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Regulating a Circadian Clock's Period, Phase and Amplitude by Phosphorylation: Insights from *Drosophila*

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Much progress has been made in understanding the molecular underpinnings governing circadian (~24 h) rhythms. Despite the increased complexity in metazoans whereby inter-cellular networks form the basis for driving overt rhythms, such as wake–sleep cycles in animals, single isolated cells can exhibit all the formal properties of a circadian pacemaker. How do these cell-autonomous rhythm generators operate? Breakthrough studies in *Drosophila melanogaster* led to the realization that the molecular logic underlying circadian clocks are highly shared. Most notably, interconnected transcriptional-translational feedback loops produce coordinated rhythms in "clock" RNAs and proteins that are required for the daily progression of clocks, synchronization to local time and transducing temporal signals to downstream effector pathways. More recent findings indicate prominent roles for reversible phosphorylation of clock proteins in the core oscillatory mechanism. In this review we focus on findings in *Drosophila* to explore the multiple levels that reversible phosphorylation plays in clock function. Specific clock proteins in this system are subjected to different phosphorylation programs, which affect three key properties of a circadian oscillator, its period, amplitude and phase. The role of phosphorylation in clocks is of clear relevance to human health because mutations that affect the PERIOD (PER) phosphorylation program are associated with familial sleep disorders. In addition, the central role of phosphorylation in the assembly of a circadian oscillator was dramatically shown recently by the ability to reconstitute a circadian phosphorylation/dephosphorylation cycle *in vitro*, suggesting that the dynamics of clock protein phosphorylation are at the "heart" of circadian time-keeping.

Key words: circadian clock, *Drosophila*, feedback loops, period, phosphorylation.

Circadian rhythms are oscillations with periods equal to or close to the 24 h solar day that are endogenously driven by life-forms (reviewed in Refs. *1* and *2*). By definition, these rhythms persist with stable ~24 h periods even in the absence of daily environmental cues but importantly can be synchronized (or entrained) by these cues, most notably the day–night cycles, such that they establish a stable phase relationship with local time. In such a manner an organism's physiology and behavior can be temporally regulated to occur at biologically advantageous times. Synchronization to local time also imparts the ability to anticipate and hence prepare for environmental changes. Another hallmark feature, although a still mysterious attribute, of circadian rhythms is that the period length is rather fixed over a wide range of physiologically relevant temperatures (termed temperature compensation), which makes biological "sense" as the solar day remains 24 h whether it is winter or summer. This does not mean that circadian clocks are insensitive to temperature. It is just that although the frequency is stable, other aspects of the rhythm such as phase and amplitude are modulated by temperature in a manner that indicates adaptive significance. For example, the timing of a rhythm relative to local time can be significantly altered by temperature and day-length, allowing for seasonal acclimation. Moreover, in many cases circadian clocks appear to measure night or day length and contribute to seasonably appropriate photoperiodic responses, such as flowering in plants.

The importance of circadian rhythms to human health and well-being are widely recognized. Malfunctions in the circadian timing system are implicated in many disorders and diseases including affective disorders such as SAD (seasonal affective disorders or "winter" depression), chronic sleep problems in the elderly, a range of metabolic syndromes and even susceptibility to cancer and alcoholism—although it is not always clear if the effects are due to *bona-fide* circadian parameters or non-clock effects of clock gene functions (*3–6*). Moreover, circadian dysfunction is accentuated in modern societies, such as the general malaise associated with jet-lag and shift-work, which are known to decrease productivity and enhance the occurrence of accidents. Finally, the fact that our physiology undergoes daily regulation is an important consideration for treatment protocols, such as chemotherapy, whereby the efficacy of a drug (benefit versus toxicity) is highly dependent on the time of administration (*7*). Clearly, understanding how circadian clocks tick, are synchronized by external cues and regulate the timing of downstream pathways are important objectives.

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Drosophila as a model system and the many roles of reversible phosphorylation in clocks

Studies in *Drosophila melanogaster* have provided some of the most important breakthroughs in understanding the molecular underpinnings of circadian clocks (reviewed in Ref. *8*). The first clock gene to be identified and molecularly characterized was the *period* (*per*) gene in that species (*9*). A conceptual breakthrough occurred when it was shown that daily fluctuations in *per* RNA levels were negatively controlled by PER protein activity in a manner that correlated with daily overt behavioral rhythms (*10*). This led to the by now standard view of transcriptional feedback loops (intertwined negative and positive) as central components in the generation of cell-autonomous circadian pacemakers (*11*).

