Review

Post-translational modifications in circadian rhythms

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The pace has quickened in circadian biology research. In particular, an abundance of results focused on post-translational modifications (PTMs) is sharpening our view of circadian molecular clockworks. PTMs affect nearly all aspects of clock biology; in some cases they are essential for clock function and in others, they provide layers of regulatory fine-tuning. Our goal is to review recent advances in clock PTMs, help make sense of emerging themes, and spotlight intriguing (and perhaps controversial) new findings. We focus on PTMs affecting the core functions of eukaryotic clocks, in particular the functionally related oscillators in *Neurospora crassa*, *Drosophila melanogaster*, and mammalian cells.

Post-translational modifications emerge on the scene

As our understanding of eukaryotic clocks has shifted focus from organismal behavior to molecular underpinnings, in the broadest description these clocks consist of a positively acting driver that is periodically suppressed by an inhibitory brake (Figure 1). More specifically, heterodimeric positively-acting PAS (named for Per-ARNT-Sim motifs) domain-containing transcription factors (+TFs) activate the transcription of negatively acting factors (-Fs). These, in turn feed back to inhibit the activity of the +TFs for long periods of time, thus setting the phase of transcription and determining the length of the feedback cycle [1–3].

This core circadian circuit is broadly controlled by post-translational modifications (PTMs). Indeed, recent evidence points to a clock based entirely on PTMs in cyanobacteria (Box 1), although this review does not focus on this system. What did we know about PTMs within these circuits circa 2004? Phosphorylation of -Fs was known to be typical, rhythmic, and phase-specific [4,5] and appeared to be the major contribution to determining the very long (circa 24 h) time constant of the feedback loop [6]. More nuanced studies showed that the activity state of +TFs that shuttled between the cytoplasm and nucleus [7] was accompanied by changes in histone acetylation [8] and chromatin remodeling [9], and there were hints that phosphorylation of +TFs [10] increased their transcriptional activity [11] and targeted them for degradation [12]. -Fs were known to dimerize and enter the nucleus [13–16] and the timing and control of this event was a regulated process [17,18]. Importantly, -Fs were known to bind and inhibit +TFs [10,16,19]. Several reports indicated that the joint activity of kinases and phosphatases (some of whose specific roles in the clock are functionally conserved across phyla, e.g. casein kinase 2 (CK2) [20–22], casein kinase 1 (CK1) [23–25] and protein phosphatase 2A (PP2A) [26,27]) caused net progressive phosphorylation of these -Fs [4,5] ultimately leading to their ubiquitylation and proteasome–dependent degradation [28–31]. These relatively slow processes of -F maturation and degradation were critical for creating a sufficiently long delay required to support a 24-h rhythm. Finally, some clues suggested that not every example of increased phosphorylation of -Fs necessarily resulted in faster degradation and period reduction, e.g. hypophosphorylation of hPER2 (human period homolog 2 (*Drosophila*)) mutants by CK1 elicited shorter periods [32]. In another case, kinases were observed to be potentiators of the inhibitory activity of -Fs [33].

An ever-increasing number of PTMs are rapidly becoming implicated in clock control. Given the diversity of experimental systems and variety of PTMs employed by clocks, here we strive to indicate how PTMs are used in common across phyla often acting on related molecules (Table 1), and to highlight occasional discrepancies in the field and real differences in clock regulation. PTMs contribute essential clock functions that are layered atop the canonical transcriptionally-controlled feedback oscillator and thus are an important and topical area of study.

An expanding view of the positive module

Recent data point to a direct link, mediated by +TF PTM activity, between chromatin remodeling and +TF transcriptional activity [34]. Mouse circadian locomoter output cycles kaput (CLOCK) has histone acetyltransferase (HAT) activity that is required for rhythmic expression of core clock and output genes [35]. CLOCK HAT activity selectively affects specific targets on histone subunits; however,

Glossary

Antimorph: typically dominant mutations that oppose the wild-type gene action.
Ataxia telangiectasia: a rare neurodegenerative disease causing ataxia (coordination defects) and telangiectasia (small blood vessels).
Circadian rhythm: a cycle of length approximately 24 h. The word originates from the Latin *circa* (about) and *dies* (day).
Negative feedback: a control process that loops back and self-limits.
Hypermorph: a mutation that causes an increase in wild-type gene action.
Hypomorph: a mutation that causes a partial loss-of-function.
Mixomorph: a mutation that has mixed character (e.g. it can act as a gain and loss-of-function depending on context).
Period: the amount of time required to complete a cycle of activity; the inverse of frequency.
Phase: any stereotypic, distinguishable and repeating single part of a cycle.
Pleiotropy: the influence of a single gene on many phenotypes.

