Post-translational modifications regulate the ticking of the circadian clock

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Abstract | Getting a good night's sleep is on everyone's to-do list. So is, no doubt, staying awake during late afternoon seminars. Our internal clocks control these and many more workings of the body, and disruptions of the circadian clocks predispose individuals to depression, obesity and cancer. Mutations in kinases and phosphatases in hamsters, flies, fungi and humans highlight how our timepieces are regulated and provide clues as to how we might be able to manipulate them.

Suprachiasmatic nucleus (SCN). Paired hypothalamic collection of neurons that receive signals from the retina and regulate circadian behaviour. Destruction of the SCN causes arrhythmic behaviour.

Circadian rhythms are ~24-hour biological cycles that allow organisms to adapt their physiology to the daily cycle of sunlight and darkness. Recently, new insights have shed light into how rhythms govern key processes — including sleep–wake cycles, glucose, lipid, bone and drug metabolism, heart rate, regulation of stress and growth hormones, and immunity — and the timing of the cell-division cycle[1–5]. Disruption of circadian rhythms, such as those experienced by humans in shift work and jet lag, might increase our risk of cancer[6]; disregulation of circadian rhythms clearly decreases fitness and survival of both prokaryotes and mammals in the wild[7,8].

Our biological clocks contain three essential elements: a central oscillator that keeps time; the ability to sense time cues in the environment and to reset the clock as the seasons change; and a series of outputs tied to distinct phases of the oscillator that regulate activity and physiology. In mammals, the central clock resides in the suprachiasmatic nucleus (SCN), which produces a rhythmic output that consists of a multitude of neural and hormonal signals that influence sleep and activity. Most importantly, these signals set the peripheral clocks present throughout the body (see REF. 9 for a recent review). The SCN clock is reset by external light, which is sensed by the ganglion cells of the retina. Remarkably, circadian oscillators are also present in all tissues of the body, where they are synchronized by unidentified signals to regulate, in a tissue-specific manner, transcriptional activity throughout the day[10]. These peripheral clocks also exist in cultured cells[11]; this finding is now being exploited for detailed molecular, biochemical and cell biology studies[12–14].

Molecular insights into the mechanisms of circadian rhythms have provided clues that post-translational modifications work hand in glove with transcriptional regulation to finely tune our days and nights. The first circadian rhythms mutant animal, the *period* fly, was reported in 1971 (REF. 15). The stability of period (PER) protein is regulated by phosphorylation, and the first mammalian circadian rhythms mutant, the *tau* hamster (which was identified in Menaker's laboratory in 1988 (REF. 16)), has a mutation in the kinase casein kinase Iε (CKIε), which regulates the degradation of the mammalian PER. The core clock is a negative-feedback loop composed of transcription factors that drive the expression of their own negative regulators. Post-translational regulation of the localization, degradation and activity of these regulators by phosphorylation, sumoylation, histone acetylation and methylation all determine the biological length of the day (and night). Once thought to be the purview of the SCN in mammals, we now understand that circadian timekeeping is ubiquitous in the tissues of the body. New insights into the kinases, phosphatases and other enzymes that control the clock make potential pharmacological interventions timely.

In this review, we briefly describe circadian rhythms and circadian clocks in *Drosophila melanogaster*, mammals and, to a lesser extent, the fungi *Neurospora crassa*, with an emphasis on the post-translational modifications that control clock function. We then review the studies that identified enzymes and defined mechanisms that post-translationally regulate the core clock. Last, we analyse inherited circadian rhythms sleep disorders to illustrate how defective phosphorylation of circadian proteins can significantly alter the rhythms of life.

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doi:10.1038/nrm2106

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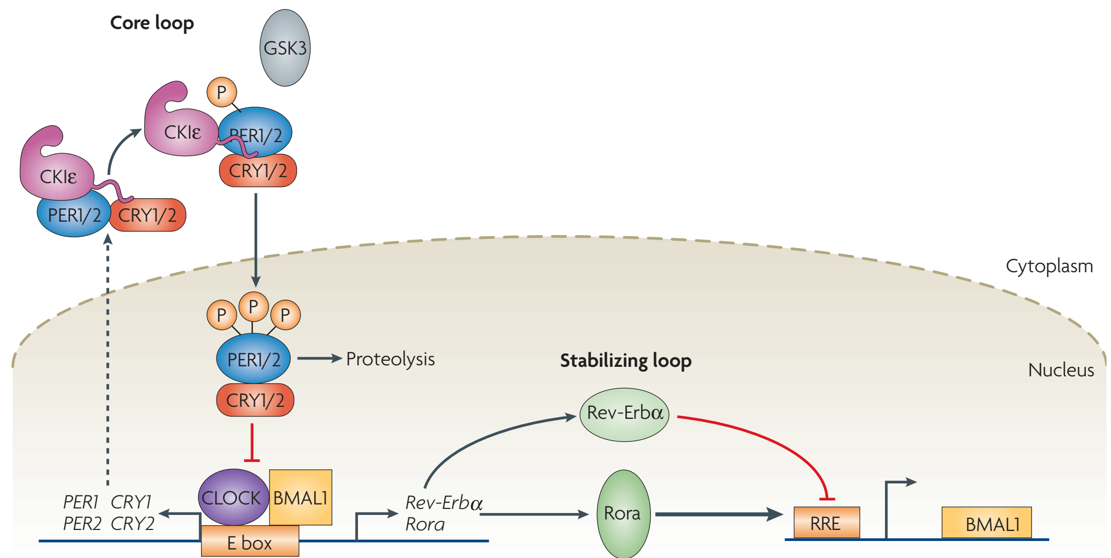

Figure 1 | **Feedback loops control the mammalian circadian core clock.** The mammalian circadian rhythms core clock is a transcription–translation negative-feedback loop with a delay between transcription and the negative feedback. It is initiated by a heterodimeric transcription factor that consists of CLOCK and BMAL1. CLOCK and BMAL1 drive expression of their own negative regulators, the period proteins PER1 and PER2 and the cryptochromes CRY1 and CRY2. Over the course of the day, the PER and CRY proteins accumulate and multimerize in the cytoplasm, where they are phosphorylated by casein kinase Iε (CKIε) and glycogen synthase kinase-3 (GSK3). They then translocate to the nucleus in a phosphorylation-regulated manner where they interact with the CLOCK–BMAL1 complex to repress their own activator. At the end of the circadian cycle, the PER and CRY proteins are degraded in a CKI-dependent manner, which releases the repression of the transcription and allows the next cycle to start. An additional stabilizing feedback loop, which involves the activator Rora and the inhibitor Rev-Erbα, controls BMAL1 expression and reinforces the oscillations. P, phosphate; RRE, R-response element.

