Post-translational Modifications are Required for Circadian Clock Regulation in Vertebrates

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Abstract: Circadian clocks are intrinsic, time-tracking systems that bestow upon organisms a survival advantage. Under natural conditions, organisms are trained to follow a 24-h cycle under environmental time cues such as light to maximize their physiological efficiency. The exact timing of this rhythm is established via cell-autonomous oscillators called cellular clocks, which are controlled by transcription/translation-based negative feedback loops. Studies using cell-based systems and genetic techniques have identified the molecular mechanisms that establish and maintain cellular clocks. One such mechanism, known as post-translational modification, regulates several aspects of these cellular clock components, including their stability, subcellular localization, transcriptional activity, and interaction with other proteins and signaling pathways. In addition, these mechanisms contribute to the integration of external signals into the cellular clock machinery. Here, we describe the post-translational modifications of cellular clock regulators that regulate circadian clocks in vertebrates.

Keywords: Circadian clock, cellular clock, clock protein, post-translational modification, transcription, clock gene.

1. INTRODUCTION

Circadian clocks regulate a number of physiological functions, such as sleep and metabolism, in a broad spectrum of organisms ranging from bacteria to humans [1]. They generate daily changes (circadian rhythms) in various biochemical, physiological and behavioral processes. Under natural conditions, circadian clocks are trained according to the 24-h cycle based on environmental time cues, such as light, to maximize an organism’s physiological efficiency [2]. Thus, disrupting circadian clocks can have a profound effect on organisms’ health and is linked to various diseases, including sleep disorders and metabolic syndromes [3, 4].

At the molecular level, circadian clocks can be divided into three conceptual components [1, 5]. The first is the pacemaker that is dedicated to generating and sustaining circadian rhythms by receiving and integrating signals from external time cues [6]. The second component is the input, which refers to the pathway through which external time cues are perceived and act upon the central pacemaker. The third element relates to how the circadian clock affects physiology and is achieved through the output pathways.

The pacemaker in the members of Neurospora and Drosophila and in vertebrates are transcription/translation-based negative feedback loops that rely on positive and negative oscillator elements [7]. In vertebrates, three basic helix-loop-helix Per-ARNT-Sim (PAS) domain-containing transcription factors, known as CLOCK, neuronal PAS domain protein 2 (NPAS2), and brain-muscle ARNT-like protein (BMAL), constitute the positive elements [1, 5, 8]. CLOCK or NPAS2 heterodimerizes with BMAL to form a transcriptionally active complex that binds to E-box elements (CACGTG) present in the promoters of the members of the period (per) and cryptochrome (cry) families. Once PER and CRY proteins are translated, they form heterodimers that can then translocate into the nucleus to repress CLOCK(NPAS2)–BMAL-mediated transcription through direct protein–protein interaction. Importantly, when active, the CLOCK(NPAS2)–BMAL complex stimulates the transcription of various other clock-controlled genes. The protein products of these genes, in turn, influence functions external to the oscillatory mechanism itself and mediate the “output” function of the clock. This partly accounts for the presence of circadian rhythms in a variety of physiological processes [3, 4].

Although the relatively straightforward mechanisms of positive and negative feedback loops are necessary to establish and maintain cellular clocks, these clocks are complex and involve processes such as the post-transcriptional regulation of cellular clock components (clock proteins) [4, 9, 10].
These modifications have essential roles in appropriately regulating clock protein stability, cellular localization, transcrip
tional activity, and interaction with other proteins. In addition, a variety of studies revealed that the post-
transcriptional modifications of clock proteins are involved in the regulation of the input and output processes of circadi-
nian clocks [11-13]. Here, we describe the roles of the post-
translational modifications of clock proteins involved in con-
trolling circadian clocks in vertebrates.