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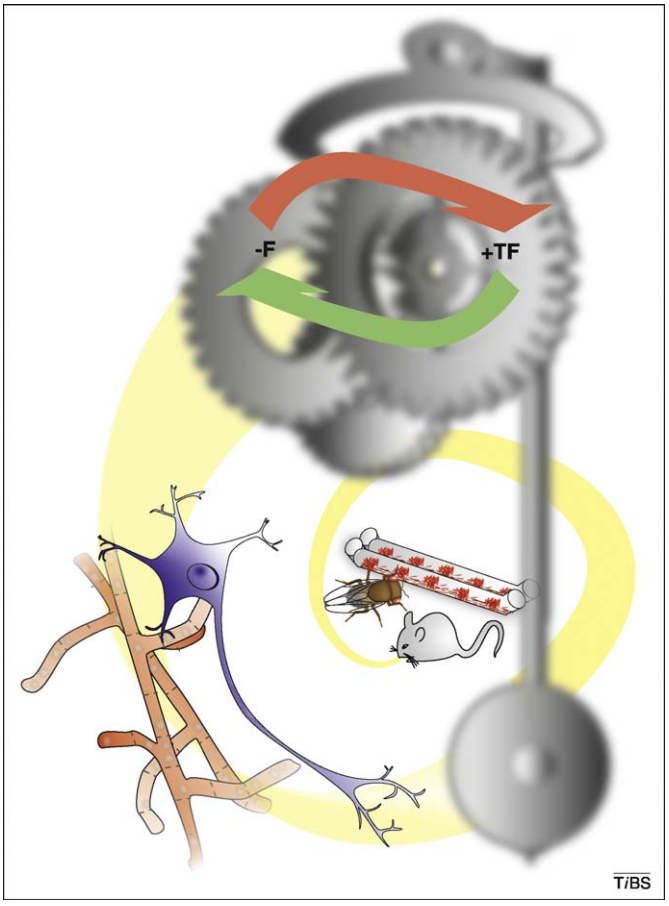

Figure 1. Circadian clocks from organism to mechanism.
Over the last several decades, our picture of circadian clocks has spiraled out from a physiological description of clock-controlled activities at the organismal level, through an understanding of clocks as being bound within single cells, to revealing a molecular mechanism that is predominantly constrained within the interplay between positive (+TF) and negative (-F) forces. A clearer picture of the clockworks of these systems is beginning to emerge, showing that many of the processes and components are controlled through post-translational modification.

CLOCK can also acetylate its partner BMAL (brain and muscle aryl hydrocarbon receptor nuclear translocator-(ARNT)-like) [36]. BMAL acetylation at Lys537 is rhythmic in liver extracts, is essential for clock function and, most interestingly, appears to be required to inactivate BMAL transcriptional activity. Further experimentation is required to determine if +TFs functionally analogous to CLOCK (*Drosophila melanogaster* CLK and *Neurospora crassa* WHITE COLLAR-2; WC–2) have similar enzymatic roles; to date there is no evidence that components of the WHITE COLLAR COMPLEX (WCC) are acetylated.

Box 1. An oscillator based only on PTMs in cyanobacteria

An outstanding case study for the importance of PTMs in circadian clocks (albeit one that is beyond the scope of this review) comes from recent work by Kondo and co-workers on the *in-vitro* KaiC oscillator (for a topical review, see [96]). Briefly, when three cyanobacterial proteins (KaiA, KaiB and KaiC) are incubated *in vitro* in the presence of ATP, a cycle of phosphorylation is observed on KaiC. This rhythmic cycle of KaiC phosphorylation does not require new production or destruction of protein, and exhibits all the hallmarks of a true circadian rhythm. Although the cyanobacterial clock has evolved independently, this exciting finding has raised interest in the possibility that non–bacterial oscillatory proteins might also be able to support rhythms based solely on PTMs.

