked line, inhibitory interaction; wavy line, *Per* and *Tim* mRNA; double arrow line, reversible phosphorylation; dashed lines, proteasomal degradation; black X, degraded proteins; P, protein phosphorylation; double line, nuclear membrane.

transcription of the *Per* and *Tim* genes (Figure 1) [4,18–20]. The levels of *Per* and *Tim* transcripts peak early in the night, whereas *Per* and *Tim* proteins do not accumulate to peak levels until late evening [16,21–26]. This delay is the result of phosphorylation dependent destabilization of *Per* by Dbt, and possibly also CK2, followed by stabilization of phosphorylated *Per* by *Tim* binding [8,9,11,27]. *Per* is also stabilized by PP2a, which is thought to remove the phosphates added by Dbt and CK2 [13].

Dbt remains bound to *Per* to form a *Per–Tim–Dbt* complex, and the entire complex (or possibly just *Tim*) is translocated into the nucleus upon Sgg-dependent *Tim* phosphorylation and CK2-dependent *Per* phosphorylation [10–12,28–30]. Once in the nucleus, *Per* continues to be phosphorylated by Dbt, and this phosphorylation potentiates *Per*'s ability to repress transcription [27]. *Per* appears to be a more potent inhibitor of Clk–Cyc dependent transcription than *Per–Tim* [29,31], consistent with the observation that *Tim* falls to low levels several hours before *Per* [26].

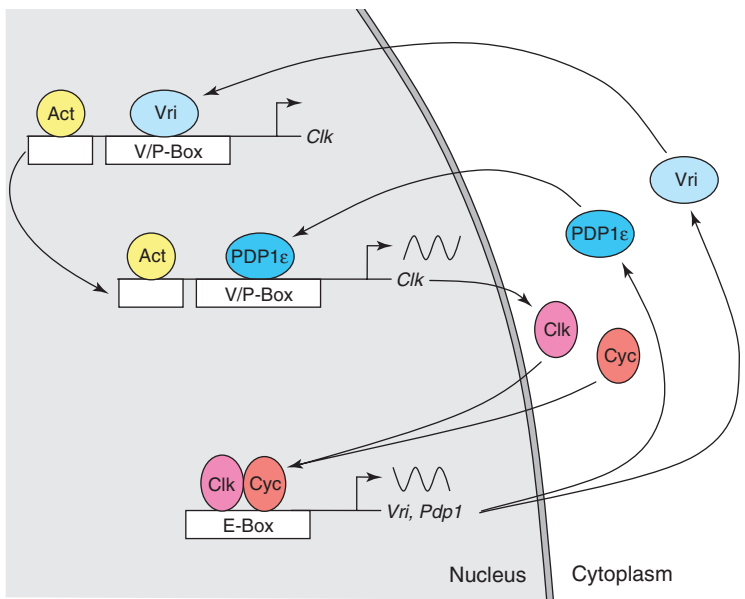
From the results of *in vitro* experiments, *Per* is thought to repress Clk–Cyc dependent transcription by binding to Clk and inhibiting the DNA binding activity of Clk–Cyc dimers [32]. Recent *in vivo* analysis has not only confirmed this mode of regulation, but also suggests that Dbt dependent phosphorylation destabilizes Clk, explaining the coincidence between phospho-*Per* and phospho-Clk levels (W. Yu, personal communication). In addition, a more stringent extraction procedure revealed that hypophosphorylated Clk accumulates in antiphase to hyperphosphorylated Clk; thus, hypophosphorylated Clk accumulates in phase with *Per*, *Tim* and other E-box/Clk-Cyc dependent transcripts (W. Yu, personal communication). This suggests a model in which hypophosphorylated, and thus stable, Clk accumulates from declining levels of

*Clk* mRNA (see below) and activates E-box dependent transcription, thus starting the next transcriptional cycle (Figure 1). In addition to activating *Per* and *Tim*, Clk–Cyc directly activates *Vri* and *Pdp1ε* within the *Clk* loop and a subset of clock output genes (see below).

