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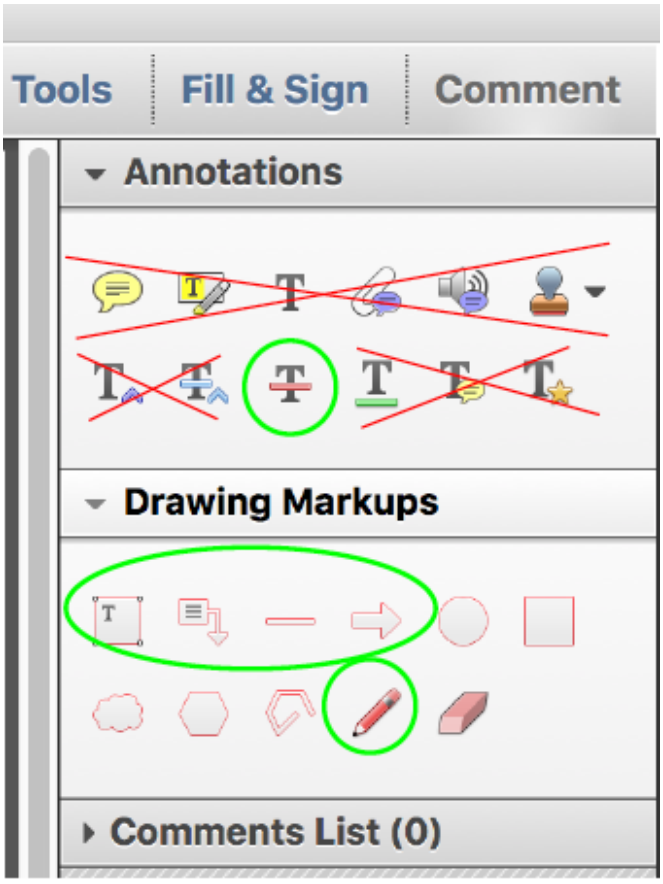

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The intricate dance of post-translational modifications in the rhythm of life

Q1 Arisa Hirano, Ying-Hui Fu & Louis J Ptáček

Endogenous biological rhythms with approximately 24-h periodicity are generated by the circadian clock, in which clock genes coordinate with one another and form a transcriptional–translational negative feedback loop. The precision of the circadian clock is further regulated by multiple post-translational modifications (PTMs), including phosphorylation, ubiquitination, acetylation and SUMOylation. Here, we review current understanding of the regulatory mechanisms of the core clock proteins by PTMs in the mammalian circadian clock.

Circadian rhythms with an approximately 24-h periodicity are present in various biological processes at molecular (e.g., hormonal rhythms and gene-expression rhythms) and behavioral (e.g., sleep–wake patterns, feeding and reproductive behavior) levels[1]. Circadian rhythms are governed by the internal body clock (circadian clock), which is a cell-autonomous oscillator. The basic structure of the circadian clock is a transcription- and translation-based negative feedback loop in which a series of clock genes are coordinately regulated[2] (**Fig. 1**). In mammals, the core loop is organized by transcriptional activators, CLOCK and BMAL1, and repressors, PERIOD1–3 (PER1–3), CRYPROCHROME (CRY) 1 and CRY2. REV-ERBs and retinoic acid receptor–related orphan receptor (ROR) proteins drive the subloop coupled with the core loop, thereby reinforcing circadian oscillation and generating various phase angles of gene-expression rhythms[2]. For the rhythmic expression of clock genes and output (clock-controlled) genes, both quantity control (amount) and quality control (localization and activity) of the clock proteins are critically important throughout the circadian day. To achieve this rhythmic expression, Q2 clock proteins are precisely controlled by multiple PTMs during the circadian cycle[3–5]. Clocks in other organisms, such as *Drosophila* and *Neurospora*, have similar feedback loops in which finely tuned PTMs contribute to circadian regulation[3,6]. A human mutation at the phosphorylation site of PER2 causes familial advanced sleep phase (FASP), owing to dramatic shortening of the circadian period[7,8], thus indicating the importance of PTMs in proper clock function. A better understanding of clock-protein modifications may provide a basis for novel therapies for diseases associated with circadian disorders.

PTMs are well-established biological phenomena and are known to regulate various physiological functions[9]. To date, over 450 unique protein modifications have been identified[10]. Here, we discuss a small number of modifications known to be critical for circadian regulation. Phosphorylation is the most common (or at least the best studied) protein modification and is catalyzed by over 500 distinct kinases in mammals[9]. Phosphorylation occurs on serine, threonine and tyrosine residues of target substrates and regulates protein stability, protein-protein interactions and protein cellular localization and enzymatic activity (depending on the substrate and the site of phosphorylation). Ubiquitination, also a well-characterized modification, is catalyzed by three enzyme families (E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases), among which E3 ligases generally determine substrate specificity[9]. Ubiquitin is a small 76–amino acid protein that is covalently bound to a lysine residue of the substrate protein. Specific regulatory mechanisms mediated by ubiquitination vary depending on the structure of the polyubiquitin chain. Polyubiquitination results when ubiquitin molecules polymerize via attachment to a lysine of the proceeding ubiquitin already bound to the substrate. Lys48-linked polyubiquitination (with polymerization at Lys48 of the preceding ubiquitin) typically targets a protein for degradation by the proteasome. Similarly to ubiquitin, small ubiquitin-related modifier 2 or 3 (SUMO2/3) forms poly-SUMO chains on lysine residues of substrate proteins[9]. Among known ubiquitin-like proteins, only SUMOylation has been reported to regulate the circadian clock. Acetylation also modifies lysine residues of substrates and hence competes with (and antagonizes the function of) ubiquitination[10]. O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) occurs on serine and threonine residues. In some cases, *O*-GlcNAc modifications and phosphorylation compete for the same target serines or threonines[9,10]. Such competition targeting the same substrate has recently been demonstrated in circadian-clock regulation[11]. Thus, there are multiple layers of regulation in complicated biological networks of protein regulation[10]. Such complexity underlies the homeostatic regulation of critically important biological processes such as circadian timing.