2. IMPORTANCE OF THE PHOSPHORYLATION OF CELLULAR CLOCK PROTEINS FOR REGULATING THE CLOCK’S PERIODICITY AND LIGHT RE-
SPONSE

The functions of various mammalian cellular clock pro-
teins, including CLOCK, BMAL1, PER1, PER2, PER3, CRY1, and CRY2, are regulated via phosphorylation by vari-
ous kinases [4, 9, 10]. The first major step towards un-
derstanding the importance of phosphorylation in vertebrate circadian clock regulation was taken when the tau mutation, which causes a short-period phenotype in the Syrian ham-
ter, was identified [14]. The tau locus encodes casein kinase I epsilon (CKIε) that phosphorylates PERs. The short-period phenotype observed in the mutant hamster was generated because of a low rate of CKI- dependent phosphorylation of
PER2. Defects in the phosphorylation of cellular clock pro-
teins have been implicated in human disorders [15, 16]. A missense mutation in the circadian clock gene Per2 is asso-
ciated with familial advanced sleep phase syndrome. The corresponding mutated PER2 protein is less effectively
phosphorylated than the wild-type PER2; moreover, the
phosphorylation-dependent stability control of the mutated
PER2 was demonstrated to have been eliminated in vitro.
Additionally, polymorphism in a region of human Per3, the
presumed CKIε-binding domain, may be associated with
delayed sleep phase syndrome [17]. CKIε specifically inter-
acts with and phosphorylates PER1, 2 and 3 proteins, and
thus regulates each of them differently [4, 9, 10, 18, 19].

A number of pharmacological studies have used lucifer-
ase-based clock reporter cells to identify the molecules that
regulate cellular clocks. These studies identified various ki-

netic and biochemical analyses reported the involvement of
these kinases in the functional regulation of clock proteins
and the maintenance of circadian clocks at the molecular,
cellular, and organismic levels [11, 22-27]. Below, we de-
scribe the role of JNKs in circadian clock regulation.

3. ROLES OF THE JNK-MEDIATED SIGNALING PATHWAY IN CIRCADIAN CLOCK REGULATION

JNK activity is regulated via the phosphorylation of par-
ticular tyrosine and threonine residues located in the kinase
domain [28]. JNK phosphorylation is catalyzed by two dual-
specificity kinases, MKK4 and MKK7, which act in a syner-
gistic manner [29, 30]. Although it is primarily activated in
response to external stress (osmolarity changes, heat shock
and UV irradiation), phosphorylated JNK has been detected
in unstressed cultured cells and in isolated mouse tissues,
such as the brain [30-32]. Notably, previous studies have
shown that JNK phosphorylation levels, and thus its kinase
activity, fluctuate in a circadian manner in both the suprachi-
asmatic nucleus (SCN), the location of the central clock in
mammals, and in cultured mammalian cells [33, 34]. These
studies indicate the importance of JNK signaling in physi-
ological processes other than cellular stress responses, includ-
ing circadian clocks.

In this context, it was previously reported that the
MKK7–JNK signaling pathway is an essential regulator of
periodicity in the cellular clocks of mammals. The MKK7–
JNK signaling pathway induces PER2 phosphorylation and
stabilizes PER2 by inhibiting its ubiquitination, which has an
effect opposite to that of CKIε-induced PER2 destabiliza-
tion [24] (Fig. 1A). Because genetically inhibiting MKK7’s func-
tion results in the extension of the cellular clock’s periodicity
in cultured cells, this phosphorylation-mediated PER2 stabil-
ity control may be necessary to maintain the normal peri-
docity of cellular clocks. In addition, a recent study generat-
ed neuron-specific Mkk7-deleted mice, in which MKK7
was genetically inactivated in the central clocks of the
SCN [32]. A behavioral analysis of these mice revealed
that the neuron-specific disruption of Mkk7 resulted in
longer periods of circadian behavioral rhythms and also
reduced the amplitude of rhythmicity compared with wild-
type mice. These findings provide evidence that the
MKK7–JNK signaling pathway is involved in the regula-
tion of the circadian pacemaker at an organismic level.

In addition to PER2, JNK phosphorylates BMAL1 and
CLOCK in mammals. In particular, Yoshitane et al. reported
that neuron-specific isofrom JNK3-deficient mice have lon-
ger free-running periods of behavioral rhythms and compro-
mised phase shifts to light [11]. In nocturnal animals, the
higher the light intensity in constant light conditions, the
longer the circadian period becomes (and vice versa in diur-
nal species). This phenomenon is known as Aschoff’s rule
[35, 36]. In JNK3-deficient mice, behavioral rhythms are
insensitive to intensity changes in constant light, thus deviat-
ing from Aschoff’s rule [11]. These findings provide solid
evidence that JNK-mediated BMAL1 phosphorylation is an
important regulatory mechanism underlying the circadian
pacemaker, as well as the light input pathway of the circadi-
an clock in vivo.
4. REGULATION OF CELLULAR CLOCKS VIA BMAL1 AND DIFFERENTIATED EMBRYOCHONDROCYTE EXPRESSED GENE 1 (DEC1) SUMOylation