A negative-feedback loop with a delay

Circadian molecular clocks have been extensively reviewed and so are discussed briefly. At their core, the clocks contain a cell autonomous oscillator that is generated by a transcription–translation negative-feedback loop with a crucial delay between stimulus and response. In mammals (FIG. 1), the transcription factors that positively regulate the clock are the proteins CLOCK (CLK in *D. melanogaster*) and BMAL1. These proteins dimerize and form the CLOCK–BMAL1 complex, which induces the expression of a large number of output genes. CLOCK and BMAL1 also induce the expression of their own negative regulators or repressors, the PER proteins (PER1 and PER2) and cryptochromes (CRY1 and CRY2)[9,17]. For the first half of the circadian day, transcription is ascendant; however, repression then begins to reign and newly synthesized inhibitors, such as the PER proteins and CRY proteins, accumulate and inhibit transcription. For a new biological day to dawn, the inhibitory proteins that repress CLOCK–BMAL1 must be removed. PER and CRY proteins are eliminated by post-translational modifications, most notably phosphorylation and degradation. Last, the core clock is stabilized by the opposing functions of the orphan nuclear receptors Rora and Rev-Erbα, which activate and repress *Bmal1* expression, respectively[17,18].

Regulation of CLOCK–BMAL1 activity seems to be central to the mammalian clock. Depending on the specific target gene, CLOCK and BMAL1 either remain bound to the promoters throughout the circadian cycle (as for the *Per* genes)[19–21] or vary rhythmically in their ability to bind DNA (for example, on the *Dbp* gene promoter)[22]. In both cases, transcription inhibition is associated with the methylation of histone H3 (REFS 21,22). Activation of transcription by CLOCK–BMAL1 is associated with histone acetylation[20,22], and CLOCK itself possesses intrinsic histone acetyltransferase (HAT) activity[23]. This finding would have made a tidy and satisfying story: CLOCK itself could be the HAT that is required for the CLOCK–BMAL1 heterodimer to activate transcription; however, it was recently shown that conditional knockout of CLOCK in mice does not eliminate circadian rhythms[24]. It is possible that other PAS-domain proteins, such as NPAS2, that can also heterodimerize with BMAL1 can largely compensate for the loss of CLOCK.

Although the components of the clock vary among phyla, the basic mechanism of the activation of transcription, translation and inhibition of transcription is highly conserved (FIG. 1,2; reviewed in REFS 25–27). In *D. melanogaster*, cycle (CYC) is the orthologue of BMAL1, and CLK–CYC dimers activate the transcription of circadian genes. In *D. melanogaster*, timeless (TIM) substitutes for both mammalian CRYs as an inhibitor, whereas *D. melanogaster* CRY functions as a photoreceptor. In flies, CLK–CYC binds to the promoters of circadian-regulated genes only at the time of transcription[28]. A stabilizing loop also exists in *D. melanogaster*; in this loop, vrille (VRI) inhibits whereas PAR-domain protein-1 (PDP1) activates *Clk* transcription[29] (FIG. 2).

In *N. crassa*, the clock mechanism is analogous, but non-orthologous, to that of mammals and flies.

PAS domain
Protein domain first identified in period, arnt, and simple-minded; it mediates protein–protein interactions.

Stabilizing loop
A separate interacting transcription–translation loop that reinforces the oscillations that are driven by a core loop.

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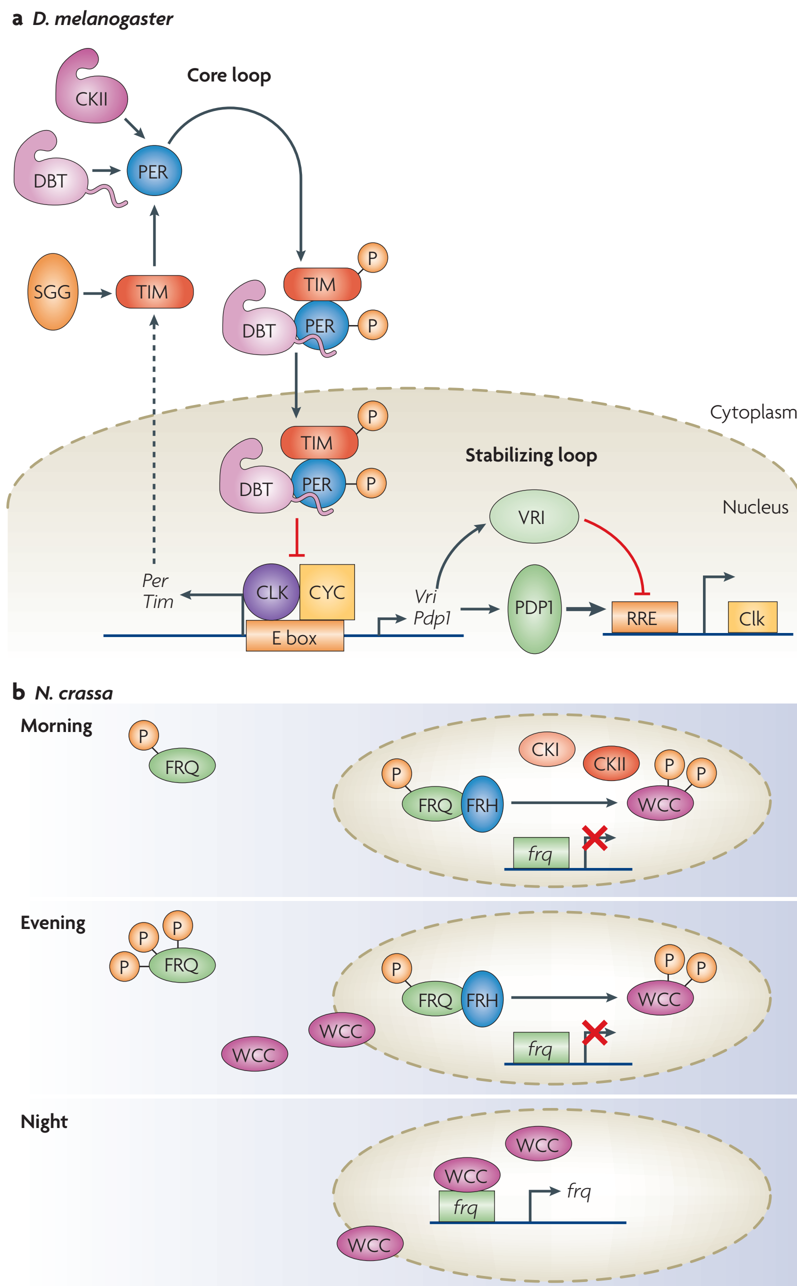