### The Clk Feedback Loop

In the *Clk* feedback loop, Clk–Cyc binds E-boxes to activate high levels of *Vri* and *Pdp1ε* expression during the late day and early night (Figure 1) [5–7]. *Vri* accumulates in phase with its mRNA and binds *Vri/PDP1ε* box (V/P box) regulatory elements to inhibit *Clk* transcription [5,7]. Consequently, *Clk* mRNA cycles in the opposite phase as Clk–Cyc/E-box regulated transcripts [5,7]. PDP1ε accumulates to high levels during the mid to late evening and activates *Clk* transcription [5]. *In vitro* experiments showed that PDP1ε can compete with *Vri* for binding to V/P-boxes, suggesting a model in which increasing levels of PDP1ε displace *Vri* from V/P-boxes and activate *Clk* transcription [5]. Though attractive, this model does not explain the constant peak levels of *Clk* expression in non-functional *Clk<sup>Jrk</sup>* and *cyc<sup>01</sup>* mutants [17], which virtually eliminate *Vri* and *Pdp1ε* expression [5]. To accommodate the *Clk<sup>Jrk</sup>* and *cyc<sup>01</sup>* results, a clock independent activator may drive constitutive *Clk* transcription, which is then rhythmically modulated by *Vri* and PDP1ε (Figure 2).

A common feature of the *Per/Tim* and *Clk* feedback loops is the activation of rhythmic transcription by Clk–Cyc. As rhythmic transcription of Clk–Cyc activated genes requires feedback by *Per–Tim*, this implies that the *Per/Tim* loop is also required for the *Clk* loop. Indeed, *per<sup>01</sup>* and *tim<sup>01</sup>* mutants abolish transcriptional rhythms in both loops [5,7,16,17]. Rhythms in the levels of *Per* and *Tim* persist if either of their respective mRNAs is constitutively expressed



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Figure 2. Model of the *Clk* feedback loop. Clk–Cyc heterodimers bind to E-boxes and activate *Vri* and *Pdp1ε* transcription. *Vri* accumulates in parallel with its mRNA, binds to V/P boxes and inhibits *Clk* transcription. *PDP1ε* accumulates in a delayed fashion and supplants *Vri* from V/P boxes to derepress *Clk* transcription. A clock independent activator (Act) constitutively activates *Clk* transcription in the absence of *Vri*, which would explain the high levels of *Clk* mRNA in the absence of Clk or Cyc. Accumulation of non-phosphorylated (or hypophosphorylated) Clk leads to heterodimerization with Cyc and another cycle of *Vri* and *Pdp1ε* transcription. Solid lines with arrow, sequential steps in the feedback loop; wavy lines, *Vri* and *Pdp1ε* or *Clk* mRNA; double line, nuclear membrane.

[33,34], but eliminating cycling of both *Per* and *Tim* mRNAs severely disrupts molecular and behavioral rhythms [34], though not to the extent seen in non-functional *per*<sup>01</sup> or *tim*<sup>01</sup> mutants. In contrast, driving *Clk* mRNA in antiphase has little effect on Clk phosphorylation or behavioral rhythms ([35] and W. Yu, personal communication), which suggests that *Clk* mRNA cycling is not necessary for circadian oscillator function.

The *Clk* loop also controls rhythmic transcription of *Cryptochrome* (*Cry*), which encodes a circadian photoreceptor that also functions as a clock component in some tissues [36–39]. In fly heads, *Cry* protein levels accumulate in the dark and decline in the light [37,40]; *Cry* abundance is thus driven by environmental light–dark (LD) cycles rather than *Cry* mRNA cycles. Moreover, *Cry* photoreceptor function can be rescued by constant levels of *Cry* mRNA [41,42]. As *Clk* and *Cry* mRNA cycling are not necessary for clock function, perhaps a major function of the *Clk* loop is to control rhythmic transcription of genes required for circadian behavior, physiology and metabolism. This possibility could be tested genetically using mutants that eliminate *Vri* and/or *Pdp1ε*, but unfortunately such mutants are developmental lethals [5,43]. Reduction or elimination of *Vri* and *Pdp1ε* via RNA interference (RNAi) in adult clock cells may be a fruitful method to test *Clk* feedback loop function.

