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The molecular ticks of the *Drosophila* circadian clock

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Drosophila is a powerful model to understand the mechanisms underlying circadian rhythms. The *Drosophila* molecular clock is comprised of transcriptional feedback loops. The expressions of the critical transcriptional activator CLK and its repressors PER and TIM are under tight transcriptional control. However, posttranslational modification of these proteins and regulation of their stability are critical to their function and to the generation of 24-h period rhythms. We review here recent progress made in our understanding of PER, TIM and CLK posttranslational control. We also review recent studies that are uncovering the importance of novel regulatory mechanisms that affect mRNA stability and translation of circadian pacemaker proteins and their output.

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Introduction

Circadian clocks are molecular oscillators that enable organisms from cyanobacteria to humans to anticipate daily events that occur in their environment. Although circadian rhythms are self-sustained, they receive input from the environment such as light and temperature, which determine their phase. However, their period is independent of ambient temperature. Circadian clocks keep time throughout the day and seasons and thus have a broad impact on the biology of most organisms. They control behavioral, physiological and molecular rhythms such as locomotor activity, feeding, sleep, reproduction and gene expression [1]. They also have a key role in sun-compass navigation, allowing migratory animals such as monarch butterflies to compensate for time-of-day when using the sun for direction [2].

Historically, *Drosophila* has been at the forefront of circadian rhythm research due to its ease of use and its advanced molecular and genetic toolkit [3]. Importantly, the molecular circadian clock is highly conserved from *Drosophila* to humans and consists of interlocked transcriptional negative feedback loops (Figure 1). At the center of these loops are the transcription factors CLOCK (CLK) and CYCLE (CYC), which form a heterodimer that drives the expression of PERIOD (PER) and TIMELESS (TIM). PER and TIM function as a heterodimeric repressor of CLK/CYC in the nucleus. In a second transcriptional feedback loop, CLK and CYC drive the expression of VRILLE (VRI) and PAR DOMAIN PROTEIN1ε (PDP1ε), which repress and activate *clk* transcription, respectively [4]. The two transcriptional feedback loops control multiple clock controlled genes (ccgs), but interestingly generate anti-phase mRNA oscillations [5]. PER and TIM synthesis and degradation is tightly regulated throughout the day, and this complex regulation is critical to set the pace of the circadian pacemaker. Indeed, delays in PER and TIM accumulation and nuclear translocation are thought to be critical for generating stable 24-h period rhythms. PER and TIM abundances are tightly regulated at the transcriptional, post-transcriptional, and post-translational levels. Important progress has recently been made on the two latter regulatory levels, which will be the focus of this review.