Figure 2 | **Conservation of mechanism in the *Drosophila melanogaster* and *Neurospora crassa* circadian clocks. a** | Homologous genes regulate the *Drosophila melanogaster* and vertebrate clocks, although some details might differ. Clock (CLK) and cycle (CYC) activate the transcription of the circadian genes in *D. melanogaster*. Period (PER) and timeless (TIM) form heterodimers in the cytoplasm where they are phosphorylated by double-time (DBT) and shaggy (SGG). They then translocate to the nucleus where PER inhibits the transcriptional activity of the CLK–CYC complex. Similarly to the mammalian clock, a number of kinases regulate PER and TIM. In the stabilizing loop, the protein vrille (VRI) inhibits, whereas PAR-domain protein-1 (PDP1) activates the transcription of *Clk*. **b** | The clock mechanism in *Neurospora crassa*. The white collar complex (WCC) activates the transcription of the frequency (*frq*) gene. The FRQ protein positively and negatively regulates the WCC. In the morning, FRQ-interacting RNA helicase (FRH) and casein kinases I (CKI) and CKII promote the FRQ-dependent phosphorylation and inactivation of the WCC, which results in the inhibition of *frq* transcription. In the evening, high amounts of hyperphosphorylated FRQ in the cytoplasm support the accumulation of WCC. At night, hyperphosphorylated FRQ is degraded, the repression on WCC is relieved and transcription of *frq* is activated. P, phosphate.

The white collar complex (WCC), which consists of the PAS-domain-containing transcription factors, white collar-1 (WC1) and white collar-2 (WC2), activates the rhythmic transcription of circadian components, including the repressor protein frequency (FRQ)[30]. The regulation of WCC by FRQ is complex and depends on the time of day and the subcellular compartment (recently reviewed in REFS 27,31). In the morning, FRQ inhibits the activity of WCC by mediating the phosphorylation of WCC in a process that requires FRQ-interacting RNA helicase (FRH), CKI and CKII[32–36]. However, later in the day, FRQ supports the cytosolic accumulation of WCC. At night, hyperphosphorylated FRQ is degraded through the ubiquitin–proteasome pathway[37,38] and the repression on WCC is relieved (FIG. 2).

Why add phosphorylation to the clock? The crucial importance of post-transcriptional regulation becomes more apparent when one considers the delay in the negative-feedback loop that is required to give the clock a 24-hour period. Transcription–translation feedback cycles generally operate on a timescale of up to a few hours. If, following synthesis, the repressor proteins PER and CRY translocated to the nucleus to repress CLOCK and BMAL1, the whole cycle would take just a few hours rather than one day. To maintain the daily oscillations of clock proteins, a significant delay between the activation and repression of transcription is required; the delay between the activation and repression of transcription is ensured by regulation through post-translational modifications. Reversible phosphorylation regulates important processes such as nuclear entry, formation of protein complexes and protein degradation. Each of these can individually contribute to introduce the delay that keeps the period at ~24 hours.

Is circadian-regulated gene expression important for the core clock to function? Although an intuitive answer is yes, several pieces of data indicate that circadian-regulated gene expression might not be important for the function of the core clock. Forced overexpression of CLK, or CLK expression 12 hours later than usual (in antiphase) still supports normal clock function[39]. In fact, CLK protein is present at constant levels during the circadian cycle, but CLK phosphorylation and its promoter-binding activity vary during the circadian cycle[28]. The oscillations in promoter binding drive rhythmic expression of *Per*; however, flies in which *Per* is expressed from a constitutive, rather than a rhythmic, promoter still have normal rhythms[40]. In both rodents and rodent cell lines, *Per1* and *Per2* can be constitutively expressed without eliminating rhythms[41–43]. And last, the *Clock*-null mouse has compromised oscillation of circadian-gene mRNA without appreciable loss of circadian-protein oscillation[24]. So, although there is substantial evidence that circadian outputs (such as sleep, metabolism and locomotor activity) are controlled by changes in output gene transcription, the core clock in flies and mammals that drives these changes in gene expression might be controlled not by circadian transcription, but by post-transcriptional regulation. Most importantly, the answer lies in regulated destruction through post-translational modifications.

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Table 1 | **The main regulators of eukaryotic circadian rhythms**

Vertebrates	*Drosophila*	*Neurospora*	Function
Positive-transcription factors			
CLOCK	CLK	WC2*	PAS-domain transcription factor
BMAL1	CYC	WC1*	PAS-domain transcription factor and histone acetyltransferase
NPAS2?			PAS-domain transcription factor
Rora	PDP1		Transcription factor
Negative-feedback elements			
PER1–3?	PER	FRQ*	PAS-domain scaffold protein
CRY1, 2	CRY		Cryptochrome
TIM?	TIM		Heterodimerization with fly PER
Rev-Erbα	VRI		Transcription factor
Regulatory enzymes			
CKIδ,ε	DBT	CKIα	Protein kinase
CKII	CKII	CKII	Protein kinase
GSK3	SGG		Protein kinase
PP1		PP1	Protein phosphatase
PP2A	PP2A	PP2A	Protein phosphatase
PP5			Protein phosphatase
βTrCP	SLMB	FWD1	Adaptor of SCF ubiquitin ligase

**Neurospora crassa* proteins that have sequence-related genes. Protein functions are indicated in the main text and Figures. CKI, casein kinase I; CLK/CLOCK, clock; CRY, cryptochrome; CYC, cycle; DBT, double-time; FWD1, F-box and WD40-repeat-containing protein-1; FRQ, frequency; GSK3, glycogen synthase kinase-3; PDP1, PAR-domain protein-1; PER, period; PP, protein phosphatase; SCF, SKP1–Cullin1–F-box protein; SGG, shaggy; SLMB, slimb; TIM, timeless; βTrCP, β-transducin repeat-containing protein; VRI, vrille; WC, white collar.

Locomotor activity
Circadian rhythms cause changes in activity (along with many other changes). Wheel running in a cage is a form of locomotor activity.