### Entraining the Oscillator to Light

The circadian oscillator must maintain synchrony with environmental cycles to drive behavioral, physiological and metabolic outputs at the appropriate time of day. Daily environmental cycles of light, temperature, food and social interactions are all capable of entraining circadian oscillators, but light is generally considered to be the strongest and most pervasive factor. Light shifts the phase of the circadian oscillator in a predictable manner. If a fly is transferred from an LD

cycle to constant darkness (DD), light pulses applied at times when lights would have been on (subjective day) produce little or no effect on oscillator phase, but light pulses applied soon after lights would have gone off (early subjective night) produce a phase delay, and light pulses applied just before lights would have come on (late subjective night) produce a phase advance [44–46]. Substantial headway has been made in identifying the molecules and mechanisms that mediate the light dependent phase shifting — entrainment — of the *Drosophila* circadian oscillator.

In general, light entrains a circadian oscillator by activating a photoreceptor, which then directly or indirectly alters the level or activity of an oscillator component. In *Drosophila*, several photoreceptors are activated by light, and these photoreceptors then trigger the ubiquitin-proteasome dependent degradation of *Tim* via tyrosine phosphorylation [47]. The light dependent degradation of *Tim* is thought to produce phase advances and delays depending on the levels of *Tim* mRNA [24–26,48]. Early in the dark phase, *Tim* levels can rebound after light induced degradation because of the high levels of *tim* mRNA, replaying a few hours of the circadian cycle and producing a phase delay. Late in the dark phase, *Tim* levels cannot rebound after light induced degradation because of the low levels of *Tim* mRNA, fast forwarding to the next phase of the circadian cycle and advancing the phase of the clock. No phase shifting is seen during the subjective day, as there is little or no *Tim* to replenish. The light dependent loss of *Tim* leads to a delay in *Per* nuclear localization and phosphorylation during the early night, and an advanced degradation of *Per* during the late night, consistent with the changes in gene expression and behavior due to light applied at these times [48].

Genetic analysis has revealed that external photoreceptors in compound eyes and ocelli, internal photoreceptors in the Hofbauer-Buchner eyelet, and

the blue light photoreceptor Cry all contribute to light dependent entrainment of behavioral rhythms in *Drosophila* [49]. The mechanism by which external and internal photoreceptors trigger light dependent degradation of Tim in neurons that control behavioral rhythms has not been characterized, and it is unlikely that these photoreceptors entrain oscillator cells elsewhere in the fly head and body (see below). In contrast, *Cry* is expressed in oscillator cells throughout the head and body ([50] and H. Zheng, personal communication), and recent work has revealed important insights into how *Cry* triggers Tim degradation in response to light (Figure 3). *Cry* contains a conserved photolyase domain and a unique carboxy-terminal domain [51]. On stimulation by light, the carboxy-terminal domain of *Cry* is thought to shift position or release an inhibitor to reveal a Tim binding site [41,42,52]. *Cry* then binds Tim [53], which is associated with the Per–Dbt or Per–Dbt–Clk–Cyc complex, and triggers its tyrosine phosphorylation and degradation by the proteasome [47]. Light also promotes *Cry* degradation, albeit more slowly, consistent with the light dependent rhythm in *Cry* levels [37,40].