Phosphorylation regulating PER-protein stability

PER1–3 proteins are major clock components, and PER-protein levels and phosphorylation levels show large-amplitude oscillations in both central and peripheral clocks[12]. Many *in vivo* and *in vitro* studies have demonstrated the physiological importance of PER regulation at the post-translational level. During circadian oscillations, PER1–3 proteins are phosphorylated by casein kinase (CK) Iδ and Iε (refs. 13–17) (**Fig. 2**, state 1 to state 2). Expression of CKIδ or CKIε in cultured

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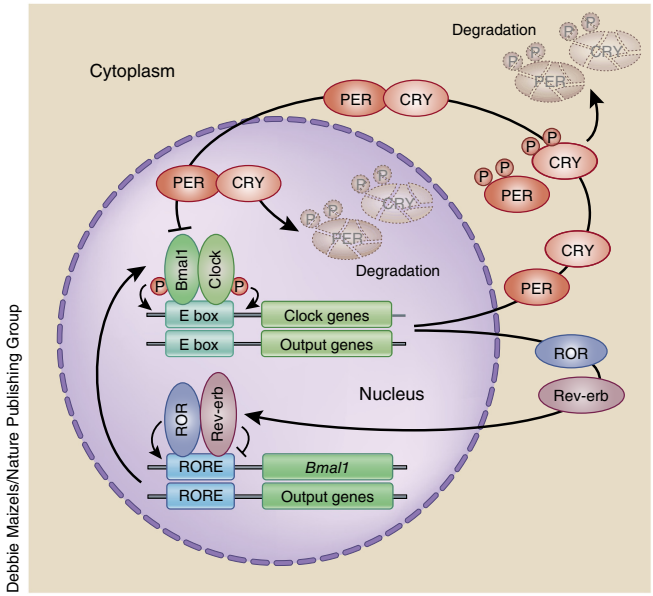

Figure 1 A basic model of the circadian molecular clock. CLOCK and BMAL1 activate the transcription of clock genes encoding PER and CRY, as well as clock-controlled genes (output genes). PER and CRY proteins accumulate gradually and repress the CLOCK–BMAL1 transactivation, thereby terminating their own transcription. In each step, clock proteins are controlled by various and intricate PTMs, thus achieving accurate circadian oscillations. REV-ERBs and RORs bind to ROR response elements (ROREs) in *cis*, thereby driving *Bmal1* transcription. P, phospho-.

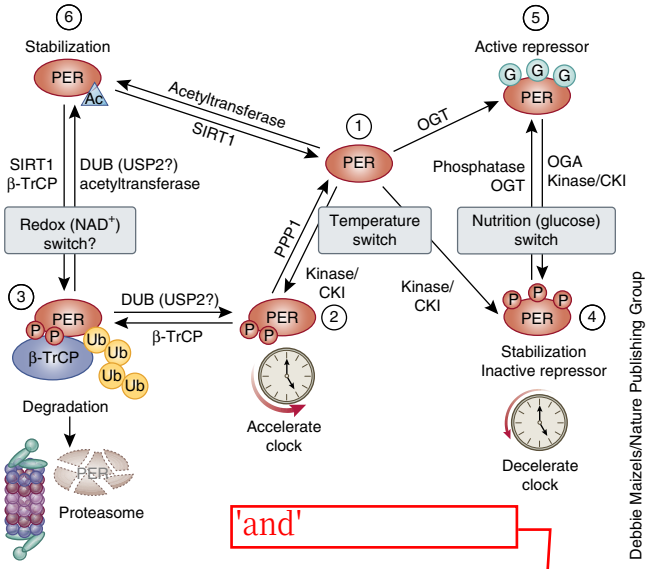

Figure 2 PER stability is controlled by cross-talk of multiple PTMs. Regulation of mammalian PER proteins by PTMs. PER proteins can exist in six distinct states. First is unmodified PER (1). CKI-mediated phosphorylation has two opposite effects on PER-protein stability, depending on the phosphorylation sites (N-terminal sites (2) or FASP sites (4)). These two mechanisms switch on the basis of temperature, and this switch is important for temperature compensation of the circadian clock. Phosphorylation at the FASP sites (4) leads to decreased repressor activity and stabilization of PER and competes with *O*-GlcNAc modification (5) at the same residues. Competitive mechanisms are also observed for acetylation (6) and ubiquitination (3), which lead to PER stabilization and proteasomal degradation, respectively. P, phospho-; Ub, ubiquitin; G, *O*-GlcNAc; Ac, acetyl; DUB, deubiquitinating enzyme; OGT, *O*-GlcNAc transferase; OGA, *O*-GlcNAc hydrolase. Slashes denote ~~'or'~~.