Eclosion
Hatching of an insect larva from an egg.

Free-running period
Rhythms observed in nature continue in the laboratory even under constant experimental conditions such as constant light or constant dark. The persistence of these rhythms is seen as proof of endogenous biological clocks. When in constant conditions away from any external cues, these rhythms are called free runs.

Familial advanced sleep-phase syndrome
(FASPS). A dominantly inherited short-circadian-period disorder.

It isn't all about destruction. The activity, rather than the abundance, of the transcription factor BMAL1 is regulated by phosphorylation by CKI. Knockdown or inhibition of CKIδ and CKIε activity concurrently decreases BMAL1 phosphorylation and reduces CLOCK–BMAL1-dependent transcription[44,45]. BMAL1 is also regulated by mitogen-activated protein kinase (MAPK). MAPK interacts with and phosphorylates BMAL1 at multiple sites, thereby decreasing the ability of the CLOCK–BMAL1 heterodimer to activate transcription from circadian promoters[46]. In *D. melanogaster*, the DNA-binding activity of CLK varies inversely with its phosphorylation (at least as assessed by mobility shift), whereas its stability is regulated (directly or indirectly) by the CKI family member double-time (DBT)[28,47]. Similarly, hyperphosphorylated *N. crassa* WCC is inactive, and therefore unable to bind to DNA[33,34].

Kinases and phosphatases in the clock

Our understanding of the molecular components of the circadian clock came initially from mutants in *D. melanogaster*, hamster and fungi that caused obvious changes in daily rhythms. Those studies identified several kinases and phosphatases (TABLE 1 and FIG. 1,2) as essential components of the clock because they directly linked mutations in these enzymes with abnormal circadian phenotypes.

The centrality of CKI. Despite its boring name, CKI has been shown to have a central role in an increasing number of crucial biological pathways (FIG. 3). DBT was the first enzyme to be identified as an essential component of the *D. melanogaster* clock. Young and co-workers[48,49] identified mutants with abnormally short (dbt^S) or long (dbt^L) periods of locomotor activity and eclosion in flies. Strikingly, they identified dbt^S and dbt^L as allelic mutations in highly conserved residues in the kinase domain of DBT. How different mutations in the same kinase can speed up or slow down the clock is now becoming clear (see below).

In humans, the closest relatives of DBT are the closely related CKI members CKIε and CKIδ[50]. These kinases function in multiple pathways, including Wnt signalling, neurotransmission, DNA-damage response[51], and as regulators of circadian rhythms. The *tau* hamster, which was identified 10 years before the *dbt* flies, has a free-running period of only 20 hours[16]. Unlike the rapid phenotype to genotype linkage in *D. melanogaster*, it took 12 years and a heroic effort to discover that the *tau* hamster phenotype was due to a mutation in CKIε[52]. Since then, mutations in CKIδ and in a potential CKI phosphorylation site in PER2 have been found in human families with familial advanced sleep-phase syndrome (FASPS)[53–55].

Last, the *N. crassa* clock is also regulated by CKIα, the homologue of DBT and CKIε; the *N. crassa* circadian rhythms (as assessed by daily alterations in growth patterns) are lost when the CKI phosphorylation site in FRQ is deleted[34,56].

Other kinases implicated in the regulation of the clock. CKII, which is unrelated to CKI except in name and in preference for acidic substrates, is one of the more recent kinases identified as a clock component. CKII is a tetramer that comprises two catalytic (α) and two regulatory (β) subunits[57,58]. Strikingly, mutations in either the regulatory or catalytic subunit cause alterations in *D. melanogaster* circadian rhythms. CKIIα-mutant flies show lengthened circadian period and delayed nuclear entry of PER[59,60]. This phenotype was also observed in flies with a mutation in the *ckIIβ* gene that causes defective assembly of the tetrameric holoenzyme[61] and in flies that carry PER with engineered mutations in the phosphorylation sites of CKII[60]. CKII has also been implicated in the *N. crassa* clock. CKIIα mutants show abnormal phosphorylation of the FRQ protein[62], and disruption of the CKII regulatory subunit CKβ1 alters conidation rhythms[63]. Despite clear evidence of involvement in the *Arabidopsis thaliana*, *N. crassa* and *D. melanogaster* clocks, there is still no evidence that CKII regulates mammalian circadian rhythms.

In *D. melanogaster*, shaggy (SGG), the orthologue of mammalian glycogen synthase kinase-3 (GSK3), regulates the circadian clock through the phosphorylation of *D. melanogaster* TIM. Overexpression of SGG shortens the period of the clock, whereas reducing SGG activity lengthens the period in flies[64]. Similarly, changes in GSK3 activity alter period length in mammalian cells[65]. The targets of GSK3 in mammals might be the PER proteins (PER phosphorylation by GSK3 might prevent nuclear entry of PER proteins), Rev-Erbα[66], and/or CRY2

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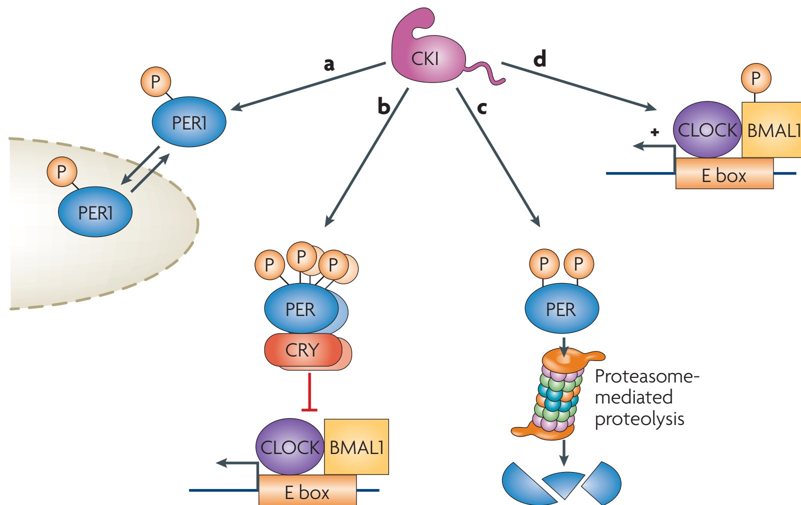

