Transcriptional Feedback Loop Regulation, Function, and Ontogeny in *Drosophila*

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The *Drosophila* circadian oscillator is composed of interlocked *period/timeless* (*per/tim*) and *Clock* (*Clk*) transcriptional feedback loops. These feedback loops drive rhythmic transcription having peaks at dawn and dusk during the daily cycle and function in the brain and a variety of peripheral tissues. To understand how the circadian oscillator keeps time and controls metabolic, physiological, and behavioral rhythms, we must determine how these feedback loops regulate rhythmic transcription, determine the relative importance of the *per/tim* and *Clk* feedback loops with regard to circadian oscillator function, and determine how these feedback loops come to be expressed in only certain tissues. Substantial insight into each of these issues has been gained from experiments performed in our lab and others and is summarized here.

INTRODUCTION

As in other organisms, the circadian clock in *Drosophila* controls daily rhythms in physiology, metabolism, and behavior through a self-sustaining oscillator that is synchronized to environmental cycles in light and temperature but keeps time even in the absence of environmental cues. The *Drosophila* circadian oscillator is composed of interlocked *period/timeless* (*per/tim*) and *Clock* (*Clk*) feedback loops that regulate rhythmic transcription in different phases of the circadian cycle (for review, see Hardin 2006; Yu and Hardin 2006). The transcriptional rhythms imparted by these feedback loops not only maintain circadian oscillator function, but also activate clock “output genes” that control physiological, metabolic, and behavioral processes in various tissues (Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Ceriani et al. 2002; Y. Lin et al. 2002; Ueda et al. 2002; Wijnen et al. 2006). Because transcriptional regulation is critically important for circadian clock function in all organisms (Young and Kay 2001; Bell-Pedersen et al. 2005), a major focus within the circadian rhythms field has been to define the mechanisms through which transcriptional feedback loops mediate rhythmic transcription.

One of the best-characterized transcriptional feedback loops is the *per/tim* feedback loop in *Drosophila* (Fig. 1). This feedback loop is initiated when CYCLE (CYC) forms a heterodimer with CLK and binds E-box regulatory sequences to activate *per* and *tim* transcription around midday or zeitgeber time 6 (ZT6) (note: Zeitgeber time refers to time during a light-dark cycle where ZT0 is lights-on and ZT12 is lights-off [Darlington et al. 1998].) Although *per* and *tim* mRNAs accumulate to high levels around dusk (ZT12) (Hardin et al. 1990; Sehgal et al. 1995), PER and TIM proteins do not feed back to inhibit CLK-CYC transcription until midnight (ZT18) because of a phosphorylation-dependent delay in their accumulation and nuclear localization (Kloss et al. 1998, 2001; Price et al. 1998; Martinek et al. 2001; J.M. Lin et al.

2002; Akten et al. 2003). Degradation of PER and TIM in the early morning (ZT4) releases transcriptional inhibition to enable the next round of CLK-CYC transcriptional activation (Hunter-Ensor et al. 1996; Myers et al. 1996; Zeng et al. 1996; Naidoo et al. 1999; Grima et al. 2002; Ko et al. 2002). The *per/tim* feedback loop is necessary for, and intrinsically linked to, the *Clk* feedback loop, which drives rhythms in *Clk* mRNA expression that peak around dawn (ZT0) (Glossop et al. 1999). The *Clk* feedback loop is initiated when CLK-CYC binds E boxes to activate *vri* (*vri*) and *PAR domain protein 1ε* (*Pdp1ε*) transcription (Cyran et al. 2003; Glossop et al. 2003). VRI protein accumulates to high levels in concert with *vri* mRNA during the early night (ZT15) and binds V/P boxes to inhibit *Clk* transcription (Cyran et al. 2003; Glossop et al. 2003). PDP1ε protein does not accumulate to high levels until late evening (ZT21), when it is thought to compete with decreasing levels of VRI for V/P-box binding to activate *Clk* transcription (Cyran et al. 2003). Although the *per/tim* and *Clk* feedback loops control rhythmic transcription within (and downstream from) the circadian oscillator, their relative contributions to oscillator function and overt rhythmicity are less well understood. Accumulating data from our lab and others suggest that the *Clk* feedback loop is not necessary for circadian oscillator function. Moreover, recent data from our lab argue that PDP1ε is a critical output factor, rather than a major *Clk* transcriptional activator, and suggests that key factors controlling rhythmic transcription within the *Clk* loop are yet to be identified.