As clock components are acetylated, so too they must be deacetylated. One research team has reported that sirtuin1 ((silent mating type information regulation 2, homolog) 1 (*S. cerevisiae*), SIRT1), an NAD^+–dependent deacetylase, has rhythmic deacetylase activity, directly interacts with CLOCK and is required for the rhythmic acetylation pattern of BMAL-Lys537 in cells and *in vivo* [37]. However, the story is complex: another research group showed rhythmic binding (but not activity) of SIRT1 to BMAL–CLOCK and SIRT1–dependent oscillatory PER2 deacetylation [38]. Interestingly, PER2 acetylation increases its stability. Resolution of these, perhaps not mutually exclusive, observations will require further experimentation. Given the involvement of SIRT1 in metabolic programs, the intriguing new connection between energy metabolism, NAD^+ levels and rhythmicity provides ample room for research.

In addition to phosphorylation and acetylation, at least one +TF is modified by the small ubiquitin-like modifier (SUMO). mBMAL1 is rhythmically SUMOylated on a highly conserved lysine residue (K259) in a CLOCK–dependent manner [39]. In tissue culture cells, BMAL is predominantly polySUMOylated, and this modification leads to ubiquitylation, localization to subnuclear foci and enhanced transcriptional activity [40].

An exploding view of the negative module

The negative module is subject to various PTMs that center around the activity of kinases and phosphatases, and the array of players is expanding. Together, the net effect of these effectors is to determine the half-life of -F factors through ubiquitin–mediated degradation and to influence period length. Emerging data have consolidated and extended this view. However, not all -F phosphorylation events lead to degradation, and we also review non–degradative effects of phosphorylation.

Kinases and phosphatases, old and new

CK1 is the 'grandfather' of circadian-associated kinases [23,41] and, as the relationships between CK1 and its -F targets become better understood, additional complexity is being uncovered. In *Drosophila*, overexpression of dominant negative CK1 (Doubletime; DBT) results in a long period or arrhythmicity, depending on the antimorph dose [42]. Although this experiment suggests a direct relationship between the dose/activity of CK1 and period length (i.e. more CK1 activity speeds up the clock), other analyses in flies and mice complicate this concept by the use of mutant alleles that could be hypomorphs, hypermorphs or even mixomorphs. For example, dbt^L and dbt^S alleles have opposite period phenotypes in flies but, curiously, both result in decreased CK1 activity *in vitro* [43].

Similar complexities that are currently only partially resolved include two mutations affecting CK1 in mammals, both of which initially appeared to contradict the dogma that increased phosphorylation of -Fs leads to shorter periods. First, although the short-period allele CK1ε (*tau*) was initially shown to be a hypomorph in *in vitro* assays [24], two research groups have recently shown that this mutation increases *in vivo* mPER2 phosphorylation, increasing its turnover [44,45]; they argue that the change

Table 1. Functional classes of clock proteins across phyla[a]

Class	Ascomycota (*Neurospora*)	Arthropoda (*Drosophila*)	Chordata (*Mus, Homo*)
+TFs	WC-1 WC-2	CYC CLK	BMAL1 CLOCK
-Fs	FRQ	 PER TIM	 PER 1,2,3 CRY
Kinases	CK-1a CKA PKA PRD-4	DBT CK2 Shaggy	CK1δ/ε CK2 PKA Chk2 Chk1 ATM
Phosphatases	PP2A PP1	PP2A PP1	PP2A
HATs, HDACs			CLOCK SIRT1
F-boxes	FWD-1	SLIMB JETLAG	β-TRCP FBXL3

[a]Proteins implicated in the operation or resetting of core circadian oscillators from three prominent model organisms are listed. Proteins in line with one another share sequence/domain structure and serve orthologous functions.

in kinase activity deriving from this mutation could be substrate-dependent. It is also unclear whether the change in mPER2 phosphorylation is a direct or indirect effect of the altered kinase; thus, it remains an open question whether CK1ε (*tau*) is a target-specific hypermorph, or alternatively, if the phenotype stems from a change in mPER2 localization [46].