Figure 3 | **Multiple roles of casein kinase I in the mammalian circadian clock.** Casein kinase I (CKI) has many roles in the circadian clock. **a** | It has a confusing role in regulating the nuclear localization of the circadian repression protein period (in this example, PER1). In some cell types, CKI activity promotes the cytoplasmic accumulation of PER1, whereas in others it mediates the nuclear translocation of PER1 (REF. 84). **b** | Time-course studies have shown that the phosphorylation of PER proteins increases over the course of the circadian day, peaking when the repression of the positive transcription factors CLOCK and BMAL1 is maximal. Mapping studies indicate that there are many CKI sites on PER proteins[45], but the function of only a subset of these sites is known. Phosphorylation of PER proteins (and the associated transcription repressor cryptochrome (CRY)) might be linked to the inhibition of transcriptional activity[19]. **c** | One clear function of the phosphorylation of PER proteins is the regulation of protein stability. Phosphorylation of one or two distinct sites on PER1 and PER2 target these proteins for ubiquitin-mediated degradation by the 26S proteasome. Degradation of PER proteins can reset the clock, allowing the CLOCK–BMAL1 complex to become active[12,83–86]. **d** | PER and CRY proteins are not the only substrates of CKI in the clock. CKIε-mediated phosphorylation of the circadian regulator BMAL1 increases its transcriptional activity[44]. P, phosphate.

(for which phosphorylation might control CRY2 degradation at the end of night)[67]. GSK3 itself is regulated by inhibitory phosphorylation at an N-terminal Ser residue — at least one study has shown that the levels of Ser9 phosphorylated GSK3β vary during the circadian cycle[67].

Given the complexities of the clock, it is perhaps not surprising that several kinases have been implicated in the regulation of circadian control. For example, the MAPK ERK2 and calmodulin-dependent protein kinase II (CaMKII) have been shown to directly phosphorylate CLK[68]. Although this review is focused on the regulation of the core clock, it is notable that light-induced clock resetting by glutamate-mediated neurotransmission in the retino-hypothalamic pathway results in activation of MAPK[69], protein kinase A (PKA) and the cyclic-GMP-activated protein kinase PKGII[70,71]. These kinases have been biochemically or genetically implicated in regulating the resetting of the clock; however, in most cases their specific targets remain ambiguous.

Conidation
Conidation is asexual reproduction in Ascomycetes by the formation of asexual, non-motile spores.

Phosphatases get in on the act. Whenever there is a crucial event regulated by phosphorylation, there is almost invariably a phosphoprotein phosphatase that participates in its regulation. As detailed in several recent reviews, phosphatases evolved along different lines than kinases[72–74]. The number of serine/threonine phosphatase catalytic subunit genes is small (probably about 5% of the number of kinases), and their activity, cellular localization and substrate specificity is determined by a large number of highly variable phosphatase regulatory subunits[72–74].

Protein phosphatase-1 (PP1) and PP2A are the most abundant intracellular serine/threonine phosphatases. In *N. crassa*, PP1 and PP2A regulate the circadian rhythms[33,75]; strains with mutations in the PP1 catalytic subunit have short periods, whereas strains with mutations in the PP2A regulatory subunit RGB1 have long periods[75]. PP2A also controls circadian rhythms in *D. melanogaster*; flies with mutations that altered protein abundance of the PP2A regulatory subunits widerborst (WDB) and twins (TWS) (B′/B56 and B/B55 in mammals) or abundance or activity of the catalytic subunit MTS resulted in either changes in the period of the molecular clock or stopped the clock altogether[76]. Most recently, the serine/threonine phosphatase PP5 has been found to interact with and be regulated by CRY proteins. Through its interaction with CRY, PP5 might regulate the phosphorylation state and so the activity of CKIε in the clock[77–79].

Control of protein stability

Throughout the phyla, an essential feature of the clock is the robust daily circadian oscillations in protein abundance of the repressors, including PER, CRY, TIM and FRQ. All of these core clock proteins are degraded through phosphorylation-regulated, ubiquitin-directed, proteasome-mediated proteolysis. Phosphorylation is required for the recruitment of ubiquitin ligases, which mediate the polyubiquitylation and the subsequent degradation of these proteins in the proteasome. This system seems to be potent enough to eliminate overexpressed factors, even when they are ectopically expressed from constitutive, rather than circadian-regulated, promoters[40].

Punching the clock versus the timing belt. In response to light, *D. melanogaster* TIM is degraded by the proteasome in a process that involves the novel ubiquitin ligase jetlag[80]. Although jetlag mutations do not affect the core clock, they render the clock less sensitive to light-induced phase shifts. The data indicate that after a light pulse, the CRY protein (in flies, a blue-light-responsive photoprotein) interacts with TIM, recruiting (possibly through phosphorylation) the leucine-rich domain of jetlag. Then, jetlag brings along the SCF (SKP1–Cullin1–F-box protein) E3 ubiquitin ligase, leading to TIM polyubiquitylation and subsequent degradation. Circumstantial data indicate that this process requires the phosphorylation of TIM[81]; however, the mechanism of TIM degradation during the free-running clock is unknown.

For the rest of the core clock proteins, it is clear that phosphorylation by serine/threonine kinases, balanced by regulated dephosphorylation, sets the stage for protein degradation. Inhibition of a phosphatase can be as powerful as activation of a kinase, because both effects result in a change in the phosphorylation of the target. Phosphorylation by *D. melanogaster* DBT reduces the stability of PER; in the absence of DBT kinase activity, the levels of PER proteins are constitutively high[48,49].

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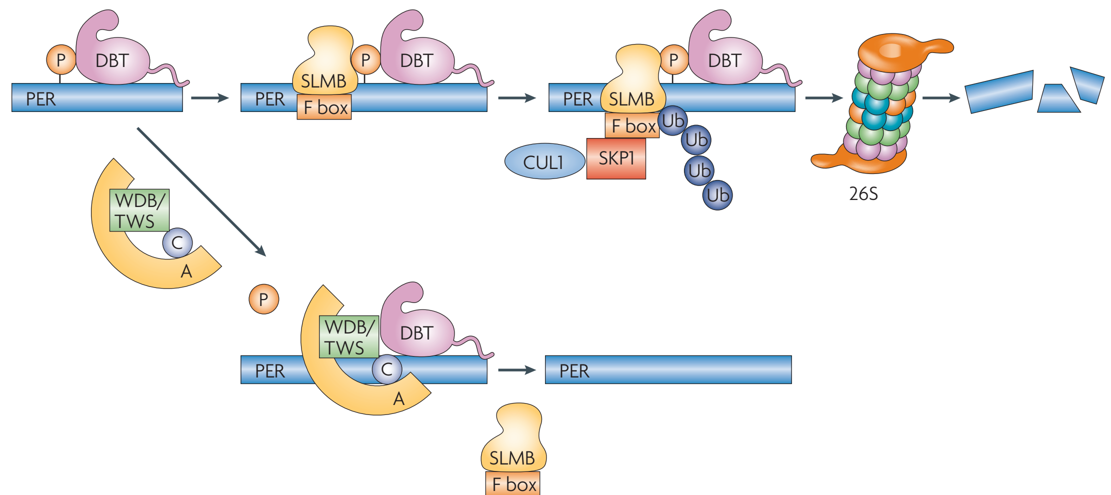