#### Organization of the *Drosophila* Circadian System

The spatial distribution of clock gene expression has been used to infer the presence of circadian oscillators in *Drosophila*. Analysis of reporter gene expression driven by the *per* promoter and Per immunolocalization revealed expression in a variety of neuronal and non-neuronal tissues in fly heads and bodies [54–56]. In heads, *Per* is expressed in photoreceptors of the compound eye (which accounts for ~75% of all *per* expression in heads), antennae, the proboscis, ocelli, the esophagus, fat bodies, brain glia and six clusters of brain neurons [54–57] (Figure 4). These clusters of *Per* expressing brain neurons have been classified according to size and position as small ventral lateral neurons (sLN<sub>V</sub>s), large ventral lateral neurons (lLN<sub>V</sub>s), dorsal lateral neurons (LN<sub>D</sub>s), dorsal neuron 1s (DN1s), dorsal neuron 2s (DN2s) and dorsal neuron 3s (DN3s) [57] (Figure 4B). In the fly body, *Per* is expressed in the gut, the cardia, salivary glands, Malpighian tubules, the rectum, legs, wings, fat bodies, ovaries and testes [55,56].

Essentially all of these tissues express *Per* gene products rhythmically [21,55,58,59], indicating that they have circadian oscillators. Although circadian oscillator cells have been defined based on the expression of *Per* gene products, other feedback loop components such as Tim and Clk are, as expected, also made in these cells [24,25,60–62]. Unlike *Per* and Tim, which appear to be expressed almost exclusively in oscillator cells, Clk is also expressed in numerous non-oscillator cells [60]. One tissue that does not express *Per* rhythmically is the ovary [58], yet even in this tissue Tim is coproduced with *Per*, though not in a Clk–Cyc dependent manner [63].

The relationship between oscillators in different tissues has been addressed using luciferase reporter genes driven by the *per* promoter (*Per-luc*), which have the advantage of allowing real-time monitoring of

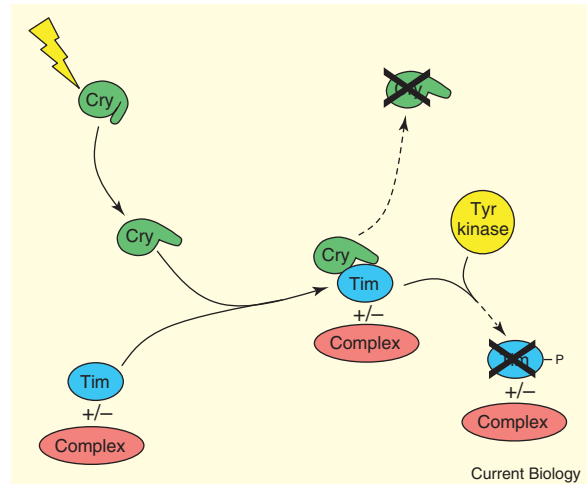


Figure 3. Model for the light dependent entrainment of the *Drosophila* oscillator.

Stimulation of CRY by light alters the carboxy-terminal domain either structurally or by releasing an inhibitor, thereby permitting binding to Tim. Cry binds to either Tim or a Tim complex — a Dbt–Per–Tim complex in the cytoplasm or a Dbt–Per–Tim–Clk–Cyc complex in the nucleus — and promotes phosphorylation of Tim by a tyrosine kinase. Phosphorylated Tim is then committed to rapid degradation in the proteasome. Prolonged light stimulation leads to the eventual degradation of Cry in the proteasome. Solid lines with arrow, sequential steps in the light response pathway; dashed lines, proteasomal degradation; black X, degraded proteins; P, protein phosphorylation.

whole animals or cultured tissues over several days [55,64,65]. Individual cultured tissues such as antennae, wings, probosci and legs rhythmically express *Per-luc* under LD and DD conditions in the same circadian phase [55]. Particularly prevalent among tissues with circadian oscillators are those with sensory function, such as the photosensory ocelli and compound eyes, chemosensory tissues of the third antennal segment, maxillary palps, wings, legs and proboscis, and mechanosensory tissues of the second antennal segment (Figure 4A). This suggests that the circadian clock controls some aspect of sensory function; indeed, at least one chemosensory function, olfaction, is under clock control in flies [66].