cells destabilizes PERs by targeting them for proteasomal degradation. Chemical inhibition with nonspecific CKI inhibitors increases PER stability and lengthens the circadian period[13–16,18,19]. CKII also triggers degradation of mammalian (m) PER2 by phosphorylation at Ser53. In contrast to CKI treatment, inhibition of this phosphorylation event shortens the period[20]; therefore, CKII-mediated phosphorylation has been proposed to occur in the PER2 accumulation phase, whereas CKI-mediated PER degradation occurs at night when PER2 levels are falling, thus facilitating transcriptional derepression[20]. Chemical screening has also identified CKIα as a modulator of PER2 stability[21]. The first evidence of the crucial role of PTMs in the circadian clock was found in *double-time* (*dbt*; official symbol *dco*) mutations in flies[22,23]. DBT is a *Drosophila* homolog of CKIδ and CKIε and phosphorylates *Drosophila* PER. Shortly after the cloning of *dbt*, an *in vivo* role of mammalian CKI was demonstrated by the identification of *tau*-mutant hamsters, which have a significantly shorter period that results from a point mutation in the gene encoding CKIε (ref. 24) and leads to increased enzymatic activity in cultured cells (for at least one substrate, mPER2)[25]. An engineered *tau* CKIε mutation in mice recapitulates the hamster phenotype, leads to destabilization of PER1 and PER2, and shortens the circadian period[26]. However, a gene-targeting study has revealed that knocking out CKIδ lengthens the period, whereas knocking out CKIε has no effect on the period, thus demonstrating the more critical role of CKIδ *in vivo*[27].

The phosphorylation of PERs (**Fig. 2**, state 2) leads to recruitment of the F-box-type E3 ligases β-TrCP1 or β-TrCP2 (FBXW1 or FBXW11) and subsequent polyubiquitination by a Skp–Cullin–F-box (SCF) complex containing β-TrCP1 or β-TrCP2; the polyubiquitinated PERs then become targets of proteasomal degradation[15,28,29] (**Fig. 2**, state 2 to state 3). Some E3 ligases including β-TrCP require a priming phosphorylation before they can bind to their substrate. The consensus sequences for phosphorylation-dependent E3 ligases are called phosphodegrons. In the circadian clock, β-TrCP1 and β-TrCP2 bind to phosphorylated mouse PER2 (477-SSGYGS-482)[15,30], which contains a CKI-phosphorylation site. Although β-TrCP1 knockdown damps cellular rhythms in cell culture, β-TrCP1 knockout has no effect on mouse behavioral rhythms[30]. The remaining β-TrCP2 may compensate for the deficiency *in vivo*, or there may still be another (yet-unrecognized) E3 ligase regulating PERs. A point mutation in the linker region between the PAS-A and PAS-B domains of PER2, denoted the early doors (*Edo*) mutation, increases the binding affinity of PER2 for β-TrCP and destabilizes PER2, thereby resulting in a shortened circadian period[31].

By using a human genetics strategy and studying families with Mendelian circadian phenotypes, we have identified a growing list of clock-gene mutations causing FASP[7,32–35], in which alteration of PER turnover is often observed. A study of families with FASP has identified mutations in *CKIδ* (official symbol *CSNK1D*) that alter the kinase activity[32,36]. Recently, we have reported that the human PER3 protein is destabilized by a mutation affecting two amino acids (P415A H417R)[33]. This mutation causes FASP accompanied with seasonal affective disorder, or 'winter depression'[33]. Notably, the human *PER3* mutation accelerates turnover of not only PER3 but also PER1 and PER2. Indeed, protein levels of PER1 and PER2 are decreased in the human *PER3* P415A H417R mouse model[33]. A mutation of a serine residue to a glycine at amino acid 662 of human PER2 (S662G) has been identified in another family with FASP. Mice with the PER2 S662G mutation show phase-advanced behavioral rhythms and a shorter circadian period[8]. The FASP mutation site is conserved in human PER1 (Ser714)[37]. Like the human PER2 mutation,

the PER1 S714G mutation in mice leads to a shortened circadian period. In addition to exhibiting advanced sleep–wake rhythms, PER1 S714G mice exhibit advanced feeding rhythms and abnormal metabolic homeostasis[37]. The S662G (S714G) mutation destabilizes PER2 (PER1) protein[8,37] (**Fig. 2**, state 4) but more importantly leads to decreased transcription of the *Per2* gene, thus resulting in lower PER2 levels. A mutation mimicking a phosphoserine (serine to aspartate) at the same residue stabilizes PER2 and increases *Per2* transcription and PER2 levels[8]. Adding to the complexity, phosphorylation of Ser662 primes PER2 for downstream CKI-mediated phosphorylation (of Ser665, Ser668, Ser671 and Ser674)[8,38,39]. Thus, CKI phosphorylates multiple sites on PER2 and consequently has different effects on PER stability and circadian timing (**Fig. 2**, states 2 and 3). Phosphorylation of an N-terminal site on PER2 targets this protein for degradation and lowers PER2 levels (**Fig. 2**, state 1 to state 2). In contrast, phosphorylation of PER2 Ser662 and four downstream serines leads to lower repressor activity and PER2 stabilization (**Fig. 2**, state 1 to state 4).

In spite of the large contribution of CKI to clock function, how CKI-mediated phosphorylation of PERs is initiated remains unsolved. It is well known that CKI requires a priming phosphorylation. Ser662 of human PER2 (FASP site) is a priming site whose phosphorylation leads to sequential phosphorylation of downstream serine residues by CKI (from Ser665 to Ser674)[8,38]. CKI also cooperates with an unknown kinase that phosphorylates a priming site (Ser477) in PER2. This priming phosphorylation leads to CKI binding and phosphorylation of downstream residues between amino acids 477 and 482 of PER2 (β-TrCP1 phosphodegron), thereby promoting degradation[15]. Intriguingly, the balance of these two antagonizing phosphorylation cascades (downstream of Ser477 and Ser662) is temperature dependent and is thought to contribute to temperature compensation in the mammalian clock[40] (**Fig. 2**. state 1 to states 2 and 3, 'temperature switch'). Higher temperatures promote phosphorylation in the region of Ser662, thus leading to PER2 stabilization. Lower temperatures favor phosphorylation of the β-TrCP1 site and consequently lead to PER2 degradation[40]. For both sites, the priming kinases have not yet been identified, but they are key molecules in the regulation of this switching.