Circadian feedback loop oscillators function in several clusters of brain neurons and numerous peripheral tissues in *Drosophila* (Hall 2003; Helfrich-Forster 2005). Because CLK-CYC heterodimers initiate both *per/tim* and *Clk* feedback loop function, factors that activate *Clk* and *cyc* would then be primary determinants of oscillator cell fate. However, the situation is more complicated because CLK is also expressed in cells that do not harbor circadian oscillators (i.e., nonoscillator cells) (Houl et al.

concert with the accumulation of phosphorylated PER in CLK-CYC complexes in the nucleus (Lee et al. 1998; Kim and Edery 2006; Yu et al. 2006). DOUBLE-TIME (DBT) kinase-dependent phosphorylation promotes PER degradation (Price et al. 1998), whereas TIM stabilizes PER by blocking DBT phosphorylation (Price et al. 1995; Ko et al. 2002). Phosphorylation of PER by casein kinase 2 (CK2) and TIM by SHAGGY (SGG) kinase promotes PER and TIM nuclear localization, respectively (Martinek et al. 2001; J.M. Lin et al. 2002; Akten et al. 2003; Nawathean and Rosbash 2004). TIM is degraded around dawn, which facilitates DBT-dependent degradation of hyperphosphorylated PER and CLK and transcriptional activation by accumulating levels of hypophosphorylated CLK (Hunter-Ensor et al. 1996; Myers et al. 1996; Zeng et al. 1996; Ko et al. 2002; Kim and Edery 2006; Yu et al. 2006).

Because posttranslational regulation of PER and TIM mediates CLK phosphorylation and transcriptional activity, is rhythmic transcription of *per* and *tim* (and by extension *per* and *tim* mRNA cycling) necessary for circadian oscillator function? Transgenic flies expressing constitutive levels of either *per* or *tim* mRNAs had little effect on molecular and behavioral rhythms (Cheng and Hardin 1998; Yang and Sehgal 2001). Likewise, about half of the transgenic fly lines that express constant levels of both *per* and *tim* mRNAs retained molecular and behavioral rhythmicity, suggesting that mRNA rhythms are not necessary for circadian oscillator function (Yang and Sehgal 2001). However, the lack of rhythmicity in half the transgenic lines and severe decrements in molecular and behavioral rhythmicity in the others suggest that mRNA cycling is important, if not essential, for circadian oscillator function. The transgenic lines used to constitutively express *per* and *tim* mRNAs (Yang and Sehgal 2001) were recently retested and found to display less than 20% rhythmicity that was generally weak and in a broad range of circadian periodicities, indicating that *per* and *tim* mRNA rhythms are indeed required for circadian oscillator function in *Drosophila* (Hall et al., this volume).

In the *Clk* feedback loop, rhythms in the VRI/PDP1 ϵ ratio are thought to control rhythmic transcription of *Clk* (Cyran et al. 2003). However, the resulting rhythm in *Clk* mRNA abundance does not give rise to cycling levels of CLK protein, although PER-dependent CLK phosphorylation cycles with a peak near dawn (Kim and Edery 2006; Yu et al. 2006). This dawn peak in CLK phosphorylation persists even after the phase of *Clk* mRNA cycling is reversed (Kim et al. 2002), indicating that *Clk* mRNA levels are not important for *per/tim* feedback loop function. In contrast, mutants that disrupt *per/tim* feedback loop function also disrupt the *Clk* feedback loop (Glossop et al. 1999), indicating that the *per/tim* loop is of primary importance. If the *Clk* feedback loop is not necessary for *per/tim* feedback loop function, then what is the purpose of the *Clk* feedback loop? One possibility is that the *Clk* loop functions to enhance the amplitude and robustness of the circadian oscillator. This possibility is supported by experiments in which disruption of the analogous feedback loop in mammals, the *Bmal1* loop, reduces the amplitude of molecular rhythms and the con-

solidation of locomotor activity rhythms (Sato et al. 2004). Another potential function for the *Clk* loop is to drive outputs that require transcriptional activation around dawn. Although no output genes have been identified that are directly under *Clk* feedback loop control (i.e., activated by PDP1 ϵ and/or repressed by VRI), microarray screens have identified a large number of potential *Clk* feedback loop target genes whose mRNAs peak at dawn (Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Ceriani et al. 2002; Y. Lin et al. 2002; Ueda et al. 2002; Wijnen et al. 2006).

