Circadian oscillators of *Drosophila* and mammals

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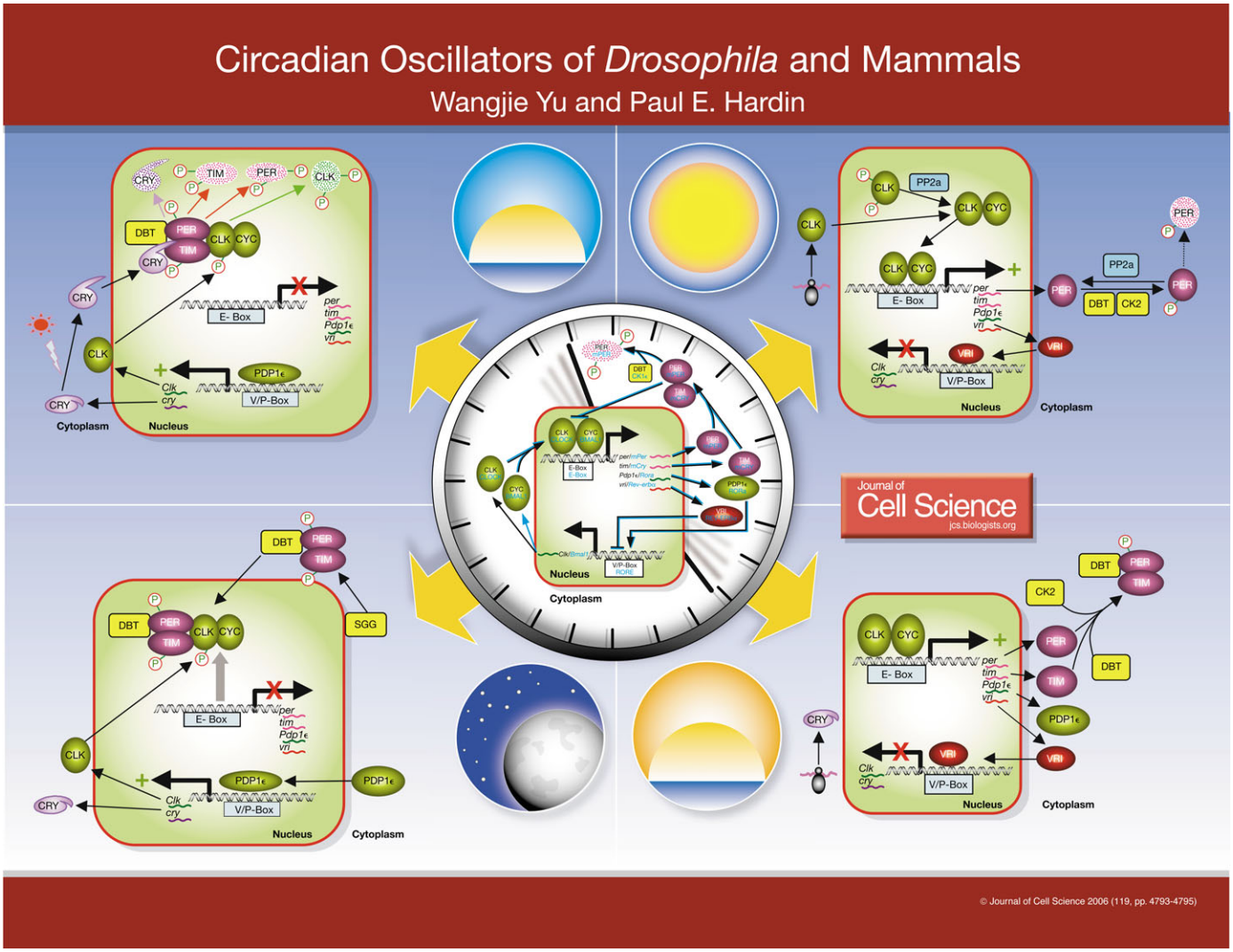
Animals, plants, fungi and even some prokaryotic organisms display daily rhythms in behavior, physiology, metabolic activity and gene expression. These rhythms are not passively driven by environmental cycles (e.g. light and temperature) but are controlled by endogenous circadian clocks that keep time even in the absence of environmental time cues. Environmental cycles are nevertheless required to entrain these clocks so that they activate rhythmic processes at the appropriate time of day. In animals, circadian clocks reside in a variety of tissues, including the brain, sensory structures and a number of internal organs (Glossop and Hardin, 2002). Although all clocks drive rhythms in gene expression, some control tissue-autonomous rhythms in physiology and metabolism, whereas others form networks of clock tissues that control rhythms in behavior (Bell-Pedersen et al., 2005; Chang, 2006).