Remarkably, oscillators in cultured *Drosophila* tissues, whether overtly sensory or not, can be directly entrained by light, indicating that they operate in a tissue autonomous manner [55]. Presumably light entrains oscillators in peripheral tissues via *Cry*, but this has been difficult to ascertain as *Cry* is important for oscillator function *per se* in many cultured peripheral tissues [38,39,67]. Although the fly is generally viewed as a collection of autonomous, light-entrainable circadian oscillators, current data cannot eliminate the possibility that there may be communication between oscillators. Indeed, there is evidence that communication between oscillator neurons within the brain is important for locomotor activity rhythms under LD and DD conditions [68–70], and that interaction between the prothoracic gland and lateral neurons is important for rhythms in eclosion [71].

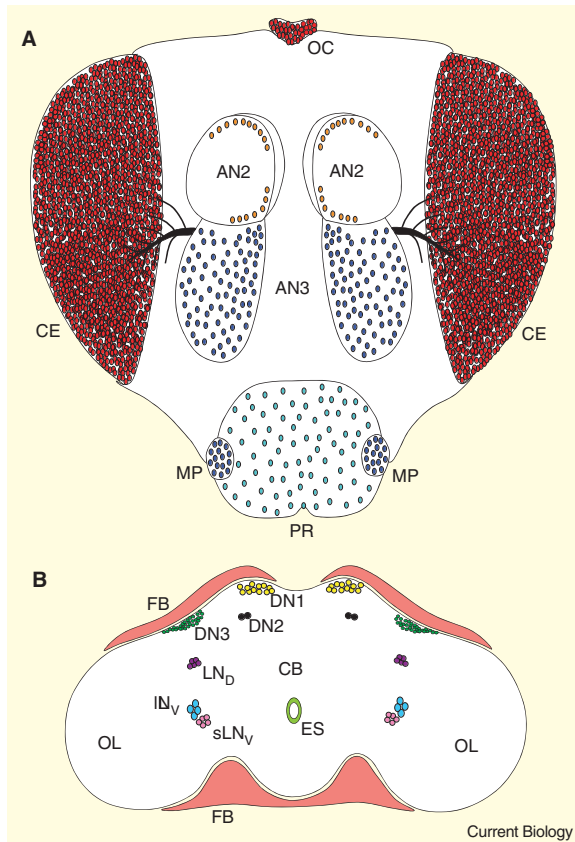


Figure 4. Circadian oscillators in *Drosophila* heads.

(A) External structures containing circadian oscillators. A frontal view of a *Drosophila* head is shown. OC, ocelli; CE, compound eyes; AN2, second antennal segment; AN3, third antennal segment; MP, maxillary palps; PR, proboscis. (B) Oscillator cells within and surrounding the brain. A frontal section through a *Drosophila* brain and surrounding tissues is shown. CB, central brain; OL, optic lobes; FB, fat body; ES, esophagus; LN<sub>D</sub>, dorsal lateral neurons; ILN<sub>V</sub>, large ventral lateral neurons; sLN<sub>V</sub>, small ventral lateral neurons; DN1, dorsal neuron 1s; DN2, dorsal neuron 2s; DN3, dorsal neuron 3s. Glia containing oscillators (not shown) are found within the central brain and optic lobes.

### Regulating Rhythmic Outputs

A fundamental question in circadian biology is how circadian oscillators regulate rhythms in behavior, physiology and metabolism. Given that the circadian oscillator is composed of transcriptional feedback loops, regulation of rhythmic outputs is likely to occur via clock dependent transcription of genes that directly control behavioral, physiological and metabolic processes. Such regulation could also be indirect; rhythmically expressed phosphatases, such as PP2a, and/or kinases could mediate rhythms in the activity of proteins that the control output processes.

The most efficient and comprehensive method of identifying transcripts that cycle in abundance has been through microarray analysis. About 150 cycling transcripts, representing genes that regulate processes such as protein degradation, detoxification, immunity, phototransduction and neurotransmission, have been identified in this way [72–76].