REGULATION OF RHYTHMIC TRANSCRIPTION WITHIN THE CLK FEEDBACK LOOP

The *Clk* feedback loop was first proposed to explain the opposite phase cycling of *Clk* mRNA with respect to *per* and *tim* mRNAs (Glossop et al. 1999). *Clk* transcription is repressed when CLK-CYC activity is high from midday to midnight in wild-type flies and in *per*⁰¹ or *tim*⁰¹ mutants, whereas *Clk* transcription is activated when CLK-CYC activity is low from midnight to midday in wild-type flies and in *Clk*^{Jrk} or *cyc*⁰¹ mutants. The high levels of *Clk* mRNA in *Clk*^{Jrk} and *cyc*⁰¹ flies predict that *Clk* is activated by a factor that is not dependent on circadian oscillator function. In contrast, the low levels of *Clk* mRNA in *per*⁰¹ and *tim*⁰¹ mutants suggest that CLK-CYC activates a repressor.

One CLK-CYC-activated transcript identified in a screen for rhythmically expressed mRNAs encodes the bZIP (basic leucine zipper) repressor VRI (Blau and Young 1999). Although circadian phenotypes cannot be measured in *vri* null mutants due to developmental lethality (George and Terracol 1997), VRI overexpression represses *Clk* and CLK-CYC-dependent transcripts such as *per* and *tim* (Blau and Young 1999; Cyran et al. 2003; Glossop et al. 2003). VRI accumulates in phase with its mRNA from midday to early evening (Cyran et al. 2003; Glossop et al. 2003), consistent with the timing of *Clk* repression and the dependence of *Clk* repression on CLK-CYC activity (Fig. 1). Another rhythmically expressed transcript that is dependent on CLK-CYC, *Pdp1 ϵ* , encodes a bZIP activator with high similarity to VRI (Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Ceriani et al. 2002; Y. Lin et al. 2002; Ueda et al. 2002; Cyran et al. 2003; Wijnen et al. 2006). *Pdp1 ϵ* mRNA and protein accumulate to high levels several hours later than *vri* mRNA and protein, suggesting that accumulating PDP1 ϵ could activate *Clk* transcription late at night as VRI levels fall (Fig. 1). Consistent with this possibility, PDP1 ϵ binds V/P-box elements in vitro, PDP1 ϵ competes with VRI to activate *Clk* transcription in cell culture, and little or no *Clk* mRNA is detected in *Pdp1 ϵ* null mutant flies (Cyran et al. 2003). Activation of *Clk* by PDP1 ϵ was unexpected because *Clk* mRNA levels are high in *Clk*^{Jrk} and *cyc*⁰¹ flies even there is little or no PDP1 ϵ . Although it is possible that small amounts of PDP1 ϵ could predominate over low levels of VRI to activate *Clk* in *Clk*^{Jrk} or *cyc*⁰¹ mutants, this would contrast with the relationship between these proteins in *per*⁰¹ and *tim*⁰¹ flies, where high levels of VRI predominate over

Figure 2. Constant low PDP1 ϵ levels do not disrupt *Clk* mRNA or VRI cycling. (A) qPCR of *Clk* mRNA from the heads of *w;+/+;timGal4/+ (timGal4)* and *w;UAS-PDP1i/+;UAS-PDP1i/timGal4* (2xPDP1i+tG4) flies collected at the indicated times under LD conditions (ZT). Relative *Clk* mRNA levels were quantified as described in Benito et al. (2007). The black and white bars represent times when lights were on or off, respectively. (B) Western blots of head extracts from 2xPDP1i+tG4 and wild-type (wt), *Clk^{trk}*, *timGal4* (tG4), and *w;UAS-PDP1i/+;UAS-PDP1i/+* (2xPDP1i) control flies collected at the indicated times under LD conditions and probed with VRI antibody. (C) Quantification of VRI levels from the western blots in B and at least two independent repeats. VRI levels were quantified as described in Benito et al. (2007), and normalized to wild type at ZT17. (Reprinted, with permission, from Benito et al. 2007 [© The Society for Neuroscience].)

high levels of PDP1 ϵ to repress *Clk* (Cyran et al. 2003). *Pdp1* produces multiple RNA and protein isoforms that are expressed in a variety of tissues including the central nervous system (CNS) (Reddy et al. 2000). Deletion of *Pdp1* leads to severe defects in growth, mitosis, and endoreplication that cause lethality during larval stages (Reddy et al. 2006). It is possible that loss of all PDP1 isoforms in the *Pdp1* deletion mutant alters clock neuron identity or development in larvae, and thus indirectly reduces *Clk* mRNA levels.