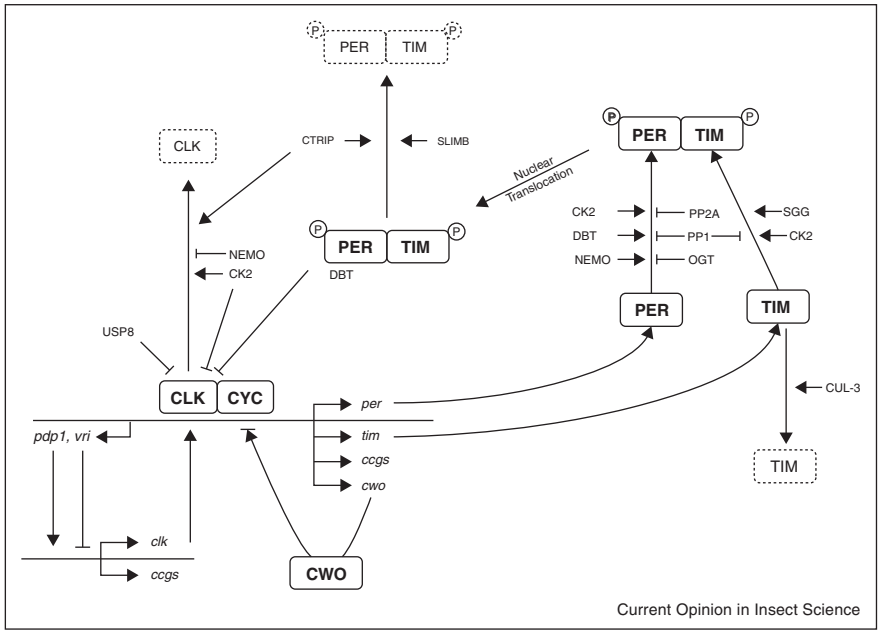
Post-translational control

PER and TIM phosphorylation and ubiquitination

Phosphorylation has been shown to have a critical role in determining the period of the clock. In fact, PER and TIM are phosphorylated at many sites throughout the day by several kinases. These include DOUBLETIME (DBT), SHAGGY (SGG), CASEIN KINASE2 (CK2) and NEMO (NMO) which regulate PER and TIM stability and nuclear entry, depending on the location and cooperativity of the target site for phosphorylation [1,6] (Figure 1). For example, mutation of T610 on PER to alanine lengthens the period by only 2–3 h while S613 mutation has minimal effect. However, simultaneous mutations of both sites to alanine results in reduced phosphorylation and increased stability of PER and a ~30 h period length, suggesting that these sites cooperate to determine circadian period [7].

DBT — the *Drosophila* homolog of CASEIN KINASE1δ/ε (CK1 δ/ε) — extensively phosphorylates PER throughout the day, which ultimately results in its degradation via the proteasome [5]. This is mediated by the F-box protein SLIMB binding to PER (Figure 1). This interaction is promoted by phosphorylation of PER's S47 [8–10]. Furthermore, this binding can be negatively regulated by NMO and DBT-mediated phosphorylation at S585, S589

Figure 1



Conceptual schematic of the *Drosophila* circadian molecular clock. Heterodimeric transcription factor complex CLK/CYC drives the expression of many clock-controlled genes (ccgs). Among them, *per* and *tim* encode critical transcriptional repressors of CLK/CYC. This core feedback mechanism is tightly regulated at the transcriptional, post-transcriptional and post-translational levels to introduce delays and checks to ensure a ~24 h cycle. This figure emphasizes transcriptional and post-translational mechanisms. Arrows (→) indicate positive/enhanced regulation, while blunt-ends (⊣) indicate negative/inhibitory regulation. CWO appears to be able to function either as a repressor or an activator [66]. Protein degradation is depicted with dashed lines.

and S596. This inhibits DBT-mediated phosphorylation of S47 and thereby delaying PER degradation by SLIMB [11]. This shows that phosphorylation does not need to be in close proximity and distant sites can influence each other in regulating PER stability and function. Phosphorylations also occur on TIM and are mediated by kinases CK2α and SGG, which regulates TIM stability, similar to PER phosphorylation [12–14]. These studies show that phosphorylation events determine the speed of the clock by regulating the rate of nuclear accumulation of PER and TIM [13,14].

Interestingly, SLIMB also controls TIM stability, with the help of another ubiquitin (UB)-ligase that contains CULLIN-3 (CUL-3) [15••] (Figure 1). CUL-3 preferentially complexes with hypo-phosphorylated TIM unbound to PER, while SLMB targets preferentially hyper-phosphorylated TIM bound to PER. Disrupting CUL-3 function through RNAi or expression of a dominant–negative mutant leads to long period phenotypes and arrhythmicity. CUL-3 downregulation results in low amplitude TIM cycling, with elevated hypo-phosphorylated TIM during the subjective day. This would fit with a simple model in which CUL-3 preferentially degrades hypo-phosphorylated TIM (Figure 1). Unexpectedly however, the abundance of the hyper-phosphorylated TIM isoform is increased with the overexpression of a dominant-negative CUL-3 mutant. CUL-3 might thus also repress TIM phosphorylation, or destabilize this isoform. These phenotypic differences could reflect different efficiency of RNAi or overexpression of a dominant negative mutant to disrupt CUL-3 activity. Further studies are clearly needed to elucidate fully the role of CUL-3 in the circadian pacemaker.

Formation of the PER/TIM heterodimer stabilizes PER, protecting it from phosphorylation and SLIMB degradation [8]. Also, PER and TIM phosphorylation is counteracted by the activity of protein phosphatases 2A (PP2A) on PER and 1A (PP1) on PER and TIM [16,17] (Figure 1). This interplay between kinases and phosphatases fine tunes the speed of the clock to ~24 h by regulating the stability of PER and TIM and the timing of PER/TIM nuclear entry. PER phosphorylation sites targeted by PP2A and PP1 have been mapped in cell culture, but precise action of PP2A and PP1 on PER (and TIM) *in vivo* still needs to be elucidated [7]. In summary, a detailed picture is emerging of cascades of phosphorylation and dephosphorylation events that regulate PER and TIM stability, dimer formation and nuclear entry. These cascades introduce delays in the progression of the circadian cycle that are critical for period determination.