Subsequent work added increased complexity to this transcriptional circuitry with the first *in vivo* biochemical characterization of a clock protein, again in this case the PER protein from *Drosophila* (*12*). PER was shown to undergo daily changes in abundance and phosphorylation; remarkably similar findings were observed for key clock proteins in other organisms (*e.g.*, *13*, *14*). The first clock-relevant kinase was also first identified in *Drosophila*, termed DOUBLETIME (DBT), a homolog of casein kinase Iε/δ (CKIε/δ) (*15*, *16*). In animal clocks, CKIε/δ and probably other kinases and protein phosphatases regulate the temporal phosphorylation of PER proteins whereby highly phosphorylated isoforms are targeted for rapid degradation by the ubiquitin/proteasome pathway (*17*, *18*). A major effect of regulating the temporal program in PER phosphorylation appears to be in setting the pace of the clock. Indeed, mutations that either affect putative phosphorylation sites on PER or CKIε/δ activity result in familial sleep disorders in humans whereby the timing of sleep/activity is very abnormal (*19*, *20*). Likewise, mutations in phosphorylation sites of other key clock proteins that undergo robust cycles in abundance, such as FREQUENCY (FRQ) in *Neurospora* (*21*), modulate the period length of the internal clock and associated behavioral rhythms.

More recent work has shown that DBT also regulates the phosphorylated state of CLOCK (CLK) (*22*, *23*), a basic-helix-loop-helix (bHLH)/PAS (Per-Arnt-Sim)-containing transcription factor that in conjunction with its partner CYCLE (CYC) stimulates the daily transcription of *per*, in addition to other clock and downstream genes (*8*). However, here recent findings suggest that CLK is regulated by a complex phosphorylation/dephosphorylation program that maintains its overall intracellular concentration within a range compatible with high-amplitude rhythm generation (*22*), an important consideration since CLK appears to be the limiting component in the transcriptional feedback loops (*24*). This viewpoint is in agreement with recent findings suggesting that CLK levels are intimately linked with the amplitude of the clock (*25*, *26*).

Finally, the role of phosphorylation also plays a prominent role in synchronization of the *Drosophila* clock to the daily light–dark cycles (reviewed in Ref. *27*). Here, the key player is TIMELESS (TIM), the second clock gene to be characterized in *Drosophila*. Light evokes the rapid degradation of TIM *via* a mechanism that also includes phosphorylation and targeting to the proteasome (*28*). Because TIM is critical for stabilizing PER (*29*), this ensures that the PER biochemical rhythms (phosphorylation, subcellular localization, levels and activity) are also synchronized to the light–dark cycle—hence, keeping the timing of the transcriptional feedback loops aligned with local time.

In this review we focus on the *Drosophila* circadian timing system to highlight various roles for reversible phosphorylation in the clock (Fig. 1). The review is centered on three different clock proteins, PER, CLK and TIM that highlight different variables of a circadian oscillator. Phosphorylation of PER appears crucial for the rate of clock progression. In the example of CLK, it was recently proposed that the CLK phosphorylation program has more to do with minimizing "molecular noise" from stochastic fluctuations in levels as a means to ensure the system maintains sufficient oscillatory potential (*22*). Finally, stimulus-driven phosphorylation of a clock protein, such as is the case with TIM, emphasizes a role in pacemaker adjustment of phase. Before describing different effects of phosphorylation on clock properties we quickly summarize the overall molecular circuitry underlying cell-autonomous circadian pacemakers in *Drosophila*.

Interconnected transcriptional-translational feedback loops

The intracellular circadian time-keeping mechanism in *Drosophila* is largely depicted as two interconnected transcriptional feedback loops with overlaying posttranslational regulatory circuits (*8*). Prominent players in the first or "major" feedback loop are PER, TIM, CLK and CYC. As noted above, a heterodimer of CLK and CYC bind E-box–containing DNA elements and coordinately stimulate transcription of *per* and *tim*. The RNA levels of *per* and *tim* begin to rise in the mid-day and peak several hours after darkness begins, the shape of the curve modulated by the photoperiod and temperature (*e.g.*, *30*). However, PER and TIM protein levels are very low during the day and peak several hours after their cognate RNAs reach maximal concentrations. In daily light-dark cycles a contributing factor to this delay between *per/tim* RNA and protein profiles is because light evokes the rapid degradation of TIM, and TIM is required for the stabilization of PER. During the day *de novo* synthesized PER is phosphorylated by DBT, which leads to its degradation. As TIM accumulates during the late day/early night it interacts with PER attenuating the effects of DBT on PER stability. The accelerated buildup of PER and TIM somehow stimulates their nuclear accumulation, beginning in the early to mid-night. Once in the nucleus PER (and perhaps TIM) binds CLK-CYC, inhibiting its activity. After several hours, nuclear TIM levels wane and hyperphosphorylation of PER increases, which eventually targets PER for degradation with concomitant relieve of autoinhibition leading to a new round of *per/tim* expression.