SUMOylation is the covalent linking of small ubiquitin-related modifier proteins to lysine residues [37, 38]. This modification is a reversible post-translational modification that has been implicated in transcriptional regulation by a number of mechanisms. Previous studies found that BMAL1 is sumoylated on a highly conserved lysine residue (Lys259) in cultured cells and that BMAL1 SUMOylation shows a circadian pattern in mouse liver tissue [39]. In addition, BMAL1 SUMOylation has been demonstrated to control BMAL protein stability and to play a critical role in the regulation of the pacemaker in the circadian clock.

Reportedly, BMAL1 SUMOylation promotes the interaction of CREB-binding protein to the CLOCK–BMAL1 complex, which resets the cellular clock in response to serum stimuli [40]. The formation of this ternary complex induces the acute activation of the CLOCK–BMAL1-mediated transcription of Per1, resetting the phase of the cellular clock. These findings clearly demonstrate that BMAL1 SUMOylation is a regulatory element for the input pathway of the circadian clock.

Differentiated embryo-chondrocyte expressed gene 1 (DEC1) is a basic mammalian helix-loop-helix protein that acts as a transcription factor [41, 42]. DEC1 inhibits CLOCK–BMAL1-mediated transcription through direct interaction with BMAL1 and/or competition for E-box elements in the promoters of cellular clock-controlled genes [43]. Moreover, DEC1 is sumoylated on highly conserved lysine residues (Lys159 and Lys279) at its C-terminal domain [44]. SUMOylation stabilizes DEC1 by inhibiting its ubiquitination and promoting the inhibition of CLOCK–BMAL1-mediated transcription. These findings suggest that SUMOylation serves as a key regulatory element of cellular clocks by controlling multiple transcriptional factors.

5. IMPORTANCE OF THE ACETYLATION OF CELLULAR CLOCK PROTEINS FOR THE MAINTENANCE OF CLOCK PERIODICITY

Chromatin, the nucleoprotein structure into which the eukaryotic genome is organized, enables the functioning of essential biological processes, such as the regulation of transcription, DNA repair, apoptosis, and cell division [45-47]. Histone acetylation plays a pivotal role in the modulation of the chromatin structure associated with transcriptional activation [48, 49]. The activation of clock-controlled genes by the CLOCK–BMAL1 complex has been shown to be coupled to circadian changes in histone acetylation at their promoters, evidence that transcription-permissive chromatin states are dynamically established in a circadian-time-specific manner [50-52].

In mammals, the core circadian regulator, CLOCK, has intrinsic histone acetyltransferase (HAT) activity [53]. Moreover, CLOCK regulates the circadian pattern of gene expression by the virtue of its HAT activity. This finding modifies the common view of the CLOCK protein, demonstrating that it operates not only as a transcription factor but also as an enzyme. The finding of CLOCK’s HAT activity suggests that the HAT enzymatic activity also targets other non-histone proteins, which is a characteristic of other HATs. In fact, CLOCK acetylates its heterodimeric partner, BMAL1 [54, 55] (Fig. 1B). This CLOCK-mediated acetylation increases the interaction of the CLOCK–BMAL1 complex with Cry1. One study reported that BMAL1 is deacetylated by Sirt1, a nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase (HDAC) [56, 57], in a time-dependent manner [58] (Fig. 1B). Accordingly, BMAL1 acetylation is significantly rhythmic in mice liver as well as in cultured cells and SCNs. Another study demonstrated that Sirt1 also deacetylates Per2, giving Sirt1 an additional function in the transcriptional regulation of the cellular clock [59].

Notably, several pharmacological studies have confirmed the importance of the acetylation of cellular clock proteins in clock regulation. For example, it has been reported that the inhibition of Sirt1 activity by its inhibitors, such as nicotinamide and the drug splitomicin, disturbs the circadian expression of clock-controlled genes and histone H3 and BMAL1 acetylations [58]. In addition, the study using several specific Sirt1 activators demonstrated that Sirt1 activation led to the suppression of clock-controlled gene expressions and H3 acetylation at corresponding promoters in vitro and in vivo [60].