Nuclear entry of PER regulated by phosphorylation and protein interaction

Beyond affecting protein stability, CKI-mediated phosphorylation regulates PER1 nuclear entry in cultured cells. CKIε expression inhibits PER1 nuclear accumulation in HEK293 cells in a manner dependent on kinase activity and binding of CKIε to PER1 (ref. 41). A mechanism for PER1 nuclear export has been proposed, in which the mouse PER1 nuclear localization signal (NLS, amino acids 824–851) is masked by CKIε-mediated phosphorylation of the serine cluster from amino acids 902 to 916. In addition, PER1–3 proteins contain nuclear export signals (NESs, amino acids 484–495 for mPER1)[42]. However, other studies have reported that expression of CKIε or CKIδ promotes nuclear accumulation of mouse PER1 and PER3 in COS-7 cells[16,43]. Thus, regulation of CKI-dependent PER nuclear transport can differ among cell lines for unknown reasons. It is possible that such variations may merely be artifacts of *in vitro* cell lines. Alternatively, these variations may reflect differential regulation that occurs in different cell types *in vivo*. Further studies have identified Ser661 and Ser663 of mouse PER1 as being important CKIε-dependent nuclear localization[44]. For PER2, PKCα and G-protein-coupled receptor kinase 2 (GRK2) promote phosphorylation and cytosolic retention, both of which are important for the light-induced phase shifts of mouse behavioral rhythms[45,46].

The nuclear entry of PER is also enhanced by dimerization with CRY or with PER proteins, although the involvement of phosphorylation in this mechanism is unknown. CRY2 has a functional NLS in the C-terminal region, but this region is not conserved in CRY1 (ref. 47). Importins α and β are nuclear transport proteins that interact with CRY2 through this NLS and consequently facilitate nuclear entry of CRY2 and PER2 expressed with CRY2. When the CRY2 NLS sequence is mutated, it can no longer facilitate the nuclear translocation of PER2 in cell culture[47]. In addition, PER2 nuclear localization is enhanced by CRY1 and is dependent on CRY1 binding[48,49]. However, nuclear entry of PER1 and PER2 is also enhanced by dimerization with PER3 via its cytoplasmic localization domain (CLD, amino acids 330–389 for mPER3) and its NLS (amino acids 726–734)[50]. Hence, CRY1 and CRY2 affect, but are not necessary for, the nuclear entry of PERs. Indeed, PER1 protein is able to enter the nucleus in *Cry1*- and *Cry2*-deficient cells through dimerization with PER3 (ref. 50). Nuclear accumulation of clock proteins is dependent on numerous protein-protein interactions via NLS, NES and CLD elements, and is controlled by phosphorylation signals for some proteins, such as PER1.

Multiple modifications antagonizing PER degradation

Dephosphorylation and deubiquitination. Many PTMs are reversible reactions: for example, phosphorylated residues can be dephosphorylated by phosphatases. Transitions between the phosphorylated and dephosphorylated states can thus be important switches regulating biological activities (for example, switching transcription from on to off). In mammals, phosphoprotein phosphatase 1 (PPP1) dephosphorylates PER proteins, thereby antagonizing CKI-mediated PER degradation[15,51,52] (**Fig. 2**, state 2 to state 1). Inhibition of PPP1 in mouse suprachiasmatic nuclei lengthens the free-running period and alters light induced phase shifts[53]. PPP5 also regulates the circadian clock in culture by preventing autophosphorylation of CKI (ref. 54). Currently, there is growing interest in the characterization of deubiquitinating enzymes. Ubiquitin specific protease 2 (USP2) deubiquitinates PER1, BMAL1, CRY1 and CRY2 (refs. 55–57). Mice with homozygous *Usp2*-null alleles display a longer circadian period accompanied by an altered response to light, particularly late in the dark period when light normally causes a phase advance[57]. PER1, BMAL1, CRY1 and CRY2 are all deubiquitinated by USP2, thus resulting in decreased proteasomal degradation and consequent stabilization of these proteins (**Fig. 2**, state 3 to state 2).

O-GlcNAcylation at FASP sites. We have previously found that O-GlcNAcylation in the FASP serine cluster (amino acids 662–674) of PER2 competes with phosphorylation at the same sites[11]. O-GlcNAcylation catalyzed by *O*-GlcNAc transferase (OGT) increases the repressor activity of PER2 and decreases protein stability (**Fig. 2**, state 1 to state 5). Because the activity of OGT is dependent on the GlcNAc donor (UDP-GlcNAc, a product of glucose and amino acid metabolism), the balance between O-GlcNAcylation and phosphorylation is regulated by glucose availability and represents a direct link between the circadian clock and metabolism[11] (**Fig. 2**, 'nutrition switch').

Acetylation. Because it modifies lysine residues, acetylation can compete for some of the same residues that are targets of ubiquitination. SIRT1 is a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase, and its expression and activity are regulated by the circadian clock[58,59]. SIRT1 binds to clock proteins in a circadian manner and promotes deacetylation of PER2, thus leading to degradation[58] (**Fig. 2**, state 6 to state 3). Knockout of SIRT1 in fibroblasts causes a