In the so-called "second" loop (which will only be briefly mentioned here), *Clk* expression is regulated by two bZip transcription factors, VRILLE (VRI) and PDP1ε. Apparently, alternative inhibition by VRI and activation by PDP1ε underlies the daily changes in *Clk* RNA levels (*31*, *32*).

A very similar molecular logic is also found in the mammalian circadian clock (reviewed in Ref. *33*). Notable

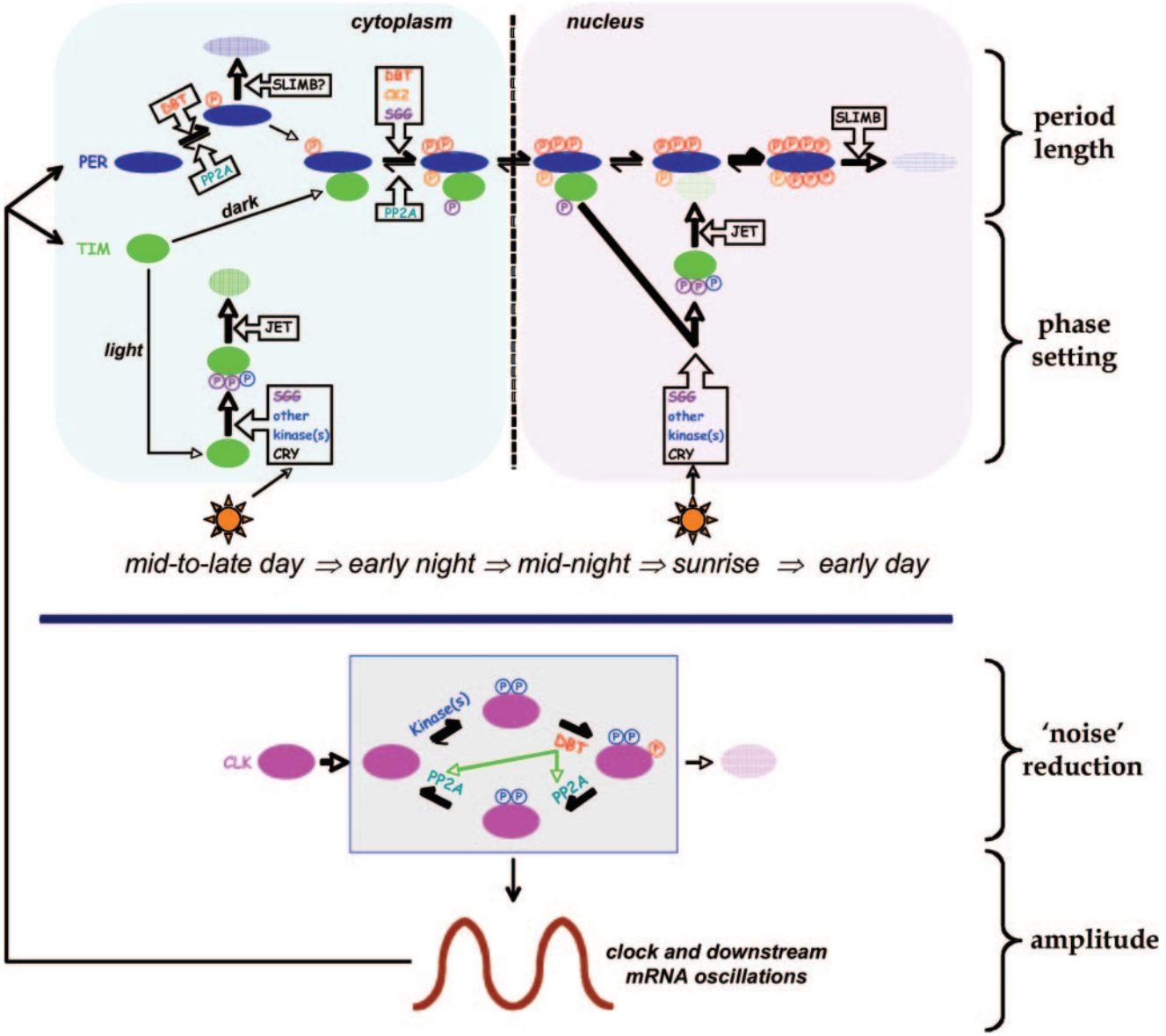

Fig. 1. **Model for the many roles of phosphorylation in the *Drosophila* clock.** The progressive phosphorylation of PER sets the overall pace of the clock. This biochemical rhythm is synchronized to the light–dark cycle by the light-mediated degradation of TIM. Finally, the highly balanced phosphorylation/dephosphorylation of CLK stabilizes the abundance of this limiting component, which affects the amplitude of the oscillator by driving cyclic gene expression. The schematic is meant to illustrate the interconnections between the different phosphorylation programs and the circadian oscillator's attribute (period, phase or amplitude) most intimately linked. See text for more details.

differences are that CRYPTOCHROMEs (CRYs) are the major negative inhibitors of CLK-BMAL1 transactivation. In addition, it is mainly the levels of *bmal1* RNA and protein that exhibit daily cycles in abundance, in contrast to the situation in *Drosophila* where *Clk* is rhythmically expressed but *cyc* is constitutively expressed. Finally, daily changes in the levels of *bmal1* are driven by a different family of negatively and positively acting transcription factors, Rev-erbα and Rora, respectively.