Figure 4 | **Reversible phosphorylation regulates the degradation of *Drosophila melanogaster* PER.** Prior to its degradation, the circadian repressor period (PER) is phosphorylated by double-time (DBT), the orthologue of the mammalian casein kinase I. Phosphorylated PER becomes a target for the SCF (SKP1–Cullin1–F-box protein) E3 ubiquitin ligases. The F-box-containing protein slimb (SLMB) recognizes DBT-phosphorylated PER and promotes its polyubiquitylation and subsequent degradation by the 26S proteasome. As phosphorylation is a reversible process, the heterotrimeric serine/threonine phosphatase-2A (PP2A) balances the effect of DBT phosphorylation and promotes the stability of PER. PP2A holoenzymes that contain either the regulatory subunit widerborst (WDB) or twins (TWS) recognize phosphorylated PER, which is dephosphorylated by the catalytic (C) subunit of the PP2A. Once the phosphate is released, PER is no longer a substrate for the SCF complex and is protected from degradation. A, A subunit of PP2A; P, phosphate; Ub, ubiquitin.

The phosphorylation of PER by DBT is balanced by the PP2A-mediated dephosphorylation of PER[76]. A robust circadian oscillation of two distinct PP2A regulatory subunits, *wdb* and *tws*, is seen in fly heads. Knockdown of either of these genes reduces PP2A activity and reduces the abundance of PER protein in S2 cells. Similar inhibition of PP2A activity by the overexpression of a dominant-negative form of the PP2A catalytic subunit in the fly clock neurons reduces PER protein levels and alters rhythms (FIG. 4). A similar balance between DBT and PP2A regulates the stability of *D. melanogaster* CLK. Phosphorylation by DBT enhances the degradation of CLK in S2 cells and hyperphosphorylated forms of CLK are absent in DBT-defective (*dbt*^{ar}) fly-head extracts. RNA interference against *wdb* and *tws* also decreases CLK abundance[47]. How two unrelated PP2A targeting subunits that presumably direct the phosphatase to distinct subunits each alters PER abundance is unclear. One possible explanation could be that WDB and TWS, which reside in the nucleus and cytoplasm respectively, dephosphorylate different sites of PER and regulate stability and nuclear localization independently. It is also possible that changes in the phosphorylation of different substrates in the clock have similar common outcomes.

In mammals, PER proteins bind to and are phosphorylated by CKI. Overexpression of CKIε or CKIδ modestly shortens the half-life of PER1 and PER2 (REFS 12, 82–87). Phosphorylation of PER1 and PER2 creates binding sites for β-transducin repeat-containing protein (βTrCP), an F-box-containing E3 ubiquitin ligase[12,88]. Protein phosphatase inhibitors such as microcystin and calyculin accelerate the degradation of mammalian PER proteins. The mammalian phosphatase that regulates PER stability is more likely to be PP1 than PP2A, as oscillations of PP2A regulators have not been observed in mammals. On the other hand, PP1 inhibitors block PER degradation in extracts, PP1 interacts with PER2 and PP1 inhibition accelerates PER2 degradation in transfected cells[89].

The ubiquitin–proteasome pathway. The ubiquitin–proteasome pathway controls degradation of most of the regulatory eukaryotic proteins. The SCF E3 ubiquitin ligases recognize specific substrates and catalyse ubiquitylation. The recognition of the phosphorylated substrate is mediated by F-box and WD40-containing proteins (reviewed in REFS 90,91).

Two groups simultaneously reported the involvement of the F-box protein slimb (SLMB) as an essential component of the *D. melanogaster* circadian clock[92,93]. Flies with mutations in the *slmb* gene are arrhythmic and analysis of head extracts of mutant flies revealed abundant (and no longer oscillating) hyperphosphorylated forms of PER and TIM, consistent with an impaired degradation of phosphorylated substrates[92]. SLMB physically interacts with DBT-phosphorylated PER and promotes its proteasome-mediated degradation[93] (FIG. 3). Also, *D. melanogaster* CRY is degraded through the ubiquitin–proteasome pathway in response to light in a mechanism that involves electron transport[94]. The degradation of *N. crassa* FRQ is also mediated by the F-box and WD40-repeat-containing protein-1 (FWD1), which is the homologue of *D. melanogaster* SLMB. Similarly to what was observed in flies, FRQ is hyperphosphorylated and more stable in a *fwd1*-null strain. *Fwd1* mutants show loss of both molecular and conidation circadian rhythms[38].

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In mammals, the stability of PER1 and PER2 is regulated by either βTrCP1 or βTrCP2, the orthologues of SLMB and FWD1. The knockdown of βTrCP or the overexpression of a dominant-negative mutant (with a deleted F-box) efficiently stabilizes PER. CKI phosphorylates PER1 and PER2 and this phosphorylation leads to the recruitment of βTrCP, which mediates the ubiquitylation and proteasomal degradation of these proteins[12,88]. Allowing for the differential regulation of stability, the βTrCP-interaction motif is not conserved between PER1 and PER2. Mutations in Ser477 and Glu479 in PER2 abolish the βTrCP binding[12], whereas the interaction motif in PER1 is located in the N-terminal region, distant from the previously characterized CKI-binding domain[88].

The functional importance of proteasome-mediated degradation in the regulation of the mammalian circadian clock has been assessed in a circadian-reporter cell line. Pharmacological inhibition of proteasome-mediated degradation causes a significant lengthening of the period in synchronized Rat-1 cells[12]. Consistent with the biochemistry, the pharmacological inhibition of CKI also caused period lengthening. Although this confirms that CKI-mediated degradation of PER proteins is a key element in speeding up the clock, this result is at odds with information derived from the *tau* hamster.