Circadian clocks have three basic parts: an input pathway that receives environmental cues and transmits them to the circadian oscillator, a circadian oscillator that keeps circadian time and activates output pathways, and output pathways that control various metabolic, physiological and behavioral processes (Eskin, 1979). Considerable effort has been focused on determining how the circadian oscillator functions to keep circadian time. Genetic and molecular studies in the fruit fly have contributed significantly to our understanding of the circadian oscillator mechanism. Identification and isolation of the first clock gene from *Drosophila*, *period* (*per*), and subsequent analysis of its expression led to the first molecular model of the circadian oscillator – an autoregulatory feedback loop in gene expression (Hall, 2003). Discovery of additional clock genes in *Drosophila* not only support the feedback loop model but add substantially to its mechanistic detail and complexity. Current analysis indicates that the *Drosophila* circadian oscillator is composed of two interlocked feedback loops – the original *per/timeless* (*tim*) loop and a *Clock* (*Clk*) loop (Hardin, 2004; Hardin, 2005; Stanewsky, 2003) – and



(See poster insert)

exhibits striking similarity to that in mammals (shown in the center of the poster).

The fly *per/tim* feedback loop

Starting from mid-day (top right), two basic-helix-loop-helix/PAS domain transcription factors, CLOCK (CLK) and CYCLE (CYC), form heterodimers and bind E-box regulatory elements (CACGTG) to activate *per* and *tim* transcription. Although *per* and *tim* mRNA levels accumulate during this phase of the circadian cycle, PER and TIM protein levels do not. TIM remains at low levels because it is destabilized by light (see below). PER is phosphorylated by DOUBLE-TIME (DBT) kinase and, without TIM, is targeted for degradation by the ubiquitin/proteasome pathway (reviewed by Harms et al., 2004). PER phosphorylation is also dependent on casein kinase 2 (CK2), which is believed to be a primer kinase for DBT, and protein phosphatase 2a (PP2a), which dephosphorylates PER (Allada and Meissner, 2005; Harms et al., 2004). The coordinated effects of kinases and phosphatases during this time keep PER at low levels and in a hypophosphorylated state because hyperphosphorylated PER is degraded owing to low TIM levels.

After sundown (bottom right), *per* and *tim* continue to be transcribed and their mRNAs reach peak levels during the early evening. TIM begins to accumulate in the dark and forms a complex with PER and DBT, thereby stabilizing PER despite continued phosphorylation by DBT and CK2. As a result, PER and TIM accumulate to high levels during the middle of the night (bottom left). As DBT-PER-TIM accumulates, phosphorylation of TIM by SHAGGY (SGG) is believed to be a crucial step that triggers DBT, PER and TIM entry into the nucleus (Harms et al., 2004). Although these proteins enter the nucleus at about the same time, PER-DBT and TIM enter the nucleus separately (Hall, 2003). Once in the nucleus, PER-DBT or re-formed DBT-PER-TIM complexes bind to CLK-CYC, which represses transcription of *per*, *tim* and other genes by removing CLK-CYC from E-boxes and promotes DBT-dependent hyperphosphorylation of CLK (Hardin, 2005). The ~6-hour delay between *per* and *tim* transcription and accumulation of PER and TIM proteins in the nucleus is thought to be a critical determinant of circadian period. By the end of the night, TIM levels begin to decline through an as yet uncharacterized mechanism.

At dawn (top left), a light-induced conformational change in the blue-light photoreceptor cryptochrome (CRY) promotes the formation of CRY-TIM complexes, TIM degradation by the ubiquitin/proteasome pathway, and CRY destabilization (Ashmore and Sehgal, 2003). PER and CLK are also degraded during the early morning, but their degradation is promoted by DBT-dependent phosphorylation (Hardin, 2005). Although PER falls to its lowest levels by the middle of the day (top right), CLK levels remain relatively constant because hypophosphorylated CLK is generated by new CLK synthesis or PP2a-dependent dephosphorylation of hyperphosphorylated CLK (Kim and Edery, 2006; Yu et al., 2006). Hypophosphorylated CLK then forms a heterodimer with CYC and binds to E-boxes to initiate a new cycle of *per* and *tim* transcription (Hardin, 2005). The *per/tim* feedback loop is a necessary component of the circadian oscillator since *per*-null and *tim*-null mutants each abolish circadian oscillator function.