A second example involves a hypomorphic version of CK1∂ caused by a T44A amino acid substitution. This mutant causes familial advanced sleep phase syndrome (FASPS) [47] and period shortening in mammals. However, transgenic flies which express the mutant kinase did not share the mammalian phenotype; instead the period lengthens. The underlying cause for this dissimilarity is an open question, but probably involves differences in regulation between flies and mammals despite conserved clock architectures—one key difference is the role of Timeless (TIM) in *Drosophila* which is not paralleled in mammals. Recent work [48] adds to the puzzle: mutations that lower *Drosophila* DBT and vertebrate CK1∂ kinase activity led to shortened rhythms. Therefore not all reduced–activity CK1 enzymes elicit opposite effects in mammals and flies, and indeed, functional conservation exists between these homologous enzymes. Overall, reduced kinase activity cannot simply explain the decreased period length, and so further analysis will be required; in the study by Fan *et al*. [48], the authors suggest that changes in site–specific phosphorylation or interaction with a regulator might be responsible for period shortening in the context of reduced kinase activity, and this perhaps represents a general solution to the conundrum.

The situation is currently more clear-cut with respect to CK2 and its role in fly clocks. *In vitro* kinase assays carried out using a dPER deletion series implicate key serines which, when altered *in vivo*, lead to period–lengthening [49]. Although some of these residues might be *bona fide* CK2 phosphosites, conclusions derived solely from consensus sequence-based approaches or *in vitro* patterns are fraught with caveats (for example, see [50]). In addition, overexpression of a dominant negative dCK2 in circadian neurons leads to dramatic period lengthening [51]; this result helps to distinguish direct from pleiotropic effects of this multifunctional enzyme.

A pressing question about CK1 and CK2 is whether the activity of these kinases is rhythmic. Various studies in many organisms have shown that bulk CK1 and CK2 levels do not cycle, as would be expected for such utility kinases. However, in *Drosophila*, rhythmic aspects of phosphatase activity—regulatory PP2A subunits are expressed in a circadian fashion—provide a nuance to this question [26]. A finding in *Arabidopsis thaliana*, by contrast, could portend possibilities for organisms with multiple CK2-beta subunits: proteasome–dependent degradation of the CKB4 isoform (a newly described fourth beta subunit) is rhythmic [52], perhaps indicating that some aspects of kinase regulation are rhythmic.

These 'veteran' kinases have been joined by some clock 'rookies': the cAMP-dependent protein kinase A (PKA) and the DNA damage/checkpoint-associated kinases Chk1, Chk2, and ATM (ataxia telangiectasia, mutated). Two independent approaches have implicated PKA in circadian function. Overexpression of the PKA catalytic subunit causes induction of hPER1 expression, and this induction is CREB (cAMP responsive element binding protein)- and CCAAT-box binding protein (C/EBP)-dependent, suggesting that cAMP-induced CREB and C/EBP are 'peripheral' modulators of hPER1 [53]. By contrast, *Neurospora* PKA has also been placed more directly in two parts of the loop: it is a priming kinase for the casein kinase action on the WCC and it stabilizes WCC and FREQUENCY (FRQ) [54]. PKA physically associates with WCC and can phosphorylate WCC and FRQ *in vitro*. Moreover, strains with reduced PKA activity show increased binding of the WCC to the *frq* promoter. On first blush, these two studies appear to contradict each other—if PKA inhibits +TF DNA binding, why should overexpression of such a transcriptional inhibitor of CLOCK–BMAL lead to increased hPER1 expression? However, if PKA works at

multiple levels, i.e. transcriptional repression and post-translational stabilization, effects of overexpression could be tricky to predict *a priori*.

Although *Chk2* null mutations have not revealed clock defects under standard conditions, this and perhaps related kinases provide conditional input to clock feedback loops. Indeed, their contribution probably would still be unknown had the co-dominant hypermorphic *Chk2* allele, *prd-4*, not been found to confer a short circadian period in *Neurospora* [55]. Molecular analyses revealed direct binding between PRD-4 and the -F FRQ; moreover, DNA damage-dependent FRQ phosphorylation triggers its destabilization—a kinase-based clock input conditional upon DNA damage. It is now clear that this response is widely conserved: Chk1 associates with hPER1 after cell irradiation [56] and DNA damage results in PER1 phosphorylation [57]. Moreover, gamma irradiation resets the clock of mice and human cell lines in an ATM–dependent manner (see [58], and also shown in MEFs with UV or MMS-induced damage [59]), as predicted from the *Neurospora* work. Moreover, other clock components, including NPAS2 (neuronal PAS domain protein 2), also appear to be involved in DNA damage responses [60].