If *Clk* mRNA cycling is driven by competition between PDP1 ϵ and VRI for V/P-box binding, then altering the PDP1 ϵ /VRI ratio by reducing or increasing PDP1 ϵ expression specifically in circadian clock cells should disrupt *Clk* mRNA cycling by producing a constant trough or peak levels of *Clk* mRNA, respectively. When PDP1 ϵ was reduced to its normal trough level or below via RNA interference (RNAi), *Clk* mRNA continued to cycle similarly to the wild-type controls with the exception of a lower *Clk* mRNA level at ZT1 (Fig. 2). VRI levels continued to cycle in flies expressing low levels of PDP1 ϵ , consistent with its role as a *Clk* repressor (Fig. 2). Similar results were seen when PDP1 ϵ levels were increased to ≥ 5 -fold above its normal peak: *Clk* mRNA and VRI protein levels continued to cycle with amplitudes comparable to wild-type controls (Fig. 3). These results demonstrate that PDP1 ϵ levels, and thus the PDP1 ϵ /VRI ratio, are not critical for *Clk* mRNA cycling or circadian oscillator function (Benito et al. 2007). Moreover, the wild-type levels of *Clk* mRNA in *Pdp1* RNAi and *Pdp1 ϵ* overexpression strains suggest that PDP1 ϵ is not the major *Clk* activator, although a minor role in *Clk* activation cannot be discounted (Benito et al. 2007).

FUNCTION OF PDP1 ϵ WITHIN THE CIRCADIAN CLOCK

Because circadian oscillator function is not disrupted in flies with constant low or high levels of PDP1 ϵ , perhaps PDP1 ϵ functions to control clock output. To test this possibility, locomotor activity rhythms were measured in *Pdp1* RNAi, *Pdp1 ϵ* overexpression, and control strains (Benito et al. 2007). Flies expressing low or high levels of PDP1 ϵ show drastic reductions in behavioral rhythmicity compared to control strains (Table 1). Despite this loss in behavioral rhythmicity, oscillator function persists in locomotor activity pacemaker cells or lateral neurons (LNs), which indicates that PDP1 ϵ controls clock output (Benito et al. 2007). Because PDP1 ϵ is a rhythmically expressed bZIP transcriptional factor, it is likely to mediate behavioral rhythms by rhythmically activating effector genes. These PDP1 ϵ -dependent rhythmic transcripts may represent some of those identified in previous microarray studies that peak in abundance around dawn (Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Ceriani et al. 2002; Y. Lin et al. 2002; Ueda et al. 2002; Wijnen et al. 2006). These data suggest a revised model for clock function in *Drosophila* in which a constitutive activator and VRI mediate *Clk* mRNA cycling within the *Clk* feedback loop (Fig. 1). PDP1 ϵ may have a minor role in regulating *Clk* transcription, but it is proposed to be a major regulator of output transcription required for (at least) behavioral rhythmicity (Benito et al. 2007). VRI may also function to control output transcription along with PDP1 ϵ , but it is worth noting that VRI's role within the clock is based on gain-of-function experiments. Eliminating VRI function within oscillator cells

Figure 3. Overexpression of PDP1 ϵ , in oscillator cells does not disrupt *Clk* mRNA or VRI cycling. (A) qPCR of *Clk* mRNA from the heads of *w*;UAS-PDP1 ϵ /+;UAS-PDP1 ϵ /+ (UPDP1 ϵ) and *w*; UAS-PDP1 ϵ /+;UAS-PDP1 ϵ /*tim*Gal4 (UPDP1 ϵ +tG4) flies were collected at the indicated times under LD conditions (ZT). Relative *Clk* mRNA levels were quantified as described by Benito et al. (2007). The black and white bars represent times when lights were on or off, respectively. (D) Western blot of head extracts from UPDP1 ϵ and UPDP1 ϵ +tG4 flies collected at the indicated times under LD conditions and probed with VRI antibody. The asterisk denotes PDP1 ϵ band that did not wash off completely from the blot in A. (E) Quantification of VRI levels in the western blot in B and at least two independent repeats. VRI levels were quantified as described by Benito et al. (2007). (Reprinted, with permission, from Benito et al. 2007 [©The Society for Neuroscience].)

will more definitively pinpoint VRI's role within the clock.