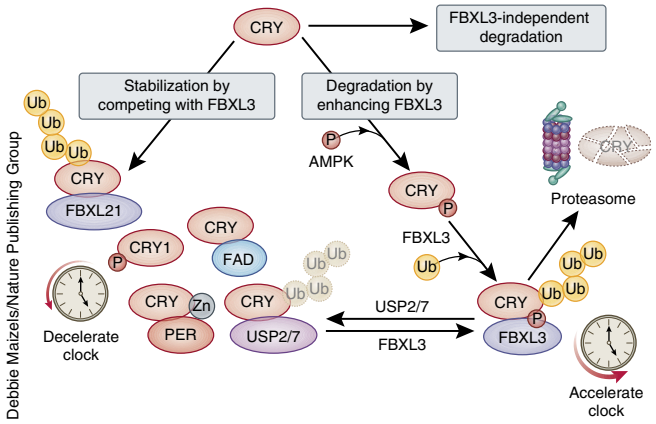

Figure 3 CRY stability is controlled by FBXL3 and multiple competing mechanisms. Regulation of mammalian CRY proteins by PTMs. CRY is subjected to ubiquitination by multiple E3 ligases. Among them, FBXL3 plays a central role in determining the circadian period, and many mechanisms antagonize FBXL3 function. FBXL21, a protein homologous to FBXL3, interferes with FBXL3 by forming ubiquitin chains with different linkages at different residues from FBXL3-mediated ubiquitination. FAD and PER (and probably FBXL21) compete with FBXL3. Phosphorylation of CRY decreases the binding affinity of FBXL3. The deubiquitinating enzymes USP2 and USP7 (USP2/7) remove ubiquitin chains from CRY. P, phospho-; Ub, ubiquitin. AMPK, AMP-activated protein kinase.

lower amplitude of circadian oscillation and stabilizes PER2. However, the details of this regulation (such as the acetylating enzyme and acetylated residues) remain to be precisely defined.

Degradation of CRY mediated by phosphorylation and ubiquitination

CRY1 and CRY2 have strong repressor activity on CLOCK–BMAL1 transactivation. Similarly to PER-protein levels, levels of CRY1 and CRY2 in suprachiasmatic nuclei and peripheral clocks fluctuate in a circadian manner[12,60]. Before 2007, there had been a focus on PER-protein degradation because of the strong effect of CKI on PER-protein stability and the conservation of CKI regulation in other organisms[3]. However, two mouse mutations, after hours (*Afh*) and overtime (*Ovtm*), identified by *N*-ethyl-*N*-nitrosourea (ENU) screening[61,62], have enabled further study of CRY regulation, especially CRY degradation. Both of these mutants have a point mutation in the mouse *Fbxl3* gene. FBXL3 (an F-box-type E3 ligase) binds both CRYs, and the FBXL3-containing SCF complex ubiquitinates CRYs, thereby promoting degradation[63] (**Fig. 3**). *Afh* and *Ovtm* mutations lead to decreased CRY degradation and extremely long free-running periods[61–63]. Interestingly, a mutation in *Fbxl21* (also known as *Psttm*) shortens the free-running period and abrogates the phenotype of the $Fbxl3^{Ovtm}$ mutant[64]. FBXL21, a homolog of FBXL3, also ubiquitinates CRY but competes with FBXL3 and consequently stabilizes CRY in mice, presumably by preventing FBXL3-mediated ubiquitination[64,65] (**Fig. 3**). Thus, despite having high sequence similarity, *Fbxl21* and *Fbxl3* have opposite effects on the regulation of CRY turnover and period[64]. But more importantly, *Fbxl3* and *Fbxl21* double-knockout mice show arrhythmic behavior in constant dark, thus indicating that stabilization and degradation of CRY cooperatively maintain robust oscillation of the clock[62]. In contrast, ovine FBXL21 promotes degradation of CRY1 in COS-7 cells[66]. FBXL3 and FBXL21 are highly similar in amino acid sequence, although only FBXL3 has an NLS in the N-terminal region and is thus localized to the nucleus[61,64,65]. In a current model, FBXL3-mediated degradation enhances nuclear clearance of CRYs, thereby leading to termination of transcriptional repression[61]. Thus, dysfunction of FBXL3-mediated CRY degradation results in a longer circadian period.

In addition to FBXL3 and FBXL21, DNA-damage-binding protein 1 (DDB1) and FBXW7 E3 ligases have recently been reported to regulate degradation of CRY1 and CRY2, respectively[67,68], thus further underscoring the complexity of CRY regulation. Similarly to PER ubiquitination, CRY degradation is also enhanced by phosphorylation. Phosphorylation at Ser71 of CRY1 (corresponding to Ser89 of CRY2) by AMP-activated protein kinase recruits FBXL3 and promotes CRY degradation[69]. The FBXW7-containing SCF complex ubiquitinates CRY2 phosphorylated at Thr300 in the FBXW7 phosphodegron (300-TPPLS-304), thus leading to proteasomal degradation[68]. Ser557 in the unique C-terminal region of CRY2 (not conserved between CRY1 and CRY2) is phosphorylated by dual-specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A)[70]. Ser557 phosphorylation is followed by secondary phosphorylation at Ser553 by glycogen synthase kinase 3 (GSK-3)[70,71], which requires a phosphorylated or negatively charged amino acid (aspartate or glutamate) four residues downstream of the target phosphorylation site. This sequential phosphorylation recruits an unknown E3 ligase, thereby leading to CRY2 degradation[70]. Mutation at the priming site (S557A) leads to decreased CRY2 turnover and a lengthened circadian period in mice[72]. However, phosphorylation at Ser588 in the C-terminal region of CRY1 prevents the FBXL3-CRY1 interaction and consequently increases CRY1 stability[73]. This phosphorylation is negatively regulated by the DNA-dependent protein kinase (DNA-PKcs), whereas the kinase responsible for Ser588 phosphorylation remains unknown.