In addition to the several known kinases, one new player from the universe of phosphatases, protein phosphatase 1 (PP1), has joined the team. Earlier evidence from *Neurospora* showed that PP1 reduction caused advanced phase and a slightly shorter period [27]. In mammals, PP1 binds and stabilizes one of the -Fs by dephosphorylation [61]. Overexpression of PP1 inhibitors and dominant negative forms of PP1 support this conclusion. By contrast, using genetic analysis, PP1 activity appears to decrease period length in flies [62]. Once again, one possibility is that these apparently contradictory results are reconciled by the presence of the -F binding factor TIM and its binding to PER, which does not appear to have a clock-specific role in mammals.

We expect to continue finding new connections between kinases and phosphatases and the clock. It is becoming clear that the relationships between phosphorylation and degradation of -Fs or activity of +TFs are not as simple as we had initially imagined. Moreover, with a given kinase/phosphatase connected on multiple levels to clock components (as in the case of PKA, which acts upon +TFs and -Fs), untangling the consequences of simple over- or underexpression will not be trivial. In fact, interconnected relationships might have evolved as an ensemble to encode aspects of post-translational rhythmicity. Along these lines, older evidence indicates that rhythmic cycling of *dper* and *dtim* mRNA is dispensable for rhythms [63,64]. A recent result consistent with this finding is that PER2-LUC protein from constitutively expressed *mPER2-luc* in Rat-1 cells might oscillate due only to PTMs [65].

Ubiquitin and the F-box

As mentioned above, a key consequence of progressive phosphorylation by clock–associated kinases is regulated destruction of their targets (typically -Fs). Seminal findings regarding degradation in *Arabidopsis* [66] have been recapitulated in *Neurospora* [31]. That F-box mediated degradation of -Fs occurs was in itself not surprising; what was perhaps surprising is that although there are many F-box containing proteins (~70 in mammals, dozens in flies, and a handful in *Neurospora*), the clock–associated F-box proteins, Supernumerary limbs (SLIMB) in *Drosophila* [29,30], FWD–1 (F-box and WD-40 repeat–containing protein 1) in *Neurospora* [31], and β–TrCP (beta–transducin repeat containing protein) in mammals [67], are all orthologs. Taken together, these results suggest that the choice of a given F-box protein for -F degradation is unlikely to have occurred through convergent evolution, but there appears to be a unique aspect to SLIMB regulation in flies. In contrast to other systems, overexpression of SLIMB in the cytoplasm leads to period lengthening [29]. The authors propose a model in which the SLIMB target, dPER, cannot accumulate to requisite critical levels and is therefore delayed in nuclear translocation.

A novel observation in this context is that phosphorylation of a key dPER phosphosite leads to SLIMB binding in flies. This site, within the first 100 aa of dPER, is rhythmically phosphorylated, leading to F-box binding and rapid degradation [68]. This result suggests that phosphorylation of specific, critical sites is more important for F-box binding than simple accumulation of electrostatic charge, and appears different from another F-box binding design in yeast cell-cycle control [69]. Moreover, the requirement for phosphorylation on several sites suggests that most phosphorylation on dPER has non-degradative consequences.

Another emerging complexity is that different F-box proteins affect the clock at different levels. For example, in *Drosophila*, Jetlag resets the clock by causing light–induced TIM degradation [70]. Mammalian FBXL3 (F-box and leucine-rich repeat protein 3), mediates destruction of an accessory mammalian -F, CRY (cryptochrome) [71–73]. It will be of interest to determine if these additional F-box protein functions are conserved across species, as observed for the SLIMB homologs.

Non-degradative consequences of -F phosphorylation

In addition to the aforementioned examples of regulated nuclear entry and -F potentiation of negative arm function, there are a growing number of examples of -F phosphorylation leading to effects other than degradation. These include new instances of -F subcellular localization and potency control as well as novel regulation of +TFs, and new insight into the regulation of progressive -F phosphorylation.