PER—a most appropriate name!

Thirty-five years ago Ron Konopka and Seymour Benzer reported the identification of *per* with three classic alleles; per^{short} (per^{S}), which has a period of ~19 h, per^{long} (per^{L}) with ~29 h periodicities and per^{0} which is arrhythmic (*9*). As the mutations were all in coding regions (with per^{0} introducing a premature translation stop codon) (*34*), this indicated that changes in the "activity" of PER can change the pace of the clock, speeding it up or slowing it down. Although it is not clear how the per^{L} and especially per^{S} mutations lead to changes in the period of the clock, overwhelming evidence indicates that the pace of the clock is strongly influenced by PER phosphorylation.

When assayed by immunoblotting of total head extracts (the usual source for *in vivo* biochemical studies), PER protein displays temporal changes in steady state levels

and phosphorylation (*12*). The relationship between phosphorylation and abundance is complex, as is the temporal phosphorylation pattern where multiple isoforms can co-exist and vary as a function of time. In general, the overall pattern is one where *de novo* synthesized PER is first phosphorylated in the cytoplasm followed by progressive increases in phosphorylation, a trend that continues in the nucleus until the early day where hyperphosphorylated isoforms are degraded.

In flies and cultured cell systems, the progressive phosphorylation of PER requires DBT (*15*, *16*, *35*). On the weight of the evidence it seems certain that DBT-mediated phosphorylation of PER in the cytoplasm targets it for rapid degradation and that TIM binding protects PER against this destruction. But we also know that highly phosphorylated isoforms of PER can accumulate in the nucleus for several hours in the absence of *de novo* synthesis (*12*, *36*). Indeed, it is thought that in the nucleus only the most highly phosphorylated isoforms are targeted for rapid degradation. Hyperphosphorylated PER is recognized by the F-box protein SLIMB, which targets it to the 26S proteasome for destruction (*35*, *37*). TIM seems to slow down but not eliminate DBT-mediated hyperphosphorylation of PER (*35*, *38*, *39*). For example, light stimulation that leads to reduction in the levels of TIM accelerates PER hyperphosphorylation and degradation (*39*, *40*). It is possible that the manner in which DBT and TIM operate to regulate the stability of PER differs in the cytoplasm and nucleus. Also, it is not known if SLIMB is involved in the turnover of cytoplasmic PER.

Thus, one can identify three phases of DBT-dependent PER phosphorylation and the role of TIM (*41*). First, *de novo* synthesized PER in the cytoplasm is very unstable and requires TIM to accumulate. Second, in the nucleus PER persists for many hours in the absence of *de novo* synthesis while undergoing progressive increases in phosphorylation, the rate of which is attenuated by TIM. Finally, TIM levels drop enhancing the hyperphorphorylation of nuclear PER and concomitant rapid downswing in its levels. In this scenario the progressive phosphorylation of PER beginning in the cytoplasm and maintained in the nucleus might function to *delay* its degradation until a critical threshold level of hyperphosphorylation is attained. Moreover, the level of PER phosphorylation might yield calibrated responses; for example, higher levels of "global" PER phosphorylation could increase the probability of targeting to the proteasome by SLIMB.

Phosphorylation of PER is not only affecting its stability but other attributes such as subcellular localization (*42*–*46*) and ability to repress CLK-CYC–mediated transcription (*47*). This introduces another level of complexity whereby kinases besides DBT directly regulate PER phosphorylation. Presently, the best characterized "other" kinase is CK2. While CK2 enhances the instability of PER (*42*, *45*, *47*), its main *in vivo* role seems weakly linked to the overall progressive phosphorylation and degradation of PER. Rather, CK2 has a prominent role in regulating the timing of PER nuclear accumulation/entry in several (but likely not all) key brain pacemaker neurons (*42*, *45*, *46*). Deficiencies in CK2 activity slow down PER nuclear staining as does mutations in putative CK2 phosphorylation sites on PER. To complicate matters further DBT also regulates the timing of PER nuclear entry/accumulation (*43*, *44*), although this might be *indirect via* regulating PER stability; *i.e.*, high levels of PER likely bypass a requirement for TIM in nuclear translocation. Additional confusion is based on the recent suggestion that highly phosphorylated isoforms of PER are better retained in the nucleus as a consequence of their increased ability to function as transcriptional repressors (*47*). Clearly, more work is required to better understand how phosphorylation regulates the timing of PER nuclear entry/accumulation. Phosphorylation has been shown to influence the subcellular distributions of mammalian PER proteins but the results seem to vary depending on the experimental system evaluated (reviewed in Ref. *17*).