The mammalian homologs of PDP1 ϵ and VRI likely have an analogous role in regulating clock output. PDP1 ϵ is related to three rhythmically expressed PAR domain-containing bZIP transcriptional activators in mammals: albumin gene site D-binding protein (DBP), thyroid embryonic factor (TEF), and hepatocyte leukemia factor (HLF) (Wuarin and Schibler 1990; Falvey et al. 1995; Fonjallaz et al. 1996). DBP knockout mice show lower locomotor activity levels and a shorter circadian period

than wild-type animals, yet robust circadian oscillator function persists (Lopez-Molina et al. 1997). These results imply that DBP functions primarily to control clock output, consistent with PDP1 ϵ function in flies. DBP binds to the same regulatory element as adenovirus E4 promoter ATF site-binding protein (E4BP4), a rhythmically expressed bZIP repressor related to VRI (Mitsui et al. 2001). Because E4BP4 is expressed in the opposite phase than DBP (Mitsui et al. 2001), sequential binding of E4BP4 and DBP could control rhythmic target gene transcription.

Table 1. Reducing and Increasing PDP1 ϵ Levels Disrupts Locomotor Activity Rhythms

Genotype	Percent rhythmic (N)	Period (h) $\pm$ S.E.M.	Power $\pm$ S.E.M.	Activity $\pm$ S.E.M.
<i>w</i> ;+/+/+/+	75.0 (172)	23.8 $\pm$ 0.03	78.9 $\pm$ 3.7	17.8 $\pm$ 0.6
<i>w</i> ;+/+/+ <i>tim</i> -Gal4/+	80.5 (41)	24.6 $\pm$ 0.07	76.7 $\pm$ 5.4	16.7 $\pm$ 0.9
<i>w</i> ;+/+/+ <i>pdf</i> -Gal4/+	57.0 (35)	24.2 $\pm$ 0.13	68.7 $\pm$ 7.7	14.0 $\pm$ 0.8
<i>w</i> ;PDP1 ϵ A/+;PDP1 ϵ B/+	77.8 (38)	23.4 $\pm$ 0.08	72.8 $\pm$ 5.3	28.3 $\pm$ 2.1
<i>w</i> ;PDP1 ϵ A/+;PDP1 ϵ B/ <i>tim</i> -Gal4	17.2 (29)	25.3 $\pm$ 0.23	35.3 $\pm$ 1.7	13.5 $\pm$ 1.0
<i>w</i> ;PDP1 ϵ A/+;PDP1 ϵ B/ <i>pdf</i> -Gal4	0 (27)	n.a.	n.a.	12.1 $\pm$ 0.7
<i>w</i> ;+/+/+/+	96.0 (25)	23.5 $\pm$ 0.15	96.1 $\pm$ 6.4	19.9 $\pm$ 2.3
<i>w</i> ;+/+/+ <i>tim</i> -Gal4/+	100 (31)	24.4 $\pm$ 0.21	78.4 $\pm$ 5.2	21.6 $\pm$ 2.0
<i>w</i> ;+/+/+ <i>pdf</i> -Gal4/+	95.5 (22)	24.3 $\pm$ 0.09	76.4 $\pm$ 9.5	13.5 $\pm$ 2.3
<i>w</i> ;PDP1 ϵ A/PDP1 ϵ B;+/+	73.9 (29)	24.5 $\pm$ 0.16	78.1 $\pm$ 11.7	27.1 $\pm$ 3.9
<i>w</i> ;PDP1 ϵ A/+;PDP1 ϵ B/ <i>tim</i> -Gal4	12.5 (31)	25.2 $\pm$ 0.12	24.5 $\pm$ 5.9	15.4 $\pm$ 1.8
<i>w</i> ;PDP1 ϵ A/+;PDP1 ϵ B/ <i>pdf</i> -Gal4	10.3 (29)	24.1 $\pm$ 0.02	27.2 $\pm$ 2.3	19.1 $\pm$ 1.5

Period and power were calculated for all rhythmic animals as described in Benito et al. (2007). Activity was calculated for all flies that survived to the end of the experiment, as described in Benito et al. (2007). n.a. indicates not applicable. (Reprinted, with permission, from Benito et al. 2007 [©The Society for Neuroscience].)