More evidence regarding -F localization and potency has come from studies in the fly. First, as in mammals, DBT keeps dPER in the cytoplasm [74], but another kinase, Shaggy, acts to oppose DBT by accelerating PER nuclear accumulation in a TIM-dependent manner. Second, certain dPER phosphosite mutants can lead to short period [75]—failure to phosphorylate these sites causes dPER to become a potent repressor of dCLK.

Next is the intriguing example of the $hPER2^{S662G}$ variant which leads to FASPS (see above). A decrease in protein stability resulting from changes in the analogous mPER2 residue (S659) was explained by premature nuclear clearance and increased sensitivity to CK1–dependent degradation [46]. One year later, a different

non-degradative explanation emerged when the same mutation, now in h*Per2*, was introduced into mice. hPER2^{S662G} in mice was associated with decreases in *Per2* mRNA levels, leading to the suggestion that phosphorylation of S662 (the pertinent residue) decreases the ability of mPER2 to inhibit its own message [76].

In *Neurospora*, phosphorylation of certain key serines in the -F FRQ appears to be required for post–translational up-regulation of +TFs [77]. When key phosphosites are ablated, FRQ cannot mediate WCC formation, and what results resembles a dampened oscillator.

Finally, accumulating evidence suggests that -Fs might harbor 'clusters' of sites responsible for distinct functions (e.g. stabilization, degradation, nuclear residency and potency of transcriptional inhibition) [68]. Some authors have speculated that clustered phosphorylation within -Fs leads to sequential unfolding and/or changes in the spectrum of interacting partners [75]. Moreover, a recent study employed heavy isotope-labeling and quantitative proteomics to follow the appearance and disappearance of >75 clustered phosphorylation sites on FRQ [78]; as seen with dPER, some domains appear to promote turnover and some influence intra- or inter- protein interactions, and their loss through mutation can accelerate or decelerate the pace of the clock. Finally, a subset of serines on FRQ appear to facilitate degradation specifically at higher temperatures—thus influencing temperature compensation [79]: another case in point that heterogeneous phosphorylation can mediate multiple consequences. Together, these findings are consistent with roles for -Fs as integrators for various cellular signals pertinent to clock function. It will be interesting to see if clustered phosphorylation is a conserved property of -Fs and whether this or some other mechanism underpins progressive phosphorylation. Overall, phosphorylation of -Fs can lead to various non–degradative effects.

Mechanistic details of negative feedback

The original model for circadian negative feedback was that -Fs simply bound +TFs and thereby inactivated them; this view is being expanded and modified. Specific details of -F action on +TFs (as well as information about temporal regulation of +TF promoter occupancy) are beginning to take shape.

Consequences of -Fs binding to +TFs

Studies in mammals have lent further support to the idea that feedback repression is a key mechanism of clock regulation [80]. The first suggestion that the model required updating came from a study in *Neurospora* in which steady-state estimates indicated there was insufficient FRQ to inactivate the WCC in a stoichiometric manner; indeed, WCC phosphorylation [81] was associated with decreased DNA binding [82]. This led to a model in which instead of constitutively binding to WCC, FRQ instead interacts with the WCC transiently and serves as a scaffold for CK1 and CK2 which in turn act enzymatically on the WCC [54,83,84]. More recent data, however, indicate that the feedback loop is closed through dynamic hypophosphorylated-FRQ-mediated export of WCC from the nucleus [85]. A similar picture has begun to emerge from studies in flies where CK1 (DBT) constitutively binds dPER and phosphorylates dCLK in a dPER–dependent manner [86–89]. Moreover, DBT cannot directly phosphorylate an N-terminal portion of dCLK *in vitro* in the absence of dPER [75]. As with *Neurospora* FRQ, a key region on dPER is required for DBT binding; when this region is altered, dPER still binds +TFs but cannot inhibit their activity [88], consistent with a scaffold model where dPER connects kinases with +TFs. In a surprising twist, in addition to its kinase activity, DBT might also act as a scaffold for another kinase because DBT catalytic activity appears unnecessary for some dCLK phosphorylation [90].