An important consideration when discussing the temporal regulation of PER phosphorylation is that differences in phosphorylation rate, although leading to period changing phenotypes, could preferentially shrink or stretch different phases of the daily cycle. This is because the different stages of PER phosphorylation are inextricably linked to different parts of the daily cycle. For example, if the stability of PER is specifically decreased during its accumulation phase such that nuclear entry is delayed but other events progress at normal rates, this would lengthen the proportion of "early night" and hence overall length of the period. However, an unstable PER in the nucleus could speed up the cycle by earlier relieve of autoinhibition, leading to a specific shortening of the "late night/early day." Indeed, different *period*-altering mutants differentially affect the relative proportion of day and night (*48*).

While kinases are obviously involved in the PER phosphorylation program, protein phosphatase activity also plays a significant role. PP2A has been shown to regulate PER stability and behavioral rhythms in *Drosophila* (*49*). Intriguingly, *twins* (*tws*)/PR55 and *widerborst* (*wdb*)/B56-2, two regulatory subunits of PP2A, exhibit specificity for PER and undergo circadian cycling in expression. As *dbt* is constitutively expressed, this raises the possibility that temporal changes in PER phosphorylation are, in part, driven by oscillations in PP2A activity. In a simplified cell culture system TWS and WDB target PER and stabilize it, consistent with hyperphosphorylation rendering PER less stable (*49*). Although it is not known if PP2A counterbalances the activities of all the PER kinases and/or has preferential effects on specific PER phosphorylation sites, it is clear that the *Drosophila* clock is sensitive to PP2A activity. Thus, the rate of progression through the PER phosphorylation program is likely to be decided by many variables including relative concentrations of the relevant players in the relevant places. Nonetheless, the metabolic fate of PER under normal conditions appears to follow a linear trajectory from hypo- to hyperphosphorylation without evidence of a reversal in the flow of phosphorylated isoforms (Fig. 1). This will become significant later when discussing CLK.

In summary, multiple kinases and phosphatases regulate the progressive and presumably hierarchical phosphorylation of PER, events that regulate its stability, subcellular distribution and perhaps ability to interact with other partners and repress CLK-CYC–mediated transactivation. The coordinated activities of TIM, DBT, CK2, PP2A and likely other kinases and phosphatases regulate the onset and duration of PER function as a

transcriptional regulator in the nucleus (it is not clear if PER has a distinct cytoplasmic function). How this happens is still a mystery as it seems that TIM and DBT (at least) are bound to PER at the same time for major portions of its life-cycle. The presence of TIM ensures that progression of the biochemical timer is synchronized to the light–dark cycle (Fig. 1). Within the context of this PER phosphorylation machinery, the progressive phosphorylation of PER yields a unidirectional and ordered chain of events, whereby different phospho-sites impart inherently different biochemical time-constraints the sum of which generates a molecular oscillator with ~24 h periods. Presumably, this temporal information is transduced to the rest of the clock machinery and ultimately downstream effector pathways by the phase-specific inhibition of CLK-CYC activity by PER.

CLK—establishing a functional range for a limiting component

About a decade ago, a similar forward genetic screen as that initiated by Konopka and Benzer using *Drosophila* was applied by Takahashi's group to identify genes involved in mammalian circadian clocks, an effort that paid its first dividends with the identification of the *Clock* gene (*50*). Subsequent work showed that *Drosophila* also contains a *Clock* gene, termed *Clk* (*51–53*). The *Clk* gene encodes a bHLH/PAS-containing transcription factor with poly-glutamine transactivation domains. As noted above, CLK forms a dimer with CYC (BMAL1 in mammals) to drive the rhythmic expression of *per*, *tim*, *vri* and *pdp1*ε in addition to clock-controlled downstream genes (*8*). By a mechanism that is still not clear VRI acts first to inhibit *Clk* expression followed later by the stimulatory activity of PDP1ε. As a result, transcripts from the *Clk* gene undergo daily rhythms in abundance that are essentially anti-phase to those of *per/tim/vri/pdp1*ε, peaking during the late night and early morning, with trough levels reached by the end of the day (*52*).