Multiple regulations antagonize FBXL3-mediated degradation

Because the degradation rate of CRY dominantly determines the circadian period[61,62], multiple mechanisms including FBXL21 counter FBXL3-mediated degradation (**Fig. 3**). Ubiquitin-specific protease 7 (USP7), also known as HAUSP, has recently been identified as a CRY1- and CRY2-deubiquitinating enzyme[74,75]. In response to DNA damage, USP7 specifically deubiquitinates and stabilizes CRY1, and advances cellular clocks, whereas CRY2 is subjected to FBXL3-mediated proteasomal degradation[74]. In the absence of DNA damage, USP7 binds and stabilizes both CRY1 and CRY2 by antagonizing the activity of multiple E3 ligases including FBXL3 (refs. 74,75). As described above, USP2 deubiquitinates CRY1, thereby decreasing proteasomal degradation and stabilizing these proteins[56].

Recently, we have observed flavin adenine dinucleotide (FAD)-mediated CRY stabilization[34] (**Fig. 3**). In a family with FASP, a mutation in the human *CRY2* gene changes the alanine residue at position 260 to a threonine in the FAD-binding domain of CRY2. The mutant CRY2 enhances FBXL3-dependent degradation because FAD sterically interferes with the FBXL3-CRY interaction[76], and the A260T mutation decreases FAD function. A large-scale chemical screen has identified the small molecule KL001, which binds CRY via the binding pocket, thus stabilizing CRY proteins[77]. These reports further support the importance of the stability of clock proteins in determining the circadian period and phase angle.

Another mechanism altering CRY stability is dimerization with PER proteins. The PAS domains of PER1–3 proteins interact with coiled-coil regions of CRY[6,48]. The polyubiquitinated form of PER2 is decreased by expression of CRY1 and CRY2, and vice versa[78]. Recent structural studies have provided a reasonable explanation for this stabilizing effect of PER on CRY. The PER2-interacting domain in

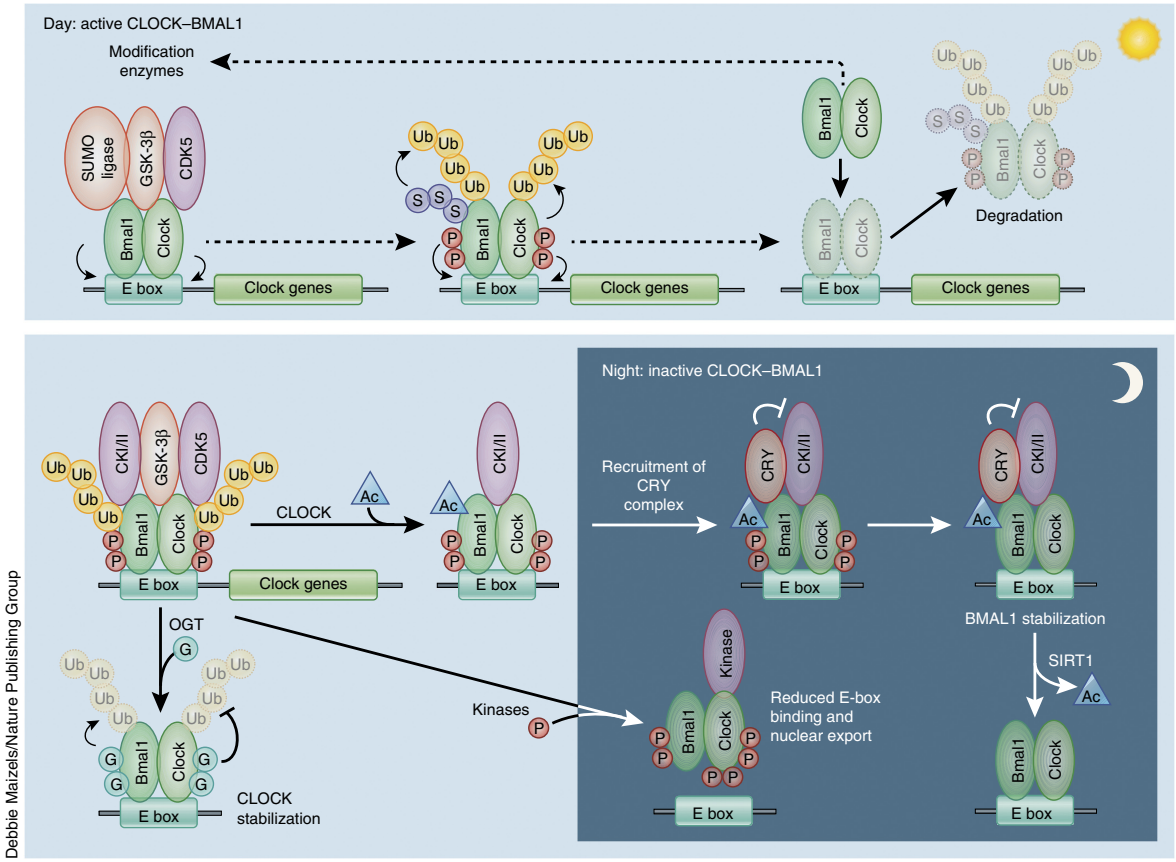

Figure 4 Temporal regulation of CLOCK–BMAL1 activity. Top, activation mechanism of CLOCK–BMAL1. When transcription of clock genes and clock-controlled genes is activated, CLOCK and BMAL1 are subjected to proteasomal degradation. CLOCK–BMAL1 recruits transcription complexes to DNA, and transcription is initiated. CLOCK and BMAL1 are modified by phosphorylation (P), SUMOylation (S) and ubiquitination (Ub) before degradation. Then another heterodimer of CLOCK–BMAL1 binds anew to DNA. This cycle enhances the transcriptional activity. (bottom) Mechanism of termination of transactivation. Activated BMAL1 (left top) is modified by acetylation (Ac), thereby recruiting CRY repressors to CLOCK–BMAL1 complexes. CRY inhibits CKI and CKII (CKI/II) kinase activity, thus stopping CLOCK–BMAL1 degradation. Acetylation of BMAL1 is removed by SIRT1. In parallel, the CLOCK–BMAL1 complex is phosphorylated by unknown kinases, thereby eliminating CLOCK–BMAL1 from DNA and the nuclei. *O*-GlcNAc (G) modification of CLOCK probably competes with phosphorylation (for degradation) and stabilizes CLOCK.