***Mathematical biology takes on* tau.** The *tau* hamster is a spontaneously arising circadian rhythms mutant[16]. *tau* heterozygotes have a period of 22 hours, whereas *tau* homozygotes have a period of only 20 hours. This short period arises from a point mutation in the kinase domain of CKIε, a mutation that decreases the activity of the kinase eightfold *in vitro*[52]. These findings led to a model that proposes that the decreased kinase activity led to a shorter period. However, this conclusion is at odds with the findings that CKI inhibitors produce longer periods, and it is difficult to reconcile with the finding that both long and short period mutants in CKI have decreased kinase activity *in vitro*.

A way out of this conundrum was provided by a comprehensive mathematical model of the clock. This model incorporates existing experimental and mechanistic data and can make testable predictions in areas in which our understanding is so far incomplete[95,96]. Importantly, this model predicts that *in vivo* decreases in kinase activity must lengthen periods. The corollary is that the *tau* mutant must be an *in vivo* gain-of-function. We have directly tested this hypothesis and found that although the *tau* mutant CKIε has decreased kinase activity on most substrates, it binds to PER-containing complexes *in vivo* and has markedly increased site-specific activity on the residues that regulate the stability of PER1 and PER2. This alteration of kinase-substrate specificity is unique, and seems to be due to two distinct types of substrate preferences of the kinase. The *tau* mutation decreases the activity of CKI on common acidic substrates, but increases activity on the non-acidic βTrCP binding site[45].

Peer pressure regulates CKI. Much of the data indicate that the enzymes in circadian rhythms are regulated by their immediate neighbours in large multiprotein complexes. This might explain the perverse activity of the *tau*-mutant CKIε; although its activity is decreased in *in vitro* assays, it has increased activity on PER proteins in cells when they form a multiprotein complex[45]. Virtually all clock components interact in large multiprotein complexes such that each protein's activity can regulate and be regulated by other members of the complex. PER proteins in particular are scaffolds that form large complexes with a number of other circadian regulators. There are several other examples that show that location is important. For example, by binding to CKI, PER can regulate the phosphorylation of CRY[44]. CRY might in turn (by binding to PP5) regulate the activity of CKI on PER[77]. In the same complex, CRY1 and CRY2 can be phosphorylated by CKIε[44]. In a separate complex, sumoylation, and so proper regulation, of BMAL1 is induced by CLK[97]. It is therefore clear that many of these reactions cannot be analysed as two component systems (for example, a kinase and a substrate) but must be understood in the context of their neighbours and complex partners.

Last but not least. Like ubiquitylation, sumoylation regulates the clock. BMAL1 has been found to be rhythmically sumoylated *in vivo*[97] through a process that requires the heterodimerization partner CLOCK. Sumoylation of BMAL1 regulates the turnover of the protein, as a mutation in the sumoylation site (K259R) of BMAL1 lengthens the half-life of BMAL1. Sumoylation is also required for proper rhythmicity. Consistent with this, the K259R mutant BMAL1 does not oscillate in synchronized cells and does not rescue a normal 24-hour period in *Bmal1*$^{-/-}$ mouse embryonic fibroblasts. Although the circadian-regulated sumoylation of BMAL1 must be controlled by SUMO ligases and proteases, these enzymes and their potential circadian regulation remain unknown.

Regulation of nuclear localization

In addition to the regulation of PER stability, phosphorylation has an important, but confusing, role in PER localization. In flies, some data indicate that DBT phosphorylation of PER promotes cytoplasmic retention, whereas dephosphorylation promotes the nuclear translocation of PER[98]. Alternatively, others have reported that DBT promotes nuclear entry of PER[99]. The interpretation might be confused by the use of mutant flies, S2 cell lines without clocks and the effects of DBT on both stability and localization. Defects in PER dephosphorylation led to delayed nuclear entry. Nuclear accumulation of PER is also delayed when its phosphorylation is enhanced in transgenic flies that carry a hypomorphic mutation in the PP2A regulatory subunit *tws*[76]. *In vivo*, DBT phosphorylation seems to hold PER in the cytoplasm, and dephosphorylation allows it to translocate to the nucleus. By contrast, SGG-dependent phosphorylation of TIM promotes nuclear localization of the PER–TIM complex[64]. In the absence of TIM, SGG fails to induce the nuclear accumulation of PER[98].

In mammals, the cellular localization of overexpressed PER proteins varies among PER proteins and cell lines. PER1 (and PER2) is found in the cytoplasm and nucleus of NIH3T3 and COS7 cells[82,100,101], whereas it is predominantly

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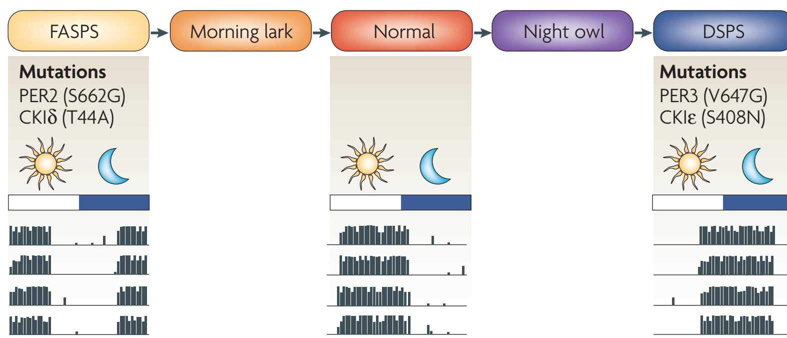
Box 1 | **Altered phosphorylation causes circadian rhythms disorders**



Familial advanced sleep-phase syndrome (FASPS)

FASPS is a autosomal dominant human behavioural disorder that causes early sleep times, early morning awakening and a short circadian period[53]. Genetic analysis in one family affected by FASPS identified a single amino-acid missense mutation in the human period-2 (*PER2*) gene as the cause of that sleep disorder variation[54]. The mutation, an S662G change, is in the casein kinase Iε (CKIε)-binding domain of PER2 and decreases PER2 phosphorylation *in vitro*.

More recently, a mutation in the human CKIδ gene was found in a family with FASPS[55]. The mutation consists of a threonine to alanine substitution, which decreases the enzymatic activity of the kinase when tested *in vitro*. Like FASPS individuals, transgenic mice carrying the CKIδ T44A mutation have shorter periods.