Dynamics of +TF promoter occupancy

Finally, our view of +TFs as being static is changing; instead, we are beginning to appreciate their dynamic nature. Though not unambiguously so, some evidence indicates that +TFs bind their promoters rhythmically. Such behavior has been shown in mammals [34], but other studies [10,36] point to constitutive promoter binding. In flies, DBT-mediated dCLK phosphorylation leads to changes in its binding to E–box–containing promoters [86], thereby eliciting changes in chromatin structure [91]. *Neurospora* appears to use both strategies; one of the two WCC members, WC–2, binds rhythmically to the *frq* promoter whereas the other, WC-1, binds constitutively [92].

In addition to their DNA binding capacity, the subcellular localization of +TFs is also highly dynamic and probably influenced by PTMs. BMAL1 phosphorylation status is reported to affect its nucleocytoplasmic shuttling, transactivation ability, and turnover [93]. Likewise, dCLK accumulates in the cytoplasm after DBT-meditated phosphorylation [87], and WCC nuclear export is promoted by hypophosphorylated FRQ [85,94]. Moreover, active destruction of +TFs appears to provide a previously underappreciated layer of regulation [95]. Overall, an emerging consensus suggests that binding, localization, and stability of +TFs are dynamic—a situation different from that originally imagined.

Concluding remarks

Our picture of the clockworks and PTMs has been focused (from the simple picture in Figure 1 to the many facets in Figure 2) in a very short timespan as appreciation of the variety and functional significance of PTMs has grown. The clock wiring diagram clearly contains a growing number of post–translational control points, and further discovery and refinement should be rapid and exciting.

One aspect of PTMs that appears destined for more prominence is the role of rhythmic PTMs in nested feedback loops which surround the core that can lead to stabilization of the core loop and to circadian output. Although the activities of utility kinases such as CK1 and CK2 appear not to be rhythmic, the deacetylase SIRT1 is reported to be clock-controlled and to have a pronounced effect on circadian output [37,38]. This could be the first example of an emerging theme.

Another question that will perhaps dominate our thinking in the near future concerns determining which