Initial biochemical analyses of CLK protein appeared to support an important role for *Clk* rhythmic expression. The levels of CLK protein were shown to undergo circadian oscillations in abundance that were in synchrony with the *Clk* RNA rhythm, suggesting the RNA cycle is critical for the CLK abundance rhythm (*54*). Moreover, multiple phospho-isoforms of CLK are detected throughout a daily cycle (*54*). This raised the possibility that similar to PER and TIM, phosphorylation regulates the stability of CLK. Although the initial biochemical characterization of CLK by western blotting showed daily oscillations in the levels of CLK, recent findings using more stringent protein extraction procedures indicate that CLK is relatively constant throughout a daily cycle (*22*, *23*), consistent with recent findings using immunohistochemical staining of key pacemaker neurons (*55*). Presumably, chromatin-bound CLK was not extracted under mild conditions giving rise to the biochemical staining rhythm (*23*). Despite the relatively constant steady-state levels of CLK using stringent extraction procedures, temporal changes in the phosphorylated state of CLK are even more readily observed. Essentially, there are three major "groups" of CLK phosphorylation isoforms that can be distinguished by electrophoretic mobility; (i) non/hypophosphorylated, (ii) phosphorylated to an intermediate range, and (iii) hyperphosphorylated isoforms that are solely observed during the second half of the night and continuing into the early day (*22*, *23*). While there are multiple CLK phosphorylation species, the overall pattern is different from that of PER where the phosphorylation program appears very unidirectional, steadily progressing from hypo- to hyperphosphorylated variants in a seemingly highly ordered manner. Despite the overall shift towards a relative increase in the proportion of higher phosphorylated variants during the late night/early day, a large fraction of CLK phospho-variants appear to be constantly present throughout a daily cycle.

The observation that highly phosphorylated variants of CLK are preferentially observed during the downswing in *per/tim* expression suggested that hyperphosphorylated CLK might be less active. Indeed, recent studies using DNA chromatin immunoprecipitation (ChIP) assays showed that CLK-CYC binds E-box elements upstream of *per* and *tim* during the late day/early night when *per/tim* expression is stimulated (*23*). Stable association between CLK and chromatin occurs during times in the day when CLK is hypophosphorylated. Presumably, under mild extraction conditions the less phosphorylated isoforms of CLK remain preferentially bound to chromatin and are removed from the final sample. Given that hyperphosphorylated variants of CLK undergo daily changes in levels this would seem to explain earlier reports showing daily oscillations in total CLK abundance. While consistent with the notion that highly phosphorylated isoforms of CLK are less competent to engage in transactivation, a causal link has not been established. Complicating matters further highly phosphorylated CLK is unstable (*22*, *23*), which might be the main reason for the reduced transcriptional activity. The instability of highly phosphorylated isoforms would enhance rapid "promoter clearance" working in concert with PER-mediated inhibition, leading to a potent inhibition of CLK-CYC–dependent transactivation during the mid-night to mid-day.

Two independent studies recently demonstrated that DBT is required for the production of highly phosphorylated and unstable variants of CLK (*22*, *23*). Moreover, arrhythmic mutants while containing intermediate phosphorylated isoforms of CLK are missing the hyperphosphorylated species. In one of the studies it was further suggested that PER itself acts as a "bridge" to enhance the phosphorylation of CLK by DBT (*23*). In this scenario PER would serve a dual function as a transcriptional repressor, blocking CLK-CYC activity and simultaneously stimulating the hyperphosphorylation and degradation of CLK. CLOCK protein is also phosphorylated in mammals but the kinase(s) has yet to be identified (*14*). Moreover, complex formation with BMAL1 is necessary for phosphorylation of the mammalian CLOCK protein (*56*). Interestingly, in a simplified cell culture system DBT-mediated CLK phosphorylation does not require its partner CYC (*22*). Although the physiological significance of this result is not clear it suggests possible differences in the phosphorylation programs for CLOCK proteins in the mammalian and *Drosophila* systems.

Besides a role for DBT, studies in a simplified cell culture system implicate the participation of PP2A (*22*). Strikingly, the same two regulatory subunits, *wdb* and *tws*, previously shown to target PER also regulate CLK levels. Despite the

obvious similarities with PER, there are stark differences in the manner in which CLK is regulated by DBT and PP2A. Most notably, it appears that DBT not only leads to hyperphosphorylation of CLK but also stimulates the concomitant appearance of a similar proportion of non/hypophosphorylated isoforms (*22*). Thus, there is an exquisite balance of CLK phosphoisoforms co-existing. This dynamic equilibrium would seem to offer an explanation for the relatively static appearance of a broad range of CLK phosphorylation variants throughout a daily cycle. Moreover, overexpression of DBT in *Drosophila* pacemaker neurons slightly shifts the phosphorylation pattern but has negligible effects on total CLK levels (*22*). This is in sharp contrast to PER where ectopic expression of DBT leads to significant reductions in PER abundance. Only when PP2A levels are dramatically reduced is the DBT-mediated enhanced degradation of CLK readily observed.