6. THE POSSIBLE ROLE OF O-LINKED ACETYLGLUCOSAMINYLATION (O-GlcNAcylation) OF CLOCK PROTEINS IN TRANSDUCING NUTRITIONAL SIGNALS TO THE CIRCADIAN CLOCK MACHINERY

O-GlcNAcylation is one of the most common post-translational protein modification with the high-energy compound, UDP-GlcNAc, as the direct donor [61]. Two enzymes regulate O-GlcNAcylation: O-GlcNAc transferase (OGT), which attaches UDP-GlcNAc to the serine and threonine residues of proteins through a β-glycosidic O-linkage, and O-GlcNAcase (OGA), which hydrolyzes O-GlcNAc in proteins [61, 62]. Analyses with O-GlcNAcylase inhibitors (the OGT inhibitor Alloxan) and activators (the OGA inhibitor PUGNAc) have revealed that the suppression of O-GlcNAcylation shortened the periodicity of cellular clocks, whereas its activation lengthened the periodicity of cellular clocks in mammalian cultured cells [13]. These findings provide evidence that O-GlcNAcylation is involved in cellular clock regulation.

Both CLOCK and BMAL1 are rhythmically O-GlcNAcylated, and this modification stabilizes both the proteins by inhibiting their ubiquitination [12, 63] (Fig. 1C). Consistent with these findings, OGT facilitates CLOCK–BMAL1-mediated transcription. Another study reported that Per2 is also O-GlcNAcylated at the region that regulates the human sleep phase in humans, competing with phosphorylation in this region [13].

Glucose flux via the hexosamine biosynthesis pathway leads to intracellular glycosylation by increasing the O-GlcNAcylation of proteins [61]. It is well established that O-GlcNAcylation regulates fundamental cellular processes in response to diverse nutritional cues. Because circadian
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![Diagram](image)

**Fig. (1). Functional regulation of clock proteins through post-translational modifications.** (A) Phosphorylation-dependent control of PER2 stability. The activity of JNK is regulated via the phosphorylation of the tyrosine and threonine residues located in the kinase domain, which is catalyzed by MKK7. MKK7-mediated JNK activation induces phosphorylation of PER2 and increases its protein half-life by competing with the CKI-induced ubiquitination and the subsequent degradation of PER2. (B) Regulation of CLOCK:BMAL-mediated transcription by BMAL acetylation. CLOCK acetylates its heterodimeric partner BMAL. This CLOCK-mediated acetylation increases the interaction of the CLOCK:BMAL complex with CRY, facilitating repression of the CLOCK:BMAL complex’s activity. SIRT1 deacetylates BMAL, which cancels the CRY-dependent repression of CLOCK:BMAL-mediated transcription. (C) O-GlcNAcylation-dependent regulation of CLOCK:BMAL complex’s stability. Both CLOCK and BMAL1 are O-GlcNAcylated, which is catalyzed by OGT and reversed by OGA. This modification stabilizes both proteins by inhibiting their ubiquitination. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

Clocks are coupled with metabolic oscillations through nutrient-sensing pathways [3, 6], these facts indicate that the O-GlcNAcylation of clock proteins plays a crucial role in transducing nutritional signals to the cellular clock machinery.