REGULATION OF *CLK* SPATIAL EXPRESSION

Circadian oscillator cells from *Drosophila* can be identified by the rhythmic expression of *per* and *tim* mRNA and protein (Hardin 1994; Plautz et al. 1997). In adults, oscillator cells have been identified in the brain, i.e., four clusters of lateral neurons (dorsal lateral neurons [LN_{DS}]; small ventral lateral neurons [sLN_{VS}]; large ventral lateral neurons [lLN_{VS}]; and a lateral posterior neuron [LPN]) and three clusters of dorsal neurons (dorsal neuron 1 [DN1]; dorsal neuron 2 [DN2]; and dorsal neuron 3 [DN3]), and in peripheral tissues (e.g., the gut, fat body, Malpighian tubules, the antenna, compound eye, proboscis, wing, and leg) (Hall 2003; Helfrich-Forster 2005; Shafer et al. 2006). Although a subset of oscillator neurons in the brain control morning and evening activity and oscillator neurons in the antenna control olfactory responses (Krishnan et al. 1999; Grima et al. 2004; Stoleru et al. 2004), outputs from other adult brain and peripheral oscillator cells have not been characterized. Circadian control of tissue-specific processes in *Drosophila* and other animals is thought to enable coordinate temporal control of behavior, physiology, and metabolism. Despite the importance of oscillator function in different tissues, relatively little is known about how this tissue-specific pattern of oscillator expression is specified.

Because CLK-CYC initiates circadian oscillator function by activating *per*, *tim*, *vri*, and *Pdp1ε* expression (Fig. 1), coexpression of CLK and CYC necessarily determines which tissues contain circadian oscillators. Such coexpression could arise by (1) expression of both CLK and CYC specifically in oscillator cells, (2) broad expression of CLK and oscillator-specific expression of CYC, (3) broad expression of CYC and oscillator-cell-specific expression of CLK, or (4) broad expression of CLK and CYC that overlaps specifically in oscillator cells. Localization of CLK has provided insight into which of these scenarios for CLK and CYC coexpression is correct. In adults, CLK is expressed in all oscillator cells as expected, but it is also expressed in nonoscillator cells (Houl et al. 2006). This result was somewhat surprising because previous work had shown that ectopic expression of CLK in certain brain neurons could generate circadian oscillators (Zhao et al. 2003). Perhaps CLK is able to induce oscillator function only in cells that already express CYC and the kinases and phosphatases that mediate feedback repression by PER and TIM. CRY expression in ectopic oscillator cells, and thus the ability of these oscillators to entrain to light, may come from CLK-CYC-dependent induction of the candidate *cry* activator PDP1ε (Cyran et al. 2003). In any case, expression of CLK in nonoscillator cells indicates that oscillator cells are determined by either oscillator-cell-specific expression of CYC or the overlapping expression of CLK and CYC specifically in oscillator cells. These possibilities can be distinguished when reagents necessary to localize CYC are available.

Circadian oscillators in the brain start to be expressed at earlier times during development than those in peripheral tissues. Brain oscillator cells can be detected as early as

the first larval instar and include precursors to the LNVs, DN2s, and a subset of DN1s (Sehgal et al. 1992; Kaneko et al. 1997; Helfrich-Forster 2005). PER expression in photoreceptors and the antenna is not detected until late during pupal development, indicating that functional oscillators are not expressed in these peripheral tissues until just before flies eclose (Liu et al. 1988). CLK expression precedes oscillator function in the larval brain and can be seen as early as embryonic stage 11, which indicates that brain oscillators are determined during mid embryogenesis (F.S. Ng et al., unpubl.).