What is the role for the balanced phosphorylation/dephosphorylation regulation of CLK? While still unclear, an insight is gained from the fact that CLK levels appear to be rate-limiting in the PER-TIM-CLK-CYC based feedback loop (*24*). Manipulations that change the overall levels/activity of CLK have profound effects on the amplitude of molecular and behavioral rhythms. For example, to address the function of the *Clk* RNA rhythm, transgenic flies were generated that express the *Clk* open reading frame (ORF) under the control of *per* regulatory elements (*26*). Surprisingly little effects were noticed on the period and phase of molecular and behavioral rhythms despite the relatively antiphase cycling of *Clk* RNA levels. However, the amplitudes of *per*/*tim* RNA rhythms were increased, most likely because overall CLK levels are higher in these transgenic animals compared to wildtype controls. In addition, a novel hypomorphic *Clk* mutant named Clk^{ar} causes behavioral arrhythmia while still maintaining reduced molecular oscillations of key clock components such as *per* and *tim* (*25*). Presumably, the lower-amplitude molecular oscillations are not sufficient to drive overt rhythms. Intriguingly, recent findings indicate that the mammalian CLOCK protein also regulates oscillator amplitude (*57*).

Thus, it appears that CLK levels need to be maintained within a certain functional range. Too much and it would be difficult to ensure robust and rapid inhibition of CLK-CYC transactivation at appropriate times in the cycle. Conversely, below a certain threshold the oscillator would either not function or have reduced amplitude unable to sustain rhythmic behavior. In this scenario, the homeostatic regulation buffering against stochastic fluctuations in CLK abundance is critical for minimizing molecular noise in the circadian feedback circuitry. The balanced phosphorylation/dephosphorylation mechanism involving DBT and PP2A (and possibly other kinases and phosphatases) likely stabilizes overall CLK levels but allows for the possibility of dynamic regulation (Fig. 1). The phase of the *Clk* expression rhythm might also contribute to the stabilization of overall CLK protein abundance because *Clk* RNA levels increase when CLK protein is hyperphosphorylated and less stable. Future research on the identification of CLK phosphorylation sites by DBT and possibly other CLK kinases (*58*) will be important to better understand the fine-tuning of CLK function and its possible role in physiologically relevant circadian responses that involve modulations in clock amplitude.

TIM—dancing with light

When evaluated by immunoblotting of head extracts across a daily cycle, TIM levels begin to increase in the early night, exhibit limited phosphorylation changes as deduced from electrophoretic mobility shifts, and rapidly disappears prior to and especially following the onset of dawn (*59–61*). Thus, the overall pattern is similar to that of its key partner PER, but likely undergoes much more limited phosphorylation changes and its stability is highly photosensitive. Much progress has been made in understanding the molecular events regulating the phosphorylation and light-mediated degradation of TIM (*27*).

A critical TIM kinase is SGG, a homolog of GSK3β. Experimental manipulations that lower the levels of SGG in clock cells leads to a delayed nuclear entry/accumulation of TIM in key pacemaker neurons, an event associated with longer behavioral periods (*62*). Conversely, upregulation of SGG accelerates the timing of when TIM's presence is first observed in the nucleus, likely contributing to the shorter periods manifested in these animals. In addition, phosphorylation of TIM by SGG leads to the detection of lower electrophoretic mobility isoforms of TIM that are less stable in the presence of light (*62*). It is not known if the effects of SGG on TIM nuclear entry and light-mediated degradation involve the same or different sites on TIM. Because slower mobility isoforms of TIM are observed in the presence of light it is possible that additional sites are phosphorylated by SGG to mark TIM for rapid degradation. However, pharmacological studies also suggest the involvement of at least one other kinase, an unidentified tyrosine kinase, in enhancing the light-mediated degradation of TIM (*28*). Thus, like in the case of PER, multiple kinases (and perhaps phosphatases) regulate the daily changes in TIM phosphorylation. Recent findings highlight another similarity; an F-box protein called JET is essential for the light-dependent degradation of TIM (*63*), consistent with prior work showing that the 26S proteasome is involved in the photosensitivity of TIM (*28*). Although not firmly established in the case of TIM, F-box proteins are known to preferentially recognize phosphorylated substrates, in agreement with the observation that higher phosphorylated isoforms of TIM are less stable in the presence of light (*61*, *62*). The putative blue-light circadian photoreceptor CRY somehow transduces the light signal to TIM (*64*). Mutations that reduce or possibly eliminate CRY function are associated with stable TIM levels throughout a daily cycle, although depending on the clock cell type other phototransduction pathways might contribute to cell-specific photosensitivity of TIM. While the flow of molecular signals from CRY to the ultimate rapid destruction of TIM are not known, it is possible that "activated" CRY enhances the activity of SGG, leading to the phosphorylation of TIM and concomitant targeting by JET. GSK3 kinases are themselves subject to signal-transduced phosphorylation events that regulate their activity. Indeed, recent work in *Drosophila* shows that serotonin signaling plays a role in TIM photosensitivity, presumably by modulating the phosphorylated status of SGG (*65*).