7. **ROLE OF POLY(ADP-ribosylation) OF CLOCK IN FEEDING-DEPENDENT CIRCADIAN CLOCK REGULATION**

Mammals have no photoreception in peripheral tissues; therefore, the effect of light on peripheral clocks is indirect [6]. SCN, the site of the master clock in mammals, integrates photic cues from the retina and transmits this information to peripheral clocks, synchronizing them through humoral signals [2, 4]. In addition to the input of the central clock (SCN), peripheral cellular clocks respond to cellular metabolism [3, 6]. When feeding in nocturnal animals is restricted to daytime, the phase of peripheral cellular clocks differs from that of SCN. Feeding cue affects peripheral cellular clocks via the poly(ADP-ribosylation) of CLOCK [64]. PARP-1, an NAD(+) dependent ADP-ribosyltransferase, has been shown to interact with and poly(ADP-ribosyl)ate CLOCK. The poly(ADP-ribosylation) of CLOCK leads to reduced DNA binding ability in the CLOCK–BMAL1 complex, and modulates the interaction of this complex with PER and CRY. These poly(ADP-ribosyl)ation-dependent functional regulations of the CLOCK–BMAL1 complex, in turn, influence the expression patterns of cellular clock-controlled genes. Notably, PARP-1-deficient mice show altered expression profiles in CLOCK–BMAL1-dependent gene expression, particularly in response to changes in feeding times [64]. In addition, PARP-1-deficient mice exhibit impaired food entrainment of peripheral cellular clocks. These findings support the idea that the PARP-1-mediated poly(ADP-ribosyl)ation of CLOCK plays a role in connecting feeding with the circadian clock system in mammals.

8. **POSSIBLE CROSSTALK BETWEEN THE CIRCADIAN CLOCK AND CELLULAR PROCESSES THROUGH SHARED POST-TRANSLATIONAL MODIFICATIONS**

Reportedly, clock proteins have important physiological roles that are not restricted to their functions as cellular clock regulators. Previous studies in mice and zebrafish reported that PER2 physically interacts with nuclear receptors such as PPARα, REV-ERβ, RORα, and PPARγ to regulate their transcriptional activities [65–67]. Particularly, the PER2-mediated regulation of PPARγ’s transcriptional activity contributes to the control of metabolism in mice [66]. It is tempting to speculate that post-translational modifications of
PER2 would regulate the interactions between PER2 and nuclear receptors, contributing to metabolic controls.

Recent studies found that BMAL1 interacts with HIF-1α to regulate the expression of HIF-1α target genes [68, 69]. The BMAL1–HIF-1α complex targets glycolysis genes, suggesting its control over cellular ATP levels. Furthermore, Bmal1−/− mice show reduced life spans with the symptoms of premature aging, implicating BMAL1 in control of aging [70]. Notably, a recent study reported that cellular clocks are not involved in the process of in vitro cellular senescence, suggesting that the BMAL1-mediated control of aging is not dependent on BMAL1’s function in cellular clock control [71]. Particularly, as various studies have reported SIRT1’s roles in aging [72], it is tempting to speculate that BMAL1 acetylation, which is regulated by SIRT1 [58], is involved in the BMAL1-mediated control of aging.

Cellular responses to the UV component of solar light and/or photo-oxidative stress have been proposed to be the evolutionary origin of circadian clocks [4, 73]. In support of this idea, the alteration of a cell’s redox–oxidation state triggers the transduction of photic signals that regulate circadian clock gene transcription [74–76]. In addition, various studies have implicated the role of core cellular clock components in the regulation of both, the cell cycle and DNA damage responses (DDRs) [4, 73]. Indeed, cellular clocks control the timing of cell proliferation by regulating the expression of key cell cycle genes in mammals and zebrafish [77, 78]. Moreover, post-translational modifications are vital for the regulation of the cell cycle and DDR. SIRT1 and casin kinase 2, already identified as being responsible for the post-translational modifications of clock proteins [22, 23, 58, 79] (Fig. 1), have also been implicated in the post-translational modifications of proteins such as p53 and E-cadherin, which are involved in the cell cycle and DDR [80, 81]. These findings support the hypothesis that circadian clock regulators may be linked to non-circadian physiologies through shared post-translational modifications.

CONCLUSION

It is now clear that the circadian-time dependent regulation of clock proteins’ phosphorylation is important for fine-tuning the period of the circadian clock. A number of kinases, such as CKIε, CK2, GSK3β and JNK, contribute to the circadian oscillation of phosphorylated clock proteins, regulating the clock proteins’ functions. Besides phosphorylation, other posttranslational modifications, such as SUMOylation, acetylation, O-GlcNAcylation and ADP-ribosylation, are essential modulators of clock proteins, regulating their transcriptional activity, subcellular localization, and protein stability. These modifications play a role in controlling the core mechanism of the circadian clock itself as well as the light signaling pathway to the circadian clock. In addition, it has been implicated that the post-translational modifications of clock proteins are import regulators of cellular physiology, apart from circadian timekeeping. The findings of new clock proteins’ post-translational modifications will reveal a yet unappreciated level of regulation within the core mechanism of the circadian clock and cellular physiology.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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