Factors that activate *Clk* and/or *cyc* in oscillator cells have not yet been identified. Given the diverse array of brain cells and peripheral tissues that contain circadian oscillators, it is likely that multiple tissue-specific factors activate *Clk* and *cyc* in oscillator cells. *Clk* is activated in locomotor activity pacemaker neurons during embryogenesis, well before oscillator function is initiated in L1 larvae, and more than 1 week before these neurons control locomotor activity rhythms. Pacemaker neurons are unique in that they appear to be dedicated to controlling activity rhythms, yet are not required for locomotor activity per se. In contrast, peripheral oscillators are thought to control outputs inherent to a given tissue, where the tissue is necessary for the process being modulated. For instance, *Drosophila* olfactory sensory neurons (OSNs) control rhythms in electrophysiological responses to odors and are necessary for odor-dependent electrophysiological responses per se (Tanoue et al. 2004; Vosshall and Stocker 2007). *Clk* is presumably activated just before *per* and *tim* when OSNs and other clock-containing peripheral tissues are differentiating in late pupae (Liu et al. 1988). The activation of *Clk* during peripheral tissue differentiation implies that oscillator function is a core property of these tissues. When these activators are identified, it will be important to determine whether they also activate *cyc* and whether they maintain *Clk* activation in adults. Because circadian oscillators in the hypothalamic suprachiasmatic nucleus and peripheral tissues of mammals are analogous to the locomotor activity pacemaker neurons and peripheral oscillator tissues in flies, respectively, similar mechanisms may be responsible for specifying clock cell identity in mammals.

CONCLUSIONS

The core circadian timekeeping mechanism operates through interlocked *per/tim* and *Clk* feedback loops that regulate rhythmic transcription peaking around dusk and dawn, respectively. Although it is well established that phosphorylation-dependent rhythms in PER and TIM abundance are necessary for circadian oscillator function, mounting evidence indicates that rhythms in *per* and *tim* transcription are also required for circadian oscillator function. Presumably high levels of *per* and *tim* mRNAs at inappropriate times alters the overall levels, cycling phase, or activity of PER and TIM, thereby disrupting oscillator function. In contrast, reversing rhythms in *Clk* mRNA abundance disrupts neither circadian oscillator function nor CLK phosphorylation rhythms, indicating that *Clk* mRNA rhythms are dispensable. Taken together

with the PER dependence of rhythms in CLK phosphorylation, these results argue that the *per/tim* feedback loop has a dominant role in maintaining circadian oscillator function.

Despite its subservient role within the *Drosophila* circadian oscillator, the *Clk* feedback loop regulates rhythmic transcription in a phase opposite to that of the *per/tim* feedback loop. *Clk* feedback-loop-dependent transcription of genes such as *Clk* was initially thought to be mediated by competition between VRI and PDP1ε for V/P boxes: VRI binds V/P boxes to repress *Clk* transcription at dusk because the VRI/PDP1ε ratio is high, and PDP1ε binds V/P boxes to activate *Clk* transcription at dawn because the VRI/PDP1ε ratio is low. However, we recently found that altering the VRI/PDP1ε ratio by reducing or increasing PDP1ε levels had little effect on *Clk* transcription or VRI cycling. Taken together with the constant high levels of *Clk* mRNA in mutants that express little if any VRI or PDP1ε (i.e., *Clk^{rk}* and *cyc⁰¹*), this result suggests a model in which VRI periodically represses the constant activation of *Clk* transcription. Although PDP1ε does not greatly affect *Clk* transcription, PDP1ε is required for rhythms in locomotor activity. We hypothesize that PDP1ε has a major role in activating genes that control rhythmic outputs, perhaps in conjunction with VRI. Such a role is consistent with that proposed for DBP and E4BP4, the mammalian homologs of PDP1ε and VRI, respectively.

Circadian oscillators are expressed in several clusters of brain neurons and a variety of peripheral tissues. This spatial distribution is controlled by factors that activate *Clk* and *cyc* because CLK-CYC initiates circadian oscillator function. However, *Clk* expression is not restricted to oscillator cells, which suggests that CYC is either specifically expressed only in oscillator cells or that CYC is broadly expressed and specifically overlaps CLK expression in oscillator cells. Moreover, expression of CLK in nonoscillator cells indicates that CLK contributes to processes other than circadian oscillator function. Nevertheless, ectopic expression of *Clk* in certain brain cells can generate circadian oscillator function, presumably because these cells already express CYC and kinases/phosphatases normally found in oscillator cells. The developmental expression of CLK suggests that locomotor activity pacemaker cells are determined during mid embryogenesis. CLK appears to be activated in peripheral oscillator tissues around the time they differentiate in late pupae. Insight into oscillator cell determination and maintenance will come with the identification and characterization of *Clk* and *cyc* activators.

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