The light-mediated degradation of TIM is considered the initial clock-specific event in the entrainment of the clock to light–dark cycle (*27*). A long established property of circadian clocks is that their phases can be perturbed by applying short pulses of external stimuli, most notably light (reviewed in Ref. *66*). Depending on when during a daily cycle the stimulus is applied the ensuing steady phase of the clock can remain unchanged, delayed or advanced. Plotting the magnitude of the changes as a function of time when the agent was administered (in most cases) yields a phase–response curve (PRC). For light-pulses, application during the subject day (refers to a dark period when light would normally be present) yield no to little phase change, whereas delays and advances are elicited in the early night and late night, respectively. This pattern is observed whether animals are diurnal or nocturnal and makes biological sense in that as the sun sets later in the day rhythms need to be delayed in order to maintain synchrony, whereas an earlier rising sun is matched by phase advances in circadian rhythms (daylight during the day leaves the clock phase unchanged as it is the *status quo*). When integrated with the known molecular circuitry underlying the *Drosophila* clock, the unidirectional light-dependent degradation of TIM can readily explain the light-PRC observed in this species (*40*, *60*, *61*) (Fig. 1). TIM levels are low during the day, explaining the "dead-zone" in the middle of the day. During the early night TIM levels accumulate prior to its translocation to the nucleus. Brief exposure to light in the early night transiently enhances TIM degradation, which after a delay can re-accumulate because high-levels of *tim* RNA are present at this time. Presumably the clock is mainly delayed because the timing of TIM and PER nuclear entry occurs later (*40*). In the late night the enhanced degradation of TIM stimulates the hyperphosphorylation and advanced degradation of PER (*39*, *40*, *61*), events that are accelerated by the low levels of *tim* and *per* RNAs at this time. The disappearance of PER in the nucleus leads to an earlier relieve of autoinhibition and advance in the phase of the clock. It is interesting to note that the break point between delays and advances in the *Drosophila* light-PRC is roughly around the time when significant amounts of PER are first observed in the nucleus.

From this light entrainment model for the *Drosophila* clock it is clear that although TIM is the primary photosensitive clock component, its partnership with PER is crucial for phase setting. Indeed, the apparently simple temporal pattern of TIM phosphorylation suggests it is less likely to play a direct role in *measuring* phase, a job better suited for specific time-of-day phosphorylation events on PER. Thus, light-mediated effects on TIM are transduced to the PER phosphorylation program, which is likely to be the heart of the ticking clock (Fig. 1). Moreover, TIM and CLK functions are also dynamically intertwined because the amplitude of a circadian oscillator modulates the light-responsiveness of the system (*e.g.*, *57*).

Perspectives

While earlier molecular studies focused on the role of transcriptional circuits in the clockworks, more recent findings indicate a crucial and broad role for clock protein phosphorylation. Studies in *Drosophila* have been highly informative in understanding the multiple roles of phosphorylation in circadian oscillators. Different clock proteins in the system are subjected to a variety of phosphorylation programs that preferentially regulate the period, phase and amplitude of circadian pacemakers. The different phosphorylation programs involve multiple kinases, phosphatases and other regulatory factors that influence a variety of clock protein attributes, including stability, subcellular localization and activity. Temporal order and direction seem to be established by the presence of at least one clock protein that undergoes intricate time-of-day dependent changes in phosphorylation status that impose a circadian time-frame and identify specific phases of the day. In the case of *Drosophila*, period, phase and amplitude are all interconnected by the co-dependent life-cycles of PER, TIM and CLK, which are heavily influenced by phosphorylation (Fig. 1). That cycles in reversible phosphorylation of a clock protein are sufficient to generate an oscillator with circadian properties was elegantly shown using a reconstituted *in vitro* system (*67*). Amazingly, incubating three purified clock proteins from cyanobacteria in the presence of ATP was sufficient to generate a self-sustaining biochemical phosphorylation rhythm exhibiting *bona fide* circadian properties. Thus, by studying clock protein phosphorylation we are peering into the biochemical nuts-and-bolts (or springs and gears) of how a clock *measures* and *keeps* time. The fact that several familial sleep disorders in humans are likely caused by mutations in the PER phosphorylation program clearly demonstrate the importance of phosphorylation in the regulation of circadian biology (*19*, *20*). It also suggests that a better understanding of how phosphorylation regulates various aspects of the clockworks could lead to the development of better treatments to combat the serious health issues associated with clock malfunction in humans.

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