Delayed sleep-phase syndrome (DSPS)

Opposite to FASPS, DSPS causes late sleep-onset and the inability to wake up at a conventional time. A polymorphism in the human *PER3* gene (V647G) has been linked to the pathogenesis of DSPS[111]. Residue 647 locates in a region similar to the CKIε-binding region of PER1 and PER2, close to the serine residue in PER2 that is disrupted by the FASPS mutation. Therefore, this polymorphism might alter the CKIε-dependent phosphorylation of human PER3.

Another polymorphism that is present in the normal population but absent in individuals with DSPS is in human CKIε. This variant (S408N) is significantly less common in DSPS patients than in control individuals. The protective function is postulated to arise from an alteration in CKIε activity, as it eliminates a putative autophosphorylation site in the autoinhibitory domain of the kinase and leads to a more active kinase, at least *in vitro*[112].

The image shows theoretical recordings of activity rhythms corresponding to individuals affected by FASPS, DSPS or normal people. Although normal individuals are active from sunrise to sunset, individuals affected by FASPS fall asleep several hours before nightfall (indicated by lack of activity) and waken before sunrise, whereas individuals with DSPS are active well into the night and sleep for hours after sunrise.

nuclear in HEK293 cells[84]. Regardless of the subcellular distribution, phosphorylation by CKIε or CKIδ can regulate the localization of PER1 and PER3, in part by regulating their nuclear localization signals (NLSs). In HEK293 cells, phosphorylation of PER1 by either CKIε or CKIδ masks the NLS of PER1. As a result, nuclear entry of PER1 is retarded and the protein accumulates in the cytoplasm[84]. However, in COS7 cells, phosphorylation by CKIε drives PER1 to the nucleus[82]. CKIε and CKIδ can also drive PER3 to the nucleus, possibly because phosphorylation unmasks its NLS[86]. Why PER proteins start in distinct compartments in different cell types and then move in different directions depending on phosphorylation state is unknown.

Phosphorylation by CKI does not seem to affect the subcellular localization of PER2 (REF. 86). Instead, nuclear entry of PER2 is regulated by GSK3β, as inhibition of GSK3β by either lithium-chloride treatment or by knockdown inhibits the nuclear entry of rat PER2 (REF. 65).

The importance of regulated nuclear entry at controlling the clock has been hinted at, but an experiment that has looked at the effect of altered nucleo–cytoplasmic shuttling on vertebrate rhythms has yet to be reported.

In the *D. melanogaster* clock, it has been thought that the association of PER and TIM masked the nuclear export signals (NES) and promoted the nuclear accumulation of the PER–TIM complex[102]. However, the different time courses of the nuclear accumulation of PER and TIM in fly neurons indicates that the formation of the heterodimer is not required[103]. Moreover, TIM seems not to be required for nuclear accumulation because PER itself can suppress circadian transcription[99]. Most recently, the association of TIM and PER has been monitored by fluorescence resonance energy transfer (FRET) in single cultured cells[104]. The data reveal that PER and TIM associate quickly in the cytoplasm, but they later dissociate and enter the nucleus separately. Although this study was performed with overexpressed protein in cultured cells, it does indicate that the rate of dissociation, rather than the rate of association, of the TIM–PER complex contributes to the delay in nuclear entry that makes the circadian cycle 24 hours long[104,105]. So now the question becomes, what controls the rate of TIM–PER dissociation? (We'll place bets on phosphorylation…)

Conclusions and perspectives

Free-running circadian rhythms have an amazing accuracy; animals left in total darkness for months have activity schedules that vary by only minutes each day. Such a precise and robust system must have significant redundancies, checks and balances. The ability of the clock to function when circadian transcriptional regulation of PER proteins is removed indicates the importance of post-transcriptional regulation in controlling the abundance and activity of the clock regulators. These data strongly indicate that the kinases, phosphatases, ligases and proteases that regulate the clock machinery can form an oscillator. In fact, the cyanobacterial circadian clock can be reconstituted *in vitro* with one autophosphorylating and dephosphorylating protein, KaiC, and two associated regulators, KaiA and KaiB[106]. Therefore, there is strong precedence for the ability of the clock to function independently of ongoing transcription.

This finding leads to the open question — what are the factors that regulate the regulators? If kinases and phosphatases regulate the clock, their activity must also be regulated. For the phosphatases, this control can be provided by regulatory subunits, as shown by Sathyanarayanan and colleagues[76]. For kinases, activation and inactivation mechanism often require kinases (either themselves or others) and phosphatases. Some suspects are CKIε and CKIδ, which both have an autoinhibitory domain that is regulated by serine/threonine phosphatases such as PP1, PP2A and PP5 (REFS 77,78). Notably, CKIε can itself be activated in neurons by metabotropic glutamate signalling[107] and in other cells by Wnt signalling[108]. One untested speculation is that neural or hormonal activity can influence the clock through changes in CKI activity. GSK3 and several other kinases are also regulated by circadian stimuli and might also participate in fine-tuning the clock.

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Delayed sleep-phase syndrome
(DSPS). Patients with DSPS have late sleep-onset and the inability to wake up at a conventional time.

Can we regulate the regulators? Will a CKI inhibitor help us get a good night's sleep? Pharmacological inhibition of CKIε was predicted to phenocopy the *tau* hamster; it would shorten the length of the period and wake us up early. Now that the *tau* mutation has been revealed, instead, to be a gain-of-function mutation, expectations must be modified. Inhibition of CKIε (and CKIδ) is likely to slow the clock by delaying the degradation of PER. These drugs might therefore be most effective at bedtime, as they will inhibit the phosphorylation of accumulating PER and prolong sleep.

As in any complex biological system, stress (be it genetic or environmental) can lead to disease. Sleep disorders are the consequence of misalignments between the endogenous circadian clock and the external environment (reviewed in REF. 109). Although some sleep disorders such as jet lag or shift-work sleep disorder are associated with social and behavioural factors, others such as the familiar advanced or the delayed sleep-phase syndromes (FASPS and DSPS, respectively) have clear genetic origins. Prolonged winter darkness disrupts circadian rhythms and causes seasonal affective disorder, with endless nights leading to depression and increased rates of suicide. Under these circumstances, pharmacological manipulation of circadian rhythms might be an effective therapy for depression[110]. In this setting, it is noteworthy that mutations and polymorphisms that are associated with FASPS and DSPS affect either the kinases CKIε and CKIδ or their substrates, the PER proteins (BOX 1). These findings indicate that the inhibition of specific protein kinases with drugs is a feasible approach to shifting circadian phases.

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Competing interests statement
The authors declare no competing financial interests.

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