mouse CRY overlaps with the FBXL3-interacting region of CRY[76,79]. Thus, PER2 interferes with the CRY-FBXL3 interaction and excludes FBXL3 from the complex, thereby protecting CRY from degradation[76] (**Fig. 3**). The CRY1-PER2 interaction is tightened by zinc binding and weakened by disulfide binding within the CRY1 molecule[80]. Furthermore, a recent study has reported that interaction with TAR DNA-binding protein 43 (TDP-43) stabilizes CRY1 and CRY2 in a manner dependent on FBXL3, thus suggesting that TDP-43 structurally competes with FBXL3 (ref. 75).

Degradation of CLOCK–BMAL1 for transactivation

In contrast to levels of PER and CRY proteins, levels of CLOCK and BMAL1 proteins do not show dramatic circadian oscillations. However, phosphorylation of CLOCK and BMAL1 proteins (as assessed by protein mobility shifts) show clear circadian oscillations and represent time-dependent post-translational regulation of CLOCK and BMAL1 (refs. 12,81). One effect of CLOCK or BMAL1 phosphorylation is altered stability, and degradation of CLOCK and BMAL1 is important for transcriptional activation of clock-controlled genes through E boxes in their promoters[82]. The concept of transcription-factor cycling (binding to DNA, recruiting transcriptional machinery and then being degraded (**Fig. 4**) in transactivation is widely accepted for other transcription factors, such as the estrogen receptor[83]. Treatment with the proteasome inhibitor MG132 leads to accumulation of CLOCK–BMAL1 proteins by preventing their degradation[82]. However, MG132 treatment also leads to decreased E-box-mediated transcription by interfering with this cycling. The *Clock* mutation was the first identified in an ENU mutagenesis screen in mice and results in deletion of exon 19 and truncation of the CLOCK protein[84]. The activity of CLOCKΔ19 in transactivation of PER-encoding genes is far weaker, and the free-running period of *Clock* mutant mice is extremely long[85,86]. A study of the heterozygous mutant has revealed that CLOCKΔ19 protein is hypophosphorylated and consequently is found in higher levels than those of wild-type CLOCK[81]. *In vitro* studies have identified several enzymes responsible for CLOCK–BMAL1 degradation (**Fig. 4**). Ser431 of CLOCK is a priming phosphorylation site allowing GSK-3-catalyzed phosphorylation at Ser427 (ref. 87). Thr21 of BMAL1 is probably the priming site for GSK-3β-catalyzed phosphorylation at Ser17 (ref. 88). Phosphorylation-deficient forms of CLOCK and BMAL1 exhibit less transcriptional activity and greater stability than the wild-type proteins, in agreement with a model in which unstable CLOCK and BMAL1 have higher activity[87,88]. Similarly, Thr451 and Thr461 in CLOCK are phosphorylated by cyclin-dependent kinase 5 (Cdk5) and consequently degraded[89], thus resulting in increased CLOCK-mediated transactivation. In the peripheral clock, knockout

of PKCγ modulates feeding-dependent gene expression in mouse liver tissue[90]. PKCγ phosphorylates and stabilizes BMAL1 by eliminating its polyubiquitination. Regulation of CLOCK–BMAL1 activity through modulation of protein turnover is not limited to phosphorylation. SUMOylation at Lys259 induces ubiquitination of BMAL1 and increases transactivation and degradation[91,92]. In a similar manner, SUMOylation at Lys67 and Lys851 of human CLOCK increases the activity of CLOCK[93], although it is unclear whether this modification destabilizes CLOCK. O-GlcNAcylation at Ser418 of BMAL1 and an unknown site of CLOCK stabilizes CLOCK and BMAL1 by inhibiting ubiquitination[94] (**Fig. 4**). O-GlcNAcylation may compete with the phosphorylation of CLOCK and BMAL1 and indirectly inhibit ubiquitination because phosphorylation of these proteins promotes proteasomal degradation[87,88]. In spite of the central role of protein degradation in regulation of CLOCK and BMAL1, little is known regarding the E3 ligases promoting ubiquitination of CLOCK and BMAL1. Recently, a HECT-type E3 ligase (UBE3A) has been shown to interact with BMAL1 and to promote its degradation[95].

Inactivation mechanisms of CLOCK–BMAL1 by sequential PTMs

CLOCK–BMAL1 is inactivated by complex sequential modifications (**Fig. 4**). CLOCK possesses intrinsic histone acetyltransferase (HAT) activity, thus resulting in histone acetylation[96] and acetylation of its partner, BMAL1 (ref. 97). Acetylated Lys537 of BMAL1 promotes recruitment of CRY1 to the CLOCK–BMAL1 complex, thereby leading to transcriptional repression. A recent study has indicated that phosphorylation at Ser90 of BMAL1 by CKII is a prerequisite for Lys537 acetylation and promotes nuclear accumulation of BMAL1 (refs. 98,99). BMAL1 acetylation leads to CRY recruitment, which in turn suppresses CKII activity[98]. Subsequently, SIRT1 binds to CLOCK and BMAL1 and deacetylates BMAL1 at Lys537 (ref. 59), thus preventing CRY1 recruitment and restarting the transactivation of clock genes. A mutation in CLOCK motif A, which is required for HAT activity, and a mutation at the BMAL1 acetylation site are unable to rescue the cellular rhythms in homozygous *Clock*-mutant mouse embryonic fibroblasts and *Bmal1* (official symbol *Arntl*)-knockout cells, respectively[96,97]. Thus, the proper timing of these sequential modifications is necessary for the generation of normal oscillations (**Fig. 4**).