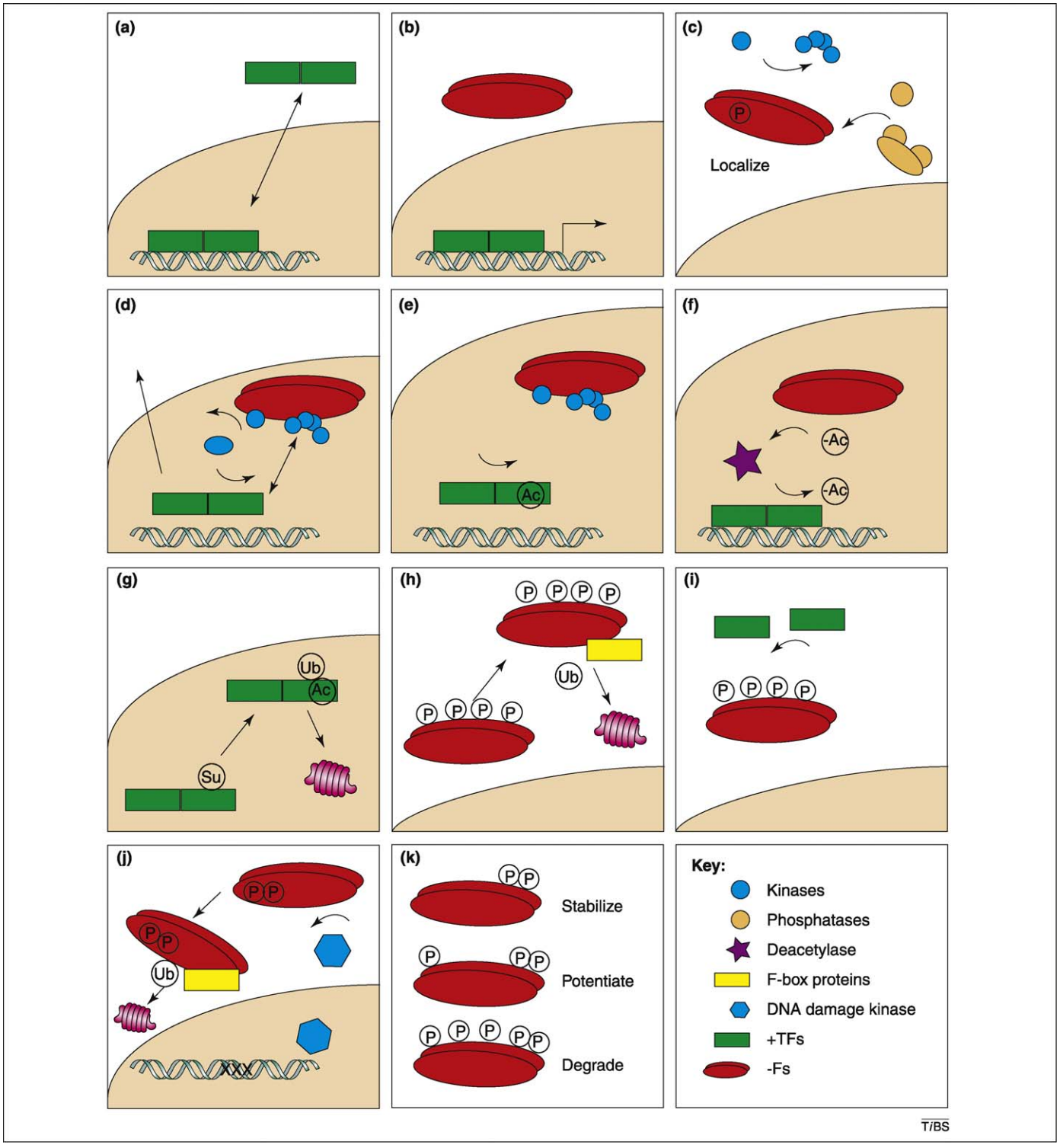

Figure 2. Vignettes of circadian post-translational modifications.
Several clock PTMs are depicted in a simplified series of cartoons. Representative PTMs are shown and do not reflect the action in all species. For more detail, please refer to specific examples throughout the text. **(a)** PAS-domain containing positively acting transcription factors (+TFs, green boxes) shuttle (bidirectional arrow) between the nucleus, on DNA, and cytoplasm. **(b)** DNA-bound +TFs activate transcription (bent arrow) of negatively acting factors (-Fs, red ovals). **(c)** Kinases (blue circles) and phosphatases (orange circles) compete to establish a pattern of progressive phosphorylation (P) which can have multiple consequences, including cytoplasmic retention of -Fs. **(d)** -Fs transport kinases which, along with independent kinases (blue oval) phosphorylate (curved arrows) +TFs (and/or -Fs) via transient interactions (bidirectional arrow) leading to inactivation of +TFs and export from the nucleus (arrow). **(e)** A mammalian +TF can act as a histone acetyltransferase (curved arrow, Ac; acetyl group) to inactivate its partner. **(f)** In mammals, a deacetylase can remove inhibitory Ac from +TFs and -Fs. **(g)** In mammals, +TFs can be sumoylated (Su), leading to their ubiquitylation (Ub) and degradation. **(h)** Progressive phosphorylation on -Fs leads to F-box binding (yellow box), ubiquitylation and subsequent degradation. **(i)** In *Neurospora,* mature -F can promote +TF complex formation. **(j)** Upon DNA damage (XXX), DNA-damage dependent kinases (blue hexagon) can phosphorylate -Fs, accelerating their degradation. **(k)** Multiple outcomes of -F degradation are depicted. Clusters of phosphosites can mediate different consequences including stabilization, potentiation of -F efficacy and degradation.

regulations are 'core' controls and which are simply consequences of indirect cascades of secondary effects. For instance, just as synthetic biology has succeeded in assembling components that can oscillate (albeit in a less than fully robust manner), it is clear that evolution can make connections in a seemingly random manner and that those that provide fitness have been maintained—as observed in the separate events which led to the three distinct clock mechanisms in the cyanobacterial (Box 1), plant and fungal/animal lineages. In this context, it is perhaps not surprising that the networks are complex. Moreover, research to date, although fruitful, has been not unlike hunting and gathering where each factoid regarding a modifier or modification has been cherished. Looking forward, we can envision the use of genomic and proteomic tools to describe entire clock-interactomes and temporal patterns of PTMs of all clock–relevant molecules.

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