These transcripts are under clock control, as their rhythmic expression is abolished in arrhythmic *Clk<sup>Jrk</sup>* flies [72,73,75,76]. In addition, the levels of many non-rhythmic transcripts are increased or decreased in *Clk<sup>Jrk</sup>* flies, which suggests that *Clk* regulates processes independently of the circadian clock [75]. Such clock independent regulation could reflect the action of *Clk* in non-oscillator cells [60].

To understand how clock controlled transcripts regulate rhythmic outputs, it is important to determine how the oscillator regulates cycling of these transcripts and to identify the behavioral, physiological and metabolic processes these transcripts control. Several clock controlled transcripts were found to be direct targets of *Clk*–*Cyc* in S2 cell culture assays [75], but direct targets of *PDP1ε* or *Vri* have not been identified. Determining what rhythmic processes these transcripts control is a major challenge, as it depends on making various behavioral, physiological or metabolic measurements over circadian time. Such analyses are nevertheless essential for determining the biological impact of the clock. Circadian rhythms in several processes have been identified in adults, including locomotor activity, mating receptivity, oviposition and olfaction [66,77–79]. Although no clock output pathway has been characterized in detail, progress has been made in understanding how locomotor activity and olfaction rhythms are regulated.

### Regulation of Locomotor Activity Rhythms

In *Drosophila*, locomotor activity under DD conditions peaks during subjective dusk [78]. As a first step towards understanding how this rhythm is controlled, the oscillator cells responsible for this rhythm were identified. Evidence from flies in which large and small LN<sub>V</sub>s were ablated by expressing proapoptotic genes, or electrically silenced by expressing constitutively active mutant K<sup>+</sup> channels showed that these neurons are necessary for free-running (DD) locomotor activity rhythms [80,81]. Surprisingly, electrically silencing large and small LN<sub>V</sub>s eliminates molecular oscillations of *Per* and *Tim*, suggesting that electrical activity is required for circadian oscillator function in these cells [80]. Restoring *Per* function in large and small LN<sub>V</sub>s of *per*<sup>01</sup> flies rescues both circadian oscillator function in these neurons and free-running locomotor activity rhythms [70]. As ILN<sub>V</sub>s do not sustain oscillator function in DD [44,70], sLN<sub>V</sub>s alone appear to be sufficient for this rhythmic output. In contrast, restoring *Cyc* function in large and small LN<sub>V</sub>s of *cyc*<sup>01</sup> flies rescues molecular rhythms, but not free-running locomotor activity rhythms [68]. It is possible that this failure of *Cyc* to rescue locomotor activity rhythms occurs because of developmental defects in sLN<sub>V</sub> projections in *cyc*<sup>01</sup> flies [82]. Taken together, these data indicate that sLN<sub>V</sub>s are necessary and sufficient for free-running locomotor activity rhythms.

The sLN<sub>V</sub>s send projections into the dorsal brain, close to the cell bodies of DN2 and DN3 clock neurons and Kenyon cells, which are required for olfactory learning [57,83]. These projections contain a neuropeptide called pigment dispersing factor (PDF),

which is required for free running locomotor activity rhythms in flies [81,84]. Circadian fluctuations in PDF content within the termini of sLN<sub>V</sub> projections suggest that PDF is rhythmically released [82]. These apparent rhythms in PDF release are sensitive to clock gene mutations, indicating they are under clock control [82]. Moreover, expressing constant levels of PDF in the vicinity of sLN<sub>V</sub> terminals disrupts locomotor activity rhythms, suggesting that rhythms in PDF release are important for free-running locomotor activity rhythms [85]. As constant levels of PDF expressed in brain areas outside the sLN<sub>V</sub> projections were found not to affect behavior [85], the site of PDF action appears to be in the dorsal brain. The presumptive G protein coupled receptor for PDF has not been identified, but it is almost certainly among the ~20 orphan G protein coupled neuropeptide receptors in the *Drosophila* genome [86]. Once the PDF receptor is in hand, PDF target cells and their projections can be defined to reveal the next piece of the neural circuit controlling free-running locomotor activity rhythms.