Some repressors act competitively through binding to DNA. How can CRY have repressor activity without binding DNA? Transcriptional repressors may also downregulate gene transcription by recruiting histone modulators (for example, by affecting methylation or acetylation) that in turn regulate transcription. Alternatively, repressors may have effects through recruitment of transcription-termination complexes. One example is the recruitment by PER2 of a transcription-termination complex that represses CLOCK–BMAL1-mediated transcription[100]. The key mechanism of CRY repression is through regulation of CLOCK–BMAL1 phosphorylation. Phosphorylation of BMAL1 and CLOCK is impaired by expression of CRY1 or CRY2 (refs. 81,101), thus leading to BMAL1 stabilization[102]. CRY has been proposed to recruit phosphatase to the CLOCK–BMAL1 complex or to inhibit the kinase activity of the complex. Regulation of CLOCK–BMAL1 phosphorylation probably affects transcription through alterations in DNA binding. Results from previous studies are consistent with this model, in which CRY1 inhibits PPP5 and consequently inhibits CKIε kinase activity through autophosphorylation of CKIε (ref. 103), which phosphorylates BMAL1 and consequently enhances BMAL1 activity[104].

In addition to protein turnover, the activity of transcription factors is regulated by other factors including nuclear–cytoplasmic shuttling, DNA binding and histone modifications around the binding region. Histone modifications in the promoter regions of clock-controlled genes (for example, *Dbp*, *Per1* and *Per2*) fluctuate in a circadian pattern, and histone modifications also are essential for normal clock function[59,96,105–108]. CLOCK–BMAL1 dimers shuttle between the nucleus and cytosol[109]. Circadian nuclear accumulation of CLOCK requires BMAL1 protein[110]. In the nucleus, CLOCK–BMAL1 rhythmically binds to E boxes[105,111–113], and CRY and PER proteins interact directly with CLOCK–BMAL1 in a rhythmic manner, thereby repressing CLOCK–BMAL1 transactivation of PER- and CRY-encoding genes at the proper time[105]. CLOCK binds to E boxes through its basic helix-loop-helix domain[114]. In this domain, CLOCK has two tandem phosphorylation sites (Ser38 and Ser42), although the kinase responsible for this phosphorylation has not been identified[81]. Mutations mimicking constitutive phosphorylation at these sites (S38D and S42D) decrease DNA binding and nuclear localization of CLOCK *in vitro*[81]. Presumably, negative charges at these sites have effects on electrostatic forces, thereby leading to decreased binding affinity toward DNA (**Fig. 4**). Phosphorylation of BMAL1 by PKC, which is stimulated by RACK1, inhibits CLOCK–BMAL1 transcriptional activity[115], thus suggesting that the activators are involved in a more complicated phosphorylation network.

PTMs of REV-ERB regulating metabolism

A recent study has identified FBXW7 as an enzyme that ubiquitinates REV-ERBα and thereby regulates the amplitude of the circadian clock[116]. FBXW7-mediated ubiquitination is dependent on the CDK1-catalyzed phosphorylation of REV-ERBα at Thr275, and liver-specific knockout of *Fbxw7* leads to dysfunction in glucose and lipid metabolism. REV-ERBα is also phosphorylated by GSK-3 at Ser55 and Ser59, and phosphorylation of these sites increases the half-life of REV-ERBα (refs. 117,118). Interactome analysis has identified two E3 ligases, Arf-bp1 (HUWE1) and Pam (Myc-bp2), as REV-ERBα-interacting proteins[118]. Furthermore, the phenotype of *Fbxl3*-knockout mice is rescued by REV-ERBα knockout, and FBXL3 inactivates the REV-ERBα–HDAC3 complex, although the substrate of FBXL3 in the complex remains unclear[119].

Concluding remarks and prospects

Here, we have summarized the current understanding of PTM-mediated regulation of clock proteins in mammals. We emphasize that the forward-genetics approach including human genetics has contributed to a large part of the important findings, thus demonstrating that the post-translational regulation of clock proteins strongly affects the circadian phenotype. In humans, altered sleep–wake cycles frequently result from abnormal modification or turnover of clock proteins (PERs and CRYs). These studies not only have provided insight into basic molecular mechanisms that play important roles *in vivo* but also have advanced clinical research. Modulation of clock proteins can aid in recovering sleep–wake patterns disturbed through genetics or shift work, as well as many disorders associated with the circadian clock. For example, destabilization of PER3 causes advanced sleep phase accompanied by seasonal affective disorder; therefore, pharmacologically increasing the stability of PER3 protein may improve winter depression sympotoms[33]. Another study has reported that KL001, identified as a period-lengthening molecule by chemical screening, indeed affects glucose homeostasis in mice by modulating CRY stability[77]. To control and manage clocks, more comprehensive understanding of PTMs of clock proteins is required. Advanced technologies such as large-scale screening, next-generation sequencing (for genome analysis) and mass spectrometry

(in applications such as phosphoproteomics and ubiquitinomics) will continue to advance PTM circadian research in the future.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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EDITORIAL SUMMARY

AOP: Clock proteins are controlled by multiple post-translational modifications during the circadian cycle. In this Review, the authors examine how post-translational modifications influence the stability, interactions and activity of mammalian clock proteins and how they contribute to proper clock function or are altered in circadian disorders.

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