The fly *Clk* loop

Interlocked with the *per/tim* feedback loop is a second feedback loop in *Clk* transcription. Two additional CLK-CYC target genes, *vrille* (*vri*) and *PAR domain protein 1ϵ* (*Pdp1ϵ*), are activated by E-box binding at mid-day (top right). Although *vri* mRNA accumulates in phase with *per* and *tim* mRNAs, *Pdp1ϵ* RNA accumulation is delayed by several hours (Hardin, 2004). In contrast to the delayed accumulation of PER and TIM, VRI levels rise in concert with *vri* mRNA. As VRI accumulates in the nucleus during the mid to late day, it binds VRI/PDP1ϵ binding sites (V/P-boxes) [consensus A(/G)TTA(/T)T(/C):GTAAT(/C)], to repress *Clk* and *cry* transcription (Hardin, 2004). VRI protein reaches peak levels during the early evening (bottom right), which is coincident with low levels of *Clk* and *cry* mRNAs. Despite the low levels of *cry* mRNA, CRY begins to accumulate because it is relatively stable in the dark (Ashmore et al., 2003).

Whereas PDP1ϵ accumulates to peak levels during the mid to late night (bottom-left), VRI levels decline during this time owing to DBT-PER-dependent repression of *vri* transcription. The rising ratio of PDP1ϵ/VRI favors binding of PDP1ϵ to V/P-boxes, which activates *Clk* and *cry* transcription (Hardin, 2004). PDP1ϵ levels start to decline during the late evening, and are low by the early morning (top left). However, small amounts of PDP1ϵ may continue to activate *Clk* and *cry* transcription until the middle of the day, when VRI starts to accumulate after the next cycle of CLK-CYC transcription is initiated (top right).

The *Clk* feedback loop necessarily drives rhythmic transcription in the opposite phase as the *per/tim* loop because CLK-CYC activates E-box transcription and represses V/P-box transcription around dusk, and DBT-PER (or DBT-PER-TIM) represses E-box transcription and activates V/P-box transcription around dawn (Hardin, 2004). In addition to driving rhythms in *per*, *tim*, *vri*, *Pdp1ϵ*, *Clk* and *cry* expression, these feedback loops drive rhythms in the expression of ~150 clock output genes (Wijnen et al., 2006). For example, the *slowpoke* (*slo*) Ca^{2+}-dependent voltage-gated potassium channel and the SLO-binding protein (*slob*) genes are rhythmically expressed (Ceriani et al., 2002; Jaramillo et al., 2004), which suggests that aspects of neurotransmission are under clock control. Since *Clk* and *cry* mRNA cycling do not control CLK and CRY levels or activity, the *Clk* feedback loop may be more important for controlling rhythmic outputs than for sustaining circadian oscillator function.

The circadian timekeeping mechanism in *Drosophila* and mammals is conserved

As in *Drosophila*, the circadian oscillator in mammals is composed of interlocked transcriptional feedback loops. Many components of the *Drosophila* circadian oscillator have orthologs and/or functional equivalents in mammals. In fact, the *Drosophila* circadian oscillator depicted in the poster can be converted to a mammalian circadian oscillator by making the following changes (see blue lettering and arrows in center): CLOCK-BMAL1 replaces CLK-CYC, mPER-

mCRY replaces PER-TIM, CK1ϵ replaces DBT, REV-ERBα replaces VRI, RORa replaces PDP1ϵ, RORE elements replace V/P-boxes, and PP2a, SGG, CK2 and CRY are removed. Several differences in the structure or function of these mammalian clock components are notable. mPER-mCRY functions to repress CLOCK-BMAL1 transcription, but mCRY is the major repressor as opposed to PER in flies (Reppert and Weaver, 2002). Although CRY functions as a circadian photoreceptor in flies, its role as a transcriptional repressor has been retained in at least some fly peripheral tissues (Collins et al., 2006). REV-ERBα and RORa are nuclear receptors rather than bZIP transcription factors like VRI and PDP1ϵ, and they regulate transcription by binding RORE elements rather than V/P-boxes (Bell-Pedersen et al., 2005). Although the circadian oscillator mechanisms of *Drosophila* and mammals show striking similarities, their entrainment to light differs markedly. In flies, light entrains the circadian oscillator by inducing TIM degradation, whereas light entrains the mammalian oscillator by inducing *Per1* transcription (Reppert and Weaver, 2002). Since light can directly entrain *Drosophila* oscillators (Ashmore and Sehgal, 2003), but indirectly entrains mammalian oscillators (Reppert and Weaver, 2002), it is not surprising that different mechanisms have evolved in these animals.

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