Locomotor activity during LD cycles differs from that in DD in that there are two activity peaks: one in the morning and one in the evening. Early studies with *Drosophila pseudoobscura* and more contemporary work with *Drosophila melanogaster* indicate that the morning and evening activity peaks are controlled by separate oscillators [87,88]. Recently, these oscillators were identified by targeting expression of clock genes to specific sets of LNs and DNs via Gal4 activation and/or Gal80 inhibition [69,70]. These experiments showed that the morning activity peak is driven by the LN<sub>V</sub>s, whereas the evening peak is driven by the LN<sub>D</sub>s and possibly a subset of DN1s. As the LN<sub>V</sub>s drive morning activity during LD cycles and are sufficient for activity rhythms in DD, it is intriguing that free-running activity peaks during the subjective evening in wild-type flies. Communication among these oscillator neurons, possibly by PDF or some other neurotransmitter, may enhance the robustness and modulate the phase of locomotor activity rhythms [69,70]. Such communication may facilitate adjustments in the phase of locomotor activity by environmental factors such as temperature and photoperiod [89].

#### **Regulation of Olfaction Rhythms**

Another circadian output that has been characterized in flies is a rhythm in olfaction. This rhythm is measured by assaying the magnitude of odor-induced electrophysiological responses in the antennae called electroantennograms (EAGs). EAG responses to the food odorant ethyl acetate show a robust rhythm in wild-type flies under both LD and DD conditions [66]. These rhythms are abolished in *per*<sup>01</sup> and *tim*<sup>01</sup> flies, confirming that they are under clock control [66]. Rhythms in EAG responses were known to require the function of oscillators in peripheral tissues [66], and recent studies in which oscillator function was either disrupted or rescued in specific cell types have shown that olfactory receptor neurons in the antenna are both necessary and sufficient for the EAG rhythms [90]. These olfactory receptor neuron oscillators behave as self-contained clocks because antennal

oscillators, including olfactory receptor neurons, can be entrained directly by light [55]. Localization of the EAG oscillator to olfactory receptor neurons suggests that components of the olfactory signal transduction pathway, such as odorant receptors and G proteins, may be targets for clock regulation. Identifying components of the olfactory signal transduction pathway that are rhythmically controlled may reveal clock outputs that effect EAG rhythms.

#### **Conclusion and Perspectives**

Substantial progress has been made in defining the molecular mechanisms underlying circadian clock function in *Drosophila*. Input pathways have been identified, and the mechanisms by which they entrain the oscillator are being revealed at an ever-increasing pace. The molecular feedback loop model of circadian oscillator function has not only withstood the test of time, but is being elaborated in fine detail. These oscillators are present in a variety of tissues, where they largely operate as autonomous clocks. A large number of clock controlled transcripts have been identified, and progress is being made in defining oscillators and, in the case of locomotor activity, molecules that mediate rhythmic outputs. But despite the substantial progress that has been made in our understanding of the *Drosophila* clock, many important questions remain.

One of the least understood aspects of circadian clock function is how oscillators regulate rhythmic outputs. There are many oscillators in the fly, yet few rhythmic outputs have been characterized. Rhythms in locomotor activity have been the most extensively characterized output in flies. Studies focusing on the clock regulation of PDF release and identifying the PDF receptor should begin to define the molecular output mechanism and reveal the underlying neural circuitry. Now that oscillator cells controlling olfaction rhythms have been identified, the mechanisms by which they control rhythms in olfactory physiology can be defined.

The small number of clock outputs is a reflection of the difficulty in developing or performing assays that can be used to measure circadian rhythms in metabolism, physiology and behavior. The presence of circadian oscillators in a host of sensory tissues nevertheless suggests a number of quantitative behavioural and physiological assays. In addition, the spatial expression and predicted function of rhythmically expressed mRNAs may highlight specific processes to target for circadian monitoring. As more clock outputs are discovered, their clock dependent regulation must be defined so that we can begin to understand the various mechanisms by which the clock imposes its control on behavior, physiology and metabolism.

In addition to controlling daily rhythms, the *Drosophila* circadian clock also controls annual rhythms in various phenomena, including the phase of locomotor activity and reproduction [91,92]. In the case of locomotor activity, flies are most active just before dawn and after dusk during the summer, but after dawn and before dusk during the winter [90]. The

clock adapts to these seasonal changes in the environment through the thermosensitive splicing of an intron in the 3' untranslated region of *Per* mRNA; enhanced splicing in cold temperatures and short photoperiods advances oscillator phase and consequently locomotor activity, whereas reduced splicing in longer photoperiods and warm temperatures delay oscillator phase and locomotor activity [89,93,94]. The molecular mechanism that regulates this splicing event is not clear, though light, clock factors and a phospholipase C are all involved [93,94]. Understanding how these factors effect splicing of this intron will provide significant insight into how the clock adapts to a changing environment.

The current model of transcriptional regulation within the *Clk* feedback loop is necessarily incomplete. First, it is surprising that *Clk* and *Cry* are rhythmically transcribed, even though *Clk* and *Cry* levels are controlled post-transcriptionally. Perhaps the primary function of *Vri* and *PDP1ε* is to control rhythmic transcription of output genes in phase with *Clk* and *Cry*. This possibility could be addressed by tissue-specific elimination or reduction of *Vri* and *Pdp1ε* function by RNAi or expressing dominant negative forms of the gene products.

Secondly, although *Vri* and *Pdp1ε* are both activated by *Clk*-*Cyc*, *Pdp1ε* mRNA accumulation is delayed compared to that of *Vri* [5]. This delay produces a concomitant delay in *PDP1ε* accumulation, which counteracts *Vri* dependent repression and consequently activates *Clk* and *Cry*. Understanding the mechanism that governs *Pdp1ε* mRNA accumulation will be important for understanding how rhythmic transcription within and downstream of the *Clk* loop is controlled.

Lastly, the high levels of *Clk* mRNA in *Clk<sup>Jrk</sup>* and *cyc<sup>01</sup>* flies suggest that *Clk* is constitutively activated. As ectopic expression of *Clk* is capable of inducing circadian clock function [95], it is possible that the constitutive activator is the same one that activates *Clk* during development. Characterizing *Clk* developmental expression and regulation might be a profitable way to resolve this question.

Recent studies have greatly enhanced our understanding of how *Cry* entrains the circadian oscillator in response to light. Once *Cry* is exposed to light it binds to *Tim*, thereby committing *Tim* to degradation by the proteasome. *Tim* degradation is preceded by tyrosine phosphorylation, which presumably occurs after *Tim* is bound by activated *Cry*. Identifying the relevant tyrosine kinase will enable a detailed analysis of the interactions that take place to destabilize *Tim*.

In contrast to *Cry*'s role in light entrainment, how it contributes to oscillator function in peripheral tissues is poorly understood. In mammals, Cryptochrome binds to *Period* homologs to inhibit *Clock*-*Bmal1* dependent transcription [96]. Whether a similar situation exists in flies can now be addressed using newly developed reagents for detecting *Cry* subcellular localization and protein interactions. The sLN<sub>γ</sub> oscillator can be entrained to light independently of *Cry*, indicating that photoreceptors in compound eyes,

ocelli and/or the Hofbauer-Buchner eyelet can mediate light entrainment. As the mammalian SCN is also entrained by photoreceptors residing in other tissues, understanding how light information is transmitted to sLN<sub>γ</sub>s and triggers a phase shift in flies is of great interest.

Analysis of the *Drosophila* circadian system has led to many breakthroughs in our understanding of circadian clock function. The studies outlined above should continue that tradition by providing the mechanistic detail needed to understand how the clock functions as an integrated set of components to drive daily rhythms in behavior, physiology and metabolism.

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